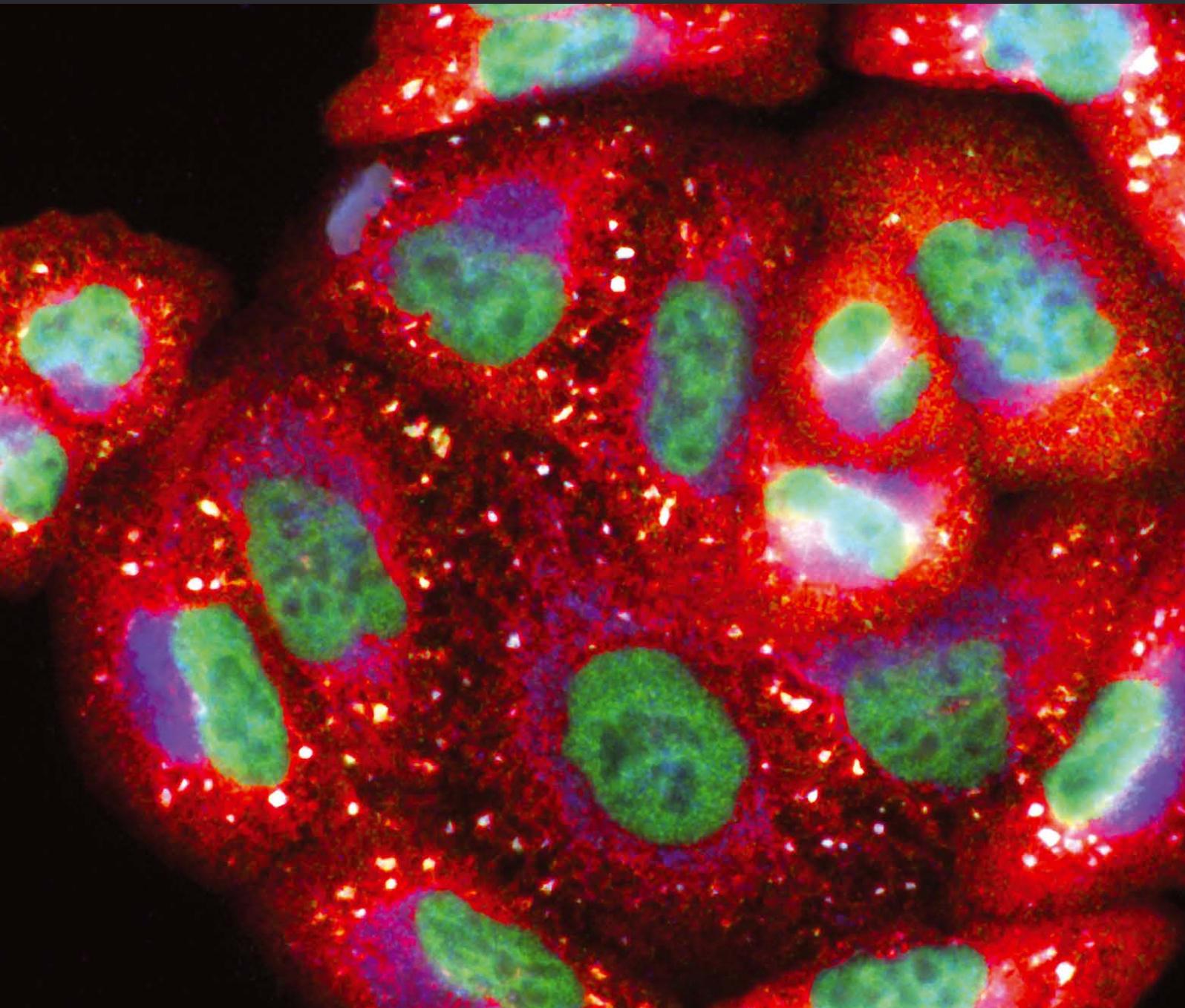


# Oxidative Stress and Cancer: Advances and Challenges

Guest Editors: Sahdeo Prasad, Subash C. Gupta, Manoj K. Pandey, Amit K. Tyagi,  
and Lokesh Deb





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## Editorial

# Oxidative Stress and Cancer: Advances and Challenges

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Received 22 December 2015; Accepted 22 December 2015

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Reactive oxygen species (ROS) constantly generated inside the body are required to drive regulatory pathways and are also a cause for several pathological conditions including cancer. Numerous lines of evidence suggest that ROS can promote as well as suppress the survival of cancer cells. *First*, ROS are known to regulate each and every step of tumor development including transformation, survival, proliferation, invasion, metastasis, and angiogenesis. *Second*, chronic inflammation, one of the major mediators of cancer, is regulated by ROS. *Third*, ROS are known to regulate signaling molecules required for cell cycle progression. *Fourth*, the expression of various tumor suppressor genes is under control of ROS. *Fifth*, a high level of ROS can suppress tumor growth through the sustained activation of the cell cycle inhibitors. *Sixth*, most of the currently available chemotherapeutic and radiotherapeutic agents kill cancer cells by increasing ROS stress. Thus, both ROS-elevating and ROS-eliminating strategies have been developed for cancer therapy.

This special issue is an effort to assess the existing concepts, recent findings, controversies, and challenges concerning the role of ROS in tumor development. In particular, the topics covered in this special issue include understanding the role of ROS in cancer initiation and progression (M. Tafani et al.), cancer cell signaling (M. Tafani et al., H. S. Khalil et al., I. Ryoo et al., L. Zong et al., and J. H. Osaki et al.), drug resistance (A. Barreiro-Alonso et al.), autophagy (L. Zhang et al.), and cancer therapy (N. Mut-Salud et al., A. M. Mileo and S. Miccadei, A. Jarosz et al., and Z.-G. Jiang et al.). The article by A. Lyakhovich and M. E. Leonart discusses various mechanisms developed by cancer stem cells to attenuate ROS levels.

Another article deals with the role of ROS in inducing polycystic ovary syndrome (PCOS), a disease condition in women associated with an increasing risk of cancers (T. Zuo et al.). The role of ROS in numerous cancer types including breast (A. C. S. A. Herrera et al., A. L. G. Júnior et al.), ovarian (A. Barreiro-Alonso et al.), prostate (A. Barreiro-Alonso et al.), hepatocellular carcinoma (B. K. Maurya and S. K. Trigun, Y.-Q. Hou et al.), pancreatic cancer (L. Zhang et al. and L. Zong et al.), bladder cancer (N. V. Savina et al.), colorectal cancer (J. Kabzinski et al.), and cervical cancer (J. H. Osaki et al.) is discussed.

It is our hope that these articles will be useful to the readers.

## Acknowledgments

We thank the contributors for their thought-provoking articles in their area of expertise. We also apologize to those whose contributions could not be solicited due to space limitations.

Sahdeo Prasad  
Subash C. Gupta  
Manoj K. Pandey  
Amit K. Tyagi  
Lokesh Deb

## Research Article

# Raman Spectroscopic Measurements of Dermal Carotenoids in Breast Cancer Operated Patients Provide Evidence for the Positive Impact of a Dietary Regimen Rich in Fruit and Vegetables on Body Oxidative Stress and BC Prognostic Anthropometric Parameters: A Five-Year Study

A. Perrone,<sup>1</sup> A. M. Pintaudi,<sup>1</sup> A. Traina,<sup>2</sup> G. Carruba,<sup>2</sup> A. Attanzio,<sup>1</sup> C. Gentile,<sup>1</sup> L. Tesoriere,<sup>1</sup> and M. A. Livrea<sup>1</sup>

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Received 28 July 2015; Revised 8 October 2015; Accepted 13 October 2015

Academic Editor: Amit Tyagi

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Dermal carotenoids are a feasible marker of the body antioxidative network and may reveal a moderate to severe imbalance of the redox status, thereby providing indication of individual oxidative stress. In this work noninvasive Resonance Raman Spectroscopy (RRS) measurements of skin carotenoids (skin carotenoid score (SCS)) were used to provide indications of individual oxidative stress, each year for five years, in 71 breast cancer (BC) patients at high risk of recurrence. Patients' SCS has been correlated with parameters relevant to BC risk, waist circumference (WC), and body mass index (BMI), in the aim of monitoring the effect of a dietary regimen intended to positively affect BC risk factors. The RRS methodological approach in BC patients appeared from positive correlation between patients' SCS and blood level of lycopene. The level of skin carotenoids was inversely correlated with the patients' WC and BMI. At the end of the 5 y observation BC patients exhibited a significant reduction of WC and BMI and increase of SCS, when strictly adhering to the dietary regimen. In conclusion, noninvasive measurements of skin carotenoids can (i) reveal an oxidative stress condition correlated with parameters of BC risk and (ii) monitor dietary-related variations in BC patients.

## 1. Introduction

A proper redox balance is essential to control signalling pathways governing cell functions, including those known to influence cell proliferation [1–4]. Indeed critical variations of the redox homeostasis, not timely compensated by endogenous systems, result in oxidative stress and concur to dysfunction of several regulatory mechanisms leading to cancer onset and development. Dietary plant constituents are now acknowledged to play a significant role in this context. The intense interaction of redox-active phytochemicals with cells at different stages of cancer development is considered essential to maintain the redox balance governing pathways

that control proliferation and evasion of cell-death, thus contrasting both cancer onset and evolution [5, 6]. All this has appeared strongly supported by epidemiological observations providing clear evidence that a dietary pattern such as the traditional Mediterranean way of eating, including large amounts of plant foods and derivatives, that is, fruits, vegetables, olive oil, red wine, and legumes, may play a primary role in promoting health and preventing onset and progression of chronic and degenerative disorders including various types of cancer [7–13].

Measuring the concentration of antioxidants in blood samples may provide indication on the individual capacity to maintain an optimal redox balance or conversely reveal

an oxidative stress status. Molecular antioxidants in the body work in concert and preserve each other; therefore the serum level of each one, including carotenoids, is predictive of the level of all others [14, 15] and is an expression of the individual antioxidant status. An optical method, based on the Resonance Raman Spectroscopy (RRS), has recently been developed for a noninvasive measurement of carotenoids in human tissues. RRS is a laser spectroscopy based on the Raman effect. When a low intensity laser monochromatic light interacts with some molecules, these diffuse the light emitting a new, higher wavelength, monochromatic light [16] that can be revealed by a scanner converting Raman intensity in counts. Because of their conjugated carbon backbone, carotenoids possess characteristic vibrational/rotational energy levels that make them particularly well suited for RRS, strongly absorbing in the blue wavelength region and emitting in the green one. RRS methodology has successfully been exploited for the quantitative measurement of carotenoids in human macula lutea, oral mucosa, and skin [17–20]. RRS measurements of carotenoids in skin have been validated by comparing with extraction and conventional high performance liquid chromatography measurements of carotenoids in skin samples [21]. Importantly, the level of dermal carotenoids has appeared significantly correlated with the blood carotenoid level [22–24]. In this context, the amount of dermal carotenoids revealed by RRS can be considered a marker of the individual antioxidative network, and its measurement was applied to assess the body redox state and eventually provide evidence of critical conditions in diseases. Indeed, RRS measurements in humans have been inversely correlated with urinary isoprostanes, known biomarkers for oxidative stress [25].

RRS has been applied in our recent investigation aimed at monitoring oxidative stress of beta-thalassemia patients and correlating the amount of skin carotenoids with iron overload [26].

In this work that comes as a complementary study of a clinical trial starting in 2009 and ending in 2014, RRS measurements of skin carotenoids have been carried out in breast cancer (BC) operated patients, to research the oxidative stress associated with this condition and monitor eventual variations from a five-year-long treatment aimed at reducing BC risk and recurrence by combining conventional therapies with dietary intervention [27, 28]. The latter was based on a traditional Mediterranean-style regimen, where a high daily consumption of fruit and vegetables was fundamental and was associated with a moderate physical activity. To substantiate the RRS approach the level of dermal carotenoids has been correlated with markers of BC risk (waist circumference, WC, and body mass index (BMI)) [29], which finally provided evidence of the effectiveness of the RRS measurements to monitor patient compliance and influence of the diet.

## 2. Patients and Methods

*2.1. Subjects and Protocol.* DIANA5 (Diet and Androgens-5) is an Italian multicenter project of alimentary education, aimed at preventing BC recurrence in patients surgically treated for BC in the previous 5 years, who have not

developed distant metastasis or second primary BC and are at high risk based on their hormonal and/or metabolic milieu; that is, they exhibit one or more of high risk traits (metabolic syndrome, oestrogen receptor negative tumor, and high serum testosterone or insulin level) and associated abnormal anthropometric parameters, WC and BMI [28]. Clinicopathological measurements, including blood level of sex hormones, insulin, IGF-1, oestrogen receptors, glucose, and lipid parameters, are determined as reported [30] to serve as inclusion criteria in the project and are monitored at baseline and yearly to detect eventual changes during the time of intervention.

BC patients have been enrolled at the Department of Oncology, ARNAS Ospedali Civico e Benfratelli G. Di Cristina e M. Ascoli, Palermo, Italy, in January 2009, and followed up to the end of 2014. Apart from conventional therapies, patients were treated with dietary intervention. This was based on a traditional Mediterranean diet, prescribed consumption of seasonal fruits and vegetables (F/V, 5 servings a day), unrefined grains, legumes, and olive oil, whereas sugared drinks, alcoholic beverages, processed meat, and animal fats were forbidden (detailed description of diet has been reported in [30]). The intervention was intended to decrease level of sex hormones, insulin and insulin growth factor 1 (IGF-1), and reduce risk factors associated with BC prognosis, BMI and WC, [31, 32], additional breast cancer events, and the risk of metastasis [11]. Moderate physical activity was also recommended. All patients received the WCRF-based guidelines for cancer prevention (see the list below) and were invited to participate in dedicated kitchen courses and physical exercise sessions; in addition, they filled in a questionnaire reporting on their own life- and dietary-style. The patients were followed up for vital status and BC related events, including BC-specific mortality, distant metastasis, local recurrences, and contro-lateral BC, which were obtained by self-reporting every six months throughout the study.

The following list is based on the World Cancer Research Fund (WCRF) and American Institute of Cancer Research (AICR).

### *WCRF/AICR 2007 Recommendation*

- (i) Be as lean as possible within the normal range of body weight.
- (ii) Be physically active as part of everyday life.
- (iii) Limit consumption of energy-dense food and avoid sugary drinks.
- (iv) Eat mostly food of plant origin, with a variety of nonstarchy vegetables and fruit every day and unprocessed cereals and/or pulses within every meal.
- (v) Limit intake of red meat and avoid processed meat.
- (vi) Limit alcoholic drinks.
- (vii) Limit consumption of salt and avoid mouldy cereals or pulses.
- (viii) Aim to meet nutritional needs through diet alone.
- (ix) Children to be breastfed by their mothers for at least six months.

- (x) Cancer survivors follow the recommendation for cancer prevention.

**2.2. The Carotenoid Study.** In 2009, a number of BC patients ( $n = 71$ ) were randomly recruited among the patients enrolled at the Centre of Palermo, aged 36 to 74, nonsmokers, accepted with informed consent, and who were to be examined for body redox status, and were invited for RRS measurement of skin carotenoids (skin carotenoid score (SCS)), as an index of oxidative stress. Each patient contributed a duplicate measurement at the baseline and then each year, with a 12-month interval, until 2014. Anthropometric measurements, weight (kg), and WC (cm) were collected each time, and BMI (body mass in kilograms/square of height in meters,  $\text{kg}/\text{m}^2$ ) was measured as reported [29]. Our observation ended in June 2014 and all patients terminated the study.

Healthy women (HW) were interviewed and examined for skin carotenoids in 2009. The mean SCS from a number of nonsmoking HW ( $n = 120$ ), reporting being used to eating high amounts of F/V (5 servings a day), aged 38 to 70, BMI between 20.5 and 31 (mean  $24.5 \pm 3$ ), and WC between 62 and 100 (mean  $80.6 \pm 6.3$ ), was taken as the reference SCS throughout our study (HW-SCS,  $39,210 \pm 9,400$ ; min HW-SCS 20,000; max HW-SCS 75,000).

**2.3. Measurement of Carotenoids in Skin.** A portable Raman spectroscope, Pharmanex® BioPhotonic Scanner S2 (NuSkin, Provo, Utah, USA), designed to monitor carotenoids in the 0.1 mm stratum corneum of the skin of the hand, has been used for the measurements. A low intensity 471.3–473 nm radiation from light emitting diodes interacts with the skin carotenoids. The scattered light is detected at 507.8–509.8 nm by the scanner that converts the Raman intensity in counts (skin carotenoid score (SCS)). A computer then transforms the scanner signals in a colored scale going from red (poor carotenoid score, <19,000) to dark blue (high carotenoid score, >50,000). SCS can be converted to laboratory measurements using the equation [ $Y = 12703 \times X + 5891.7$ ], where “Y” is the SCS value and “X” is the carotenoid concentration expressed as micrograms/mL of serum.

**2.4. Measurement of Lycopene.** Blood lycopene of patients who submitted themselves to skin carotenoid evaluation was measured. Duplicate measurements from the same sample were carried out. Lycopene was extracted from 500  $\mu\text{L}$  serum samples, diluted 1:2 with 0.15 mM NaCl, with 1 volume of methanol and 3 volumes of hexane: diethyl ether (1:1, vol: vol). The extracts were then dried under nitrogen, resuspended with a mixture of acetonitrile: methanol: tetrahydrofuran (58.5:35:6.5, vol: vol: vol), and analyzed with the same solvent [33] by a HPLC Supelco Supelcosil LC-18 column (0.46  $\times$  25 cm) (Bellefonte, PA), at a flow rate of 2.5  $\text{mL min}^{-1}$ . Under these conditions lycopene eluted at 8.2 minutes. Revelation was at 450 nm.

**2.5. Statistical Analysis.** BMI, WC, and SCS are expressed as means and standard deviation (SD) of the patients' values. Differences in SCS among groups of fruit and vegetable

intakes and BMI were tested by analysis of variance (ANOVA) and Bonferroni post hoc tests, with  $p < 0.05$  being taken as significant. Pearson's correlations were used to determine the relationship between SCS and BMI or WC. All statistical analyses were done using GRAPHPAD PRISM v5 (GraphPad Software, San Diego, California, USA), SYSTAT version 10.0 (SPSS, Chicago, IL, USA), and Microsoft Excel.

### 3. Results and Discussion

Cancer control can be achieved by decreasing the rate of oxidative stress and enhancing antioxidant defense mechanisms. In this context the role of F/V and dietary redox-active phytochemicals in reducing the risk of cancer including BC [11, 27, 30, 34], by regulating antioxidant defense mechanisms and redox signaling, has long been described [35–37]. Dermal carotenoids can be regarded as biomarkers of the body antioxidant status [16, 38], as well as of fruit and vegetable intake in nutritional studies [21]. On this basis, dermal carotenoids have been monitored for five years in 71 BC operated patients at high risk of recurrence, to assess oxidative stress status and influence of dietary treatment based on high daily consumption of fruit and vegetables.

**3.1. Dermal Carotenoids Are Correlated with Plasma Lycopene in BC Patients.** Relatively high concentrations of carotenoids accumulate in human skin. The RRS-based measurement of dermal carotenoids has been validated in healthy adults by pairing blood and skin levels of carotenoids [21]. Feasibility of the RRS approach to measure skin carotenoids as a reflection of their plasma concentration was at first explored in BC patients. Since lycopene is the major carotenoid in the skin [17, 18], the individual correlation between SCS and the plasma level of lycopene was examined. Figure 1(a), reporting data at the baseline in 2009, shows a net positive correlation, thus validating the use of this spectroscopic method to evaluate the body carotenoid level even in BC patients. The positive correlation was also confirmed at the end of the observation in 2014 (Figure 1(b)).

**3.2. Variations of Dermal Carotenoids in BC Patients during the 5 y Dietary Intervention.** To the best of our knowledge, only a few trials investigated skin carotenoid response by Raman Spectroscopy to controlled diets [39] including increase of fruit and vegetables [24, 40], and none was carried out on cancer patients. BC patients underwent dermal carotenoids measurements with a 12-month interval to assess the evolution of the oxidative stress status during the 5 years of observation. SCS (means  $\pm$  SD of the values recorded at the end of each year) are reported in Figure 2(a). The SCS increased significantly during the first and second year of observation; thereafter only a nonsignificant positive trend was observed (Figure 2). However, while the SCS of patients at starting ( $28,580 \pm 10,060$ ,  $n = 71$ ) was approximately 28% lower than the reference value of healthy women (HW-SCS =  $39,210 \pm 9,400$ ,  $n = 120$ ) ( $p < 0.001$ , Student's  $t$ -test), no significant difference was found at the end of observation (mean SCS  $38,590 \pm 9,920$ ,  $n = 71$ ) ( $p = 0.67$ ), indicating improvement of the antioxidant status. On the other hand,

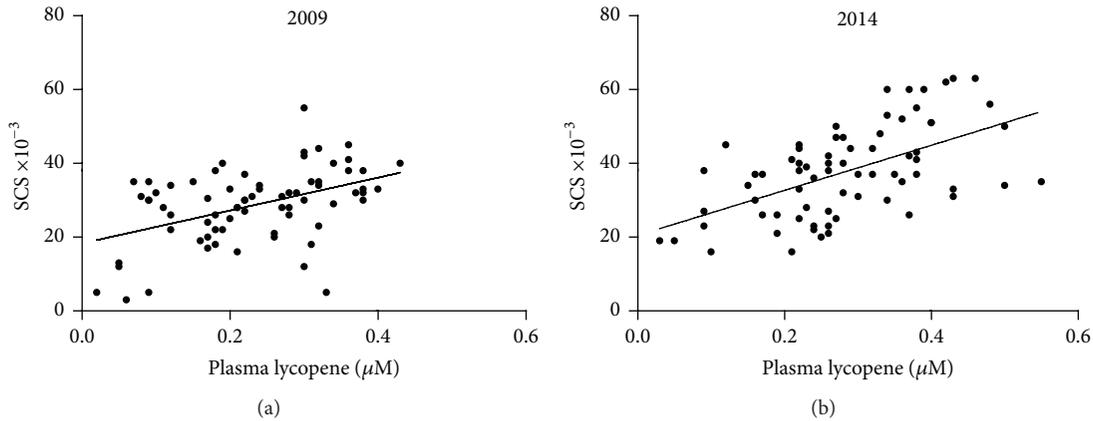


FIGURE 1: Correlation between plasma lycopene and skin carotenoid score (SCS) in BC patients ( $n = 71$ ): (a) before ( $r = 0.450$ ;  $p < 0.0001$ ) and (b) after ( $r = 0.559$ ;  $p < 0.0001$ ) five years of dietary intervention. SCS and lycopene values are the mean of duplicate measurements ( $n = 71$ ;  $p < 0.0001$ ).

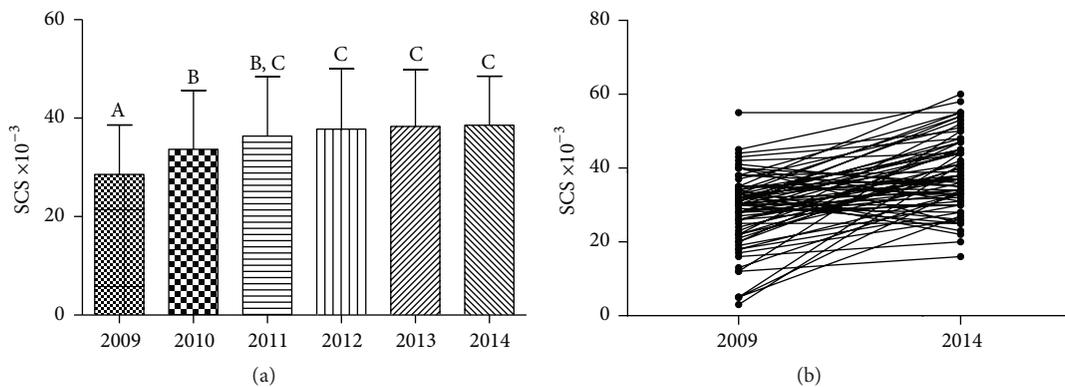


FIGURE 2: Skin carotenoid score (SCS) of BC patients ( $n = 71$ ) during the five years of dietary intervention. (a) Data are the mean  $\pm$  SD of measurements carried out in duplicate on the patients once a year. Values of columns labelled with different letters are statistically different ( $p < 0.05$ , Anova one way followed by Bonferroni's multiple comparison test). (b) Values of single patients at starting (2009) observation and at the end (2014) of observation.

a very high interindividual variability in the SCS progress and evolution was evident when the values of each patient in 2009 ( $n = 71$ ) were judged against the relevant measurements 5 years later (Figure 2(b)): the increase varied from 101% to 1300% ( $n = 56$ ); 3 of the patients did not change their level of carotenoids, and 12 of them exhibited a decrease varying between 3% and 30%. It may be interesting to mention that the major percent increments were observed in patients showing the highest redox imbalance at starting ( $n = 9$ , SCS 3,000 to 15,000), whereas the patients exhibiting a decrease of the carotenoid score were in a medium-high SCS range (30,000 to 40,000). A number of reasons including adiposity [16] and/or concomitant therapies may account for the differences between the individual response to the intervention, including a higher or lower adherence of the patients to the dietary rules.

**3.3. Variations of BMI and WC in BC Patients before and after the 5y Dietary Intervention.** The importance of BMI and WC as prognostic factors in BC is well documented [31, 32];

then measurements of these parameters can help to follow the evolution in the status of BC patients. The Mediterranean dietary pattern is intended to reduce risk factors for breast cancer and then affect positively BMI and WC [41]. This was also observed in the patients under our observation. Figure 3 compares the mean values of BMI and WC in patients in 2009 and at the end of the intervention in 2014. Both BMI and WC were significantly reduced.

**3.4. Correlation between Oxidative Stress and BC Risk Factors.** Since both oxidative stress and anthropometric parameters, either WC or BMI, are associated with the BC pathology and changed positively at the end of the trial, we hypothesized that a correlation existed between these factors. The correlation between SCS and either BMI or BC is shown in Figure 4. Only a negative trend existed at starting (Figures 4(a) and 4(d)), whereas a significant inverse correlation was observed two years later (Figures 4(b) and 4(e)) and at the end of the intervention in 2014, with an even greater significance (Figures 4(c) and 4(f)), indicating that a higher number of patients had

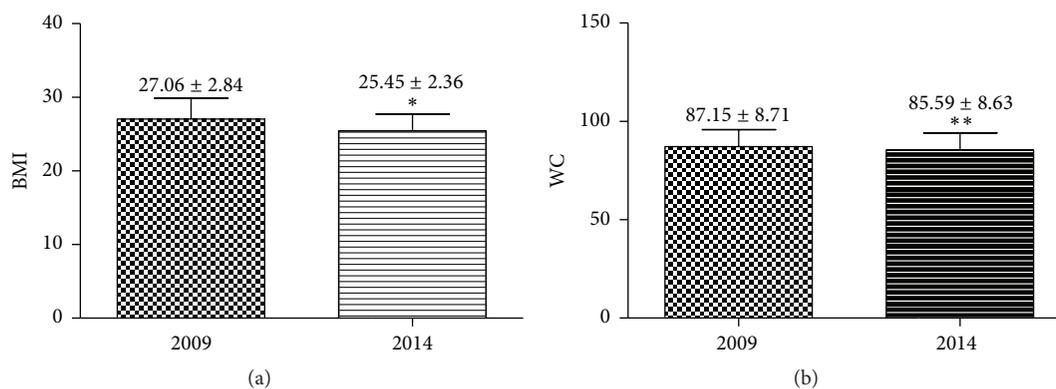


FIGURE 3: Body mass index (BMI) (a) and waist circumference (WC) (b) of BC patients at starting (2009) and at the end (2014) of five years of dietary intervention. Values are the mean  $\pm$  SD of measurements carried out on all patients ( $n = 71$ ). With respect to the relevant values in 2009, values are significant with \*  $p < 0.0001$  or \*\*  $p = 0.0072$  (paired  $t$ -test).

increased SCS, that is, achieved a better control of redox status and reduced oxidative stress, with concomitant reduction of BMI and WC. The poor correlation at starting may be an expression of particular additional factors besides the BC status, concurring to an oxidative stress high or very high (e.g., some of the patients had undergone recent surgery and chemotherapy ( $n = 4$ ) and some had been smoker before starting the trial ( $n = 8$ ), which prevents a clear correlation of SCS with BMI and/or BC). The inverse correlation observed after an equilibrium condition was getting on is further indicative of the great importance of a good redox balance and low WC and BMI in the BC pathology. Indeed, a negative correlation between SCS and BMI and WC was also observed in HW (Figures 4(d) and 4(h)), in accordance with data reported for healthy adults [21, 42].

**3.5. Oxidative Stress and Compliance with Dietary Rules.** Whether and/or to what extent compliance with the dietary intervention was involved in improving the antioxidant balance was evaluated. At the end of the intervention in 2014, all questionnaires filled in by patients, yearly reporting on their own life- and dietary-style, were examined. Patients were then divided into two groups according to whether they reported to have strictly adhered to the dietary rules and constantly consumed five F/V servings per day ( $n = 39$ ) or whether they had not been steady in observing constantly the dietetic pattern ( $n = 32$ ). The SCS values measured for both groups at the end of observation were then compared with values at starting. The data as means  $\pm$  SD are reported in Figure 5. At the end of the study, SCS was remarkably lower for the patients that had less than five servings per day, with respect to patients having five, although both groups of patients did not differ significantly at starting (Figure 5). While showing that the better was the compliance of patients with the dietetic pattern, the higher was the body level of antioxidants; these data suggested that diet had a major role in ameliorating their body redox balance and then positively affected oxidative stress.

**3.6. Follow-Up.** Our observation stopped in June 2014. The patients were followed up for vital status and occurrence of new BC events by clinicians. We were informed that, within December 2014, one patient out of 71 who underwent the SCS measurements passed away and five patients had recurrence. We may also report that 43 of all patients recruited at the centre in Palermo ( $n = 391$ ) had recurrence, whereas 13 passed away. A thorough knowledge and analysis of data gathered from all Italian centres participating in the DIANA5 project are necessary to determine to what extent the dietetic pattern and lifestyle adopted may affect the outcome of BC patients.

## 4. Conclusions

The development of biomarkers for oxidative stress as a diagnostic, prognostic, and therapeutic approach has attracted a lot of interest recently. This was the only study designed to evaluate the antioxidant balance as an index of oxidative stress in BC operated patients at high risk of recurrence, by RRS measurement of dermal carotenoids, and monitor the skin carotenoid response to a 5 y long dietary intervention intended to decrease BC risk factors. The treatment indeed caused a decrease of BMI and WC, while the dermal carotenoid level appeared inversely correlated with both these parameters. To the best of our knowledge, skin carotenoids have never been correlated to risk factors for cancer. The observed correlation between SCS and BMI and WC, two prognostic factors for breast cancer, appears to reflect the importance of all these parameters on this condition and allows SCS to be considered as a reliable additional tool to monitor at-risk individuals and/or effectiveness of interventions. In addition, our findings confirm the effectiveness of the RRS methodology to reveal body redox state even in patients whose antioxidant status is remarkably altered [26].

Breast cancer (BC) is one of the most common in women, with over 400,000 deaths worldwide every year [43]. Surgery, radiotherapy, chemotherapy, and/or hormone therapy are shown to be only partially effective in reducing

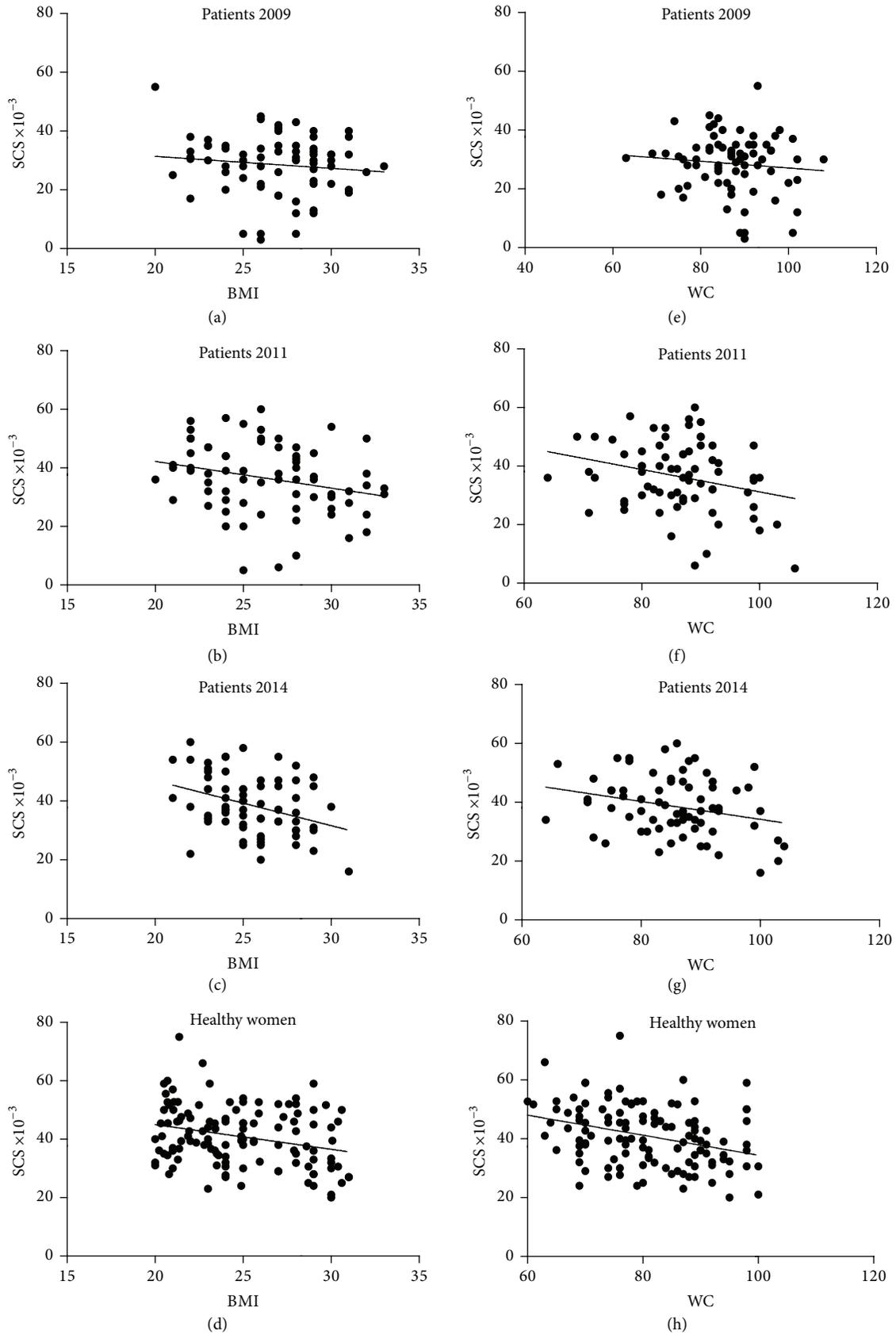


FIGURE 4: Correlation between SCS and either BMI (a–c) or WC (e–g) in BC patients ( $n = 71$ ) at starting and during the course of five years of dietary intervention and in healthy women ( $n = 120$ , (d) and (h)). Each SCS value is the mean of duplicate measurements. Patients: (a) ( $r = -0.118$ ;  $p = 0.326$ ); (b) ( $r = -0.259$ ;  $p = 0.028$ ); (c) ( $r = -0.351$ ;  $p = 0.0027$ ); (e) ( $r = -0.099$ ;  $p = 0.408$ ); (f) ( $r = -0.267$ ;  $p = 0.023$ ); (g) ( $r = -0.265$ ;  $p = 0.025$ ); healthy women: (d) ( $r = -0.276$ ;  $p < 0.0022$ ); (h) ( $r = -0.33$ ;  $p < 0.0002$ ).

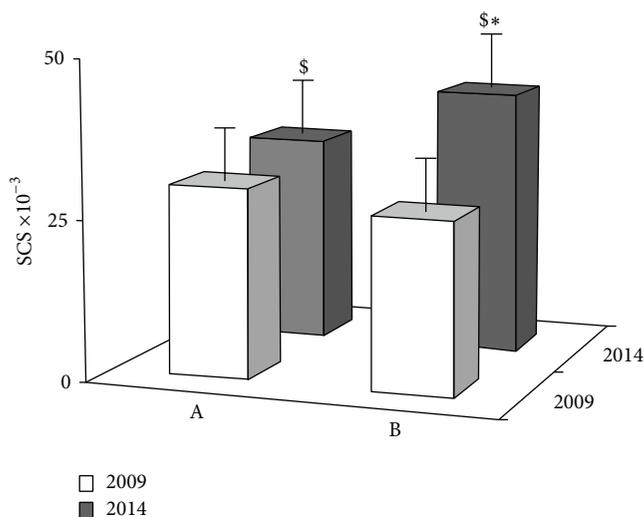


FIGURE 5: Variations of SCS after five years of dietary intervention in BC patients that had consumed less than five (A,  $n = 39$ ) or five (B,  $n = 32$ ) F/V servings per day. The values are the mean  $\pm$  SD of duplicate measurements at starting (white bar) and at the end (grey bar) of observation. (\$) with respect to the relevant value in 2009, significant with  $p < 0.0001$  (paired  $t$ -test). (\*) with respect to A in 2014, significant with  $p < 0.0001$  (unpaired  $t$ -test).

morbidity, which emphasizes the importance of prevention. To this aim lifestyle factors, including diet, are considered fundamental [7–13]. Our work highlights the importance of a firm adhesion to a recommended dietary pattern and suggests a useful tool to help the patients' motivation. The SCS value has appeared an objective tool to evaluate the effectiveness of the dietary intervention in BC patients. Such a simple and noninvasive measurement may be useful to improve patient awareness of the importance of adhesion to healthy lifestyle and alimentary regimen to positively affect BC risk factors. In our experience these measurements allowed each patient to appraise the effectiveness of her own strains, helping her to understand the accuracy of the diet performed or whether corrections had to be made.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## Research Article

# Targeting TRPM2 Channels Impairs Radiation-Induced Cell Cycle Arrest and Fosters Cell Death of T Cell Leukemia Cells in a Bcl-2-Dependent Manner

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Received 22 June 2015; Accepted 15 October 2015

Academic Editor: Lokesh Deb

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Messenger RNA data of lymphohematopoietic cancer lines suggest a correlation between expression of the cation channel TRPM2 and the antiapoptotic protein Bcl-2. The latter is overexpressed in various tumor entities and mediates therapy resistance. Here, we analyzed the crosstalk between Bcl-2 and TRPM2 channels in T cell leukemia cells during oxidative stress as conferred by ionizing radiation (IR). To this end, the effects of TRPM2 inhibition or knock-down on plasma membrane currents, Ca<sup>2+</sup> signaling, mitochondrial superoxide anion formation, and cell cycle progression were compared between irradiated (0–10 Gy) Bcl-2-overexpressing and empty vector-transfected Jurkat cells. As a result, IR stimulated a TRPM2-mediated Ca<sup>2+</sup>-entry, which was higher in Bcl-2-overexpressing than in control cells and which contributed to IR-induced G<sub>2</sub>/M cell cycle arrest. TRPM2 inhibition induced a release from G<sub>2</sub>/M arrest resulting in cell death. Collectively, this data suggests a pivotal function of TRPM2 in the DNA damage response of T cell leukemia cells. Apoptosis-resistant Bcl-2-overexpressing cells even can afford higher TRPM2 activity without risking a hazardous Ca<sup>2+</sup>-overload-induced mitochondrial superoxide anion formation.

## 1. Introduction

*Transient Receptor Potential (TRP) Cation Channels.* The TRP superfamily comprises a diverse range of Ca<sup>2+</sup>-permeable cation channels [1]. TRP channels contribute to changes in cytosolic free Ca<sup>2+</sup> ( $_{free}[Ca^{2+}]_i$ ) by directly acting as Ca<sup>2+</sup> entry channels in the plasma membrane or by changing membrane potentials, modulating the activity and/or driving forces for the Ca<sup>2+</sup> entry channels [2]. The melastatin subfamily (TRPM) has been subdivided into three subgroups on the basis of sequence homology (TRPM1/TRPM3, TRPM4/TRPM5, and TRPM6/7) with TRPM8 and TRPM2 being distinct proteins [3]. The Ca<sup>2+</sup>-permeable TRPM2 channels, formerly known as TRPC2 and LTRPC2, were first identified

in 1998 [4]. Reactive oxygen species (ROS) have been demonstrated to induce TRPM2 currents and increase  $_{free}[Ca^{2+}]_i$  in various cell types transfected with TRPM2 [5], as well as in pancreatic  $\beta$ -cells [6], neutrophil granulocytes [7], and U937 monocytes [8].

*TRPM2 and Cell Death.* By increasing  $_{free}[Ca^{2+}]_i$ , TRPM2 may increase the susceptibility to cell death suggesting that TRPM2 channels function as “death channels.” As a matter of fact, heterologous expression of TRPM2 in human embryonic kidney cells [9] or A172 human glioblastoma cells [10] facilitates oxidative stress-induced cell death. Moreover, expression of TRPM2 has been demonstrated in several tumor entities such as insulinoma [6], hepatocellular carcinoma [6],

prostate cancer [11], lymphoma [12], leukemia [13], and lung cancer cell lines [14] in which TRPM2 reportedly may foster cell death [15].

*Ca<sup>2+</sup>-Signaling by TRPM2, Bcl-2, and Mitochondria.* ROS-induced TRPM2 channel activation most probably occurs indirectly via formation of adenosine diphosphate ribose (ADPR) which activates the channel by binding to a special domain located at the C-terminus of the channel [16]. ADP-ribose polymers are formed during DNA damage response by poly(ADP-ribose) polymerases (PARPs). Upon DNA repair ADPR is released from the ADPR polymers by glycohydrolases [17, 18]. Another main source of ADPR is the mitochondria [19].

Mitochondrial Ca<sup>2+</sup> absorbance exerts Ca<sup>2+</sup> buffering function (for review see [20]). The mitochondrial respiratory chain and the mitochondrial permeability transition pore (PTP) are regulated by Ca<sup>2+</sup>. Moderate mitochondrial Ca<sup>2+</sup> increase may disinhibit the respiratory chain leading to  $\Delta\Psi_m$  hyperpolarisation [21] which in turn is accompanied by increasing superoxide anion formation [22]. Mitochondrial Ca<sup>2+</sup> overload, in contrast, opens the PTP leading to  $\Delta\Psi_m$  dissipation, cytochrome C release, and apoptotic cell death [20].

The antiapoptotic protein Bcl-2 is a key player in cellular Ca<sup>2+</sup> homeostasis. In some cell models, overexpression of Bcl-2 reportedly may increase the Ca<sup>2+</sup> leakage through IP<sub>3</sub> receptor subtypes in the ER membrane and decrease the ER Ca<sup>2+</sup> filling. More recent studies, in contrast, suggest an inhibition of IP<sub>3</sub>-receptor-mediated Ca<sup>2+</sup> release by Bcl-2. Like Bcl-2-caused Ca<sup>2+</sup> store depletion, Bcl-2-mediated IP<sub>3</sub>-receptor inhibition is thought to prevent proapoptotic bulk Ca<sup>2+</sup> release from the ER (for review see [23–26]).

*Direct and Indirect Oxidative Stress Conferred by Ionizing Radiation.* Most energy of ionizing radiation (IR) is absorbed by cell water leading to formation of hydroxyl radicals (for review see [27]). Oxidative stress- and DNA repair-associated release of ADP-ribose is supposed to increase the plasma membrane Ca<sup>2+</sup> permeability by activating TRPM2 channels. Subsequent changes in  $_{\text{free}}[\text{Ca}^{2+}]_i$  and mitochondrial function are under the control of Bcl-2. Together, this hints to a crosstalk between Ca<sup>2+</sup> and ROS signaling involving TRPM2 Ca<sup>2+</sup>-permeable channels in the plasma membrane, the Ca<sup>2+</sup>-regulated  $\Delta\Psi_m$  across the inner mitochondrial membrane, and the antiapoptotic protein Bcl-2 in the ER and outer mitochondrial membrane of irradiated cells.

*Aim of the Study.* The present study aimed to define this crosstalk in human T cell leukemia cells subjected to ionizing radiation. To this end, Jurkat cells stably transfected with Bcl-2 or the empty control vector were irradiated with 0, 5, or 10 Gy by 6 MV photons. Ion channel activity, Ca<sup>2+</sup> signaling, mitochondrial superoxide anion formation, cell cycle control, and cell death were assessed by patch-clamp whole-cell recording, fura-2 Ca<sup>2+</sup> imaging, immunoblotting, and flow cytometry in irradiated and nonirradiated cells, respectively. In addition, mRNA data of hematopoietic and lymphoid tissue cancer cell lines of the Novartis and Broad

Institute Cancer Cell Line Encyclopedia were queried for TRPM2 and Bcl-2 mRNA abundance.

## 2. Material and Methods

*2.1. Cell Culture.* Jurkat E6.1 T cell leukemia cells were from ATCC (Bethesda, Maryland, USA). Jurkat cells stably expressing Bcl-2 (Jurkat-Bcl-2) or a control vector (Jurkat-vector) were prepared as described before [28, 29]. Inducible Bcl-2 transfectants were generated as described [30]. To suppress Bcl-2 expression in Tet-off Jurkat cells, Bcl-2 transfectants were treated with 1  $\mu\text{g}/\text{mL}$  doxycycline (Clontech, Heidelberg, Germany) for 48 h. As control cells, the maternal Jurkat Tet-off cells were used. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Life Technologies, Eggenstein, Germany) and maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

*2.2. Transfection with siRNA.* Transfection with siRNA was performed as described [31]. In brief, cells were cultured at a low density to ensure log phase growth. For transfection,  $2 \times 10^6$  cells were resuspended in 200  $\mu\text{L}$  RPMI 1640 without phenol red. Shortly before transfection, TRPM2 or nontargeting siRNA was added at a concentration of 1  $\mu\text{M}$ . TRPM2 ON-TARGET SMARTpool and the siCONTROL NON-TARGETING pool siRNA were purchased from Dharmacon (Chicago, IL, USA). Cells were electroporated in a 4 mm cuvette in an EPI2500 electroporator (Fischer, Heidelberg, Germany) at 370 V for 10 ms. Immediately after transfection, cells were resuspended in 6 mL prewarmed medium and continued to be cultured as described above. Transfection efficiency as well as viability was determined by transfecting the cells with 400 nM green fluorescence siRNA (siGLO from Dharmacon, Chicago, IL, USA) followed by propidium iodide exclusion dye and flow cytometric analysis.

*2.3. Patch-Clamp Recording.* Maternal, Bcl-2-overexpressing, and control vector-transfected Jurkat cells were irradiated (IR) with 0, 5, or 10 Gy 6 MV photons by the use of linear accelerator (LINAC SL25 Philips) at a dose rate of 4 Gy/min at room temperature. Whole-cell currents were evoked by 9–11 voltage pulses (700 ms each) to voltages between –100 (–80) mV and +100 (+80) mV delivered in 20 mV increments. Mean steady state current values were analyzed 2–49 h after IR. The liquid junction potentials between the pipette and the bath solutions were estimated according to [32], and data were corrected for the estimated liquid junction potentials. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces.

Cells were superfused at 37°C temperature with NaCl ringer solution (in mM: 125 NaCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 KCl, 5 D-glucose, 1 MgCl<sub>2</sub>, and 1 CaCl<sub>2</sub>, titrated with NaOH to pH 7.4). Upon G $\Omega$ -seal formation and entry into the whole-cell recording

mode, cells were recorded with NaCl bath solution (in mM: 140 NaCl, 10 HEPES titrated with NaOH to pH 7.4), KCl bath solution (in mM: 140 KCl, 10 HEPES titrated with KOH to pH 7.4), CaCl<sub>2</sub> bath solution (in mM: 100 CaCl<sub>2</sub>, 10 HEPES, titrated with Ca(OH)<sub>2</sub> to pH 7.4), or *N*-methyl-*D*-glucamine-Cl (NMDG-Cl) bath solution (in mM: 180 mM *N*-methyl-*D*-glucamine titrated with HCl to pH 7.4). The K-*D*-gluconate/KCl pipette solution contained (in mM) 60 K-*D*-gluconate, 80 KCl, 5 HEPES, 1 MgCl<sub>2</sub>, 1 K<sub>2</sub>-EGTA, and 1 K<sub>2</sub>-ATP, titrated with KOH to pH 7.4. To activate TRPM2 channels, ADP-ribose (1 μM, Sigma, Taufkirchen, Germany) was added to the pipette solution. To inhibit TRPM2 and IK channels *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA, 0 or 20 μM) and TRAM-34 (0 or 10 μM, both from Sigma, both prepared from a 10 mM stock solution in DMSO) were added to the bath solution, respectively.

**2.4. Querying the Cancer Genome Atlas (TCGA) Data Sets.** Via the cBioportal Web resource [33, 34], 178 hematopoietic and lymphoid tissue cancer cell lines of the Novartis and Broad Institute Cancer Cell Line Encyclopedia [35] were queried for TRPM2 and Bcl-2 mRNA abundance.

**2.5. Western Blotting.** Irradiated Jurkat cells (0, 5, or 10 Gy, 2–4 h after IR) were lysed in a buffer (containing in mM 50 HEPES, pH 7.5, 150 NaCl, 1 EDTA, 10 sodium pyrophosphate, 10 NaF, 2 Na<sub>3</sub>VO<sub>4</sub>, 1 phenylmethylsulfonyl fluoride (PMSF) additionally containing 1% triton X-100, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 3 μg/mL pepstatin) and separated by SDS-PAGE under reducing condition. In some experiments, cells were preincubated (0.25 h), irradiated (5 Gy), and postincubated (4 h) in the presence of the TRPM2 channel inhibitor ACA (20 μM). Segregated proteins were electrotransferred onto PVDF membranes (Roth, Karlsruhe, Germany). Blots were blocked in TBS buffer containing 0.05% Tween 20 and 5% nonfat dry milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with the following primary antibodies: rabbit anti-phospho-CaMKII (Thr286) antibody (Cell Signaling #3361, New England Biolabs, Frankfurt, Germany, 1:1000), rabbit anti-CaMKII (pan) antibody (Cell Signaling #3362, 1:1000), rabbit anti-phospho-cdc25b (Ser187) antibody (Epitomics #T1162, Biomol Hamburg, Germany, 1:1000), rabbit anti-cdc25b antibody (Cell Signaling #9525, 1:1000), rabbit-anti TRPM2 (Bethyl Laboratories Inc., #A300-414A-2, Montgomery, TX, USA, 1:300), or mouse anti-Bcl-2 antibody (Santa Cruz Biotechnology, sc-509, Heidelberg, Germany, 1:1000). Equal gel loading was verified by an antibody against β-actin (mouse anti-β-actin antibody, clone AC-74, Sigma #A2228 1:20,000). Antibody binding was detected with a horseradish peroxidase-linked goat anti-rabbit or horse anti-mouse IgG antibody (Cell Signaling #7074 and #7076, resp.; 1:1000–1:2000 dilution in TBS-Tween/5% milk) incubated for 1 h at room temperature and enhanced chemoluminescence (ECL Western blotting analysis system, GE Healthcare/Amersham-Biosciences, Freiburg, Germany) was detected by film autoradiography.

**2.6. Fura-2 Ca<sup>2+</sup> Imaging.** Fluorescence measurements were performed using an inverted phase-contrast microscope (Axiovert 100; Zeiss, Oberkochen, Germany). Fluorescence was evoked by a filter wheel (Visitron Systems, Puchheim, Germany) mediated alternative excitation at 340/26 or 387/11 nm (AHF, Analysentechnik, Tübingen, Germany). Excitation and emission light was deflected by a dichroic mirror (409/LP nm beamsplitter, AHF) into the objective (Fluar x40/1.30 oil; Zeiss) and transmitted to the camera (Visitron Systems), respectively. Emitted fluorescence intensity was recorded at 587/35 nm (AHF). Excitation was controlled and data acquired by Metafluor computer software (Universal Imaging, Downingtown, PA, USA). The 340/380 nm fluorescence ratio was used as a measure of cytosolic free Ca<sup>2+</sup> concentration ( $_{free}[Ca^{2+}]_i$ ). The cells were irradiated (0 or 5 Gy) and loaded with fura-2/AM (2 μM for 30 min at 37°C; Molecular Probes, Goettingen, Germany) in supplemented RPMI medium.  $_{free}[Ca^{2+}]_i$  was determined 1.5–5 h after IR at 37°C during superfusion with NaCl ringer (see above), upon Ca<sup>2+</sup> depletion with Ca<sup>2+</sup>-free NaCl ringer solution (in mM: 125 NaCl, 32 HEPES, 5 KCl, 5 *D*-glucose, 1 MgCl<sub>2</sub>, and 0.5 EGTA, titrated with NaOH to pH 7.4), and during Ca<sup>2+</sup> readdition in NaCl ringer solution additionally containing ACA (0 or 20 μM).

**2.7. Flow Cytometry.** To test for mitochondrial production of superoxide anion, Jurkat cells were irradiated (0 or 10 Gy), further cultured for 6 h, harvested, washed, and incubated for 10 min at 37°C in NaCl ringer solution (see above) containing 5 μM of the superoxide anion-sensitive dye MitoSOX (Invitrogen) and 0 or 20 μM ACA, and superoxide anion-sensitive fluorescence was recorded by flow cytometry in fluorescence channel FL-2 (logarithmic scale, 488 nm excitation and 564–606 nm emission wavelengths). To confirm equal fluorescence dye loading, samples were oxidized (10 mM *tert*-butylhydroperoxide) for 12 min and recorded (data not shown).

To monitor mitochondrial function, Jurkat cells were irradiated (0 or 10 Gy) and further cultured for 6 h. Thereafter, cells were harvested, washed, and incubated for 30 min at 37°C in NaCl ringer solution (see above) containing 25 nM of the inner mitochondrial membrane potential ( $\Delta\Psi_m$ ) specific dye tetramethylrhodamine ethyl ester perchlorate (TMRE, Invitrogen) and  $\Delta\Psi_m$  was analyzed by flow cytometry in fluorescence channel FL-2 (logarithmic scale).

For cell cycle analysis, Jurkat cells were preincubated (0.25 h), irradiated (0, 5 or 10 Gy), and incubated for further 24 h in supplemented RPMI 1640 medium additionally containing either ACA or clotrimazole (Sigma, 0 or 20 μM, each). Cells were permeabilized and stained (0.5 h at room temperature) with unsteril (i.e., not RNase-free) propidium iodide (PI) solution (containing 0.1% Na-citrate, 0.1% triton X-100, 10 μg/mL PI in phosphate-buffered saline, PBS), and the DNA amount was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany, 488 nm excitation wavelength) in fluorescence channel FL-3 (linear scale, >670 nm emission wavelength). In parallel, cells with degraded DNA were defined by the subG<sub>1</sub> population of the

PI histogram recorded in fluorescence channel FL-2 (logarithmic scale).

For determination of  $[\text{Ca}^{2+}]_i$  cells were loaded in NaCl ringer solution (see above) for 0.5 h with fluo-3-AM ( $2 \mu\text{M}$  in NaCl ringer, Calbiochem; Bad Soden, Germany) and recorded in fluorescence channels FL-1 (logarithmic scale, 515–545 nm emission wavelengths). As loading control for fluo-3, cells were incubated with the  $\text{Ca}^{2+}$  ionophore ionomycin ( $1 \mu\text{M}$  for 10 min) prior to analysis by flow cytometry. Data were analyzed with the FCS Express 3 software (De Novo Software, Los Angeles, CA, USA).

**2.8. Statistics.** Data are expressed as means  $\pm$  SE and statistical analysis was made by normal or Welch-corrected two-tailed *t*-test or ANOVA where appropriate using InStat software (GraphPad Software Inc., San Diego, CA, USA).

### 3. Results and Discussion

#### 3.1. Modulation of on Channel Activity by Ionizing Radiation.

To assess the effect of ionizing radiation (IR) of ion channel activity, Jurkat cells were irradiated with 10 Gy and whole-cell currents were recorded at different time periods after IR. As shown in Figures 1(a) and 1(b), IR induced an increase in whole-cell currents 2–6 h after IR. Substitution of  $\text{Na}^+$  in the bathing solution by  $\text{Ca}^{2+}$  or the impermeable  $\text{Na}^+$  substitute NMDG<sup>+</sup> indicated both cation-selectivity and  $\text{Ca}^{2+}$  permeability of the IR-induced currents (Figures 1(c)–1(e)).

Next, the functional expression of TRPM2 channels and its dependence on Bcl-2 was determined in Jurkat cells. Such dependence was suggested by a positive correlation of the TRPM2 and Bcl-2 mRNA abundances in 178 hematopoietic and lymphoid tissue cancer cell lines of the Novartis and Broad Institute Cancer Cell Line Encyclopedia (Figure 2(a)). In the Jurkat cell model, in contrast, TRPM2 protein abundance seemed to be lower in Bcl-2-overexpressing (Jurkat-Bcl-2) cells as in the control vector-transfected (Jurkat-vector) cells as suggested by immunoblotting (Figure 2(b)). IR did not modify total TRPM2 protein content of the cells (Figure 2(b)).

To activate TRPM2 in Jurkat cells, whole-cell currents were recorded with the TRPM2 agonist ADP-ribose in the pipette and compared in unpaired experiments with those recorded under control conditions. Intracellular ADP-ribose stimulated a whole-cell current fraction which was sensitive to the unspecific TRPM2 inhibitor ACA [36] (Figures 2(c) and 2(d)). Importantly, ADP-ribose-stimulated currents exhibited unitary current transitions with a unitary conductance of some 50 pS as reported for heterologously expressed TRPM2 channels [37] (Figure 2(e)). Together, these data indicated functional expression of TRPM2 in Jurkat cells.

#### 3.2. Mitochondrial Superoxide Anion Formation: Effect of Ionizing Radiation, Bcl-2 Overexpression, and TRPM2 Inhibition.

To assess IR-stimulated formation of superoxide anion by mitochondria and to estimate a potential role of TRPM2 channels herein, Jurkat-Bcl-2 and Jurkat-vector cells were irradiated (0 or 10 Gy), postcultured for 6 h, and incubated

for 10 min with the superoxide anion-sensitive fluorescence dye MitoSOX. The dye incubation was performed in the absence or presence of the TRPM2 inhibitor ACA. As shown in Figure 3(a), upper panel, and Figure 3(b), three distinct cell populations with low, intermediate, or high MitoSOX fluorescence were apparent. The latter two showed lower cell sizes as compared to the low-fluorescent population, suggestive of superoxide anion formation-associated cell shrinkage. Staining of the cells in parallel experiments with the inner mitochondrial membrane potential ( $\Delta\Psi_m$ ) specific dye TMRE indicated dissipation of  $\Delta\Psi_m$  in most of the shrunken cells (Figure 3(a), lower panel) suggesting that the vast majority of cells with intermediate and high MitoSOX fluorescence underwent apoptotic cell death.

The low-fluorescent, nonshrunken cell population was larger and exhibited significant lower superoxide anion formation in Jurkat-Bcl-2 cells as compared to this population in Jurkat-vector cells (open bars in Figures 3(c) and 3(d), left). In the low-fluorescent populations, irradiation significantly increased the superoxide formation only in Jurkat-Bcl-2 cells to levels which still remained below those of control or irradiated Jurkat-vector cells (compare open and closed bars in Figure 3(c)). Importantly, ACA slightly but significantly ( $p \leq 0.05$ , ANOVA) decreased superoxide anion formation in unirradiated (from  $20.9 \pm 0.21$  to  $18.2 \pm 0.22$  relative units,  $n = 4$ ) and irradiated (from  $23.8 \pm 0.26$  to  $21.6 \pm 0.79$  rel. units,  $n = 4$ ) low-fluorescent Jurkat-Bcl-2 cells while having no inhibiting effect on superoxide anion formation in low-fluorescent Jurkat-vector cells (data not shown). IR effects on the intermediate- or high-fluorescent populations of both cell clones, in contrast, were not apparent (Figure 3(a)). ACA markedly decreased the superoxide formation of the intermediate- or high-fluorescent populations in all control or irradiated cells (compare Figures 3(a) and 3(b)) resulting in the disappearance of the high-fluorescent cells (Figure 3(e)).

Combined, these data demonstrate lower mitochondrial superoxide anion formation in Jurkat-Bcl-2 cells as compared to Jurkat-vector cells. Only in the former cells, IR induced an increase in superoxide anion formation. In addition, superoxide anion formation was lowered by the TRPM2 inhibitor ACA in cells of both clones independent of IR stress. This might suggest a contribution of TRPM2-mediated  $\text{Ca}^{2+}$  uptake to mitochondrial ROS formation.

#### 3.3. Ionizing Radiation-Stimulated $\text{Ca}^{2+}$ Uptake: Regulation by Bcl-2 and Involvement of TRPM2 Channels.

To determine IR-induced changes in TRPM2 activity, irradiated (0 or 5 Gy) Jurkat-Bcl-2 and Jurkat-vector cells were whole-cell recorded in the absence and presence of ACA (Figures 4(a)–4(c)). The ACA-sensitive current fraction of nonirradiated cells was higher in Jurkat-vector than in Jurkat-Bcl-2 cells (compare 1st with 3rd and 5th with 7th bar in Figure 4(c), resp.) which might reflect the observed differences in TRPM2 protein abundance and which is in accordance with the observed differences in mitochondrial ROS formation. IR (5 Gy) stimulated an increase in the ACA-sensitive currents predominately in Jurkat-Bcl-2 cells (Figure 4(c)) which again might be mirrored by the observed IR sensitivity of mitochondrial ROS formation exclusively in Jurkat-Bcl-2 cells.

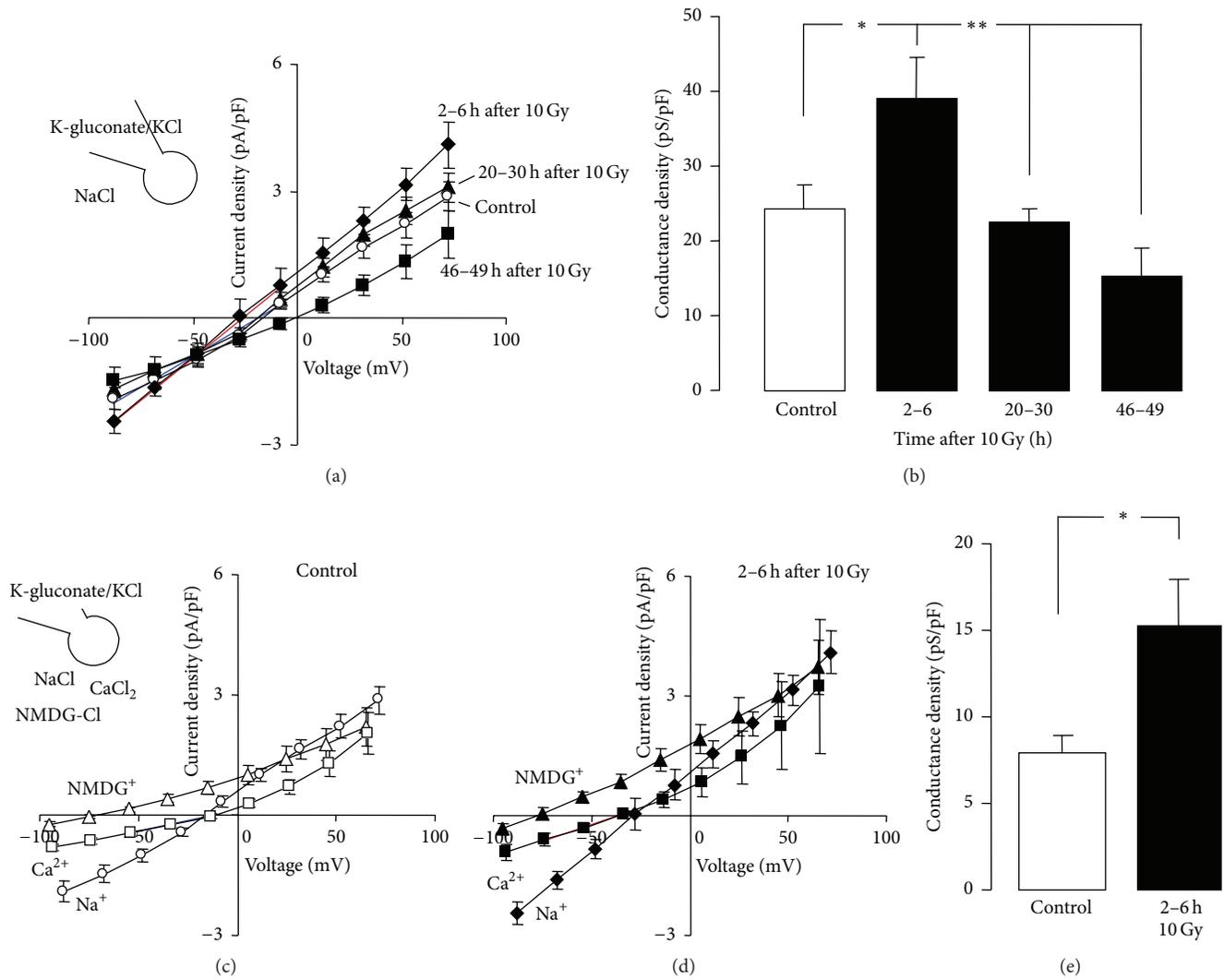
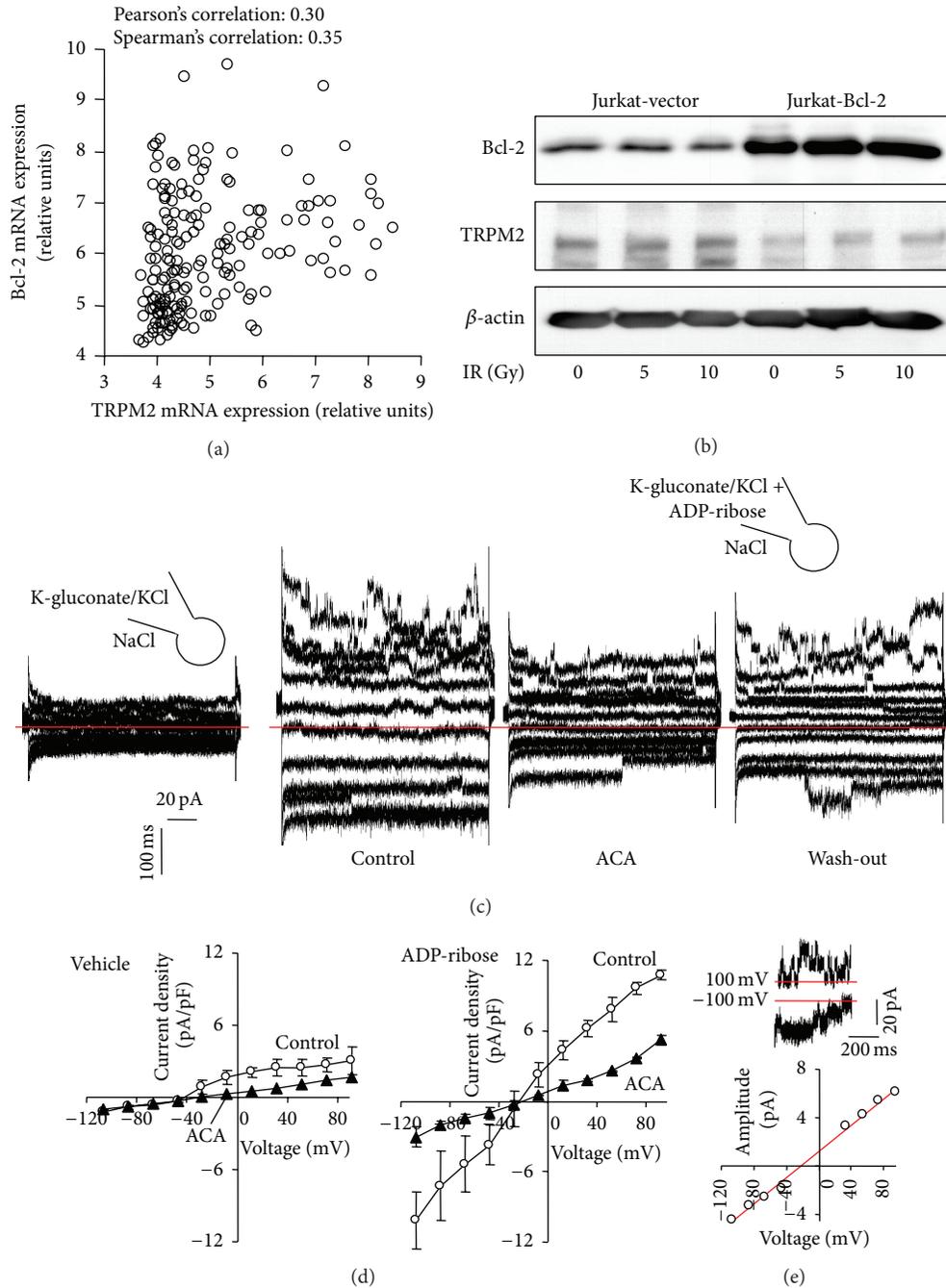


FIGURE 1: Ionizing radiation (IR) increases the cation and the Ca<sup>2+</sup> conductance of the plasma membrane in human Jurkat T cell leukemia cells. (a, b) Current density-voltage relationships (*I/V* curves, a) and conductance densities (b) of Jurkat cells at different time periods (as indicated) after IR with 0 Gy (control, open circles and bar) or 10 Gy (closed symbols and bars). Currents were recorded in whole-cell voltage-clamp mode with K-gluconate/KCl pipette and NaCl bath solution and elicited by 9 voltage square pulses to voltages between -80 mV and +80 mV (20 mV increments). Conductance densities were calculated for the inward currents as shown by the blue and red line in (a) for control cells and irradiated cells (2-6 h after IR), respectively. (c, d) *I/V* curves of control (c) and irradiated Jurkat cells (2-6 h after 10 Gy, d) recorded as in (a) with NaCl bath solutions (circles) or after replacement of Na<sup>+</sup> with Ca<sup>2+</sup> (squares) or the impermeable cation *N*-methyl-D-glucamine (NMDG<sup>+</sup>, triangles). (e) Ca<sup>2+</sup> conductance density of control cells (open bar) and irradiated Jurkat cells (2-6 h after 10 Gy, closed bar). The blue and red line in (c) and (d), respectively, show the voltage range used for calculation of the Ca<sup>2+</sup> conductance densities. Data are means ± SE, *n* = 5 for the 46-49 h values in (a) and *n* = 8-15 for all other data. \* and \*\* indicate *p* ≤ 0.05 and 0.01 as tested by ANOVA (b) and Welch-corrected *t*-test (e), respectively.

In accordance with an IR-induced increase in TRPM2 activity, IR stimulated an ACA-sensitive Ca<sup>2+</sup> uptake as measured by fura-2 Ca<sup>2+</sup> imaging using an extracellular Ca<sup>2+</sup> depletion/repletion protocol (Figure 4(d)). In contrast to the ACA-sensitive basal whole cell currents (Figure 4(b)), basal (ACA-sensitive) Ca<sup>2+</sup> uptake was higher in Jurkat-Bcl-2 than in Jurkat-vector cells (compare 1st with 5th bar in Figure 4(e)). Similarly to the whole-cell currents, IR (5 Gy) stimulated a larger Ca<sup>2+</sup> uptake in Jurkat-Bcl-2 as compared to Jurkat-vector cells (compare 2nd with 6th bar in Figure 4(e)). In the presence of ACA, Ca<sup>2+</sup> uptake did not

differ between control and irradiated Jurkat-vector and Jurkat-Bcl-2 cells (3rd, 4th, 7th, and 8th bar in Figure 4(e)). Together, these observations indicated an IR-stimulated Bcl-2-regulated Ca<sup>2+</sup> uptake in Jurkat cells which probably involves TRPM2 channels.

**3.4. Role of TRPM2 Channels in Ionizing Radiation-Stimulated Activation of Ca<sup>2+</sup> Effector Proteins Involved in Cell Cycle Arrest.** This IR-stimulated Ca<sup>2+</sup> uptake might be hazardous for the cells leading to Ca<sup>2+</sup> overflow and subsequent cell death. In fact, 24 h after IR with 10 Gy, some 25% of the Jurkat cells



**FIGURE 2:** T cell leukemia cells functionally express TRPM2  $\text{Ca}^{2+}$ -permeable cation channels and TRPM2 expression correlates with that of the antiapoptotic protein Bcl-2. (a) Dot blot showing the relative mRNA abundances of TRPM2 and Bcl-2 in 178 hematopoietic and lymphoid tissue cancer cell lines. Data are from the Novartis and Broad Institute Cancer Cell Line Encyclopedia. (b) Immunoblots from whole lysates of irradiated (0, 5, or 10 Gy, 4 h after IR) stably transfected control (Jurkat-vector) and Bcl-2-overexpressing (Jurkat-Bcl-2) cells probed against Bcl-2, TRPM-2, and  $\beta$ -actin. (c) Current tracings recorded as in Figure 1(a) in Jurkat-Bcl-2 cells with vehicle alone (1st tracings) or in an unpaired experiment with the TRPM-2 activator ADP-ribose ( $1 \mu\text{M}$ ) in the pipette (2nd–4th tracings). The recordings with ADP-ribose were performed before (2nd tracings, control), during (3rd tracings, ACA), and after (4th tracings, wash-out) bath application of the TRPM-2 inhibitor *N*-(*p*-amylcinnamoyl)-anthranilic acid (ACA,  $20 \mu\text{M}$ , zero currents are shown by red lines). (d)  $I/V$  curves of the mean whole cell currents ( $\pm$  SE,  $n = 3$ ) of Jurkat-Bcl-2 cells recorded the absence (left) or presence of the TRPM2-activator ADP-ribose (right) in the pipette before (open circles) and after bath superfusion with the TRPM2 inhibitor ACA (closed triangles). (e) Single channel characteristics of the ADP-ribose-stimulated channel. Unitary current transitions were apparent in whole-cell currents tracings as depicted here for  $-100 \text{ mV}$  and  $+100 \text{ mV}$  clamp-voltage in the upper panel. The lower panel shows the relationship between unitary current transitions and voltage indicating a unitary conductance of about 50 pS.

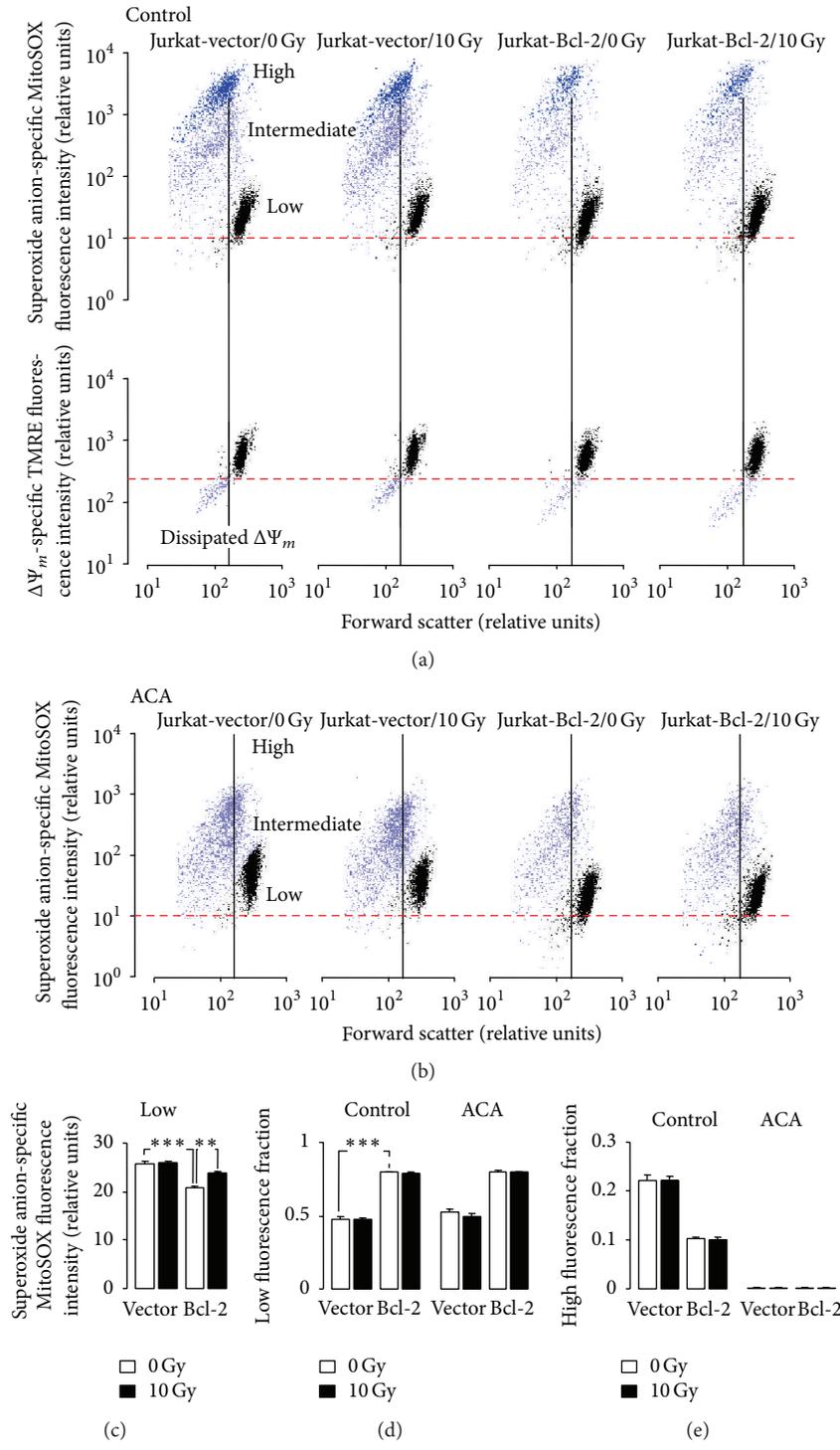
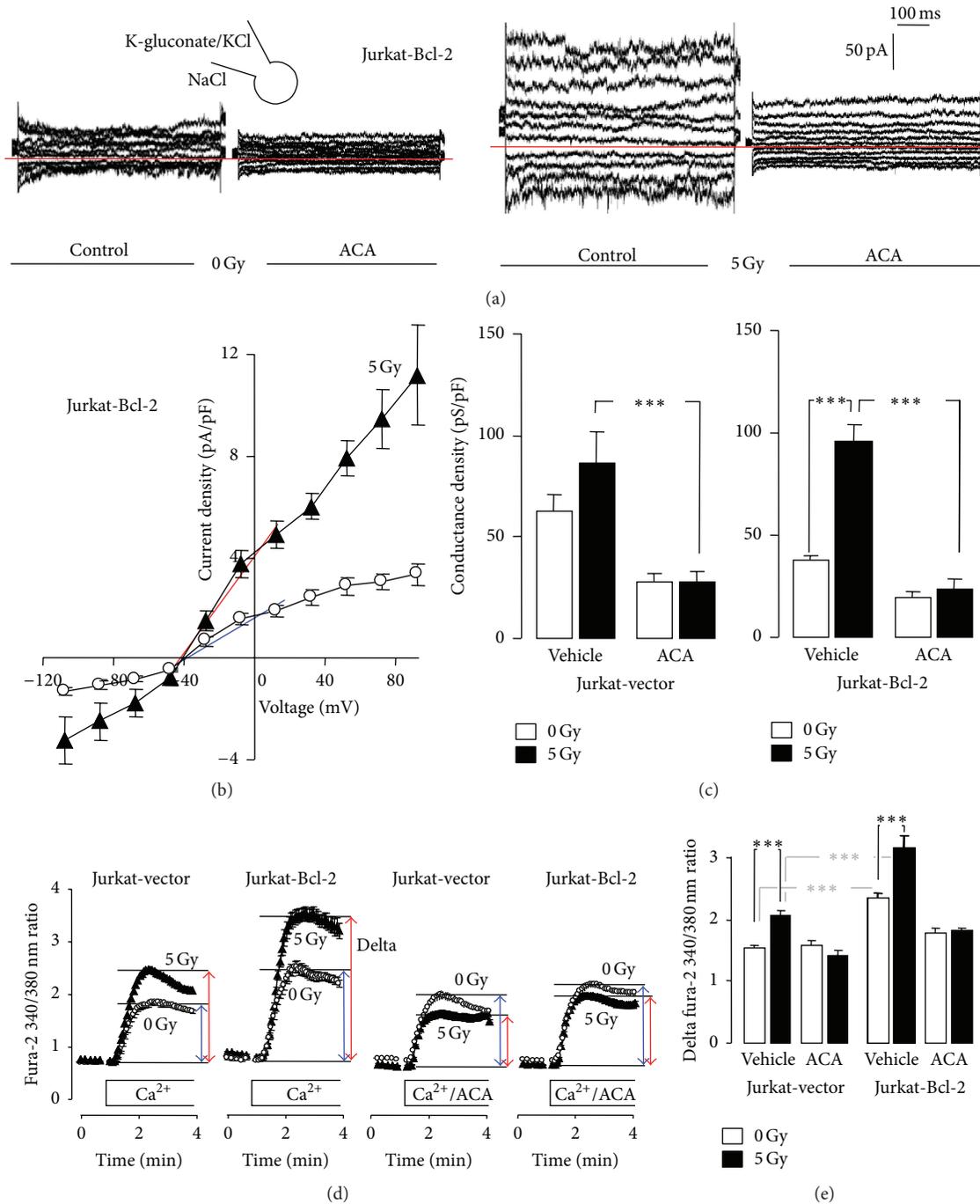


FIGURE 3: IR increases mitochondrial superoxide anion formation in Bcl-2-overexpressing cells. (a, b) Dot plots showing forward scatter and the superoxide anion-selective MitoSOX fluorescence (a, upper panel, and b) as well as the inner mitochondrial membrane potential  $\Delta\Psi_m$ -specific TMRE fluorescence (a, lower panel) of Jurkat-vector- (1st and 2nd panels) and Jurkat-Bcl-2 cells (3rd and 4th panels) 6 h after irradiation with 0 Gy (1st and 3rd panels) or 10 Gy (2nd and 4th panels). Incubation (10 min at 37°C) with the superoxide anion-sensitive fluorescence dye was carried out in the absence (a) or presence (b) of the TRPM2 inhibitor ACA (20  $\mu$ M). Three distinct cell populations with low (black), intermediate (lilac), or high (blue) superoxide anion formation were apparent. The majority of intermediate and high superoxide anion-forming cells exhibited a low forward scatter which was associated with dissipation of  $\Delta\Psi_m$  (a, lower panel). (c) Mean ( $\pm$  SE,  $n = 4$ ) MitoSOX fluorescence intensity in the low-fluorescent populations of 0 Gy- (open bars) or 10 Gy-irradiated (closed bars, 6 h after irradiation) Jurkat-vector- (left) and Jurkat-Bcl-2 cells (right). (d, e) Mean ( $\pm$  SE,  $n = 4$ ) fraction of MitoSOX low-fluorescent (d) and high-fluorescent Jurkat-vector- (1st, 2nd, 5th, and 6th bars) and Jurkat-Bcl-2 cells (3rd, 4th, 7th, and 8th bars) 6 h after irradiation with 0 Gy (open bars) or 10 Gy (closed bars). The incubation with the fluorescence dye was carried out in the absence (1st–4th bars) or presence (5th–8th bars) of ACA (20  $\mu$ M). \*\* and \*\*\* indicate  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, ANOVA.



**FIGURE 4:** IR stimulates Ca<sup>2+</sup> entry through TRPM2 channels especially in Bcl-2-overexpressing Jurkat cells. (a) Whole-cell current tracings recorded in Jurkat-Bcl-2 cells irradiated with 0 Gy (1st and 2nd tracings) or 5 Gy (3rd and 4th tracings, 2 h after IR). Records were obtained in unpaired experiments as described in Figure 1(a) before (1st and 3rd tracings) and during bath application of the TRPM2 inhibitor ACA (20  $\mu$ M, 2nd and 4th tracings). (b) Relationship of the mean ( $\pm$  SE,  $n = 7-10$ ) current density and the voltage recorded as in (a) in Jurkat-Bcl-2 cells irradiated with 0 Gy (open circles) or 5 Gy (closed triangles). (c) Mean ( $\pm$  SE,  $n = 6-12$ ) conductance density of control (0 Gy, open bars) and irradiated (5 Gy, 2-5 h after IR) Jurkat-vector (left) and Jurkat-Bcl-2 cells (right) recorded as in (a) in the absence or presence of ACA. Conductance densities were calculated for the outward currents as shown by the blue and red line in (b) for control and irradiated cells, respectively. (d, e) Mean ( $\pm$  SE,  $n = 197-336$ ) fura-2 340/380 nm ratio (d) and delta fura-2 ratio (e) as measures of cytosolic free Ca<sup>2+</sup> concentration and Ca<sup>2+</sup> entry in Ca<sup>2+</sup>-depleted cells, respectively. Ca<sup>2+</sup>-specific fura-2 fluorescence was recorded by imaging in control (0 Gy, open circles and bars) and irradiated (5 Gy, closed triangles and bars, 1.5-5 h after IR) Jurkat-vector and Jurkat-Bcl-2 cells using extracellular Ca<sup>2+</sup> removal/readdition protocol. Ca<sup>2+</sup> readdition was performed in the absence (vehicle) or presence of ACA (20  $\mu$ M). \* \* \* indicates  $p \leq 0.001$ , ANOVA.

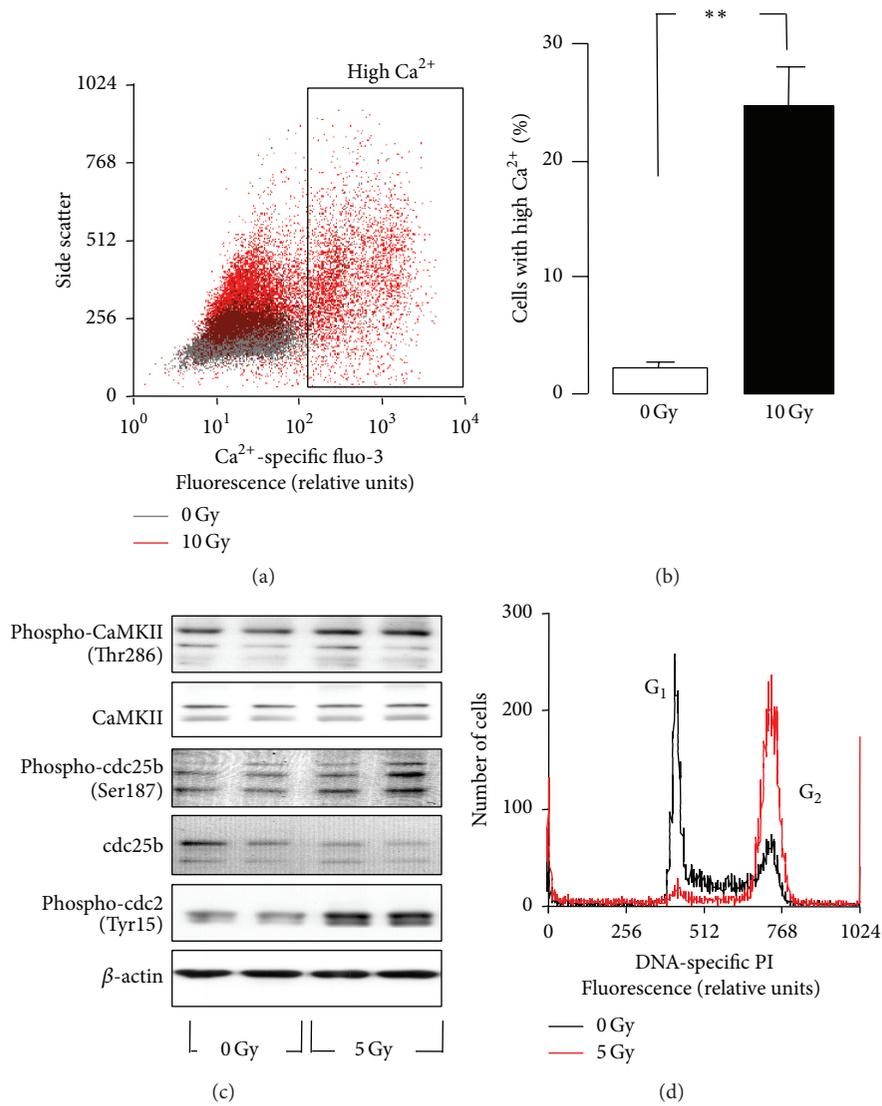


FIGURE 5: IR induces Ca<sup>2+</sup> signaling and a G<sub>2</sub>/M cell cycle arrest in Jurkat cells. (a) Dot plot recorded by flow cytometry showing the Ca<sup>2+</sup>-specific fluo-3 fluorescence intensity in dependence on side scatter of Jurkat cells 24 h after IR with 0 Gy (grey) or 10 Gy (red). (b) Mean (± SE, *n* = 4) percentage of control and irradiated Jurkat cells with high cytosolic free Ca<sup>2+</sup> concentrations (determined by fluo-3 fluorescence in flow cytometry as described in (a)), \*\* indicates *p* ≤ 0.01, Welch-corrected *t*-test). (c) Immunoblots from whole lysates of irradiated (0 or 5 Gy, 4 h after IR) Jurkat cells probed against phosphorylated and total Ca<sup>2+</sup>/CaM-dependent kinase II (CaMKII) isoforms, against the phosphorylated and total phosphatase cdc25b, the phosphorylated cell division cycle protein 2 (cdc2), and β-actin for loading control. (d) Flow cytometry histogram depicting the fluorescence intensity of the DNA-specific dye propidium iodide (PI) in Jurkat cells 24 h after IR with 0 (black) or 5 Gy (red).

exhibited a highly increased  $_{free}[Ca^{2+}]_i$  as deduced from fluo-3 flow cytometry (Figures 5(a) and 5(b)). Ca<sup>2+</sup> uptake might also contribute to Ca<sup>2+</sup> signaling that is required for DNA damage response of the irradiated T cell leukemia cells. IR (5 Gy) stimulated autophosphorylation and activation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) isoforms and phosphorylation-dependent inactivation of the CaMKII downstream target cdc25b as suggested by immunoblotting (Figure 5(c)). Inactivation of the phosphatase cdc25b was parallel by radiation-induced phosphorylation-dependent inactivation of the cdc25b substrate cdc2

(Figure 5(c)). This might hint to an involvement of Ca<sup>2+</sup> effector proteins such as CaMKII in G<sub>2</sub>/M arrest as observed in PI flow cytometry 24 h after IR (5 Gy, Figure 5(d)).

To confirm an involvement of TRPM2 and CaMKII in the stress response of Jurkat cells, Jurkat-Bcl-2 and Jurkat-vector cells were irradiated (0 or 5 Gy) and postincubated in the presence or absence of the TRPM2 inhibitors ACA or clotrimazole [38, 39] and kinase activities of the CaMKII isoforms and cdc2 and cell cycle distribution and cell death were analyzed by immunoblotting and PI flow cytometry, 4 h and 24 h after IR, respectively. ACA decreased the basal and

radiation-induced abundance of phosphorylated CaMKII in Jurkat-Bcl-2 and Jurkat-vector cells (Figure 6(a), 1st and 2nd blot). Most importantly, ACA blocked the radiation-induced phosphorylation-dependent inactivation of cdc2 in both genotypes (Figure 6(a), 3rd blot) suggesting a functional significance of ACA-sensitive  $\text{Ca}^{2+}$  entry for  $G_2/M$  cell cycle arrest. Accordingly, ACA and clotrimazole decreased the number of irradiated cells arrested in  $G_2/M$  (Figures 6(b)–6(e)) and increased the number of dead cells. ACA- and clotrimazole-induced cell death was more pronounced in irradiated Jurkat-vector than in Jurkat-Bcl-2 cells (sub $G_1$  population, Figures 6(b)–6(e)).

Finally, the function of TRPM2 in radiation-induced  $G_2/M$  arrest of Jurkat cells was directly tested by TRPM2 knock-down. Transfection of Jurkat-vector cells with TRPM2 siRNA resulted in downregulation of TRPM2 protein level as compared to nontargeting (nt) RNA-transfected cells (Figure 6(f), insert). Transfected Jurkat cells were irradiated (0 or 5 Gy) and  $G_2/M$  arrest and cell death analyzed 24 h thereafter. TRPM2 knock-down exerted small but significant effects on radiation-induced  $G_2/M$  arrest and cell death mimicking those of ACA and clotrimazole (Figure 6(f)).

**3.5. Regulation of TRPM2-Mediated  $\text{Ca}^{2+}$  Influx by Mitochondria and Bcl-2.** ADP-ribose is liberated in the mitochondria from, for example, NAD-dependent deacetylation intermediates, from mono- or polyADP-ribosylated proteins, or from  $\text{NAD}^+$ , and released into the cytosol [19]. Oxidative and nitrosative stress have been demonstrated to stimulate the mitochondrial release of ADP-ribose into the cytosol which in turn activates TRPM2 channels in the plasma membrane resulting in  $\text{Ca}^{2+}$  entry and depolarization of the membrane potential [16]. Since an elevated  $_{\text{free}}[\text{Ca}^{2+}]_i$  may disinhibit the respiration change leading to  $\Delta\Psi_m$  hyperpolarisation and superoxide anion formation and, eventually, to mitochondrial  $\text{Ca}^{2+}$  overload and  $\Delta\Psi_m$  dissipation, TRPM2 activation has been proposed to amplify signals that trigger cell death (for review see [27]).

The present study demonstrates that irradiated human T cell leukemia cells may utilize the TRPM2 “death channel” for pro-survival  $\text{Ca}^{2+}$  signaling. Noteworthy, IR-induced TRPM2 currents and  $\text{Ca}^{2+}$  entry were larger in cells overexpressing Bcl-2 pointing to a crosstalk between Bcl-2 in the ER and outer mitochondrial membrane and TRPM2 in the plasma membrane. The correlation between TRPM2 and Bcl-2 mRNA abundances in a panel of lymphohematopoietic cancer cell lines (see Figure 2(a)) further suggests a functional interdependence between both proteins.

In some cell models, Bcl-2-overexpressing cells have been proposed to counteract the Bcl-2-mediated  $\text{Ca}^{2+}$  leakage from the stores by downregulating  $\text{Ca}^{2+}$  uptake through the plasma membrane (for review see [19]). In line with such compensatory mechanism might be the observation of the present study that Bcl-2-overexpressing Jurkat cells exhibited under basal conditions lower TRPM2 protein abundance, smaller ACA-sensitive currents in patch-clamp whole-cell recordings than the control vector-transfected cells (see Figure 2(b)).

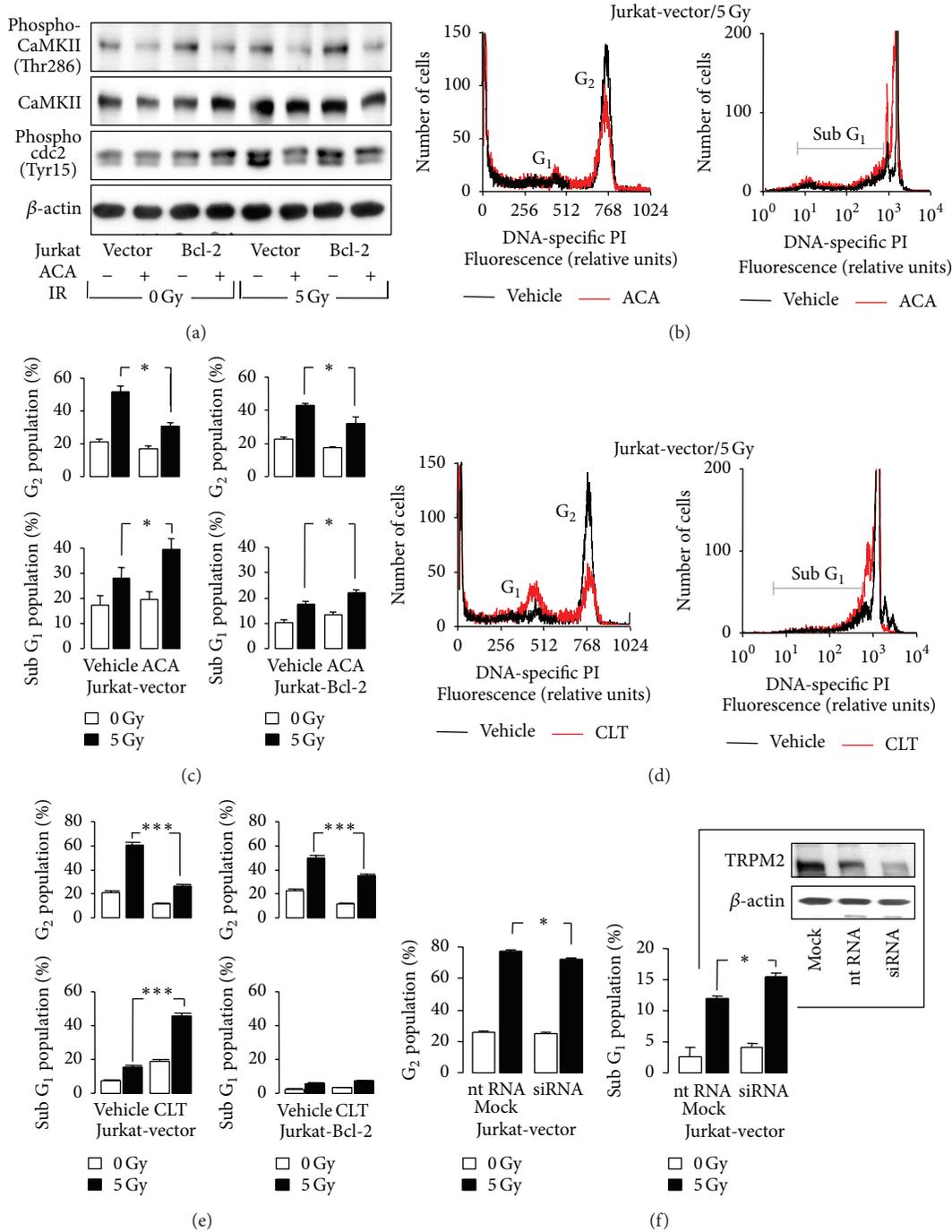
In intact cells (i.e., in fura-2  $\text{Ca}^{2+}$  imaging experiments, see Figure 4(e)), however, a basal ACA-sensitive  $\text{Ca}^{2+}$  uptake fraction was only apparent in Bcl-2-overexpressing cells suggestive of a TRPM2 inactivity in control cells under resting conditions. Compared to control cells, the more sustained  $\text{Ca}^{2+}$  uptake in Bcl-2-overexpressing cells (see Figures 4(d) and 4(e)) suggests that Bcl-2 overexpression might be associated with a set-point shift of the resting  $_{\text{free}}[\text{Ca}^{2+}]_i$  towards higher levels. Fura-2  $\text{Ca}^{2+}$  imaging and fluo-3 flow cytometry recordings of the present study indeed demonstrated a higher basal  $_{\text{free}}[\text{Ca}^{2+}]_i$  in constitutively and inducibly Bcl-2-overexpressing Jurkat cells as compared to the respective control cells (see Supplementary Figure A, in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/8026702>). Elevated  $_{\text{free}}[\text{Ca}^{2+}]_i$  levels reportedly facilitate TRPM2 activation by ADP-ribose [40]. The observed basal ACA-sensitive  $\text{Ca}^{2+}$  uptake that occurred exclusively in Bcl-2-overexpressing cells might, therefore, be simply explained by a higher basal  $_{\text{free}}[\text{Ca}^{2+}]_i$  in Bcl-2-overexpressing as compared to control cells.

Noteworthy, despite higher basal  $_{\text{free}}[\text{Ca}^{2+}]_i$ , Bcl-2-overexpressing cells exhibited lower basal mitochondrial ROS formation than control cells (see Figure 3(c)) suggestive of a Bcl-2-mediated protection of mitochondrial superoxide anion formation. As a matter of fact, a direct promoting function of mitochondrial superoxide anion formation has been attributed to the Bcl-2 opponent Bax in neuronal cells [41].

**3.6. Rearrangements of the  $\text{Ca}^{2+}$  Signalosome in Tumor Cells: Functional Significance for Cell Cycle Control and Stress Response.** In many tumor entities rearrangements of the  $\text{Ca}^{2+}$  signalosome have been reported. In prostate cancer, for instance, malignant progression is reportedly accompanied by TRPM8-mediated  $\text{Ca}^{2+}$  store depletion and downregulation of store-dependent  $\text{Ca}^{2+}$  entry across the plasma membrane. In exchange, TRP channels such as TRPV6 are upregulated in the plasma membrane of advanced prostate cancer cells which have been proposed to generate in concert with IK  $\text{K}^+$  channels survival and growth factor-independent  $\text{Ca}^{2+}$  signaling (for review see [42]).

In the present study, IR stimulated the ACA-sensitive currents of Jurkat cells in patch-clamp recordings and the ACA-sensitive  $\text{Ca}^{2+}$  uptake in fura-2 imaging experiments suggesting an IR-induced increase in TRPM2 activity. IR-induced modifications of ion channel activity have been reported in different tumor entities where they contribute to stress evasion [43], glucose fueling [44, 45], cell cycle control [46, 47], or radioresistance [48].

The p53-mutated Jurkat cells [49] accumulate in  $G_2/M$  cell cycle arrest upon IR-mediated DNA damage (see Figure 5(d)). The proposed IR-stimulated  $\text{Ca}^{2+}$  entry through TRPM2 channels most probably contributed to the  $G_2/M$  cell cycle arrest. This was evident from the observation of the present study that two nonspecific TRPM2 inhibitors or TRPM2 knock-down decreased the number of cells accumulating in  $G_2$  and increased the number of dead cells (see Figure 6). One might speculate that TRPM2 inhibition or



**FIGURE 6:**  $\text{Ca}^{2+}$ -signaling via ACA-sensitive  $\text{Ca}^{2+}$  entry contributes to IR-induced  $\text{G}_2/\text{M}$  cell cycle arrest and decreases IR-induced cell death of Jurkat cells. (a) Immunoblots from whole lysates of irradiated (0 or 5 Gy, 4 h after IR) Jurkat-vector and Jurkat-Bcl-2 cells probed against phospho-CaMKII and total CaMKII isoforms, against phospho-cdc2, and against  $\beta$ -actin. Cells were irradiated and postincubated in the presence of ACA (0 or 20  $\mu\text{M}$ ). (b, d) Histograms showing the DNA-specific PI fluorescence intensity of irradiated (5 Gy, 24 h after IR) Jurkat-vector cells pre- (0.25 h) and postincubated (24 h) with 0  $\mu\text{M}$  (black) or 20  $\mu\text{M}$  ACA (red) in (b) or 0  $\mu\text{M}$  (black) or 20  $\mu\text{M}$  clotrimazole (CLT, red) in (d). (c, e) Mean ( $\pm$  SE,  $n = 9-12$ ) percentage of irradiated (0 Gy, open bars, or 5 Gy, closed bars) and ACA- in (d) or CLZ- in (e) (both 0 or 20  $\mu\text{M}$ ) cotreated Jurkat-vector and Jurkat-Bcl-2 cells arrested in  $\text{G}_2$  phase of cell cycle (upper line) or belonging to the dead cells accumulating in the sub $\text{G}_1$  population (lower line). (f) Knock-down of TRPM2 by RNA interference mimics the effect of ACA. Electroporation with TRPM2-specific siRNA decreases the TRPM2 protein abundance in Jurkat-vector cells to about a half of that of nontargeting RNA- (nt RNA-) transfected control cells as analyzed by TRPM2 and  $\beta$ -actin immunoblots, 48 h after electroporation (insert, mock: electroporation without RNA). Lower line: mean ( $\pm$  SE,  $n = 3-6$ ) percentage of irradiated (0 Gy, open bars, or 5 Gy, closed bars, 24 h after IR) in  $\text{G}_2/\text{M}$  arrest (left) or in sub $\text{G}_1$  population as analyzed by PI staining and flow cytometry as shown in (b, d). Cells were either mock-electroporated or transfected with nt RNA or TRPM2-specific siRNA. Mock and nt RNA data did not differ and were pooled. \* and \*\*\* indicate  $p \leq 0.05$  and  $p \leq 0.001$ , ANOVA, respectively.

knock-down overrides G<sub>2</sub>M cell cycle arrest and forces cells with unrepaired DNA damage into mitosis.

This scenario is strengthened by the observation that IR promoted the  $_{free}[Ca^{2+}]_i$ -dependent phosphorylation of CaMKIIs and their downstream targets *cdc25b* and *cdc2* in an ACA-sensitive manner (see Figures 5(c) and 6(a)). CaMKIIs phosphorylate and thereby inactivate the phosphatase *cdc25b* which results in accumulation of the phosphorylated, inactive form of the mitosis promoting factor subunit *cdc2* [47]. Combined, these observations suggest that IR-dependent TRPM2 activation contributes to Ca<sup>2+</sup> signals that are able to induce autophosphorylation and thereby activation of CaMKIIs.

Likewise, irradiated human myeloid leukemia cells have been shown to generate Ca<sup>2+</sup> signals by the concerted action of TRPV5/6 and Kv3.4 K<sup>+</sup> channels in the plasma membrane. These Ca<sup>2+</sup> signals program G<sub>2</sub>M cell cycle arrest similarly to proposed mechanism of the present study via CaMKIIs, *cdc25b*, and *cdc2* [46, 47]. K<sup>+</sup> channel activity in close vicinity to Ca<sup>2+</sup> entry pathways maintains a high inwardly directed driving force for Ca<sup>2+</sup> and, thus, is indispensable for robust Ca<sup>2+</sup> signals. In analogy to the leukemia cells [47], IR induced the coactivation of IK K<sup>+</sup> channels in the plasma membrane of Jurkat cells (see supplementary Figure B). This points to both a common signaling in irradiated myeloid and lymphoblastic leukemia cells and the possibility that functionally equivalent Ca<sup>2+</sup> signals can be generated during DNA damage response by different sets of TRP and K<sup>+</sup> channels in the plasma membrane.

**3.7. Conclusions.** Plasma membrane TRPM2 channels have been attributed tumor suppressor function in several tumor entities. The Ca<sup>2+</sup> signalosome of human T cell leukemia cells comprises TRPM2 channels that are activated during DNA damage response. In particular, irradiated Jurkat cells utilize TRPM2 to control the G<sub>2</sub>/M cell cycle arrest probably via activation of the Ca<sup>2+</sup> effector protein CaMKII and subsequent inhibition of *cdc25b* and *cdc2*. The antiapoptotic protein Bcl-2 in the ER or outer mitochondrial membrane even fosters TRPM2 activity presumably by inducing higher  $_{free}[Ca^{2+}]_i$  levels and decreases at the same time mitochondrial ROS formation. By doing so, Bcl-2-overexpressing cells may harness TRPM2-generated Ca<sup>2+</sup> signals without running into the risk of hazardous mitochondrial ROS formation. Thus, Bcl-2 function on mitochondrial integrity and stress-induced TRPM2-mediated Ca<sup>2+</sup> signaling cooperate in resistance to radiation therapy in T cell leukemia cells.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Dominik Klumpp cultured the cells and carried out patch-clamp, Ca<sup>2+</sup> imaging, flow cytometry, TRPM2 knock-down, and immunoblotting. Milan Misovic performed patch-clamp recording and Ca<sup>2+</sup> imaging. Kalina Sztejn did patch-clamp

experiments. Ekaterina Shumilina analyzed the patch-clamp and imaging data, did the statistics, and compiled the figure parts with these methods. Justine Rudner designed and supervised the study, analyzed the flow cytometry and immunoblotting data, did the statistics, and wrote part of the paper draft. Stephan M. Huber co-conceived and co-supervised the study, compiled the remaining figure parts, and wrote the remaining paper draft. All authors read and approved the final paper. Justine Rudner and Stephan M. Huber contributed equally to this work and, thus, share senior authorship.

## Acknowledgments

This work was supported by grants from the German Research Foundation DFG (RU 1641/1-1) donated to Justine Rudner and from the Wilhelm Sander Stiftung (2011.083.1) donated to Stephan M. Huber. Dominik Klumpp was supported by the DFG International Graduate School 1302 (TP T9 SH). The authors thank Heidrun Faltin and Ilka Müller for excellent technical assistance.

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## Research Article

# From Six Gene Polymorphisms of the Antioxidant System, Only GPX Pro198Leu and GSTP1 Ile105Val Modulate the Risk of Acute Myeloid Leukemia

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Received 21 May 2015; Accepted 25 October 2015

Academic Editor: Subash Chandra Gupta

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Oxidative stress might contribute to the occurrence of cancers, including the hematological ones. Various genetic polymorphisms were shown to increase the quantity of reactive oxygen species, a phenomenon that is able to induce mutations and thus promote cancers. The purpose of the study was to evaluate the association between *CAT* C262T, *GPX1* Pro198Leu, *MnSOD* Ala16Val, *GSTM1*, *GSTT1*, and *GSTP1* Ile105Val gene polymorphisms and acute myeloid leukemia risk, in a case-control study comprising 102 patients and 303 controls. No association was observed between AML and variant genotypes of *CAT*, *MnSOD*, *GSTM1*, and *GSTT1* polymorphisms. Our data revealed a statistically significant difference regarding the frequencies of *GPX1* Pro198Leu and *GSTP1* Ile105Val variant genotypes between AML patients and controls ( $p < 0.001$ ). Our results showed no association in the distribution of any of the *CAT* C262T, *GPX1* Pro198Leu, *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms regarding age, gender, FAB subtype, cytogenetic risk groups, *FLT3* and *DNMT3* gene mutations, and overall survival. Our data suggests that the presence of variant allele and genotype of *GPX1* Pro198Leu and *GSTP1* Ile105Val gene polymorphisms may modulate the risk of developing AML.

## 1. Introduction

Acute myeloid leukemia (AML) is a complex disease characterized by the accumulation of blasts in the bone marrow and uncontrolled proliferation, in which excess production of oxygen derived radicals compromises the antioxidant defense system thereby leading to oxidative stress [1]. Excessive

cellular reactive oxygen species (ROS) or deficiencies in antioxidant defenses are generators of oxidative stress [2], and as previously demonstrated, tumor cells show higher susceptibility to oxidative stress, compared to normal cells.

Oxidative stress has been frequently observed in cancer and was also reported in several hematopoietic malignancies including acute lymphoblastic leukemia [3], acute myeloid

leukemia [4], myelodysplastic syndrome (MDS), and chronic myeloid leukemia (CML) [5].

Elevated and persistent ROS levels produce oxidative DNA damage and lead to single-stranded and double-stranded DNA breaks, thus promoting mutagenesis [2].

According to Udensi and Tchounwou, ROS may lead to cancer development by causing gene mutations and/or chromosomal aberrations [6]. Increased production of ROS can lead to acquisition of genomic changes, thereby producing genomic instability. This environment can sustain tumor formation and disease progression [7, 8]. Sallmyr et al. suggested that *FLT3/ITD* mutations (FMS-like tyrosine kinase 3, internal tandem duplications) in acute myeloid leukemia (AML) result in ROS production [7]. This may lead to DNA damage and defective repair mechanisms in myeloid leukemia, besides additional chromosomal aberrations and gene mutations.

The antioxidant defense system includes MnSOD (manganese superoxide dismutase), GPX (glutathione peroxidase), and catalase (CAT) which inactivate ROS [1, 9].

Manganese superoxide dismutase (MnSOD), an antioxidant enzyme, has a critical role in protecting cells against oxidative stress by eliminating superoxide radicals after their conversion to  $H_2O_2$  and oxygen [5, 10, 11].

Several *MnSOD* polymorphisms were found to cause an increased superoxide dismutase activity and increased  $H_2O_2$  (hydrogen peroxide) quantities that may produce high levels of ROS, which if not subsequently neutralized represent contributing factors to the neoplastic transformation of cells [5, 12].

Glutathione peroxidase (GPX1), an antioxidant defense enzyme, detoxifies hydrogen peroxide into water and oxygen [13]. It has been reported that *GPX1* gene polymorphism (*GPX1* Pro198Leu) is associated with decreased enzyme activity, thereby conferring an increased risk of developing cancer in Caucasians [14].

Catalase (CAT), an important enzyme of the antioxidant system, converts  $H_2O_2$  to water and molecular oxygen [15]. The expression of catalase is influenced by gene polymorphisms, which may lead to differences in susceptibility of individuals to oxidative damage caused by ROS [15]. According to an up-to-date meta-analysis performed by Shen et al., *CAT* C262T polymorphism may be a risk factor for cancer, with cancer type-specific effects [16].

Glutathione S-transferase (GST) enzymes are involved in the metabolism and detoxification of a wide variety of oxidative stress products, xenobiotics, and carcinogens [3].

Glutathione S-transferases (GSTs), phase II metabolizing enzymes, are encoded by *GST* genes and have an important role in cellular defense and therefore in protecting tissues against oxidative damage [5, 17].

*GST* gene polymorphisms are associated with deficiencies in enzyme activity and have been implicated in susceptibility to acute leukemia, but study results are still controversial [4, 18, 19].

To the best of our knowledge, even though the role of oxidative stress and *GSTM1*, *GSTT1*, *GSTP1*, *CAT*, and *GPX1* as well as *MnSOD2* in the pathogenesis of cancer have been previously investigated, no studies on the association of all

these six polymorphisms with acute myeloid leukemia have been previously published.

The purpose of the study was to investigate possible associations between glutathione S-transferases (*GSTM1*, *GSTT1*, and *GSTP1*), superoxide dismutase (*MnSOD* Ala16Val), glutathione peroxidase (*GPX1* Pro198Leu), catalase (*CAT* C262T) gene polymorphisms, and AML susceptibility in a Romanian population.

## 2. Material and Method

**2.1. Patients and Controls.** The research was conducted at the Department of Genetics, University of Medicine and Pharmacy of Targu Mures. The study group consisted of 102 unrelated AML patients (46 males and 56 females; mean age  $51.70 \pm 16.761$  SD, standard deviation, years) and 303 (174 males and 129 females; mean age  $46.46 \pm 14.501$  SD years) unrelated healthy controls with no known malignancies.

The patients were diagnosed with AML according to French-American-British (FAB) subtype and also according to WHO standards [20, 21] at the Hematology Clinics from Targu Mures, Romania, between 2010 and 2013. AML patients were stratified by French-American-British (FAB) subtype as follows: 9 M0, minimally differentiated AML (8.8%); 25 M1, AML without maturation (24.6%); 26 M2, AML with maturation (25.5%); 3 M3, acute promyelocytic leukemia (2.9%); 19 M4, acute myelomonocytic leukemia (18.6%); 15 M5, acute monocytic leukemia (14.7%); 1 M6, erythroleukemia (0.9%); and 4 M7, megakaryoblastic leukemia (3.9%).

Based on WHO 2008 standards and available data, AML cases included in the present study were classified as follows: 14 AML with recurrent cytogenetic anomalies (13.72%), 9 AML dysplasia related (8.82%), 0 myeloid neoplasia therapy related (0%), 0 myeloid sarcoma (0%), 0 myeloid proliferations Down syndrome related (0%), and 79 AML not otherwise specified (77.45%).

Regarding the cytogenetic risk group, favorable group comprised 10 AML patients (9.9%) and intermediate risk group comprised 66 cases (64.7%) while unfavorable (or high risk) cytogenetic group comprised 12 patients (11.7%).

For induction treatment, the standardized protocol consisting of a combination of anthracycline and cytarabine-ara (Ara-C) in the well-known 7 + 3 days' protocol was used. The anthracycline used was daunorubicin or idarubicin. Ara-C was administered as a bolus every 12 hours or continuous infusion over 7 days. All the transretinoic acid (ATRA) was administered in patients with a diagnosis of acute promyelocytic leukemia (APL). In AML, intensive consolidation was used, the standard dose for consolidation being  $1.5 \text{ g/m}^2$  every 12 hours. Reduced intensity conditioning was used in some cases in patients above 60 years of age or in those with poor performance (due to the associated diseases) before the start of chemotherapy.

Both patients and healthy controls were from the central region of Romania. AML cases were followed-up till their death or the beginning of 2015. From the investigated AML patients, 57 cases achieved complete remission (CR) from which 31 relapsed, 23 attained partial remission (PR), and 21 were refractory to treatment.

**2.2. Genotyping Procedures.** Fresh whole blood samples were collected at the time of diagnosis in tubes containing ethylene diamine triacetic acid (EDTA). Genomic DNA was extracted using Quick-gDNA MiniPrep kits (ZymoResearch, USA) and Wizard Genomic DNA Purification kits (Promega, Madison, WI, USA) according to the manufacturers' instructions.

*FLT3* (fms-like tyrosine kinase 3) and *DNMT3A* (DNA methyltransferase) mutations were assessed using PCR-based methods in all AML patients, as previously described [22, 23].

*CAT* C262T, *GPXI* Pro198Leu, *MnSOD* Ala16Val, and *GSTPI* Ile105Val polymorphisms were genotyped by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) methods as previously described with minor modifications to the PCR protocol and digestion step for superoxide dismutase gene which consisted of exposing PCR amplicons obtained by Thermo Scientific FastDigest HaeIII (BsuRI) for 20 minutes [15, 24–26].

Genotyping of the *GSTMI* and *GSTTI* polymorphisms was carried out by multiplex polymerase chain reaction as described by Sharma et al. [27].

**2.3. Statistical Analysis.** Nominal variables were described as absolutes and relative frequencies (%) and the association between them was analyzed by Pearson's Chi-square test or Fisher's Exact Test. The size effect for statistically significant associations was expressed as an odds ratio (OR) with 95% confidence interval associated. Each polymorphism of interest was analyzed univariately as a possible predictor for AML using simple binary logistic regression. The independent effect of a polymorphism was tested using multiple binary logistic regression. Multivariable model was defined considering all exogenous variables whose estimated significance level in univariate logistic regression was  $p < 0.25$ , the polymorphism's effects being adjusted for possible confounders (gender variable). The best predictive model was chosen comparing nested models based on Akaike information criterion (AIC), Bayesian information criterion (BIC), and Likelihood Ratio Test (LR). All the regression models were additive models that resulted from no significance of interaction terms.

The performance of the final logistic model was evaluated with respect to goodness of fit (Nagelkerke  $R^2$  coefficient, Brier score), discrimination (predictive and classificatory ability) [28], and calibration aspects [29]. Discrimination was established using  $c$ -index which is equal to the area under receiver operating characteristic curve (AUC), while Somers'  $D$  index, discrimination slope, and calibration were analyzed by graphical representation of the concordance between predicted model probabilities and observed proportions of criterion variable. The unreliability index ( $U$ ), quality index ( $Q$ ), maximal error, and mean squared error were used for measuring model miscalibration. In order to assess the reproducibility of the logistic model we performed internal validation by bootstrap resampling method [30]. Using 1000 bootstrap resamples, we calculated unbiased optimism corrected for all estimates of model performance indices.

The Kaplan-Meier estimates were determined for each polymorphism and survival distributions were compared using DeLong test.

The level of statistical significance for all two-sided tests was set at  $p < 0.05$ .

Statistical analysis was performed with the R software version 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria) using rms libraries.

The study protocol was approved by the Ethics Committees of the University of Medicine and Pharmacy Tirgu Mures and informed consents were signed by the patients.

### 3. Results

*FLT3* mutations were found in 24 AML cases while *DNMT3A* mutations were identified in 12 patients. The frequency of *CAT* 262T allele in AML cases was 25.9% while in controls it was 26.5% ( $p = 9.269$ ). The frequency of *GPXI* 198Leu allele was significantly higher in AML cases (83.3%) compared to healthy controls (57.2%) ( $p < 0.0001$ , OR = 3.707, and 95% CI: 2.48–5.541). Variant allele frequencies of the *MnSOD* Ala16Val polymorphism in case and control groups were 47.3% and 54.5%. The frequency of variant *GSTPI* 105Val allele was 33.8% in AML group and 26.9% in control group. There was a significant difference between distributions of *GSTPI* Ile105Val allele frequencies in AML group and control subjects ( $p < 0.0001$ , OR = 2.357, and 95% CI: 1.649–3.368). In AML patients, the frequencies of *GSTMI* and *GSTTI* null genotypes were 57.8% and 23.5%, respectively. The genotype distributions of *CAT*, *GPXI*, *MnSOD*, *GSTPI*, *GSTMI*, and *GSTTI* gene polymorphisms in AML cases and controls are shown in Table 1.

There were no significant differences between variant genotype and AML risk for *CAT* C262T (Chi-square test,  $\chi^2 = 0.03$ , df = 2, and  $p = 0.985$ ), *MnSOD2* (Chi-square test,  $\chi^2 = 4.022$ , df = 2, and  $p = 0.134$ ), *GSTMI* (Chi-square test,  $\chi^2 = 0.026$ , df = 1, and  $p = 0.873$ ), and *GSTTI* (Chi-square test,  $\chi^2 = 0.339$ , df = 1, and  $p = 0.560$ ) polymorphisms.

Our data revealed a statistically significant difference regarding the frequencies of *GPXI* Pro198Leu genotypes between AML patients and controls (Chi-square test,  $\chi^2 = 62.399$ , df = 2, and  $p < 0.001$ ).

Also, a significant difference in the frequency of *GSTPI* Ile105Val variant genotype was found between AML group and controls (Chi-square test,  $\chi^2 = 27.606$ , df = 2, and  $p < 0.001$ ).

The distribution of investigated polymorphisms' combined variant (heterozygous and homozygous) genotype in AML patients stratified by age, gender, FAB subtype, cytogenetic risk group, and *FLT3* and *DNMT3A* mutations criteria is presented in Table 2.

Presence of the variant genotype of *CAT* C262T, *GPXI* Pro198Leu, *MnSOD* Ala16Val, and *GSTPI* Ile105Val, as well as null *GSTMI* and *GSTTI* genotype in AML group was further analyzed in relation to gender and age. There was no significant difference regarding the variant genotype according to gender and age in AML patients ( $p > 0.05$  for all these comparisons).

Furthermore, genotype frequencies for all six gene polymorphisms were compared with French-American-British (FAB) subtype and cytogenetic risk group.

TABLE 1: Distribution of *CAT*, *GPX*, *MnSOD*, *GSTP1*, *GSTM1*, and *GSTT1* genotypes among AML patients and controls.

Polymorphism	AML patients <i>n</i> (%)	Controls <i>n</i> (%)	<i>p</i> value, OR (95% CI) AML versus controls
<i>CAT C262T</i>			
CC	55 (53.9)	161 (53.1)	Reference
CT	41 (40.2)	123 (40.6)	0.985, 0.98 (0.61–1.56)
TT	6 (5.9)	19 (6.3)	0.985, 0.92 (0.35–2.43)
CT + TT	47 (46.1)	142 (46.9)	0.89, 0.969 (0.618–1.519)
<i>GPX1 Pro198Leu</i>			
Pro/Pro	3 (2.9)	34 (11.2)	Reference
Pro/Leu	28 (27.5)	190 (62.7)	0.588, 1.670 (0.480–5.80)
Leu/Leu	71 (69.6)	79 (26.1)	<0.0001, 10.186 (2.997–34.622)
Pro/Leu + Leu/Leu	99 (97.1)	269 (88.8)	0.012, 4.171 (1.252–13.886)
<i>MnSOD Ala16Val</i>			
Ala/Ala	24 (23.5)	54 (17.8)	Reference
Ala/Val	60 (58.8)	168 (55.4)	0.464, 0.803 (0.457–1.413)
Val/Val	18 (17.6)	81 (26.7)	0.074, 0.50 (0.248–1.009)
Ala/Val + Val/Val	78 (76.5)	249 (82.2)	0.206, 0.705 (0.409–1.214)
<i>GSTP1 Ile105Val</i>			
Ile/Ile	39 (38.2)	205 (67.7)	Reference
Ile/Val	57 (55.9)	88 (29.0)	<0.001, 3.405 (2.111–5.491)
Val/Val	6 (5.9)	10 (3.3)	0.0393, 3.154 (1.083–9.183)
Ile/Val + Val/Val	63 (61.8)	98 (32.3)	<0.0001, 3.379 (2.120–5.386)
<i>GSTM1</i>			
Present	43 (42.2)	125 (41.3)	Reference
Null	59 (57.8)	178 (58.7)	0.873, 0.964 (0.612–1.518)
<i>GSTT1</i>			
Present	78 (76.5)	240 (79.2)	Reference
Null	24 (23.5)	63 (20.8)	0.56, 1.172 (0.686–2.002)

No association was found between FAB subtype and some of the investigated gene polymorphisms, namely, *CAT C262T*, *GPX1 Pro198Leu*, *GSTP1 Ile105Val*, *GSTM1*, and *GSTT1*. A positive association was obtained between *MnSOD Ala16Val* variant heterozygous and homozygous genotype and FAB subtype ( $p = 0.014$ ), precisely acute monocytic leukemia (M5).

In the case of cytogenetic classification according to the risk group, it was not associated with variant genotype of the investigated gene polymorphism ( $p > 0.5$  for all comparisons performed).

Furthermore, we analyzed whether there are any associations between *DNMT3A* and *FLT3* gene mutations and investigated gene polymorphisms in AML patients. We observed no significant differences ( $p > 0.05$ ) between AML patients with *DNMT3A* or *FLT3* gene mutations and variant genotype in the case of *CAT C262T*, *GPX1 Pro198Leu*, *MnSOD Ala16Val*, *GSTP1 Ile105Val*, *GSTM1*, and *GSTT1*.

We also investigated if there are any associations between AML patients' outcome and different parameters such as *DNMT3A*, *FLT3* gene mutations, and variant genotypes of all investigated polymorphisms. Only *FLT3* gene mutations

were associated with patient outcome (Pearson Chi-Square test,  $p = 0.011$ ). In addition, from multinomial logistic regression, the presence of *FLT3* mutation was a predictor only for relapse ( $p = 0.005$ , OR = 4.95, 95% CI: 1.63–15.06, and reference category = CR) not for PR ( $p = 0.451$ , OR = 1.60, 95% CI: 0.47–5.45, and reference category = CR).

In addition, we analyzed the frequency of combined variant genotypes of *CAT C262T*, *GPX1 Pro198Leu*, *MnSOD Ala16Val*, *GSTP1 Ile105Val*, *GSTM1*, and *GSTT1* in AML cases and controls.

Taking into account the observed frequencies of different combined genotypes of the investigated polymorphisms in relation to the number of variant genotypes, we tested the hypothesis of leukemia association with the presence of more than two variant genotypes. Our data revealed an association between the presence of leukemia and the presence of more than two variant gene polymorphisms (Chi-square test,  $\chi^2 = 12.16$ ,  $df = 4$ , and  $p = 0.016$ ).

In case of the presence of 3 variant genotypes in our AML patients, the crude OR was 2.45 (95% CI crude OR: 1.23–4.88), in case of 4 variant genotypes the crude OR was 1.79 (95% CI crude OR: 0.89–3.60), in case of 5 variant genotypes

TABLE 2: AML patient characteristics according to the CAT C262T, GPXI Pro198Leu, MnSOD Ala16Val, GSTP1 Ile105Val, GSTML, and GSTT1 genotypes.

	CAT C262T		GPXI Pro198Leu		MnSOD Ala16Val		GSTP1 Ile105Val		GSTML		GSTT1							
	CC	CT + TT	p value	Pro/Pro	Pro/Leu + Leu/Leu	p value	Ala/Ala	Ala/Val + Val/Val	p value	Ile/Ile	Ile/Val + Val/Val	p value	Present	null	p value			
Gender																		
Female	22	24	Ref	0	46	Ref	13	33	Ref	23	23	Ref	18	28	Ref	36	10	Ref
Male	33	23	0.264	3	53	0.055	11	45	0.308	16	40	0.026	25	31	0.574	42	14	0.699
Age																		
<60 years	37	31	Ref	1	67	Ref	17	51	Ref	24	44	Ref	24	44	Ref	53	15	Ref
>60 years	18	16	0.888	2	32	0.257	7	27	0.805	15	19	0.387	19	15	0.057	25	9	0.805
Age (median, range)																		
<50 years	27	20	Ref	1	46	Ref	13	34	Ref	16	31	Ref	17	30	Ref	35	12	Ref
>50 years	28	27	0.554	2	53	1.00	11	44	0.483	23	32	0.540	26	29	0.316	43	12	0.815
FAB classification																		
M0	2	4	0.698	0	6	0.565	2	4	0.014	2	4	0.333	1	5	0.149	6	0	0.461
M1	15	10		2	23		2	23		10	15		10	15		18	7	
M2	16	10		0	26		10	16		5	21		12	14		19	7	
M3	2	1		0	3		1	2		1	2		1	2		3	0	
M4	11	8		0	19		6	13		11	8		5	14		16	3	
M5	6	9		1	14		0	15		6	9		11	4		10	5	
M6	1	3		0	4		1	3		2	2		1	3		3	1	
M7	2	2		0	4		2	2		2	2		2	2		3	1	
Cytogenetic risk group																		
Favorable	6	6	0.058	0	12	0.549	4	8	0.067	4	8	0.925	6	6	0.630	11	1	0.194
Intermediate	38	26	0.751	2	62	1.00	11	53	0.238	25	39	1.00	27	37	0.753	48	16	0.279
Unfavorable	2	8	0.204	0	10	—	5	5	0.665	4	6	1.00	3	7	0.730	6	4	0.136
ND	9	7	1.00	1	15	1.00	4	12	0.691	6	10	1.00	7	9	1.00	13	3	0.613
FLT3 mutations																		
FLT3-	40	38	Ref	3	75	Ref	17	61	Ref	31	47	Ref	36	42	Ref	57	21	Ref
FLT3+	15	9	0.93	0	24	1.00	7	17	0.457	8	16	0.572	7	17	0.141	21	3	0.145
DNMT3A mutations																		
DNMT3A-	48	42	Ref	2	88	Ref	21	69	Ref	31	59	Ref	39	51	Ref	69	21	Ref
DNMT3A+	7	5	0.744	1	11	0.316	3	9	1.00	8	4	0.054	4	8	0.510	9	3	1.00

ND: not determined; Ref. reference.

TABLE 3: The effect of predictors on outcome variable (results from univariate logistic regression).

Variables	Statistics Z	$p^*$	Crude OR	95% CI for crude OR
Gender	2,152	0,031	1,64	1,05–2,58
CAT262	−0,138	0,89	0,97	0,62–1,52
MnSOD2	−1,260	0,208	0,70	0,41–1,21
GPXI98	2,328	0,02	4,17	1,25–13,88
GSTM1	−0,160	0,873	0,96	0,61–1,52
GSTT1	0,582	0,561	1,17	0,69–2,00
GSTP1	5,118	<0,001	3,38	2,12–5,39

\*Wald's test crude  $p$  values; reference categories: gender = women; CAT262 = normal; SOD2 = normal; GPXI98 = normal; GSTM1 = present; GSTT1 = present; GSTP1 = present.

TABLE 4: The final multivariate logistic model.

Variables	$b^*$	SE	$p^+$	Adjusted OR	95% CI for adjusted OR
Gender	0,41	0,24	0,092	1,5	0,94–2,41
SOD2	−0,38	0,29	0,196	0,69	0,39–1,22
GPXI98	1,09	0,63	0,081	2,98	0,88–10,15
GSTP1	1,12	0,24	<0,0001	3,08	1,92–4,94
Constant	−2,54	0,67	0,0001	0,08	0,02–0,26

\*Estimated unstandardized regression coefficients; SE = standard error;  $^+$ Wald's test adjusted  $p$  value.

the crude OR was 2.72 (95% CI crude OR: 1.20–6.19), and in case of 6 variant genotypes the crude OR was 5.79 (95% CI crude OR: 1.63–20.52).

The level of statistical significance of the estimated regression coefficient associated with each of the independent variables was estimated by univariate logistic regression and is described in Table 3. A statistically significant dependency relation was observed between three variables and leukemia ( $p < 0.05$ ).

Table 4 presents the final logistic model, considered the best predictor of leukemia, consistent with the concerning data. From the set of all considered predictors, only *GSTP1* Ile105Val polymorphism can be considered an independent predictor or an independent risk factor for leukemia ( $p < 0.0001$ ) while *GPXI* Pro198Leu and patient gender had a positive effect on the risk of leukemia but only with a tendency towards statistical significance ( $p = 0.09$ ;  $p = 0.08$ , resp.).

Figure 1 points out the intensity of the relation between model predictors (gender, *GPXI98*, and *GSTP1*) and presence of AML leukemia, the existence of such a relationship being highlighted by a significance level of 0.05 and 0.10.

The model goodness-of-fit indices showed acceptable data fit, the discrimination indices revealed that the model had a good capacity to differentiate between AML subjects and healthy persons, the values close to zero of miscalibration indices showed a well-calibrated nomogram, and the internal validation procedure demonstrated the stability of selection of independent variables (Table 5).

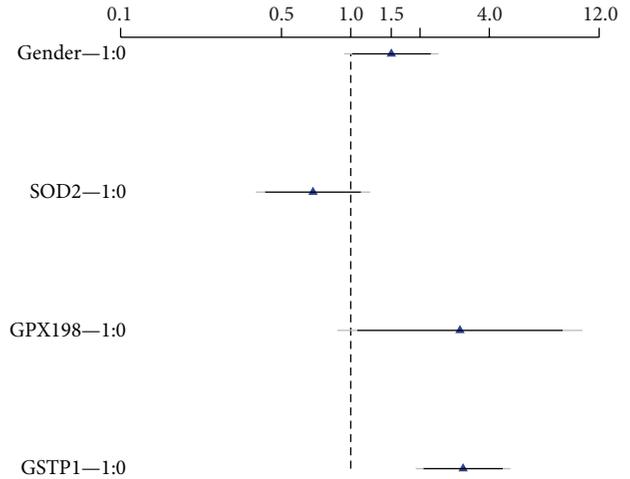


FIGURE 1: Graphical representation of estimated effects (OR) and associated confidence interval for each factor. Note: black = 90% confidence interval of multivariable adjusted OR; grey = 95% confidence interval of multivariable adjusted OR; 0 = reference category; 1 = variant category.

In Figure 2, the calibration graph suggested a predictive model with an acceptable level of concordance between predicted and observed probabilities.

#### 4. Discussion

Certain genetic disorders, environmental carcinogens, physical (ionizing radiation) and chemical exposure, and chemotherapy may lead to acute myeloid leukemia, a heterogeneous disease [18, 31]. Sustained environmental stress may lead to overproduction of ROS and thus significant cell damage and occurrence of somatic mutations. This in turn favors the neoplastic transformation. MnSOD2, GPX, and CAT enzymes are involved in the prevention of DNA damage by ROS [32]. It was reported that *MnSOD2*, *GPX*, and *CAT* gene polymorphisms decrease the enzymatic activity and therefore may increase the risk of cancer by inducing oxidative DNA damage [14, 33].

Also, *GST* gene polymorphisms were associated with a decreased capacity of detoxification for certain mutagens and carcinogens [34].

In the present study, we investigated the oxidative stress enzyme polymorphisms on AML risk in a Romanian population, from the country's central region.

*GST* gene polymorphisms have been extensively studied in AML and *GSTM1* and *GSTT1* null genotypes have been found to increase the risk of AML in both Caucasians and Asians [4, 17, 18, 35, 36].

In the current study, we observed no significant differences between distributions of *GSTM1* and *GSTT1* null genotypes in patients with AML and controls. Similar results were found when we analyzed combined *GSTM1* and *GSTT1* null genotypes. Therefore, we may consider that *GSTT1* and *GSTM1* null genotypes are not associated with the risk of AML in Romanian patients. Our findings are similar to those

TABLE 5: Assessment of final model fit.

Performance measure	Final predictive model	Internal validation*
Global measure of goodness of fit		
Brier	0,17	0,18
$R^2$ (Nagelkerke)	0,13	0,10
Discrimination		
AUC = C stat (95% CI)	0,68 (0,63–0,75)	0,67
Somers' $D$ index	0,36	0,34
Discrimination slope	1,00	0,90
Calibration		
Hosmer-Lemeshow goodness-of-fit test ( $\chi^2$ , $p$ value)	6,29 (0,39)	
Unreliability index $U$	-0,005	0,004
Quality index $Q$	0,09	0,06
Maximal error	<0,001	0,04
Mean squared error	0,00061	0,00057

\* Evaluated by bootstrapping method (number of resampling,  $B = 1000$ ); the optimism corrected indices values.

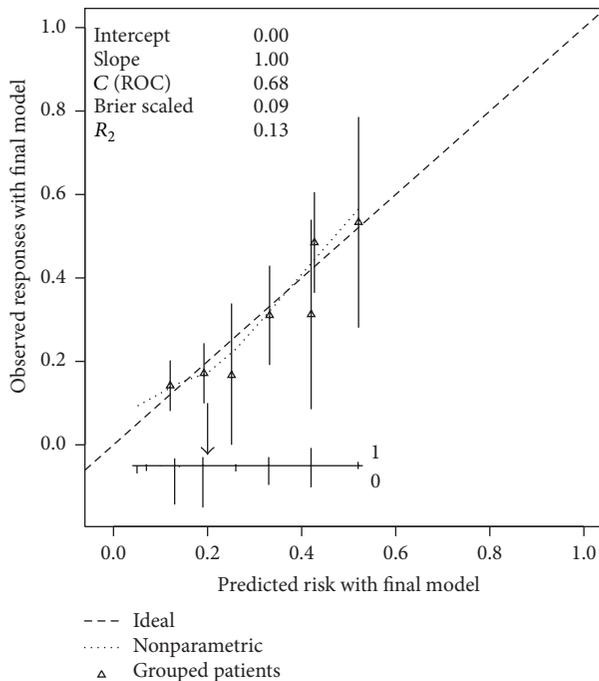


FIGURE 2: Calibration of the nomogram for AML prediction. The horizontal axis contains the predicted probability of AML; the vertical axis described the observed probability of AML. Perfect prediction corresponds to the dashed oblique line ( $45^\circ$ ). Bars correspond to CI of estimated grouped proportions and the arrow corresponds to the cut-off of 20% risk of AML.

reported in a recent study performed on chronic myeloid leukemia (CML) in a Romanian population [5].

In contradiction, a recent meta-analysis performed by He et al. showed that *GSTM1* null genotype was associated with the risk of developing AML in East Asians while *GSTT1* null genotype was a risk factor for AML in Caucasians [37]. The same study revealed that the presence of both *GSTM1* and *GSTT1* null genotypes might increase significantly the risk of AML in both Asians and Caucasians [37].

In a study performed on 147 ALL and 143 AML patients by Dunna et al., the homozygous variant genotype of the *GSTP1* Ile105Val polymorphism was associated with the risk of developing acute leukemia and was associated with poor prognosis [19]. In agreement with the previous study, our research indicated that the presence of variant *GSTP1* Ile105Val genotype significantly increases the risk of AML. In contradiction, in a meta-analysis performed by Tang et al. *GSTP1* polymorphism was not associated with acute leukemia risk in Asians [18]. Similar results to those reported by Tang et al. were observed by He et al. [37].

Taking into account that the relationship between all six gene polymorphisms and overall survival has not been previously investigated, we analyzed the effect of predictors (variant genotype) on survival time by using DeLong's test crude  $p$  values.

Our findings revealed that *GSTP1* Ile105Val variant genotypes and *GSTT1* and *GSTM1* null genotypes did not modify overall survival in AML patients.

Data obtained from our study show that the presence of variant genotypes of *CAT* C262T and *MnSOD* Ala16Val gene polymorphisms is not associated with the risk of AML. Our findings are consistent with a previous study performed on patients with CML from Romania [5]. Similarly, a recent case-study performed on Persian (Caucasians) Muslims living in Shiraz (Iran), a heterogeneous population, and a meta-analysis reported no significant association between *CAT* C262T gene polymorphism and susceptibility to breast cancer [38].

Results from Kaplan-Meier analysis showed no difference in overall survival between patients with variant and wild-type genotype for *CAT* C262T gene polymorphism or for *GPXI* Pro198Leu gene polymorphism.

Similar results were reported in a research performed on 89 AML patients regarding overall survival between carriers of the variant and wild-type genotypes of *CAT* C262T and *GPXI* Pro198Leu polymorphisms [11].

Regarding *MnSOD* Ala16Val gene polymorphism and overall survival, our results showed that variant genotypes

were not associated with significantly shorter survival in AML, compared to the wild-type genotype. Of the studied variables, only *FLT3* mutation has a significant effect on survival time (Cox regression,  $p < 0.001$ ).

In contradiction to our findings, in the study conducted by Koistinen et al. a significant overall survival ( $p = 0.02$ ) was observed for AML patients carrying Val allele of *MnSOD* Ala16Val polymorphism compared to cases with Ala/Ala genotype [11].

Regarding *GPXI* Pro198Leu genotypes, we observed that the presence of variant genotype is associated with a statistically significant risk of AML. Our findings are in contradiction with those previously observed on CML patients from the same region (central part) of Romania [5].

According to Liu et al. in a meta-analysis which included 8,102 patients with breast cancer no statistically significant association was found between *MnSOD* gene polymorphism and risk for breast cancer, excepting the variant allele in premenopausal women [39].

Our study is the first to evaluate the association of all six genes' polymorphisms (*CAT* C262T, *GPXI* Pro198Leu, *MnSOD* Ala16Val, *GSTT1*, *GSTM1*, and *GSTP1* Ile105Val) with AML among de novo patients.

Univariate logistic regression revealed that combined variant (heterozygous + homozygous) genotype of *CAT*, *GPXI*, *MnSOD*, and *GSTP1* gene polymorphisms is associated with an increased risk of developing AML ( $p = 0.003$ , OR = 12.68, and 95% CI: 2.36–68.01).

A positive association was observed between combined variant (heterozygous + homozygous) genotype of *CAT*, *GPXI*, *MnSOD*, and *GSTP1* and null genotype for *GSTM1* and AML risk ( $p = 0.017$ , OR = 7.34, and 95% CI: 1.43–37.67). We have found a statistically significant correlation between variant genotypes for *GPXI* Pro198Leu, *MnSOD* Ala16Val, *GSTP1* Ile105Val, and *GSTM1* null genotype and AML in our study ( $p = 0.004$ , OR = 10.33, and 95% CI: 2.12–50.26). Based on our data, we may suggest that the presence of one of the three possible combined genotypes may represent a risk factor for AML.

We could not find a statistically significant association in the distribution of any of the six gene polymorphisms regarding gender, cytogenetic risk group, and *FLT3* and *DNMT3A* gene mutations.

To our knowledge, the relationship between the investigated gene polymorphisms and *FLT3* and *DNMT3A* gene mutations and AML FAB subtype has not been previously investigated.

In addition, this study is the first on the distribution of *CAT* C262T, *GPXI* Pro198Leu, *MnSOD* Ala16Val, *GSTT1*, *GSTM1*, and *GSTP1* Ile105Val polymorphisms in Romanian AML patients.

Our study has some limitations, such as the relatively small group size, the lack of investigation of *RUNX1* gene mutation (and other mutations), and the lack of antioxidant enzyme activity determination.

In conclusion, our present study reveals that the presence of variant allele and genotype of *GPXI* Pro198Leu and *GSTP1* Ile105Val gene polymorphisms may increase the risk of developing AML. As the number of patients is small,

additional studies performed on larger cohorts are required to establish the relationship between these polymorphisms and AML risk.

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

Claudia Bănescu designed the study, performed genetic analyses, and wrote the paper. Adrian P. Trifa contributed to the design of the study and performed critical revision of the paper. Erzsebet Benedek Lazar and Marcela Căndea collected blood samples and data for the AML patients. Carmen Duicu, Valeriu G. Moldovan, Florin Tripon, and Andrei Crauciuc performed genetic analysis and collected the data for controls. Mihaela Iancu performed statistical analysis. Minodora Dobreanu designed the research and performed critical revision of the paper. All authors read and approved the final version of the paper. Claudia Bănescu, Mihaela Iancu, Adrian P. Trifa, and Erzsebet Benedek Lazar equally contributed to this paper.

## Acknowledgment

This work was supported by Internal Research Grants of the University of Medicine and Pharmacy of Targu Mureș, Romania, Project no. 19/11.12.2013.

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## Review Article

# Oxidative Stress and Carbonyl Lesions in Ulcerative Colitis and Associated Colorectal Cancer

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Received 3 June 2015; Revised 14 October 2015; Accepted 25 October 2015

Academic Editor: Lokesh Deb

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Oxidative stress has long been known as a pathogenic factor of ulcerative colitis (UC) and colitis-associated colorectal cancer (CAC), but the effects of secondary carbonyl lesions receive less emphasis. In inflammatory conditions, reactive oxygen species (ROS), such as superoxide anion free radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $HO^{\cdot}$ ), are produced at high levels and accumulated to cause oxidative stress (OS). In oxidative status, accumulated ROS can cause protein dysfunction and DNA damage, leading to gene mutations and cell death. Accumulated ROS could also act as chemical messengers to activate signaling pathways, such as NF- $\kappa$ B and p38 MAPK, to affect cell proliferation, differentiation, and apoptosis. More importantly, electrophilic carbonyl compounds produced by lipid peroxidation may function as secondary pathogenic factors, causing further protein and membrane lesions. This may in turn exaggerate oxidative stress, forming a vicious cycle. Electrophilic carbonyls could also cause DNA mutations and breaks, driving malignant progression of UC. The secondary lesions caused by carbonyl compounds may be exceptionally important in the case of host carbonyl defensive system deficit, such as aldo-keto reductase 1B10 deficiency. This review article updates the current understanding of oxidative stress and carbonyl lesions in the development and progression of UC and CAC.

## 1. Introduction

Reactive oxygen species (ROS) refer to a class of special oxygen chemical forms or oxygen-containing compounds that have much higher chemical activity than the oxygen. Oxidative stress (OS) occurs if the generation of ROS exceeds the defensive capability of the antioxidant system in the cell [1]. As a largest endocrine and immune organ, the intestinal tract abundant with microorganisms is important in stress response, such as oxidative stress [2]. Superoxide anion free radical ( $O_2^{\cdot-}$ ) and nitric oxide free radical ( $NO^{\cdot}$ ) are two main endogenous reactive oxygen/nitrogen species (ROS/RNS), from which other reactive free radicals, such as hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $HO^{\cdot}$ ), and peroxynitrite anion ( $ONOO^-$ ), are derived [3]. There are

several sources of ROS in the digestive tract [4]. Luminal microbes produce a large amount of ROS; inside cells, superoxide anion, hydrogen peroxide, and hydroxyl radicals are produced as byproducts of mitochondrial respiration in aerobic metabolism and in cytochrome P450 detoxifying reactions; and in the process of chronic inflammation, a large amount of ROS is produced by neutrophil phagocytosis of bacteria, granular materials, or soluble irritants [5, 6].

In normal condition, intestinal ROS have bactericidal effects, participating in the intestinal defensive function. However, oxidative stress derived from excessive ROS production over the buffering capability of antioxidant defense in the host would cause lipid peroxidation, intestinal mucosal barrier damage, bacterial translocation, and inflammatory response [2, 7]. Ulcerative colitis (UC) is a type of chronic

inflammatory bowel disease (IBD) in which oxidative stress plays a critical role in its pathogenesis and malignant progression to colorectal cancer (CRC) [8, 9]. UC affects the distal colon and rectum but often extends to the proximal colon and eventually to the whole colon. Clinically, patients with UC usually experience an intermittent course for a lifetime and colectomy is the only curative option [10, 11]. A worse scenario of UC is the increased risk of developing colorectal cancer, so-called colitis-associated colorectal cancer (CAC) [12]. This review article focuses on the oxidative stress and secondary carbonyl (lipid peroxide) lesions in the pathogenesis of UC and CAC.

## 2. Oxidative Stress and Carbonyl Lesions in Ulcerative Colitis

UC is essentially an immune-inflammatory disease. Inflammation is a process that consists of a series of protective responses, such as immune cell infiltration and cytokine expression, to eliminate pathogens/insults and initiate damage repair of the tissue. Acute inflammation is the immediate response of the body to pathogens and characterized with recruitment of leukocytes, particularly granulocytes. Chronic inflammation is a prolonged inflammatory process and characterized by simultaneous damage and healing of tissues at the inflammatory spot, resulting in a progressive shift of cell types. Therefore, chronic inflammation often leads to progressive diseases in the host [13].

Ulcerative colitis (UC) is a chronic inflammation described with remission and reactivation [10]. In active phase, UC is characterized with diffusive inflammatory cell infiltration and small intestinal mucosal crypt abscesses. In the inflammatory colon, mucosa, submucosa, and lamina propria are often infiltrated with neutrophils, lymphocytes, plasma cells, and eosinophils [14]. The infiltrated neutrophils produce a large amount of ROS, triggering oxidative stress, and proteolytic enzymes. The proteolytic enzymes and ROS act on endothelial cells and cause cell injury and subsequent epithelial barrier permeability and luminal pathogen invasion, which in turn exaggerate inflammatory cell infiltration and inflammatory damage, eventually leading to intestinal mucosal necrosis and ulceration [15]. Meanwhile, epithelial regeneration starts to cover the ulcerative area under stimulation of mitogenic cytokines and prostaglandins produced in inflammatory response. In this circumstance, intestinal mucosal hyperemia, edema, and hyperplasia polyps may appear.

Etiopathology of UC is complicated, including bacterial or viral infection, changes of colon microbiota, excessive immune response, and oxidative stress injury [16, 17]. Host genetic factors also play an etiological role in the development and progression of UC. It has been reported that the chromosomal loci 3, 7, and 12 in humans are associated with individual sensitivity to inflammatory bowel disease, including UC [18]. Recent studies from our laboratory have demonstrated that aldo-keto reductase 1B10 (AKR1B10) is a potential etiopathogenic factor of UC and CAC [19]. Among these etiopathological factors, the abnormal immune

response is considered a key of UC. The normal colon mucosa plays an immune, endocrine, and barrier function. Injuries occurring in the intestinal mucosa insult its barrier function; increased intestinal mucosal permeability allows microbes and antigens to invade and excessively stimulate immune response, triggering intestinal inflammation. Excessive ROS are produced leading to oxidative stress during the inflammatory response, exaggerating inflammatory lesions in the pathogenesis of UC.

*2.1. Redox in the Intestine.* In the intestine, main ROS include hydroxyl free radical ( $\text{OH}^{\bullet}$ ), superoxide anion radical ( $\text{O}_2^{\bullet-}$ ), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) while superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and catalase (CAT) are main antioxidant enzymes [20]. Peroxisomes are important organelles in biological oxidation in cells and participate in production and clearance of free radicals. Peroxisomes are enriched with hydrogen peroxide enzymes, oxidases, and peroxidases [21, 22]. Oxidases catalyze  $\beta$ -oxidation of fatty acids for energetic metabolism, producing  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  is in turn transformed into  $\text{OH}^{\bullet}$  or other active free radicals [23]. In addition, xanthine oxidase and uric acid oxidase produce electronics in oxidative metabolic pathways [24, 25]. Hydrogen peroxide enzyme and SOD reduce  $\text{H}_2\text{O}_2$  into  $\text{H}_2\text{O}$ . In the process of chronic intestinal inflammation, a large amount of ROS, such as  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$ , is produced by neutrophils during phagocytosis. This phagocytic process activates nicotinamide adenine dinucleotide phosphate oxidase (NOX) in the membrane, leading to rapid depletion of oxygen and production of superoxide anion [5, 6].

Cells evolve an antioxidant defense system to maintain homeostasis between the oxidant and antioxidant species [26, 27]. Excessive generation of free radicals beyond the capacity of defense leads to failure of this homeostatic process and oxidative injuries, such as lipid peroxidation and DNA damage, so-called oxidative stress. The antioxidant defense system in cells consists of enzymatic and nonenzymatic antioxidant molecules (Table 1). In addition to the endogenous cellular antioxidant species, natural food is also an important resource of antioxidants. For example, quercetin (3,5,7,3',4', pentahydroxyflavone), a flavonoid present in numerous fruits and vegetables, demonstrates appreciable antioxidant activity by eliminating free radicals and quenching singlet oxygen [28]. Resveratrol, a phenolic substance in red wines, is also a natural antioxidant and anti-inflammatory molecule [29].

*2.2. Oxidative Stress Insults in Ulcerative Colitis.* While a basal level of ROS may play a protective role in the intestine, the oxidative stress derived from imbalance between ROS production and antioxidant system is harmful, being an important pathogenic factor of UC. ROS are highly active chemical forms that target macromolecules, such as proteins, lipids, and nucleic acids, leading to lipid peroxidation, protein dysfunction, and DNA mutations (Figure 1). Therefore, excessive ROS cause cell and tissue damage, exaggerate inflammation, and lead to far-reaching effects, such as carcinogenesis. Herein we will discuss the protein and lipid damage and cellular effects induced by oxidative stress. Nuclei

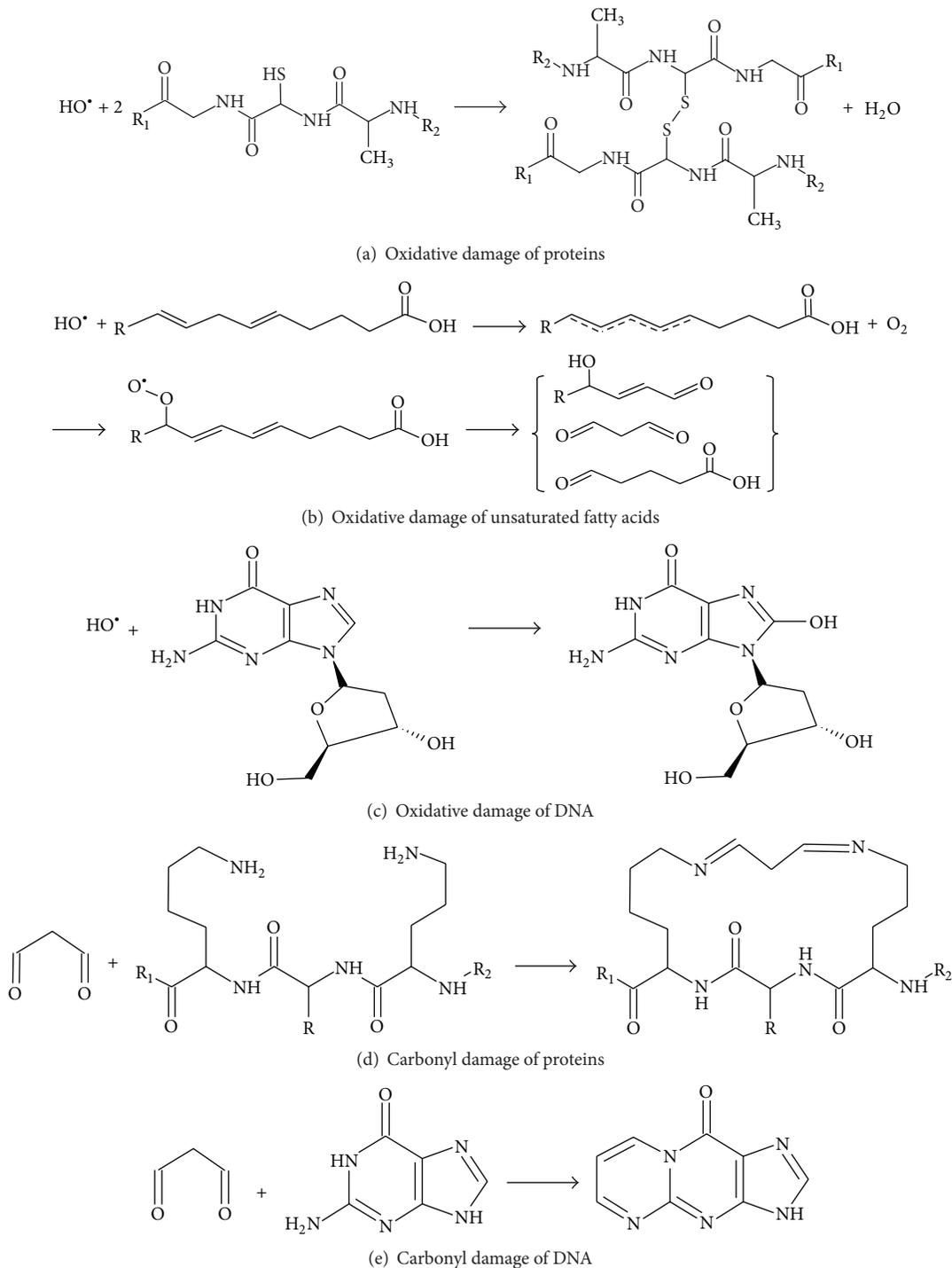


FIGURE 1: Schematic formulas. Reactive oxygen species and carbonyl compounds are highly reactive, causing protein, lipids, and DNA damage.

acid damage and carcinogenic effects of oxidative stress will be addressed in Section 3.

**2.2.1. Protein Damage Induced by Oxidative Stress.** Oxidative stress insults proteins. Highly active ROS can readily interact with protein amino acid residues, such as His, Pro, Trp, Cys, and Tyr residues, and cause protein structure changes, polypeptide chain cracking, and loss of the biological activity.

For example, ROS can oxidize sulfhydryl groups (-SH) in the amino acid residues to form disulfide bonds (-S-S-) and cross-link (Figure 1). ROS could also attack the methyl-sulfide group (CH<sub>3</sub>-S-) in methionine (Met) and affect hydrolysis and carbonylation of proteins [30, 31].

**2.2.2. Lipid Peroxidation Triggered by Oxidative Stress.** Lipid peroxidation (LPO) is a serious cellular damage. Reactive

TABLE 1: Oxidant species and antioxidant defense.

	Oxidant species
Reactive oxygen species (ROS)	Superoxide anion ( $\cdot\text{O}_2^-$ )
	Peroxide ( $\text{O}_2^{-2}$ )
	Hydrogen peroxide ( $\text{H}_2\text{O}_2$ )
	Hydroxyl radical ( $\cdot\text{OH}$ )
	Hydroxyl ion ( $\text{OH}^-$ )
Reactive nitrogen species (RNS)	Nitric oxide ( $\cdot\text{NO}$ )
	Peroxynitrite ( $\text{ONOO}^-$ )
	Nitrogen dioxide ( $\cdot\text{NO}_2$ )
	Dinitrogen trioxide ( $\text{N}_2\text{O}_3$ )
	Nitrosoperoxycarbonate ( $\text{ONOOCO}_2^-$ )
ROS resources	Mitochondrial electron transition
	Enzyme reactions: peroxisomal oxidases, cytochrome P-450, NAD(P)H oxidases, and xanthine oxidase
	Xenobiotics, drugs, and radiation: cisplatin, doxorubicin, and so forth.
RNS resources	Nitric oxide ( $\cdot\text{NO}$ ) from nitric oxide synthase 2 (NOS2)
	Other RNS from reaction of nitric oxide ( $\cdot\text{NO}$ ) with superoxide anion ( $\cdot\text{O}_2^-$ ) and other reactive species.
	Antioxidant defense
Nonenzymatic antioxidants	Glutathione (GSH)
	Cysteine
	Metallothionein
	Coenzyme Q (CoQ)
	Uric acid
Enzymatic antioxidants	Superoxide dismutase (SOD)
	Catalase (CAT)
	Peroxiredoxin (Prx)
	Glutathione reductase (GR)
	Glutathione peroxidases (GPx)
	Glutathione-S-transferases (GST)
	Thioredoxin and thioredoxin reductase
Natural (food) antioxidants	Vitamin A/C/E
	Flavonoid
	Resveratrol

ROS readily bind to unsaturated fatty acids in lipids that contain multiple double bonds, “steal” electrons, and trigger a free radical chain reaction (Figure 1). This oxidative process usually consists of initiation (production of a fatty acid radical), propagation (creation of a peroxy-fatty acid radical), and termination (production of electrophilic carbonyls) [32]. This lipid peroxidation process produces two major biological effects, that is, direct membrane damage and permeability and production of lipid peroxides [33]. The common lipid peroxides created by lipid peroxidation include malondialdehyde (MAD), 4-hydroxynonenal (HNE), crotonaldehyde, and acrolein [34]. These lipid peroxides are  $\alpha,\beta$ -unsaturated and highly reactive to cellular proteins and nucleic acids. In UC pathogenesis, lipid peroxides are important secondary injury factors of oxidative stress.

Phospholipids are primary ingredients of cell and organelle membrane and are enriched with unsaturated fatty acids. Therefore, the lipid peroxidation induced by oxidative stress mainly occurs in the membrane, and attacking by ROS would lead to direct structural and functional changes of membranes [33]. Mitochondrial membrane is the site of the respiratory chain that generates ROS in the normal cells. Therefore, mitochondria are the main organelles that are produced and attacked by ROS [35]. In the status of oxidative stress, excessive ROS attack oxidation respiratory chain and lead to obstacle of oxidative phosphorylation, producing more ROS. Excessive ROS also make  $\text{Ca}^{2+}$  overload in the mitochondria and lead to mitochondrial membrane depolarization and permeability, releasing free radicals into cytoplasm and causing cellular damage in general. Increased

membrane permeability also releases cytochrome C (Cyt-C) and apoptosis inducing factor (AIF) into cytoplasm and activates caspase cascade for apoptosis [36, 37]. Therefore, in oxidative status ROS production by respiratory chain, mitochondrial membrane insults, and ROS release into cytoplasm form a vicious cycle, causing cell death and tissue injury. We will discuss the lesions induced by lipid peroxides in Section 2.3.

**2.2.3. Cell Signaling Triggered by Oxidative Stress.** ROS could function as second messengers to activate intracellular signaling pathways, such as NF- $\kappa$ B, a major modulator of UC [38–42]. In the normal intestinal epithelium, NF- $\kappa$ B maintains intestinal epithelial barrier function and coordinates epithelial immune response to microorganisms. On the other hand, as transcription factors, deregulation of NF- $\kappa$ B signaling, such as oxidative activation, stimulates expression of a variety of proinflammatory cytokines in the intestinal epithelial cells, such as TNF- $\alpha$ , IL-1, IL-8, and COX-2, and promotes inflammation and carcinogenesis. In static state, NF- $\kappa$ B in the cells is bound to I $\kappa$ B, inhibitors of  $\kappa$ B, and hooked in the cytoplasm. Activation of NF- $\kappa$ B consists of I $\kappa$ B kinase (IKK) activation, I $\kappa$ B phosphorylation and ubiquitinated degradation by 26S proteasomes, and nuclear translocation and DNA binding of free NF- $\kappa$ B, finally promoting target gene expression [43]. Oxidative stress can activate IKK and stimulate nuclear translocation of NF- $\kappa$ B (Figure 2). In the diseased colon tissues of UC patients, NF- $\kappa$ B expression, particularly the p65 (RelA) and p52/p100 (NF- $\kappa$ B2), is increased, and blockade of NF- $\kappa$ B activity is considered practical treatment of UC [44]. In addition, the activation of p50, c-Rel, and p65 is documented in macrophages in the lamina propria of UC patients [45].

Oxidative stress also activates mitogen-activated protein (MAP) kinase (MAPK) signaling pathways. MAPKs are highly conserved serine/threonine protein kinases functioning in various fundamental cellular processes, such as growth/proliferation, differentiation, motility, and apoptosis/survival, as well as stress response [46]. Conventional MAPKs include the extracellular signal-regulated kinases 1 and 2 (Erk1/2), the c-Jun N-terminal kinases 1–3 (JNK1–3)/stress activated protein kinases (SAPK), the p38 isoforms (p38 $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), and the Erk5. These MAPKs can be activated by growth factors and mitogens, as well as various stresses. These stimuli activate MAPKK kinases (MAPKKKs) via receptor dependent and independent mechanisms, followed by phosphorylation and activation of a downstream MAPK kinase (MAPKK) and then MAPKs. Activated MAPKs phosphorylate and activate specific target protein kinases, such as RSK, MSK, or MNK to mediate biological processes [47]. The increased ROS can activate ERKs, JNKs, or p38 MAPKs [48, 49]. The exact mechanism by which the ROS activate these kinases is unclear, but a plausible mechanism may be relative to oxidative modifications and resultant activation of the signaling effector proteins and inactivation and/or degradation of MAPK phosphatases (see [50] for more details). Nevertheless, the p38 and JNK signaling activated by ROS is involved in the disease progression of UC [51–54]. In UC tissues, p38 MAPK signaling changes are

a molecular signature of UC and proportional to the degree of inflammation [55, 56].

**2.3. Carbonyl Stress and a Vicious Cell Damage Cycle.** A class of carbonyl compounds is called  $\alpha,\beta$ -unsaturated carbonyls, also referred to as electrophilic carbonyls. These include acrolein, glyoxal, methylglyoxal, crotonaldehyde, malondialdehyde, and 4-hydroxynonenal (Table 2). As byproducts, these electrophilic carbonyl compounds are constantly produced during the metabolism of lipids, carbohydrates, amino acids, biogenic amines, vitamins, and steroids, as well as some antitumor agents, such as cyclophosphamide [57–63]. Besides endogenous production, daily food consumption may represent the most dangerous exposure of human gastrointestinal (GI) tract to exogenous electrophilic carbonyls which are pervasively present in various beverages and foodstuffs [64–66]. For instance, humans are exposed to crotonaldehyde through the consumption of vegetables (1.4–100  $\mu$ g/kg), fruits (5.4–78  $\mu$ g/kg), fish (71.4–1000  $\mu$ g/kg), meat (10–270  $\mu$ g/kg), and alcoholic beverages, such as wine (300–700  $\mu$ g/L) and whisky (30–210  $\mu$ g/L) [66]. Furthermore, methylglyoxal is a constituent of coffee [67, 68], and acetaldehyde is a carcinogenic metabolite of alcohol consumed [69, 70]. Therefore, human GI tract is repeatedly exposed to carbonyl threats, which are important factors of GI inflammatory and neoplastic lesions (Table 3).

In organisms, there are three main pathways responsible for elimination of intracellular carbonyls, through which carbonyls are oxidized to carbonic acids, conjugated with glutathione, or reduced to less toxic alcohols. Aldehyde dehydrogenases mediate the oxidative pathway of carbonyls, forming carbonic acids [71, 72]; glutathione-S-transferases (GST) catalyze the conjugation of carbonyls with glutathione [73–75]; and aldehyde reductase and aldo-keto reductases (AKRs) are responsible for the reduction of carbonyls to alcohols with NAD(P)H as a coenzyme [75–77]. AKR1B10 is the sole carbonyl-detoxifying enzyme with intestine-specific expression identified thus far [78] and plays a critical role in the inflammatory lesions and malignant progression of the colon [19]. Therefore, in normal conditions human consumption or endogenous production of the cytotoxic carbonyls may be subcytotoxic. However, in oxidative stress, excessive ROS oxidize unsaturated fatty acids and produce a large amount of highly reactive  $\alpha,\beta$ -unsaturated carbonyl compounds, that is, lipid peroxides. For instance, 4-hydroxynonenal (HNE) is at 0.1 to 3.0  $\mu$ M in normal tissues but increases to  $\sim$ 10  $\mu$ M in the condition of oxidative stress [79]. Carbonyl accumulation due to overproduction and/or impaired clearance, such as AKR1B10 deficiency [19], would lead to carbonyl stress.

Due to their high reactivity,  $\alpha,\beta$ -unsaturated lipid peroxides are highly cytotoxic and genotoxic. They can interact with free amino groups of proteins (e.g., lysine residue), peptides, and amino acids, with sulfhydryl groups of amino acid residues (e.g., cysteine residue), and with histidine and other residues, forming covalently modified adducts [57, 80–86]. The covalent modifications could lead to protein dysfunction, resistance to proteolysis, or depolymerization. Protein adducts can also act as special second

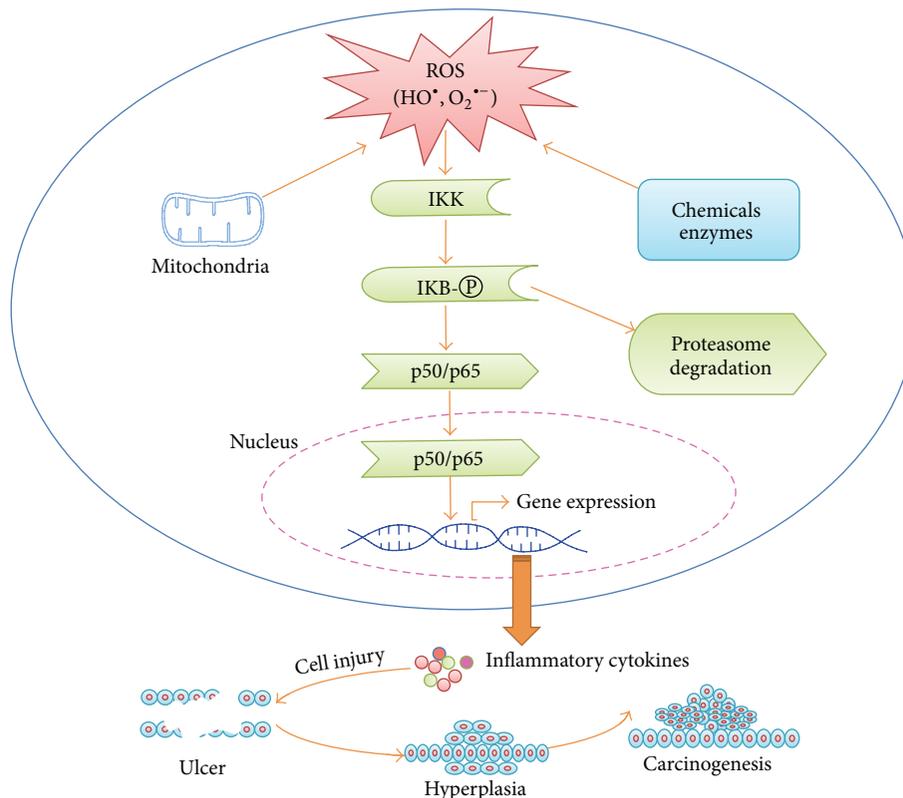


FIGURE 2: NF- $\kappa$ B signaling pathway, inflammation, and carcinogenesis induced ROS. Excessive reactive oxygen species (ROS) derived from mitochondrial membrane, xenobiotics, and enzyme reactions activate IKK. Activated IKK phosphorylates I $\kappa$ B and leads to ubiquitination and proteasome degradation of I $\kappa$ B, releasing NF- $\kappa$ B proteins, such as p50 and p65. The free p50 and p65 translocate into nuclei and drive target gene expression, such as inflammatory cytokines, leading to inflammatory lesions and carcinogenesis.

TABLE 2: Carbonyl compounds and clearance.

Carbonyl compounds	Carbonyl clearance
Acrolein (CH <sub>2</sub> =CHCHO)	
Glyoxal (OHCCHO)	
Methylglyoxal (CH <sub>3</sub> COCHO)	(1) Glutathione-S-transferases (GST) catalyze carbonyl-glutathione conjugation
Crotonaldehyde (CH <sub>3</sub> CH=CHCHO)	(2) Aldehyde reductase and aldo-keto reductases (AKRs) catalyze reduction to alcoholic forms
Malondialdehyde (OCHCH <sub>2</sub> CHO)	(3) Aldehyde dehydrogenases catalyze oxidation to carbonic acids
4-Hydroxynonenal (OCHCH=CHCH(-OH)(CH <sub>2</sub> ) <sub>4</sub> CH <sub>3</sub> )	

messengers or autoantigens, promoting macrophage accumulation, retention, and activation, thus increasing ROS generation. Furthermore, carbonyl-induced protein dysfunction may impair mitochondrial respiratory chain reactions and membrane potential, leading to increased ROS production and release into cytosol. Therefore, in inflammatory conditions (i.e., UC), the carbonyl lesions may create a vicious loop with oxidative stress, aggravating cell and tissue damage [19, 87].

### 3. Oxidative Stress and Carbonyl Lesions in Colitis-Associated Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer worldwide with mortality ranked within top four [88, 89].

According to International Agency for Research on Cancer of WHO ([http://globocan.iarc.fr/Pages/fact\\_sheets\\_cancer.aspx](http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx)), about 1.36 million of new CRC cases were diagnosed globally in 2012, accounting for approximately 69,000 deaths. Clinically, there are two main types of CRC, that is, sporadic colorectal cancer (SCC) and hereditary colorectal cancer (HCC). The latter includes familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Colorectal adenoma, colorectal nonadenomatous polyposis, and inflammatory bowel disease are precancerous lesions associated with CRC. The UC patients have an increased risk of developing colorectal cancer, so-called colitis-associated colorectal cancer (CAC) [90], and the cancer risk increases exponentially with the duration of disease [91–93]. A UC patient with 10 years of

TABLE 3: Carcinogenic role of carbonyl compounds.

Diseases/genotoxicity	Species	Carbonyl association	References
Colitis-associated colorectal neoplasms	Mice	Coupled with high carbonyl levels, for example, malondialdehyde	[19, 153]
Stomach hyperplasia, squamous papilloma, and carcinoma	Rats	2,4-Hexadienal exposure	[73]
Precancerous gastritis and gastric cancer	Humans	High serum malondialdehyde levels	[154, 155]
Colorectal cancer	Humans	High serum lipid peroxide levels	[156]
Colon and gastric cancers	Humans	Acetaldehyde from alcohol	[69, 70]
Colorectal adenocarcinoma	Humans	High protein carbonyl levels	[157]
Precancerous colorectal adenopolyps	Humans	High protein carbonyl levels	[158]
Colorectal cancer	Humans	High lipid peroxide levels in tissues	[159–161]
Genotoxicity	Humans	High carbonyl DNA adduct levels in tissues	[58, 162, 163]
Genotoxicity	Cell lines/ <i>in vitro</i> studies	Production of carbonyl DNA adducts	[164–167]

disease duration has 10-fold higher CRC risk than the general population.

Etiopathogenesis of CAC is complex. In UC, intestinal epithelial and immune cells produce and secrete a variety of mitogenic cytokines that stimulate cell growth and proliferation. Massive ROS and inflammatory cytokines produced in UC tissues activate multiple signal pathways, such as NF- $\kappa$ B, STAT3, p38 MAPK, and Wnt/ $\beta$ -catenin pathways, which mediate cell proliferation, differentiation, and apoptosis/survival [94]. Finally, DNA damage induced by oxidative and carbonyl stresses plays an essential role in the carcinogenic transformation of the disease. Therefore, malignant progression of UC to CAC is a complicated process and oxidative and carbonyl stresses are key factors in this process.

**3.1. Sporadic Colorectal Cancer and Colitis-Associated Colorectal Cancer.** CRC is a multistaged, complicated disease associated with multiple oncogene and tumor suppressor gene mutations, such as *p53*, *K-ras*, and adenomatous polyposis coli (*APC*) mutations [95]. In pathogenesis, sporadic CRC often demonstrates an “adenoma-carcinoma” progression, but the CAC experiences a unique sequence of “inflammation-dysplasia-carcinoma” [96]. Patients with UC may experience a long course of dysplasia. Three types of atypical hyperplasia may appear in the carcinogenic process of UC: (1) normal mucosa or mucous membrane with regeneration, also named dysplasia negative type, (2) dysplasia uncertain type, (3) dysplasia positive type. UC patients with high or moderate grade dysplasia are at high risk of developing CAC [97].

CAC also demonstrates a different time line and involvement of gene mutations. In sharp contrast to sporadic CRC, *p53* mutation occurs early and is an important step in the progression of CAC. The *p53* mutations are often detected in mucosa that is even nondysplastic [98, 99], but *APC* mutations are present at the late stage of CAC [100–103]. *K-ras* mutation plays a rare role in CAC development [104], but

DNA methylation is an early event in UC [105], although less common than in sporadic CRC [106, 107].

**3.2. Inflammatory Cytokines and CAC Progression.** Inflammatory cytokines produced by intestinal epithelial cells and infiltrated inflammatory cells in UC include IL-1, IL-6, TNF- $\alpha$ , and TGF- $\beta$ . These cytokines activate mitogenic signaling pathways, stimulate cell proliferation and survival, and thus promote inflammation-associated tumorigenesis. For instance, the plasma level of IL-6 is significantly elevated in patients with IBD, and the increased IL-6 activates STAT3/JAK1 signaling, promoting cell proliferation, evolution, and tumorigenic progression [94]; inhibition of JAK1 signaling or IL-6 deficiency by targeted disruption diminishes CRC incidence and progression [108, 109].

Tumor necrosis factors (TNF) are proinflammatory cytokines which are produced and secreted mainly by monocyte-macrophages. In this family, TNF- $\alpha$  is an important member that functions in inflammation, immune response, and tumorigenesis. Animal experiments have demonstrated that TNF- $\alpha$  can increase the plasma level of IL-6 [110] and initiate colorectal carcinogenesis mediated by chronic inflammation [111]. To date, TNF- $\alpha$  monoclonal antibody is used for IBD treatment and has demonstrated promising results; this antibody may also be effective in prevention of CAC [112].

TGF- $\beta$  and family members are secretory signal transduction peptides that regulate cell proliferation and apoptosis. In the normal cells, the major function of TGF- $\beta$  is to arrest cell division in the early stage of DNA synthesis, induce cell differentiation, or promote apoptosis. Literature reports indicate that mutations in TGF- $\beta$  signal transduction pathway occur in patients with UC before the formation of colorectal cancer [113]. For example, TGF- $\beta$ RII mutations have been detected in UC dysplasia and are associated with CAC progression [114].

Finally, inflammatory cytokine IL-1 $\alpha$  increases in UC and may be involved in CAC development [115], but compared

to other cytokines, the role of IL-1 $\alpha$  in the development and progression of CAC is more complicated. IL-1 $\alpha$  may promote cancer progression by stimulating angiogenesis [116]; IL-1 $\alpha$  may also promote epithelial repair and prevent CAC by inducing the expression of cyclooxygenase 2 (COX-2), a key enzyme of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis from arachidonic acid (AA) [117]. PGE<sub>2</sub> is a prominent prostaglandin in the intestine; through binding to E prostanoïd (EP) receptor, PGE<sub>2</sub> mediates intestinal epithelial cell proliferation and apoptosis [118, 119]. This is considered favorable to injury repair and remission of UC. In fact, ulcerogenic response of nonsteroidal anti-inflammatory drugs (NSAIDs) in the intestine is ascribed to inhibition of cyclooxygenases and resultant PGE<sub>2</sub> deficiency [120]. In dextran sodium sulfate-(DSS-) induced colitis, COX-2/PGE<sub>2</sub> promotes epithelial cell proliferation; inhibition of COX-2 decreases epithelial proliferation, exacerbates colitis, and prolongs injury phase, thus promoting intestinal injury and dysplasia [121–123]. Therefore, evaluation of IL-1 $\alpha$  in CAC development and progression needs to be more cautious.

**3.3. Oxidative DNA Damage in CAC Progression.** DNA mutations and resultant protooncogene activation and/or tumor suppressor gene inactivation are a hallmark of cell carcinogenesis, which reprograms cell growth, division, and gene transcription. The high risk of UC patients to develop colorectal cancer is essentially attributed to the increased DNA damage induced by inflammatory oxidative stress and carbonyl lesions. DNA is a ready target of active oxygen free radicals, leading to oxidative DNA damage. Through abstractions and addition reactions, highly reactive hydroxyl radicals react with the heterocyclic DNA bases and sugar moiety, producing carbon-centered sugar radicals and OH- or H-adduct radicals of heterocyclic bases [124]. Further reactions of these radicals yield numerous effects, such as 8,5'-cyclopurine-2'-deoxynucleosides, tandem lesions, clustered sites, and DNA-protein cross-links [124, 125]. Among types of oxidative DNA damage induced by ROS, 8-hydroxy-2'-deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is a predominant form and a valuable biomarker widely used for endogenous oxidative damage to DNA (Figure 1). For instance, the urinary 8-OHdG is used as a biomarker for risk assessment of cancers and degenerative diseases [126, 127].

GC to TA transversion is a major type of DNA mutations resulting from 8-OHdG adducts [128]; two common target genes of the 8-OHdG damage are *Ras* and *p53*, leading to activation of the protooncogene *Ras* and inactivation of *p53* tumor suppressor, driving tumorigenesis [129, 130]. ROS also cause DNA methylation, single- and double-strand breaks, and shortening of telomeres. DNA methylation is an early event in the progression of UC to CAC [105], but less common than in sporadic CRC [106, 107]. Oppositely, DNA breaks and telomere shortening occur more often in the UC-associated tumorigenesis [131, 132]. The telomere shortening induced by ROS could induce chromosome instability, leading to chromosomal loss, heteroploid, amplification, and translocation, driving tumorigenesis [133, 134].

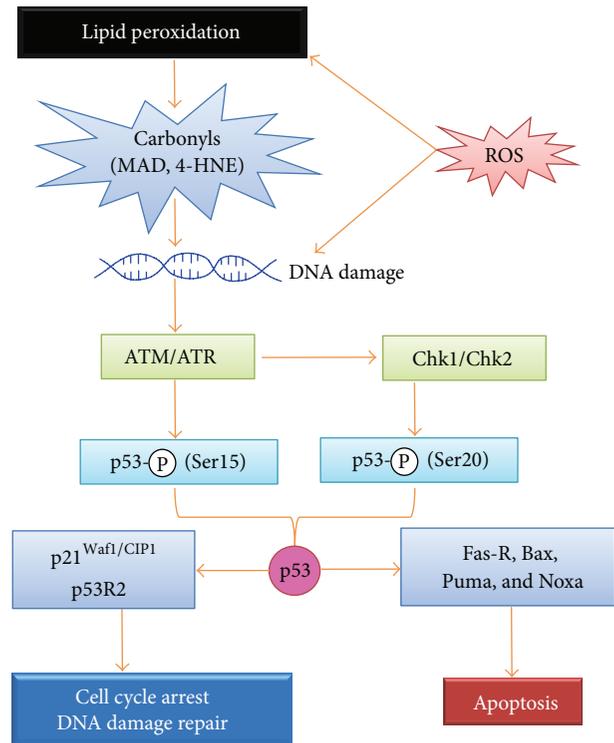


FIGURE 3: DNA damage induced by oxidative and carbonyl stresses and p53-dependent DNA damage response (DDR). Reactive oxygen species (ROS) and  $\alpha,\beta$ -unsaturated carbonyl compounds produced by lipid peroxidation, such as MDA and HNE, trigger DNA damage, such as double-strand DNA breaks. ATM/ATR senses the breaks and activates p53 by phosphorylating Ser15; ATM/ATR also phosphorylates Ser345 of Chk1/Chk2 and activates Chk1/Chk2, which further activates p53 by phosphorylating Ser20. In cells with mild DNA damage, p53 drives expression of p21<sup>Waf1/CIP1</sup> and p53R2, leading to cell cycle arrest and DNA damage repair. In cells with severe DNA damage, p53 drives Fas-R, Bax, Puma, Noxa, Apaf-1, and Pidd expression, activating intrinsic and extrinsic apoptotic pathways.

**3.4. Carbonyl DNA Damage in CAC Progression.** Carbonyl stress derived from lipid peroxidation is also an important DNA damage factor in UC. Electrophilic carbonyls can readily react with DNA forming covalently modified DNA adducts (Figure 1). The DNA adducts can block DNA semi-conservative replication performed by DNA polymerases or arrest transcription driven by RNA polymerases [58, 135–137]. DNA adducts can also cause miscoding and induce DNA breaks [58, 137–139]. For instance, malondialdehyde (MDA) can react with deoxyguanosine in DNA to form an exocyclic adduct, pyrimido[1,2- $\alpha$ ]purin-10(3H)-one (MIG), which is mutagenic by resulting in frameshift mutations and base pair substitutions [140]. The 4-HNE-dG polymer derived from 4-hydroxynonenal can lead to GC to TA transversion at codon 249 of *p53* gene, driving UC progression to CAC [141, 142].

Of note, DNA breaks induced by carbonyl compounds may activate cellular DNA damage response (DDR), inducing cell cycle arrest for DNA repair or apoptosis (Figure 3). In

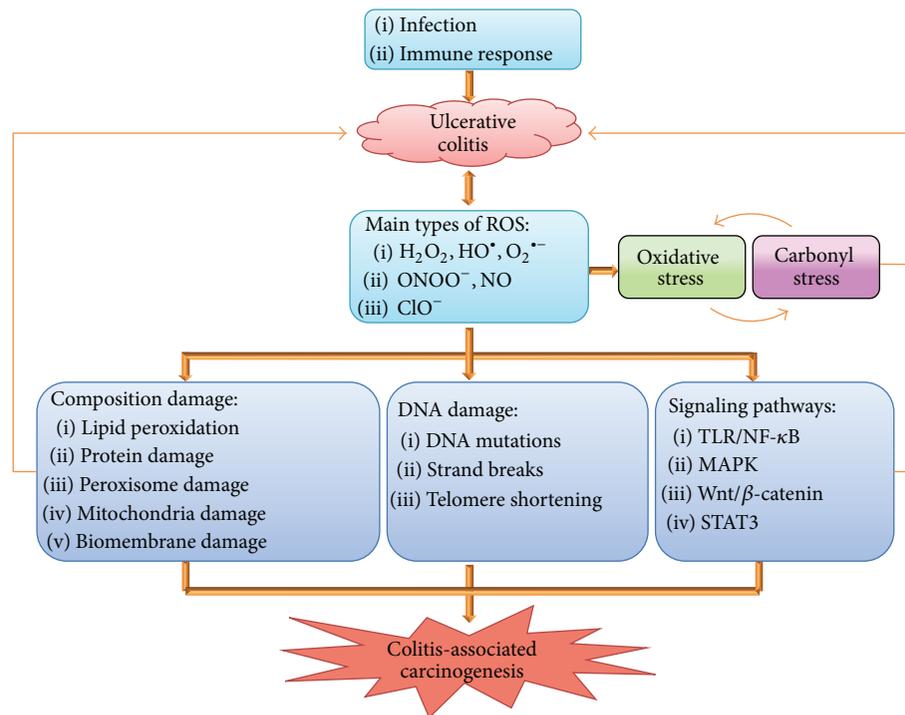


FIGURE 4: Hypothetic model of oxidative stress and carbonyl lesions in ulcerative colitis and associated colorectal cancer. Infection and immune response act as primary initiators to trigger inflammation and inflammatory cell infiltration. In this process, intestinal mucosal crypt abscesses occur and vast reactive oxygen species (ROS) are produced, thus leading to oxidative stress. Excessive ROS exaggerate inflammatory lesions and stimulate epithelial cell proliferation through oxidative insults to proteins, lipids, and DNA and also by activation of cell signaling pathways, eventually leading to ulcerative colitis (UC) and colitis-associated colorectal cancer (CAC). Electrophilic carbonyl compounds play as important secondary factors of oxidative stress to cause cellular and macromolecular lesions, which, together with oxidative stress, may form a vicious cycle. Meanwhile, proinflammatory cytokines produced by epithelial cells and infiltrated inflammatory cells may promote the progression of UC and CAC.

this DDR process, ATM/ATR functions as a sensor of DNA breaks, and p53 acts as a key mediator [143, 144]. Sensing the DNA double-strand breaks, ATM/ATR is activated by phosphorylation, which reaches the peak within 30 minutes [145]. The activated ATM/ATR phosphorylates p53 at Ser15 and/or Chk1/Chk2 at Ser345, and Chk1/Chk2 further phosphorylate p53 at Ser20 [146]. Activated p53 triggers cell cycle arrest for DNA damage repair or apoptosis to eliminate cells with severe DNA damage through selective activation of target gene expression, such as apoptotic genes Fas-R, Bax, Puma, and Noxa or cell cycle monitoring and DNA repair genes p21<sup>Waf1/CIP1</sup> and p53R2 [147]. Therefore, DDR is considered a barrier of carcinogenesis, and mutations of genes in this pathway are carcinogenic. In fact, p53 mutation is an early event in CAC and occurs even in noncancerous UC tissues [148, 149].

#### 4. Conclusion and Perspective

Early in 1863, a German pathologist Virchow proposed that tumor might be derived from chronic inflammation tissues; in 2009, Hanahan and Weinberg proposed tumor-related inflammation as the seventh hallmark of cancer. To date, the role of chronic inflammation in cancer development

and progression has become an important research focus in tumor microenvironment. In UC, the pathogenesis of CAC is a classical path of nonresolving inflammatory progression to cancer, featured with a unique sequence of “inflammation-dysplasia-carcinoma.” Oxidative stress and secondary carbonyl lesions are key factors in the development and progression of UC and CAC; the ROS take an important part in multiple stages of initiation, promotion, and progression of UC and CAC and the secondary carbonyl lesions play an exaggerating role both in oxidative stress itself and in progression of UC and CAC (Figure 4).

To date, antioxidant prevention and treatment have been investigated in experimental animals of colitis and in clinical patients of UC. In animals, antioxidant *G. biloba* extract (EGb 761) showed effectiveness in prevention and treatment of DSS-induced colitis in mice [150], and the *Zingiber officinale* extract demonstrated efficacy in modulating extent and severity of colitis in rats [151]. In humans, consumptions of antioxidant food, such as blueberries, cherries, tomatoes, squashes, and bell peppers have been suggested as supplementary treatment of active UC and prevention of reactivation. More impressively, a clinical trial of rectal d-alpha tocopherol, a powerful vitamin E antioxidant, has shown that “all 14 patients responded clinically to the therapy and remission was induced in 9 of them (64%)”; no adverse

events were reported and no patients were hospitalized for “worsened disease activity” [152]. These preclinical and clinical approaches suggest that antioxidant treatment may be a novel mode of UC management and prevention of malignant progression. Further studies are warranted.

## Abbreviations

AIF:	Apoptosis inducing factor
AKR1B10:	Aldo-keto reductase 1B10
APC:	Adenomatous polyposis coli
CAC:	Colitis-associated colorectal cancer
CAT:	Catalase
COX-2:	Cyclooxygenase 2
CRC:	Colorectal cancer
DSS:	Dextran sodium sulfate
GSH-PX:	Glutathione peroxidase
IBD:	Inflammatory bowel disease
MAD:	Malondialdehyde
NOX:	Nicotinamide adenine dinucleotide phosphate oxidase
OS:	Oxidative stress
Raf:	Root abundant factor
ROS:	Reactive oxygen species
SCC:	Sporadic colorectal cancer
SOD:	Superoxide dismutase
TGF- $\beta$ :	Transforming growth factor $\beta$
TNF- $\alpha$ :	Tumor necrosis factor $\alpha$
UC:	Ulcerative stress.

## Conflict of Interests

Authors declare no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Zhiqi Wang wrote draft. Sai Li draw figures. Duan-Fang Liao, Yu Cao, Xuefei Tian, and Rong Zeng contributed to literature search and discussion. Deliang Cao revised the paper. All authors read and approved the final version of this paper.

## Acknowledgments

This work was supported in part by Hunan Engineering Center for Rapid Test and Removal of Toxic and Harmful Substances in Chinese Medicine (201303 for Zhiqi Wang) and by National Natural Science Foundation of China (81503492 for Zhiqi Wang; 81272918 and 81472465 for Deliang Cao).

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## Review Article

# The Interplay of Reactive Oxygen Species, Hypoxia, Inflammation, and Sirtuins in Cancer Initiation and Progression

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Received 23 July 2015; Accepted 29 September 2015

Academic Editor: Sahdeo Prasad

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The presence of ROS is a constant feature in living cells metabolizing O<sub>2</sub>. ROS concentration and compartmentation determine their physiological or pathological effects. ROS overproduction is a feature of cancer cells and plays several roles during the natural history of malignant tumor. ROS continuously contribute to each step of cancerogenesis, from the *initiation* to the *malignant progression*, acting directly or indirectly. In this review, we will (a) underline the role of ROS in the pathway leading a normal cell to tumor transformation and progression, (b) define the multiple roles of ROS during the natural history of a tumor, (c) conciliate many conflicting data about harmful or beneficial effects of ROS, (d) rethink the importance of oncogene and tumor suppressor gene mutations in relation to the malignant progression, and (e) collocate all the cancer hallmarks in a mechanistic sequence which could represent a “physiological” response to the initial growth of a transformed stem/pluripotent cell, defining also the role of ROS in each hallmark. We will provide a simplified sketch about the relationships between ROS and cancer. The attention will be focused on the contribution of ROS to the signaling of HIF, NFκB, and Sirtuins as a leitmotif of cancer initiation and progression.

## 1. Introduction

ROS (Reactive Oxygen Species) production has been strictly associated with cancer [1], ageing [2], diabetes [3], obesity [4], neurodegeneration [5], and other age-related diseases such as age-related retinopathy, cochlear degeneration, and chronic inflammatory diseases [6]. How can ROS contribute to so many apparently different clinical entities and what are the common molecular targets and pathways altered by ROS? In recent years, a great amount of information has been produced to answer these questions. Interestingly, such information stems from the study of the roles of ROS along the tumorigenesis sequence [7].

The complexity of relationships between ROS and cancer pathogenesis is primarily due to the diverse species of ROS produced by O<sub>2</sub> metabolism and their properties, such as chemical nature, half-life, reactivity and specificity for

their biological targets, ability to diffuse and travel among subcellular compartments, type of changes produced in target molecules, and, finally, the importance of affected biological functions [8]. Moreover, it is difficult to identify the molecular targets and the numerous redundant pathways modified by ROS, with a significant role in cancerogenesis. Besides, biologically active or toxic concentrations of ROS resulting from the ratio between production and detoxification introduce additional important variables to be considered in describing the ROS/cancer relationships [9].

Cancer pathogenesis may be described as a multistep process including *transformation*, *growth promotion* and, in clinically evident tumors, *malignant progression* [10]. During the natural history of cancer a large number of genes, molecules, and pathways contribute first to transformation and promotion then to the manifestation of the malignant cancer phenotype; most of these molecules and pathways

interact with ROS in the cytosol, nucleoplasm, and intra-organelle space.

A transformed cell is identified by the loss of control of proliferation and deregulation of apoptosis producing an excess of cell number and forming a mass (tumor). The disruption of cell cycle and apoptosis regulation is due to mutations of genes with a gain-of-function (oncogenes) and a loss-of-function (oncosuppressor genes), both leading to an excessive proliferative signal [11, 12]. The deregulation of apoptosis is due to mutations of genes involved in the signaling controlling programmed cell death, with a gain-of-function of genes (oncogenes) protecting from apoptosis and a loss-of-function (oncosuppressor genes) promoting apoptosis. Upstream, alterations of DNA repair mechanisms may often facilitate the accumulation of crucial mutations in a single stem cell giving rise to the transformed stem cell responsible for the growth of the early small tumor [13].

The initial growth of a small tumor occurs with absent, insufficient, or abnormal angiogenesis. This produces areas of hypoxia of different severity in which ROS increases, favoring tumor cell survival, adaptation, and progression [14]. Even though the precise mechanism through which hypoxia increases ROS is still a matter of debate, it seems that ROS production is due to the effects of hypoxia on the mitochondria electron transport chain (ETC). In particular, hypoxia would drive ROS increase by acting on complexes I, II, and III of the ETC [15, 16]. In fact, the use of inhibitors for each one of these complexes resulted in the inhibition of ROS accumulation [15, 16]. Moreover, such ROS are mainly represented by  $H_2O_2$  since forced expression of catalase or glutathione peroxidase-1 completely reversed hypoxia-induced ROS expression in isolated pulmonary artery myocytes [15, 16]. Interestingly, hypoxia-driven ROS increase would then leave the mitochondria causing destabilization of Prolyl Hydroxylases (PHD) and stabilization of HIF1 $\alpha$  [15, 16].

HIF is the major transcription factor responsible for triggering tumor progression [17]. In addition, in this phase, ROS further increases contributing to the involvement of NF $\kappa$ B and Sirtuins in the full acquisition of malignant phenotype [18].

Here we will shortly review the contributions and mechanisms of ROS from cell transformation to the acquisition of every single hallmark of a clinically significant malignant tumor, trying to correlate specific molecular targets to ROS role.

## 2. ROS Compartmentation and Production

Five main compartments contain ROS: mitochondria, cytosol, single membrane-bound organelles (peroxisomes, endosomes, and phagosomes), exosomes released by plasma membranes by shedding, and extracellular fluids including plasma [9]. As schematized in Table 1 and Figure 1, ROS are produced in different subcellular compartments by the action of different enzymes and then they can travel through channels or vesicles. In particular, mitochondria produce large amount of ROS that can be either detoxified or can leave the organelle through channels such as voltage dependent

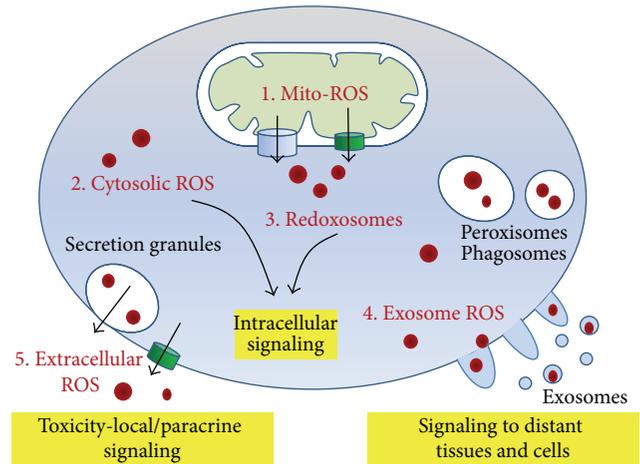


FIGURE 1: Subcellular compartmentation of ROS. 1. Mitochondrial ROS which can travel to cytoplasm through VDAC (superoxide) or through aquaporin (peroxides). 2. Cytosolic ROS. 3. Redoxosomes, such as peroxisomes and endoplasmic reticulum derived vesicles. 4. ROS included into exosomes and vesicles shedding from damaged plasma membranes. 5. Extracellular ROS in extracellular fluids and plasma, partly crossing the plasma membrane through aquaporin, partly secreted with granules (i.e., activated leukocytes).

anion channel (VDAC) or aquaporin. Similarly ROS can be produced by NADPH-oxidases (NOX) and other cytosolic enzymes as well as by peroxisomes. Finally, ROS can be released in the extracellular space through aquaporin or exosomes (Figure 1).

Three broad classes of ROS may be produced: hydroxyl radicals, superoxides, and hydroperoxides, with distinctive characteristics regarding their reactivity, half-life, target specificity, localization, and, very importantly, biological and pathological effects (Table 1). At present, the acronym ROS may include also several nitrogen-containing compounds or RNS (Reactive Nitrogen Species), such as nitric oxide (NO), nitroxyl anion ( $NO^-$ ), and peroxyxynitrite ( $ONOO^-$ ). NO is produced by the activity of inducible nitric oxide synthase (iNOS) and reacts with superoxide to give rise to the other RNS. ROI (Reactive Oxygen Intermediates) and RNI (Reactive Nitrous Intermediate) are additional acronyms used to indicate ROS [8, 19].

ROS are produced in the *mitochondria* as by-products of fatty acid (FA) metabolism and oxidative phosphorylation for ATP synthesis [8, 19]. *Hydroxyl anion* half-life is extremely short ( $10^{-9}$  sec) interacting with and sometimes damaging any biological molecule in its range. *Supeeroxides* encounter two destinies: rapid detoxification by mitochondrial MnSOD (Mn-dependent superoxide dismutase) as hydrogen peroxide or mitochondrial membrane crossing through the VDAC. *Hydroperoxides* travel easily to cytosol through membrane aquaporin [8] (Figure 2).

*Cytosol* can produce ROS from many endogenous (growth factors, cytokines, and metabolisms) or exogenous sources (nutrients, radiation, microbiome, and xenobiotics). On the other hand, cytosol can accumulate ROS produced by mitochondria and redoxosomes, especially superoxide

TABLE 1: Classes of ROS and their properties.

Radical	Structure	Reactivity	Half-life	Production/localization	Diffusion	Targets	Biological effect	Pathological effect
Hydroxyl radical	$\text{OH}^\bullet$	High	$10^{-9}$ sec	Mitochondria Phagosome Endoplasmic reticulum (ER)	Highly localized where is produced	Any cell component	Unknown	Toxicity
Superoxide	$\text{O}_2^-$	Low	1-15 minutes	Mitochondria ER Cytosol Peroxisome	Localized, it can diffuse through an anion channel	Fe-S centers Nitric oxide	Protein modification (activation or inhibition)	Protein damage
Hydrogen peroxide	$\text{H}_2\text{O}_2$	Moderate Reversible	Hours to days	Mitochondria ER Cytosol Peroxisome	Diffuse, it can travel through aquaporins	Iron-sulphur Cysteine residues	Activation of signaling	Mutation, accumulation, and genomic instability

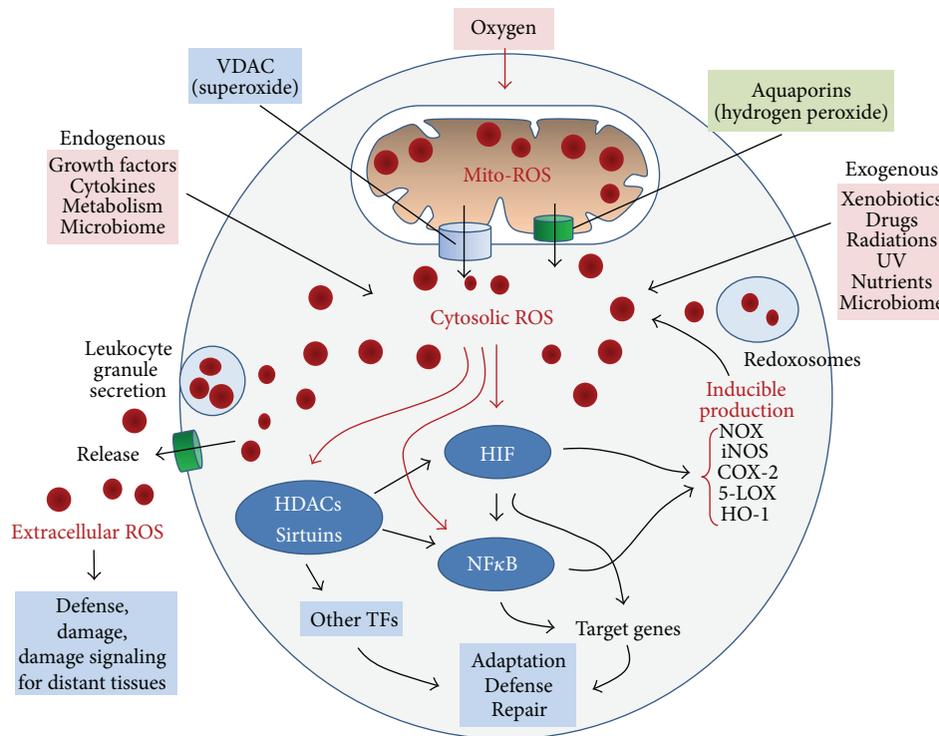


FIGURE 2: ROS are produced mainly in the mitochondria. Superoxides are rapidly detoxified by mitochondrial MnSOD as hydrogen peroxide or can cross mitochondrial membranes through the VDAC. Hydroperoxides travel easily to cytosol through membrane aquaporin. In addition to ROS coming from mitochondria, cytosolic ROS can originate from many endogenous or exogenous sources, including nutrients, radiation, microbiome, growth factors, cytokines, and other metabolisms. Proinflammatory inducible enzymes such as NADPH-oxidases (NOX), inducible nitric oxide synthase (iNOS), inducible cyclooxygenase (COX2), 5-lipoxygenase, and inducible heme-oxygenase-1 (HO-1) may produce an additional burst of ROS. HIF1 $\alpha$ , NF $\kappa$ B, and HDACs, especially Sirtuins, are activated by ROS in synergy with the specific signaling from receptors and metabolism. Target genes of activated TFs are aimed at adaptation to hypoxia, proinflammatory harmful agents' inactivation, and damage repair. ROS are also released in the extracellular space by secretion of granules of activated leukocytes or crossing plasma membrane through anionic channels (superoxides) or aquaporins (hydroperoxides). Extracellular ROS are important for defense (as in case of ROS released by eosinophils against macroparasite) and produce collateral damage not only in adjacent healthy tissues but also in distant tissues and organs, signaling the local damage and activating improper mechanisms of adaptation, remodeling, and chronic damage.

and hydroperoxides. ROS and RNI, accumulating into the cytosol, can diffuse easily (depending on half-life) into the nucleoplasm, interacting with nucleic acids and other nuclear components [20].

In the cytosol and redoxosomes, proinflammatory inducible enzymes such as NADPH-oxidases (NOX), inducible nitric oxide synthase (iNOS), inducible cyclooxygenase (COX2), inducible 5-lipoxygenase (5-LOX),

and inducible heme-oxygenase-1 (HO-1) may produce an additional burst of ROS.

In particular, the different isoforms of NOX, identified in many tissues and cells, are an important source of ROS in response to different stimuli including hypoxia [21]. NOX is a multisubunit enzyme complex generating superoxide by one-electron reduction of oxygen using reduced NADPH as the electron donor [21]. NOX is widely distributed among different species, suggesting that such enzyme plays an important role in the cell. However, the precise physiological role of NOX is still unclear, whereas its pathophysiological role is definitely lined to ROS production and ROS-induced damage [22]. Finally, as for HIF (see below), mitochondrial ROS accumulation following hypoxia can, in turn, activate NOX through a mechanism requiring protein kinase C $\epsilon$  and leading to further ROS increase and cellular damage [23].

Hypoxia-induced ROS accumulation also increases expression and activity of 5-LOX in pulmonary artery endothelial cells with production of leukotrienes and induction of cell proliferation [24].

The presence of the cytosolic CuSOD (Cu-dependent superoxide dismutase) and of a number of scavenging molecules, that is, peroxiredoxins and glutathione peroxidase, [22, 25, 26] detoxifies the excess of cytosolic ROS (Figure 2).

A special case of ROS production and utilization occurs in the *redoxosomes*. A number of oxidases are localized in specialized stable (peroxisomes) or transient (phagosomes, multivesicular bodies, endosomes, etc.) single-membrane bound organelles that can produce substantial amount of ROS as typically occurs in the respiratory burst of activated leukocytes (macrophages and eosinophils) or during peroxisome proliferation in response to xenobiotics [27].

Members of NOX family (NADPH-oxidases) and myeloperoxidase are induced and confined in the vacuole microenvironment where they produce a large amount of ROS mainly aimed at killing bacteria and inactivating harmful substances. This represents an efficient defense mechanism against bacteria and parasites [28].

Variable ROS concentrations have been measured in many extracellular fluids, such as blood plasma and spermatic, peritoneal, and pleural fluid [29, 30]. Free *extracellular ROS* have two origins: from cytosol crossing the plasma membrane through aquaporins (hydroperoxides) and some anion channels (superoxides) and by secretion (external opening of phagosomes and granules) as typically occurs in activated degranulating leukocytes [31, 32]. The range of action of extracellular ROS is determined by their half-life, reactivity, velocity of diffusion, and the possibility to travel with plasma. More reactive and short-living ROS (hydroxyl anion and superoxide) act in a short range damaging local biological structures (i.e., macroparasites and adjacent tissue cells), while hydroperoxides may travel with plasma contributing to determining the redox levels of the blood and thus influencing the activity and the life of blood cells and of important plasma proteins [33, 34]. ROS are also released in the extracellular space by secretion of granules of activated leukocytes or crossing plasma membrane through chloride and other anion channels (superoxides) and aquaporins (hydroperoxides)

[35]. Extracellular ROS are important for defense (as in case of ROS released by eosinophils against macroparasite) and produce collateral damage not only in adjacent healthy tissues but also in distant tissues and organs, signaling the local damage and activating improper mechanisms of adaptation, organ remodeling, and chronic damage (Figure 2).

Recent literature has recognized the functional importance of *exosomes*. Exosomes are small (50–90 nm) vesicles originating from invagination of multivesicular bodies and plasma membrane and are released in small amount by normal cells and in large number by cell under various types of stress and by cancer cells, diffusing and traveling through extracellular biological fluids [36, 37]. Their content is largely determined by the local cytosolic composition where exosomes are formed and therefore, their content, includes water, ions, soluble metabolites proteins nucleic acid, and ROS. Their membrane contains membrane-associated proteins including ligands which can allow exosomes to interact with distant cells and tissues expressing the corresponding receptor. After this interaction external and internal molecules can enter target cells initiating signal cascades that can influence cell physiology and pathology. In particular, exosomal microRNA and proteins have been demonstrated to play a role in distant organ remodeling and damage developing multiorgan diseases as observed in complex patients [37].

### 3. ROS Biological Functions and Damaging Effects

The increase of ROS following hypoxia has been extensively documented using different techniques. However, precise numbers indicating the level of ROS generation in tumors are difficult to obtain due to the multiple antioxidant pathways and molecular mechanisms activated by tumors to survive to such an increase and to thrive. An important aspect is the interaction of ROS with different cellular components that produces different types of changes depending on the classes of ROS. In particular, as shown in Table 1, hydroxyl radicals are highly reactive causing sublethal or lethal degradation (toxicity), whereas superoxide and hydrogen peroxide have a lower reactivity but can cause local damage or activation of signaling cascade when present at physiological concentration. Lipids, proteins, and nucleic acids (sugar backbone and N-bases) are the most significant targets of ROS-induced damage [38].

Chronic oxidative stress exerts detrimental effects during the multistage process of carcinogenesis, including DNA damage, impaired DNA repair, mutations in tumor suppressor genes, epigenetic changes, altered apoptosis, disruption of signal transduction pathways responsible for maintaining the normal cellular homeostasis, angiogenesis, and metastasis. For a comprehensive description of ROS-induced DNA damage we refer the reader to the reviews of Ziech et al. and Caputo et al. [39, 40].

*Lipoperoxidation* has the most significant impact on plasma membrane structure and permeability. Plasma membrane damage disrupts ionic gradients: the entry of Na<sup>+</sup> and water leads to cell swelling (one of the most frequent

cell alterations in mammalian tissue pathology). However, the necrotic catastrophe is associated with the entry of extracellular  $\text{Ca}^{++}$ . Disruption of  $\text{Ca}^{++}$  homeostasis leads to a rapid cell degradation through (1) a further increase of ROS production and damage [41], (2) an abnormal function of cytoskeletal components (supercontracture) [42, 43], and (3) an abnormal activation of  $\text{Ca}^{++}$ -dependent proteases, such as calpains, caspases, and proteasomes [44]. To this effect it is important to consider that  $\text{Ca}^{++}$  homeostasis maintenance depends on the cellular compartment roughly as follows: (i) cytosolic ( $[\text{pCa}_i^{++}] = 10^{-9}$  M); (ii) endoplasmic reticulum cisternae ( $[\text{pCa}^{++}] = 10^{-6}$  M); (iii) mitochondrial ( $[\text{pCa}_m^{++}] = 10^{-5}$  M); (iv) extracellular ( $[\text{pCa}_e^{++}] = 10^{-3}$  M).

*Superoxides* react rapidly with iron-sulphur groups of proteins or with NO generating peroxynitrite, which, in turn, acts on proteins (tyrosine nitration and S-glutathionylation) [45]. Hydroperoxides act by oxidizing cysteine residues in proteins and influencing deeply their activity. Mitochondrial DNA and nuclear DNA undergo several alterations that may result in mutation accumulation and genomic instability [46]. Mitochondria undergo mtDNA alterations and metabolic dysfunction with increase in ROS production. Nuclear DNA undergoes point mutations, breaks and consequent deletion, inversion, and translocation, all conditions that can activate oncogenes or inactivate oncosuppressor genes.

Several proteins, targets of ROS, play a crucial role during tumor progression. Herewith we will focus the attention on HIF,  $\text{NF}\kappa\text{B}$ , and Sirtuins. It is interesting to note that a full activation of HIF,  $\text{NF}\kappa\text{B}$ , and Sirtuins occurs in synergy with specific signaling from receptors or other pathways following a previous interaction with ROS.

**3.1. ROS May Induce Cell Transformation through Mutations.** ROS are at the early origin of cancer. Radiations, UV, xenobiotics, chemical carcinogens, nutrients, and chronic inflammation are sources of mitochondrial and cytosolic ROS. In the nucleus they damage in different ways DNA producing random mutations including those that allow a normal cell to lose the control of cell cycle and of the apoptosis [47]. Most of the times, mutations are corrected by one of the DNA repair mechanisms such as double strand break (DSB) repair, base excision repair (BER), mismatch repair (MMR), and, possibly, nucleotide excision repair (NER) [48]. Alternatively, the cell can undergo apoptosis. In these conditions, the chances to select a transforming combination of mutations are substantially increased by defective DNA repair mechanisms, by predisposing germline mutations and by defective ROS detoxifying systems. In conclusion, ROS mediate the mutagenic action of a number of carcinogenic agents playing a prevalent role in the initial transformation of a normal cell into a tumor cell.

**3.2. ROS Promote Growth and Genomic Instability in Already Transformed Cells.** A second contribution to cancerogenesis is given by additional ROS constitutively produced in transformed cells by mutated oncogenes. In particular, oncogenes such as Ras and Myc, often overexpressed in tumor cells, have been linked to deregulation of cell proliferation with increase

of ROS that, in turn, cause DNA damage [49]. In fact, both Ras and Myc induce metabolic reprogramming of cancer cells with increased glucose and glutamine metabolism and, consequently, increased proliferation and ROS production. In this case, ROS species are represented by superoxide that accumulates after Ras and Myc overexpression. However, the precise mechanism through which Ras and Myc induce ROS increase is still unknown and does not depend on mitochondrial superoxide production [50]. Another important family of transcription factors linked to Ras is represented by the STAT family that is inactivated by increased ROS through oxidation of cysteine residues [51]. Alternatively, increased STAT3 and 5 determine a decrease in mitochondrial ROS production [51]. Interestingly, malignant transformation of mouse embryo fibroblasts by activated Ras oncogene also requires mitochondrial STAT3 and decreased ROS accumulation [52].

#### 4. ROS and Hypoxia: Tumor Necrosis and Adaptation

Initial growth of transformed cells, leading to the initial tumor mass, occurs in the absence of or with inefficient angiogenesis. When tumor diameter and the intercapillary distances reach  $200\ \mu\text{m}$  (which is the diffusion limit of the oxygen from blood) the tumor tissue becomes hypoxic, with important effects for the tumor microenvironment and for the metabolism of transformed cell itself that becomes more glycolytic [53] (Figure 3). In order to better understand the characteristics of the tumor microenvironment, it is important to consider that average oxygen partial pressure ( $\text{pO}_2$ ) of tumors, measured by a polarographic  $\text{pO}_2$  sensor, is about 8–10 mmHg or 1.1–1.3%. By contrast,  $\text{pO}_2$  in various human tissues has an average of 35 mmHg or 4.6% [54]. Therefore, it is now a consolidated fact that hypoxia is a characteristic of solid tumors and represents a negative prognostic indicator [54].

As previously described, during this phase, a third prominent role of ROS is evident: hypoxia produces ROS which activate HIF1 $\alpha$ , by inactivating its inhibitor, PHD (Prolyl Hydroxylase Domain) [53]. Even though this review is focused on the interplay among ROS, HIF1 $\alpha$ , and  $\text{NF}\kappa\text{B}$ , it is important to keep in mind that hypoxia-driven ROS activates also other transcription factors such as NRF2. NRF2 has an important role in regulating transcription of proteins involved in antioxidant defense thereby reducing ROS accumulation [55]. Importantly, NRF2 and HIF1 $\alpha$  may act together or independently in regulating, for example, HO-1 expression. Moreover, HIF1 $\alpha$  regulates NRF2 in some colorectal cell lines [55], whereas silencing NRF2 expression results in HIF1 $\alpha$  and VEGF reduction indicating a complex and yet unraveled network between these players [55]. In addition, the situation gets complicated by the observation that many human cancers show a significant upregulation of NRF2 correlating with a poor prognosis [55]. For an exhaustive description of NRF2 function in physiological and pathological conditions, we remind the reader to the recent review by Moon and Giaccia [56].

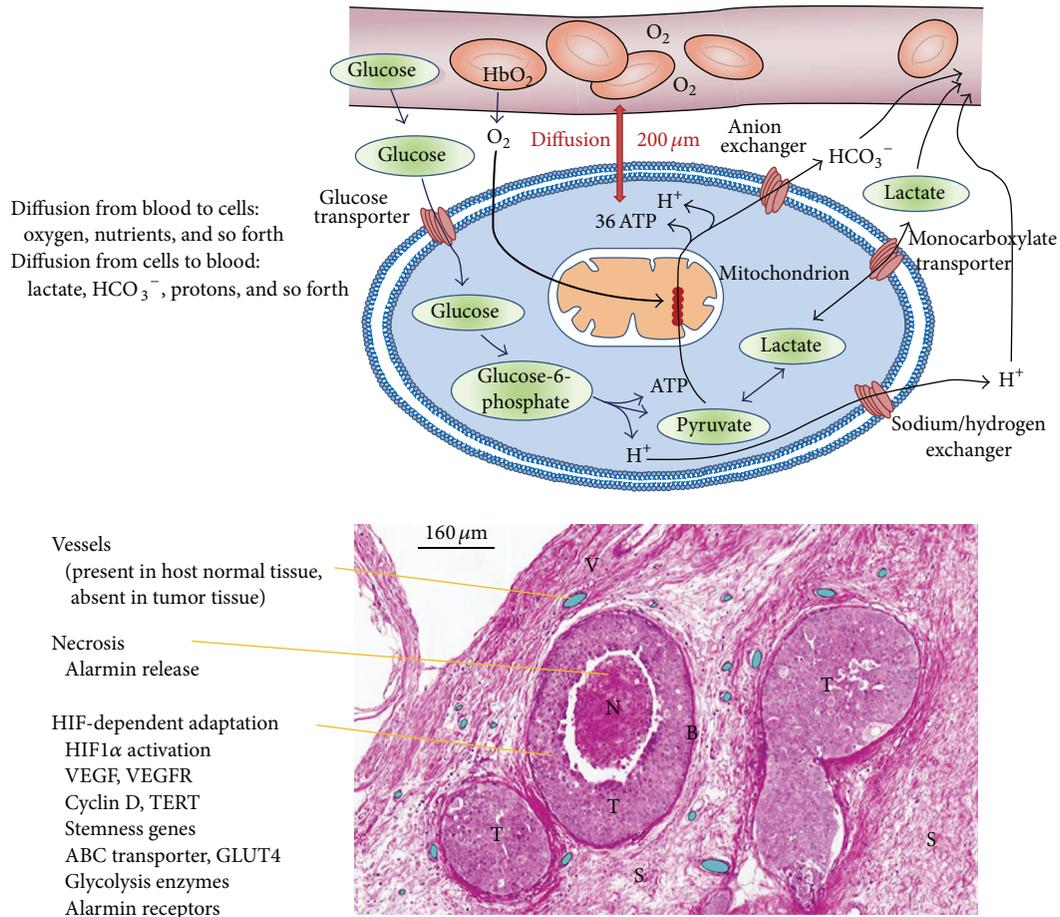


FIGURE 3: Hypoxia in cancer cells. Exchanges between blood in the vessels and cells are limited by distance and diffusion rate. Oxygen, glucose, and other nutrients diffuse from blood to feed the cells. Lactate, protons, carbonate,  $\text{CO}_2$ , and catabolites reach the blood for their disposal. In the early tumor growth, in the absence of angiogenesis, the central regions of tumoral mass, more distant from vessels, undergo necrosis, while peripheral regions survive and adapt to the hypoxia thanks to the HIF-dependent gene expression. Cancer stem cells seem to adapt more easily than differentiated cancer cells [53].

A further ROS increase causes DNA double strand breaks with increase in mutations (genomic instability) and cell damage (lipoperoxidation) leading to *necrosis* of cells that are more distant from vessels. However, the *activation of HIF1 $\alpha$*  by ROS in sublethally damaged tumor cells closer to the vessels allows the expression of HIF1 $\alpha$ -driven genes that contribute to their survival and growth thereby increasing their commitment to malignancy.

*Necrotic damage* includes plasma membrane fragmentation and release of intracellular molecules, some of which constitute alarmins or DAMPs (Damage-Associated Molecular Patterns) [57]. The interaction of released alarmins with their receptors triggers a proinflammatory gene expression in various cell types: resident innate immunity cells or leukocytes, usually expressing a number of alarmin receptors [58]. Importantly, tumor cells may also express alarmin receptors following hypoxia and HIF1 $\alpha$  activation. Alarmin receptor signaling leads to the activation of NF $\kappa$ B and then to the proinflammatory gene expression. This proinflammatory microenvironment can contribute to tumor progression (see below).

*Activation of HIF1 $\alpha$*  leads to the expression of hundreds of genes. Some important HIF1 $\alpha$ -dependent genes with their role in cancer cell as well as the effect of ROS are reported in Table 2. Many of these genes provide a first impulse (commitment) toward tumor progression. For example, VEGFs and their receptors are responsible for neoangiogenesis and for the possibility to grow above the limit of 400 microns in diameter [59]. Telomerase activation increases the proliferative potential [60]. Finally, changes in intermediate and energy metabolism provide a growth advantage to tumor cells that can quickly use glucose and glutamine [61, 62].

## 5. ROS, HIF1 $\alpha$ , and HIF1 $\alpha$ -Dependent Genes

ROS produced during hypoxia have a central role in stabilizing and activating HIF1 $\alpha$  which in turn triggers the molecular mechanisms important, for instance, to sustain survival, growth, motility, metastasis, and metabolic changes of a transformed cell. However, in some cases, ROS can also directly influence the activity of a number of gene families

TABLE 2: HIF-dependent genes in hypoxia adaptation in determining malignancy hallmarks.

HIF-dependent genes	Adaptation phenotype	ROS effect	References
VEGFs and VEGFRs	Neoangiogenesis, repair	Indirect	[63–65]
TERT (telomerase)	↑ telomere length and proliferative potential	Direct and indirect	[66–69]
Cyclin D1, cyclin D2	Increased proliferation	Indirect	[70]
TERT; c-Myc, SOX2, OCT4, KLF4, Notch	Stem cell renewal, differentiated cell reprogramming	Indirect	[71, 72]
ABC transporter	Drug resistance	Indirect	[75–77]
ALDA, PGK, GLUT-1	Changes in energy metabolism	Indirect	[61, 78]
PDGF, chemokine receptors	Motility and polarized migration	Indirect	[104, 105]
MMP9, MMPs	Integrity of basement membrane; invasiveness	Direct and indirect	[97–100]
Alarmin (DAMPs) receptors	NFκB activation; IRR gene express	Indirect	[80–82]

playing a critical role in pushing a transformed cell toward the acquisition of many hallmarks of malignancy.

**5.1. ROS and VEGFs and VEGFRs.** Increased expression of VEGFs and their receptors VEGF-R1 and R2 is due to the activation of HIF1 $\alpha$  by ROS and has the fundamental role of activating a tumor-specific neoangiogenesis, allowing the early tumor to grow over the dimensions (200–300  $\mu$ m), imposed by the simple diffusion of oxygen and nutrients [63]. Alternatively, ROS can also activate the MAPK pathway leading, again, to the increased expression of VEGF [64]. Interestingly VEGF, VEGF-R1, and R2 are expressed in human colorectal samples as well as in human colon cancer cell line, whereas no expression is observed in human normal colonic cell lines. This suggests that VEGF can be produced and secreted by cancer cells to sustain their proliferation and migration. Accordingly, VEGF silencing in colon cancer cells resulted in decreased growth and motility of colon cancer cells [65].

**5.2. ROS and Telomerase.** There are indications about a direct role of ROS on telomerase activity in hepatocellular carcinoma [66]. However, it is believed that the role of ROS may depend on their amount in the cells with low or mid levels being able to activate and high levels to inhibit telomerase activity [67]. Moreover, the effect of ROS on telomerase activity may depend on HIF1 $\alpha$  as previously demonstrated [68]. Recently, a role of telomerase in regulating cell survival, signaling, and mitochondrial function has been also proposed [69].

**5.3. ROS and Proliferation.** A further contribution to the proliferative potential is given by the HIF1 $\alpha$ -dependent activation of typical proproliferative genes such as c-Myc and cyclin D1 [70]. As discussed below, the increased proliferation of tumor cells, in which HIF1 $\alpha$  is active, is also linked to the metabolic reprogramming of these cells.

**5.4. ROS and Stem Cell Maintenance and Reprogramming.** In addition, HIF1 $\alpha$  activates OCT4 and Notch facilitating stem cell renewal, contributing to the immortalization and increasing survival of cancer stem cells [71, 72]. These observations derive from studies conducted using hematopoietic stem cells

(HSC). In fact, HSC pool is present in hypoxic regions of the bone marrow and shows a high expression of HIF1 $\alpha$  that is essential to maintain stem cell cycle quiescence through a mechanism involving p16/p19 proteins [73]. Moreover, SOX2 and KLF4 can also be activated along with ROS accumulation in glioblastoma cells thereby increasing the number of stem cells [74]. The observation that the canonical stemness genes are all overexpressed suggests the possibility that reprogramming differentiated tumor cells can have a role in increasing and maintaining the tumor stem cell compartment.

**5.5. ROS and Resistance to Chemotherapy.** Resistance of tumor cells and particularly of cancer stem cells is achieved by overexpression of ABC transporters driven by the HIF1 $\alpha$  transcription factor activated by reduced oxygen tension and/or ROS in the tumor microenvironment [75]. In particular, HIF1 $\alpha$  has been shown to increase expression of MDR1 [76]. Interestingly, also in this case, the level of ROS achieved inside tumor cells plays an important role. In fact, high levels have been shown to reduce HIF1 $\alpha$  and MDR expression and survival in spheroids from prostate tumor cells [77].

**5.6. ROS and Changes in Tumor Metabolism.** ROS and HIF1 $\alpha$  activation are responsible for the large metabolic reprogramming of cancer cells that requires other transcription factors such as Myc and proceeds with the overexpression of proteins such as glucose transporter 1 (GLUT1) for glucose uptake, glutaminase for glutamine usage, hexokinase II (HKII) for glycolysis, and carbonic anhydrase IX (CAIX) for control of intracellular pH, which assures the glucose and glutamine dependency and the fast growth of tumors [61, 78].

**5.7. ROS Contribution to Invasion and Metastasis.** ROS increase in tumor cells contributes to the activation of proteases involved in the recognition and degradation of basement membrane as well as in the formation of invadopodia [79]. Importantly, most of the invasion and metastasis genes are cocontrolled by HIF1 $\alpha$  and by NFκB, as also discussed in the next paragraph. In particular, ROS activates intracellular signaling mechanisms involving MAPK that depend on NFκB and are upstream of MMPs [79]. Moreover, ROS also activates the recruitment of a series of actin-associated proteins such as cofilin and fascin as well as adhesion (integrins) and signaling (c-Src tyrosine kinases)

TABLE 3: NF $\kappa$ B-dependent genes and their role in tumorigenesis.

NF $\kappa$ B-dependent gene families	Proinflammatory phenotype and malignancy hallmark	ROS effect	References
Inducible enzymes	Vasodilatation, migration	Indirect	[87–89]
Cytokines and receptors	Local amplification of IRR	Direct and indirect	[90–94]
MMPs and TIMPs	Invasion, migration	Direct and indirect	[97–100]
Adhesion molecules and their counterreceptors	Detachment, homing	Indirect	[101–103]
Chemokines and receptors	Migration, homing	Direct and indirect	[104, 105]
VEGFs and VEGFRs	Angiogenesis, repair	Indirect	[63–65, 106]
Growth and survival factors	Proliferation, antiapoptosis, repair	Indirect	[107, 108]
Acute-phase proteins	IRR amplification, chemotaxis, repair	Indirect	[109–113]
SOCS-1	Negative regulator of IRR	Indirect	[114–116]

proteins that assemble together to form the invadopodia [79]. Therefore, the metastatic potential of transformed tumor cells can be increased following upregulation of ROS production.

**5.8. ROS and Receptors for Alarmins.** In the presence of a hypoxic environment a number of cell types, including cancer and normal stem cells, express *de novo* or overexpress different alarmin receptors (similar to those present in activated leukocytes or CD45<sup>+</sup> cells) [80, 81]. RAGES, P2X7, TLRs, and others, upon activation by alarmins released by necrotic cells, converge in the activation of NF $\kappa$ B with a robust proinflammatory gene expression. This represents the key event to bridge the hypoxia to the adaptation with the expression of hundreds of genes related to the IRR and, very importantly, to the acquisition of classical properties of the malignant phenotype. This picture includes also the so-called EMT (epithelial-mesenchymal transition), in which involved genes can be HIF1 $\alpha$ - and/or NF $\kappa$ B-dependent target [82].

## 6. ROS, NF $\kappa$ B Activation, and the Full Acquisition of Hallmarks of Malignancy

The inflammatory response is finalized to defend cells by eliminating or detoxifying the harmful agents and to repair cell/tissue damage through differentiation of resident or recruited stem cells or by forming a scar of connective tissue. ROS are important players in both defensive and repairing functions of the inflammatory-reparative response (IRR). However, ROS can also cause cell damage, depending on the type, on the local concentration, and on how long and how specifically they interact with cell components [38, 83].

In the classical (“physiological”) IRR, defense and repair are efficiently coordinated by NF $\kappa$ B [84]. This transcription factor becomes fully activated through many synergic and confirming signals, such as cytosolic ROS, exogenous alarmins (i.e., virus, bacteria, other parasites, crystals, and fibers), and endogenous alarmins released by damaged and necrotic cells [85]. This signaling leads to NF $\kappa$ B nuclear translocation and activation and expression of ROS producing enzymes such as NADPH-oxidases, COX2, iNOS, and 5-lipoxygenases [86].

Once NF $\kappa$ B has been activated, a complex gene response occurs, with the expression of genes belonging to specific gene families including a large number of members

functionally related to the inflammatory and reparative response (see Table 3). Individually most of these genes have been implicated in the acquisition of crucial properties of the malignant phenotype, providing a coherent theoretical framework to explain the acquisition of most of the malignant hallmarks as an integrated response and adaptation to the tumor environment.

**6.1. ROS and Inducible Enzymes (NOX, COX2, 5-LOX, and iNOS).** Inducible enzymes produced in activated leukocytes upon activation of NF $\kappa$ B are responsible for the production of mediators such as prostaglandins, leukotrienes, plasmalogens, and NO, leading to the manifestation and amplification of the IRR. Their presence in tumor microenvironment and their expression by tumor cells have been two of the earliest observations involving inflammation in the pathogenesis and progression of cancer [87]. Molecules produced by these enzymes contribute to many aspects of tumor progression such as neoangiogenesis, recruitment of leukocyte to the tumor microenvironment, and changes for EMT [88]. Almost 15 years ago a landmark epidemiological study suggested that the use of low-dose aspirin for cardiovascular prevention drastically reduced the risk for colon cancer [89]. These epidemiological observations stimulated a number of other retrospective studies and controlled clinical trials on aspirin and other COX2 inhibitors in preventing tumors and their progression, giving rise to a new era in understanding the role of inflammation in tumor pathogenesis.

**6.2. ROS, Cytokines, and Their Receptors.** ROS have been shown to induce cytokine synthesis in different systems either directly or following activation of NF $\kappa$ B [90, 91]. Cytokines have a direct influence on IRR by targeting leukocytes, by polarizing the response as Th1 or Th2 and by stimulating the proliferation of target cells (CD45<sup>+</sup>) to reinforce and amplify the IRR [92]. Cytokines are present in most human tumor microenvironment, being produced by cancer cells themselves and/or by leukocyte infiltrate [93, 94]. Interestingly, tumor cells also express receptors for various cytokines in parallel with their degree of malignancy [92]. Therefore, thanks to the presence of cytokine receptors, tumor cells can be strongly influenced in their biology, such as proliferation rate (IL-2), and in their polarization (Th1 cytokines) and, probably, in the expression of adhesion molecules and their

counterreceptors, thus influencing the homing for metastasis [95, 96].

**6.3. ROS and MMPs and TIMPs.** Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) are HIF1 $\alpha$ - and NF $\kappa$ B-dependent genes normally expressed in activated leukocytes. ROS can activate MMP synthesis either directly [97] or, more frequently, through NF $\kappa$ B [98, 99]. It is well known that disruption of the MMP/TIMP activity ratio with a gain-of-function of protease activity over basement membrane and extracellular matrix proteins is present in malignant tumors and parallels the invasive potential [100]. Therefore, the key event for demolishing the physiological tissue barrier and starting invasion is basically controlled by both HIF1 $\alpha$  and NF $\kappa$ B through the expression of these genes.

**6.4. ROS, Adhesion Molecules, and Their Counterreceptors.** The activation of NF $\kappa$ B in leukocytes finely reprograms the expression of adhesion molecules for migration and for homing at constitutive district tissue or at damaged site. A ROS-induced NF $\kappa$ B-dependent and/or cytokine-dependent new expression of adhesive molecules occurs also in tumor cells, allowing for a number of biological changes typically related with malignancy [101, 102]. These changes include the ability to detach from the original tissue (i.e., cadherins), the ability to migrate following a specific chemotactic gradient and a path of ECM molecules (receptors for chemokines and integrins), and, finally, the identification of the homing site represented by activated endothelial or other tissue cells (ICAM-1, selectins, and their counterreceptors) [103].

**6.5. ROS, Chemokines, and Their Receptors.** Tumor cells express both chemokines and their receptors in parallel with their degree of malignancy [104]. The production of chemokines gives rise to a gradient which is probably the main responsible for the attraction of leukocytes and mononuclear infiltration in advanced tumors [104]. More importantly, the expression of chemokine receptors is a crucial event for the occurrence of metastasis. In fact, detachment from the primary tumor tissue must be followed by a vectorial migration along a chemotactic gradient, which implies the presence of specific receptors for the chemoattractant. CXCR4, a receptor for SDF1 $\alpha$ , is the best characterized receptor in tumor cells and has been definitely associated with progression and prediction of metastasis in many human tumors [104]. Interestingly, ROS can enhance CXCR4 function in prostate cancer cells [105]. Moreover, chemokines and their receptors are under the control of NF $\kappa$ B and can be, therefore, induced by ROS.

**6.6. ROS, VEGFs, and VEGFRs.** As also described above when talking about the role of ROS in inducing HIF1 $\alpha$  and VEGF, in order to be clinically relevant and detectable by the present imaging techniques, a tumor needs to grow at the dimension of a few mm in diameter. At the same time, this tumor must activate a process of neoangiogenesis, with an adequate expression of VEGFs and VEGFRs. VEGFs can

be produced by activated leukocytes and mesenchymal cells present in the tumor microenvironment or, more importantly, by tumor cells themselves under the influence of activated HIF1 $\alpha$  and NF $\kappa$ B [63, 106]. In the last case, it has been demonstrated that cancer cells (probably tumor stem cells and progenitors) may express also VEGFRs, suggesting the possibility that tumor cells can contribute to the formation of their new vascular tree [65].

**6.7. ROS, Growth, and Survival Factors.** HIF1 $\alpha$  and NF $\kappa$ B control a number of growth and survival factors and their receptors. This has been demonstrated in activated leukocytes (involved in tissue repair) and in hypoxia activated tumor cells. This is an additional advantage for tumor growth and a prerequisite for the establishment of a secondary metastatic tumor. The “seed and soil” hypothesis predicts that a favorable tissue environment is relevant for the occurrence of a metastasis [107]. In this case, growth and survival factors can be provided by activated leukocytes or mesenchymal cells of the microenvironment and by tumor cells themselves in which proliferative pathways are already activated (transforming oncogenes) or in which these genes are overexpressed upon ROS-dependent NF $\kappa$ B activation [108].

**6.8. ROS and Acute-Phase Proteins.** Acute-phase proteins have been considered as plasma markers useful to evaluate the systemic IRR. They include soluble and cell bound isoforms, such as C reactive protein, pentraxin-3, and other pentraxins; their functions are only partially elucidated. Similar to the other NF $\kappa$ B-dependent genes, they are expressed or overexpressed in hypoxia-activated tumor cells and in activated leukocytes. Their function in tumor progression is still debated. On one hand, they seem to inhibit tumor cell proliferation and to decrease with progression [109]; on the other hand they can be highly expressed in malignant cells compared to the host normal tissue [110–113].

**6.9. ROS, SOCS, and Negative Regulators.** NF $\kappa$ B activation includes also the expression of a number of proteins that function as negative key-regulator of IRR, such as SOCS-1 [114]. This latter protein is a member of SOCS family that suppresses the cytokine signaling via JAK/STAT, downregulates TLR expression and signaling, and decreases NF $\kappa$ B activity and duration [115]. This family and other negative regulators are considered as part of the normal feedback control of the IRR. As predicted by our hypothesis, SOCS-1 decreased in hypoxia-activated cells, as a physiological response of HIF1 $\alpha$ -NF $\kappa$ B integrated activation [116].

## 7. ROS and Sirtuins in Modulating Cell Redox Status and HIF1 $\alpha$ /NF $\kappa$ B Pathway

In mammals there are seven Sir2 homologs (SIRT1–7). Sirtuins are either class III nicotinamide adenine dinucleotide-(NAD<sup>+</sup>-) dependent deacetylase, desuccinylase, demalonylase, deglutarylase, or ADP-ribosyltransferases [117, 118]. Their dependence on NAD<sup>+</sup> directly links Sirtuins activity

to the metabolic state of the cells and to ROS. For this reason, Sirtuins have been implicated in many physiological functions such as gene silencing, cell death, longevity, inflammation, cancer and, importantly, the regulation of ROS levels through both ROS production and detoxification [117]. In addition, Sirtuins deacetylate and then directly regulate the activity of both HIF1 $\alpha$  and NF $\kappa$ B. However, while only for SIRT1, 2, 3, and 6 this regulatory function has been clearly demonstrated, it is now clear that also the other Sirtuins influence a number of metabolic pathways, converging in ROS regulation.

**7.1. SIRT1.** SIRT1 can be a target of damaging ROS and this may cause its relocalization, inactivation, and degradation. In particular, ROS can oxidize SIRT1 cysteine residues thereby inhibiting its activity and targeting the protein towards proteasomal degradation [119]. In fact, oxidative stress associated with inflammation downregulates the expression and the activity of SIRT1 and [119, 120] SIRT1 can be cleaved in inflammatory conditions [121, 122].

Another mechanism through which ROS can reduce SIRT1 activity involves NAD<sup>+</sup>. In fact, oxidative stress reduces the cellular level of NAD<sup>+</sup> suppressing the SIRT1-mediated signaling [123]. Interestingly, the increase of oxidative stress observed during aging in several rat tissues is accompanied by a concurrent decrease in the level of NAD<sup>+</sup> and in the activity of SIRT1 [124]. Similar changes were observed in human skin [125]. Recently, it was shown that, in mammalian cells, oxidative stress (H<sub>2</sub>O<sub>2</sub>) causes a cytosol to nucleus translocation of SIRT1 followed by its chromatin binding [126]. At least part of the SIRT1 pool appears to be targeted to double strand breaks, where it promotes repair and genomic stability. Genes that are normally silenced by SIRT1 become derepressed, leading to an altered pattern of transcription that resembles that of the aging brain, which is known to be subjected to significant oxidative stress. Finally, oxidative stress also activates PARP-1, which consumes cellular NAD<sup>+</sup> storage thereby decreasing SIRT1 activity [127].

On the other hand, downstream effects of SIRT1 on various transcription factors can affect directly ROS production and decrease or increase ROS resistance by influencing ROS detoxifying/scavenging systems. Importantly, SIRT1 deacetylates both HIF1 $\alpha$  and NF $\kappa$ B. In the case of NF $\kappa$ B, SIRT1 has been shown to deacetylate and inactivate the p65/relA component with inhibition of the NF $\kappa$ B complex [128]. In fact, both *in vitro* and *in vivo* observations have shown that SIRT1 or activation of SIRT1 by resveratrol and other polyphenols decreases inflammatory response by deacetylating and inhibiting NF $\kappa$ B [129]. These results are particularly interesting considering the central role of NF $\kappa$ B in many cellular pathways involved, for instance, inflammation, aging, and cancer. Controversial results have been reported, instead, for SIRT1/HIF1 $\alpha$  signaling. In fact, it is not yet clear if SIRT1 is influenced or not by hypoxia. Some reports indicate that hypoxia increases SIRT1 levels, whereas others indicate that hypoxia decreases SIRT1 [130, 131]. Under hypoxia SIRT1 deacetylates HIF1 $\alpha$ ; however, such reaction in some cases decreases HIF1 $\alpha$  activity, whereas in others

it increases HIF1 $\alpha$  activity. Obviously, more data must be accumulated on different cell lines, tissue, *in vivo* models, and tumors before the real function of SIRT1 on HIF1 $\alpha$  can be delineated. Moreover, it is also possible that SIRT1 action of HIF1 $\alpha$  differs in different tissues and organs. Given the widespread actions of SIRT1 in mammalian cells, it is likely that we have only scratched the surface of how this Sirtuin influences and interacts with ROS.

**7.2. SIRT2.** The connection between SIRT2 and ROS is still at the beginning. However, some results have shown that oxidative stress increases SIRT2 expression and nuclear accumulation. Nuclear SIRT2 then deacetylates and activates DNA binding of Foxo3a transcription factor that, in turn, results in increased transcription of its target genes and finally a decrease of ROS [132]. SIRT2 has also been shown to inhibit ROS production following LPS treatment of macrophages by suppressing NF $\kappa$ B activation [133]. In fact, SIRT2 has been shown to deacetylate subunit p65 of NF $\kappa$ B on lysine 310 (K310) in the cytoplasm [134]. In this way SIRT2 inhibits NF $\kappa$ B activation and transcription of NF $\kappa$ B target genes following TNF stimulation [134]. In fact, SIRT2 silenced cells have an increased activation of NF $\kappa$ B and a lower percentage of cell death following TNF exposure [134]. Finally, addition of a cell permeable PEP-1-SIRT2 protein to murine macrophages resulted in a reduction of ROS due to an increase in antioxidant enzymes such as MnSOD and catalase [135]. The precise role of SIRT2 in tumors is still a matter of debate with some reports showing a correlation between SIRT2 levels and poor prognosis in non-small-cells lung cancer or progression of cervical cancer [136, 137], whereas others report a correlation between low levels of SIRT2 and non-small lung cancer [138]. However, the current literature points to an oncogenic role of SIRT2 since its inhibition results in an impaired growth of lung, cervical, sarcomas, gliomas, and so forth by regulating cell cycle and autophagy [139, 140].

**7.3. SIRT3.** The expression and deacetylating activity of the mitochondrial Sirtuin SIRT3 have been extensively associated with a decrease of oxidative stress and an increase of cell vitality and lifespan. In particular, in arsenic-treated adipocytes, reduction of ROS by SIRT3 is due to the activation of transcription factors such as FOXO3a that, in turn, increases expression of ROS scavenging enzymes [141]. Deacetylation of FOXO3 by SIRT3 decreases proteasomal degradation of the former and increases resistance to ROS [141]. Decrease in ROS production after SIRT3 overexpression or activation (resveratrol) has been documented in different systems and pathologies such as age-related dysfunction of the auditory system [142], doxorubicin toxicity of cardiomyocytes due to oxidative stress [143], and hypoxic stress of endothelial cells [144]. In fact, SIRT3 control of HIF protein stability is achieved by controlling ROS levels as well as other metabolic pathways [145]. In particular, by decreasing ROS levels, SIRT3 stabilizes HIF degrading enzyme Prolyl Hydroxylase (PHD) lowering HIF1 $\alpha$  levels [146]. Interestingly, SIRT3 deficiency is associated with tumor growth in xenografts and SIRT3

expression is lowered in several cancers and cancer cell lines [146].

**7.4. SIRT4.** Very little is known about this mitochondrial Sirtuin and its role in the regulation of oxidative stress response. However, SIRT4 ADP-ribosylates and inactivates glutamate dehydrogenase 1 (GDH-1) decreasing insulin secretion in pancreatic cells [147]. Interestingly, SIRT4 seems to increase sensitivity of HeLa cells to oxidative agents and such effect has been linked to GDH-1 inhibition [148]. The mechanism involves a SIRT4-dependent opening of the permeability transition pore in the mitochondria with increased cell death following exposure of cells to oxidative stress [148]. Given the fact that SIRT4 is involved in the regulation of mitochondrial metabolism, it has been postulated that this Sirtuin must play an important role during metabolic reprogramming of cancer cells [149]. In particular, SIRT4 has been reported to have a tumor suppressive role because of its ability to suppress glutamine metabolism by ADP-ribosylation and inhibiting GDH [150]. In fact, SIRT4 suppresses Myc-induced B cell lymphoma and survival of human colorectal cancer cells [151, 152].

**7.5. SIRT5.** As in the case of SIRT4, the study of the role of this mitochondrial Sirtuin in oxidative stress response is still at the beginning. One study has shown that SIRT5 desuccinylates and activates Cu/ZnSOD, an effect that is accompanied by a reduction of ROS levels [153]. Other studies have, instead, linked SIRT5 desuccinylating activity to the inhibition of glutamine metabolism that produces glutamate necessary for the production of the antioxidant glutathione [154]. Therefore, in this case, SIRT5 could determine an increase in ROS. Of note, many tumors show a decreased expression of SIRT5 and an increased glutamine metabolism [155, 156]. Moreover, increased glutamine metabolism determines the production and diffusion of ammonia that, in turn, stimulates autophagy that limits ROS and DNA damage and inhibits tumor initiation [157]. However, autophagy has also a central role in the survival of established tumors by removing damaged organelles and toxic agents [158]. It must be concluded that the role of glutamine metabolism, mitochondrial Sirtuins, and ROS depends on the cancer type and, more interestingly, on the stage and context of the tumor.

**7.6. SIRT6.** SIRT6 has been linked to ROS, inflammation, and cancer by several studies. In particular, the expression of this nuclear Sirtuin is reduced in endothelial cells in the presence of ROS with acquisition of a senescent phenotype [159]. On the other hand, SIRT6 deacetylation of histone H3 regulates genes important for metabolism and telomeres maintenance thereby promoting resistance to oxidative stress damage [160]. SIRT6 controls cell metabolism by deacetylating and inactivating transcription factors such as HIF, NF $\kappa$ B, and Myc [161]. In fact, SIRT6 protects cardiomyocytes from hypoxia by increasing Bcl-2 and decreasing NF $\kappa$ B expression [162]. The inhibition of NF $\kappa$ B by SIRT6 determines its control over inflammation. Accordingly, SIRT6 downregulation is followed by an increase of NF $\kappa$ B transcriptional activity and

release of inflammatory cytokines such as IL-1 $\beta$  or synthesis of COX2, MMPs, and adhesion molecules [163]. Moreover, overexpression of SIRT6 prevented inflammation in a mouse model of collagen-induced arthritis [164]. Finally, SIRT6 has also been linked to malignancy. To this effect, SIRT6 is considered as a tumor suppressor because it deacetylates and inactivates HIF and NF $\kappa$ B but, more importantly, because it regulates the activation of the DNA repair machinery after both double strand breaks (DSB) and base excision repair (BER). In fact, SIRT6 declines with age or SIRT6 downregulation is associated with a decrease of BER [165]. On the other hand, however, the increased lifespan associated with SIRT6 could imply that SIRT6 may promote tumor formation and, in fact, recently an increase of SIRT6 has been associated with enhancement of tumorigenicity of hepatocellular carcinoma cells in the presence of TGF- $\beta$ 1, H<sub>2</sub>O<sub>2</sub>, and HOCl [166].

**7.7. SIRT7.** Initially identified as an activator of RNA polymerase I [167], SIRT7 is now also linked to tumor transformation by controlling cellular proliferation and survival. In fact, SIRT7 has been shown to reduce DNA damage markers following doxorubicin treatment of osteosarcoma cells as well as cell cycle arrest markers such as p21. Moreover, SIRT7 decreased apoptosis and p53 response pathway [168]. Furthermore, SIRT7 inactivation suppresses migration of cancer cells and tumor metastasis formation in a mouse model [169]. However, SIRT7, at least in cardiomyocytes, has an important role for cell survival and function because of its ability to deacetylate and inhibit p53, to protect from oxidative stress, and to reduce inflammation. In fact, SIRT7-deficient mice develop cardiac hypertrophy and inflammation and have a shorter lifespan [170].

In conclusion, giving the fact that Sirtuins regulate both HIF1 $\alpha$  and NF $\kappa$ B and the central role that these two transcription factors have during tumor progression, the possibility to act on Sirtuins in order to control HIF1 $\alpha$  and NF $\kappa$ B has drawn much attention. Therefore, presently, great deals of efforts have been put in producing Sirtuins modulators. Several natural compounds such as resveratrol, quercetin, piceatannol, and other polyphenols have been shown to modulate Sirtuins function and particularly SIRT1 [171, 172]. However, their action is not limited to SIRT1 but influences other enzymes such as phosphodiesterases (PDEs) and AMP kinase (AMPK) [173]. Unfortunately, so far, no specific inhibitors or activators for other Sirtuins are available.

## 8. Conclusions

This review has been an occasion to summarize evidences that cell redox status is the milieu where many players can contribute *initially* to the cell transformation and *successively* to the progression of the malignancy. Initially there is the formation of early small tumors in the absence of angiogenesis which then progress to grow as a vascularized clinically evident tumor, with the acquisition of all the hallmarks of malignant phenotype. From a molecular point of view, two transcription factors, namely, HIF1 $\alpha$  and NF $\kappa$ B, may be

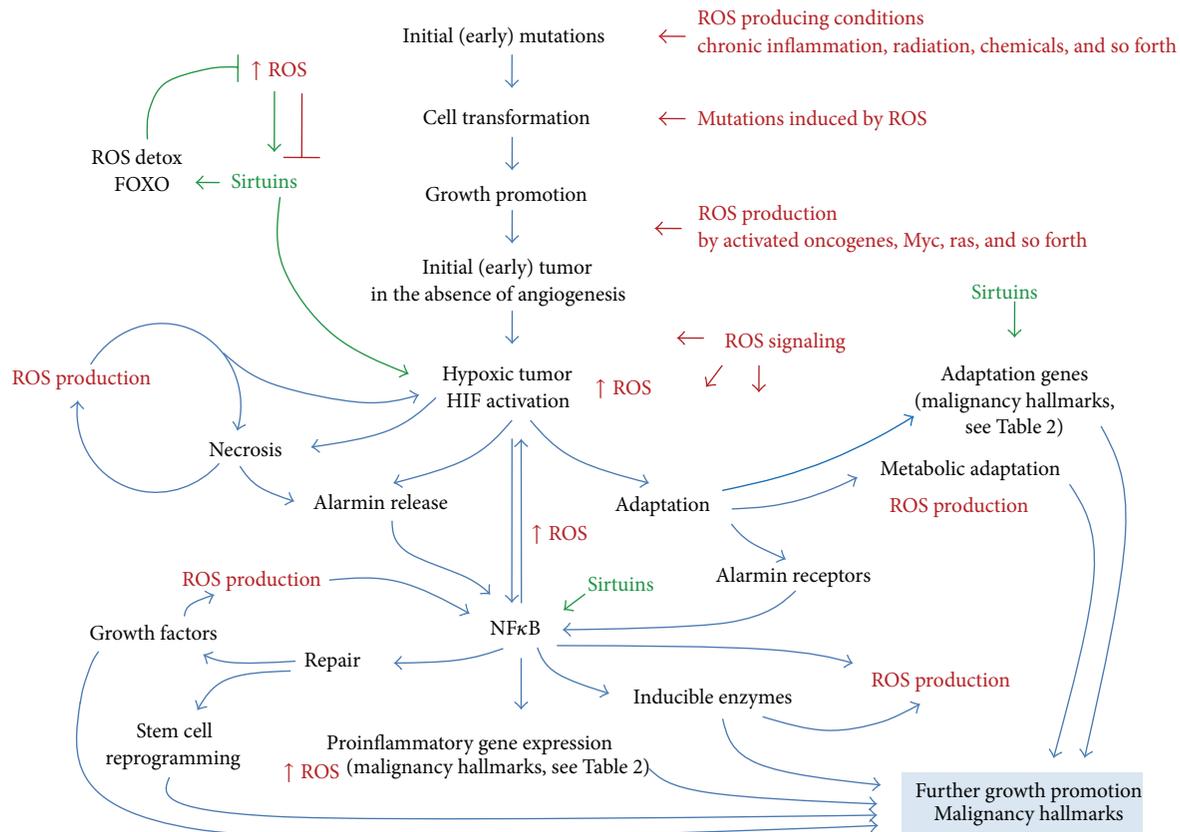


FIGURE 4: The natural history of a tumor from initial cell transformation to progression occurs and develops in a ROS-rich milieu which deeply influences, reinforces, and amplifies the different steps of the progression, the role of various molecular players, especially DNA repairing mechanisms, HIF, NF $\kappa$ B, and Sirtuins, and the full acquisition of all hallmarks of malignant phenotype.

considered as master regulators of tumor cell adaptation to ROS. In fact, both HIF1 $\alpha$  and NF $\kappa$ B are induced by ROS and, in turn, can regulate ROS production to sustain tumor cell survival and growth. An overview of the different aspects discussed in this review is summarized in Figure 4 in which ROS production and signaling as well as ROS effect on tumor cell metabolism and behavior are indicated. Figure 4 also indicates the important role of hypoxia and transcription factors HIF1 $\alpha$  and NF $\kappa$ B in orchestrating the tumor cell response to ROS. Therefore, it is conceivable that a number of exogenous agents and strategies aimed at influencing their activity could be used to reduce tumor transformation and progression.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

### Acknowledgments

The authors acknowledge the support of research Grant no. RF-2011-02349126 from Ministero della Salute, Italy. This work was also supported by Fondazione Roma.

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## Research Article

# NRF2 Regulates HER2 and HER3 Signaling Pathway to Modulate Sensitivity to Targeted Immunotherapies

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Received 5 June 2015; Revised 23 August 2015; Accepted 25 August 2015

Academic Editor: Amit Tyagi

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NF-E2 related factor-2 (NRF2) is an essential transcription factor for multiple genes encoding antioxidants and detoxification enzymes. NRF2 is implicated in promoting cancer therapeutic resistance by its detoxification function and crosstalk with proliferative pathways. However, the exact mechanism of this intricate connectivity between NRF2 and growth factor induced proliferative pathway remains elusive. Here, we have demonstrated that pharmacological activation of NRF2 by *tert*-butylhydroquinone (tBHQ) upregulates the HER family receptors, HER2 and HER3 expression, elevates pAKT levels, and enhances the proliferation of ovarian cancer cells. Preactivation of NRF2 also attenuates the combined growth inhibitory effects of HER2 targeting monoclonal antibodies, Pertuzumab and Trastuzumab. Further, tBHQ caused transcriptional induction of HER2 and HER3, while SiRNA-mediated knockdown of NRF2 prevented this and further caused transcriptional repression and enhanced cytotoxicity of the HER2 inhibitors. Hence, NRF2 regulates both HER2 and HER3 receptors to influence cellular responses to HER2 targeting monoclonal antibodies. This deciphered crosstalk mechanism reinforces the role of NRF2 in drug resistance and as a relevant anticancer target.

## 1. Introduction

The receptor tyrosine kinases (RTKs) are key drivers of normal cellular proliferation, differentiation, and survival, as well as determinants of cancer initiation, maintenance, and progression [1–4]. Dimerization and stimulation of the intrinsic tyrosine kinase in RTKs lead to the phosphorylation of tyrosine residues in the intracellular domain of the receptors. The phosphotyrosine residues serve as docking sites to recruit a number of signal adapter proteins containing the so-called SH2 and PTB domains, which link RTKs to different cellular signaling pathways such as PI3K/AKT/mTOR, MAPK, and STAT pathways [5, 6]. Among the RTK superfamily receptors are the type I RTKs that belong to the epidermal growth factor receptor (EGFR or HER) family. The HER receptor network contains four members (HER1, HER2, HER3, and HER4) whose activation kinetics depend significantly on their expression levels which vary across different cells and cancers [7]. Likewise it is these variations combined with

receptor interaction that drive and confer complexity in the HER receptor family behaviour.

The two receptors, HER2 and HER3, are nonautonomous and possess certain defining features, in that HER2 has autokinase activity but no known ligands, and HER3 is a pseudokinase receptor that lacks tyrosine kinase activity. These features define the interaction between the HER2 and HER3 receptors and for forming active homodimer and heterodimer complexes. Specifically, mutation or increased gene copy number leads to overexpression of HER2 receptors in cancer cells causing constitutive activation of proliferative pathways in the absence of ligand through homodimerization and RTK autophosphorylation [1, 5, 6, 10]. HER2 functions as the shared coreceptor for EGFR, HER3, and HER4 receptors, and these heterodimeric complexes are activated by the partner ligands [11, 12]. Moreover, HER2 is the preferred heterodimerization partner of HER3, whilst HER2 overexpression is believed to enhance the signaling

from HER3 receptor in response to binding of its specific ligand, neuregulin. As such, HER2-HER3 heterodimers are known to be the strongest elicitors of the PI3K/AKT/mTOR pathway [13–17]. Coexpression of the different receptors, the diversity in their ligand-independent and ligand-dependent activation, variation in their preference towards dimerization partners, and receptor-dependent specificity in cells play a major role in both redundancy in the HER network interaction and effective drug target identification [17–21]. Further complexity in HER2/HER3 activation and signaling arises from the complex transcriptional and posttranslational coregulation of HER2/HER3 receptors and their ligands following HER receptor specific targeted therapies which often lead to inconsistent tumour responses [13, 17, 21].

Nuclear factor- (erythroid-derived 2-) like 2 (NRF2) is a leucine zipper transcription factor and the master regulator of the antioxidant response (AR) pathway. It drives both basal and oxidative stress-induced transcription of a battery of phases I, II, and III detoxification enzymes and cytoprotective genes [22–24] as well as other genes of the metabolic and signal transduction pathways [14, 23, 25]. This is achieved by heterodimerization of NRF2 with small MAF proteins and binding to some genome *cis*-acting factors called antioxidant response elements (ARE) or electrophile response elements (EpREs) within the promoters of its target genes [26, 27]. Under basal conditions, only a low level of free NRF2 is available in the cytoplasm with some translocating into the nucleus to drive the basal transcription of target genes.

Like the HER receptors [28–31], NRF2 is a recognised agent in cellular proliferation and adaptation to reactive oxygen species (ROS) and in conferring therapeutic resistance to cancers [32–34]. Importantly, NRF2 activation and KEAP1 inactivation mutations leading to permanent constitutive adaptive activation of the NRF2 pathway are frequently observed in cancers [35–37]. Also several therapeutic strategies such as anticancer radio- and chemotherapy greatly depend on ROS manipulation to induce cytotoxicity. Paradoxically, there is a growing body of evidence implicating HER2/HER3, NRF2, and ROS in the promotion of cellular proliferation and therapeutic resistance in cancer cells [38, 39]. Cancer cells have been shown to evolve intricate mechanisms of cellular resistance towards both ROS and other cellular damaging agents as demonstrated by a very robust antioxidant sensing and ROS neutralising mechanisms as well as a highly efficient cytoprotective systems [33, 34, 40–42].

ROS is long not only recognised as the regulator of NRF2 stability and activity but has also been shown to trigger both the AR and the HER family receptor pathways with concomitant transcriptional upregulation of HER2/HER3 complexes and subsequent activation of their functions [30, 31, 43, 44]. Hence, ROS might serve as the point of convergence and as such establish cross relationship between the two pathways. Furthermore, components of the receptor regulated PI3K and MAPK have been shown to regulate NRF2 function [45–47], while many aspects of RTK signaling are regulated by ROS whose levels are directly modulated by NRF2 function [48, 49].

Since NRF2 is a transcription factor to several hundreds of genes, including proto-oncogenes, it is feasible that

HER2/HER3 receptors are transcriptional targets of NRF2 via direct or indirect means involving ROS. Thus this study aims to investigate this and identify crosstalk between the NRF2 dependent AR pathway and the HER2/HER3 receptors signaling pathway, in order to determine their potential interdependence in eliciting cellular proliferation, cytoprotection, and responses to therapies.

By generating gene transcriptional reporter assays, carrying out pharmacological activation or siRNA knockdown of NRF2, and performing HER2/HER3 functional inhibition and activation strategies, we have identified a direct node of functional integration of the two pathways in our ovarian cancer cell model which converges at NRF2. We demonstrated that inhibition of NRF2 leads to disruption of the antioxidant pathway and attenuation of HER2/HER3 signaling and that this is as a consequence of transcriptional repression of both *HER2* and *HER3* genes. Furthermore, we have demonstrated that this functional link could be utilised to either sensitise or reproduce resistant responses in our cell model. Thus, this study reveals a new mechanism of crosstalk between AR and HER2/HER3 pathways and opens up novel avenues of targeting and manipulating the NRF2-AR to uncouple and sensitise HER2/HER3 pathways resistant ovarian cancer cells to targeted immunotherapeutics.

## 2. Materials and Methods

**2.1. Cell Lines, Culture Conditions, and Treatments.** Human ovarian cancer cell lines PEO1 and SKOV3 were maintained in RPMI 1640 media (Gibco Invitrogen) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin in an atmosphere of 5% CO<sub>2</sub> and incubated at 37°C. Before experimental treatments, cells were grown for 24 h in RPMI 1640 media prepared as above but replacing FBS with 5% double charcoal stripped FBS (Fisher). Heregulin- $\beta$ 1 (HRG, Sigma) was used by preparing 1  $\mu$ mol/L stock solution made with 5% trehalose and 10% FBS in phosphate buffered saline (PBS) and diluted to a final concentration of 1 nmol/L with media during treatments. Monoclonal antibodies targeting HER2 receptor, Pertuzumab and Trastuzumab (RTKi), were used by directly diluting the drugs in media to a final concentration of 20  $\mu$ g/mL. Tert-butylhydroquinone (tBHQ) stock solution (Sigma) was made with Dimethylsulfoxide (Fisher) and diluted to a final concentration as required with media. For ROS detection, 2',7'-Dichlorofluorescein diacetate (DCFDA, Sigma) solution was prepared with Dimethylsulfoxide in amber tubes to a concentration of 50 mM and stored at -20°C in the dark until used. For cytotoxicity assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was needed by making a stock solution of 5 mg/mL in PBS and filter sterilising it. The solution was stored at 4°C in the dark until used.

**2.2. Reactive Oxygen Species (ROS) Detection.** ROS detection assay was performed by using 2',7'-Dichlorofluorescein diacetate (DCFDA) staining (Sigma). Briefly, cells were seeded in triplicate at a density of  $0.2 \times 10^5$  cells/well of opaque

flat bottom 96-well tissue culture plates in 100  $\mu\text{L}$  media without phenol red and allowed to grow for 18 h. Following transfection, cells were washed with PBS and maintained in 100  $\mu\text{L}$  of phenol red-free medium and further incubated for 24 h. A 50 mM stock solution of DCFDA was added to each well containing 100  $\mu\text{L}$  preexisting media to achieve a final concentration of 25  $\mu\text{M}$  and incubated for 45 min at 37°C. Fluorescence signal intensities indicating ROS levels were recorded by taking readings using 96-well fluorescent multiplate reader (MODULUS, Promega) using excitation and emission spectra of 485 nm/535 nm. To normalise the fluorescence signal, cells in the same wells were stained with Coomassie brilliant blue stain (Sigma) for 1 h and washed with distilled water and 10% sodium dodecyl sulphate (SDS) solution was added to release the absorbed dye for 10 min while shaking. The absorbance values at 595 nm were then recorded using a multiplate absorbance reader (MODULUS, Promega) data used after normalising the fluorescence values.

### 2.3. Cloning and Expression Vectors Used in the Study.

Closely 1.5 kb proximal promoter regions of *HER2* and *HER3* were cloned and used in the current study. The *HER2* primer sequences used for each construct were *HER2* forward: 5'-GTGCTCGAGGCAAGAAGGGTGCATTTTGAAG-3' and *HER2* reverse: 5'-GTCAAGCTTGTCTCTTGGATGGCCATC-3'. The *HER3* primer sequences used for each construct were *HER3* forward: 5'-GTGCTCGAGGCCCTCTAGGTTGCATATCAATAGG-3' and for *HER3* reverse: 5'-GTCAAGCTTGAAAAGCAAGCCCAGCAC-3'. For cloning *HER2* and *HER3* promoters (pr*HER2* and pr*HER3*, resp.), total genomic DNA was isolated from human cells using DNeasy Blood and Tissue Kit (Qiagen) and quantified using AstraGene microvolume spectrophotometer (AstraNet). 100 ng of the genomic DNA was used to amplify the promoter sequences (MyFi mix, Bionline) using relevant primers that incorporated *XhoI* and *HindIII* restriction endonuclease sites 5' and 3' ends of the amplified promoters, respectively. PCR conditions for promoter amplification were initial denaturation of 95°C for 7 min followed by 35 cycles of 95°C for 30 s for denaturation, 50°C for 30 s for annealing, and 72°C for 90 s for extension and a final extension for 10 min at 72°C. The PCR products were run and extracted from agarose gel (Qiagen), digested using *XhoI* and *HindIII* restriction enzymes (Promega), and ligated into PGL3 vector (Promega) to create *HER2* and *HER3* promoter constructs (pr*HER2* and pr*HER3*, resp.) driving the expression of luciferase gene for utilisation in dual luciferase reporter assay (Promega). The integrity of cloned sequences was determined by sequencing the plasmids using commercial sequencing service (<http://www.dnaseq.co.uk/>). All cloned constructs were transfected into relevant cell lines using Lipofectamine 3000 (Life Technologies).

**2.4. Protein Extraction and Immunoblotting.** For immunoblotting, cells were seeded in 60 mm tissue culture plates and grown until being 70% confluent. At the time of protein harvest, cells were trypsinized (Gibco Invitrogen) and washed with PBS. Protein lysates were prepared using radio immune

precipitation assay buffer (Pierce Biotech) supplemented with protease and phosphatase inhibitor cocktail (Pierce Biotech) and subjected to sonication of 2 cycles for 10 s at 50% pulse. The final mixture was shaken gently on ice for 15 min and the protein supernatant was obtained following centrifugation of the lysates at 14000 g for 15 min. Proteins obtained were quantified by Bradford assay (Sigma-Aldrich) using bovine serum albumin as a standard and sample buffer (Nupage LDS, Invitrogen) was added to protein lysates, heated at 70°C for 20 min, and stored at -20°C until further use. Once the protein lysates were prepared, they were loaded into wells of 4–12% gradient SDS-polyacrylamide gels (Nupage Bis-Tris gels, Life Technologies) and subjected to electrophoresis at 200 V for 1–2 h. Following this, proteins were transferred to polyvinylidene difluoride membranes (PVDF, GE Amersham) using the XCell SureLock Mini-Cell system (Invitrogen) at 50 V for 90 min and processed using a commercially available kit (WesternBreeze Chromogenic Immunodetection Kit, Invitrogen). Nonspecific reactivity was blocked by incubation with the blocking reagent supplied in the kit. Membranes were further treated by incubating with primary antibodies (Table 1) for 2 h at room temperature or overnight at 4°C, followed by incubation for 30 min at room temperature with appropriate secondary anti-rabbit antibody supplied in the kit. Bands were visualized with the 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride based chromogenic substrate. For loading control, immunoblotting of the same lysates was performed using either Beta-actin ( $\beta$ -actin) antibody (Abcam Bioscience, UK) or the PVDF membranes with transferred proteins visualised using Ponceau stain (Sigma).

**2.5. Luciferase Reporter Assay.** For the analysis of promoter activities and transcriptional regulation, the 1.5 kb promoter regions of *HER2* and *HER3* genes cloned in pGL3 basic vector (Promega) were transfected into relevant cell lines. Briefly, cells were seeded in triplicate in 24-well plates at a density of  $2 \times 10^5$  cells per well and allowed to attach for 18 h. Following this, cells were transfected with either 1  $\mu\text{g}$  of empty pGL3 basic vector (Promega) or pGL3 basic vector with cloned fragments of *HER2* or *HER3* promoters driving the expression of luciferase gene, using Lipofectamine 3000 as transfection reagent according to manufacturer's instructions (Life Technologies). Cotransfection was also performed with 0.2  $\mu\text{g}$  of pRL-CMV vector (Promega) to provide for an internal control of transfection. Following this, cells were allowed to grow for 24 h, subjected to desired treatments and lysed and protein lysates transferred to opaque white bottom 96-well plates. The dual luciferase activity of fire fly luciferase (from cloned promoters) and Renilla (internal control) in the harvested lysates was measured sequentially by following manufacturer's instructions (Promega) and taking luminescence readings in luminometer (MODULUS, Promega). To determine the transcriptional activity of NRF2 in PEO1 and SKOV3 cell lines, basic pGL3 vector (Promega) containing cloned 8 x *cis* regulatory ARE promoter elements was transfected into the cell lines grown in 24-well plates and subjected to dual luciferase reporter assay (Promega) as described above.

TABLE 1: Antibodies used in the study.

Antibody	Host	Catalogue number	Company
NRF2	Rabbit	Sc-722	Santa Cruz
Phospho-NRF2 S-15	Rabbit	ab76026	Abcam
HER2	Rabbit	2165S	Cell Signalling
HER3	Rabbit	4754S	Cell Signalling
Phospho-HER2 T877	Rabbit	2241S	Cell Signalling
Phospho-AKT 473	Rabbit	4060S	Cell Signalling
BID	Rabbit	2002	Cell Signalling
Phospho-ERK p44/p22	Rabbit	4379	Cell Signalling
Heme oxygenase-1 (HO-1)	Rabbit	Sc-10789	Santa Cruz
Alexa Fluor 488 conjugated secondary antibody	Rabbit	ab150077	Abcam
Alexa Fluor 568 conjugated secondary antibody	Rabbit	ab175471	Abcam

**2.6. SiRNA Transfection.** Small inhibitory RNA (SiRNA) was used to knockdown NRF2 (Hs\_NFE2L2.6, Qiagen). For SiRNA transfection, cells were seeded in triplicate either in 24-well plates ( $0.5 \times 10^5$  cells), in 60 mm plates with cells grown on poly-L lysine coated coverslips ( $0.5 \times 10^6$  cells), or in 96-well plates in triplicate ( $2 \times 10^4$ ) and allowed to grow for 24 h. Following this, cells were cotransfected using either 20 pmol SiRNA and 1  $\mu$ g of different PGL3 promoter constructs (24-well plate) or 75 pmol and 100 pmol SiRNA only (60 mm plates) or 7 pmol of SiRNA (96-well plate) and incubated for a further 24 h. Cells transfected in 24-well plate were further processed for dual luciferase assay and those in 60 mm plates were harvested for immunoblotting or used for imaging analysis while those in 96-well plates were processed for cytotoxicity assay. In all cases, scrambled SiRNA was used as a control while transfection was performed using Lipofectamine 3000 (Life Technologies) according to manufacturer's instructions.

**2.7. Cytotoxicity Assay.** For cytotoxicity (or cell viability) assay, cells were seeded in triplicate at a density of  $0.5 \times 10^4$  cells in 96-well plate and allowed to attach for 18 h. On the day of treatment, old media were removed and 80  $\mu$ L of media containing relevant drugs was added and the plate was incubated for the required period of time. On the day of assay, 20  $\mu$ L of the 5 mg/mL MTT stock was added to each well and plate was further incubated for 4 h. Following this, the old media with MTT were removed, cells were gently washed with prewarmed PBS, and 100  $\mu$ L of DMSO was added to solubilise the internalised MTT by shaking over an orbital shaker for 15 min. Absorbance of the released dye was measured and recorded using multiplate reader (MODULUS, Promega) at 540 nm.

**2.8. Immunocytochemistry/Immunolabelling.** For immunocytochemistry, exponentially growing cells were seeded at a density of  $5 \times 10^4$  cells in complete media onto poly-L lysine (Sigma-Aldrich) coated cover slips placed in a 12-well tissue culture plates. Next day, following relevant treatments, cells were washed three times with ice cold PBS

and fixed in 3.5% paraformaldehyde in a standard PBS at room temperature for 30 min. Following this, cells were gently washed twice with 1 mL of PBS, permeabilized with 0.3% Triton X-100 for 10 min, and, following three washes with PBS, blocked with a solution containing 1% goat serum, 1% bovine serum albumin, and 0.05% Triton X-100 in PBS for 30 min. Cells were then incubated with relevant primary antibody (Table 1) diluted in blocking solution for 1 h, washed three times with 0.1% Triton X-100/PBS for 5 min, and then incubated with Alexa Fluor 488 or 568 conjugated goat anti-rabbit (Table 1) for 30 min. After subsequent three washes with the 0.1% Triton X-100 in PBS for 5 min, cover slips with cells were mounted on slide using 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) containing mounting reagent (Life Technologies) and imaged under relevant filters with a Leica DMiRe2 electronic microscope.

**2.9. Statistical Analysis.** All statistical analyses were performed using statistical software SPSS (IBM, version 22). Test for normality of data was determined by Shapiro-Wilk and Kolmogorov and Smirnov tests. The significance ( $p$  value) of differences of pooled results was determined by either independent  $t$ -tests or One WAY ANOVA followed by post hoc Tukey's tests. Significance was defined as \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , and \*\*\* =  $p < 0.001$ .

**2.10. Imaging and Analysis.** Quantitative analysis of raw immunoblots was performed by capturing the images in high resolution TIFF format files using a charge-coupled-device camera (AxioCam MRc, Carl Zeiss) and subjected to Gelpro analysis software, version 3.1 (Gelpro Media Cybernetics) for integrated optimal densitometry. Fluorescence images of immunocytochemistry were collected under relevant excitation and emission filters depending on the fluorotype under Leica DMiRe2 electronic microscope equipped with iXonEM +897 EMCCD camera (ANDOR Technologies Ltd.). Images were analysed using multidimensional microscopy software Andor Module iQ Core. Colocalization assay was performed and determined with software integral features supplied by Andor iQ Core software. Data were generally expressed as mean  $\pm$  S.D. for individual sets of experiments.

### 3. Results

**3.1. Pharmacological Activation of NRF2 Enhances Ovarian Cancer Cell Growth and Protects from Cytotoxicity Caused by HER2-Targeted Immunotherapeutic Agents.** Numerous studies have shown that NRF2 promotes resistance to chemotherapeutic agents [50, 51] and contributes to general cytoprotection, metabolic reprogramming, and cell survival [52–55]. On the other hand, targeted immunotherapy involving inhibitory monoclonal antibodies against HER2 receptor has generated interest in recent years as a potential strategy to overcome ovarian cancer cell therapeutic resistance [17, 56]. Using HER2 overexpressing and low expressing ovarian cancer cell lines SKOV3 and PEO1, respectively [57], we first examined whether preactivation of NRF2 would change the cytotoxic responses of these cells to HER2-targeted immunotherapeutic agents Pertuzumab and Trastuzumab. For this, cells were grown in media containing 5% charcoal stripped FBS and 1 nmol/L Heregulin (HRG), a ligand for the HER3 receptor [58] for relevant treatments. Firstly, we found that pharmacological activation of NRF2 by tBHQ alone was sufficient to enhance the proliferation of both cell lines for six days (Figure 1). On the other hand and as expected, exposure of cells to HER2 inhibitors, Pertuzumab and Trastuzumab, inhibited the proliferation of both cell lines for up to 4 days of treatment, while losing its inhibitory effect on day 6. Interestingly, pretreatment of cells with 200  $\mu$ M tBHQ for 5 h before the introduction of the HER2 inhibitors significantly protected cells from the inhibitory action of the subsequently added HER2 targeting monoclonal antibodies. This was consistent for both cell lines and for all the treatment days tested (Figure 1). Furthermore, inclusion of tBHQ with the inhibitors not only protected the cells but increased survival even beyond the untreated levels on days 2, 4, and 6 in PEO1 and days 1, 2, and 6 in SKOV3 cell lines (Figure 1). This demonstrated that NRF2 activation is not only implicated in resistance to genotoxic agents as previously demonstrated [55] but can also lead to resistance to immunotherapies involving Pertuzumab and Trastuzumab whose actions otherwise are very specific to HER2 receptors and unrelated to antioxidant pathway.

**3.2. TBHQ Treatment Causes Protein Induction of HER2 and HER3 and Parallel Increase in Phospho-AKT S473.** Previous studies have examined the crosstalk between growth promoting MAPK and PI3K pathways and NRF2 antioxidant pathway in numerous cell systems. However, in the majority of such studies, the focus was regulation of NRF2 by these kinases [46, 47, 59, 60]. The observation that preactivation of NRF2 led to resistance against agents of targeted therapy (Figure 1) suggested potential regulation of HER2 dependent growth pathways by NRF2. Hence, we next exposed PEO1 and SKOV3 cell lines to a single concentration of tBHQ for 4 h and examined the effects of such treatment on the protein levels of HER2 and HER3 and their downstream substrate pAKT Ser 473. Firstly, higher levels of HER2 receptor were confirmed in SKOV3 cell line that was also accompanied by induced basal pAKT, consistent with previous reports [61]. Secondly, following tBHQ treatment, we saw induction of

total HER2 and parallel consequential induction of pAKT levels in both cell lines. Further, HER3 was found to decrease in PEO1 while being induced in SKOV3, demonstrating a differential regulation of the receptors in the two cell lines (Figures 2(a) and 2(b)). This is consistent with the increased proliferation seen following tBHQ treatment, as an enhanced cell surface expression of receptors would lead to a greater degree of binding to their HRG ligand and triggers growth promoting signaling.

To explore further HER2 and pAKT induction by tBHQ at a single cell level, we performed subcellular localisation by fluorescent double immunolabelling of these proteins in PEO1 and SKOV3 cell lines following the same treatments (Figure 2(c)). Consistent with Figure 2(a), we saw higher expression of HER2 in SKOV3 as compared to PEO1. For both cell lines, pAKT was found uniformly distributed in the cytoplasm and nucleus. This could be indicating the constitutively active nature of this pathway and could be explained by the presence of HRG in the media. Following tBHQ treatment for 4 h, we saw an increase in HER2 expression and an accompanying increase in pAKT levels as well. Superimposition and colocalisation of the images captured in the red and green fluorescence channels to indicate HER2 and pAKT, respectively, were performed and showed increased localisation of the two proteins, as demonstrated by the appearance of yellow fluorescence following tBHQ as compared to untreated controls (Figure 2(c)). To confirm and measure the enhanced colocalisation following treatments, we also performed further imaging analysis by generating cytofluorograms and found that Pearson's coefficient of correlation ( $r$ ) increased in both cell lines following tBHQ exposure (Figure 2(d)). Altogether, these data illustrated effects of tBHQ treatment on RTK mediated growth signaling. This was demonstrated by induction of HER2 and HER3 and activation of AKT following pharmacological activation of NRF2, which supported the enhanced proliferation seen before (Figure 1).

**3.3. Pharmacological Activation of NRF2 Causes Transcriptional Induction of HER2 and HER3 Genes.** Previous studies have shown transcriptional perturbation of HER2 and HER3 following different targeted therapy treatments [17]. In some contexts, this was proposed to be used as a biomarker for treatment response [61]. After finding the modulatory effects of tBHQ treatment on protein levels of HER2 and HER3 receptors, we next wanted to identify the mechanism of this upregulation. Specifically, we wanted to examine whether the protein inductions seen in Figure 2 result from transcriptional regulation. To determine this, we generated transcriptional reporter assays for both HER2 and HER3 receptors. This involved developing HER2 and HER3 promoter driven luciferase reporter system (named prHER2 and prHER3, resp.). We transfected these luciferase reporter systems carrying 1.5 kb of the upstream promoter regions of the two receptors into both PEO1 and SKOV3 cell lines to first determine their basal level of transcription and then studied the effects of tBHQ treatment. Figure 3(a) interestingly revealed that SKOV3 cell line exhibited enhanced basal transcription of both *HER2* and *HER3* genes as compared to

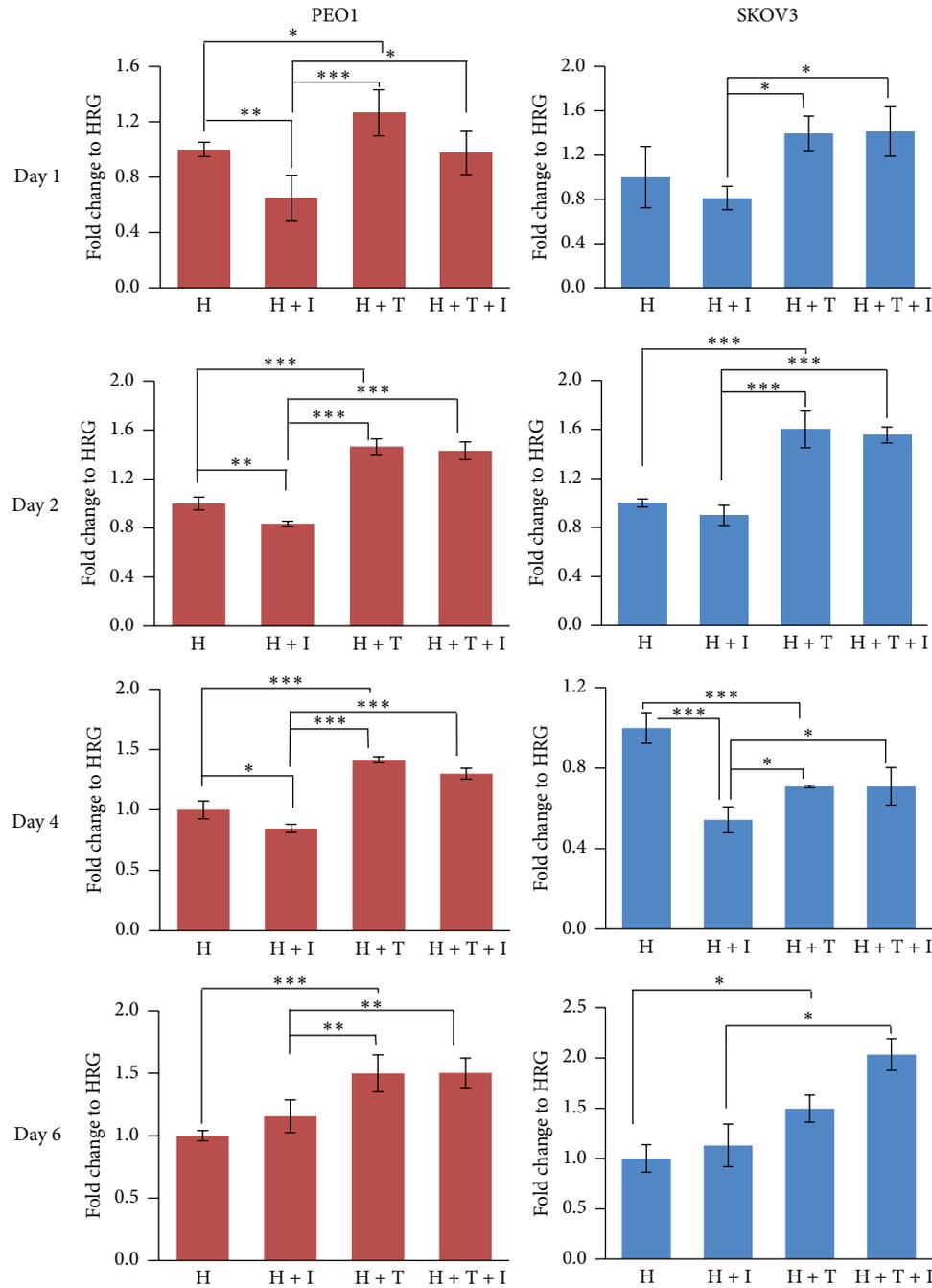


FIGURE 1: NRF2 activation causes cytoprotection from HER2-targeted agents, Pertuzumab and Trastuzumab. PEO1 or SKOV3 cells in the presence of 1 nM HRG were either left untreated (H) or treated with 20  $\mu\text{g}/\text{mL}$  of HER2 inhibitors Pertuzumab and Trastuzumab (H + I), 200  $\mu\text{M}$  tBHQ (H + T), or combination of inhibitors and tBHQ (H + T + I). TBHQ was added 5 h in advance. Cell number was assessed indirectly by use of the MTT assay. Values shown are means  $\pm$  S.D. of triplicates normalised to untreated controls expressed as 1. Statistical significance was calculated between H + I, H + T, and H + T + I groups by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , and \*\*\*:  $p < 0.001$ .

PEO1 cell line (Figure 3(a)). However, the previous western blot analysis in Figure 2 showed higher basal levels of HER3 in PEO1 as compared to SKOV3 whereas HER2 levels were consistently higher in SKOV3. This illustrated that the over-expression of HER2 in SKOV3 cell line could be explained by both gene amplification [62] and higher basal transcription.

We next exposed cells transfected with the prHER2 and prHER3 reporter assays to increasing concentrations of tBHQ to further explore the nature of this transcriptional regulation. Strikingly, both PEO1 and SKOV3 cell lines exhibited significant dose-dependent transcriptional induction of HER2 (Figure 3(b), blue bars). Interestingly, prHER3

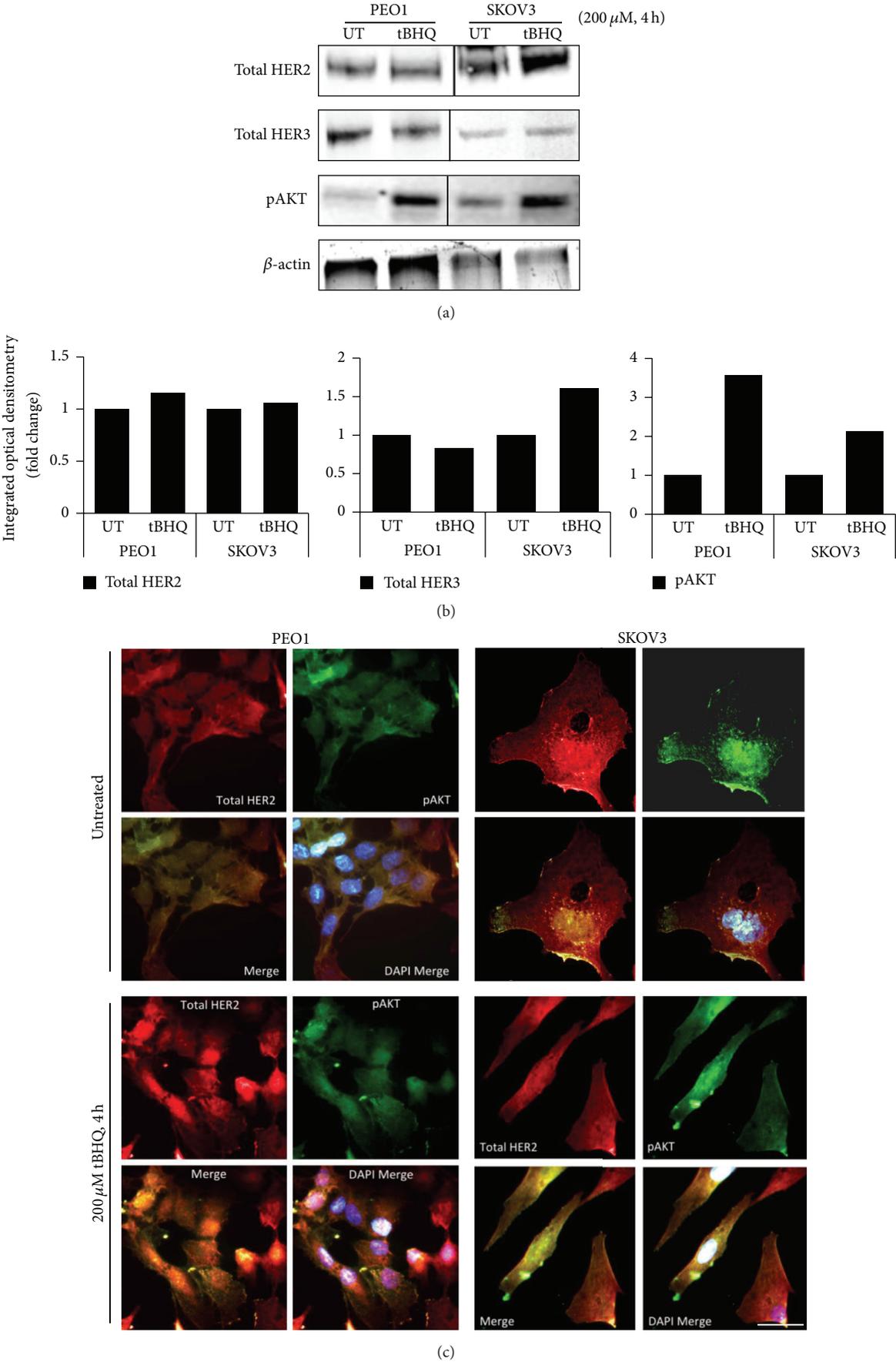


FIGURE 2: Continued.

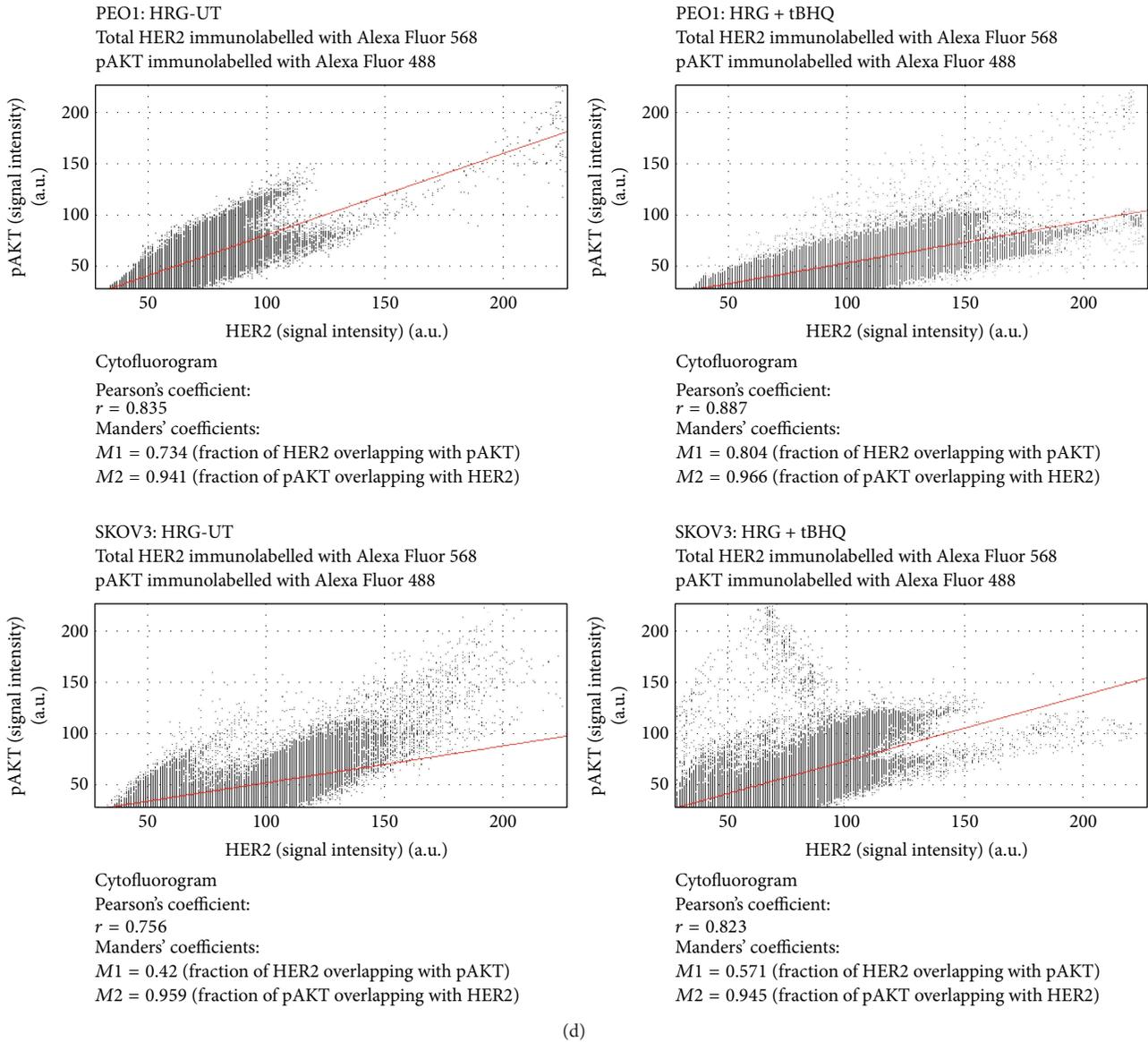


FIGURE 2: TBHQ treatment causes protein induction of HER2 and HER3 and upregulation of pAKT levels in PEO1 and SKOV3 cells. (a) Immunoblot analysis following treatment with tBHQ demonstrated protein induction of both HER2 and HER3 receptors and increase of pAKT. Exponentially growing cells were either left untreated (UT) or treated with 200  $\mu\text{M}$  tBHQ for 4 h before being harvested and processed for immunoblotting using relevant antibodies (Table 1). (b) Bar chart showing total HER2, total HER3, and phospho-AKT levels in PEO1 and SKOV3 cell lines by quantifying immunoblot signal intensities obtained in (a) and normalised to the value of UT and expressed as fold change. (c) Immunofluorescent labelling of endogenous HER2 and phospho-AKT reveals protein induction following tBHQ treatment. Cells were processed for immunocytochemistry and immunolabelled using anti HER2 (red fluorescence) or phospho-AKT (green fluorescence) primary antibodies followed by Alexa Fluor conjugated secondary antibodies. Nuclear reference was provided by costaining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Scale bar indicates 10  $\mu\text{m}$ . (d) Analysis of colocalisation between immunostained HER2 and pAKT in the images obtained in (c). Spatial correlation between the two fluorescent signals was obtained by generating cytofluorograms and performing Pearson's correlation analysis.

exhibited a varying response. While prHER3 activity was significantly induced in PEO1 following 50  $\mu\text{M}$  tBHQ, increasing dosage beyond 50  $\mu\text{M}$  led to its repression. In SKOV3 cells on the contrary, 50  $\mu\text{M}$  tBHQ repressed prHER3 activity while increasing dosage of 100 and 200  $\mu\text{M}$  led to subsequent induction (Figure 3(b), brown bars). This complex regulation

of HER3 is reminiscent of recent reports that revealed that induction of HER2 might repress HER3 expression while its inhibition could lead to transcriptional induction of HER3 [13, 14, 63]. This set of results confirmed the transcriptional basis of induction of HER2 and HER3 protein levels, which concomitantly also led to pAKT induction.

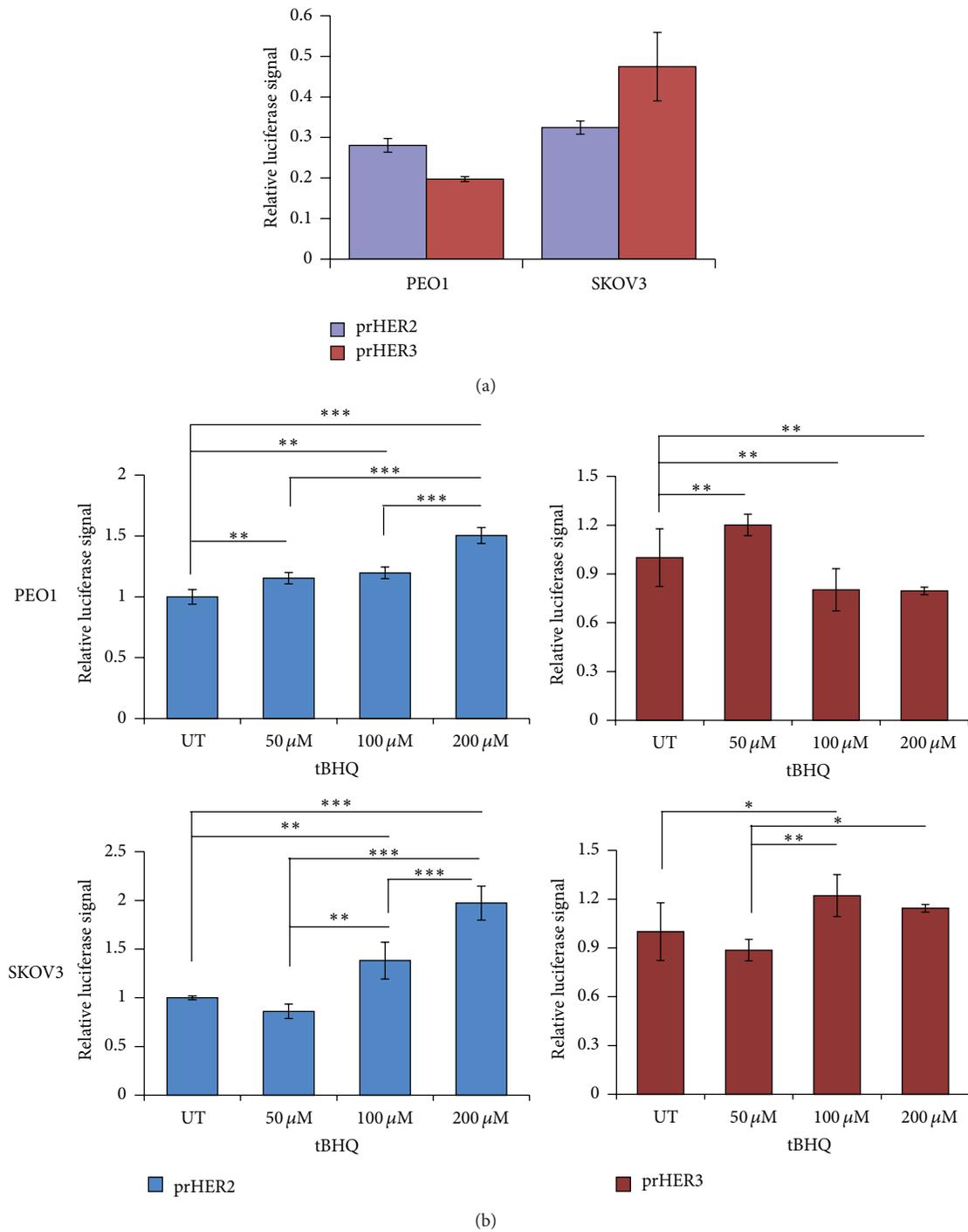


FIGURE 3: Nrf2 activation leads to transcriptional induction of HER2 and HER3 in a concentration dependent manner. (a) SKOV3 cells exhibit higher basal transcription of both *HER2* and *HER3*. PEO1 and SKOV3 cells were transfected with either empty PGL3 basic vector or 1 μg PGL3 basic vector with cloned 1.5 kb fragments of either *HER2* (prHER2) or *HER3* (prHER3) promoter driving the expression of luciferase gene. Cotransfection with 0.2 μg pRL-CMV plasmid was performed as an internal transfection control. (b) TBHQ causes transcriptional induction of *HER2* and *HER3* in a concentration dependent manner. PEO1 and SKOV3 cell lines were transfected in triplicate as in (a) but were treated with different concentrations of tBHQ as indicated for 4 h. Data shown are the means ± S.D. of triplicates, normalised to untreated (UT) controls and expressed as fold change with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , and \*\*\*:  $p < 0.001$ ).

**3.4. NRF2 Activation Desensitises RTK Signaling Pathway to Combination of HER2 Targeting Monoclonal Antibodies Pertuzumab and Trastuzumab.** The observation that tBHQ treatment led to transcriptional induction of HER2 and HER3 suggests that NRF2 may be directly involved in regulating the receptor expression and as such may influence responses to targeted therapies involving HER2 inhibitors. This important question was next investigated by treating PEO1 or SKOV3 cells either with the combination of Pertuzumab and Trastuzumab alone or by cotreatment with tBHQ to examine the consequences of NRF2 activation on drug responses. Interestingly, some features of the signaling response were similar between these two cell lines while others were more distinct. In the PEO1 cell line, treatment with inhibitors alone induced both HER2 and HER3 levels consistent with the parallel increase in phospho-HER2 T877 (Figure 4) in this cell line. In contrast, for SKOV3, only HER3 expression showed a minor induction while total HER2 levels were reduced explaining the decrease in phospho-HER2 T877 levels as well. In order to better understand the effect of these inhibitors on RTK signaling, we normalised the blot signal of phospho-HER2 in both cell lines to the corresponding values of total HER2 (Figure 4, blue bars). This analysis interestingly revealed that while the inhibitors reduced the ratio of phospho-HER2 to total HER2, cotreatment with tBHQ restored the ratio back to that of untreated controls. This effect was more pronounced in the SKOV3 cell line. Importantly, in terms of pAKT S473, while 4 h treatment with inhibitors led to minor repression of its levels as revealed by the densitometry analysis, cotreatment with tBHQ protected this repression and increased pAKT levels beyond that of untreated controls (Figures 4(a) and 4(b)). These results revealed that tBHQ can protect RTK signaling against the inhibitory action of the drugs. We also included phospho-ERK p44/p22 levels in our analysis as ERK was previously shown to be inhibited by drugs targeting HER2 receptor [56]. We saw a very minor repression of phospho-ERK levels only in SKOV3 cells following 4 h of inhibitor treatment. However, tBHQ dependent induction for ERK was seen in PEO1 cells. Inhibitor treatment did not influence pNRF2 S15 levels in either of the cell lines, but as expected, its levels increased following tBHQ treatment. Finally, we examined intact and cleaved levels of proapoptotic protein BID in order to further support our conclusions drawn from Figure 1. By determining the ratio of cleaved BID over intact, we observed that while treatment with inhibitors induced levels of cleaved BID, tBHQ cotreatment led to a minor repression, further explaining the cytoprotective effect of tBHQ treatment (Figure 4). These results revealed important consequences of tBHQ treatment on targeted therapy using HER2-targeted monoclonal antibodies and showed that treatment with NRF2 activator attenuated the inhibitory action of these monoclonal antibodies.

**3.5. Knockdown of NRF2 by Small Inhibitory RNA (SiRNA) Elevates ROS, Represses pNRF2 and Heme Oxygenase-1 (HO-1) Levels, and Disrupts tBHQ Dependent Induction of ARE.** In order to confirm the direct role of NRF2 in tBHQ dependent induction of HER2 and HER3 receptors, we next knocked

down NRF2 using SiRNA. To this end, we first optimized and verified sufficient knockdown of NRF2 using specific SiRNA and then studied the effects of this knockdown on antioxidant pathway. As shown in Figure 5(a), 75 pmol of NRF2 SiRNA produced maximum depletion of NRF2 both following 24 and 48 h of transfection, while 100 pmol showed lesser depletion (Figure 5(a), black bars indicating band intensities). We next determined whether this depletion is sufficient to cause repression of the antioxidant pathway by examining NRF2 substrates. We found that 75 pmol SiRNA sufficiently downregulated phospho-NRF2 and HO-1 levels as well (Figure 5(b)). Efficiency of internalisation of NRF2 targeting SiRNA in SKOV3 cells using different amounts was confirmed and verified (Figure 5(c)). We next quantified total basal ROS following NRF2 knockdown to determine whether NRF2 depletion caused elevation of ROS. Loading of cells with 2',7'-Dichlorofluorescein diacetate dye which is a fluorescent marker of intracellular ROS confirmed elevation of ROS resulting from NRF2 knockdown (Figure 5(c)). Finally, we performed immunostaining of endogenous pNRF2 and HO-1 following transfection with either scrambled or NRF2 targeting SiRNA and as consistent with Figure 5(b), we verified repression of pNRF2 and HO-1 levels at single cell level (Figure 5(d)). Having confirmed the effectiveness of our SiRNA-mediated NRF2 knockdown, we next examined whether depletion of NRF2 would also disrupt tBHQ dependent induction of antioxidant pathway in PEO1 and SKOV3 cell lines and thus confirm the direct involvement of NRF2 in this mechanism. To do this, we exposed cells to tBHQ either in the presence of endogenous NRF2 or following its genetic depletion. Figure 6(a) revealed that NRF2 protein induction seen in tBHQ treatment was disrupted following its SiRNA transfection. Next, in order to confirm that NRF2 depletion also caused inhibition of transcriptional antioxidant response program and to further confirm the conclusions drawn from Figure 5(b), we transfected cells with *cis*-antioxidant response elements (ARE) in luciferase reporter vector driving the expression of luciferase to report transcriptional activity of NRF2. Firstly, we saw repression of ARE signal supporting our conclusions drawn from Figure 5. Secondly, we saw that the tBHQ treatment regime that had caused induction of NRF2 protein levels (Figure 6(a)), and those of NRF2 substrate HO-1 (Figure 5(b)) and HER2 and HER3 receptor expressions (Figures 2 and 3), also significantly enhanced the activity of the NRF2 dependent antioxidant transcriptional programme in both PEO1 and SKOV3 cell lines (Figure 6(b)). Finally, we saw that such induction was inhibited following knockdown of NRF2 to significant levels as compared to tBHQ treatment alone. Altogether, Figures 5 and 6 provide evidence of knockdown of NRF2, repression of the antioxidant response pathway, and disruption of tBHQ mediated pathway induction.

**3.6. NRF2 Depletion Causes Transcriptional Inhibition of HER2 and HER3 Leading to Repression of HER2, HER3, and pAKT Proteins and Sensitisation to Targeted Immunotherapeutics.** As shown in the previous sections, NRF2 activation by tBHQ not only induced the NRF2 dependent antioxidant response pathway as expected, but surprisingly also induced

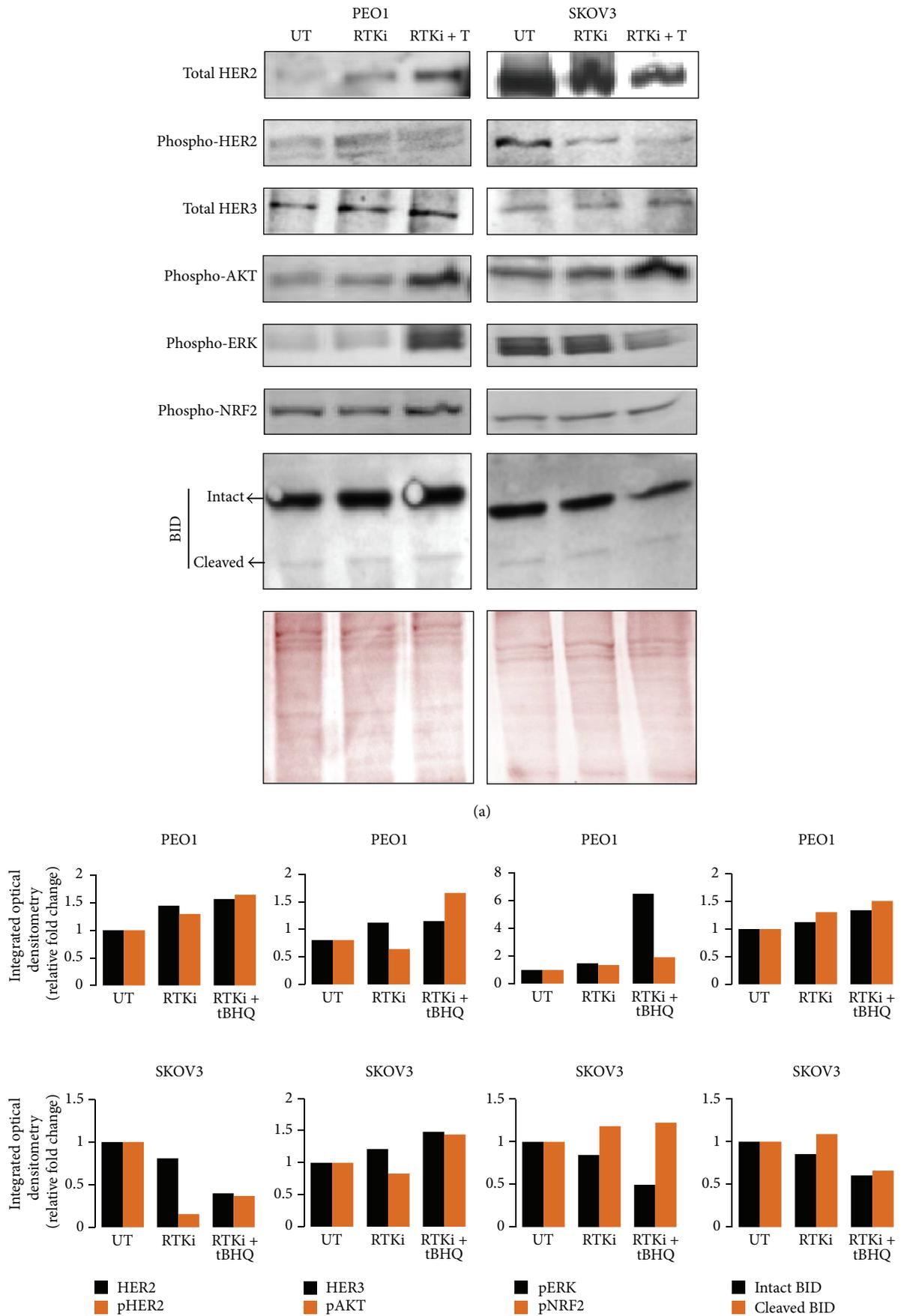


FIGURE 4: Continued.

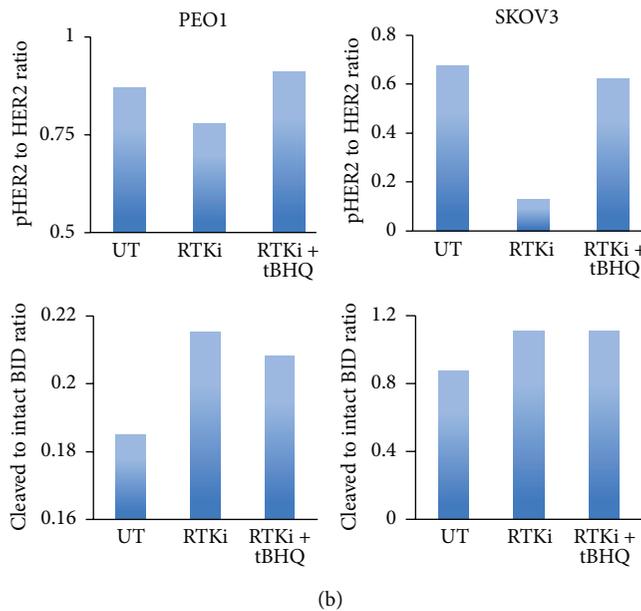


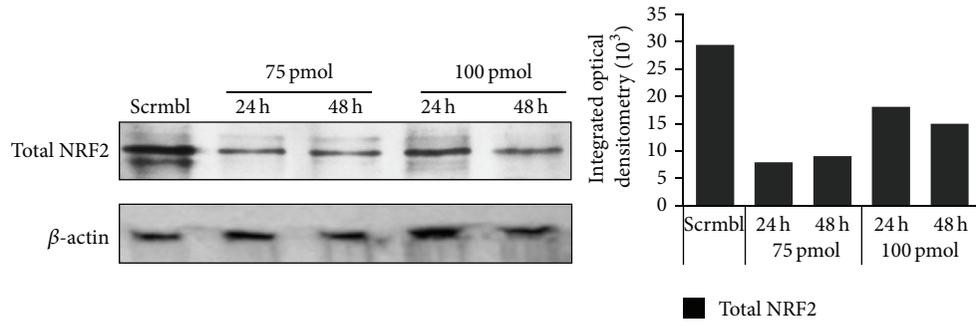
FIGURE 4: NRF2 activation desensitises RTK signaling pathway to HER2 inhibitors Pertuzumab and Trastuzumab. (a) Immunoblot analysis showing tBHQ dependent recovery of RTK signaling following its inhibition by HER2 inhibitors. Exponentially growing cells were either left untreated (UT) or treated with combination of HER2 inhibitors, Pertuzumab and Trastuzumab at concentration of  $20 \mu\text{g}/\text{mL}$  (RTKi), or with cotreatment of  $200 \mu\text{M}$  tBHQ (RTKi + T) for 4 h before and processed for immunoblotting using relevant antibodies (Table 1). Ponceau stain of the same blot was used as loading control. (b) Bar chart showing total HER2, phospho-HER2, total HER3, phospho-AKT, phospho-ERK, phospho-NRF2, and BID levels in PEO1 and SKOV3 cell lines by quantifying immunoblot signal intensities obtained in (a) and normalised to the value of UT and expressed as fold change. Blue bars show ratio of phospho-HER2 to HER2 (upper panels) and cleaved BID to intact BID (lower panels).

protein levels of HER2 and HER3 (Figure 2) and we further confirmed that the protein upregulation was as a result of their transcriptional induction (Figure 3). These findings were important because such receptor induction attenuated the inhibitory responses of HER2-targeted drugs (Figure 4). As an alternative approach to study the regulation of HER receptors by NRF2, we knocked down NRF2 in our cell lines and firstly studied the protein levels of the receptors and their downstream substrate, pAKT. We found significant protein repression of HER2 and HER3 as well as pAKT. Quantification of the resulting immunoblot signals revealed a greater repression with  $75 \text{ pmol}$  NRF2 SiRNA (Figure 7(a)). Interestingly, we could also detect and capture such repression at single cell level by performing immunostaining for HER2 and pAKT following either scrambled or NRF2 specific SiRNA (Figure 7(b)). Immunolabelling also revealed localisation features of total HER2 and pAKT. HER2 was mostly localised at the cell membrane as expected and apparently without any nuclear staining. The pAKT on the other hand was localised at the cell membrane, general cytosol and nucleus, as revealed by immunostaining and the merger with DAPI staining (Figure 7(b)). This is consistent with previous reports of nucleocytoplasmic shuttling of pAKT that could have physiological consequences [63–65].

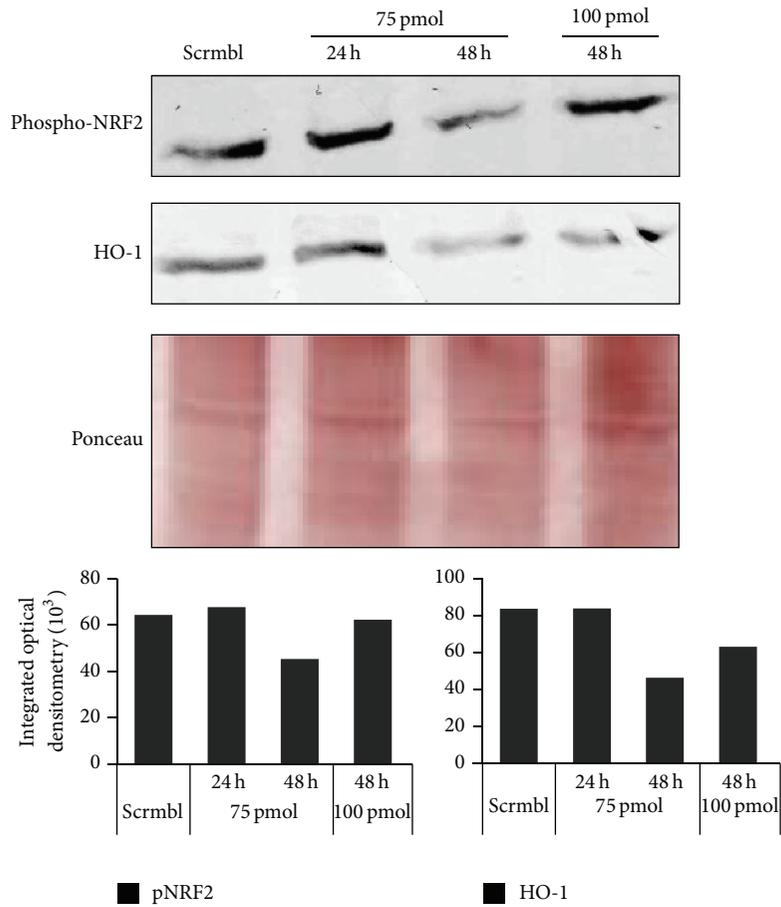
We next wanted to determine and confirm any transcriptional mechanism of NRF2 specific SiRNA dependent repression of HER2 and HER3. We thought this could be a likely explanation as we earlier showed tBHQ dependent transcriptional upregulation of HER receptors (Figure 3). To

address this, we again utilised our transcriptional reporter assays for both HER2 and HER3 receptors that were established for this study. Using our ovarian cell line models, we individually transfected the reporter systems but, this time, cotransfecting with NRF2 specific SiRNA as well. Following this, cells were either left untreated or treated with tBHQ. We found that following NRF2 knockdown, HER2 transcription was significantly repressed in both PEO1 (Figure 7(c)) and SKOV3 (Figure 7(d)) cell lines. In terms of HER3, while NRF2 knockdown significantly repressed transcription in SKOV3, such repression was not seen in PEO1 (compare Figures 7(c) and 7(d) for prHER3). Interestingly, the tBHQ dependent transcriptional induction of prHER2 and prHER3 gene reporters as seen in Figure 4 was disrupted following depletion of NRF2 in both PEO1 and SKOV3 cell lines to significant levels (Figures 7(c) and 7(d)). These important findings confirmed that tBHQ mediated protein and transcriptional induction of HER receptors was dependent on NRF2 and not by any off NRF2 target effect of tBHQ treatment. Finally, we repeated knockdown of NRF2 either alone or with parallel knockdown of KEAP1 and exposed such cells to targeted immunotherapeutics for 24 and 48 h (Figure 7(e)). We found significant increase in cell death in NRF2 knockdown cells upon exposure to the immunotherapeutics and significant reversal of this response with parallel KEAP1 knockdown.

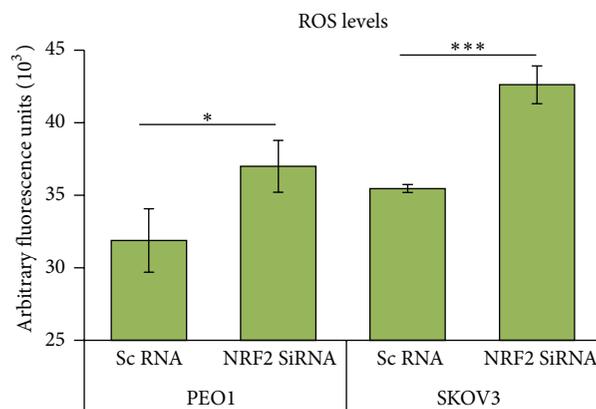
These results confirmed the transcriptional regulatory role of NRF2 for HER2 and HER3 receptors and illustrated alteration of protein abundance as a result of such transcriptional regulation. These data also confirmed



(a)



(b)



(c)

FIGURE 5: Continued.

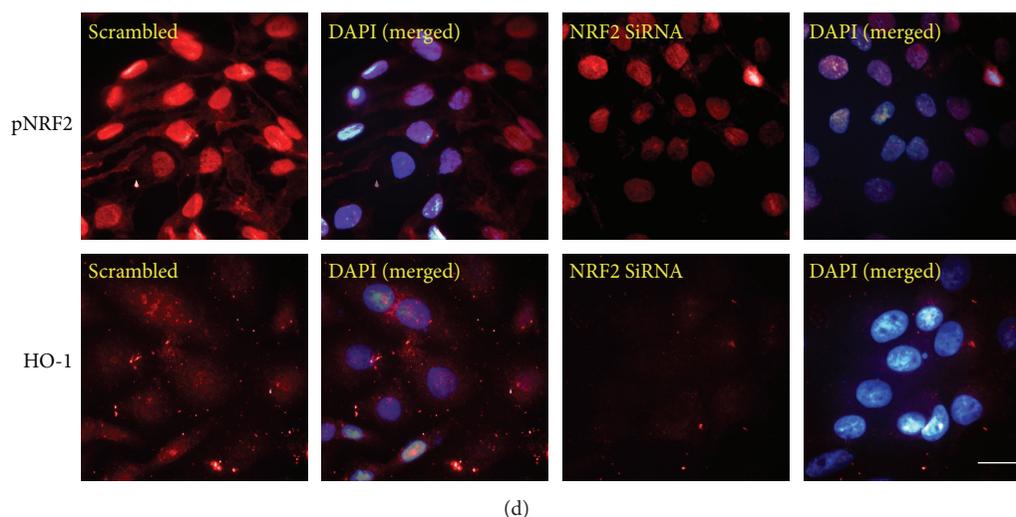


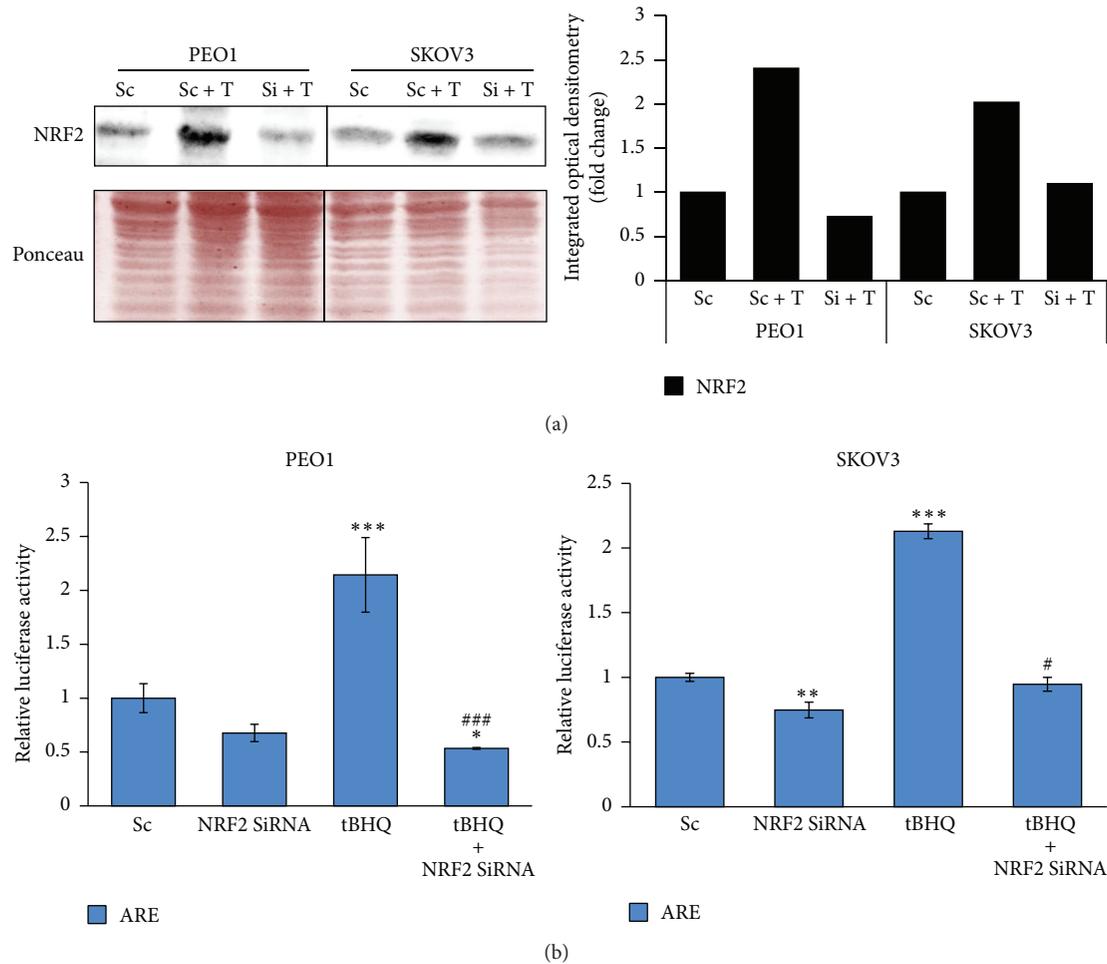
FIGURE 5: Knockdown of NRF2 by SiRNA causes repression of phospho-NRF2 and HO-1 levels and elevation of reactive oxygen species (ROS). (a) Optimization of SiRNA-mediated NRF2 knockdown. Exponentially growing cells were transfected either with scrambled RNA (scrmbl) or with different amounts of SiRNA for either 24 h or 48 h before being processed for immunoblotting. (b) NRF2 knockdown results in repression of its substrates. The same lysates as in (a) were blotted for phospho-NRF2 and HO-1 levels. Bar charts in (a) and (b) show total NRF2, phospho-NRF2, and HO-1 levels in SKOV3 cell lines by quantifying immunoblot signal intensities obtained in respective blots and normalised to the value of UT and expressed as fold change. (c) NRF2 knockdown leads to ROS accumulation. SKOV3 cells were seeded in triplicate for 18 h and transfected with NRF2 SiRNA. Following 48 h incubation, cells were assayed for total ROS by loading them with DCFDA for 45 min and measuring fluorescence using fluorescence multiplate reader (MODULUS, Promega) with excitation and emission spectra of 485 nm/535 nm. The fluorescence reading was normalised to total cell abundance within the same wells as described in Materials and Methods. Data are the means with  $\pm$ S.D. of triplicates, normalised to untreated (UT) control and expressed as fold change with statistical significance determined by Student's *t*-test according to the scale \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , and \* \* \*:  $p < 0.001$ . (d) Immunofluorescent labelling of endogenous phospho-NRF2 and HO-1 exhibits repression following NRF2 knockdown. Cells were transfected as in (a) and processed for immunocytochemistry. Relevant primary antibodies followed by Alexa Fluor conjugated secondary antibodies were used for immunolabelling for phospho-NRF2 and HO-1 (red fluorescence). Nuclear reference was provided by costaining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Images were captured with Leica DMiRe2 electronic microscope with 100x objective while merging, colocalisation, and further analysis were performed by using integrated features of Andor iQ Core software (ANDOR Technologies Ltd.). Scale bar indicates 10  $\mu$ m.

the role of NRF2 in determining overall treatment responses to HER2 targeting immunotherapeutics and hence defining the balance between resistance and sensitivity.

#### 4. Discussion

The receptor tyrosine kinases (RTKs), exemplified by HER2/HER3 family receptors, are key regulators of cellular proliferation, differentiation, and survival, as well as determinants of cancer initiation, maintenance, and progression [1–4]. Complexity in understanding the HER2/HER3 activation and signaling arises from the intricate and complex regulation of coexpression of HER2/HER3 receptors and their ligands and the broad spectrum of tumour biochemistry, heterogeneity, and range of sensitivities and resistance exhibited to drugs targeting the HER receptor system [13, 14, 17, 21]. Furthermore, clinical data on HER2/HER3 coexpression profile correlates to some degree with disease-free survival, not only regarding anti-RTK treatment outcome [66], but also by other therapeutic agents [43, 67, 68]. However, it has been suggested that sustained and complete inhibition of HER3 and its output to PI3K/Akt is required for the maximal antitumour effect of HER2 inhibitors [13, 16] and

that inhibition of HER2 receptor alone might not generate sufficient anticancer response [16]. Lately, data have accrued to evidence and implicate NRF2 and ROS, in addition to HER2/HER3, in the promotion of cellular proliferation and therapeutic resistance in cancer cells [31, 69, 70]. It is also known that ROS can trigger both the AR and the HER family receptor pathways with concomitant transcriptional upregulation of HER2/HER3 and NRF2 and subsequent elevation and activation of their functions [31, 68–70]. Thus, the HER2/HER3 family receptor signaling pathway is upstream of PI3K/AKT/mTOR pathway [13–16] and has likewise been shown to be upstream of the NRF-AR pathway as well [46, 47, 59, 60, 71, 72]. These highlight the possibility of a more direct, rather than indirect, contact and cross relationship between the HER2/HER3 and NRF2-AR pathways. This crosstalk could be likely and even necessary as RTK dependent growth and metabolism creates ROS, which would require parallel NRF2 dependent antioxidant pathway for its neutralisation. Likewise, the implication of NRF2 in proliferative and cytoprotective pathways may involve RTK dependent signaling [73]. RTK-targeted cancer therapies are compromised or limited when tumour cells circumvent the action of a single agent, and multiple agents due to the readjustments in



**FIGURE 6: Knockdown of NRF2 by SiRNA represses both basal and induced antioxidant response pathway in PEO1 and SKOV3 cell lines.** (a) Immunoblotting analysis showing repression of NRF2 following NRF2 knockdown by SiRNA in PEO1 and SKOV3 cell lines. Cells were either transfected with scrambled SiRNA (Sc) or transfected with 75 pmol of NRF2 SiRNA (Si). After 48 h, cells were either left untreated or treated with 200  $\mu$ M tBHQ (T) for 4 h, before being processed for immunoblotting using relevant antibodies (Table 1). Ponceau stain of the same blot was used as loading control. Bar chart shows NRF2 levels by quantifying immunoblot signal intensities obtained in (a) and normalised to the value of untreated (UT) control and expressed as fold change. (b) Knockdown of NRF2 causes inhibition of its transcriptional antioxidant program in both constitutive and tBHQ induced states. PEO1 and SKOV3 cells were transfected with either empty PGL3 basic vector or 1  $\mu$ g PGL3 basic vector with a cloned 8 x *cis*-antioxidant response elements (ARE) driving NRF2 dependent expression of luciferase gene. Cotransfection with 0.2  $\mu$ g pRL-CMV plasmid was performed as an internal transfection control. Where required, cotransfection with either scrambled RNA (Sc) or NRF2 SiRNA was performed using 20 pmol SiRNA. At 24 h after transfection, treatment with 200  $\mu$ M tBHQ was performed where indicated for 4 h following which, cells were processed for dual luciferase reporter assay (Promega) to record luciferase activity in multiplate reader (MODULUS, Promega). Data are the means with  $\pm$ S.D. of triplicates normalised to the value of scrambled SiRNA (Sc) and expressed as fold change with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test. \* indicates significance of scramble versus treatment groups while # indicates significance of tBHQ versus tBHQ + NRF2 SiRNA groups according to the scale symbolised by \* or #:  $p < 0.05$ , \*\* or ##:  $p < 0.01$ , and \*\*\* or ###:  $p < 0.001$ .

coexpression of HER2/HER3 receptors, their ligand binding dynamics, or changing preference for the dimerizing partner [17, 21, 61, 74, 75] suggest that the anticancer effect of these agents might be further optimized or be better predicted by effectively limiting HER2/HER3 expression at the DNA level or at least identifying a common regulatory centre of HER2 and HER3 transcription. Thus the identification of factors that mediate or modulate the transcriptional expression of HER2/HER3 will be paramount.

NRF2 has already been implicated in numerous reports as a key contributor to resistance towards anticancer drugs. However, most of these past studies have explored the role of NRF2 in resistance against DNA damaging agents [50, 51, 55]. The present study demonstrates that NRF2 may regulate cancer cell proliferation, susceptibility, and resistance to targeted therapy via transcriptional regulation of *HER2/HER3*. To demonstrate the role of NRF2 in RTK signaling and thus in determining responses to targeted therapies, we used HER2

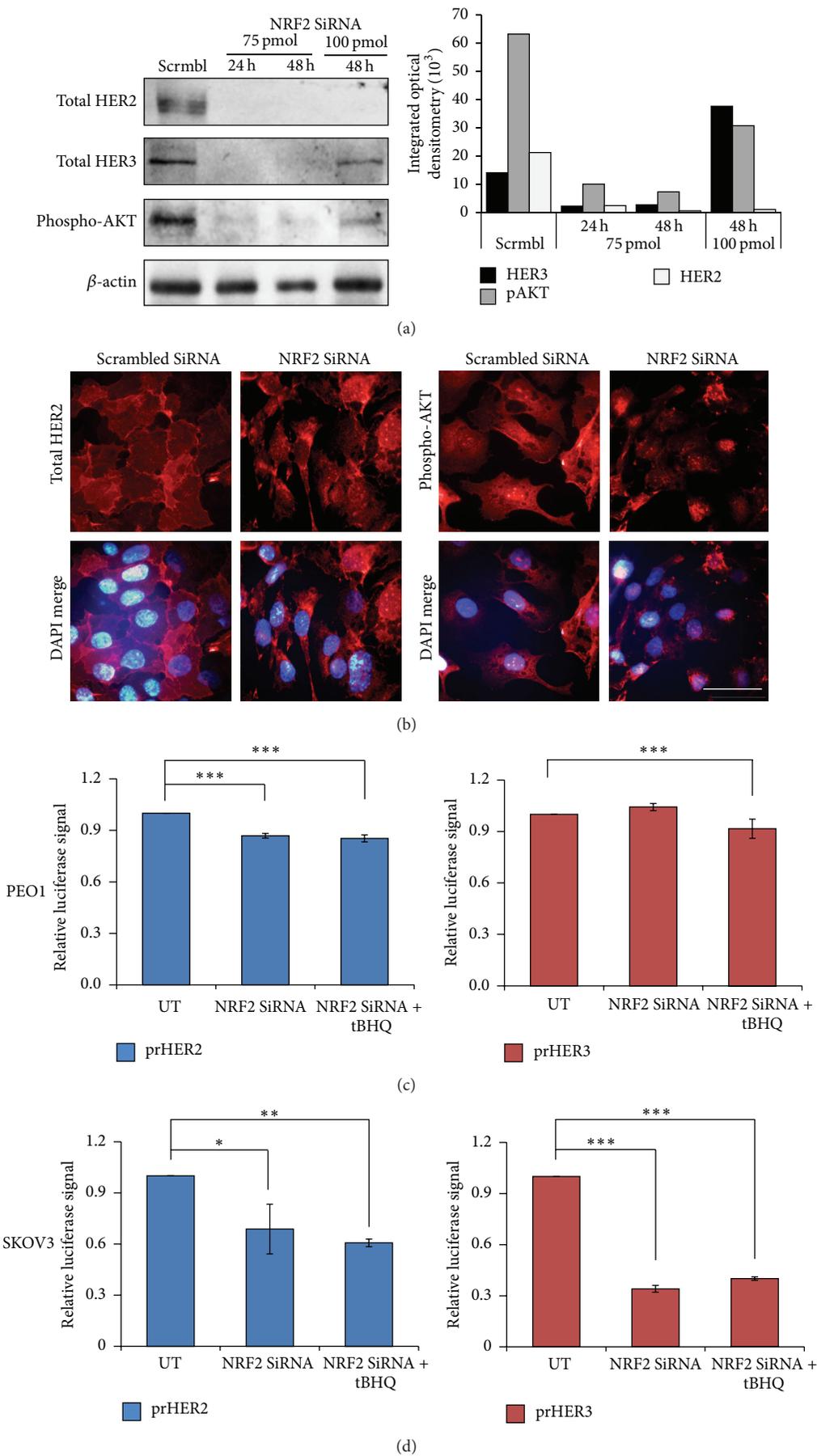


FIGURE 7: Continued.

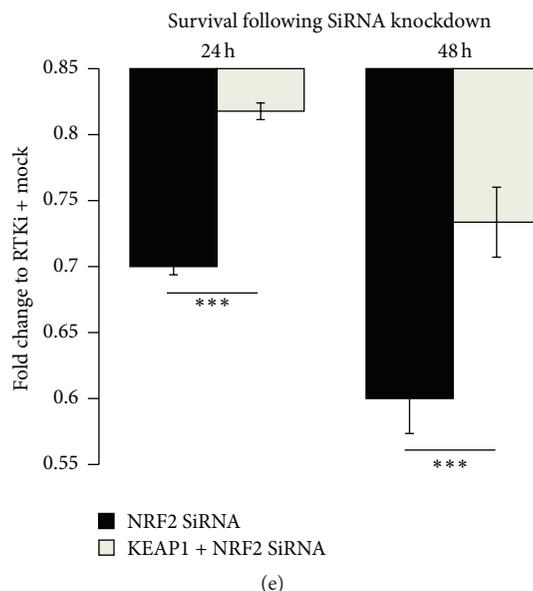


FIGURE 7: NRF2 knockdown causes downregulation of HER2 and HER3 levels, repression of pAKT, and sensitisation to targeted immunotherapeutics. (a) Immunoblotting analysis showing inhibition of RTK signaling following depletion of NRF2 mRNA by SiRNA in SKOV3 cell line. Exponentially growing cells were either transfected with scrambled SiRNA (Scrambl) or transfected with 75 pmol of NRF2 SiRNA for either 24 or 48 h or 100 pmol of NRF2 SiRNA for 48 h and processed for immunoblotting using relevant antibodies (Table 1).  $\beta$ -actin was used as a loading control. Bar chart shows protein levels by quantifying immunoblot signal intensities obtained and normalised to the value of untreated (UT) control and expressed as fold change. (b) Immunofluorescent labelling of endogenous total HER2 or phospho-AKT exhibits repression following NRF2 knockdown. Cells were transfected as in (a) and processed for immunocytochemistry. Relevant primary antibodies were used to stain HER2 or phospho-AKT followed by Alexa Fluor conjugated secondary antibody (red fluorescence). Nuclear reference was provided by costaining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Images were captured with Leica DMiRe2 electronic microscope at 100x objective while merging, colocalisation, and further analysis were performed by using integrated features of Andor iQ Core software (ANDOR Technologies Ltd.). Scale bar indicates 10  $\mu$ m. (c and d) HER2 and HER3 downregulation following NRF2 knockdown is caused by their transcriptional repression. Exponentially growing PEO1 cells (c) or SKOV3 cells (d) were transfected with either empty PGL3 basic vector or 1  $\mu$ g PGL3 basic vector with cloned 1.5 kb fragments of either HER2 (prHER2) or HER3 (prHER3) upstream promoter regions driving the expression of luciferase gene. Cotransfection with 0.2  $\mu$ g pRL-CMV plasmid was performed as an internal transfection control. At 24 h after transfection, cells were either left untreated (UT) or treated with 200  $\mu$ M tBHQ as indicated for 4 h following which, cells were processed for dual luciferase reporter assay (Promega) to record luciferase activity in multiplate reader (MODULUS, Promega). (d) The same was done for SKOV3 cell lines. (e) Knockdown of NRF2 through SiRNA sensitises cancer cell to RTK inhibitors while parallel knockdown of KEAP1 partially relieves this sensitisation. Cells were transfected with scrambled SiRNA or SiRNA targeting NRF2 either alone or with the inclusion of KEAP1 SiRNA. Following further 24 h incubation, cells were either left untreated or treated with 25  $\mu$ g/mL of HER2 inhibitors Pertuzumab and Trastuzumab. Cytotoxicity assay was performed as in (a). In (c–e), data are the means with  $\pm$ S.D. of triplicates and expressed as fold change with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test (for c and d), or Student's *t*-test (for e) according to the scale \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , and \* \* \*:  $p < 0.001$ .

overexpressing (SKOV3) and low expressing (PEO1) ovarian cancer cell lines [57] grown in HER receptor ligand Heregulin and employed pharmacological and genetic activation or inhibition of both NRF2-AR and HER2/HER3 signaling pathways.

Firstly, pharmacological activation of NRF2 with tBHQ enhanced ovarian cancer cell growth and protected cells from cytotoxicity caused by combined HER2-targeted immunotherapeutic agents, Pertuzumab and Trastuzumab. This was also concomitant with the induction of HER2, HER3, and pAKT proteins in oscillatory and dose-dependent fashions, which is consistent with current emerging concepts of transcriptional control and gene expression [70, 76–80]. Furthermore, NRF2 activation-dependent induction of the receptors and their signaling pathway was governed and executed by NRF2 at the transcriptional level of *HER2* and

*HER3* genes. Our results from both immunocytochemistry and gene reporter assays of HER2 and HER3 expressions were further supportive and reminiscent of recent reports that revealed that induction of HER2 might repress HER3 expression while its inhibition led to transcriptional induction of HER3 ([13, 14, 63], also see Figure 3(b)). It is clear that tBHQ treatment led to induction of NRF2, its associated antioxidant transcriptional program, and transcriptional and signaling activation of HER2 and HER3 and that this tBHQ response was evidently dependent on NRF2. Thus, NRF2 activation by tBHQ desensitised RTK signaling pathway to inhibitory action of the HER2 targeting immunotherapeutic agents Pertuzumab and Trastuzumab.

Next, to further investigate and confirm the involvement of NRF2 in the elevation of HER2 and HER3, we took a genetic approach to deplete NRF2 status and function using

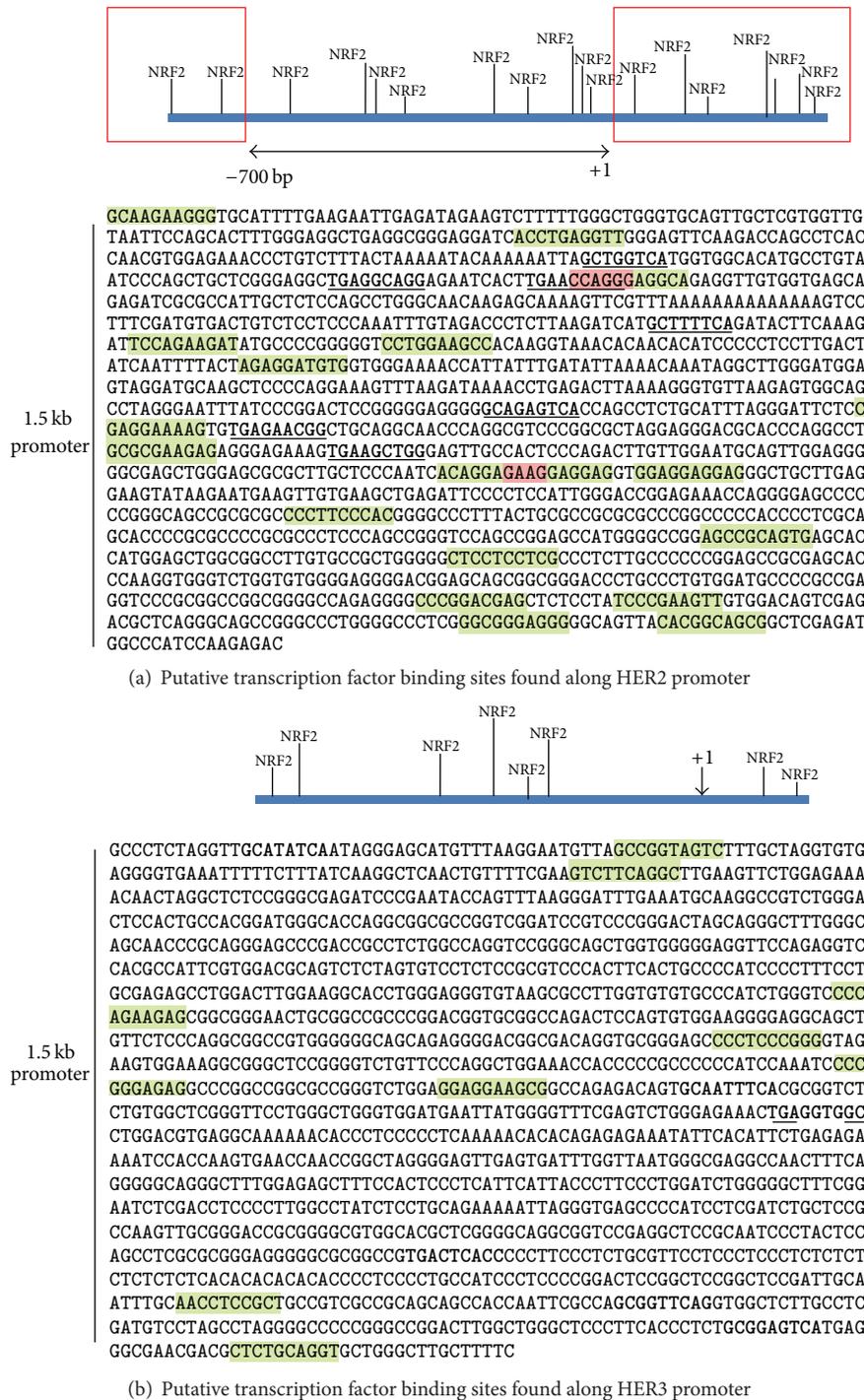


FIGURE 8: *In silico* analysis of HER2 and HER3 promoter sequences. (a) 1.5 kb promoter region of *HER2* gene was fetched from database (ensemble.org) and subjected to transcriptional factor binding prediction program (<http://consite.genereg.net/>) to predict for putative NRF2 binding sites as indicated. Line with arrowheads shows the 0.7 kb sequence of HER2 promoter [8], while regions enclosed in rectangles show additional sequences included and cloned in the PGL3 luciferase reporter vector (Promega) because of carrying additional NRF2 binding sites. (b) The same analyses were performed for HER3 promoter. In (a) and (b), +1 indicate the transcriptional start site, sequences highlighted in green show NRF2 binding sites as predicted by ConSite, and sequences in bold represent manual identification of putative NRF2 binding sites based on ARE consensus sequence [9] while those highlighted in pink show overlapping NRF2 binding sites by the two methods mentioned above.

SiRNA. This approach increased cellular ROS, repressed pNRF2 and HO-1 levels, and even disrupted the tBHQ dependent induction of our ARE reporter system (Figures 5 and 6). In addition, NRF2 depletion by SiRNA caused transcriptional repression of *HER2* and *HER3* leading to lowered expression of HER2, HER3, and pAKT proteins. As an alternative approach, we also cloned and overexpressed individually both NRF2 and KEAP1 genes in our cancer cell lines and found these in either cytoprotection or sensitisation to targeted therapies, respectively (data not shown). Moreover, we illustrated that while knockdown of NRF2 significantly sensitised ovarian cancer cells to targeted immunotherapy, parallel knockdown of KEAP1 reversed this sensitisation. These results support and confirmed our earlier inferred regulatory role of NRF2 in the transcription of *HER2* and *HER3* receptors and its association with alteration of HER2 and HER3 proteins abundance. A recent study has suggested a similar role for NRF2 in regulating the expression of HER2 [73] but fell short of evidencing direct transcriptional regulation as shown in this study. To demonstrate transcriptional modulation of these receptors, we generated and utilised luciferase reporter assays of their proximal promoter sequencing spanning 1.5 kb regions. We performed *in silico* analysis of these upstream regulatory regions for the presence of NRF2 binding and ARE like consensus sequences and found a number of such binding sites (Figure 8). Moreover, a direct interaction of NRF2 and HER2 in regulating the expression of NRF2 target genes, including *HO-1*, via binding of the complex to the ARE of the target genes has been reported [45] which adds credence to our observed downregulation of HER2, HER3, and pAKT as well as HO-1 and pNRF2 levels following our SiRNA-mediated depletion of NRF2. However, further experiments are necessary to confirm the role of NRF2 as a transcription factor for HER receptors.

Thus we have shown that NRF2 regulates HER2 and HER3 signaling pathway to modulate sensitivity to targeted therapies. This demonstrates that NRF2 activation is not only implicated in resistance to genotoxic agents as previously shown [55] but can also lead to resistance to immunotherapies involving Pertuzumab and Trastuzumab, whose actions are very specific to HER2 receptors and unrelated to antioxidant pathway until this study.

## 5. Conclusion

The effectiveness of current anticancer therapies that involve DNA damaging and ROS producing agents is limited, because of NRF2 dependent emergence of cellular resistance to genotoxic agents. On the other hand, targeted anticancer therapeutic agents, while being initially found to be promising, have their own limitations. These include predicting their action and outcome owing to their tight dependence on properties such as cell surface expression of receptors, their dimerizing preferences, presence of ligands, and dynamics of recycling/degradation. This study has found a novel node of regulation between the AR and RTK signaling pathway. As such, the central regulatory node that converges at transcription factor NRF2 presents itself as a very attractive drug

target especially in both scenarios of resistance described above. We have presented evidence at the gene expression, protein induction, localisation, and cytotoxicity levels that the two pathways are coregulated and together predict and inform outcomes to targeted immunotherapies and that such responses could be controlled by modulating NRF2 function.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgment

This work was partly funded by The Northwood Trust.

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## Review Article

# Roles of Oxidative Stress in Polycystic Ovary Syndrome and Cancers

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Received 22 June 2015; Revised 28 August 2015; Accepted 6 September 2015

Academic Editor: Sahdeo Prasad

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Oxidative stress (OS) has received extensive attention in the last two decades, because of the discovery that abnormal oxidation status was related to patients with chronic diseases, such as diabetes, cardiovascular, polycystic ovary syndrome (PCOS), cancer, and neurological diseases. OS is considered as a potential inducing factor in the pathogenesis of PCOS, which is one of the most common complex endocrine disorders and a leading cause of female infertility, affecting 4%–12% of women in the world, as OS has close interactions with PCOS characteristics, just as insulin resistance (IR), hyperandrogenemia, and chronic inflammation. It has also been shown that DNA mutations and alterations induced by OS are involved in cancer pathogenesis, tumor cell survival, proliferation, invasion, angiogenesis, and so on. Furthermore, recent studies show that the females with PCOS are reported to have an increasing risk of cancers. As a result, the more serious OS in PCOS is regarded as an important potential incentive for the increasing risk of cancers, and this study aims to analyze the possibility and potential pathogenic mechanism of the above process, providing insightful thoughts and evidences for preventing cancer potentially caused by PCOS in clinic.

## 1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders of women at reproductive age and the major cause of anovulatory infertility [1]. It was first described as the change of ovarian morphology by Chereau in 1844 [2], and the diagnostic criteria were established by the European Society for Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) in 2003 based on the extensive studies during the last decades, which is the so-called Rotterdam Consensus Criteria [3]. PCOS is a disease with high heterogeneity, and its clinical features mainly include menstrual disorder, secondary amenorrhea, serum hormone abnormality, hairiness, acne, obesity, and infertility [3].

PCOS has been regarded as a chronic systemic disease instead of the simple local disease, and it is frequently associated with insulin resistance (IR), hyperandrogenemia, chronic inflammation, and oxidative stress (OS), though the pathogenesis mechanism has not been well defined [4–8]. A lot of investigations have revealed that OS level is significantly increased in patients with PCOS compared with the normal, when oxidative status is evaluated by circulating markers, such as malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [4]. However, OS level is also observed to be significantly correlated with obesity, insulin resistance, hyperandrogenemia, and chronic inflammation [9–12]. Though OS is considered as a potential inducement of PCOS pathogenesis [4], it is still undetermined whether the abnormal OS levels of patients with PCOS

derive from PCOS itself or if they are related to the potential complications.

Besides the above complications, PCOS is probably accompanied with some malignant lesions as well, such as endometrial cancer, breast cancer, and ovarian cancer [13, 14]. Several investigations indicated that PCOS perhaps could increase the risk of developing endometrial cancer, and abnormal hormone level, IR, hyperinsulinemia, and even obesity were suggested as the potential inducements of endometrial cancer pathogenesis in PCOS patients [15–18]. What is more, OS, altered in PCOS, is discovered to play pivotal roles in cancer pathogenesis [19–21]. ROS could cause genetic changes by attacking DNA, leading to DNA damages, such as DNA strand breaks, point mutations, aberrant DNA cross-linking, and DNA-protein cross-linking [22]. As a result, the mutations in protooncogenes and tumor suppressor genes probably hijacked cell proliferation out of control, when the DNA repair mechanism has been disrupted [23, 24]. On the other hand, OS could cause epigenetic changes as well by DNA methylation, silencing tumor suppressor genes [25, 26]. Therefore, OS could be one of the major underlying inducements of the increasing risk of gynecological cancers in PCOS patients.

## 2. Altered Oxidative Stress in Polycystic Ovary Syndrome

Oxidative stress (OS) reflects an imbalance between production and scavenging of reactive oxygen/nitrogen species (ROS/RNS) [27], and excess ROS accumulated in vivo would induce cell [28, 29], protein [30–32], and lipid damage [33]. ROS includes both free radical and non-free radical oxygenated molecules, such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^{\bullet-}$ ), singlet oxygen ( $1/2 O_2$ ), and the hydroxyl radical ( $\bullet OH$ ). Reactive nitrogen, iron, copper, and sulfur species are also involved in OS [34, 35]. Free radicals are the species possessing unpaired electron in the external orbit and could exist independently [35, 36]. In general, chemical substances used for evaluating oxidative status could be divided into chemical components modified by reactive oxygen, ROS scavenging enzymes or antioxidative chemicals, and transcription factors regulating ROS production. However, it is hard to reflect OS status accurately with the same biomarkers in various diseases, because OS usually plays different roles and triggers different signaling pathways in different diseases, so biomarkers used to evaluate OS in a particular disease are limited and should be always filtrated carefully [32, 37–40].

According to the modified criteria defined at Rotterdam meeting, polycystic ovary syndrome (PCOS) would be determined when two of the following three criteria have been discovered: (1) clinical and/or biochemical evidence of androgen excess after the exclusion of other related disorders; (2) oligoovulation or anovulation; (3) ultrasound appearance of the ovaries: presence of more than 12 follicles in each ovary measuring 29 mm and/or increased ovarian volume (>10 mL) [3]. Though the full pathophysiology of PCOS is still not determined, hyperandrogenemia and insulin resistance

(IR) are frequently involved. The hyperandrogenemia that accompanies PCOS may be caused by the abnormal ovaries, adrenal glands, peripheral fat, and hypothalamus-pituitary compartment. Insulin resistance, frequently appearing in PCOS as well, results in a compensatory hyperinsulinemia, which augments luteinizing hormone- (LH-) stimulated androgen production, either via its own receptors or via insulin growth factor (IGF-1) receptors [41]. As a syndrome, PCOS is usually treated based on detailed clinical symptoms, and therapeutic schedules mainly include ovulation induction, downregulating androgen and LH levels, attenuating IR, and operation [41].

OS is also intimately involved in PCOS pathogenesis, since PCOS patients show more serious OS compared with the normal [4] (Table 1). However, results would not be consistent absolutely, when different markers are employed and the same marker is evaluated in different sources and even with different investigation methods [42–44]. In addition, OS is involved in the pathological processes of IR, hyperandrogenemia, and obesity as well, which accompany PCOS frequently but not absolutely [45]. Thus, appropriate markers should be chosen to evaluate the OS levels in PCOS for the particular circumstance. Current employed circulating markers majorly include homocysteine, malondialdehyde (MDA), asymmetric dimethylarginine (AMDA), superoxide dismutase (SOD), glutathione (GSH), and paraoxonase-1 (PON1) [4]. Because of the complicated cross-link of OS and physiological and clinical characteristics of PCOS, the interactions of OS and PCOS would be described below from major nodes linking OS and PCOS.

*2.1. Oxidative Stress, Obesity, and Polycystic Ovary Syndrome.* Obesity, a popular endocrine disease in the world, was firstly divided into visceral obesity and peripheral obesity by Vague in 1956 [46], also called central obesity and lower body obesity. Visceral obesity, the so-called abdominal obesity, in which visceral adipose tissues are mainly accumulated in the abdomen and distributed widely on omentum and mesenterium, around viscera, and in skeletal muscle, could be determined by the increased waist circumference (WC). Compared with visceral obesity, peripheral adipose tissues are mainly accumulated under the peripheral skin, especially in buttocks and legs, and are usually evaluated by body mass index (BMI). About 42% of patients with polycystic ovary syndrome (PCOS) have the complication of obesity [47]. Abdominal adipose tissue is considered to be correlated with metabolic diseases more significantly than subcutaneous adipose tissue [48]. Diagnostic method of abdominal obesity has not been defined yet, but the size and the thickness of visceral fat determined by electronic computer X-ray tomography technology (CT) are often regarded as the golden standard [49]. In addition, WC is a simple and reliable criterion usually applied to evaluate abdominal obesity in clinic. Abdominal obesity is regarded as a common complication of PCOS, and the risk of abdominal obesity in PCOS women ranges from 40% to 80% because of the differences of people and nations [50, 51]. Body mass index (BMI) is used as a popular criterion in clinic to evaluate obesity; however, about 50% of PCOS patients with normal BMI still have abdominal obesity [51].

Therefore, both BMI and WC should be considered when considering the contribution of obesity to PCOS etiology.

Obese patients are expected to have more serious oxidative stress (OS) levels [52], and significant correlations of OS markers with obesity indexes, such as BMI and WC, are discovered [53, 54]. Levels of markers that could reflect the degrees of lipid peroxidation and protein peroxidation, such as oxidized low density lipoprotein (ox-LDL), malondialdehyde (MDA), thiobarbituric reactive substances (TBARS), and advanced oxidation protein products (AOPP), increase significantly in the obese patients compared with the normal, and levels of markers that could reflect the antioxidant ability, such as glutathione peroxidase (GSH-Px) and copper- and zinc-containing superoxide dismutase (CuZn-SOD), decreased significantly [55–57]. As an important pathological and physiological process, OS is associated with a number of chronic diseases, which are the main complications of obesity. What is more, the investigation of Khan et al. [58] reported that systemic OS levels of obese females without smoking history, diabetes, hypertension, dyslipidemia, dysfunctions of liver and kidney, and tumor history were still significantly higher than nonobese females, and GSH concentrations of erythrocytes were significantly lower. In addition, obese patients have more serious oxidative stress as well while PCOS patients are ruled out [9, 59]. Thus, obesity, besides abdominal obesity, is directly associated with OS and contributes to the increased OS levels in PCOS [60].

However, obesity is not the only factor leading to the more serious oxidative status of PCOS, and other factors are considered to have contributions as well. While obese patients are ruled out according to BMI, nonobese women with PCOS still have more serious oxidative stress compared with those without PCOS (Table 1). What is more, when PCOS patients with abdominal obesity are excluded instead of peripheral obesity, the result remains the same [61]. In conclusion, obesity is a one of the impact factors contributing to the increased OS levels in PCOS but not the only one.

**2.2. Oxidative Stress, Insulin Resistance, and Polycystic Ovary Syndrome.** Insulin resistance (IR) is a physiological condition in which a given concentration of insulin produces a less-than-expected biological effect, because cells fail to respond to the normal actions of the hormone insulin, leading to dysfunctions of glucose transfer and utilization [62, 63]. Andres clamp technique is the most accurate method to diagnose IR, but its high cost limits the clinical acceptance; therefore, fasting insulin (FINS) and homeostasis model assessment of insulin resistance (HOMA-IR) are usually employed in clinic [64, 65]. IR is regarded as the core mechanism of polycystic ovary syndrome (PCOS) pathogenesis [3], and the IR rate of PCOS patients ranges from 50% to 70% [66, 67]. In fact, IR markers of women with PCOS, such as HOMA-IR, increase significantly compared with normal women and are usually significantly correlated with oxidative stress (OS) markers [10, 68, 69].

IR encourages OS because hyperglycemia and higher levels of free fatty acid lead to reactive oxygen species (ROS) production [45, 70]. When excess glucose or free fatty acid are absorbed in the cell, a large number of reducing

metabolites, just like pyruvic acid and acetyl coenzyme A, will be transferred into mitochondria for oxidization, leading to enhancing the activity of electron transport chain and single electron transfer, finally resulting in increasing ROS production. Furthermore, OS would be caused if reducing enzymes, just like super oxidative dismutase (SOD), peroxidase, and catalase, fails to scavenge the excess ROS in the cell [27, 71]. In the IR model of animals induced by high fructose, OS is observed to be enhanced, with the increased protein carbonyl, nonesterified fatty acid (NEFA) and malondialdehyde (MDA),  $O_2^-$ , reduced glutathione (GSH), and so on [72–74]. As it is known, IR is frequently accompanied with obesity and exists in about half of the obese [47], so IR is also regarded as one of the core mechanisms by which obesity contributes to OS. In the study of Huber-Buchholz et al., reducing the body weight by 11%, obese women were demonstrated to increase insulin sensitivity by 71% and decrease fasting insulin levels by 33% [75]. However, the correlation of oxidative stress and IR is still significant independent of obesity [10].

Though the full mechanism of OS-induced IR remains unclear, OS has been demonstrated to play crucial roles in IR pathogenesis [70, 76]. In multiple studies, it was reported that exposure to oxidative stress inhibits the metabolic pathways induced by insulin in L6 myotube and 3T3-L1 adipocyte models [77, 78]. According to the investigation of Bloch-Damti and Bashan, insulin-stimulated glucose uptake, glycogen synthesis, and protein synthesis would be inhibited after exposure to  $50 \mu\text{M}$   $H_2O_2$  for 2 hours [70]. Oxygen radical plays an important role in glucose regulation [79]. For example,  $H_2O_2$  could regulate the insulin release of  $\beta$  cell stimulated by glucose and participate in the regulation of insulin signaling pathway [80]. In general, insulin receptor substrate (IRS) is the key player of IR pathogenesis [81]. With the increased OS, various protein kinases are activated to induce serine/threonine phosphorylation of IRS and inhibit normal tyrosine phosphorylation of IRS, reducing the capacity of IRS to combine with insulin receptor, suppressing IRS to activate the downstream phosphatidylinositol 3-kinase (PI3K); and finally insulin signal to the effector via insulin receptor (InsR)/IRS/PI3K pathway is interfered with. In addition, serine/threonine phosphorylation of IRS could also induce the degradation of IRS and make IRS become the inhibitor of InsR kinase [82, 83]. Insulin signaling pathways could also be activated by OS mainly through Jun N-terminal kinase/Stress Activated Protein Kinase (JNK/SAPK) signaling pathway and inflammatory signaling pathway ( $I\kappa\text{B}$  kinase/nuclear factor  $\kappa\text{B}$ , IKK/NF- $\kappa\text{B}$ ), leading to IR via post-insulin receptor defect [84–86].

IR in PCOS is alternative for glycometabolism, and the synthesis of sex hormones is enhanced [87, 88]. The mechanism of the alternative IR in PCOS still remains unclear, but post-insulin receptor defect in insulin signaling is regarded as the major pathogenesis mechanism of IR in PCOS [89]. Levels of Ser-phosphorylated IRS-1 of adipose tissue and serum in PCOS women are significantly higher than those in controls, whereas IRS-1 tyrosine phosphorylation levels in PCOS women are lower than in controls [90, 91]. The amount of IRS-1 decreases in adipose tissue and granulosa cells but increases in PCOS theca cells [61, 92]. Levels of activated

TABLE 1: Oxidative stress (OS) markers employed in polycystic ovary syndrome (PCOS) patients are shown in the table.

Biomarkers evaluating OS level	Location and source	OS levels of PCOS patients compared with the normal		References
		Independent of obesity		
<i>Markers reflecting oxidative levels</i>				
Malondialdehyde (MDA)	Serum; erythrocyte	Higher	Higher	[4, 42, 43, 69, 126, 158, 177–182]
Advanced glycosylated end products (AGEs)	Serum	Higher		[177, 183]
Xanthine oxidase (XO)	Serum	Higher		[184]
8-Hydroxydeoxyguanosine (8-OHdG)	Serum	Lower	Lower	[185]
Lipid peroxidation (LPO)	Follicular fluid; serum	Higher		[178, 186]
Protein carbonyl	Serum	Higher		[187]
Reactive oxygen species (ROS)	Follicular fluid; granulose cell; mononuclear cell	Higher		[186, 188, 189]
Total oxidant status (TOS)	Serum	Higher	Higher	[190, 191]
Oxidative stress index (OSI)	Serum	Higher		[190]
Homocysteine (Hcy)	Serum	Higher	Higher	[4]
Asymmetric dimethylarginine (ADMA)	Serum	Higher	Higher	[4]
Prolidase (PLD)	Serum	Higher		[190]
Nitrotyrosine (Ntyr)	Serum		Higher	[192]
Uric acid	Serum		Higher	[192]
Neopterin (NEO)	Serum	Higher	Higher	[193]
<i>Markers reflecting antioxidative levels</i>				
Superoxide dismutase (SOD)	Serum; erythrocyte; follicular fluid	Higher	Higher	[4, 42–44, 182, 194, 194]
Glutathione (GSH)	Serum	Lower	Lower	[4, 43]
Paraoxonase 1 (PON1)	Serum	Lower	Lower	[4, 69, 179, 184]
Heme oxygenase-1 (HO-1)	Serum		Lower	[195]
Total antioxidant status (TAS)	Serum	Lower	Lower	[126, 187]
Total antioxidant capacity (TAC)	Follicular fluid; serum	Lower		[69, 186]
Vitamin E	Serum	Lower		[178]
Vitamin C	Serum	Lower		[178]
Thiol	Serum	NS	Lower	[94, 184]
L-Carnitine	Serum		Lower	[196]

extracellular signal-regulated kinase 1/2 (ERK1/2) of adipose tissue and serum in PCOS women are observed to be higher than those in controls, but levels of insulin receptor, glucose transporter-4 (GLUT4), and PI3K are lower [61, 90].

Thus, OS is intimately associated with IR and is possible to be the major inducement of IR in PCOS via post-insulin receptor defect. In addition, studies with antioxidants such as vitamin E,  $\alpha$ -lipoic acid, and N-acetylcysteine indicate a beneficial impact on insulin sensitivity and offer the possibility of new treatment approaches for IR [93]. So, IR is certainly involved in the physiological process of PCOS but may well be a noninitial factor caused by OS. However, OS still remains increased in PCOS independent of obesity and IR [94, 95].

**2.3. Oxidative Stress, Chronic Inflammation, and Polycystic Ovary Syndrome.** Chronic low-grade inflammation is considered as an important feature of polycystic ovary syndrome

(PCOS) and has been suggested to participate in the pathogenesis and development of PCOS [96, 97]. Inflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-18 (IL-18), monocyte chemoattractant protein-1 (MCP-1), and acute phase serum amyloid A (APSAA), increased in women with PCOS compared with the normal [98–102]. It has been accepted that there is a tight link of oxidative stress (OS) and inflammation, and it is hard to distinguish inflammation from OS absolutely; they are usually accompanied with each other [37]. Reactive oxygen species (ROS) could induce releasing inflammatory factors and inflammatory response, via activating the associated signaling pathways of nuclear factor- $\kappa$ B (NF- $\kappa$ B), activated protein-1 (AP-1), and hypoxia-inducible factor-1 (HIF-1) [103]. On the other hand, ROS could be generated by rheumatoid synovial cells via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Nox), during exposure to two major rheumatoid arthritis (RA) cytokines, interleukin-1 $\beta$  (IL-1 $\beta$ ) and TNF- $\alpha$  [104, 105].

Inflammation has also been demonstrated to be associated with IR in PCOS [106]. It was reported that adipose-derived TNF- $\alpha$  levels in mice were increased during the advancement of obesity, but when TNF- $\alpha$  was neutralised, insulin sensitivity was improved [107]. As well as OS, inflammation could induce insulin resistance (IR) mainly via interfering with post-insulin receptor signaling pathway, insulin receptor substrate 1-phosphatidyl inositol 3 kinase-protein kinase B (IRS1-PI3K-PKB/Akt) pathway [108].

**2.4. Oxidative Stress, Hyperandrogenemia, and Polycystic Ovary Syndrome.** Hyperandrogenemia is a classical feature of polycystic ovary syndrome (PCOS), and 70%–80% of women with hyperandrogenemia are diagnosed with PCOS [109]. Hyperandrogenemia is regarded as the core pathogenesis of PCOS, as PCOS models of animals could be established by excess androgen administration [110, 111]. For the increased androgen levels in PCOS, insulin resistance (IR) is regarded as the primary factor, by compensatory hyperinsulinemia [112]. Insulin is reported to stimulate ovarian androgen secretion directly alone and/or augment luteinizing hormone- (LH-) stimulated androgen secretion [113–115]. In addition, insulin may also enhance the amplitude of gonadotropin-releasing hormone- (GnRH-) stimulated LH pulses, decrease hepatic production of serum sex hormone-binding globulin (SHBG), and/or decrease insulin-like growth factor binding protein-1 (IGFBP-1) [116–121]. Finally, the availability of free insulin-like growth factor-1 (IGF-1) is increased to stimulate androgen production [122, 123].

However, oxidative stress (OS) and inflammation seem to contribute to hyperandrogenemia in PCOS, but detailed interactions still remain unclear, as few investigations have been discovered to focus on the subject. In multi-investigations, OS and inflammation markers are discovered to be positively correlated with androgen levels in PCOS patients [124–126]. In vitro, OS was reported to enhance the activities of ovarian steroidogenesis enzymes, which could stimulate androgen generation, and antioxidative chemicals, just as statins, inhibit the activities [127]. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), an inflammatory marker associated with tissue inflammation, was reported to have the ability to promote the proliferation of mesenchymal cells of follicular membrane and the synthesis of androgen in the rat [128].

Hyperandrogenemia seems to have the ability to cause obesity, IR, and OS in females and female animals. Compared with controls, PCOS models induced by excess androgen have increased weights, triglycerides, nonesterified fatty acid (NEFA), fasting serum insulin (FINS), fasting blood glucose (FBG), homeostasis model assessment of insulin resistance (HOMA-IR), and altered oxidative stress markers, such as malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) [129–132]. In addition, after women with normal body mass index (BMI) of reproductive age were administered with oral dehydroepiandrosterone (DHEA) to increase the androgen levels in vivo, blood samples were obtained both under fasting state and after glucose stimulation, and leukocytic reactive oxygen species (ROS) generation, p47(phox) gene expression, and plasma thiobarbituric reactive substances (TBARS) were discovered to be increased

to promote oxidative stress [101]. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is the potential crucial mediator of inflammation induced by hyperandrogenemia [133–135]. Expression and phosphorylation level of NF- $\kappa$ B increased, and interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) synthesis was enhanced in adipose cells after administering testosterone, but IL-6 and MCP-1 levels decreased when NF- $\kappa$ B inhibitors were administered as well [136].

It is interesting to note that androgen may also play a role in protecting cells or tissues from inflammation and oxidative stress. In the obese PCOS patients, body mass, free fatty acid level, IL-6 level, and C-reactive protein (CRP) level increased, while androgen level was downregulated with GnRH agonist for a long term [137]. In addition, androgen was reported to have the ability to enhance the activity of hormone-sensitive lipase (HSL) to promote lipolysis and inhibit adipose tissue further growth [138]. Thus, a hypothesis was raised that androgen may contribute to anti-inflammation by promoting lipolysis, limiting adipose tissue addition, and further reducing inflammatory factor synthesis [137, 139]. In human decidual endometrial stromal cells, expressions of forkhead box protein O1 (FOXO1) and superoxide dismutase 2 (SOD2) could be promoted by dihydrotestosterone (DHT) to enhance the resistance to oxidative stress [140]. It indicates that the functions of androgen may perform multiformity in different circumstances and depend on the dosage.

### 3. Polycystic Ovary Syndrome and Cancers

A higher risk for cancers of the reproductive tract, especially endometrial cancer, seems to be related to polycystic ovary syndrome (PCOS) [141–144]. In addition, PCOS women also manifest clinical features, correlated with risk factors for breast cancer and ovarian cancer [13, 14, 145]. However, defined associations of PCOS, breast cancer, and ovarian cancer have not been found yet until recently [14]. The association of PCOS and endometrial was firstly reported in 1949, and the complicated interrelationship between endometrial cancer and PCOS has been recognized for several years, involving multiple risk factors, such as obesity, diabetes, hypertension, anovulation, nulliparity, and family history [16, 17, 146]. The meta-analysis of the data collected by Chittenden et al. [145] suggests that women with PCOS are more likely to develop cancer of the endometrium (OR 2.70, 95% CI 1.00–7.29), and the risk would increase to 3-fold, which was confirmed by Haoula et al. [143]. While the same meta-analysis was done by Fearnley et al., a similar conclusion was obtained, but the risk of endometrial cancer in PCOS women was enhanced to 4-fold (OR 4.0, 95% CI 1.7–9.3) compared with controls in another study based on Australian women younger than 50 years [147]. In addition, the increased risk for endometrial cancer in PCOS women is modified to 2.7-fold (95% confidence interval 1.0–7.3) by Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group [148].

**3.1. Contributions of Oxidative Stress to Cancer Pathogenesis.** Oxidative stress (OS), which is altered in PCOS, increases in malignant cells compared with normal cells in culture and in

vivo [149, 150]. OS could induce directly genetic variation by DNA damage, such as DNA chain rupture, base modification, DNA-DNA crosslinking, DNA-protein crosslinking, and epigenetic change, including elevated DNA methylation level, which both play important roles in the pathogenesis of cancer [12, 22]. Most modifications of DNA bases locate on the eighth carbon atom of deoxy guanine, forming 8-hydroxydeoxyguanosine (8-OHdG). The formation of 8-OHdG could make the modified guanine replaced by thymine, leading to gene mutation and resulting in the base pairing error of “G-C → T-A” in the process of DNA replication [22, 151]. The 8-OHdG level of tumor cell is found to be significantly higher than that of normal cell and further regarded as a classical biomarker of oxidative DNA damage [152]. Though 8-OHdG could not kill cells directly, it could induce the nearby DNA bases to be modified singularly, aggravating genome instability and tumor cell transfer [153]. While adducts, just as 8-OHdG, avoid DNA self-repair by 8-oxoguanine glycosylase (OGG1) and mutY DNA glycosylase (MUTYH), genetic mutations (point mutations mainly) could be caused, and cancer would initiate if the DNA mutations locate in cancer-related genes, such as Ras protooncogene and p53 cancer suppressor gene [25, 26, 151, 154].

DNA methylation refers to the process that the methyl group of S-adenosyl-L-methionine (SAM) is transferred to adenine base or cytosine base of DNA catalyzed by DNA methylase (Dnmt) after DNA replication, modifying the DNA [155]. DNA methylation is involved in expression and control of genes and acts specifically according to tissue and gene. In the normal cells, the normal state of genome is held by hypomethylation levels of the promoter region of tumor suppressor genes and hypermethylation levels of some repetitive sequences, such as long interspersed nuclear element (LINE1) and Alu element [156]. DNA damage induced by reactive oxygen species (ROS), especially  $\cdot\text{OH}$ , could influence the connection of DNA, as a substrate, with Dnmt, decreasing the methylation levels of the whole genome [26]. However, ROS also could induce hypermethylation of the promoter regions of cancer suppressor genes, promoting cell malignant transformation [157].

**3.2. Oxidative Stress-Induced DNA Damage in Polycystic Ovary Syndrome.** Micronucleus (MN) frequency, evaluated by cytokinesis block micronucleus index, which reflects genomic instability, is increased in PCOS patients compared with controls [158–161]. Furthermore, women with PCOS show a significant increase in DNA strand breakage and  $\text{H}_2\text{O}_2$ -induced DNA damage [162]. In addition, elevated chromosome malsegregation (assessed by X chromosome chromogenic in situ hybridisation) and reduced mitochondrial DNA (mtDNA) copy number (reflecting mitochondrial metabolism) are also found in PCOS [159, 163].

Serum MDA levels, an OS marker, were observed to be positively correlated with MN in PCOS patients but not the normal [158]. In addition, mtDNA copy number was negatively correlated with indices of insulin resistance, waist circumference, and triglyceride levels and positively correlated with sex hormone-binding globulin levels [163]. Significant correlations were also found between free testosterone

and DNA strand breakage and  $\text{H}_2\text{O}_2$ -induced DNA damage [162]. As stated above, there are intimate interactions between OS and IR and obesity. It seems that the altered oxidative stress in PCOS has increased the instability of genes and the risk of DNA mutations and potentially contributes to the pathogenesis of gynecological cancers.

**3.3. Obesity and Endometrial Cancer.** Obesity could significantly aggravate OS and is usually accompanied with PCOS and is well known to be associated with endometrial hyperplasia and endometrial cancer, thus being regarded as one of the most significant risk factors for endometrial cancer [15]. Approximately 70–90% of Type 1 (estrogen-dependent) endometrial cancer patients are obese [164], and Schouten et al. demonstrated that obesity increased the risk of endometrial cancer by 4.5 times [165]. In fact, several studies show that adiposity contributes to the increased incidence and/or death from cancers of not only endometrium but also colon, breast, kidney, ovary, esophagus, stomach, pancreas, gallbladder, and liver [166, 167]. Furthermore, this increased endometrial cancer risk related to PCOS is reduced by almost one-half when adjusted for body mass index (BMI) (OR 2.2, 95% CI 0.9–5.7), emphasizing that obesity plays a key role in endometrial cancer pathogenesis, possibly via oxidative stress [15].

**3.4. Insulin Resistance and Endometrial Cancer.** Insulin resistance (IR), which is also significantly associated with OS regardless of obesity, is another common feature of PCOS and endometrial cancer and is regarded as the potential mechanism of endometrial hyperplasia and endometrial cancer pathogenesis in PCOS [14]. Elevated fasting serum insulin levels and insulin responses after glucose administration have been found in postmenopausal women with endometrial cancer [168]. In the study of Zhang, it is statistically significant that 12 of 19 PCOS patients with IR show endometrial hyperplasia or endometrial canceration compared to 4 of 15 PCOS patients without IR [169].

Just as stated above, IR would induce compensatory hyperinsulinemia, and excess insulin would increase insulin growth factor-1 (IGF-1). Insulin and IGF have been shown to accelerate the growth of endometrial cancer cells in vitro, and the mitogenic effect of hyperinsulinemia may be mediated by activation of the mitogen-activated protein kinase (MAPK) pathway [170], increasing expression of vascular endothelial growth factor (VEGF) [171]. Conversely, when endometrial cancer cells are exposed to serum from metformin-treated women with PCOS, cell growth is attenuated, and signaling pathways associated with inflammation and tumor invasion are altered [172]. Hyperinsulinemia reduces insulin-mediated glucose uptake and also enhances steroidogenesis. As a result, excessive insulin stimulates theca cell androgen secretion activity and elevates serum-free testosterone levels through the pathways stated above [173]. Testosterone level has been shown to be positively correlated with p-ERK and p-AKT, which are significantly higher in endometrial tissue of PCOS patients with endometrial hyperplasia or canceration compared with the normal controls, and play key roles in tumor proliferation [169]. In addition, just as discussed above, OS is

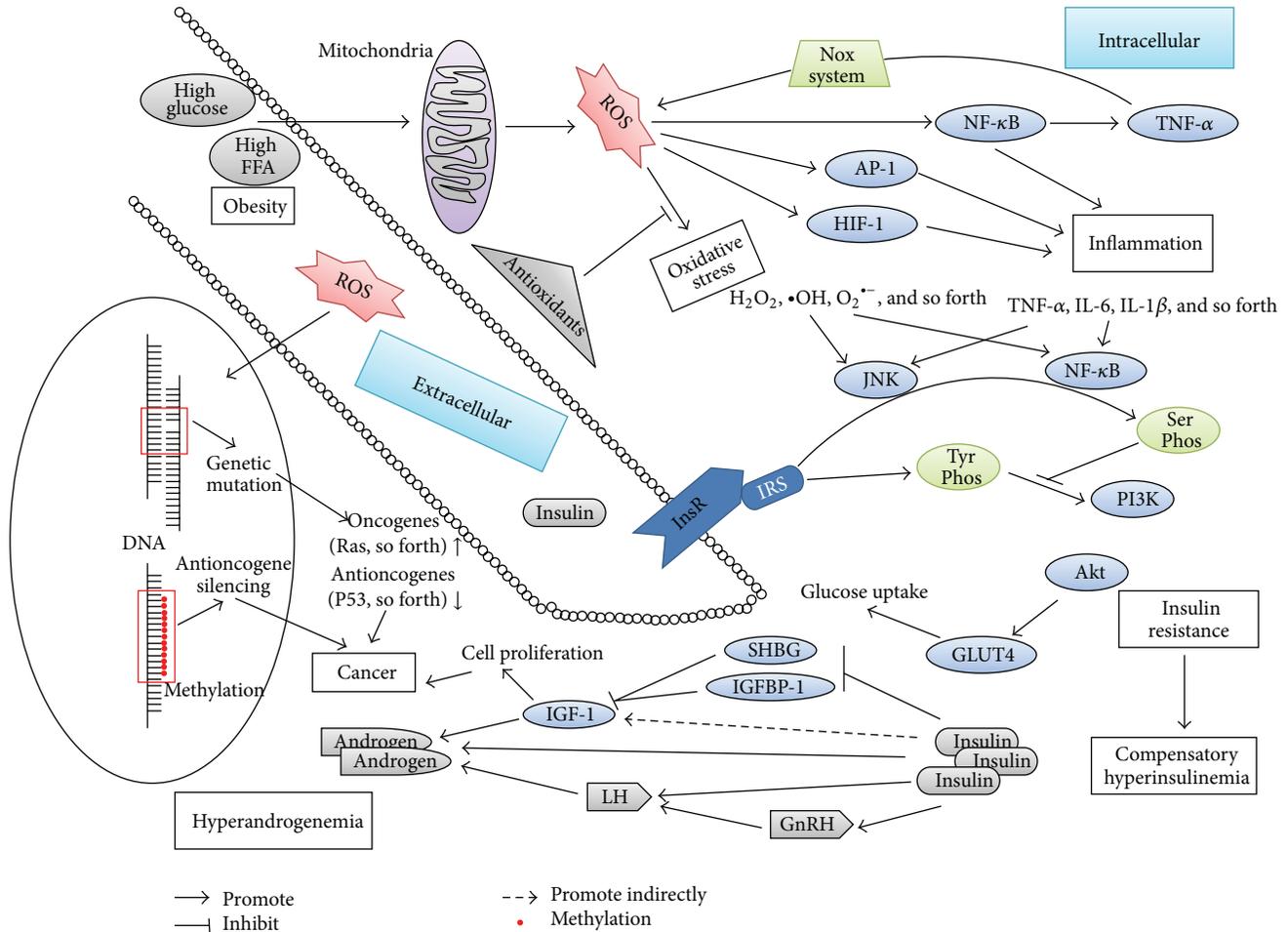


FIGURE 1: Interactions of oxidative stress, inflammation, insulin resistance, and hyperandrogenemia are described briefly in the figure, which are all involved in polycystic ovary syndrome physiopathology. Oxidative stress seems to induce cancer through genetic variation and cell signaling pathway. FFA, free fatty acid; ROS, reactive oxygen species; NF-κB, nuclear factor kappa B; AP-1, activator protein-1; HIF-1, hypoxia-induced factor-1; TNF-α, tumor necrosis factor-α; Nox, nicotinamide adenine dinucleotide phosphate oxidase system; IL, interleukin; JNK, c-Jun N-terminal kinase; InsR, insulin receptor; IRS, insulin receptor substrate; Tyr Phos, tyrosine phosphorylation; Ser Phos, serine phosphorylation; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; GLUT4, glucose transporter-4; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; IGFBP-1, insulin growth factor binding protein; IGF-1, insulin growth factor-1.

an important inducer of IR by post-insulin signaling defects and has interassociation with hyperandrogenemia. Consequently, IR and hyperandrogenemia may be the potential converged mechanisms that oxidative stress influences on during endometrial canceration process.

**3.5. Estrogen and Endometrial Cancer.** The prolonged exposure to unopposed estrogen in the absence of sufficient progesterone, which is induced by denominator anovulation, is also regarded as a major factor causing endometrial hyperplasia and canceration in PCOS [174–176]. Estrogen could bind to its nuclear receptor, stimulating secretions of various growth factors, such as IGF, and epidermal growth factor (EGF), and activate ERK signaling pathway, to promote endometrial proliferation and even canceration. In addition, metabolites of estrogen also could be the inducers of endometrial canceration by binding to DNA and causing further

DNA damage, and the procedure is associated with oxidative stress. Under oxidative stress, estrogen intermediate metabolites, including 2-hydroxyl estrone (2-OHE1), 4-hydroxyl estrone (4-OHE1), and 16α-hydroxyl estrone (16αOHE1), could not be methylated and eliminated from the body and would be oxidized to semiquinonoid compounds and quinonoid compounds. The two abnormal types of metabolites of estrogen with electron affinity bind to nucleophilic group of DNA by covalent bond, causing DNA mutation, and further lead to endometrial canceration process.

**3.6. Polycystic Ovary Syndrome and Other Cancers.** In the investigation of Schildkraut et al. [142], ovarian cancer risk is found to increase to 2.5-fold (95% confidence interval [CI] 1.1–5.9) among women with PCOS, and the association is found to be stronger among women who never used oral contraceptives (odds ratio [OR] 10.5, 95% CI 2.5–44.2) and

women who were in the first quartile of body mass index (13.3–18.5 kg/m<sup>2</sup>) at the age of 18 (OR 15.6, 95% CI 3.4–71.0). Though PCOS perhaps could increase the risk of ovarian cancer based on the limited few studies, the association of them has also been under the doubt and needs more evidences to be proved. On the other hand, breast cancer seems to be not associated with PCOS based on the current limited data [14]. In addition, powerful evidences are needed to evaluate the associations between PCOS and vaginal, vulvar, and cervical cancer or uterine leiomyosarcoma. Nevertheless, obesity and estrogen excess are suggested as the two important factors inducing cancers besides endometrial cancer [14].

#### 4. Conclusion

It is known that DNA damage and methylation induced by oxidative stress (OS) play key roles in the early stage of tumor pathogenesis and tumor conversion by activating protooncogene and silencing antioncogene. Mechanistically, the abnormal oxidative stress in polycystic ovary syndrome (PCOS) patients could cause genetic instability and raise the risk of cancers. OS has been demonstrated to be significantly associated with obesity, insulin resistance (IR), inflammation, and hyperandrogenemia, which are the common characteristics and potential inducers of PCOS and endometrial cancer and could participate and be induced in an interweaving way during disease physiology (Figure 1). ROS and proinflammatory factors, produced under OS, could induce IR majorly through IRS-PI3K-Akt by activation of associated signaling pathways, such as NF- $\kappa$ B and JNK. Hyperinsulinemia, compensatory for IR, contributes to cancer pathogenesis by activating cell proliferation signaling pathways and finally leads to malignant transformation. In addition, OS, IR, and inflammation could be induced by excess androgen in vivo and involved in obesity. Thus, OS is considered as an initial factor, leading to cancers in PCOS. It remains to be determined whether other potential pathways mediated by oxidative stress could play roles in the pathogenesis of PCOS related cancers.

#### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## Review Article

# Bypassing Mechanisms of Mitochondria-Mediated Cancer Stem Cells Resistance to Chemo- and Radiotherapy

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Received 19 June 2015; Revised 24 August 2015; Accepted 25 August 2015

Academic Editor: Amit Tyagi

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Cancer stem cells (CSCs) are highly resistant to conventional chemo- and radiotherapeutic regimens. Therefore, the multiple drug resistance (MDR) of cancer is most likely due to the resistance of CSCs. Such resistance can be attributed to some bypassing pathways including detoxification mechanisms of reactive oxygen and nitrogen species (RO/NS) formation or enhanced autophagy. Unlike in normal cells, where RO/NS concentration is maintained at certain threshold required for signal transduction or immune response mechanisms, CSCs may develop alternative pathways to diminish RO/NS levels leading to cancer survival. In this minireview, we will focus on elaborated mechanisms developed by CSCs to attenuate high RO/NS levels. Gaining a better insight into the mechanisms of stem cell resistance to chemo- or radiotherapy may lead to new therapeutic targets thus serving for better anticancer strategies.

## 1. Introduction

One of the hypotheses explaining tumor progression suggests the existence of a group of cells with a stem phenotype which preserves tumors through a continuous production of progeny [1]. In recent years, the CSCs hypothesis has gained ground in several cancers [2]. The CSCs mediate tumor resistance to chemo- and radiation therapy and are also capable of invading and migrating to other tissues [3]. Similarly to cancer cells (CCs), the CSCs features include self-renewal capacity, the ability of proliferation, migration to and homing at distant sites, and resistance to toxic agents. Accordingly, CSCs identification and isolation include *in vitro* (sphere forming, Hoechst dye exclusion, aldehyde dehydrogenase ALDH enzymatic activity, surface markers, colony forming, label retention, and migration) and *in vivo* (tumor propagation, xenotransplantation) assays. This theory has been recently supported by the findings that, among all malignant cells within a particular tumor, only CSCs have

the exclusive potential to generate tumor cell population [4]. Given these shared attributes, cancer was proposed to originate from transforming mutation(s) in normal stem cells that deregulate their physiological programs [5]. In turn, intrinsic or acquired resistance of CSCs involves mechanisms such as genetic aberrations, quiescence, overexpression of drug transporters, DNA repair ability, and overexpression of antiapoptotic proteins [6]. Intrinsic resistance to chemotherapy is emerging as a significant cause of treatment failure and evolving research has identified several potential causes of resistance most of which end up in increased apoptosis [7]. Among the mechanisms of CSC-related therapy resistance may include ROS resistance, activation of ALDH, active developmental pathways (Wnt, Notch), enhanced DNA damage response, deregulated autophagy, altered metabolism, and microenvironmental conditions [8]. Surprisingly, most of the above-mentioned pathways in CSCs are mediated by redox misbalance and involvement of mitochondria-mediated antioxidant capacity [9].

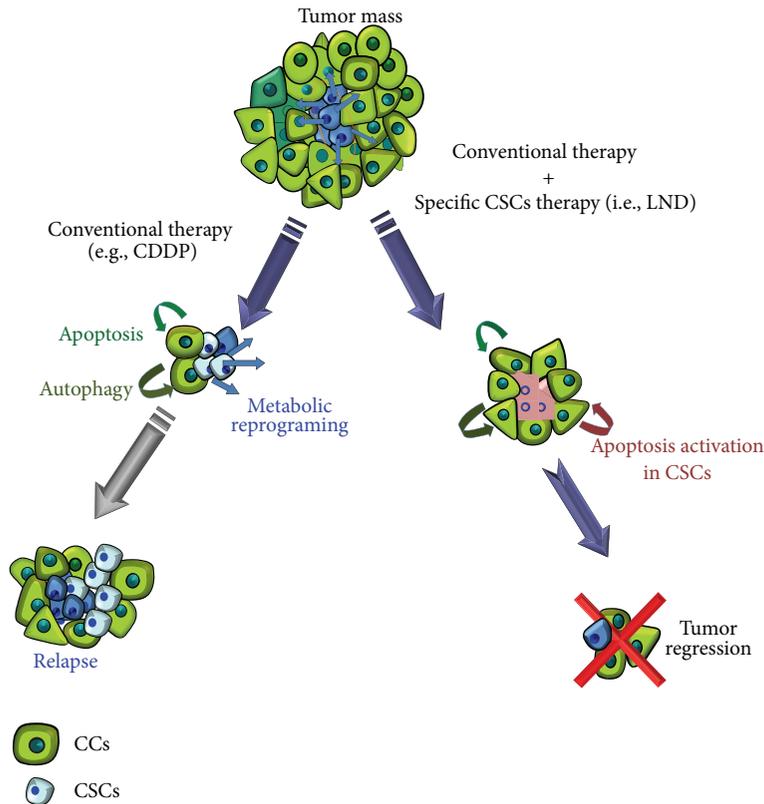


FIGURE 1: CSCs survival after chemo-/radiotherapy. The percentage of CSCs in a tumor varies depending on tumor type and tumor stage but generally comprises 0.5–5%. Most CCs in a tumor are killed after radiation or conventional chemotherapy (i.e., CDDP). The most important consequence of this is that although the tumor disappears in some cases (i.e., by image such as nuclear magnetic resonance), the percentage of CSCs has not diminished; quite the contrary it increased in proportion to the whole number of microscopically tumoral cells (reaching till 50% or more). CSCs left behind unaffected, due to their chemo- and radioresistance, eventually will experience metabolic reprogramming to give rise to new CCs and CSCs, nesting the gap left by the tumor often with more aggressive phenotype. The cotreatment of conventional therapy with a more specific drug against CSCs (i.e., LND) in parallel will solve this problem.

The major exogenous source of reactive species in eukaryotic cells is mitochondria. In normal cells, RO/NS concentration is maintained at certain threshold required for signal transduction or immune response mechanisms, and CSCs, which exhibit an accelerated metabolism, demand high ROS concentrations to maintain their high proliferation rate [10]. The imbalance between ROS generation and detoxification, known as OS, is thought to be involved in cancer development and progression [11, 12].

Chemo-/radioresistance to cancer therapy is an unsolved problem in oncology [13]. Numerous studies have attempted to explain mechanisms of resistance over the last decades. CSCs may be innately resistant to many standard therapies due to a high antioxidant capacity and inability to perform apoptosis thus surviving cytotoxic or targeted therapies (Figure 1) [14]. Here we review the progress of CSCs studies made for the last years focusing on possible mechanisms of CSCs radio- and chemoresistance in connection to oxidative stress (OS) and summarizing some therapeutic approaches to overcome that issue.

## 2. Resistance of CSCs to Conventional Chemo- and Radiotherapeutic Regimes in Connection to Oxidative Stress (OS)

Although conventional chemotherapy kills most cells in a tumor, it is believed to leave CSCs behind causing chemo- and radioresistance (Table 2). As a consequence, CSCs persist in the body of cancer patients and in the middle-long term will migrate to the blood to nest in distal organs to metastasize. In the last five years, several protective CSC pathways have been proposed. The multifunctional efflux transporters from the superfamily of human ATP-binding cassette (ABC) are among them. They comprise seven subfamilies with 49 genes grouped into seven families (from A to G) with various functions, and at least 16 of these proteins are implicated in cancer drug resistance [15]. These ABC proteins have been known to also participate in multidrug resistance (MDR) of tumor cells [16]. Recent data demonstrate their role in protection of CSCs from chemotherapeutic agents [17]. Importantly, they are engaged

in redox homeostasis and protection from OS in mammals [18]. Malfunction of the ABCD1 gene impairs oxidative phosphorylation (OXPHOS) triggering mitochondrial ROS production from electron transport chain complexes [19]. ABCC9 is required for the transition to oxidative metabolism [20]. Deficiency of a transregulator of mitochondrial ABC transporters PAAT decreases mitochondrial potential and sensitizes mitochondria to OS-induced DNA damages [21]. Drug resistance in colon CSCs is mediated by the ABC G member 2 (ABC-G2) and regulated by Apel redox protein [22]. Overall, one may conclude that redox dysregulation of one or several ABC members may significantly impact CSCs survival after chemotherapeutic treatment. Decreasing the activity of ABC transporters may therefore overcome drug resistance [23].

On the other hand, developmental pathways such as the Epithelial-Mesenchymal Transition (EMT) play crucial roles in tumor metastasis and recurrence. EMT process resembles very much the fate of CSCs and is involved in de novo and acquired drug resistance [24]. Altered production of RO/NS is involved in the regulation of CSC and EMT characteristics [25]. Moreover, microRNAs play also key roles in this aspect. For example, miR-125b suppressed EMT by targeting SMAD2 and SMAD4 [26]. Moreover, secreted frizzled-related protein 4 (sFRP4) chemosensitized CSC-enriched cells to the most commonly used anti-glioblastoma drug, temozolomide (TMZ), by the reversal of EMT. Significantly, the chemosensitization effect of sFRP4 was correlated with the reduction in the expression of drug resistance markers ABCG2, ABCC2, and ABCC4 [27]. These findings could be exploited for designing better targeted strategies to improve chemoresponse and eventually eliminate CSCs.

### 3. Apoptosis and CSCs Resistance due to Increased Antioxidative Properties

An increasing number of conventional and novel generation chemotherapeutic drugs induce apoptosis through the induction of OS. If decreased RO/NS detoxification in CSCs is indeed a prime factor for chemo- or radioresistance prooxidant chemicals as, for example, malonohydrazides, targeting the redox state of pathogenic versus nonpathogenic cells may represent a challenging solution. The most developed drug of this class, STA-4783 (elesclomol), targets OS by Hsp70 induction and induces ROS within CCs [28]. Shepherdin is one of the first rationally designed mitochondrial drugs targeting Hsp90/TRAP1 functions through inhibiting ATPase activities. The tumor necrosis factor (TNF) receptor-associated protein 1 (TRAP1) is a mitochondrial homologue of Hsp90 [29]. Phosphorylation of TRAP1 by PTEN is responsible for the protection of ROS-mediated cell death [30]. Therefore, blocking the ATP pocket in the Hsp90 by shepherdin or geldanamycin causes inhibition of the TRAP1 chaperone function and may provide a novel strategy to design anti-CSCs drugs [31]. SMIP004 (N-(4-butyl-2-methyl-phenyl) acetamide), a novel anticancer drug, induces mitochondrial ROS formation and disrupts the balance between redox and bioenergetics states [32].

Recent works by Kim et al. identified CD13(+) liver CSCs surviving in hypoxic lesions after chemotherapy, presumably through increased expression of CD13/aminopeptidase N, a ROS scavenger [45]. CD13 also enhances the generation and accumulation of mutations following DNA damage. Therefore, the CD13(+) dormant cancer stem cells must be eradicated fully to achieve complete remission of cancer [46]. The resistance of CD133 positive CSCs to chemotherapy can also be linked with higher expression of BCRP1 and MGMT, as well as the antiapoptosis protein and inhibitors of apoptosis protein families [47, 48].

Resistance of glioma to chemo- or radiotherapy is associated to inability of glioma CSCs to undergo apoptosis. Combined therapy aiming to inhibit AKT/mTOR signalling pathway and reactivate TP53 functionality allowed triggering cellular apoptosis [49]. Rottlerin (ROT) is widely used as a protein kinase C-delta (PKC- $\delta$ ) inhibitor has been found to induce apoptosis via inhibition of PI3K/Akt/mTOR pathway and activation of caspase cascade in human pancreatic CSCs [33].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential component of cellular defense against a variety of endogenous and exogenous stresses [50]. NRF2 is an inducible transcription factor that activates a battery of genes encoding antioxidant proteins and phase II enzymes in response to oxidative stress and electrophilic xenobiotics [51]. NRF2-silencing in CSCs models, known as mammospheres, demonstrated increased cell death and lack of anticancer drug resistance [52]. Moreover, dedifferentiated cells upregulate MDR genes via Nrf2 signaling and suggest that targeting this pathway could sensitize drug-resistant cells to chemotherapy [53]. Interestingly, bardoxolone methyl (also known as CDDO-Me or RTA 402) is one of the derivatives of synthetic triterpenoids acting via Nrf2 and has been used for the treatment of leukemia and solid tumors [34].

### 4. Chemo- and Radioresistance of CSCs due to Impaired Autophagy: Novel Therapeutic Targets

Autophagy, also referred as “cell cannibalism,” is the degradation of cytoplasmic components, protein aggregates, and organelles through the formation of autophagosomes, which are degraded by fusion with lysosomes [54]. This process depends on a group of evolutionarily conserved autophagy-related (ATG) genes [55]. Although autophagy and apoptosis are apparently two different mechanisms, one promoting cell survival and the latter cell death, they are quite coordinated in the cells. For example, Beclin-1 (Bec1), the mammalian orthologue of yeast Atg6, is part of the class III phosphatidylinositol 3-kinase (PI3K) complex that induces autophagy. Beclin-1 interacts with the antiapoptotic protein Bcl-2 and its dissociation is essential for its autophagic activity [56].

Hypoxia-mediated autophagy has been previously suggested to promote the survival of CSCs of various origin. Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), one of the key players of cell survival response to hypoxia, was shown to convert non-stem pancreatic cancer cells into pancreatic cancer

stem-like cells through autophagic mechanisms [57]. HIF1 induction and NF $\kappa$ B activation are sufficient to induce the autophagic degradation of breast CSCs [58]. Inhibition of Wnt by resveratrol in breast CSC [35] and Notch by honokiol in melanoma SCs [36] suggests involvement of these autophagy-related players in regulation of CSCs signaling pathways.

Autophagy plays a critical role in adaptation to stress conditions in CCs and can enhance the radio- and chemoresistance of CSCs by limiting OS and protecting CSCs stemness properties [59]. Although mechanisms inducing autophagy are not fully understood, the connection of the CSCs resistance to the chemo- and radiotherapy is supported by a number of indirect evidences. Platin-derived drugs, which are used commonly in the conventional chemotherapeutic treatments, have a role in autophagy. For example, cisplatin (CDDP) preferentially induces autophagy in resistant esophageal CCs EC109/CDDP but not in EC109 cells (parental or sensitive to CDDP) [60]. Moreover, abolition of autophagy by pharmacological inhibitors or knockdown of ATG5/7 resensitized EC109/CDDP cells. In particular, the chemotherapeutic drug oxaliplatin induced autophagy, enriched the population of colorectal CSCs, and participated in maintaining the stemness of colorectal CSCs, thus making the cells more resistant to chemotherapy [61].

The Janus-activated kinase 2- (Jak2-) signal transducer and activator of transcription 3 signaling pathway may play a role in autophagy-dependent chemoresistance of CSCs derived from triple-negative breast tumors. In a recent study by Choi et al., chloroquine (CQ), an antimalarial reagent which blocks autophagy, was identified as a potential CSC inhibitor [37]. The CQ is known to evoke mitochondrial ROS and ROS scavengers may decrease CQ-induced mitochondrial autophagy [62]. All these facts support the note of ROS-dependent autophagic survival of CSCs. Recently explored inducible mouse model of mutated Kras revealed that a subpopulation of dormant tumor cells surviving oncogene ablation have features of CSCs and their tumor relapse is dependent on expression of genes governing OXPHOS, mitochondrial respiration, and autophagy [63].

Highly synergistic growth inhibition was observed in patient-derived lung CSCs exposed to a multitarget folate antagonist pemetrexed followed by a histone deacetylase inhibitor ITF2357, a known autophagy inducer [38]. A few studies using cultured cells found that melatonin promoted the generation of ROS at pharmacological concentrations [64]. Treatment with melatonin induced glioma CSCs death with ultrastructural features of autophagy [65].

Reduced glutathione (GSH) is considered to be one of the most important scavengers of reactive oxygen species (ROS), and its ratio with oxidised glutathione (GSSG) may be used as a marker of oxidative stress [39]. The side population (SP) cells from bladder cancer cell lines which resemble characteristics of CSCs had low ROS levels and high GSH/GSSG ratio and might contribute to radioresistance of CSCs [66]. The SP cells also showed substantial resistance to gemcitabine, mitomycin, and cisplatin compared with the non-SP counterparts and revealed a high autophagic flux associated with the ABCG2 expression. Importantly, pharmacological

and siRNA mediated inhibition of autophagy potentiated the chemotherapeutic effects of gemcitabine, mitomycin, and CDDP in these CSCs. This may represent a potent target for the treatment of bladder carcinoma [67]. Screening studies by Jangamreddy et al. identified molecules that were preferentially toxic to CSCs, in particular, K<sup>+</sup>-ionophore salinomycin [40]. Salinomycin causes mitochondrial dysfunction, decreases ATP production, and induces autophagy [68]. Under hypoxia or/and low glucose level (the primary energy source for CCs) its toxicity towards CCs is amplified [69]. The mechanism includes activation of the AMP activated protein kinase (AMPK) that triggers autophagy making salinomycin to be anti-CSCs chemical [70]. The combination of AMPK agonist such as metformin and a glycolysis inhibitor 2-deoxyglucose (2DG) led to significant cell death associated with a sustained autophagy inhibiting tumor growth in mouse xenograft models [71]. Since AMPK activation was shown to mediate the metabolism reprogramming in drug-resistant CCs including promoting Warburg effects and mitochondrial biogenesis, both salinomycin and corresponding inhibitors of AMPK are now suggested to combat chemo- and radiotherapeutic resistance of CSCs [72].

Another type of selective autophagy, called mitophagy is served to the removal of dysfunctional mitochondria from the cells and is often controlled by moderate level of ROS [73, 74]. During mitophagy dysfunctional mitochondria are engulfed by a double-layered membrane (phagophore) that forms so-called autophagosome followed by degradation [75]. Among several drugs inducing mitophagy proton pump inhibitor ESOM damages mitochondria through NADPH oxidase and ROS accumulation [76]. The ESOM may work as a synthetic lethal reagent which increases cytotoxicity if used upon knockdown of Beclin-1 [77]. Another drug DCA (dichloroacetate) is a small molecule and a mitochondria-targeting agent. In CCs, the DCA induces mitophagy through accumulation of ROS and reduction of lactate excretion followed by the increase of NAD(+)/NADH ratio [78]. Importantly, paclitaxel-resistant cells contained sustained mitochondrial respiratory defect. DCA specifically acts on cells with mitochondrial respiratory defect to reverse paclitaxel resistance. DCA could not effectively activate oxidative respiration in drug-resistant cells but induced higher levels of citrate accumulation, which led to inhibition of glycolysis and inactivation of P-glycoprotein [79].

Overall, the above data provide multiple lines of evidence supporting the idea that impaired autophagy coupled with OS plays an essential role in the development of drug resistance, self-renewal, differentiation, and tumorigenic potentials of CSCs, implying the therapeutics potential of autophagy inhibitors to overcome that issue (Table 2).

## 5. OS, Mitochondria, and CSCs

In mammalian systems RO/NS presumably include so-called free ( $\cdot$ OH, RO $\cdot$ , ROO $\cdot$ , NO $\cdot$ , hydroxyl, alkoxy, peroxy, and nitroxy), superoxide (O<sub>2</sub><sup>-</sup>) radicals, and peroxides (H<sub>2</sub>O<sub>2</sub>, RO<sub>2</sub>H) and are mainly generated by OXPHOS in mitochondria, whereas, in pathological conditions, high level

of RO/NS can be mitochondria dependent (ischaemia, loss of cytochrome c, low ATP demand and consequent low respiration rate, diabetes, DNA damage, and mutations), independent or indirect (cancers, tissue injuries, and inflammatory events) [80, 81]. Importantly, being the main source of RO/NS generation, mitochondria are also their primary and the most susceptible target. This may evoke a “secondary wave” of OS generated by damaged mitochondria followed by formation of extra RO/NS or by inhibition of detoxifying enzymes and generation more RO/NS flux thus forming a vicious cycle [82]. In fact, decreased mitochondrial priming in colon CSCs responsible for resistance to conventional chemotherapy has been recently determined [83]. The relevance of OXPHOS has also been shown in glioblastoma (GBM) sphere cultures (glioma spheres). Insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) expression provides a key mechanism to ensure OXPHOS maintenance by delivering respiratory chain subunit-encoding mRNAs to mitochondria and contributing to complex I and complex IV assembly [84]. Several antioxidant enzymes such as Mn, Cu, Zn-containing superoxide dismutases (SODs), glutathione peroxidase, glutathione reductase (GPx), glutathione S-transferase (GSTs), and catalase protect DNA from OS [85]. Unlike CSCs, CCs have higher bioenergetic metabolism, higher ROS level, and higher capacity to detoxify RO/NS [86]. These facts may explain overall better cancer survival. In CSCs, the level of RO/NS is not that high, comparatively to surrounding CCs [87–90]. There can be several reasons to that. The mitochondrial mass can be higher in CSCs or mitochondrial functions (ATP production,  $\Delta\psi_m$ ) can be impaired. However, in the recent experiments with lung CSCs no difference in mitochondrial mass between CSCs and non-CSCs was found [91]. The  $\Delta\psi_m$  level and the intracellular concentrations of ATP and ROS were also lower than in non-CSCs. Another possible scenario of low ROS in CSCs could be metabolic reprogramming, which is critical to sustain self-renewal and enhance the antioxidant defense mechanism. This fact is closely related to the adaptation of CSCs to hypoxia requiring a biochemical trim characterized by a glycolytic-oriented metabolism that counterbalances a poor mitochondrial apparatus. In metabolic shift, CSCs showed a greater reliance on glycolysis for energy supply compared with the parental cells [92]. On the other hand, ALDH are a group of enzymes that oxidize aldehydes formed in the process of alcohol metabolism. High levels of the detoxifying enzyme ALDH1 were frequently associated with CSCs, and this marker was used for the identification of CSCs [93]. Recently, Honoki et al. evaluated the cancer spheroid subpopulation of cells from human sarcoma with high ALDH1 activity and found that these cells possess strong chemoresistance and detoxifying capability [94]. The identification of CSCs from human lung CCs identified cells with high ALDH1 activity, which was attributed to high self-renewal capacity, differentiation, and resistance to chemotherapy [95]. Breast CSCs identified as ALDH1-positive play a significant role in resistance to chemotherapy [96]. It seems like ALDH protects the drug-tolerant subpopulation of cells, including CSCs, from the potentially toxic effects of elevated levels of RO/NS. Not surprisingly, pharmacologic disruption of ALDH activity

leads to accumulation of ROS to toxic levels, even within the drug-tolerant subpopulation [97].

## 6. Suggested Principles of Drugs Design towards CSCs Resistance

Some physiological metabolites such as pyruvate, tetrahydrofolate, and glutamine act as powerful cytotoxic agents on CSCs when supplied at doses that perturb the biochemical network, sustaining the resumption of aerobic growth after the hypoxic dormant state [98]. This indicates that the metabolic state of CSCs must be crucial for their resistant to therapy because when CSCs need to differentiate and proliferate, they shift from anaerobic to aerobic status.

The principles of drug resistance in CCs can be also applicable to CSCs. Cells can be resistant to the drug by (1) active drug efflux by drug transporters, such as Pgp, MRP, and BCRP; (2) loss of cell surface receptors and/or drug transporters or alterations in membrane lipid composition; (3) compartmentalization of the drug in cellular vesicles; (4) altered/increased drug targets; (5) metabolic disruption due to OXPHOS; (6) alterations in cell cycle; (7) increased drug metabolism/enzymatic inactivation; (8) active damage repair; (9) inhibition of apoptotic pathways. However, targeting RO/NS upon designing novel therapeutic strategy to overcome chemo- and/or radioresistance of CCs is associated with some difficulties and should be considered with extra care. This is because antioxidant systems not only remove oxidants but also maintain them at an optimum level [99]. Therefore, besides obvious pharmacological properties (low toxicity, subnanomolar active concentrations, solubility, and oral bioavailability), the following principles should be taken into account when rationally designing such drugs: (i) they should transiently interact with proteins that block autophagy or promote apoptosis to allow sufficient RO/NS accumulation; (ii) ideally, those drugs should have an antagonist with higher affinity to the drug and lower affinity to surrounding molecules; (iii) specific moiety for selective delivery to these organelles should be considered; (iv) low adverse side effects should be taken into account. Although a number of drugs triggering apoptotic or autophagic events have been produced for the treatment of cancer, only few of them can meet the above criteria and are summarized in Table 1. In addition, few other drugs have to be added. Alpha-tocopheryl succinate ( $\alpha$ -TOS), an anionic analogue of vitamin E [100] of which mechanism of action involves interaction with ubiquinone-binding site of mitochondrial complex II and concomitant inhibition of succinate dehydrogenase (SDH) activity [101]. It is accompanied by recombination with molecular oxygen to yield ROS and permeabilization of mitochondria [102]. Proapoptotic drug BMD188 (cis-1-hydroxy-4-(1-naphthyl)-6-octylpiperidine-2-one) generates mitochondrial ROS and triggers apoptosis by activation of caspase-3. It was reported to inhibit the primary growth of prostate CSCs [41, 103]. Anti-neoplastic drug LND, ionidamine, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid has been shown to inhibit glycolysis and induce mitochondria-mediated apoptosis by activation of caspase-9, caspase-3, and Akt/mTOR pathways

TABLE 1: Drugs targeting specific CSCs and their modes of action.

Compounds	Mechanism of action	Type of CSCs	References
Temozolomide (TMZ)	Reversal of EMT and chemosensitizing CSCs	Glioblastoma	[27]
STA-4783	Targets OS by Hsp70 induction and induces ROS within CCs	Breast	[28]
Geldanamycin	Inhibition of the TRAP1 chaperone function	Breast, lung, and neural	[31]
Rottlerin (ROT)	Inhibitor of PI3K/Akt/mTOR pathway and inducer of apoptosis	Pancreatic	[33]
Bardoxolone methyl	Nrf2 inhibitor	Leukemia	[34]
Resveratrol	Wnt inhibitor	Breast	[35]
Honokiol	Notch inhibitor	Melanoma	[36]
Chloroquine (CQ)	Autophagy inducer	Colorectal	[37]
Pemetrexed	Folate antagonist	Lung	[38]
Melatonin	Induces autophagy by increasing ROS	Glioma	[39]
Salinomycin	K <sup>+</sup> -ionophore and triggers autophagy	General CSCs	[40]
BMD188	Proapoptotic	Prostate cancer	[41]
Gossypol	Hsp70 induction, ROS induction	Breast, leukemia	[42]
PUFAs	Induces apoptosis and autophagy	Colorectal	[43]
TrxR	ROS scavenger	Cervical	[44]

TABLE 2: Difference in CSCs and CS survival after chemo-/radiotherapy. Higher apoptosis, autophagy/mitophagy, ROS, and lower metabolic activity in CSCs versus CCs may be predominant factors in explaining chemo-/radiotherapy.

Survival properties	CCs	CSCs
RO/NS	↑	↓
Metabolic activity	↑	↓
Autophagy/mitophagy	↓	↑
Apoptosis	↓	↑
Chemo-/radioresistance	↑	↓

[104]. Natural terpenoid aldehyde gossypol has been shown to increase ROS and induce apoptosis and necrosis via inhibition of Bcl-2, activation of caspase-3, cytochrome c release from mitochondria, and displacing BH3-only proteins from Bcl-2 [42]. Both gossypol and its derivative, apogossypolone (ApoG2), were also shown to induce autophagy in several CCs through Beclin-1-mediated ROS upregulation [105–108]. Polyunsaturated fatty acids (PUFAs) induce apoptosis and autophagy by means of mitochondrial ROS-mediated Akt-mTOR signaling [43, 109]. Finally, inhibitors of oxidoreductase thioredoxin (TrxR) scavenging ROS provide a promising therapeutic target for CSCs intervention. In particular, Ru(II) polypridyl complexes inducing ROS-mediated apoptosis have been suggested [44]. Organoselenium compound BBSKE (1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane) is a TrxR inhibitor which induce apoptosis via Bcl-2/Bax pathway. Since TrxR mediates resistance to irradiation of a non-small cell lung cancer, BBSKE has been proposed as a radiosensitizer in some clinical trials [110].

## 7. Concluding Remarks

It is becoming clearer that a single drug against cancer would not be effective to cure the disease as CCs learn how to become resistant in the middle-long term along the treatment and persist hidden in the body of cancer patients upon reactivation. In principle, conventional treatments are effective to induce apoptosis or autophagy in the bulk of the tumor particularly on CCs but without affecting the CSCs. The fatal consequences of this are not only that conventional therapy favors the presence of the CSC but also that they resume growth more aggressively. The reasons of CSCs resistance to the induction of apoptosis, autophagy, or hypoxia are closely related to their metabolic status which in turn depends on mitochondria as the main source of energy. A synthetic lethality or combinatorial therapy followed by animal studies to specify the dose and timing and minimize side effects should be considered for effective targeting of CSCs.

## Abbreviations

$\Delta\psi_m$ :	Mitochondrial membrane potential
CCs:	Cancer cells
CSCs:	Cancer stem cells
EMT:	Epithelial-Mesenchymal Transition
GSH:	Glutathione
GSSG:	Oxidised glutathione
mTOR:	Mammalian target of rapamycin
OS:	Oxidative stress
RO/NS:	Reactive oxygen/nitrogen species
ROS:	Reactive oxygen species.

## Conflict of Interests

Matilde E. Lleonart is a FIS Investigator (CP03/00101). Alex Lyakhovich visit is sponsored by ICRC/Masaryk University, Brno, Czech Republic.

## Acknowledgment

This work was supported by the FIS Project PI12/01104.

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## Research Article

# Can Breast Tumors Affect the Oxidative Status of the Surrounding Environment? A Comparative Analysis among Cancerous Breast, Mammary Adjacent Tissue, and Plasma

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Received 22 May 2015; Revised 4 August 2015; Accepted 12 August 2015

Academic Editor: Manoj K. Pandey

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In this paper, we investigated the oxidative profile of breast tumors in comparison with their normal adjacent breast tissue. Our study indicates that breast tumors present enhanced oxidative/nitrosative stress, with concomitant augmented antioxidant capacity when compared to the adjacent normal breast. These data indicate that breast cancers may be responsible for the induction of a prooxidant environment in the mammary gland, in association with enhanced TNF- $\alpha$  and nitric oxide.

## 1. Introduction

Redox imbalance is a process reported in most of the chronic diseases [1]. The constant activity of reactive species (RS) on lipids, DNA, and proteins promotes critical modifications in cell physiology [2], which can interfere with its normal functioning under chronic inflammatory conditions, such as cancer [3].

Cancer cells constantly experience moderate to high levels of oxidative stress, but curiously moderate to high oxidative stress does not cause immediate cell death [4]. This fact indicates that cancer cells are able to overcome and adapt against redox changes. The sustained oxidative stress promoted during chronic inflammation supports pivotal events to cancer survival, including most of the hallmarks of cancer. This fact has been associated with aberrant activation of transcription factors, induction of protooncogenes, and

cumulative acquisition of mutations, which perpetuates the genomic instability of cancer cells [5].

Tumors with enhanced proliferative capacity, as breast cancer, produce high levels of RS during their chronic cycles of ischemia, reperfusion, and angiogenesis, resulting in exceeding growth signaling [6]. It is reported that DNA obtained from breast carcinomas presents greater oxidative damage than the adjacent nontumoral breast [7], suggesting that the tumor cells are more exposed to *in situ* oxidative stress than the proximal or distant nontumoral tissues. On the other hand, this fact suggests that cancer can potentially induce oxidative damage in surrounding normal cells.

Therefore, nontransformed epithelial cells located adjacently to the tumoral tissue may experience variable concentrations of RS generated by the constitutive activation of mitogenic pathways arising from surrounding tumor cells [8]; however, the impact of this event on the homeostasis

of nontumoral adjacent cells is unclear. What is known so far is that tumors are “oxidatively stressed” and that in some extension it could be related with the systemic redox changes reported in patients bearing breast tumors [9–11].

Although growing evidence highlights the occurrence of persistent oxidative stress in breast tumors, most of studies have not focused on reporting the redox modifications of breast cancer regarding its healthy counterpart tissue and whether there is a relationship between the tumor oxidative status and the systemic redox profiling. In this context, we proposed to map the oxidative and inflammatory profiles of fresh nonfixed breast tumors and their paired adjacent mammary nontumoral tissue, as well as their respective plasma. To reach these goals, we designed our analysis employing high-sensitivity oxidative stress approaches to investigate the functional redox changes that occurred in tumor microenvironment and its correlation with the circulating levels of proinflammatory/oxidative mediators.

## 2. Material and Methods

**2.1. Study Design.** A series of 321 women with breast cancer were screened from March 2011 to December 2012 at Londrina Cancer Institute, Londrina, Paraná, Brazil. A total of 50 women were included based on the inclusion and exclusion criteria. Inclusion criteria embraced women bearing unilateral tumor with histopathological diagnosis of primary ductal infiltrative carcinoma of the breast, before starting the chemotherapeutic regimen. Exclusion criteria included current smoking, hepatic, cardiac, or renal dysfunction, obesity, use of drugs, hypertension, autoimmune disorders, and diabetes, among other chronic conditions.

Adjacent mammary tissue and tumoral tissue were surgically resected at the moment of tumor withdrawal according to standard procedures, before chemotherapy starting. The adjacent tissue was collected from the most distant point in relation to the tumoral tissue (3 to 4 cm of distance from the macroscopic tumor). Adjacent breast was confirmed as nontumoral by conventional histopathological analysis. Heparinized blood was further collected for analysis. Samples were kept frozen at  $-86^{\circ}\text{C}$  until analysis, by at most 2 weeks.

All recommendations of the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria [12] were followed throughout this study regarding patient selection, assays performance, and data analysis. Institutional board previously approved all practice and all participants signed informed consent terms. This study is in accordance with the ethical principles for medical research involving human subjects from the Declaration of Helsinki.

Clinicopathological data of cancer patients was collected from medical records and included age at diagnosis, TNM staging, tumor histological type, histological tumor grade, lymph nodal status, tumor size, and presence of distant metastasis.

**2.2. Immunohistochemical Labeling for Nitrotyrosine (NT).** Nitrotyrosine (NT) is a residue formed by the action of peroxynitrite (derived from the reaction of nitric oxide

and superoxide anion) on proteins. Paraffin-embedded sections were heated (30 min,  $65^{\circ}\text{C}$ ), deparaffinized, and rehydrated. Sections were treated at room temperature with 2% bovine serum albumin and incubated overnight at  $4^{\circ}\text{C}$  with primary mouse anti-human antibodies against NT-labeled residues (diluted 1 : 300, Santa Cruz Biotechnology, clone sc-32757, USA), previously validated for human samples [13]. The secondary antibody, horseradish peroxidase, and 3,3'-diaminobenzidine (DAB) were provided by the commercial kit (Dako LSAB, Germany). In the last step, sections were weakly counterstained with Harry's hematoxylin (Merck). For each case, negative controls were performed on serial sections by omitting the primary antibody incubation step. The intensity and localization of the immunoreactivity were examined with a photomicroscope (Leica DM 2500 and Leica DFC280, Leica, Germany).

**2.3. Sample Processing for Determining the Tissue Oxidative Status.** Frozen tissue samples were thawed, precisely weighted, and homogenized in sterile saline phosphate buffer 10 mM pH 7.4, at a final concentration of 100 mg tissue/mL. The mixture was centrifuged at  $5000\times g$ ,  $4^{\circ}\text{C}$  during 10 minutes. Supernatants were collected and kept in ice bath until analysis. All described methods used this concentration of tissue homogenate, except when some specific dilution is highlighted. Heparinized blood samples were centrifuged at  $1500\times g$ ,  $4^{\circ}\text{C}$  during 5 minutes. Plasma samples were separated for further analysis. All measurements were conducted at the same day of sample processing.

**2.4. Measurement of Tissue Lipoperoxidation by High-Sensitivity Chemiluminescence.** Lipoperoxidation of adjacent and tumoral tissue was evaluated as previously published, with some adaptations [14]. Aliquots of  $250\ \mu\text{L}$  of tissue homogenate (100 mg/mL) were added to  $750\ \mu\text{L}$  of saline phosphate buffer 10 mM pH 7.4, with addition of  $10\ \mu\text{L}$  of t-butyl 3 mM solution. For plasma analysis,  $200\ \mu\text{L}$  of sample was mixed with  $780\ \mu\text{L}$  of saline phosphate buffer 10 mM pH 7.4, with addition of  $20\ \mu\text{L}$  of t-butyl 3 mM solution. Readings were carried out in a Glomax luminometer (Glomax, Promega). The results were expressed as relative light units (RLU) and the entire curve profile was used as indicator of lipoperoxidation. The area under the curve (AUC) was obtained by area integration.

**2.5. Determination of Lipid Hydroperoxide Level by the Ferrous Oxidation-Xylenol Orange (FOX) Method.** Lipid hydroperoxide concentrations in samples were estimated by the FOX method as published by Victorino et al. [15]. Plasma or tissue homogenate aliquots of  $50\ \mu\text{L}$  were mixed with  $50\ \mu\text{L}$  of FOX reagent. Samples were incubated for 30 minutes in the dark at room temperature and the absorbance of the supernatant was measured at 550 nm and the results were expressed as  $\mu\text{M}$  hydroperoxide/mg tissue.

**2.6. Carbonyl Content and Antimyeloperoxidase Determination.** Carbonyl content was measured as estimate of oxidative injury in proteins [16]. Tissue homogenate or plasma aliquots of  $200\ \mu\text{L}$  were added in 2 tubes. Test tubes received 1 mL

of dinitrophenylhydrazine (DNPH) 10 mM and blank tubes received 1 mL of HCL 2.5 M. Tubes were incubated during 1 hour in ice bath. After that, samples were successively incubated with 1.25 mL of trichloric acetic acid 20% and 10% in ice bath during 20 minutes each, with centrifugation between incubations (1400  $\times$ g/15 minutes). Supernatants were discarded and pellets were twice treated with 1 mL of an ethanol/water solution (1:1). The final precipitates were dissolved in 1 mL of guanidine 6 M and were left for 24 hours at 37°C. Carbonyl content was calculated by obtaining the spectra at 355–390 nm of DNPH-treated samples, employing one blank tube for each test. The obtained peaks were employed to calculate carbonyl concentration using a molar extinction coefficient of 22 M<sup>-1</sup>cm<sup>-1</sup>. Results were expressed as nmol/mL/mg tissue. Myeloperoxidase was detected by a commercial ELISA kit following manufacturer's instructions (IBL International, Germany), and the data were expressed as U anti-MPO/mL.

**2.7. Total Radical-Trapping Antioxidant Parameter (TRAP).** TRAP was measured in pure breast tissue homogenates (100 mg/mL) or plasma samples (diluted 1:50). For TRAP calculation, the induction time of the sample (time for which the sample antioxidants can inhibit the ABAP action) was compared to that of the standard antioxidant (trolox) and expressed as  $\mu$ M trolox/g tissue [17].

**2.8. Evaluation of Nitrite as Estimate of Nitric Oxide (NO) Levels.** NO was estimated by measuring nitrite as previously described [18]. Homogenate or plasma samples (60  $\mu$ L) were deproteinized, and the supernatants were recovered and incubated with cadmium granules. After 10 minutes, the Griess reagent (Sigma) was added to 200  $\mu$ L of the supernatants, and the reactions were incubated for 10 minutes at room temperature. The absorbance was read at 550 nm using a standard microplate reader (Multiskan EX, LabSystems, Minnesota, USA). The final results were expressed as  $\mu$ M nitrite/mg tissue.

**2.9. Estimation of MDA Levels by High-Performance Liquid Chromatography (HPLC).** MDA determinations were made using equipment HPLC-20AT Shimadzu equipped with a LC20AT pump and SPD20A UV, diode array absorbance detector employing a C18 reverse phase column, as described by [15]. Aliquots of 160  $\mu$ L of plasma samples, tissue homogenate, or standard solution reacted with 100  $\mu$ L of 0.5 M perchloric acid. Samples were centrifuged for 5 minutes, 5000  $\times$ g at 4°C. About 180  $\mu$ L of supernatant was recovered to react with 100  $\mu$ L of thiobarbituric acid for 30 minutes, at 95°C, and transferred to ice bath to stop reaction. 100  $\mu$ L of 1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, was added to stabilize sample pH. Further, samples were centrifuged for 10 minutes, 5000  $\times$ g at 4°C. Mobile phase was composed of 65% 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.0, and 35% methanol HPLC grade. Readings were executed at 535 nm during 12 minutes with isocratic flow of 0.8 mL/minute and results were expressed as MDA peak height.

TABLE 1: Clinicopathological characterization of patients.

Number of patients	N = 50
Mean age at diagnosis (range, years)	53.8 (31–77)
Histological type	
Ductal infiltrative carcinoma	100%
Histological grade	
1 or 2	70%
3	30%
TNM classification	
I/II stage	83.3%
III stage	16.7%
IV stage	None
Tumor size	
Mean (range) cm	2.88 (0.9–5)
Mean BMI (kg/m <sup>2</sup> )	24.2

TNM = tumor-node-metastasis classification, BMI= body mass index.

**2.10. Homocysteine and TNF- $\alpha$  Levels.** Homocysteine (Axis-Shield Diagnostics, Abbott Diagnostics Division, UK) and TNF- $\alpha$  levels (e-Bioscience, USA) were determined in aliquots of 200  $\mu$ L of plasma or tissue homogenates by using commercial kits. Homocysteine was expressed as  $\mu$ mol/L and TNF- $\alpha$  as pg/mL.

**2.11. Statistical Analysis.** All analyses were conducted in triplicate sets. Statistical analysis was performed using GraphPad Prism 5.0, Microsoft Office Excel 2007, and OriginLab 7.5 software. Results were expressed as arithmetic means and errors of the means. Differences among groups were assessed by two-way analysis of variance (ANOVA) with *post hoc* Bonferroni's test for the lipid peroxidation curves and by Student's paired *t*-test for the other parametric parameters. Nonparametric data was analyzed by Mann-Whitney or Wilcoxon matched-pairs tests. Correlations among parameters in plasma and tumoral tissue were also performed using Pearson or Spearman tests. All data were checked using the Grubbs test (GraphPad Quickcalcs) to eliminate significant outliers ( $p < 0.05$ ).  $p < 0.05$  was considered statistically significant.

### 3. Results

Table 1 shows the clinicopathological characterization of the 50 patients enrolled in this study. The mean age at diagnosis was 53.8 years, ranging from 31 to 77 years. Most of tumors presented histological grade 2 and the mean tumor size was 2.9 cm. Regarding tumor subtype, a prevalence of luminal tumors was found (30% of patients presented luminal A tumors, 20% were triple negative, 26.6% had HER2 enriched, and 23.4% were typed as luminal B). Most women presented local or locoregional disease (TNM I/II, 83.3%; TNM III, 16.7%), without any presence of distant metastasis. None of the included women were overweight/obese at diagnosis. The number of patients did not allow dividing the groups regarding the molecular subtype.

Aiming at characterizing the redox status of adjacent and tumoral breast tissue samples, we performed the analysis of

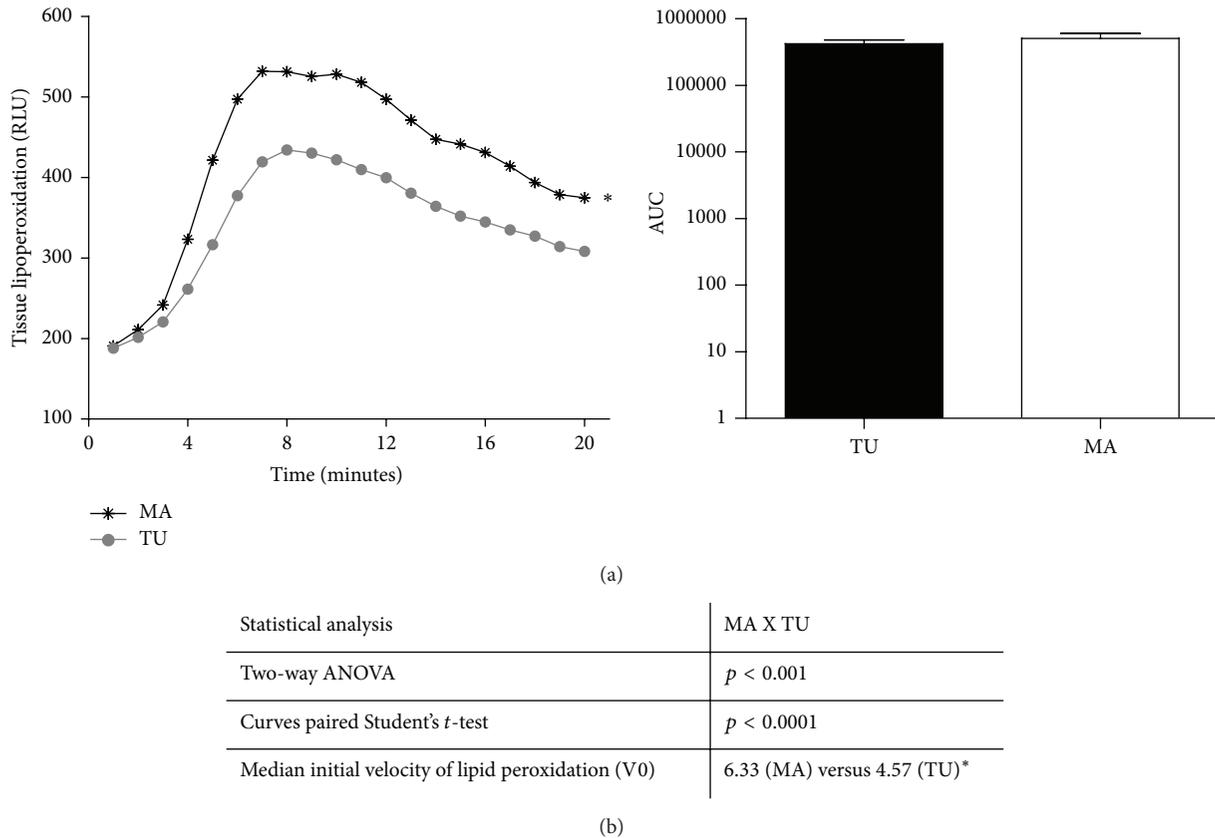


FIGURE 1: Lipid peroxidation profile of mammary adjacent tissue (MA) and tumoral tissue (TU).

some markers of oxidative damage in lipids and proteins. Figure 1 shows the lipid peroxidation profile determined by high-sensitivity chemiluminescence. This method allows identifying the oxidative damage of RS on lipidic components located at the plasmatic membrane. As shown, the adjacent mammary tissue presented higher lipid peroxidation status than the tumoral tissue ( $p < 0.0001$ ). Other lipid peroxidation-derived metabolites did not vary (FOX and MDA levels, Figures 2(a) and 2(b)).

Elevated homocysteine levels (from  $7.26 \pm 0.31 \mu\text{M}/100 \text{ mg}$  tissue in adjacent breast to  $9.49 \pm 1.08 \mu\text{M}/100 \text{ mg}$  tissue in tumoral tissue,  $p = 0.0221$ , Figure 2(c)) were found in the tumoral tissue when compared to the adjacent mammary breast. Antioxidant capacity of tumoral tissue was significantly higher than the adjacent breast ( $5532 \pm 1041 \text{ nM trolox/g}$  tissue in adjacent breast and  $9181 \pm 1041 \text{ nM trolox/g}$  tissue in tumor,  $p = 0.0068$ , Figure 2(d)).

Tumor samples displayed increased TNF- $\alpha$  levels ( $239.8 \pm 13.07 \text{ pg/mL}$  of homogenate in adjacent breast and  $418.1 \pm 19.6 \text{ pg/mL}$  of homogenate in tumoral tissue,  $p < 0.001$ , Figure 3(a)) and NO ( $4.64 \pm 0.32 \mu\text{M}/\text{mg}$  tissue in adjacent breast and  $6.89 \pm 0.32 \mu\text{M}/\text{mg}$  tissue in tumoral tissue,  $p < 0.001$ , Figure 3(b)).

The protein-induced oxidative modifications are represented in Figure 4. All tumor samples presented moderate/intense labeling for nitrotyrosine labeling, suggesting a prooxidant role for NO in breast cancer (Figure 4(a)).

High carbonyl content (from  $12.33 \pm 2.56 \text{ nmol}/100 \text{ mg}$  tissue in adjacent breast to  $22.39 \pm 3.95 \text{ nmol}/100 \text{ mg}$  tissue in tumoral tissue,  $p = 0.0274$ , Figure 4(b)) was found in breast tissue. Anti-MPO levels did not vary between groups ( $2.3 \pm 0.35 \text{ U}/100 \text{ mg}$  tissue in adjacent breast and  $1.83 \pm 0.44 \text{ U}/100 \text{ mg}$  tissue in tumoral tissue,  $p = 0.6129$ ). Semi-quantitative analysis of nitrotyrosine (Figure 4(d)) showed augmented levels in TU samples when compared to the adjacent normal breast ( $0.625 \pm 0.18$  arbitrary unities in MA samples and  $2.125 \pm 0.226$  arbitrary unities in TU samples,  $p = 0.0025$ ).

We further performed Spearman analysis to investigate whether there was some correlation between the oxidative status of tumors and its respective plasma obtained from the same patient (Table 2). All parameters were compared. Significant positive correlations were found with plasmatic versus tumoral TNF- $\alpha$  ( $p < 0.001$ ), tumoral nitrotyrosine versus plasmatic NO ( $p = 0.0456$ ), and plasmatic versus tumoral carbonyl contents ( $p = 0.0302$ ).

#### 4. Discussion

It is known that oxidative stress is active during the carcinogenic process and correlates with disease prognosis in breast cancer patients [19, 20]. In spite of that, this is the first characterization of the oxidative status of human tumor samples in comparison with matched nontumoral

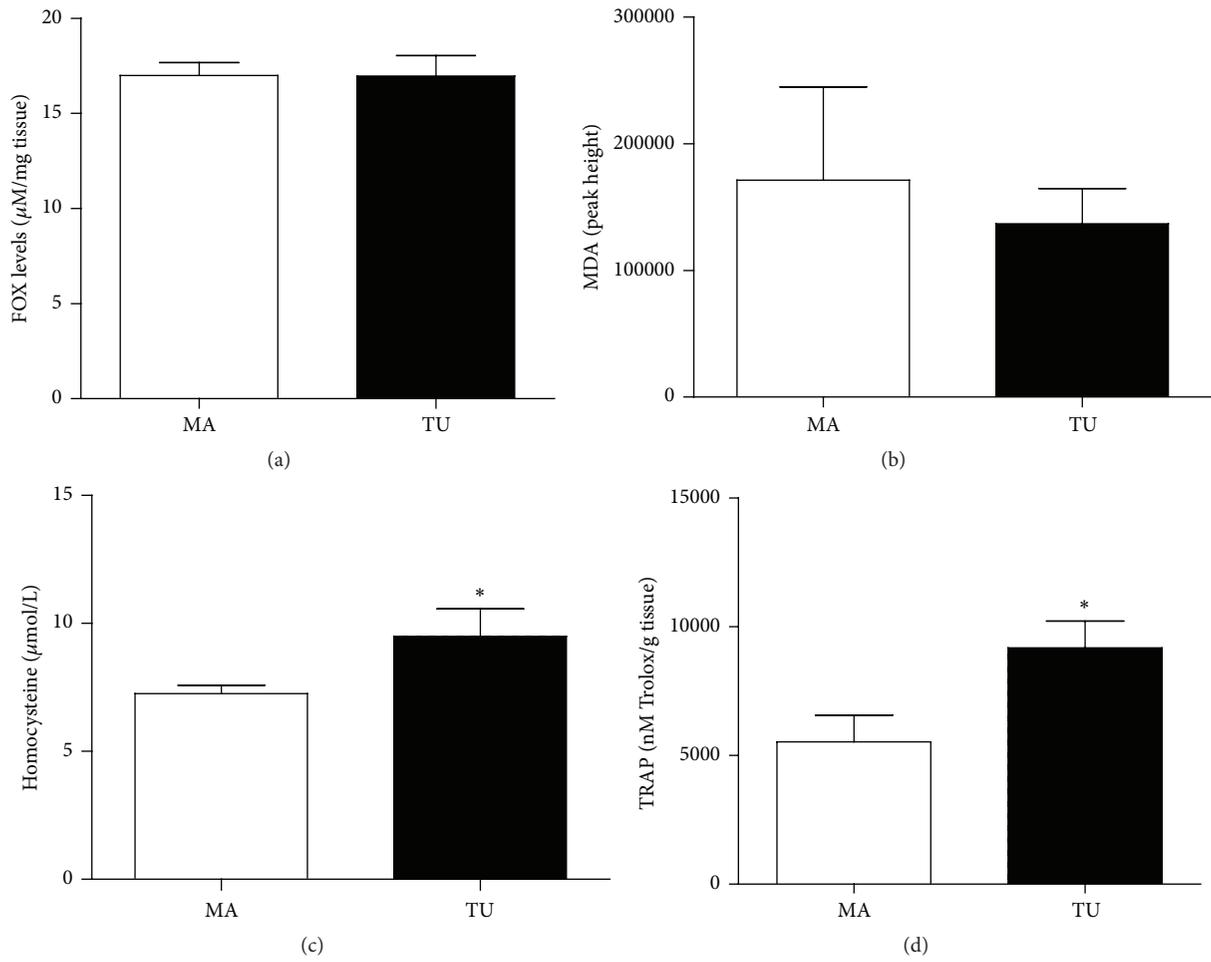


FIGURE 2: Oxidative stress markers in the mammary adjacent tissue (MA) and the tumoral tissue (TU).

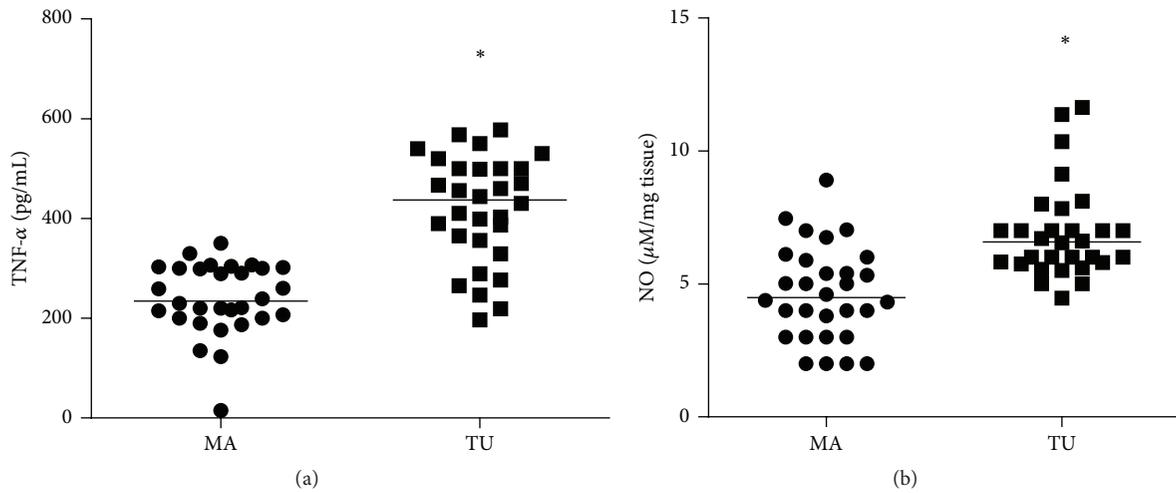


FIGURE 3: Tumor necrosis factor-alpha (TNF- $\alpha$ ) and nitrite as estimate of nitric oxide levels (NO) from mammary adjacent tissue (MA) and tumoral tissue (TU).

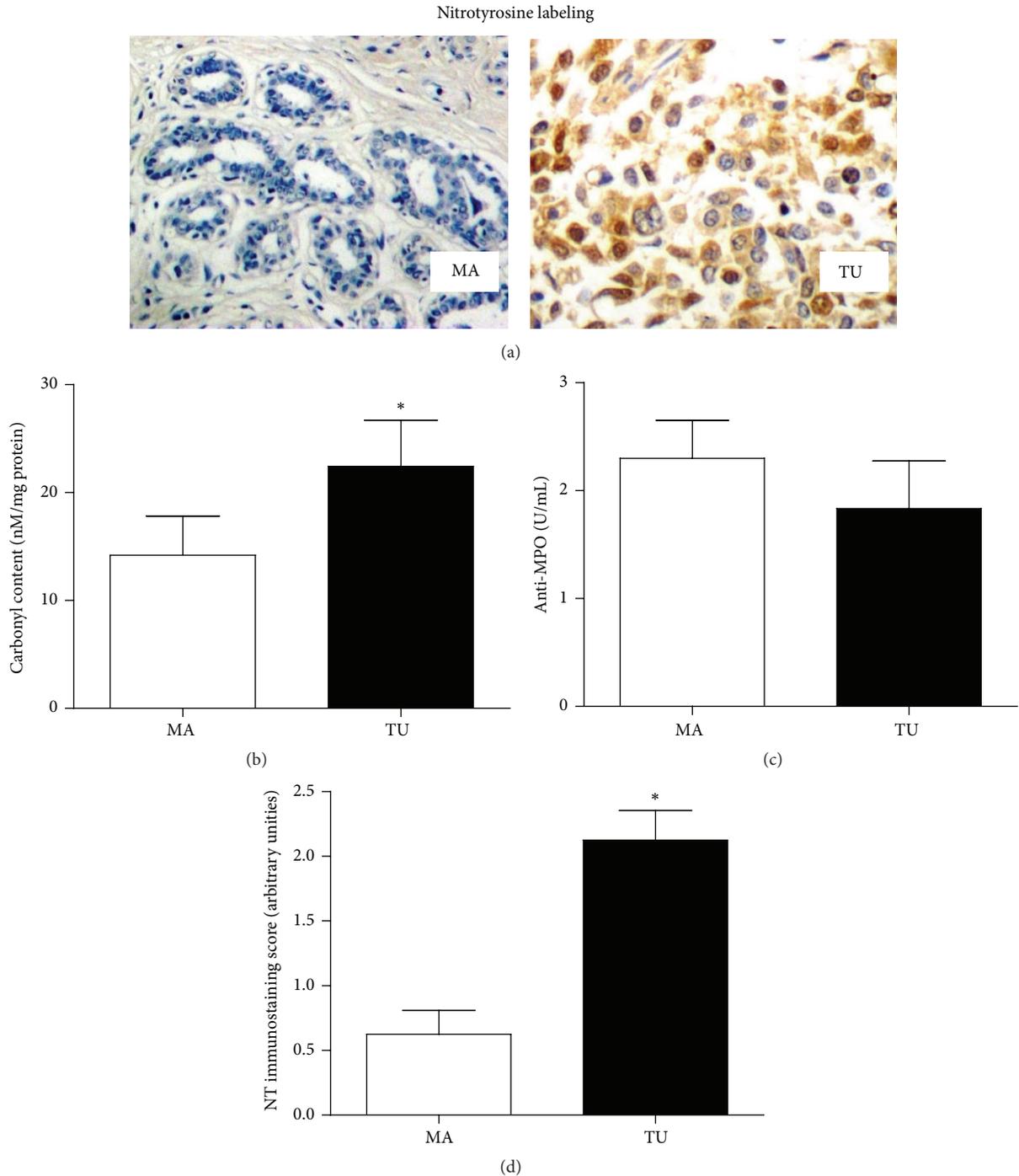


FIGURE 4: Protein-induced oxidative modifications and MPO detection in mammary adjacent tissue (MA) and tumoral tissue (TU).

adjacent mammary tissue. Our findings indicate that the breast tumor presents a variable oxidative profile. There was reduced oxidative stress as demonstrated by reduced lipid peroxidative reaction, revealed by the decrease of chemiluminescence and MDA levels and increased total antioxidant capacity (TRAP). Contrarily, nitrosative stress increased as nitrotyrosine labeling and NO was shown to be augmented in tumoral tissue with significant correlation between them.

Protein oxidation also was elevated in tumoral tissue possibly as a consequence of nitrosative stress. Our results showing high levels of TNF besides increased levels of NO and nitrotyrosine suggest a possible cross talk between nitrosative stress and inflammatory mediators in tumor tissue. This cross talk is enforced by the significant correlation between TNF- $\alpha$ , NO, nitrotyrosine, and the carbonyl content between tumor and its matched plasma sample.

TABLE 2: Spearman's correlations among the levels of oxidative parameters in plasma and plasma matched tumoral tissue.

Plasma versus tumor	<i>r</i> value	<i>p</i> value
TNF- $\alpha$	0.8322	<i>p</i> < 0.001*
NO $\times$ nitrotyrosine	0.7771	<i>p</i> = 0.0456*
Carbonyl content	0.7082	<i>p</i> = 0.0302*
TRAP	-0.09790	<i>p</i> = 0.7621
Homocysteine	0.2562	<i>p</i> = 0.5680
MDA	0.5691	<i>p</i> = 0.8954
Anti-MPO	0.6598	<i>p</i> = 0.6977
Lipid peroxidation	0.5870	<i>p</i> = 0.2314
NO	0.5477	<i>p</i> = 0.7894

TNF- $\alpha$  = tumor necrosis factor-alpha, TRAP = total antioxidant capacity, MDA = malondialdehyde, and MPO = myeloperoxidase.

\* indicates significant statistical difference (*p* < 0.05).

The mammary tissue presents a large content of adipocytes, which provides abundant feedstock for the occurrence of lipid peroxidation reactions. Polyunsaturated fatty acids that contain two or more double bonds are more susceptible to peroxidation [21]. Several RS may abstract the first hydrogen atom to produce a lipid peroxy radical [22]. In this context, we first evaluated the lipid peroxidation chain by using a chemiluminescence-based analysis. Here, the initiation step is characterized by the ascending part of the curve, which is dependent on the antioxidant content of the tissue [23]. The analysis of this initial reaction revealed that the lipids from adjacent nontumoral tissue were more oxidized than the lipidic content of tumor samples. The occurrence of reduced lipid peroxidation in the breast cancer environment has been reported in nipple aspirate fluids, suggesting a role for the downregulation of lipid peroxidation products in carcinogenesis [24]. Low lipid peroxidation activates several redox signaling pathways that recruit antioxidant induction from the organism to the site of increasing RS production and this configures a survival adaptation [25, 26].

In association with the reduced lipid peroxidation profile of tumors, we further found unaltered levels of lipid peroxidation products in tumor samples, as hydroperoxides (FOX) and malondialdehyde (MDA), suggesting that other low molecular weight substances may affect the liperoxidative status of cells. These findings indicate that, in some instance, tumors are protected against lipid peroxidation, potentially by accumulating membrane and intracellular antioxidants that neutralize this process. This fact was corroborated by the enhanced antioxidant capacity detected in tumor samples when compared to normal adjacent tissue, which explains the reduced lipid peroxidation chain observed in breast tumors. Therefore, high antioxidant content inside breast tumors may be able to retard the initiation of lipid peroxidation process, which may have a regulatory role in cell adaptation to oxidative changes.

In spite of the enhanced antioxidant capacity of tumors, we found significant augmented protein carbonylation in the tumoral tissue (but not in the adjacent mammary breast). The carbonylation reaction is an irreversible posttranslational

modification that occurred in protein structure that results from the reaction between amino acid residues with the low molecular weight aldehydes generated during the lipid peroxidation process [27–30] and it is reported as an oxidative marker locally produced in the cancerous breast [31].

We further detected increased homocysteine levels in breast tumors, suggesting the attack of thiol residues by RS originated in tumor microenvironment. Homocysteine regulates cell cycle, apoptosis, and oxidative stress responses [32]. A direct relationship between high homocysteine and enhanced carbonyl content has been demonstrated [33], indicating that the augmented carbonylation of tumor proteins can be induced by high homocysteine concentration.

In cancer cells, enhanced activity of proinflammatory cytokines leads to RS production [27]. A recent study of nipple aspirate fluids obtained from patients with breast cancer indicated that high levels of cytokines, as TNF- $\alpha$ , are concomitant with augmented protein carbonylation [34] and correlated with elevated systemic homocysteine [35], corroborating the present findings in tumor samples. Therefore, enhanced carbonyl content, homocysteine, and TNF- $\alpha$  level seem to comprise a network communicating with each other in the breast cancer microenvironment.

The prooxidative status found here in the tumoral tissue was probably sustained by its proinflammatory nature, as shown by TNF- $\alpha$  and NO levels in tumor samples. NO is a multifunctional molecule in cancer. Our data indicate a nitrosative function for this molecule inside breast tumor, as nitrotyrosine was found only in the tumor analysis and not detected in the nontumorous counterpart. In addition, the TNF-driven pathway is constitutively activated in breast cancer [8], inducing NO and increasing [36] and modulating oxidative stress in breast cancer cells [37]. Our previous publications have demonstrated that these patients carrying breast tumors present a variety of oxidative plasmatic modifications, and we have questioned if this fact could be related with tumor oxidative status. Therefore, we performed a correlation analysis aiming to understand the putative association between levels of oxidative stress parameters in plasma versus tumoral tissue. We have found significant correlations between plasmatic versus tumoral levels of TNF- $\alpha$ , NO/nitrotyrosine, and carbonyl content. Altogether, these data support that the systemic prooxidative status reported for several studies in breast cancer patients may be correlated with tumor-driven inflammation.

A recent study from our group [16] has demonstrated that the presence of the primary tumor mass is determinant for the sustained proinflammatory systemic status found in women with breast cancer, which included high NO, enhanced oxidative stress, and augmented TNF- $\alpha$ . Therefore, in spite of the reduced size of the tumor mass, it seems that the breast tumor microenvironment (cancer cells, infiltrated macrophages, and endothelial cells) is endowed with an enormous capability to promote profound modifications in the host organism, resulting in the persistent inflammatory status provoked here by TNF- $\alpha$ , NO, and RS production. This fact cannot be satisfactorily explained by the immune response against the tumor, since women presenting local breast disease have systemically established a Th2 immune status [11].

Another contributing factor to this redox scenario may be the components of tumor stroma. Tumor-associated fibroblasts can affect metabolically their adjacent cells, and cancer cells use the oxidative environment as advantage to obtain nutrients from the surrounding environment [38]. Further, sustained oxidative stress allows cancer-associated fibroblasts to become myofibroblasts, which secrete growth factors and cytokines [39] and yield high ROS generation [40]. This vicious cycle may also affect distant mammary cells by propagating the prooxidant signaling in a paracrine manner, which helps to explain the altered oxidative profile observed here in the distant mammary adjacent tissue.

In conclusion, this set of redox alterations found in breast tumors seems to be necessary to ensure the hallmarks of cancer biology, since RS have been implicated in oncogene activation, genomic instability, chemotherapy resistance, and the metastatic process [5]. Furthermore, it is known that enhanced antioxidant capacity has been strongly associated as an innate tumoral mechanism for acquiring chemoresistance [41]. The presented data point to the existence of a correlation between tumor proinflammatory mediators and their circulating levels, suggesting that the tumor may be a putative source that stimulates the onset of such substances in blood.

## Conflict of Interests

Authors declare no conflict of interests.

## Acknowledgments

The authors would like to thank Jesus Antônio Vargas for excellent technical assistance and CAPES, CNPq, and Fundação Araucária for providing financial support.

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## Review Article

# High Mobility Group B Proteins, Their Partners, and Other Redox Sensors in Ovarian and Prostate Cancer

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Received 2 June 2015; Accepted 27 July 2015

Academic Editor: Sahdeo Prasad

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Cancer cells try to avoid the overproduction of reactive oxygen species by metabolic rearrangements. These cells also develop specific strategies to increase ROS resistance and to express the enzymatic activities necessary for ROS detoxification. Oxidative stress produces DNA damage and also induces responses, which could help the cell to restore the initial equilibrium. But if this is not possible, oxidative stress finally activates signals that will lead to cell death. High mobility group B (HMGB) proteins have been previously related to the onset and progressions of cancers of different origins. The protein HMGB1 behaves as a redox sensor and its structural changes, which are conditioned by the oxidative environment, are associated with different functions of the protein. This review describes recent advances in the role of human HMGB proteins and other proteins interacting with them, in cancerous processes related to oxidative stress, with special reference to ovarian and prostate cancer. Their participation in the molecular mechanisms of resistance to cisplatin, a drug commonly used in chemotherapy, is also revised.

## 1. Introduction

Reactive oxygen species (ROS), generated as consequence of oxidative metabolism, activate signal transduction pathways, which contribute to cellular homeostasis [1]. Metabolically active cells, neutrophils, and macrophages from the immune system produce high levels of ROS. Consequently, the recruitment of immune cells during chronic inflammation increases oxidative stress (OS) in the microenvironment [2]. Exogenous sources, such as cigarette smoke and UV-light, also contribute to increasing the total cellular ROS content. The maintenance of the steady-state equilibrium between ROS generation and elimination is crucial for cell survival, while its loss causes cell death by different mechanisms triggered by oxidative damage. Cancer cells demand high energy production to sustain their pathological increase in proliferation rate. Thus, in order to avoid excessive ROS generation, they switch the utilization of metabolic pathways that

require mitochondrial respiration to fermentation [3]. They also develop specific strategies to increase ROS resistance, which include deviation of the glycolytic flux into the pentose phosphate pathway (PPP) or changes in other enzymatic mechanisms enhancing ROS detoxification [3, 4]. In cancer cells, ROS production is mainly due to overexpression of the NADPH oxidase [3]. Paradoxically, the antioxidant enzymes necessary for ROS elimination use the NADPH coenzyme; therefore, the PPP is important as a source of NADPH reducing power [3]. While a balance between enhanced ROS production and detoxification can be maintained, cancer cells will proliferate and survive. Commonly used radio- and chemotherapies are prooxidant strategies that alter cancer cells through ROS modulation and induce cell death [5, 6].

Changes in the redox state of cells affect proteins, lipids, and nucleic acids in different ways. HMGB1 is an abundant protein,  $10^6$  molecules per cell [7], which has been postulated as a redox sensor [8]. HMGB1 is also related to the hallmarks

of cancer as described by Hanahan and Weinberg [9]. These are as follows: sustained proliferative signalling, cell death resistance, replicative immortality, genome instability and increased mutations, tumour-promoted inflammation, insensibility to growth repressors, deregulation of cellular energetics, evasion of immune destruction, induction of metastasis, and promotion of angiogenesis. The biological functions of HMGB1 are diverse in normal cells and during the start and progression of cancer. Remarkably, these functions change depending on its redox state and cellular compartment. In the nucleus it behaves as a DNA chaperone, sustains nucleosome dynamics and chromosomal stability, and contributes to telomere maintenance [10]. It also modulates gene transcription and recombination [7]. Besides, HMGB1 participates in DNA repair by different mechanisms such as nucleotide excision repair, NER, mismatch repair, MMR, base excision repair, BER, and double strand break repair, DSBR [11]. In the cytoplasm, HMGB1 binds the protein beclin1, increases autophagy, inhibits apoptosis, and regulates mitochondrial morphology and function [12]. HMGB1 can be secreted by activated macrophages, monocytes, natural killer cells, and dendritic cells or can be released from necrotic or injured cells mainly during oxidative stress [13, 14]. Once HMGB1 becomes an extracellular signal, it binds to several cell surface receptors, principally to the receptor for advanced glycation end products (RAGE) and toll-like receptors (TLRs) and activates nuclear factor kappa B (NF- $\kappa$ B) signalling [15] and other downstream signalling pathways [12]. As a result, HMGB1 modulates immune and inflammatory responses and promotes cell proliferation, angiogenesis, and cell adhesion and migration. Curiously, oncogenic and tumour-suppressive activities have been assigned to HMGB1 at different stages of tumour genesis and therapy [12]. Moreover, it has been reported that Tax1, an oncogenic protein of viral origin, upregulates HMGB1 levels, which suggests that cancers of viral origin could also be related to HMGB1 deregulation [16].

Different isoforms of the human protein HMGB1, encoded by the *HMGB1* gene, have been reported [17] and other genes (*HMGB2* and *HMGB3*—alias HMG2a—and *HMGB4*), encoding similar although less studied HMGB proteins, are present in the human genome [18–20]. This review describes recent advances in the biological functions of human HMGB proteins and other proteins interacting with them, in cancerous processes related to OS, with special reference to ovarian and prostate cancer. These two malignancies have been previously related to redox imbalance and deregulation of the nuclear factor erythroid 2-related factor 2 gene, *NRF2*, encoding a transcription factor that binds to antioxidant response elements (AREs) and that is regarded as a promising therapeutic target [21, 22]. The molecular mechanisms of resistance to cisplatin, commonly used in chemotherapy of ovarian and prostate cancers, and their interplay with HMGB proteins are also reviewed.

## 2. HMGB Proteins as Redox Sensors

HMGB1 is so far the most studied member among the human HMGB protein family. It has many different functions that

depend on its redox state and posttranscriptional modifications, like acetylation, which determine its cellular or extracellular localization. HMGB1 is polyacetylated near its nuclear-localization sequences (NLSs) and this modification blocks the interaction with the nuclear importer [23]. Acetylated cytosolic HMGB1 is incorporated into cytoplasmic secretory vesicles that allow the regulated secretion of the protein [24]. The four human HMGB proteins have two positively charged DNA binding domains, HMG A-box and HMG B-box, folded in the characteristic L-shaped architecture (Figure 1(a)). Each domain is formed by three alpha-helix-stretches which are indicated in Figure 1(b). In HMGB1, the HMG A-box includes amino acids 1–79, and the HMG B-box is formed by amino acids 89–163. The acidic carboxyl terminus, amino acids 186–215, is negatively charged and has an extended and flexible structure, which interacts with residues within and between the two HMG boxes [25] although it has the highest affinity for the HMG B-box [26]. Many of the redox changes, associated with different functions of HMGB1, are conditioned by the environment and, therefore, HMGB1 is considered a master redox sensor. This function depends on three cysteine residues at positions 23, 45, and 106, which can be in reduced state, as thiols, or oxidized, as disulphide bonds. In moderate oxidative conditions, Cys23 and Cys45 easily form an intramolecular disulphide bridge, while Cys106 remains reduced (the semioxidized HMGB1 form). Nuclear magnetic resonance (NMR) spectroscopy studies of HMG A-box have shown that the redox potential of the Cys23-Cys45 pair is within the physiological intracellular range [8]. The formation of the disulphide bond is favoured with a standard redox potential as low as  $-237$  mV [27]; in consequence, a significant fraction of HMGB1 is expected to be in the semioxidized form within cells [8]. This Cys-Cys bond is a target of glutathione-dependent reduction by glutaredoxin [28]. The proximity of these Cys residues to amino acids that are necessary for DNA binding [29] explains the importance of redox-regulated conformational changes in HMGB1, which may modulate their affinity for DNA. Redox changes may also affect the interaction with other proteins and receptors and modify their biological functions. Cysteines can be further oxidized to sulfenic (RSOH), sulfinic (RSO<sub>2</sub>H), or sulfonic (RSO<sub>3</sub>H) acids under increased OS pressure [28].

## 3. Structural and Functional Similarities and Differences between Human HMGBs

The tertiary structure of HMGB1 A-box [29] reveals that Cys23 and Cys45 are located at the centre of helix I and helix II, respectively, opposing each other and at a distance that allows the formation of a disulphide bond under appropriate oxidative conditions (Figure 1(a)). The proteins HMGB1, HMGB2, HMGB3, and HMGB4 share a great similarity in their amino acid sequences as shown in the CLUSTALW alignment (Figure 1(b)). Only HMGB4 has some remarkable differences with the others, but even so, it conserves high similarity. Cys23 and Cys45 are conserved in HMGB2 and

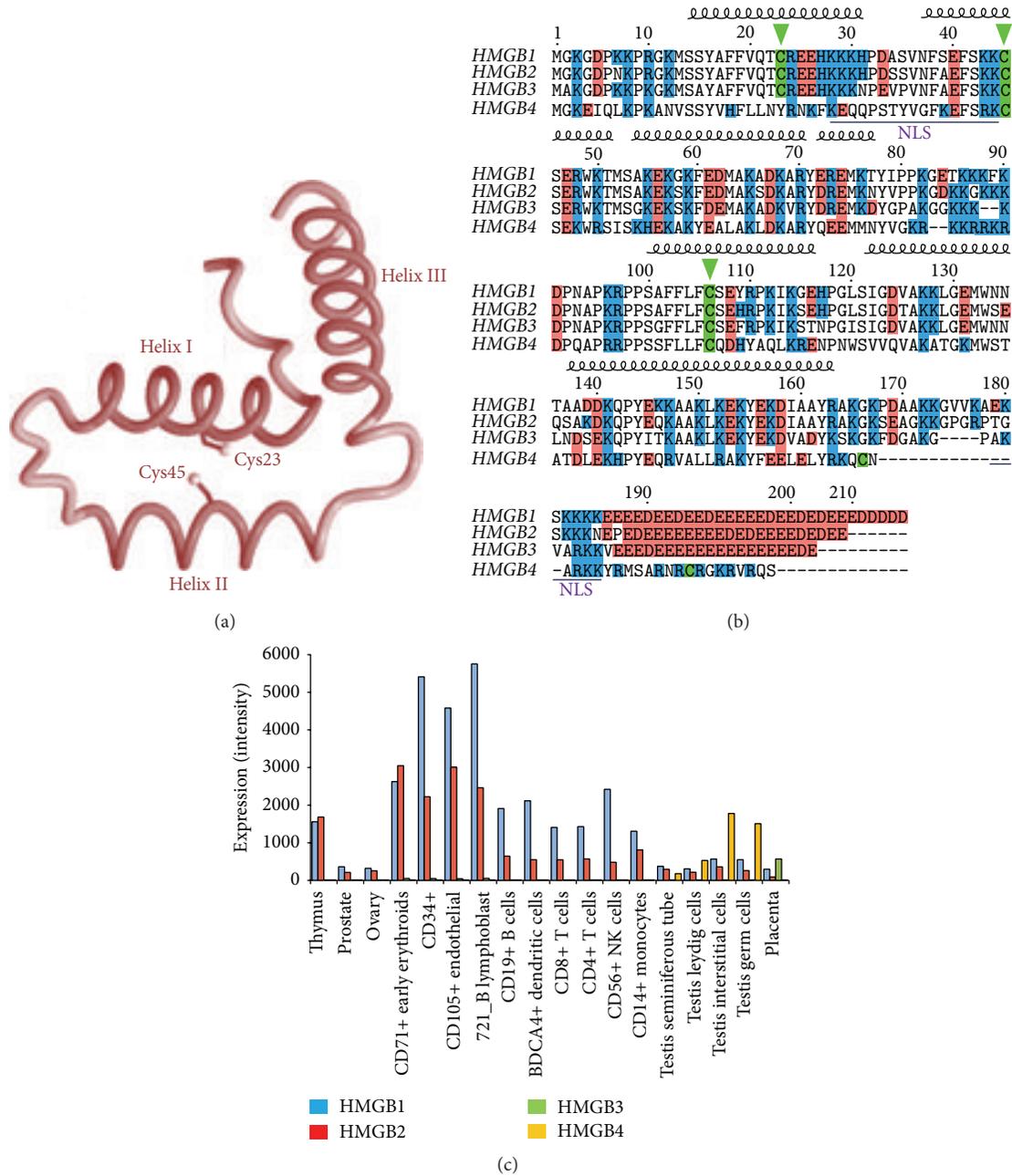


FIGURE 1: Comparative structure and expression of human HMGB proteins. (a) The HMG box folding characteristic of HMGB proteins showing the two Cys that form the disulphide bond. (b) CLUSTAL alignment of the human HMGB proteins. The three alpha-helix-stretches of HMG box-A and Box-B are indicated by their secondary structure; the acidic tail in the carboxylic end is signalled in red; cysteines are in green; the two NLSs characterized in HMGB1 are underlined in yellow. (c) Levels of expression of mRNAs from HMGB proteins are according to data from BioGPS (<http://BioGPS.org>).

HMGB3. Cys23 is absent in HMGB4, and certainly the DNA binding capacity of HMGB4 is independent of redox changes [30].

Cys106 is involved in the nuclear localization of HMGB1 [28] and this residue is conserved in the four human HMGB proteins (Figure 1(b)). Two nuclear location signals (NLSs), which are rich in lysine residues and extend from amino

acids 28–44 and 179–185, respectively, have been described in HMGB1 [31]. The NLSs are well conserved as shown in the alignment (Figure 1(b)) although in HMGB4 they show more variation. Although Cys106 is not present inside the NLS, thiols may participate in nuclear transport by a number of indirect mechanisms such as nuclear pore complex binding [32], ubiquitination [33], or transporter interaction [34, 35].

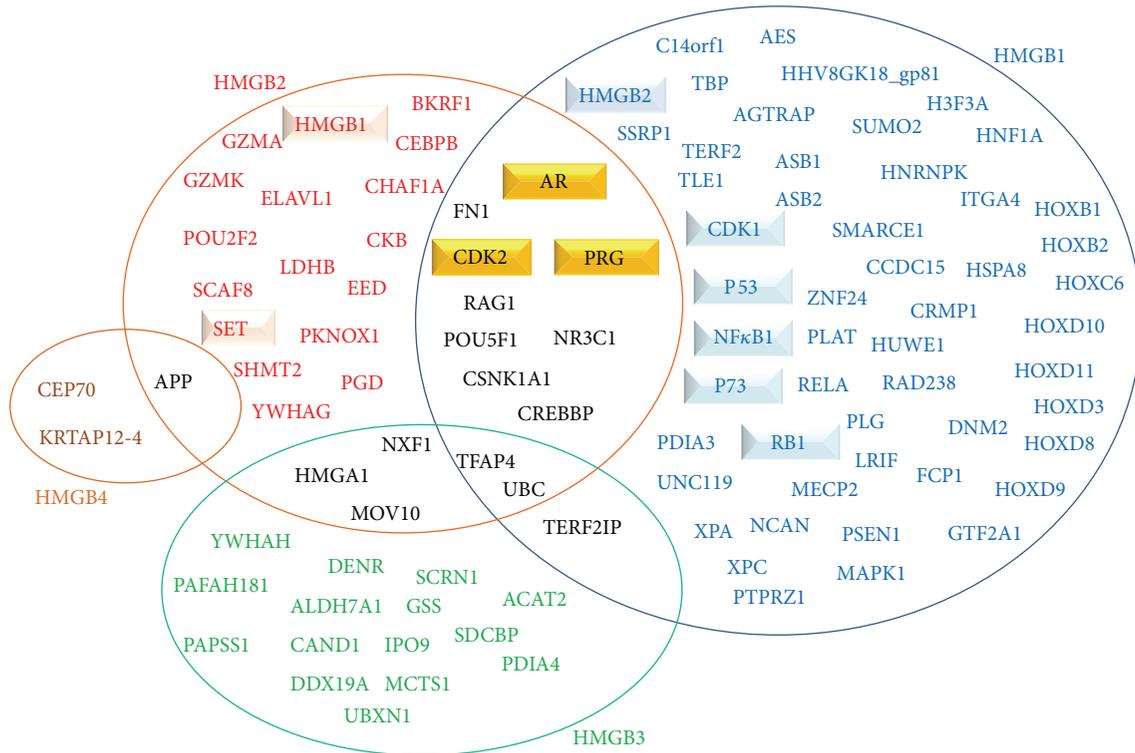


FIGURE 2: Venn diagram of HMGB protein interaction partners. Those reported as modified during ovarian or prostate cancer progression are highlighted inside the boxes. The figure has been done considering the public results from BioGRID (<http://thebiogrid.org/>), as available on date 05/31/2015).

Consequently, Cys106 conservation may be important to preserve the nuclear functions of these proteins.

Besides the absence of Cys23, the two most outstanding features of HMGB4, in comparison to the other HMGB proteins, are the presence of two additional cysteines at positions 164 and 178 and the absence of the acidic tail in the carboxylic end (Figure 1(b)). To our knowledge the biological significance of Cys164 and Cys168 in HMGB4 has not been studied yet. The function of the acidic tail in HMGB proteins is related to the interaction with other proteins, like nucleosome core histone H3 [36], and also to the stabilization of specific HMGB folding forms, because in HMGB1 it can interact with basic residues present in HMG B-box or in the interconnection of the two HMG boxes [25, 26].

HMGB proteins are widely expressed, except in cells without nucleus [37]. Data from microarrays reveal that *HMGB1* and *HMGB2* genes are the highest expressed in immune cells. *HMGB3* expression is relatively high in placenta and *HMGB4* expression is specific of testis (Figure 1(c)). The functional significance of these variations is unknown, since specific studies have not been reported. One possible explanation is that they may have different functions in different tissues, which may be associated with binding to tissue-specific protein partners. Remarkably, abnormal mRNA and protein levels of these proteins have been detected in numerous cancers, including ovarian and prostate [38–41].

#### 4. HMGB Interactions with Nuclear Proteins

After ribosomal synthesis, HMGB1 is imported into the nucleus where it interacts with the minor groove of free DNA through the HMG boxes [42] and it behaves as a DNA chaperone [43]. HMGB1 also binds to packed DNA, relaxes the structure of nucleosomes, promotes their sliding, and relaxes chromatin; thus, by its ability to bend DNA, HMGB1 favours the accessibility of other proteins to chromatin [44]. The C-terminal unstructured acidic tail of HMGB1 interacts with the N-terminal unstructured tail of histone H3, which is close to the DNA entry/exit points around the nucleosome dyad, thus positioning HMGB1 on the linker DNA [36]. This DNA chaperone function would explain the implication of HMGB proteins in wide variety of nuclear processes such as DNA replication, recombination, transcription, telomere maintenance, and diverse mechanisms of DNA repair [45–47]. OS causes DNA damage and it also affects proteins involved in these DNA-related processes. The OS induced responses could help the cell to restore the initial equilibrium or if the feedback to the initial status is not possible, they could activate pathways that would lead to cell death.

Several proteins have been recognised as HMGB1, HMGB2, HMGB3, or HMGB4-interactants by diverse approaches and results are deposited in BioGRID (<http://thebiogrid.org/>). A summary of these interactions is presented by a Venn diagram (Figure 2). The results in BioGRID include more interactions detected for HMGB1 or HMGB2

than for HMGB3 or HMGB4 proteins, a feature that will probably change with the progression of ongoing interactome projects in the near future. HMGB1 and HMGB2 interact with each other and they have common interactors like the nuclear hormonal receptors which are deregulated in prostate and ovarian cancers [48–50]. The functions of the HMGB partners as well as their sensibility to OS could help us to understand the role of HMGB proteins in the response to oxidative damage and their implications in the origin and progression of cancer.

In the nucleus, HMGB proteins interact with a number of transcription factors, among them tumour suppressors like P53 [51–53] or its homolog P73 [54]. It has been reported that nuclear retention of HMGB1 and P53 depends on the formation of a complex between them and, without their binding partner, HMGB1 or P53 can return more easily to the cytoplasm [55]. The interaction with P53 is of particular importance in the relation of HMGB1 with OS and cancer since P53 also functions as a redox sensor in the cell [56]. It has been recently reported that P53 can directly sense OS through DNA-mediated charge transport and that purine regions with lower redox potential facilitate higher P53-DNA dissociation [57]. The association *in vivo* and *in vitro* of each of the four HMGB proteins with the retinoblastoma protein (RB) occurs through a common LXCXE/D motif that is necessary for modulation of cancer cell growth [58, 59].

HMGB1 interacts differentially with members of the REL family of transcription factors (RELA/P65, c-REL, RELB, P50/NF- $\kappa$ B1, and P52/NF- $\kappa$ B2) like NF- $\kappa$ B1 [60]. In the nucleus NF- $\kappa$ B1 promotes cell proliferation and antiapoptosis by transcriptional regulation, playing a key role in tumour genesis and progression [61]. HMGB1 and HMGB2 interact with nuclear steroid hormone receptors including estrogen, androgen, and glucocorticoid receptors [48–50] favouring the binding to their DNA targets [62, 63]. The interactions with hormone receptors are of relevance taking into account the hormonal dependence of several cancers [40].

HMGB1 binds to cyclin-dependent kinases like CDK2 that control transcriptional regulation of genes related to cell cycle progression [64]. HMGB1 also interacts with topoisomerase II alpha, highly expressed in tumours and involved in replication and chromosomal segregation and recombination, and stimulates its catalytic activity [47]. In absence of RB, HMGB1 and HMGB2 modulate the binding of the transcription factor NF-Y to the topoisomerase II alpha promoter [65]. NF-Y recognizes CCAAT boxes and has been related to different types of cancer [66].

The high mobility group A (HMGA) proteins belong, as HMGB proteins, to the HMG family and are characterized by the “AT hook” domain for DNA binding, instead of the HMG box present in HMGB proteins. The HMGA proteins alter chromatin structure and thereby regulate the transcription of several genes, being also implicated in the development of benign and malignant neoplasms [67]. HMGA proteins have been related to the process by which epithelial cells change to mesenchymal type (the epithelial-to-mesenchymal transition, or EMT). During EMT, epithelial cells lose their cell polarity and cell-cell adhesion capacity, which leads to constriction caused by the two vicinal cells and extrusion

of a new mesenchymal cell. This stromal mesenchymal cell has both migratory and invasive capacities and also has the potential to differentiate into a variety of cell types. EMT is essential for numerous developmental processes and also occurs in the initiation of metastasis, being very important in tumours of epithelial origin. Carcinoma cells in the primary tumour lose cell-cell adhesion mediated by E-cadherin and gain access to the bloodstream through extravasation [68]. HMGA2, once induced by transforming growth factor  $\beta$  (TGF $\beta$ ), associates with SMAD complexes and induces expression of the SNAIL transcription factors, which controls epithelial-mesenchymal transitions by repressing E-cadherin [69] and Twist [70] expression. No direct effect of the HMGB proteins in this process has been described and although HMGB1, HMGB2, and HMGB3 interact with HMGA1 [71], a direct interaction with HMGA2 has not been reported. The study of HMGB-HMGA interactions is an interesting area to explore in relation to EMT.

HMGB proteins are able to bind to other nuclear proteins that do not have DNA binding capacity but that have a role in modifying transcription and in the onset of cancer. HMGB1 interacts with the aminoterminal enhancer of split, AES, [72], which plays an important role in tumour metastasis by regulating cell adhesion through changes in RND3 expression [73]. RND3 (alias RHOE) is a member of the small GTPases and regulates actin cytoskeleton organization and cell migration [74], as well as proliferation, differentiation, and apoptosis [75–77].

Besides the effects caused by direct interactions between HMGB proteins and other regulators of gene expression, we also have to consider the cross-regulation that operates to modulate the expression of all these factors. In this sense, it has been recently shown that the enhanced ectopic expression of HMGB1 decreases BCL-2-like protein 4 (BAX) and P53 expression, while it enhances B-cell lymphoma extra large (BCL-XL), B-cell lymphoma 2 (BCL-2), cyclin D1, and NF- $\kappa$ B expression [78]. This causes activation of cell growth and diminishes cell death [78].

## 5. HMGB Proteins in Survival versus Cell Death Control

Redox imbalance in the cells could lead to oxidative damage in the nucleus, with the consequent genome instability, and other processes related to malignancy along cancer origin and propagation. The progression of the OS response in the cell is accompanied by changes that might affect cell survival, providing reparative mechanisms, or promoting cell death. However, death or survival of a single cell could be good or bad for the organism. If survival affects a cell without a serious compromise in genome integrity and stability, the tissue will probably restore its healthy status. But if a cell with previous hallmarks for cancerous progression survives, its success is paradoxically detrimental for the tissue and the organism.

Cell death can occur by different mechanisms and the oxidative state of the cell and its microenvironment is a key determinant for their selection (Figure 3). When oxidative

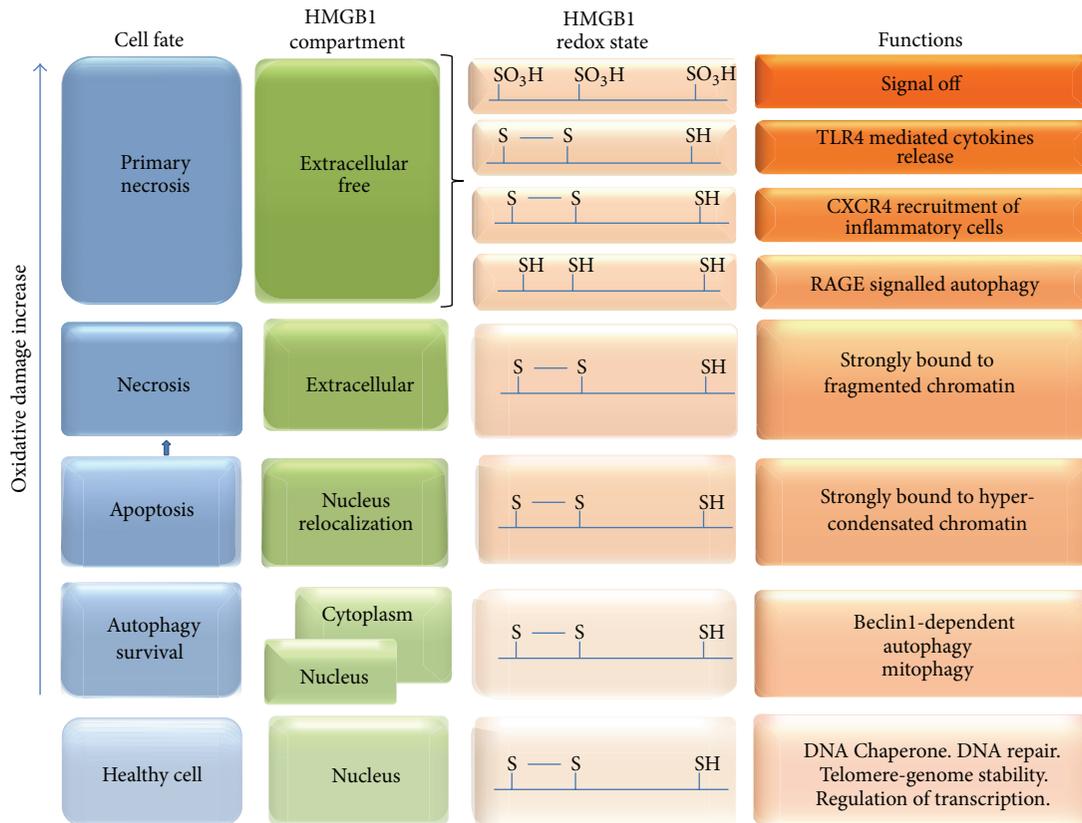


FIGURE 3: Relationship between oxidative stress progression, cell fate, HMGB1 redox states, and HMGB1 functions.

damage starts and ROS production is enough for starting the mitochondrial permeability transition (MPT), mitophagy and autophagy allow the cell to recycle damaged elements and survive. Autophagy and its selective form mitophagy that destroys damaged mitochondria require the formation and progression of the phagophore to finally produce the autophagosome. Then, the autophagosome fuses to the lysosome to constitute the autolysosome, where the degradation of the sequestered elements occurs [79]. If the oxidative damage persists, the integrity of the mitochondria is affected and cytochrome *c* is released; this molecule signals apoptosis and, consequently, cell death without immunogenic activation. Finally, with the highest levels of oxidative damage, necrosis is established and with it the possibility of a wide immunogenic activation [80].

As shown in Figure 3, in viable cells, HMGB1 is mostly localized in the nucleus associated with DNA and proteins in chromatin. Low acetylation of histones, observed during apoptosis, causes a hypercondensation of chromatin and the irreversible HMGB1 binding; this binding is a canonical characteristic of alarmins like HMGB1 [80]. If the apoptotic cell is not cleared by macrophages, secondary necrosis is produced and the instability of cellular membranes allows HMGB1 to be released to the extracellular media strongly bound to DNA [37]. If necrosis is primary, not derived from previously apoptotic cells, HMGB1 release is also observed, but in this case the protein is free, not associated with

DNA [81]. ATP depletion mediated by poly[ADP-ribose] polymerase 1 (PARP1) also regulates HMGB1 release during necrosis [82].

HMGB1 has important functions controlling the balance between autophagy and apoptosis. In the nucleus, as a regulator of transcription, and in the cytoplasm, by binding to regulator proteins, HMGB1 controls these processes. Under OS or other types of stress, hyperacetylation of NLSs promotes HMGB1 translocation from the nucleus to the cytoplasm [83]. The export from the nucleus is mediated by the chromosome-region maintenance 1 protein, CRM1 [31]. In the cytoplasm, semioxidized HMGB1 (Cys23-Cys45 disulphide and Cys106 thiol) leads to the activation of caspase-3 and caspase-9 and promotes the induction of the mitochondrial pathway of apoptosis. But it also binds to the protein beclin1 and favours the formation of the autophagosome [84]. Under proautophagic conditions beclin1 forms a complex with the proteins ambra1, VPS34, and VPS15 that initiates the formation of the phagophore [85]. The binding of HMGB1 to beclin1 favours autophagy and simultaneously inhibits apoptosis [27, 86]. Moreover, P53 is a negative regulator of HMGB1-beclin1 interactions in the cytoplasm, and loss of P53 increases interactions between HMGB1 and beclin1 [55].

HMGB1 also controls autophagy as a direct transcriptional regulator of the heat shock protein  $\beta$ 1 (HSP $\beta$ 1), which is a regulator of actin cytoskeleton dynamics [86]. Therefore, the suppression of HSP $\beta$ 1 expression avoids the dynamics

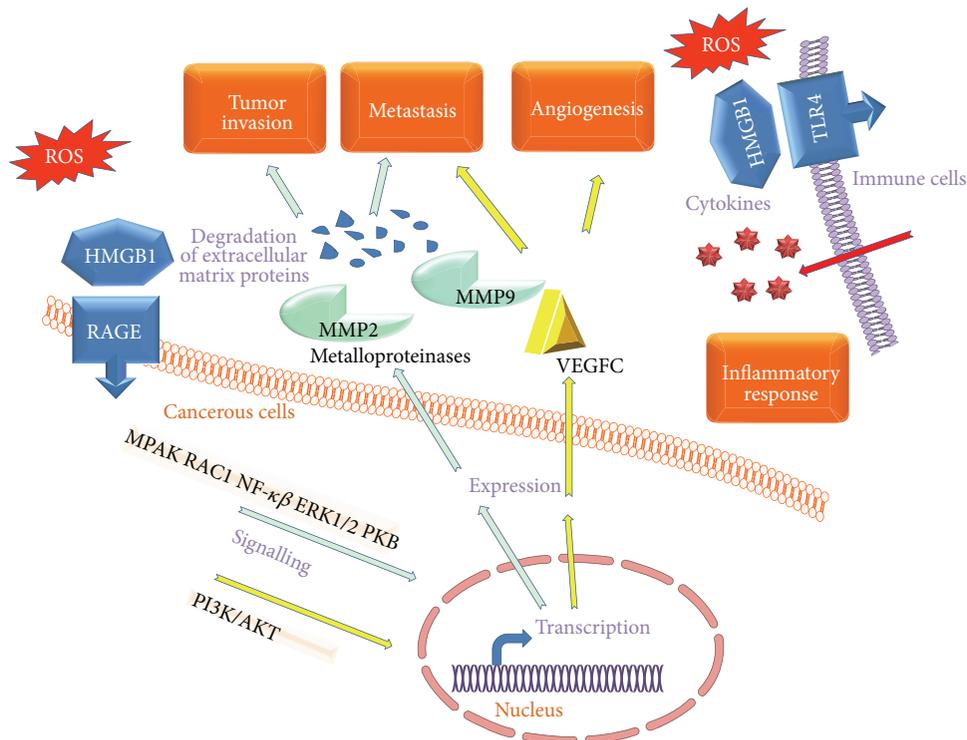


FIGURE 4: Simplified model of the effect caused by extracellular HMGB1, after oxidative stress, and upon the inflammatory response, invasion, metastasis, and angiogenesis.

necessary to the progression of the autophagosome and consequently inhibits autophagy as well as mitophagy [86]. Class III phosphatidylinositol-3 kinase (PI3K III) activity is required for the activation of autophagy [87] and HMGB1 promotes the formation of the beclin1-PI3K III complex [88], which is necessary for triggering autophagosome nucleation [89], probably by mitogen-activated protein kinase kinase (MEK) and protein-serine/threonine kinases (ERK1/2) signalling [88].

## 6. Extracellular HMGB1 Functions and Effects on Other Cells That Contribute to Cancer Progression

If cancer cells do not cope with redox imbalance and undergo necrosis, the released HMGB1 induces diverse responses over the cells in the microenvironment (Figure 4), which contribute to tumour cell survival and the development of metastases [90]. These effects of extracellular HMGB1 are linked to poor prognosis in several cancers including prostate, colon, pancreas, and breast [80].

The extracellular HMGB1 binds to diverse receptors in several cells, alone or forming heterocomplexes with other immunogenic molecules. Reduced HMGB1 (three thiols in Cys23, Cys45, and Cys106) binds to RAGE and induces beclin1-dependent autophagy [84]. RAGE is expressed in macrophages, cancer cells, and cells in the microenvironment

of tumours such as leukocytes, endothelial cells, and fibroblast [91]. Overexpression of RAGE and HMGB1 has been observed during cancer progression, invasion, and metastasis [92]. Conversely, blockade of RAGE-HMGB1 signalling suppresses tumour growth and metastases [93].

Semioxidized HMGB1 binds to TLR4 receptors in the immune cells and produces the release of cytokines, whereas reduced HMGB1 does not bind to TLR4. However, the reduced form binds to CXCR4 receptor forming a heteromer with the C-X-C motif chemokine 12 (CXCL12) and this interaction signals cell migration, thus promoting recruitment of motile inflammatory cells [94]. When all the thiol groups of HMGB1 have been oxidized to sulfonates, the molecule loses both the cytokine-inducing and chemoattractant activity [95]. In addition, HMGB1 forms complexes with other immune-stimulatory molecules as the lipopolysaccharide (LPS), the TLR2 ligand Pam3CSK4, nucleosomes, interleukin-1 $\beta$  (IL-1 $\beta$ ), RNA, and DNA, which bind to diverse receptors in the cellular membrane or in the membrane of endosomes [37].

The migration of endothelial cells is necessary for angiogenesis and tumour growth and HMGB1 overexpression is associated with an increased angiogenic potential of the endothelial cells [96]. The molecules by which HMGB1 stimulates this proangiogenic response in the endothelial cells include targets of the vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) as well as increased activity of matrix metalloproteinases, integrins, and NF- $\kappa$ B [96].

## 7. The Function of HMGB Proteins and Other Redox Sensors during Oxidative Stress in Ovarian Cancer

Distinct cyto- and histopathology disorders in ovaries have been related to cancer malignancies and the epithelial origin (epithelial ovarian cancer or EOC) is the most frequent (80%) cause. There is some controversy about whether EOC is initiated in the ovarian surface epithelium or in the fallopian tube, since both share a common embryogenic origin [97].

OS has been proposed as a cause of ovarian cancer. ROS are generated during ovulation, and indeed several factors that reduce the number of ovulatory cycles along women life (oral contraceptive pills, pregnancies, and lactation) diminish the risk to have this type of cancer [98, 99]. Two hypotheses have been formulated to explain how the increase of ROS production accompanying ovulation might induce the carcinogenesis. In the “incessant ovulation” hypothesis, it is assumed that repeated cycles of apoptotic cell death and repair at the ovarian surface epithelium eventually generate OS and irreparable genetic damage; tumour suppressor genes become mutated and cells become malignant. The major epithelial origin of ovarian cancer could be a consequence of the less robust DNA repair mechanisms in the surface epithelial cells of the ovary [100]. In a second view, the “incessant menstruation” hypothesis, ROS are generated through the Fenton reaction supported by the iron present in heme released after lysis of red blood cells by macrophages [101].

Common gene mutations associated with OS, and which are found in the surface epithelial cells of the ovary, affect in 50–80% of ovarian cancers to the protein P53 and in 30% of ovarian cancers to RB. Other frequent mutations affect the small GTPases, RAS proteins, whose mutations produce resistance against OS-induced apoptosis, 8-oxoguanine DNA glycosylase (OGG1) whose mutation prevent the repair of oxidized guanine and increase C to T transitions, and the mutS homolog 2 (MSH2), involved in DNA mismatch repair [102, 103].

Enzymatic and nonenzymatic oxidative defence systems are necessary to cope with the oxidative environment that persists in the ovary. Among the enzymatic systems, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase have been described in ovary [104]. The transcription factor NRF2 in healthy cells senses the redox state and activates the expression of genes related to protection against ROS damage through binding to AREs that are present in the promoters of the target genes. Although NRF2 is not a molecular redox sensor by itself, its translocation to the nucleus depends on the dissociation of its partner, the redox sensor KEAP1, which is E3 ligase adapter that in absence of ROS retains NRF2 in the cytoplasm and targets it for degradation in the proteasome [105–107]. NRF2 is also targeted for degradation in the proteasome by a KEAP1-independent mechanism that implies the phosphorylation of specific serines in the NEH6 domain of NRF2 by glycogen synthase kinase-3 (GSK3) and the interaction with the ubiquitin ligase adapter TrCP and the Cullin1/Ring-Box 1, E3 ubiquitin protein ligase (RBX1) complex [108]. OS affects

the redox state of cysteine residues of KEAP1 and prevents NRF2 ubiquitination; in these conditions NRF2 enters the nucleus where it binds, together with the MAF proteins [109, 110], to AREs in the promoters of its target genes [111]. After restoration of the redox balance SRC-kinases will promote the export of NRF2 again to the cytoplasm for degradation [112]. The KEAP1-NRF2 pathway regulates both mitochondrial and cytosolic ROS production through NADPH oxidase [113]. Abnormal activation of NRF2 is a major event during ovarian carcinogenesis [22] and it is frequently due to RBX1 alterations [114]. A direct interaction between the two major redox sensors, KEAP1-NRF2 and HMGB1, which are implicated in the onset and progression of cancers related to OS, has not been reported; however they might converge in several signalling pathways. A cross talk between NRF2 and HMGB1 during the response to DNA damage has been proposed; it is thought that the NRF2-ARE pathway may regulate time kinetics of HMGB1 release; ROS and HMGB1 levels will then modulate the response to DNA damage [115].

OS activates the oncoprotein AKT in several cell types; the activation of serine/threonine kinase AKT is achieved either by a direct phosphorylation cascade or by inactivation of the phosphatase and tensin homolog protein PTEN [116]. Signalling pathways for AKT activation include those elicited by the EGF receptor, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and integrins [117–119]. Activated AKT controls apoptosis and cellular proliferation and migration, as well as DNA repair [120]. However, active AKT also down-regulates the antioxidant systems; this causes an increase in ROS generation that, in turn, stimulates AKT activation and produces further OS in a vicious cycle [121]. Activation of the PI3K/AKT pathway is indeed associated with 40% of human ovarian cancers in The Cancer Genome Atlas Network [102, 103, 122, 123]. A triple association of oxidative stress, AKT activation, and ovarian cancer has not yet been proved in humans, although it has been found in surface epithelial cells of mouse ovary [118]. It has been demonstrated that the extracellular signalling of HMGB1 through RAGE and TLR4 receptors activates the PI3K-AKT/ERK1/2 pathway and contributes to proliferation of lung cancer cells [124]. A connection between NRF2 and AKT has also been recently reported [108].

HMGB1 is considered a biomarker for ovarian cancer [38, 39] and increased levels of interleukin-8 protein (IL-8) and HMGB1 correlate with poor prognosis in prostate and ovarian cancer cells [125]. Targeting HMGB1 by RNA interference inhibits ovarian cancer growth and metastasis [126]. The relevance of HMGB1 is of particular importance to hormone-related cancers, including ovarian origin [40]. In this sense, the interaction between the estrogen receptor (ER) and the estrogen responsive element (ERE) in the promoters of target genes is markedly minor (60-fold) in nucleosome DNA compared to that in free DNA and diverse approaches have shown that HMGB1 restructures the canonical nucleosome to facilitate strong ER binding [40]. Lymph node is a probable channel by which ovarian cancer cells may spread and invade other tissues. In human epithelial ovarian cancer, the protein HMGB1, together with tumour-associated macrophages, enhances lymphangiogenesis [127].

HMGB2 is also deregulated in EOC [128]. HMGB2 is part of the SET complex, which is composed of NM23, P32, SET, HMGB2, and APE1. This complex is also implicated in apoptosis and response to OS and DNA repair [128]. Tumours expressing low levels of SET, but high levels of NM23, or, alternatively, low levels of APE1, but high levels of HMGB2, have a better prognosis compared to other tumours [128]. Although the mechanisms producing these patterns are still unknown, the authors postulated that specific combinations of markers from the SET complex could be useful to classify patients for treatment [128].

## 8. Oxidative Stress in Prostate Cancer and the Function of HMGB Proteins and Other Redox Sensors

The human prostate anatomy displays a zonal architecture, corresponding to central, periurethral transition, peripheral zone, and anterior fibromuscular stroma. The majority of prostate carcinomas are derived from the peripheral zone, while benign prostatic hyperplasia arises from the transition zone [129]. Prostate contains a pseudostratified epithelium formed by three cell types: luminal, basal, and neuroendocrine [130]. However, a histopathological classification of prostate cancer subtypes, which differ in their prognosis or treatment, has not been possible. The majority of the diagnosed prostate cancers correspond to acinar adenocarcinomas that originate in the prostate gland and express the androgen receptor [129].

Increased ROS production in prostate cancer cells has been linked to diverse processes. The first one is the change observed in mitochondrial function. Frequently, the mitochondrial DNA isolated from prostate cancer cells contains an increased rate of mutations [131], which compromise the stability of the genome and the mitochondrial function, thus increasing ROS production. Upregulation of members of the membrane-bound NADPH oxidase protein complex (NOX1-5 and DUOX), which catalyses the production of superoxide from oxygen using NADPH as a cofactor [132], is another important source of intracellular ROS production. In human prostate cancer cells the levels of NOX2, NOX4, and NOX5 are increased [133]. As an additional source during prostaglandin biosynthesis, the catalytic activities of the cyclooxygenase enzymes (COXs) also produce ROS. The COXs proteins are present in two isoforms, COX1, constitutively and ubiquitously expressed, and COX2 that is overexpressed in cancerous prostate tissues [134]. Androgens, which are very important in prostate cancer development, also contribute to increasing ROS levels by signalling the transcription factor JUND [135] and the mitochondrial redox regulator P66SHC, a 66 kDa SRC homologous-collagen homologue (SHC) adaptor protein [136]. However, ROS levels could also be increased due to androgen deprivation [137, 138]. These results indicate that physiological levels of androgens are necessary to maintain the cellular redox equilibrium, and deviations caused by high or low production cause OS. Chronic inflammation, proliferative inflammatory atrophy (PIA), and infectious prostatitis constitute a prior stage to

prostate malignancy [139, 140] and, in these conditions, activated inflammatory cells and secreted inflammatory cytokines contribute to ROS generation and therefore to carcinogenesis [139, 141].

Antioxidant defences are diminished in prostate cancer cells, oppositely to what could be expected taking into account the increased production of ROS. Superoxide dismutase (SOD1, SOD2) and catalase activities are downregulated [142, 143] and the master redox regulator NRF2 is significantly downregulated in human prostate cancer [21]. As a consequence of higher levels of ROS production and diminished antioxidant defences, several indicators of oxidative damage have been found and tested as diagnosis and prognosis markers in prostate cancer. These include increased F2-isoprostane [144] or 8-hydroxydeoxyguanosine [145] in urine and increased peroxide levels [137] or decreased levels of the antioxidant  $\alpha$ -tocopherol [146] in serum.

Recently, functional links between OS and prostate cancer have been reviewed [138]. Oxidative damage and DNA damage, which may produce changes favouring the invasive behaviour of epithelial cells, have been described [147] as well as the shortening of telomeres, which may lead to chromosomal instability [148]. The levels of the tumour suppressor homeobox protein NKX3.1 are diminished in nearly all prostate cancers and metastases studied [149]; it has been suggested that NKX3.1 has a protective role against DNA damage [150]. This protein also links OS with prostate cancer in animal models; mutation of the homologous protein in mice displays deregulated expression of several antioxidant and prooxidant enzymes; in this model, progression to prostate adenocarcinoma is correlated with decreased superoxide dismutase activity and accumulation of oxidative damage in DNA and proteins [151].

Diverse cellular signalling pathways have been reported to play significant roles in the progression of prostate cancer [152]. Among them those regulated by the androgen receptor (AR) [153–155], estrogen receptors [156], PI3K/Akt/mTOR [157, 158], PTEN [159], NF- $\kappa$ B [160], the epidermal growth factor receptor EGFR [161], and PDGF [162]. Also, ROS-activated matrix metalloproteinases, which promote invasion and metastasis, are activated in prostate cancer cells [133]. RND3, which contributes to cell migration, is also deregulated in prostate cancer [76]. Finally, it has been suggested that, during prostate cancer progression, genes expressed in embryonic developmental programs are reactivated [163]. In particular, elevated canonical Wnt signalling may play a role in the emergence of castration resistance [164, 165]. Activation of Hedgehog signalling [166, 167] and Notch [168] and fibroblast growth factor (FGF) signalling [169, 170] may also play significant roles in prostate cancer.

There are many interconnections between these signalling pathways. For instance, PTEN functions as a tumour suppressor by negatively regulating the PI3K/AKT signalling and, in 30–50% of prostate cancer cases, loss of PTEN function causes PI3K/AKT signalling upregulation [158]. In an early step of prostate carcinogenesis, PTEN undergoes copy number loss and this event is correlated with progression of prostate cancer to a more aggressive, castration-resistant, stage that does not respond to hormone therapy [171].

The upregulation of AKT/mTOR signalling pathway in prostate cancer occurs primarily through activation of AKT1 [172]. The consequences of AKT activation are mediated in part by activation of NF- $\kappa$ B signalling via stimulation of inhibitor NF- $\kappa$ B kinase, IKK [173]. The stimulation of AR signalling leads to activation of SRC oncogenic kinases that phosphorylate AR in prostate cancer cells and cause castration resistance and cellular proliferation and invasiveness [174]. PI3K/AKT signalling [175] and AR signalling [155] increase SKP2 abundance in prostate cancer cells. SKP2 is the S-phase kinase associated protein 2 involved in cell cycle progression; it is the component of the SCF complex that confers substrate specificity to E3 ligase for ubiquitination of many targets that are tumour suppressors, which are marked for degradation in the proteasome [176]. Remarkably, as explained along the review in precedent sections, several among these signalling pathways are elicited by the redox sensor NRF2 or by the HMGB proteins.

Finally, several research lines outline the direct importance of HMGB proteins in prostate cancer and their implications in therapy. Increased HMGB2 expression [177], HMGB1 expression [41], or coexpression of RAGE and HMGB1 [178, 179] has been associated with prostate cancer progression and has been correlated to poor patient outcome. Consequently, silencing of *HMGB1* [180] or *RAGE* [181] genes in prostate cancer cells resulted in decreased cellular viability.

## 9. Cisplatin, Chemoresistance, Oxidative Stress, and HMGB Proteins

Cisplatin (cis-diamminedichloroplatinum(II)) is commonly used in prostate, ovarian, and other cancers therapy. It binds to DNA and forms majorly intrastrand cross-links with guanines. This produces cytotoxicity by inducing a DNA damage stress response [182, 183]. Emodin, an effective ROS generator, in cotreatment with cisplatin remarkably enhances chemosensitivity in prostate cancer cells, compared with cisplatin alone [184]. Cisplatin also generates OS response in the cells [185] that, together with the OS response generated as a consequence of cancer disease, might affect the functions of HMGB proteins. Steroid hormones that induce HMGB1 overexpression sensitize cancer cells to cisplatin and carboplatin [186]. In the LNCaP prostate cancer cell line, combined treatment with estrogen and cisplatin increases HMGB1 expression and apoptosis more than cisplatin alone and this effect is mediated by interaction between estrogen and ER-alpha [187].

Indeed, cisplatin and HMGBs proteins are functionally related, since these proteins bind with higher affinity to platinated DNA than to unmodified DNA [188]. The reduced (three-thiol) form of HMGB1 has a higher affinity for platinated DNA than the semioxidized form [189]. In this sense, the success of cisplatin chemotherapy toward testicular tumours has been attributed to the specific expression in testis of HMGB4 that lacks one of the cysteine residues that forms the disulphide bond in the other HMGB proteins [30].

The initial positive response to cisplatin treatment is frequently limited by development of broad resistance against

radio- and chemotherapies. Therefore, there is much interest in understanding the mechanisms responsible for development of resistance in the treatment of ovarian and prostate cancers and other types of cancers. The proteins HMGB1, HMGB2, HSC70, GRP58, and GAPD form a nuclear complex, which alters DNA conformation, and they have been associated *in vivo* with resistance to chemotherapeutic drugs in ovarian cancer patients [190]. In an ovarian cell line resistant to platinum-treatment some genes were overexpressed including those encoding for matrix metalloproteinases (MMP3 and MMP12) and HMGB2, while genes that encode for extracellular matrix proteins were downregulated as well as genes involved in the regulation of cell cycle and growth [191]. In a wide-genome study of genes associated with platinum-based chemotherapy resistance in ovarian cancer, several connections with the OS response have been found; these include the response mediated by NRF2, P53, and TGF $\beta$  signalling [192, 193], which have many links to HMGB proteins as already explained. Nucleus accumbens-1 (NAC1), a nuclear factor belonging to the BTB/POZ gene family, also modulates sensitivity of ovarian cancer cells to cisplatin by altering the HMGB1-mediated autophagic response [194].

Clusterin, a chaperone protein upexpressed in prostate cancer, stabilizes Ku70/BAX complexes, sequestering BAX from its ability to induce mitochondrial release of cytochrome *c*, thus avoiding subsequent apoptosis and promoting resistance to cisplatin; the secreted clusterin form is expressed in aggressive late stage tumours, and although its high expression may be considered an adaptive response to OS, it enhances the survival potential of cancerous cells [195]. Overexpression of riboflavin kinase, necessary for synthesis of FAD and glutathione reduction, is upregulated in cisplatin-resistant cells and it is related to prostate cancer progression [196]. The ubiquitin-specific protease 2a (USP2A), a deubiquitinating enzyme overexpressed in prostate adenocarcinomas, confers resistance to cisplatin; USP2A increases intracellular reduced glutathione content, reduces ROS production, and impairs the activation of apoptosis [197].

Resistance to cisplatin has been also attributed to DNA repair enzymes, which are able to remove lesions caused by cisplatin on DNA [182]. The mechanism of DNA repair is however inhibited by HMGB proteins that contribute to cytotoxicity both *in vitro* [198–200] and *in vivo* assays [201].

## 10. Conclusions and Perspectives

ROS overproduction and imbalance are a primary cause of malignancy in the onset of cancer. Cells have evolved multiple strategies in response to ROS production and HMGB proteins play a major role in many molecular mechanisms participating in these responses. In the nucleus, HMGB proteins affect DNA repair, transcription, and chromosomal stability; in cytoplasm they determine key decisions that finally lead towards autophagy or apoptosis; as extracellular signals they produce changes that affect the microenvironment of the tumour and attract cells from the immune system. In turn, the inflammatory onset can increase ROS production and therefore enhances the response. HMGB1

and HMGB2 are expressed at the highest levels in immune cells and, besides, they have been related to cancers, which are hormone-responsive, such as ovarian and prostate cancers. Since HMGB proteins have many different functions and are necessary in healthy cells, an improved strategy to modulate their role in cancer progression could be to act through other proteins interacting specifically with them. The identification of HMGB partners, which could be univocally associated with specific cancerous processes or with mechanism of cisplatin resistance, is a field of interest for ongoing translational cancer research. Interactome strategies are outstanding for the development of these research lines.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

Funding is acknowledged both from the Instituto de Salud Carlos III under Grant Agreement no. PI14/01031 and from Xunta de Galicia (Consolidación D.O.G. 10-10-2012. Contract no. 2012/118) cofinanced by FEDER. Aida Barreiro-Alonso was funded by a predoctoral fellowship from Xunta de Galicia-2013 (Spain).

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## Research Article

# Fisetin Modulates Antioxidant Enzymes and Inflammatory Factors to Inhibit Aflatoxin-B1 Induced Hepatocellular Carcinoma in Rats

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Received 19 June 2015; Accepted 11 August 2015

Academic Editor: Sahdeo Prasad

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Fisetin, a known antioxidant, has been found to be cytotoxic against certain cell lines. However, the mechanism by which it inhibits tumor growth *in vivo* remains unexplored. Recently, we have demonstrated that Aflatoxin-B1 (AFB1) induced hepatocarcinogenesis is associated with activation of oxidative stress-inflammatory pathway in rat liver. The present paper describes the effect of *in vivo* treatment with 20 mg/kg b.w. Fisetin on antioxidant enzymes *vis-a-vis* oxidative stress level and on the profile of certain proinflammatory cytokines in the hepatocellular carcinoma (HCC) induced by two doses of 1 mg/kg b.w. AFB1 i.p. in rats. The reduced levels of most of the antioxidant enzymes, coinciding with the enhanced level of reactive oxygen species in the HCC liver, were observed to regain their normal profiles due to Fisetin treatment. Also, Fisetin treatment could normalize the enhanced expression of TNF $\alpha$  and IL1 $\alpha$ , the two proinflammatory cytokines, reported to be involved in HCC pathogenesis. These observations were consistent with the regression of neoplastic lesion and declined GST-pi (placental type glutathione-S-transferase) level, a HCC marker, in the liver of the Fisetin treated HCC rats. The findings suggest that Fisetin attenuates oxidative stress-inflammatory pathway of AFB1 induced hepatocarcinogenesis.

## 1. Introduction

In general, genotoxic agents are known to initiate neoplastic lesions by inducing DNA damage [1]. Aflatoxin-B1 (AFB1), produced by *A. flavus* and *A. parasiticus* fungi, is a genotoxic agent which causes hepatocellular carcinoma (HCC) by making AFB1-DNA adducts mainly in the liver cells [2]. This is because it is metabolized by the liver specific CYP450 (3A4) enzymes to produce highly reactive AFB1-8,9-epoxides that bind at N<sup>7</sup> of guanine, thus creating lesions mainly in hepatocytes DNA [3]. Moreover, only those DNA aberrations drive hepatocytes to become tumorigenic which allow generation of tumor supportive microenvironment around [4].

Using diethylnitrosamine (DEN) induced HCC model, it has been described that the genotoxic damage of DNA is likely to induce oxidative stress to initiate hepatocytes necrosis resulting in release of the proinflammatory cytokines

to drive HCC progression [5]. This process might implicate alterations in the cellular antioxidant enzymes of the cells undergoing genotoxic necrosis [6, 7]. The three antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), mainly constitute antioxidant defense system of the cells [8, 9]. In particular, SOD1 (Cu-Zn SOD) is considered more relevant, as it catalyzes committed step of the antioxidant pathway [10] and has been reported to exist at a very low concentration in most of the growing tumors [11, 12]. Downstream to SOD1, catalase and GPx play important roles in removing H<sub>2</sub>O<sub>2</sub> produced by SOD activity. GPx, in particular, is responsible not only for metabolizing H<sub>2</sub>O<sub>2</sub> but also for maintaining rapid turnover of GSH, a critical cellular antioxidant. Importantly, modulations in GPx isoforms, GPx1 and GPx2, have been found associated with the tumor development [13, 14].

During the interplay of oxidative stress-inflammatory pathway, a number of proinflammatory cytokines have been

identified to drive genotoxically affected hepatocytes to undergo compensatory proliferation [5, 15, 16]. TNF $\alpha$  has attracted much attention in this respect [17, 18]. IL1 $\alpha$  is another member of inflammatory cascade which has been found to be associated with the tumor development and cancer cells metastasis [19, 20]. Some experimental data also suggest that prooxidative condition and inflammatory cytokines potentiate each other's effects in many ways during tumor progression. For example, enhanced TNF $\alpha$  level has been found to be associated with increased ROS generation by declining the level of SOD1 in U937 cells [21]. Similarly, downregulation of IL1 $\alpha$  and IL1 $\beta$  in melanoma cell lines has been observed to normalize ROS level in those cells [22]. Thus, it is reasonable to examine modulation of oxidative stress-inflammatory pathway as a therapeutic target in AFB1 induced hepatocarcinogenesis.

Indeed, antioxidant enzymes have been found to serve as relevant pharmacological targets in a number of tumor models [11]. Modulation of all the key antioxidant enzymes by Emodin, an anthraquinone, in Dalton's lymphoma (DL), resulting in regression of the tumor *in vivo* [23], is a relevant example in this context. Certain exogenous antioxidants have been reported to prevent HCC development during DEN induced [24] and against AFB1 induced hepatocarcinogenesis as well [25].

Recently, we have reported that AFB1 toxicity declines all the antioxidant enzymes to activate oxidative stress-inflammatory pathway as main initiator of hepatocarcinogenesis in those rats [15]. This necessitated investigation on whether modulation of antioxidant enzymes and proinflammatory cytokines could serve as a therapeutic target to regress AFB1 induced HCC progression.

During the recent past, natural products have attracted much attention for their anticancer roles. Fisetin is a dietary polyphenol which was primarily predicted to serve as a strong ROS scavenger compound [26]. Indeed, by activating glutathione system and by scavenging cellular ROS, Fisetin has been described to prevent growth of the lung fibroblast cells [27]. However, besides its ROS scavenging ability, the data, derived mainly from *in vitro* studies, suggest that this compound shows cytotoxicity against a number of cell lines by modulating some of the tumor associated biochemical/molecular targets like inhibition of CDKs by downregulating NF $\kappa$ B [28], inhibition of PI3K/Akt pathway in prostate cancer cells [29], and retarding angiogenic mechanisms in the endothelial cells [30].

Since Fisetin has been shown to induce apoptosis by downregulating bcl2 in the Huh7 cells (HCC cell line) as well [31], this compound deserves special merit to evaluate its anticancer activity against HCC *in vivo*. Though information is limited about *in vivo* anticancer activities of this compound, a report does indicate its role as a modulator of oxidative stress factors against benzopyrene induced lung carcinoma in mice [32]. We have also observed that AFB1 intoxication implicates alterations in the antioxidant enzymes and proinflammatory cytokines to develop HCC in rats [15] and that Fisetin treatment is able to normalize the level of GST-pi (glutathione-S-transferase, placental type), a HCC marker,

and to regress HCC lesions in those HCC livers (data of the present report).

Therefore, the present study aimed to investigate whether a nontoxic dose of Fisetin is able to modulate the HCC growth supportive profiles of the antioxidant enzymes *vis-a-vis* oxidative stress markers and proinflammatory cytokines in AFB1 induced HCC rat liver.

## 2. Material and Methods

**2.1. Chemicals.** Fisetin (3,3',4',7-tetrahydroxyflavone) and *Aspergillus* extracted Aflatoxin-B1 were procured from Sigma-Aldrich, USA. SOD1 and GST-pi polyclonal antibody and primers for TNF $\alpha$  and IL1 $\alpha$  were obtained from Santa Cruz Biotechnology, USA, and from Genetix, India, respectively. All other chemicals were purchased from Sisco Research Laboratory (SRL) Chemicals, India.

**2.2. Animals.** Male Charles foster rats (18–20 weeks old), procured from central animal house IMS-BHU, India, and maintained under controlled condition of temperature and humidity with alternate 12 h light/dark cycle, were used for this study. Rats were fed with standard pellet diet and water *ad libitum*. The study was conducted according to the ethical norms approved by the Institutional Animal Care and Use Committee (IACUC), Animal Ethical Committee of Banaras Hindu University, Varanasi (reference number Dean/10-11/169).

**2.3. Experimental Protocol.** Rats were randomly divided into three groups consisting of 5-6 rats each. Based upon the results of pilot experiments and as described in the previous report [15], the HCC group rats were administered intraperitoneally (ip) with two consecutive doses of 1.0 mg/Kg b.w. AFB1 dissolved in DMSO on the same day and monitored for development of HCC up to the 10th week. Fisetin treated HCC group (HCC + F) rats were administered with 20 mg/Kg b.w. Fisetin/day i.p. after the 6th week of AFB1 intoxication and this continued up to the 10th week. Based on pilot experiments and as reported in case of the mouse model [32], this dose of Fisetin was found to be nontoxic to the normal rats. The control group rats were similarly administered with DMSO throughout the treatment period.

At the end of the treatment period, rats from all the three groups were sacrificed by cervical dislocation after anesthesia (as per IACUC guidelines) and liver was excised. Livers from all the three groups were then subjected to histopathological and biochemical/molecular studies.

**2.4. Liver Histology.** Liver histology was performed as described earlier by our lab [15]. In brief, the liver sections were sliced into 0.3–0.5 cm pieces from control, HCC, and HCC + F groups rats. They were fixed in Bouin's fluid for 16–18 h and were transferred to 70% ethanol. This was followed by alcoholic dehydration and embedding in paraffin. Liver sections of 7  $\mu$ m thickness were cut and spread on the poly-L-lysine precoated slides. Slides were then subjected to

Hematoxylin-Eosin (HE) staining. After mounting in DPX, slides were analyzed under Leica 2000 microscope.

### 2.5. Biochemical Analysis

**2.5.1. Extract Preparation.** For native PAGE analysis, 10% liver extract was prepared in 0.2 mM Tris-HCl buffer (pH 7.4) and centrifuged at 10,000 ×g to collect partially enriched cytosolic fraction. For western blot analysis, 10% homogenate was prepared in 0.1 mM HEPES buffer (pH 7.4) containing 0.3 M KCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 0.001 mM PMSF, and 0.002 mM benzamidine and centrifuged at 10,000 ×g to collect partially enriched cytosolic fraction.

### 2.5.2. Biochemical Estimations

ROS. NBT reduction assay was performed for ROS measurement in the liver extracts as reported previously [33]. Briefly, 2% liver extract, diluted in PBS, was added with the NBT-PBS (1 mg NBT/mL) solution in the ratio of 0.5 mL/mL and incubated at 37°C for 4 h. After centrifugation, the pellet was washed thrice with methanol and dissolved in 2.0 mL mix of 2 M KOH and DMSO. Absorbance was recorded at 630 nm. The OD obtained was compared with a standard plot constructed against NBT and values were expressed as mole of NBT/mg protein.

**Total Glutathione.** Total glutathione was estimated in the liver extracts following a previously reported procedure [15]. Briefly, 0.1 mL liver extract was mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2) followed by addition of 0.1 mL of 0.01 M 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The mixture was made 10 mL with methanol and incubated for 30 min. After centrifugation, absorbance of the supernatant was read at 412 nm and values were expressed as nM/mg protein.

Protein content was estimated following the method of Lowry et al., 1951 [34].

**2.6. Native PAGE Analysis of SOD1, Catalase, and GPx.** As described previously [15], for nondenaturing PAGE analysis of SOD1, CAT, and GPx, the extract containing 40 µg protein was subjected to 8% nondenaturing PAGE followed by development of substrate specific achromatic bands against dark background for the specific antioxidant enzyme. Gels were scanned and the enzyme bands were quantified by the gel densitometry software Alpha Imager 2200.

**2.7. Western Blot Analysis.** Western blot analysis was performed as described previously [15]. Briefly, samples containing 60 µg proteins were subjected to 10% SDS PAGE followed by transferring the protein bands to nitrocellulose membrane and probing them against anti-GSTpi and SOD1 antibody (1:1000 dilutions). The membrane was then probed with HRP conjugated secondary antibody (1:5000 dilutions). ECL SuperSignal West Pico kit was used to develop protein bands on X-ray films. HRP conjugated monoclonal β-actin antibody (1:10,000) was used as the loading control.

**2.8. Semiquantitative RT-PCR.** As described previously [15], total RNA was isolated from the liver tissue using TRI reagent following the protocol of the kit supplied from Sigma-Aldrich. Briefly, 2 µg RNA sample was used for cDNA synthesis using random hexamer primer from the Revert Aid first strand cDNA synthesis kit (MBI Fermentas). Reaction mixture contained 19 µL of Taq polymerase buffer, 0.2 mM dNTPs, 1 U of Taq polymerase, and 10 pmol of primer. The rat gene specific primers used were TNFα (forward: 5'-ACT-CCCAGAAAAGCAAGCAA-3'; reverse: 5'-AGCAGGAAT-GAGAAGAGG-3'), IL1α (forward: 5'-AATCCTCTGAGC-TTGCCAGG-3'; reverse: 5'-GAGGGCAAAGACTGACCA-3'), and β-actin (forward: 5'-TCTACAATGAGCTGCGTGTG-3'; reverse: 5'-AATGTCACGCACGATTCCCC-3'). Amplification products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining and quantified by the gel densitometry software Alpha Imager 2200.

**2.9. Statistical Analysis.** Experimental data was subjected to Student's *t*-test analysis for statistical significance. The probability values of less than 0.05 were considered statistically significant and results were presented as mean ± SD, where *n* = 4–6 for each group.

## 3. Results

**3.1. Effect of Fisetin on Histopathology and HCC Marker.** In general, appearance of foci of altered hepatocytes (FAH) regions, in histological preparations, is considered to represent neoplastic lesions during HCC development. Figure 1(a) illustrates that, in comparison to the normal trabecular arrangement of hepatocytes seen in the liver section from the normal rats, regions of compressed trabeculae with compact hepatocytes, representing discrete FAH areas, could be seen in case of the liver from HCC group rats. Moreover, after Fisetin treatment, FAH regions are seen to be reduced remarkably with fewer number of compact hepatocytes within in the HCC liver.

Recently, GST-pi has been demonstrated to serve as a reproducible marker for AFB1 induced HCC progression. Therefore, to ascertain effect of Fisetin on HCC progression, the profile of GST-pi protein was also compared in the liver from the control, HCC, and Fisetin treated HCC group rats. As compared to the control group rats, ~4x increase (*p* < 0.001) in GST-pi level could be observed in the liver from the HCC group rats, which was brought back to its control level in the liver from the Fisetin treated HCC rats (Figure 1(b)).

**3.2. Effect of Fisetin on Oxidative Stress and HCC Markers.** Enhanced level of ROS is associated with AFB1 induced HCC progression. To ascertain whether Fisetin could suppress oxidative stress in AFB1 treated rats, ROS levels in the liver from control and experimental group rats were compared. A significant rise in ROS level (*p* < 0.05) was observed in the liver from the HCC rats as compared to the control counterparts. However, after Fisetin treatment, the enhanced

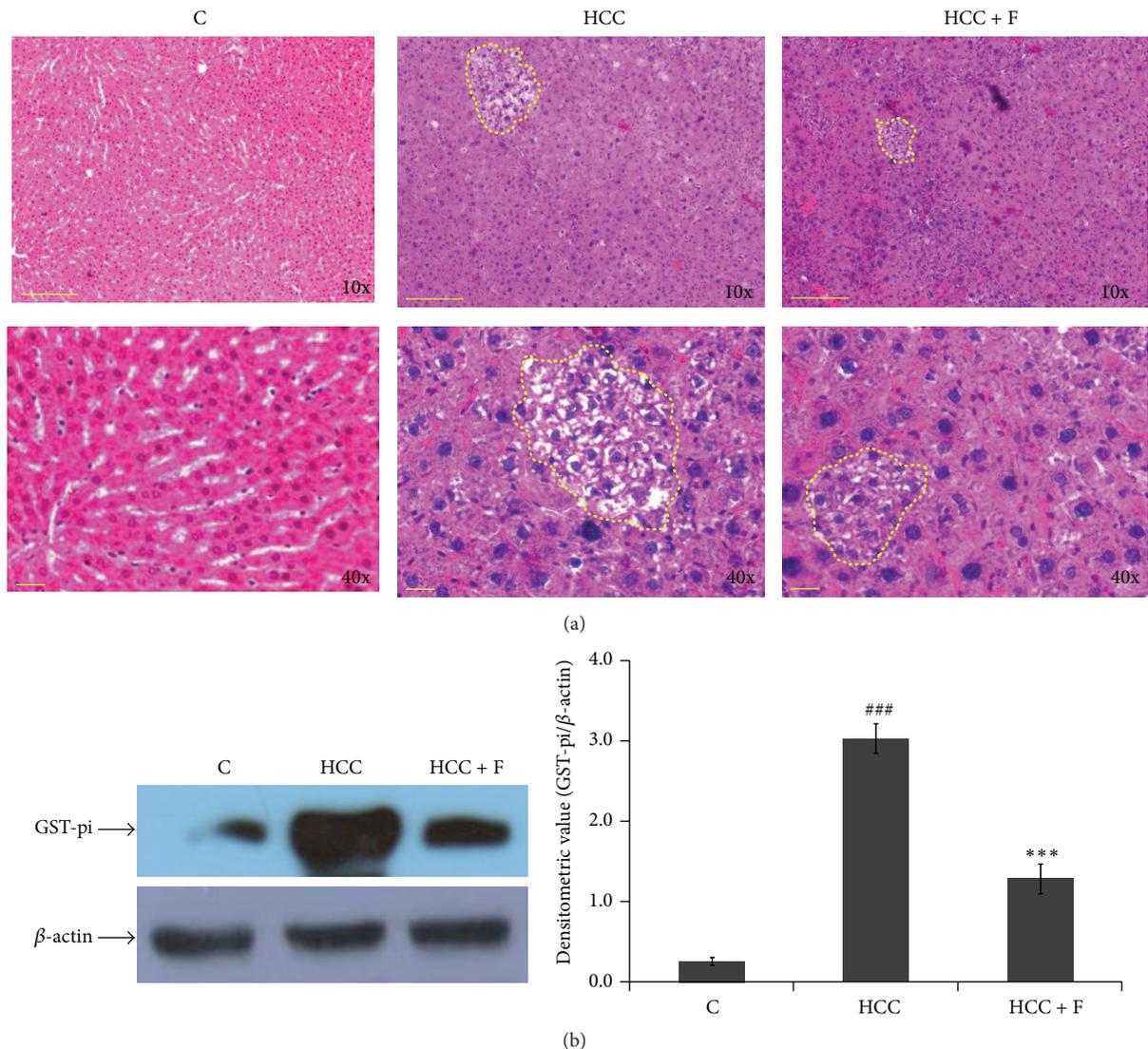


FIGURE 1: Effect of Fisetin on histopathology of HCC liver. Representative photomicrographs of liver sections from control (C), HCC, and HCC + F groups at 10 W stage have been shown. In (a), upper panel shows 10x magnification with scale bar of 200  $\mu$ m and lower panel shows 40x magnification with scale bar of 50  $\mu$ m. Dotted line encircles FAH area as a mark of neoplastic lesion. (b) shows level of GST-pi in the liver from control, HCC, and Fisetin treated HCC rats, wherein liver extract containing 60  $\mu$ g protein in each lane was subjected to 10% SDS-PAGE followed by western transfer and detection of GST-pi bands against a polyclonal anti-GST-pi. The photograph is representative of the three western blot repeats. Normalized densitometry values of GST-pi/ $\beta$ -actin have been presented as mean  $\pm$  SD from three western repeats. <sup>###</sup> $p < 0.001$  (control versus HCC group) and <sup>\*\*\*</sup> $p < 0.001$  (HCC versus HCC + F group).

ROS level in HCC liver was seen to be decreased significantly ( $p < 0.05$ ) to regain its normal value (Figure 2(a)).

Enhanced level of glutathione, a nonenzymatic antioxidant, is considered critical for maintaining reducing equivalence in the cells facing oxidative stress. According to Figure 2(b), the liver from HCC group rats showed a significant decrease ( $p < 0.05$ ) in glutathione level as compared to the liver from the control group rats. Moreover, such a decline of glutathione in the HCC liver could be recovered to its normal value in the HCC group rats administered with Fisetin.

**3.3. Effect of Fisetin on the Profile of Antioxidant Enzymes: SOD, Catalase, and GPx.** Declined SOD1 level is often

considered accountable for the rise of ROS at cellular level. In the present context, the expression and activity of SOD1 were measured in the liver from the control and the two experimental group rats. There was a significant decline ( $p < 0.05$ ) in the expression of SOD1, as compared to the control group rats, in the liver from the HCC group rats (Figure 3(a)). This pattern of SOD1 expression was seen to be consistent with the activity profile of this enzyme in those livers (Figure 3(b)). Moreover, both the expression (Figure 3(a)) and activity of SOD1 (Figure 3(b)) could regain their normal values in the liver from the Fisetin treated HCC rats.

SOD is the committed enzyme of the antioxidant pathway that neutralizes  $O_2^-$  by converting them into  $H_2O_2$ . Catalase

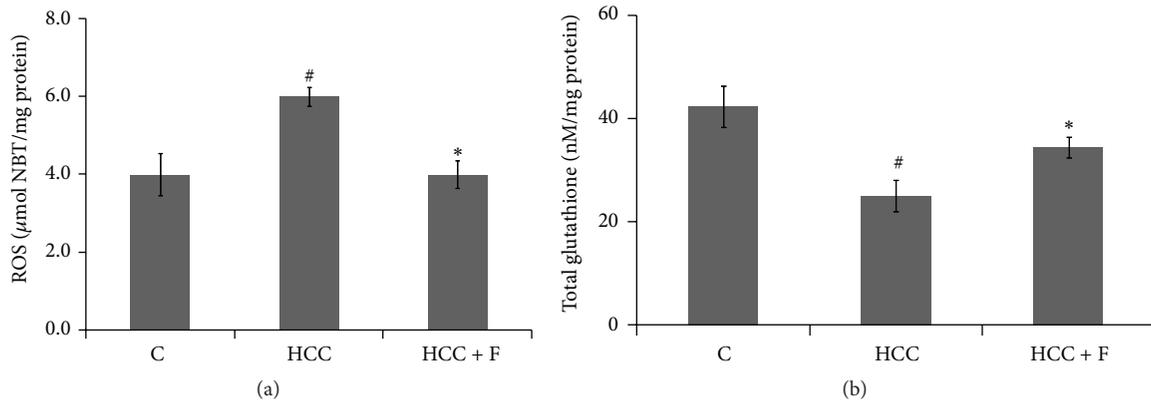


FIGURE 2: Effect of Fisetin on ROS (a) and glutathione (b) levels in the liver from control (C), HCC, and Fisetin treated HCC rats. Values have been represented as mean  $\pm$  SD, where  $n = 6$  and each experiment is done in triplicate. <sup>#</sup> $p < 0.05$  (control versus HCC group) and <sup>\*</sup> $p < 0.05$  (HCC versus HCC + F group).

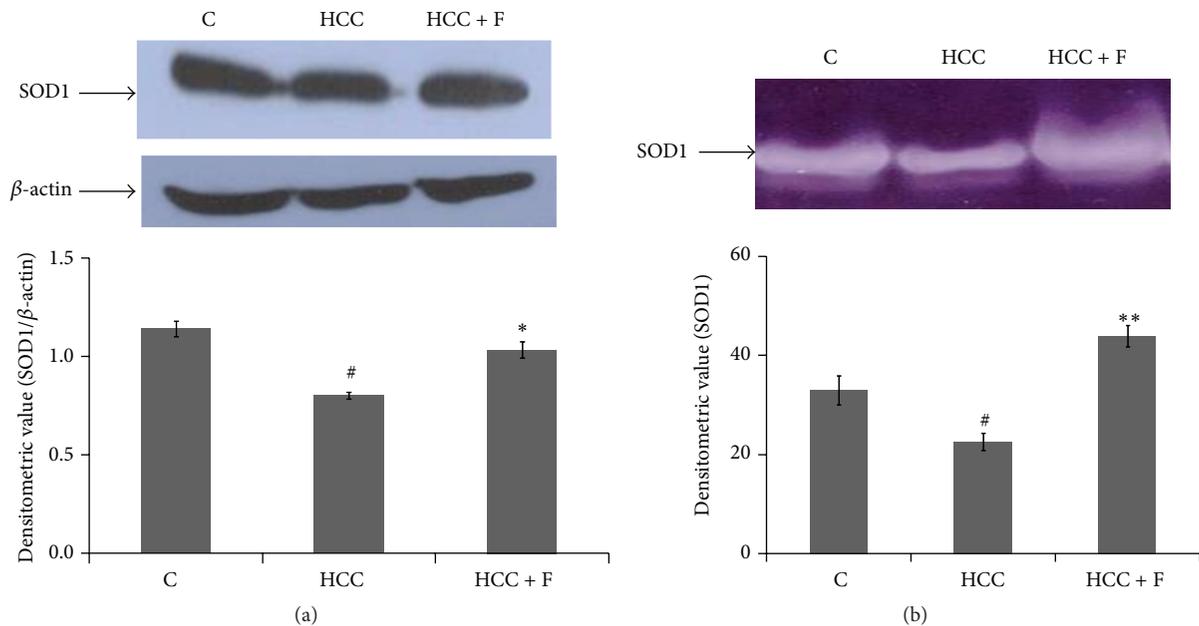


FIGURE 3: Effect of Fisetin on expression (a) and activity profile (b) of SOD1 in the liver from control (C), HCC, and Fisetin treated HCC rats. In (a), liver extract containing 60  $\mu$ g protein in each lane was subjected to 10% SDS-PAGE followed by western transfer and detection of SOD1 bands against a polyclonal anti-SOD1. The photograph is representative of the three western blot repeats. Normalized densitometry values of SOD1/ $\beta$ -actin have been presented as mean  $\pm$  SD. (b) shows 8% nondenaturing PAGE results of 40  $\mu$ g protein loaded in each lane. After electrophoresis, gel was subjected to development of substrate specific SOD1 band. The gel photograph is representative of the four PAGE repeats. The relative densitometric values of SOD1 band have been presented as mean  $\pm$  SD from the four PAGE repeats. <sup>#</sup> $p < 0.05$  (control versus HCC group) and <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$  (HCC versus HCC + F group).

and GPx together metabolize  $H_2O_2$  by utilizing GSH. Thus, catalase and GPx both play important roles in maintaining ROS homeostasis in the cells. According to Figures 4(a) and 4(b), the active levels of both of these enzymes were found to be declined significantly ( $p < 0.05$ ) in the HCC liver as compared to the liver from the control group rats. However, after the treatment with Fisetin, the profiles of both catalase and GPx were found to be enhanced significantly to finally regain their values around the control liver.

**3.4. Effect of Fisetin on Inflammatory Cytokines (TNF $\alpha$  and IL1 $\alpha$ ).** It has been demonstrated that AFB1 intoxication enhances TNF $\alpha$  and IL1 $\alpha$  levels to finally support oxidative stress-inflammatory pathway of hepatocarcinogenesis. Since we observed that Fisetin administration is able to normalize oxidative stress parameters (Figures 2–4) in the HCC liver, it was speculated that such a biochemical change may diminish upregulated profile of TNF $\alpha$  and IL1 $\alpha$  as well. Indeed, Figure 5 illustrates that the enhanced levels of TNF $\alpha$  and IL1 $\alpha$  mRNA

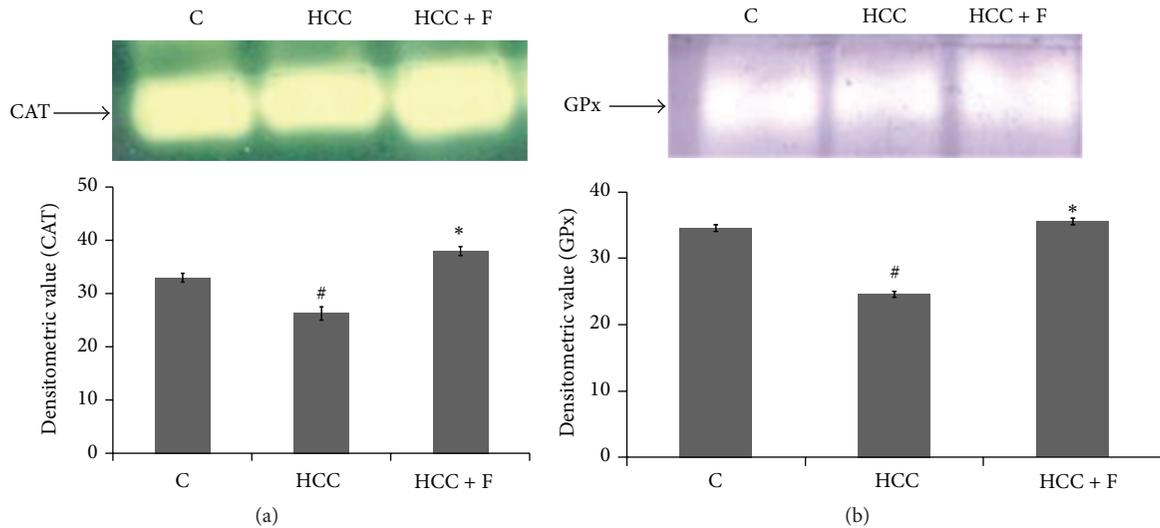


FIGURE 4: Effect of Fisetin on  $H_2O_2$  metabolizing enzymes, catalase (a) and GPx (b), in the liver from control (C), HCC, and Fisetin treated HCC rats. The upper panels of (a) and (b) show 8% nondenaturing PAGE results of  $40 \mu\text{g}$  protein loaded in each lane. After electrophoresis, gels were subjected to development of substrate specific catalase and GPx bands, respectively. The gel photographs are representative of the four PAGE repeats. The relative densitometric values of catalase and GPx bands have been presented as mean  $\pm$  SD from the four PAGE repeats. #  $p < 0.05$  (control versus HCC group) and \*  $p < 0.05$  (HCC versus HCC + F group).

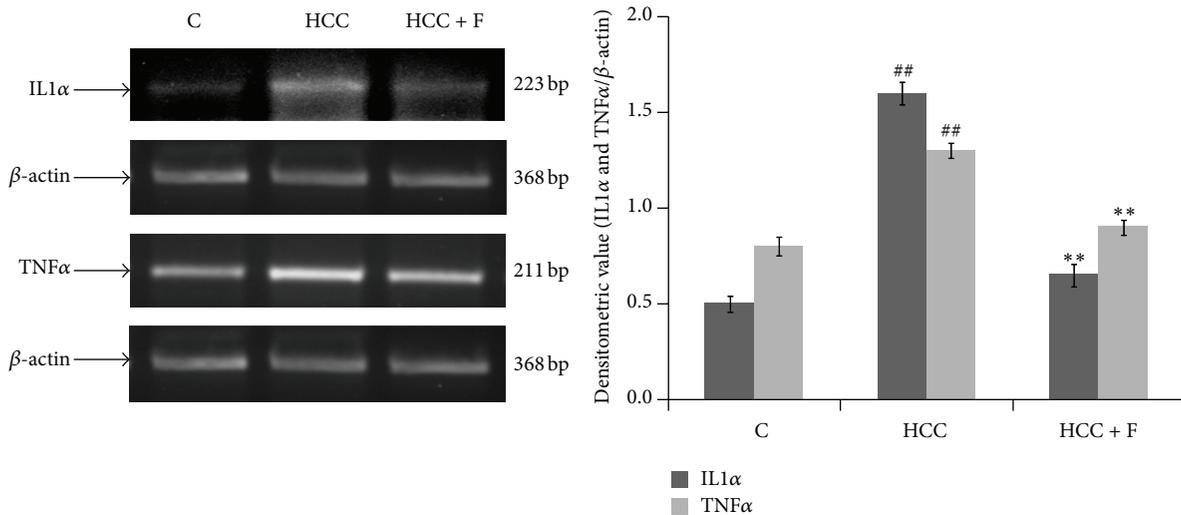


FIGURE 5: Effect of Fisetin on the level of proinflammatory cytokines, TNF $\alpha$  and IL1 $\alpha$ , in the liver from control (C), HCC, and Fisetin treated HCC rats. The figure shows representative RT-PCR photographs from four repeats with the normalized densitometric values of TNF $\alpha$ / $\beta$ -actin and IL1 $\alpha$ / $\beta$ -actin as mean  $\pm$  SD from four RT-PCR repeats. ##  $p < 0.01$  (control versus HCC group) and \*\*  $p < 0.01$  (HCC versus HCC + F group).

in HCC liver ( $p < 0.05$ ) could be recovered to the values observed in case of the normal liver due to the Fisetin treatment to the HCC group rats.

#### 4. Discussion

With regard to tumor development, oxidative stress is now evident to act as a double edged sword; some amount of oxidative stress stimulates tumor growth [35]; however, persistently enhanced oxidative stress, generated mainly

due to depleted endogenous antioxidant system, has been demonstrated to induce apoptosis in the tumor cells *in vitro* [36] and *in vivo* as well [37, 38]. As such, this mechanism, although it needs to be confirmed in case of a higher number of *in vivo* tumor models, provides a biochemical basis to design therapy targeted to modulate antioxidant system in the tumor cells. In a recent report from this lab, it has been demonstrated that AFB1 induced hepatocarcinogenesis also implicates oxidative stress imposed due to decrease in the levels of all the antioxidant enzymes [15]. Moreover, it was interesting to observe that Fisetin, a dietary flavonol, is

able to bring down the enhanced level of GST-pi, a HCC marker (Figure 1(b)), and could reduce the neoplastic lesions (FAH areas) in the HCC liver significantly (Figure 1(a)). In general, both GST-pi level and appearance of FAH regions are considered end point parameters to ascertain genotoxin induced HCC development in rat models [5, 15] and, thus, it was argued that Fisetin is able to regress AFB1 induced HCC in rats. However, since this pattern was consistent with the reversal of the enhanced ROS level in those HCC livers (Figure 2(a)), attempt was made to explore whether Fisetin regresses HCC by modulating antioxidant enzymes in the HCC liver *in vivo*.

Structurally, due to possession of three OH groups around, Fisetin is primarily known as a potent free radical scavenger [39]. In the present context, however, Fisetin was found not only to normalize ROS level but also to regain the level of depleted glutathione in the HCC liver (Figure 2(b)). This hinted at the multimodal action of Fisetin towards maintaining antioxidant milieu in the HCC cells. Indeed, some *in vitro* studies suggest that this compound is able to modulate different tumorigenic factors [27] including endogenous antioxidant factors at cellular level [30].

Mainly, the three antioxidant enzymes, SOD, catalase, and GPx, constitute central antioxidant mechanism to prevent oxidative insult at cellular level. SOD is the first enzyme of the antioxidant pathway that catalyzes dismutation of  $O_2^-$  into a less toxic  $H_2O_2$  compound [12]. Out of the two main SOD isoforms, SOD1 and SOD2, the level of SOD1 is found to be more critical in ROS mediated cancer progression [11]. It has been reported that reduced level of SOD1 facilitates genotoxin induced tumorigenesis via maintaining a high level of  $O_2^-$  whereas increased SOD1 level has been demonstrated to attenuate this process [40, 41]. In the present context also, the HCC associated low level of SOD1 and consequently increased level of ROS in the AFB1 induced HCC liver could be recovered to their respective normal levels due to the treatment with Fisetin (Figures 3(a) and 3(b)).

Although  $H_2O_2$ , produced by SOD1, is considered relatively less toxic, its rise has been found to support the genotoxin induced tumorigenesis including AFB1 induced HCC as well [15, 42]. Therefore, its degradation by the two downstream enzymes, catalase and GPx, becomes equally critical for preventing oxidative stress induced tumorigenesis. It has been demonstrated that enhanced level of GPx alone can prevent oxidative insult in SOD1 and catalase dual suppressed cells, thereby suggesting concordant roles of these three antioxidant enzymes in tumor progression/regression [43, 44]. Since declined levels of both GPx and catalase, reported in case of AFB1 induced HCC [15], are recovered back to their normal levels due to the Fisetin treatment (Figures 4(b) and 4(a)), it may be discerned that Fisetin modulates all the three antioxidant enzymes to prevent rise in tumor supportive ROS level in the HCC liver.

In addition to preventing a rise in ROS level, GPx activity contributes to maintaining the level of reducing equivalents in the form of glutathione in the cell [45, 46]. This is because GPx catalyzed reaction involves glutathione turnover in the cells [47]. A relative decrease in glutathione level has also been found to be associated with the rise in ROS level during

genotoxins induced tumorigenesis, which could be prevented due to the exogenous glutathione administration [48]. We could also observe that AFB1 induced HCC progression is accompanied with the declined glutathione level in the AFB1 induced HCC liver [15]. However, its level in those HCC livers was recovered due to the Fisetin treatment (Figure 1(b)).

Taken together, the findings of Figures 3 and 4 suggest that Fisetin modulates concordantly all the enzymes of antioxidant pathway which could account for prevention of ROS mediated HCC progression in the AFB1 treated rats. Though information is scanty about alterations in antioxidant enzymes by Fisetin in *in vivo* tumor models, it has been described that this compound does modulate oxidative factors against benzopyrene induced lung carcinoma in mice [32].

There could be more than one mechanism by which oxidative stress drives a cell towards neoplastic progression. Moreover, in case of genotoxin induced tumorigenesis, implication of oxidative stress-inflammatory pathway has been found to be the most plausible one [23, 49]. Using DEN induced HCC model, it has been speculated that oxidative stress is likely to induce local hepatocytes necrosis, thereby secreting certain proinflammatory cytokines around, which ultimately drives the neighboring cells to undergo compensatory proliferation and thus HCC progression [5]. Indeed, this pathway has recently been found to drive AFB1 intoxicated hepatocytes towards HCC progression as well [15]. Therefore, it was reasonable to examine whether Fisetin is able to modulate HCC associated inflammatory factors in the AFB1 induced HCC liver.

Among the inflammatory cytokines,  $TNF\alpha$  has been given much attention because its deficiency has been demonstrated to prevent formation of neoplastic lesions during DEN induced hepatocarcinogenesis [17, 18].  $IL1\alpha$  is another cytokine which has been found to be implicated in the oxidative stress led necrotic death and consequently tumor progression [5]. Concordant with the declined levels of all the antioxidant enzymes, both of these cytokines have also been found to be overexpressed in the AFB1 induced HCC liver [15]. Not much information is available to derive a mechanistic link for reciprocal changes between antioxidant enzymes and the inflammatory factors; in a human cell line, it has been reported that enhanced  $TNF\alpha$  represses SOD1 promoter activity via JNK/AP-1 signaling pathway [21]. It is reported here that Fisetin treatment could decline the enhanced level of both  $TNF\alpha$  and  $IL1\alpha$  (Figure 5), which is consistent with recovery in SOD1 activity (Figure 3), declined ROS level (Figure 2(a)), and HCC regression (Figure 1). Thus, it is argued that this compound could normalize proinflammatory-antioxidant pathway by declining  $TNF\alpha$  expression and thus preventing SOD1 depletion in the HCC liver.

## 5. Conclusion

Targeting tumor growth associated biochemical events by nontoxic compounds is an evolving concept in cancer

chemotherapy. In case of genotoxicity mediated tumorigenesis, imposition of oxidative stress is considered critical for driving a normal cell to undergo neoplastic progression *in vivo*. This paper describes that a nontoxic dose (examined on normal rats) of Fisetin, a natural flavanol, is able to normalize ROS led inflammatory pathway of AFB1 induced hepatocarcinogenesis in rats. And it does so by modulating the enzymes of the main antioxidant pathway in the HCC cells. The findings suggest that modulating antioxidant enzymes and proinflammatory factors could be the relevant mechanisms to inhibit tumor development *in vivo*.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgments

This work was financially supported by a UGC project (P-01/663) to Surendra Kumar Trigun. The award of CSIR JRF & SRF to Brajesh Kumar Maurya and the facilities of BHU-DBT ISLS in Faculty of Science, DST-FIST, UGC UPE, and UGC-CAS programs to the Department of Zoology are also acknowledged.

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## Research Article

# The Relevance of Nrf2 Pathway and Autophagy in Pancreatic Cancer Cells upon Stimulation of Reactive Oxygen Species

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Received 22 May 2015; Revised 15 July 2015; Accepted 28 July 2015

Academic Editor: Sahdeo Prasad

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Nrf2 (NF-E2-related factor 2) pathway and autophagy both can respond to oxidative stress to promote cancer cells to survive in the tumor microenvironment. We, therefore, explored the relevance between Nrf2 pathway and autophagy in pancreatic cancer cells upon stimulation of reactive oxygen species (ROS). Pancreatic cancer cells were cultured under controlled ROS stressing condition or basal condition. Different inhibitors were used to prevent autophagy at particular stages. Nrf2 siRNA was used to inhibit Nrf2 pathway activation. Ad-mRFP-GFP-LC3 infection was used to monitor autophagic flux. The result shows that a small amount of exogenous hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can significantly improve the level of intracellular ROS. Moreover, our findings indicate that ROS promotes the activation of both Nrf2 pathway and autophagy in pancreatic cancer cells. Moreover, our data demonstrate that suppression of autophagic activity at particular stages results in an increased promotion of Nrf2 pathway activation upon ROS stimulation. Furthermore, we found that silencing of Nrf2 promotes autophagy upon ROS stimulation. In addition, Nrf2 interference effectively promotes autophagic flux upon ROS stimulation. In summary, our findings suggest that Nrf2 pathway and autophagy have a negative interaction with each other upon ROS stimulation.

## 1. Introduction

Autophagy is an evolutionary conserved lysosomal degradation process in which the cells degrade long-lived proteins, misfolded proteins, and damaged cytoplasmic organelles for recycling [1]. Autophagy has been considered to maintain the cellular homeostasis and adaptation to stressed conditions such as oxidative stress, nutrient starvation, and hypoxia [1–4]. For its many important roles, it is not surprising that impaired autophagic function promotes the progression of cancer. However, cancer cells break down cellular damaged organelles and accumulated proteins by autophagy, allowing the catabolites to be recycled and thus used for biosynthesis and energy metabolism to cope with the stressing conditions, which is essential to enable cancer cells to survive [5, 6].

It has been considered that Nrf2, a transcriptional factor, is an adaptive cellular response to protect cells against oxidative stress. Nrf2 is targeted by Keap1 (Kelch-like ECH-associated protein1) for ubiquitylation and proteasomal degradation under normal condition [7]. When faced with cellular stressing signals (e.g., oxidative stress), the interaction between Nrf2 and Keap1 is disrupted, resulting in Nrf2 stabilization and translocation from cytoplasm to the nucleus, which is regarded as canonical way of Nrf2 activation [8]. As a result, the nuclear Nrf2 binds to antioxidant response elements (AREs) or electrophile response elements (EpREs) to deal with the stressing signals [9–11]. High levels of Nrf2 have been observed in many cancers, including head and neck, gall bladder, lung cancer, and colorectal cancer [12–14],

which promote the growth and survival of cancer cells under stressing conditions. Nrf2 pathway and autophagy both have the ability to antagonize cellular stressing signals by promoting a series of antioxidant programs. Furthermore, studies have shown that Nrf2 and autophagy both contribute to the chemoresistance [15–18].

The relationship between the Nrf2 pathway and autophagy has been explored in recent years, and researchers found that the association between them mainly relied on p62/SQSTM1, an adaptor for selective autophagy, and Keap1 [19–21]. Inhibition of autophagy leads to accumulation of p62. P62 works to sequester Keap1 into the autophagosomes, inhibiting the ubiquitylation of Nrf2, resulting in the noncanonical activation of Nrf2 [19–21]. Many studies have shown that cancer cells accumulate more reactive oxygen species (ROS) than normal cells [22, 23]. Consistent with other researchers, we found that the level of ROS in pancreatic cancer cells had elevated along with the increasing metastatic ability [24]. With the increased ROS, cancer cells induce antioxidant programs to set a new redox balance, resulting in cellular adaptation. Studies have shown that autophagic inhibitor (chloroquine) caused accumulated ROS in cells, and downstream of Atg1, FIP200-(Atg17 homologue) knockout livers, and Atg5- and Atg7-knockout cells both increase ROS production [25–30]. On the basis of these results, we hypothesized that there is a possibility that autophagy inhibition not only leads to accumulation of p62 to activate Nrf2 pathway by a noncanonical way but also increase ROS production to directly activate Nrf2 pathway.

In addition, another study indicated that Nrf2 could lower the level of intracellular ROS [31]. Thus, we speculated that the relationship between Nrf2 pathway and autophagy could not be a simple upstream or downstream. The mechanism of the interaction between Nrf2 pathway and autophagy is needed to be investigated to facilitate the discovery of new therapies. In this study, we set pancreatic cancer cells at an increased ROS level to simulate oxidative stress condition and explore the relevance between Nrf2 pathway and autophagy.

## 2. Materials and Methods

**2.1. Cell Culture and Reagents.** Human pancreatic cancer cell lines BxPc-3, PANC-1, SW1990, AsPC-1, and MiaPaCa-2 were obtained from and validated by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured as per their instructions. Briefly, cells were cultured with a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) added with 100 µg/mL ampicillin and 100 µg/mL streptomycin. In experiments designed to elevate the level of intracellular ROS, H<sub>2</sub>O<sub>2</sub> was added in the serum-free media with a final concentration of 100 µmol/L for 24 h. Different autophagic inhibitors were used in this study: 3-MA of 5 mmol/L or CQ of 40 µmol/L for 24 h. Antibodies were obtained from the following resources: anti-β-actin antibody (Santa Cruz, USA), anti-Nrf2 antibody (Abcam, USA), anti-Beclin1 antibody (Abcam, USA), anti-LC3 antibody (Sigma, USA), and anti-p62 antibody (Proteintech Group, USA).

Autophagic inhibitors were obtained from the following resources: 3-MA (HanBio, China) and CQ (Sigma, USA).

**2.2. Cell Proliferation Analysis.** PANC-1 cells were seeded in 96-well plates at the point of 24 h prior to the serum free incubation at an amount of 5–10 × 10<sup>3</sup> cells per well. Following the serum starvation of 24 h, cells were maintained in medium with concentrations of H<sub>2</sub>O<sub>2</sub> ranging from 100 to 400 µmol/L. At the time point of 24 h, 48 h, or 72 h, the medium in each well was removed, and then MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added. After incubation of 4 h at 37°C, DMSO was added to each well with an amount of 150 µL. The microplate reader (BIO-TEC Inc, VA) was used to measure the optical densities (OD) at 490 nm. The proliferation rate was determined as OD (cells plate)/OD (blank plate).

**2.3. Detection of Intracellular ROS.** The level of intracellular ROS was detected using an oxidation-sensitive fluorescent probe (DCFH-DA). After treatment with H<sub>2</sub>O<sub>2</sub> 100 µmol/L or not, PANC-1 cells were washed twice with PBS. Then cells were incubated with 10 µmol/L DCFH-DA for 20 min at 37°C according to the manufacturer's instructions. DCFH-DA was oxidized to the fluorescent compound 2, 7-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence was detected by FACScan flow cytometer (Becton Dickinson). 10 000 events were collected for each sample.

**2.4. RNA Extraction and Quantitative Real-Time PCR.** Total RNA was extracted from pancreatic cancer cells by Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and the real-time PCR assay was performed with an iQ5 Multicolor real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and a SYBR Green PCR Kit (TaKaRa) followed by their manufacturer's instructions. The PCR primer sequences of Nrf2, Beclin1, LC3 and β-actin are shown in Supplementary Table S1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/3897250>. For all real-time PCR analyses, β-actin was used as a normalization control to quantify the relative expression of the target gene.

**2.5. RNA Interference.** Six siRNAs for NRF2 (Supplementary Table S2) were purchased from GenePharm (Shanghai, China). Cells were seeded in six orifice plates with an amount of 2 × 10<sup>5</sup> per well and transfected with a final concentration of 100 nM siRNA using Lipofectamine RNAi MAX Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After transfection, cells were used for further study at the time point of 48 h.

**2.6. Western Blot.** The protein levels of Nrf2, Beclin1, LC3, and p62 were quantified by semiquantitative densitometric analysis. Cell samples were lysed in RIPA, and a BCA protein assay kit was used to determine the protein concentration. Briefly, the total protein lysates were separated on 12% SDS-PAGE gels for detecting Nrf2, Beclin1, LC3, and p62. Then

the proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% skimmed milk for 2 h at room temperature, and then immunoblotted with rabbit polyclonal anti-human antibodies against  $\beta$ -actin (1:1000), Nrf2 (1:1000), Beclin1 (1:1000), LC3 (1:1000), and p62 (1:1500) overnight at 4°C. This was followed by application of a goat anti-rabbit peroxidase conjugated secondary antibody (1:5000, Santa Cruz) for 2 h at 37°C. Then the bands were detected using the enhanced chemiluminescence system and Quantity One image analysis software was used to measure the band intensity. The housekeeping protein  $\beta$ -actin was used for loading control. For result analysis, we normalized the band intensity of target protein to  $\beta$ -actin in each sample, and then we normalized the relative target protein expression level of treated group to its control group.

**2.7. Immunofluorescence.** The cells were washed with phosphate-buffered saline (PBS) for three times, fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 for 10 min, and then blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Next, cells were incubated with rabbit polyclonal anti-human antibodies against Nrf2 (1:150), Beclin1 (1:150), and LC3 (1:150) at 4°C overnight, respectively. After that, cells were washed and incubated with goat anti-rabbit dylight 594 (red) IgG antibody (QENSHARE BIOLOGICAL Inc., Xi'an, China) or goat anti-rabbit FITC (green) IgG antibody (ZSGB-BIO Inc., Beijing, China) at 1:150 dilution for 60 min at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole for 5 min. Cells were visualized with the fluorescent microscope (Nikon Eclipse Ti-s, Japan) using appropriate excitation wavelength.

**2.8. Autophagy Detection Using mRFP-GFP Adenoviral Vector.** Ad-mRFP-GFP-LC3 was purchased from HanBio Technology Co. Ltd. (HanBio, Shanghai, China) and the process of adenoviral infection was implemented according to the manufacturer's instructions. PANC-1 cells were plated in six orifice plates and determined to reach at the desired confluency of 50%–70% at the time of infection. Then, cells were cultured in DMEM supplemented with 2% FBS with the adenoviruses at a final MOI of 60 for 2 h at 37°C. After infection, cells were grown in medium with 10% FBS and used for further study at the time point of 48 h. Autophagy was observed under a fluorescence microscope (Nikon Eclipse Ti-s, Japan). Autophagic flux was determined by evaluating the number of GFP and RFP puncta (puncta/cell were counted).

**2.9. Live Cell Microscopy and Imaging.** For live cell imaging, PANC-1 cells infected with Ad-mRFP-GFP-LC3 were grown on glass-bottom dishes (MatTek). Then, the cells were transfected with Nrf2 siRNA. After transfection, cells were treated with ROS stimulation and observed by Live Cell Imaging Confocal Scanner System according to the manufacturer's instructions. Briefly, cells were maintained in a 5% CO<sub>2</sub> chamber at 37°C through the acquisition. Three random positions of the cells were selected to monitor the process of autophagy. Cells were visualized with the fluorescent

microscope using appropriate excitation wavelength, and images for the three selected positions were acquired at the beginning and the time point of 24 h.

**2.10. Statistical Analysis.** All experiments were repeated at least three times. Data are presented as Mean  $\pm$  SD. Statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 17.0 (SPSS Inc., Chicago, IL, USA). Student's *t*-test and one-way ANOVA with the LSD post hoc test were used to evaluate the differences of presented data. The overall *P* values presented here were two-sided. The significance level was set at a *P* value less than 0.05. In all figures, (\*) denotes *P* < 0.05.

### 3. Results

**3.1. Expression of Nrf2 in Pancreatic Cancer Cell Lines.** To explore the possible roles of Nrf2 pathway and autophagy under oxidative stress in pancreatic cancer cell lines, we first detected the expression of Nrf2 in 5 pancreatic cancer cell lines (BxPc-3, PANC-1, SW1990, AsPC-1, and MiaPaCa-2) by Western blot and real-time PCR analyses, respectively (Figures 1(a)–1(b)). We found that Nrf2 expression and transcription were the strongest in PANC-1 cells but the weakest in BxPc-3 and AsPC-1 cells.

**3.2. Expression of Autophagic Related Protein Level in Pancreatic Cancer Cells.** Beclin1 and LC3 were conducted to detect the autophagic activity of pancreatic cancer cells for their important roles in autophagy. We detected the expression of Beclin1 and LC3 in 5 pancreatic cancer cell lines (BxPc-3, PANC-1, SW1990, AsPC-1, and MiaPaCa-2) by Western blot and real-time PCR analyses, respectively (Figures 2(a)–2(c)). We found that the expression of Beclin1 and LC3-II protein and also Beclin1 and LC3 transcription were all the strongest in PANC-1 cells. Therefore, we chose the cell line of PANC-1 for further study.

**3.3. H<sub>2</sub>O<sub>2</sub> Induces Increased Generation of ROS in PANC-1 Cells.** To elevate the level of intracellular ROS in PANC-1 cells, exogenous hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was used to treat cells. H<sub>2</sub>O<sub>2</sub> is a prototypic reactive oxygen species (ROS) generated as a by-product of the normal oxidative metabolism. At low concentrations, H<sub>2</sub>O<sub>2</sub> acts as a survival molecule, but at high concentrations it can lead to irreversible damage, followed by cell death. With the increased concentration of H<sub>2</sub>O<sub>2</sub> in serum-free medium, the capacity of inhibiting proliferation for PANC-1 cells became more apparent. Ultimately, we selected 100  $\mu$ mol/L as the optimum concentration through the MTT assay (Figure S1). Then, we observed that a large number of cancer cells died after 48 h when treated with 100  $\mu$ mol/L H<sub>2</sub>O<sub>2</sub>. Thus, we choose 24 h as the optimal H<sub>2</sub>O<sub>2</sub> treated time. Flow cytometry was used to detect the intracellular ROS level in PANC-1 cells (Figure S2). The results showed that a small amount of exogenous H<sub>2</sub>O<sub>2</sub> can significantly improve the level of intracellular ROS.

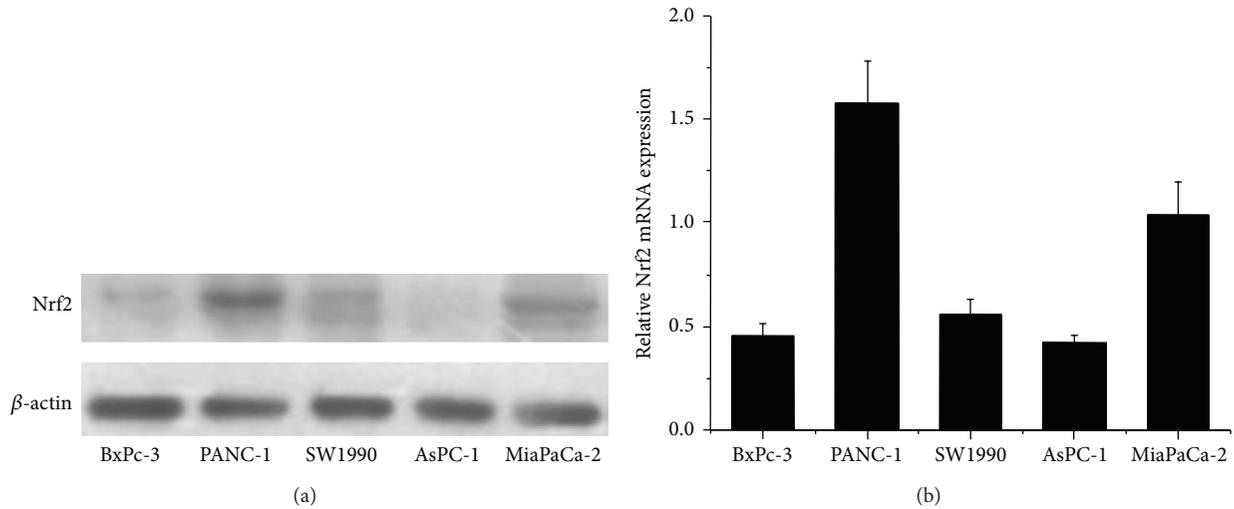


FIGURE 1: The expression of Nrf2 in pancreatic cancer cells. (a) The expression of Nrf2 at protein level in BxPc-3, PANC-1, SW1990, AsPC-1, and MiaPaCa-2 cells was evaluated by Western blotting. (b) The expression of Nrf2 mRNA level was estimated in 5 pancreatic cancer cell lines by qRT-PCR. The data are presented as Mean  $\pm$  SD for three independent experiments. The bar graph below shows the relative mRNA expression levels among the cell lines. Column: Mean; bar: SD.

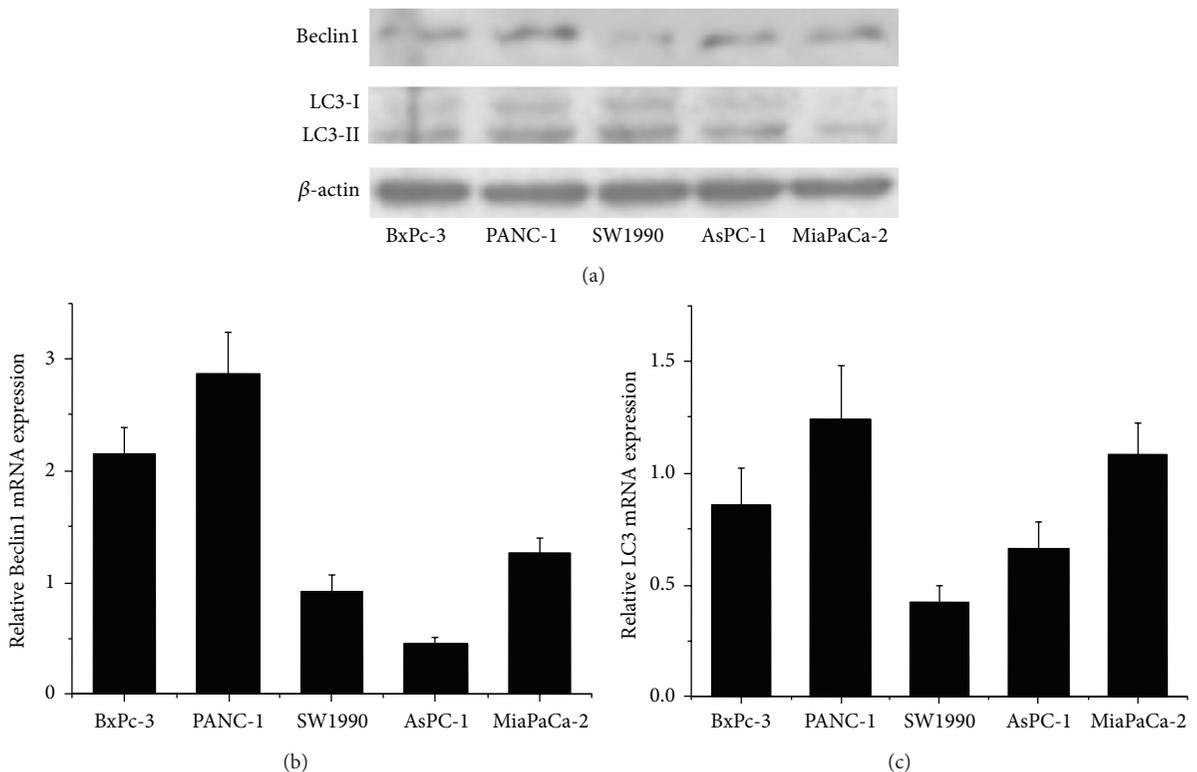


FIGURE 2: The expression of Beclin1 and LC3 in pancreatic cancer cells. (a) The expression of Beclin1 and LC3-II protein in BxPc-3, PANC-1, SW1990, AsPC-1, and MiaPaCa-2 cells was evaluated by Western blotting. (b-c) The expression of Beclin1 and LC3 mRNA level was estimated in 5 pancreatic cancer cell lines by qRT-PCR. The data are presented as Mean  $\pm$  SD for three independent experiments. The bar graph shows the relative mRNA expression levels among the cell lines. Column: Mean; bar: SD.

3.4. *The Influence of ROS on Nrf2 Expression and Autophagy in PANC-1 Cells.* The expression of Nrf2 and autophagic related proteins were explored by Western blot. As shown in Figure 3(a), treatment with  $H_2O_2$  (ROS) could significantly

improve the expression of Nrf2, Beclin1, and LC3-II and also the level of p62 in PANC-1 cells. Immunofluorescence analyses indicated a marked increase of Nrf2 immunofluorescence signal in both the cytoplasm and the nucleus, suggesting that

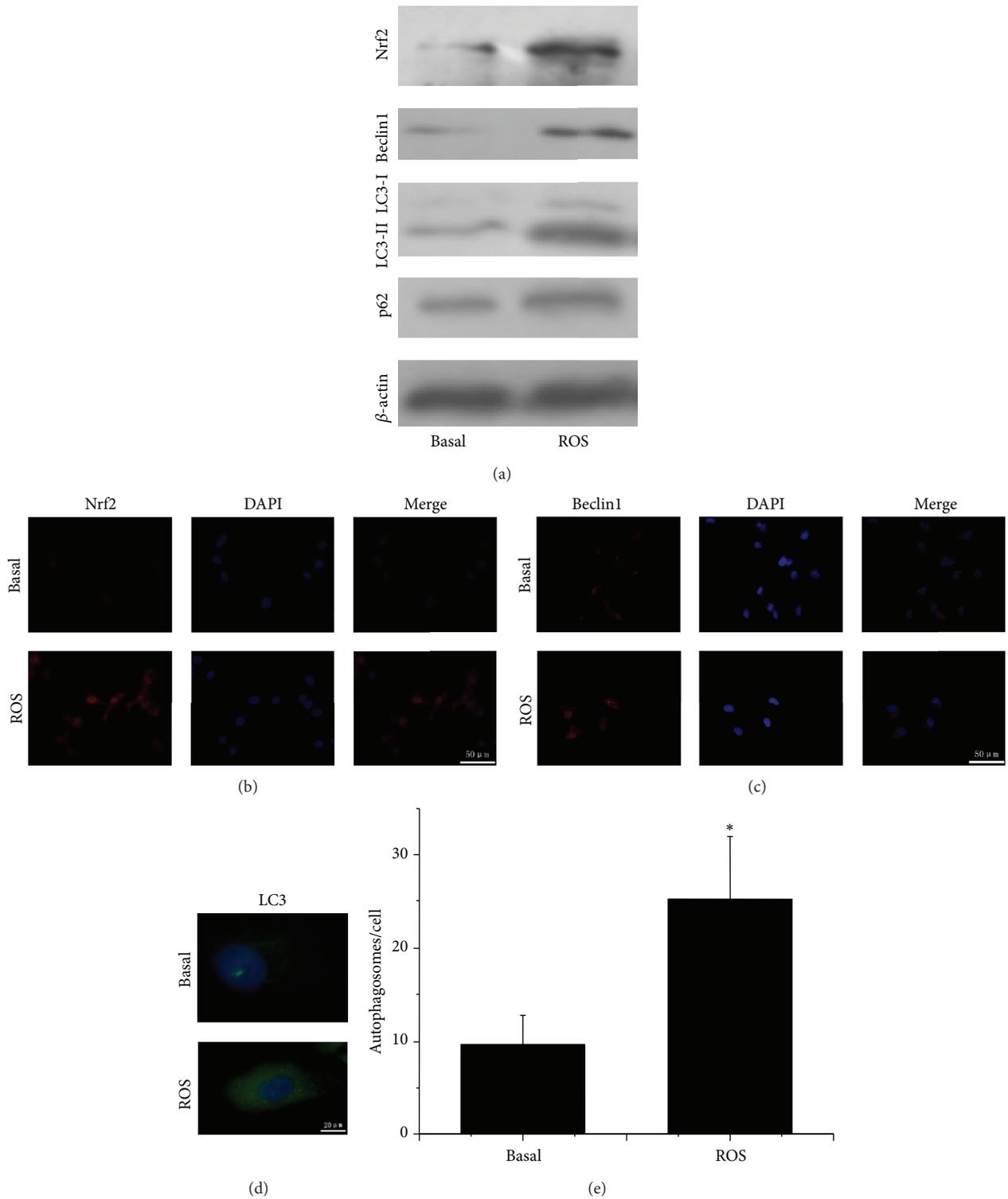


FIGURE 3: ROS stimulation induces translocation of Nrf2 to the nucleus and autophagy in PANC-1 cells. (a) The expression of Nrf2, Beclin1 and LC3-II and also the level of p62 in PANC-1 cells under basal condition and upon ROS stimulation. (b–d) Immunofluorescence images of PANC-1 cells for Nrf2, Beclin1 and LC3 under basal condition and upon ROS stimulation. (e) Numbers of autophagosomes per cell were counted in 10 random fields. The data are presented as Mean  $\pm$  SD for three independent experiments. Column: Mean; bar: SD.

the expression of Nrf2 and nuclear translocation of Nrf2 were enhanced as an effect of increased ROS (Figure 3(b)). Furthermore, we found a stronger immunofluorescence signal for Beclin1 in the cytoplasm of cells upon ROS stimulation compared with control cells, indicating that the expression of Beclin1 was elevated by ROS (Figure 3(c)). Additionally, LC3 changed from the diffuse state to gathered granular in the cytoplasm (Figure 3(d)). We quantitated the occurrence of autophagy and found that autophagosomes were significantly promoted by ROS (Figure 3(e)). In addition, for the accumulation of p62 reflects the inhibition of autophagy, the increased level of p62 in our study indicated that ROS induces the p62 transcription.

**3.5. The Influence of ROS on Nrf2 Expression in PANC-1 Cells Under Suppressed Autophagy.** To explore the effect of ROS on Nrf2 pathway when the autophagic activity was suppressed, we used different inhibitors (3-methyladenine: 3-MA and chloroquine: CQ) to prevent autophagy in PANC-1 cells at particular stages. 3-MA has been used to prevent the formation of autophagosomal precursors at the early stage of autophagy as an inhibitor of class III phosphatidylinositol 3-kinase and CQ, a lysosomotropic weak base, could inhibit the fusion of autophagosome with lysosome to prevent the process of autophagy [32]. As shown in Figure 4(a), we found that the treatment of 3-MA inhibited the expression of Beclin1, and CQ lead to accumulation of LC3-II in basal state. These results indicated that 3-MA and CQ both can inhibit the autophagic activity. Moreover, Western blot was used to detect the expression of Nrf2, Beclin1, and LC3-II and also the level of p62 when cells were under basal condition and upon ROS stimulation with or without 3-MA or CQ. We found that the treatment of 3-MA effectively arrested the ROS-induced autophagic activation determined by low Beclin1 expression and accumulation of p62. Similarly, CQ prevented the ROS-induced autophagy characterized as accumulation of LC3-II and p62 (Figure 4(b)).

Both inhibition and blockage of autophagy at particular stages caused a marked increase in the expression level of Nrf2 upon ROS stimulation (Figure 4(b)). Additionally, the expression of Nrf2 in the cytoplasm and the nuclear translocation of Nrf2 were both enhanced as an effect of autophagic inhibitor treatment upon ROS exposure, as demonstrated by immunofluorescence (Figure 4(c)). These findings indicated that suppression of autophagic activity results in an increased promotion of Nrf2 pathway upon ROS stimulation in PANC-1 cells.

**3.6. The Influence of ROS on Autophagy in PANC-1 Cells Transfected with Nrf2 siRNA.** To confirm if Nrf2 pathway could regulate autophagy under the treatment of ROS, Nrf2 siRNA was applied to knock down Nrf2 in PANC-1 cells (Figure 5(a)). Using fluorescence microscope, we found that the transfection efficiency is up to 98% (Figure S3A). Next, we screened appropriate siRNA sequence with RT-PCR and Western blot from six designed Nrf2 siRNAs. The result showed that the inhibition efficiency of NRF2-homo-1498 was the most efficient (Figure S3B-C).

We detected the expression of autophagic related proteins when Nrf2 siRNA-PANC-1 cells were faced with ROS stimulation by Western blot and immunofluorescence. As shown in Figure 5(b), silencing of Nrf2 resulted in significant increase in the expression of Beclin1 and LC3-II after ROS intervention; similar results were detected using immunofluorescence (Figure 5(c)). Furthermore, we found a significant increase in gathered granular of LC3 in the cytoplasm when PANC-1 cells transfected with Nrf2 siRNA compared with NC siRNA upon ROS exposure, as demonstrated by immunofluorescence (Figure 5(d)). Additionally, we quantitated the occurrence of autophagy and found that autophagosomes were significantly promoted as a result of Nrf2 knockdown (Figure 5(e)).

P62, a link between LC3 and ubiquitinated substrates, could be successfully degraded by autophagy [33]. To determine whether silencing of Nrf2 promotes the exact autophagic flux of PANC-1 cells upon ROS stimulation, Western blot detection of p62 was used to assess the capacity of autophagic flux. As shown in Figure 5(b), the level of p62 protein was decreased as an effect of silencing of Nrf2 in PANC-1 cells upon ROS exposure. These findings indicated that silencing of Nrf2 promotes autophagic flux upon ROS stimulation in PANC-1 cells.

**3.7. Silencing of Nrf2 in PANC-1 Cells upon ROS Stimulation Enhances Autophagic Flux by Promoting Autolysosome Formation.** As autophagy is a dynamic process, the detection of LC3 processing by Western blot and formation of autophagosomes by fluorescence to monitor autophagic activity is insufficient to determine the entire autophagic system. For example, the increased autophagosomes could suggest either autophagic activation or an inhibition of lysosomal degradation [34]. Therefore, to further confirm that the silencing of Nrf2 promotes the progression of complete process of autophagy (autophagic flux) upon ROS stimulation in PANC-1 cells, we observed live cells infected with the Ad-mRFP-GFP-LC3 to differentiate the autophagosome and autolysosome during autophagy. The assay takes advantage of the stability of red fluorescent protein (RFP) under acidic conditions and the acid-sensitive green fluorescent protein (GFP) and also the pH difference between autophagosome and autolysosome. Quenching of GFP and maintaining of RFP which exhibits red puncta could represent the autolysosome by indicating the fusion of autophagosome with acidic lysosomal compartment. However, maintaining of both GFP and RFP which exhibits yellow puncta could represent the autophagosome. Live Cell Imaging Confocal Scanner System was used to observe the autophagic flux of the same cells treated with or without ROS. As shown in Figures 6(a)-6(b), we found a successful introduction of this adenovirus displaying both fluorescent proteins when cells were infected with the Ad-mRFP-GFP-LC3. The results indicated marked fold increases of the autophagosomes and autophagosomes in both Nrf2 siRNA and NC siRNA-transfected PANC-1 cells when faced with ROS stimulation, but the Nrf2 siRNA-transfected PANC-1 cells tend to have the higher fold increase which suggests that inhibition of Nrf2 further promote the autophagic flux upon ROS stimulation (Figures 6(c)-6(d)). These results further

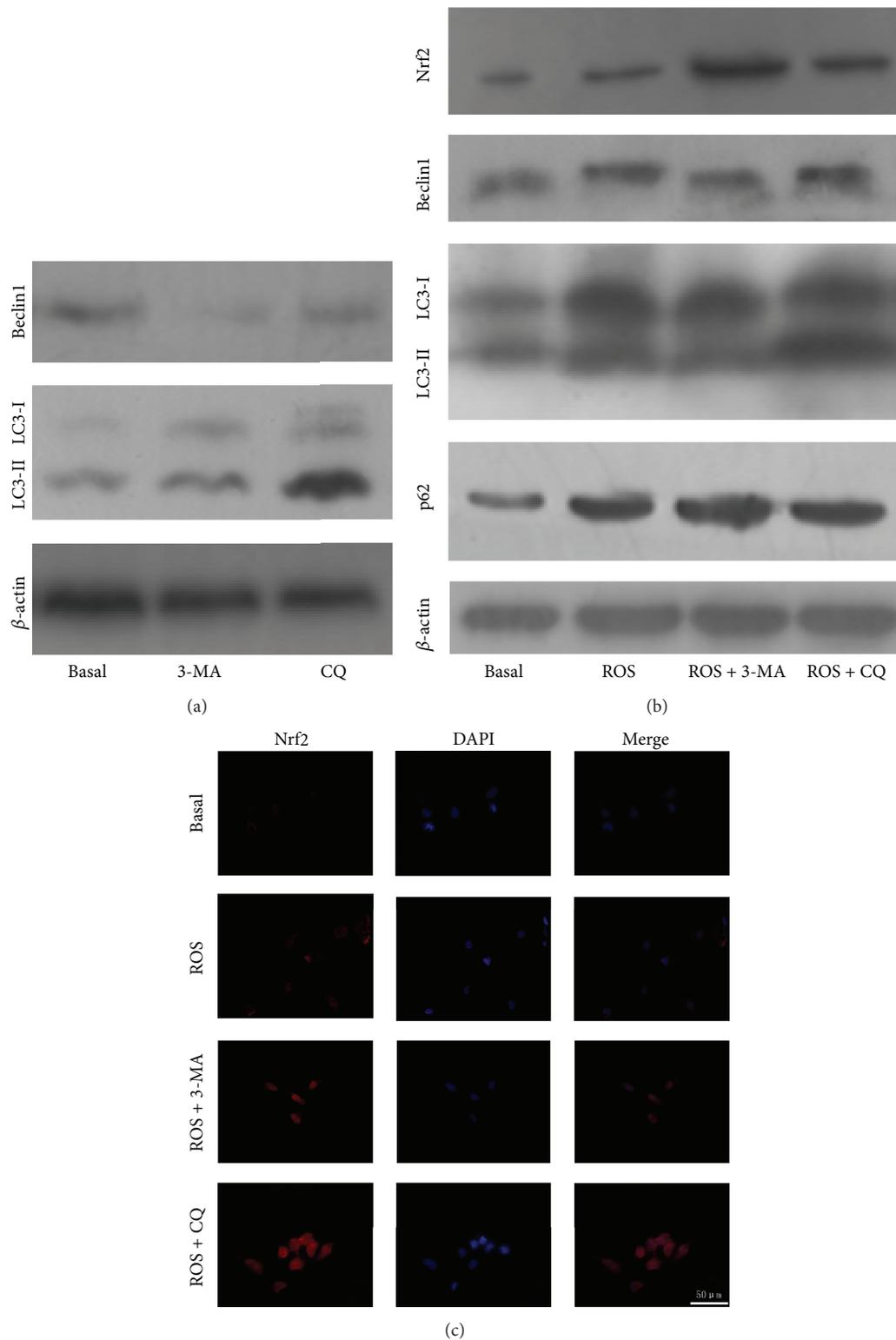


FIGURE 4: Suppression of autophagic activity enhances Nrf2 expression and translocation of Nrf2 to the nucleus upon ROS stimulation in PANC-1 cells. (a) The expression of Beclin1 and LC3-II treated with or without 3-MA or CQ in the absence of ROS. (b) The expression of Nrf2, Beclin1, and LC3-II and also the level of p62 in pancreatic cancer cells under basal condition and upon ROS stimulation with or without 3-MA or CQ. (c) Immunofluorescence images of PANC-1 cells for Nrf2 under basal condition and upon ROS stimulation with or without 3-MA or CQ.

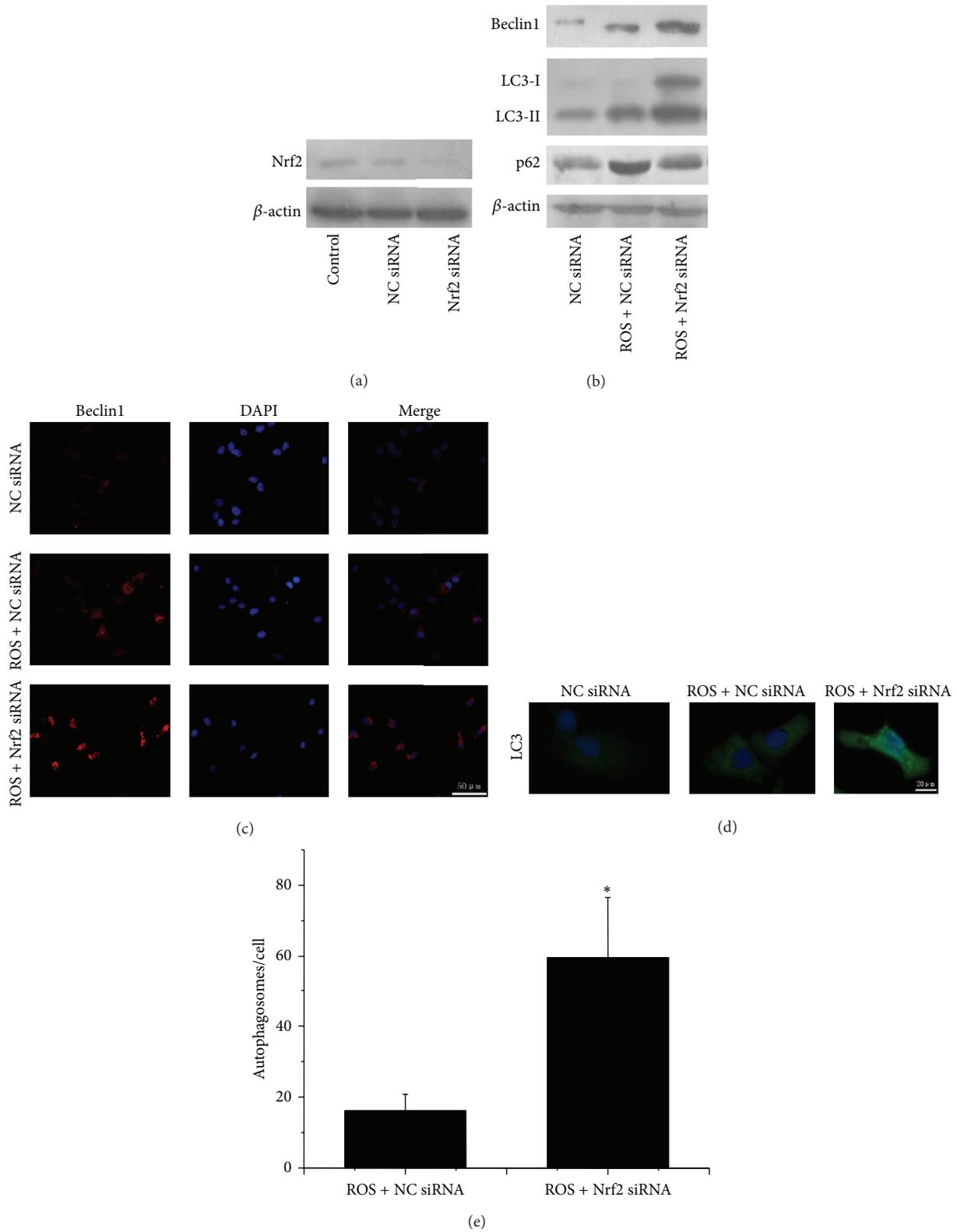


FIGURE 5: Silencing of Nrf2 promotes autophagy upon ROS stimulation in PANC-1 cells. (a) Western blot detection of Nrf2 in siRNA-transfected PANC-1 cells. (b) The expression of Beclin1 and LC3-II and also the level of p62 in Nrf2 siRNA-transfected PANC-1 cells upon ROS stimulation. (c-d) Immunofluorescence images of PANC-1 cells for Beclin1 and LC3 upon ROS stimulation. (e) Numbers of autophagosomes were counted in 10 random fields. The data are presented as Mean  $\pm$  SD for three independent experiments. Column: Mean; bar: SD.

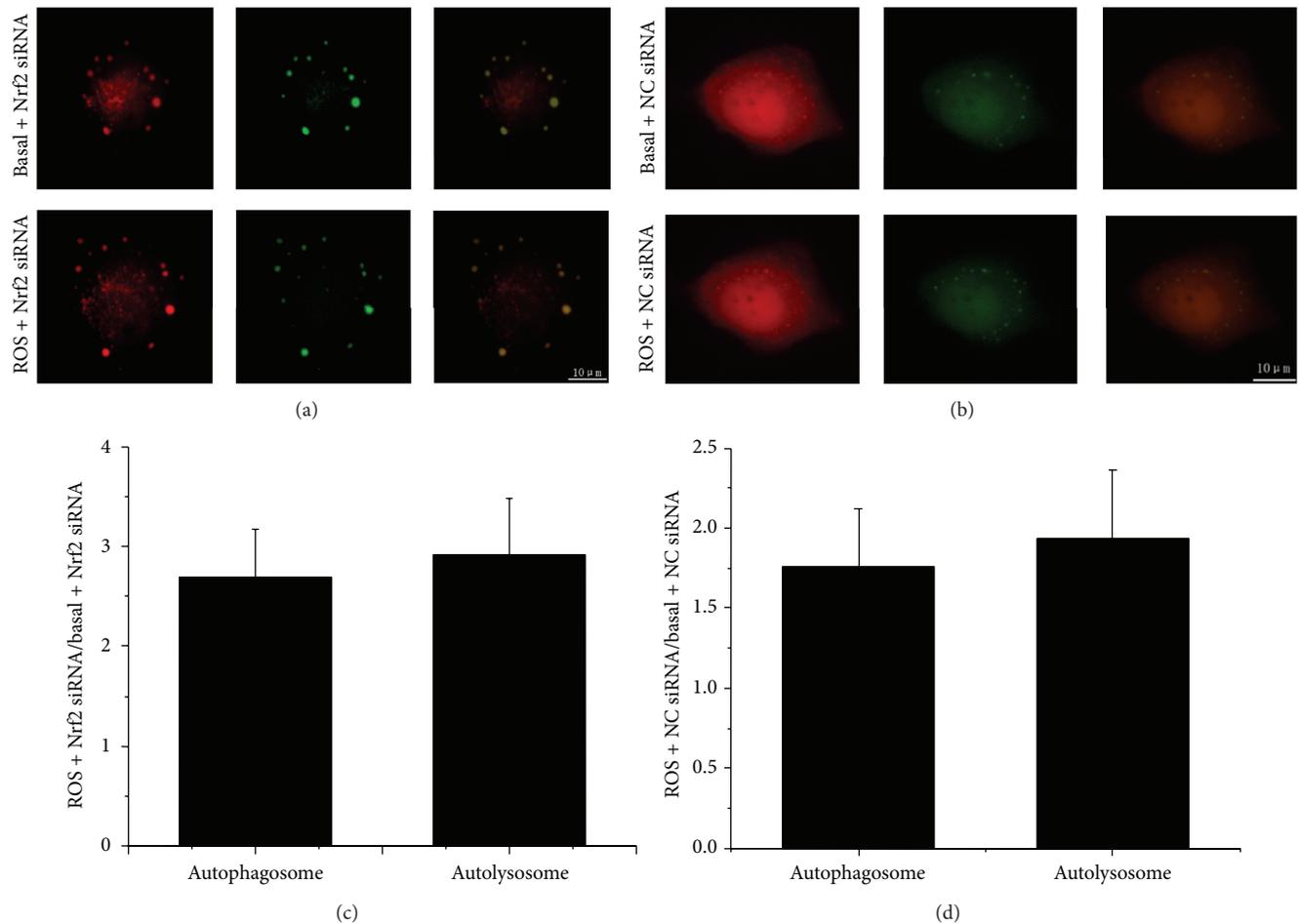


FIGURE 6: Silencing of Nrf2 in PANC-1 cells upon ROS stimulation promotes autolysosome formation. (a) PANC-1 cells were infected with the Ad-mRFP-GFP-LC3. Then, the cells were transfected with Nrf2 siRNA, treated with ROS stimulation, and observed by Live Cell Imaging Confocal Scanner System. (b) PANC-1 cells were infected with the Ad-mRFP-GFP-LC3. Then, the cells were transfected with NC siRNA, treated with ROS stimulation, and observed by Live Cell Imaging Confocal Scanner System. (c-d) Numbers of puncta in the same cell was determined when cells were under basal condition and upon ROS stimulation. Cells were counted in 10 random fields. The data of fold increase are presented as Mean  $\pm$  SD for three independent experiments. Column: Mean; bar: SD.

confirmed that silencing of Nrf2 promotes autophagic flux upon ROS stimulation in PANC-1 cells.

#### 4. Discussion

In the present study, we have provided direct evidence that Nrf2 pathway and autophagy have a negative interaction with each other in pancreatic cancer cells upon ROS stimulation. We have successfully set a model with ROS stress to better study the relevance of Nrf2 pathway and autophagy in pancreatic cancer cells.

It has been shown that high expression levels of Nrf2 have been observed in many cancers, including head and neck, gall bladder, lung, pancreas, and colorectal cancer [12, 14, 35, 36]. Moreover, the research of Yang et al. indicates a higher autophagic level in pancreatic cancer [6]. Nrf2 and autophagy both benefit the progression of pancreatic cancer. As we know, loss of autophagic function leads to accumulation of p62 which acts to sequester Keap1 into

the autophagosomes, inhibiting the ubiquitylation of Nrf2, resulting in the noncanonical activation of Nrf2. However, it has been demonstrated that persistent activation of Nrf2 is critical for liver tumorigenesis that occur in mice with autophagy-deficient hepatocyte [37]. Moreover, Riley et al. consider that the accumulation of poly-Ub chains in circumstances with defects in autophagy is an indirect consequence of activation of Nrf2 [38]. Persistent activation of Nrf2 results in tumorigenesis, in this way, the Nrf2 inhibitors can be used to inhibit the persistent Nrf2 activation induced by loss of autophagic function to prevent the progression of pancreatic cancer. Meanwhile, a study showed that inhibition of autophagy leads to robust tumor regression and prolonged survival in pancreatic cancer xenografts and genetic mouse models. It seems that the inhibition of autophagy combined with Nrf2 inhibitors would be more effective to prevent the progression of pancreatic cancer due to its dark side of activating Nrf2 pathway.

Since the nutrient-limited environment in pancreatic cancer cells could lead to elevated ROS level, followed by activation of both Nrf2 pathway and autophagy, they may be involved in the protection of cells against oxidative stress. Low level of ROS could promote the antioxidant production and the tumor growth in cancer cells, but further increased production of ROS and/or decrease in antioxidant capacity would lead to the imbalance in oxidant-antioxidant system of cancer cells, resulting in cell death [39]. Some evidence indicated that Nrf2 has the ability to lower intracellular ROS by its antioxidant program [31]. For autophagy, its function of removing damaged organelles and accumulated proteins could prevent the further increase of ROS. Taken together, Nrf2 pathway and autophagy are required coordinately reducing the intracellular ROS accumulation to ensure the survival of pancreatic cancer cells, but their inner relationship is unclear. It is puzzling whether Nrf2 pathway or autophagy is the upstream regulator to modulate the other one. To make it clear, we first used exogenous  $H_2O_2$  to improve the level of intracellular ROS, which resulted in activation of both Nrf2 pathway and autophagy. Then, for the several steps of autophagic process, different inhibitors (3-MA and CQ) were used to prevent autophagy at early or late stage, and the result showed that suppression of autophagic activity at different stages lead to an increased promotion of Nrf2 pathway. This indicates that when faced with oxidative stress, if the autophagic function of cancer cell is suppressed, the further activation of Nrf2 pathway is required to respond to excessive ROS to help cancer cell to survive.

On the other hand, we showed that silencing of Nrf2 resulted in significant increase in the expression of Beclin1 and LC3-II, which suggest that Nrf2 pathway regulates autophagy at both the initial and the final steps. As autophagy is a dynamic process, Western blot detection of p62 and AdmRFP-GFP-LC3 infection were used to monitor the change of autophagic flux after Nrf2 knockdown. Interestingly, our findings showed that silencing of Nrf2 promotes the exact autophagic flux. This indicates that when faced with oxidative stress, if the activation of Nrf2 pathway in cancer cell is repressed, the further promotion of effective autophagic flux is required to deal with the ROS mediated damage to avoid cell death. Thus, the relationship between Nrf2 pathway and autophagy cannot be a simple upstream or downstream. Taken together, when we regard ROS as the key factor, Nrf2 pathway and autophagy would be in a negative interaction with each other, and autophagy inhibition not only leads to accumulation of p62 to activate Nrf2 pathway by a noncanonical way but also increase ROS production to directly activate Nrf2 pathway.

Antioxidant programs inhibit excessive ROS production to maintain pancreatic cancer cells at quiescent state, causing chemotherapeutic and radio therapeutic resistance [40, 41]. We suggest that cancer cells with higher activated Nrf2 pathway and autophagy may have stronger ability to survive under oxidative stress due to their capacity to lower intracellular ROS. Combined with their negative interaction with each other, we suggest that novel drug targets for coinhibition of Nrf2 pathway and autophagy may be a potential therapy for preventing the progression of pancreatic cancer.

## 5. Conclusions

In summary, our findings suggest that Nrf2 pathway and autophagy have a negative interaction with each other upon ROS stimulation, and autophagy inhibition not only leads to accumulation of p62 to activate Nrf2 pathway by a noncanonical way but also increase ROS production to directly activate Nrf2 pathway. The demonstrated relationship between Nrf2 pathway and autophagy will advance our understanding of the progression of pancreatic cancer induced by ROS. Thus, novel drug targets for coinhibition of Nrf2 pathway and autophagy may be a potential therapy for preventing the progression of pancreatic cancer.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (nos. 81201824, 81172360, 81301846, and 81402583).

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## Research Article

# Juglanthraquinone C Induces Intracellular ROS Increase and Apoptosis by Activating the Akt/Foxo Signal Pathway in HCC Cells

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Received 5 May 2015; Revised 10 July 2015; Accepted 27 July 2015

Academic Editor: Subash Chandra Gupta

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Juglanthraquinone C (JC), a naturally occurring anthraquinone extracted from *Juglans mandshurica*, could induce apoptosis of cancer cells. This study aims to investigate the detailed cytotoxicity mechanism of JC in HepG2 and BEL-7402 cells. The Affymetrix HG-U133 Plus 2.0 arrays were first used to analyze the mRNA expression exposed to JC or DMSO in HepG2 cells. Consistent with the previous results, the data indicated that JC could induce apoptosis and hyperactivated Akt. The Western blot analysis further revealed that Akt, a well-known survival protein, was strongly activated in HepG2 and BEL-7402 cells. Furthermore, an obvious inhibitory effect on JC-induced apoptosis was observed when the Akt levels were decreased, while the overexpression of constitutively active mutant Akt greatly accelerated JC-induced apoptosis. The subsequent results suggested that JC treatment suppressed nuclear localization and increased phosphorylated levels of Foxo3a, and the overexpression of Foxo3a abrogated JC-induced apoptosis. Most importantly, the inactivation of Foxo3a induced by JC further led to an increase of intracellular ROS levels by suppressing ROS scavenging enzymes, and the antioxidant *N*-acetyl-L-cysteine and catalase successfully decreased JC-induced apoptosis. Collectively, this study demonstrated that JC induced the apoptosis of hepatocellular carcinoma (HCC) cells by activating Akt/Foxo signaling pathway and increasing intracellular ROS levels.

## 1. Introduction

*Juglans mandshurica* Maxim (Juglandaceae) is one of the rare species of trees used as a traditional medicine, and many studies have reported on the screening of apoptosis-inducing compounds isolated from *J. mandshurica* [1, 2]. Juglone, a major chemical constituent of *J. mandshurica* Maxim [3], induces the increase of intracellular reactive oxygen species (ROS) levels, mitochondrial dysfunction, and elevated ratio of Bax/Bcl-2, triggering events responsible

for mitochondrial-dependent apoptosis in human leukemia cell HL-60 [4, 5]. Plumbagin, another naphthoquinone, reduces a change in Bcl-2/Bax ratios, resulting in mitochondrial membrane potential loss, Cytochrome *c* release, and caspase-9 activation, triggering the mitochondrial apoptosis [6]. Juglanthraquinone C (JC), a new naturally occurring anthraquinone compound isolated from the stem bark of *J. mandshurica*, was reported to have significant anticancer effects by inducing S-phase arrest and mitochondrion-dependent apoptosis [7]. However, the underlying signal

transduction pathways that mediated JC-induced cell apoptosis were still unknown.

The induction of apoptosis is a major mechanism of cancer therapeutics, and it is a constitutive suicide program triggered by a variety of extrinsic and intrinsic signals. The tumor necrosis factor (TNF) acts via the tumor necrosis factor receptor (TNFR) and is a part of the extrinsic pathway for triggering apoptosis [8]. TNFR can recruit the adaptor proteins Fas-associated death domain (FADD) that can trigger the caspase cascade, irreversibly sensitizing the cell to apoptosis [9]. Mitochondrial apoptosis is the best-known intrinsic apoptosis pathway [10]. Mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-regulated protein kinase 1/2 (ERK1/2), *c-Jun* N-terminal kinase (JNK), and p38 MAPK (p38), can trigger mitochondrial apoptosis. High glucose also can induce apoptosis in HepG2 cells through activating the ASK1-p38/JNK pathway [11].

Akt or protein kinase B, a 57-kDa Ser/Thr kinase, is activated by extracellular signals. Akt is frequently activated in cancer cells, and its activation promotes cell proliferation and provides protection from apoptosis [12]. But hyperactivated Akt induces premature senescence and sensitizes cells to ROS-mediated apoptosis by increasing intracellular ROS through increased oxygen consumption and by inhibiting the expression of ROS scavengers downstream of Foxo [13]. Foxo is directly phosphorylated by Akt, and then its transcriptional activity is inhibited. Foxo3a is a member of forkhead transcription factors (Foxos) and plays an important role in protecting cells against oxidative stress through regulating ROS scavengers, including superoxide dismutase 2 (SOD2) and catalase. In normal cells, low amounts of ROS are eliminated by nonenzymatic and enzymatic antioxidizing agents such as glutathione, thioredoxin, SOD2, catalase, and peroxidases [14]. So the inhibition of ROS scavenger activation could cause an increase of ROS levels. High levels of ROS cause changes in cellular adenosine triphosphate (ATP) and  $\text{Ca}^{2+}$  levels and lead to the release of Cytochrome *c* and mitochondrion-dependent apoptosis [15].

Hepatocellular carcinoma (HCC) constitutes one of the most prevalent malignant diseases. The purpose of this study is to clarify the molecular mechanisms by which JC induced the apoptosis of HepG2 and BEL-7402 cells. Interestingly, JC was found to induce mitochondrion-dependent apoptosis by activating the Akt/Foxo signaling pathway, resulting in the apoptosis of HCC cells; this was contradictory to the conventional role of Akt in apoptosis. Further studies revealed that the hyperactive Akt induced by JC inhibited Foxo transcription factors, impaired ROS scavenging, and eventually resulted in the apoptosis of HCC cells.

## 2. Materials and Methods

**2.1. Chemicals, Antibodies, Kits, and Reagents.** JC was isolated from the stem bark of *J. mandshurica*, and its chemical structure was described by Lin et al. [16]. The purity of JC was greater than 98% as determined by the high-performance liquid chromatography-mass spectrometry. Antibodies

against p65, p38, p-p38, JNK, p-JNK, and Histone H1 were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against ERK, p-ERK, Akt, p-Akt (Ser473), caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, Foxo3a, p-Foxo3a, and catalase were purchased from Cell Signaling Technology (MA, USA). Antibody against SOD2 was purchased from EMD Millipore Corporation Division (MA, USA). A mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from KangChen Bio-tech (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), RPMI medium 1640 (1640), *N*-acetyl-L-cysteine (NAC), PEG-catalase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 were purchased from Sigma Chemical (MO, USA). Dimethyl sulfide (DMSO) was purchased from Ameresco (MA, USA). The one-step TUNEL apoptosis assay kit, phorbol 12-myristate 13-acetate (PMA), ROS assay kit, and fluorescence probe dihydroethidium (DHE) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The Annexin V-FITC Apoptosis Detection Kit was purchased from Becton, Dickinson and Company (NJ, USA). An Akt siRNAs Kit, including negative control (Nc), GAPDH positive control, 438, and 1191, was purchased from GenePharma (China).

**2.2. Cell Culture.** Human HCC cell lines HepG2 and BEL-7402 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HepG2 cells were cultured in DMEM and the BEL-7402 cells in 1640. Both of them were supplemented with 10% fetal bovine serum (FBS) (Sijiqing, China) at 37°C with 5%  $\text{CO}_2$ .

**2.3. Cytotoxicity and Proliferation Assay.** Cytotoxic effects of JC on BEL-7402 cells were tested by the MTT cell viability assay. Briefly, BEL-7402 cells were plated in 96-well plates ( $8 \times 10^3$  cells/well) and routinely cultured for 12 hours. Then, the cells were treated with various concentrations of JC or DMSO in a medium supplemented with 3% FBS. After 48 hours of treatment, the cells were analyzed by the MTT assay (as described in [7]).

**2.4. siRNA Transfection.** HepG2 and BEL-7402 cells ( $2 \times 10^5$ ) were seeded into each well of 12-well plates with an appropriate complete growth medium 24 hours prior to transfection. Cells were incubated at 37°C with 5%  $\text{CO}_2$  overnight and treated with 150 pmol siRNA per well. The siRNAs were incubated with the Lipofectamine 2000 Transfection Reagent, which was purchased from Invitrogen (CA, USA), according to the manufacturer's instructions. After incubation for 12 hours, cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced by another medium with 3% FBS and JC. Then HCC cells were harvested and assayed. This study included two human siRNAs (GenePharma, China) designed against Akt (GenBank NM\_001288.4) and two control siRNAs. The sequences for control siRNAs and Akt siRNAs were as

follows: negative control (sense: 5'-UUCUCCGAACGU-GUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGG-AGAATT-3'); GAPDH positive control (sense: 5'-GUA-UGACAACAGCCUCAAGTT-3', antisense: 5'-CUUGAG-GCUGUUGUCAUACTT-3'); Akt siRNA 1191 (sense: 5'-GACGGGCACAUAAGAUCATT-3', antisense: 5'-UGA-UCUUAUGUGCCCGUCTT-3'); and Akt siRNA 438 (sense: 5'-GCACCUUCAUUGGCUACAATT-3', antisense: 5'-UUGUAGCCAAUGAAGGUGCTT-3').

**2.5. Plasmid Construction and Transfection.** Human full-length Akt and Foxo3a were cloned into the pcDNA3 basic vector. Akt (S473D) and Akt (S473A), which contain mutations at the phosphorylation site (Ser473 to alanine or aspartate), were generated by site-directed mutagenesis. The primer sequences used were as follows: Akt (S473D) (sense: 5'-CCGCTGGCCGAGTAGTCAACTGGGGGAAGTG-3', antisense: 5'-CACTTCCCCAGTTCGACTACTCG-GCCAGCGG-3'); Akt (S473A) (sense: 5'-CGCTGGCCG-AGTAGGCCGAAGTGGGGGAAGTG-3', antisense: 5'-CACTTCCCCAGTTCGCTACTCGGCCAGCG-3'). To knockdown the expression of Akt, two Akt short interfering RNA (shRNA) vectors were created. The two shRNA targeting Akt sequences were synthesized by GenScript. The shRNA sequences targeting Akt were as follows: Akt#1 (sense: 5'-GATCCGCTACTTCCCTCCTCAAGAATGTTCAAGA-GACATTCTTGAGGAGGAAGTAGCTTTTTTGGAAA-3', antisense: 5'-AGCTTTTCCAAAAAAGCTACT-TCCCTCCTCAAGAATGTCTCTTGAACATTCT-TGAGGAGGAAGTAGCG-3'); Akt#2 (sense: 5'-GATCCGCTGGGAGAACCTCATGCTGTTCAAGAGA-CAGCATGAGGTTCTCCAGCTTTTTTGGAAA-3', antisense: 5'-AGCTTTTCCAAAAAAGCTGGGAGAACCT-CATGCTGTCTTGAACAGCATGAGGTTCTCCAGC-G-3'). The oligonucleotides were inserted into the pRNAT-U6.1/Hygro vector, which expresses hairpin sequences utilizing the U6 RNA pol III promoter (GenScript Corporation, NJ, USA). All constructs were confirmed by DNA sequencing. HepG2 and BEL-7402 cells ( $4 \times 10^5$  or  $2 \times 10^5$ ) per well were seeded in 6-well plates or 12-well plates, respectively. After incubating overnight with a medium containing 10% FBS, the aforementioned different plasmids were transfected into the cells using Lipofectamine 2000, according to the manufacturer's instructions.

**2.6. Apoptosis Analysis by DAPI Staining.** JC-induced apoptosis was detected by DAPI staining. The cells were harvested and stained with DAPI (as described in [17]). Images were acquired using an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan). Apoptotic cells were morphologically defined by chromatin condensation and fragmentation.

**2.7. Apoptosis Analysis by TUNEL Staining.** Commercially available one-step TUNEL apoptosis assay kits (BD Biosciences, NJ, USA) were used to evaluate the apoptotic response of HepG2 cells, according to the manufacturer's instructions. HepG2 cells were stained with DAPI (as described in [17]).

**2.8. Apoptosis Analysis by Annexin V-FITC/PI Double Staining.** The apoptosis and necrosis ratio was analyzed using the Annexin V-FITC Apoptosis Detection Kit, according to the manufacturer's instructions. Fluorescence was measured by flow cytometer (BD Biosciences).

**2.9. Western Blot Analysis.** The cells were harvested, and cytosolic and nuclear extracts were prepared. Western blotting was performed as described in [18]. The membranes were incubated overnight at 4°C with primary antibodies specific for p65, p38, p-p38, JNK, p-JNK, ERK, p-ERK, Akt, p-Akt (Ser473), caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, Foxo3a, p-Foxo3a, catalase, SOD2, GAPDH, and Histone H1. The membranes were subsequently visualized using an ECL reagent (TransGen Biotech, China) and analyzed with DNR Bio-Imaging Systems (Israel).

**2.10. ROS Measurement.** The production of ROS was monitored using the nonfluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA diffuses into cells and is deacetylated by esterases to form the nonfluorescent product 2',7'-dichlorodihydrofluorescein (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product 2',7'-dichlorofluorescein (DCF). HepG2 cells were treated with 8 µg/mL of JC for 12, 24, 36, and 48 hours. BEL-7402 cells were treated with 6.7, 8.7, and 1.05 µg/mL of JC for 12 hours. DCFH-DA was diluted in a medium at a final concentration of 10 µM and incubated with microglia for 30 minutes at 37°C in the dark. The cells were washed twice with PBS, trypsinized, and then resuspended in 250 µL of PBS. Fluorescence was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm by flow cytometry.

The intracellular ROS levels of HepG2 cells were assayed using the fluorescence probe DHE. Intracellular DHE is oxidized to ethidium, which binds to DNA and stains nuclei with a bright fluorescent red color. HepG2 cells were seeded in 6-well plates and pretreated with JC as previously described. DHE was diluted in a medium at a final concentration of 10 µM and incubated with microglia for 30 minutes at 37°C in the dark. The cells were washed twice with PBS, trypsinized, and then resuspended in 250 µL of PBS. Fluorescence was measured at an emission wavelength of 370 nm and an excitation wavelength of 420 nm by flow cytometry.

**2.11. RNA Extraction and Affymetrix Microarray.** HepG2 cells were seeded at  $3.5 \times 10^5$  cells per well in 6-well plates and treated with DMSO or 8 µg/mL of JC for 4 or 10 hours. The total RNA was isolated from HepG2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The RNA quantity and quality were assessed by e-Spect (Malcom, Tokyo, Japan). The 260/280 ratios of all samples were between 2.13 and 2.18. The total RNA (50 ng) were used to generate amplified and biotinylated cRNA. Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix, CA, USA) were hybridized for 16 hours in a 45°C incubator, rotated at 60 rpm, and washed and stained according to the Affymetrix GeneChip Expression Analysis Manual. Finally,

Affymetrix HG-U133 Plus 2.0 arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix).

**2.12. Fold Change and Gene Ontology Analysis.** To screen out the differentially expressed genes between the treatment group and the control group, the CEL files were analyzed. The raw data was normalized using the robust multiarray average method [19, 20]. Fold changes of gene expression  $\geq 1.5$  were considered significant and used in the following Gene Ontology (GO) analysis [21]. GO term scores with  $P \leq 0.05$  were considered significant. The GO analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov>). Heat maps were made using the freely available statistical computing software R (<http://mirror.bjtu.edu.cn/cran/>). A probe set is a group of probe pairs used together to interrogate a sequence that represents a gene on the array. The median value of several probe sets, which represent one gene, was taken.

**2.13. Statistical Analysis.** Experiments were repeated at least three times. Statistical analysis of the data was performed using the Student *t*-test and two-tailed distribution. The significance level was set at  $*P < 0.05$  and  $**P < 0.01$ . Error bars denote the standard deviation.

### 3. Results

**3.1. Role of JC in Inducing Apoptosis.** Previous studies have suggested that JC showed strong cytotoxicity in HepG2 cells. In this study, JC was found to reduce the cell viability of HCC BEL-7402 cells in a dose-dependent manner (Figure 1(a)). For a 48-hour exposure, the IC<sub>50</sub> was 10.5  $\mu\text{g}/\text{mL}$  in BEL-7402 cells. Some previous studies have suggested that JC could selectively inhibit cancer cell viability by inducing apoptosis. To further confirm the ability of JC to induce the apoptosis of human liver cancer cells, the chromatin condensation and DNA fragmentation by DAPI staining in HepG2 and BEL-7402 cells were analyzed (Figures 1(b) and 1(c)). HCC cells exposed to JC showed chromatin condensation and fragmented nuclei in a time-dependent manner.

To study the impact of JC-induced cytotoxicity on gene expression and reveal the mechanisms responsible for JC-induced apoptosis in HepG2 cells, the mRNA expression was analyzed by Affymetrix HG-U133 Plus 2.0 arrays. Genes with an expression ratio  $\geq 1.5$ -fold were regarded as differentially transcribed genes [21]. After treating HepG2 cells with JC for 10 hours, 2494 individual probe sets were differentially expressed, and 1271 of these 2494 probe sets were downregulated while 1223 were upregulated due to JC treatment.

To identify different gene clusters among the differentially transcribed genes, the DAVID Functional Annotation Tool was used for GO analysis. After treating HepG2 cells with JC for 10 hours, the data of probe sets, which were upregulated by JC, were enriched by GO. Based on this analysis, three main clusters encoding genes involved in cell death, including GO: 0043068, positive regulation of programmed cell death (82 probe sets); GO: 0010942, positive regulation of cell death (83 probe sets); and GO: 0043065, positive regulation of

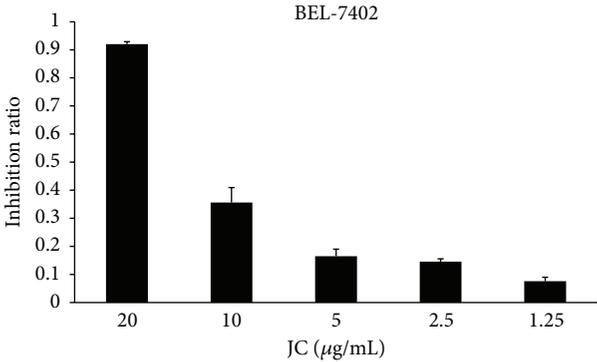
apoptosis (81 probe sets), were identified in the upregulated data of the JC treatment group. Moreover, 81 probe sets were shared by the three main cluster-enriched genes that were upregulated by JC (Figure 1(d)). These data further verified that JC could induce apoptosis of HepG2 cells, and the results were consistent with a previous study [7].

**3.2. Activation of Akt Signaling Pathway Caused by JC.** To investigate the differentially expressed genes involved in signaling pathways that mediated JC-induced apoptosis, the mRNA expression was analyzed after treating HepG2 cells with JC for 4 hours. The results of this study suggested that 1113 individual probe sets were differentially expressed after treating HepG2 cells with JC for 4 hours. Out of these 1113 probe sets, 482 were downregulated and 631 were upregulated due to JC treatment. The probe sets, which were upregulated, were used for GO analysis. A significant GO term (GO: 0007167, enzyme-linked receptor protein signaling pathway) was found. As shown in Figure 1(e), out of the upregulated probe sets, 54 were involved in the enzyme-linked receptor protein signaling pathway. An enzyme-linked receptor, also known as a catalytic receptor, is a transmembrane receptor, and the binding of an extracellular ligand triggers an enzymatic activity on the intracellular side. Receptor tyrosine kinases (RTKs) are the main types of enzyme-linked receptors, and MAPK cascade, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), and PI3 K/Akt are the main signaling pathways triggered by RTKs [22–24]. Surprisingly, four genes related to the PI3 K/Akt signaling pathway were found to be significantly upregulated in HepG2 cells treated with JC compared to those treated with DMSO (Figure 1(f)). Importantly, it was found that all of the four genes and one uncharacterized probe set play a role in positively regulating the Akt signaling pathway.

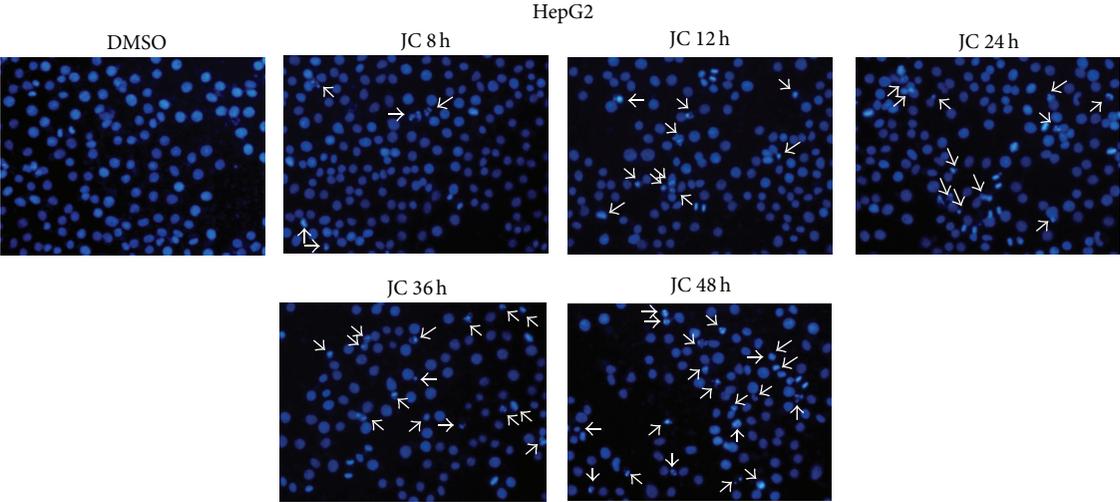
To validate the microarray data, the effects of JC on NF- $\kappa$ B, MAPK, and Akt signaling pathways were analyzed by Western blot. Compared to the control, JC had no obvious effect on the nuclear translocation of p65 and the phosphorylated level of p38, JNK, and ERK; however, the level of Akt phosphorylation on Ser473, which has been shown to be an important driver of human cancer [25], was greatly increased (Figures 2(a)–2(d) and 2(g)). After culturing in a medium without FBS for 12 hours, JC induced a higher level of Akt phosphorylation (Figure 2(e)). Also, Akt was found to be activated in BEL-7402 cells after being treated with JC (Figure 2(f)).

To further determine the activation of Akt after JC treatment, HepG2 and BEL-7402 cells were pretreated with the PI3 K inhibitor LY294002 for 1 hour and then treated with JC. In addition, rapamycin, a selective mTOR inhibitor that could induce the activation of Akt signaling through an IGF-1R-dependent mechanism [26], was used as a positive control. The increased level of Akt phosphorylation induced by JC was found to be dramatically reversed by LY294002 (Figures 2(h) and 2(i)). Collectively, these data indicated that the PI3 K/Akt signaling pathway was activated by JC.

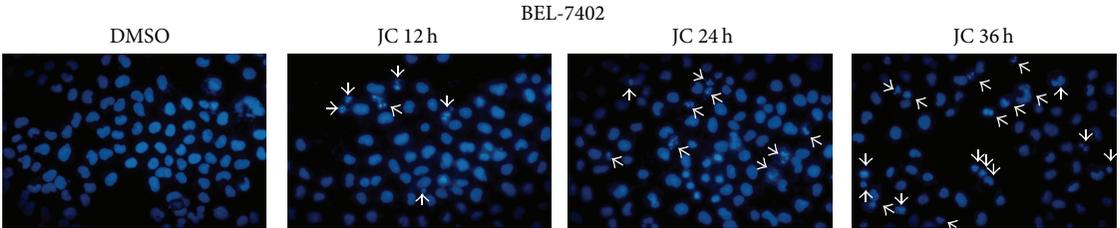
**3.3. Effect of Akt Deficiencies on JC-Induced Apoptosis of HCC Cells.** Akt is overactivated in a wide range of tumor



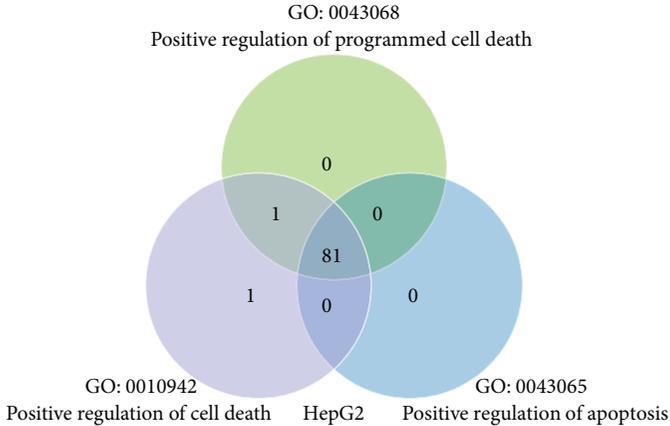
(a)



(b)



(c)



(d)

FIGURE 1: Continued.

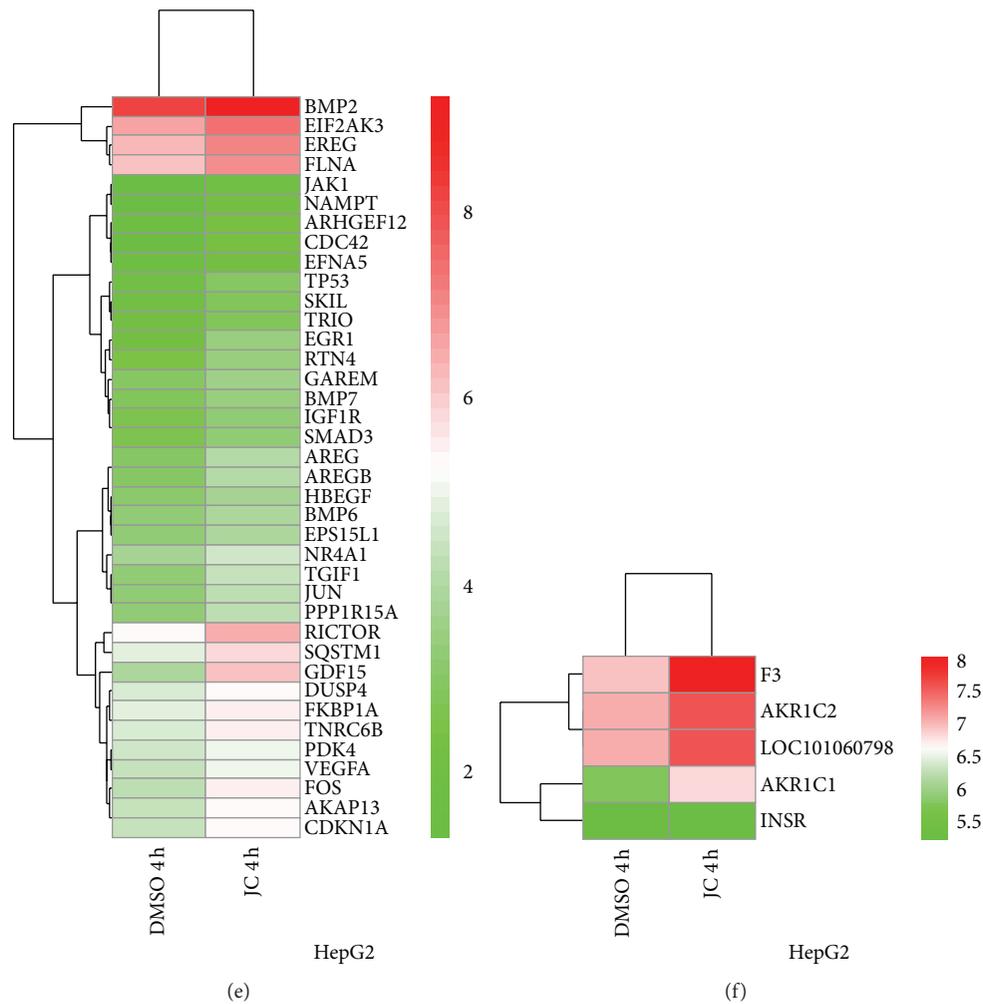


FIGURE 1: JC induces apoptosis. (a) BEL-7402 HCC cells were treated with indicated concentrations of JC for 48 hours and then subjected to the MTT cell viability assay. (b, c) Detection of apoptosis in JC-induced HCC cells by DAPI staining. HepG2 cells were treated with DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for the indicated times (b). BEL-7402 cells were treated with 8.7  $\mu\text{g}/\text{mL}$  of JC for the indicated times (c). Then, the cells were fixed and stained with DAPI. Arrows are used to indicate apoptotic bodies in apoptotic HCC cells. (d) DNA microarray analysis of the genes enriched in cell death, programmed death, and apoptosis after HepG2 cells were treated with JC for 10 hours. (e) GO classification for upregulated genes after HepG2 cells were treated with JC for 4 hours. (f) Clustering analysis of genes that can positively regulate Akt signaling after HepG2 cells were treated with JC for 4 hours.

types, and it triggers a cascade of responses, including cell growth, proliferation, survival, and motility, and drives tumor progression [25]. However, the aforementioned results have suggested that JC could induce apoptosis and increase the Akt phosphorylation level in HCC cells, which was contrary to the antiapoptotic effect of Akt. To verify whether Akt activation is required for JC-induced apoptosis, a shRNA interference approach was developed to selectively downregulate cellular Akt expression. Two shRNA plasmids against Akt were constructed and designated as Akt#1 and Akt#2, and Nc was a negative control. HepG2 cells were transfected with Akt#1 and Akt#2 and exposed to 8  $\mu\text{g}/\text{mL}$  of JC. As shown in Figure 3(a), compared with the control group, Akt deficiency obviously reduced the cleaved caspase-3 level and chromatin condensation induced by JC (Figures 3(a) and 3(b)).

To achieve a better interference effect than Akt shRNAs, HepG2 and BEL-7402 cells were infected with Akt siRNA,

designated as 438 and 1191, and exposed to JC. This study suggested that Akt siRNAs resulted in an obvious reduction of Akt expression (Figures 3(c), 3(d), and 3(f)). Furthermore, the knockdown of Akt by siRNA greatly decreased the JC-induced cleaved caspase-3 level, as shown in Figure 3(d). Consistent with the preceding results, the TUNEL staining and flow cytometry analysis confirmed that Akt deficiencies significantly reduced the JC-induced apoptosis of HCC cells (Figures 3(e) and 3(g)). These results suggested that activating the Akt signaling pathway was required in JC-induced apoptosis in HCC cells.

**3.4. Role of Akt Phosphorylation in JC-Induced Apoptosis.** Akt is a Ser/Thr kinase having a wide range of substrates. It is activated by recruitment to the plasma membrane, where it is phosphorylated at Thr308 and Ser473 [25]. It

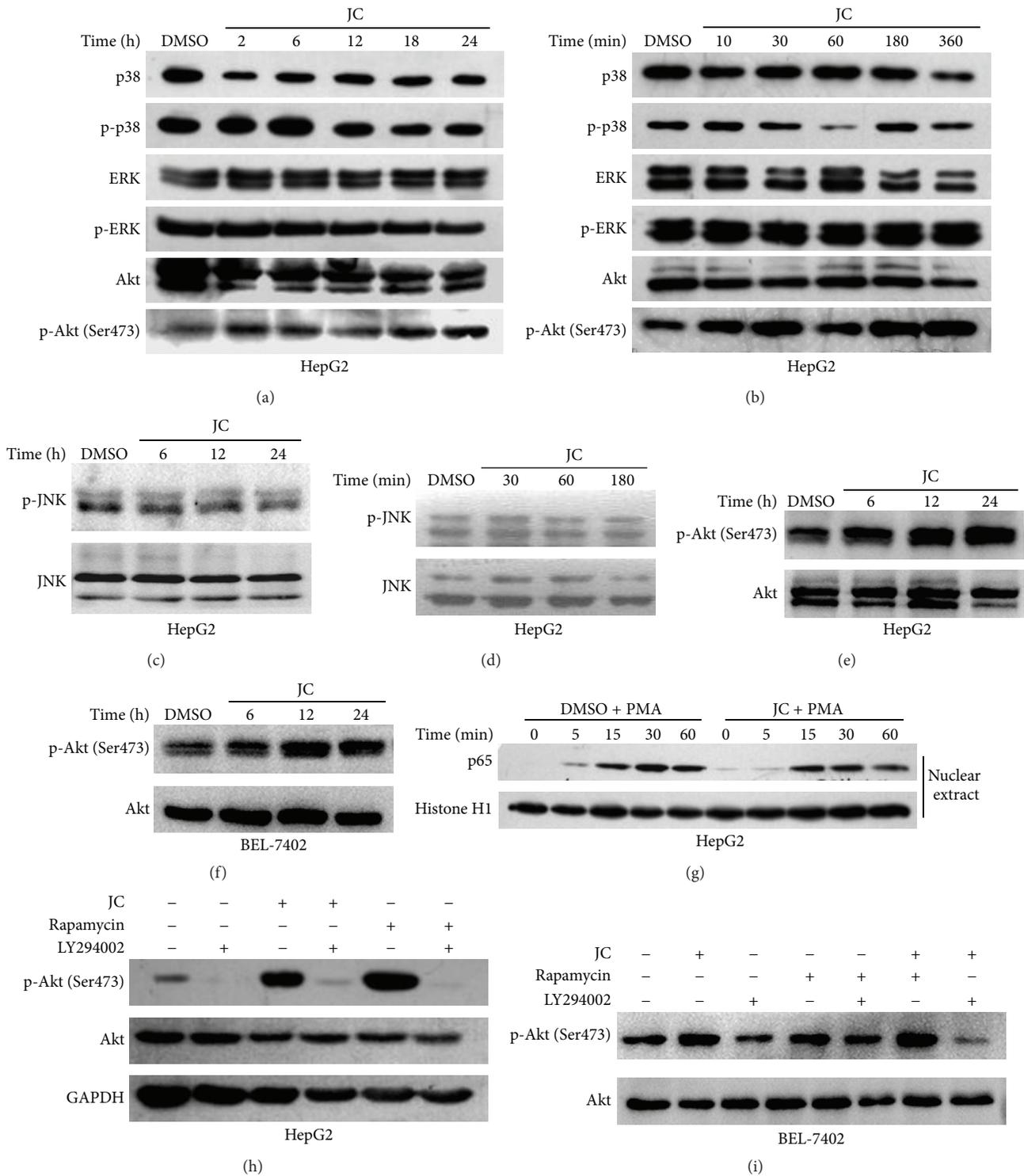
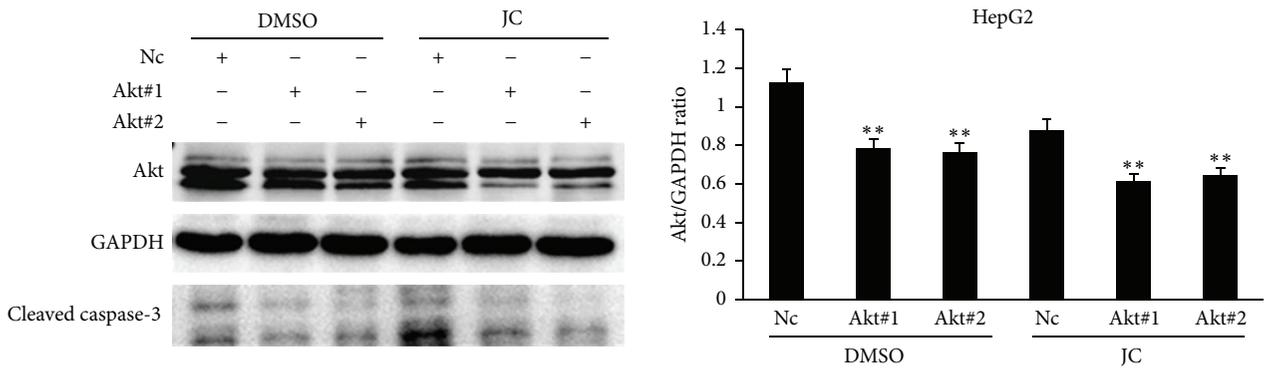
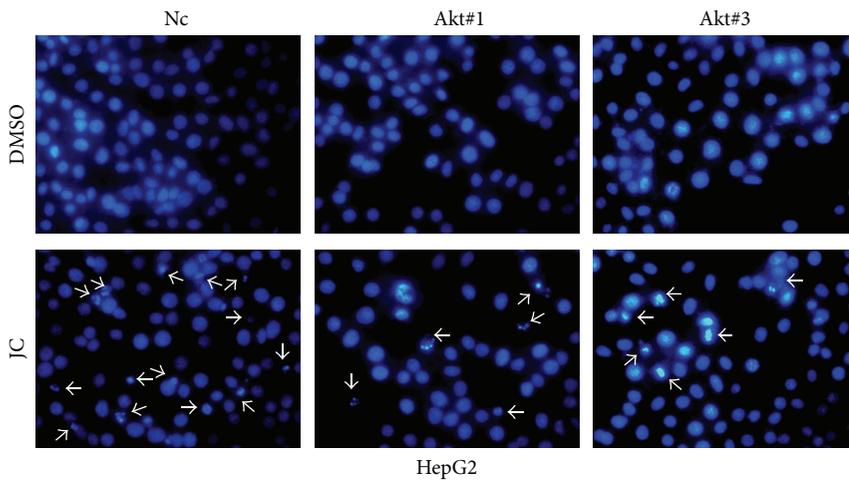


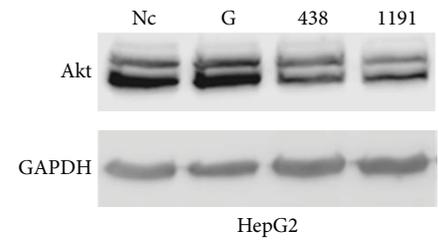
FIGURE 2: JC activates Akt signaling pathway. (a–d) Effects of JC on MAPK and Akt signaling pathways were determined by Western blot analysis. HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for different time periods. (e, f) Effects of JC on the Akt signaling pathway were determined by Western blot analysis. HepG2 cells were cultured in a medium without FBS for 12 hours and then treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for the indicated times (e). BEL-7402 cells were treated with either DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for the indicated times (f). (g) Effects of JC on the NF- $\kappa\text{B}$  signaling pathway were determined by Western blot analysis. HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 12 hours and then treated with PMA, which is an activator of NF- $\kappa\text{B}$ , for 5, 15, 30, and 60 minutes. Histone H1 was used as a loading control. (h, i) Effects of LY294002 and JC on Akt activation were determined by Western blot analysis. HepG2 cells were pretreated with 50  $\mu\text{M}$  of LY294002 for 1 hour and then treated with 15 nM of rapamycin and 8  $\mu\text{g}/\text{mL}$  of JC separately for 3 hours (h). BEL-7402 cells were treated with 30  $\mu\text{M}$  of LY294002 for 1 hour and then treated with 15 nM of rapamycin and 8.7  $\mu\text{g}/\text{mL}$  of JC separately for 3 hours (i). GAPDH was used as a loading control.



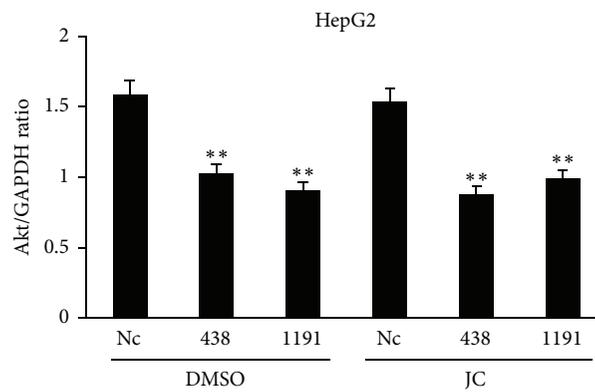
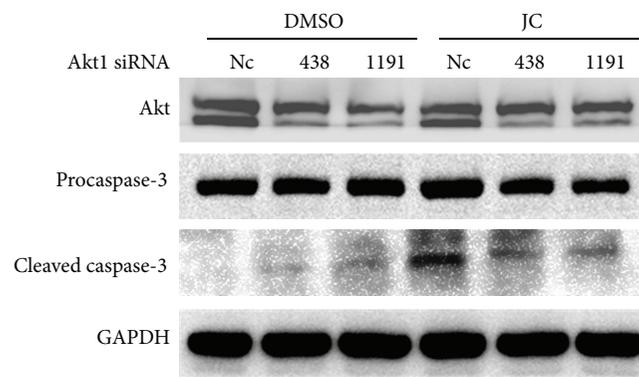
(a)



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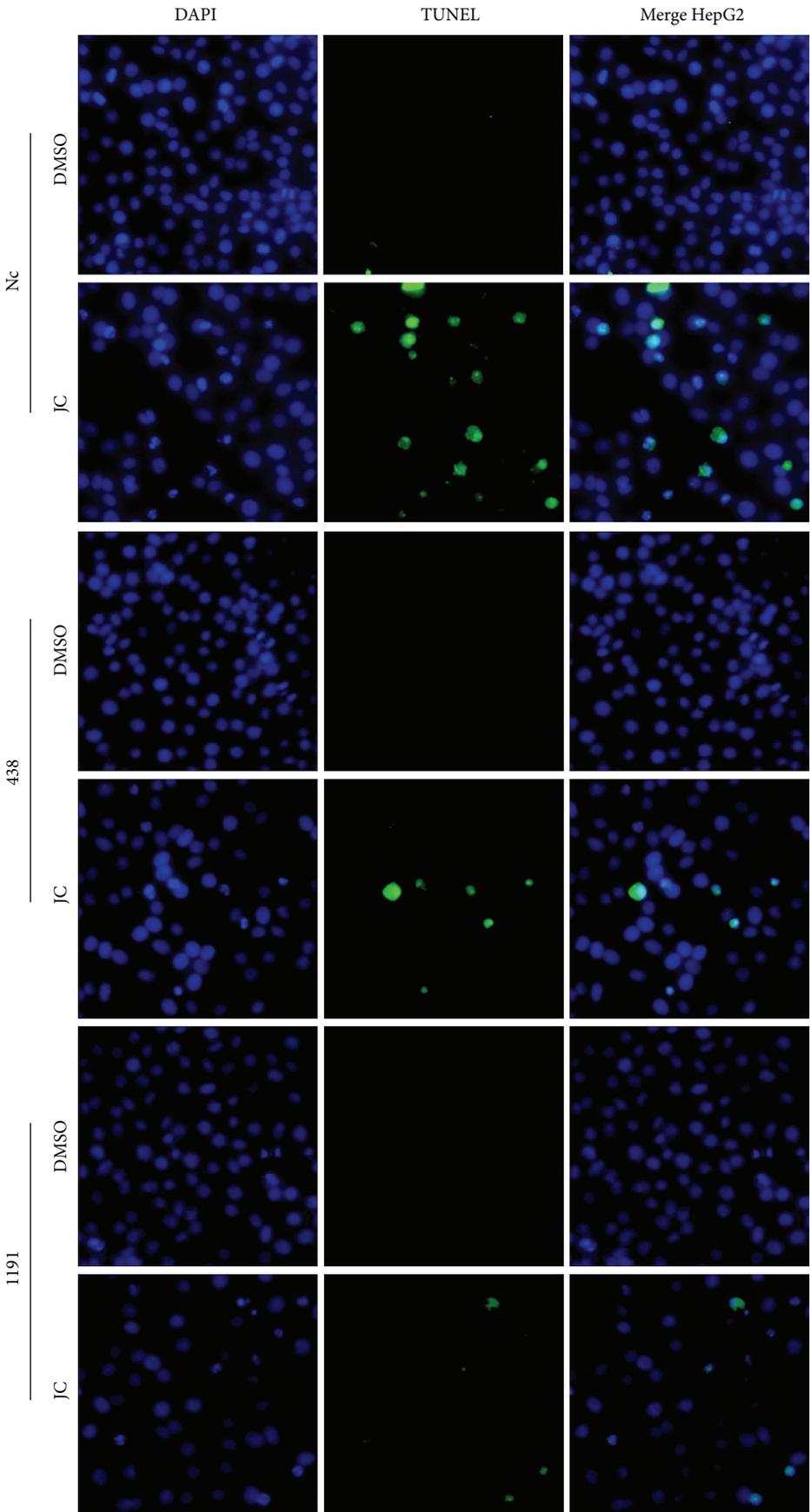


(c)



(d)

FIGURE 3: Continued.



(e)

FIGURE 3: Continued.

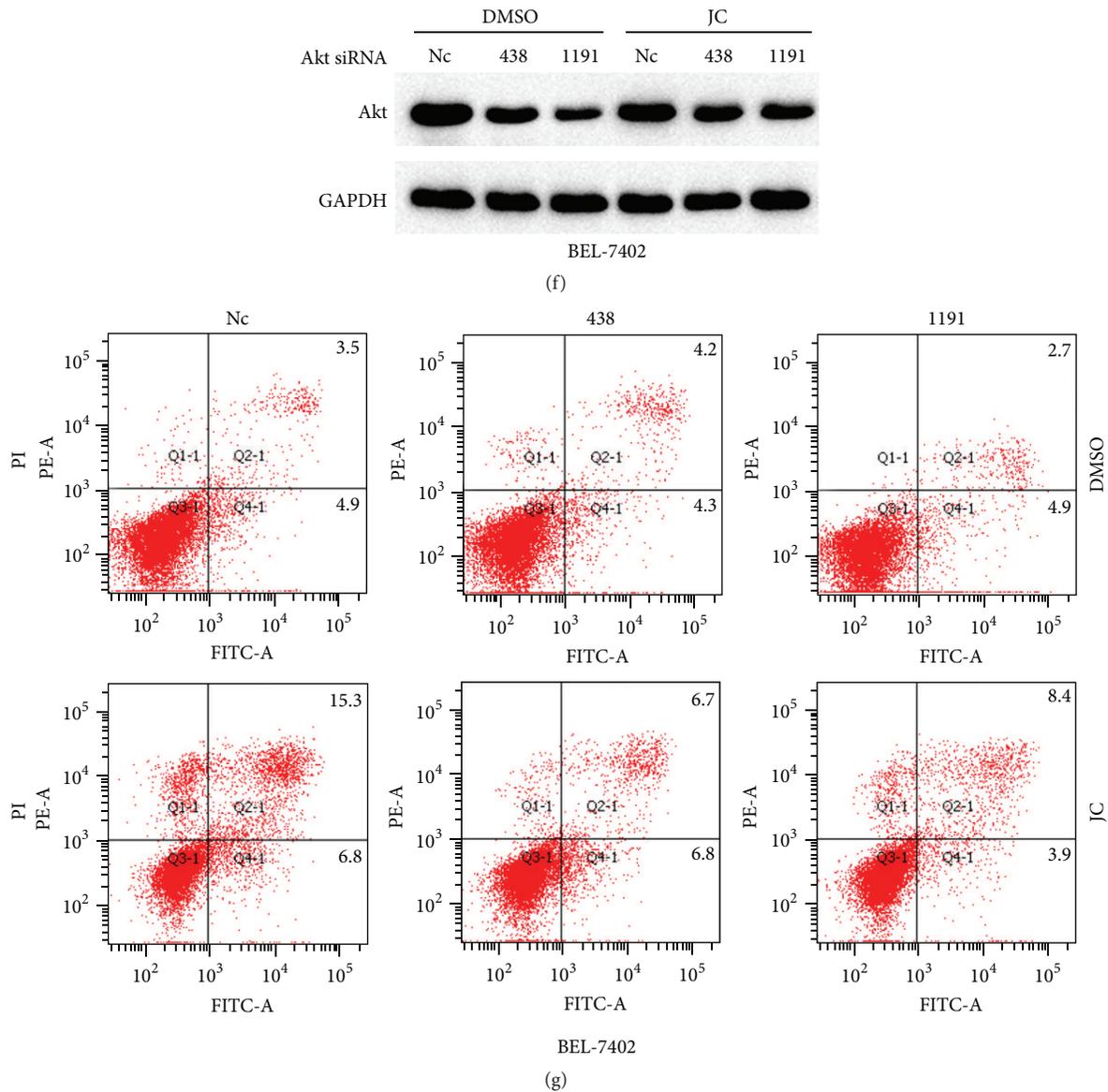


FIGURE 3: Akt deficiency abrogates HepG2 cell apoptosis induced by JC. (a) Effect of Akt deficiency on JC-induced apoptosis was determined by Western blot analysis. HepG2 cells were transfected with scrambled shRNA (Nc) or Akt shRNAs (Akt#1, Akt#2). Twelve hours after transfection, the cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours. GAPDH was used as a loading control. The ImageJ software was used to quantify Akt levels. (b) Detection of apoptosis in JC-treated HepG2 cells by DAPI staining. HepG2 cells were transfected with scrambled shRNA (Nc) or Akt shRNAs (Akt#1, Akt#2). Twelve hours after transfection, the cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours. The cells were fixed and stained with DAPI. Arrows are used to indicate apoptotic bodies in apoptotic HepG2 cells. (c) Effect of Akt siRNA on Akt expression was determined by Western blot analysis. HepG2 cells were transfected with scrambled siRNA (Nc) or Akt siRNAs (438, 1191) for 48 hours. G is GAPDH siRNA and is used as a positive control. GAPDH was used as a loading control. (d, e) Effect of Akt deficiency on JC-induced apoptosis. HepG2 cells were transfected with scrambled siRNA (Nc) or Akt siRNAs (438, 1191). Twelve hours after transfection, the cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours. Then, cell apoptosis was detected by both Western blot analysis (d) and TUNEL staining (e). The ImageJ software was used to quantify Akt levels. (f, g) Effect of Akt deficiency on JC-induced apoptosis in BEL-7402 cells. The cells were transfected with scrambled siRNA (Nc) or Akt siRNAs (438, 1191). Twelve hours after transfection, the cells were treated with either DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for 24 hours. Then, BEL-7402 cells were detected by both Western blot analysis (f) and flow cytometry analysis (g).

was shown that Akt was phosphorylated at Ser473 in JC-induced apoptosis. To further investigate the role of Akt phosphorylation in the apoptosis of HepG2 cells caused by JC, inactivated mutant of Akt (Akt-S473A), constitutively

active Akt (Akt-S473D), and wild-type Akt (Akt-WT) were introduced in HepG2 cells to examine their effects on JC-induced apoptosis. Notably, the overexpression of Akt-S473A prevented apoptosis treated with JC in HepG2 cells, whereas

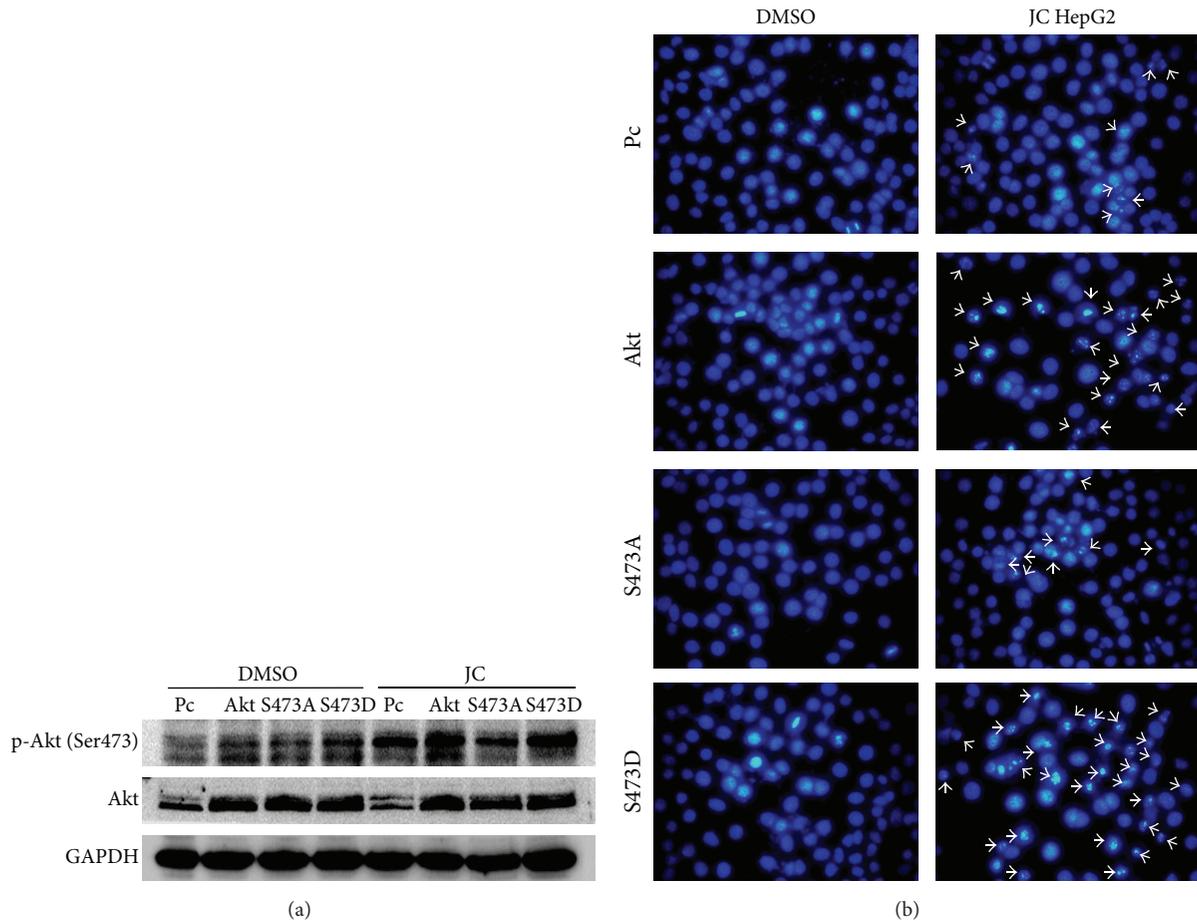


FIGURE 4: Akt activation promotes HepG2 cell apoptosis induced by JC. (a) Effects of wild-type and mutant Akt on JC-induced apoptosis were determined by Western blot analysis. HepG2 cells were transiently transfected with pcDNA3 vector control or pcDNA3-Akt. Twelve hours after transfection, the cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours. In the Akt-WT group, the p-Akt (S473) blot is for both endogenous and exogenous Akt. In the other groups (PC, S473A, and S473D), the p-Akt (S473) blot is for the endogenous Akt. GAPDH was used as a loading control. (b) Effects of wild-type and mutant Akt on JC-induced apoptosis were determined by DAPI staining. Twelve hours after transfection, the cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours. The cells were fixed and stained with DAPI. Arrows are used to indicate apoptotic bodies in apoptotic HepG2 cells.

the overexpression of Akt-WT and Akt-S473D caused higher levels of apoptosis, elucidating that JC regulated apoptosis at least partially through promoting the phosphorylation of Akt at Ser473 (Figures 4(a) and 4(b)).

**3.5. Role of JC in Inducing Apoptosis, Activating Akt, and Inhibiting Foxo3a Transcriptional Activity.** The Foxo family is a key downstream target of the PI3 K/Akt pathway and is directly phosphorylated by Akt [27]. The phosphorylation of Akt at Ser473 is required for its activation and inactivation of the Foxos [28]. The phosphorylation of Foxo factors by Akt triggers inactivation and rapid relocalization of Foxo proteins from the nucleus to the cytoplasm [29, 30]. The Foxo family participates in diverse processes including cell proliferation, apoptosis, stress resistance, differentiation, and metabolism [31]. The inactivation of Foxo transcription factors can induce apoptosis and regulate the cellular production of ROS [32]. Based on the aforementioned results and recent reports, whether Foxo3a translocated to the nucleus in JC-treated

HepG2 and BEL-7402 cells was analyzed. As expected, it was found that JC treatment activated Akt, inhibited nuclear localization of Foxo3a, and increased phosphorylated Foxo3a levels (Figures 5(a)–5(d)).

To confirm the contribution of Foxo3a to the apoptosis induced by JC, a Foxo3a expression construct (pcDNA3-Foxo3a) was transfected into HepG2 and BEL-7402 cells. As shown in Figures 5(e) and 5(f), the overexpression of Foxo3a abrogated the apoptosis of HCC cells induced by JC, indicating that Foxo3a was necessary for the apoptosis induced by JC. All these data suggested that JC activated Akt signaling, inhibited the transcriptional activity of Foxo3a, and finally induced the apoptosis of HCC cells.

**3.6. Effect of JC on ROS Levels.** Foxos have effects on detoxification of ROS by upregulating the free radical scavenging enzymes, including SOD2 and catalase [33]. Results from Nogueira suggested that Akt had the ability to inhibit apoptosis induced by multiple apoptotic stimuli excluding ROS, and

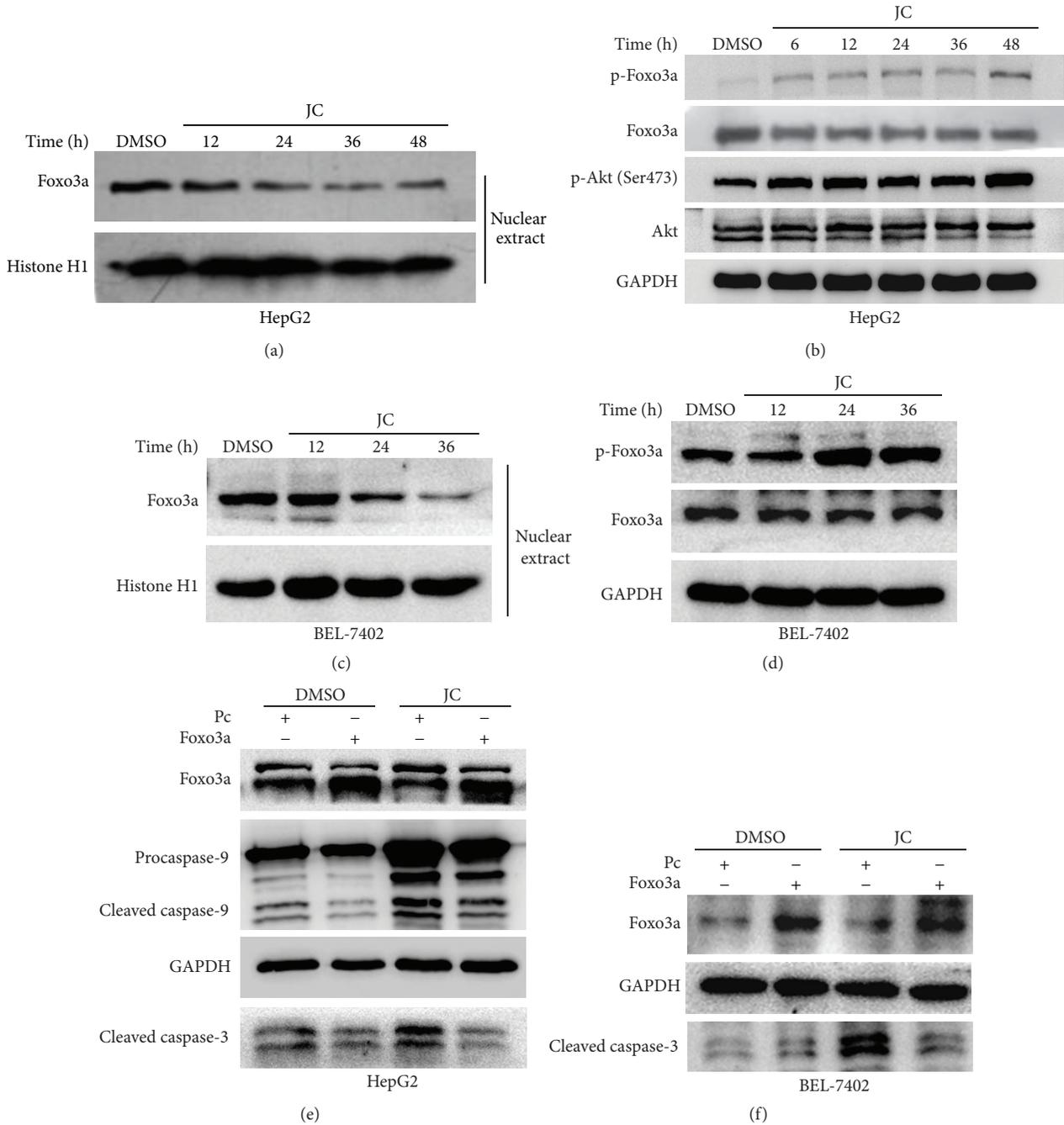


FIGURE 5: JC induces apoptosis by inhibiting the transcriptional activity of Foxo3a. (a, c) Effect of JC on Foxo3a levels in the nucleus was determined by Western blot analysis. HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for the indicated times (a). BEL-7402 cells were treated with either DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for the indicated times (c). Histone H1 was used as a loading control. (b, d) Effect of JC on the Akt/Foxo3a signaling pathway was determined by Western blot analysis. HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for the indicated times (b). BEL-7402 cells were treated with either DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for the indicated times (d). GAPDH was used as a loading control. (e, f) Overexpression of Foxo3a inhibits JC-induced apoptosis. HepG2 and BEL-7402 cells were transiently transfected with pcDNA3 vector control or pcDNA3-Foxo3a. Twelve hours after transfection, HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours (e). BEL-7402 cells were treated with either DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for 24 hours (f). Then, cell apoptosis was detected by Western blot analysis. GAPDH was used as a loading control.

Akt activation sensitized cells to ROS-mediated apoptosis [13]. So, in this study, the effects of JC on the ROS levels in HepG2 and BEL-7402 cells were analyzed. After treating HepG2 cells with JC, the levels of intracellular ROS were evaluated by DCFH-DA or DHE staining separately. Compared with the control, treatment with JC significantly increased the level of ROS in a time-dependent manner (Figures 6(a) and 6(b)). Additionally, JC significantly increased ROS levels in BEL-7402 cells in a dose-dependent manner (Figure 6(c)).

Under normal conditions, low amounts of ROS levels were eliminated by scavenging enzymes such as SOD2 and catalase, which converted  $H_2O_2$  to  $H_2O$  and  $O_2^-$  [34]. To verify whether JC increased ROS production by influencing SOD2 and catalase, their expressions were examined. As shown in Figures 6(d)–6(f), SOD2 and catalase were significantly decreased when cells were treated with JC for different times.

It was reported that treatment with antioxidant NAC and catalase was capable of restoring normal levels of intracellular ROS [33, 34]. To further determine whether the JC-induced apoptosis of HepG2 and BEL-7402 cells was mediated by increased ROS level, HCC cells were pretreated with NAC and PEG-catalase for 1 hour before incubating them with JC. This study showed that, compared to JC, NAC or catalase treatment could significantly reduce JC-induced apoptosis in HCC cells (Figures 6(g)–6(j)). These data suggested that treating HepG2 and BEL-7402 cells with JC resulted in high levels of ROS by inhibiting ROS scavenging, which severely damaged the cells and eventually led to the apoptosis of HCC cells.

#### 4. Discussion

Natural products have long been a fertile source for cancer treatment drugs. At least 250,000 species of plants exist, out of which more than 1000 plants were found to possess significant anticancer properties [35]. Many molecules obtained from the nature have shown anticancer activity and have become anticancer agents in clinical practice, such as paclitaxel [36], podophyllotoxin [37], camptothecin [38], and so on. This study has shown that JC, an anthraquinone compound extracted from *J. mandshurica* Maxim, could induce the apoptosis of cancer cells. In this study, a comparison of the three GO terms related to cell death showed that the shared 81 probe sets were identified in the three terms (Figure 1(d)). It can be inferred that these genes may be related to the apoptosis of HepG2 cells.

RTKs are the main type of enzyme-linked receptors that played an important role in the development and progression of cancer [39]. RTKs can activate MAPK, NF- $\kappa$ B, and PI3K/Akt signaling pathways. In cancer cells, Akt activation promotes cell proliferation, regulates cellular energy metabolism, and provides protection from apoptosis, which could partly explain that it is frequently activated in human cancers [12]. Here, whether JC induced apoptosis by inhibiting these signaling pathways was investigated. Surprisingly, the results from microarrays showed that enzyme-linked receptor protein signaling pathways were activated and the genes positively regulating Akt signaling were upregulated,

after treatment with JC. The Western blot analysis further confirmed that Akt was activated after treatment with JC, which induced the apoptosis of HepG2 and BEL-7402 cells (Figure 2). This study also demonstrated that Akt deficiency obviously inhibited apoptosis while the overexpression of a dominant-active mutant of Akt accelerated apoptosis induced by JC (Figures 3 and 4), suggesting that HepG2 cells that were transfected with Akt shRNA and siRNA were less sensitive than WT cells to JC-induced apoptosis. All these results suggested that JC-induced apoptosis was mediated by Akt activation, and this result was different from that of a previous study [12].

Akt normally acted as a proliferative signal, but the role of Akt is also a double-edged sword. Hyperactivated Akt also attenuates G2 arrest in Rat1a cells following DNA damage and induces premature senescence and sensitizes cells to ROS-mediated apoptosis [13, 40]. Aberrant loss or gain of Akt activation underlies the pathophysiological properties of a variety of complex diseases, including cancer [27, 41]. So the hypothesis that Akt activation was required for JC-induced apoptosis was investigated.

Akt is recruited to the plasma membrane by phosphatidylinositol-3,4,5-triphosphate and phosphorylated by 3-phosphoinositide-dependent protein kinases 1 and 2 at Thr308 and Ser473, respectively, which causes the full activation of Akt [25]. Activated Akt phosphorylates a wide range of direct intracellular targets containing a minimal Akt recognition motif, including Bad, Tsc2, Gsk3, and Foxos, which contribute to the diverse cellular roles of Akt, including cell survival, growth, proliferation, metabolism, and migration [27, 30]. Akt phosphorylation at Ser473 is required for the inactivation of the Foxos [28]. After being activated, Akt directly phosphorylates Foxos, and this phosphorylation excludes Foxos from the nucleus, thereby inhibiting their transcriptional activity [42]. It was found that the transcription activity of Foxo3a was inhibited after treatment with JC (Figures 5(a)–5(d)). Additionally, the apoptosis of HCC cells induced by JC was abrogated by the overexpression of Foxo3a (Figures 5(e) and 5(f)), suggesting that Foxo3a was a key factor in regulating JC-induced apoptosis and Akt activation.

ROS are generally small, short-lived, and highly reactive molecules, formed by incomplete one-electron reduction of oxygen [14]. The damage induced by the accumulation of ROS is considered a major determinant of life span at both the organismal and cellular levels. ROS can damage proteins, nucleic acids, and intracellular membranes, which lead to oxidative stress and impairment of cellular functions [14]. Excessive ROS causes the release of Cytochrome *c* from mitochondria to the cytosol and triggers caspase-9 activation and apoptosis [15]. In this study, the levels of intracellular ROS were evaluated after cells were treated with JC (Figures 6(a) and 6(c)). The increase of apoptosis induced by JC was reversed by the antioxidant NAC and PEG-catalase (Figures 6(g)–6(j)). Therefore, these results indicate that JC-induced mitochondrial apoptosis is mediated by ROS.

ROS scavengers SOD2 and catalase are known to be Foxo target gene. Under normal conditions, ROS are reduced by nonenzymatic and enzymatic antioxidizing agents, such as glutathione, thioredoxin, SOD, catalase, and peroxidases [14,

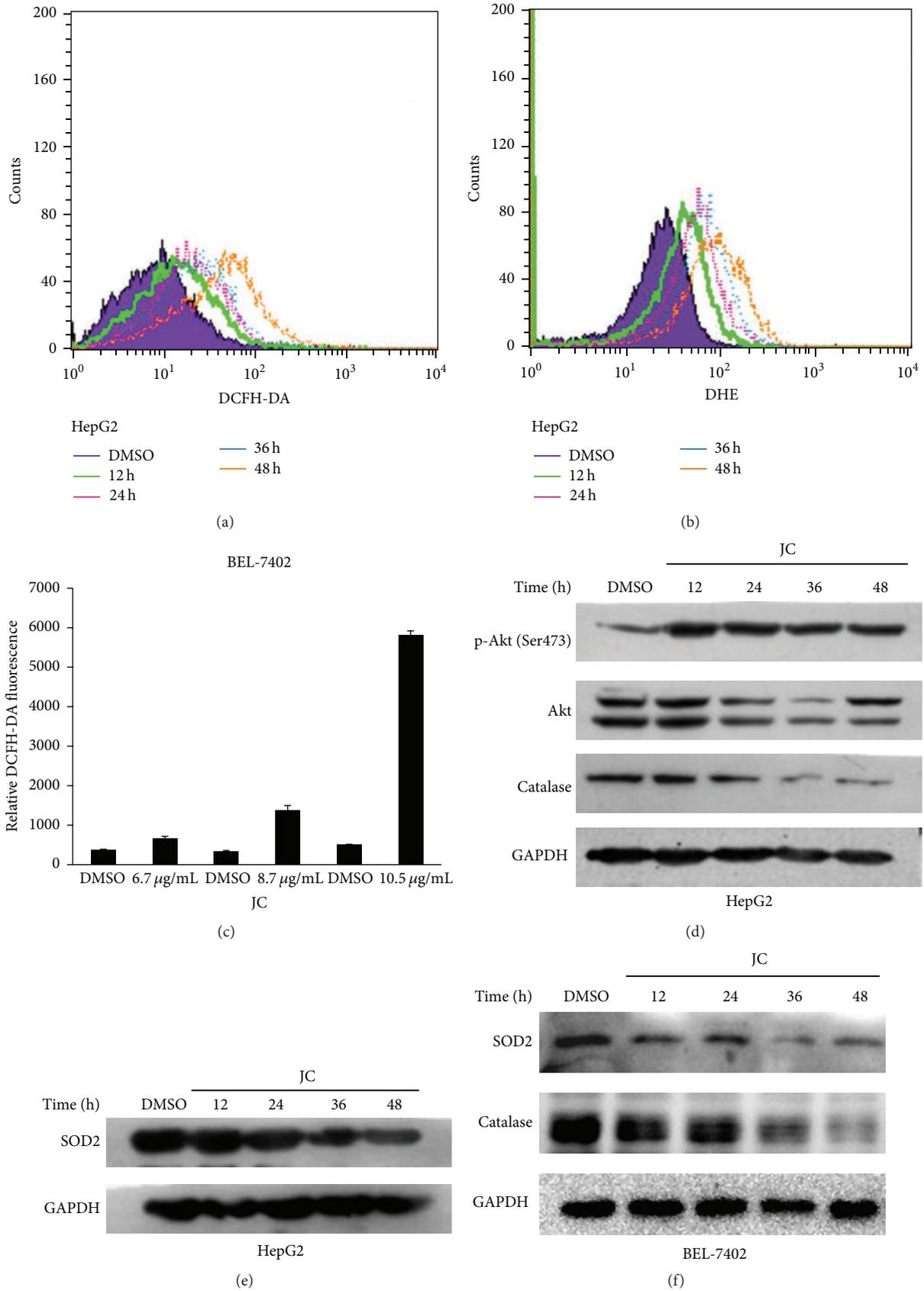
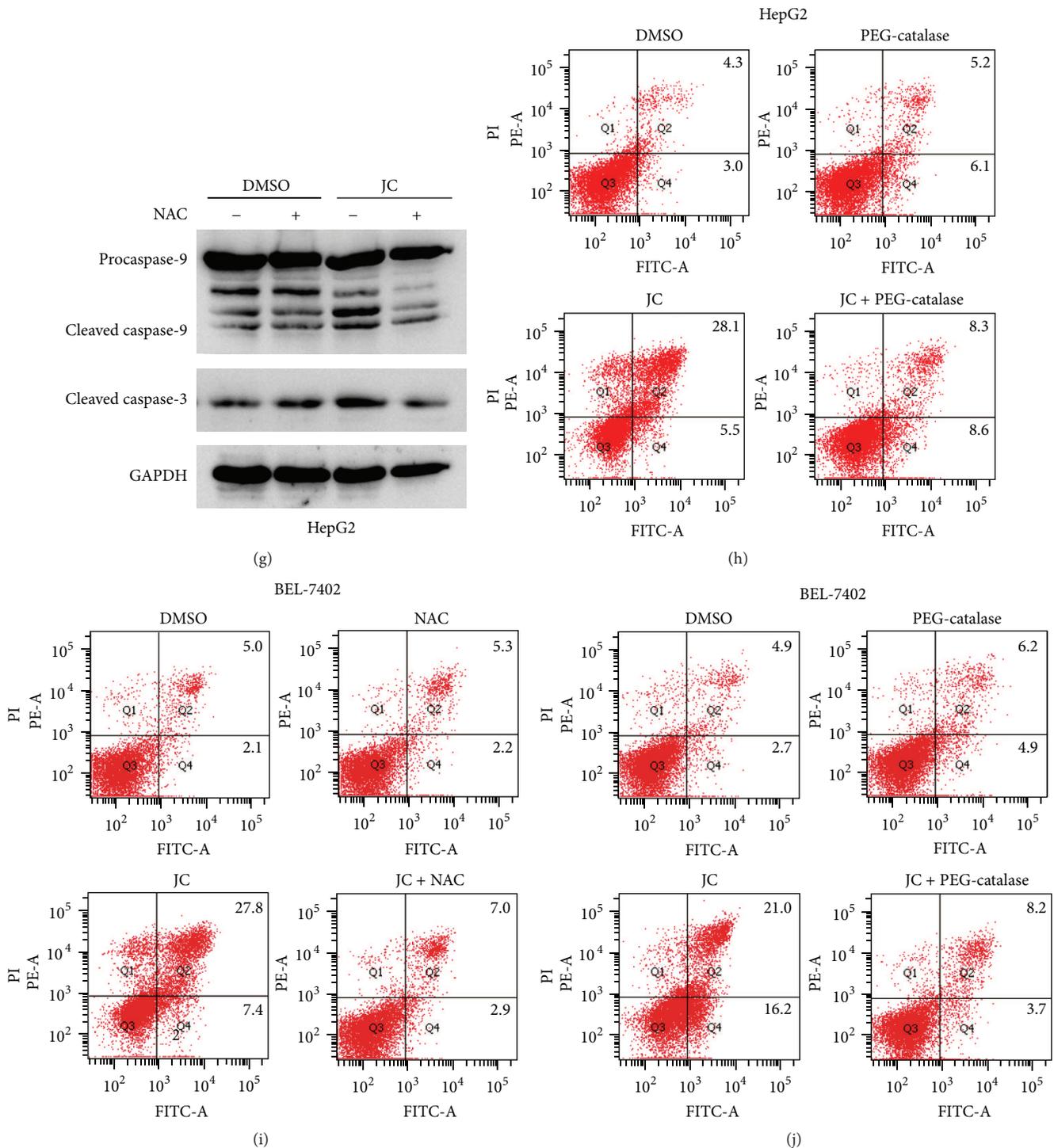


FIGURE 6: Continued.



**FIGURE 6:** JC induces apoptosis by increasing intracellular ROS. (a, c) Effect of JC on the total ROS levels in HepG2 and BEL-7402 cells was examined by flow cytometry. HepG2 cells were treated with DMSO or 8  $\mu\text{g}/\text{mL}$  JC for 12, 24, 36, and 48 hours (a). BEL-7402 cells were treated with DMSO or 6.7, 8.7, and 10.5  $\mu\text{g}/\text{mL}$  of JC for 12 hours (c). (b) Effect of JC on superoxide anion levels in HepG2 cells was examined by flow cytometry. The levels of ROS in HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 12, 24, 36, and 48 hours and analyzed by flow cytometry. (d–f) Effects of JC on catalase and SOD2 expression and Akt activation were determined by Western blot analysis. HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for the indicated times (d and e). BEL-7402 cells were treated with DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for the indicated times (f). GAPDH was used as a loading control. (g–j) NAC and PEG-catalase abrogate JC-induced apoptosis. HepG2 cells were pretreated with NAC and PEG-catalase for 1 hour. HepG2 cells were treated with either DMSO or 8  $\mu\text{g}/\text{mL}$  of JC for 36 hours and detected by Western blot analysis (g) or flow cytometry analysis (h). GAPDH was used as a loading control. BEL-7402 cells were treated with either DMSO or 8.7  $\mu\text{g}/\text{mL}$  of JC for 24 hours and detected by flow cytometry analysis (i and j).

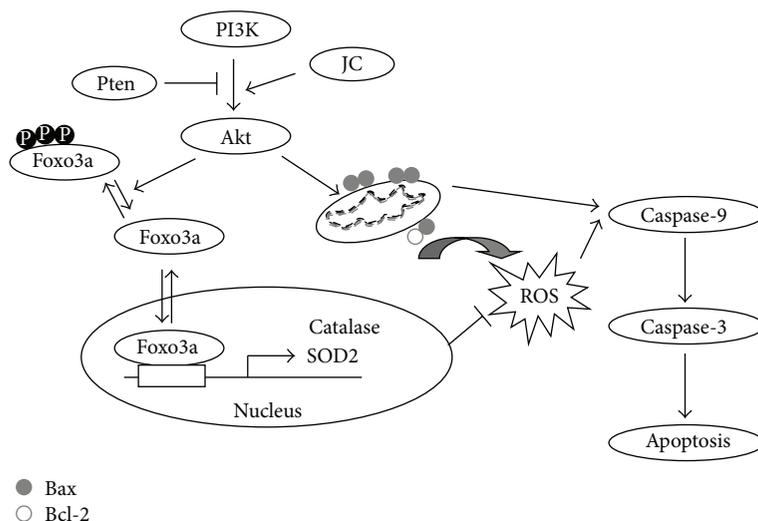


FIGURE 7: A proposed model to illustrate mechanisms by which JC induced the apoptosis of HCC cells. Akt hyperactivation caused by JC-inhibited Foxo transcriptional activity, which resulted in highly increased ROS levels via impairment of ROS scavenging. The upregulation of ROS, in turn, promoted apoptosis of HepG2 and BEL-7402 cells.

34]. In this study, both SOD2 and catalase were significantly decreased, while ROS levels were increased, when HCC cells were treated with JC (Figure 6). These results suggest that SOD2 and catalase are related to the increased ROS levels induced by JC. Akt could also increase ROS levels by increasing oxygen consumption. Most of ROS are products of mitochondrial respiration and generated at Complexes I and III of the respiratory chain [14, 43]. Akt can increase cellular ATP production by accelerating both glycolytic and oxidative metabolism [44], which contributes to an increase of ROS levels.

Given that activating the PI3 K/Akt pathway is frequently implicated in human cancer, many intracellular components of the PI3 K/Akt pathway have been targeted as anticancer drug discovery [45]. However, existing drugs against various components of the PI3 K/Akt pathway possibly exhibit undesired physiological consequences such as diabetes. Compared with normal cells, cancer cells normally contain higher levels of ROS, which can stimulate cell proliferation and induce genetic instability [46]. It was reported that abnormal increases in ROS can be exploited to selectively kill cancer cells [47]. Thus, using hyperactivated Akt and high levels of ROS as targets is a strategy to selectively kill cancer cells. It was demonstrated that JC can selectively eradicate HepG2 and BEL-7402 cells with hyperactivated Akt by inducing excessive ROS, suggesting that JC is a potentially effective anticancer drug.

It was reported that the activation of Akt is frequently implicated in resistance to anticancer drugs [48]. Moreover, this study proved that JC can selectively kill HCC cells with hyperactivated Akt. So the combination of JC and anticancer drugs, such as PEITC and rapamycin, could be an effective strategy to selectively eradicate tumors that display hyperactive Akt and resistance to anticancer drugs.

Overall, these findings suggest a model (Figure 7) in which JC increases Akt Ser473 and Foxos phosphorylation.

Foxos were excluded from the nucleus, thereby inhibiting the expression of their target genes SOD2 and catalase, resulting in the intracellular ROS accumulation, and eventually leading to cell apoptosis.

## Disclaimer

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the paper.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Ya-Qin Hou and Yao Yao contributed equally to this work.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grants nos. 31170324 and 31070318), the Fundamental Research Funds for the Central Universities, and the Research Foundation of Jilin Provincial Science and Technology Development (Grants nos. 20140203008YY and 20110938).

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## Review Article

# Redox Modulating NRF2: A Potential Mediator of Cancer Stem Cell Resistance

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Received 5 June 2015; Accepted 27 July 2015

Academic Editor: Amit Tyagi

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Tumors contain a distinct small subpopulation of cells that possess stem cell-like characteristics. These cells have been called cancer stem cells (CSCs) and are thought to be responsible for anticancer drug resistance and tumor relapse after therapy. Emerging evidence indicates that CSCs share many properties, such as self-renewal and quiescence, with normal stem cells. In particular, CSCs and normal stem cells retain low levels of reactive oxygen species (ROS), which can contribute to stem cell maintenance and resistance to stressful tumor environments. Current literatures demonstrate that the activation of ataxia telangiectasia mutated (ATM) and forkhead box O3 (FoxO3) is associated with the maintenance of low ROS levels in normal stem cells such as hematopoietic stem cells. However, the importance of ROS signaling in CSC biology remains poorly understood. Recent studies demonstrate that nuclear factor-erythroid 2-related factor 2 (NRF2), a master regulator of the cellular antioxidant defense system, is involved in the maintenance of quiescence, survival, and stress resistance of CSCs. Here, we review the recent findings on the roles of NRF2 in maintenance of the redox state and multidrug resistance in CSCs, focusing on how NRF2-mediated ROS modulation influences the growth and resistance of CSCs.

## 1. Introduction

Reactive oxygen species (ROS) are highly proactive molecules derived from molecular oxygen and include free radicals such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^-$ ), and hydroxyl radical ( $OH^*$ ). Under normal physiological conditions, low-to-moderate levels of ROS play a critical role in cellular development and signaling. However, excess ROS levels, which can be caused by metabolic dysfunction or environmental stress conditions, can lead to peroxidation of cellular macromolecules such as lipids, proteins, and nucleic acids [1, 2]. These ROS-induced byproducts eventually trigger cellular senescence, carcinogenesis, or cell death. Interestingly, mammalian cells have developed tightly regulated antioxidant systems for protection against ROS-induced oxidative damage. For example, the superoxide anion, a product of mitochondrial dysfunction, is converted to  $H_2O_2$  by superoxide dismutases (SODs).  $H_2O_2$  is then decomposed to oxygen and water by catalase or glutathione peroxidases (GPXs) [3, 4].

Multiple lines of evidence suggest that cancer cells possess higher levels of intracellular ROS than normal cells [5, 6]. Elevated ROS levels in cancer can be utilized to promote cell proliferation, invasiveness, and metastasis [6–9]. There are several underlying mechanisms involved in ROS elevation in cancer cells. First, activated oncogenes can trigger ROS production through upregulation of ROS-generating enzymes such as NADPH oxidases (NOXs) [10, 11]. The *RAS* oncogene increases NOX1 expression via the extracellular signal-regulated kinases (ERK) [10] or mitogen-activated protein kinase (MAPK) signaling pathways [11] in human cancers. Overexpression of the *c-MYC* oncogene in normal human fibroblasts induces DNA damage by increasing ROS levels [12]. Mutation of mitochondrial DNA (mtDNA) is a major cause of ROS elevation in cancer cells. Polyak et al. found that seven out of ten colorectal cancer cell lines retained somatic mutations in mtDNA; most of these mutations were detected in mitochondrial genes such as those encoding cytochrome c oxidases 1–3, which has potential implications with respect to increase in mitochondrial ROS [13]. Cancer cells have their

own adaptation mechanisms against increased ROS, such as upregulation of ROS scavenging systems. As a result of these systems, malignant transformed cells can utilize ROS as a signal for tumor progression and metastasis [5, 14].

Recent studies are expanding our knowledge about the biological implications of ROS in cancer stem cells (CSCs), which are small subpopulation of cancer cells responsible for tumorigenesis and tumor progression and relapse. Based on increasing evidence for the role of ROS in stem cell biology, lower levels of cellular ROS are considered beneficial for the maintenance of quiescence and chemo/radioresistance of CSCs [15]. In this review, we show current findings illustrating the relationship between ROS and CSC biology and present emerging evidence that nuclear factor-erythroid 2- (NF-E2-) related factor 2 (NRF2) may play a role in CSC growth and resistance.

## 2. CSCs and Resistance to Environmental Stress and Chemotherapy

Tumors contain a small population of cells with stem cell properties, namely, CSCs or tumor-initiating cells (TICs) [16, 17]. These cells are known to play a crucial role in tumor maintenance and relapse. In the 1990s, the first experimental evidence of CSCs was introduced by Bonnet and Dick [18]. In acute myeloid leukemia (AML), it appeared that 0.1 to 1% of the total cell population had tumor-initiating activity. This subpopulation exhibited a CD34<sup>+</sup>/CD38<sup>-</sup> phenotype and was capable of tumor reconstitution after transplantation into nonobese diabetic/severe combined immune-deficient (NOD/SCID) mice [18]. Since then, multiple lines of evidence have revealed that the CSC population exists in different types of solid tumors, including brain, breast, and colon cancers [19–21].

CSCs are characterized by their self-renewal and differentiation capacity, similar to normal stem cells [16]. Markers of embryonic stem cells (ESCs) such as octamer-binding transcription factor 4 (OCT4), Nanog homeobox (NANOG), and SRY (sex determining region Y)-box 2 (SOX2) are expressed in CSCs, and the Wnt/ $\beta$ -catenin, Hedgehog, and Notch pathways are implicated in the self-renewal of CSCs [22–26]. Several CSC-specific surface markers have been identified for the detection and isolation of CSCs from the tumor mass. CD44<sup>+</sup>/CD24<sup>-</sup> phenotypic cells were isolated from breast cancer tissues and breast carcinoma cell lines and were shown to exhibit self-renewal and high tumorigenic capacity [27]. The CD133<sup>+</sup> subpopulation from brain tumors demonstrated stem cell properties and showed tumor-initiating capability in NOD/SCID mouse brains [20].

CSCs are considered to be one of the main causes of tumor recurrence after therapy. CSC resistance to conventional anticancer drug therapies and radiotherapy is attributed to increased expression of ROS scavenging molecules, drug transporters, and enhanced DNA repair capacity [28–30]. It has been reported that CSCs contain low levels of endogenous ROS compared to those seen in non-CSCs [31, 32]. In primary AMLs, a subpopulation of low ROS-producing cells demonstrated characteristics of CSCs including quiescence

and a CD34<sup>+</sup>/CD38<sup>-</sup> phenotype [31]. Moreover, Chang et al. observed that this population of low ROS-producing cells exhibited increased expression of stem cell markers (OCT4 and NANOG) and higher chemoresistance and tumorigenicity than the population of high ROS-producing cells in head and neck cancer [32].

The ATP-binding cassette transporter (ABC transporter) family is known to induce multidrug resistance by actively transporting intracellular drugs to outside of the cell [33, 34]. P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and breast cancer associated protein (BCRP) belong to the ABC transporter family. Since many types of anticancer agents are substrates of these ABC transporters, enhanced expression of P-gp, MRPs, and BCRP is strongly associated with the chemoresistant phenotype of CSCs. Based on this, ABC transporters are often used as a CSC surface marker [28, 35]. The side population (SP), which is a fraction of cells that expresses a high level of BCRP, can be isolated from cancer cells using fluorogenic dye Hoechst 33342. As Hoechst 33342 dye is a substrate of BCRP, the BCRP overexpressing cells exclude this fluorescent dye and thereby a fraction of cells with low fluorescence can be isolated from non-SP cells. This method is now widely used to isolate CSCs from cancer cell lines and specimens using a flow cytometry [36].

## 3. Role of ROS in Stem Cells

Stem cells can be broadly classified into two categories: adult stem cells (e.g., haematopoietic stem cells (HSCs) and neural stem cells) and ESCs. Under homeostatic conditions, these stem cells, particularly adult stem cells, are generally maintained in a quiescent state. However, stem cells are able to escape quiescence and enter the cell cycle for proliferation when they are exposed to metabolic changes [37–40].

ROS are considered as important signaling molecules in stem cell biology. They play a key role in stem cell maintenance by preserving quiescence and protecting against environmental stress [37, 38]. Recent stem cell studies have demonstrated that stem cells contain low levels of intracellular ROS, and this redox status was found to be critical for regulation of stem cell quiescence and self-renewal. Murine ESCs exhibited low levels of intracellular ROS compared to differentiated murine cells, due to the increased level of GSH and thioredoxin (TXN) system [41]. Furthermore, HSCs containing low levels of ROS (i.e., cells with low fluorescent activity following the incubation with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), a cell-permeable ROS sensing fluorogenic dye) were highly quiescent and expressed relatively high levels of NOTCH1 and BCRP compared to high DCFDA fluorescent cells. High levels of ROS are cytotoxic, since ROS accumulation in HSCs can lead to cellular prematurity and senescence [42, 43].

ROS have been reported to be involved in stem cell differentiation. Bone marrow mesenchymal stem cells (MSCs) are found in the bone marrow together with HSCs and have the potential to differentiate to adipocytes, osteocytes, and chondrocytes. It was shown that human MSCs are highly resistant

to ROS. This phenomenon was linked to low levels of cellular ROS and high levels of SODs, catalase, GPX1, and GSH in MSCs [38, 40]. Elevated antioxidant molecules appear to play a crucial role in the protection of stem cells against oxidative stress. However, under certain circumstances, NOX-derived ROS are associated with MSC differentiation. The treatment of MSCs with antioxidants or interfering RNA of NOX4 prevented adipocyte differentiation of MSCs via cAMP response element-binding protein (CREB) inhibition. Similarly, ESC differentiation to the cardiac lineage was dependent on NOX4-derived ROS [44]. These findings indicate that ROS are important for stem cell fate determination for quiescence or differentiation.

#### 4. Redox Signaling Molecules in Stem Cells

It has been reported that multiple signaling molecules are involved in ROS-mediated regulation of stem cells (Figure 1). First, ataxia telangiectasia mutated (ATM) plays a critical role in controlling ROS levels in stem cells. ATM, a serine/threonine protein kinase, is a known regulator of the DNA damage response and contributes to the regulation of cellular ROS. ATM is known to regulate ROS via modulation of AMPK-mTOR pathway or NADPH production [45, 46]. Ito et al. showed that *atm*<sup>-/-</sup> mice developed bone marrow failure after 24 weeks of age due to a depletion of HSCs. In this study, HSCs in *atm* knockout mice showed higher levels of ROS than wild type mice, which presumably caused a reduction in the self-renewal activity of HSCs. However, the treatment of mice with antioxidant *N*-acetylcysteine (NAC) restored HSC reconstitution in *atm* knockout mice by reducing ROS in HSCs, confirming the critical role of ROS in HSCs maintenance [47]. Similarly, in another study, NAC treatment prevented hypersensitivity of *atm*<sup>-/-</sup> mice to X-ray irradiation and senescence of *atm*<sup>-/-</sup> embryonic fibroblasts [48]. Cosentino et al. have presented a molecular mechanistic role for ATM, demonstrating that ATM activation promotes the binding of heat shock protein 27 (HSP27) to glucose-6-phosphate dehydrogenase (G6PDH), which can result in G6PDH activation and subsequent NADPH increase [46].

The forkhead box O (FoxO) transcription factor family is also implicated in redox regulation of stem cells. The FoxO family, including FoxO1, FoxO3, FoxO4, and FoxO6, is a key regulator of cell survival, proliferation, DNA repair, and apoptosis. FoxO1 and FoxO3 are reported to upregulate the expression of GSH biosynthetic enzymes and SODs and therefore are associated with cellular protection against oxidative stress [49, 50]. Particularly, FoxO3 has been known to play crucial roles in cytoprotection of stem cells including HSCs [51–53]. Loss of FoxO3a, which regulates the expression of antioxidant enzymes such as catalases and SOD2, led to ROS accumulation and thus a higher rate of cell cycling and a loss of quiescence in HSCs [51]. In *foxo1/foxo3a/foxo4* triple-knockout mice, the number of HSCs was substantially decreased and apoptotic HSCs were increased through ROS elevation [52]. Notably, Yalcin et al. provided a link between ATM and the FoxO protein in ROS regulation of stem cells. In *foxo3*<sup>-/-</sup> HSCs, ATM expression was diminished compared to

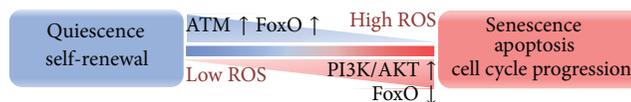


FIGURE 1: Involvement of ROS in normal stem cell quiescence and self-renewal. In normal stem cells, modulation of ROS levels can determine quiescence and cell fate progression. At low ROS levels, which are maintained by ATM and FoxO signaling, stem cells remain quiescent and self-renewal activity is enhanced. On the other hand, increased ROS levels result in cell cycle progression, cellular senescence, and apoptosis. The PI3K-AKT pathway is known to elevate ROS levels by negative regulation of FoxO.

normal HSCs, suggesting that FoxO3 repressed ROS via ATM regulation [53]. Similar to HSCs, *foxo*-deficient neural stem cells demonstrated a decline in self-renewal capacity due to increased cellular ROS levels [54, 55].

The phosphoinositide 3-kinase (PI3K)/AKT pathway is another ROS regulator in normal stem cells. In particular, PI3K/AKT signaling associates with FoxO transcription factors to mediate ROS regulation. Activated AKT promotes FoxO phosphorylation, resulting in the nuclear export and cytoplasmic degradation of FoxO through the proteasome [56, 57]. Therefore, *akt1/2* double knockout HSCs displayed increased quiescence and low cellular ROS levels [58]. Consistently, persistent activation of the PI3K/AKT pathway in phosphatase and tensin homolog (PTEN) deleted HSCs led to defective quiescence, resulting in cellular senescence [59]. Based on the above observations, the PI3K/AKT pathway and FoxO/ATM pathway exhibit opposite roles in ROS regulation of stem cells.

Hypoxia-inducible factors (HIFs) are transcription factors that respond to hypoxic conditions [60]. They are also critical factors for the maintenance of stem cells. HSCs cultured in hypoxic conditions displayed a higher colony formation capacity, and high HIF levels positively regulated the pluripotency of human ESCs by activating stemness transcription factors such as OCT4, SOX2, and NANOG [61, 62]. Moreover, in neuronal stem cells in a hypoxic environment, accumulated HIF1 $\alpha$  promoted Wnt/ $\beta$ -catenin pathway activation [63]. The involvement of HIFs in stem cell biology is mediated by ROS. Takubo et al. observed that *hif1 $\alpha$* <sup>-/-</sup> HSCs contain high levels of ROS, which could be associated with a loss of HSC quiescence and an induction of cellular senescence [64]. In agreement with this finding, the suppression of HIF1 $\alpha$  and HIF2 $\alpha$  in HSCs led to increased ROS generation via mitochondrial metabolic shift and consequently induced cellular senescence and apoptosis. Scavenging ROS by NAC treatment could restore HSC quiescence and function in stem cells with HIF1 $\alpha$  and HIF2 $\alpha$  suppression [65].

#### 5. Involvement of ROS in CSC Biology

Very few studies have investigated the involvement of redox change in CSC biology compared to that in cancer cells or normal stem cells. However, it has been reported that

CSCs appear to share several ROS-associated properties with normal stem cells. CSCs are known to contain lower levels of ROS compared to non-CSCs. CD44<sup>+</sup>/CD24<sup>-/low</sup> breast cancer CSCs, isolated from MDA-MB-231 and MCF7 mammospheres, were relatively more resistant to radiation and this was associated with lower levels of ROS after radiation [66]. Diehn et al. observed that ROS levels in CD44<sup>+</sup>/CD24<sup>-</sup> breast cancer CSCs were lower than in non-CSCs, and the expression levels of the modulatory subunit of glutamate cysteine ligase (GCLM, the rate-limiting enzyme of GSH synthesis) and FoxO1 were high. Treatment of the CD44<sup>+</sup>/CD24<sup>-</sup> subpopulation with buthionine sulphoximine (BSO), an inhibitor of GSH synthesis, resulted in reduced colony forming capacity and increased sensitivity to radiation therapy through an increase in ROS [67]. In a study using human AML specimens, leukemic stem cells with low levels of ROS majorly contributed to stem cell quiescence by maintaining a low rate of oxidative phosphorylation and metabolism [31]. A leukemia with a high amount of leukemic stem cells showed low levels of ROS and increased expression of GPX3 compared to tumors with a low frequency of leukemic stem cells. This study demonstrated that GPX3 levels positively correlated with poor prognostic outcome in AML patients [68]. Glioma stem cells within the tumor mass have low levels of cellular ROS, although they are located in a hypoxic environment. A proposed molecular mechanism of this phenomenon was the significantly upregulated expression of peroxiredoxin 4 (PRDX4) in glioma stem cells [69].

Evidence is indicating that low ROS levels in CSCs result from the intrinsic characteristics of CSCs. Cell surface markers of CSCs, including CD44 and CD13, are found to be involved in ROS regulation. Ishimoto et al. demonstrated that a variant isoform of CD44 (CD44v) can bind to the cystine/glutamate exchange transporter xCT and activates cysteine uptake to enhance GSH synthesis in gastrointestinal CSCs [70]. The expression of antioxidant genes such as GPX1/2 was significantly increased in CD44<sup>+</sup> gastric tumor cells. In addition, knockdown of CD44 in mice led to ROS increase, p38MAPK activation, and cellular senescence that are related to p21 expression. In a subsequent study, the same group demonstrated that the number of CD44<sup>+</sup> cells increased with neoadjuvant chemotherapy in head and neck squamous cell carcinoma (HNSCC) patients. These CD44<sup>+</sup> undifferentiated cancer cells displayed high xCT expression, GSH upregulation, and low cellular ROS levels. Ablation of xCT by siRNA or sulfasalazine treatment (xCT-mediated cystine transport inhibitor) induced differentiation of HNSCC CSCs both *in vitro* and *in vivo* [71]. CD13 has been identified as a surface marker for liver CSCs. In liver cancer cell lines including Huh7 and PLC/PRF/5, CD13 positive cells predominated the SP fraction and were mainly in the G0/G1 phase of the cell cycle. Additionally, resistance to anticancer drugs or radiation in the CD13 positive cell fraction was much higher than that observed in the CD13 negative cell fraction. Direct comparison of ROS levels between the two cell fractions revealed that the CD13 positive cell fraction contains lower levels of ROS and expresses higher levels of GCLM [72]. In another study by the same group, CD13 expression reduced transforming growth factor- $\beta$  (TGF- $\beta$ )

induced ROS production and promoted survival of liver CSCs [73].

It has been demonstrated that signaling pathways involved in ROS regulation of normal stem cells also play a role in CSC biology. The nuclear expression levels of FoxO3a was high in chronic myeloid leukemia-initiating cells, and the transplantation of leukemic stem cells derived from *foxo3a* knockout mice significantly reduced their ability to cause myeloid leukemia in an animal model [74]. This study also revealed that TGF- $\beta$  is a crucial regulator of FoxO3a activity. In the SP of MCF-7 breast cancer cells, activation of the PI3K/mammalian target rapamycin (mTOR) signaling pathway was important for tumorigenicity of these CSCs, and knockdown of PI3K or mTOR led to ablated tumorigenicity [75]. When CD133<sup>+</sup>/CD44<sup>+</sup> prostate cancer cells were grown in sphere-forming conditions, activated PI3K/AKT signaling was found to be critical for maintaining CSCs [76]. CD44<sup>+</sup>/CD24<sup>-or low</sup> cells isolated from breast cancer cell lines and breast cancer patient specimens were radioresistant, and this resistant phenotype was associated with ATM signaling activation [77].

## 6. NRF2 as a Key Molecule for Redox Homeostasis

In 1990, Rushmore and Pickett discovered the enhancer sequence in the rat *gsta2* gene promoter as a response element to  $\beta$ -naphthoflavone and *t*-butylhydroquinone (*t*-BHQ) and named it antioxidant responsive element (ARE) [78]. Subsequent studies revealed that ARE is commonly involved in the transcription of multiple antioxidant and detoxifying genes, including glutamate-cysteine ligase (GCL), glutathione S-transferase (GST), and NAD(P)H quinone oxidoreductase-1 (NQO-1) [79, 80]. Based on sequence homology between ARE and MAF-recognition element (MRE), further studies hypothesized that small MAF and bZIP cap'n'collar (CNC) transcription factors may interact with ARE [81, 82]. Among bZIP CNC transcription factors, NRF2 was found to play a crucial role in ARE regulation, in which inducible expression of NQO1 and GST was ablated in *t*-butylhydroxy anisole-treated *nrf2* null mice, in contrast to the observation in wild type mice [83]. After this report, numerous studies have elucidated a wide spectrum of protective effects of NRF2 signaling against various stressors. For example, sensitivity to benzo[*a*]pyrene-induced carcinogenesis was significantly greater in *nrf2*-knockout mice than in wild type mice [84].

To account for the protective effects of Nrf2, comparative analyses of gene expression patterns were carried out in *nrf2*-deficient and wild type mice following treatment with Nrf2 activators. In global gene analysis of dithiolethione-administered mouse livers, Nrf2 was found to govern the expression of xenobiotic-detoxifying enzymes, GSH-generating systems, antioxidant proteins, and the molecular chaperone-26S proteasome [85]. Similarly, Hu et al. demonstrated that detoxifying enzymes, antioxidants, drug transporters, stress response proteins, and some signaling molecules serve as Nrf2-dependent and isothiocyanate-inducible genes in mouse liver [86]. It has now been firmly

established that NRF2 regulates divergent genes to coordinate xenobiotic detoxification and redox homeostasis [85, 87–89]. In its function as a regulator of cellular redox homeostasis, NRF2 elevates the expression of GCL and the cysteine transporter xCT to increase cellular GSH levels. NRF2 also enhances regeneration of reduced GSH by upregulating GPX and GSH reductase (GSR). Expression of thioredoxin 1 (TXN1), thioredoxin reductase 1 (TXNRD1), and peroxiredoxin 1/6, which can reduce oxidized protein thiols, is also under the control of NRF2. In addition, the levels of NADPH, a cofactor of many antioxidant enzymes such as GSR and TXNRD, can be increased by NRF2 (reviewed in Hayes and Dinkova-Kostova [90]). The expression of multiple NADPH generating enzymes such as G6PDH and 6-phosphogluconate dehydrogenase is upregulated by NRF2. Additionally, the role of NRF2 in ABC transporter expression for xenobiotic detoxification is notable. The basal expression level of Mrp1 was relatively lower in *nrf2*-deficient fibroblasts than that in wild type fibroblasts, and the treatment of mice with *Nrf2* activating diethyl maleate increased Mrp1 expression in the liver [91]. Levels of MDRI, MRP2/3, and BCRP were elevated following oltipraz treatment in primary human hepatocytes [92]. Sulforaphane (SFN) treatment enhanced the levels of MDRI, BCRP, and MRP2 in the blood-brain barrier of rats [93]. Our recent study showed that genetic activation of *NRF2* via KEAP1 silencing increases the expression of MDRI, MRP2/3, and BCRP in human proximal tubular epithelial cells [94]. As direct molecular evidence, functional AREs have been identified in human *MRP3* [95] and *BCRP* genes [96].

Kelch-like ECH-associated protein 1 (KEAP1), a cysteine-rich actin-binding protein, is the main negative regulator of NRF2 activity [83, 97]. Under quiescent conditions, NRF2 remains inactive by forming a complex with KEAP1 in the cytoplasm. NRF2 is subject to ubiquitination and KEAP1-induced proteasomal degradation through the Cullin 3 (CUL3) based E3 ligase. KEAP1 has three major domains as follows: (i) The BTB domain is associated with KEAP1 homodimerization, (ii) the IVR domain plays a role in regulation of KEAP1 activity, and (iii) the Kelch/DGR domain mediates binding with NRF2 [83, 90, 98–101]. The binding of NRF2 with KEAP1 has been described as the “hinge and latch” model, where one molecule of NRF2 interacts with the Kelch/DGR domains of the KEAP1 dimer through conserved motifs called ETGE (D/N-X-E-T/S-G-E) and DLG (L-X-X-Q-D-X-D-L-G) [102–104]. In these reports, it was shown that the binding affinity of the ETGE motif to KEAP1 is much higher than that of the DLG motif. It has therefore been shown that “latch” binding of the NRF2 DLG motif is easily broken by modifications of KEAP1 cysteine residues by ROS or electrophiles. In turn, disrupted DLG binding of NRF2 to KEAP1 leads to the blockade of ubiquitination and further degradation of NRF2, resulting in nuclear translocation of NRF2. It has been demonstrated that the sulfhydryl groups of multiple cysteine residues of KEAP1 can be directly modified by oxidation/reduction or alkylation. In particular, Cys151, Cys273, and Cys288 were found to be essential for the regulation of NRF2 activity [99, 104–106]. Mutation of the Cys273 or Cys288 residue of KEAP1 ablated its ability to

suppress NRF2 activity, leading to accumulation of the NRF2 protein [99, 104].

In addition to KEAP1-mediated stability regulation, NRF2 activity can be modulated at multiple steps. First, it is noticeable that NRF2 activity is regulated at the transcriptional step. Functional AREs were identified in the murine *nrf2* gene promoter and were involved in the autoregulation of NRF2 through transcriptional activation [107]. Moreover, a single nucleotide polymorphism in the ARE-like sequences of the human *NRF2* promoter was associated with increased lung cancer susceptibility [108]. Second, it was shown that NRF2 activity is regulated by posttranslational modifications. Studies indicate that NRF2 activation involves phosphorylation signaling mediated by multiple kinase pathways such as MAPK, protein kinase C (PKC), PI3K, and protein kinase RNA-like endoplasmic reticulum kinase (PERK) [109–111]. Meanwhile, glycogen synthase kinase-3 (GSK-3), a constitutively active serine/threonine kinase, was found to inhibit NRF2 activity [112]. Last, NRF2 activity is increased by several intrinsic proteins such as p21 and p62 [113, 114]. For example, p62, a linker protein of ubiquitinated proteins to autophagy degradation, binds to the KEAP1 protein and interferes with the binding of NRF2 to KEAP1, resulting in NRF2 stabilization.

## 7. Emerging Role of NRF2 in Cancer Biology

Continuous or fatal stimuli such as toxic chemicals and excess ROS disrupt cellular homeostasis, causing macromolecular damage and alterations in cell cycle and growth signaling, which can eventually result in carcinogenesis. The NRF2 pathway deserved significant attention in the area of cancer biology because numerous studies have demonstrated that activation of the NRF2 pathway decreases the sensitivity of cells to carcinogens [115–117]. For instance, the burden of gastric neoplasia caused by benzo[a]pyrene was effectively attenuated by the *Nrf2* activator oltipraz in wild type mice, whereas *nrf2* knockout mice did not show any protective effect of oltipraz [84]. Similarly, the incidence of N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) induced urinary bladder carcinoma was greater in *nrf2* knockout mice than in wild type mice, and oltipraz treatment reduced tumor incidence only in wild type mice [118].

Although it has been firmly confirmed that NRF2 activation can protect cells against a wide range of toxicants and stressors, aberrant activation of NRF2 has been associated with several types of cancers. NRF2 levels were constitutively elevated in cancer cell lines and tumor samples of the lung, breast, esophagus, endometrial cancers, and prostate cancers [119–125]. Molecular mechanisms involved in constitutive NRF2 activation include the following: (i) somatic mutations of *KEAP1* or *NRF2*, (ii) epigenetic silencing of the *KEAP1* gene, (iii) aberrant accumulation of proteins that compete with NRF2 for KEAP1 binding, and (iv) oncogene-mediated overexpression of NRF2 [101, 126]. First, somatic mutations of *KEAP1-NRF2* have been reported in an initial study by Padmanabhan et al. [127]. This study identified mutations

in the Kelch/DGR domain of *KEAP1* in lung cancer cell lines as well as lung cancer tissue samples and demonstrated that these mutant *KEAP1* proteins lost their NRF2 repressive function, which resulted in NRF2 accumulation. Singh et al. also demonstrated that the Kelch/DGR and IVR domains of the *KEAP1* gene contain multiple somatic mutations and these mutations were identified in 19% of tumor specimens from non-small cell lung cancer patients [123]. In gallbladder cancer, 4 of 13 patients harbored *KEAP1* mutations [122]. Shibata et al. reported that *NRF2* somatic mutations were found in 10.7% of primary lung cancer patients and 27.2% of primary head and neck cancer patients [122]. Notably, these mutations were primarily located in the DLG and ETGE motifs, and eventually led to the loss of a proper interaction between the NRF2 protein and *KEAP1*. Second, CpG island hypermethylation in the *KEAP1* promoter resulted in low *KEAP1* expression in lung cancer cell lines and tumor samples [124]. Third, in human hepatocellular carcinoma, p62 positive cellular aggregates were found with a frequency of 25%, and most of these tumors retained higher levels of NRF2 and its target gene expression [128]. Fourth, oncogenes have also been shown to play a role in NRF2 signaling. Oncogenic activation of KRAS (KRAS<sup>G12D</sup>), c-MYC (c-MYC<sup>ERT12</sup>), and BRAF (BRAF<sup>V619E</sup>) elevates the transcript levels of NRF2 and its target gene expression [129].

It is now widely accepted that aberrant activation of NRF2 can enhance cancer cell survival and growth in oxidizing tumor environments, and further promote chemo/radioresistance. Indeed, the prognosis of cancer patients negatively correlated with NRF2 levels in the tumor [122, 130]. The favorable effect of NRF2 overexpression on tumor survival and growth can be attributed to the increase in NRF2 target antioxidant proteins and their counteractive effect on oxidative stress. For instance, GSH, which is a direct target molecule of NRF2, has been shown to be critical for cell proliferation [131, 132]. In addition to its antioxidant contribution, Mitsuishi et al. provided direct evidence, demonstrating that NRF2 alters the cellular metabolism in relation to anabolic pathways to accelerate cell proliferation [133]. Multiple metabolic genes, such as those involved in the pentose phosphate pathway, were upregulated by NRF2 through ARE, and these changes promoted purine synthesis, glutamine metabolism, and NADPH production for enhanced cell proliferation.

Constitutively high levels of NRF2 have been associated with chemoresistance as well as radioresistance. Cancer cells with high NRF2 activity were less sensitive to cytotoxic chemotherapeutics such as cisplatin, doxorubicin, and 5-fluorouracil through facilitated detoxification of anticancer agents and enhanced antioxidant capacity [101]. It is therefore hypothesized that NRF2 inhibition can enhance the chemosensitivity of cancers. NRF2 siRNA could suppress cancer resistance to cisplatin, topoisomerase inhibitors, and 5-fluorouracil [101, 122, 134, 135]. Cancer cells with constitutively high NRF2 were protected against  $\gamma$ -radiation induced toxicity. Moreover, siRNA-mediated inhibition of NRF2 in non-small cell lung cancer cell lines substantially enhanced radiosensitivity [136]. Additionally, NRF2 expression was

increased during the acquisition of chemoresistance. In our previous study, doxorubicin-selected ovarian cancer cells demonstrated increased expression of NRF2 and its target genes for GSH synthesis, and NRF2 inhibition in this resistant cell line could restore doxorubicin sensitivity [137].

Our understanding of the role of NRF2 in cancer cell signaling has expanded. In particular, the relationship between oncogenic signaling and NRF2 is noteworthy. As mentioned earlier, activation of oncogenes such as *KRAS* and *c-MYC* increased the expression of NRF2 presumably through oncogene-mediated ROS increase, and this phenomenon appears to contribute to the maintenance of reduced redox homeostasis in cancer cells [129]. In ERBB2 (Her2/Neu) overexpressing ovarian cancer cells, the stable silencing of NRF2 repressed ERBB2 expression and its downstream signaling and retarded tumor growth. Therefore, the inhibition of NRF2 could sensitize these cells to taxol therapy by repressing ERBB2 expression [138]. Moreover, NRF2 was shown to be associated with HIF signaling, which is a critical factor for tumor angiogenesis. When NRF2 was stably knocked down in colon carcinoma cell lines, hypoxia-inducible HIF-1 $\alpha$  accumulation was abrogated and consequently, angiogenesis and tumor growth were significantly suppressed in NRF2 knockdown tumors compared to the control group [139]. In type 2 papillary renal cell cancer, which is characterized by loss of the fumarate hydratase gene and consequent metabolic alteration, accumulated fumarate was associated with tumor progression via NRF2 signaling. Fumarate was shown to modify *KEAP1* cysteine residues and elevate NRF2 levels, which contributed to the growth and progression of type 2 papillary renal cell cancer [140]. These accumulating lines of evidence suggest that once cells are transformed to the neoplastic stage, cancer cells utilize NRF2 signaling to adapt to the stressful tumor environment and to promote survival and further cancer progression (Figure 2).

## 8. Involvement of NRF2 Signaling in Stem Cell Quiescence and Differentiation

There is considerable evidence to suggest that NRF2 plays a role in normal stem cell biology [141–145]. For example, NRF2 activation in HSCs plays a critical role in not only the maintenance of quiescence but also in the determination of differentiation fate [141, 145]. In *Drosophila* intestinal stem cells, constitutive Nrf2 activation sustained quiescence by reducing the levels of ROS via upregulation of antioxidant genes such as *gclc*. However, in the case of *KEAP1*-mediated Nrf2 repression, high levels of intracellular ROS facilitated an ablation of the quiescent state in intestinal stem cells and age-related degeneration in the intestinal epithelium [146]. Similarly, low intracellular ROS levels are required for the maintenance of quiescence in human airway basal stem cells (ABSCs). When exposed to exogenous ROS, quiescent ABSCs enter the proliferation stage. Changes in ROS levels activate the NRF2-Notch pathway, which results in self-renewal and protection of ABSCs from ROS-induced hyperproliferation and senescence. Moreover, the quiescent

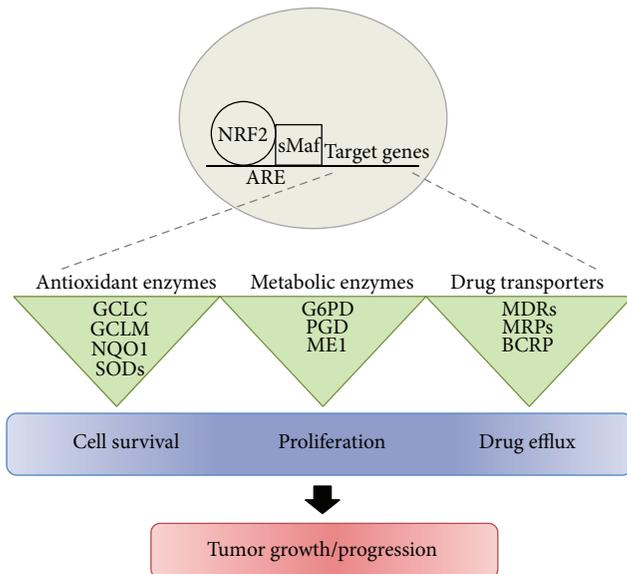


FIGURE 2: Implications of NRF2 signaling in cancer. NRF2 coordinates the expression of genes associated with cellular redox regulation, metabolism, and xenobiotic efflux, and thereby its aberrant activation promotes cancer cell survival, proliferation, and anti-cancer drug resistance. GCLM, glutamate cysteine ligase modifier subunit; GCLC, glutamate cysteine ligase catalytic subunit; NQO1, NAD(P)H:quinone oxidoreductase 1; SODs, superoxide dismutases; G6PDH, glucose-6-phosphate dehydrogenase; PGD, phosphogluconate dehydrogenase; ME1, malic enzyme 1, MDRs, multidrug resistance proteins; MRPs, multidrug resistance-associated proteins; BCRP, breast cancer resistance protein.

state of ABSCs was maintained by NRF2 activation [147]. In osteoclast progenitor cells, hydrogen sulfide ( $H_2S$ ) inhibited human osteoclast differentiation by NRF2-dependent induction of peroxiredoxin 1 and NQO1. These results were further confirmed using NRF2 activators including sulforaphane and *t*-BHQ [148]. It is also notable that NRF2 participates in the regulation of cell fate determination of HSCs. Murakami et al. demonstrated that HSCs derived from *KEAPI*-deficient mice exhibited preferred differentiation into the granulocyte-monocyte lineage rather than differentiating into the erythroid-lymphoid lineage [145].

Up to now, numerous studies have demonstrated that NRF2 plays a protective role against various stressors in stem cells. In neural stem cells, overexpression of NRF2 or pharmacological NRF2 activation prevented necrotic cell death [149]. In an animal study, *nrf2*-deficient mice showed defective stem cell function. HSCs from *nrf2*<sup>-/-</sup> mice expressed lower levels of pro-survival cytokines and exhibited spontaneous apoptosis [150]. Ionizing radiation-induced myelosuppression and mortality were mitigated through NRF2-mediated Notch signaling activation in HSCs [142]. Similarly, resveratrol-induced NRF2 expression improved the survival of cardiac stem cells and consequently regenerated infarcted myocardium [151, 152]. The heme oxygenase 1 (HO-1) inducer, cobalt protoporphyrin (CoPP) elicited an antiapoptotic effect on cardiac stem cells via activation of the

ERK-NRF2 pathway [153]. In neural stem cells, NRF2 activation by melatonin or *t*-BHQ ameliorated lipopolysaccharide (LPS) or  $H_2O_2$  induced cell death [149, 154]. In addition, amyloid  $\beta$ -mediated neural stem cell death could be alleviated by exogenous NRF2 transduction, which was accompanied by increased expression of GCLC, NQO-1, and HO-1. This study also demonstrated that neuronal differentiation of neural stem cells is enhanced by NRF2 activation [155]. Similar to neural stem cells, NRF2 has a protective role against hypoxic and oxidative stress conditions in undifferentiated MSCs. Treatment of the murine mesenchymal stem cell line with adrenaline increased the mRNA expression of *nrf2*, *gclc*, and *xCT*, leading to an increase in GSH levels and the prevention of ROS-induced cytotoxicity [156, 157].

## 9. Potential Implication of NRF2 in CSC Maintenance and Resistance

The role of NRF2 in CSC biology is now beginning to be unveiled. Similar to the case of normal stem cells, it was shown that NRF2 contributes to CSC stemness by maintaining their self-renewal capacity and protecting them from chemo/radiotherapy. Achuthan et al. established stable chemotherapy-resistant breast cancer cells and observed that these cells expressed higher levels of CD133 and OCT-4, indicating that these cells exhibit CSC phenotype [34]. Of note, it was shown that ROS levels were relatively low in these drug-selected cells, presumably due to higher levels of antioxidant enzymes such as SOD1 and GPX1/2. NRF2 protein stabilization was associated with high levels of antioxidant enzymes. As an underlying molecular mechanism, diminished proteasome activity and increased p21 levels appear to stabilize the NRF2 protein in these stem-like cells. Evidently, p21 knockdown repressed the mammosphere-forming potential of these stem-like breast cancer cells. Similarly, a study by Zhu et al. showed the involvement of NRF2 in glioblastoma stem cells that were isolated from human surgical glioblastoma specimens. *NRF2* knockdown in glioblastoma stem cells inhibited cell proliferation and neurosphere formation and further suppressed SOX2 expression. Moreover, *NRF2* knockdown changed the cell cycle distribution to the G2 phase and significantly attenuated the tumorigenicity of glioblastoma stem cells [158, 159]. These results are providing evidence that NRF2 is necessary for maintenance of the self-renewal capacity of glioblastoma stem cells.

On the other hand, activation of NRF2 signaling has been demonstrated in different types of CSC models, including lung, esophageal, breast, ovarian, and colon CSCs, and this is closely correlated with the maintenance of low intracellular ROS levels and chemoresistance of CSCs [160–165]. In lung and esophageal cancer cells, cigarette smoke condensate increased the SP as well as BCRP expression, which are hallmarks of CSCs [165]. Promoter analysis revealed that BCRP expression was associated with elevated levels of NRF2, aryl hydrocarbon receptor (AhR), and specificity protein 1 (SP1). Additionally, this study demonstrated that mithramycin diminished BCRP expression via repression of

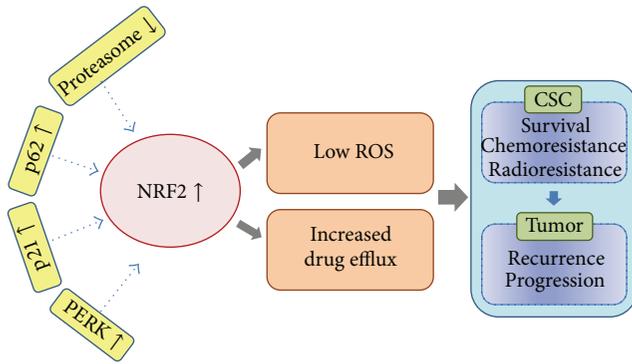


FIGURE 3: Potential roles of NRF2 signaling in CSCs. In CSC-like cell models, NRF2 is activated through multiple molecular mechanisms in a context-dependent manner. The upregulation of competing proteins such as p62 and p21, activation of PERK, or repressed proteasome function were shown to enhance NRF2 activity in these models. Elevated NRF2 levels in CSCs can contribute to the maintenance of low ROS by upregulating multiple antioxidant genes. In addition, NRF2-mediated expression of ABC transporters elicits efflux of anticancer drugs from cancer cells. Overall, activated NRF2 signaling facilitates CSCs survival and stress resistance, and consequently, it can be suggested that CSCs with high NRF2 activity play a crucial role in tumor recurrence and further progression.

NRF2, AhR, and SP1, and thereby inhibiting the expression of genes associated with CSC-related pathways, resulting in reduced proliferation and tumorigenicity. Similarly, it was reported that lung cancer SP cells exhibited high levels of NRF2 and BCRP expression. These SP cells were highly tumorigenic and possessed self-renewal capacity, compared to non-SP cells [164]. Emmink et al. performed a proteome analysis on collected secretome from highly tumorigenic CSCs and their corresponding nontumorigenic differentiated cells, both of which were established from human colorectal specimens [160]. Subsequent bioinformatic analysis revealed that the CSC secretome contained a large amount of proteins associated with cell survival and protein quality control, compared to differentiated tumor cells. Notably, the CSC secretome contained an NRF2 antioxidant and detoxifying protein signature, in that it included elevated levels of GCLC, GPX2/3, and TXNRD1. This study provided novel evidence that CSCs secrete NRF2 target antioxidant proteins to counteract extracellular stressors and chemotherapeutics. In patients with ovarian clear cell carcinoma, the expression of the CSC marker aldehyde dehydrogenase-1 (ALDH1) was strongly correlated with an advanced clinical stage and reduced progression free survival [161]. Ovarian clear cell carcinoma cells with high ALDH1 expression maintained low levels of ROS compared to ALDH-low cells, and these cells were shown to express higher levels of NRF2 and its target genes.

Two recent studies have demonstrated NRF2 activation in sphere cultures of breast cancer cells, that is one of models of CSCs. Wu et al. showed that mammospheres derived from MCF7 and MDA-MB231 breast cancer cell lines exhibited lower ROS levels compared to their monolayer counterparts. They also showed that levels of NRF2 and target genes such

as NQO1 and GCLM were elevated in mammospheres [163]. Similarly, our group has shown substantially elevated NRF2 protein levels along with increased expression of antioxidant genes (e.g., *HO-1* and *GPX2*) and drug efflux transporters (e.g., *MRP2* and *BCRP*) in sphere cultures of breast cancer cells. NRF2 accumulation was also observed in sphere-cultured ovarian and colon cancer cells. However, shRNA-mediated downregulation of NRF2 led to decreased chemoresistance of mammospheres presumably due to reduced levels of antioxidant genes and drug transporters. High ROS levels in NRF2 knockdown mammospheres caused sphere growth retardation and apoptosis. Coherently, ablation of ABC transporter induction in NRF2 knockdown mammospheres sensitized to anticancer agents [162]. Additionally, this study provided evidence that increased NRF2 protein expression in mammospheres can be linked to 26S proteasome reduction and p62 accumulation. In particular, knockdown of p62 in MCF7 mammospheres significantly attenuated NRF2 elevation.

Surviving dedifferentiated breast cancer cells after chemotherapy treatment retained high levels of NRF2 activation, similar to other CSCs. However, NRF2 activation was mediated by a noncanonical pathway. Levels of PERK were high in dedifferentiated cancer cells and this in turn phosphorylated and activated NRF2 signaling to maintain low cellular ROS levels and to express ABC transporters. In agreement with these findings, clinical observations revealed that the PERK pathway gene signature is related to chemoresistance and reduced patient survival [166].

## 10. Concluding Remarks

Recent studies have started to uncover the role of ROS signaling in the biology of CSCs, which is related to tumorigenicity, tumor progression, and relapse. Expression of the transcription factor NRF2, a master regulator of antioxidant genes expression, is increased in different models of CSCs, and this elevation is likely to promote CSC maintenance and survival in an oxidizing tumor microenvironment. In addition, NRF2-mediated overexpression of ABC transporters, particularly the CSC marker BCRP, may play a critical role in the multidrug resistance of CSCs. These findings, combined with the increasing evidence showing the alteration of KEAP1-NRF2 signaling in cancer cells, suggest a novel role of NRF2 in CSC maintenance and survival (Figure 3).

One important question that arises from the current studies is whether it is possible to design CSC-targeted therapies through regulation of the NRF2 pathway and its related redox homeostasis in CSCs. Recent studies provide several potential clues for addressing this question: the naturally occurring alkaloid brusatol could reduce the growth and chemoresistance of breast CSCs [163]. Treatment of mammospheres with brusatol elevated ROS levels and promoted taxol-induced growth retardation and cell death. The NRF2 repressive mechanism of brusatol has not been clearly elucidated, but it appears to be independent of KEAP1-mediated degradation [167]. In addition to brusatol, natural compounds such as

chrysin, apigenin, luteolin, and trigonelline are known to inhibit NRF2 signaling in several types of cancer cells [102, 168–170] and therefore the development of NRF2 inhibitors with characterized modes of action will enable efficient targeting of the redox homeostasis system as well as multidrug resistance systems in CSCs.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgment

This study was financially supported by the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2013RIA2A2A01015497).

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## Review Article

# Antioxidant Intake and Antitumor Therapy: Toward Nutritional Recommendations for Optimal Results

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Received 22 June 2015; Accepted 12 August 2015

Academic Editor: Sahdeo Prasad

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The role of the induction of oxidative stress as the mechanism of action of many antitumor drugs is acquiring an increasing interest. In such cases, the antitumor therapy success may be conditioned by the antioxidants present in our own body, which can be synthesized *de novo* (endogenous) or incorporated through the diet and nutritional supplements (exogenous). In this paper, we have reviewed different aspects of antioxidants, including their classification, natural sources, importance in diet, consumption of nutritional supplements, and the impact of antioxidants on health. Moreover, we have focused especially on the study of the interaction between antioxidants and antitumor therapy, considering both radiotherapy and chemotherapy. In this regard, we found that the convenience of administration of antioxidants during cancer treatment still remains a very controversial issue. In general terms, antioxidants could promote or suppress the effectiveness of antitumor treatment and even protect healthy tissues against damage induced by oxidative stress. The effects may depend on many factors discussed in the paper. These factors should be taken into consideration in order to achieve precise nutritional recommendations for patients. The evidence at the moment suggests that the supplementation or restriction of exogenous antioxidants during cancer treatment, as appropriate, could contribute to improving its efficiency.

## 1. Introduction

The first definition of antioxidant was proposed by Halliwell et al. in 1989 as “any substance that, present in low concentrations compared to oxidizable substrates (carbohydrates, lipids, proteins or nucleic acids), significantly delays or inhibits the oxidation of the mentioned substrates” [1]. Later, other definitions of antioxidant were proposed, such as “any substance that prevents, delays or eliminates oxidative damage of a target molecule” [2] or “any substance that can eliminate reactive oxygen species directly or indirectly, acting as a regulator of the antioxidant defense, or inhibiting the production of those species” [3].

Reactive oxygen species (ROS) are a group of molecules produced by some metabolic processes, due to the action of oxidases in the mitochondria or other cellular compartments. ROS have high reactivity because they possess unpaired

electrons that can interact with oxidizable substrates through redox reactions. The main ROS involved in the biological systems are superoxide anion, hydroxyl radical, hydroperoxyl and peroxy radical, nitric oxide, and other species such as hydrogen peroxide, singlet oxygen, and hypochlorous acid [4, 5]. However, there are other reactive molecules derived from the reaction of ROS with nitric oxide (reactive nitrogen species, RNS) or thiols (reactive sulfur species, RSS) [6] (Figure 1).

The balance between oxidants and antioxidants (redox balance) is essential in maintaining a healthy cellular microenvironment. The generation of oxidative stress is caused by an alteration in the balance between ROS production and the efficiency of the cell antioxidant defense system. Cells and tissues are continuously being exposed to free radicals derived from the metabolism or external factors, such as pollution, microbes, allergens, radiation, cigarette

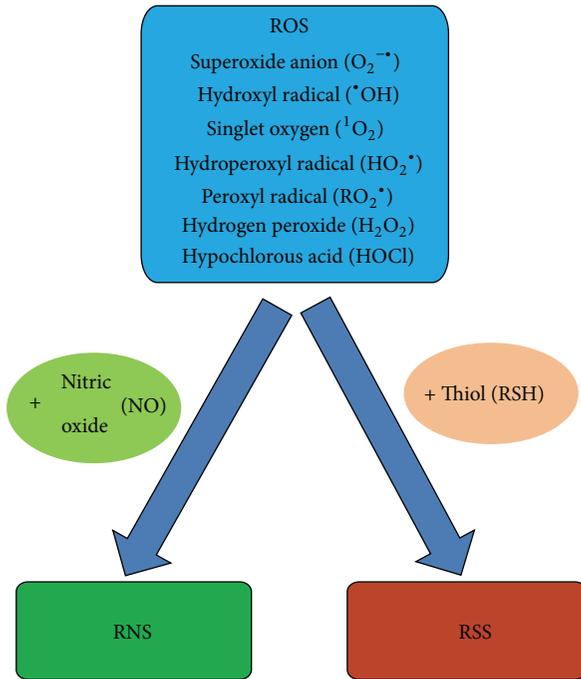


FIGURE 1: Oxygen reactive species (ROS) and derivatives. ROS includes superoxide anion, hydroxyl radical, singlet oxygen, hydroperoxyl radical, peroxyl radical, hydrogen peroxide, and hypochlorous acid. There are other reactive species which result from the reaction between ROS and nitric oxide (reactive nitrogen species, RNS), or with thiols (reactive sulfur species, RSS) [4–6].

smoke, and pesticides [7]. However, ROS can play a dual role, acting as beneficial or harmful factors [8]. On the one hand, the increase in ROS production generates oxidative stress, a damaging process that can alter cell structures and influences the expression of genes related to accelerated cell aging [9]. Nevertheless, ROS derived from the mitochondrial respiratory chain, at low or moderate concentrations, participate in physiological functions such as in the defense against infections and in the maintenance of redox balance [9, 10].

Cells have several mechanisms to transform and eliminate ROS to avoid their harmful effects. The synergistic action of both antioxidant proteins and enzymes and exogenous antioxidants neutralize free radicals and modulate cell signaling [11]. In fact, numerous studies suggest that antioxidants exert a protective effect against radiation and also prevent the development of many diseases such as cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration, and diabetes [12, 13].

## 2. The Antioxidant Defense

The natural antioxidant defense is composed of endogenous antioxidants, which are enzymatic and nonenzymatic antioxidants produced by our own body, and exogenous antioxidants, which can be incorporated through the diet or nutritional supplements [14]. Furthermore, there is another group that comprises synthetic antioxidants widely used in

the food industry, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). Several *in vivo* studies carried out in the 80s and the 90s reported some health risks associated with the consumption of synthetic antioxidants [15]. However, this is a controversial issue. A trial conducted in 1993 suggested that the toxic effects produced by BHA and BHT occur only at high doses in long-term treatments [16]. Another study found that the usual intake of BHA and BHT at low doses is not associated with stomach cancer risk [17]. More recently, the European Food Safety Authority (EFSA) studied in depth all the contradictory published data and established that the acceptable daily intakes of 0,25 mg/kg/day for BHA and 1,0 mg/kg/day for BHT are safe for adults and children [18].

Antioxidants can be classified into three lines of defense according to their mechanism of action. The first line includes antioxidants that prevent the formation of new free radicals. It is a very heterogeneous group which includes enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX); proteins that bind metals such as ferritin and ceruloplasmin; and minerals such as Se, Cu, and Zn. The second group of antioxidants is responsible for capturing free radicals, and thus they prevent oxidative chain reactions. This group is formed by the glutathione enzyme, albumin, vitamins C and E, carotenoids, and flavonoids. The third line of defense includes antioxidant enzymes that repair the damage caused by free radicals to biomolecules, such as lipases, proteases, DNA repair enzymes, transferases, and methionine-sulfoxide reductases [19–21]. Most exogenous antioxidants are produced by vegetables. Therefore, they are often called phytochemicals, although this is a concept which refers to any chemical compound derived from plants [22] (Figure 2).

## 3. Classification of Exogenous Antioxidants

Exogenous antioxidants constitute a very large and diverse group of molecules in terms of chemical structure and biological properties [23, 24]. Due to the abundance and diversity of members, this group can be divided into three subgroups: polyphenols, vitamins and derivatives, and antioxidant minerals [18].

Polyphenols are the most abundant natural antioxidants. The two main types of polyphenols are flavonoids and phenolic acids. For its part, flavonoids can be classified into several groups: flavonols, flavanones, flavones, catechins, anthocyanins, and isoflavones. Polyphenols are usually secondary metabolites involved in the defense against UV radiation or pathogens [25]. They are found in all plant products such as fruits, vegetables, juices, tea, and wine, and they contribute to their color, taste, smell, and oxidative stability [26]. Numerous epidemiological studies in the late twentieth century have suggested that polyphenols confer some protection against the development of prevalent diseases, including diabetes, infections, cancer, cardiovascular diseases, asthma, and osteoporosis [23, 27, 28].

Within the family of vitamins and derivatives, we want to highlight vitamins C, E, and K and carotenoids. Carotenoids

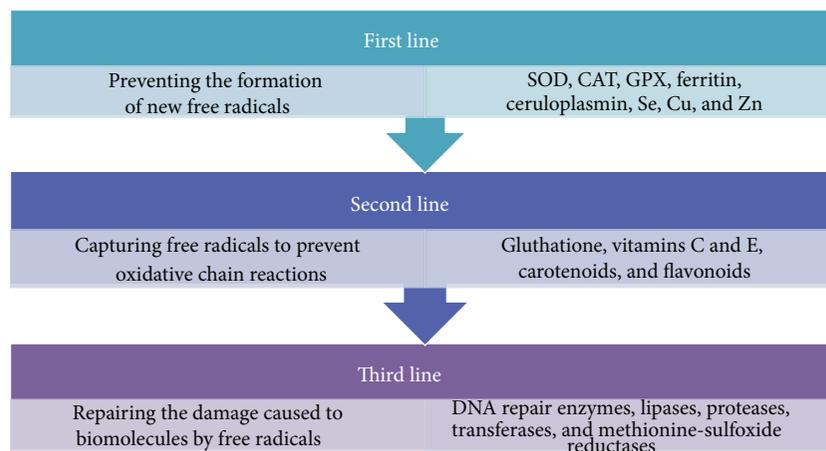


FIGURE 2: The antioxidant defense. The human antioxidant defense is composed of exogenous and endogenous antioxidants that can be classified into three different lines regarding their mechanism of action. The first line prevents the formation of new free radicals and includes SOD, CAT, GPX, ferritin, ceruloplasmin, Se, Cu, and Zn. The second line captures free radicals to prevent the oxidative chain reactions and includes glutathione, vitamins C and E, carotenoids, and flavonoids. The third line repairs the damage caused to biomolecules by free radicals and includes DNA repair enzymes, lipases, proteases, transferases, and methionine-sulfoxide reductases [19–21].

are a group of pigments present in many fruits and vegetables. There are more than 600 types, but only a few of them have demonstrated biological properties, as is the case of  $\beta$ -carotene and lycopene.  $\beta$ -Carotene is the most studied antioxidants for the prevention of diseases [29]. A product of the hepatic catabolism of  $\beta$ -carotene is vitamin A or retinol, which has beneficial effects on the skin, eyes, and internal organs, and that has the ability to combine and neutralize peroxyl radicals before they produce lipid peroxidation [30, 31].

Vitamin C or ascorbic acid is known by its electron-donating ability, thanks to which it prevents the accumulation of oxidizing agents and free radicals. It is especially efficient in eliminating superoxide anion radicals, hydrogen peroxide, hydroxyl, singlet oxygen, and RNS [32, 33]. Vitamin E family includes tocotrienols and tocopherols. They are highly lipophilic molecules that exert an antioxidant action due to their ability to join biological membranes, stabilizing and protecting them against lipid peroxidation [29]. Vitamin K is also lipophilic and it is involved in the blood clotting process. There are two known natural isoforms of vitamin K.  $K_1$  is present in green plants and is called phyloquinone, while  $K_2$  types are produced by bacteria of the intestinal flora and are called menaquinones. Although vitamin K is not considered a classic antioxidant, various studies have demonstrated its ability to slow the depletion of glutathione caused by oxidative stress [34].

Within the group of antioxidant minerals, selenium has a special importance because it is a cofactor of antioxidant enzymes such as GPX and thioredoxin reductase, among others [35]. Its role as part of the superoxide dismutase (SOD) and its capacity of inhibiting the NADPH oxidases that catalyze the transformation of oxygen into singlet oxygen radical are also relevant [18]. Similarly, it has been found that zinc can prevent lipid peroxidation and therefore protect cell membranes [36–38].

Apart from the antioxidants mentioned above, in recent years, the importance attributed to melatonin and N-acetylcysteine (NAC) as antioxidants has risen. Melatonin is the main product produced by the pineal gland. It exerts antioxidant activity both directly and indirectly, and it also has anti-inflammatory properties. Melatonin can directly eliminate free radicals such as hydroxyl radical, oxygen singlet, hydrogen peroxide, and peroxyxynitrite, and indirectly it induces the production of antioxidant enzymes, including GPX, glutathione reductase, Glucose 6P-DH, and SOD. Moreover, unlike classic antioxidants, melatonin does not produce a dose-dependent prooxidant effect, and it is able to cross the blood brain barrier [39]. For its part, NAC has mucolytic properties, it is the precursor of L-cysteine, and it is able to eliminate ROS and restore intracellular glutathione levels. In addition, recent studies indicate that NAC could cross the blood brain barrier, although depending on the dose and method of administration. Both melatonin and NAC also stand out for their low toxicity [40].

#### 4. Sources of Exogenous Antioxidants and Diet

Numerous studies have focused on determining the antioxidant content of foods, which conclude that the food with more antioxidant is derived from the plant kingdom (fruits, vegetables, and cereals), while meat and fish are poor in antioxidants. Comparing the group of meat and meat products, with plant foods such as fruits, nuts, cocoa, and berries, the latter are 5- to 33-fold richer in antioxidants than the former [41]. Therefore, diets mainly composed of animal source foods may not provide sufficient antioxidants, which could increase the oxidation of biomolecules and cell damage [42]. Nevertheless, proteins and hydrolysates derived from milk and eggs have shown some antioxidant activity [43].

TABLE 1: Classification of biologically relevant exogenous antioxidants and their natural sources [13, 41].

Exogenous antioxidants	Sources
Vitamins and derivatives	
Vitamin C	Berries, citrus fruits, some vegetables (peppers, cabbage), pulses, and some herbs and spices
Vitamin E	Seeds, vegetable oils, peanuts, nuts, and some fruits
Vitamin K	Green leafy vegetables, some herbs and spices
Carotenoids	
$\beta$ -Carotene	Many vegetables (spinach, carrots, pumpkins, and red pepper) and fruits (mango, apricots, and peaches)
Lycopene	Tomatoes, ketchup, and watermelon
Polyphenols	
Flavonoids	
Quercetin	Fruits (apples, citrus), onions, parsley tea, red wine, and green leafy vegetables
Catechins	Green tea, cocoa, and berries
Proanthocyanidins	Many fruits and vegetables, nuts, seeds, cocoa, and some medicinal herbs
Genistein and daidzein	Soy
Hesperetin	Citrus fruits
Resveratrol	Red grapes, red wine, peanuts, and berries
Phenolic acids	
Caffeic and chlorogenic acids	Coffee
Ferulic acid	Cereals, seeds, citrus fruits, and some vegetables

As stated above, polyphenols are the most abundant group of natural antioxidants. One of them, resveratrol, stands out for its antitumor properties, an aspect that will be discussed later in this paper. This molecule can be synthesized by a large number of plants, in which it seems to protect against different forms of stress such as heat, insects, bacteria, and fungi.

Resveratrol is present in common foods like red grapes and wine, peanuts, and berries [44]. Other important dietary polyphenols are catechins, present in green tea and some fruits [45, 46]; proanthocyanidins, present in many fruits and vegetables, nuts, and seeds [47]; quercetin found in fruits, vegetables, tea, and wine [48, 49]; genistein and daidzein in soy [50]; the phenolic acids in many fruits and vegetables; the hesperetin present in some citrus [51]; the chlorogenic and caffeic acids which abound in coffee [52]; and ferulic acid, found in cereals, citrus fruits, and some vegetables [53]. Cereals, pulses, and nuts also have important polyphenol content [41, 54] (Table 1).

Tea and coffee are very important sources of antioxidants for humans. They are rich in polyphenols and also the two most consumed beverages on the planet after water [54, 55]. Cocoa has a high content of flavanols and procyanidins, and *in vitro* studies have shown that it possesses anti-inflammatory, antiallergic, antiviral, and even antitumor properties [56–58].

Traditional medicinal plants are especially rich in antioxidants, for example, the sap from the trunk of *Croton lechleri* in Peru, known as “sangre de grado” or blood of dragon. This sap has been used for a long time by the Indians of South America to heal wounds, demonstrating antifungal, antiseptic, antiviral, and antihemorrhagic properties. Its main components are proanthocyanidins that have the ability to accelerate the healing of stomach ulcers and to induce

apoptosis in some tumor cells [59, 60]. Another interesting plant is *Triphala*, from India, which seems to possess anti-inflammatory, antibacterial, and anticancer properties [61].

Although herbs and spices constitute only a small percentage of the daily food intake, they can be an important source of exogenous antioxidants, especially in cultures where spices are regularly used for cooking [22]. Curcumin is extracted from the plant *Curcuma longa*, and it is commonly used in India. Curcumin has shown anti-inflammatory, antimicrobial, cardioprotective, and neuroprotective properties, among others [62–64]. Recently, it has been found that its mechanism of action involves the expression of antioxidant enzymes such as glutathione transferases, glutathione reductase, and catalase in liver, kidney, and small intestine [64–66]. The content of vitamin C and tocopherols in rosemary (*Rosmarinus officinalis*) [67], sage (*Salvia officinalis*) [68], and cat’s claw (*Uncaria tomentosa*) [69] extracts, which are currently used as alternatives sources to synthetic antioxidants in the food industry, has to be highlighted.

Vegetables, fruits, olive oil, and red wine are basic ingredients of the Mediterranean diet. These foods provide a wide variety of antioxidants such as vitamins C and E, polyphenols, and carotenoids [13]. In fact, beneficial effects of the Mediterranean diet, regarding the prevention of cardiovascular diseases and the improvement in cognitive status, have been reported [70, 71].

It is important to note that the antioxidant content of natural products and foods can vary for many reasons, such as the environmental and climatic conditions of growth, storage conditions, and the existence of genetically different varieties [72]. Similarly, the antioxidant content may be substantially modified after processing or cooking. This is the case of berries, which are an important source of flavonoids like tannins, stilbenes, lignans, and phenolic acids. However,

during the transformation process of berries into jams and syrup, the content of phenols is reduced up to half of the original amount [72, 73]. However, in other cases, the processing liberates elements included in the food matrix, leading to an increase in the content of certain antioxidants. An example is the tomatoes, since their content in lycopene is available only when they are processed by heat [74, 75].

## 5. Antioxidant Supplementation

Although cells possess a large repertoire of enzymes and antioxidants, sometimes these agents are insufficient to normalize the redox state produced by an intense oxidative stress [76]. In these cases, exogenous antioxidant supplements may be required to restore the cell redox homeostasis [77].

A nutritional or dietary supplement can be defined as any product directed to improve human nutrition and which must contain at least one dietary ingredient. The classic mode of administration of nutritional supplements is orally, in all physical forms, liquid, powder, tablets, capsules, drops, and ampoules. However, in some experimental trials, supplements can be administered by other routes, such as parenteral [78].

The composition of the supplements is very variable. They can contain only vitamins (vitamins C, B and/or multivitamins), only minerals (selenium, zinc, iron, or multimineral), or a combination of vitamins and minerals (multivitamin and multimineral; MVM). Other supplements are mixtures of oils and vitamins or minerals, or plant extracts (ginseng, fiber). Both in Europe and in the United States, the most consumed supplements are MVM, and the most consumed antioxidants are vitamins C, E, D, and A [79, 80]. Additionally, vitamin E is usually added as an antioxidant to preserve different formulations of supplements.

It has been suggested that antioxidant supplementation may protect against oxidative stress associated with the development of certain diseases or that it may reverse the oxidative stress produced during their course. This knowledge has contributed to the fact that the consumption of antioxidant supplements had become an increasingly common practice in the population for the maintenance of physical and mental health [81]. However, the reasons that justify antioxidant supplements consumption vary according to people's age and sex. Usually, older people take them to treat ailments or health problems, while young people consume supplements to achieve higher levels of body energy and to strengthen the immune system. Moreover, women are more likely to use supplements to prevent bone and colon diseases, while men take them to prevent cardiovascular diseases [79, 80].

AHRQ (Agency for Healthcare Research and Quality) in USA conducted a review of all the articles published between 1996 and 2006 related to the preventive effect of MVM supplements on the development of chronic disease [82]. The nutrients considered in the supplements were vitamins D, E, and A, folic acid, calcium, iron, selenium, and  $\beta$ -carotene; the diseases were hepatitis, AIDS, rheumatoid arthritis, renal failure, dementia, Parkinson, type II diabetes, cancer, and some ocular diseases (cataracts and macular degeneration), among others. Interestingly, they did not find significant

benefits of antioxidant intake for prevention of these diseases and very limited benefit of MVM supplements on primary cancer prevention. The only significant relation found was a reduction in the progression of macular degeneration in smokers who had taken zinc-based supplements.

However, several clinical trials demonstrated that some antioxidant supplements improve the recovery of patients who suffer diseases associated with an excessive production of ROS, for example, premature infants with bronchopulmonary dysplasia (vitamins A, E, recombinant human SOD, Zn, and Se), necrotizing enterocolitis (glutamine, arginine, and human recombinant SOD), periventricular leukomalacia (vitamin E, lactoferrin, and cysteine), or retinopathy (resveratrol, caffeic acid, and epicatechin) [83], and also in cases of idiopathic male infertility (vitamins C, E, coenzyme Q10, glutathione, and selenium) [84]. According to these results, in an experimental assay it was observed that pomegranate juice and resveratrol, orally administered to mice mothers, provide significant protection to their newborn pups against the brain damage caused by hypoxic-ischemic insult. Similarly, supplementation with omega-3 fatty acids could reduce brain damage from rodents, even five weeks after hypoxic-ischemic insult [85, 86].

We have found numerous studies focused specifically on the effect of antioxidant supplementation on cancer and its treatment. These themes will be discussed in the next two sections of this paper.

## 6. Role of Antioxidants in Human Health

Attending to the literature, we believe that antioxidants have impact on health. The questions to be answered are what is the right antioxidant for each particular physiological or pathological condition? And how the antioxidants must be taken, through food or as nutritional supplements? [87].

Many oxidative substances that penetrate into our body through ingestion, inhalation, or skin can be harmful. These substances can generate free radicals that are being accumulated. This accumulation can cause damage and even death due to the biological consequences, whether the antioxidant defense is sufficient or not. Currently, the main causes for reducing the plasma level of antioxidants are smoking and chronic alcoholism [21]. In the skin, for example, there is an antioxidant defense against UV radiation. It is formed by melanin and antioxidant enzymes but also by food antioxidants. This defense prevents swelling, wrinkling, and skin cancer. For that reason, some authors recommend the use of skin protective creams together with antioxidants orally or by topical application, to avoid the damaging effect of sun [88].

The benefit of antioxidant uptake has been demonstrated in the course of some diseases and certain conditions as diabetes, asthma, hemodialysis, thalassemia, rheumatoid arthritis, systemic attack, postmenopause, schizophrenia, depression, and leukemia [89, 90] (Figure 3).

The consumption of polyphenols has been associated with the prevention of the development of atheromatous lesions [91], the reduction of the size of such lesions *in vivo* [92, 93], and the inhibition of platelet aggregation *in vitro* [94] and *in vivo* [95]. In addition, polyphenols seem to reduce

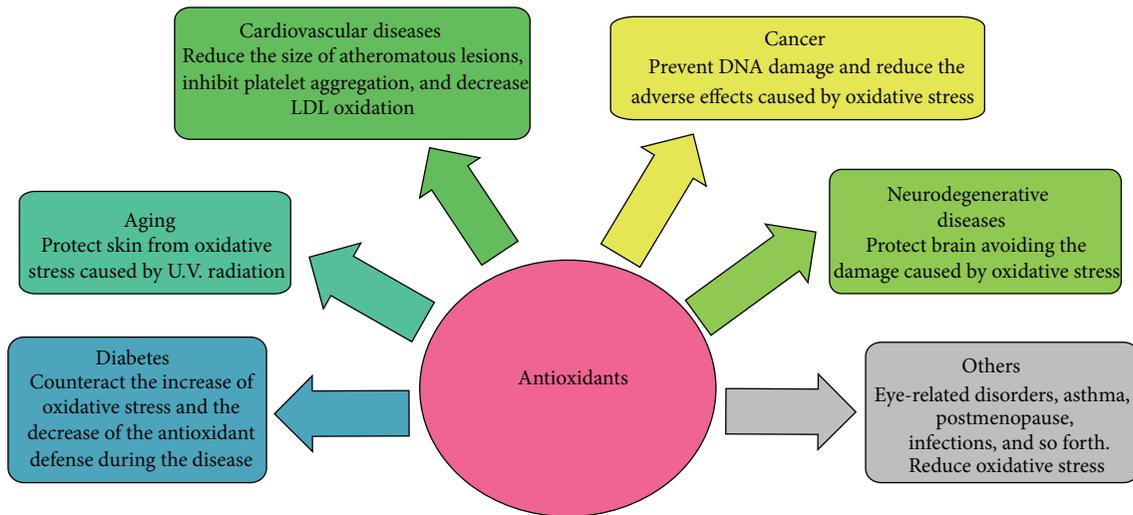


FIGURE 3: Influence of antioxidants on human health. Antioxidants can influence many aspects of human health such as diabetes, aging, cardiovascular and neurodegenerative diseases, cancer, and other illnesses. Antioxidants produce several beneficial effects, promoting a healthy status, reducing the oxidative stress caused by ROS [89, 90].

the oxidation of LDL, a process that may be responsible for atherosclerosis development. For its part, tea catechins inhibit proliferation and invasiveness of smooth muscle cells in the artery walls of experimental animals. This effect could contribute to reducing the formation of atheromatous lesions. However, this effect has not been fully clarified in humans [91].

Oxidative stress and the damage it causes in the brain are involved in the pathophysiology of highly prevalent neurodegenerative diseases. Several studies suggested that the consumption of foods rich in polyphenols can prevent the development of these diseases [96, 97]. Green tea provides protection against Parkinson [10], and daily consumption of wine has been linked to a lower incidence of dementia and Alzheimer [98]. In fact, it has been shown that dietary polyphenols act against hydrogen peroxide, being more effective than vitamins [99]. Similarly, the consumption of fruit and vegetable juices may also play an important role in delaying the development of neurodegenerative disease [100].

In the area of our interest, that is, cancer, antioxidants are acquiring great importance. It is believed that antioxidants can prevent the development of cancer due to their effects on cell cycle regulation, inflammation, the inhibition of tumor cell proliferation and invasiveness, the induction of apoptosis, and the stimulation of the detoxifying enzyme activity [29, 101]. The antitumor effect of some polyphenols, such as catechins, isoflavones, lignans, flavanones, resveratrol, ellagic acid, quercetin, and curcumin, has been extensively studied. It has been found that these compounds are able to reduce tumor growth through various action mechanisms, in different locations such as mouth, stomach, liver, lung, duodenum, colon, mammary gland, and skin [102–104].

One important antioxidant is resveratrol, since it has demonstrated both *in vivo* and *in vitro* ability to slow down tumor progression in experimental models of lung, skin, breast, and colon cancer, it interferes with the inflammatory

mechanisms, and it has antiangiogenic and antimetastatic properties [104–106]. These findings, coupled with the fact that high doses of oral resveratrol seem to be nontoxic, make resveratrol a promising antioxidant for cancer therapy [107].

Regarding the prevention of cancer, there are numerous studies that often provide conflicting conclusions. As an example, a systematic review of lung cancer concluded that there was evidence to recommend supplements of vitamins A, C, and E and selenium, both individually and in combination, to prevent lung cancer. This study also could indicate that the intake of  $\beta$ -carotene supplements may be associated with a small increase in the incidence and mortality from cancer in active and passive smokers [108]. This unfavorable aspect of the intake of supplements confirms the results of earlier trials as “The  $\alpha$ -Tocopherol and  $\beta$ -Carotene Trial” (ATBC) and “The Carotene and Retinol Efficacy Trial” (CARET), which also were conducted with smokers. Both studies had to be suspended after observing an increase in the incidence of lung cancer besides an increased mortality due to this cancer [14, 109].

However, some studies have shown reported benefits of consuming antioxidant supplements, such as the trial made by Lappe et al., which showed that supplementation with vitamin D and calcium could reduce the overall risk of cancer in postmenopausal women older than 55 years [110]. In this regard, there is an outstanding study of primary prevention, conducted in large-scale, named NIT (Linxian General Population Nutrition Intervention Trial), which initially involved 29,584 adults of both sexes. This trial evaluated the effect of the intake of supplements of  $\beta$ -carotene, selenium, and vitamins E and D, over 10 years in the Chinese town of Linxian. Interestingly, they found a decrease in mortality caused by cancer, especially in stomach cancer. Similarly, it was found that the treatment with supplements tends to be more beneficial in young people. So, the individual’s age appears to play a crucial role in the effects obtained [111].

There are some trials that suggest that flavonoids may have a preventive role against colorectal cancer recurrence. One of these studies involved a population of 87 patients who had undergone colon resection or polypectomy and took a supplement composed of a mixture of apigenin and epigallocatechin 3-gallate (EGCG). After 3-4 years of treatment and monitoring by colonoscopy, the results with this long-term treatment appeared to decrease the recurrence of colon cancer in patients with resected colon [112]. Likewise, a similar study found that a high intake of flavonoids was associated with a reduced risk of advanced adenoma recurrence [113]. Among flavonoids, the mentioned EGCG is the major green tea catechin that has been studied more intensively in recent years. Some studies have shown that the intake of this compound can inhibit the disease progression in lung, cervix, breast, stomach, liver, and colon cancer [114]. In addition, numerous clinical trials have been conducted to study the effects of this catechin. One of them involved 8000 patients with stage I or II of breast cancer. Its results revealed that daily consumption of green tea could reduce the recurrence of breast cancer and increase the disease-free survival [114, 115].

The SU.VI.MAX trial is a valuable study that showed controversial results. This study took place in France and included 7876 women aged between 35 and 60 years and 5141 men aged between 45 and 60 years. They were given daily oral supplements in capsules including 6 mg  $\beta$ -carotene, 120 mg vitamin C, 30 mg  $\alpha$ -tocopherol, 100  $\mu$ g selenium, and 20 mg zinc, individualized or mixed form [140]. The effects of each supplement were evaluated separately and combined. Individual selenium supplementation was associated with some protection against the development of cancer in general in both sexes. This fact has been confirmed by other studies [141]. However, combined therapy consisting of vitamins C and E,  $\beta$ -carotene, selenium, and zinc appeared to reduce the incidence of any type of cancer in men but not in women. Researchers attributed this result to the fact that the group of women was younger and less smokers and enjoyed better health than men. Furthermore, previous to the trial, blood tests showed that female samples were higher in vitamin C and  $\beta$ -carotene than male samples [140].

Interestingly, supplementation reduced the risk of prostate cancer in 94% of men, while the remaining 6%, who had a higher level of prostate-specific antigen (PSA) in serum, showed an increased risk of developing the disease. It is believed that this beneficial effect would be provided by selenium. This antioxidant mineral may be effective in healthy people or in early stages of the disease, but not in later stages, as in the case of prostate cancer associated with elevated levels of PSA [14, 142]. The intake of selenium as a supplement has shown no effect on the incidence of prostate cancer in patients at high risk for the disease, either with elevated PSA levels or under suspicion of cancer after a digital rectal examination [143]. However, selenium through diet has been associated with a lower risk of pancreatic cancer (up to 20  $\mu$ g/day), although this effect seems to disappear if there is an additional intake of MVM supplements that increase the levels of selenium [144].

There is evidence to suggest that the intake of tea and coffee antioxidants, especially vitamin E in form of gamma-tocopherol, would provide some protection against the development of prostate cancer [145, 146]. Similarly, in a clinical trial conducted in Canada, a group of men suffering prostate neoplasia were given daily supplements compound of soy proteins (40 g), vitamin E (800 IU), and selenium (200 mg) for 3 years. It was observed that this supplement appeared to reduce the incidence of prostate cancer [147]. Also, the effect of other minerals, such as zinc, could produce on this disease was assessed. Both in *in vitro* and *in vivo* studies, the ability of zinc was found to inhibit the proliferation of prostate tumor cells [148, 149]. Other studies have provided more data about the role of zinc in the course of the disease [150]. Furthermore, the epidemiological study conducted by Leitzmann et al. [151] showed that a high intake of zinc supplementation (>100 mg/day) would increase the risk of prostate cancer, while, according to Ho, the dietary deficiency of this mineral would increase the production of oxidative stress, and, thus, it would increase cell damage both *in vitro* and *in vivo* [152].

A study conducted in Bangladesh, which began in 2006, was to prove the administration of vitamin E and selenium for five years, individually and in combination, to offset the adverse effects of exposure to arsenic suffered by the population. The aim was to improve the skin lesions and reduce the incidence of skin cancer caused by arsenic toxicity. However, they found that although the treatment improved the evolution of lesions, there was an increase in mortality and skin dysplasia in the supplemented patients [153]. Similarly, recent *in vivo* studies conducted in mice have shown that the intake of supplements with vitamin E and NAC led to greater progression of lung cancer [154].

## 7. Antitumor Therapy, Oxidative Stress, and Interactions with Antioxidants

Some evidence suggests that cancer cells have a higher level of oxidative stress compared to normal cells. This stress is associated with an increased production of ROS and some changes in the metabolic activity related to oncogenic transformation [155]. Therefore, tumor cells may be more sensitive to drugs that generate big amounts of ROS, or drugs that damage the ROS scavenging capacity of cells, leading these cells to death by apoptosis [156]. Apoptosis is conducted by proteases called caspases, of which there are two main waterfalls, and acts to produce cellular DNA damage and disruption of microtubules [157].

In a multifactorial disease as cancer, an important aspect to consider is the relation between antioxidants and gene expression. Tumor cells show elevated levels of ROS, which may alter prooncogenic signaling pathways that contribute to the malignant phenotype of cells. In this sense, some of the most studied routes are Nrf2 and p53. Nrf2 belongs to an important signaling pathway that controls the expression of genes involved in the neutralization of oxidant agents [158], and the p53 pathway protects the DNA from the oxidation induced by ROS [159, 160]. Many signaling pathways associated with carcinogenesis are related directly or

indirectly to ROS metabolism. Thus, these pathways may also be influenced by the presence of antioxidants [161].

Increased ROS during cancer development makes tumor cells become highly dependent on antioxidant agents. For this reason, low concentrations of free radicals due to an excessive administration of antioxidants may promote the proliferation of harmful cells in the neoplastic state, promoting the development of cancer rather than interrupting it [101]. Another aspect to consider is that the intense generation of ROS in tumor cells could damage DNA, promoting the genetic instability and the development of drug resistance. However, it seems interesting to develop new therapeutic strategies to eliminate tumor cells using ROS-mediated mechanisms [155].

Radiation therapy is based on the ability of the ionizing radiation to kill cells. This therapy involves the generation of ROS, including hydroxyl radicals, superoxide anion, and other organic radicals, and also producing lipid peroxidation [126, 162]. In the presence of oxygen, these radicals cause increased formation of other ROS such as peroxides [163]. Therefore, radiation adverse effects would be influenced by these increased radicals, affecting the cellular antioxidant status [164]. In the trial conducted by Bairati et al. with head and neck cancer patients, who were treated with radiotherapy and supplemented with high doses of vitamin C and E, they seemed to improve the adverse effects, but also a loss of effectiveness of the treatment was observed, even an increased mortality in patients who received the treatment with antioxidants [116, 117]. There are several studies that have linked the consumption of these vitamins with improved adverse effects during both chemotherapy and radiotherapy [165–167]. However, other trials showed that the intake of vitamins does not improve the side effects and could even reduce the efficacy of the treatment [168] (Table 2(a)).

Moreover, some studies have reported that curcumin could have synergistic effect with radiotherapy, whether administered separately or in combination [136, 169]. It was observed that, using cell lines of head and neck squamous cell carcinoma (HNSCC), SCC1, SCC-9, A431, and KB, the combination of curcumin and radiation resulted in a greater antitumor effect [124]. The role of curcumin as a radiosensitizer has been supported by the results from other studies, such as the cases of prostate [170], breast [171], colorectal [172], and ovarian tumors [173], among others [62, 174].

Another promising radiosensitizer is EGCG. This catechin has shown synergistic effects with radiation on radioresistant glioblastoma multiforme, multiple myeloma (IM-9), leukemia (K-562), and cancer cervix (HeLa) cells [125]. Moreover, a recent clinical trial showed that EGCG may improve the prognostic of breast cancer patients under radiotherapy [119].

Melatonin is one of the most studied antioxidants in recent years, both in *in vitro* and *in vivo* assays. As it was hypothesized by Vijayalaxmi et al. [175], melatonin may slow the saturation of repair enzymes. This fact would lead to repairing the damage caused by oxidative stress and also would allow the use of higher doses of radiation in the treatment, making melatonin an ideal protective agent during radiotherapy. Although, in most studies, melatonin has been used at very high doses (it is not toxic up to 250 mg/kg), it

was found that its administration at low doses in mice, over a period of time (e.g., 0.1 mg/kg/day for 15 days before receiving radiation), appeared to be quite effective, so that the suitable dose of melatonin for humans in radiotherapy treatments is an issue that has to be investigated in more depth [126] (Table 2(b)).

As for chemotherapy, there are numerous agents that induce cell death by oxidative stress either directly, leading to the disruption of redox signaling and ROS scavenging, or indirectly by reducing intracellular levels of antioxidants and deactivating the cellular defense. Numerous articles have reported on many chemotherapeutic agents whose effects involve the induction of oxidative stress. Some of them are new molecules as Meroxest, a synthetic merosquiterpene derivative of the *trans*-communic acid, plentiful in *Cupressus sempervirens* [176], or Jadomycin, which is synthesized by the bacteria *Streptomyces venezuelae* [177]. Other compounds are part of the current therapeutic repertoire, like oxaliplatin [178], bleomycin [179], gemcitabine [180, 181], cyclophosphamide [182], celecoxib [183], capecitabine [184, 185], bortezomib (a proteasome inhibitor, approved for the treatment of multiple myeloma) [186, 187], and arsenic trioxide (ATO). ATO, which is used in the treatment of acute promyelocytic leukemia (APL), can produce a loss of permeability of the outer mitochondrial membrane and impair the function of the respiratory chain, leading to an increase in superoxide anion [188–191]. However, many of the agents that induce oxidative stress have hardly any studies about the interaction between their antineoplastic activity and antioxidants.

Then, we present the information about various antitumor drugs which have been selected according to their utility, therapeutic efficacy, and involvement in studies that were focused on the evaluation of the interaction with antioxidants during chemotherapy.

Anthracyclines are antitumor antibiotics commonly used in chemotherapy. They have been linked to the generation of oxidative stress and increased ROS levels and could act as mediators of apoptosis by the activation of caspases 3 and 9 [192–194]. Doxorubicin (Adriamycin) is a widely anthracycline used in the treatment of various cancers, including solid breast and prostate tumors. It exerts its antitumor activity by inhibiting topoisomerase II and generating ROS, hereby producing DNA damage and cell death by apoptosis [195, 196]. This increasing of ROS seems to play an important role in the cardiotoxicity caused by doxorubicin [197].

There are *in vitro* studies which have indicated that the administration of antioxidants could counteract the toxicity of this drug in cardiomyoblasts, although other studies have shown different results. For example, vitamin E could exert a cardioprotective effect but only against chronic cardiotoxicity, not against the development of chronic cardiomyopathy [198]. Recently, Wu et al. were able to reduce apoptosis in cardiomyocytes and also the oxidative stress in a model of heart failure in Japanese white rabbits, using intravenous injections of doxorubicin after being treated with NAC [127]. In another experimental trial, it was intended to evaluate the influence of vitamin C on the cytotoxicity caused by antineoplastic agents, such as doxorubicin. As a result, it was observed that when the level of vitamin C was increased, there was

TABLE 2: Antioxidants and antitumor therapy: (a) clinical evidence, (b) preclinical evidence.

(a)			
Clinical evidence			
Treatment	Disease	Results	Reference
High dose of vitamins C and E + radiotherapy	HNSCC	Improve adverse effects but decrease effectiveness of the treatment	[116, 117]
Normal dose of vitamins C, E and $\beta$ -carotene + cisplatin + radiation	Cervical cancer	Decrease oxidative damage, increased muscle strength, and less fatigue	[118]
EGCG + radiotherapy	Breast cancer	Decrease the levels of angiogenic factors and HGF	[119]
Uncaria tomentosa + FAC	Breast cancer	Decrease the adverse effects without interfering with the efficacy of treatment	[120]
NAC and vitamin E + vincristine, doxorubicin, cytosine arabinoside, cyclophosphamide, and 6-mercaptopurine + radiation	ALL	Decrease the incidence of toxic hepatitis Decrease the requirement of blood and platelet transfusions during treatment	[121]
Melatonin + cisplatin plus etoposide or cisplatin plus gemcitabine	NSCLC	Increase the rate of tumor regression and greater two-year survival rate	[122]
Melatonin + oxaliplatin and 5-FU	Gastrointestinal cancer		
Melatonin in combination with chemotherapy	Advanced NSCLC	Decrease the side effects with no better rates of survival	[123]
HNSCC: head and neck squamous cell carcinoma; ALL: acute lymphoblastic leukemia; NSCLC: non-small-cell lung carcinoma.			
(b)			
Preclinical evidence			
Treatment	Experimental model	Results	Reference
Curcumin + radiotherapy	SCCI, SCC-9, A431, and KB of HNSCC	Increase the antitumor effect of radiation	[124]
EGCG + radiotherapy	Tumor cervical cells (HeLa), multiple myeloma (IM-9), and leukemic (K-562)	Decrease cell proliferation Increase apoptosis and necrosis	[125]
Melatonin + radiotherapy	CD2-F1 mice	Increase the survival of animals	[126]
NAC + doxorubicin	Model of heart failure in Japanese white rabbits	Decrease apoptosis in cardiomyocytes	[127]
Vitamin C + doxorubicin	Cell lines of chronic myelogenous leukemia (K562) and lymphoma (RL) Mice with RL cell xenografts	Increase the resistance to treatment Larger tumors in mice	[128]
Suppression of Prdx + doxorubicin	MCF-7 human breast tumor cells	Increase the apoptotic effect of the drug	[129]
EGCG + doxorubicin	Colorectal tumor cells (BEL-7404/DOX)	Increase cell death and the sensitivity to the drug	[130]
Resveratrol + paclitaxel	Human breast tumor cells	Decrease the antitumor action of the drug	[131]
Nitroxide + docetaxel or doxorubicin	Mice with breast tumor cells xenografts	Decrease the side effects without interfering with the efficacy of treatment	[132]
Quercetin + cisplatin or 5-FU, taxol, or pirarubicin	Ovarian tumor cells (C13* and SKOV3)	High concentrations of quercetin: proapoptotic effect Low concentrations of quercetin: decrease the damage caused by ROS	[133]
Quercetin at low doses + cisplatin, 5-FU, taxol, or pirarubicin	Athymic nude mice with ovarian tumor cells (C13*) xenografts	Inefficiency in the treatment	[133]
High dose of vitamins A, E and selenium + cisplatin	Tumor cells of colon (COLO-205-GFP) induced in mice	Significant lower growth of tumors compared to the control tumors	[134]
Curcumin + cisplatin	Liver tumor cells (HA22T/VGH) HNSCC tumor cells (CAL27, UMSCC)	Increase the cytotoxic effect of the drug	[135] [136]
NAC before or up to 1 hour after the drug + cisplatin	Human ovarian carcinoma cells (SKOV3), human SCLC tumor cells (B.5 LX-1), human glioblastoma cells (U87), and rat Rat1 fibroblasts	Blocks the proapoptotic effect of the drug	[137]
NAC up to 4 hours after drug + cisplatin	Long-Evans rats	Otoprotective without interfering with the efficacy of treatment	[138]
Lycopene + cisplatin	Adult male Sprague-Dawley rats	Decrease the renal toxicity without interfering with the efficacy of treatment	[139]

a greater resistance to treatment in two cell lines of chronic myelogenous leukemia (K562) and lymphoma (RL). It also occurred in mice with RL cell xenografts. Moreover, after 32 days of treatment, when vitamin C was given to mice 2 hours before being treated with doxorubicin, the tumors became almost four times larger than the tumors of mice treated with just doxorubicin. So, they concluded that vitamin C seemed to interfere with the cytotoxic effect of doxorubicin [128].

In other cases, antioxidant supplements have shown positive effects, without affecting the effectiveness of treatment. The combination of 5-fluorouracil, doxorubicin, and cyclophosphamide (FAC) appears to involve a decrease in antioxidant levels, as a result of the lipid peroxidation produced in the cell membrane [199]. In a clinical trial conducted with patients treated with FAC who were in stage II of invasive ductal carcinoma of breast, the aim was to test the effectiveness of *Uncaria tomentosa*. It was observed that the patients who received chemotherapy along with 30 mg/day of the extract of the plant experienced a decrease of the adverse effects from chemotherapy such as neutropenia, without affecting the effectiveness of drugs [120]. Similarly, tannins (a type of polyphenols) administered during the treatment with doxorubicin showed their capacity of lowering the cardiotoxicity caused by the drug, without reducing its antitumor efficacy.

The ability of EGCG as an adjuvant in chemotherapy has also been investigated both *in vitro* and *in vivo* [125]. This catechin exerts synergistic effects with doxorubicin in chemoresistant models of hepatocellular carcinoma (HCC). In addition, *in vivo* studies showed that mice receiving EGCG with doxorubicin experienced a lower growth rate of liver tumors than mice that received only doxorubicin [130]. Similarly, other trials evaluated the combination of EGCG with other drugs such as 5-FU and cisplatin, and their conclusions also suggest the great potential of this catechin as adjuvant in anticancer therapy [200, 201].

A very important topic in antitumor therapy based on doxorubicin is the development of drug resistance. In this regard, the relationship between this resistance and the presence of endogenous antioxidants was recently described. So, McDonald et al. managed to demonstrate the involvement of peroxiredoxins (Prdx) in the doxorubicin resistance of MCF-7 breast tumor cells. Prdx are a family of six proteins expressed in mammals which are thiol-specific antioxidants. This trial showed that MCF-7 had elevated levels of Prdx compared to nontumor cells MCF-10A, and the levels of these proteins in line MCF-7 resistant to doxorubicin were higher. This study also reported that the suppression of the expression of four of these six Prdx led to increasing the apoptotic effect of doxorubicin [129].

Taxanes are anticancer cytotoxics that include paclitaxel, which is a natural antitumor drug used to treat various types of tumors. Numerous studies have indicated that it induces ROS and alters the permeability of the mitochondrial membrane producing  $H_2O_2$ . A recent study reported a reduction of glutathione levels in blood samples collected from patients treated with Paclitaxel, which implies that there was a decrease of the antioxidant potential of cells [202]. *In vitro* studies also point in the same direction. T47D

and MDA-MB231 breast tumor cells, treated with scavengers (NAC, catalase, or SOD), were able to maintain their viability. It was discovered how another agent, such as 2-deoxy-D-glucose (a competitive inhibitor of glycolysis), was able to promote a prooxidant effect of paclitaxel [203]. In other trials, it was shown that the administration of resveratrol, during the treatment with paclitaxel, decreased its antineoplastic action against breast tumor cells both *in vitro* and *in vivo* [131].

Docetaxel (Taxotere) is a derivative of paclitaxel that is often used as a first-line drug to treat prostate cancer and other types of tumors. According to some research, its way of inducing cell death would be due to microtubule depolymerisation [204]. It has also been reported that this drug is able to induce oxidative stress by activating caspase 3 [205, 206]. Recently, the prooxidant effect of docetaxel on breast tumor cells (MDA-231 and MCF-7) was demonstrated, which could be enhanced with the addition of C6 ceramide (a cell-permeable-short-chain ceramide), increasing the drug toxicity [207].

Attending to reduce the side effects of this drug, a reduction of oxidative stress in blood levels of mice with breast tumor cells xenografts was found, due to the supplementation of a nitroxide (3-carbamoylpyrrolidine nitroxyl derivative pirolin) when they were treated with docetaxel and doxorubicin. It also was found that this compound did not interfere with the antitumor activity of these drugs [132].

Cisplatin was the first heavy metal used for treating cancer and it has been widely used to treat solid tumors of lung, ovary, testes, and lymphoma, among others [208, 209]. Its mechanism of action involves the generation of an intense oxidative stress but also causes numerous side effects due to their toxicity [133, 210]. Its mechanism of action is associated with the expression of p53 (tumor suppressor gene), anti-apoptotic Bax proteins, p21 protein (cell cycle regulator), and the cleavage of PARP and caspases 3 and 9 [137]. After an extensive review, it has come to our attention that there is a large literature focused on the study of interactions between treatment with cisplatin and antioxidant supplementation, so this fact may be a reflection of the importance of this drug in the treatment of cancer. Here we report some of the most clarifying studies about this drug.

The role of quercetin is remarkable, since it has been reported in several studies that it seemed to act as an adjuvant in the treatment with cisplatin. In a recent study on the treatment with cisplatin in ovarian tumor cells (C13\* and SKOV3), it was found that high concentrations of quercetin ( $40\ \mu\text{M}$ – $100\ \mu\text{M}$ ) appeared to have a proapoptotic effect, while low concentrations ( $5\ \mu\text{M}$ – $30\ \mu\text{M}$ ) seemed to reduce the damage caused by ROS. This reduction of the damage was due to the increase of SOD, and therefore the antineoplastic effect of cisplatin was attenuated. Similarly, the interaction of quercetin with commonly used drugs in the treatment of ovarian cancer (5-FU, taxol, and pirarubicin) was analyzed and the results were alike. Moreover, *in vivo* studies using athymic nude mice with C13\* cells xenografts showed that low doses of quercetin could cause inefficiency in the treatment with cisplatin, 5-FU, taxol, or pirarubicin [133].

Other studies have reported that some antioxidants help to slow the progression of tumor cells. It was discovered

that tumor cells of colon (COLO-205-GFP) induced in mice that were treated with cisplatin and received high-dose supplements of vitamins A, E and selenium (5 times higher than the standard diet) along with fish oil experienced a significant lower growth compared to the control tumors [134]. However, the mechanism responsible for this effect has not been explained.

An interesting clinical trial evaluated the effect of vitamin supplementation on the quality of life of patients with cervical cancer, at different stages of the disease and undergoing treatment with cisplatin. In this case, chemotherapy was combined with radiation and cisplatin, and parallelly patients took vitamins C, E and  $\beta$ -carotene. Most of patients, aged between 29 and 73, displayed lower antioxidant levels than recommended (except for vitamin C and zinc) in the pretrial serum analysis. The results showed that women who took supplements during the treatment had less oxidative damage (lower concentration of free carbonyls in serum), their muscle strength was increased, and they showed less fatigue than women who did not take them. It is noteworthy that, in this study, the dose of supplement contained the recommended daily doses, not like other studies in which doses were much higher [118].

Curcumin is another antioxidant that, in addition to its mentioned radiosensitizer potential, has also been investigated in the role of adjuvant therapy with cisplatin. In an *in vitro* assay performed with liver tumor cells HA22T/VGH, it was reported that curcumin enhanced the cytotoxic activity of the drug [135, 211] and so it did against HNSCC tumor cells (CAL27 and UMCC lines) both *in vitro* and *in vivo* [136].

There have been numerous studies focused on the study of the effects of NAC during the treatment with cisplatin. It has been reported that the administration of NAC can reverse the cytotoxicity and the proapoptotic effects exerted by cisplatin, in human SKOV3 ovarian carcinoma cells, human B.5 LX-1 SCLC, human U87 glioblastoma cells, and rat Rat1 fibroblasts, reaching values of up to 99% reduction in its efficacy. Interestingly, they found that the proapoptotic effect of cisplatin was blocked by NAC if it was administered before or up to 1 hour later than the drug. In case of adding the antioxidant 8 hours after the cisplatin applying, no changes occurred in the proapoptotic effects [137]. Similarly, thanks to other studies *in vivo*, it has been found that NAC can be otoprotective when it is administered up to 4 hours after cisplatin [138]. It was also found that the best route of administration of NAC, in order to improve protection against the renal damage caused by cisplatin, is the intra-arterial (compared to the oral, intravenous, and intraperitoneal routes) [212]. According to these trials, it appears that both the timing and the route of administration of the antioxidant may be important factors to provide some nutritional recommendations associated with cancer treatment. In addition to NAC, other antioxidants have been evaluated to reduce the toxicity of cisplatin, such as lycopene, which has demonstrated its capability of reducing the renal toxicity induced by this drug [139].

The effect of NAC has also been evaluated in patients treated with combinations of chemotherapy agents plus radiotherapy. A clinical trial, in which 40 children with acute lymphoblastic leukemia (ALL) took part, was intended to assess whether the intake of NAC and vitamin E (400 IU/day)

orally would counteract the high toxicity from chemotherapy (vincristine, doxorubicin, cytosine arabinoside, cyclophosphamide, and 6-mercaptopurine) and the prophylactic cranial irradiation during the first two months of treatment. After analyzing blood levels of GPx, malondialdehyde (MDA), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and liver enzymes, the results indicated that children who received antioxidants showed lower incidence of toxic hepatitis and less probabilities of requirement of blood and platelet transfusions during treatment [121].

As mentioned earlier, melatonin has a great antioxidant capacity through different mechanisms of action. Therefore, we have referred to its role as adjuvant in chemotherapy to treat various cancers. In a clinical trial conducted by Lissoni, the effect of administration of 20 mg/day of oral melatonin was evaluated in patients with NSCLC treated with cisplatin plus etoposide or cisplatin plus gemcitabine, or gastrointestinal cancer treated with oxaliplatin and 5-FU. In both cases, patients who received melatonin had a higher rate of tumor regression and a greater two-year survival rate [122]. And more recently, Sookprasert et al. revealed the results of the MIRCIT trial, which concluded that advanced NSCLC patients receiving melatonin in combination with chemotherapy did not get better levels of survival, though the side effects were fewer [123]. It would be necessary to continue further studies to clarify the role of melatonin and to be able to compare these two clinical trials. This would allow us to establish the similarities and differences between the advanced and nonadvanced NSCLC patient, their appropriate treatment, and their basal serum levels of antioxidants, in order to compare the results of both studies.

## 8. Conclusions and Future Perspectives

Considering all the results exposed above, we conclude that antioxidant intake seems to influence the effectiveness of antitumor therapy and its adverse effects. However, we believe that at the moment it cannot be possible to give a general recommendation on whether or not to take antioxidants during treatment. This is because the final effect will depend on the type of cancer, the mechanism of action of the drug or drugs used in the treatment, and the type of antioxidants.

More studies are needed to clarify the results of the clinical trials, which sometimes are contradictory to each other. It is necessary to define the most appropriate patient profiles to adopt a nutritional regimen that could contribute to a better result of antitumor therapy. Aspects such as disease stage, treatment resistance, and previous cycles of chemotherapy and/or radiotherapy may be important factors in this regard.

The variable influence of antioxidants on antitumor therapy can be clearly illustrated considering lung cancer patients, for whom the intake of supplements with high doses of  $\beta$ -carotene is harmful, especially in smokers, and it has been correlated with a worse prognosis. However, mineral antioxidants seem to produce a beneficial effect on prostate cancer. Thus, the intake of selenium together with vitamin E, at physiologic doses and for a long period of time, appears to decrease the incidence of the disease. Similarly, zinc appears to inhibit the proliferation of prostate tumor cells. Paradoxically, zinc

must be ingested through the diet or as a supplement at physiologic doses (below 100 mg/day), because higher doses can produce the opposite effect. Furthermore, the administration of different antioxidants with the same antitumor drug can also produce very diverse effects. Thus, the intake of vitamin C supplements during the doxorubicin treatment has been associated with an acceleration of the malignant process, whereas if some polyphenols are administered, such as tannins, the systemic toxic effects of the drug could be reduced, without interfering with the efficacy of doxorubicin.

Some clinical studies have shown that certain antioxidants could have synergy with some drugs, enhancing its activity. In this regard, NAC, melatonin, and some flavonoids appear to be the most promising antioxidant candidates for cancer therapies. Since the antioxidant environment has been associated with a reduction of the activity of some drugs, it would be very interesting to conduct new trials, in which one of these antioxidants is administered to patients under nutritional restriction of antioxidants. The antioxidant status of cells has been correlated with the resistance against drugs that exert their antitumor effects through the induction of oxidative stress. Therefore, it would be interesting to evaluate the basal antioxidant status of the patients, by determining serum levels of antioxidants. This study could serve in the future to adjust the doses of exogenous antioxidants. The moment of administration of the exogenous antioxidants seems to be another important factor. In fact, and according to the results of the studies reviewed in this paper, it could be possible that dietary recommendations to patients should not be needed to be followed long time after receiving the drug dose. This is due to the fact that the interaction of antioxidant and drugs seems to disappear a few hours after their administration. Similarly, other important topics are the route of administration and the dosage of antioxidants, since they may affect the effectiveness of the treatment.

It is also important to deepen the understanding of the biochemistry of endogenous antioxidants which are responsible for the failure of some treatments, as in the case of Prdx proteins, which reduce the therapeutic efficacy of doxorubicin. This study would allow the discovery and development of specific inhibitors of such proteins and therefore improve the treatments. In fact, there are already specific inhibitors of SOD and other endogenous enzymes, such as DDT (sodium diethyldithiocarbamate) or ellagic acid, which are being studied to be used to enhance the effect of different cytotoxic agents.

Although it is possible to find in the literature numerous articles that discuss the interaction between cancer and antioxidant therapy, there are many drugs for which this kind of studies has not been performed yet. Future research would be helpful to establish concrete recommendations for patients, in order to improve the response to cancer treatment. In our opinion, it is necessary to define specific guidelines for each type of patient, which have to take into account the following: type of cancer; molecular subtype; stage of the disease; therapy, considering the type of drugs, their mechanisms of action, and the cycles; previous treatment; basal antioxidant status; the type of antioxidants that appear to produce a better response with the selected

treatment; route of administration; dosage; and the diet of the patient and habits, with the purpose of correcting the antioxidant intake if some restriction or supplementation is necessary. The main problems related to antioxidants clinical trials may be to define homogeneous groups of patients regarding the histopathological and molecular classification and treatment and to control the pool of serum antioxidant that results from the intake of supplements and habits (diet, alcohol, smoking, etc.). Therefore, these clinical trials should be addressed by multidisciplinary teams comprising at least oncologists and nutritionists.

We believe that nutritional recommendations about exogenous antioxidant supplementation or restriction, as appropriate, carried out in parallel to cancer treatment, could contribute to improving its efficiency.

## Disclosure

This paper is related to the Ph.D. thesis of Nuria Mut-Salud.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgment

This study was supported by the Regional Government of Andalusia (Project P11-CTS-7651 and assistance to the CTS-107 group).

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## Research Article

# Lipoxin A<sub>4</sub> Attenuates Cell Invasion by Inhibiting ROS/ERK/MMP Pathway in Pancreatic Cancer

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Received 21 May 2015; Accepted 27 July 2015

Academic Editor: Amit Tyagi

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Lipoxin A<sub>4</sub> (LXA<sub>4</sub>), an endogenous arachidonic acid metabolite, was previously considered an anti-inflammatory lipid mediator. But it also has the potential to inhibit cancer progression. To explore the therapeutic effect of LXA<sub>4</sub> in pancreatic cancer, we used Panc-1 cells to investigate the mechanism by which LXA<sub>4</sub> can attenuate pancreatic cancer cell invasion. Our data showed that LXA<sub>4</sub> significantly inhibited both cell invasion and the expression of matrix metalloproteinase- (MMP-) 9 and MMP-2. Further experiments implied that LXA<sub>4</sub> decreased the levels of intracellular reactive oxygen species (ROS) and the activity of the extracellular signal regulated kinases (ERK) pathway to achieve similar outcome to ROS scavenger N-acetyl-L-cysteine (NAC). However, a decreased level of intracellular ROS was not observed in cells treated with the specific ERK pathway inhibitor FR180204. The blocking of either intracellular ROS or ERK pathway caused the downregulation of MMP-9 and MMP-2 expression. Furthermore, tests revealed that LXA<sub>4</sub> inhibited MMP-9 and MMP-2 at the mRNA, protein, and functional levels. Finally, LXA<sub>4</sub> dramatically limited the invasion of CoCl<sub>2</sub>-mimic hypoxic cells and abrogated intracellular ROS levels, ERK activity, and MMPs expression. These results suggest that LXA<sub>4</sub> attenuates cell invasion in pancreatic cancer by suppressing the ROS/ERK/MMPs pathway, which may be beneficial for preventing the invasion of pancreatic cancer.

## 1. Introduction

Pancreatic cancer is the fourth-leading cause of cancer-related death in the United States [1]. Although biochemical and clinical studies have led to significant advances, the five-year survival rate remains less than 7% [1]. High invasive and metastatic tendencies are important characteristics of pancreatic cancer, which partially result in rapid progression and poor prognosis. However, the mechanisms that lead to invasion and metastasis in pancreatic cancer are still poorly understood.

Lipoxin A<sub>4</sub> (LXA<sub>4</sub>) is a type of metabolite that is derived from endogenous arachidonic acid (AA). Lipoxygenases (LOX), especially 5-LOX, 15-LOX, and 12-LOX, are key enzymes that contribute to LXA<sub>4</sub> biosynthesis [2]. Interestingly, aspirin tends to acetylate cyclooxygenase-2 (COX-2),

which changes its product from prostaglandin to an analogue of LXA<sub>4</sub> or aspirin-triggered lipoxin (ATL) [2]. Previously, LXA<sub>4</sub> was regarded as an anti-inflammatory, proresolution lipid that plays important roles in the programmed switch from inflammation to resolution [3, 4]. However, its various anticancer effects have been investigated in recent years. On the one hand, with its anti-inflammatory function, LXA<sub>4</sub> may block carcinogenesis through the attenuation of chronic inflammation, which usually presents as premalignant lesions; on the other hand, cancer cell proliferation, apoptosis, migration [5], and angiogenesis [6] can also be influenced by LXA<sub>4</sub> independent of its function in the resolution of inflammation.

Endogenous reactive oxygen species (ROS), including hydroxyl radical, superoxide anion, and hydrogen peroxide,

are mainly produced on the mitochondrial inner membrane during the process of oxidative phosphorylation via the electron transport chain. Generally, ROS can be scavenged by antioxidant systems. However, in cancer cells, excessive ROS overwhelms the capacity of antioxidant systems, which leads to oxidative stress; this in turn has been demonstrated to promote cell migration, invasion, and metastasis [7, 8].

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade extracellular matrix components. Specifically, MMP-9 and MMP-2 are thought to facilitate cancer invasion and metastasis. In pancreatic cancer, these two proteins are secreted by both pancreatic cancer cells and pancreatic stellate cells [9]. Our previous study demonstrated that miR-106a and miR-221/222 induced the overexpression of MMPs, which can significantly promote cell invasion [10, 11]. Additionally, the expression of MMPs is downregulated when the ROS/extracellular signal regulated kinases (ERK) pathway is blocked in breast [12] and prostate [13] cancers.

In this study, we demonstrate that LXA<sub>4</sub> can effectively attenuate cell invasion and MMP-9/MMP-2 expression in pancreatic cancer by inhibition of intracellular ROS accumulation and ROS-induced ERK activation. Furthermore, LXA<sub>4</sub> also reverses CoCl<sub>2</sub> mimetic hypoxia-induced MMP-9/MMP-2 overexpression as well as cell invasion.

## 2. Materials and Methods

**2.1. Materials.** The reagents used in this study include 5(S), 6(R)-Lipoxin A<sub>4</sub> (Cayman Chemical, Ann Arbor, MI, USA), N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, MO, USA), and FR180204 (Sigma-Aldrich). The following antibodies were purchased from Bioworld (St. Louis Park, MN, USA): anti-MMP-9, anti-MMP-2, anti-ERK1/2, anti-phospho-ERK1/2; an anti- $\beta$ -actin antibody was obtained from Sigma-Aldrich.

**2.2. Cell Culture.** The Panc-1 human pancreatic cancer-derived cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37°C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ExCell, South America) plus 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Gibco).

**2.3. Western Blot Analysis.** Panc-1 cells cultured under each experimental condition were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl-fluoride, 10  $\mu$ g/mL aprotinin, and 10  $\mu$ g/mL leupeptin), proteinase inhibitors (Roche, Mannheim, Germany), and phosphatase inhibitors (Roche) on ice for 30 min. The extracts were centrifuged at 12,000 rpm for 20 min at 4°C. Total protein (100  $\mu$ g) was electrophoresed in a 10% SDS-PAGE gel and then transferred to PVDF membranes (Roche), which were then blocked with 10% nonfat dry milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20). The membranes were incubated

with primary antibodies overnight at 4°C. After five washes of 10 min each in TBST, the membranes were incubated with HRP-conjugated secondary antibodies for 2 hours at 20°C and then washed again. The peroxidase reaction was performed using an enhanced chemiluminescence detection system to visualize the immunoreactive bands.

**2.4. Cell Invasion Assay.** A chamber-based cell invasion assay (Millipore, Billerica, USA) was performed to evaluate pancreatic cancer cell invasion. Briefly, the upper surface of the membrane was coated with Matrigel (BD Biosciences, Franklin Lakes, USA). Panc-1 cells ( $1 \times 10^5$ ) were suspended in the upper chamber in FBS-free media and allowed to migrate down a serum gradient (10%) in the lower chamber. The medium was aspirated from the inside of the insert and the noninvasive cells on the upper side were removed by scraping with a cotton swab. The membrane was fixed in 4% paraformaldehyde and was stained with crystal violet. The number of invasive cells was counted in 10 random fields on each membrane and photographed at 200x magnification. The values reported here are averages of triplicate experiments.

**2.5. Quantitative Real-Time RCR Assay (qRT-PCR).** Total RNA was extracted from Panc-1 cells with the Fastgen200 RNA isolation system (Fastgen, Shanghai, China), and reverse transcription was performed with a PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufactures' instructions. Real-time PCR was conducted as previously reported [14]. The PCR primer sequences for MMP-9, MMP-2, and  $\beta$ -actin are shown in Supplemental Table 1 (in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/6815727>). To quantitate the expression of each target gene, the expression was normalized to  $\beta$ -actin, and the comparative Ct method was used [15].

**2.6. Assay of Intracellular ROS.** The presence of intracellular ROS was tested as in a previous study. Panc-1 cells were incubated with 5  $\mu$ g/mL 2'-7'-dichlorofluorescein diacetate (DCF-DA) for 20 min. After washes with PBS, the cells were lysed in 1 mL RIPA buffer and were analyzed immediately by fluorimetric analysis at 510 nm. The data were normalized to the total protein content.

**2.7. Enzyme-Linked Immunosorbent Assay (ELISA).** The cells were conditioned in serum-free medium for 24 h. The culture supernatants were collected and centrifuged at 1,500 rpm for 5 min to remove particles; the supernatants were frozen at -80°C until use. The MMP-9 and MMP-2 levels in the supernatants of Panc-1 cells were assessed using a commercially available ELISA kit (R&D Systems, USA) according to the manufacturer's recommendations.

**2.8. Statistical Analysis.** The data are presented as the mean  $\pm$  the standard deviation (SD). The differences were evaluated by Student's *t*-test with SPSS 13.0. *P* values below 0.05

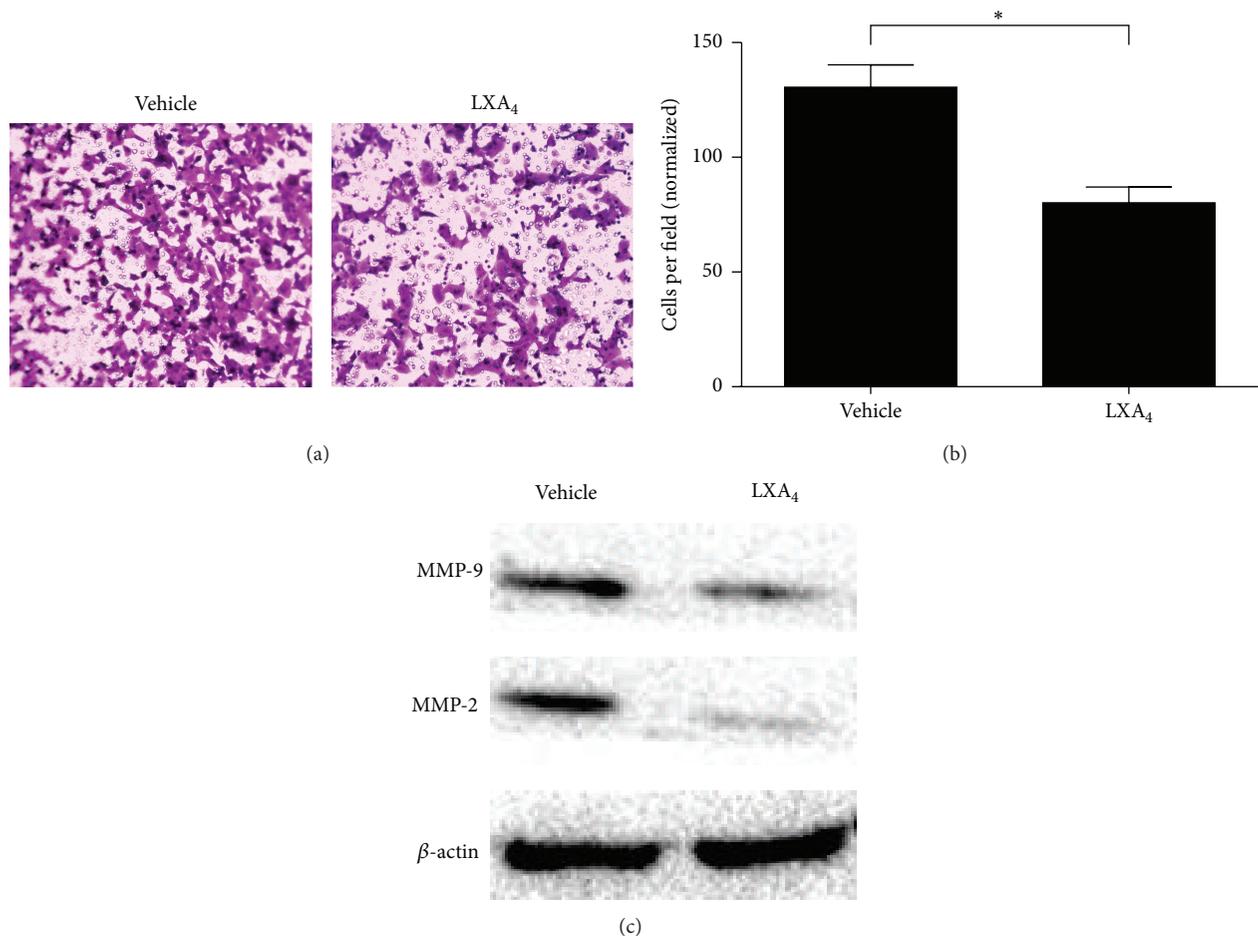


FIGURE 1: LXA<sub>4</sub> inhibited cell invasion and decreased expression of MMP-9 and MMP-2. (a) Effect of LXA<sub>4</sub> on cell invasion in Panc-1 cells. Cells were treated with either vehicle (methanol) or LXA<sub>4</sub> (400 nM) and incubated for 24 hours. Then  $1 \times 10^5$  cells were transferred into transwell chambers covered with Matrigel. Cultured for 48 hours, cells were stained with 0.1% crystal violet and finally observed and counted under microscope. (b) The quantified results of (a). (c) Representative western blot analysis of MMP-9 and MMP-2 in cells treated like above. \*  $P < 0.05$  versus vehicle control.

were considered statistically significant. All experiments were repeated independently at least three times.

### 3. Results

**3.1. LXA<sub>4</sub> Inhibits Cell Invasion and Decreases Expression of MMP-9 and MMP-2.** To test the influence of LXA<sub>4</sub> on pancreatic cancer *in vitro*, we chose the pancreatic cell line Panc-1, which was treated with either the vehicle control (methanol) or 400 nM LXA<sub>4</sub> for 24 hours. Then, to test the invasive capability of the treated cells, a transwell assay was performed, which showed that  $130.6 \pm 9.7$  cells in the vehicle control group passed through the Matrigel, whereas  $80.2 \pm 8.5$  cells in the LXA<sub>4</sub> group passed through the Matrigel (Figures 1(a) and 1(b)). This suggests that LXA<sub>4</sub> could significantly suppress cell invasion. MMP-9 and MMP-2 are two widely accepted proteinases that facilitate cell invasion and metastasis. We also observed that compared

with the vehicle control lower levels of MMP-9 and MMP-2 were expressed in Panc-1 cells after they were treated with LXA<sub>4</sub> (Figure 1(c)).

### 3.2. LXA<sub>4</sub> Attenuates Cell Invasion by Inhibiting ROS Pathway.

It has been reported that elevated intracellular ROS tends to enhance cell invasion [16], whereas LXA<sub>4</sub> can decrease intracellular ROS [17–19]. We treated Panc-1 cells with vehicle, LXA<sub>4</sub>, and ROS scavenger NAC at 20 mM. Then, we performed cell invasion assay, which demonstrated that fewer cells passed through the Matrigel after they were treated with LXA<sub>4</sub> and NAC compared with cells that were treated with vehicle (Figures 2(a) and 2(b)). This demonstrated that ROS might be involved in the regulation of cell invasion. At the same time, based on the intracellular ROS levels that were detected in Panc-1 cells that were treated with vehicle, LXA<sub>4</sub>, and NAC, the data suggest that LXA<sub>4</sub>, similar to NAC, decreased the amount of intracellular ROS compared with the vehicle control (Figure 2(c)). These data supported the

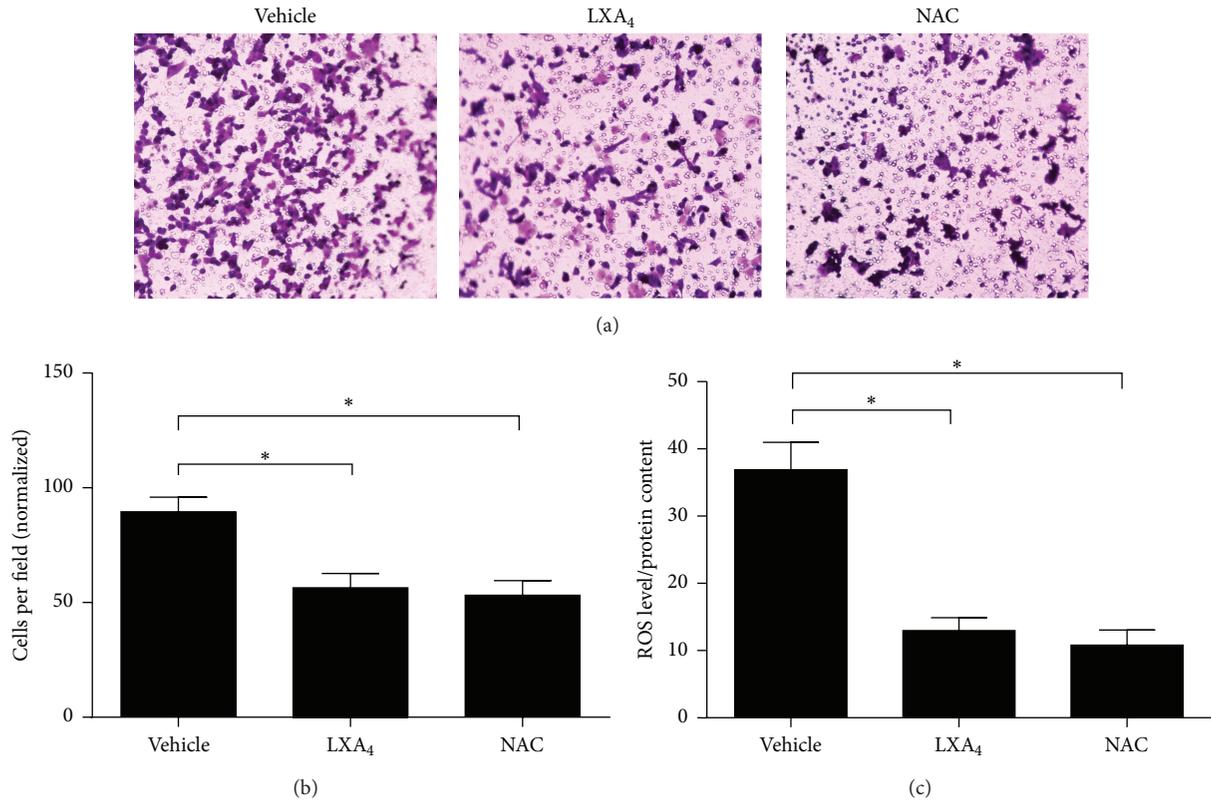


FIGURE 2: LXA<sub>4</sub> attenuated cell invasion via inhibiting ROS pathway. (a) Cell invasion tested by transwell chamber in Panc-1 cells treated with vehicle (methanol), LXA<sub>4</sub> (400 nM), or ROS scavenger NAC (20 mM). (b) The quantified results of (a). (c) Intracellular ROS determined in cells treated in (a). Cells incubated with DCF-DA for 20 min were washed with PBS three times and then lysed by RIPA lysis buffer and tested by fluorimetry at 510 nm. It was normalized by total protein. \**P* < 0.05 versus vehicle control.

concept that the suppression of ROS pathway by LXA<sub>4</sub> was responsible for attenuated cell invasion.

**3.3. LXA<sub>4</sub> Negatively Regulates Cell Invasion by Inhibiting ROS/ERK Pathway.** The ERK pathway, which is overactive in pancreatic cancer, is widely accepted to affect cell invasion [20]. When exposed to the specific ERK pathway inhibitor FR180204 (10 μM), cells present less aggressive invasion as LXA<sub>4</sub> and NAC (Figures 3(a) and 3(b)), which suggests that ERK might mediate LXA<sub>4</sub> attenuated cell invasion. Because ERK is reported to be a downstream pathway of ROS [12, 13], we detected ERK activity and showed phospho-ERK accounted for a lower proportion of total ERK when the cells were treated with LXA<sub>4</sub> and NAC (Figure 3(c)). However, cells that were exposed to FR180204 failed to show a decrease in intracellular ROS (Figure 3(d)). Our data confirmed that ROS could induce ERK activation, which suggests that LXA<sub>4</sub> could inactivate the ERK pathway via decreasing intracellular ROS. This in turn further downregulates cell invasion.

**3.4. LXA<sub>4</sub> Downregulated MMP-9/MMP-2 on Transcriptional Level rather than Translation or Secretion.** Our previous data demonstrated that LXA<sub>4</sub> could inhibit cell invasion via the downregulation of MMP-9/MMP-2 and the suppression of ROS/ERK pathway. However, it still needed to investigate

how LXA<sub>4</sub> influenced the expression of MMPs. Thus we performed ELISA assay to test secreted MMPs, which showed fewer amounts MMP-9 and MMP-2 were secreted by cells treated with LXA<sub>4</sub> (Figure 4(a)). At the protein level, as previous data (Figure 4(b)) have shown, MMPs were expressed to a lesser extent in the LXA<sub>4</sub>-treated group. Eventually, RT-qPCR demonstrated that LXA<sub>4</sub> could downregulate MMP-9 and MMP-2 at the transcriptional level (Figure 4(c)).

**3.5. LXA<sub>4</sub> Reverses CoCl<sub>2</sub>-Induced Cell Invasion through the ROS/ERK/MMP Pathway.** According to our previous study [21, 22], pancreatic cancer is a type of malignancy that demonstrates poor perfusion, and consequently a hypoxic microenvironment can dramatically increase intracellular ROS which may promote cell invasion and epithelial-mesenchymal transition (EMT). To test whether hypoxia could increase MMP-9 and MMP-2 levels and whether LXA<sub>4</sub> could reverse this overexpression, we added 0.15 mM CoCl<sub>2</sub> to mimic the cellular hypoxic state. In cell invasion assay, after a comparison with cells that were treated with vehicle control, we found that cells treated with CoCl<sub>2</sub> became more aggressive in nature. However, when they were treated with CoCl<sub>2</sub> + LXA<sub>4</sub>, the number of cells that passed through the Matrigel decreased (Figures 5(a) and 5(b)), which suggested LXA<sub>4</sub> reversed CoCl<sub>2</sub>-induced cell invasion. Next, the

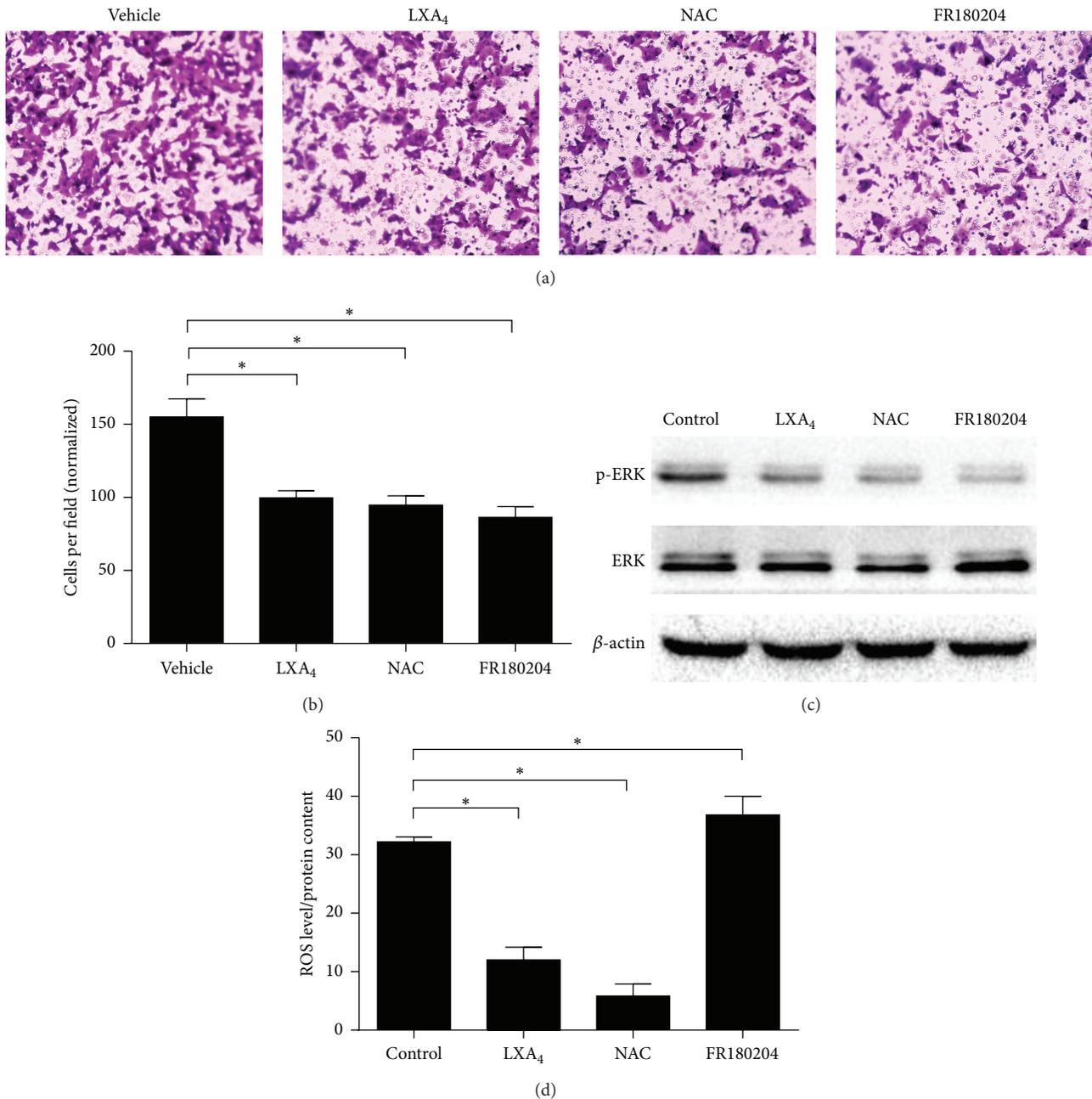


FIGURE 3: LXA<sub>4</sub> negatively regulated cell invasion by inhibiting ROS/ERK pathway. (a) Influence of LXA<sub>4</sub> on cell invasion in Panc-1 cells. Cells were treated with vehicle (methanol), LXA<sub>4</sub> (400 nM), ROS scavenger NAC (20 mM), or ERK specific inhibitor FR180204 (10 μM) for 24 hours. Then  $1 \times 10^5$  cells were transferred into transwell chambers covered with Matrigel. After forty-eight hours, cells were stained with 0.1% crystal violet, observed, and counted under microscope. (b) The quantified results of (a). (c) Representative western blot analysis of activated p-ERK and total ERK in cells treated as in (a). (d) Intracellular ROS determined in cells treated in (a). Cells incubated with DCF-DA for 20 min were washed with PBS three times and then lysed by RIPA lysis buffer and tested by fluorimetry at 510 nm. It was normalized by total protein. \* $P < 0.05$  versus vehicle control.

expression of MMP was measured. Cells that were treated with CoCl<sub>2</sub> overexpressed MMP-9 and MMP-2, which was reversed by CoCl<sub>2</sub> + LXA<sub>4</sub> (Figure 5(c)). This demonstrates that LXA<sub>4</sub> could reverse the CoCl<sub>2</sub>-induced overexpression of MMPs. Furthermore, an assay to determine intracellular ROS assay showed that CoCl<sub>2</sub> upregulated intracellular ROS

while LXA<sub>4</sub> could attenuate that effect (Figure 5(d)). In addition, the cellular ERK pathway was activated when the cells were cultured with CoCl<sub>2</sub>, but it was inactivated by LXA<sub>4</sub> (Figure 5(e)). These data implied that inactivation of the ROS/ERK/MMP pathway might be involved in the reversal of CoCl<sub>2</sub>-induced cell invasion.

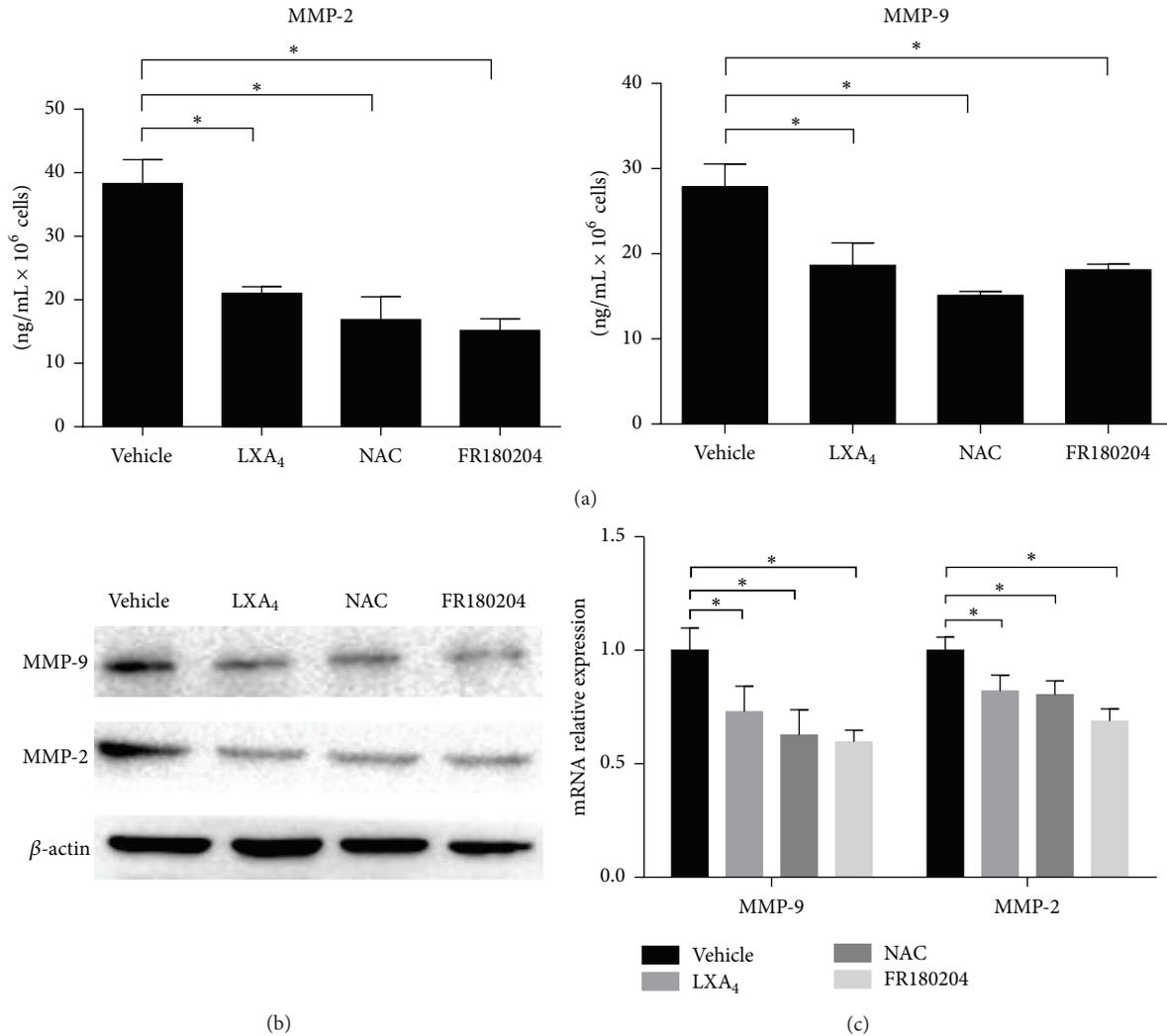


FIGURE 4: LXA<sub>4</sub> downregulated MMP-9 and MMP-2 mRNA transcription. (a) Secretion of MMP-9 and MMP-2 influenced by LXA<sub>4</sub>, NAC, or FR180204. Cells were cultured with FBS-free medium for 24 hours and then MMP-9 and MMP-2 secreted into mediums normalized by cell number were tested by ELISA. (b) Western blot analysis of MMP-9 and MMP-2 in Panc-1 cells treated like above. (c) Transcription of MMP-9 and MMP-2 tested by RT-qPCR. \**P* < 0.05 versus vehicle control.

#### 4. Discussion

Pancreatic cancer is characterized by early invasion and metastasis, which partially account for a compromised therapeutic effect and poor outcome [23]. Therefore, it is necessary to establish new methods to control cell invasion and metastasis. In the present study, we show that LXA<sub>4</sub> tends to attenuate cell invasion *in vitro*.

LXA<sub>4</sub> has been described as an anti-inflammatory and proresolution small lipid mediator. Over the last few decades, several studies have reported that LXA<sub>4</sub> might exert powerful anticancer effects. Here, our results demonstrate that LXA<sub>4</sub> downregulates intracellular ROS to inhibit cell invasion, which is in agreement with data of previous studies on inflammation [18, 19] and endothelial cells [17]. Some studies have revealed that the LXA<sub>4</sub> analog ATL acts as a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor,

and thus it can block the production of intracellular ROS [17, 19]. In addition, LXA<sub>4</sub> can also block neutrophil-platelet interactions; this reduces neutrophil-derived ROS, which is a characteristic of inflammation [18]. However, the results of another study contradict the aforementioned results. That study showed that LXA<sub>4</sub> activates rather than blocks NADPH oxidase and COX-2 to elevate ROS production in rat aortic cells [24], which indicates that LXA<sub>4</sub> may have different functions in different tissues.

ROS were originally regarded as promoters of cancer because of their role in tumor initiation, promotion, progression, and tissue destruction [25]. However, accumulating evidence indicates that ROS may play dual roles in cancer in a dose-dependent manner [7, 8]. On the one hand, mild intracellular ROS orchestrates various cell signals to promote cancer advancement, and therefore the suppression of ROS can attenuate cancer progression, including invasion. Our

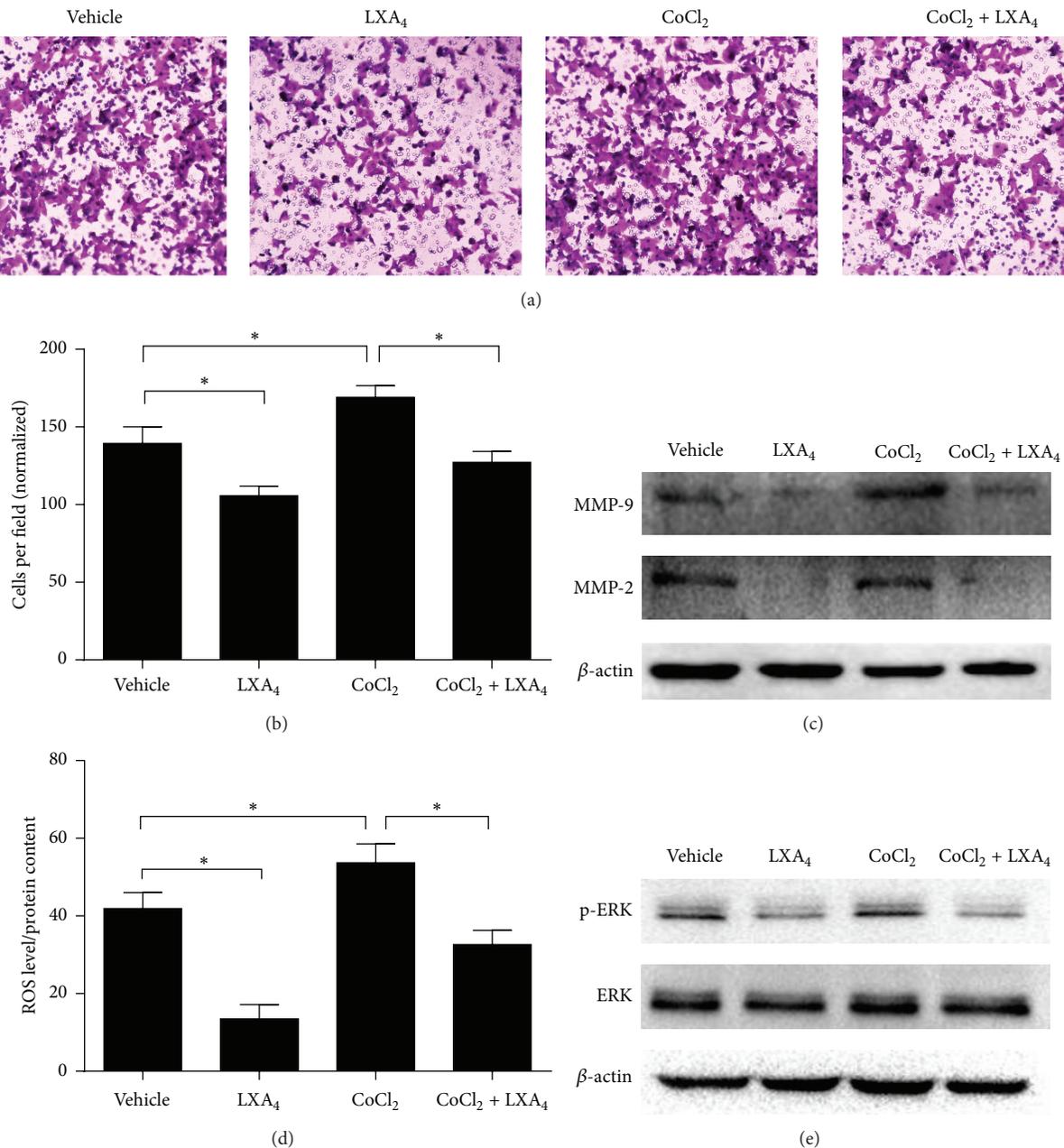


FIGURE 5: LXA<sub>4</sub> reverses CoCl<sub>2</sub>-induced cell invasion through ROS/ERK/MMP pathway. (a) Effect of LXA<sub>4</sub> on CoCl<sub>2</sub>-induced cell invasion. Panc-1 cells were treated with vehicle (methanol), LXA<sub>4</sub>, CoCl<sub>2</sub> (0.15 mM), or CoCl<sub>2</sub> + LXA<sub>4</sub>. Cell invasion assay was performed when cells had been transferred into transwell chamber for 48 hours. (b) The quantified data of (a). (c) Western blot analysis of cells treated as above. (d) Intracellular ROS determined in cells treated in (a). Cells incubated with DCF-DA for 20 min were washed with PBS three times and then lysed by RIPA lysis buffer and tested by fluorimetry at 510 nm. The absorbance was normalized by total protein. (e) Expression of activated p-ERK and total ERK detected by western blot. \**P* < 0.05 versus corresponding control.

data show that the intracellular ROS inhibited by LXA<sub>4</sub> or NAC reduce cell invasion and thus support this perspective, as in our previous study, where we illustrated that the depletion of H<sub>2</sub>O<sub>2</sub> by catalase limits pancreatic cell invasion [22]. On the other hand, extremely high levels of ROS, which are usually induced by radiation therapy or chemotherapeutic agents, destroy almost all cellular components, which then triggers cell death. The present study is not concerned

with radiation and other therapeutic agents, and thus the intracellular ROS level is not so high as to limit cancer progression; hence, the scavenging of ROS by LXA<sub>4</sub> induces anticancer effects.

Invasion is widely accepted as a hallmark of cancer [26], especially in pancreatic cancer. Studies have been conducted in this field for several decades, but invasion is still responsible for the poor outcome of patients with

pancreatic cancer. In recent years, studies that have focused on the tumor microenvironment revealed that a remodeled tumor extracellular matrix (ECM), which is affected by cancer cells and stroma, facilitates cancer cell invasion [25, 27, 28]. MMPs secreted by cancer cells play a key role in the degradation of the ECM, which weakens the natural barrier and inhibits cell invasion [29]. However, MMPs are regulated by different cellular signals. Several studies have demonstrated that mitogen-activated protein kinase (MAPK) pathways, especially ERK, regulate MMP expression [12, 13, 30–32]. In fact, most patients with pancreatic cancer carry mutational activation of the KRAS oncogene [23] which partially accounts for a dramatically activated ERK pathway, overexpression of MMPs, and obvious invasive potential [20]. Additionally, an overactivated ERK pathway in cancer may also be regulated by ROS [33]. In our study, through a comparison of intracellular ROS and ERK activation between the NAC- and the FR180204-treated groups, we can conclude that ROS acts upstream of ERK. This result is in accordance with that of other studies discussed above. Furthermore, our data also elucidate that the inhibition of the ROS/ERK pathway by LXA<sub>4</sub> efficiently downregulates the expression of MMP-9 and MMP-2, which attenuates cell invasion. Finally, we confirmed that the inhibitory effect of LXA<sub>4</sub> on the expression of MMPs is implemented at the transcriptional level.

Poor perfusion is another characteristic of pancreatic cancer [23], which typically is associated with a hypoxic microenvironment. Hypoxia promotes pancreatic cancer progression through various means including the enhancement of cell invasion [21, 34]. In the last part of our study, we treated the cells with CoCl<sub>2</sub> to mimic a hypoxic environment. Our results show that ROS production dramatically increases with hypoxia and that consequent ERK pathway activation leads to the overexpression of MMP-9 and MMP-2, which promotes cell invasion. Encouragingly, the protective effect of LXA<sub>4</sub> exists even in this hypoxic model, which indicates that LXA<sub>4</sub> is more likely to be effective against pancreatic cancer *in vivo*.

In summary, our present study showed that the endogenous AA metabolite LXA<sub>4</sub> could attenuate pancreatic cancer cell invasion via the inhibition of the ROS/ERK/MMP pathway. Our data also revealed that in a CoCl<sub>2</sub>-induced hypoxic model cancer cells tended to upregulate the ROS/ERK/MMP pathway to obtain aggressive, invasive behavior and that this effect could be reversed by LXA<sub>4</sub>. This implies that LXA<sub>4</sub> may be a novel agent that targets the ROS/ERK/MMP pathway to prevent or control cancer cell invasion.

## 5. Conclusion

Our work demonstrates that LXA<sub>4</sub> attenuates cell invasion in pancreatic cancer by suppression of the ROS/ERK pathway and consequent MMP-9/MMP-2 transcription not only in a pancreatic cancer cell line but also in a CoCl<sub>2</sub>-induced model of hypoxia. This suggests that LXA<sub>4</sub> may be a novel agent that targets the ROS/ERK/MMP pathway to prevent or control cancer cell invasion.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Acknowledgment

This study was supported by grants from the National Natural Science Foundation of China (no. 81301846 and no. 81402583).

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## Research Article

# The Cellular Response to Oxidatively Induced DNA Damage and Polymorphism of Some DNA Repair Genes Associated with Clinicopathological Features of Bladder Cancer

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Received 18 May 2015; Revised 26 June 2015; Accepted 21 July 2015

Academic Editor: Amit Tyagi

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Genome instability and impaired DNA repair are hallmarks of carcinogenesis. The study was aimed at evaluating the DNA damage response in H<sub>2</sub>O<sub>2</sub>-treated lymphocytes using the alkaline comet assay in bladder cancer (BC) patients as compared to clinically healthy controls, elderly persons, and individuals with chronic inflammations. Polymorphism in DNA repair genes involved in nucleotide excision repair (NER) and base excision repair (BER) was studied using the PCR-RFLP method in the Belarusian population to elucidate the possible association of their variations with both bladder cancer risk and clinicopathological features of tumors. The increased level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage and a higher proportion of individuals sensitive to oxidative stress were found among BC patients as compared to other groups under study. Heterozygosity in the *XPB* gene (codon 751) increased cancer risk: OR (95% CI) = 1.36 (1.03–1.81),  $p = 0.031$ . The frequency of the *XPB* 312Asn allele was significantly higher in T ≥ 2 high grade than in T ≥ 2 low grade tumors ( $p = 0.036$ ); the *ERCC1* 1097Val/Val genotype was strongly associated with muscle-invasive tumors. Combinations of homozygous wild type alleles occurred with the increased frequency in patients with non-muscle-invasive tumors suggesting that the maintenance of normal DNA repair activity may prevent cancer progression.

## 1. Introduction

Oxidized DNA base lesions induced by environmental pollutants and endogenous metabolites lead to a variety of mutations and consequently to genetic instability, which is a hallmark of cancer [1, 2]. As shown in European populations, increased frequencies of chromosome aberrations and micronuclei are closely associated with cancer risk [3, 4]. When monitoring genomic alterations in the urothelial carcinomas in individual patients for a long time, the increased level of the mitotic recombination was found at the early carcinogenesis stage, and extensive genetic damage was accumulated during the evolution of the tumors [5]. When studying the cellular response to DNA damage in different types of cancer, activation of the ATM–Chk2–p53 signal pathway was observed in early human tumorigenesis

[6] indirectly indicating accumulation of DNA lesions that, in turn, might be considered as the primary trait of upcoming genome instability and cell malignancy. Thus, the oxidatively induced DNA damage initiating genome instability is one of the principal factors of carcinogenesis, whereas the other one seems to be DNA repair deficiency or impairment.

The oxidatively damaged bases are predominantly removed via the BER pathway initiated with their excision by DNA glycosylases [7]. Among them, 8-oxo-guanine DNA glycosylase 1 (OGG1) is responsible for elimination of the highly mutagenic DNA lesion, 8-oxo-7,8-dihydroguanine (8-oxoGua). Another functionally important protein, X-ray repair cross-complementing protein 1 (XRCC1), interacts with DNA glycosylases, AP endonuclease-1 (APE-1), DNA polymerase  $\beta$  (POL $\beta$ ), DNA ligase III (Lig III), poly (ADP-ribose) polymerase 1 (PARP-1), and polynucleotide kinase

(PNK) at the damaged site, by modulating their activities and coordinating the subsequent enzymatic BER steps [8, 9]. The reduced BER activity has been newly discussed to trigger the development of sporadic cancers [10]. The performed proteomic analysis of BER deficient human cells has demonstrated that BER deficiency, leading to genome instability, results in dramatic changes in gene expression, resembling changes found in many cancers. These findings suggest that genetically unstable BER deficient cells may be a source of precancerous cells [10].

The majority of chemically induced DNA adducts are removed by the NER pathway that operates globally throughout the genome (global genome, GG NER) or during transcription (transcription coupled, TC NER); both subsets differ only in their initial recognition of the helix-distorting DNA damage [11, 12]. In this multistep repair process, DNA helicases unwind the double helix, thus opening access to the lesion site for other repair enzymes [13]. The XPD helicase, mutated in the cancer-prone xeroderma pigmentosum (XP), is part of the TFIIH complex that is essential for signaling events triggering transcription, cell cycle checkpoints, and DNA repair [14]. TC NER requires specific factors, including Cockayne syndrome (CS) protein B (CSB). The latter belongs to both the helicase superfamily 2 and to the SWI/SNF complex maintaining and remodeling chromatin structure [15, 16] and it acts at the crossroads of transcriptional networks [17, 18]. In the context of the present study, the recently reported data confirming involvement of NER-initiating proteins in the elimination of oxidatively generated DNA damage [19, 20] take on special significance.

We attempted to estimate the cellular response to oxidatively induced DNA damage and polymorphism in some DNA repair genes in bladder cancer. The frequencies of *OGGI* Ser326Cys (rs1052133), *XRCC1* Arg399Gln (rs25487), *XPD* Asp312Asn (rs1799793), and *ERCC6* Met1097Val (rs2228526) polymorphisms have been recently determined in the bladder cancer (BC) patients as compared with clinically healthy residents of Belarus [21]. Our results indicated the association of the *XPD* 312Asp/Asn heterozygous genotype with an increased risk of bladder cancer, whereas the *OGGI* 326Ser/Cys heterozygous genotype has exhibited the protective effect. Here, genome integrity and stability was analyzed in peripheral blood lymphocytes using the comet assay. Besides, isolated DNA samples were genotyped for polymorphism of DNA repair genes involved in BER and NER to elucidate both the possible impact of some other genetic variations (*XPD* Lys751Gln and *ERCC6* Gly399Asp) on bladder cancer susceptibility and the association of all six polymorphisms with clinicopathological parameters of tumors for evaluating their prognostic relevance.

## 2. Materials and Methods

**2.1. Study Populations.** The study included two independent experimental sets. In the first experimental set, the control group comprised 35 clinically healthy volunteers aged 22–63 years old who had no chronic and acute diseases and contacts with occupational hazards. They were recruited among residents of regions that were not affected by the Chernobyl

fallout. Forty individuals with histologically verified bladder cancer (BC) were randomly selected among patients of the Department of Urology of N.N. Alexandrov National Cancer Centre of Belarus in 2011. The average age of BC patients was about 70 years; males and smokers amounted to 85% and 89% of the sample, respectively. Besides, the group of elderly people was represented by fifteen clinically healthy persons over 60 years; among them, 73% were males. Fifteen individuals comprised the group of chronic inflammatory diseases including chronic obstructive pulmonary disease (9 cases), chronic pyelonephritis (4 cases), and rheumatic disease and polyarthritis (2 cases); all of them were beyond the exacerbation phase. Their average age was about 50 years, and males amounted to 53% of the sample. The cellular response to oxidatively induced DNA damage was compared in these four groups using the comet assay.

In the second experimental set, the case group comprised 418 BC patients who were treated at the Department of Urology of N.N. Alexandrov National Cancer Centre of Belarus over 2011–2014. All urothelial carcinoma diagnoses were verified histologically after transurethral resection of tumors. The T stages were determined using the international Tumor-Node-Metastases (TNM) classification, and the grade of tumor tissue differentiation was established according to WHO classifications of 1973 and 2004 [22, 23]. Blood samples (3–5 mL) were collected by venal puncture by the qualified medical personnel in accordance with the Declaration of Helsinki (1964) [24]. The blood samples were accompanied with a demographic profile of patients and the clinicopathological description of tumors (Table 1).

370 individuals were randomly recruited as controls among healthy volunteers involved in blood donation at the Republic Research and Production Center for Transfusiology and Medical Biotechnologies (Minsk) and elderly people who were observed at the Department of Gerontology and Geriatrics at the Belarusian Medical Academy of Postgraduate Education. Individuals from both control subgroups had no positive cancer history or acute diseases and should be considered the population-based controls. The control population was predominantly represented by males (68.7%). Like the BC patients, noncancer individuals were between 31 and 94 years old, with the average age of  $64.5 \pm 13.5$  years as opposed to  $66.7 \pm 10.9$  years in the case group. The controls were matched to the cases by the recruitment period, the ethnic origin (both were predominantly Belarusians or other Eastern Slavs), and age. However, they differed from each other in the smoking status, since smokers amounted to 31% among controls and to 68% among patients. It should be mentioned that the same control population was used in the previous work in order to study the possible impact of *OGGI* Ser326Cys (rs1052133), *XRCC1* Arg399Gln (rs25487), *XPD* Asp312Asn (rs1799793), and *ERCC6* Met1097Val (rs2228526) polymorphisms on susceptibility to bladder cancer [21]. Herein, the control population was used for the similar purpose concerning *XPD* Lys751Gln (rs13181) and *ERCC6* Gly399Asp (rs2228528) polymorphisms, while the next steps of the study were carried out using the enlarged case group stratified into several categories depending on tumor stages and grades.

TABLE 1: The demographic features of BC patients and clinicopathological parameters of tumors.

Features	Patients	
	<i>n</i>	Frequency %
Gender		
Males	344	82.3
Females	74	17.7
Age (years)		
Min	31	
Max	93	
Mean ± SD		66.7 ± 10.9
Median	67	
Smoking		
Smokers	283	67.7
Nonsmokers	117	28.0
Not specified	18	4.3
Tumor stages		
TIS	1	0.2
Ta	91	21.8
T1	198	47.4
T2	72	17.2
T3	27	6.5
T4	27	6.5
Not specified	2	0.4
Tumor grades		
1973		
CIS	1	0.2
G1	139	33.3
G2	186	44.5
G3	86	20.6
Not specified	6	1.4
2004		
PUNLMP	11	2.6
CIS	1	0.2
Low	241	57.7
High	156	37.3
Not specified	9	2.2
Recurrence		
No	268	64.1
Yes	150	35.9

Informed consent was obtained from each participant included in the study before the collection of blood samples. All participants were interviewed to complete a questionnaire covering medical, residential, and occupational history as well as age, gender, and the tobacco smoking status. The smoking status was summarized as “smokers” (combining current smokers and ex-smokers) or “nonsmokers” (including never smoking persons).

**2.2. Estimation of Genome Integrity in Freshly Isolated Lymphocytes Using the Comet Assay.** The approach for evaluation of genome integrity in order to diagnose genome instability in isolated lymphocytes was earlier described in detail [25–27].

In this investigation, 2–3 mL peripheral blood was collected into the heparinized Vacutainer tubes and kept at 4°C for no longer than 2 h. Lymphocytes were isolated from whole blood samples by centrifugation over 2.5 mL Histopaque at 1500 rpm for 30 min. Then lymphocytes were washed twice with RPMI 1640, suspended in cold PBS, and exposed to hydrogen peroxide (100 μM H<sub>2</sub>O<sub>2</sub>) at 4°C for 1 min, followed by washing with cold PBS. Intact and treated cells were incubated in RPMI 1640 with 10% fetal bovine serum (FBS) during a 3-h period at 37°C. Their viability was traditionally evaluated with the trypan blue exclusion test and usually varied in the range of 96–98%.

All the reagents and procedures were used according to the admitted protocol of the alkaline comet assay (single cell gel electrophoresis) [28]. Briefly, procedures included slide preparation, lysis of cell membranes for DNA elution by keeping the slides in the cold lysing solution (2.5 M NaCl, 10 mM Na<sub>2</sub>EDTA, 10 mM Tris, 1% Triton-X100, pH 10) for 1 h, DNA unwinding in fresh electrophoresis buffer (1 mM Na<sub>2</sub>EDTA, 300 mM NaOH) for 20 min, and horizontal electrophoresis for 20 min at 1 V/cm, 300 mA and pH > 13. After electrophoresis, slides were washed twice for 5 min with 0.4 M Tris buffer (pH 7.5) for neutralization and fixed in ice-cold 96% ethyl alcohol for 10 min. After staining with ethidium bromide, slides were analyzed with a fluorescence microscope Olympus BX-50. Visual estimation of DNA damage in arbitrary unites (a.u.) was carried out according to published recommendations [29]. Two slides were prepared for each point of analysis, and at least 100 cells were scored per each of two replicate slides by one researcher that provided the concordance between the results. The levels of DNA damage were calculated as average values.

Basal DNA damage was determined after 180 min incubation of intact lymphocytes in RPMI 1640 with 10% FBS. The initial level of oxidatively induced DNA damage was estimated immediately after mutagenic treatment, and the residual level of DNA damage was measured 180 min after exposure. To estimate DNA repair kinetics, samples of H<sub>2</sub>O<sub>2</sub>-treated lymphocytes were collected at 0, 30, 60, and 180 min of their incubation.

**2.3. Genotyping.** DNA for genotyping procedures was extracted using the traditional phenol-chloroform technique. Single nucleotide polymorphisms (SNPs) in some DNA repair genes were determined by the PCR-RFLP method under conditions used in the previous work [21]. In addition to polymorphisms *OGG1* Ser326Cys, *XRCC1* Arg399Gln, *XPB* Asp312Asn, and *ERCC6* Met1097Val, *XPB* Lys751Gln (rs13181) and *ERCC6* Gly399Asp (rs2228528) were analyzed in the present study. These polymorphisms were detected at conditions described elsewhere [30, 31]. The PCR products were digested with restriction enzymes, electrophoresed through 2.5% agarose gels containing ethidium bromide, and visualized under UV light. DNA repair genes and corresponding genotypes are shown in Table 2.

**2.4. Statistical Analysis.** Pearson’s  $\chi^2$  test (or Fisher’s exact test when necessary) was used to verify the significance of differences between the groups of BC patients and controls

TABLE 2: Characteristics of allelic variants and some conditions for their detection.

Gene polymorphisms	Primer sequences	Restriction enzyme	PCR products (bp)
Nucleotide excision repair			
<i>ERCC2/XPD</i> Asp312Asn rs1799793	(F) 5'-CTG TTG GTG GGT GCC CGT ATC TGT TGG TCT-3' (R) 5'-TAA TAT CGG GGC TCA CCC TGC AGC ACT TCC T-3'	StyI	Asp/Asp: 507 + 244; Asp/Asn: 507 + 474 + 244 + 33; Asn/Asn: 474 + 244 + 33
<i>ERCC2/XPD</i> Lys751Gln rs13181	(F) 5'-GCC CGC TCT GGA TTA TAC G-3' (R) 5'-CTA TCA TCT CCT GGC CCC C-3'	Pst I	Lys/Lys: 290 + 146; Lys/Gln: 290 + 127 + 146; Gln/Gln: 227 + 146
<i>ERCC6/CSB</i> Met1097Val rs2228526	(F) 5'-CCT GCT T CT AAC ATA TCT GT-3' (R) 5'-AAT CAC TGA CAA CTC TTC TG-3'	Nla III	Met/Met: 123 + 78; Met/Val: 201 + 123 + 78; Val/Val: 201
<i>ERCC6/CSB</i> Gly399Asp rs2228528	(F) 5'-TGA AGA GTC TGA GTA TTT CC-3' (R) 5'-ATC TTC ATC TCC ATC ATC TC-3'	RsaI	Gly/Gly: 180 + 91; Gly/Asp: 271 + 180 + 91; Asp/Asp: 271
Base excision repair			
<i>XRCC1</i> Arg399Gln rs25487	(F) 5'-GGA CTG TCA CCG CAT GCG TCG G-3' (R) 5'-GGC TGG GAC CAC CTG TGT T-3'	MspI	Arg/Arg: 115 + 34; Arg/Gln: 149 + 115 + 34; Gln/Gln: 149
<i>OGG1</i> Ser326Cys rs1052133	(F) 5'-CTG TTC AGT GCC GAC CTG CGC CGA-3' (R) 5'-ATC TTG TTG TGC AAA CTG AC-3'	MboI	Ser/Ser: 224 + 23; Ser/Cys: 247 + 224 + 23; Cys/Cys: 247

TABLE 3: The cellular response to the oxidative stress *in vitro* in the case group as compared to controls.

Features under study	Exposure, time of lymphocyte incubation (min)	BC patients (n = 40)	Controls/healthy donors (n = 35)
Basal DNA damage	180	Intact lymphocytes	9.2 ± 0.8
		Exposure to H <sub>2</sub> O <sub>2</sub>	
Oxidatively induced DNA damage	0	117.1 ± 7.1	85.6 ± 4.4 <sup>a</sup>
	30	41.8 ± 3.8	31.7 ± 3.2 <sup>a</sup>
	60	29.9 ± 2.7	22.8 ± 2.1 <sup>a</sup>
	180	17.7 ± 1.6	13.3 ± 1.1 <sup>a</sup>
DNA repair efficiency	30	65.2	63.0
	60	74.5	73.4
	180	84.3	84.4

<sup>a</sup>Significant differences are observed between the levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in BC patients and healthy controls ( $p = 0.00035, 0.045, 0.037, \text{ and } 0.026$  at 0, 30, 60, and 180 min after mutagenic exposure according to two sided Student's  $t$ -test, and  $0.01 < p < 0.05$  according to the nonparametric Mann-Whitney  $U$  test).

as well as between groups of different tumor stage and grade categories in genotype/allele frequencies. Student's  $t$ -test was used for comparison of the groups by age and other continuous variables, including the DNA damage levels. Nonparametric Mann-Whitney  $U$  test was also used in the latter case. DNA repair efficiency (RE) was calculated as percentage of DNA lesions eliminated at consequent time points relative to their initial level. The DNA repair rate in different groups was compared by the coefficients of linear regression ( $\beta$ ) [25–27].

When genotyping the DNA samples for DNA repair gene polymorphisms, the statistical significance for deviation from Hardy-Weinberg equilibrium was determined using  $\chi^2$  test.  $p \leq 0.05$  values were considered significant. The relative risk was estimated as odds ratio (OR) with 95% confidence intervals (CI).

### 3. Results

**3.1. Genome Integrity In Isolated Lymphocytes after Oxidative Stress In Vitro.** To estimate adequately the cellular response to oxidized DNA damage in bladder cancer, it was compared among several groups and first of all between the cases and controls (Table 3, Figure 1). The results indicated the absence of statistically significant differences between the levels of basal DNA damage in the control and case groups, whereas the levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in BC patients exceeded those in healthy volunteers during the whole period of observations. The greatest differences were observed immediately after lymphocyte mutagenic exposure. Nevertheless, the slopes of repair kinetics closely resembled each other (the insert in Figure 1), and, being compared by means

TABLE 4: The cellular response to DNA damage in the groups of BC patients, elderly people, and individuals with chronic inflammatory diseases.

Features under study	BC patients ( <i>n</i> = 40)	Individuals older than 60 years ( <i>n</i> = 15)	Individuals with chronic inflammations ( <i>n</i> = 15)
Average age (mean ± SE)	69.55 ± 1.57 <sup>a</sup>	62.8 ± 0.74	48.87 ± 2.86
Sex ratio females/males (% of males)	6/34 (85)	4/11 (73.33)	7/8 (53.33)
Smokers/nonsmokers (% of smokers)	5/35 (89)	6/9 (60)	2/13 (13.33)
Basal DNA damage (a.u.) at 180 min	11.38 ± 1.09	9.93 ± 2.61	6.0 ± 1.32
H <sub>2</sub> O <sub>2</sub> -induced DNA damage (a.u.) at 0 min	117.13 ± 7.01 <sup>b</sup>	89.33 ± 11.55	92.47 ± 7.97
Residual level of H <sub>2</sub> O <sub>2</sub> -induced DNA damage (a.u.) at 180 min	17.7 ± 1.59	18.2 ± 4.21	12.77 ± 3.03
DNA repair efficiency for 30 min incubation	65.24 ± 2.08	69.91 ± 3.82	66.23 ± 4.03
DNA repair efficiency for 180 min incubation	84.25 ± 1.33	81.27 ± 3.27	84.72 ± 3.22

<sup>a</sup>Significant differences concerning age were revealed between BC patients and elderly persons ( $p = 0.0004$ ) and between BC patients and individuals with chronic inflammatory diseases ( $p = 0.0001$ ).

<sup>b</sup>Significant differences concerning the initial level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage were observed between BC patients and elderly persons ( $p = 0.05$ ) and between those and individuals with inflammations ( $p = 0.027$ ).

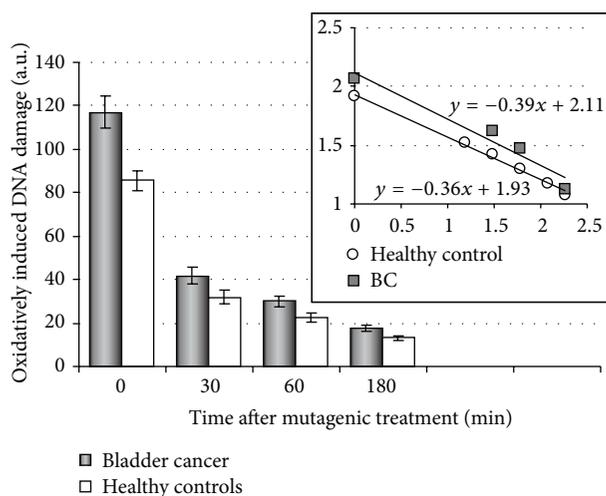


FIGURE 1: The oxidatively induced DNA damage and DNA repair kinetics in isolated lymphocytes from BC patients as compared to healthy donors. The case group included 40 BC patients; the control group comprised 35 clinically healthy donors. The insert reflects the DNA repair kinetics on a logarithmic scale. The coefficients of linear regression in the groups of patients ( $\beta = -0.39$ ) and controls ( $\beta = -0.36$ ) are approximately equal.

of regression analysis, these data revealed no differences between two groups with respect to the DNA repair velocity. DNA repair efficiency was also equal in lymphocytes from patients and controls (Table 3), suggesting that induced DNA damage was eliminated in a similar manner in both groups.

Then the cellular response to H<sub>2</sub>O<sub>2</sub> exposure in BC patients was compared with that in elderly persons and individuals with chronic inflammatory diseases (Table 4). The significant differences were found between all the groups with respect to the initial levels of oxidatively induced DNA

damage, with the highest level in the BC patients indicating increased cellular sensitivity to oxidative stress in bladder cancer.

In another approach, the frequency of sensitive individuals (with an enhanced DNA damage response) was estimated in the same groups using the earlier established reference intervals for all the parameters under study in the control population of 172 residents of Belarus [32]. In brief, the normal lymphocyte response to DNA damage was determined due to calculating 10th and 90th percentiles for levels of basal and exogenous DNA damage as well as for DNA repair efficiency measured at certain time points after mutagenic exposure. The marginal values were determined as follows: 15 a.u. for basal DNA damage, 110 a.u. for the initial level, 25 a.u. for the residual level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage, and 70% for DNA repair efficiency by the end of cell incubation. Subjects with the levels of DNA damage exceeding these values as well as with DNA repair efficiency, which is lower than the normal parameter, were attributed to the group of “sensitive” individuals. It is seen from Table 5 that half of the BC patients sample manifested the increased sensitivity of lymphocytes to H<sub>2</sub>O<sub>2</sub> immediately after treatment as opposed to 14.3% in the control group and 26.7% among elderly persons and individuals with chronic inflammations, respectively. Both approaches have demonstrated that the cellular responses to oxidatively induced DNA damage in bladder cancer strongly differed from those in healthy donors and to a lesser degree in aging and inflammations. Consequently, the increased initial level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in isolated lymphocytes might serve as a potential biomarker of genome instability predisposing to cancer.

3.2. Association of DNA Repair Gene Polymorphisms with Bladder Cancer Risk and Clinicopathological Characteristics of Tumors. Polymorphism in some DNA repair genes has

TABLE 5: The frequency of individuals with increased lymphocyte sensitivity to DNA damage in various study groups.

Study groups	Proportion of sensitive subjects (%) with respect to			Total
	Basal DNA damage	H <sub>2</sub> O <sub>2</sub> -induced DNA damage Initial level	Residual level	
BC patients ( <i>n</i> = 40)	17.5	50 <sup>a</sup>	10	62.5 <sup>b</sup>
Individuals older than 60 years ( <i>n</i> = 15)	20.0	26.67	26.67	46.67
Individuals with chronic inflammatory diseases ( <i>n</i> = 15)	6.67	26.67	6.67	46.67
Controls ( <i>n</i> = 35)	8.57	14.29	5.71	40.0

<sup>a</sup>Significant differences were revealed between all the groups by criterion  $\chi^2$  ( $p = 0.009$ ) and between the case group and controls ( $p = 0.001$ ).

<sup>b</sup>Significant differences were observed between BC patients and controls ( $p = 0.05$ ).

TABLE 6: Distribution of allelic variants of some DNA repair genes in the group of BC patients as compared to controls.

Genotypes/variant alleles	BC cases		Controls		<i>p</i>
	<i>n</i>	%	<i>n</i>	%	
<i>ERCC2/XPD</i> Lys751Gln (rs13181)					
Lys/Lys	120	29.2	132	36.2 <sup>a</sup>	0.039
Lys/Gln	212	51.6	160	43.8 <sup>b</sup>	0.031
Gln/Gln	79	19.2	73	20.0	>0.05
Lys/Gln + Gln/Gln	291	70.8	233	63.8 <sup>c</sup>	0.039
Gln	370/822	45.0	306/730	41.9	>0.05
<i>ERCC6/CSB</i> Gly399Asp (rs2228528)					
Gly/Gly	283	68.0	259	71.0	>0.05
Gly/Asp	121	29.1	101	27.7	>0.05
Asp/Asp	12	2.9	5	1.4	>0.05
Gly/Asp + Asp/Asp	133	32.0	106	29.0	>0.05
Asp	145/832	17.4	111/730	15.2	>0.05

The genotypic distribution is in accordance with Hardy-Weinberg equilibrium in the control and case groups:  $\chi^2 = 3.63$  and  $0.72$  ( $p = 0.06$  and  $0.39$ ) for *ERCC2/XPD* Lys751Gln polymorphism;  $\chi^2 = 1.95$  and  $0.05$  ( $p = 0.16$  and  $0.83$ ) for *ERCC6/CSB* Gly399Asp polymorphism.

<sup>a</sup>OR [95% CI] = 0.73 [0.54–0.98],  $p = 0.039$ ; <sup>b</sup>OR [95% CI] = 1.36 [1.03–1.81],  $p = 0.031$ ; <sup>c</sup>OR [95% CI] = 1.37 [1.02–1.86],  $p = 0.039$ . OR values describe a homozygous wild type genotype of the *XPB* gene as a protective factor, whereas the heterozygous genotype and sum of genotypes containing a variant allele seem to be risk factors for developing bladder cancer.

been recently reported to affect susceptibility to bladder cancer in Belarus [21]. Herein, the results of genotyping for *XPB* Lys751Gln (rs13181) and *ERCC6* Gly399Asp (rs2228528) polymorphisms are added (Table 6). The *ERCC6* Gly399Asp polymorphisms were found to be neutral unlike the *XPB* polymorphisms. In the latter case, cancer risk was mainly associated with the *XPB* 751Lys/Gln heterozygous genotype (OR (95% CI) = 1.36 (1.03–1.81) ( $p = 0.031$ )), which indicated that heterozygosity in this codon predisposes to tumorigenesis as it was earlier noticed for the *XPB* codon 312.

The comparison of the genotype distribution depending on the tumor stages and tumor tissue differentiation (Table 7) revealed lack of differences, except for the *ERCC6* Met1097Val polymorphism (rs2228526). The frequency of the *ERCC6* 1097Val/Val genotype was significantly increased in muscle-invasive tumors as compared to non-muscle-invasive ones ( $p = 0.0045$ ), and the similar trend concerned the Val allele frequency, which was almost doubled in patients with T2 tumors as compared to T<sub>a</sub> neoplasms (37.5% and 20.9%, resp.;  $p = 0.0009$ ). These data suggested that the carriers of the *ERCC6* 1097Val allele, predominantly in the homozygous state, have a higher probability of developing advanced

cancer, what is also indicated by the odds ratio: OR (95% CI) = 2.86 (1.36–6.05) ( $p = 0.0061$ ) for the Val/Val genotype.

The distribution of genotypes/alleles for polymorphisms of DNA repair genes did not depend on tumor grades. However, the analysis of genetic variations in papillary neoplasms of low malignant potential (PNLMP) as compared with high grade or poorly differentiated cancer revealed some peculiarities concerning *ERCC6* Met1097Val and *OGG1* Ser326Cys polymorphisms (Figure 2). The frequencies of homozygous wild type genotype of *ERCC6* gene and heterozygous genotype of the *OGG1* gene were significantly increased in LMP tumors, whereas the genotypes containing at least one variant allele of the *ERCC6* gene occurred more often in patients with G3 or high grade urothelial carcinomas. Thus, neoplasms of low malignant potential were distinct from others with respect to genotype distribution of both the *OGG1* Ser326Cys and the *ERCC6* Met1097Val polymorphisms.

When dividing tumors into four categories (T<sub>a</sub>/T<sub>1</sub> low, T<sub>a</sub>/T<sub>1</sub> high, T<sub>2</sub>  $\geq 2$  low, and T<sub>2</sub>  $\geq 2$  high), evident differences were found only in muscle-invasive carcinomas, with the homozygous wild type genotype of the *XPB* gene (codon 312) being associated with low grade cancer, whereas

TABLE 7: Distribution of genotypes of some DNA repair genes in non-muscle-invasive (Ta/T1) and muscle-invasive (T ≥ 2) tumors depending on their differentiation.

DNA repair gene polymorphisms	Genotype frequency (%) depending on the tumor stages and grades						
	Ta/T1	T ≥ 2	G1	G2	G3	low	high
<i>OGG1</i> 326 rs1052133	<i>n</i> = 288	<i>n</i> = 126	<i>n</i> = 135	<i>n</i> = 185	<i>n</i> = 86	<i>n</i> = 240	<i>n</i> = 156
Ser/Ser	67.0	69.0	71.1	65.9	67.4	67.5	69.2
Ser/Cys	28.8	27.0	24.4	30.8	27.9	28.8	26.3
Cys/Cys	4.2	4.0	4.4	3.2	4.7	3.8	4.5
<i>XRCCI</i> 399 rs25487	<i>n</i> = 288	<i>n</i> = 126	<i>n</i> = 135	<i>n</i> = 186	<i>n</i> = 86	<i>n</i> = 241	<i>n</i> = 156
Arg/Arg	39.6	44.4	41.5	38.2	46.5	39.0	44.2
Arg/Gln	49.0	44.4	48.9	48.4	43.0	48.5	44.9
Gln/Gln	11.5	11.1	9.6	13.4	10.5	12.5	10.9
<i>XPD</i> 312 rs1799793	<i>n</i> = 288	<i>n</i> = 126	<i>n</i> = 135	<i>n</i> = 185	<i>n</i> = 86	<i>n</i> = 240	<i>n</i> = 156
Asp/Asp	29.2	32.5	24.4	35.7	30.2	31.3	30.8
Asp/Asn	54.5	49.2	62.2	44.9	52.3	53.7	49.3
Asn/Asn	16.3	18.3	13.3	19.5	17.5	15.0	19.9
<i>XPD</i> 751 rs13181	<i>n</i> = 280	<i>n</i> = 124	<i>n</i> = 131	<i>n</i> = 181	<i>n</i> = 84	<i>n</i> = 234	<i>n</i> = 152
Lys/Lys	28.9	30.6	28.2	30.4	31.0	31.2	28.3
Lys/Gln	53.2	51.6	57.3	49.2	51.2	52.1	52.0
Gln/Gln	17.9	17.7	14.5	20.4	17.8	16.7	19.7
<i>ERCC6</i> 1097 rs2228526	<i>n</i> = 289	<i>n</i> = 126	<i>n</i> = 135	<i>n</i> = 186	<i>n</i> = 86	<i>n</i> = 241	<i>n</i> = 156
Met/Met	50.9	46.8	57.0	45.2	47.7	48.1	49.3
Met/Val	44.3	40.5	37.8	47.3	43.0	45.6	41.7
Val/Val	4.8	12.7 <sup>a</sup>	5.2	7.5	9.3	6.2	9.0
<i>ERCC6</i> 399 rs2228528	<i>n</i> = 283	<i>n</i> = 126	<i>n</i> = 133	<i>n</i> = 183	<i>n</i> = 85	<i>n</i> = 236	<i>n</i> = 155
Gly/Gly	67.8	67.5	65.4	69.4	67.0	65.7	69.7
Gly/Asp	29.0	30.2	31.6	27.3	30.6	31.4	27.7
Asp/Asp	3.2	2.4	3.0	3.3	2.4	2.9	2.6

<sup>a</sup>Significant differences were observed between Ta/T1 and T ≥ 2 tumors with respect to frequencies of the homozygous *ERCC6* 1097 Val/Val genotype according to  $\chi^2$  test ( $p = 0.0045$ ).

the frequencies of genotypes containing a variant Asn allele were significantly increased in high grade neoplasms (Figure 3(a)). Two other polymorphisms (*XPD* Lys751Gln and *ERCC6* Gly399Asp) showed similar trends, but the differences between T ≥ 2 low grade tumors and T ≥ 2 high grade carcinomas were not statistically confirmed (Figure 3(b)).

Based on the assumption that the wild type of DNA excision repair genes may provide elimination of mutagenic/carcinogenic DNA lesions thus promoting both decrease in cancer risk and inhibition of tumor expansion/malignancy, the frequencies of combined homozygous wild type alleles were estimated depending on T stages. The combination involving Ser/Ser, Arg/Arg, Asp/Asp, Lys/Lys, Met/Met, and Gly/Gly genotypes of *OGG1* (codon 326), *XRCCI* (codon 399), *XPD* (codons 312 and 751), and *ERCC6* (codons 1097 and 399), respectively, was a rare event occurring only in Ta/T1 tumors (2.1%). As shown in Figure 4(a), the total frequency of combinations represented by any five homozygous wild type genotypes was significantly higher in non-muscle-invasive carcinomas (18.7%) as compared to advanced tumors (7.9%) and together with the former combination containing all six wild type homozygotes they achieved 20.8% in Ta/T1 neoplasms as opposed to 7.9% in T ≥ 2 tumors (Figure 4(b)). Accordingly, homozygosity for

the wild type alleles of the DNA repair genes under study seemed to prevent tumor expansion. The distribution of these combinations did not depend on tumor grades.

#### 4. Discussion

Before discussing the data, it should be noted that evaluation of bladder cancer risk implies a case-control study, but the comparison of the demographic profiles of BC patients and controls (Section 2.1 and Table 1) shows the significant differences between groups concerning tobacco smoking status that is likely to limit interpretation of the results. On the other hand, the random selection of sizeable populations, which are matched by the recruitment period, age and ethnicity, allows a higher frequency of smokers among BC patients to be considered as a disease-specific feature in support of findings that bladder cancer is an age-, gender-, and smoking-related disease [33, 34]. It should also be mentioned that the percentage of smokers in the control population reflects the situation with tobacco consumption in Belarus, and the gene-smoking relationship in bladder cancer has been previously characterized [21, 35]. A close association of bladder cancer with age and a tobacco smoking habit suggests that oxidative stress contributes to its development.

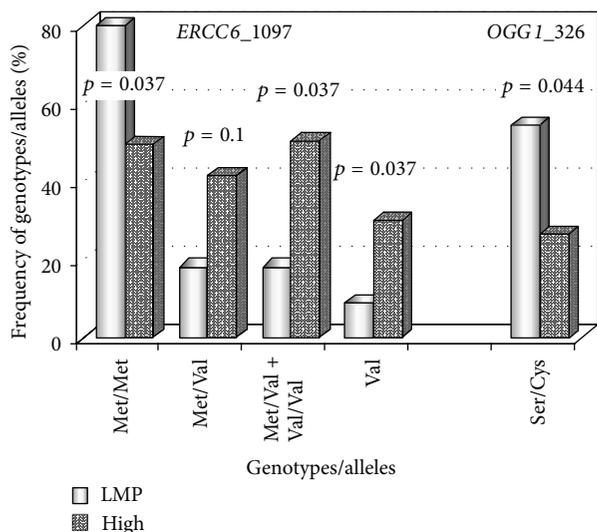


FIGURE 2: Distribution of some genotypes/alleles in patients with papillary urothelial neoplasms of low malignant potential (LMP, 11 samples) as compared to high grade carcinomas (high, 156 samples). Four coupled bars correspond to *ERCC6* Met1097Val polymorphisms. The frequencies of the Met/Met genotype are 81.8% and 49.4% in LMN and high grade tumors, whereas the frequencies of the Met/Val + Val/Val genotypes are 18.2% and 50.6% in the same types of urothelial carcinomas, respectively. The last coupled bars reflect the frequencies of the *OGGI* (codon 326) heterozygous genotype, which are 54.5% in LMP neoplasms and 26.3% in high grade tumors.

The comet assay used in the first experimental set remains a widespread and efficient tool in biomonitoring studies [36, 37]. Our approach resembles a challenge assay, which is based on detecting chromosome breakage and has developed for revealing exposure-induced DNA repair deficiency as a functional biomarker of cancer risk [38]. In our studies, basal and exogenous DNA lesions were identified as potential biomarkers of genome destabilization, and their levels as well as DNA repair kinetics after oxidative stress *in vitro* were measured as average group values and individually. Using this approach, we diagnosed and specified genome instability in lymphocytes of patients with some genetic disorders and occupationally exposed subjects [25–27]. Taking into account conflicting data on the relationship between age and the yield of DNA damage in the comet assay [36, 39], the age differences, in particular between groups of BC patients and individuals with chronic inflammations (Table 4), might be a limitation of the present study. However, lack of such correlation for basal and H<sub>2</sub>O<sub>2</sub>-induced DNA damage [27] confirms the reliability of our observations.

Herein, the significantly increased levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage were found in lymphocytes of BC patients as compared to controls with the pronounced effect immediately after mutagenic treatment due to dysfunction of antioxidant defense and disturbance of redox homeostasis [40, 41] rather than a reduced DNA repair rate or efficiency (Figure 1 and Table 3). However, reactive oxygen species (ROS) are known to contribute to inflammations [42, 43],

aging, and related diseases [44, 45], whereas inflammations, resulting from and triggering ROS production, forego and accompany carcinogenesis [46–48]. Therefore, it was reasonable for comparing the cellular response to oxidative stress in different conditions. The initial levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage as well as the proportion of individuals with increased cellular sensitivity to hydrogen peroxide in the group of BC patients exceeded those among elderly persons and subjects with chronic inflammatory diseases (Tables 4 and 5). The results seemed to demonstrate an essential role of the abnormal cellular response to oxidatively generated DNA damage in bladder cancer.

Among various underlying mechanisms, mutations or polymorphisms in genes responsible for antioxidant defense, redox regulation, and oxidatively damaged base repair are currently studied. Our second experimental set was focused on the latter mechanism, and it would be interesting to discuss involvement of DNA helicases in removing oxidized DNA lesions. An inability to repair oxidatively generated damage accumulating in the brain was hypothesized to cause the neurological degeneration in xeroderma pigmentosum [49]. As recently reported, the neurodegeneration in Cockayne syndrome is associated with ROS-induced damage in the mitochondria, independent of nuclear transcription coupled repair [50]. Moreover, CSB protein appears to behave as an electron scavenger in the mitochondria whose absence leads to increased levels of ROS in CSB-mutated cells [50]. The CSA and CSB proteins, in addition to their basic role in TC NER, can participate in BER directly by interaction with BER proteins and indirectly by modulating gene expression [51]. Using high performance liquid chromatography coupled to electrochemical detection (HPLC-EC) to measure the genomic 8-oxoGua levels in mouse NER- or BER-deficient embryo fibroblasts [20] as well as the immunofluorescence method to detect binding of CSB and XPC to oxidative lesions in different nuclear compartments in fibroblast cell lines derived from patients [19], the experimental evidence for a direct involvement of some XP and CS gene products in repair of oxidatively induced damage has been provided. In spite of the fact that the *OGGI* DNA glycosylase dominates in 8-oxoGua repair, NER (XPC and XPA) and transcription-coupled repair proteins (CSB and CSA) are similar but are minor contributors [19].

In our studies, the *OGGI* (codon 326) heterozygous genotype decreased bladder cancer risk, especially in smokers with OR = 0.55 (0.34–0.89) ( $p = 0.014$ ) [21] and prevented high grade tumors as compared to neoplasms of low malignant potential (Figure 2). Our findings, at least with respect to cancer predisposition, are in line with some other data [52, 53]. The *ERCC6/CSB* 1097Val/Val genotype enhanced susceptibility to advanced ( $T \geq 2$ ) urothelial carcinoma (Table 7), and the *ERCC2/XPD* 312Asn allele seemed to promote tumor malignancy, since its frequency was increased in patients with  $T \geq 2$  high grade tumors as compared to  $T \geq 2$  low grade neoplasms (Figure 3(a)). The effects associated with impaired activity of CSB and XPD proteins might be mediated by accumulation of ROS, which act as the second messengers in intracellular signaling cascades inducing and maintaining the oncogenic phenotype of cancer cells [54, 55].

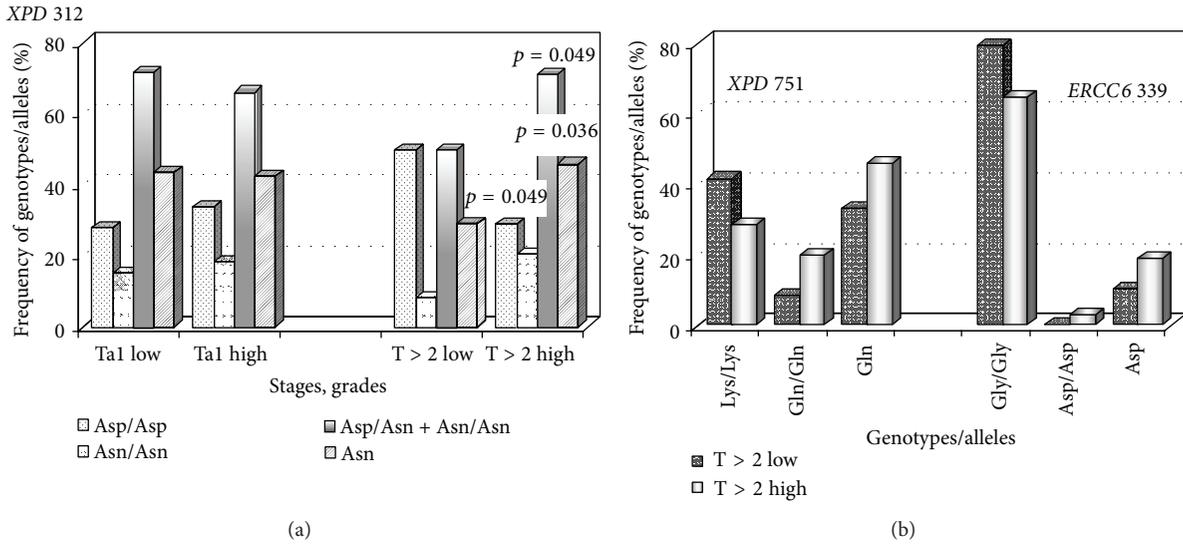


FIGURE 3: Distribution of some genotypes/alleles of DNA repair genes in non-muscle-invasive and muscle-invasive tumors depending on their grades. (a) The frequencies of *XPD* Asp312Asn polymorphisms in Ta/T1 low grade tumors (227 samples) as compared to Ta/T1 high grade neoplasms (59 samples) as well as in T ≥ 2 low grade tumors (24 samples) as opposed to T ≥ 2 high grade neoplasms (97 samples). In the latter case, the frequencies of the Asp/Asp, Asn/Asn, and Asn/Asn+Asp/Asn genotypes and the Asn alleles were as follows: 50% and 28.9% ( $p = 0.049$ ), 8.3% and 20.6% ( $p = 0.16$ ), 50% and 71.1% ( $p = 0.049$ ), and 29.2% and 45.9% ( $p = 0.036$ ) in T ≥ 2 low grade and T ≥ 2 high grade tumors, respectively. (b) The frequencies of *XPD* (codon 751) and *ERCC6* (codon 399) polymorphisms in T ≥ 2 low grade tumors (24 samples for each polymorphisms) as compared to T ≥ 2 high grade neoplasms (95 and 97 samples, resp.).

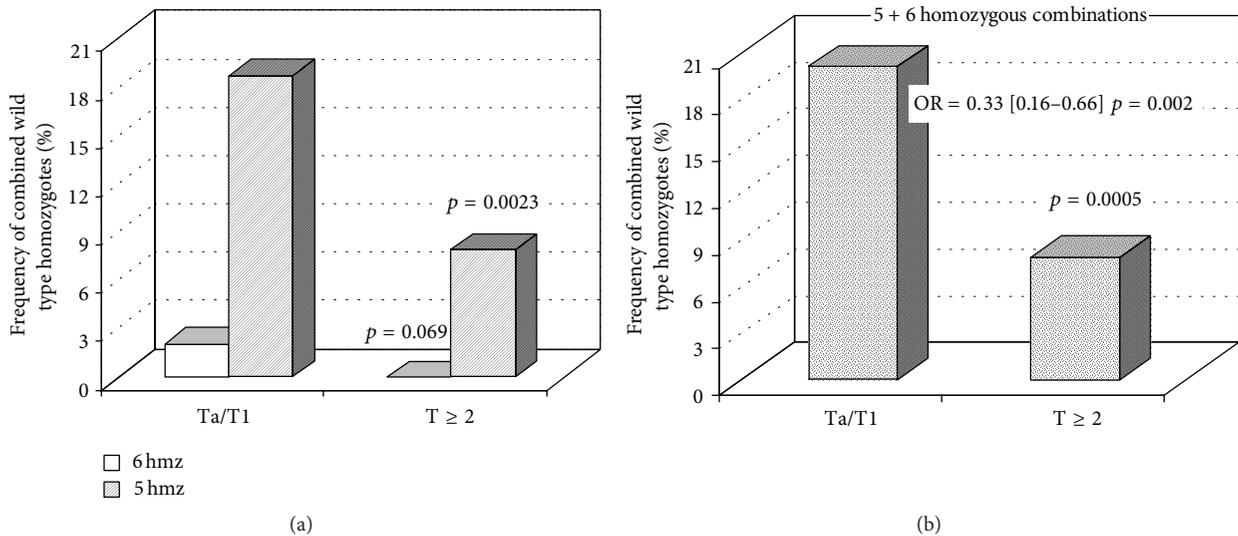


FIGURE 4: Distribution of wild type homozygous combinations in non-muscle-invasive and muscle-invasive urothelial carcinomas. 6 hmz correspond to combination of all six wild type homozygotes; 5 hmz correspond to total combinations containing any five wild type homozygotes. The Ta/T1 group was represented by 289 samples, whereas the T ≥ 2 group consisted of 120 samples.

It is generally accepted that the “driver” mutations in a few key genes trigger certain (sometimes alternative) pathways of cancer pathogenesis. In bladder cancer, the mutations in *FGFR3* gene are strongly associated with superficial tumors, whereas mutations in *TP53* gene lead to muscle-invasive cancer [56]. However, the molecular analysis of tumor tissue samples from the same Belarusian patients has shown that about 30% among them are of the wild type

genotype with respect to both genes suggesting multiple genetic origins of urothelial carcinomas [57]. The current molecular-genetic analysis of bladder cancer includes the whole genome sequencing, detection of genome-wide gene expression profiles, studies of DNA repair and replication processes, the immune and inflammatory responses, and other common hallmarks of human cancers [58–62]. These investigations are aimed at revealing novel molecular markers

with high predictive and prognostic relevance. In the context of our study, the results concerning a set of mutations, which were not earlier recognized as significant events for bladder cancer, are of great interest. Specifically, among 32 identified genes, there was the NER *ERCC2/XPD* gene, and its fifteen of sixteen genetic variations were represented by deleterious missense mutations with dominant negative effects [63].

In spite of the fact that genetic variations in excision repair genes are not attributed to driver mutations in bladder cancer [62], they may modulate susceptibility to cancer initiation and cancer progression. For example, genome-wide association studies (GWAS) have identified more than 300 validated associations between genetic variants and risk of approximately 70 common diseases [64]. The functions of genes identified as relevant for bladder cancer focus on detoxification of carcinogens, maintenance of DNA integrity, control of the cell cycle, and apoptosis. Our data indicate both the accumulation of oxidatively induced DNA damage and impact of modified XPD and CSB proteins on risk and clinical course of bladder cancer. It is typical that all known SNPs are associated with bladder cancer with odds ratios lower than 1.5; however, when interacting, they may collectively result in a substantial cancer risk [64]. Combinations of the homozygous wild type alleles are expected to exert a reverse effect. Indeed, the combined wild type homozygotes for some DNA repair genes reduced susceptibility to bladder cancer [35] and even prevented tumor expansion (Figure 4(b)).

The impact of excision repair gene polymorphisms on susceptibility to different cancers, including urothelial carcinoma, has been widely discussed in literature [21, 35]. Their associations with clinicopathological parameters of tumors are still poorly understood, although there are intriguing findings indicating the dual effects of DNA repair gene polymorphisms with respect to bladder cancer risk/recurrences/progression and clinical outcomes. Improved overall and disease-specific survival as well as decreased mortality risk of BC patients after chemotherapy and radiotherapy was observed in carriers of variant alleles of the *XPC* gene [65] and the *XPD* 751Gln allele combined with the *XPC* 939Gln allele [66]. The clinical outcomes were also affected by a series of *XRCC1* polymorphisms [67] and by the *OGG1* 326 Cys/Cys genotype [68]. Hence, so-called “risky” genotypes/alleles of some DNA repair genes decreased tumor resistance to radiation or chemical treatment, thereby improving clinical outcomes. At the same time, the “risky” *ERCC6* 1097Val allele increased the frequency of urothelial carcinoma recurrences [69] and the *XRCC1* 399 A/A (Gln/Gln) genotype greatly reduced recurrence free survival of BCG treated patients [70]. The higher frequency of muscle-invasive tumors was observed in carriers of *XRCC1* 194 CT+TT genotypes as compared to the wild type CC genotype [70] and in carriers of the mutated *APE1* 148Glu allele [71]. Our results indicating the association of some polymorphic variants of *ERCC6/CSB* and *ERCC2/XPD* genes with advanced bladder cancer ( $T \geq 2$  as compared to Ta/T1 tumors or  $T \geq 2$  high as opposed to  $T \geq 2$  low grade carcinomas) fit into an overall picture, but the problem needs to be further explored to confirm some regularities arising from our own and literature data.

## 5. Conclusion

Using the alkaline comet assay, the increased level of  $H_2O_2$ -induced DNA damage was found in isolated lymphocytes of BC patients as compared to healthy controls, elderly people, and individuals with chronic inflammatory diseases. The proportion of individuals with the enhanced cellular response to oxidative stress was also significantly higher among BC patients than among healthy subjects. These results showed that accumulation of oxidatively induced DNA damage might serve as a potential biomarker of genome instability predisposing to cancer.

Some excision repair gene polymorphisms modified the susceptibility to bladder cancer and were associated with clinicopathological parameters of tumors. Polymorphisms in *XPD* gene (codons 312 and 751) increased cancer risk, and at the same time the variant *XPD* 312Asn allele was significantly associated with muscle-invasive high grade tumors. Polymorphisms in *ERCC6* gene (codon 1097), especially the Val/Val homozygous genotype, occurred with the higher frequency in muscle-invasive tumors as compared to non-muscle-invasive ones, and polymorphic variants in the *XPD* (codon 751) and *ERCC6* (codon 399) genes manifested the trends resembling effects of the *XPD* Asp312Asn polymorphisms with respect to  $T \geq 2$  high grade tumors as compared to  $T \geq 2$  low grade carcinomas. Interestingly, the combinations of homozygous wild type genotypes were associated with non-muscle-invasive tumors and their frequency was more than twice lower in  $T \geq 2$  carcinomas suggesting that the maintenance of normal DNA repair activity, specifically of some XP and CS gene products, seems to inhibit cancer initiation and/or cancer progression. Based on the literature data, one can assume that their positive effects, at least in part, are mediated through elimination of mutagenic/carcinogenic oxidatively induced DNA damage.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## Review Article

# Polyphenols as Modulator of Oxidative Stress in Cancer Disease: New Therapeutic Strategies

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Received 22 May 2015; Accepted 21 July 2015

Academic Editor: Amit Tyagi

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Cancer onset and progression have been linked to oxidative stress by increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation and therefore antioxidant agents could interfere with carcinogenesis. It is well known that conventional radio-/chemotherapies influence tumour outcome through ROS modulation. Since these antitumour treatments have important side effects, the challenge is to develop new anticancer therapeutic strategies more effective and less toxic for patients. To this purpose, many natural polyphenols have emerged as very promising anticancer bioactive compounds. Beside their well-known antioxidant activities, several polyphenols target epigenetic processes involved in cancer development through the modulation of oxidative stress. An alternative strategy to the cytotoxic treatment is an approach leading to cytostasis through the induction of therapy-induced senescence. Many anticancer polyphenols cause cellular growth arrest through the induction of a ROS-dependent premature senescence and are considered promising antitumour therapeutic tools. Furthermore, one of the most innovative and interesting topics is the evaluation of efficacy of prooxidant therapies on cancer stem cells (CSCs). Several ROS inducers-polyphenols can impact CSCs metabolisms and self-renewal related pathways. Natural polyphenol roles, mainly in chemoprevention and cancer therapies, are described and discussed in the light of the current literature data.

## 1. Introduction

Many epidemiological studies suggest that diet particularly rich in fruits and vegetables have cancer preventive properties [1–3]. The beneficial effects of diet are attributable, at least in part, to polyphenols which have antitumour activities both in animal models and in humans [4, 5].

During the past few decades the growing interest in natural polyphenols has contributed to understanding these compounds in terms of their chemical and biological functions and beneficial effects on human health [6, 7]. With the advent of cellular, molecular experimental systems, and transgenic/knockout mice models, relevant advances in understanding the mechanisms involved in the action of polyphenols have been achieved. Most of the beneficial effects of natural polyphenols are considered to reflect their ability to scavenge-free radicals endogenously generated [8] or formed by radiation and xenobiotics [9]. However, some

data in literature, suggest that the antioxidant properties of the phenolic compounds may not fully account for their chemopreventive effects [10]. Emerging evidence indicates that these polyphenols may also behave as prooxidants to initiate a reactive oxygen species mediated cellular DNA breakage and consequent cell death [11]. It has been reported that such a prooxidant mechanism is a result of redox-active microenvironment in cancer cells due to elevated levels in copper [12]. Copper is an important redox-active metal ion present in chromatin, closely associated with DNA bases and can be mobilized by metal chelating agents. Several studies have established that serum, tissue, and cellular copper levels in cancer patients are significantly elevated. Given that aberrant redox system is frequently observed in many tumour cells [13], it was hypothesized that polyphenols may selectively affect tumour cells behaviour based on their differential redox status [12].

The protective mechanisms that block the initiation of carcinogenesis can be defined as chemoprevention, a concept that was originally introduced by Wattenberg [14]. Interestingly, natural polyphenols could induce apoptotic cell death in preneoplastic or neoplastic cells through various growth inhibitory mechanisms as the activation of cytochrome c and caspases, the arrest of cell cycle, and the modulation of signalling pathways (NF- $\kappa$ B, JAK/STAT) which result in the inhibition of tumour progression [15, 16].

Research on the anticancer activities of dietary polyphenols, identified new antitumour molecules that can be used in cancer prevention and treatment, both alone and in combination with current chemotherapy/radiotherapy [17–19].

Cellular senescence is a physiological process of irreversible cell-cycle arrest that contributes to various physiological and pathological processes of aging [20]. Replicative senescence (RS) is associated with telomere erosion after repeated cell divisions, whereas stress-induced premature senescence (SIPS) is a telomere-independent process and occurs in response to aberrant oncogenic signalling, oxidative stress, and DNA damage. Although senescent cells have irreversibly lost their capacity for cell division, they are viable and remain metabolically active [21, 22].

Induction of cellular senescence can be considered a relevant mechanism of tumour suppression. The concept of prosenescence therapy has emerged over the past few years as a novel therapeutic approach to treat cancers. Emerging evidence has demonstrated that therapy-induced senescence (TIS) is a critical mechanism through which many anticancer drugs inhibit tumour progression [23]. TIS may be viewed either as an independent approach to treat cancer cells or as a combined strategy with conventional chemo-/radiotherapy. In a neoadjuvant setting, prosenescence therapy could be used with traditional treatments in order to reduce tumour mass before surgery. Furthermore, the engagement of prosenescence as an adjuvant therapy could be helpful in reducing the statistical risk of cancer relapse.

Epigenetic alterations, such as DNA methylation, histone acetylation level, and gene expression miRNA-regulated cancer stem cells biology, and induction of premature senescence in tumour cells have been identified as relevant anticancer features of many dietary polyphenolic compounds [24–28]. Increasing data from both cancer epidemiology and experimental attempts support the bright future of polyphenols as epigenetic modulators, prosenescence inducers, and cancer stem cells metabolism regulators in new anticancer approaches.

In this review, we will discuss the current progress in the study of polyphenols as very promising tools for the management of cancer prevention and treatment.

## 2. Oxidative Stress and Cancer

*2.1. Cellular Transformation Mechanisms Mediated by ROS.* Cancer is currently one of the most deadly diseases worldwide. According to a report by the World Health Organization (WHO) (<http://who.int/cancer/en>) 8.2 million people died of cancer in 2012; however 30% of cancer can be

prevented and some of the most common cancers such as breast, colorectal, and cervical cancer are curable if treated promptly. Among many factors that cause cancer, oxidative stress is one of the most important and well-studied event that gives rise to the conditions leading to tumour onset and progression [29].

It has been demonstrated that continuous inflammation may lead to a preneoplastic situation [30–32]. In chronically inflamed cells, the secretion of a large amount of reactive oxygen/nitrogen species (ROS/RNS) recruits more activated immune cells, which leads to the amplification of dysregulated processes and eventually to a preneoplastic condition. If the amount of cellular ROS/RNS produced is high enough to overcome endogenous antioxidant response, an irreversible oxidative damage to nucleic acids, lipids, and proteins may cause genetic and/or epigenetic alterations leading to the dysregulation of oncogenes and tumour suppressor genes. Hence, the oxidative stress and chronic inflammation processes are tightly coupled and the failure to block these processes could result in genetic/epigenetic changes that drive the initiation of carcinogenesis [33]. Furthermore, several studies have shown that oxidative stress affects several signalling pathways associated with cell proliferation [34]. Among them, the epidermal growth factor receptor signalling pathway (EGFR) can be mentioned and key signalling proteins, such as the nuclear factor erythroid 2-related factor 2 (NRF2), Ras/Raf, the mitogen activated protein kinases (MAPKs) ERK1/2, and MEK, phosphatidylinositol 3-kinase (PI3K), phospholipase C, and protein kinase C are affected by oxidative stress [35–37]. Moreover, ROS alter the expression of the p53 suppressor gene that is a key factor in apoptosis. Thus, oxidative stress causes changes in gene expression, cell proliferation, and apoptosis and plays a significant role in tumour initiation and progression [37–40].

Intriguingly, it is believed that, on the one hand, ROS contribute to the carcinogenesis, but, on the other hand, excessive amounts of ROS may act as cellular toxicants which lead to cancer cell growth arrest, apoptosis, or necrosis [41] (Figure 1). It is speculated that the malignant cells which are under increased level of oxidative stress would be more vulnerable to further ROS attack [42].

The evaluation of tissue redox status has great diagnostic potential in oncology. There are studies showing that low antioxidant status and increased oxidative stress levels are detected in cancer patients, even before oncology treatment starts [43]. Moreover, the redox status has a prognostic relevance for cancer therapy and could significantly contribute to the planning of an appropriate patient treatment regime. The conventional therapeutic strategy is based on drugs that increase ROS generation and induce apoptotic damage in cancer cells. However, this therapeutic approach has a serious disadvantage such as the development of various toxic side effects in normal tissues.

It has been reported [44] that normal cells compared to cancer cells show a low steady-state level of ROS and constant level of reducing equivalents. The different redox status of normal and cancer cells allows the use of this parameter for the design of new promising therapeutic strategies based on the regulation of redox signalling.

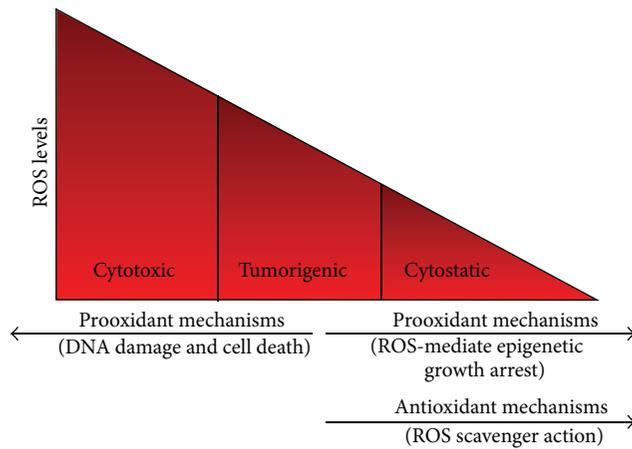


FIGURE 1: Dual prooxidant role of ROS level in cancer cells. Prooxidant mechanisms associated with different cellular ROS levels: high levels could induce DNA damage and cell death whereas low levels could induce epigenetic alterations and senescence-like growth arrest. In the figure the classical role of ROS scavengers as antioxidants is also reported.

There is preliminary evidence suggesting that certain antioxidant supplements may reduce adverse cancer therapeutic reactions including neurotoxicity, asthenia, stomatitis/mucositis, and weight loss [45]. Significant reductions in toxicity may alleviate dose-limiting side effects so that more patients are able to complete prescribed chemo-/radiotherapy regimens successfully, suggesting an improved therapeutic index.

Furthermore, from a chemopreventive point of view, antioxidants have been shown to play an important role. Many epidemiological studies concluded that people who eat more vegetables, fruits, and other types of food rich in phenolic antioxidants may have a lower risk of developing some types of cancer. It is well established that the Mediterranean dietary pattern has beneficial effects on the prevention of cancer incidence and mortality. The Mediterranean diet is characterized by high antioxidant content capable of affecting inflammatory progress, cell cycle, proliferation and apoptosis process, and gene expression modulation [3, 46].

**2.2. Oxidative Stress: Role of Epigenetic Alterations in Cancer.** Since 1940, epigenetics has been defined as heritable changes in gene expression without changes in the DNA sequence and described the interactions between the genome and the environment that leads to the formation of the phenotype [47, 48]. Traditional epigenetic changes such as DNA methylation and histone modifications are able to affect gene expression mostly by interfering with the accessibility of transcription factors with DNA or may lead to structural rearrangement of chromatin thus promoting the expression of particular genes. Recent evidence has shown the association of altered expression of noncoding RNAs in general and microRNAs (miRNAs) in particular with epigenetic modifications [25]. miRNAs are small RNA molecules, ~22 long nucleotides, that can negatively control their target gene expression at

a posttranscriptional level. miRNAs bind to their target mRNAs and downregulate their stabilities and/or translation. Accumulating evidences have shown that epigenetic alterations can largely contribute to the carcinogenesis [49] and are considered a hallmark of cancer [50]. The onset and progression of cancer are driven not only by acquired genetic alterations but also epigenetic modifications of gene expression [51, 52]. In cancer cells, hypermethylation on certain promoter regions of tumour suppressor genes causes gene silencing, thereby blocking the expression of these pivot genes [53]. Oxidative stress and inflammatory damage play an important role in epigenetic reprogramming of expression of cytokines, oncogenes, and tumour suppressor genes, thereby setting up a ground for chronic inflammatory diseases and carcinogenesis [30, 31]. On the other hand, global hypomethylation of DNA causes global chromosome instability leading to various mutations and, eventually, to cancer progression [54]. Since epigenetic aberrations occur in early stages of cancer, interventional approaches targeting the epigenome have been proposed as preventive and therapeutic strategies. Unlike genetic defects, epigenetic modifications are reversible and represent a promising avenue for therapeutic intervention [55]. Current epigenetic therapies aim to reverse cancer-associated epigenetic changes and restore normal gene expression. In this regard, two groups of drugs are approved for treatment by the Food and Drug Administration (FDA) [56, 57]: DNA methyl transferase (DNMT) and histone deacetylase (HDAC) inhibitors. Although some of them have even shown promising results in clinical trials [58], epigenomic therapies have several challenges ahead. To this purpose, it is relevant to note that various new DNMT as well as HDAC inhibitors are under development [59]. A synergistic combination of epigenetic modifying agents, including miRNAs, may provide a clinically important reversal of epigenomic cancer states.

### 3. Advances and Novelty in Cancer Chemoprevention and Treatment: Role of Polyphenols

**3.1. Epigenetic and Antioxidant Treatment in Cancer.** In addition to the standard anticancer treatment options such as surgery and chemo-/radiotherapy, several natural polyphenols have been identified as having potential for cancer prevention [60] and treatment [61] (Table 1).

Within the last few years most of the studies reporting on polyphenols have focused on their antioxidant properties [62]. In addition to their antioxidant ability to prevent damage caused by oxidative stress, polyphenols exert some of their biological effects via chromatin remodelling and other epigenetic modifications [63]. The beneficial effects of polyphenols in cancer treatment can be linked to their ability to modulate, in a reversible manner, epigenetic mechanisms involved in tumorigenesis leading to gene expression activation or silencing [64]. Many polyphenols are reported to regulate nuclear factor kappa B (NF- $\kappa$ B) expression and chromatin remodelling through either activation or inhibition of epigenetic-related enzymes such as HDACs, histone acetyltransferases

TABLE 1: Natural polyphenols as anticancer agents.

Bioactive components	Plants	Cancer models	Molecular mechanisms	References	Clinical trials references
Artichoke polyphenols	Artichoke	(i) Hepatocellular carcinoma and breast cancer cell lines	Apoptosis	[118, 119]	
		(ii) Breast cancer cell line	ROS/senescence, histone modifications, and DNA methylation	[28]	
Chlorogenic acid	Coffee	Breast cancer cell lines	DNA methylation	[100]	
Curcumin	Curcuma	(i) Lung cancer in mice	Apoptosis	[104]	Breast cancer Phase 2, [191]
		(ii) Pancreatic, prostate, and lung cancer cell lines	Histone modifications, DNA methylation, and miRNAs	[66]	Colon rectal cancer Phase 1, [191]
Daidzein	Soy	Breast cancer cell lines	Apoptosis	[109]	
Epigallocatechin-3-gallate	Green tea	(i) Hepatocellular carcinoma	miRNAs/apoptosis	[99]	Prostate cancer, [192]
		(ii) Skin cancer cell line	Histone modifications and DNA methylation	[92]	Leukaemia Phase 2, [193]
		(iii) Breast cancer stem cells	Inhibition of mammosphere formation	[153]	Cancer prevention, [194]
		(i) Prostate cancer cells and esophageal cell carcinoma	Histone modifications and DNA methylation	[111]	
Genistein	Soy	(ii) Renal carcinoma cell line	Histone modifications and DNA methylation	[112]	
		(iii) Breast cancer cell lines	Apoptosis	[109]	
		(iv) Breast cancer cell lines	Oxidative stress	[110]	
		Glioma cell lines	ROS/senescence	[135]	
Lycopene	Tomato	Breast cancer cells	DNA methylation	[66]	
Phenethyl isothiocyanate	Broccoli, cabbage, Brussels sprouts, and cauliflower	Prostate cancer cell lines	Histone modifications and DNA methylation	[82]	
		Breast cancer stem cells	NF- $\kappa$ B/miRNA488	[184]	
Resveratrol	Red grapes, cranberries, blueberries, and nuts	(i) Gastric cancer cell lines	Sirtuins/senescence	[138]	Colorectal cancer Phase 1, [191]
		(ii) Lung cancer cell lines	ROS/senescence	[134]	Multiple myeloma Phase 2, [191]
		(iii) Colon cancer cell lines	miRNAs	[106]	Melanoma Phase 1, [191]
Sulforaphane	Broccoli, cabbage, and kale	(i) Colon cancer cells	Histone modifications	[79]	
		(ii) Colon cancer in mice	Histone modifications	[80]	
		(iii) Breast cancer stem cells	Wnt/ $\beta$ catenin self-renewal pathway modulation	[166]	
		(iv) Pancreatic cancer stem cells	Hedgehog pathway activation	[165, 167]	
Quercetin	Onions, buckwheat, and citrus	(i) Oral carcinoma in hamster	Apoptosis, histone modifications, and DNA methylation	[116]	
		(ii) Pancreatic cancer cell lines and cancer stem cells	Apoptosis Inhibition self-renewal cell property	[169, 170]	

(HATs), and DNMTs [65]. In particular some polyphenols with antitumoural activity, such as genistein, phenethyl isothiocyanate, curcumin, sulforaphane, and resveratrol, act on the inhibition of deacetylation of histone proteins whereas other polyphenols, including epigallocatechin-3-gallate (EGCG), genistein, and curcumin, act on the inhibition of acetylation of histone proteins during epigenetic modifications [66]. Furthermore, dietary polyphenols, such as EGCG, genistein, lycopene, curcumin, and resveratrol, inhibit DNA methylation process by affecting DNA methyltransferase activity.

Cancer cells are distinguished by several distinct characteristics due to cumulative epigenetic changes of multiple genes and associated cell signalling pathways, some of which are linked to inflammation. Immune cells infiltrate tumours and are engaged in a cross talk with neoplastic cells; thus, inflammation might affect responses to cancer therapy [67]. Studies on a wide spectrum of various bioactive polyphenols that regulate multiple cancer-inflammation pathways and epigenetic cofactors exhibit low toxicity and are readily available [68]. The anti-inflammatory properties of many reported polyphenols are associated with their ability to induce HDAC activity [69].

Since several polyphenols can modulate both HDAC and HAT, there may be a common underlying mechanism. For instance, curcumin, a known antioxidant as well as a free radical, may regulate both acetylation and deacetylation through the modulation of oxidative stress. Rahman et al. [70] have shown that oxidative stress can induce NF- $\kappa$ B pathway through the activation of intrinsic HAT activity, resulting in the expression of proinflammatory mediators, but it can also inhibit HDAC function.

The role of polyphenols in regulation of epigenetic pathways including sirtuin 1 (SIRT1) modulation has been investigated [71]. Sirtuins are a subclass of HDACs that have been shown to modify metabolism, inflammation, aging, or cellular apoptosis in many pathological processes. The epigenetic effect of SIRT1 is due to its ability to deacetylate many transcriptional factors such as p53, NF- $\kappa$ B, forkhead box class O (FOX), and histone proteins [72].

Sulforaphane (SFN) is a bioactive polyphenol present in cruciferous vegetables such as broccoli, cabbage, and kale [73]. It has been previously shown that SFN induces the expression of phase-II detoxification enzymes [74] and expression of glutathione transferase in murine hepatocytes [75]. Furthermore SFN stimulates phase-II detoxification through activation of nuclear factor E-related factor 2 (NRF2) localized in the cytoplasm [76]. In response to oxidative stress, NRF2 translocates to the nucleus and binds to the antioxidant responsive element (ARE) promoting expression of antioxidant enzymes [77]. In a xenograft murine model, oral administration of SFN significantly reduces tumour size and increases apoptosis. These results indicated that SFN anticancer effects are exerted via inhibition of oxidative stress induced by NRF2-mediated pathways [77, 78]. In addition, SFN promotes anticancer effects through the inhibition of HDAC activity. For instance, in HCT116 colon cancer cell line, SFN inhibits HDAC activity in a dose-dependent manner [79]. A ten-week diet supplementation with SFN induces acetylation of histones in the ileum, colon, and prostate

C57BL/6J mice tissues [80]. Moreover, sulforaphane-N-acetylcysteine (SFN-NAC) and sulforaphane-cysteine (SFN-Cys), two metabolites of SFN generated via the mercapturic acid pathway, may mediate the inhibitory effects on HDAC activity [80].

Isothiocyanate, such as phenethyl isothiocyanate (PEITC), has been shown to inhibit carcinogenic process by growth arrest of many types of cancer cells through induction of apoptotic pathway [81]. Treatment of human prostate cancer cell lines, with PEITC modulated histone acetylation and methylation pathways, in particular restored GSTP1 expression through demethylation of specific gene promoter and inhibited the activity of HDACs [82].

Curcumin, a polyphenol extracted from the most popular Indian turmeric spice (*Curcuma longa*), has antioxidant and anti-inflammatory properties which have been associated with multiple health benefits including cancer prevention [83]. In liver of lymphoma bearing mice long term effect of curcumin leads to prevention of cancer, by inducing phase-II antioxidant enzymes via activation of NRF2 signalling, restoration of tumour suppressor p53, and modulation of inflammatory mediators like TGF- $\beta$  and COX2. These results suggest antioxidant and anti-inflammatory properties of curcumin [83].

Curcumin is a potential modulator of histones affecting both the HAT and HDAC enzyme activities [84]. Several *in vitro* studies, performed on cancer cell lines derived from various tissues, have demonstrated that curcumin has the potential to specifically downregulate p300/CBP HAT activity. In particular, such inhibition suppresses histone acetylation as well as acetylation of nonhistone protein like p53 [85]. Furthermore, curcumin exposure led to a significant reduction of histone acetylation via inhibition of HAT activity without changing HDAC levels in hepatoma cultured cells [84]. In hematopoietic cell lines, curcumin repressed the HAT activity of p300/CBP as well as the activity of various classes of HDACs, which in turn limits the proliferative capacity of cells and induces apoptosis [86]. Antitumour activity of curcumin has been also linked to its ability to modulate miRNA expression level in cancer cells. To this purpose, curcumin has shown to reduce the expression of the antiapoptotic protein Bcl-2 in a breast cancer cell line, MCF7, by upregulating miR-15a and miR-16 [87].

Green tea polyphenols are known to have high antioxidant properties and consequent beneficial functions, including anti-inflammation and cancer prevention. On the other hand, some studies have demonstrated their gastrointestinal toxicity when used at high doses, presumably due to their prooxidant properties [88]. Among green tea polyphenols, EGCG has been extensively studied. A treatment of high doses of this catechin may aggravate colon carcinogenesis in mice and induce hepatotoxicity in experimental animals and in humans as reported by epidemiological observations [88]. Importantly, it has been reported that EGCG can reduce cisplatin-mediated side effects treatment, in particular nephrotoxicity. Cisplatin, a cancer chemotherapeutic drug, induces kidney specific mitochondrial oxidative stress and impaired antioxidant defense enzyme activity. Treating mice with EGCG reduces cisplatin induced mitochondrial oxidative stress leading to an improved renal function compared

to the counterparts. EGCG may be a potential and promising adjuvant agent for cisplatin cancer therapy [89]. Additionally these bioactive compounds are extensively studied from an epigenetically point of view. It has been shown that treatment on a human prostate cancer cell line altered DNA methylation levels and chromatin modelling and reduced the activity of Class I HDACs [90]. EGCG remarkably inhibits HAT activity, whereas other polyphenols derivatives, such as catechin, epicatechin, and epigallocatechin, exhibited low anti-HAT effects. EGCG acted as a HAT inhibitor and reduced the binding of p300/CBP to the promoter region of *interleukin-6* gene with an increased recruitment of HDAC3, which highlights the importance of the balance between HATs and histone deacetylases in the NF- $\kappa$ B-mediated inflammatory signalling pathway [91]. Nandakumar and colleagues [92] demonstrated that EGCG-treatment of skin cancer cells modulated the levels of DNA methylation and histone modifications. These findings resulted in reexpression of tumour suppressor genes *p16<sup>INK4a</sup>* and *p21<sup>CIP/WAF1</sup>*.

A combination of green tea polyphenols, a dietary DNA methyltransferase inhibitor and sulforaphane, a dietary histone deacetylase inhibitor leads to the epigenetic reactivation of silenced tumour suppressor genes such as *p21<sup>CIP/WAF1</sup>* and KLOTHO through active chromatin modifications in breast cancer cell lines [93]. These findings are relevant for understanding the potential of synergistical activity of polyphenol therapeutic combinations.

Treatment of various human cancer cell lines with EGCG caused a concentration and time-dependent reversal of hypermethylation of *p16<sup>INK4a</sup>*, *p15*, *RAR $\beta$* , *MGMT*, and *hMLH1* genes [94, 95]. Furthermore, EGCG partially reversed the hypermethylation status of tumour suppressor gene *RECK* and enhanced the expression of *RECK* mRNA, which correlated with reduced expression of matrix metalloproteinases MMP-2 and MMP-9 involved in the invasive ability of cancer cells [96].

Aberrant promoter methylation of Wnt inhibitory factor-1 (WIF-1) is a fundamental mechanism of epigenetic silencing in human cancers. EGCG has been reported to directly reactivate the WIF-1, through the promoter demethylation in lung cancer cell lines [97].

EGCG modulated miRNAs in lung cancer and hepatocellular carcinoma where the expression of several miRNAs was changed [98, 99]. One of the upregulated miRNAs, miR16, specifically targets antiapoptotic protein Bcl-2 [99]. Altogether these pieces of data indicate that EGCG may be effective in different cancer cell types through different epigenetic pathways.

Coffee and tea polyphenols are also demethylating agents in human breast cancer cell lines where caffeic acid or chlorogenic acid inhibited DNMT1 activity, in a concentration-dependent manner [100].

Resveratrol (RV), a natural polyphenol found in blueberries, cranberries, nuts, red grapes, and wine, exerts anti-inflammatory and anticancer effects [101]. It has the ability to modulate signalling pathways that control cell growth, apoptosis, angiogenesis, and tumour metastasis processes [102]. Furthermore, RV is gaining attention for its antioxidant capabilities and influence on glucose metabolism. Oxidative

stress and high glycolytic flux are common characteristics of cancer cells. It has been demonstrated that RV inhibits intracellular ROS level and suppresses cancer cell glycolytic metabolism [103].

Since anticancer biological activities are already demonstrated for RV and curcumin, to investigate the combined chemopreventive potential of these two polyphenols has been of great interest. It has been shown by Malhotra and colleagues [104] that curcumin and RV when supplemented in combination regulate drug-metabolizing enzymes and antioxidant enzymes, during lung carcinogenesis in mice.

RV activates the protein deacetylase SIRT1 leading to the formation of inactive chromatin and changes in gene transcription [103]. On the other hand, RV activates p300/CBP HAT that participates in the formation of an active chromatin structure [105]. Furthermore, Tili and his group [106] have shown that RV also inhibits oncogenic miRNAs while inducing tumour suppressor miRNAs. These multiple epigenetic alterations by RV exposure can partially explain the activation of some tumour suppressor genes.

In breast cancer, the tumour suppressor gene *BRCA1* is associated with lower levels of SIRT1s expression. It has been reported that in *in vitro* and *in vivo* experimental models RV can increase the expression of *BRCA1* by inhibiting Survivin expression and activating SIRT1. These findings suggest that resveratrol treatment serves as a potential strategy for targeted therapy for *BRCA1*-associated breast cancer [107]. Furthermore, RV in combination with black tea polyphenols suppresses growth and development of skin cancer in mice by inhibiting the MAPK and p53 pathways [108].

Isoflavones are compounds found in soy beans and act like estrogens. Among them, genistein and daidzein have gained the most research attention. Many studies have reported that genistein can be used as a chemopreventive agent in several types of cancers, especially for hormone-dependent breast cancer [109]. Genistein has been shown to bind both the estrogen receptor alpha (ER $\alpha$ ) and the estrogen receptor beta (ER $\beta$ ). The ER $\alpha$ /ER $\beta$  ratio is a prognostic marker for breast tumours, and ER $\alpha$  expression could indicate the presence of malignant tumours. It has been reported that in human breast cancer cell lines genistein effects depend on ER $\alpha$ /ER $\beta$  ratio for oxidative stress regulation, mitochondrial functionality, and modulation of antioxidant enzymes, and sirtuins [110]. Genistein is also involved in the regulation of gene transcription by modification of epigenetic events including DNA methylation and histone modifications. Genistein has been shown to cause reversal of DNA hypermethylation and reactivated methylation-silenced genes, including tumour suppressor gene *p16<sup>INK4a</sup>* in human esophageal squamous carcinoma cell line [111]. In renal carcinoma, the cell tumour suppressor gene *BTG3* is transcriptionally downregulated. This inhibition is due to promoter CpG island methylation. The methylation-silenced *BTG3* gene can be reactivated by genistein treatment that causes CpG demethylation, inhibition of DNMT activity, and induction of active histone modifications [112].

Moreover, genistein treatment has shown the ability to modulate miRNAs expression level. For instance, in prostate cancer cells, genistein caused an increase of miRNA-1296

and accumulation of cells in the S phase of the cell cycle along with a significant downregulation of minichromosome maintenance gene (*MCM-2*), target of miRNA-1296 [113].

Quercetin, a dietary polyphenol present primarily in buckwheat and citrus and onions [114], is known to reduce intracellular ROS levels in various cell types by modulating detoxifying enzymes, such as superoxide dismutase 1 (SOD1) and catalase (CAT). Low concentration of quercetin attenuates the therapeutic effects of cisplatin and other antineoplastic drugs in ovarian cancer cells, by reducing ROS damage. The study concluded that quercetin supplementation during ovarian cancer treatment may detrimentally affect therapeutic response [115].

Quercetin activates SIRT1 deacetylase, through inhibition of HDAC and DNMT1, and has been shown to inhibit the cell cycle and induce apoptosis, thus suppressing tumour growth and angiogenesis [116].

Artichoke polyphenolic extracts had cytotoxic and apoptotic effects on colorectal cancer cells. It has been found that the proapoptotic *BAX* gene expression and a cell cycle inhibitor *p21<sup>CIP/WAF1</sup>* were induced in the presence of artichoke polyphenols [117]. Polyphenolic extracts from the edible part of artichoke (AEs) exhibited cancer cytotoxic activity on a human hepatoma cell line [118] as well as on other cell lines derived from various human tissues. It triggered apoptosis in a dose-dependent manner on a human breast cancer cell line without any effects on normal breast epithelial cell line. Furthermore, cell motility and invasion capabilities were remarkably inhibited by AEs treatment [119]. Furthermore AEs induce DNA hypomethylation and increase lysine acetylation levels in total proteins [28]. Importantly, the authors have shown that AEs have a prooxidant activity in breast cancer cells [28] and an antioxidant effect on normal hepatocytes [118].

From another point of view, chemopreventive polyphenols may indirectly modulate chromatin dynamics and epigenetic effects upon interference with global cancer metabolism [68]. To this purpose, the important role of sirtuins as principal intracellular mediators of the beneficial effect of the Mediterranean diet has been recently highlighted [46].

**3.2. Therapy-Induced Senescence (TIS).** Cancer therapy has traditionally relied on cytotoxic treatment. This approach may produce complete cell death within neoplastic tissues; however such cancers often develop therapy-resistance and recur or progress to advanced primary and metastatic tumours. An alternative strategy to the cytotoxic treatment is the induction of cytostasis which disables the proliferation capacity of cells without inducing cancer cell death [120, 121] (Figure 1). This therapeutic approach could give an equivalent or prolonged survival with fewer or no side effects related to treatment toxicity in patients and may provide a more realistic goal for the chronic management of some cancers. To this purpose, a promising tool for generation of cytostasis is therapy-induced senescence (TIS) which promotes the induction of a permanent growth-arrest cellular phenotype with distinct morphological and biochemical characteristics [23]. The main features include development of a flattened

and enlarged morphology *in vitro* and increased senescence-associated  $\beta$ -galactosidase activity in both cultured cells and tissues [122]. Unlike cells undergoing apoptosis in response to conventional cytotoxic drugs, senescent cells may persist almost indefinitely [121]. Several crucial genes, including tumour suppressors *p53* and *Rb*, have a well described growth inhibitory role [21, 123]. Cells with functional *Rb* and *p53* appear more sensitive to stress and oncogene activities that stimulate senescence [124]. However, it is noteworthy from a therapeutic point of view that cancer cells lacking functional *Rb*, *p53*, and other tumour suppressor proteins display TIS responsiveness. Notably, in cancer cell lines lacking *Rb* and *p53*, doxorubicin induced senescence in more than 50% of cells without the direct involvement of these classic tumour suppressor genes [125].

Importantly, the combined activity of *p53* and *pRb* could determine whether cells enter senescence or cell death pathways [126, 127]. However, the active role of these tumour suppressor proteins in senescence process is complex and actually not completely understood. Beside *Rb* and *p53*, several cell cycle involved genes including the cyclin-dependent kinase inhibitors (CDKIs) *p16<sup>INK4a</sup>*, *p21<sup>CIP/WAF1</sup>*, and *p27* [128] are active during senescence and promote senescent state when overexpressed in cancer cell lines. Overexpression of *p21<sup>CIP/WAF1</sup>* can induce a senescence-like cell-cycle arrest, whereas depletion of *p21<sup>CIP/WAF1</sup>* can delay senescence-associated arrest. Moreover, *p16<sup>INK4a</sup>* acts in *Rb* pathway by inhibiting the activation of CDK4 and CDK6 which is the initial step of *Rb* phosphorylation [129]. The function of *p16<sup>INK4a</sup>* is to keep *Rb* in its active, hypophosphorylated form, which blocks the expression of genes regulated by E2F transcription factors leading to a G1 cell-cycle arrest. Numerous studies have provided important insights into the *p53/p21* and *Rb/p16* pathways that promote cellular senescence: the first one is primarily responsible for senescence induced by telomere shortening or DNA damage; the second one is involved in mediated stress-induced premature senescence (SIPS).

The combination of prosenescence induction with already established treatment protocols in order to take into account the prosenescence approaches in the development of novel cancer therapies has been considered of interest. For instance, both neoadjuvant and adjuvant therapies have a more and more relevant role in the treatment of some neoplasias, including breast, prostate, and colon cancer, where such approaches significantly increase the disease-free survival and the overall survival of patients. In a neoadjuvant protocol, a prosenescence approach could be combined with traditional treatments in order to reduce tumour mass before surgery. Furthermore, senescence-inducing molecules such as natural compounds may be used in combination with radiotherapy in cancer patients who are not suitable for surgery because of their age or advanced stage of disease [130, 131]. Such a treatment may be expected to have two potentially positive outcomes. First, the induction of senescence itself may reduce tumour growth and trigger the immune system to clear senescence cells, contributing to reduction of the tumour mass. Second, since both senescence and apoptosis responses share key effector molecules (such

as p53), the combination of pro-senescence approaches with traditional chemo-/radiotherapeutic protocols may have the added effect of address cancer cells, which are en route to becoming senescent, toward apoptotic death.

A number of promising pro-senescence agents are currently under consideration for cancer clinical management [132]. To this purpose, natural compounds targeting the epigenetic control of senescence are under investigations to develop additional pro-senescence cancer therapeutic strategies [133, 134].

Several anticancer polyphenolic compounds from fruit and vegetables induce cellular growth arrest largely through the induction of a ROS-dependent premature senescence. Among them, 20(S)-ginsenoside Rg3 [135], a compound extracted from ginseng, at a subapoptotic concentration, caused senescence-like growth arrest and increased ROS production in chronically treated human glioma cells [135]. Furthermore, bisdemethoxycurcumin, a natural derivative of curcumin, suppresses human breast cancer cell proliferation by inducing oxidative stress senescence. A relevant role of ROS was also demonstrated for the phenethyl isothiocyanate-induction of apoptosis and senescence in tumours [136].

Polyphenolic extracts from the edible part of artichoke (AEs) have been shown to be potential chemopreventive and anticancer dietary compounds. High doses of AEs induce apoptosis and decrease the invasive potential of the human breast cancer cell line, MDA-MB231 [119]. Chronic and low doses of AEs treatment at sublethal concentrations suppress human breast cancer cell growth via the induction of premature senescence through epigenetic and ROS-mediated mechanisms [28]. In addition to the widely accepted antioxidant properties of the artichoke polyphenols [118], it has been demonstrated that one causative stimulus for senescence induction by chronic treatment of AEs is an increased level of reactive oxygen species. These results show a crucial role of ROS as effectors of polyphenol-induced prooxidant damage in cancer cells. To confirm this important contribution of ROS, the antioxidant NAC attenuates the effect of AEs on MDA-MB231. Importantly, the authors have shown that AEs have a prooxidant activity in breast cancer cells [28] and an antioxidant effect on normal hepatocytes [118]. Given that aberrant redox system is frequently observed in many tumour cells, the authors hypothesized that AEs may selectively inhibit the growth of tumour cells with little or no toxicity on normal cells based on their differential redox status.

Low doses treatment of RV exerts its anticancer and chemopreventive effects through the induction of premature senescence in lung cancer cells. This event correlates with increased DNA double strands breaks and ROS production through the upregulation of NAPDH oxidase-5 expression [137].

Furthermore, low doses of RV treatment arrested gastric cancer cells in the G1 phase and led to senescence instead of apoptosis which is initiated by high doses treatment [138]. The inhibitory effect of resveratrol on gastric cells was also verified *in vivo* using a nude mice xenograft model. RV exerted inhibitory activities on gastric development and significantly decreased the fraction of Ki67-positive cells in the nude mice tumour specimens. After the RV treatment, the induction of

senescence and the changes in the expression of the regulators involved in the cell cycle and senescence pathways were similar to what was observed *in vitro*.

The propensity of tumour cells to undergo senescence in response to low and chronic exposure of RV treatment and to apoptosis with high doses of RV was confirmed in C6 rat glioma cells and further investigated in cooperation with quercetin. Chronically administered, RV and quercetin in subapoptotic doses can induce senescence-like growth arrest. These results suggested that the combination of these agents could be a good candidate treatment for glioma tumours [139].

#### 4. Cancer Stem Cells: Potential Targets for Polyphenols

In the few last years, many studies have highlighted the existence of CSCs in most solid and nonsolid tumours, including brain, head and neck, breast, colon, and leukaemia among others [140–143]. These cells are considered responsible for tumour relapse and resistance to therapy [144–147]; thus novel therapeutic approaches, targeting the cancer stem cells pool, are under investigation [148]. Despite differentiated cancer cells, CSCs exhibit low ROS levels due to high expression of scavenger molecules, more efficient DNA repair responses, and promotion of glycolysis and autophagy [149, 150].

Recently, many strategies targeting cancer stem cells have been proposed, namely, (a) inhibiting their self-renewal ability and chemoresistance related pathways, (b) inducing their differentiation [151, 152], (c) targeting some of their cell-surface molecular markers [153], (d) impacting their energetic metabolism via inhibition of glycolysis [154] and/or by targeting mitochondria [155], and (e) designing miRNA-based strategies to block cancer stemness [156]. Theoretically in all tumours, cancer stem cells might reside within specific microenvironments distinguished by the presence of hypoxia [157], oxidative stress [158], chronic inflammation [159], and a peritumoural acidic pH [160]. Thus, many investigators have suggested that cancer can be overcome either by inhibiting the CSCs metabolisms or by targeting the surrounding cancer environment [161].

Novel anticancer strategies should be designed to selectively target cancer stem cells and to this purpose natural compounds might have a relevant role. We provide a revision of the most recent literature addressing the CSCs-regulation role of some of the most investigated polyphenols.

(a) *Role of Polyphenols in the Regulation/Inhibition of Cancer Stem Cells Self-Renewal.* It has been shown that polyphenols can impact cancer stem cells self-renewal related pathways, such as Wnt/ $\beta$ -catenin, Hedgehog, and notch [162]. In particular isothiocyanates (ITCs) have been described to have positive effects in the prevention of human tumours [163]. Beside several mechanisms of action, including activation of carcinogen-detoxifying enzymes, modulation of apoptotic pathway, cell-cycle arrest of cellular proliferation, and modulation of epithelial-mesenchymal transition (EMT), CSCs self-renewal suppression was reported, thus inhibiting

oncogenic signalling pathways such as NF- $\kappa$ B and STAT3 [164].

Between cruciferous family natural compounds, SFN has been demonstrated to be capable of targeting cancer stem cells in different types of cancer, by regulating pathways such as NF- $\kappa$ B, Hedgehog, and Wnt/ $\beta$ -catenin also contributing to the induction of epithelial-mesenchymal transition. For these properties, SFN has been proposed as an adjuvant of chemotherapy in several preclinical studies [165]. Previous reports have demonstrated that SFN reduced the cancer stem cells population in human breast cancer cells as shown by decrease of aldehyde dehydrogenase (ALDH) + cells and reduction of primary mammospheres *in vitro* [166]. Furthermore, the antiproliferative property of SFN has been reported on pancreatic CSCs *in vitro* and *in vivo* models; such effect strongly depends on the activation of Hedgehog pathway for cancer stem cells self-renewal activity [167, 168].

Pancreatic CSCs studies carried out *in vitro* models, showed that quercetin, a polyphenol present in many fruit and vegetables [169], decreased ALDH1 activity, induced apoptosis, and decreased the expression of EMT-proteins. Whereas, in *in vivo* experiments, quercetin inhibited cancer stem cells-derived xenografts, reducing the expression of proliferation, stemness, and angiogenesis related genes [169].

Remarkably, quercetin effects were amplified in the presence of SFN, suggesting the importance to combine different polyphenols for designing synergistic anticancer strategies [169, 170].

The use of soy foods has been shown to be beneficial for the reduction of mammary tumour risk. The intake of these natural compounds was demonstrated to be beneficial for the modulation of body weight and adiposity associated with breast cancer both in humans and in animal models [171–174]. Moreover, human MCF-7 breast cancer cells cultured in a genistein-treated adipocytes conditioned medium generated a lower number of mammospheres [172].

*(b) Polyphenols Affecting Cancer Stem Cells Metabolism.* Cancer stem cells, like physiological stem cells, are characterized by a hyperglycolytic metabolism [175] and, in parallel, by a lowered mitochondrial respiration, compared to more differentiated cells within the tumour bulk [154, 176]. Thus a possible strategy to counteract CSCs could be to impair their metabolism either by inhibiting glycolysis or by forcing cancer stem cells into mitochondrial metabolism and oxidative phosphorylation [177]. To this purpose, many polyphenols have been shown to play a role in the regulation of cancer metabolism. Some plant derived polyphenols in relation to cancer cell metabolism are described. Genistein was shown to affect the pentose phosphate pathway (PPP), without modulating the synthesis of fatty acids in pancreatic adenocarcinoma cells [178]. Moreover, the green tea polyphenol, EGCG, is known to activate AMP-activated protein kinase (AMPK) in human breast cancer cells [179]. Activation of AMPK, a key actor in the control cellular energy status, cell cycle, protein synthesis, and cell viability, led to cell proliferation inhibition, upregulation of the CDK inhibitor  $p21^{CIP/WAF1}$ , downregulation of the mammalian target of rapamycin pathway, and suppression of the cancer stem cells

population [179]. Moreover, polyphenols naturally present in extra olive oil have been shown to have anticancer effects by suppressing the expression of genes involved in both aerobic glycolysis (Warburg effect) and CSCs self-renewal [180].

Hyperglycolytic cancer stem cells have an increased basal level of ROS, although they result as being more vulnerable than physiological cells to a further increase in oxidative damage elicited by prooxidant polyphenol action [181]. One of these compounds is curcumin which, promoting ROS production, reducing the mitochondrial membrane potential, and inducing apoptotic pathways, leads to cell death in many cancer models [182, 183].

Cancer stem cells are localized within specific niches, normally characterized by the presence of lower oxygen tension (hypoxia), inflammation, oxidative stress, and a lower pH. Polyphenols can be exploited to regulate the CSC niche, by targeting signalling pathways that are implicated in the maintenance of tumour microenvironmental features. Among the polyphenols targeting hypoxia, pterostilbene, a stilbene isolated from blueberries, has been recently reported to have anticancer properties.

Breast cancer cell lines such as MCF-7 and MDA-MB231 were cocultured with tumour-associated macrophages, known to enhance malignancy promoting metastasis. In these experimental conditions, a large subpopulation of cancer stem cells, characterized by an increased level of HIF1 $\alpha$ ,  $\beta$ -catenin, Twist1, and NF- $\kappa$ B and by a high ability to produce mammospheres, was present. By adding pterostilbene to the cell medium, the percentage of cancer stem cells was significantly reduced. The effects of such a polyphenol were confirmed *in vivo* experiments where tumorigenesis and metastasis were inhibited [184].

*(c) Polyphenols Targeting Proinflammation Signalling Pathways.* The presence of chronic inflammation could be a characteristic of the neoplastic niche [159]. Some polyphenols have been suggested to be potential therapeutic molecules to counteract chronic inflammatory status that eventually leads to several diseases including cancer [185]. Flavonoids, present in fruit and vegetables, are demonstrated to be suppressors of NF- $\kappa$ B pathway, which is involved in inflammation, cellular transformation, tumour cell proliferation, invasion/metastasis, and angiogenesis [186]. Moreover, some carotenoids have been demonstrated to inhibit NF- $\kappa$ B signalling and thus have anti-inflammatory and anticancer properties [187]. Recently, on a murine model of inflammation-triggered colon carcinogenesis, remarkable anti-inflammatory and antitumoural properties of glucosinolates, extracted from Brassicaceae, have been shown [188].

*(d) Polyphenols Regulating the Peritumoural Acidic pH.* Cancer stem cells extracellular microenvironment is often characterized by an acidic status caused by CSCs metabolic dependence on aerobic glycolysis. Buffering the acidic cancer pH with the use of sodium bicarbonate inhibited tumour growth and cancer cell invasion in a preclinical animal model [189, 190]. To this purpose, high potassium intake coming from a diet rich in vegetables and fruits and a lowered consumption of animal proteins could be a natural strategy

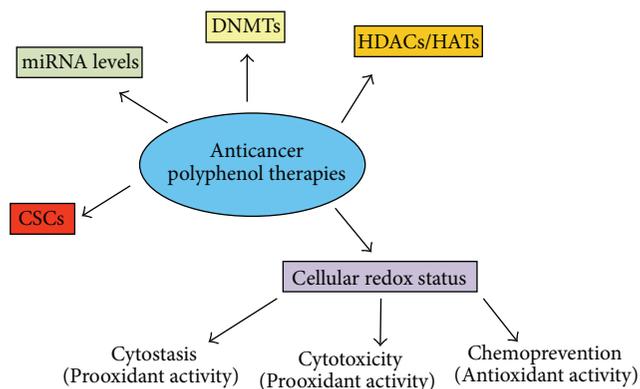


FIGURE 2: Targets of polyphenol anticancer therapies. Epigenetic pathways, cellular redox status, and cancer stem cells as therapeutic targets of polyphenol anticancer therapies as extensively discussed in the text. Depending on acute or chronic treatment a prooxidant activity may induce, respectively, high ROS-mediated cytotoxicity or low ROS-mediated cytostasis. According to the figure, several natural compounds as resveratrol, artichoke polyphenols, ginsenoside Rg-3, and quercetin induce a prooxidant apoptotic mechanism at high concentrations whereas low doses and chronic exposure trigger a ROS-epigenetic mediated cellular senescence.

to neutralize cancer acidosis. An intriguing chemopreventive and therapeutic approach to raise pH could be the use of polyphenols such as genistein, EGCG, and RV in order to impair the cancer stem cells metabolism either by inhibiting aerobic glycolysis or by forcing them into oxidative phosphorylation, as previously described in this review.

Furthermore, several plant compounds have been shown to increase pH values by inhibiting proton pump activity and consequently elicited apoptosis in cancer [190]. This might represent another valid approach to counteract cancer cell growth.

## 5. Conclusions

Compared to normal cells, cancer cells have an increased rate of ROS production and have aberrant regulation mechanisms to deal with their particular redox status. ROS have a well-defined role in promoting and maintaining tumorigenicity indicating that dietary antioxidants have an active role in preventing or reducing tumorigenesis. On the other hand, high levels of ROS can also be toxic to neoplastic cells and can potentially induce cell death. Accumulating evidence shows that ROS levels in tumour cells are crucial for designing advanced therapies and future challenge in anticancer treatments. To this purpose, increasing knowledge from epidemiological and experimental data supports the bright future of natural polyphenols as anticancer tools [191–194] (Figure 2). The complex balance among cell proliferation, apoptosis, and senescence induced by polyphenols could be exploited therapeutically to improve the efficacy of conventional cancer treatment and to develop new antitumour strategies (Table 1).

Much attention is currently focused on the role of natural polyphenols on modulating intracellular ROS levels leading

to epigenetic modifications of pivotal genes in tumorigenesis. It is important to stress that DNA methylation and posttranslational histone modifications are crucial actors in epigenomic landscape playing a relevant role in the structure and function of chromatin. Several polyphenols were demonstrated to interfere with enzymes driving the epigenetic alterations which modulate inflammation process that might hesitate in cancer. As such, it will be a challenge for future anti-inflammatory therapies to deeply evaluate the anticancer role of polyphenols as epigenetic modulators. However, there are some concerns that anticancer therapies with polyphenol regulators of DNMT and HAT/HDAC may suffer from a lack of specificity. To overcome this limitation, an alternative strategy may be to synergistically combine nonselective epigenetic treatments with low doses of conventional targeted therapies which lead to less toxicity comparing to a high dose standard treatments. Furthermore, microRNAs molecules are promising actors in the epigenetic combination therapies, as their target specificity may bridge the gap between genetic and epigenetic changes. To this purpose, natural polyphenols may indirectly modulate the epigenome by affecting levels of microRNAs which target specific epigenetic modifier enzymes.

## 6. Future Perspectives

The future of polyphenol-epigenomic therapy has several challenges ahead and it is a promising field for clinical cancer interventions.

In developing novel anticancer strategies, prosenescence has a relevant role. The current knowledge of senescence, as a major mechanism of tumour suppression as well as a determinant of the outcome of cancer treatment, leads to the concept of prosenescence therapy, which could be an important alternative or addition to conventional chemo-/radiotherapy. To this aim, prosenescence-polyphenols treatment may minimize toxicity and side effects of conventional therapies in cancer patients. On the other hand, some investigators suggest caution in the clinical management of this therapy because the induction of senescence might give rise to quiescent tumour cells, mainly cancer stem cells, which represent a potential niche for cancer recurrence. Thus, deeper understanding of the biological mechanisms responsible for cellular senescence is required in order to better characterize the role of polyphenols in prosenescence therapy for more efficient management of cancer treatment in the future.

The association of cancer stem cells and the resistance to chemo-/radiotherapy stimulate a critical consideration regarding the efficiency of prooxidant therapy on CSCs. Most conventional anticancer therapies are ineffective in killing this cell population. It is for this reason that there has been a growing interest to develop new strategies based on identifying agents able to directly target quiescent cancer stem cells. Since low ROS levels has been suggested to be critical for maintaining cellular stemness, an increase of these reactive species polyphenol-mediated might sensitize cancer stem cells to therapy. However, evaluating novel treatment

approaches also require the development of assays or identification of biomarkers able to identify CSCs population in order to select and assess cancer patients.

## Abbreviations

DNMTs:	DNA methyl transferases
HDACs/HATs:	Histone deacetylases/histone acetyltransferases
CSCs:	Cancer stem cells
miRNAs:	MicroRNAs.

## Conflict of Interests

The authors have declared no conflict of interests.

## Acknowledgments

The authors thank Dr. Lucia Monaco (University of Rome “Sapienza”) for her helpful comments and advice. The authors acknowledge Ms. Tania Merlino for her grammatical suggestions of the English paper. Contract grant sponsor is Lega Italiana per la Lotta contro i Tumori (LILT). Contract Grant no. is 08/12/C/73.

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## Research Article

# Efficiency of Base Excision Repair of Oxidative DNA Damage and Its Impact on the Risk of Colorectal Cancer in the Polish Population

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Received 21 May 2015; Revised 8 July 2015; Accepted 27 July 2015

Academic Editor: Subash Chandra Gupta

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DNA oxidative lesions are widely considered as a potential risk factor for colorectal cancer development. The aim of this work was to determine the role of the efficiency of base excision repair, both in lymphocytes and in epithelial tissue, in patients with CRC and healthy subjects. SNPs were identified within genes responsible for steps following glycosylase action in BER, and patients and healthy subjects were genotyped. A radioisotopic BER assay was used for assessing repair efficiency and TaqMan for genotyping. Decreased BER activity was observed in lymphocyte extract from CRC patients and in cancer tissue extract, compared to healthy subjects. In addition, polymorphisms of *EXO1*, *LIG3*, and *PolB* may modulate the risk of colorectal cancer by decreasing (*PolB*) or increasing (*LIG3* and *EXO1*) the chance of malignant transformation.

## 1. Introduction

Colorectal cancer (CRC) is a common neoplasia in both men and women and is ranked as the second most common type of cancer. The causes of colorectal cancer have not yet been established and its incidence is known to be increasing, with approximately 1.4 million new cases diagnosed each year [1]. CRC occurs mainly in three specific forms: sporadic form, which accounts for about 80% of all cases, a familial form, which represents about 15%, and inherited forms, observed in 5% of all cases, which include familial adenomatous polyposis (FAP), and hereditary nonpolyposis colorectal cancer (HNPCC) [2]. Despite the cause of most colorectal cancers being environmental factors, studies show that individual predispositions for developing this cancer may depend on mutations of certain genes, including those involved in the process of DNA repair. Several DNA repair mechanisms have evolved to protect the genome from DNA damage caused by endogenous or environmental factors, which if unrepaired could lead to the initiation of carcinogenesis. The efficiency

of DNA repair varies between individuals, and the reasons for this should be sought in polymorphisms within the DNA repair genes. An increasing number of DNA repair gene polymorphisms are being correlated with increased risk of cancer occurrence. Although an irrefutable link has already been established between colorectal cancer and the presence of mutations in mismatch repair (MMR) genes [3], other polymorphisms of DNA repair genes (BER and NER) are undergoing investigation for a potential influence on CRC.

Base excision repair (BER) is DNA repair system that operates on small lesions such as oxidized or reduced bases. A single damaged base is removed by base-specific DNA glycosylases. The abasic site is then rebuilt by endonuclease action, removal of the sugar residue, DNA synthesis using the other strand as a template, and then ligation. The molecules involved with the process include Exonuclease I, which cleaves the nucleotides from the end of DNA strand, DNA polymerase beta, which is involved in gap filling, and DNA ligase 3, which seals interruptions in the phosphodiester backbone of duplex DNA [4]. Polymorphisms in the genes

encoding these proteins are suspected to influence the efficiency of the whole BER process and thus modulate the risk of CRC.

The present study has two major aims. The first is to evaluate the influence of the presence of polymorphisms within the tested genes with an elevated risk of colorectal cancer. The second is to make an *in vitro* assessment of the efficiency of restoring DNA continuity via the BER pathway. As all protein products of the genes chosen for SNP screening are involved in the BER stages directly following glycosylase action, the BER assay was adjusted to measure only the gap-filling step, when *PolB*, *EXO3*, and *LIG3* play crucial roles.

## 2. Materials and Methods

DNA for genotyping was isolated from lymphocytes of the peripheral blood. The blood samples were taken from 235 unrelated patients hospitalized in the Military Medical Academy University Teaching Hospital-Central Veterans' Hospital in Lodz. Each patient had histopathologically confirmed colorectal cancer. The studied group included 137 men and 98 women (average age 61 years  $\pm$  8 years). The stage of the tumors was established according to TNM scale. The control group included 240 individuals not diagnosed with cancer and with ages corresponding to the age of the studied group ( $p < 0.05$ ). Permission to conduct research was granted by the bioethics committee of the Medical University of Lodz.

DNA isolation was carried out with a commercial kit QIAamp DNA Blood Mini Kit for isolation of high-molecular-weight DNA (Qiagen).

The occurrence of polymorphic variants of 242Pro/Arg of *PolB* gene, 780Arg/His of *LIG3* gene, and 589Glu/Lis of *EXO1* gene was studied with TaqMan technique. Briefly, 25  $\mu$ L of reaction mixture was used for analysis, containing 1  $\mu$ L of genomic DNA solution, 1  $\mu$ L of probes designed specifically for each polymorphism, 13  $\mu$ L of premix with polymerase, and 10  $\mu$ L of water. The PCR reaction was performed in a Stratagene Mx3005P Real Time PCR Thermocycler. The RS numbers for polymorphisms and thermal conditions of reaction are shown in Table 1. For 10% of the randomly selected samples, genotyping was repeated to confirm reproducibility. Cases and controls were genotyped randomly and researchers were blinded to the case/control status during genotyping.

BER efficiency was evaluated according to Matsumoto et al. [5] with some minor modifications to improve the preparation of synthetic lesion site. A plasmid construct with radioactively labeled single-strand breaks was incubated with protein extract isolated from peripheral blood lymphocytes and slices of cancerous tissue removed during surgical procedures. Repair capability was assessed by densitometric analysis of DNA fragments which had been electrophoretically separated and depicted on X-ray film: these fragments vary with regard to length and can identify repaired or unrepaired fractions. The course of procedure is outlined in Figure 1.

The blood donors were a 79-year-old man with histopathologically confirmed adenocarcinoma and a cancer-free woman of the same age. A colorectal cancer tumor had been removed from the 86-year-old female with

TABLE 1: The refSNP and thermal conditions used in the PCR reaction.

Gene	<i>PolB</i>	<i>LIG3</i>	<i>EXO1</i>
Polymorphism	242Pro/Arg	780Arg/His	589Lys/Glu
refSNP	3136797	3136025	1047840
Thermal conditions	(1) 95°C—10 min (2) 92°C—15 sec (3) 60°C—1 min (4) Step 2 and 3—45x		
Dyes	ROX, HEX, and FAM		
Ref. dye	ROX		

cecum carcinoma. Control tissue samples of the colon were taken from patients with primary inguinal incarcerated hernia from the macroscopically unchanged tissue during the operation. All individuals enrolled in this experiment were hospitalized in the Military Medical Academy University Teaching Hospital-Central Veterans' Hospital, Lodz.

### 2.1. Details of the BER Assay Procedure

**2.1.1. Preparation of DNA Substrate.** The Vector, a pBSII plasmid, was multiplied in *E. coli* DH5 $\alpha$  and isolated by Qiagen Maxiprep and underwent double digestion with 2.5 U of XbaI and 2.5 U XhoII fast digest enzymes for 1 hour (Fast digest, ThermoScientific, Rochester, USA). SAP (ThermoScientific, Rochester, USA) enzyme was applied to avoid self-ligation. Insert 5'-TCGAGAATUCGATATCAT-3' was labeled in kinase reaction 2 U T4 kinase polynucleotide (thermo) with 2  $\mu$ L of [ $\gamma$ -<sup>32</sup>P] ATP (6000  $\mu$ Ci) whereas the second oligo (5'-CTTAAGCTATAGTAGGATC-3') was incubated under the same conditions but with unlabeled ATP. Equal amounts of the two oligonucleotides were mixed and annealed through heating to 95°C before being left to slowly cool down. A 1:5 vector:insert molar ratio (established previously) was applied to allow ligation with 1 U of T4 ligase (ThermoScientific, Rochester, USA) overnight. The construct was purified by elution (GenJet Maxi prep kit, ThermoScientific) from 1% agarose gel.

Preparation of whole-cell protein extract is as follows: Minute Total Protein Extraction Kit (Invent) was used to isolate proteins from peripheral blood lymphocytes or tissue slices. All protein samples were adjusted to 2  $\mu$ g/mL.

**2.1.2. Repair Assay.** A 100 ng/reaction of unaltered native pBSII (load control) was mixed with prelabeled plasmid (1000 cpm/sample) carrying an AP site generated by digestion with 1 U of UDG glycosylase (ThermoScientific) for 4 h. This mixture was made up to a total volume of 15  $\mu$ L by adding the reagents 0.4  $\mu$ L of 1 M HEPES-KOH, pH 7.5, 0.2  $\mu$ L of 1 M MgCl<sub>2</sub>, 0.5  $\mu$ L of 3 M KCl, 0.2  $\mu$ L of 0.1 M DTT, 1  $\mu$ L of 0.1 M ATP, and 0.4  $\mu$ L of 1 mM dNTP and adjusted to a final volume with H<sub>2</sub>O. The reaction was launched by adding 5  $\mu$ L (10  $\mu$ g) protein extract. Repair incubation was carried out in a thermocycler (Bio-Rad) and took 90 min at 25°C. The reaction was stopped by adding of 6  $\mu$ L of 2% SDS to

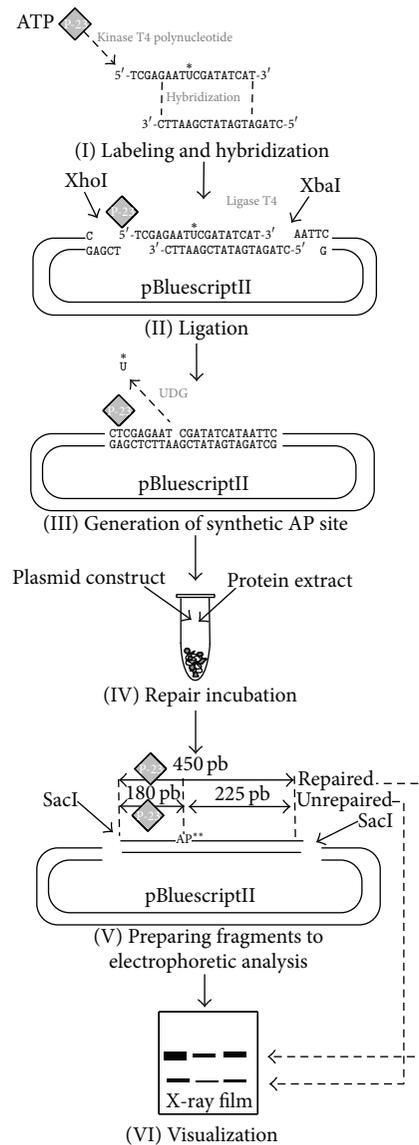


FIGURE 1: (I) A uracil-containing oligonucleotide was subjected to action of polynucleotide kinase to attach a radioactive phosphate group from [ $\gamma$ - $^{32}$ P] ATP. It was hybridized with a second oligonucleotide whose sequence was adjusted to obtain sticky ends referring to XhoI and XbaI digestion site. (II) The short DNA fragment prepared in stage I was cloned into a pBluescriptII plasmid. (III) Uracil-DNA glycosylase was utilized to remove uracil and, as consequence, create a single gap in DNA to act as a synthetic lesion. (IV) A plasmid with single AP site constituted a substrate for the protein extract in 90-minute repair incubation. (V) Two SacI recognition sites of the pBluescriptII plasmid were used to excise 450 pb-long fragment covering the lesion site and radioactive label for analysis on 8% urea/acrylamide gel. (VI) Interpretation of outcomes was based on detection of two bands. The full-length 450 pb fragment reflects restored DNA fraction, whereas presence of short 180 pb fraction indicates the amount of unrepaired DNA. \*U: uracil; \*\*AP: apurinic/aprimidinic.

each tube. Thereupon, 2  $\mu$ L of 1 mg/mL proteinase K and 2  $\mu$ L of 0.2 mg/mL carrier tRNA were added to each tube and incubated at 37°C for 30 min, and then treated with 150  $\mu$ L stop solution (10 mM Tris-HCl, pH 7.5, 300 mM sodium acetate, 10 mM EDTA pH 8.0, 0.5% SDS). DNA was recovered by phenol/chloroform extraction (1:1) and overnight ethanol precipitation. A 450 pb length DNA fragment was excised from plasmid by 1 U of SacI enzyme (ThermoScientific, Rochester, USA) incubated at 37°C for 1 h. To allow the repaired and unrepaired fractions to be differentiated, the

remaining unrepaired AP site was treated with 1 U of AP-recognizing endonuclease IV for 1 h. All samples were run on 8% urea-containing polyacrylamide gel for 3 h in 120 V. The accurate electrophoresis was preceded by 1 h preelectrophoresis with loading buffer.

**2.1.3. Visualization.** The bands were detected by autoradiography. The gels were dried and stored at -20°C with X-ray film for 2 h, 6 h, or overnight exposure. Bromidium ethidium staining was used to visualize load control. Optical density

TABLE 2: The distribution of genotypes, allele frequencies, and the analysis of the odds ratio (OR) for 589Lys/Glu polymorphism of *EXO1* gene in patients with colorectal cancer (CRC) and the control group.

Genotype/allele	Patients <i>n</i> = 309	Controls <i>n</i> = 304*	OR (95% CI)	<i>p</i>
Lys/Lys	57	69	1 (ref.)	—
Lys/Glu	<b>203</b>	<b>147</b>	<b>1.672 (1.109–2.519)</b>	<b>0.014</b>
Glu/Glu	49	88	0.674 (0.411–1.106)	0.118
Lys	317	285	1 (ref.)	—
Glu	301	323	0.838 (0.670–1.048)	0.121

\*Genotype distribution in Hardy-Weinberg equilibrium;  $\chi^2 = 0.612$ .

TABLE 3: The distribution of genotypes, allele frequencies, and the analysis of the odds ratio (OR) for 242Pro/Arg polymorphism of *PolB* gene in patients with colorectal cancer (CRC) and the control group.

Genotype/allele	Patients <i>n</i> = 303	Controls <i>n</i> = 302*	OR (95% CI)	<i>p</i>
Pro/Pro	147	121	1 (ref.)	—
Pro/Arg	123	142	0.713 (0.507–1.003)	0.052
Arg/Arg	33	39	0.697 (0.413–1.174)	0.174
Pro	417	384	1 (ref.)	—
Arg	<b>189</b>	<b>220</b>	<b>0.772 (0.601–0.994)</b>	<b>0.044</b>

\*Genotype distribution in Hardy-Weinberg equilibrium;  $\chi^2 = 0.791$ .

TABLE 4: The distribution of genotypes, allele frequencies, and the analysis of the odds ratio (OR) for 780Arg/His polymorphism of *LIG3* gene in patients with colorectal cancer (CRC) and the control group.

Genotype/allele	Patients <i>n</i> = 310	Controls <i>n</i> = 305*	OR (95% CI)	<i>p</i>
Arg/Arg	101	121	1 (ref.)	—
Arg/His	<b>173</b>	<b>132</b>	<b>1.570 (1.109–2.224)</b>	<b>0.011</b>
His/His	36	52	0.829 (0.503–1.368)	0.462
Arg	375	374	1 (ref.)	—
His	245	236	1.035 (0.823–1.302)	0.764

\*Genotype distribution in Hardy-Weinberg equilibrium;  $\chi^2 = 0.125$ .

quantification of bands was performed with GeneTools software (Invitrogen).

### 3. Results

The genotyping results indicate that the Lys/Glu genotype of the *EXO1* gene (Table 2) may increase the risk of colorectal cancer (OR = 1.672 (1.109–2.519),  $p = 0.014$ ). The investigated *PolB* gene polymorphism was not found to increase the risk of CRC; however, our analysis suggests that occurrence of Arg allele may have a protective effect, since it decreases the risk of colorectal cancer (OR = 0.772 (0.601–0.994),  $p = 0.044$ ) as shown in Table 3. The 780Arg/His polymorphism of the *LIG3* gene was found to contribute to an increase in the risk of CRC (OR = 1.570 (1.109–2.224),  $p = 0.011$ ) (Table 4).

In order to investigate the interaction of the polymorphisms of the studied genes and to evaluate their mutual influence on the risk of colorectal cancer, gene-gene interactions were analyzed. The simultaneous occurrence of the Lys/Glu genotype of the *EXO1* gene and the Pro/Pro genotype of the *PolB* gene was found to possibly increase the risk of colorectal cancer (OR = 2.265 (1.193–4.301),  $p = 0.011$ ) (Table 5).

In case of gene-gene interactions between 589Lys/Glu *EXO1* SNP and 780Arg/His *LIG3* SNP, the simultaneous occurrence of Lys/Glu and Arg/His genotypes may increase risk of colorectal cancer (OR = 1.970 (1.041–3.731),  $p = 0.036$ ) while concomitant presence of Glu/Glu and Arg/Arg genotypes may decrease the risk (OR = 0.402 (0.178–0.906),  $p = 0.026$ ) (Table 6). Finally, the analysis of gene-gene interactions for 242Pro/Arg *PolB* gene and 780Arg/His *LIG3* gene indicated that the cooccurrence of genotypes Pro/Pro and Arg/His may increase the risk of CRC (OR = 2.154 (1.265–3.667),  $p = 0.004$ ) (Table 7).

In general, optical density detection of particular DNA bands revealed higher BER repair efficiency among cancer-free individuals than CRC patients in both lymphocytes and colon tissue samples. The percentage ratio of repaired to damaged fractions was found to be 89.67%/10.32% in lymphocytes taken from healthy subjects and 70.5%/29.5% in those of CRC patients. Examination of the ability of tissue protein extract to perform BER indicated a significantly greater repair level in normal tissue (68.11%/31.89%) than CRC tissue (58.36%/41.64%). The results of the BER assay analysis are presented in Figure 2.

TABLE 5: The distribution of genotypes and the analysis of the odds ratio (OR) for gene-gene interactions: 589Lys/Glu *EXO1* and 242Pro/Arg *PolB* in patients with colorectal cancer (CRC) and the control group.

Genotype	Patients <i>n</i> = 302	Controls <i>n</i> = 302	OR (95% CI)	<i>p</i>
Lys/Lys-Pro/Pro	24	28	1 (ref.)	—
Lys/Lys-Pro/Arg	20	26	0.897 (0.404–1.994)	0.791
Lys/Lys-Arg/Arg	11	15	0.856 (0.331–2.212)	0.752
Lys/Glu-Pro/Pro	<b>99</b>	<b>51</b>	<b>2.265</b> <b>(1.193–4.301)</b>	<b>0.011</b>
Lys/Glu-Pro/Arg	81	72	1.313 (0.698–2.467)	0.396
Lys/Glu-Arg/Arg	19	23	0.964 (0.426–2.180)	0.920
Glu/Glu-Pro/Pro	23	42	0.639 (0.303–1.346)	0.238
Glu/Glu-Pro/Arg	22	44	0.583 (0.276–1.232)	0.156
Glu/Glu-Arg/Arg	3	1	—	—

TABLE 6: The distribution of genotypes and the analysis of the odds ratio (OR) for gene-gene interactions: 589Lys/Glu *EXO1* and 780Arg/His *LIG3* in patients with colorectal cancer (CRC) and the control group.

Genotype	Patients <i>n</i> = 302	Controls <i>n</i> = 302	OR (95% CI)	<i>p</i>
Lys/Lys-Arg/Arg	21	27	1 (ref.)	—
Lys/Lys-Arg/His	31	27	1.476 (0.684–3.185)	0.320
Lys/Lys-His/His	3	15	—	—
Lys/Glu-Arg/Arg	64	46	1.789 (0.902–3.547)	0.094
Lys/Glu-Arg/His	<b>118</b>	<b>77</b>	<b>1.970</b> <b>(1.041–3.731)</b>	<b>0.036</b>
Lys/Glu-His/His	17	23	0.950 (0.407–2.218)	0.920
Glu/Glu-Arg/Arg	<b>15</b>	<b>48</b>	<b>0.402 (0.178–0.906)</b>	<b>0.026</b>
Glu/Glu-Arg/His	18	26	0.890 (0.389–2.038)	0.777
Glu/Glu-His/His	15	13	1.484 (0.582–3.784)	0.409

#### 4. Discussion

All cells in the human body are permanently exposed to the negative effects of reactive oxygen species. Virtually all kinds of cell components, including proteins, lipids, and nucleic acids, can be targets for attack by ROS, which may interfere with the proper functioning of cellular biochemical processes. Oxidative stress has been confirmed to play a role in carcinogenesis by a number of previous studies [6]. Oxidative damage to DNA has significant mutagenic potential, and

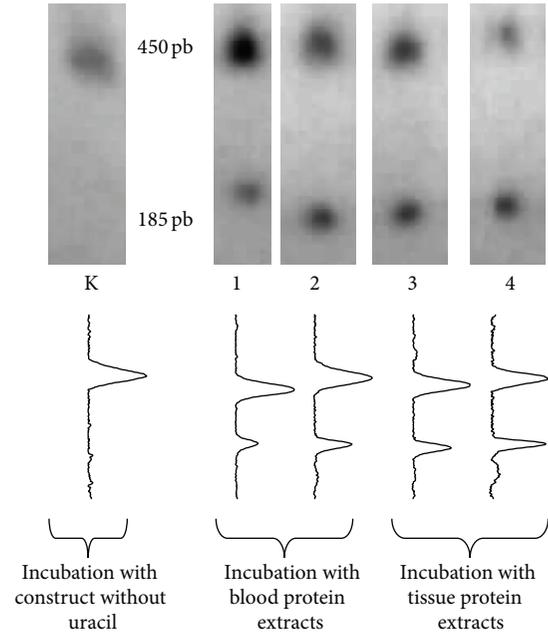


FIGURE 2: A comparison of BER activity in the lymphocytes and tissue of CRC patients and healthy controls. Each electropherogram shows two fractions of DNA: 450 pb repaired and 185 pb unrepaired. Lanes 1-2 indicate lymphocyte BER efficiency while lanes 3-4 refer to BER in tissue. Samples are presented in the following order: K: positive control; DNA substrate did not contain uracil and so reflects 100% of repair; 1: healthy control; 2: colorectal cancer; 3: unchanged colon tissue; 4: colorectal cancer.

TABLE 7: The distribution of genotypes and the analysis of the odds ratio (OR) for gene-gene interactions: 242Pro/Arg *PolB* and 780Arg/His *LIG3* in patients with colorectal cancer (CRC) and the control group.

Genotype	Patients <i>n</i> = 302	Controls <i>n</i> = 302	OR (95% CI)	<i>p</i>
Pro/Pro-Arg/Arg	52	56	1 (ref.)	—
Pro/Pro-Arg/His	<b>82</b>	<b>41</b>	<b>2.154 (1.265–3.667)</b>	<b>0.004</b>
Pro/Pro-His/His	12	24	0.539 (0.245–1.185)	0.121
Pro/Arg-Arg/Arg	38	48	0.853 (0.483–1.506)	0.584
Pro/Arg-Arg/His	66	73	0.974 (0.589–1.611)	0.920
Pro/Arg-His/His	19	21	0.974 (0.471–2.015)	1.000
Arg/Arg-Arg/Arg	9	15	0.646 (0.261–1.603)	0.343
Arg/Arg-Arg/His	20	17	1.267 (0.599–2.679)	0.538
Arg/Arg-His/His	4	7	—	—

excessive accumulation of damage to DNA leads to cell necrosis or apoptosis, the most abundant types of lesion being 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and

4,6-diamino-5-formamidopyrimidine (FapyA) and 8-oxo-7,8-dihydroguanine (8-oxoG).

By virtue of its high stability and relative simplicity of detection, 8-oxoG is a vital biomarker of DNA oxidative damage [7]. Hence, colorectal cancer was extensively examined in terms of 8-oxoG presence. An analysis performed on a Spanish population indicated a twofold higher level of 8-oxoG in colorectal tumors than in normal mucosa [8]. In other studies, immunohistochemical tests complemented by high-performance liquid chromatography (HPLC) have revealed considerable higher levels of 8-oxoG in colorectal carcinoma than nontumorous colon epithelial cells [9]. Furthermore, the same team reports the presence of an elevated 8-oxoG level accompanied by 8-oxoG lyase overexpression [10]. Increased levels of 8-oxoG have been observed in CRC patient lymphocytes [11] and plasma [12] compared to those of healthy controls.

These high levels create a very challenging environment for base excision repair processes, which have to operate with high efficiency to maintain genome integrity. Omission of oxidized forms of guanine may lead to incorrect base-pairing, resulting in G:C to T:A transversion mutation [13, 14]. However, no evaluation of BER system activity can be performed purely on the basis of 8-oxoG level. In addition, the majority of previous studies tests have been based on the total 8-oxoG content including unbound 8-oxoG and that bound to DNA. A high level of free 8-oxoG might occur as consequence of efficient performance of the BER initial step, when the damaged bases are being recognized and excised by glycosylase. Conversely, higher numbers of damaged bases remaining in a DNA-associated form may indicate that BER activity is insufficient to cope with repairs.

A more precise tool to evaluate the level of DNA single-strand breaks and repair capacity is the comet assay. An alkaline version of the comet assay used in a previous investigation indicated statistically significant differences in repair efficiency between CRC patients and healthy subjects. After a 240-minute repair incubation, the level of single-strand DNA breaks was significantly diminished in lymphocytes from a cancer-free control group in comparison to CRC subjects. A similar difference was observed in a comparative analysis of cells from normal colon mucosa tissue and a CRC tumor [15]. In the course of a comet assay, cells are incubated intravitaly after hydrogen peroxide treatment, whereby the BER process, consisting of the excision of the damaged base and restoration of the DNA sequence, can be tracked holistically.

Glycosylase activity in initial stages of BER is an issue that has been the focus of a great degree of research interest. Thus far, eukaryotic cells have been found to possess several glycosylases such as NEIL1-3, UNG, NTH1, MUTYH, APE1, and OGG1 [16–19]. OGG1 is the primary BER enzyme capable of cleaving *N*-glycosyl bond between the sugar component and 8-oxoG. Studies based on a mouse model with *OGG1*<sup>-/-</sup> knock-out revealed this deficiency to have minor or even marginal importance in pathogenesis and cancer frequency [20]. *MUTYH* has a unique ability to remove normal adenines misincorporated opposite to 8-oxoG. Similar to *OGG1*, studies based on biallelic *MUTYH* mutation implied no significant increase in sensitivity to oxidative stress [21].

Surprisingly, an additive effect has been observed in mice with the double mutation *OGG1*<sup>-/-</sup> and *MUTYH*<sup>-/-</sup>, where higher tumor appearance frequencies have been noted [22].

Both *OGG1* and *MUTYH* have numerous polymorphic variants which are being eagerly examined in the context of carcinogenesis. The common polymorphisms of the *OGG1* gene, S326 C and R46Q, have been found to slightly decrease the activity of the enzyme [23, 24]. Regarding population screening, definitely more attention has been paid to the screening of S326 C, especially its involvement in lung cancer development. However, several investigations summarized in a meta-analysis do not reveal any linkage with lung cancer [25]. In contrast, certain variants of *MUTYH*, a polymorphism-rich gene, have been shown to elevate the risk of CRC 28-fold [26]. A great deal of current research into genetic variation of proteins has focused on the *XRCC1* gene. *XRCC1* is an important protein due to its participation in the recruitment of the other BER proteins, making it a binder of all stages [27]. However, several large scale meta-analyses display contradictory conclusion about its role in carcinogenesis. To be specific, while the Arg194Trp polymorphism was found to have a protective effect on tobacco smoking with regard to cancer risk [28], it was found to have no such role for other examined cancers [25, 29, 30].

As the literature shows no consensus on role of the early stages of BER, the present study addresses the gap-filling stage. The present study is so far the only one aimed to evaluate the effectiveness of BER in CRC. Undoubtedly, although our findings show an interesting trend, they should be treated with great caution. As a BER deficiency can be observed in lymphocytes from a CRC individual, it can be inferred that the native repair system is also deficient, which may result in a slow, gradual accumulation of damage that, at some critical moment, may contribute to the development of cancer.

It is important to determine whether some difficult to exclude factors can interfere with the result. To minimize this risk of appearance of additional undesirable damaging agents, the primary inclusion criteria for the BER assay were place of residence (the same city), the subject not taking medication, including cancer therapy for CRC, and the lack of any smoking addiction or alcohol abuse. However, it is difficult to predict the influence of other significant factors such as ionizing radiation, UV light, diet, or stress associated with everyday situations. There is some risk that any of these factors could put BER on standby, while it is forced to repair more cellular proteins which had been produced as a response to greater exposure [31]. In the follow-up phase of the experiment, the tumor cell extract demonstrated a similar reduction of BER activity in comparison to normal tissue.

However, it is unclear whether this reduced repair ability is innate and this phenotype is maintained after tumorigenesis, as differences could emerge due to the presence of mutations which were nested during malignant transformation. In addition, weak BER capacity may help exacerbate the genotoxic effect of ROS, allowing malignancy to progress. Chan et al. report that the presence of hypoxia in CRC provokes changes in BER [32]. Other reports note that some characteristics of colon tissue factor may induce oxidative

stress: an increased amount of free radicals may occur as result of diet rich in red meat [33] or with low calcium or vitamin D levels [34]. What is more, bacteria living in the intestine can also be an important extracellular source of ROS which promotes increased DNA damage in colonic epithelial cells [35]. The available evidence seems to suggest that BER has a possible impact on both the development and progression of CRC.

A similar concept has recently been presented by Stanczyk et al., who, by using a similar methodology to the present study, report significantly lower BER efficiency in lymphocytes taken from children suffering from childhood acute lymphoblastic leukemia in comparison to healthy controls [36]. Although CRC and leukemia are virtually incomparable due to their totally different natures, the BER system was found to play a crucial role in both and may also be involved in the pathogenesis of several other diseases.

It is undeniable that polymorphisms of DNA repair systems participate in the carcinogenesis process, as mentioned before in the first part of Section 4. Extensive studies suggest that these polymorphisms play a role in almost all types of cancer [37–40], including colorectal cancer [29, 41]. The genetic polymorphisms of MMR system appear to participate in the pathogenesis of hereditary nonpolyposis colorectal cancer [42, 43], and a growing body of evidence suggests their involvement in the BER system [44, 45], but reports concerning the NER system are inconclusive, with some confirming the link [46] and others denying it [47].

The present paper examines the impact of polymorphisms of *PolB*, *LIG3*, and *EXO1* of the BER repair system on the modulation of the risk of colon cancer. The genes were selected on the basis that the products of these three genes do not have glycosylase activity, thus avoiding any negative impact on the first part of the experiment. All three proteins are involved in stages of BER directly following the glycosylase action. Therefore, BER assay was adjusted to measure only the gap-filling step where *PolB*, *EXO1*, and *LIG3* play crucial roles. The protective effect of the Arg allele for the 242Pro/Arg gene polymorphism of *PolB* demonstrated in our work (Table 3) has been shown in previous publications [46]. In addition, the 242Pro/Pro genotype of the *PolB* gene in combination with the genotype 780Arg/His of *LIG3* gene increases the risk of CRC (Table 7), and the risk is much higher than in case of the 780Arg/His SNP of *LIG3* (OR = 2.154; 1.265–3.667,  $p = 0.004$  versus OR = 1.570; 1.109–2.224,  $p = 0.011$ ) (Table 4). This clearly shows the important role of gene-gene interactions in modulating the risk of malignant transformation, which has been confirmed in many other publications [48, 49].

Our finding that the 589Lys/Glu SNP of *EXO1* is associated with an increased risk of CRC is contrary to those of Akbari et al. [50]. However, it should be noted that the previous study was performed on an Iranian population, while our tests were carried out on a Polish population. Ethnic group has been repeatedly demonstrated to have a significant impact on the modulation of the risk of particular diseases [51, 52]. Yamamoto et al. [53] suggest that the potential impact of polymorphism 589Lys/Glu on increased risk of

carcinogenesis may depend on the presence of cigarette smoking by the patient. Again, however, these studies concern a Japanese population, which may exert an influence on the results.

In our opinion, it is important to note interaction of polymorphisms 589Lys/Glu of the *EXO1* gene and 780Arg/His of the *LIG3* gene (Table 6), whose coexistence increases the risk of CRC compared to the presence of polymorphism 589Lys/Glu itself (OR = 1.970; 1.041–3.731,  $p = 0.036$  versus OR = 1.570; 1.109–2.224,  $p = 0.011$ ). As no extant publications describe the influence of the 780Arg/His polymorphism of *LIG3* on the risk of CRC, our own findings in this regard showing an elevated risk (Table 4) are significant. In addition, attention should be once again directed to the mentioned earlier gene-gene interaction of *LIG3* with the 589Lys/Glu polymorphism of the *EXO1* gene. Furthermore, not only does the potential protective effect of the Arg allele of the 242Pro/Arg *PolB* SNP and the increased CRC risk associated with the 780Arg/His SNP of *LIG3* and 589Lys/Glu SNP of *EXO1* merit attention, but also, more importantly, the modulation of risk induced by gene-gene interactions identified in this study can significantly affect individual predisposition to the development of cancer.

## 5. Conclusions

Decreased BER activity may play a crucial role in the pathogenesis, development, and progression of colorectal cancer, as the activity of BER is distinctly reduced in lymphocytes and cancer tissue from CRC individuals. In addition, the genotyping of SNPs which have so far not been thought to be associated with CRC (*LIG3*, *PolB*, and *EXO1*) suggests that potential BER dysfunction may lay not only in its first steps, but equally or at even greater level in the gap-filling events. We believe that our results are promising, yet further studies are needed on this subject to establish a link between a given polymorphism and its phenotypic effect in the modulation of BER activity and thus its impact on carcinogenesis.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

This work has been supported by Umed in Lodz Grants 502-03/5-108-05/502-54-158 and 502-03/5-108-05/502-54-144 and by Polish Ministry of Science and Higher Education Grants N402422138 and NN403250340.

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## Research Article

# Modulation of RhoA GTPase Activity Sensitizes Human Cervix Carcinoma Cells to $\gamma$ -Radiation by Attenuating DNA Repair Pathways

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Received 16 May 2015; Accepted 21 July 2015

Academic Editor: Manoj K. Pandey

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Radiotherapy with  $\gamma$ -radiation is widely used in cancer treatment to induce DNA damage reducing cell proliferation and to kill tumor cells. Although RhoA GTPase overexpression/hyperactivation is observed in many malignancies, the effect of RhoA activity modulation on cancer radiosensitivity has not been previously investigated. Here, we generated stable HeLa cell clones expressing either the dominant negative RhoA-N19 or the constitutively active RhoA-V14 and compared the responses of these cell lines with those of parental HeLa cells, after treatment with low doses of  $\gamma$ -radiation. HeLa-RhoA-N19 and HeLa-RhoA-V14 clones displayed reduced proliferation and survival compared to parental cells after radiation and became arrested at cell cycle stages correlated with increased cellular senescence and apoptosis. Also, Chk1/Chk2 and histone H2A phosphorylation data, as well as comet assays, suggest that the levels of DNA damage and DNA repair activation and efficiency in HeLa cell lines are correlated with active RhoA. In agreement with these results, RhoA inhibition by C3 toxin expression drastically affected homologous recombination (HR) and nonhomologous end joining (NHEJ). These data suggest that modulation of RhoA GTPase activity impairs DNA damage repair, increasing HeLa cell radiosensitivity.

## 1. Introduction

Radiotherapy is widely used in the clinic to inhibit cancer progression and can be administered as a monotherapy or combined with chemotherapy, surgery, and other alternatives. During  $\gamma$ -radiation radiotherapy, the ionizing radiation applied to tumors is absorbed directly by DNA, inducing DNA damage (including single- and double-strand breaks) [1], which leads to tumor cell death or decreases the effect of tumor cells on adjacent tissues.

In the human cervical carcinoma cell line HeLa, treatment with the bacterial toxin HdCDT induces DNA double-strand breaks similar to those resulting from  $\gamma$ -radiation [2]. In this system, induction of DNA double-strand breaks activates the small GTPase RhoA, which regulates a variety of cellular activities involving cytoskeletal reorganization (including cell motility and actin stress fiber formation), as

well as cell cycle progression [3]. RhoA overexpression has been reported in breast, colon, lung, and gastric tumors, and it facilitates cancer progression by inducing increased tumor cell motility, proliferation, and survival, as well as a loss of cell polarity [4]. Rho family enzymes interchange between an active form (Rho-GTP) and an inactive form (Rho-GDP). GTPase activation by GTP binding is aided by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP by GTP in the active site. The intrinsic activity of GTP hydrolysis of Rho enzymes (including RhoA) is then activated by GAPs (GTPase activating proteins), leading to conversion of GTP into GDP and Rho inactivation [5].

In HeLa cells, RhoA activation by HdCDT treatment increases HeLa cell survival, and this effect depends on the activity of the ataxia telangiectasia mutated (ATM) serine/threonine protein kinase [2], a DNA damage repair

protein activated as a response to DNA double-strand breaks (such as those induced by ionizing radiation) [6]. Although these data suggest the existence of a “cross talk” between RhoA and DNA repair pathways, the effect of RhoA activity modulation on the sensitivity of cancer cells to radiotherapy has not been examined to date.

In this study, we analyzed the effect of modulation of RhoA activity in the response to  $\gamma$ -radiation (0.5, 5, and 15 Gy) treatment, in HeLa cells. We generated stable HeLa cell lines that express a constitutively active RhoA (HeLa-RhoA-V14) or a dominant negative RhoA (HeLa-RhoA-N19). These mutants are analogous to the Ras-V12 (constitutively active) and Ras-N17 (dominant negative) mutants found in ~25% of all human cancers, in marked contrast to RhoA mutations, which are rarely found [7, 8]. Our results show that cells expressing either the constitutively active or the dominant negative RhoA mutants are less resistant to the effects of  $\gamma$ -radiation than parental HeLa cells and have reduced ability to proliferate and survive after treatment. These data correlated with the reduced activation of DNA damage response and repair pathways and efficiency of DNA damage repair, in cells with reduced RhoA activity.

## 2. Materials and Methods

**2.1. Cell Lines.** The human cervical carcinoma cell line HeLa (CCL-2; ATCC, Manassas, VA, USA) was maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), at 37°C and 5% CO<sub>2</sub>, in a humidified incubator.

**2.2. Cell Treatments by  $\gamma$ -Radiation.** HeLa cells and clones were treated with three different doses (0.5, 2, 5, and 15 Gy) of gamma ( $\gamma$ ) ionizing radiation (Co60-Gammacell 220, Atomic Energy of Canada Limited (AECL), Ontario, Canada) at the Nuclear and Energy Research Institute (IPEN, SP, Brazil). But for some experiments dose-response curves were performed, while for others only one dose was used, according to the cell viability (unpublished results, not shown) and the duration of the experiment.

**2.3. Generation of Sublines of RhoA-N19 and RhoA-V14 Mutants from HeLa Cells.** To produce HeLa cell sublines stably expressing RhoA mutants, constructs containing the recombinant retroviral pCM vector and cDNA sequences for the constitutively active RhoA-V14 (Ala to Val substitution at position 14) or the dominant negative RhoA-N19 (Thr to Asp substitution at position 19) were packaged into recombinant retrovirus particles using the Phoenix system ( $\phi$ NX-cells, kindly donated by Gary P. Nolan, Stanford University, CA, USA). Subconfluent HeLa cells seeded in 10 cm dishes (in DMEM/10% FBS) were infected with the recombinant retrovirus particles in the presence of 8  $\mu$ g/mL of polybrene [9]. After infection, cells were selected for approximately 30 days with 500  $\mu$ g/mL of G418, and isolated colonies, representing clones of HeLa-RhoA-N19 and HeLa-RhoA-V14, were collected and maintained in DMEM/10% FBS with 100  $\mu$ g/mL of G418 until freezing or further use.

**2.4. Active RhoA Pull-Down Assay.** To measure RhoA activity, we used a RhoA-GTP pull-down protocol adapted from Ren et al., 1999 [10]. HeLa cells were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.2, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2  $\mu$ g/mL leupeptin, pepstatin, aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride, or PMSF) (all from Sigma-Aldrich, Saint Louis, MO, USA), and cell lysates were incubated with Glutathione-Sepharose beads (GE, Healthcare, Cleveland, OH, USA) bound to the RBD-GST fusion protein (RhoA binding domain of the Rhotekin protein, kindly donated by Gary M. Bokoch, The Scripps Research Institute, La Jolla, CA, USA) for 90 min at 4°C. Then, beads were recovered by centrifugation (3000 rpm, for 3 min at 4°C) and washed 3 times with buffer B (50 mmol/L Tris-HCl, pH 7.2, containing 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 10  $\mu$ g/mL leupeptin, aprotinin, and 0.1 mmol/L PMSF). RhoA-GTP bound to RBD-GST-Sepharose beads was resolved on 13% SDS-PAGE gels, transferred to nitrocellulose membranes and analyzed using a monoclonal anti-RhoA antibody (26C4, from Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described below (see Section 2.11).

**2.5. Growth Curves.** For population growth analysis, HeLa cells were seeded in 35 mm dishes ( $3.5 \times 10^4$  cells/dish) and allowed to adhere at 37°C (with 5% CO<sub>2</sub>), for 24 h. Then cells were exposed to 0.5 or 5 Gy of  $\gamma$ -radiation and reincubated at 37°C. Cell samples were collected in duplicate every 24 h after  $\gamma$ -radiation, for five consecutive days, and cells were counted manually in a Fuchs-Rosenthal chamber.

**2.6. Clonogenic Assays.** For clonogenic assays, HeLa cells were seeded in 60-mm dishes ( $2 \times 10^3$  cells/dish) and allowed to adhere at 37°C (with 5% CO<sub>2</sub>) for 24 h. Then, cells were exposed to 0.5, 5, or 15 Gy of  $\gamma$ -radiation and reincubated at 37°C for 10–12 days. Colony foci were fixed in 10% formaldehyde in PBS for 10 min, stained with 0.5% crystal violet in PBS for 5 min (both at room temperature), and counted manually.

**2.7. Cell Cycle Analysis.** For cell cycle analysis, HeLa cells were plated in 35-mm dishes ( $3.5 \times 10^5$  cells/dish) and allowed to adhere at 37°C (with 5% CO<sub>2</sub>) for 24 h. After  $\gamma$ -radiation, cells were harvested by trypsinization, washed in PBS, and fixed in 80% ethanol in PBS. Then, cells were stained with 10  $\mu$ g/mL propidium iodide (PI) and stored at 4°C. Samples were run in a Beckman Coulter FC500 MPL cytometer (Brea, CA, USA), and flow cytometry data were analyzed using WinMDI 2.8 software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA).

**2.8. Apoptosis Assay.** To estimate apoptosis, HeLa cells were plated in 35-mm dishes ( $1.5 \times 10^5$  cells/dish), for 24 h, and treated with 5 Gy or 15 Gy of  $\gamma$ -radiation, or with 60 J/m<sup>2</sup> ultraviolet C (UVC; positive control for apoptosis induction). Then, adhered and suspended cells were harvested by

successive rounds of PBS washing-trypsinization-centrifugation, 48 h or 72 h after  $\gamma$ -radiation. Harvested cells were resuspended in Annexin-V binding buffer (50 mM HEPES, pH 7.4, containing 0.7 M NaCl and 12.5 mM  $\text{CaCl}_2$ ) for a final density of  $1 \times 10^6$  cells/mL, and 5  $\mu\text{L}$  Annexin-V-FITC (BD Biosciences, Franklin Lakes, NJ, USA) and 1.5  $\mu\text{L}$  propidium iodide (1 mg/mL) were added to 100- $\mu\text{L}$  aliquots of cell suspension ( $1 \times 10^5$  cells). Samples were incubated for 15 min at room temperature (and protected from light), and then 400  $\mu\text{L}$  of Annexin-V binding buffer was added to each sample, and cells were analyzed by flow cytometry in a FACSVerser (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry data were analyzed on the Kaluza 1.3 Flow Analysis software (Beckman Coulter, Brea, CA, USA).

**2.9. Senescence-Associated  $\beta$ -Galactosidase Assay.** Cell senescence was estimated using a senescence-associated  $\beta$ -galactosidase assay, as described by Dimri et al., 1995 [11]. HeLa cells ( $3.0 \times 10^4$  cells/dish, in 35-mm dishes) (Corning, New York, NY, USA) were allowed to adhere at 37°C (with 5%  $\text{CO}_2$ ) for 24 h, prior to treatment with 0.5, 5, or 15 Gy of  $\gamma$ -radiation. Then, cells were incubated for 96 h at 37°C, fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 3 min, washed in PBS, and stained for 18 h at 37°C with 2 mL/dish of X-gal staining solution (30 mmol/L PBS/citric acid (pH 6) containing 5 mmol/L  $\text{K}_3\text{Fe}(\text{CN})_6$ , 2 mmol/L  $\text{MgCl}_2$ , 150 mmol/L NaCl, 5 mmol/L  $\text{K}_4\text{Fe}(\text{CN})_6$ , and 1 mg/mL X-gal). Then, samples were washed twice in PBS and kept at 4°C prior to analysis, by direct counting of  $\beta$ -galactosidase-positive/negative cells ( $1 \times 10^3$  cells/dish, in duplicate), in an inverted Olympus microscope (Olympus, Tokyo, Japan).

**2.10. Alkaline Comet Assay.** The alkaline comet assay was performed as described by Singh et al., 1998 [12], with modifications. HeLa cells were seeded in 35-mm dishes ( $2 \times 10^5$  cells/dish) and were allowed to adhere at 37°C (with 5%  $\text{CO}_2$ ) for 24 h, before  $\gamma$ -radiation with 5 Gy. After treatment, cells were harvested by trypsinization, mixed with 0.5% low-melting point agarose, and 100  $\mu\text{L}$  of this mixture was pipetted onto glass slides with 1.5% normal-melting point agarose. Then, cells were lysed with lysis buffer (10 mmol/L Tris, pH 10, containing 2.5 mmol/L NaCl, 100 mmol/L EDTA, 1% Triton X-100, and 10% DMSO, all from Sigma-Aldrich, Saint Louis, MO, USA) for 24 h at 4°C and in the dark. Samples were denatured in alkaline electrophoresis buffer (300 mmol/L NaOH, 1 mmol/L EDTA, pH >13) for 25 min, and then electrophoresis was performed at 25 V and 300 mA, for 30 min. After electrophoresis, slides were washed 3 times (5 min/wash) in neutralizing buffer (0.4 mmol/L Tris-HCl, pH 7.5), DNA was stained with 2  $\mu\text{g}/\text{mL}$  ethidium bromide, and comets (from 50 cells/slide, in duplicate) were imaged using a fluorescence microscope Olympus IX51 (Olympus, Shinjuku, Tokyo, Japan). Comet assay data were analyzed using the software Komet 6.0 (Andor, Technology, Belfast, BT, UK).

**2.11. Western Blotting.** For Western blotting, HeLa cells were lysed with RIPA buffer (see Section 2.4), and 50  $\mu\text{g}$  of protein was mixed with Laemmli sample buffer [13] and resolved in 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane (Millipore, Billerica, MA, USA), and membranes were blocked in TBS-T with 5% milk, for 1 h at room temperature. Then, membranes were incubated with one of the following primary antibodies diluted in TBS-T: anti-phospho-Chk1 Ser345 (Cat. number 2341), anti-phospho-Chk2 Thr-68 (Cat. number 2661), or anti-phospho-H2AX Ser139 (Cat. number 9718) polyclonal/monoclonal antibodies from Cell Signaling (Danvers, MA, USA) or an anti- $\alpha$ -Tubulin polyclonal/monoclonal antibody (B-7, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were incubated with appropriate species-specific IRDye (Infrared Dye) secondary antibodies (680 or 800 nm, diluted to 1:15000 in TBS-T) for 1 h and visualized and analyzed (by band density quantification) using an Odyssey Infrared Imaging System and the Odyssey V3.0 software (both from Li-COR Biosciences, Lincoln, NE, USA).

**2.12. Inhibition of RhoA Activity by the C3 Toxin.** HeLa cells were transiently transfected with the eukaryotic expression vector pEF-myc (Invitrogen) containing the C3 toxin coding sequence (plasmid kindly provided by Professor Dr. Gary Bokoch, The Scripps Research Institute, La Jolla, CA, USA). HeLa cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, and then plated into 100-mm dishes (for immunoblotting experiments) or 35-mm dishes (for comet assays) and allowed to grow until ~80% confluence. The cells were incubated for 24 hours, prior to RhoA activation analysis (see Section 2.4), and after this time the cells were exposed to 5 Gy of  $\gamma$ -radiation and analyzed according to the previously described experiments.

**2.13. Homologous Recombination (HR) and Nonhomologous End Joining (NHEJ) Assays.** The rates of HR and NHEJ were estimated using HeLa cells stably expressing DR-GFP and EJ-GFP, respectively, as described by Gunn and Stark with modifications [14]. To produce HeLa-DR-GFP and HeLa-EJ5-GFP stable cell lines, subconfluent HeLa cells grown in 60-mm dishes were transfected using 7.5  $\mu\text{g}$  Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) and 3.5  $\mu\text{g}$  of DR-GFP or EJ5-GFP plasmids [14], in DMEM with 10% FBS. Transfectants were selected and isolated using 5  $\mu\text{g}/\text{mL}$  of puromycin and maintained in DMEM/10% FBS supplemented with 1  $\mu\text{g}/\text{mL}$  of puromycin.

For HR and NHEJ assays, approximately  $2 \times 10^5$  HeLa-DR-GFP and HeLa-EJ5-GFP cells were seeded in 35-mm dishes and allowed to adhere at 37°C (with 5%  $\text{CO}_2$ ) for 24 h. Then, cells were transfected with 4  $\mu\text{g}$  of the I-SceI expression vector or an empty vector (EV), alone or in combination with 2  $\mu\text{g}$  of pEF-myc-C3 (using 2  $\mu\text{g}$  of Lipofectamine 2000; Invitrogen, Waltham, MA, USA). Cells were harvested 72 h after transfection, and the percentage of GFP-positive cells was determined by flow cytometry in a FACSVerser cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**2.14. Statistical Analysis.** Comparisons between treatments were performed by Student's *t*-test (for paired data) or by ANOVA (for multiple groups), using the Prism 6.0 software, and differences were considered statistically significant when  $P < 0.05$ .

### 3. Results

**3.1. Expression of Dominant Negative or Constitutively Active RhoA Prevents the Increase in Active RhoA Levels by  $\gamma$ -Radiation.** To evaluate if the activation of the small GTPase RhoA has a role in the response to  $\gamma$ -radiation in cancer cells, we generated stable clones of HeLa cells expressing either the constitutively active HeLa-RhoA-V14 or the dominant negative HeLa-RhoA-N19 RhoA mutants. Cells from both clones appeared to spread on the surface of culture flasks more effectively than parental HeLa cells (Figure 1(a)). Analysis of RhoA activity by a pull-down assay for the active RhoA-GTP form [15] showed that HeLa-RhoA-N19 and parental HeLa cells had similar basal levels of active RhoA, while HeLa-RhoA-V14 cells had higher levels of active RhoA, as expected for cells expressing a constitutively active RhoA mutant (Figure 1(b)). RhoA-GTP levels increased after  $\gamma$ -radiation in parental HeLa cells (Figure 1(b)). In contrast, we detected no further RhoA activation in cells expressing RhoA-N19 or RhoA-V14, after  $\gamma$ -radiation (Figure 1(b)).

The RhoA GTPase is a key regulator of cell migration via cytoskeletal reorganization [16]. Thus, we also performed scratch assays in confluent cell monolayers, to evaluate the effect of mutant RhoA expression on cell migration (see Supplementary Figure S1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/6012642>). For cells grown in medium containing 10% FBS, the migration rate of those expressing the dominant negative RhoA-N19 was considerably reduced (43%) compared with that of parental HeLa or HeLa-RhoA-V14 cells (100% migration). In serum-free conditions (0% FBS), HeLa-RhoA-N19 migrated only 5%, 24 h after serum starvation, but migration rates in 10% FBS (after serum starvation) were similar to those observed in cells that had not been serum-starved prior to migration. However, in starving conditions, HeLa-RhoA-V14 cells displayed reduced migration (27%) compared with parental HeLa cells (Supplementary Figure S1). These results suggest that the expression of either RhoA-V14 or RhoA-N19 promotes an imbalance in the RhoA activity in HeLa cells, despite the presence of normal to high basal levels of active RhoA. While the migration of parental or HeLa-RhoA-V14 cells was only significantly affected by high doses of  $\gamma$ -radiation (15 Gy), treatments as low as 5 Gy of  $\gamma$ -radiation reduced significantly the migration of HeLa-RhoA-N19 cells in medium with 10% FBS (Supplementary Figure S2). Overall, the scratch assay data suggest that, despite the persistent levels of RhoA-GTP in cells expressing either dominant negative or constitutively active RhoA mutants, these cells had altered RhoA activity, judging from their reduced migration ability, and this effect was particularly evident in cells expressing the dominant negative RhoA-V14 mutant, after  $\gamma$ -radiation treatment. Thus, the migration data also indicate that the HeLa-RhoA-V14 and HeLa-RhoA-N19 cell lines are valid

models for the study of RhoA activity modulation after radiation.

**3.2. Expression of RhoA Mutants Alters HeLa Cell Proliferation and Survival Rates after  $\gamma$ -Radiation.** To investigate the effect of RhoA activity modulation on cell proliferation and survival after exposure to low doses (0.5 and 5 Gy) of  $\gamma$ -radiation, we performed growth curves and clonogenic assays of HeLa cells expressing mutant RhoA proteins. HeLa-RhoA-N19 and HeLa-RhoA-V14 displayed a reduced doubling time compared to parental HeLa cells ( $\sim 3.1$  and  $\sim 2.6$  days, resp.). We observed a clear reduction in the proliferation of all cell lines after exposure to 5 Gy of  $\gamma$ -radiation, and proliferation inhibition was observed earlier (between 2 and 3 days after radiation) in HeLa-RhoA-N19 and HeLa-RhoA-V14 cultures, compared with parental HeLa cells (Figure 1(c)). No significant reduction in cell proliferation was observed after exposure to 0.5 Gy of  $\gamma$ -radiation (Figure 1(c)).

In clonogenic (colony formation) assays, both HeLa-RhoA-N19 and HeLa-RhoA-V14 displayed decreased survival, with a reduction of  $\sim 50\%$  in the number of colonies in untreated cells, and this sensitivity to the effects of 0.5 Gy of  $\gamma$ -radiation was relatively well maintained compared with HeLa cells. When exposed to 5 Gy of  $\gamma$ -radiation, HeLa-RhoA-V14 and HeLa-RhoA-N19 were significantly more sensitive than parental HeLa cells, with a reduction of  $\sim 70\%$  and  $\sim 80\%$ , respectively, in the number of colonies, compared with parental cells subjected to the same treatment (Figure 1(d)). These data suggest that HeLa cells expressing either a dominant negative or a constitutively active RhoA mutant are more sensitive to low doses of  $\gamma$ -radiation than parental HeLa cells.

**3.3. Expression of RhoA Mutants Leads to Differential Cell Cycle Arrest with Increased Senescence and Apoptosis Induction.** Cell cycle analysis (by flow cytometry using PI) of irradiated cells suggested that, after exposure to 15 Gy of  $\gamma$ -radiation, cells with constitutively high levels of activated RhoA (HeLa-RhoA-V14) remain arrested in S and G2/M, whereas HeLa cells expressing the dominant negative RhoA-N19 remain predominantly in the G1 and S phases of the cell cycle (Figure 1(e)). As expected, we observed a marked arrest of HeLa cells in the G2/M phase of the cell cycle after treatment with high (15 Gy) dose of  $\gamma$ -radiation (Figure 1(e)).

The radiation-induced arrest at different cell cycle stages correlates with the distinct types of antiproliferative effects observed in HeLa cell lines expressing RhoA mutants, after radiation treatment (Figure 2). Cells expressing HeLa-RhoA-N19, expected to be deficient in RhoA activity, display higher senescence levels at lower doses of 0.5 and 5 Gy of  $\gamma$ -radiation, which correlates with their preferential arrest at G1 and S phases (Figure 2(a)). These cells also showed increased apoptosis 48 and 72 h after exposure to the highest (15 Gy) dose of  $\gamma$ -radiation (Figure 2(b)). Similarly, all doses of radiation treatment led to increased senescence in cells expressing the constitutively active HeLa-RhoA-V14 mutant, compared with parental HeLa cells (but not with cells expressing RhoA-N19), although the highest senescence levels were observed only after treatment with the highest dose (15 Gy) of  $\gamma$ -radiation (Figure 2(a)). These results correlate with the preferential

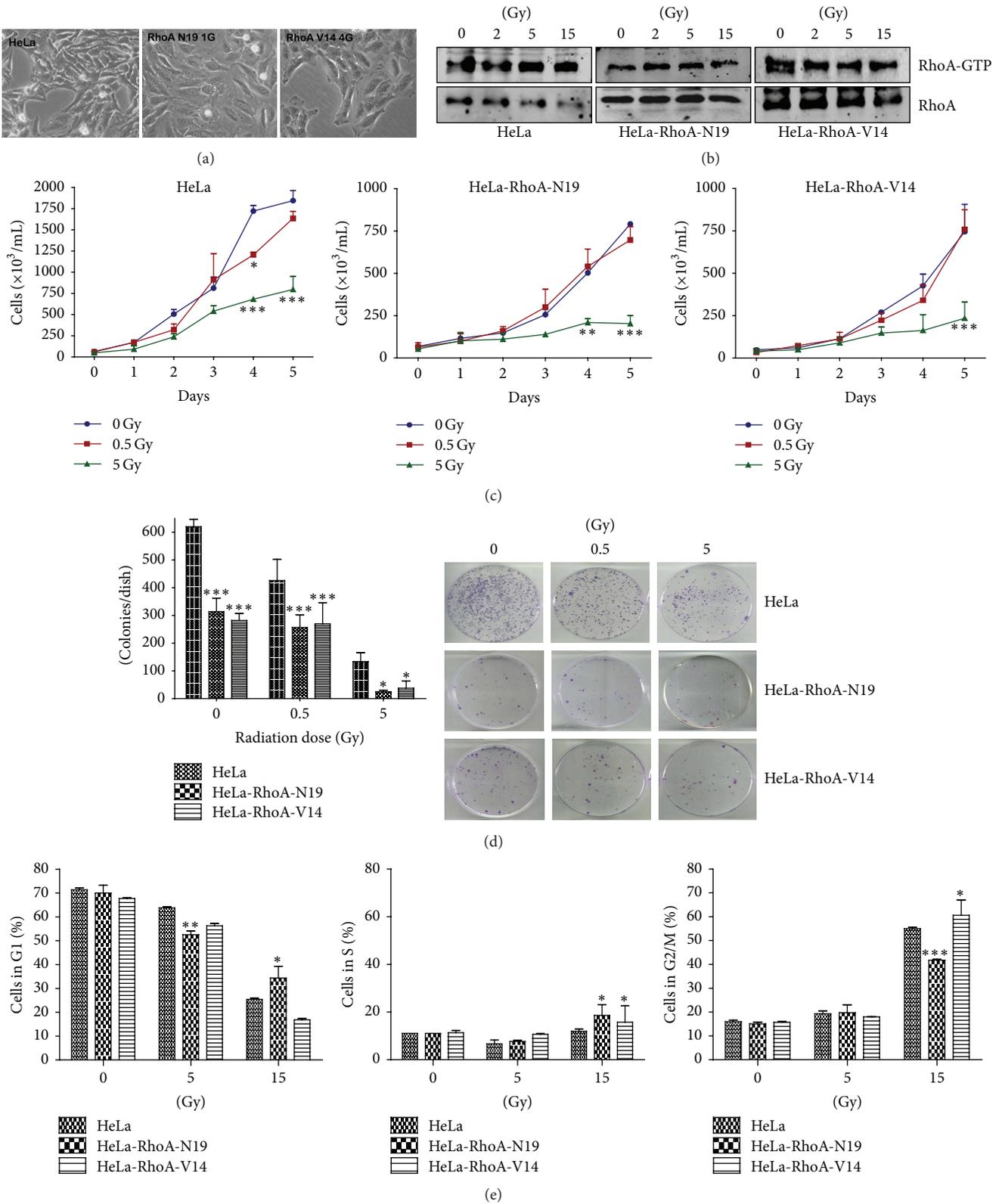


FIGURE 1: Morphological Rho activity and proliferation analyses of parental and clonal HeLa cell lines expressing RhoA-N19 or RhoA-V14 mutants after  $\gamma$ -radiation. (a) Morphology of parental and derived HeLa cell lines. (b) Immunoblotting of pull-down assays for active RhoA (RhoA-GTP) in different cell lines. (c) and (d) Growth curves (c) and clonogenic assays (d) in cell lines under positive or negative modulation of RhoA activity. (e) Cell cycle profiles by flow cytometry analysis (using PI staining) of HeLa cell lines after exposure to different doses of  $\gamma$ -radiation. Graphs display mean  $\pm$  SD of at least three independent experiments. \* $P < 0.05$ , \*\* $P < 0.005$ , and \*\*\* $P < 0.001$  between clones and parental HeLa cells in the same treatment conditions (by ANOVA).

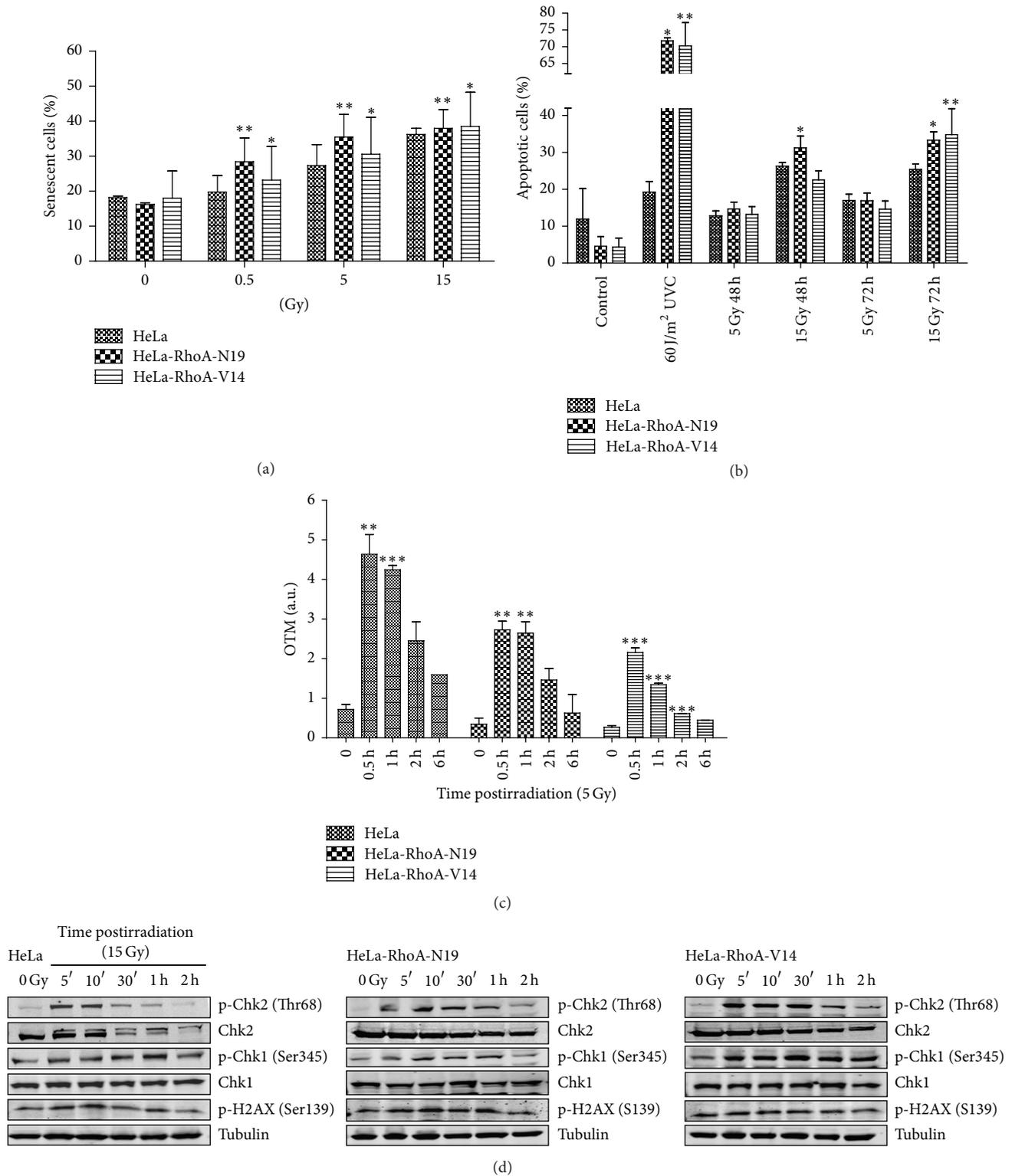


FIGURE 2: Cell death and DNA damage response and repair analyses in HeLa cells expressing RhoA mutants, after  $\gamma$ -radiation treatment. (a) Quantification of senescent cells, by a senescence-associated  $\beta$ -galactosidase assay, performed 96 h after radiation. (b) Quantification of apoptotic cell death after radiation, by Annexin-V and propidium iodide staining (Annexin V<sup>+</sup>/PI<sup>+</sup> cells were considered apoptotic). (c) Estimation of DNA damage and repair efficiency following radiation, by the olive tail moment (OTM, in arbitrary units) measurements from comet assays. (d) Immunoblotting analysis of phosphorylated Chk1/Chk2 and histone H2AX levels in the different HeLa cell lines, after exposure to 15 Gy of  $\gamma$ -radiation (and using  $\alpha$ -Tubulin as loading control). Graphs and immunoblots are representative of three independent experiments. \* $P < 0.01$ , \*\* $P < 0.05$ , and \*\*\* $P < 0.001$  between clones and parental HeLa cells and \* $P < 0.005$  between treated and untreated conditions (by ANOVA).

arrest of HeLa-RhoA-V14 at S and G2/M and with an increase in apoptotic cell death after exposure to 15 Gy of  $\gamma$ -radiation, especially at the longer time-point of 72 h posttreatment (Figure 2(b)). Taken together, the cell cycle analysis, apoptosis, and senescence data suggest that modulation of RhoA activity leads to arrest at different stages of the cell cycle, leading to the induction of different levels of senescence or apoptosis.

**3.4. Modulation of RhoA Activity in HeLa Cells Affects DNA Damage Repair Induction and DNA Damage Response (DDR) Protein Activation.** To investigate if modulation of RhoA activity affects DNA repair after radiation treatments, we performed comet assays at different time-points after exposure to 5 Gy of  $\gamma$ -radiation. All three HeLa cell lines exhibited a peak of fragmented DNA (i.e., an increase in the olive tail moment, or OTM) 0.5 h after radiation, and this peak was ~6- and ~8-fold higher than the basal levels (in nonirradiated cells), for parental HeLa and RhoA mutant-expressing cells, respectively (Figure 2(c)). Although fragmented DNA levels decreased up to 6 h after radiation in all three HeLa cell lines, DNA repair (i.e., a statistically significant reduction in OTM) could be detected as early as 1 h after radiation treatment in parental HeLa and in HeLa-RhoA-V14 cells (Figure 2(c)). In contrast, DNA repair could be detected from 2 h after  $\gamma$ -radiation treatment in HeLa-RhoA-N19 cells, suggesting that DNA damage repair is delayed in this cell line, which is expected to have reduced RhoA activity (Figure 2(c)).

To examine a possible correlation between the efficiency of DNA repair and the phosphorylation of DNA damage responses (DDR) proteins, we exposed parental HeLa, HeLa-RhoA-N19, and HeLa-RhoA-V14 cells to 15 Gy of  $\gamma$ -radiation and monitored the activation (by phosphorylation) of the checkpoint proteins Chk1 and Chk2, as well as the appearance of a marker for double-strand DNA breaks (p-Ser139 H2AX), for up to 2 h after radiation (Figure 2(d)). After treatments, we observed a reduction in the phosphorylation levels of both Chk1 (Ser345) and Chk2 (Thr-68) in cells expressing the dominant negative RhoA-N19 mutant, while cells expressing the constitutively active RhoA-V14 displayed overactivation of Chk1/Chk2; both responses were different from those observed in parental HeLa cells. Thus, the levels of Chk1/Chk2 phosphorylation obtained for the three cell lines correlate with their RhoA-GTP levels. In contrast, the phosphorylation of H2AX (Ser139), which peaked between 5 min and 1 h after radiation and returned to basal levels 2 h after treatment, was not significantly affected by the modulation of RhoA activity (Figure 2(d)).

**3.5. C3 Toxin-Mediated Downregulation of RhoA Activity Impairs DNA Repair and Overactivates DDR Proteins.** To confirm that decreased RhoA activity reduces DNA repair efficiency, as suggested by the comet assay data on HeLa cells expressing dominant negative RhoA, we performed a potent and persistent inhibition of RhoA in parental HeLa cells by transfection with a plasmid encoding the C3 toxin. To that we transfected HeLa cells with a plasmid driving constitutive expression of the C3 toxin, an exoenzyme secreted by the bacterium *Clostridium botulinum* and capable of selectively

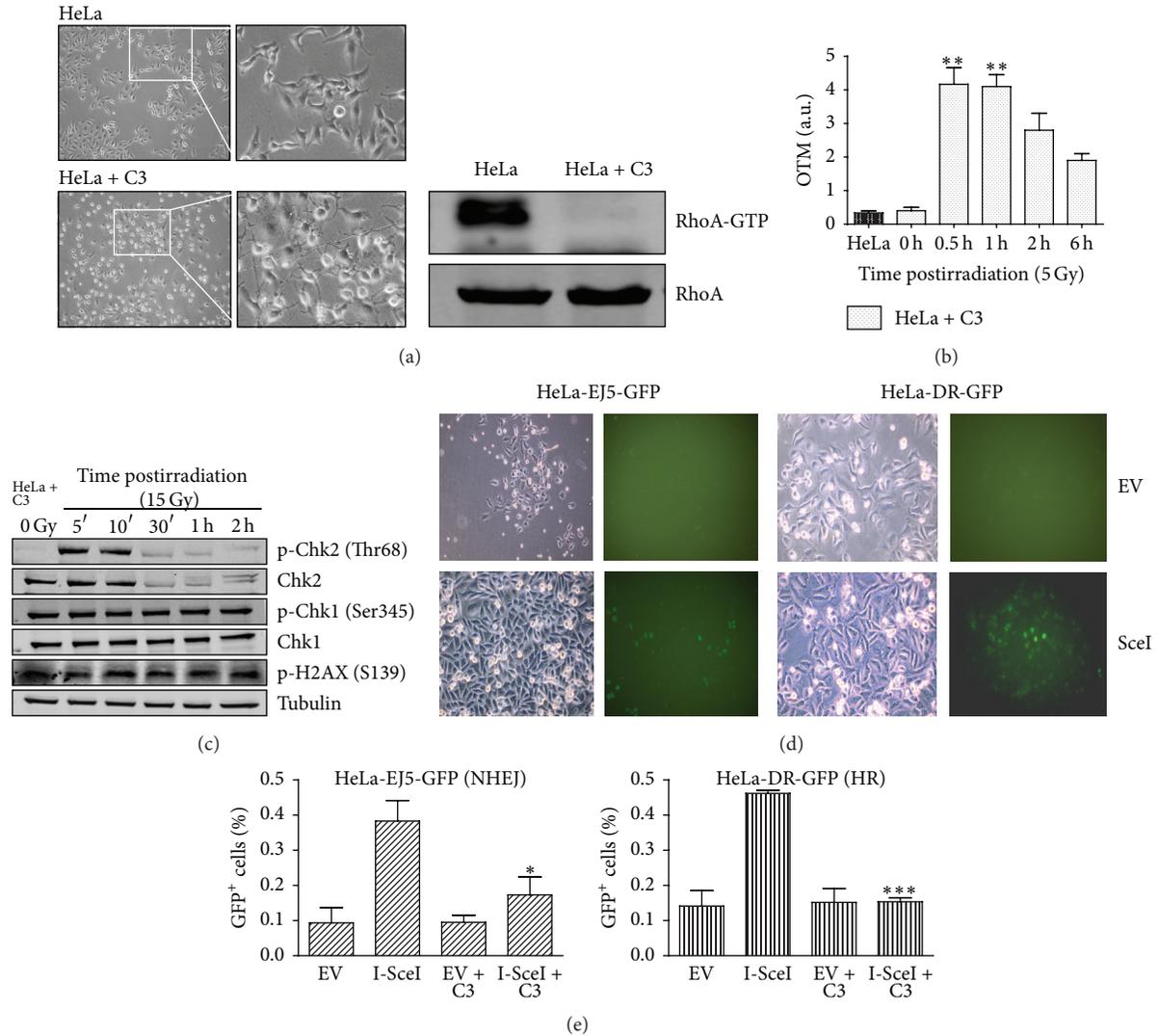
inhibiting the activation of RhoA, RhoB, and RhoC GTPases [17–19]. As expected, expression of the C3 toxin had a strong effect on cell morphology, 24 h after transfection, and reduced RhoA-GTP to residual levels (Figure 3(a)). Also, comet assay results suggest that C3 toxin expression increased HeLa cell sensitivity to DNA damage by  $\gamma$ -radiation (5 Gy) (Figure 3(b)). After C3 toxin expression, the levels of DNA breaks increased by ~10-fold at 0.5 h after radiation, and DNA damage repair could be detected from 2 h after radiation, similar to the response observed in the HeLa Rho-N19 cells (Figure 2(c)); however, DNA damage appeared more persistent in HeLa cells expressing C3, judging from the levels of damage remaining up to 6 h after  $\gamma$ -radiation (Figure 3(b), compared with HeLa, in Figure 2(c)). These results are in agreement with the overactivation of phospho-Chk1 (Ser345) after  $\gamma$ -radiation (15 Gy) treatment, in cells expressing the C3 toxin, which seems to reflect the persistence of high phospho-H2AX (Ser139) levels in HeLa cells (Figure 3(c)).

To investigate the effect of C3 toxin-mediated RhoA inhibition on the activity of specific DNA repair pathways, we generated HeLa cell lines capable of GFP-based detection of homologous recombination (HR, via the reporter EJ5-GFP) or nonhomologous end joining (NHEJ), via the reporter gene DR-GFP, after expression of the endonuclease I-SceI, which cleaves on specific sequences in the reporter gene plasmidial DNA [14]. Interestingly, in cells expressing both the C3 toxin and I-SceI, the levels of double-strand break repair by either HR or NHEJ were significantly reduced compared with those observed in cells expressing the I-Sce-I enzyme only, reaching similar levels to those observed in controls (empty vector, or EV, and EV + C3) (Figures 3(d) and 3(e)). Endogenous RhoA inhibition by C3 expression affected both repair pathways: while HR was completely inhibited, NHEJ was partially disrupted in cells where the endogenous repair machinery was specifically recruited to reporter gene sequences (EJ-GFP and DR-GFP, resp.) integrated in the genome. Altogether, these results strongly support the involvement of RhoA in DNA damage response and repair mechanisms.

## 4. Discussion

RhoA GTPase is overexpressed and overactivated in cancer and is involved in cancer progression, directly regulating cell proliferation, survival, and invasion [3, 4]. Our results, using stable HeLa cell lines expressing either a constitutively active RhoA (RhoA-V14) or a dominant negative version of this protein (RhoA-N19), suggest that RhoA GTPase activity also regulates cancer cell sensitivity to  $\gamma$ -radiation, by affecting basic DNA repair mechanisms. Despite the fact that HeLa cells have been used as a good model for our hypothesis and this whole work has been done solely on it, we believe that our results do not reflect a cell line-dependent phenomenon because unpublished results (not shown) performed in metastatic melanoma MeWo cell line culminate in similar cellular responses.

We observed that HeLa cells have high basal level of RhoA GTPase in the active state (RhoA-GTP) and that the activity of RhoA was modulated accordingly (up or down) in both mutant clones. RhoA-GTP levels increased



**FIGURE 3: Inhibition of RhoA activity by C3 toxin expression strongly affects DNA damage response, including global and specific DNA repair mechanisms in HeLa cells, following  $\gamma$ -radiation.** (a) Dendritic morphology of HeLa cells (HeLa + C3 images) associated with decreased RhoA-GTP levels (on the right), 24 h after transfection with a plasmid for C3 toxin expression. Images on the right are insets from those on the left, 200x. (b) Estimates of DNA damage and repair efficiency (by olive tail moment, or OTM, measurements from comet assays) in HeLa cells expressing the C3 toxin, following  $\gamma$ -radiation. (c) Immunoblotting analysis of the effects of  $\gamma$ -radiation (15 Gy) on phosphorylated Chk1/Chk2 and histone H2AX levels in HeLa cells expressing the C3 toxin (using  $\alpha$ -Tubulin as a loading control). (d) and (e) Assays for GFP-based detection of homologous recombination (HR, using HeLa-DR-GFP) or nonhomologous end joining (NHEJ, using HeLa-EJ5-GFP) after DNA damage induced by I-SceI restriction enzyme expression. (d) Phase contrast (left) and green fluorescence (right) images of cells transfected with a plasmid for I-SceI expression (I-SceI), or with an empty vector (EV), showing the appearance of GFP-positive cells indicative of HR (HeLa-EJ5-GFP) or NHEJ (HeLa-DR-GFP), 72 h after transfection. (e) Quantification of HR and NHEJ assays, with (EV + C3 and I-SceI + C3 groups) or without (EV and I-SceI groups) concomitant C3 toxin expression. Graphs (with mean  $\pm$  SD values) and immunoblots are representative of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.001$ , and \*\*\* $p < 0.005$ , between treated and untreated conditions (by ANOVA).

in response to either  $\gamma$ -radiation activation or serum stimuli (not shown). The high basal levels of RhoA-GTP observed here in cervical adenocarcinoma HeLa cells are similar to those reported for other cancer cell lines, including the breast cancer cell line MDA-MB-231 [20], and also in colorectal cancer cell lines and tumor samples [21]. The RhoA GTPase directly regulates cytoskeletal dynamics via actin polymerization, mediating cell adhesion and migration [16, 22].

In glioblastoma multiforme tumors, radiation-induced activation of RhoA increases cell migration and invasive potential [23]. Our study extends these results, showing that cells expressing the dominant negative RhoA-N19 display decreased migration rates, both in the presence and in the absence of FBS, and also following  $\gamma$ -radiation. The opposite was observed for HeLa cells expressing the constitutively active RhoA-V14, indicating that in HeLa cells with decreased

RhoA activity migration is inhibited by ionizing radiation, while RhoA overactivation enables cells to migrate after radiation treatment, in agreement with the results reported by Ridley in 2006 [24].

When compared with those displayed by parental HeLa cells, the proliferation and survival responses to  $\gamma$ -radiation of both HeLa cell lines expressing RhoA mutants are interesting, since they suggest that “fine-tuning” of RhoA activity impacts on DNA repair efficiency. Similar results were reported for canine T23 MDCK cells, where down-regulation of RhoA activity by expression of RhoA-N19 decreased cell survival after toxin-mediated DNA double-strand break induction [2], showing that RhoA GTPase activity is important for survival after DNA damage in these cells. However, we observed that cells expressing the dominant negative RhoA-N19 and those expressing the constitutively active RhoA-V14 were equally susceptible to decreases in proliferation and survival, following  $\gamma$ -radiation. Interestingly, differential modulation of RhoA activity in HeLa cells led to population arrest at distinct stages of the cell cycle, which correlated with changes in the levels of cellular senescence and apoptosis observed in each cell line, following  $\gamma$ -radiation. These different “cell-fate” decisions seem to depend on the levels of RhoA activity, which in turn affect DNA damage sensing by the DDR pathway, directly reflecting in cell cycle phase-dependent triggering of cell proliferation inhibition followed by cell death. These data are in agreement with studies showing that DNA damage activates RhoA in an ATM-dependent manner and that RhoA activation is important for cell survival and proliferation, after treatment with low doses of  $\gamma$ -radiation [2, 25].

The effectiveness of radiotherapy treatment of human tumors is based (almost entirely) on the inability of cancer cells to repair radiation-induced DNA damage [2]. Given that the presence of DNA damage induces RhoA activation and triggers DNA repair mechanisms [2, 26], it is not surprising that DNA repair was more efficient in cells expressing the constitutively active RhoA-V14 mutant than in those expressing the dominant negative RhoA-N19, although DNA repair in HeLa-RhoA-V14 was still less efficient than that observed in parental HeLa cells. We detected increased levels of DNA damage relative to basal conditions for both mutant clones. We also observed increased levels of DNA damage and slow repair after inhibition of endogenous RhoA activity by C3 toxin expression, and RhoA inhibition drastically reduced the activity of the DNA repair pathways HR and NHEJ in HeLa cells. These data strongly suggest that RhoA GTPase is involved (possibly indirectly) in the regulation of DNA repair pathways, particularly in early repair. The similarities between our results and those obtained with HdCDT-induced DNA damage provide further support to our hypothesis that cytosolic RhoA signaling modulates nuclear genome integrity mechanisms [2, 27, 28].

Finally, our data on the effects of RhoA activity modulation on classical DNA damage response pathways suggest that RhoA is indirectly involved in the regulation of Chk1 and Chk2 activation after  $\gamma$ -radiation, because Chk1 (Ser345) and Chk2 (Thr-68) phosphorylation appeared attenuated

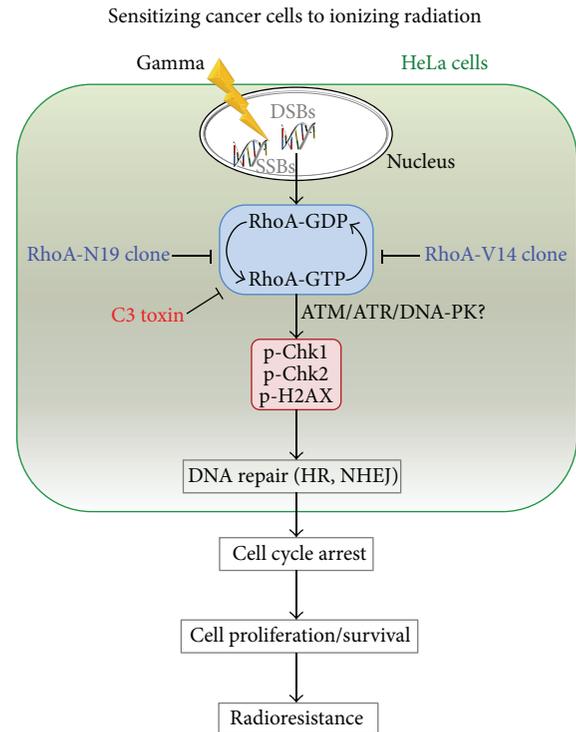


FIGURE 4: Model of the effects of RhoA activity modulation on global and specific DNA repair mechanisms, leading to increased cell proliferation or cell death, and reflecting in the radioresistance levels of cancer cells.

in HeLa-RhoA-N19 cells and increased in HeLa-RhoA-V14, after exposure to  $\gamma$ -radiation.

Chk1/Chk2 protein kinases are activated in response to DNA damage and are involved in DNA damage repair [29]. Pharmacological inhibition of Chk1/Chk2 induces cellular radiosensitivity, impairing DNA repair and triggering mitotic catastrophe, in the human colon cancer cell line HT-29 [30]. Thus, the attenuated phosphorylations of Chk1 and Chk2 in cells deficient in RhoA signaling may have impaired DNA repair (by HR and NHEJ) in these cells, which would explain the reductions in survival and proliferation, the specific cell cycle arrest pattern, and the increased levels of senescence and apoptosis observed in these cells. Overall, our data support the existence of a “cross talk” between RhoA signaling and DNA damage response and repair pathways in cancer cells (Figure 4), which may contribute to increased radioresistance. Importantly, these findings raise the interesting possibility that, in the clinic, the combination of chemotherapy using RhoA inhibitors followed by radiotherapy may lead to positive associations, for specific stages of cervical cancers.

## 5. Conclusions

Our findings provide strong evidence that positive or negative modulation of RhoA activity increases HeLa cell's sensitivity to  $\gamma$ -radiation treatment and therefore points to a possible clinical association of chemotherapy, using RhoA inhibitors,

followed by radiotherapy sections for different stages of cervical cancers.

### Conflict of Interests

The authors declare that there is no potential conflict of interests.

### Authors' Contribution

Conception and design were done by Fabio L. Forti. Development of methodology was done by Juliana H. Osaki, Yuli T. Magalhaes, and Gisele Espinha. Acquisition of data was done by Juliana H. Osaki, Yuli T. Magalhaes, and Gisele Espinha. Analysis and interpretation of data were performed by Juliana H. Osaki, Gisele Espinha, and Fabio L. Forti. Writing, review, and/or revision of the paper were made by Juliana H. Osaki and Fabio L. Forti. Study supervision was done by Fabio L. Forti.

### Acknowledgments

The authors thank Andressa P. Costa, Juliana R. Domingos, Benedita Oliveira, and Viviane Q. Machtura for technical assistance. They also thank Dr. Margarida M. Hamada, Dr. Elizabeth Somessari, and the staff from CTRD-IPEN/CNEN (São Paulo, SP, Brazil) for conducting  $\gamma$ -radiation on their samples. They thank Professor Marisa Gennari de Medeiros for allowing them to perform comet assays in her laboratory. Additionally, they thank the Publicase staff and editors for their editing services. This research was supported by the Brazilian agency FAPESP through a Young Investigator Fellowship, no. 2008/58264-5, to Fabio L. Forti. Juliana H. Osaki is the recipient of an FAPESP Ph.D. Fellowship (142668/2009-5), GE is the recipient of a CAPES Master Fellowship (2011/05822-3), and Yuli T. Magalhaes is an undergraduate student recipient of CNPq Scientific Initiation Fellowship. All research projects in our laboratory have been also supported by the Brazilian agency CAPES through the Graduate Program in Biochemistry of the Chemistry Institute. The final publication of this paper was supported by the CAPES/PROEX funds and by the Sao Paulo State Research Foundation (FAPESP) through Grant no. 2015/18341-4 to Fabio L. Forti.

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## Review Article

# Oxidative Stress and Mitochondrial Activation as the Main Mechanisms Underlying Graphene Toxicity against Human Cancer Cells

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Received 22 April 2015; Revised 10 June 2015; Accepted 21 July 2015

Academic Editor: Sahdeo Prasad

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Due to the development of nanotechnology graphene and graphene-based nanomaterials have attracted the most attention owing to their unique physical, chemical, and mechanical properties. Graphene can be applied in many fields among which biomedical applications especially diagnostics, cancer therapy, and drug delivery have been arousing a lot of interest. Therefore it is essential to understand better the graphene-cell interactions, especially toxicity and underlying mechanisms for proper use and development. This review presents the recent knowledge concerning graphene cytotoxicity and influence on different cancer cell lines.

## 1. Graphene: Properties and Applications

Novoselov et al. first described graphene in 2004 as monocrystalline graphitic film and received Nobel Prize in 2010 for the exploration of its exceptional properties [1]. The discovery of graphene became a new driving force in the development of nanoindustry [2, 3]. Graphene is a single-atom-thick, two-dimensional sheet of  $sp^2$ -hybridized carbon atoms arranged in a regular hexagonal pattern like in honeycomb structure (Figure 1) [4–9]. Graphene conducts heat and electricity extremely well [2] and as one of the carbon allotropes it is considered the thinnest and strongest known material [10]. The ratio of thickness of graphene sheet to the size of its surface differentiates this material from all other known nanomaterials [10]. The unique physicochemical properties of graphene are large surface area ( $2630\text{ m}^2/\text{g}$ ), extraordinary electrical (mobility of charge carriers,  $200,000\text{ cm}^2\text{ V}^{-1}\text{ s}^{-1}$ ) and thermal conductivity ( $\sim 5000\text{ W/m/K}$ ), extremely high mechanical strength (Young's modulus  $\sim 1100\text{ Gpa}$ ), and possibility of mass-production at low cost [4, 11–13]. The perfect electronic transport properties and high surface-to-volume ratios are responsible for its exceptional mechanical and rheological properties and resistance to degradation. Graphene has two

active sides which are surfaces and edges that improve the attachment of biological molecules to graphene and its adhesion to the cells [11]. Graphene has higher ratio of peripheral to central carbon atoms than similar nanomaterials. Consequently atoms at the edge allow better interaction with cell membranes and interference with cell metabolism [14]. Unlike other carbon allotropes, that is, fullerenes or carbon nanotubes, graphene exhibits unique chemical and physical properties closely related to the possibility of its surface functionalization which makes it more biocompatible and less toxic [15].

Graphene and graphene-based nanomaterials are today applied in numerous fields for purposes including nanoelectronics and energy technology (supercapacitors, batteries, composite materials, transistors, solar cells, fuel cells, matrix for mass spectra, and hydrogen storage), energy storage, sensors, catalysis, and biomedicine [2, 4, 11, 12]. Due to their unique mechanical properties, such as high elasticity, flexibility, and adaptability for tissue engineering graphene family nanomaterials (GFNs) have been investigated in several biomedical applications especially cancer therapy, drug delivery, and diagnosis [5, 16, 17]. Other biomedical applications comprise gene delivery, antibacterial and antiviral materials, tissue engineering, and biocompatible scaffolds for

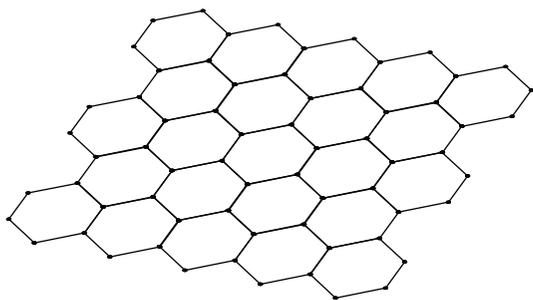


FIGURE 1: The graphene structure: single layer of  $sp^2$ -hybridized carbon atoms arranged in 2D crystal honeycomb lattice (adapted from [9]).

cell cultures. Graphene-based materials are promising in the field of biosensing and bioimaging (optical sensing, fluorescence imaging probes, and electrochemical sensing) [4, 5, 12, 18]. Furthermore, graphene nanomaterials have been used in advanced therapeutic techniques such as photothermal and photodynamic therapies [3, 16].

Graphene and its derivatives, referred to as graphene family nanomaterials (GFNs), include graphene oxide (GO), its reduced form (rGO) and single- or few-layer graphene, graphene nanosheets (GNS), and graphene nanoribbons [4, 11, 19]. Graphene nanoparticles, depending on the method of synthesis, can show different morphologies and chemical or physical properties [20]. So far various approaches have been developed to synthesize graphene and its derivatives such as mechanical exfoliation, epitaxial growth, or unzipping carbon nanotubes. The mechanical exfoliation, firstly used by Novoselov in 2004, resulted in few-layer graphene from highly oriented pyrolytic graphite. Graphene samples with the lateral size up to millimeter-range were obtained after many method modifications but still are too large and cannot be produced on a large scale, hence the inability to be used in most practical applications. Chemical vapor deposition (CVD) based on dissolving carbon atoms into a metal substrate allows producing large scale graphene films. Graphene nanoribbons (GNRs) of precise dimensions and 100% yield can be obtained by the novel strategy based on longitudinal unzipping carbon nanotubes. However, the most developed method for the mass-production of graphene is the exfoliation of graphene oxide (GO). Oxygen functional groups on the graphene surface make GO and rGO sheets strongly hydrophobic although the electrical conductivity is lower than that of pristine graphene. Poor conductivity can be bypassed in the process of liquid phase exfoliation of graphite where high-quality monolayer graphene at significant yield can be produced [15]. In our previous article we have described numerous methods of graphene synthesis related with the development of various forms of graphene which differ in the quality, number of layers, and the amount of the structure defects [21]. Lots of the possible applications of graphene derivatives obtained in different conditions make it problematic to use graphene safely in biomedicine or tissue engineering. In this paper we have focused on the impact of graphene family nanomaterials (GFNs) on the different

cancer cells, the possible mechanisms of graphene toxicity, and available applications of graphene in cancer therapy or drug delivery.

## 2. Graphene Family Nanomaterials (GFNs)

Among other members of graphene family nanomaterials (GFNs) graphene oxide (GO) is one of the most important chemical graphene derivatives. GO is a highly oxidized form of graphene [4, 22, 23] produced mainly by chemical methods through energetic oxidation of graphite using different oxidant agents or known procedures as in Hummers method [10, 12]. GO nanosheets present hydroxyl and epoxide functional groups on their basal surface and carboxyl functional groups on their plane edges [3]. GO has usually 1–3 layers (1–2 nm thick), with size ranging from a few to several hundred nanometers [12]. GO is hydrophilic and forms stable suspensions in pure water but in salt and other biological solutions it creates aggregates [24, 25]. Reactive COOH and OH groups in GO facilitate connection with various materials, such as polymers, biomolecules, DNA, protein, quantum dots, or  $Fe_3O_4$  nanoparticles which improve the solubility and prevent aggregation in salt-containing physiological buffers [3, 12].

Improved properties of graphene oxide make it useful in biological and medical applications, as a surface coating material for implants and also as a stimulator of growth and differentiation of the cells [12, 17, 18]. The large aromatic surface of graphene oxide with lots of functional groups allows adsorbing molecules with high affinity and creating stable complexes which make GO an ideal nanocarrier for effective drug and gene delivery [23, 26]. Different targeting molecules such as folic acid or antibodies can be conveniently immobilized on GO which allows precise and efficient delivery of GO into targeted cells. Solid tumor cells are more acidic (pH  $\sim$  6.8) than normal cells (pH 7.4) and are ideal candidates for controlled release of anticancer drugs [27]. Lowered pH in some drug molecules additionally increases their solubility and decreases their tendency to stay adsorbed which eventually leads to the controlled endocytosis and the release in lysosomes [15]. pH-responsive and integrin  $\alpha_v\beta_3$  monoclonal antibody functionalized graphene oxide is an example of the nanocarrier for targeted delivery and controlled release of doxorubicin (DOX) into cancer cells [27].

Reduced graphene oxide (rGO) is the product of thermal or chemical modification of graphene oxide (GO) with reducing agents (e.g., hydrazine) [3, 4]. rGO possess lower number of oxygen containing functional groups than GO [28]. The reducing conditions greatly influence the properties of GO such as electrical conductivity, surface charge, or water dispersibility (increase hydrophobicity) [4]. rGO possesses high capacity for hydrophobic interactions among various functional molecules but it leads to creation of aggregates with weak stability under physiological conditions. Surface modification of rGO with polymers or biopolymers has been used to stabilize and improve the properties of rGO and use it as a nanocarrier [29].

Graphene platelets (GPs) are produced by physical methods directly by exfoliation of graphite without the initial

stage of oxidation. GPs are hydrophobic and form stable hydrocolloids [10]. Zero-dimensional, single-atom layer graphene quantum dots (GQDs) have lateral dimensions below 100 nm and size of 10 nm or less [16]. GQDs are biocompatible due to their small size and high oxygen content which improves solubility and stability in water or serum [12, 16]. Graphene quantum dots due to their excellent photoluminescent properties are promising agents for optical probes in bioimaging [6]. Graphene nanoparticles, referred to as graphene nanoribbons, are formed by the longitudinal unzipping of multiwalled carbon nanotubes [30].

### 3. Graphene and Cells

The potential toxic effects of graphene materials on the environment and on the human health have recently attracted considerable attention among researchers. Understanding of the interactions of GFNs with living systems and their adverse effects in vitro and in vivo is essential for further development and safe use of graphene-based nanomaterials [11]. Cytotoxicity studies of graphene include the influence on the cell viability and morphology, membrane integrity, ROS generation, DNA damage, gene expression, DNA damage, and mechanism of uptake (Figure 2) [4, 10, 13]. The interactions of graphene nanoparticles with the cells depend on the physicochemical and electrical properties [5, 12, 40–43]. The reports indicate that morphology (size, shape, and sharp edges), surface charge, surface functionalization, dispersibility, state of aggregation, number of layers, purity, and method of synthesis (e.g., CVD [21], arc-discharge [30], and biological methods [31]) are the key factors that influence the mechanism of uptake (passive diffusion and endosomal uptake) and tissue response to graphene-based nanomaterials [2, 4, 5, 20, 30]. Moreover, the toxic effect of graphene highly depends on the conditions of the experiment, which include the time of exposure, dose, type of the cells, and the method used to establish the cell viability [19, 24, 30, 31, 33].

The chemical methods used in the production of graphene nanomaterials including oxidation or reduction of graphene oxide bring harsh conditions and toxic agents, such as hydrazine or its derivatives, which influence the structure of graphene and its safety. One of the approaches used to decrease the toxicity of graphene involves aqueous and environmentally friendly reduction strategy based on bacterial and yeast respiration [31]. Recently used microbial biomass for the reduction of GO including *Escherichia coli* [44], *Bacillus marisflavi* [31], and *Ganoderma* extract [5] has significantly increased biocompatibility of graphene.

The majority of GFNs have poor solubility and create aggregates in salt-containing physiological buffers due to electrostatic charge and nonspecific binding to proteins [11]. Functionalization of pristine graphene via covalent or noncovalent coatings by various materials such as polymers, DNA, proteins, and nanoparticles greatly improves the biocompatibility [3]. Surface modifications of graphene nanomaterials also improve their solubility and significantly reduce toxic interactions with living systems. Significant changes in biocompatibility have been achieved by producing graphene reinforced composite materials with polyethylene

glycol (PEG) or other biopolymers such as chitosan, hyaluronan (HA), or dextran [3, 11, 13, 17, 19].

Most of the members of graphene family nanomaterials easily enter the living cells because of the small size, sharp edges and rough surface [11]. Additionally, negatively charged ( $-30.89$  eV) GO can easily accumulate inside the cell [40]. The uptake can be also affected by the shape and the aggregation state of GO sheets [35]. The presence of carboxyl, epoxy, and hydroxyl groups in GO reduces its cytotoxicity [40] and the small size (smaller than 5 nm) and the high content of oxygen improve the solubility and increase biocompatibility [16]. However, the mechanism of cellular uptake and the fate of graphene inside the living cells are still not fully understood. This process may depend on the cell type, on the properties of graphene, or on both of these factors. Some researchers suggest endocytosis as a basic mechanism of cellular uptake for PEG-GO while others combine endocytosis and macropinocytosis depending on the formation of smaller or larger aggregates of PEG-graphene nanoribbons [3].

The physical interactions of graphene with the cell membranes are one of the major causes of GFNs cytotoxicity [5, 19, 45]. Hydrophobic forms of graphene interact with the cell membrane lipids [19] while the other forms may bond to the cell receptors and interfere with the cell metabolism, inhibit nutrient supply, and induce stress or cell death [10]. Moreover, graphene itself can bind the micronutrients and amino acids from the cell culture medium which limits their availability and inhibits cellular growth and viability [24]. GO is smaller and less toxic than rGO because of the high oxygen content, smoother edges, and hydrophilic properties. Reduced graphene oxide has high affinity to the cell membranes and the irregular and sharp edges affect their integrity, stimulate receptors, and activate mitochondrial pathways which may cause apoptosis [46].

Oxidative stress and generation of reactive oxygen species (ROS) can be involved in the toxic effects of graphene-based nanomaterials [16, 19, 45]. When the cell homeostasis is disrupted and the enzymes responsible for reducing ROS (superoxide dismutase and glutathione peroxidase) fail, the macromolecules, such as proteins, DNA, and lipids, can be damaged, which greatly influence the cell metabolism and signaling [19, 42]. The interactions of the GO with the cells can lead to excessive ROS generation, which is the first step in the mechanisms of carcinogenesis, ageing, and mutagenesis [22].

Except for the plasma membrane damage and oxidative stress induction graphene can cause apoptosis and/or cell necrosis through the direct influence on the cell DNA or mitochondrial activity [18]. Graphene nanoparticles can induce dissipation of the mitochondrial membrane potential which subsequently increases the generation of intracellular ROS and eventually triggers apoptosis by activating the mitochondrial pathway [31]. The interactions of graphene with cell genetic material are based on DNA-intercalation and cleavage mechanisms [13]. Difference in the structure of rGO and GO makes rGO more potent to penetrate cell compartments and directly interact with the nuclear DNA resulting in genotoxic effects [46].

Additionally, graphene can directly interact with different genes encoding important proteins and enzymes [4, 13].

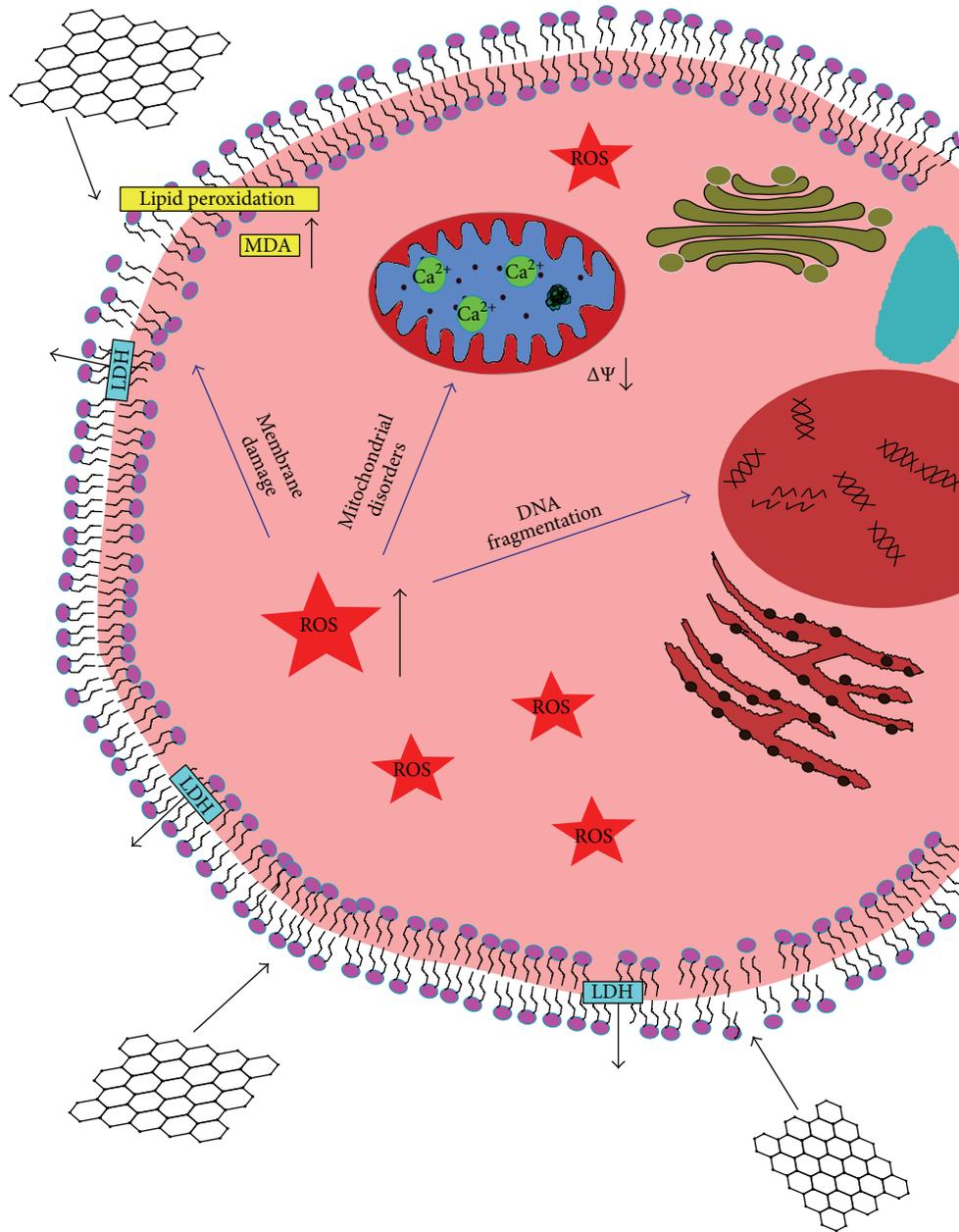


FIGURE 2: Schematic toxicity mechanisms of graphene on human cancer cells. Graphene provides the formation of reactive oxygen species (ROS) which are the cause of DNA (fragmentation and condensation) and cell membrane damage (release of LDH, lipid peroxidation, and increase in MDA-malondialdehyde), mitochondrial disorders (reduction of mitochondrial membrane potential  $\Delta\Psi$ , increase in  $\text{Ca}^{2+}$ ), and cell death.

Other indirect mechanisms of GO cytotoxicity involve DNA damage caused by ROS [13], inhibition or activation of specific enzymes [22], or reaction with other cell components such as proteins and polysaccharides [13]. For better understanding of the mechanisms of graphene action inside the cell further studies are required, particularly to explain the cellular interactions of graphene materials with proteins and cell membrane lipids on a molecular level [19].

**3.1. Breast Cancer Cell.** Many of the currently available methods for producing graphene are not environmentally friendly

and rGO obtained by these methods is not safe enough to use in biological and medical applications. Therefore researchers developed a novel and simple approach for rGO synthesis using microorganisms which is cost-effective and safe for the environment. Gurunathan et al. compared the cytotoxicity of GO obtained from graphite powder using a modified version of Hummers and Offeman's method with rGO synthesized by *Bacillus marisflavi* biomass on human breast adenocarcinoma cells (MCF-7) using WST-8 assay. Incubation of MCF-7 cells with both B-rGO (biogenic rGO) and GO at concentrations ranging from 0 to 100  $\mu\text{g}/\text{mL}$

showed dose-dependent graphene cytotoxicity. In concentrations higher than 60  $\mu\text{g}/\text{mL}$  graphene markedly decreased the cell viability and increased ROS generation and release of LDH. Surprisingly, bacterial rGO had stronger cytotoxic effect on MCF-7 cells compared to GO [31]. In another experiment Gurunathan and colleagues used mushroom extracts (*Ganoderma*) to reduce graphene oxide. They examined the influence of GO and GE-rGO on MDA-MB-231 human breast cancer cells using WST-8 viability assay, membrane integrity test (LDH assay), and DCFH-DA assay as a quantitative method for oxidative stress assessment. The cytotoxicity of graphene was dose-dependent (0–150  $\mu\text{g}/\text{mL}$ ) especially at the higher concentrations where elevated levels of ROS induced membrane damage and LDH leakage in the presence of GE-rGO [5]. These studies indicate that rGO synthesis with the use of bacteria and fungi is easier, less expensive and works better for the development of a potential therapeutic agent that targets breast cancer cells.

In vitro anticancer activity of GO was examined in various concentrations (10, 20, 40, and 80  $\mu\text{g}/\text{mL}$ ) on human breast cancer cells MCF-7 using MTT viability assay. GO showed approximately 13% inhibition of cell viability of MCF-7 cells and the cytotoxicity at dose-dependent manner [32]. Other tests concerning cytotoxicity of GO were carried on human adenocarcinoma breast cancer cells (MDA-MB-231) using Cell Counting Kit-8 (CCK-8) assay. 48 h incubation with GO in concentrations ranging from 100  $\mu\text{g}/\text{mL}$  to 500  $\mu\text{g}/\text{mL}$  showed increasing cytotoxicity against MDA-MB-231 cells together with the increasing amount of graphene in the medium. Further studies showed that GO reacts directly with genomic DNA and inhibits cell replication with complete blockage of human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) gene at the concentration of 1  $\mu\text{g}/\text{mL}$ . MDA-MB-231 cells treated with GO even at low concentration (10  $\mu\text{g}/\text{mL}$ ) after 24 h incubation showed signs of apoptosis. Hence, scientists tested 30,000 genes to examine the impact of GO on the gene expression at the cellular level. The results revealed 101 genes (mainly responsible for DNA-damage control, cell apoptosis, cell cycle, and metabolism) that showed 2-fold or even greater expression changes after GO treatment at the concentrations of 10  $\mu\text{g}/\text{mL}$  and 100  $\mu\text{g}/\text{mL}$ . Additionally, GO increased expression of ATM and Rad51 genes (DNA repair proteins) which can explain the influence of graphene on the cell DNA [13].

Zhou and coworkers evaluated the cytotoxicity of GO modified with polyethylene glycol (PEG) using three cell lines derived from human breast cancers: MDA-MB-231, MDA-MB-436, and SK-BR-3. PEG-GO had no apparent influence on the cell viability but inhibited cancer cell migration and invasion. PEG-GO disrupted F-actin filaments responsible for cell migration by depleting ATP levels through downregulation of mitochondrial energy metabolism [47]. Another research on the human breast cancer cells MDA-MB-231 with pristine graphene and graphene oxide also showed no apparent influence on the cell viability at low concentrations but prominent inhibition of migration and invasion [48].

Recent findings have proved that the functionalization of the graphene surface makes it less toxic. Mullick Chowdhury et al. investigated the cytotoxicity of oxidized-graphene

nanoribbons coated with the amphiphilic polymer PEG-DSPE (O-GNR-PEG-DSPE) at various concentrations (0–400  $\mu\text{g}/\text{mL}$ ) on Sloan Kettering breast cancer (SKBR3) cells and Michigan Cancer Foundation-7 (MCF-7) breast cancer cells using Alamar blue assay. Both cell lines showed the reduction in viability by about 10%–15% at the highest concentrations after 24 h incubation with the copolymer. SKBR3 cells incubated with O-GNR-PEG-DSPE demonstrated slight increase in the LDH release while MCF-7 cells did not show any statistically significant LDH leakage. Additionally, SKBR3 and MCF-7 cells showed small or no uptake of O-GNR-PEG-DSPE. The results indicate that graphene copolymer has no toxic effect on the tested cells up to 10  $\mu\text{g}/\text{mL}$  and exhibits low cytotoxicity even at the highest concentrations (400  $\mu\text{g}/\text{mL}$ ) [30].

The toxicity of covalently pegylated nano-GO with unmodified rGO was compared using MTS assay and MCF-7 human epithelial breast cancer cells. The half maximal inhibitory concentration (IC<sub>50</sub>) of nano-rGO was established at the concentration of approximately 80 mg/L, while for pegylated nano-GO it was at about 99 mg/L [32]. According to MTT assay fluorinated form of graphene oxide (FGO) even at the concentration of 576  $\mu\text{g}/\text{mL}$  showed no toxicity to human breast cancer cells (MCF-7) [49]. Waiwijit and coworkers investigated the toxicity of graphene-carbon paste (GCP) in four different concentrations (1, 2.5, 5, and 10 wt%) on MDA-MB-231 breast cancer cells also using MTT assay. The cell viability decreased after longer incubation periods (48 and 72 h) and at the presence of the highest concentration of GCP in comparison to the cultures with CP alone. Moreover, MDA-MB-231 cancer cells exhibited increased ROS generation with the increasing time of incubation and the amount of GCP in the culture medium [42]. Together, these studies demonstrate the different impact of graphene nanomaterials on breast cancer cells including the cell viability and cytotoxicity connected with the generation of ROS, loss of the membrane integrity, and DNA damage which may have potential clinical advantage pertaining to increased therapeutic efficacy and decreased local toxicity of the used nanomaterial.

**3.2. Cervical Cancer Cell.** Remarkably durable and prolific HeLa cells derived from cervical cancer are more sensitive to the graphene than other cell lines. According to Zhang et al. GO showed high cytotoxicity to HeLa cells even at low concentrations. The biological responses induced by GO were evaluated by series of assays, including MTT, malondialdehyde (MDA), superoxide dismutase (SOD), lactate dehydrogenase (LDH), and reactive oxygen species (ROS). HeLa cells were treated with different concentrations of GO ranging from 0 to 80  $\mu\text{g}/\text{mL}$  and cultured for 3 h and 24 h. MTT test results showed dose-dependent GO cytotoxicity with the cell viability at about 50% at the concentration of 80  $\mu\text{g}/\text{mL}$ . To evaluate the lipid peroxidation and oxidant stress the levels of MDA and SOD enzyme activity were measured in the cell lysates. The results showed an obvious increase in MDA production after exposure to 80  $\mu\text{g}/\text{mL}$  of GO and decreased SOD activity. Moreover, the incubation of HeLa cells with 80  $\mu\text{g}/\text{mL}$  of GO for 24 h increased the

levels of ROS 17 times. The researchers suggested that the cytotoxicity of GO is not associated with the cell uptake [33].

Instead of the biological assays measuring cell activity and viability there are available more selective, more sensitive, and faster electrochemical approaches to evaluate the toxicity of graphene. Yoon et al. used cell-based electrochemical impedance biosensing with interdigitated indium tin oxide (ITO) electrodes to analyze toxicity of graphene nanoflakes in HeLa cells. Researchers used two different sizes of graphene flakes (80 nm and 30 nm) in the concentration of 400  $\mu\text{g}/\text{mL}$  and monitored the cytotoxicity for 1 day. The studies showed greater cytotoxic effect of the smaller 30 nm graphene nanoflakes due to their higher uptake, while 80 nm graphene nanoflakes agglomerated on cell membranes causing less harm to the cells [34].

Liu's group conjugated graphene oxide with dextran, a widely used surface coating biopolymer. They cultured HeLa cells with different concentrations (10, 50, and 200 mg/L) of GO and GO-DEX and studied in vitro toxicity for 24 h, 48 h, and 72 h. The cell counting data showed dose-dependent decrease in the cell proliferation after incubation with GO and notably smaller influence on the cell count after GO-DEX treatment. The calcein AM/propidium iodide (PI) staining was carried out to further determine graphene toxicity. The results revealed that GO did not induce significant cell death even at high concentrations up to 200 mg/L, while GO-DEX showed no influence on the cell growth and viability. All the evidence demonstrates that dextran coating may improve the biocompatibility of GO [50].

In other studies the cytotoxicity of graphene polymer (GQD-PEG) was evaluated on HeLa cells using WST-1 assay. GQD-PEG did not induce apoptosis or necrosis even at the concentration of 160  $\mu\text{g}/\text{mL}$ . LDH release and ROS level measurements showed no impact of GQD-PEG on the cell membrane integrity and oxidative stress generation probably because of the small size of the particles (smaller than 5 nm) and the presence of PEG polymer [16]. However, HeLa cells showed a reduction of the cell viability by 60% after 24 h incubation with 400  $\mu\text{g}/\text{mL}$  O-GNR-PEG-DSPE (oxidized-graphene nanoribbons (O-GNRs) with the amphiphilic polymer). As the dose increased, the survival rate of the cells decreased together with the release of LDH. On the images of the cells lots of swollen intracellular vesicles were observed together with disrupted plasma membranes which are a characteristic feature in necrotic cells [30].

**3.3. Lung Cancer Cell.** The biological effect of GFNs on lung cancer cells depends mainly on the size and concentration of graphene [22, 35]. Hu et al. investigated the cellular effect of different concentrations (0 to 100  $\mu\text{g}/\text{mL}$ ) of GO nanosheets on human alveolar adenocarcinoma cell line (A549). MTT assay showed concentration-dependent cytotoxicity and about 50% decrease in cell viability after incubation with GO at the concentration of 100  $\mu\text{g}/\text{mL}$ . Interestingly, the cell viability was greatly mitigated after addition of 10% FBS (fetal bovine serum) into the culture medium. TEM imaging demonstrates that precoating of GO with FBS prevents cell membranes from the damage, the outflow of cytoplasm, and eventually cell death. GO nanosheets possess

high adsorption capability for proteins in the medium and therefore cytotoxic effect of GO precoated with 10% FBS was largely reduced [36].

In other experiments scientists compared the cytotoxicity of GO nanosheets and reduced with hydrazine rGO nanosheets characterized by lower thickness and less surface defects. The metabolic activity assays based on succinate dehydrogenase activity in the mitochondria showed that GO in the concentration of 20  $\mu\text{g}/\text{mL}$  slightly influenced the viability of A549 cells (20%) but in higher concentration (85  $\mu\text{g}/\text{mL}$ ) reduced the cell viability to 50% within 24 h. rGO nanosheets reduced the A549 cell viability to 47% and 15% with 20 and 85  $\mu\text{g}/\text{mL}$ , respectively. Therefore, rGO nanosheets are significantly more cytotoxic than GO's which is because of different surface charge and functional groups on the nanosheet surfaces. Transmission electron microscopy (TEM) showed that graphene nanosheets could be internalized within A549 cells via endocytosis. However, flow cytometric analysis demonstrated no apoptosis in A549 cells treated with GO nanosheets (20 and 85  $\mu\text{g}/\text{mL}$  for 24 h) but cell cycle arrest in the G2 phase (mitosis metaphase). These data suggest that the observed small decrease in the cell viability is not because of the cell death but rather might arise from GO-retarded cell cycle which restrains the proliferation rate [37].

The group of scientists investigated also the cytotoxicity of graphene oxide (GO) and highly hydrogenated graphene (HHG) in concentrations that ranged from 3.125  $\mu\text{g}/\text{mL}$  to 400  $\mu\text{g}/\text{mL}$ . The results from MTT and WST-8 assays indicated that HHG was more toxic to A549 cells than GO and that the toxicity was dose-dependent. The percentage of viable cells after 24 h treatment with GO and HHG in the concentration of 400  $\mu\text{g}/\text{mL}$  was 43% and 26%, respectively [24].

In contrast, Chang et al. reported that graphene oxide (GO) is a reasonably safe material at the cellular level. Researchers examined the toxicity of GO at the concentration range from 0 to 200  $\mu\text{g}/\text{mL}$  on human lung carcinoma epithelial cell line A549. In this comprehensive study the morphology, viability, apoptosis, ROS production, and membrane integrity were examined. The CCK-8 assay used to estimate the GO toxicity showed dose- and size-dependent loss of the viability with little influence of the culture period. However, the level of apoptosis was not relevant to the dose or the size of the GO samples and exposure to GO did not induce LDH leakage. The LDH levels of GO-treated cells (for 200  $\mu\text{g}/\text{mL}$  was 6%) were even slightly lower than those of the control cells (7.5%). GO induced oxidative stress in A549 cells even at low concentrations, but with no obvious toxicity. The results showed that the cells grow on the GO films very well and there is no considerable difference in the morphology and density of the GO-treated and control cells. There is no impact on the ultrastructure of A549 cells and no signs of GO sheets inside the cells. These results indicate that GO is biocompatible and has a great potential for being the substrate for the cell growth [35].

de Marzi et al. with the same cell line investigated the impact of graphene oxide on the viability using MTT assay. Graphene oxide was used at various concentrations (10, 50, and 100  $\mu\text{g}/\text{mL}$ ) and in two different flake sizes (1.32  $\mu\text{m}$  and

130 nm). The results showed slight loss in the viability of the A549 cells after 24 h incubation with both types of GO. The comet assay showed size-dependent genotoxic effect on the cells with high degree of toxicity even at the low concentrations with 130 nm GO flakes [22]. Cytotoxicity and distribution of GO inside the A549 cells were evaluated by Jin and coworkers using CCK-8 assay and transmission electron microscopy (TEM), respectively. After 4 h incubation with GO in concentrations of 100 and 300  $\mu\text{g}/\text{mL}$  there was no significant decrease in the cell viability. GO was present inside the cells in the cytoplasm and nucleus but cellular organelles were not affected [40].

Yuan et al. examined the cytotoxicity of graphene quantum dots (GQDs) with various surface modifications ( $\text{NH}_2$ ,  $\text{COOH}$ , and  $\text{CO-N}(\text{CH}_3)_2$ ) in human lung carcinoma cells (A549 cells) using MTT assay. GQDs with different functional groups had low cytotoxicity even when the concentration reached 200  $\mu\text{g}/\text{mL}$ . Moreover, the three kinds of GQDs did not induce cell apoptosis and/or necrosis. GQDs (50  $\mu\text{g}/\text{mL}$ ) were localized in the cytoplasm and did not enter into the cell nucleus. GQDs are smaller and provide less damage to cell membranes than GO and therefore are more biocompatible and less cytotoxic to cells even when modified with different chemical groups [6]. Other studies showed that pegylated graphene quantum dots (GQDs-PEG) are practically not toxic to A549 cells at all [16]. The inconsistency of these results might come from the different methods of preparation or synthesis of GO and distinct testing models.

**3.4. Liver Cancer Cell.** The increasing number of possible applications of graphene nanomaterials triggers considerable concerns about the impact on health and environment though further more thorough investigations are vital. Chatterjee et al. investigated toxicity of various concentrations of graphene oxide (GO) and reduced graphene oxide (rGO) on HepG2 cells for 24 h. According to EZ-Cytox assay the cells viability was clearly dose- and time-dependent for both nanomaterials but rGO indicated higher cytotoxicity with unclear converse change after 16 h of exposure. EC20 and EC50 for rGO were 8 mg/L and 46 mg/L, respectively, whereas they were 10 mg/L and 81 mg/L for GO. The microscopic images showed increased internal granularity of the GO-treated cells which indicates that GO was internalized by HepG2 cells through endocytosis. The rGO treated cells showed outsized aggregation and accumulation of rGO on the cell membrane due to its hydrophobic nature. Difference in the uptake efficiency explains various modes of cytotoxicity [4].

One of the principal mechanisms underlying nanomaterial toxicity involves oxidative stress. In the experiments both GO and rGO induced release of reactive oxygen species (ROS) in HepG2 at dose-dependent manner. However, rGO mediated ROS production was the result of physical interaction while oxidative stress induced by GO involved NADPH oxidase and significant increase in the antioxidative enzyme genes (SOD1, SOD2, CAT, GSTA1, and GSTA4) expression. The toxicity of graphene can also be caused by direct interaction with the cell DNA. GO and rGO induced both single and double stranded DNA damage. rGO did not significantly influence the DNA repair gene expression and

DNA damage resulted from physical interactions rather than biological one. Moreover, GO and rGO both caused increase in the apoptosis rate of HepG2 cells. However, apoptosis induced by GO was dose- and time-dependent and involved alterations in expression of the key apoptotic genes whereas rGO elicited apoptosis only at lower dose and early time of exposure. The cytotoxicity of rGO is probably caused by the strong hydrophobic interactions with the cell membranes and eventual destruction by extremely sharp edges and highly depends on their uptake by HepG2 cells [4].

The objective of the study of Lammel and his coworkers was to evaluate the cytotoxicity and underlying mechanism of two different graphene derivatives: graphene oxide (GO) and carboxyl graphene (CXYG) towards human hepatoma cell line. It was observed that cells exposed to GO and CXYG in concentrations of 16  $\mu\text{g}/\text{mL}$  for 24 h were completely covered with the nanomaterial and further increase in the concentration caused unspecific cell damage due to mechanical stress. TEM and scanning electron micrographs demonstrated that both GO and CXYG were able to penetrate the plasma membrane and cumulate in the intracellular vesicles resulting in altered cell morphology and an augmented number of apoptotic cells. Exposure of HepG2 to GO (1–16  $\mu\text{g}/\text{mL}$ ) and CXYG (2–32  $\mu\text{g}/\text{mL}$ ) for 72 h caused dose-dependent increase in the fluorescence intensity indicating an elevated metabolic activity of the cells which suggests plasma membrane damage. Loss of the membrane integrity was associated with a strong physical interaction of GO with the phospholipid bilayer and increased metabolism was probably associated with energy-dependent process involved in plasma membrane repair. Elevated fluorescence intensity at the high exposure concentrations can be also explained by oxidative stress increase. However, the underlying ROS-generating mechanisms were distinct after GO and CXYG treatment. Exposure to GO and GXVG indicates mitochondrial membrane depolarization and/or a decrease in the amount of mitochondria which leads to increased intracellular ROS. The authors concluded that plasma membrane damage and oxidative stress are the key factors in graphene-induced cytotoxicity of HepG2 cells [18].

Yuan et al. applied the iTRAQ-coupled 2D LC-MS/MS approach to analyze the protein profile change of HepG2 cells treated with graphene oxide. They observed only a moderate variation of protein levels within the cells [45, 51]. Moreover, MTT assay resulted in 17% loss of the cell viability in the cells treated with GO [45].

**3.5. Nerve Cell Cancer.** Graphene toxicity and biocompatibility were further established by Jaworski et al. who examined the influence of graphene platelets (GPs) on two different human glioma cell lines (U87 and U118) with high degree of malignancy. The GP-treated cells were more oval and denser and in both cases graphene platelets created agglomerates close to the cell bodies but did not enter the cells. GPs caused cell membrane disruption higher in U87 than in U118 cells. Exposure to graphene at the concentration of 100  $\mu\text{g}/\text{mL}$  for 24 h resulted in 54% and 58% decrease in the cell viability in U87 and U118 cells, respectively. The degree of apoptosis was higher in both glioma cell lines (68% in

U87 and 99% in U118) together with necrosis present only in U87 (24%). The results indicate that the high concentration and the direct physical contact with the cells are the main cause of graphene toxicity. Difference in the activity of genes involved in a cell cycle regulation of the U87 and U118 cells is responsible for the susceptibility to programmed cell death indicating the potential applicability of GP in anticancer therapy [10]. Similar results of nano-rGO were obtained in U87MG glioblastoma cell line using MTS assay where half maximal inhibitory concentration (IC<sub>50</sub>) reached 85 mg/L [52]. Jaworski et al. using the same glioma cells (U87 and U118) as previously mentioned investigated cytotoxicity and genotoxicity of GO and rGO platelets. In vitro analysis showed that both GO and rGO enter glioma cells and reduce the cell viability and the proliferation with increasing doses. However, the lower cell vitality and the higher degree of apoptosis were observed after rGO treatment which indicates that GO is less toxic to glioma cells than rGO [14]. The scope of another experimental in vitro study on glioblastoma cancer cells U87 was to determine the cell viability and DNA fragmentation after exposure to different carbon allotropes. All studied nanoparticles did not alter the cell morphology; however pristine graphene (GN) and reduced graphene oxide (rGO) led to a significant decrease in the cell viability. The comet assay results demonstrated that DNA damage was caused by GN, rGO, graphite, and ultradispersed detonation diamond (UDD) and only GO had no genotoxic effect on U87 cells. These findings indicate the potential use of GO as a drug nanocarrier and GN, rGO, graphite, and UDD in the direct elimination of glioblastoma multiforme cells because of their higher toxicity [46].

Moore and coworkers investigated the impact of nanographene (nGr) in U-138 glioblastoma cells. Cytotoxicity was measured in vitro using PrestoBlue cell viability assay after 24 h incubation. The results showed significant increase in the number of dead cells and the decrease in cell density after graphene treatment in the concentrations higher than 50  $\mu\text{g}/\text{mL}$  [17].

Yuan et al. examined the cytotoxicity of graphene quantum dots (GQDs) with different surface modifications ( $\text{NH}_2$ ,  $\text{COOH}$ , and  $\text{CO-N}(\text{CH}_3)_2$ ) in human neural glioma cells (C6) using MTT assay. Conversely, data analysis showed low cytotoxicity and good biocompatibility for all tested graphene nanomaterials even at the very high concentrations (200  $\mu\text{g}/\text{mL}$ ) [6]. Carboxylated graphene oxide (GO-COOH) and chlorotoxin-conjugated graphene oxide (CTX-GO) both had negligible toxic effects on C6 cells (80% of viability at concentrations of 3.0  $\mu\text{g}/\text{mL}$ , 7.5  $\mu\text{g}/\text{mL}$ , and 15.0  $\mu\text{g}/\text{mL}$ ) [23]. Coating graphene with the multifunctional PLA-PEG (poly(lactide) and poly(ethylene glycol)) reduced the toxicity of uncoated graphene and did not show signs of dose-dependent toxicity up to 250  $\mu\text{g}/\text{mL}$  [17].

Interesting results were obtained by Oh et al. Scientists used MTT assay to examine the viability of SH-SY5Y cell line grown on partially functionalized graphene sheets with oxygen or fluorine. SH-SY5Y cells cultured on the oxygenated graphene sheets showed approximately 138% viability but only 50% viability on the fluorinated graphene compared to pristine graphene samples. The increase in cell proliferation

can be explained by adhesion of the hydrophilic oxygenated graphene sheets to the cell surface [8].

**3.6. Other Cancer Cells.** Except described cancer cell lines where cytotoxic effect was predominant, some reports show only slight decrease in the cell viability with improved influence of graphene on the cell proliferation and survival [22, 25, 53]. The cytotoxicity of graphene depends on various possible mechanisms including interactions with the cells or culture medium. de Marzi et al. using graphene oxide at growing concentrations (10, 50, and 100  $\mu\text{g}/\text{mL}$ ) and in two different flake sizes (1320 nm and 130 nm) investigated the cytotoxic effect on CaCo2 human colorectal adenocarcinoma cell line. Both micro- and nano-GO exhibited high biocompatibility and increased CaCo2 cell proliferation slightly decreasing with higher concentrations of nano-GO. The 24 h comet assay showed that micro-GO flakes genotoxicity rose together with the used concentration, while nano-GO had no significant genotoxic effect on treated cells [22].

Beyond exerting little cytotoxic effects on the cells, Ruiz et al. observed morphological changes, cell enlargement, and better attachment to GO-coated slides of HT-29 mammalian colorectal adenocarcinoma cells (control glass slides and glass slides coated with 10  $\mu\text{g}$  of GO). The results indicated promotion of mammalian cell proliferation, spreading, and growth after graphene oxide exposure [53].

Wu et al. evaluated the cytotoxicity of graphene oxide (GO) on human multiple myeloma cells (RPMI-8226). Increasing GO concentration from 10 to 100 mg/L after 24 h treatment reduced the cell viability from 95.6% to 79.6%, respectively. Cells treated with GO were round with little cell shrinkage but with no typical apoptotic features. Annexin V-FITC/PI staining by flow cytometry showed no significant differences in the cell apoptotic rate between the untreated and GO-treated cells suggesting only slight cytotoxicity of GO [38].

Sun and his group examined toxicity of single-layer pegylated graphene oxide sheets (NGO-PEG) soluble in buffers and serum. Incubation of Raji cells (Burkitt's lymphoma B lymphocytes) in various concentrations of NGO-PEG for 72 h showed no obvious toxicity except a slight delay of the cell growth at the highest concentration (150 mg/L) [25].

Human prostate cancer cells (PC3) were incubated in the presence of different concentrations (0–180  $\mu\text{g}/\mu\text{L}$ ) of chemically reduced graphene oxide (CRGO) and chitosan magnetic graphene nanoparticles (CMG) for 72 hours. The cytotoxicity was evaluated using the WST-1 assay and the results revealed dose-dependent increase in graphene oxide cytotoxicity while CMG nanoparticles did not show any toxicity at all the tested concentrations. Chitosan-coated graphene oxide is soluble in both organic and acidic aqueous solutions and less toxic than nonfunctionalized GO and hence has higher therapeutic efficacy [39]. New insights into specific cancer treatment were presented in the research on metastasis of prostate cancer cells PC3. With the low influence on the cell viability pristine graphene and GO effectively inhibited migration and invasion of these cancer cells with no apparent effect on the induction of apoptosis [48].

Conventional therapeutic approaches to eradicate all cancer cells fail because of the presence of tumor-initiating cells that are resistant to drugs, chemotherapy, and radiation. Cancer stem cells (CSCs) constitute a minority of the overall cancer cell population, although they are highly invasive and tumorigenic and the inability of their efficient elimination results in disease relapse and formation of metastases [54]. Fiorillo et al. used flakes of GO to inhibit selectively CSCs proliferation in multiple cell lines including breast, lung, ovarian, prostate, and pancreatic cancers. Two different grades of GO were used, small GO (0.2–2  $\mu\text{m}$ ) and big GO (5–20  $\mu\text{m}$ ). Both small and big GO flakes inhibited tumor-sphere formation in all independent cancer cells. They did not affect the viability of non-CSCs but selectively targeted cancer stem cells. Analysis of these targeted actions showed that GO inhibited a number of several key signal transduction pathways related to cancer stem cells including antioxidant and interferon responses [55].

#### 4. Conclusion

Graphene was first isolated in 2004 and since then its properties have been studied widely [2]. Graphene-based nanomaterials have boosted the development of the interdisciplinary research caused by their unique properties and possible applications in electronics and biotechnology. Single-atom-thick, two-dimensional sheet of  $\text{sp}^2$ -hybridized carbon atoms arranged in a regular hexagonal pattern [3, 4] owns extraordinary electrical and thermal properties, mechanical strength, and capability of biofunctionalization [11–13]. Graphene nanoparticles have been used as drug and gene delivery agents in multimodal imaging and could be useful in biomedicine and cancer therapy [20]. Graphene is a nanomaterial whose chemical, physical, or mechanical properties and structure permit the active tissue integration of desirable cell types and tissue components suggesting the potential use in tissue engineering [12, 17, 18]. Besides the research confirming graphene biocompatibility there are reports of dose-dependent graphene toxicity against cultured cells. However, most of these reports concentrate mainly on graphene oxide and reduced graphene oxide (rGO) prepared in solutions [56]. Graphene family nanomaterials include ultrathin graphite, few-layer graphene (FLG), graphene oxide (GO; from monolayer to few layers), reduced graphene oxide (rGO), and graphene nanosheets (GNS) [4]. Among the most frequently used graphene derivatives in the cytotoxicity study are GO, rGO and graphene quantum dots (GQD) with various surface modifications. Mainly studied cancer cells include lung, breast, cervical, liver, and nerve cancer cell lines (Table 1).

Depending on the cell line and type of the nanomaterial, graphene can increase the viability [22, 53] or cause the cell death [33]. In the study of de Marzi et al. GO shows a slight decrease in A549 cells viability while the same concentration and time of exposure result in increased cell viability in CaCo2 colorectal carcinoma cells [22]. Oxidized-graphene nanoribbons (O-GNRs) water-solubilized with the amphiphilic polymer PEG-DSPE (O-GNR-PEG-DSPE) show significantly higher toxic effect on cervical cancer cells

(HeLa) than on other cancer or normal tested cells [30]. We can assume that reduced graphene oxide (rGO) is more cytotoxic than graphene oxide (GO) to lung, liver, and breast cancer cells [4, 31, 37]. However, the influence of rGO is similar among U87 nerve cancer cells and MCF-7 breast cancer cell line ( $\text{IC}_{50} = 85 \text{ mg/L}$  and  $80 \text{ mg/L}$ , resp.) [52]. Graphene surface functionalization with different groups of various biomaterials such as PEG or dextran results in better nanomaterial biocompatibility. Pegylated graphene quantum dots (GQDs-PEG) exhibit very low or no toxicity against lung and cervical cancer cells even at very high concentrations (200  $\mu\text{g/mL}$ ) [6, 16]. Pegylated graphene oxide (GO-PEG) [25, 52], dextran covered graphene oxide (GO-DEX) [50] and fluorinated graphene oxide (FGO) [49] are more biocompatible than other graphene derivatives such as highly hydrogenated graphene (HHG) which after 24 h incubation reduce the viability of lung cancer cells (A549) to 26% [24].

Therefore, each graphene derivative may have diverse effect on the same cell type and the same graphene form can cause different reaction depending on the cell origin. Evaluations of the cytotoxicity and biocompatibility are an essential step in developing of any new biomaterial for in vivo biomedical applications. This review reveals that the toxicity of graphene nanomaterials depends not only on the graphene chemical structure, functionalization, size, concentration, and time of exposure but also on various possible mechanisms including interactions with different types of cells or culture medium components. Moreover the diversity of the samples and methods of the production hinder establishing of the biological impact of graphene [21].

One of the proposed mechanisms underlying graphene cytotoxicity involves reactive oxygen species [19, 45] while the others include plasma membrane damage, impairment of mitochondrial activity, DNA damage, and interaction with biomolecules which finally lead to apoptotic and/or necrotic cell death [19, 31, 56]. Toxicity of graphene is desirable when used against cancer cells but not in case of surrounding healthy ones. It would be best to use graphene as a delivery agent for water insoluble drugs, antigens, antibodies, or nucleic acids and unload therapeutic molecules selectively inside the cancer cells to impair their activity [57, 58]. Use of graphene as a drug delivery agent has been recently the subject of numerous scientific researches [7, 12, 17, 23, 25, 27, 29, 38, 39, 41, 58–61]. However, the mechanisms of cellular uptake and modes of action are still under investigation. In vitro studies regarding the influence of GFNs on mammalian cells give only a slight overview on the possible interactions with living organisms. The inconsistency of available data and the lack of sufficient information make it impossible to fully assess the suitability of graphene as a biomaterial. To understand better the impact of graphene further studies should be performed especially in vivo on the mechanisms of cell uptake and signaling combined with the results of long term effects of the materials internalization. More thorough research concerning graphene hemo- and biocompatibility together with the impact on immunological system would be essential to establish safe administration or implantation of GFNs. Yet the most important thing in graphene technology is to establish one universal and recurrent method of

TABLE 1: Influence of graphene-based nanomaterials on cancer cells.

Cell/tissue	Graphene-based nanomaterials	Dose and time incubation	Percentage of inhibition	Effects	Reference
<b>MCF-7</b> breast	<b>O-NNR-PEG-DSPE</b> (oxidized-graphene nanoribbons O-GNRs with amphiphilic polymer PEG-DSPE)	10–400 $\mu\text{g}/\text{mL}$	24 h: 400 $\mu\text{g}/\text{mL}$ = 15%	Slight reduction of cell viability	Mullick Chowdhury et al. [30]
		24 h 48 h	48 h: 400 $\mu\text{g}/\text{mL}$ = 20%		
		0–100 $\mu\text{g}/\text{mL}$	100 $\mu\text{g}/\text{mL}$ = 42%	(i) Reduction of cell viability	Gurunathan et al. [31]
		24 h	100 $\mu\text{g}/\text{mL}$ = 64%	(ii) ROS generation (iii) Released LDH	
<b>MDA-MB-231</b> breast	<b>B-rGO</b> (3833 nm)	10–80 $\mu\text{g}/\text{mL}$	80 $\mu\text{g}/\text{mL}$ = 13,1%	Reduction of cell viability	Chaudhari et al. [32]
		24 h	24 h: 150 $\mu\text{g}/\text{mL}$ = 40%		
		0–150 $\mu\text{g}/\text{mL}$	48 h: 150 $\mu\text{g}/\text{mL}$ = 50%	(i) Reduction of cell viability	Gurunathan et al. [5]
		24 h 48 h	150 $\mu\text{g}/\text{mL}$ = 50%	(ii) ROS generation (iii) Released LDH	
<b>SKBR3</b> breast	<b>GE-rGO</b> (3200 nm)	100–500 $\mu\text{g}/\text{mL}$	500 $\mu\text{g}/\text{mL}$ = 40%	(i) Reduction of cell viability	Liu et al. [13]
		48 h	48 h: 150 $\mu\text{g}/\text{mL}$ = 70%	(ii) DNA damage (iii) Interfered with gene expression (iv) Apoptosis	
		10–400 $\mu\text{g}/\text{mL}$	24 h: 400 $\mu\text{g}/\text{mL}$ = 10%	(i) Slight reduction of cell viability	Shim et al. [29]
		24 h 48 h	48 h: 400 $\mu\text{g}/\text{mL}$ = 22%	(ii) Slight released LDH	
<b>HeLa</b> cervix	<b>GO</b> (graphene oxide)	0–160 $\mu\text{g}/\text{mL}$	160 $\mu\text{g}/\text{mL}$ = <5%	Slight reduction of cell viability	Chong et al. [16]
		24 h	24 h: 400 $\mu\text{g}/\text{mL}$ = 60%	(i) Reduction of cell viability	Mullick Chowdhury et al. [30]
		10–400 $\mu\text{g}/\text{mL}$	48 h: 400 $\mu\text{g}/\text{mL}$ = 63%	(ii) Released LDH	
		24 h 48 h	80 $\mu\text{g}/\text{mL}$ = 50%	(i) Reduction of cell viability (ii) Released LDH (iii) Increased MDA (iv) Decreased SOD (v) ROS generation	Zhang et al. [33]
<b>Graphene nanoflakes</b> 80 nm 30 nm	<b>GO</b> (graphene oxide)	0–80 $\mu\text{g}/\text{mL}$	80 nm: 400 $\mu\text{g}/\text{mL}$ = 14%	Reduction of cell viability	Yoon et al. [34]
		24 h	30 nm: 400 $\mu\text{g}/\text{mL}$ = 29%		
		400 $\mu\text{g}/\text{mL}$			
		24 h			

TABLE I: Continued.

Cell/tissue	Graphene-based nanomaterials	Dose and time incubation	Percentage of inhibition	Effects	Reference
A549	GQDs with modified groups NH <sub>2</sub> , COOH, and CO-N (CH <sub>3</sub> ) <sub>2</sub>	0–200 µg/mL 24 h	200 µg/mL = <20%	Slight reduction of cell viability	Yuan et al. [6]
	GO 1320 nm 130 nm	10; 50; 100 µg/mL 24 h	100 µg/mL = 10%	(i) Slight reduction of cell viability (ii) Genotoxic effect	de Marzi et al. [22]
	GO	0–400 µg/mL 24 h	400 µg/mL = 57%	Reduction of cell viability	Chng et al. [24]
	HHG (highly hydrogenated graphene)		400 µg/mL = 74%		
	GO		s-GO:		
	s-GO (160 ± 90 nm)	0–200 µg/mL 24 h	200 µg/mL = <33%	(i) Reduction of cell viability (ii) ROS generation	Chang et al. [35]
	m-GO (430 ± 300 nm)		m-GO, l-GO:		
	l-GO (780 ± 410 nm)		200 µg/mL = <20%		
	GO nanosheets	0–100 µg/mL 24 h	100 µg/mL = 50%	Reduction of cell viability	Hu et al. [36]
	GO nanosheets rGO nanosheets	20 µg/mL 85 µg/mL 24 h	85 µg/mL = 50% 85 µg/mL = 85%	Reduction of cell viability	Hu et al. [37]
HepG2 liver	GO	0–200 µg/mL 24 h	EC20 = 10 µg/mL EC50 = 81 µg/mL	(i) Reduction of cell viability (ii) Increased MDA (iii) ROS generation	Chatterjee et al. [4]
	rGO		EC20 = 8 µg/mL EC50 = 46 µg/mL	(iv) DNA damage (v) Mitochondrial disorders (vi) Increase in Bax (vii) Decrease in Bcl2	
	GPs (graphene platelets)	0–100 µg/mL 24 h	100 µg/mL = 46%	(i) Reduction of cell viability (ii) Released LDH (iii) Apoptosis	
	GO 100 nm–10 µm rGO 100 nm–1,5 µm	0–100 µg/mL 24 h	100 µg/mL = 28% 100 µg/mL = 64%	(i) Reduction of cell viability (ii) Apoptosis (iii) Reduction of cell proliferation	
U87 nerve	GPs (graphene platelets)	0–100 µg/mL 24 h	100 µg/mL = 42%	(i) Reduction of cell viability (ii) Released LDH (iii) Apoptosis	Jaworski et al. [10]
	GO 100 nm–10 µm rGO 100 nm–1,5 µm	0–100 µg/mL 24 h	100 µg/mL = 22% 100 µg/mL = 51%	(i) Reduction of cell viability (ii) Apoptosis (iii) Reduction of cell proliferation	Jaworski et al. [14]
	GPs (graphene platelets)	0–100 µg/mL 24 h	100 µg/mL = 42%	(i) Reduction of cell viability (ii) Released LDH (iii) Apoptosis	Jaworski et al. [10]
U118 nerve	GO 100 nm–10 µm rGO 100 nm–1,5 µm	0–100 µg/mL 24 h	100 µg/mL = 22% 100 µg/mL = 51%	(i) Reduction of cell viability (ii) Apoptosis (iii) Reduction of cell proliferation	Jaworski et al. [14]
	GPs (graphene platelets)	0–100 µg/mL 24 h	100 µg/mL = 42%	(i) Reduction of cell viability (ii) Released LDH (iii) Apoptosis	Jaworski et al. [10]
	GO 100 nm–10 µm rGO 100 nm–1,5 µm	0–100 µg/mL 24 h	100 µg/mL = 22% 100 µg/mL = 51%	(i) Reduction of cell viability (ii) Apoptosis (iii) Reduction of cell proliferation	Jaworski et al. [14]

TABLE I: Continued.

Cell/tissue	Graphene-based nanomaterials	Dose and time incubation	Percentage of inhibition	Effects	Reference
<b>U-138</b> nerve	<b>nGr</b> (nanographene)	50; 100; 250 $\mu\text{g}/\text{mL}$ 24 h	250 $\mu\text{g}/\text{mL}$ = 35%	Reduction of cell viability	Moore et al. [17]
<b>RPMI-8226</b> peripheral blood	<b>GO</b>	0–100 $\mu\text{g}/\text{mL}$ 24 h	100 $\mu\text{g}/\text{mL}$ = 20%	Slight reduction of cell viability	Wu et al. [38]
<b>PC3</b> prostate	<b>CRGO</b> : chemically reduced graphene oxide	0–180 $\mu\text{g}/\mu\text{L}$ 72 h	160 $\mu\text{g}/\text{mL}$ = 60%	Reduction of cell viability	Wang et al. [39]

production which would allow obtaining graphene with the same properties on large scale and in cost-effective manner. Therefore, detailed studies are required to explain the toxicity pathways of GFNs which would allow not only establishing the effect of graphene on cancer cells but also facilitating their proper use in medicine and cancer therapy.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## Research Article

# PAN-811 Blocks Chemotherapy Drug-Induced *In Vitro* Neurotoxicity, While Not Affecting Suppression of Cancer Cell Growth

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Received 7 April 2015; Revised 2 July 2015; Accepted 6 July 2015

Academic Editor: Swaran J. S. Flora

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Chemotherapy often results in cognitive impairment, and no neuroprotective drug is now available. This study aimed to understand underlying neurotoxicological mechanisms of anticancer drugs and to evaluate neuroprotective effects of PAN-811. Primary neurons in different concentrations of antioxidants (AOs) were insulted for 3 days with methotrexate (MTX), 5-fluorouracil (5-FU), or cisplatin (CDDP) in the absence or presence of PAN-811-Cl-H<sub>2</sub>O. The effect of PAN-811 on the anticancer activity of tested drugs was also examined using mouse and human cancer cells (BNLT3 and H460) to assess any negative interference. Cell membrane integrity, survival, and death and intramitochondrial reactive oxygen species (ROS) were measured. All tested anticancer drugs elicited neurotoxicity only under low levels of AO and elicited a ROS increase. These results suggested that ROS mediates neurotoxicity of tested anticancer drugs. PAN-811 dose-dependently suppressed increased ROS and blocked the neurotoxicity when neurons were insulted with a tested anticancer drug. PAN-811 did not interfere with anticancer activity of anticancer drugs against BNLT3 cells. PAN-811 did not inhibit MTX-induced death of H460 cells but, interestingly, demonstrated a synergistic effect with 5-FU or CDDP in reducing cancer cell viability. Thus, PAN-811 can be a potent drug candidate for chemotherapy-induced cognitive impairment.

## 1. Introduction

One of the most common complications of chemotherapeutic drugs is toxicity to the central nervous system (CNS), namely, chemotherapy-induced cognitive impairment or chemo-brain. This toxicity can present in many ways, including encephalopathy syndromes and confusional states, seizure activity, headache, cerebrovascular complications and stroke, visual and hearing loss, cerebellar dysfunction, and spinal cord damage with myelopathy [1]. Mild to moderate effects of chemotherapy on cognitive performance occur in 15–50% of the survivors after treatment [2, 3]. The cognitive problems can last for many years after the completion of chemotherapy in a subset of cancer survivors. Up to 70% of patients with cancer report that these cognitive difficulties persist well beyond the duration of treatment [4–6]. Chemobrain can seriously affect quality of life and life itself in cancer patients.

Among possible candidate mechanisms, oxidative stress (OS) may play a key role in cognitive disorders caused

by broad types of anticancer drugs, such as antimetabolites, mitotic inhibitors, topoisomerase inhibitors, and paclitaxel [7]. These chemotherapeutic agents are not known to rely on oxidative mechanisms for their anticancer effects. Among the antimetabolite drugs, methotrexate (MTX) and 5-fluorouracil (5-FU), widely used chemotherapeutic agents, are most likely to cause CNS toxicity [1]. Although there are yet no reports of 5-FU increasing CNS OS, it has been observed to induce apoptosis in rat cardiocytes through intracellular OS [8], to increase OS in the plasma of liver cancer patients [9], and to decrease glutathione (GSH) in bone marrow cells [10]. MTX can also cross the blood-brain barrier as well [11] and result in an increase of OS in cerebral spinal fluid and executive dysfunction in MTX-treated patients of pediatric acute lymphoblastic leukemia [12, 13]. It is well known that ROS, such as H<sub>2</sub>O<sub>2</sub>, can result in neuronal cell death [14, 15]. Cisplatin (CDDP) is an alkylating agent. Its cytotoxic effect is thought to be mediated primarily by the generation of nuclear DNA adducts, which,

if not repaired, cause cell death as a consequence of DNA replication and transcription blockage. However, oxidative damage has been observed *in vivo* following exposure to CDDP in several tissues including nervous tissue, suggesting a role for OS in the pathogenesis of CDDP-induced dose-limiting toxicities [16–18]. Cotreatment with antioxidants (AOs) suppresses the toxic effects of CDDP on several organs [19, 20].

Currently, there are no proven treatments for chemotherapy-induced cognitive impairment. Some efforts have been focused on correcting cognitive deficits rather blocking the neurotoxic pathway of chemotherapeutic drugs [21]. Since ROS mediates neurotoxicity in a number of neurodegenerative disorders, one strategy in disease control has been focused on development of antioxidants as preventive and therapeutic molecules. These include vitamin C, vitamin E, glutathione, coenzyme Q (CoQ), carotenoids, melatonin, and green tea extract [22, 23]. In contrast to the minimal positive effects of these efforts, antioxidative therapy could be a promising strategy for the treatment of neurotoxicity. Several preclinical studies have shown that AO treatment prevents chemotherapy-induced OS and cognitive deficits when administered prior to and during chemotherapy [24, 25].

Our previous research has demonstrated that PAN-811 (known as 3-aminopyridine-2-carboxaldehyde thiosemicarbazone or Triapine), a bioavailable small molecule (MW 195) currently in phase II clinical trials for the treatment of patients with cancer, can efficiently block neurodegeneration. Major underlying mechanisms for the neuroprotection of PAN-811 are blockage of both excitatory pathway and OS [22]. Hence we hypothesized that PAN-811 could protect neurons from anticancer drugs, such as MTX, 5-FU, and CDDP. Since PAN-811 is an anticancer drug targeting ribonucleotide reductase, which is distinctive from intracellular targets of MTX, 5-FU, or CDDP, coadministration of PAN-811 with any of these may also have a synergistic effect on suppression of cancer cell growth.

## 2. Materials and Methods

**2.1. Neuronal Cell Culture.** Mixed cortical and striatal neurons from embryonic day 17 male Sprague-Dawley rats (tissue obtained from NIH) were seeded into poly-D-lysine coated 96-well plates at density of 50,000 cells/well and initially cultured at 37°C, 5% CO<sub>2</sub>, in neurobasal medium (NB) with B27 supplement (Invitrogen) containing full strength of AOs to obtain highly enriched (95%) neurons [26]. Since AOs, including vitamin E, vitamin E acetate, superoxide dismutase (SOD), catalase (CAT), and GSH, are additives to culture medium, reduction of AO concentration in culture medium provides an approach to determine the level of OS involvement in a neurotoxic process. In our study, the culture medium was replaced at a 50% ratio with NB plus B27 minus AOs twice at days 7 and 9 to set AO concentrations as 50% and 25%, respectively. At 16 days *in vitro* (d.i.v.), a fraction of the culture medium was harvested for lactate dehydrogenase (LDH) assay, and then AO concentration was reduced to 12.5% or 17.5%, and cultured for a further 5 hours prior to ending the experiment.

**2.2. Cancer Cell Culture.** The mouse liver cancer cell line BNL3 (gift of Dr. Jack Wands, Brown University) and the human lung cancer cell line H460 (ATCC) were seeded into 96-well plates at a density of 4,000 cells/well and cultured at 37°C, 5% CO<sub>2</sub>, in DMEM (11965, Gibco) supplemented with 10% fetal bovine serum, 20 mM HEPES, 1 mM sodium pyruvate, and 24 ng/mL gentamycin (all reagents came from Gibco).

**2.3. Cell Insults and Treatments.** Determination of concentration for each anticancer drug in our experiments was based on its reported concentration in human cerebral spinal fluid (CSF) in chemotherapy, literature report of its neurotoxicity in culture, and our preliminary *in vitro* experimental data. At 13 d.i.v., the neuronal cell cultures were insulted with 100 μM of MTX (M9929, Sigma) [27–30], 25 μM of 5-FU (F6627, Sigma) [31–33], or 3.5 μM of CDDP (sc-200896, Santa Cruz) [34, 35] for 3 days in absence or presence of PAN-811·Cl·H<sub>2</sub>O. For ROS examination, the neurons were insulted with both 100 μM of MTX and 25 μM of 5-FU by 15 d.i.v. PAN-811·Cl·H<sub>2</sub>O was added to cultures to final concentrations of 1.25, 2.5, 5, and 10 μM at the same time as addition of the anticancer drugs. For cancer cell lines, the cells were insulted by the second day of cell seeding with the same concentration of MTX, 5-FU, or CDDP as used in neuronal culture in the absence or presence of 10 μM PAN-811·Cl·H<sub>2</sub>O for another 3 days.

**2.4. Quantitative Assays and Morphological Assessment.** Cell membrane integrity and mitochondrial function of either neurons or cancer cells were measured with LDH and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS] analyses, respectively. The latter has been used to quantify cell survival. For the LDH assay, a mixture of a 35 μL aliquot of culture supernatant and 17.5 μL of Mixed Substrate, Enzyme and Dye Solutions (Sigma) was incubated at room temperature (RT) for 30 minutes. For the MTS assay, 10 μL of MTS reagent (Promega) was added to a culture well containing neurons in 50 μL of medium. The preparations were incubated at 37°C for 2 hours. The preparations for both assays were then spectrophotometrically measured at 490 nm using a 96-well plate reader (Mode 550, Bio-Rad). Neuronal cell death was morphologically determined based on the integrity of the cell soma and continuity of neuronal processes. The change in number of cancer cells was judged directly by cell density. Cells were photographed under an inverted phase contrast microscope (IX 70, Olympus) using 10x or 20x objective.

**2.5. ROS Examination.** Neurons were incubated in 15 μM dihydrorhodamine 123 (DHR123, Molecular Probes) for 30 min at 37°C to determine intramitochondrial ROS levels. Fluorescence was photographed by using a fluorescent microscope and quantified by excitation at 485 nm and emission at 520 nm using a 96-well plate reader (Model 550, Bio-Rad).

**2.6. Data Analysis.** Data were generated from 4–6 replicate wells, expressed as mean ± standard deviation (SD), and

statistically evaluated at a significance level of 1% with one-factor ANOVA or Student's *t*-test by using software VASARSTATS (<http://vassarstats.net/>) followed by the Tukey HSD test. Figure symbols are as follows: #,  $P < 0.05$ , and ##,  $P < 0.01$ , compared with control; \*,  $P < 0.05$ , and \*\*,  $P < 0.01$ , compared with the insulted group; §§,  $P < 0.01$ , compared with PAN-811 treated group by Student's *t*-test.

### 3. Results

**3.1. MTX, 5-FU, or CDDP Elicited Neurotoxicity in an AOs-Dependent Manner.** By 3 days following the insults, neither MTX at 100  $\mu\text{M}$ , 5-FU at 25  $\mu\text{M}$ , nor CDDP at 3.5  $\mu\text{M}$  caused morphological changes, LDH release, or MTS reduction when neurons were cultured in the medium containing 100% or 50% AOs (data not shown). However, MTX, 5-FU, or CDDP at the same concentrations elicited significant LDH increase (indicating cell membrane leakage, Figure 1(c)) in the culture supernatant when the AO concentration was reduced to 25%, although no cell damage was visible (Figure 1(a)), and no change in MTS level was detectable (data not shown) under these conditions. When the AO concentration was reduced to 12.5% for 5 hours at 16 d.i.v., extensive neuronal cell death occurred in the cultures insulted with 25  $\mu\text{M}$  5-FU or 3.5  $\mu\text{M}$  CDDP, as indicated by loss of cell bodies, together with interruption of neurite networks on the background (Figure 1(b)). Corresponding to the morphological cell death, the MTS readings for 5-FU- and CDDP-insulted groups were reduced by 27% and 66%, respectively (Figure 1(d)). MTX at 100  $\mu\text{M}$  did not elicit significant MTS reduction (Figure 1(d)) under 12.5% AO condition. Thus, the neurotoxicities elicited with MTX, 5-FU, or CDDP were dependent on AO reduction.

**3.2. PAN-811 Dose-Dependently Suppresses MTX-, 5-FU-, or CDDP-Induced Neurotoxicity.** We then examined PAN-811 for its effect on the anticancer drug-induced neurotoxicity at the 12.5% AO condition. MTX at 100  $\mu\text{M}$  did not result in significant loss of cell number, while 5-FU at 25  $\mu\text{M}$  or CDDP at 3.5  $\mu\text{M}$  caused robust loss of neurons in culture (Figure 2(a)). Correspondingly, MTX insult did not significantly affect the MTS reading, while 5-FU- and CDDP-insulted cultures showed significant reduction in MTS readings (Figure 2(c)). PAN-811 dose-dependently inhibited 5-FU- or CDDP-induced MTS reduction. PAN-811 at 10  $\mu\text{M}$  completely blocked 5-FU-induced MTS reduction and inhibited CDDP-induced MTS reduction by 48%. The LDH release assay demonstrated that each of MTX at 100  $\mu\text{M}$ , 5-FU at 25  $\mu\text{M}$ , and CDDP at 3.5  $\mu\text{M}$  resulted in significant increases in LDH reading (Figure 2(b)). PAN-811 dose-dependently suppressed LDH increase caused by each anticancer drug. PAN-811 at 5  $\mu\text{M}$  fully blocked LDH release in MTX-, 5-FU-, or CDDP-insulted cultures (with no statistically significant difference from untreated control culture by ANOVA analysis). Thus, PAN-811 was demonstrated as a potential neuroprotective compound for anticancer drug MTX-, 5-FU-, or CDDP-induced neurotoxicity.

**3.3. PAN-811 Suppresses Cell Membrane Leakage When MTX and 5-FU Are Coadministered.** Since MTX and 5-FU are coadministered for cancer therapies in many cases, we were interested to know if PAN-811 can block neurotoxicity that is elicited with a combined insult with both MTX and 5-FU. An insult with a combined 100  $\mu\text{M}$  MTX and 25  $\mu\text{M}$  5-FU resulted in a 109% increase in LDH reading by comparison with noninsulted control group ( $P < 0.05$  by ANOVA; Figure 3(a)). PAN-811 showed concentration-dependent suppression of LDH release within the tested range from 1.25 to 10  $\mu\text{M}$ . PAN-811 at 10  $\mu\text{M}$  fully inhibited MTX/5-FU-elicited LDH increase.

**3.4. PAN-811 Inhibits MTX- and 5-FU-Elicited OS.** To understand the underlying mechanism for MTX- and 5-FU-induced neurotoxicity, a cell-permeable fluorogenic probe DHR123 was used for the detection of intramitochondrial ROS. Neuronal insult with coadministered 100  $\mu\text{M}$  MTX and 25  $\mu\text{M}$  5-FU greatly increased intensity of DHR123 fluorescence (Figure 3(b)), resulting in a 33.4% increase in DHR123 level in comparison with noninsulted group ( $P < 0.05$  by *t*-test, data not shown). PAN-811 at 10  $\mu\text{M}$  provided significant suppression to the increased ROS, showing a 62.3% suppression rate (Figure 3(c)).

**3.5. PAN-811 Shows No Antagonistic Effect on MTX-, 5-FU-, or CDDP-Induced Cytotoxicity in BNL3 Cells.** To understand whether PAN-811 could interfere with anticancer efficacy of tested anticancer drugs, the mouse liver cancer cell line BNL3 was cotreated with each anticancer drug at the concentrations used for elicitation of neurotoxicity and 10  $\mu\text{M}$  PAN-811, the highest concentration used for neuronal protection in these experiments.

A 3-day insult with 100  $\mu\text{M}$  MTX severely reduced the cancer cell number (Figure 4(a)). In the culture treated with 10  $\mu\text{M}$  PAN-811 alone or cotreated with 100  $\mu\text{M}$  MTX and 10  $\mu\text{M}$  PAN-811, cell density was also much lower than that in no-insult control. Quantitatively, MTX at 100  $\mu\text{M}$  reduced MTS reading by 85% (Figure 4(d)), while PAN-811 at 10  $\mu\text{M}$  reduced MTS reading to the same level as MTX. A cotreatment with both did not cause any further reduction in MTS reading when comparing with MTX alone.

Similarly, 5-FU at 25  $\mu\text{M}$  significantly reduced the cell density of the cancer cells, and a cotreatment with both 25  $\mu\text{M}$  5-FU and 10  $\mu\text{M}$  PAN-811 significantly decreased the cell number as well (Figure 4(b)). Quantitatively, 5-FU at 25  $\mu\text{M}$  reduced MTS reading by 84%, which was less efficient than 10  $\mu\text{M}$  PAN-811 group (Figure 4(e)). A cotreatment with both caused a further reduction in MTS reading when comparing with 5-FU alone. No synergistic effect between 5-FU and PAN-811 could be detected.

An insult with 3.5  $\mu\text{M}$  CDDP also caused a decrease in the cell density (Figure 4(c)), while a treatment with PAN-811 alone or a cotreatment with both 3.5  $\mu\text{M}$  CDDP and 10  $\mu\text{M}$  PAN-811 introduced a significant reduction in the cell density. Quantitatively, 3.5  $\mu\text{M}$  CDDP reduced MTS reading by 44%, while 10  $\mu\text{M}$  PAN-811 caused a 94% reduction in MTS reading (Figure 4(f)). A cotreatment with both did not introduce an extra reduction in MTS reading by comparing with PAN-811

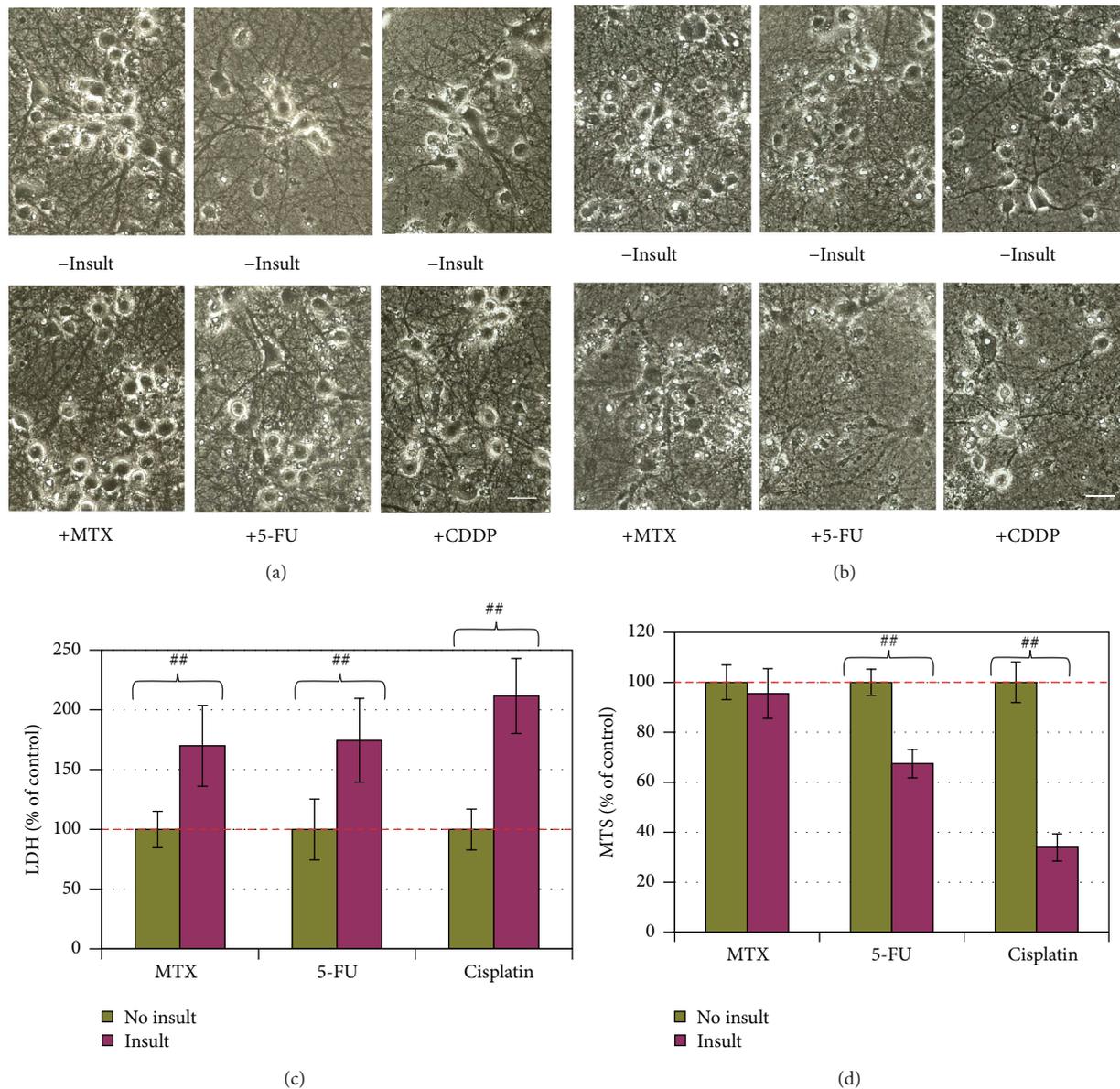


FIGURE 1: Neurotoxicity of MTX, 5-FU, or CDDP in an AO-dependent manner. (a, b) Phase contrast photographs for neurons in 25% AO and 12.5% AO, respectively (bar = 25  $\mu$ m). (c) Cell membrane leakage was determined via the LDH analysis at the end of experiment for neurons in 25% AO ( $n = 5$ ). (d) Cell viability was determined with MTS analysis for neurons in 12.5% AO ( $n = 5$ ). The bar in green and bars in other colors indicate the cultures without an insult and with anticancer drug insults, respectively. LDH and MTS data are expressed as % of noninsulted control. Figure symbol is ##,  $P < 0.01$ , compared with noninsult control group by Student's  $t$ -test.

alone, despite showing much lower reading than CDDP alone ( $P < 0.01$ ).

In general, PAN-811 did not show any inhibition in the effect of MTX, 5-FU, or CDDP on BNL3 cells, neither did it demonstrate any synergistic effect with each tested anticancer drug on BNL3 cell growth.

**3.6. PAN-811 Shows No Antagonistic Effect on MTX-, 5-FU-, or CDDP-Induced Cell Death of H460 Cells, While Demonstrating a Synergistic Effect with 5-FU or CDDP on Suppression of the Cell Growth.** To understand whether there is any negative effect of PAN-811 on the efficacy of tested

anticancer drugs in humans, the human lung cancer cell line H460 was treated with each of these anticancer drugs at the concentrations used for elicitation of neurotoxicity, in the absence or presence of 10  $\mu$ M PAN-811.

A 3-day insult with 100  $\mu$ M MTX, 10  $\mu$ M PAN-811, or both robustly decreased the cell density of H460 in culture (data not shown). Quantitatively, 100  $\mu$ M MTX and 10  $\mu$ M PAN-811 reduced MTS readings by 67% and 76%, respectively. The MTS reading for a cotreatment with both 100  $\mu$ M MTX and 10  $\mu$ M PAN-811 was about the same as 10  $\mu$ M PAN-811 alone (Figure 5(b)). In membrane integrity analysis (Figure 5(e)), 100  $\mu$ M MTX resulted in a 95% increase in LDH reading in

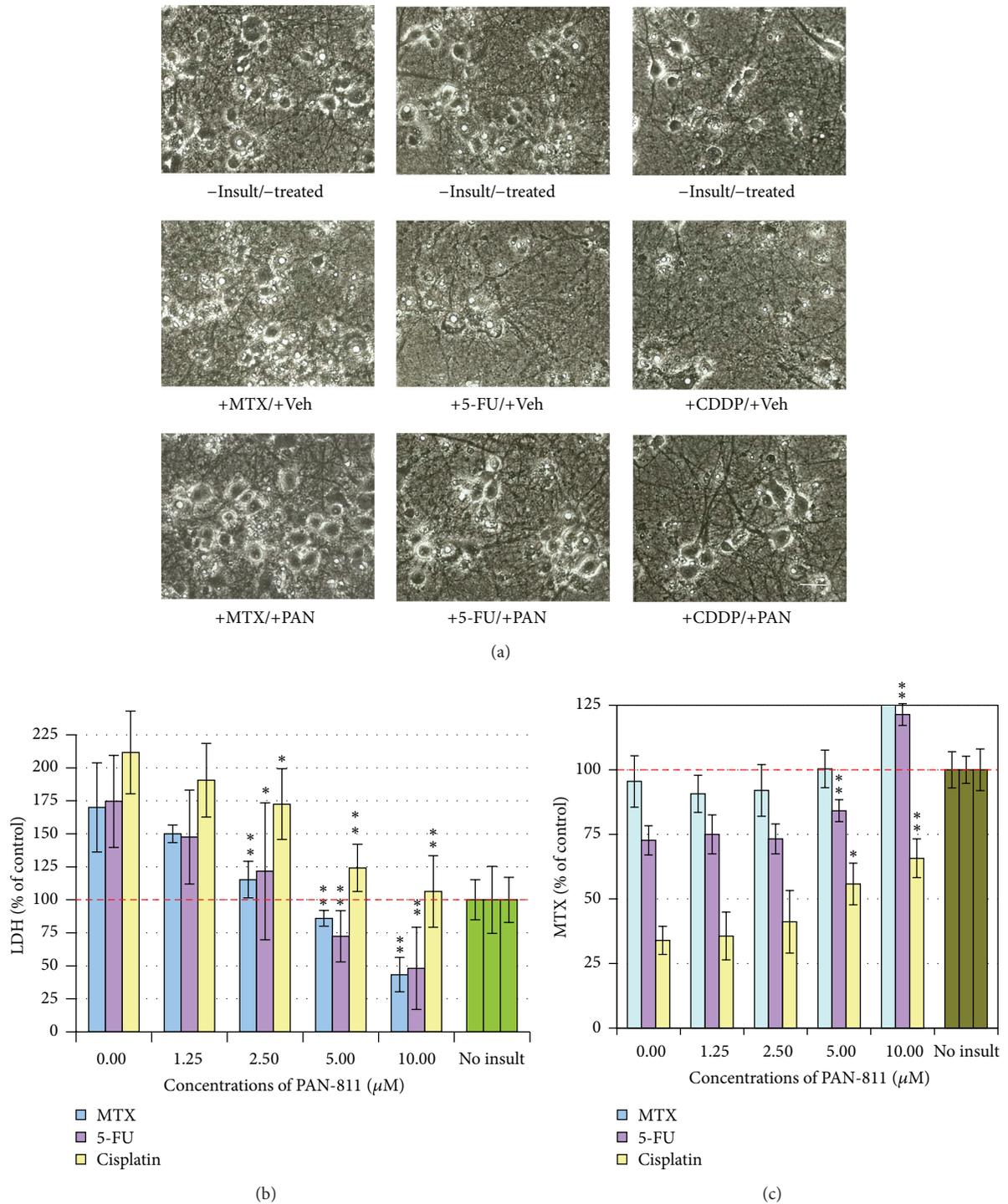


FIGURE 2: Dose-dependent neuroprotection of PAN-811-Cl·H<sub>2</sub>O against anticancer drug-induced neurotoxicity. (a) Phase contrast photographs for neurons in 12.5% AO (bar = 25  $\mu\text{m}$ ; PAN: PAN-811-Cl·H<sub>2</sub>O). (b) LDH analysis for (a) ( $n = 5$ ). (c) MTS analysis for (a) ( $n = 6$ ). The bar in green and bars in other colors in the graphs indicate the cultures without an insult and with anticancer drug insults, respectively. Data are expressed as % of noninsulted control. Figure symbols are \*,  $P < 0.05$ , and \*\*,  $P < 0.01$ , compared with insult group alone (without PAN-811 treatment) by one-factor ANOVA followed with Tukey HSD test.

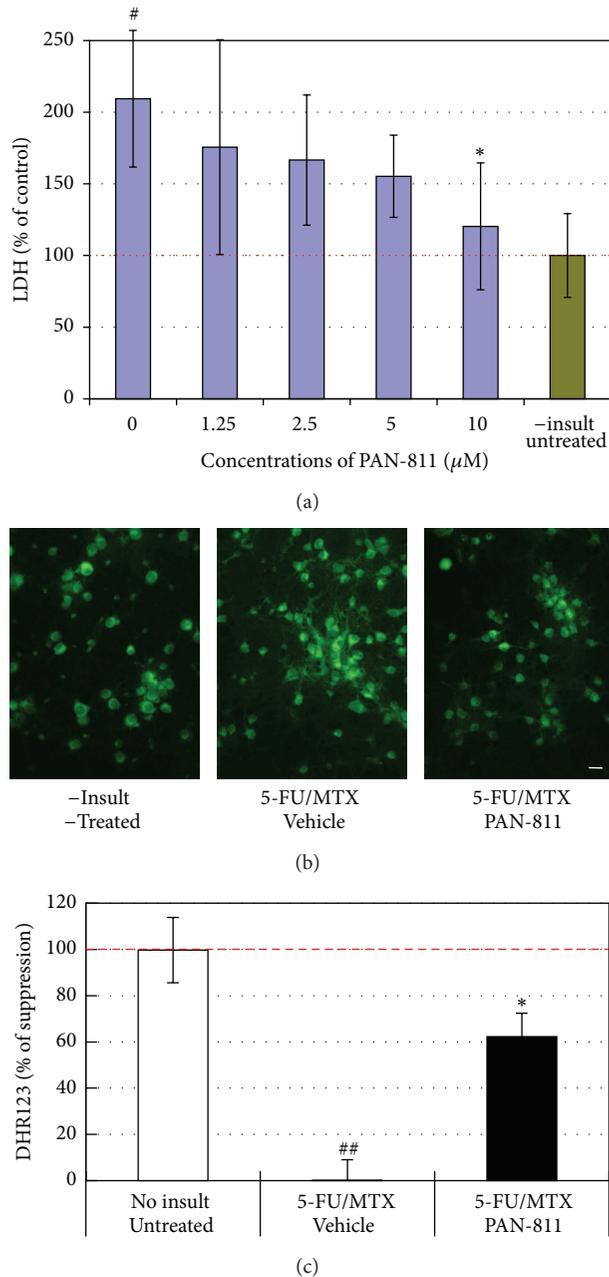


FIGURE 3: Suppression of 5-FU/MTX-induced increases in LDH and DHR123 readings by PAN-811. (a) LDH release analysis for neurons that were cultured in 17.5% AOs-containing medium and insulted with both 100  $\mu\text{M}$  MTX and 25  $\mu\text{M}$  5-FU in the absence or presence of PAN-811-Cl $\cdot$ H $_2$ O at different concentrations for 1 day (blue bars  $n = 6$ ); Green bar represents noninsult/untreated control. (b) Fluorescent microscope for neurons in 17.5% AOs-containing medium insulted with both 100  $\mu\text{M}$  MTX and 25  $\mu\text{M}$  5-FU in the absence or presence of 10  $\mu\text{M}$  PAN-811-Cl $\cdot$ H $_2$ O for 1 day and incubated with DHR123 for 30 min (bar = 50  $\mu\text{m}$ ). (c) Quantification of (b) at excitation at 485 nm and emission at 520 nm ( $n = 4$ ). Data are expressed as % suppression = [(Insulted&Untreated - Insulted&Treated)/(Insulted&Untreated - NonInsulted&Untreated) \* 100%]. Figure symbols are \*,  $P < 0.05$ , compared with insult group alone (without PAN-811 treatment) by Student's  $t$ -test (one tail) and one-factor ANOVA followed by Tukey HSD test; #,  $P < 0.05$ ; ##,  $P < 0.01$ , compared with noninsult/untreated control group by one-factor ANOVA followed with Tukey HSD test.

the culture supernatant, while 10  $\mu\text{M}$  PAN-811 led to a 31% increase in the LDH reading. A cotreatment with 100  $\mu\text{M}$  MTX and 10  $\mu\text{M}$  PAN-811 reduced LDH reading by 70% when compared with MTX group ( $P < 0.01$  by ANOVA), indicating an inhibitory effect of PAN-811 on MTX-caused membrane leakage.

Similarly, a 3-day treatment with 25  $\mu\text{M}$  5-FU, 10  $\mu\text{M}$  PAN-811, or both robustly decreased the cell density of H460 in culture (Figure 5(a)). Quantitatively, 25  $\mu\text{M}$  5-FU and 10  $\mu\text{M}$  PAN-811 reduced MTS readings by 57% and 74%, respectively (Figure 5(c)). In contrast, a cotreatment with 25  $\mu\text{M}$  5-FU and 10  $\mu\text{M}$  PAN-811 reduced MTS readings by

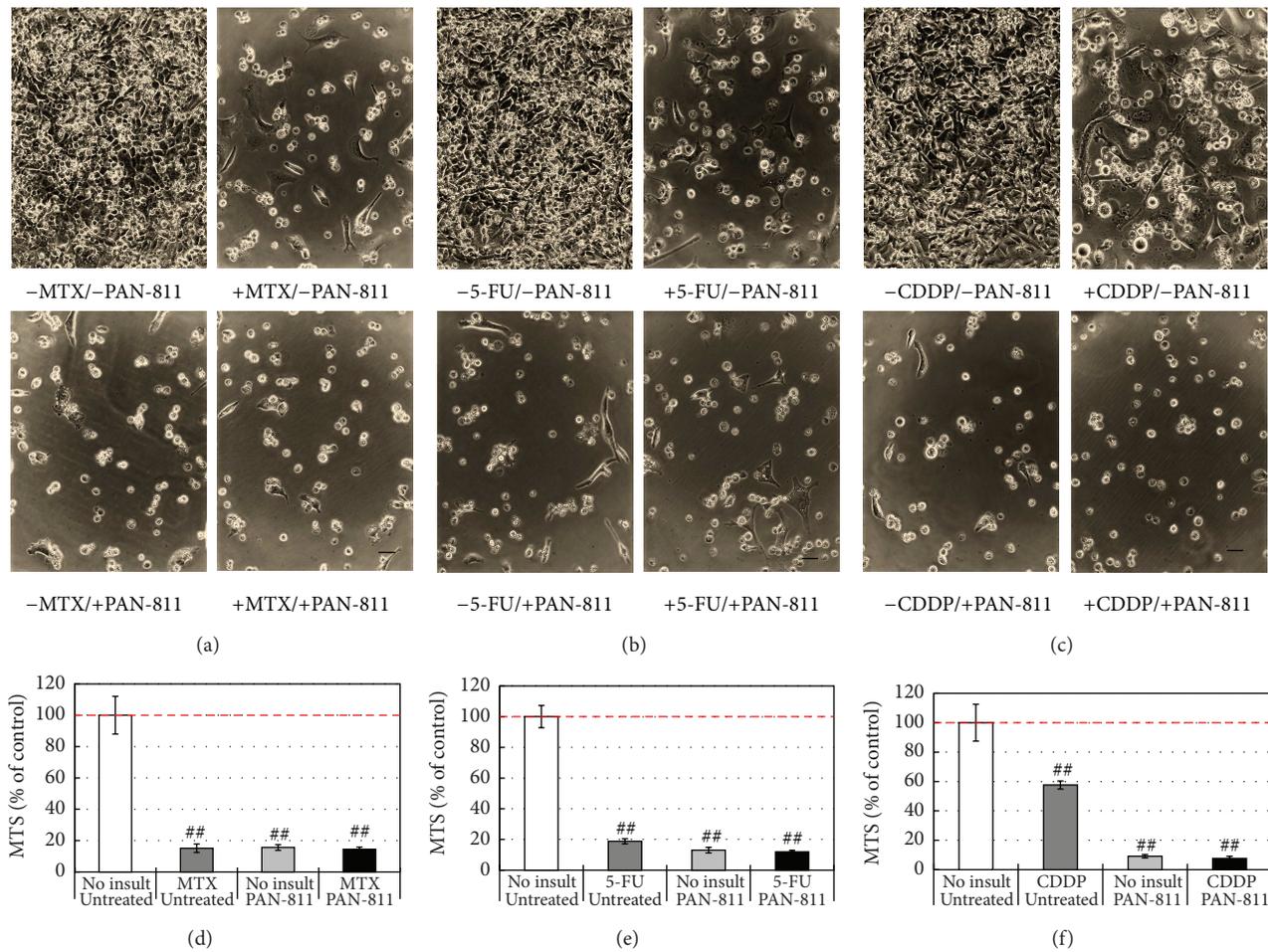


FIGURE 4: No interference of PAN-811-Cl-H<sub>2</sub>O with anticancer drug-induced cytotoxicity to mouse cancer cell line BNL3. (a)–(c) Phase contrast photographs for BNL3 cells that were treated without or with 10  $\mu$ M PAN-811-Cl-H<sub>2</sub>O and insulted with 100  $\mu$ M MTX, 25  $\mu$ M 5-FU, or 3.5  $\mu$ M CDDP for 3 days, respectively (bar = 50  $\mu$ m). (d)–(f) MTS analysis corresponding to (a)–(c) ( $n = 6$ ). Data are expressed as % of noninsulted/untreated control. Figure symbol is ##,  $P < 0.01$ , compared with noninsult/untreated control group by one-factor ANOVA followed with Tukey HSD test.

84%, which shows a statistically significant difference from 5-FU ( $P < 0.01$ ) or PAN-811 alone ( $P < 0.01$ ), indicating a synergistic effect of 5-FU and PAN-811 on suppression of growth of human lung cancer cell H460. In membrane integrity analysis (Figure 5(f)), 25  $\mu$ M 5-FU resulted in a 124% increase in LDH reading in the culture supernatant, while 10  $\mu$ M PAN-811 led to a 30% increase in the LDH reading. A cotreatment with 100  $\mu$ M 5-FU and 10  $\mu$ M PAN-811 enhanced LDH reading by 40%, which is much lower than that in the group with 25  $\mu$ M 5-FU alone. It indicates an inhibitory effect of PAN-811 on 5-FU-caused membrane leakage.

A 3-day treatment with 3.5  $\mu$ M CDDP, 10  $\mu$ M PAN-811, or both greatly decreased the cell density of H460 in culture (data not shown). Quantitatively, 3.5  $\mu$ M CDDP and 10  $\mu$ M PAN-811 reduced MTS readings by 22% and 75%, respectively (Figure 5(d)). A cotreatment with 3.5  $\mu$ M CDDP and 10  $\mu$ M PAN-811 reduced MTS readings by 85%, which shows a statistically significant difference from CDDP ( $P < 0.01$  by ANOVA) or PAN-811 alone ( $P < 0.01$  by  $t$ -test), indicating a synergistic effect of CDDP and PAN-811 on suppression

of growth of human lung cancer cell H460. In membrane integrity analysis (Figure 5(g)), 3.5  $\mu$ M CDDP resulted in a 71% increase in LDH reading in the culture supernatant, while 10  $\mu$ M PAN-811 led to a 30% increase in the LDH reading. A cotreatment with 3.5  $\mu$ M CDDP and 10  $\mu$ M PAN-811 enhanced LDH reading by 57%, which is a statistically significant difference from that in the group with 3.5  $\mu$ M CDDP alone ( $P < 0.01$ ), demonstrating an inhibitory effect of PAN-811 on CDDP-induced membrane leakage.

In general, PAN-811 did not show any inhibition in the effect of MTX, 5-FU, or CDDP on cell growth of H460 cells, although it manifested an inhibitory effect on MTX-, 5-FU- or CDDP-induced membrane leakage. A synergistic effect between 5-FU and PAN-811 or between CDDP and PAN-811 occurred on suppression of H460 cell survival.

#### 4. Discussion

The fate of neurons under an OS condition is dependent on the balance between production of ROS and strength of AO

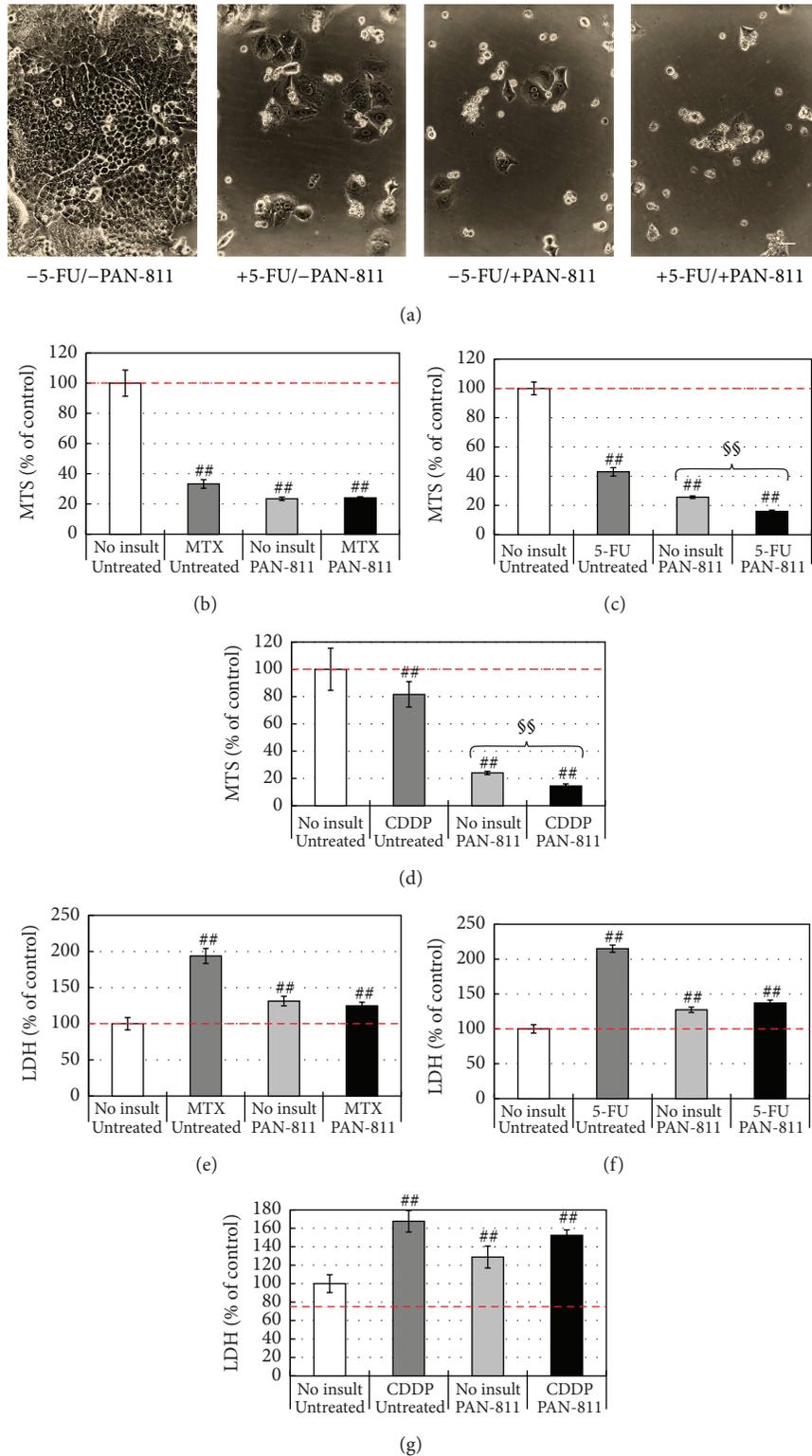


FIGURE 5: Effects of PAN-811-Cl·H<sub>2</sub>O on anticancer drug-induced cytotoxicities to human cancer cell H460. (a) Phase contrast photographs for the H460 cells that received 25  $\mu$ M 5-FU, 10  $\mu$ M PAN-811, or both for 3 days (bar = 50  $\mu$ m). (b)–(d) MTS analysis for the H460 cells that received 10  $\mu$ M PAN-811-Cl·H<sub>2</sub>O, one of 100  $\mu$ M MTX, 25  $\mu$ M 5-FU, and 3.5  $\mu$ M CDDP, or both 10  $\mu$ M PAN-811-Cl·H<sub>2</sub>O and one of these anticancer drugs for 3 days, respectively ( $n = 6$ ). (e)–(g) LDH analysis for (b)–(d) ( $n = 6$ ). Data are expressed as % of noninsulted/untreated control. Figure symbol is ##,  $P < 0.01$ , compared with noninsult/untreated control group by one-factor ANOVA followed with Tukey HSD test. §§,  $P < 0.01$  between no insult and insult groups given PAN-811 by Student's  $t$ -test.

defense systems *in vivo*. Enzymatic AOs, such as SOD and CAT, and nonenzymatic AOs, exemplified with vitamin E and GSH, are both involved in the defenses [23]. Loss of the balance, under condition such as chemotherapy, can elicit cytotoxicity and organ toxicity in experimental animals [24, 36] and in humans [9, 12, 13]. Administration of anticancer drugs is accompanied by not only an increase in ROS level, but also a decrease in antioxidative enzymes [37, 38]. In comparison with the *in vivo* studies, it is rare to find an *in vitro* study that examines the direct effects of anticancer drug on neurons in an enriched neuronal culture system. The presence of AOs in the culture medium may sufficiently block the effect of an anticancer drug and therefore the system is not suitable for examining ROS-mediated neurotoxicity and may not reflect the real conditions under chemotherapy in animals and humans. To mimic *in vivo* conditions under chemotherapy, we reduced AO concentrations in a double-diluted manner to a final AO concentration of 12.5%. It was observed that neurotoxicity of MTX, 5-FU, or CDDP occurred only when neurons were bathed in low AO-containing medium. Cell membranes seemed to be more fragile to these anticancer drugs under these conditions. When the AO content was reduced to 25%, MTX, 5-FU, and CDDP all resulted in robust LDH release, but neither notable morphological changes nor MTS reading differences in anticancer drug-insulted groups were detected. Only when the AO content was further reduced to 12.5% was there observation of morphological cell death in 5-FU- or CDDP-insulted groups and corresponding 33% and 66% reductions in MTS readings, respectively. These data, together with the phenomenon where coadministration of MTX and 5-FU resulted in a significant intramitochondrial ROS increase, indicate a key role of OS in mediation of the *in vitro* neurotoxicity.

Our study demonstrated that under low AO conditions MTX insult only resulted in membrane leakage but did not show significant detrimental effects on cell viability of neurons in our neuron-enriched culture. This is identical to the previous findings that excitatory neurotoxicity marks MTX-mediated cell death, which only occurs in the presence of glial cells, and is protected by N-methyl-D-aspartate receptor antagonists MK-801 and memantine [39].

PAN-811 can suppress neurotoxicity of all tested anticancer drugs in the present study. Under 12.5% AO condition, PAN-811 dose-dependently blocked MTX-, 5-FU-, or CDDP-induced membrane leakage. In addition, PAN-811 at 10  $\mu$ M fully inhibited 5-FU-induced MTS reduction and elevated MTS reading by 48% for CDDP-insulted neurons under 12.5% AO condition. Furthermore, neurons that were treated with PAN-811 looked to have a healthy appearance even when they were insulted with MTX, 5-FU, or CDDP. Our previous studies have demonstrated that, besides inhibiting excitatory neurotoxicity, PAN-811 can protect neurons from cell death under different OS-involved conditions, such as hypoxia [22], and hydrogen peroxide insult [14, 15]. In a cell-free and metal-free system, PAN-811 demonstrated an activity in direct scavenging of stable radical diphenylpicrylhydrazyl (DPPH) [22]. Taken together, the neuroprotection provided by PAN-811 in the anticancer drug-insulted condition is most

likely due to its activity in inhibition of intracellular ROS accumulation.

In this study, the blockage of oxidative damage by PAN-811 was shown by not only its neuroprotective effect but also its inhibitory role in anticancer drug-induced membrane leakage. Our results showed that each of MTX, 5-FU, and CDDP can induce membrane leakage of cancer cell H460. Theoretically, ROS can be produced in plasma membrane and other cell compartments [40]. Free radicals can pass freely through cellular and nuclear membranes and oxidize biomacromolecules, including lipids. Lipid peroxidation caused by ROS leads to membrane leakage [41]. Efficient inhibition of membrane leakage of H460 cells by PAN-811 indicates its role in suppression of ROS signal not only in neurons but also in other cell types.

Our result demonstrated that PAN-811 did not suppress anticancer efficacy of anticancer drugs MTX, 5-FU, and CDDP despite suppressing anticancer drug-induced membrane leakage. This indicates that the anticancer activity of the tested anticancer drugs does not rely on intramitochondrial ROS accumulation they induced, which provides a basis for using PAN-811 as a neuroprotectant in chemotherapy. In addition, PAN-811 manifested a synergistic effect with 5-FU or CDDP on suppression of cancer cell growth. Both MTX and 5-FU are antimetabolites or antifolate drugs. MTX inhibits DNA synthesis by competitively binding to dihydrofolate reductase, an enzyme that converts dihydrofolate into tetrahydrofolate [42]. 5-FU acts predominantly as a thymidylate synthase inhibitor and suppresses synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication [43]. CDDP is an alkylating agent. It binds to and causes cross-linking of DNA, which ultimately triggers apoptosis [44]. PAN-811 is an anticancer drug by itself with a different intracellular target from MTX, 5-FU, and CDDP. PAN-811 divalently chelates the ferrous ions of ribonucleotide reductase and blocks its bioactivity in conversion of ribonucleotides to deoxynucleotides and therefore inhibits DNA synthesis [45]. The synergistic effect by cotreatment with PAN-811 and 5-FU or CDDP may be due to affecting more than one intracellular target. The synergistic effect by cotreatment with PAN-811 may provide an opportunity in reduction of dose usage of 5-FU or CDDP. In this way, the neurotoxicity of 5-FU or CDDP could be further reduced while retaining equal strength of anticancer efficacy. PAN-811 can be a potential neuroprotective drug for chemotherapy-induced cognitive impairment due to its *in vitro* inhibition of MTX-, 5-FU-, or CDDP-induced neurotoxicity. A pharmacodynamic study for the effect of PAN-811 on cognitive functions will be carried out in a chemobrain animal model in near future.

## Conflict of Interests

Zhi-Gang Jiang, Steven A. Fuller, and Hossein A. Ghanbari are employees of and own stock in Panacea Pharmaceuticals, Inc.

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## Clinical Study

# Serum Oxidative Stress Markers and Genotoxic Profile Induced by Chemotherapy in Patients with Breast Cancer: A Pilot Study

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Received 29 November 2014; Revised 2 March 2015; Accepted 2 March 2015

Academic Editor: Amit Tyagi

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The aim of this study was to evaluate the oxidative parameters of erythrocytes and genotoxicity in leukocytes of patients with breast cancer. Oxidative parameters were detected by spectrophotometry and genotoxic damage by single cell gel electrophoresis. Twenty-eight women with breast cancer were monitored before chemotherapy and after the second and fourth cycles of therapy with cyclophosphamide and doxorubicin. After the fourth cycle, increases ( $P < 0.05$ ) in the reactive substances to thiobarbituric acid levels, nitrite content, and superoxide dismutase activity and high rates of DNA damage in leukocytes were observed when compared with healthy women group and baseline levels. Similarly, after the second cycle, the same parameters were increased ( $P < 0.05$ ) when compared with baseline levels. Increase in catalase activity was detected only after the fourth cycle and reduced glutathione levels and glutathione peroxidase activity were decreased in all cycles when compared with healthy women, as well as after the second and fourth chemotherapy cycles compared to baseline ( $P < 0.05$ ). Patients with breast cancer presented an indicative of oxidative stress before, during, and after chemotherapy, as well as increased genotoxic damage in all stages of treatment, demonstrating the clinical applicability of this investigation.

## 1. Introduction

The etiology of breast cancer possesses a multifactorial origin [1, 2], showing as risk factors reproductive age, early menarche, late menopause, nulliparity, exogenous hormones, smoking, obesity, diet, alcohol consumption, physical inactivity, and genetic and environmental factors [1–5].

Most chemotherapeutic agents are not specific against neoplastic cells, also affecting normal cells [6], which results in a wide range of adverse reactions in virtually all tissues of body such as bone marrow suppression, alopecia, fatigue, generalized rash, diarrhea, and dizziness [7, 8]. Cyclophosphamide, one of the most used anticancer compounds, is a bifunctional alkylating member of the nitrogen mustard

family that induces various types of DNA damage, such as DNA adducts, gene mutations, and chromosomal aberrations [6, 9]. In clinical and trials protocols, cyclophosphamide is used in combination with doxorubicin, an anthracycline agent capable of intercalating into DNA [10]. Their mechanism of cytotoxicity includes intracellular production of free radicals, DNA intercalation, and subsequent inhibition of DNA topoisomerase II [6, 9, 10].

Reactive oxygen species (ROS) represent important factor in carcinogenesis and may play a role in initiation and progression of tumors. Free radicals stimulate oxidative DNA damage, contributing to mutagenesis, which is essential for the process of tumor initiation [11–13]. Unrepaired DNA damage has been associated with a variety of human disorders including cancer and neurodegenerative diseases. When DNA is properly repaired, the injuries are inactivated and the cells return to normal cell cycle operation. If this damage is not repaired, specific cellular responses such as cell death, senescence, or uncontrolled proliferation could result. This damage may consist of small lesions in very specific sites within the DNA molecule, as adducts, cross-links, abasic sites, and points of gross abnormalities [14, 15]. The extent of this damage caused by ROS can be maximized or minimized by enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) or nonenzymatic (vitamins A, C, and E, selenium, and reduced glutathione (GSH)) [2, 16–18] mechanisms of antioxidant defense. Based on this approach, the present study evaluated the antioxidant and genotoxic profile in blood cells of patients receiving a combined chemotherapy of adriamycin (doxorubicin) and cyclophosphamide (AC).

## 2. Materials and Methods

**2.1. Study Population and Sample Collection.** The subjects were patients diagnosed with ductal breast cancer under treatment at the Department of Oncology, São Marcos Hospital, Teresina, Piauí, Brazil, from August 2012 to February 2013. This clinical study was approved by the Research Ethics Committee of University Center UNINOVAFAPI (registration number 0406.0.043.00011). This study involved a total of 56 individuals including 28 patients exposed to chemotherapy by the AC protocol (adriamycin 60 mg/m<sup>2</sup> and cyclophosphamide 600 mg/m<sup>2</sup>) and 28 patients not exposed to the chemotherapy. The patients were exposed to four 21-day cycles with intravenous AC. The unexposed group consisted of individuals who had not been exposed to genotoxic agents (including radiation and chemicals) and who were free of any malignant neoplasm or clinical, biochemical, hematological, hepatic, cardiovascular, renal, or endocrine manifestations. Blood samples were collected with EDTA or heparin by venipuncture using vacutainers, maintained at 4°C during transport to the laboratory, and immediately processed. Three collections of the peripheral blood were carried out during four cycles of chemotherapy: the first collection was performed before the beginning of treatment (C0), 21 days after the second cycle of chemotherapy (C2), and 21 days after the fourth cycle (C4).

All individuals in this study were submitted to a questionnaire from International Commission for Protection against Environmental Mutagens and Carcinogens [19], which included questions regarding standard demographic data (e.g., age and gender), medical issues (e.g., exposure to X-rays, vaccinations, and medications), lifestyle (e.g., smoking, coffee and alcohol consumption, diet, etc.) and occupation, such as number of working hours per day and protective measures adopted (PPE). In all groups, individuals who smoked more than 20 cigarettes per day were considered smokers [20].

After the questionnaires, data were analyzed using SPSS 17.0. (Chicago: SPSS Inc.) and the demographic, medical and lifestyle were summarized in Table 1.

Details about clinical features, such as cancer site, clinical stage and HER-2/neu, ER (estrogen receptor), and PR (progesterone receptor) *status*, were obtained and analyzed from medical records. The descriptive statistics for such variables are listed in Table 2.

**2.2. Comet Assay.** The alkaline comet (*single cell gel electrophoresis* (SCGE)) assay was performed as described by Singh et al. [21] with modifications suggested by Tice et al. [22]. Blood cells (5  $\mu$ L) were embedded in 95  $\mu$ L of 0.75% low-melting point agarose, which was immediately added to the surface of a precoated (1.5% agarose) microscope slide. When the agarose had solidified, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris; pH 10.0–10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulfoxide (DMSO) for a minimum of 1 day and a maximum of 7 days. After treatment with lysis buffer, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA; pH N 13) for 20 min and the DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA after which the buffer was neutralized with 0.4 M Tris (pH 7.5) and dried overnight. Gels were rehydrated for 5 min in distilled water and then stained for 15 min (37°C) with a solution containing the following sequence: 34 mL of Solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, and 5% w/v sodium carbonate) and 66 mL of Solution A (5% sodium carbonate). The staining was stopped with 1% acetic acid and the gels were air dried. Analyses (100 cells/patient) were carried out by light microscopy (Olympus CX40) at 100x magnification with immersion oil. Images of cells (50 cells/slide in two replicates) were evaluated for the following: (i) damage index (DI), in which each cell was classified into classes (no damage = 0, maximum damage = 4) according to tail size and cell shape [23], with resulting values for each individual ranging from 0 (0  $\times$  100) to 400 (4  $\times$  100); (ii) damage frequency (DF), calculated as the percentage of injured cells. International guidelines and recommendations for the comet assay consider the visual scoring of comets to be a well-validated evaluation method. Although the DI parameter is often subjective, it has high correlation with computer-based image analysis [22, 24, 25].

**2.3. Nitrite Content.** The determination of nitrite content was based on the Griess reaction [26] in which 500  $\mu$ L of Griess

TABLE 1: Demographic, medical, and lifestyle data of the patients.

Characteristics	Control group	Breast cancer
Total of patients	28 (100.0)	28 (100.0)
Age in years (mean $\pm$ SD)	48.1 $\pm$ 11.8	50.8 $\pm$ 12.8
Race [ <i>n</i> (%)]		
Caucasian	20 (71.4)	22 (78.6)
Non-Caucasian	8 (28.6)	6 (21.4)
Menopause [ <i>n</i> (%)]		
Premenopausal	19 (67.9)	20 (71.4)
Postmenopausal	9 (32.1)	8 (28.6)
Family history of breast cancer [ <i>n</i> (%)]		
Yes	6 (21.4)	15 (53.6)
No	22 (78.6)	13 (46.4)
Physical exercises [ <i>n</i> (%)]		
Yes	11 (39.3)	9 (32.1)
No	17 (60.7)	19 (67.9)
Smoker [ <i>n</i> (%)]		
Never smoked	20 (71.4)	12 (42.9)
Ex-smoker	8 (28.6)	13 (46.4)
Smoking	0 (0.0)	3 (10.7)
Marital status [ <i>n</i> (%)]		
Single	7 (25.0)	6 (21.4)
Married	16 (57.1)	15 (53.6)
Divorced	1 (3.6)	3 (10.7)
Widow	4 (14.3)	4 (14.3)

SD: standard deviation.

TABLE 2: Clinical characteristics of patients with breast ductal carcinoma (*n* = 28).

Characteristics	Breast cancer
Cancer sites [ <i>n</i> (%)]	
Left breast	11 (39.3)
Right mama	17 (60.7)
Clinical stage [ <i>n</i> (%)]	
Grade 1	4 (14.3)
Grade 2	10 (35.7)
Grade 3	14 (50.0)
Estrogen receptor [ <i>n</i> (%)]	
Negative	7 (25.0)
Positive	21 (75.0)
Progesterone receptor [ <i>n</i> (%)]	
Negative	7 (25.0)
Positive	21 (75.0)
HER2/neu [ <i>n</i> (%)]	
Score 0	9 (32.1)
Score +1	10 (35.7)
Score +2	1 (3.6)
Score +3	8 (28.3)

HER2/neu: human epidermal growth factor receptor 2.

reagent was added in white tube plus 500  $\mu$ L of distilled water (Blank) was added in white tube. In another test tube

500  $\mu$ L of Griess reagent and 500  $\mu$ L of the homogenate at 10% of the erythrocytes (sodium phosphate buffer 50 mM pH 7.4) (Test) were added. The spectrophotometric measurement was performed at 560 nm. Results were expressed in  $\mu$ M/mg protein.

**2.4. Thiobarbituric Acid Reactive Substances (TBARS) Levels.** Blood samples were centrifuged at 3000 rpm at 4°C during 5 minutes. Plasma was removed and a pellet of erythrocytes was washed with a cold solution of NaCl 0.9% and centrifuged. An erythrocytes' homogenate 10% diluted in phosphate buffer sodium 50 mM and pH 7.4 was stored at -20°C. Lipid peroxidation was measured by TBARS levels, a method previously described by Draper and Hadley (1990) [27, 28]. 250  $\mu$ L of homogenate, 1 mL of trichloroacetic acid 10%, and 1 mL of thiobarbituric acid 0.67% were mixed and stirred. Subsequently, this mixture was maintained in a bath of boiling water for 15 min and freshened under running water. After cooling, 2 mL of n-butanol was added and centrifuged at 1.200 rpm/5 min and the butanol phase was read spectrophotometrically at 535 nm. Results were expressed as nmol/mL.

**2.5. Reduced Glutathione (GSH) Levels.** Determination of GSH was based on the Ellman reaction (5,5'-dithiobis-2-nitrobenzoic acid) with some modifications described by Sedlak and Lindsay [29]. Four hundred microliters of erythrocytes' homogenate (EDTA pH 5.4 buffer) was mixed with

320  $\mu\text{L}$  of distilled water and 80  $\mu\text{L}$  of trichloroacetic acid 50%. After centrifugation at 3,000 rpm for 15 min, 400  $\mu\text{L}$  from the supernatant was collected and added to 800  $\mu\text{L}$  of Tris-HCl 0.4 M, pH 8.9, and 20  $\mu\text{L}$  of DTNB 0.01 M. One minute later, spectrophotometric measurement was performed at 412 nm. Concentration of GSH was expressed in mg/g of hemoglobin.

**2.6. Glutathione Peroxidase (GPx) Activity.** The glutathione peroxidase activity coupled assay was determined by Paglia and Valentine [30]. GPx catalyzes the reduction of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP<sup>+</sup> (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GPx is limiting, the decrease in absorbance at 340 nm is directly proportional to the GPx concentration. 1 unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by  $\text{H}_2\text{O}_2$ ) of 1.0  $\mu\text{mole}$  GSH to GSSG, per minute at 25°C, pH 7.0. Results were expressed in U/g of hemoglobin.

**2.7. Catalase (CAT) Activity.** Erythrocytes' homogenate in pH 7.4 was centrifuged (800 g, 20 min) and the supernatant was used to quantify catalase activity. The reaction medium was prepared with  $\text{H}_2\text{O}_2$  (18 mL), Tris HCl 1 M, EDTA pH 8.0 5 mM (1.0 mL), and  $\text{H}_2\text{O}$  (0.8 mL). The reading was carried out in a quartz cuvette at 230 nm with 980  $\mu\text{L}$  of reaction medium plus 20  $\mu\text{L}$  erythrocytes' homogenate prepared in sodium phosphate buffer 50 mM, pH 7.4 [31].

**2.8. Superoxide Dismutase (SOD) Activity.** Erythrocytes homogenate prepared in sodium phosphate buffer 50 mM, pH 7.4, was centrifuged (800 g, 20 min) and supernatants were used for testing superoxide dismutase (SOD) activity. Cytochrome *c* reduction rate was determined by superoxide radicals using the xanthine-xanthine oxidase system as a source of superoxide anion ( $\text{O}_2^-$ ) [32]. Results were expressed as U/mg protein. One unit (U) of SOD activity corresponds to the inhibition of 50% of  $\text{O}_2^-$  in the presence of cytochrome *c*.

**2.9. Statistical Analyses.** In order to determine statistical differences, data expressed as mean  $\pm$  standard error of the mean (S.E.M.) were compared by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test ( $P < 0.05$ ) using the Graphpad program (Intuitive Software for Science, San Diego, CA) and SPSS (version 19, SPSS Inc.). Correlations among data obtained were calculated using Spearman's correlation coefficient.

### 3. Results

**3.1. Evaluation of Oxidative Stress.** Evaluation of oxidative stress in patients with breast cancer in AC chemotherapy was performed by analyzing enzymatic and nonenzymatic parameters in erythrocytes by serum thiobarbituric acid reactive substances (TBARS) level, nitrite content, GSH concentration, and GPx, CAT, and SOD activities.

Results showed that the status of oxidative stress ( $P < 0.05$ ) increased, as demonstrated by basal TBARS ( $1.42 \pm 0.45$  nM/mg of protein) and nitrite ( $1.16 \pm 0.62$   $\mu\text{M}$ /mg protein) contents in erythrocytes of patients with breast cancer when compared with the control group ( $0.37 \pm 0.09$  nM/mg of protein and  $0.16 \pm 0.05$   $\mu\text{M}$ /mg of protein, resp.) ( $P < 0.05$ ). When these same patients were submitted to chemotherapeutics (combination of cyclophosphamide and doxorubicin), such increases in both TBARS ( $4.76 \pm 0.68$  and  $11.98 \pm 0.65$  nM/mg of protein) and nitrite ion levels ( $1.81 \pm 0.02$  and  $3.49 \pm 0.07$   $\mu\text{M}$ /mg of protein) were also detected in C2 and C4, respectively ( $P < 0.05$ ; Table 3).

Red blood cells of the patients revealed decrease in reduced glutathione concentration at 36.1% ( $24.94 \pm 1.51$  U/g protein) in comparison with control group ( $36.13 \pm 7.65$  U/g protein) ( $P < 0.05$ ). With AC chemotherapy, there was decrease in GSH levels in C2 (46.6%) and C4 groups (50.9%) ( $P < 0.05$ ). Similarly, baseline levels (C0) also presented diminution of 21.4% and 27.7% in C2 and C4 groups, respectively (Table 3).

GPx activity (Figure 1(a)) showed reduction (63.32, 78.31, and 81.0%) in erythrocytes of the patients with breast cancer activity in all groups analyzed ( $101.90 \pm 29.48$ ,  $60.25 \pm 4.66$ , and  $52.77 \pm 3.26$  U/g for C0, C2, and C4, resp.) when compared to the control group ( $277.8 \pm 15.88$  U/g), respectively ( $P < 0.05$ ).

In relation to the catalase levels, only treated patients (C4,  $22.83 \pm 1.17$   $\mu\text{M}$ /mg) exhibited a significant increase (44.3%) when compared to the control group ( $15.82 \pm 1.21$   $\mu\text{M}$ /mg), C0, and C2 ( $18.44 \pm 1.24$  and  $18.77 \pm 0.96$   $\mu\text{M}$ /mg, resp.) (Figure 1(b)). Similarly, superoxide dismutase activity (Figure 1(c)) also increased (33.1%) after the second cycle of chemotherapy ( $1.81 \pm 0.63$   $\mu\text{M}$ /mg) when compared with control group. After chemotherapy ( $2.63 \pm 0.65$   $\mu\text{M}$ /mg), its activity increased about 93.4 and 54.7% in relation to the control group ( $1.36 \pm 0.62$   $\mu\text{M}$ /mg) and baseline ( $1.70 \pm 0.43$   $\mu\text{M}$ /mg), respectively.

**3.2. Index and Frequency of DNA Damage.** DNA in the tail was organized into five classes: (i) class 0: undamaged, with no tail; (ii) class 1: with tail shorter than the diameter of the head (nucleus); (iii) class 2: with tail length between one and two times the diameter of the head; (iv) class 3: with tail longer than two times the diameter of the head; and (v) class 4: comets with no heads [33] (Figure 2).

With the application of alkaline comet assay it was possible to observe an increase ( $P < 0.05$ ) in the classes of DNA damage in lymphocytes of patients with breast cancer (C0) in the control group. This condition is increased ( $P < 0.05$ ) in C2 and C4 (Figure 3).

DNA damage in lymphocytes of patients with breast cancer increased by 122.6% ( $98.89 \pm 5.56$ ) compared to the control group ( $44.43 \pm 1.67$ ). After AC chemotherapy, there was an increase of 66.25 and 105.2% of damage index in C2 ( $164.4 \pm 6.36$ ) and C4 groups ( $202.9 \pm 5.34$ ) in comparison with C0 group ( $98.89 \pm 5.56$ ). In a similar way, an increase of 23.2% after the fourth cycle was noted when compared to the C2 group (Figure 4(a)).

An increase of 171.1% in frequency of DNA damage before chemotherapy (C0) ( $61.00 \pm 2.01$ ) was observed

TABLE 3: Biomarkers levels of oxidative stress in antioxidant enzymatic system of patients with breast cancer before (C0), during (C2), and after chemotherapy (C4) and control group.

Groups	TBARS levels (nM/mg de protein)	NO <sub>2</sub> <sup>-</sup> content (μM/mg protein)	GSH concentration (U/g protein)
Control	0.37 ± 0.09	0.16 ± 0.05	36.13 ± 7.65
C0	1.42 ± 0.45 <sup>a</sup>	1.16 ± 0.62 <sup>a</sup>	24.94 ± 1.51 <sup>a</sup>
C2	4.76 ± 0.68 <sup>a,b</sup>	1.81 ± 0.02 <sup>a,b</sup>	19.30 ± 0.74 <sup>a,b</sup>
C4	11.98 ± 0.65 <sup>a,b,c</sup>	3.49 ± 0.07 <sup>a,b,c</sup>	17.75 ± 0.46 <sup>a,b</sup>

TBARS: thiobarbituric acid reactive substances levels, NO<sub>2</sub><sup>-</sup>: nitrite content, and GSH: reduced glutathione concentration. Values represent mean ± S.E.M. <sup>a</sup>*P* < 0.05 when compared with control group (CG) by ANOVA followed by *t*-Student-Newman-Keuls. <sup>b</sup>*P* < 0.05 when compared with C0 group (before chemotherapy) and <sup>c</sup>*P* < 0.05 when compared with C2 group (second cycle of chemotherapy).

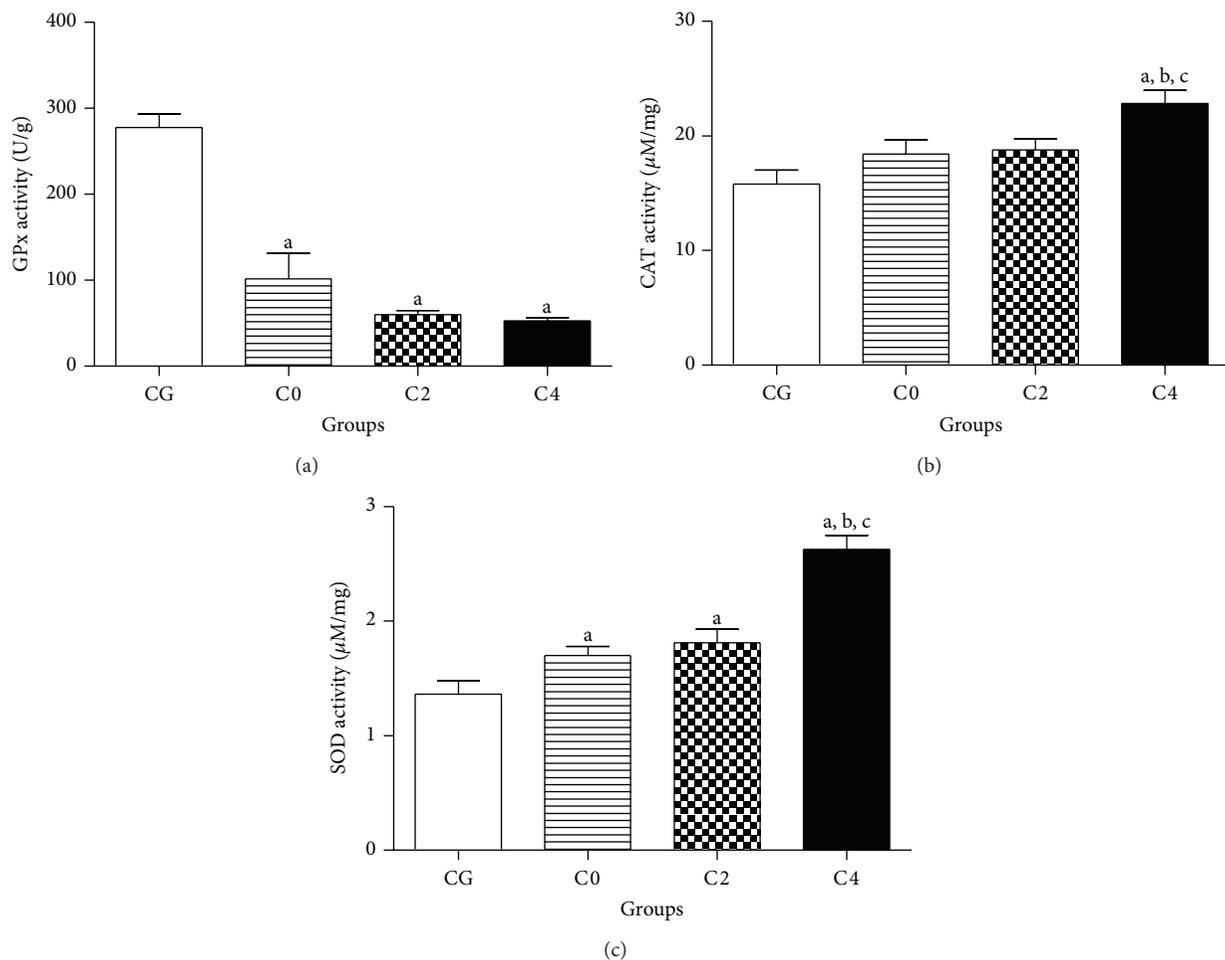


FIGURE 1: Antioxidant enzymes activity in erythrocytes of patients with breast cancer before (C0), during (C2), and after (C4) AC chemotherapy. Control group (CG) is represented by healthy patients. Values represent mean ± S.E.M. <sup>a</sup>*P* < 0.05 when compared with control group (CG) by ANOVA followed by *t*-Student-Newman-Keuls. <sup>b</sup>*P* < 0.05 when compared with C0 group (before chemotherapy) and <sup>c</sup>*P* < 0.05 when compared with C2 group (second cycle of chemotherapy).

when compared to the CG (22.50 ± 0.94) (Figure 4(b)). AC chemotherapy raised this frequency by 34.9 and 56.3% in C2 (82.32 ± 2.08) and C4 (95.36 ± 0.99) groups, respectively, in comparison with the base *status* (C0). Similarly, in C4 patients, an increase of 15.8% was observed in comparison with the frequency of C2.

There was no correlation between sperm risk factors, age, smoking, and family history with the disease, as well as the levels of oxidative stress assessed by measurements of enzymes catalase, superoxide dismutase and glutathione peroxidase and malondialdehyde levels, nitrite, and reduced glutathione (*P* > 0.05). However, a significant positive

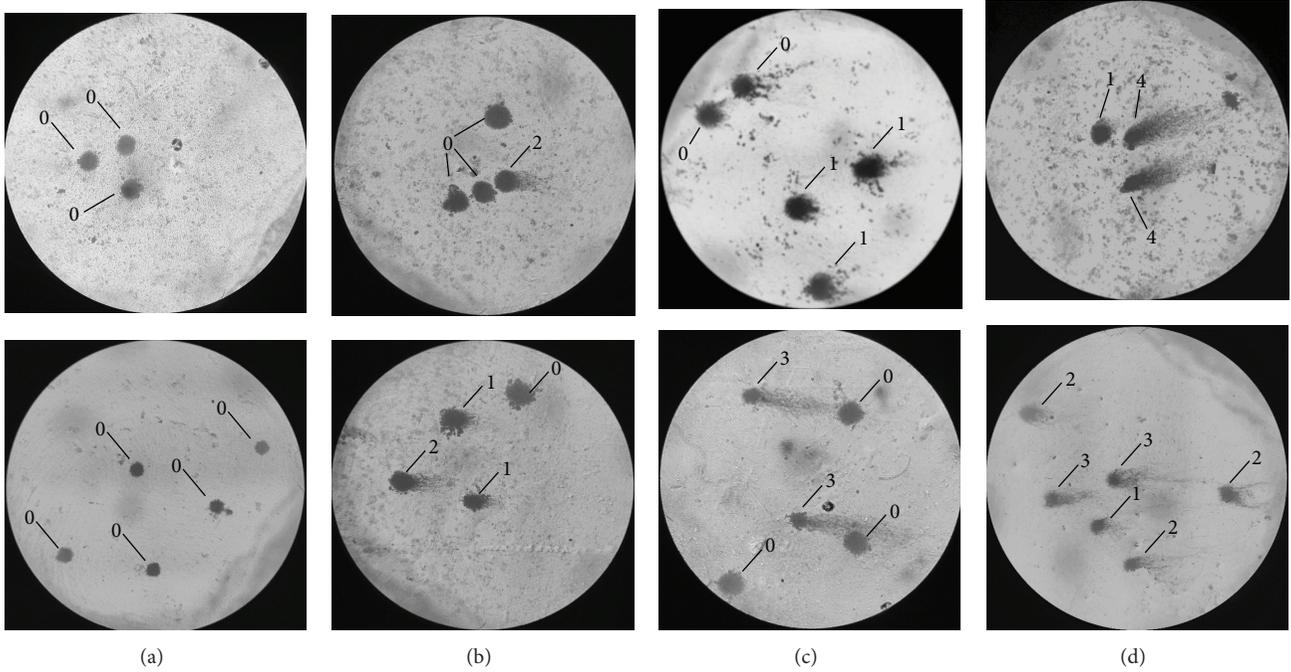


FIGURE 2: Photomicrograph of the comet test indicative of the types of damage. (a) Control group (CG); (b) beginning of treatment (C0); (c) 21 days after second cycle of chemotherapy (C2); (d) 21 days after the fourth cycle (C4).

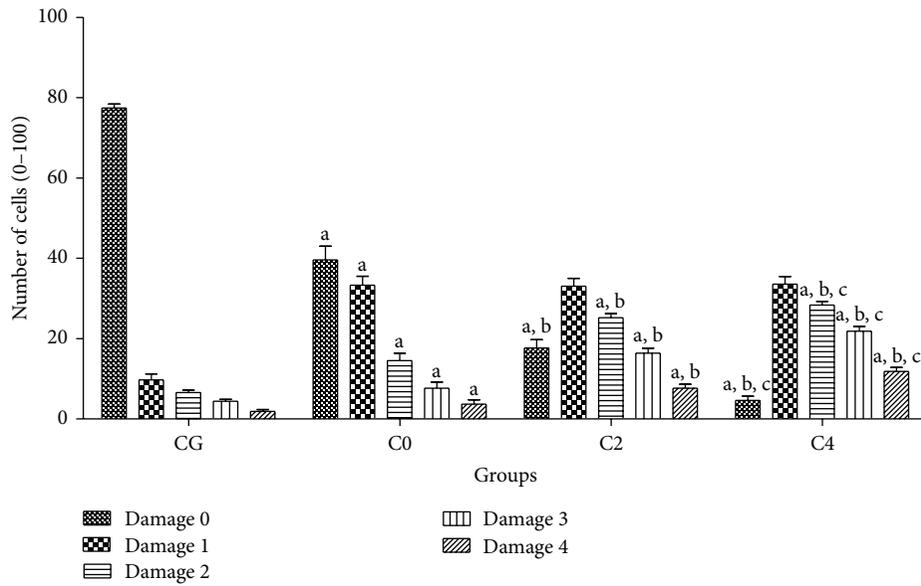


FIGURE 3: Profile of DNA damage in lymphocytes evaluated by the alkaline comet assay (single cell gel electrophoresis) for each phase of chemotherapy. Control group (CG) is represented by healthy patients. Values represent mean  $\pm$  S.E.M. <sup>a</sup> $P < 0.05$  when compared with control group (CG) by ANOVA followed by *t-Student-Newman-Keuls*. <sup>b</sup> $P < 0.05$  when compared with C0 group (before chemotherapy) and <sup>c</sup> $P < 0.05$  when compared with C2 group (second cycle of chemotherapy).

correlation (correlation factor = 0.389 and  $P = 0.041$ ) was observed between race and nitrite levels after chemotherapy (Q4) and a negative correlation was observed with nitrite levels (correlation factor =  $-0.474$ ,  $P = 0.011$ ) in the diagnosis and activity of superoxide dismutase (correlation factor =

$-0.389$ ,  $P = 0.041$ ) after chemotherapy (Q4) to marital status. There was also a negative correlation (correlation factor =  $-0.460$ ,  $P = 0.014$ ) between the practice of physical exercises and malondialdehyde levels during chemotherapy. No correlation was observed between ER and PR receptors

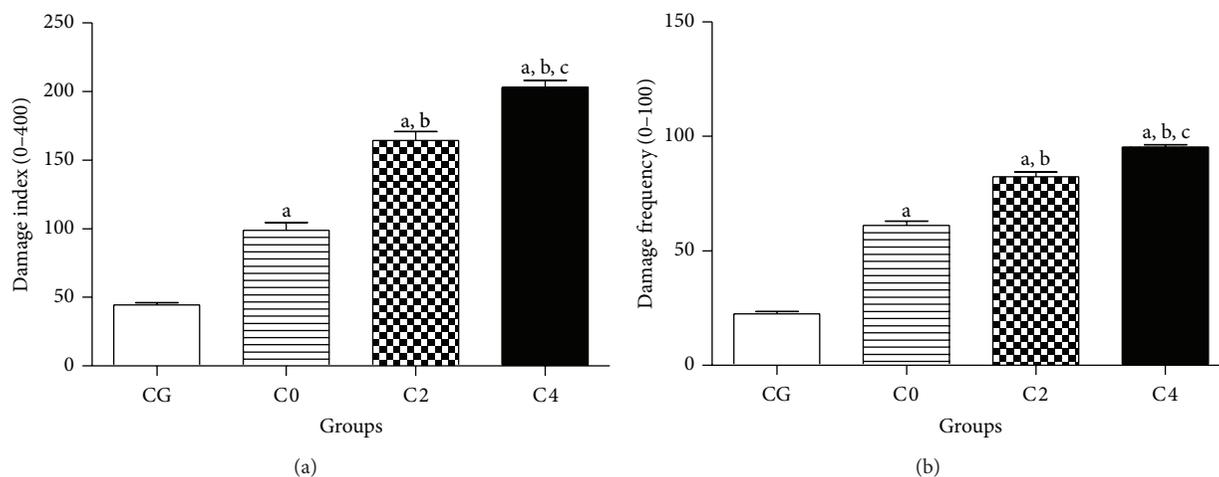


FIGURE 4: DNA damage investigation by the alkaline comet assay (single cell gel electrophoresis) carried out in lymphocytes of patients with breast cancer before (C0), during (C2), and after AC (C4) chemotherapy. Control group (CG) is represented by healthy patients. Analyses were performed by light microscopy at 100x magnification with immersion oil. Values represent mean  $\pm$  S.E.M. <sup>a</sup> $P < 0.05$  when compared with control group (CG) by ANOVA followed by *t-Student-Newman-Keuls*. <sup>b</sup> $P < 0.05$  when compared with C0 group (before chemotherapy) and <sup>c</sup> $P < 0.05$  when compared with C2 group (second cycle of chemotherapy).

with oxidative stress, except between HER2 and glutathione peroxidase in Q2 group with 0.412 correlation factor  $P = 0.29$ .

Regarding the genotoxicity and oxidative stress, positive correlations were observed for the contents of DNA damage assessed at diagnosis (QD) compared to those obtained during (Q2) and after (Q4) chemotherapy, with 0.663 correlation factor 0.537 and  $P = 0.000$  and 0.003, respectively. A negative correlation was observed between levels of DNA damage during chemotherapy (Q2) and nitrite levels (Q4), as well as between frequency of damage (QD) and nitrite in group Q4 and Q2 catalase group.

#### 4. Discussion

Breast cancer is the second most common cancer in women over the age of 50. It is often first detected as an abnormality on a mammogram before the patient or health care provider feels it. Early cases may be asymptomatic, and pain and discomfort are typically not present. Breast cancer can begin in different areas of the breast, such as ducts and lobules. Ductal carcinoma *in situ* (DCIS) is the most common noninvasive or preinvasive type with chances of a recurrence under 30% within 5–10 years after initial diagnosis. On the other hand, invasive ductal carcinoma (IDC), known as infiltrating ductal carcinoma, is the most common type of invasive breast cancer, representing around 80% of cases. About two-thirds of women are 55 or older when they are diagnosed with an IDC [34].

Many studies have reported that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the etiology and progression of various cancers [35–38]. These reactive species have been associated with the development of carcinogenesis by activating diverse types of DNA damage, contributing to the emergence of mutations

and chromosomal aberrations in the inflammatory process and leading to intense tissue disorganization and injuries [39].

An alternative method of analyzing oxidative stress is achieved by quantification of lipid peroxidation. The lipid radical is unstable and degrades very rapidly into secondary products. Most of them are electrophilic aldehydes, such as TBARS, which is the main marker of oxidative injury in the unsaturated lipids in cell membranes, leading to oxidation of fatty acids (LH) and formation of the lipid radical ( $L\bullet$ ) [40]. Therefore, TBARS is an important indicator of oxidative stress [16]. The present study demonstrated an elevation of TBARS levels in AC-treated breast cancer patients compared to controls, corroborating previous studies [1, 35] and suggesting severe lipid peroxidation. These changes may be attributed to the production of hydroxyl radicals, which participate directly in the lipid peroxidation process, inducing a disturbance in membrane structure [41].

The evaluation of nitrite concentration has been used as an index of endogenous NO production in biological systems in distinct pathological processes beyond its physiological properties such as vasodilation, neurotransmission, and immune response [3, 18]. It was noted that serum nitrite content determined by the Griess method increased in patients with breast cancer. Higher levels of nitrite and nitrate are related to inflammation caused by diseases and pharmacotherapies [39]. Interestingly, the results of present study showed that, even before the chemotherapy cycles, the disease itself induced  $\text{NO}_2^-$  generation and revealed an increase according to the treatment when compared to the baseline. Previous findings found analogous results, exhibiting increases in lipid oxidation activated by  $\text{NO}_2^-$  levels or nitric oxide [5, 37]. Nitric oxide has a dual role in tumor invasion and metastasis, inducing tumor growth or promoting tumoricidal activity [3]. Our results support the

hypothesis that breast cancers are associated with increased nitric oxide levels whose changes are linked to inflammatory process [38]. Prior analyses in 14 patients with breast carcinomas showed no elevation of serum TBARS. However, increased NO concentrations were detected [42].

The extent of oxidative damage depends not only on ROS levels, but also on mechanisms of cellular antioxidant defenses. Low level of GSH, a molecule of critical importance in maintaining the stability of erythrocytes membranes, is related to cellular defense against xenobiotics and harmful compounds such as free radicals and hydroperoxides [43]. This drop in GSH was also observed in erythrocytes of the patients. An additional reduction in GSH levels was observed in healthy patients and those under chemotherapy. Glutathione acts as the first line of defense against free radicals produced by antitumor molecules. Decreased GSH levels can be explained by a decrease in GSH synthesis and/or increased consumption to remove peroxides and xenobiotics [44].

Metabolites generated by CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) induced lipid peroxidation by inactivation of GSH levels and SOD, CAT, GPx, and GST activities in erythrocytes of patients with breast cancer, thereby rendering the system inefficient in management of the free radical attack. Acrolein and phosphoramidate mustard are the metabolites of cyclophosphamide that are among the causative agents, which reduce the activity of SOD, CAT, GPx, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase in erythrocytes of CMF treated breast cancer patients [45]. In the present study, GSH concentration and GPx activity were also observed just before AC chemotherapy. Our data demonstrate that GPx activity decreased, compared to the control group. However, this decrease was seen before the start of chemotherapy, suggesting no change in the activity of this enzyme for the therapeutic protocols used, since the reductions for during and after chemotherapy evaluation were similar to those observed prior to chemotherapy. Furthermore, these results suggest that the establishment of the pathophysiology of breast cancer may be a compromise in the activity of this enzyme. Present results and outcomes of Singh et al. [46] and Prabasheela et al. [47] also revealed, during chemotherapy FAC (5-fluorouracil, doxorubicin, and cyclophosphamide) or AC, a decrease in the nonenzymatic antioxidant GSH levels in patients with breast cancer before chemotherapy. On the other hand, additional studies did not find decrease in GPx activity before or after administration of chemotherapeutics [47, 48].

In relation to the CAT levels, our findings did not show differences before and during chemotherapy, presenting only increasing activity after treatment. On the other hand, while some studies found increases only after chemotherapy [48, 49], others observed decreases in CAT activity before and after chemotherapy [5, 40]. Since antioxidants can activate gene expression via the antioxidant response element [50], overexpression of enzymatic activities can explain these findings [40]. Similarly, SOD activity was elevated in patients with breast cancer before, during, and after chemotherapy. Hasan et al. [51] also showed plasma SOD activity increasing

in patients with breast carcinoma compared to patients with benign tumors, suggesting that elevated total SOD might reflect a response to oxidative stress and then may predict a state of excess reactive oxygen species in the carcinogenesis process. Analogous outcomes were described by Badid et al. [52] before chemotherapy in erythrocytes of 38 patients with ductal breast cancer. Nevertheless, Gupta et al. [53] found a decrease in SOD activity in serum from 30 women. Patients with breast cancer in chemotherapy with epirubicin (90 mg/m<sup>2</sup>) and cyclophosphamide (600 mg/m<sup>2</sup>) also showed reduced CAT, SOD, GSH, and GPx activity and increased TBARS levels [54]. Some of these parameters are contradictory when compared to the outcomes in the present study. These differences are probably explained by the fact that enzymatic activity of antioxidant defenses is more expressed at the cytoplasmic and mitochondrial cellular level, especially for SOD [46].

In this study, the genotoxic profile assay of the patients with breast cancer under treatment with AC was also investigated. This evaluation was carried out by alkaline comet assay, a well-established, simple, versatile, rapid, visual, and sensitive tool used to assess DNA damage and repair quantitatively as well as qualitatively in individual cell populations [55]. Some other forms of DNA damage such as DNA cross-links (e.g., thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the comet assay. This technique has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies, genotoxicity testing, and human biomonitoring [56]. Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution, and micronucleus assay, the advantages of the comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 10<sup>10</sup> Da of DNA) [57].

The pathological condition significantly raised the damage indices and frequencies in lymphocytes when compared with the normal control group, confirming previous investigations performed by Sánchez-Suárez et al. [6] and Agnoletto et al. [4]. These effects on DNA structure remained elevated up to 80 days after the end of exposure to FEC (5-fluorouracil, epirubicin, and cyclophosphamide) [6]. As seen in this work, Vaghef et al. [9] showed significant increase in DNA damage on lymphocytes of patients treated with cyclophosphamide.

In fact, antineoplastic agents are currently used in clinical studies which induce breaks in mammalian DNA strands as seen with topoisomerase I (camptothecin) and topoisomerase II (etoposide) inhibitors and 5-FU [58]. This, for example, is an antimetabolite widely used to treat breast adenocarcinoma and cancers of the gastrointestinal tract, head, and neck due to its inhibitory action on the enzyme thymidylate synthase, among other mechanisms, despite their *in vivo* clastogenic activity [59, 60]. Moreover, doxorubicin, beyond inhibiting topoisomerase II, also induces apoptosis and free radical formation [10, 45, 61]. These can cause DNA adducts, cross-links, double strand breaks, and single strand breaks. So, any biological reaction has potentiality to induce carcinogenesis [6, 62].

## 5. Conclusion

Patients with breast cancer under chemotherapy presented antioxidant status indicative of oxidative stress before, during, and after chemotherapy, as well as increasing genotoxic damage in all stages of the treatment. These results highlight the importance of monitoring patients in chemotherapy, especially using cytogenetic and molecular markers in order to provide new prognostic findings to the treatment as a strategy to reduce recurrences and to improve quality of life.

## Ethical Approval

This study was previously approved by Committee in Ethical Research at UNINOVAFAPI (N. 0406.0.043.00011) and is in accordance with Brazilian research guidelines (Law 466/2012, National Council of Health, Brazil) and with Declaration of Helsinki.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgments

The authors are grateful to the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo e Pesquisa do Estado do Piauí (FAPEPI) for financial support in the form of grants and fellowship awards.

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