Journal of Oncology

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Lead Guest Editor: Everly Conway de Macario
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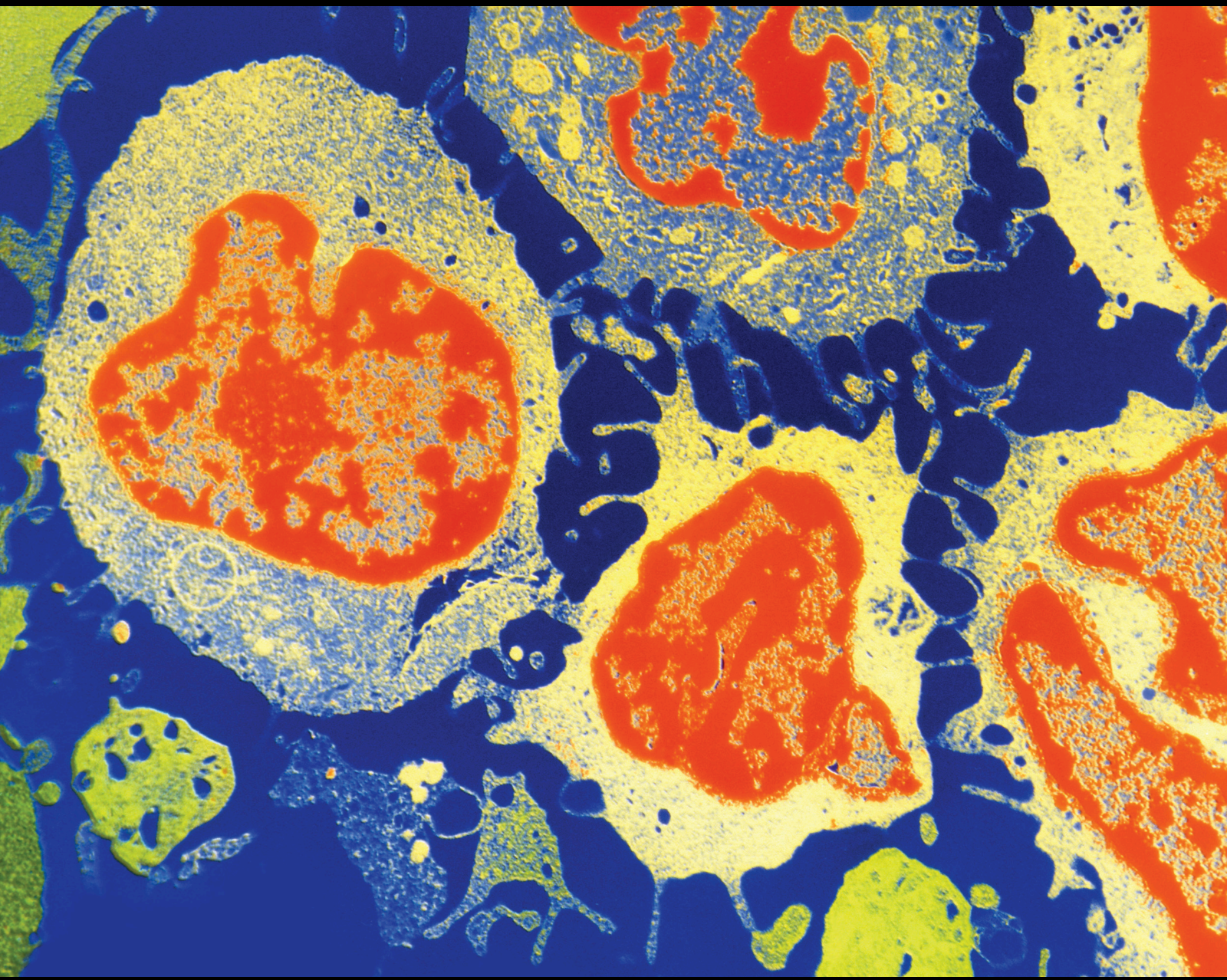

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Editorial

Role of Molecular Chaperones in Carcinogenesis: Mechanism, Diagnosis, and Treatment

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The role of molecular chaperones in carcinogenesis is a central theme of many current research efforts worldwide. It is pertinent to clarify that while various chaperones are Hsps, many are not, and vice versa not all Hsps are chaperones. An example of the latter is Hsp32, better known as heme oxygenase-1 (HO-1), which is dealt with in one of the articles in this Special Issue because of its probable involvement in certain types of carcinogenesis. Likewise, long noncoding RNAs are currently emerging as modulators of metastasization, and one contribution deals with this interesting new issue in Oncology.

While the involvement of chaperones in cancer progression has been extensively reported, the mechanism of their participation in carcinogenesis is largely unknown. Nevertheless, extant information is enough for considering these molecules as a promising biomarker for diagnosis and patient monitoring in some types of cancer and also promising to be used as therapeutic targets or agents. The aim of this Special Issue is to gather information about these themes.

Characterization of mechanisms underlying the role of molecular chaperones in cancer is an emerging issue in the oncology field. Molecular chaperones are involved in many biochemical pathways essential for tumor cell survival, and this makes them candidates for consideration as key players in the biochemistry of cancer. Therefore, the development of tools for diagnosis and treatment targeting chaperones is currently an active discipline within oncology.

Some malignant tumors can be classified as "chaperonopathies by mistake" because the molecular chaperones in the cancer cells contribute to their proliferation and mediate their resistance against antitumor defenses and facilitate metastasization. Chaperones are thus helping the "enemy" so to speak and are therefore "mistaken." These chaperones work for the tumor rather than to defend the host. Efforts must be directed toward finding ways to eliminate or block these "mistaken" chaperones. This strategy of negative chaperonotherapy is currently being incorporated to the battery of other approaches such as chemo- and immunotherapy to treat cancer.

In this Special Issue, various examples of tumors in which Hsp-chaperones and a Hsp that is not a chaperone play a noteworthy role are discussed along with the potential of chaperonotherapy.

The review by Das et al. highlights recent advances and perspectives in Hsp-based cancer immunotherapy. The importance of research on the role of Hsp-chaperones, specifically Hsp27, Hsp60, Hsp70, and Hsp90, in modulating carcinogenesis is discussed, emphasizing their critical role in determining the balance between protective and destructive

immunological responses within the tumor microenvironment; the possibilities involving Hsp27, Hsp70, and Hsp90 are clearly schematized in the first figure of the article, while pathways of presentation of tumor antigens by Hsps to antigen-presenting cells (APCs) are summarized in the second figure. Along this line of thought, Hsps are seen as an effective therapeutic option for some malignant tumors, including melanoma. Recent progress in this field is discussed, including anticancer vaccines, specifically those based on Hsp70 and Hsp90 are summarized in Table 1 of the article. These vaccines have been shown to be active against a spectrum of tumor antigens since they induce T-lymphocyte activation as well as stimulation of antigens uptake by APCs.

In the review by Fucarino et al., emphasis is given to the study of the molecular mechanisms in carcinogenesis that involve chaperones. Specifically, they focus on the chaperonin Hsp60 alone or in complex with Hsp10, and its implication in lung cancer, and also analyze the broad set of Hsp60 interactors, some of which are listed in Table 1 of the article. Noteworthy is the relationship of the fragile histidine triad (FHIT) protein, a tumor-suppressor factor, with Hsp60 and Hsp10 in complex, in mitochondria. FHIT, like many other mitochondrial proteins, depends for its correct folding on the Hsp60/Hsp10 chaperoning complex; it is, therefore, possible that a chaperonopathy affecting either one or the other of these two chaperonins will result in a decrease of tumor suppression, namely, will favor tumor growth. This is certainly a topic for future investigation because it may help find ways to apply positive chaperonotherapy to oppose tumor progression by administering Hsp60 and/or Hsp10 or boosting their activities, thereby reenforcing the chaperoning of FHIT protein correct folding, which will thus become an effective tumor suppressor. Continuous stress conditions in the respiratory mucosa, such as cigarette smoking, increase Hsp60 chaperonin levels outside mitochondria. High levels of this chaperonin are associated with tumor deterioration in some cancer types, but in other types, the reverse is seen, namely, tumor progression is favored probably due to inhibition of apoptosis and senescence in tumor cells by the chaperonin.

The paper by Wu et al. examines the effects of long noncoding RNA lnc-TLN2-4:1 in gastric cancer (GC). Forty-nine patients were recruited for this study who had not been submitted to chemotherapy before and were followed for four years. The authors demonstrate that lnc-TLN2-4:1 is decreased in GC tissue compared with matched normal tissue and is involved in poor overall survival rates of GC patients. Moreover, they show that lnc-TLN2-4:1 overexpression inhibits GC cell migration and invasion, but does not affect GC cell proliferation, suggesting its involvement as a tumor suppressor of GC metastases. In conclusion, Inc-TLN2-4:1 is a suppressor of metastasization and when it is under-regulated like in GC the survival rate of patients is poor.

The paper by Castruccio Castracani et al. presents data on the effect of estradiol E2 in human glioblastoma-multiforme (GBM) cells, focusing on proliferative capacity and mitochondrial functions. Expression of genes involved in mitochondrial biogenesis, oxidative phosphorylation, and dynamics was measured. Also, nuclear translocation of Nrf2 was assessed. The results showed that E2 increases the proliferation of glioblastoma cells and changes the expression of various genes among those investigated, e.g., those involved in oxidative phosphorylation. E2 also increased nuclear translocation of Nrf2 resulting in the induction of one of its target genes, hemeoxygenase-1, which is associated with the increase of both, chemoresistance and tumor-cell proliferation. These data show that E2 induces GBM proliferation and enhances its mitochondrial physiology, both of which certainly play a role in the well-known resistance of this tumor to all kinds of therapies, and open new avenues for developing novel treatment strategies.

In the paper by Kam et al., the authors report on the compound 2-methoxyestradiol as a potential anticancer agent, using as a model the A375 melanoma cells. They also studied the effect of polyphenol ferulic acid using the same cells. By applying MTT, flow cytometry, and Western blotting, the authors examined the molecular mechanisms underpinning the compounds' actions. These are partly related to the reduction of Hsp60 and Hsp90 levels and the induction of nitric oxide in A375 melanoma cells. The authors did not observe any changes in Hsp70 levels after 2-methoxyestradiol and ferulic acid treatment separately or in combination. This information is pertinent to evaluate chemoresistance mechanisms since Hsp70 accumulation reduces induction of cancer-cell death and decreases the antitumor efficacy of some therapeutic agents.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this Special Issue.

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Review Article

Role of HSP60/HSP10 in Lung Cancer: Simple Biomarkers or Leading Actors?

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Cancers are one of the major challenges faced by modern medicine both because of their impact in terms of the amount of cases and of the ineffectiveness of therapies used today. A concrete support to the fight against them can be found in the analysis and understanding of the molecular mechanisms involving molecular chaperones. In particular, HSP60 and HSP10 seem to play an important role in carcinogenesis, supporting tumours in their proliferation, survival, and metastasis. Efforts must be directed toward finding ways to eliminate or block this "mistaken" chaperone. Therefore, the scientific community must develop therapeutic strategies that consider HSP60 and HSP10 as the possible target of an anti-tumoural treatment and not only as diagnostic biomarkers, since they contribute to the evolution of pre-cancerous respiratory pathologies in lung tumours. HSP60 acts at the mitochondrial, cytoplasmic, and extracellular levels in the development of cancer pathologies. The molecular mechanisms in which these chaperones are involved concern cell survival, the restoration of a condition of absence of replicative senescence, the promotion of pro-inflammatory environments, and an increase in the ability to form metastases. In this review, we will also present examples of interactions between HSP60 and HSP10 and different molecules and ways to exploit this knowledge in anticancer therapies for lung tumours. In order to improve not only chances for an earlier diagnosis but also treatments for patients suffering from this type of disease, chaperones must be considered as key agents in carcinogenesis and primary targets in therapeutics.

1. Introduction

Lung cancer incidence has been increasing in the last years, in both developing and developed countries. It is one of the main causes of death worldwide, and it has become a very frequent malignant tumour for mankind. Although there are several possible ways to treat lung cancer (chemotherapy, radiotherapy, surgery, etc.), the patient survival rate at 5 years is 15% [1]. The survival rate increases when patients are subjected to surgical treatment earlier, but only a small proportion of subjects who have been diagnosed with lung cancer can undergo this procedure [2]. Therefore, it is necessary to optimize diagnostic procedures and to understand the molecular mechanisms of metastasization to reduce the mortality of this pathology. Understanding the molecular mechanism and signalling pathways in lung cancer is also of fundamental importance for the creation of new therapeutic strategies that can assist surgical treatment. Although the number of possible molecular biomarkers is high, the scientific community pays increasing attention to the possible involvement of heat shock proteins in the establishment of lung cancer and its pathological progression. Heat shock proteins (HSPs) are a group of highly conserved proteins that help protect cells from various type of stress (heat, cold, and abnormal levels of glucose or oxygen). They help the correct folding of many proteins and protect cells from deleterious consequences as protein misfolding, premature degradation, or aggregation [3–6]. HSPs normally support other protein functions in normal cells, but they may be present at high levels in cancer cells. This

deregulation in the levels of HSPs produced in cancer cells alone could be the cause of metastatic progression, not only in lung tumours but, more generally, in various types of carcinoma [7]. Tumours usually appear as a result of several factors, and, as mentioned above, HSPs should be considered among the genes involved in their progression. This means that some tumours can be considered chaperonopathies. In particular, regarding lung tumours, the scientific evidence of a possible role of HSPs in molecular pathways keeps growing. HSPs localization occurs in various subcellular compartments such as mitochondria, endoplasmic reticulum, microvesicles, and even the nucleus [8]. They can be released out of the cells through different ways (via Golgi or inside extracellular vesicles, such as exosomes), acting as cross cellular messengers. Both a paracrine effect in the proximity of the releasing cell and an endocrine effect through the blood stream are to be considered as possible effector pathways [9]. A massive production of HSPs by neoplastic cells leads this class of proteins to favour the tumour at the expense of the individual [10]. In fact, "protumour" HSPs support cancer cells in different processes, such as their proliferation, growth, and resistance to chemotherapy and radiotherapy treatments, and favour their metastasization [9, 11]. Therefore, the study and development of chaperonotherapy models is of fundamental importance if contextualized within a treatment that already includes classical approaches such as chemo-, radio-, and immunotherapy in order to arrest the progression of tumoural pathology. In addition to the possibility of using HSPs as therapeutic targets in the fight against lung tumours, this protein class appears to be an excellent candidate for predicting disease onset [12–14]. This is of the utmost importance, considering what has been previously stated on the benefits of an early diagnosis of lung tumour.

2. HSP 60

The heat shock protein 60 or HSP60 is a protein, weighing of 60 kDa, belonging to the chaperone family. It is used by the cell for the correct folding of other proteins [15]. HSP60 is a highly conserved protein that is present in many species of living organisms [16]. In addition to its main biological function, this class of proteins is the subject of a growing study regarding tumour progression [17, 18]. In different tumour types, the levels of HSPs are altered; therefore, their variation from standard values could be associated to those changes occurring during the processes of carcinogenesis [19].

Under normal conditions, HSP60 and its biological partner (co-chaperonin) HSP10 are two molecular chaperones with mitochondrial localization that, like the other proteins of their family, protect cells from different types of stress, closely related to mitochondrial integrity [20]. HSP60 and HSP10 form a folding cage through their rings and produce large and efficient protein-folding machinery that facilitates proper folding and assembling of mitochondrial imported proteins and corrects misfolded polypeptides [21]. HSP60 assembles into an oligomer with a precise quaternary structure to perform its characteristic role of chaperone. In addition, participation in the process of seven HSP10 subunits and ATP hydrolysis is necessary, with a final assembling of a bell-shaped form [22, 23]. In this review, we are going to illustrate the link between the alterations of HSP60 and HSP10 levels or localization and the occurrence of lung tumours, a relationship which has already been found in other carcinogenic processes such as colorectal, pancreatic, tongue, urinary bladder, prostatic, vesical, and exocervical tumours. It is curious how in some of these tumour forms the levels of HSP60 are increased, while, in others, the expression of the chaperone is reduced [24–44]. This alternation of conflicting results is also maintained when we observe the relationship between HSP60 and the prognosis of several tumours: an apparently anomalous behaviour that can be explained by the dual pro- and anti-apoptotic activity of an over-expression of HSP60. Another property linked to excess of HSP60 is the loss of replicative senescence of tumour cells [45, 46]. Similarly, an increase in synthesis levels of HSP10 is associated with different tumour types. Prostate cancer, exocervical cancer, large bowel cancer, and serous ovarian cancer are all cases in which this condition is present. On the contrary, as with HSP60, it is possible to find examples of tumours in which the levels of HSP10 are reduced, an example being lung cancer [47–51].

Another important factor in the functioning of HSP60 is its location within the cellular compartment [52]. As mentioned, normally, the HSP60/HSP10 complex finds its natural localization within the mitochondrial compartment, while in tumour cells it is not unusual to find HSP60 at the level of the cytoplasm. HSP60 has also been localized in lipid rafts that are rich in cholesterol and glycosphingolipids [26, 53, 54]. Even HSP10 that is normally localized inside the mitochondrial matrix can be found in other localizations, such as microvesicles or cell cytoplasm [55]. It is curious how high extracellular levels of HSP10 can be found during pregnancy. In fact, this is referred to as early pregnancy factor, and there are studies that have shown its importance in cell proliferation and differentiation [56, 57].

As mentioned above, when the chaperonins take the extracellular pathway, they can influence different cytotypes, in particular, HSP60 has an immunomodulatory effect on cells such as macrophages [58, 59] and neutrophils [60, 61]. An increased number of neutrophils and macrophages is a characteristic key to a pathology that usually evolves into lung tumour pathology: the chronic obstructive pulmonary disease (COPD). COPD is a pathology that owes its development mainly to cigarette smoking, and patients affected by this condition are characterized by a progressive and irreversible loss of their lung function. Several epidemiologic studies have showed that, in smokers with COPD, the incidence of lung cancer is five times higher than in smokers without COPD. At the same time, there is a greater ease of onset of lung cancer in patients with a severe level of airway obstruction. The ability of extracellularly released HSPs to modulate the secretions of proinflammatory cytokines probably plays a key role in the progression of COPD itself and in its potential evolution to a carcinogenic level [61–64].

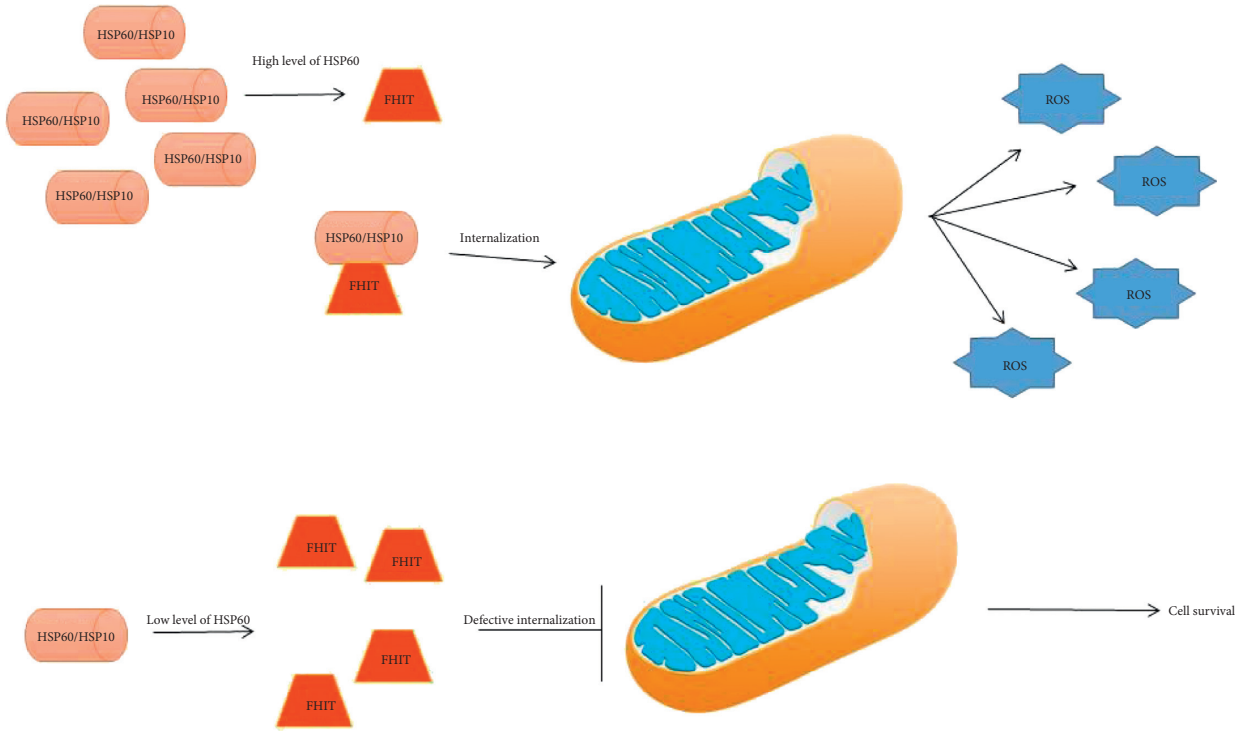

Figure 1: The tumor-suppressor FHIT protein interacts with HSP60, and possibly is folded by the chaperoning complex HSP60/HSP10 inside mitochondria. Therefore, any quantitative or qualitative defect of HSP60 (chaperonopathy by defect) may decrease the level of functional FHIT protein, causing a failure of tumor suppression and favoring carcinogenesis.

3. Lung Cancer: HSP60 and HSP10 Molecular Interactions

Analysing the broad set of proteins with which HSP60 and HSP10 interact, there are several of them that can play a role in the development and progression of lung cancer. However, there are further interactions that may have a protective effect in counteracting the onset of tumours.

3.1. Fragile Histidine Triad (FHIT) Protein. Fragile histidine triad protein (FHIT), also known as bis (5′-adenosyl)-triphosphatase, is a member of the histidine triad gene family involved in purine metabolism. FHIT is characterized by an unusual genomic fragility. As a consequence of this condition, its expression is reduced, if not absent, in many cancers. Although the precise function of FHIT is still not fully understood, it is clear that this protein acts as a tumour suppressor. The interaction between FHIT and the molecular chaperone complex HSP60/HSP10 has been demonstrated, and it is probably fundamental for the entry of FHIT into the mitochondria, where it plays an important role in electron transportation. FHIT has been shown to have considerable relevance in lung cancer. Studies conducted in vitro on tumour cells have shown that its expression, associated with a condition of cellular stress, leading tumour cells to trigger apoptotic processes. Furthermore, reduced expression levels, mainly due to genomic damage, have been found in hyperplastic lung lesions, but also in precancerous conditions in those smokers who maintain an apparently normal bronchial epithelium. The entry at the mitochondrial level of FHIT, made possible by the interaction with HSP60/HSP10, is crucial for the antitumour activity of FHIT itself. In fact, through the interaction with Fdxr, at 54-k flavoprotein, it is able to trigger the apoptotic process generating reactive oxygen species (ROS) via p53. A reduced expression of HSP60/HSP10 is therefore linked with a defective mitochondrial internalization of FHIT with consequent loss of apoptotic function by ROS. Otherwise, recent studies have brought to light a new possibility: exploiting the importance of oxidative phosphorylation for a particular cell population, cancer stem cells. Lung tumour cells are sensitive to treatments based on selective inhibitors of oxidative phosphorylation that act on the mitochondrial complex III, while these are ineffective when FHIT is present. Therefore, an assessment of HSP60/HSP10 levels (in association with the expression of FHIT) appears fundamental in choosing the kind of therapy to be used against lung cancer [65–69] (Figure 1).

3.2. Toll-Like Receptor (TLR). As previously stated, the different levels of expression (high or low) of HSP60 present a dichotomy when compared to precancerous conditions, such as COPD, or lung tumours. Human bronchial epithelial cells subjected to a high level of oxidative stress (such as cigarette smoke) increase the release of HSP60 in extracellular compartment. Once HSP60 is released outside the cell, it can bind different receptors present on immune cells, usually promoting an inflammatory state. Among the HSP60

target molecules are toll-like receptors, in particular TLR-4 and TLR-2. Specifically, the expression of TLR-4 is directly associated with the epithelial tissue, allowing it to feed the inflammatory condition via cytokine release. Among the possible ways of activation, the one via MyD88 appears to be the most likely since high levels of this TLR adapter have been found in association with the extracellular release of HSP60. In close association with TLR activation, an increase in extracellular HSP60 levels leads to an upregulation of CREB1 (a nuclear transcription factor) which results in a massive production of IL-8, thus creating the ideal conditions for a proinflammatory environment [62, 70–74]. A condition of this type, often closely associated with continuous external insults such as cigarette smoke, can easily develop a preneoplastic state in lung tumours.

3.3. p53/HSP60 Complex. Acting against the inactivation of the replicative senescence is one of the possible strategies to use in the fight against cancerous pathologies. Factors such as oxidative stress, telomere shortening, and DNA damage can trigger a state of replicative senescence that blocks the normal cell cycle. Cancer cells have lost this inhibition to cell proliferation, and the possible restoration of a replicative senescence would guarantee a greater success rate in anticancer treatments. Several studies have demonstrated how important is HSP60 in the loss of replicative senescence in many different tumours. In fact, a reduction of these proteins is associated with the appearance of senescence features and a reduction/arrest of tumour-cell expansion. It is possible to explain this function of chaperonins, if we consider how they are not implicated exclusively in protein folding, but focusing on their antiapoptotic role. Among the proteins with which HSP60 forms complexes, one of the most relevant is certainly p53. The formation of an HSP/p53 complex leads to a reduction in the interaction of p53 itself with the promoters of cell cycle arrest genes, thus preventing the onset of a state of replicative senescence in tumour cells. A further therapeutic strategy could therefore be to modify the interaction domains between HSP60 and p53. Previous studies on mucoepidermoid cell lines in human lungs have already shown the benefits of a doxorubicin treatment that promotes HSP60 acetylation with a consequent reduction of its levels and ability to form a stable complex with p53, leading to a restoration of replicative senescence [29, 46, 75–78].

3.4. SAHA (Suberoylanilide Hydroxamic Acid). The effects of Suberoylanilide hydroxamic acid (SAHA) as an anti-tumour molecule are already known. It is a member of the histone deacetylase inhibitor family (HDACi), and as the name suggests, this class of compounds is involved in the acetylation of histones resulting in a modification of the expression of the chromatin and the consequent transcriptome. SAHA and similar compounds also acetylate other proteins, among these, one of the targets is HSP60. Previous work had already shown that SAHA was able to acetylate other chaperonins such as HSP90 and HSP70. This does not happen, however, for HSP60: in fact, it has been revealed that, in this chaperonin, the induced posttranslational modification is a nitration at the level of the tyrosines 222 and 226, two amino acids present on the apical domain of HSP60. A study conducted on a cell line derived from human lung cancer (H292) has shown that, as a result of this nitration, intracellular HSP60 levels are reduced exclusively at a post-translational level. The nitration of tyrosine probably depends on the reactive nitrogen species created by SAHA activity inside tumoural cells. As a result, there is a reduced capacity of ATP-hydrolysis by HSP60 and an increased difficulty in binding to the co-chaperonin HSP10. In the future, antitumour treatments involving various HDAC is, and SAHA in particular, must take in consideration their ability to interact with HSP60 indirectly, with a pathway that is not linked to their principal mechanism. The property of SAHA to edit post-translational molecules like HSP60 can open new strategies for antitumour therapeutic protocols at a design level [79–84].

3.5. Lipid Rafts and Plasma Membrane. In this section, rather than single interactions between HSP60 and target proteins, some anomalous localizations of chaperonin in lung cancer cells will be analysed. On two different cell lines deriving from lung tumours, HSP60 molecules have been detected at the level of the plasma membrane and not at the usual mitochondrial location. The cell lines in question were A549, derived from a human lung adenocarcinoma, and H292, created from a human lung mucoepidermoid. Furthermore, a temporal analysis shows a different presence of HSP60 at the level of the cytoplasmic membrane, probably the result of different stages leading to its secretion. This hypothesis is further confirmed by pointing out that there is an accumulation of HSP60 in the cytoplasmic compartment near the membrane itself, suggesting an active localization of the molecular chaperone in this precise cellular district. Analysing the localization on the plasma membrane of the tumour cells in even more detail, the HSP60s have a preferential aggregation at the level of lipid rafts. Since lipid rafts are often the "departure stations" from which microvesicular bodies originate, the use of treatments with lipid raft pathway inhibitors would reduce the amount of HSP60 released at the extracellular level by cancer cells [55, 85–87].

3.6. Pro-Caspase 3. As already explained in the cases analysed above, HSP60 concentration levels can suffer variations, if compared to a basal expression, in case of lung tumours or conditions that can evolve into cancerous diseases (such as COPD). Various examples have been mentioned, both of cases in which expression levels were increased or decreased, and of cases of ectopic localizations. As a result of oxidative stress, tests on mucoepidermoid carcinoma cell lines have shown an increase in the cytoplasmic concentration of HSP60; if paired with a massive release from mitochondrial compartment, the role of HSP60 is pro apoptotic; otherwise, if the mitochondrial release is absent, the chaperone performs an anti-apoptotic action. Studies have shown that HSP60 is able to bind to the inactive form of caspase 3, pro-Caspase 3 (p-C3). This shows how it

plays an anti-apoptotic action at the level of lung cancer cells. Indeed, subsequent work has shown that by inhibiting the binding of HSP60 with p-C3, tumour cells prove to be more susceptible to apoptosis. Tumour growth is then interrupted by the activation of the caspase cascade following the activation of p-C3 in caspase 3 (its active form). Therefore, it is necessary to consider the use of drugs and compounds which reduces the binding that stabilizes the inactive form of caspase as a possible therapeutic option, in order to favour a better prognosis and successful treatment in patients suffering from lung cancer [27, 45, 88–90].

4. Conclusions

The examples reported in this review and the molecular interactions that will be discovered in the future will constitute an important weapon for doctors, not only in the treatment of lung tumours, as they will also lead to better diagnostic methods, but also the creation of therapies against precancerous conditions that can evolve into lung cancer. Continuous stress conditions in the respiratory mucosa (a clear example is cigarette smoking) increase the levels of chaperonins at the extra-mitochondrial level; the high concentration of HSP60 has a positive effect on cell survival, by inhibiting both apoptotic processes and cellular senescence. This increase in the cytoplasmic levels of the molecular chaperone leads to its release through different mechanisms (multivesicular bodies, exosomes, etc.), feeding a proinflammatory state by acting on the immune cells. Thanks to this positive feedback an ideal environment is created for the development of lung tumours.

The number of cases of individuals with lung cancer is set to increase in the years to come. Both its impact on the world population and its costs on national health systems will be influenced by this trend. The development of new compounds and new therapeutic strategies, associated with an increased knowledge of pathways, will provide a new weapon in the fight against lung tumours, in which the contribution of the HSP60 and HSP10 chaperonins appears increasingly relevant. The time has come to assign a much more important role to these molecular chaperones, making them evolve from simple biomarkers to leading actors in the development and evolution of lung tumours.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Table 1: molecular interactions of HSP60/10 complex in lung cancer cells. (*Supplementary Materials*)

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Research Article

Long Noncoding RNA Lnc-TLN2-4:1 Suppresses Gastric Cancer Metastasis and Is Associated with Patient Survival

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Gastric cancer (GC) is one of the most common malignancies worldwide, and the tumor metastasis leads to poor outcomes of GC patients. Long noncoding RNAs (lncRNAs) have emerged as new regulatory molecules that play a crucial role in tumor metastasis. However, the biological function and underlying mechanism of numerous lncRNAs in GC metastasis remain largely unclear. Here, we report a novel lncRNA, lnc-TLN2-4:1, whose expression is decreased in GC tissue versus matched normal tissue, and its low expression is involved in the lymph node and distant metastases of GC, as well as poor overall survival rates of GC patients. We further found that lnc-TLN2-4:1 inhibits the ability of GC cells to migrate and invade but does not influence GC cell proliferation and confirmed that lnc-TLN2-4:1 is mainly located in the cytoplasm of GC cells. We then found that lnc-TLN2-4:1 increases the mRNA and protein expression of TLN2 in GC cells and there is a positive correlation between the expression of lnc-TLN2-4:1 and TLN2 mRNA in GC tissue. Collectively, we identified a novel lncRNA, lnc-TLN2-4:1, in GC, where lnc-TLN2-4:1 represses cell migration and invasion. The low expression of lnc-TLN2-4:1 is associated with poor overall survival rates of GC patients. These suggest that lnc-TLN2-4:1 may be a tumor suppressor during GC metastasis.

1. Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer mortality worldwide [1]. Patients with early GC who have been subject to operation have satisfactory outcome. However, for patients with advanced GC, in spite of the successful surgery and optimized chemotherapy, the survival time remains still poor [2]. The major reason that leads the patient to die is GC metastasize [3], but the underlying mechanism remains largely unclear.

Long noncoding RNAs (lncRNAs) are a class of single RNAs with more than 200 nucleotides in length and fail to encode protein [4]. In the past decade, lncRNAs have been demonstrated to play important roles in a variety of diseases, including cancer. For example, lncRNAs can affect cell proliferation, apoptosis, migration, invasion, adherence, etc, in the development of malignancy [5]. There are several regulatory mechanisms involved in lncRNAs, such as (1) lncRNAs interact with proteins, resulting the functional change of the proteins or their locations in the cell organs [6]; (2) lncRNAs serve as competitive endogenous RNAs that absorb miRNAs, thereby controlling the expression of miRNAs' target genes [7]; (3) lncRNAs also bind to mRNAs and then prevent mRNAs from degradation, or influence their translation [8]. A recent report showed that lncRNA GMAN promotes translation of ephrin A1 (EFNA1) mRNA into protein via binding to the antisense GMAN-AS, which is complementary to EFNA1 mRNA, resulting in the enhancing ability of GC cells to metastasize and invade, so that it leads to GC metastasis and poor patient survival [9]. Even so, for GC, there are numerous lncRNAs which have not been identified and their biological functions and the underlying mechanisms have not been explored yet. Interestingly, after analyses of our previous microarray data

(GSE58828), we found an unidentified lncRNA, lnc-TLN2-4:1, whose expression is significantly decreased in GC tissue compared with matched normal tissue. However, the role and mechanism of this lncRNA in GC remains unknown.

Talin (TLN) plays a crucial role in cell migration, invasion, and cancer metastasis [10]. TLN gene encodes two TLN isoforms, TLN1 and TLN2. TLN2 is composed of 2532 amino acids that are 74% identical (86% similar) to human TLN1 which contains 2541 amino acids, and the complete sequencing has indicated that lower eukaryotes encode only one TLN gene corresponding to TLN1, whereas vertebrate animals possess two TLN genes [11], suggesting that TLN2 has a specific function in these species. In the past decades, a large number of studies have demonstrated the biological function of TLN1 in the development of several types of cancers [12–15], including GC [16], but there is little evidence with regard to the role of TLN2 in GC metastasis.

In the present study, we found a novel lncRNA, lnc-TLN2-4:1, located in the cytoplasm of GC cells, whose expression is decreased in GC tissue compared with matched normal tissue and is involved in poor overall survival rates of GC patients. We further found that lnc-TLN2-4:1 overexpression inhibits GC cell migration and invasion, but does not affect GC cell proliferation. These suggest that lnc-TLN2-4:1 may be a tumor suppressor during GC metastasis.

2. Materials and Methods

2.1. Patients and Specimens. Forty-nine pairs of fresh human GC samples in this study were collected from the consenting individuals based on the instructions approved by the Ethics Review Board at Xinqiao Hospital, Army Medical University (Third Medical University), from 2013 to 2017. The GC tissues were processed in the operating room and stored in liquid nitrogen within 10 min. The matched normal tissues were collected at a distance of >5 cm from the tumor tissues, and all tissues were identified histologically. None of the patients underwent chemotherapy or radiotherapy before operation. A four-year follow-up of the 49 GC patients was performed.

2.2. Cell Culture. Six human GC cell lines (AGS, MKN45, MGC803, BGC823, SGC7901, and MKN74) were purchased from BeNa culture Collection (BNCC). AGS cells were cultured in the F12 medium (HyClone Logan, UT, USA) supplemented with 10% FBS (Gibco BRL), and the other cells were cultured in the DMEM/HIGH GLUCOSE medium (HyClone Logan, UT, USA) supplemented with 10% FBS (Gibco BRL) at 37°C in an atmosphere of 5% CO_2.

2.3. RNA Extraction, Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR), and Immunoblotting. The procedures and reagents of RNA extraction, qRT-PCR, and immunoblotting are described in our previous study [17]. For qRT-PCR experiments, the expression of lnc-TLN2-4:1 and TLN2 was normalized to an internal control, β-actin, using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are as the follows: TLN2 sense: 5′ACGGCGGAACCAGAGGAGAT3′, TLN2 antisense: 5′GGTGTCCAGGTCGGCAATGAT3′; lnc-TLN2-4:1 sense: 5′GCTGGCTGCTTCTGAGACTTAC3′, lnc-TLN2-4:1 antisense: 5′TGGAGCAACAGACTGAGGACAT3′. The parameter of PCR running is 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. For immunoblotting, the anti-TLN2 antibody (ab108967) was purchased from Abcam, China (Shanghai, China), and HRP-conjugated secondary antibody was purchased from Zhongshan Biotechnology (Beijing, China), and all antibodies were used according to the manufacturer's instructions.

2.4. Vector and Lentivirus Construction. The LV5-V6256-1 vector containing lnc-TLN2-4:1 cDNA sequence was synthesized from a company, GenePharma (Shanghai, China). The lentivirus construction is described in our previous study [18].

2.5. Cell Migration, Invasion, and Proliferation. The procedures and reagents of cell migration, invasion, and proliferation experiments are described in our previous study [18]. BGC823 and SGC7901 cells which were transfected with control or lnc-TLN2-4:1-overexpressing vector were used to perform the cell migration, invasion, and proliferation experiments. The statistics of cell migration and invasion are based on three different-area images from each transwell.

2.6. Statistical Analysis. All data are presented as the means ± standard deviation or standard error. The difference between two groups was analyzed using Student's t test or Mann–Whitney U test. The one-way ANOVA was used to analyze the difference among three or more groups. Receiver operating characteristic curve (ROC) was used to assess the power of distinguishing two groups. The patient survival was analyzed using the Kaplan–Meier method and log-rank test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 19.0 (Chicago, IL, USA) and GraphPad Prism 8.0 (Graphpad Software Inc, California).

3. Results

3.1. Lnc-TLN2-4:1 Expression Is Frequently Decreased in GC Tissues Compared with Matched Normal Tissues and Is Associated with GC Metastasis. To find a novel lncRNA which may be of regulatory function in the development of GC, we analyzed the data from an lncRNA microarray (GSE58828) that was performed in our previous study [19]. Based on stringent filtering criteria (fold change > 2, $P < 0.01$, and the lengths of lncRNAs are between 1000 nt and 2000 nt), we found an unidentified lncRNA, AF070527, whose expression was decreased in three GC tissues compared with the matched normal tissues (Figure 1(a)). The name of this lncRNA has been updated to lnc-TLN2-4:1 in LncBook, a curated knowledgebase of human lncRNAs (https://bigd.big.ac.cn/lncbook/index). Lnc-TLN2-4:1 is an intergenic lncRNA and shown to have no encoding capacity (LncBook).

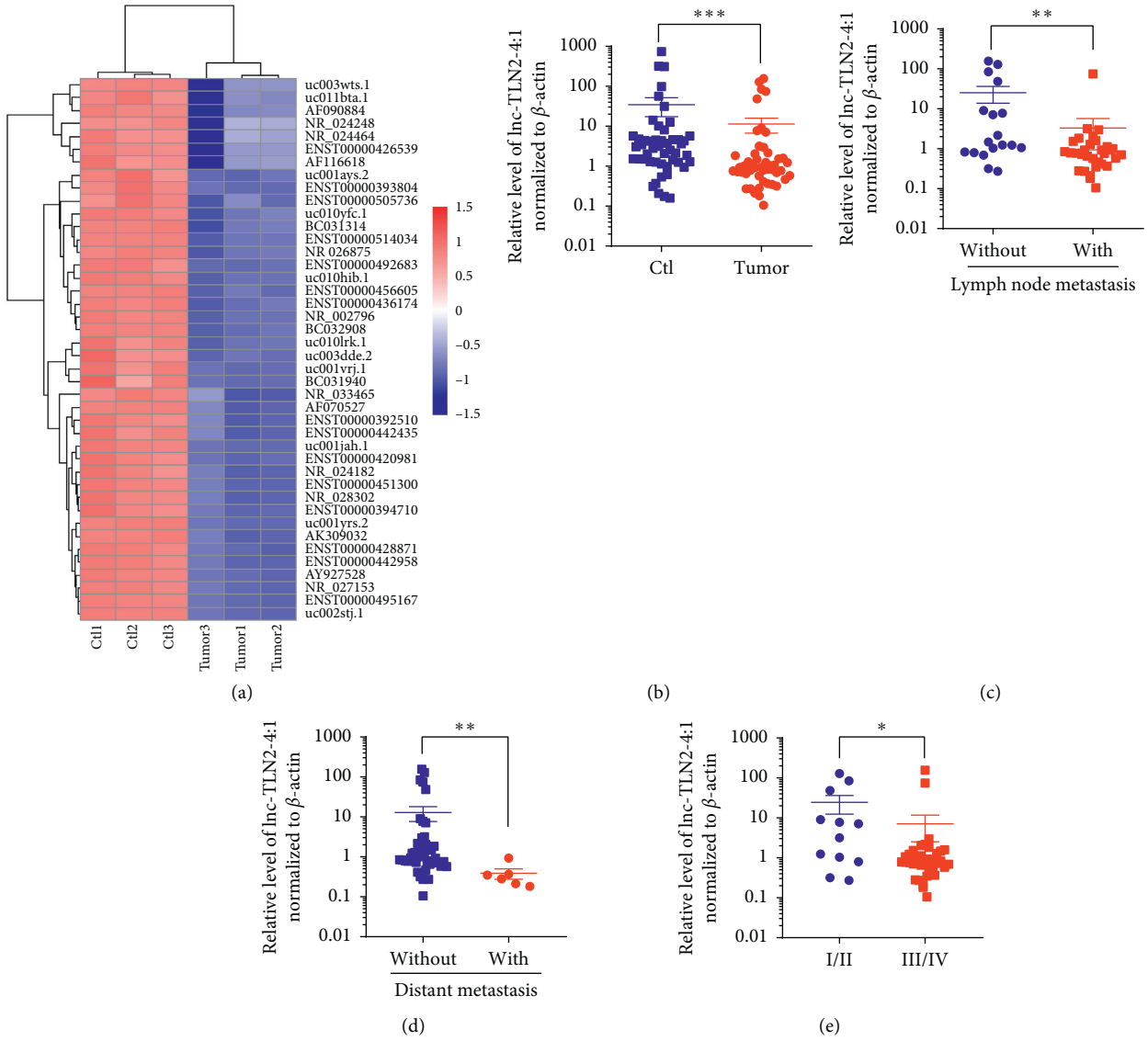

Figure 1: Lnc-TLN2-4:1 expression is frequently decreased in GC tissues compared with matched normal tissues and is associated with GC metastasis. (a) A heatmap shows the aberrant expression of lncRNAs in three pairs of GC and matched normal tissues detected by a Human LncRNA Microarray. (b) Scatter plots show the expression of lnc-TLN2-4:1 in 49 pairs of GC and matched normal tissues, detected by qRT-PCR, and β-actin serves as the internal control. (c) Scatter plots show the expression of lnc-TLN2-4:1 in 49 GC tissues, 31 of which are of lymph node metastasis and 18 of which are not. (d) Scatter plots show the expression of lnc-TLN2-4:1 in 49 GC tissues, 6 of which are of distant metastasis and 43 of which are not. (e) Scatter plots show the expression of lnc-TLN2-4:1 in 49 GC tissues, 12 of which are the sum of TNM stage I and II, and 37 of which are the sum of TNM stage III and IV. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$.

To further determine the exact expression of lnc-TLN2-4:1 in GC, we collected 49 pairs of GC tissues and matched normal tissues from the enrolled GC patients. QRT-PCR experiments revealed that the expression of lnc-TLN2-4:1 is significantly decreased in GC tissue versus matched normal tissue (Figure 1(b)). We next analyzed the correlation of the clinical characteristics of GC patients with the expression of lnc-TLN2-4:1 and found that the expression of lnc-TLN2-4:1 is significantly decreased in GC tissue with lymph node metastasis, distant metastasis, or TNM stage I and II compared to that without lymph node metastasis, distant metastasis, or with TNM stage III and IV (Figures 1(c)–1(e)). However, there is no correlation of the expression of lnc-TLN2-4:1 with the patients' gender and age, as well as tumor size and differentiation (data not show). These data suggest that lnc-TLN2-4:1 is a novel lncRNA significantly decreased in GC and associated with GC metastasis.

3.2. Lnc-TLN2-4:1 Expression Is Associated with Overall Survival Rates of GC Patients. We next determined whether the expression of lnc-TLN2-4:1 in GC tissue has a diagnostic power for GC. Receiver operating characteristic curve (ROC) analyses revealed an AUC of 0.7071, with the sensitivity of 65.31% and the specificity of 81.25% and the cutoff value of 1.246, in discriminating GC tissues from matched

normal tissues (Figure 2(a)), and an AUC of 0.733, with the sensitivity of 70.97% and the specificity of 72.22% and the cutoff value of 1.016, in distinguishing GC tissues with lymph node metastasis from those without lymph node metastasis (Figure 2(b)). We further analyzed the correlation of lnc-TLN2-4:1 expression with overall survival rates of the GC patients based on the different cutoff values obtained from the two above ROC curves and found that when the cutoff value of 1.246 was used to define the low or high expression of lnc-TLN2-4:1, there are no significant difference of overall survival rates between GC patients with low and high expression of lnc-TLN2-4:1 (Figure 2(c)). However, when the cutoff value of 1.016 was used, the significant difference of overall survival rates was observed (Figure 2(d)). These data suggest that lnc-TLN2-4:1 expression may be a prognostic marker for GC.

3.3. Lnc-TLN2-4:1 Represses GC Cell Migration and Invasion In Vitro. As abovementioned, aberrant expression of lnc-TLN2-4:1 was associated with GC metastasis; therefore, we directly investigated whether lnc-TLN2-4:1 could influence the migration and invasion of GC cells. To perform the gain-of-function, we measured the expression of lnc-TLN2-4:1 in six GC cell lines, including AGS, MKN45, MGC803, BGC823, SGC7901, and MKN74 and found that BGC823 and SGC7901 almost have the lowest expression of lnc-TLN2-4:1 (Figure 3(a)); thus, we performed the ectopic expression of lnc-TLN2-4:1 in the two GC cells using a lentivirus containing lnc-TLN2-4:1-overexpressing vectors (Figure 3(b)). Wound healing and transwell assays showed that upregulation of lnc-TLN2-4:1 significantly inhibits the migration and invasion of BGC823 and SGC7901 cells *in vitro* (Figures 3(c) and 3(d)). Because cell proliferation commonly occurred in GC development, including GC metastasis, we also determined whether lnc-TLN2-4:1 could affect GC cell proliferation. However, an assay based on a CCK-8 kit revealed that lnc-TLN2-4:1 overexpression cannot modify the proliferative ability of BGC823 and SGC7901 cells (Figure S1). These data suggest that lnc-TLN2-4:1 may be a tumor suppressor which represses GC cell metastasis but not proliferation.

3.4. Lnc-TLN2-4:1 Is Located in GC Cell Cytoplasm, and Its Expression Is Positively Correlated with TLN2 Expression in GC Tissues. To well understand the underlying mechanism of lnc-TLN2-4:1 in GC metastasis, we determined the location of lnc-TLN2-4:1 in GC cells because the regulatory mechanism of the lncRNA is constrained by its location. We found that lnc-TLN2-4:1 is mainly located in the cytoplasm of BGC823 cells, and the expression of lnc-TLN2-4:1 is significantly increased in the cytoplasm of BGC823 cells with the ectopic expression of lnc-TLN2-4:1 compared to those with wild-type expression of lnc-TLN2-4:1 (Figure 4(a)–4(c)), suggesting that the location of the ectopic expression of lnc-TLN2-4:1 in GC cells is corresponding to its natural location and reflecting its real function. TLN2 is a coding gene which has been reported to be involved in cancer metastasis. By the nucleotide blast, we found that in lnc-TLN2-4:1 and TLN2 mRNA exist a large number of overlapped nucleotides. Therefore, we supposed whether lnc-TLN2-4:1 could regulate the expression of TLN2 mRNA in GC cells. QRT-PCR and western blotting experiments showed that lnc-TLN2-4:1 upregulation significantly increases the mRNA and protein expression of TLN2 (Figures 4(d) and 4(e)). We further analyzed the expression of lnc-TLN2-4:1 and TLN2 mRNA in GC tissues and found that there is a positive correlation between their expressions in 49 GC tissues (Figure 4(f)). These data suggest that lnc-TLN2-4:1 inhibits GC metastasis through regulating the expression of TLN2 mRNA.

4. Discussion

Lnc-TLN2-4:1 is predicted to have no encoding capacity and has 1558 nt in length. In this study, we found that lnc-TLN2-4:1 expression is significantly decreased in GC tissue versus matched normal tissue and is associated with the GC cell lymph node and distant metastases. ROC analyses revealed that lnc-TLN2-4:1 expression has a potentially predictive power in distinguishing GC tissue from matched normal tissue, and the decreased expression of lnc-TLN2-4:1 is closely involved in poor overall survival rates of GC patients. So far, there are hundreds of lncRNAs that have been identified to have aberrant expression in GC development and also be considered as potential biomarkers for GC detection. For example, Zhuo et al. report an lncRNA, GMAN, which is overexpressed in GC tissue versus nontumor tissue and its upregulation is also associated with poor overall survival rates of GC patients [9]; Zhang et al. report an lncRNA, HOXC-AS3, whose expression is increased in GC tissue versus nontumor tissue and correlated with clinical outcomes of GC [20]. These instances suggest that lncRNAs may be a potential biomarker for GC detection, and our findings also suggest that lnc-TLN2-4:1 may be a novel biomarker for the diagnosis and prognosis of GC and hint that it may have an important role during GC metastasis.

To determine the biological function of lnc-TLN2-4:1 in GC, we selected two GC cell lines which have low expression of lnc-TLN2-4:1 and constructed BGC823 and SGC7901 cells with stably ectopic expression of lnc-TLN2-4:1 using a lentivirus containing lnc-TLN2-4:1-overexpressing vectors. This effect of ectopic expression of lnc-TLN2-4:1 was identified by qRT-PCR and immunofluorescence. Stably modified expression of lncRNAs using lentivirus is widely used in studying their biological functions, such as in our previous study [18]. The biological functions of lncRNAs are strongly associated with their location in cells, and the results of immunofluorescence experiments in our study indicate that the location of lnc-TLN2-4:1 with ectopic expression is corresponding to its natural location. Our findings revealed that lnc-TLN2-4:1 upregulation can significantly inhibit the migration and invasion of GC cells but does not affect GC cell proliferation, suggesting that lnc-TLN2-4:1 acts as a tumor suppressor in GC metastasis.

To address the underlying mechanism by which lnc-TLN2-4:1 represses GC metastasis, we searched for the candidate target genes of lnc-TLN2-4:1. Because lnc-TLN2-4:1 is located in the cell cytoplasm, we considered that

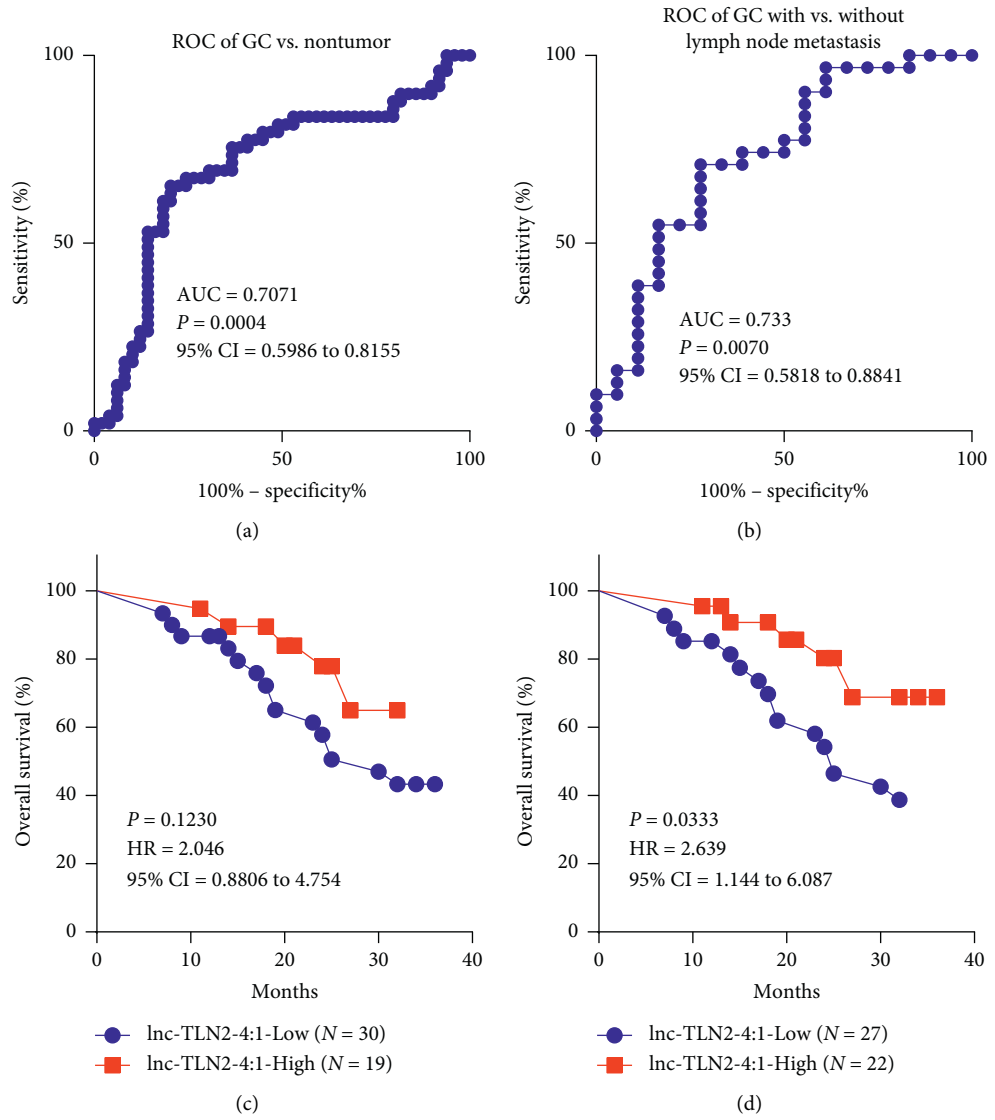

Figure 2: Lnc-TLN2-4:1 expression is associated with overall survival rates of GC patients. (a) ROC curve shows that the expression of lnc-TLN2 has an AUC of 0.7071 in distinguishing GC tissue from nontumor tissue, with the sensitivity of 65.31% and the specificity of 81.25% and the cutoff value of 1.246. (b) ROC curve shows that the expression of lnc-TLN2 has an AUC of 0.733 in distinguishing GC tissue from nontumor tissue, with the sensitivity of 70.97% and the specificity of 72.22% and the cutoff value of 1.016. (c) Overall survival analysis shows the survival rates of GC patients with low or high expression of lnc-TLN2-4:1, which is defined by the cutoff value of 1.246. (d) Overall survival analysis shows the survival rates of GC patients with low or high expression of lnc-TLN2-4:1, which is defined by the cutoff value of 1.016.

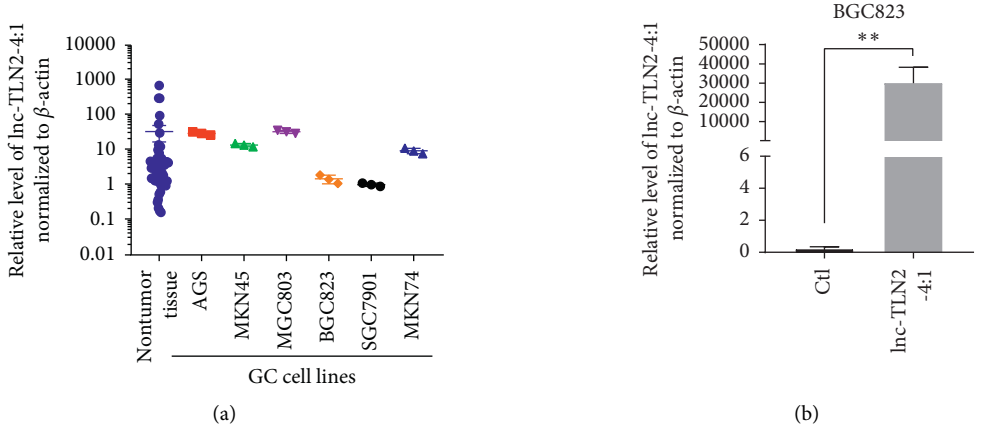

Figure 3: Continued.

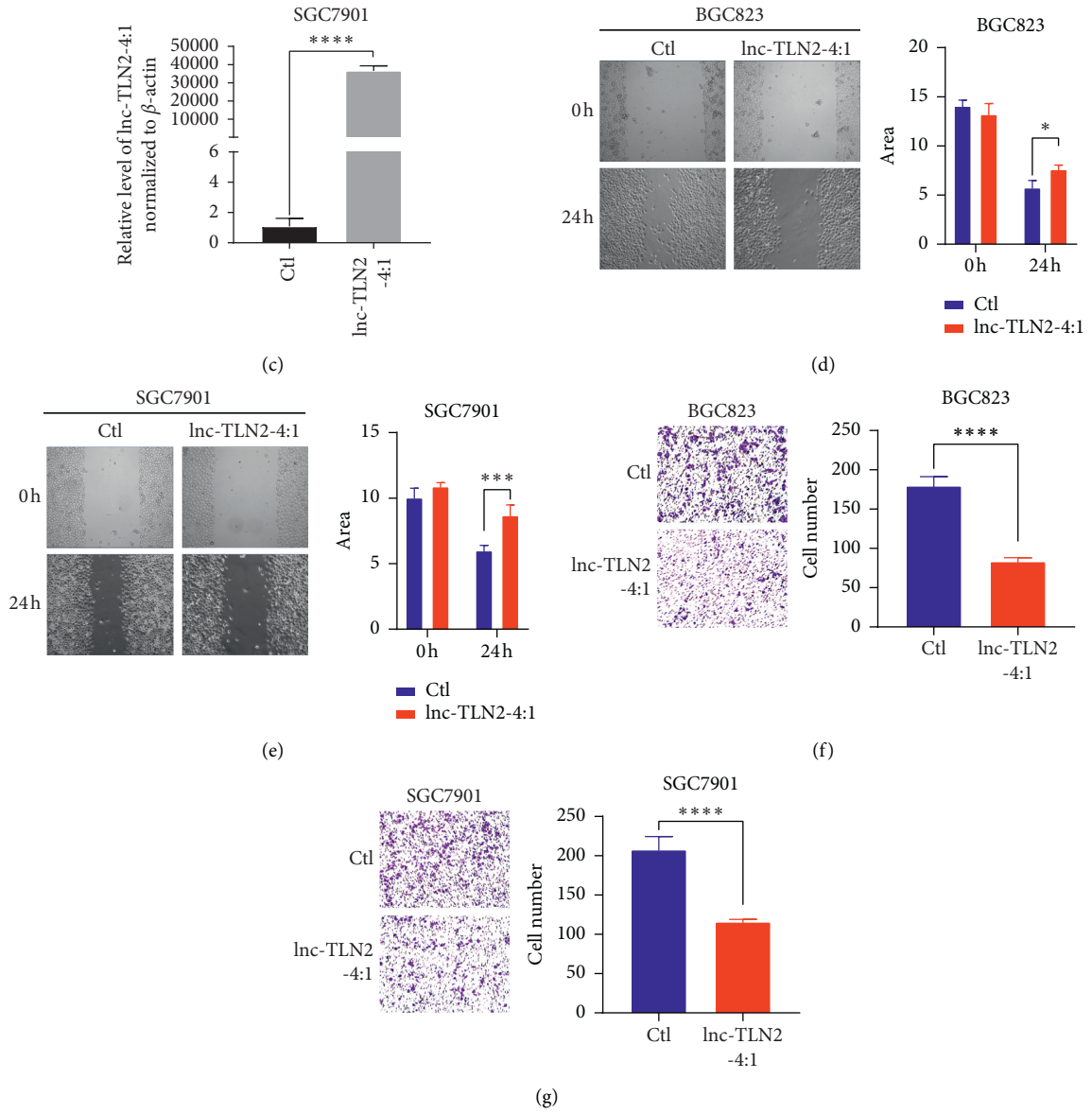

Figure 3: Lnc-TLN2-4:1 represses GC cell migration and invasion *in vitro*. (a) Scatter plots show the expression of lnc-TN2-4:1 in 49 nontumor tissues and GC cell lines, detected by qRT-PCR, and β-actin serves as the internal control. (b and c) Bars show the expression of lnc-TLN2-4:1 in BGC823 and SGC7901 cells which were transfected with lnc-TLN2-4:1-overexpressing vectors, detected by qRT-PCR, and β-actin serves as the internal control. (d and e) Wound healing experiments show the abilities of BGC823 and SGC7901 cells which were transfected with lnc-TLN2-4:1-overexpressing vectors to migrate. Bars show the statistics based on three independent experiments. Area indicates the area without cells in the images, calculated by Image J. (f and g) Transwell experiments show the ability of BGC823 and SGC7901 cells which were transfected with lnc-TLN2-4:1-overexpressing vectors to invasive. Bars show the statistics based on three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

lnc-TLN2-4:1 has a possibility of regulating TLN2 mRNA stability. A large number of studies have reported that lncRNAs can protect mRNAs from degradation [21, 22]. The specific stability effect of lncRNAs on mRNAs is based on the complementary base pairing, and our findings revealed that the nucleotide sequence of lnc-TLN2-4:1 completely overlaps the 3′end fragment of TLN2. Our further investigation showed that lnc-TLN2-4:1 upregulation increases the mRNA expression of TLN2 in GC cells and there is a positive correlation between the expression of lnc-TLN2-4:1 and TLN2 mRNA in 49 GC tissues. These data suggest that lnc-TLN2-4:1 inhibits GC metastasis through regulating the expression of TLN2 mRNA, but the underlying mechanism needs to be identified in the future.

In conclusion, we identified a novel lncRNA, lnc-TLN2-4:1, which is downregulated in GC tissue versus matched normal tissue and whose low expression is associated with GC metastasis and poor overall survival rates of GC patients. We further found that lnc-TLN2-4:1 represses the ability of GC cells to migrate and invade, and lnc-TLN2-4:1 promotes

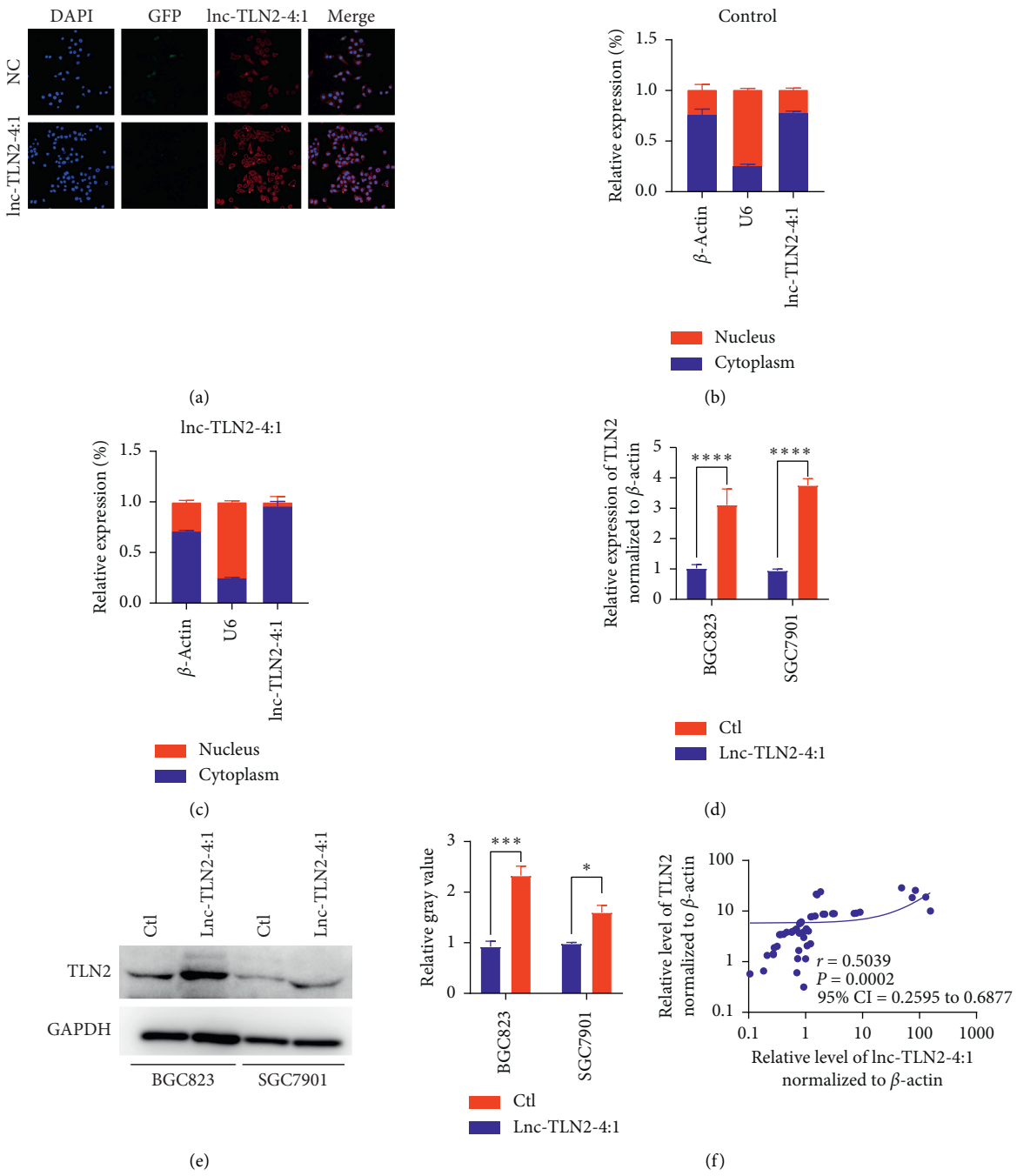

Figure 4: Lnc-TLN2-4:1 is located in the GC cell cytoplasm, and its expression is positively correlated with TLN2 expression in GC tissues. (a) Fluorescence in situ hybridization (FISH) shows the expression and location of lnc-TLN2-4:1 in BGC823 cells. DAPI indicates cell nucleus. GFP indicates the expression status of the vectors (negative control and lnc-TLN2-4:1-overexpressing vectors). Red fluorescence indicates the expression of lnc-TLN2-4:1. (b and c) Bars show the relative expression % of lnc-TLN2-4:1 in the cytoplasm and nucleus of BGC823 cells which were transfected with the control or lnc-TLN2-4:1-overexpressing vectors, detected by qRT-PCR, and β-actin serves as the internal control in the cytoplasm and U6 serves as the internal control in the nucleus. (d) Bars show the expression of TLN2 in BGC823 and SGC7901 cells which were transfected with control or lnc-TLN2-4:1-overexpressing vectors, detected by qRT-PCR, and β-actin serves as the internal control. (e) Western blotting shows the protein expression of TLN2 in BGC823 and SGC7901 cells which were transfected with the control or lnc-TLN2-4:1-overexpressing vectors, and GAPDH serves as the internal control. Bars show the statistics based three independent experiments. (f) The correlation between the expression of TLN2 mRNA and lnc-TLN2-4:1 in 49 pairs of GC tissues, detected by qRT-PCR, and β-actin serves as the internal control. $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

the expression of TLN2 in GC cells and there is a positive correlation between the expression of lnc-TLN2-4:1 and TLN2 in GC tissues. These data suggest that lnc-TLN2-4:1 may be a therapeutic target for GC.

Data Availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files), and more detailed data are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yuyun Wu and Ningbo Hao contributed equally to this work.

Acknowledgments

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Supplementary Materials

Figure S1. Lnc-TLN2-4:1 doses not affect the abilities of BGC823 and SGC7901 cells to proliferate. (A and B) the proliferative abilities of BGC823 and SGC7901 cells which were transfected with the control or lnc-TLN2-4:1-overexpressing vectors were analyzed using a CCK-8 kit. (*Supplementary Materials*)

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Research Article

Role of 17β-Estradiol on Cell Proliferation and Mitochondrial Fitness in Glioblastoma Cells

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Gliomas are the most common primary tumors of the central nervous system (CNS) in the adult. Previous data showed that estrogen affects cancer cells, but its effect is cell-type-dependent and controversial. The present study aimed to analyze the effects of estradiol (E2, 5 nM) in human glioblastoma multiforme U87-MG cells and how it may impact on cell proliferation and mitochondrial fitness. We monitored cell proliferation by xCELLigence technology and mitochondrial fitness by assessing the expression of genes involved in mitochondrial biogenesis (PGC1α, SIRT1, and TFAM), oxidative phosphorylation (ND4, Cytb, COX-II, COX IV, NDUFA6, and ATP synthase), and dynamics (OPA1, MNF2, MNF1, and FIS1). Finally, we evaluated Nrf2 nuclear translocation by immunocytochemical analysis. Our results showed that E2 resulted in a significant increase in cell proliferation, with a significant increase in the expression of genes involved in various mechanisms of mitochondrial fitness. Finally, E2 treatment resulted in a significant increase of Nrf2 nuclear translocation with a significant increase in the expression of one of its target genes (i.e., heme oxygenase-1). Our results suggest that E2 promotes proliferation in glioblastoma cells and regulate the expression of genes involved in mitochondrial fitness and chemoresistance pathway.

1. Introduction

Gliomas are the most common primary tumors of the central nervous system (CNS) in the adult. Glioblastoma is the most frequent and aggressive brain tumor in humans with a median survival from 14 to 17 months after the diagnosis [1, 2]. Targeted therapies directed to ubiquitous cancer-associated targets (i.e., erlotinib and gefitinib) had limited success [3–5], further reinforcing the need for the identification of glioma-specific novel molecular targets. With the advent of new technologies, several recent studies have reiterated the importance of metabolic reprogramming in various cancers. The importance of glycolysis in the survival and progression of certain cancers is undeniable, and it is increasingly evident that cancer cells may use many alternative metabolic pathways to drive their phenotype [6]. Previous data showed that estrogen affects glioblastoma cells since certain glioblastomas express estrogen receptors (ERs) [7, 8]. Consistently with this evidence, the ER-modulator tamoxifen inhibits the growth of certain glioblastomas [9–11].

Furthermore, a previous study showed that high concentrations of 17-β-estradiol causes apoptosis in the human breast cancer cell line MCF-7: this result is not shown with low growth-stimulated conditions in the ER-negative human breast cancer cell line MDA-MB 231 [12]. In addition to its nuclear functions, estradiol also plays an essential role in the mitochondria. The mitochondrial electron transport

chain comprises several complexes formed by proteins that are encoded by the nuclear or mitochondrial genome. Moreover, E2 plays a role in mitochondrial bioenergetic function, modulating the microviscosity of the inner membrane [13] and inducing mitochondrial biogenesis genes in hepatic cells [14]. Others reported that long-term E2 treatment increased nuclear respiratory factor-1 (NRF-1) protein in cerebral blood vessels of ovariectomized rats [15]. Interestingly, high estradiol concentrations (about 10-8 M) decrease the mitochondrial DNA contents, and ATP formation and these effects were not showed at minor concentrations [16, 17]. For this reason, in this work, we study the effect of estradiol at low concentrations.

Given that, estradiol may produce cell growth or death under different conditions, depending on the expression of ERs in the brain and other tissues and the concentration of estradiol [18–20]. We analyzed the effects of estradiol in human glioblastoma multiforme U87-MG cells and how it may impact on cell proliferation and mitochondrial fitness.

2. Materials and Methods

2.1. Cell Culture and Pharmacological Treatments. Human glioblastoma cells (U87-MG) were purchased from ATCC Company (Milan, Italy). Cells were suspended in DMEM (Gibco, Cat. # 11965092) culture medium containing 10% fetal bovine serum (FBS, Gibco, category no. 10082147), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco, category no. 15070063). At 80% confluency, cells were passed using trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA, Gibco, category no. 25300054) [21]. 20 μg/mL 17β-estradiol (E2) (category no. E2758 Sigma-Aldrich, Milan, Italy) solution was prepared in 1 mL absolute ethanol (category no. 51976 Sigma-Aldrich, Milan, Italy), and it was added separately to the cell culture of all experiments at final concentrations of 5.0 nM.

2.2. Real-Time Monitoring of Cell Proliferation. xCELLigence experiments were performed using the RTCA (Real-Time Cell Analyzer) DP (Dual Plate) instrument according to manufacturers' instructions (Roche Applied Science, Mannheim, Germany, and ACEA Biosciences, San Diego, CA). The RTCA DP instrument includes three main components: (i) RTCA DP analyzer, which stays inside a humidified incubator maintained at 37°C and 5% CO_2, (ii) RTCA control unit with RTCA software preinstalled, and (iii) E-plate 16 for proliferation assay. First, we defined the optimal seeding number by cell titration and growth experiments to obtain a significant cell index value and a constant cell growth (data not shown). We added 100 μl of cell culture media in the E-plate 16, and we left it in the tissue culture hood for 30 minutes at room temperature: this procedure ensures the equilibrium between the culture media and E-plate surface. We inserted the E-plate 16 into a cradle pocket of the RTCA DP analyzer, and we performed blank reading to measure the background impedance of cell culture media. We added 100 μl of a cell solution with a final concentration of 2500 cells/well in the E-plate 16, and, as recommended, we waited 30 minutes before starting the automatic monitoring every 15 min for 24 h.

2.3. Real-Time PCR for Gene Expression Analysis. RNA was extracted by Trizol® reagent (category no. 15596026, Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was then synthesized with High-Capacity cDNA Reverse Transcription kit (category no. 4368814, Applied Biosystems, Foster City, CA, USA). High cDNA quality was checked, taking into consideration the housekeeping gene Ct values. Quantitative real-time PCR was performed in Step-One Fast Real-Time PCR system, Applied Biosystems, using the SYBR Green PCR MasterMix (category no. 4309155, Life Technologies, Monza, Italy). The specific PCR products were detected by the fluorescence of SYBR Green, the double-stranded DNA binding dye. Primers were designed using BLAST® (Basic Local Alignment Search Tool, NBCI, NIH), considering the shortest amplicon proposed: primers' sequences are shown in Table 1, and β-actin was used as the housekeeping gene. Primers were purchased by Metabion International AG (Planneg, Germany). The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with β-actin by using a comparative $2^{-\Delta\Delta Ct}$ method.

2.4. Immunocytochemistry. Cells were grown directly on coverslips before immunofluorescence and treated with 17β-estradiol (E2) at the final concentration of 5 nM. After washing with PBS, cells were fixed in 4% paraformaldehyde (category no. 1004968350 Sigma-Aldrich, Milan, Italy) for 20 min at room temperature. Subsequently, cells were incubated with primary antibody against TFAM at dilution 1 : 200, overnight at 4°C. The next day, cells were washed three times in PBS for 5 min and incubated with secondary antibodies: TRITC (anti-goat, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilution 1 : 200 for 1 h at room temperature. The slides were mounted with medium containing DAPI (4′,6-diamidino-2phenylindole, category no. sc-3598, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to visualize nuclei. The fluorescent images were obtained using a Zeiss Axio Imager Z1 microscope with Apotome 2 system (Zeiss, Milan, Italy). As a control, the specificity of immunostaining was verified by omitting incubation with the primary or secondary antibody. Immunoreactivity was evaluated considering the signal-to-noise ratio of immunofluorescence.

2.5. Statistical Analysis. Statistical analysis was performed using SPSS11.0 software. Statistical significance ($p < 0.05$) of differences between experimental groups was determined by the Fisher method for analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by either single-factor analysis of variance (ANOVA) for multiple groups or the unpaired t-test for two groups, and the data are presented as mean ± SD.

Table 1: List of qRT-PCR primers.

Gene of interest	Forward primer (5′ ⟶ 3′)	Reverse primer (5′ ⟶ 3′)
PGC1α	ATGAAGGGTACTTTTCTGCCCC	GGTCTTCACCAACCAGAGCA
SIRT1	AGGCCACGGATAGGTCCATA	GTGGAGGTATTGTTTCCGGC
TFAM	CCGAGGTGGTTTTCATCTGT	AGTCTTCAGCTTTTCCTGCG
ND4	CCAGTGGAATGCCTTGCCTA	TTGATCGCGGTGAGATTCCC
CyB	ACGAGCCACCGAAACAGAAT	ACGATTTTCGCCAGTCACCT
COX II	ACGACCTCGATGTTGGATCA	ATCATTTACGGGGGAAGGCG
COX IV	GCGGCAGAATGTTGGCTAC	AGACAGGTGCTTGACATGGG
NDUFA6	CAGTCGGGACATGAACGAGG	GAATTGGTGCACAGTGTTCG
ATP synthase	CCGCCTTCCGCGGTATAATC	ATGTACGCGGGCAATACCAT
OPA1	AGGAGCTCATCTGTTTGGAGTC	GCTCACCAAGCAGACCCTTT
MNF2	GCGGAGACTCATAATGGCAGA	TCCGAGATAGCACCTCACCA
MNF1	ATGCAGTGGGAGTCCGAGC	CAGGGACATTGCGCTTCAC
FIS1	AAGAAAGATGGACTCGTGGGC	CCGCGTCTCCTTCAGGATTT
HO-1	AAGACTGCGTTCCTGCTCAA	GGGCAGAATCTTGCACTTTGT
β-Actin	CCTTTGCCGATCCGCCG	AACATGATCTGGGTCATCTTCTCGC

3. Results

3.1. E2 Induces Glioblastoma Cell Proliferation and Mitochondrial Metabolism Gene Expression. We firstly aimed at studying the effect of E2 on cell proliferation. As shown in Figure 1, E2 treatment resulted in a significant increase in cell proliferation in U87-MG cells, as showed by cell index performed by xCELLigence technology. Increased cell index was already significant following 3 hours ($p < 0.001$) of treatment with E2, and such effect was still evident following 9 hours of treatment ($p < 0.001$). We, therefore, investigated the effect of E2 on mitochondrial metabolism concerning mitochondrial biogenesis, oxidative phosphorylation, and dynamics. As shown in Figure 2(a), E2 resulted in a significant increase of PGC1α gene expression following 1 hour of treatment ($p < 0.001$), and such an expression decreases in a time-dependent manner reaching the control levels following 24 h. Consistently, we observed a significant increase in two additional biomarkers of mitochondrial biogenesis (i.e., SIRT1 and TFAM) (Figures 2(b) and 2(c)). This set of experiments showed that E2 resulted in a significant ($p < 0.001$) increase in SIRT1 and TFAM gene expression following 1 hour of E2 treatment, and such an increased expression was sustained during all other times of observation. As shown in Figure 3, E2 treatment also resulted in a significant change in the expression of genes involved in oxidative phosphorylation. E2 treatment significantly increased ND4, Cyb4, COXII, COXIV, COX, and NDUFA6 gene expression following 1-hour treatment of E2 (Figures 3(a)–3(e)). Similarly, ATP synthase gene expression significantly increased treatment and peaked 3 h following E2 pharmacological treatment (Figure 3(f)). Besides, E2 exhibited a significant effect on the expression controlling mitochondrial dynamics. E2 treatment resulted in a significant ($p < 0.001$) increase of OPA1, MNF2, and MNF1 gene expression following 1 hour of treatment (Figures 4(a)–4(c)). Consistently, E2 treatment resulted in a significant ($p < 0.010$) increase in FIS1 gene expression following 3 hours of E3 treatment (Figure 4(d)). Finally, these results were further confirmed by immunocytochemistry analysis, demonstrating increase TFAM protein expression and increased mitochondrial network as measured by mitotracker staining (Figures 5(a)–5(d)).

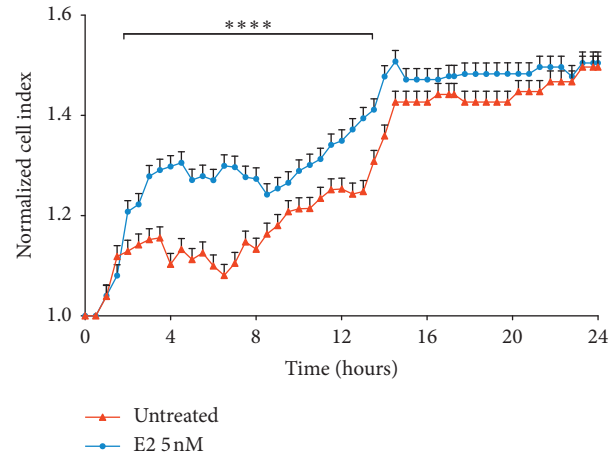


Figure 1: Effect of E2 in glioblastoma cell proliferation. E2 treatment resulted in a significant increase in cell proliferation in U87-MG cells following E2 5 nM treatment. A normalized cell index was performed for 24 hours by xCELLigence RTCA technology.

3.2. E2 Induces Nrf2 Nuclear Translocation and Increases Heme Oxygenase-1 Expression. To assess the effect of E2 on the activation of pathways involved in chemoresistance mechanisms, we evaluated the nuclear translocation of Nrf2. Our data showed that E2 treatment resulted in a significant increase in nuclear translocation following 24 h treatment when compared to untreated cells (Figures 6(a) and 6(b)). Consistently with this observation, we also showed that HO-1, an Nrf2-targeted gene, was upregulated following E2 treatment ($p < 0.001$) (Figure 6(a)).

4. Discussion

Previous studies showed that high concentrations of estradiol, under low growth-stimulated conditions, inhibit cell proliferation and increase apoptosis in ER-positive breast cancer cells through the sustained activation of the JNK pathway [12, 22]. These findings emphasize the basis for the antitumor effects of high-dose estrogen therapy in postmenopausal

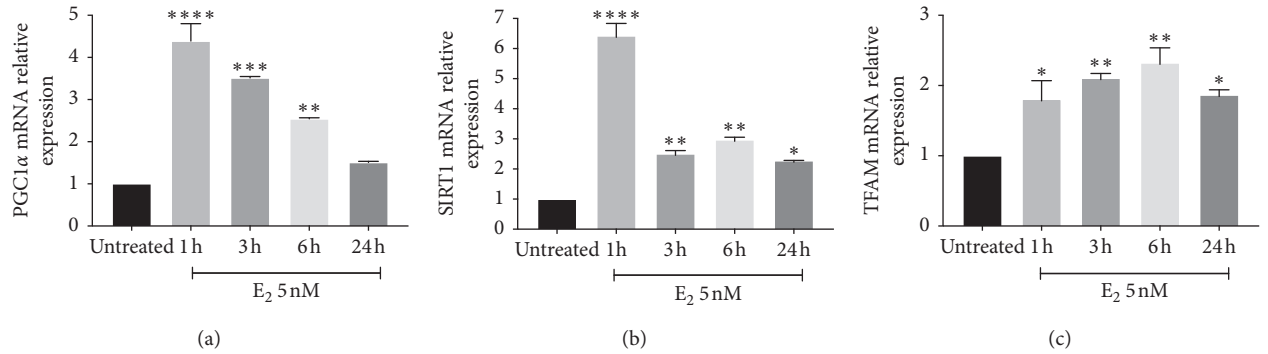


Figure 2: Effect of E2 on mitochondrial biogenesis. E2 resulted in a significant increase of PGC1α gene expression following 1 h treatment, and such an expression decreases in a time-dependent manner reaching the control levels following 24 h (Figure 2(a)). Consistently, SIRT1 and TFAM show a significant increase following 1 h of E2 treatment (Figures 2(b) and 2(c)). The calculated value of $2^{-\Delta\Delta}C^t$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.001$; and $^{***}p < 0.0001$.

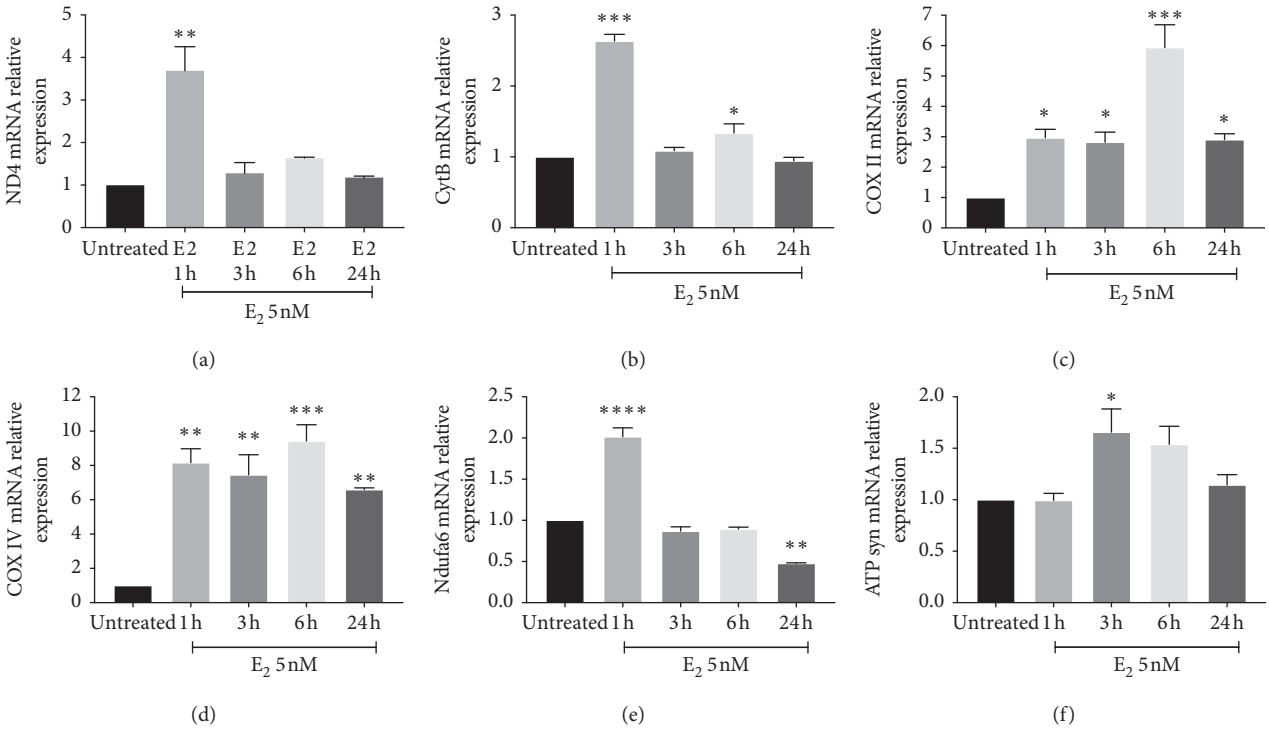


Figure 3: Effect of E2 on mitochondrial OXPHOS gene. E2 treatment significantly increased ND4, Cyb4, COXII, COXIV, COX, and NDUFA6 gene expression following 1 h treatment of E2 (Figures 3(a)–3(e)). Similarly, the treatment increases ATP synthase gene expression and peaks at 3 h (Figure 3(f)). The calculated value of $2^{-\Delta\Delta}C^t$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.001$; and $^{***}p < 0.0001$.

women approximately 40 years ago [20]. Recently, high concentrations of estradiol were shown to trigger apoptosis in adrenal carcinoma cells [23], indicating that the mechanisms of these cytotoxic effects of estradiol remain to be further elucidated. Glioblastomas are the most aggressive type of brain tumors, with a poor prognosis and a limited response to chemotherapy and other therapeutic strategies [24, 25]. Failure of therapy arises from the resistance of tumor cells to therapy-induced apoptosis [26]; therefore, new drugs targeting alternative pathways are required. In the present study, E2 induces cell proliferation and the expression of genes involved in mitochondrial metabolism in glioblastoma cells. Estradiol, the predominant form of estrogen, mediates its effects via the activation of intracellular signaling pathways on neurons and glial cells [27]. Previous studies concerning the effects of estrogens in cancer cells exhibited controversial results [28]. With regard to glioblastoma, epidemiological evidence suggests an E2 tumor suppressor role [29]. The rate of the development of glioblastoma is increased in men: women aged 15–49 years (women of reproductive age) have a

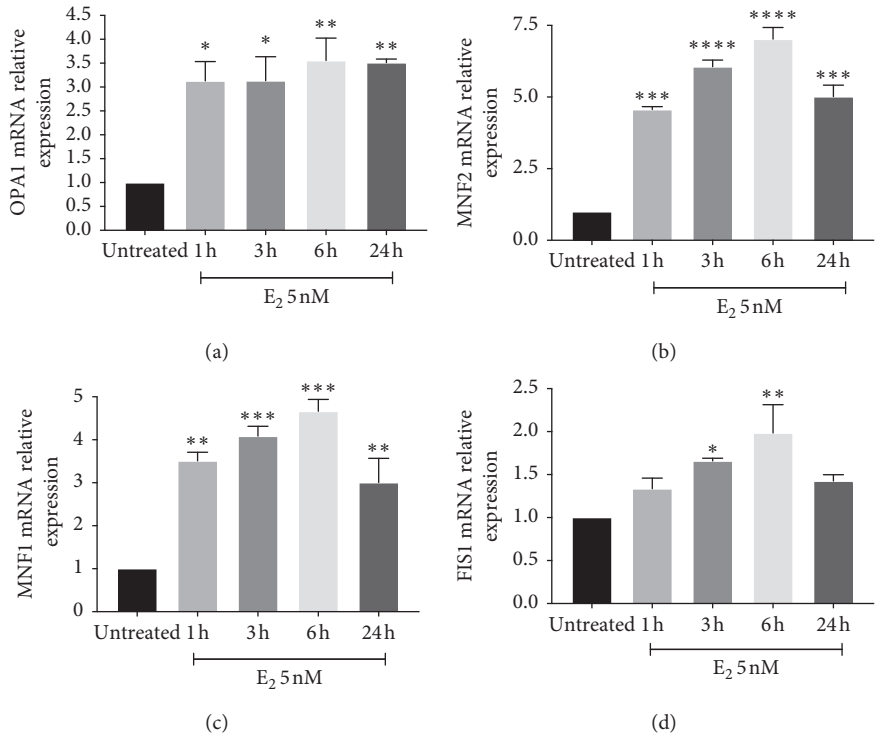


Figure 4: Effect of E2 on mitochondrial dynamics. E2 treatment increases OPA1, MNF2, and MNF1 gene expression following 1 hour of treatment (Figures 4(a)–4(c)). Consistently, E2 treatment increases FIS1 gene expression following 3 hours of treatment (Figure 4(d)). The calculated value of $2^{-\Delta\Delta}C^{t}$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.001$; and $^{***}p < 0.0001$.

survival advantage compared with men and postmenopausal women [29–31]. These results suggest that estrogens are involvement in the suppression of glioblastoma, but how they could do it is poorly understood. By contrast, our data suggest that E2 induces cell proliferation in the U87-MG glioblastoma cell line. However, a different expression of ERβ may explain, at least in part, the discrepancy with previously published reports. In this regard, multiple ERβ isoforms exist and may have distinct roles in various cancers [32–34]. The ERβ2 isoform is increased in chronic lymphatic leukemia, prostate cancer, non-small-cell lung cancer, breast cancer, and ovarian cancer [35]. The worsening disease-free survival and overall survival of patients were correlated with ERβ2 expression in patients treated with tamoxifen [36]. Moreover, ERβ2 is involved also in the metastasis of prostate cancer [37]. In addition, ERβ3 has restricted to testis [38]. ERβ5 is overexpressed in ovarian cancer and prostate cancer and associated with poor prognosis [39], while ERβ5 expression is associated with good prognosis in non-small-cell lung cancer and confers sensitivity to chemotherapeutic agent-induced apoptosis in breast cancer cells [39]. Several authors advanced that ERβ5 was highly expressed in primary and established GBM cells compared to ERβ1 and ERβ2, with ERβ4 [34, 40]. The data regarding the effect of E2 on glioblastoma progression are further supported by our results showing that E2 induces Nrf2 nuclear translocation and HO-1 expression. Estradiol also exerts nongenomic rapid actions via direct interaction of estradiol with plasma-associated ERs and the activation of second messenger pathways [41]. The late and sustained effects of estradiol described in this study suggest that nongenomic rapid actions of estradiol are not involved. In this regard, it has become evident that malignant cells benefit from having increased Nrf2 pathway activity: this was first observed in lung cancer [42], as well as subsequently in many other cancer types, such as pancreatic, ovarian, liver, and gallbladder cancers [43]. Aberrant Keap1-Nrf2 signaling leads to radio- and chemoresistance and provides a growth advantage to cancer cells, due to the constitutive expression of cytoprotective genes [44]. Multiple mechanisms for Nrf2 overactivation have been found, such as somatic mutations in either KEAP1 or NFE2L2, deletion of exon 2 of NFE2L2, aberrant expression of inhibitory proteins, and transcriptional induction by oncogenes and hormones [45]. Previous results demonstrated that, in GBM cells, inhibition of Nrf2 and p62 decreased tumorigenic properties, such as cell invasion and anchorage-independent growth [46]. Furthermore, Nrf2 could also function as a key balancing factor in metabolic reprogramming, as Nrf2 can regulate both energy metabolism and antioxidant response to ROS to favor glioma growth and development. Our results are consistent with these observations and showed that E2 resulted in a significant increase in the expression of genes involved in mitochondrial metabolism, biogenesis, and dynamics. Furthermore, our results showed that E2 resulted in a significant increase of HO-1, which is associated with increased chemoresistance and proliferative phenotype [47, 48], thus further confirming our

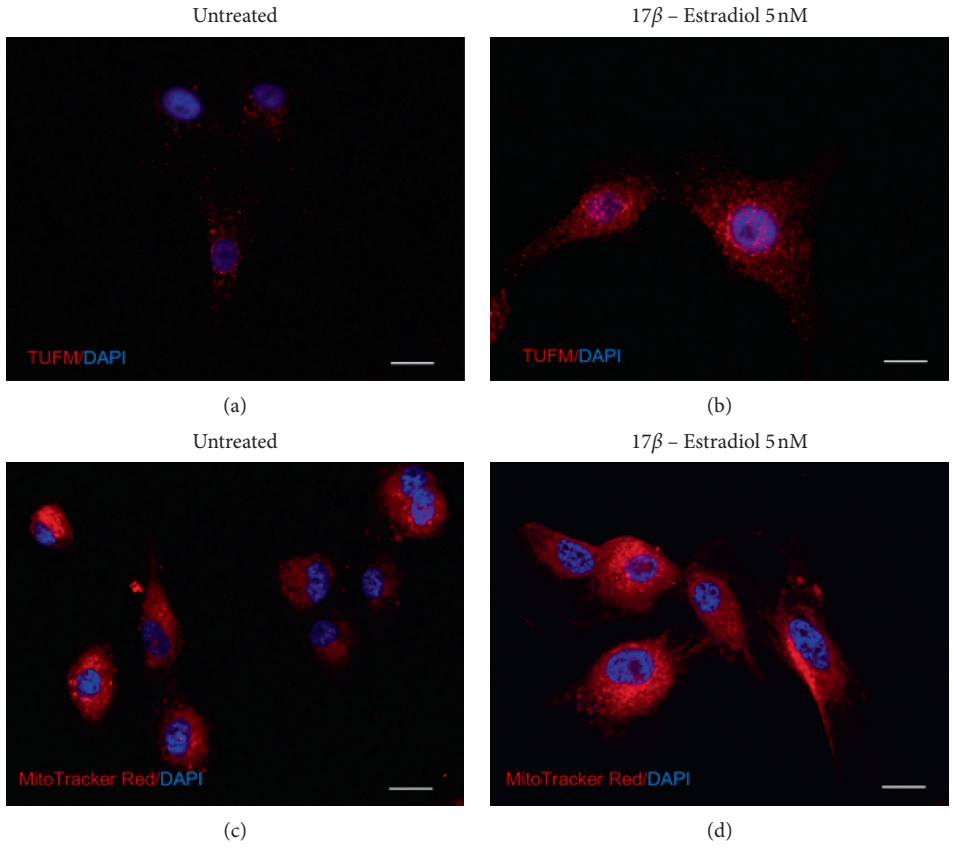


Figure 5: E2 increases mitochondrial mass in glioblastoma cells. The immunocytochemistry analysis demonstrates an increase in TFAM protein expression and mitochondrial network. Immunofluorescence staining of TFUM (red) was performed in U87-MG human glioblastoma cells in basal condition (Figure 5(a)) and after 24 hours of treatment with E2 (Figure 5(b)). The Mitotracker Red staining was performed in U87-MG human glioblastoma cells at basal condition (Figure 5(c)) and after 24 hours of treatment with E2 (Figure 5(d)). DAPI was used to stain the cell nucleus, and the scale bar is set as 10 μm.

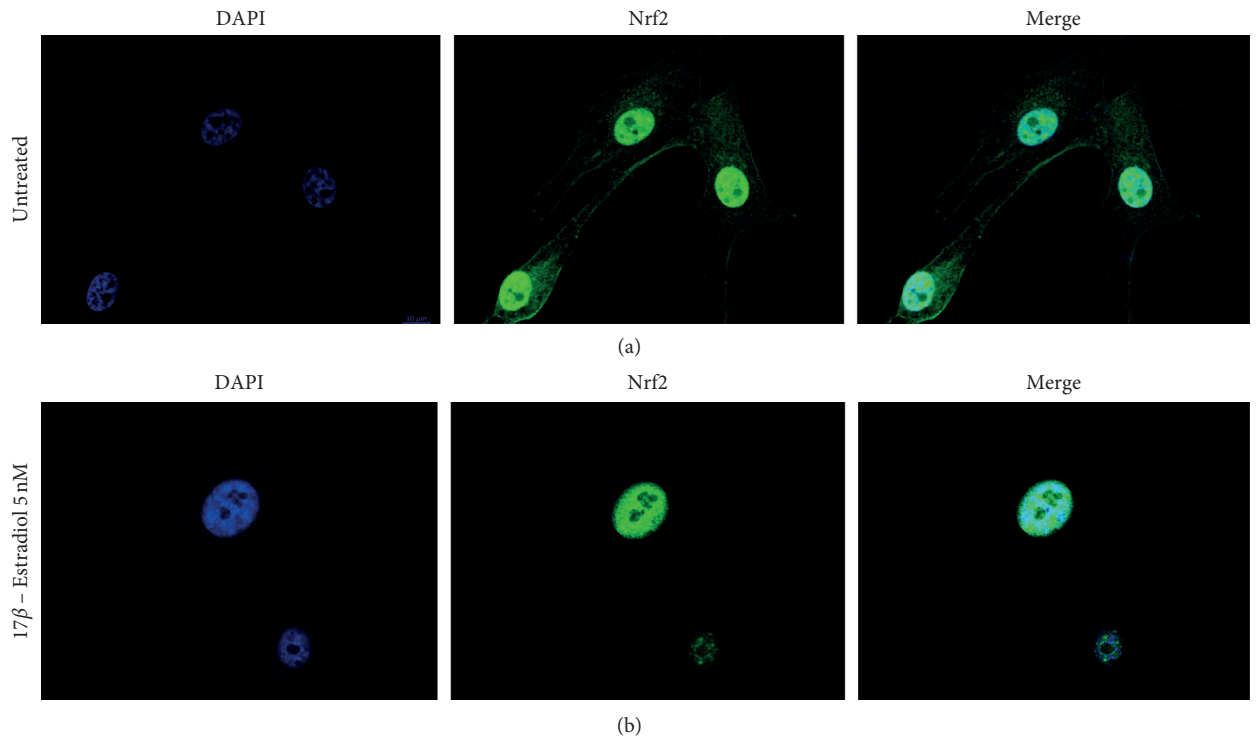
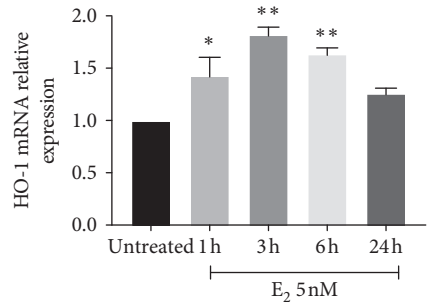


Figure 6: Continued.



(c)

Figure 6: E2 increases HO-1 expression and induces Nrf2 nuclear translocation. E2 treatment increases Nrf2 nuclear translocation following 24 h treatment when compared with untreated cells (Figures 6(a) and 6(b)). Consistently, HO-1, one of the Nrf2-targeted genes, is upregulated following E2 treatment ($p < 0.001$) (Figure 6(c)). Immunofluorescence staining of Nrf2 (green) was performed in U87-MG human glioblastoma cells at basal condition (Figure 6(a)) and after 24 hours of treatment with E2 (Figure 6(b)). DAPI was used to stain the cell nucleus, and the scale bar is set as 10 μm. Gene expression analysis of HO-1 was performed after 24 hours of treatment with E2 (5 nM) in glioblastoma cells. The calculated value of $2^{-\Delta\Delta}C^t$ in untreated controls is 1. Data are expressed as mean ± SD of at least four independent experiments (Figure 6(c)). $^*p < 0.05$; $^{**}p < 0.001$.

observations. Our data showed that E2 plays an important role in GBM progression, improving the mitochondrial fitness, highlighting its role in resistant mechanisms to the therapies: this can lead to a new therapeutic strategy for future studies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This work was part of the Ph.D. thesis of Dr. Carlo Castruccio Castracani (Neuroscience International Ph.D. program).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

CCC, MA, and GLV made a substantial contribution to the concept and design, acquisition of data or analysis, and interpretation of data; GLV, CCC, and DT drafted the article and revised it critically for relevant intellectual content; LL, AD, DA, SK, MC, DT, RA, and GLV performed in vitro experiments; all the authors approved the final version of the manuscript. Carlo Castruccio Castracani, Lucia Longhitano, Daniele Tibullo, and Giovanni Li Volti contributed equally to this work.

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Review Article

Heat Shock Proteins in Cancer Immunotherapy

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Heat shock proteins (HSPs) are highly conserved molecular chaperones with divergent roles in various cellular processes. The HSPs are classified according to their molecular size as HSP27, HSP40, HSP60, HSP70, and HSP90. The HSPs prevent nonspecific cellular aggregation of proteins by maintaining their native folding energetics. The disruption of this vital cellular process, driven by the aberrant expression of HSPs, is implicated in the progression of several different carcinomas. Many HSPs are also actively involved in promoting the proliferation and differentiation of tumor cells, contributing to their metastatic phenotype. Upregulation of these HSPs is associated with the poor outcome of anticancer therapy in clinical settings. On the other hand, these highly expressed HSPs may be exploited as viable immunotherapeutic targets for different types of cancers. This review discusses recent advances and perspectives on the research of HSP-based cancer immunotherapy.

1. Introduction

Cells respond to stressful conditions by activating stress response proteins that promote cellular sustenance. Heat shock proteins (HSPs) are highly conserved stress response chaperone proteins, which are synthesized in response to various stresses. These HSPs have cryoprotective and other critical cytoprotective functions. The ability of the HSPs to protect cells from damaging stress has been attributed to their chaperoning activity through which they prevent misfolding and expedite the refolding and renaturation of proteins [1, 2]. However, when reaching the limit of stress tolerance, the cells invoke programmed cell death (apoptosis or autophagy) to prevent irrevocable systemic damage to the organism. HSPs also play critical roles in inhibiting proapoptogenic molecules through modulation of several signaling cascades such as JNK, AKT, and NF-κB [3]. The HSPs are therefore at the core of maintaining a fine balance between cell death and survival, significantly impacting the biological consequences. The overwhelming evidence on the emerging role of HSPs in modulating carcinogenesis has precisely extended their relevance from simple diagnostic biomarkers to central targets in cancer therapeutics.

2. Role of HSPs in Cancer

HSPs are highly expressed in various types of carcinomas. The levels of circulating HSPs along with the antibodies to HSPs are excellent biomarkers for analyzing the stage and aggressiveness of certain types of cancer. HSPs are implicated in tumor cell proliferation, differentiation, invasion, and metastasis [4]. Some HSPs like HSP27 have been shown to contribute to the poor prognosis of osteosarcomas and gastric carcinomas [5]. HSP70 has been found to significantly influence the prognosis of breast cancer [6]. Expressions of HSP27 and HSP70 were reported to affect the response of tumor cells to conventional anticancer treatment [7, 8]. For instance, increased expression of HSP27 leads to poor outcome of chemotherapy in breast cancer and leukemia patients; by contrast, increased expression of HSP70 in osteosarcomas results in an improvement of the outcome of chemotherapy [9]. HSP60 is another key heat shock chaperonin protein which predominantly localizes in the

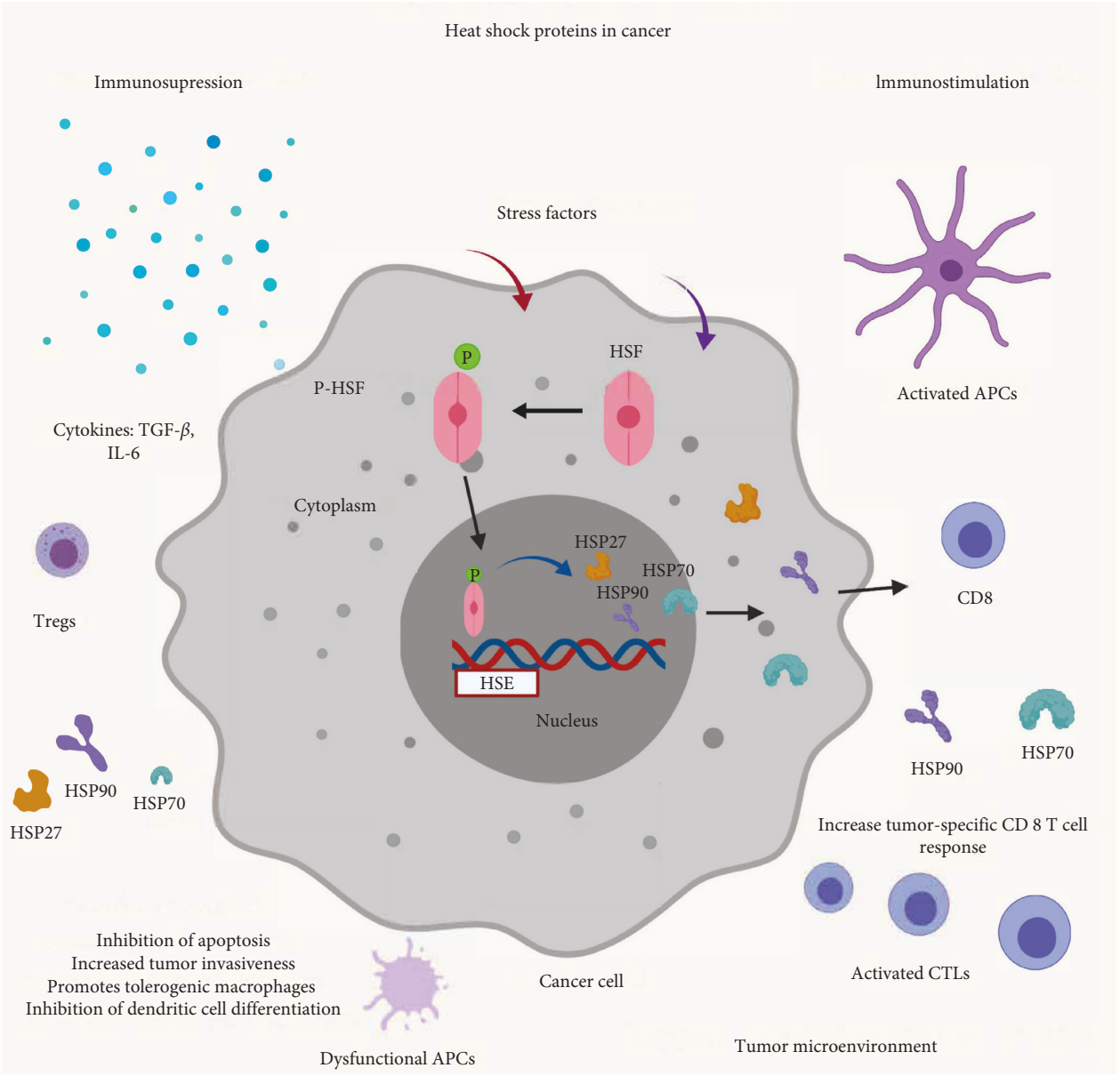

Figure 1: Heat shock proteins in cancer. Cancer cells are exposed to several stress factors from the extracellular milieu of tumor microenvironment. These stress factors activate heat shock transcription factors (HSFs) by facilitating their dissociation from heat shock proteins and phosphorylating them. The heat shock transcription factors are then translocated into the nucleus where they bind with heat shock elements (HSE) and initiate the transcription of heat shock proteins like HSP27, HSP70, and HSP90. The HSPs are exported into the tumor microenvironment modulating the immune response against cancer cells. In immunosuppressive conditions, the HSPs enhance the survival and proliferation of cancer cells by activating their cellular protection machinery. The HSPs may also stimulate the anticancer immune response under optimal conditions, thereby maintaining a fine balance between cell death and survival.

mitochondria and aids in the folding and transport of mitochondrial proteins. HSP60 has also been implicated in the progression of cancer, as the levels and the cellular localization of HSP60 were found to be altered in several different carcinomas [10]. The HSP60 chaperonopathic carcinomas could be inherited or acquired, in which this chaperone plays a significant etiologic-pathogenic role. Like the other HSPs, the high-molecular weight HSP90 chaperone is also a significant regulator of the process of tumor progression. The association of HSP90 with its client proteins has been explored extensively in cancer research. Several inhibitors of HSP90 have shown to be very effective against carcinogenesis [11, 12].

HSPs are not only involved in tumor progression but also in determining their response to treatment. Some of the critical HSPs intricately regulate the fine balance between the protective and destructive immunological responses within the tumor microenvironment (Figure 1), thereby making it imperative to comprehend their central roles in oncoimmunology. Modulating the expression or activity of HSP

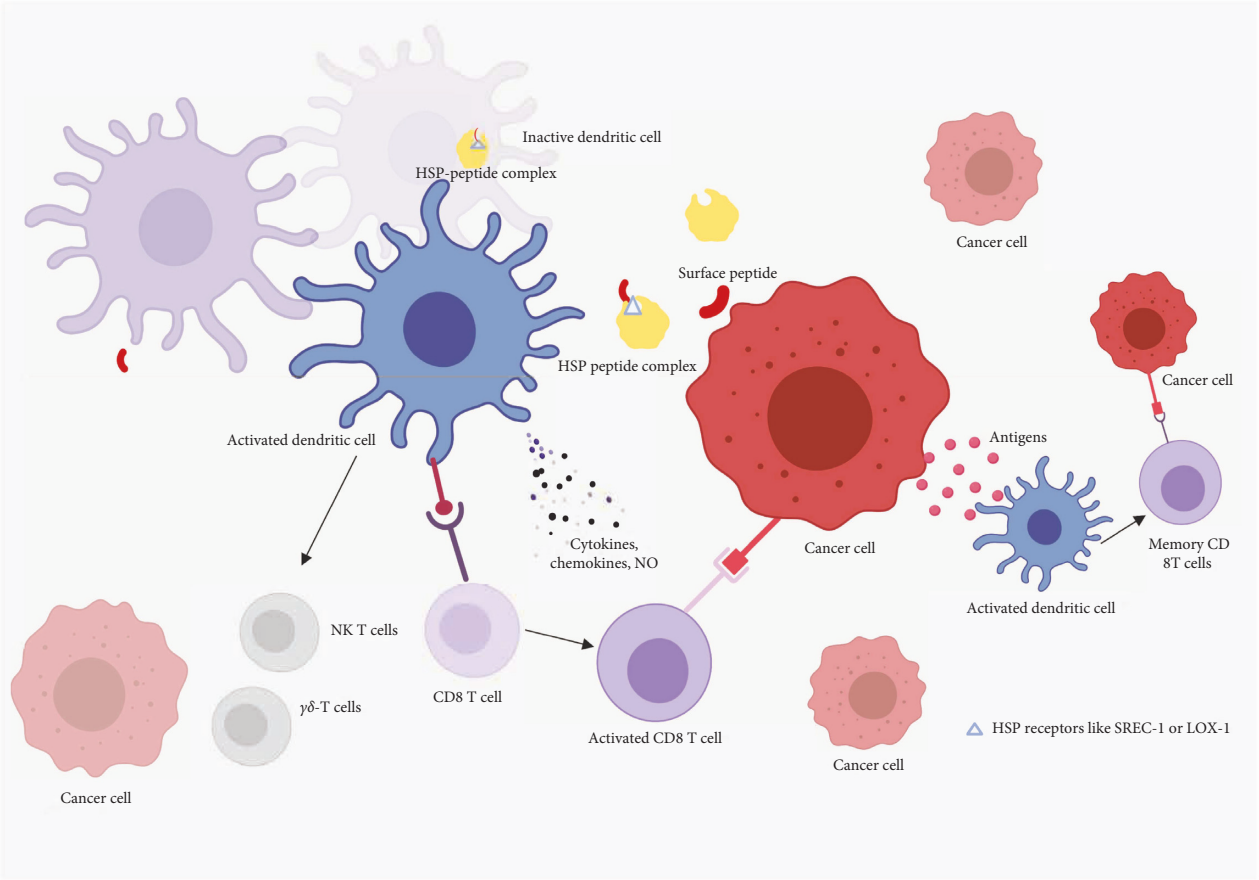

Figure 2: Overview of pathways in tumor antigen cross-presentation by the HSPs to the APCs. Cancer cells display limited surface peptides or antigens which are released into the extracellular milieu. These antigens are recognized by HSPs through HSP-receptors, such as SRECI and LOX-1. The HSP peptide complex may be either engulfed into the DCs through CD91 receptor-mediated endocytosis or recognized by the cognate receptors on the surface of these DCs, resulting in their activation. This leads to a cascade of subsequent innate and adaptive immunological responses against cancer cells. The activated DCs activate the $\gamma\delta$ T cells and NK T cells which may facilitate the lysis of the cancer cells. These DCs also produce inflammatory cytokines, chemokines, and nitric oxide. The activation of APCs results in the recognition and killing of cancer cells through cytotoxic CD8+ T-lymphocytes response. The lysis of cancer cells releases cancer antigens into the extracellular milieu leading to the formation of memory CD8+ T cells. The cross-presentation of HSP peptide complex to APCs is therefore an effective process bridging innate and adaptive immune response and mounting an optimal anticancer immunity. The inactive DCs/CD8+ T cells are represented in light color while the activated cells are represented in dark color. This illustration has been created with Biorender.com. DC-dendritic cells.

chaperones has been explored as an anticancer therapeutic strategy. The use of HSP-based immunotherapeutic approaches and development of anticancer vaccines have also greatly contributed to the enrichment of the field of oncoimmunology and assisted in devising more effective anticancer regimens. Cancer immunotherapy adopts a number of approaches including, but not limited to, targeted antibody therapies, immune-checkpoint inhibitors, cytokines, and adoptive cell transfer methodologies. HSPs are now considered as viable targets for cancer immunotherapy.

3. HSPs as Immunomodulants

Extracellular HSPs can bind to specific receptors on dendritic cells to promote the cross-presentation of their peptides [13]. Among the known receptors for HSPs, SRECI and LOX-1 are two of the most important receptors in this category. Stimulation of SRECI (a member of scavenger receptor family of receptors) and LOX-1 (a member of both the scavenger receptor and c-type lectin receptors family) enables the cross-presentation of HSPs with their associated peptides. LOX-1 mainly binds to HSP60 and HSP70, but SRECI binds to a wide range of common heat shock proteins including HSP60, HSP70, HSP90, HSP110, gp96, and GRP170 [14]. This cross-presentation of peptides is critical in immunosurveillance (Figure 2), as the bound peptide is not only protected against degradation but the efficiency of cross-presentation is also higher in the dendritic cells [15]. Also, the internalization of HSP-peptide complex is comparatively more efficient than the exclusive internalization of soluble antigens only. Moreover, there are very few neoantigens expressed on some tumor cells, limiting the amount of target antigens available for antigen-presentation. The cross presentation of HSP-antigenic peptide complex therefore broadens the spectrum of available HSP-peptide complexes as targets for the immune system. HSPs are also

known to bind antigenic peptides displayed on cancer cells, making the HSP-peptide complexes ideal vaccination targets for cancer therapy [14]. The binding of unknown peptides by HSPs in vitro also induces peptide specific vaccine response in HSP-based anticancer immunotherapy. The success of HSP derived anticancer vaccines largely stems from their efficiency in cross-priming across the diversity of human leucocyte antigen (HLA) barrier [16]. The binding of peptides with nonpolymorphic HSPs is a process similar to the association of MHC class 1 molecules with peptides in the antigen-presentation pathway. They can make use of different HLA haplotype cases and are not limited by their specificity. This remarkable phenotype of the HSP-peptide complex makes the prospect of generating general or quasi-general HSP vaccines a distinct possibility. Some HSPs like HSP70 and HSP90 are also involved in the intracellular cytosolic pathway of cross-presentation and transportation of antigens from the endosome into the cytosol [17].

HSPs regulate the production of a range of inflammatory cytokines including TNF-α, IL-6, IL-10, and IL-12 [18]. The modulation of the production of these cytokines determines the anticancer as well as anti-infective immune response by the host. Besides proinflammatory and anti-inflammatory cytokines, HSPs also regulate the production of nitric oxide (NO) and chemokines [19]. Innate and adaptive immune receptors recognize and bind HSPs initiating signal transduction and production of cytokines as well as effector cells. The immunomodulatory functions of HSPs have led to their classification as "Chaperokines" or molecular chaperones. The growing evidence of the roles of HSPs in immunomodulation makes these proteins potential therapeutic targets for ameliorating immunopathies, especially metastatic immunopathies. Here, we outline the current understanding of the role of the critical HSPs, such as HSP27, HSP60, HSP70, and HSP90, in cancer therapy. We also summarize the immunomodulatory activities of HSPs and recent advances in utilization of HSPs in anticancer immunotherapy.

4. HSP27

HSP27 belongs to the small HSP family, and its function is regulated by phosphorylation at serine residues. The phosphorylations of Ser 15, Ser 78, and Ser 82 regulate the growth, differentiation proliferation, and migration of cells. HSP27 can reverse epithelial to mesenchymal transition and decrease the matrix metalloproteinase activity. Thus, dysregulated expression of HSP27 drives tumor development and progression. It has been shown that the expression of HSP27 is correlated with an increase in the transcription of vascular endothelial growth factor gene [20], thereby promoting angiogenesis and cellular migration in metastasis of cancer.

Several studies have shown that HSP27 is significantly higher in different malignancies such as ovarian, prostate, and breast cancer [7, 21]. HSP27 was found to be present both inside and outside the cancer cells and binds to cytochrome c, inhibiting the activation of caspases and preventing apoptosis. A study has shown that HSP27 expression was significantly correlated with the Ki-67 index in brain tumors [22]. In another study, expression of HSP27 was shown to be associated with poor prognosis in patients with meningioma [23]. The cytoplasmic immunoreactivity of HSP27 is lowered in patients with meningioma, although the chaperone protein is detected ubiquitously in all the meningioma tissues [24]. Involvement of HSP27 in tumor invasion and metastasis signaling cascades is one of the factors contributing to the poor survival rates among patients. HSP27 expression was found higher in the biopsy tissues of prostate cancer and in the serum and tumor microenvironment of female breast cancer [25]. HSP27 levels were also significantly higher in the interstitial fluid isolated from primary breast tumor tissue [26].

The structural complexity of HSPs (e.g., HSP27) makes the design of their inhibitors challenging. However, there is compelling evidence suggesting that HSP27 could be an attractive target for cancer therapy. For instance, quercetin and RP101 are two small molecule inhibitors of HSP27, which have been investigated for their anticancer properties [27, 28]. Quercetin is a bioflavonoid compound that demonstrates anticancer activity. However, currently, there are no undergoing clinical trials with quercetin, which may be due to its potent cytotoxic activity [29]. RP101 is an antiviral nucleoside that has been successfully used as an anticancer HSP27 inhibitor in clinical studies [28, 30]. Similarly, several peptide and antisense oligonucleotide inhibitors of HSP27 have been devised by several groups [31, 32], but none of them have been approved by FDA for clinical use. Therefore, the HSP27-based immunotherapeutic approaches could play an important role in the treatment of cancer. For instance, an interesting study by Straume et al. showed that HSP27 was critical in maintaining the balance between the progression and dormancy of tumor, suggesting that immunological targeting of HSP27 could be a useful strategy [8]. They showed that HSP27 was upregulated significantly in the angiogenic cells of a MDA-MB-436 breast tumor xenograft model, and those results were validated in cell lines, mouse models, and clinical datasets. They also showed that stable downregulation of HSP27 in the angiogenic tumor cells resulted in long-term tumor dormancy, and remarkably, none of the tumor cells could escape dormancy. Similarly, Mahvi et al. showed that overexpression of HSP27 in the estrogen receptor-positive MCF-7 cells stimulated the proliferation of peripheral blood lymphocytes and promoted the lysis of MCF-7 cells by $\gamma\delta$ T cell clones. The role of HSP27 in modulation of vascular inflammation and chronic inflammatory disorders has been well-studied and established [33]. These studies, along with other similar evidences, indicate a great potential of the HSP27-targeted immunotherapeutic approach in treatment of cancer.

5. HSP60

HSP60 is an extensively studied heat shock protein, especially in the immunological context. Like other HSPs, it is an intracellular chaperone that facilitates homeostatic protein

folding and transportation [34]. HSP60 is particularly well studied in the context of autoimmune diseases [35]. Self HSP60 reactive lymphocyte clones were found in healthy and physiological conditions in mammals [36, 37], demonstrating that HSP60 are indeed the key players in physiological autoimmunity. Self-HSP-reactive T and B cell clones can be categorized as significant players in immunological signal transduction pathways. These molecules control inflammation by limiting clonal expansion and are also involved in maintenance and repair of tissue. The HSP60 chaperones are thereby vital components involved in maintaining cellular homeostasis through their immunomodulatory activities [38].

HSP60 chaperone is known to play an important role in the pathogenesis of cancers. It was reported that the oncogenic HSP60 drives the development of pancreatic ductal adenocarcinoma through modulation of mitochondrial oxidative phosphorylation (OXPHOS) [39]. Tumors promoted by HSP60 were classified as "chaperonopathies by mistake," as these molecular chaperones help promote the growth, proliferation, and metastasis of tumor cells and mediate their resistance to stressors, rather than protecting the host [40]. HSP60 is also known to be a dual regulator of apoptosis and has both pro- and antitumoral effects. Recently, a clinical study found that expressions of HSP60 and HSP70 are associated with a long-term outcome in patients with T1 high-grade urothelial bladder tumor following Bacillus Calmette–Guérin immunotherapy [41]. Also, It has been shown that immunization with a recombinant HSP60 of *Histoplasma capsulatum* elicits a protective immune response that is mediated by a subset of Vβ8.1/8.2 + T cells in a murine model [42]. Similarly, Yamazaki et al. showed that HSP60-reactive T cells accumulate in the gingival tissues of periodontitis patients [43]. Our own ongoing study has been focusing on cloning of the HSP60 reactive T-cell receptor alpha and beta chains for facilitating the directed differentiation of T lymphocytes from induced pluripotent stem cells (iPSs). Our study may have great potential to generate the HSP60-based novel immunomodulatory strategy for the treatment of various diseases including cancer.

6. HSP70

HSP70 is a high-molecular weight ubiquitous chaperone protein, which has a significant role in regulating cellular homeostasis, by controlling protein folding, translocation, biogenesis, and degradation [44]. Although HSP70 is primarily induced as a stress response protein, it is also constitutively expressed as a housekeeping gene in different types of cells. HSP70 contains a 44 kDa amino-terminal nucleotide binding domain with ATPase activity, 18 kD substrate binding domain, and a 10 kDa C-terminal lid [45]. HSP70 can be classified into two subfamilies: the canonical DNAk-like protein and the higher-molecular weight HSP110 [46]. The canonical HSP70s refolds misfolded proteins and suppresses protein aggregation, promoting the growth of cancer cells [47]. HSP110 members are structurally and functionally distinct from the canonical HSP70s, with a limited role in carcinogenesis, and will not be discussed in detail in this review.

The canonical HSP70 inhibits apoptosis through preventing the activation of Bax. However, HSP70 also promotes the release of proapoptotic factors and facilitates mitochondrial membrane permeabilization. Furthermore, HSP70 prevents the assembly of death inducing signaling complex (DISC) [48]. In addition, cellular senescence is induced through the p53-mediated downregulation of the canonical HSP70. In experimental models, overexpression of HSP70 was shown to increase the tumorigenicity of transformed cells, while the downregulation of HSP70 significantly decreased the tumorigenicity of the cells.

As constitutively increased expression of HSP70 leads to various types of cancers, neutralizing HSP70 has emerged as an attractive anticancer strategy. Chemotherapy increases HSP70 expression, which contributes to the resistance of cells to anticancer therapy and other cell-death inducing stimulus. Schmitt et al. have shown that a protein designated as ADD70 sensitized different human cancer cells to apoptosis by interacting with HSP70, suggesting that selective neutralization of HSP70 is beneficial in inducing apoptosis in drug-resistant cells [49]. Despite the success of targeting HSP70 as an anticancer therapeutic strategy, this approach has some limitations. For example, the inhibition of HSP70 results in undesirable cytotoxicity for normal cells, owing to its ubiquitous expression in physiological conditions. Therefore, there has been a concerted effort towards using HSP70-based targeted anticancer immunotherapy.

HSP70 anticancer vaccines have been successfully used in clinical settings with positive impact on cancer patients. For instance, a human fusion protein vaccine composed of HSP70-HPV16 oE7 antigen was shown to elicit effective CD8+ antitumor cell-mediated response [50]. Another study by Abkin et al. showed that the purified HSP70-based gel diffused effectively through the outer layer of B16 tumor, promoting intratumoral antitumor effects. Intratumorally derived HSP70 showed significant antitumor efficacy when combined with phloretin in a murine melanoma model [51]. In addition, Sato et al. reported that the leukemia cell-derived HSP70 has immunization effects and improved the survival of BALB/c mice after syngeneic bone marrow transplantation [52]. HSP70 on the cancer cell surface elevated NK cell toxicity [53] and enhanced dendritic cell maturation besides promoting the activation of T cells [54]. HSP70 is also known to initiate the functions of both the innate and adaptive immunity through the production of a range of cytokines [55]. These HSPs function as classical chaperones, enabling cross-presentation of antigenic peptides to APCs. The increased translocation of HSP70 into the extracellular milieu, which is triggered by the delivery of purified HSP70 into tumor microenvironment, enhances the sensitivity of cancer cells to conventional treatment options. The broad range of the aforementioned immunomodulatory activities of HSP70 makes it one of the most versatile HSPs for anticancer immunotherapy, with immense potential for future development.

7. HSP90

HSP90 is a molecular chaperone that facilitates the maturation of substrates. Several kinases, transcription factors, E3 ubiquitin ligases, and steroid hormone receptors are the partners of HSP90 and bind to HSP90 in highly dynamic conformations. The pleiotropic effects of HSP90 on several of its partner proteins implicate them in various diseases including neurodegeneration and cancer. Many of the client proteins of HSP90 are oncogenic drivers; therefore, inhibition of HSP90 is believed to have a therapeutic impact in treatment of cancer. Although the HSP90 inhibitors have been shown to be effective in solid tumors and hematological malignancies, these agents are not efficacious as stand-alone single agents in cancer patients. Combining HSP90 inhibitors with immunotherapy has been proposed as a promising strategy for exploration. For instance, Mbofung et al. have shown that inhibition of HSP90 effectively enhanced T-cell-based anticancer immunotherapy through upregulation of the interferon response genes. They observed that the combination of HSP90 inhibition with CTLA4 blockade greatly enhanced CD8+ T cell functions in tumor microenvironment [56].

Several lines of experimental evidence have established the essential role of HSP90 in antigen presentation with MHC I molecules on the cellular surface [57]. Recent studies have also shown that extracellular HSP90 binds to its peptide substrate, which is recruited by the heat shock protein receptors on antigen-presenting cells. The HSP90-peptide substrate complex is then internalized through the vesicles and processed by the proteasomes. These processed antigenic peptides are displayed on MHC II complex and released into the endoplasmic reticulum. The MHC II-peptide complex are subsequently transported to the cellular surface and presented to CD-4+ T cells, thereby activating a canonical cascade of anticancer immune response [58]. These immunological features of HSP90 indicate that inhibition of HSP90 might dampen natural immune responses, particularly anticancer immunity. Surprisingly, several independent studies have shown that HSP90 "clients proteins" like HIF-1α and JAK2 modulate immune-checkpoint blockade through induction of PD1 and PD-L1 expression [59, 60], suggesting that HSP90 inhibition could be used as an effective approach to enhancing anticancer immunotherapy. The efficacy of HSP90 inhibitors have since been validated in preclinical and clinical studies. Combined use of ganetespib (a HSP90 inhibitor) and STI-A1015 (an anti-PD-L1 antibody) in a syngeneic mice model bearing colon cancer or melanoma was proven to be an effective antitumor combination therapy [60]. Similarly, the HSP90 inhibitor, SNX-5422, also proved to be an effective antitumor agent when used in combination with monoclonal antibodies against PD-1, PD-L1, or CTLA4, in a colorectal cancer model system [61].

Development of anticancer vaccines has been tested with glycoprotein96 (gp96), an ER residing member of the HSP90 family of proteins. Immunogenic peptides chaperoned with gp96 were shown to elicit specific anticancer immune response, making this protein an ideal vaccine candidate. Several clinical trials in patients suffering from malignant melanoma have been conducted with gp96 chaperonic protein for testing its proposed efficacy between the years of 2000 to 2014 [62–65]. Clinical trials have also been undertaken with gp96 protein for gastric carcinoma, pancreatic carcinoma, and Hodgkin lymphoma and glioblastoma [66].

8. Overview of HSP70 and HSP90 Vaccines in Tumor Immunity

The HSP70 and gp96 (HSP90) vaccines are the most successful and widely used HSP vaccines.

Some common HSP70 and HSP90 vaccines are listed in Table 1. The interaction of these classical HSP vaccines with tumor immunological signaling network is complex; thus, a better understanding of this interaction is fundamental towards the development of improved and more effective anticancer HSP vaccines. Several studies have indicated that HSPs have the ability to induce T-cell tolerance, via not only shifting the cytokine response from a Th1- to a Th2-type but also promoting the suppression of the Th17 based inflammatory cytokine IL-17, with the simultaneous expansion of CD4+ CD25+ (Treg) cells [35]. The Th17 and Treg cells originate from a common precursor naïve CD4+ T cells. The disruption of the delicate Treg/Th17 balance creates an immune-suppressive environment conducive to the progression of carcinogenesis [70]. Treg cells promoted by HSPs inhibit immunological responses through the production of anti-inflammatory interleukin-10 (IL-10) cytokine. The shifting of the immune response from the inflammatory IL-17 towards the HSP driven IL 10 production may impede the mounting of an optimal immune response and induce T-cell tolerance. This environment may result in inhibition of the activity of cytotoxic T-lymphocytes and lead to the prevention of maturation of dendritic cells, thereby reducing the antigen presentation capacity [71]. However, despite these apparent limitations of using autologous and purified HSP vaccines, several HSP70 and gp96 vaccines have been successful. The interaction of HSPs with the immune system may result in a very complex outcome, which has been previously demonstrated with the inverse dose-immune response relationship of gp96 vaccines [72].

In the case of HSP70-based vaccines, the immune response is biased away from the generation of the Tregs towards the Th17-based killing of cancer cells by the CTLs [73]. Cytokines are the most potent determinants of the Treg/Th17 homeostatic balance. For instance, although the initial differentiation of Th17 and Tregs is driven by the common tumor growth factor (TGF)-β signal, IL-6 is critical in maintaining the subsequent homeostasis of Treg/Th17 balance [74]. Therefore, the presence of a suitable spectrum of cytokine milieu composed of IL-6 and TGF-β [71, 73] appears to be essential towards the functioning of HSP70 vaccines. The requirement of IL-6 for the optimal functioning of HSP70 vaccines has been demonstrated by a study showing that exclusive elevation of HSP70 was not sufficient for elimination of pancreatic tumors. In the same study, the authors found that the immune response was skewed towards the Treg cells rather than the Th17 cells [75], and this

Table 1: Common HSP70 and HSP90 vaccines.

HSP vaccine	Target carcinoma	Clinical trial	Reference
Vitepsin-Gp96-based vaccine	Several-liver, ovarian, glioma, melanoma, etc.	Phases II and III, approved in some places.	[67]
HSP.PC 96-Gp96-based vaccine	Renal carcinoma	Phases II and III	[68]
HSP. 70PC-HSP70-based vaccine	Breast cancer	Phase I	[69]
HPV16oE7-HSP70-based vaccine	Cervical cancer		[50]

may be attributed to the lack of IL-6 in this specific tumor microenvironment. The recent developments in understanding antitumor HSP vaccine-mediated immunity has a promising potential to improve adoptive T cell transfer therapy, using patient's own T cells in conjunction with or independently of HSP anticancer vaccines, to recognize and kill tumor cells.

9. Conclusion

Cancer immunotherapy has gained great success as an effective therapeutic option in our fight against some malignant tumors such as melanoma. The potential of HSPs as therapeutic targets for immunotherapy has been increasingly appreciated in the past decade. Recently, several important advances have been made in the field of HSP-based oncoimmunology, including the usage of anticancer vaccines. The HSP-based anticancer vaccines have been shown to be effective against a spectrum of antigen-expressed tumors, as they not only promote the uptake of antigens by APCs but also trigger the activation of T lymphocytes. Nevertheless, improving the efficacy of penetration of the activated CTLs into tumor microenvironment remains a challenge. A better understanding of the role of HSPs in the modulation of tumor microenvironment may help greatly in designing more effective immunotherapeutic strategies. Notably, the safety and efficacy of anticancer vaccines have been improved through combination therapies, including the use of chaperone-based immunotherapy in combination with immune-checkpoint inhibitors such as the inhibitors of CTLA-4, PD-1, and PD-L1. Targeting of HSPs may also sensitize cancer cells to conventional treatments such as chemotherapy and radiotherapy. It is anticipated that the HSP-based immunotherapy shall remain a major focus in the cancer therapeutic area, with the hope for more discoveries that can be exploited as therapeutic interventions in treating patients with cancer.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

Acknowledgments

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Research Article

2-Methoxyestradiol and Its Combination with a Natural Compound, Ferulic Acid, Induces Melanoma Cell Death via Downregulation of Hsp60 and Hsp90

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Melanoma is an aggressive type of skin cancer with one of the highest mortality rates. Notably, its incidence in the last few decades has increased faster than any other cancer. Therefore, searching for novel anticancer therapies is of great clinical importance. In the present study, we investigated the anticancer potential of 2-methoxyestradiol, potent chemotherapeutic, in the A375 melanoma cellular model. In order to furthermore evaluate the anticancer efficacy of 2-methoxyestradiol, we have additionally combined the treatment with a naturally occurring polyphenol, ferulic acid. The results were obtained using the melanoma A375 cellular model. In the study, we used MTT assay, flow cytometry, and western blot techniques. Herein, we have evidenced that the molecular mechanism of action of 2-methoxyestradiol and ferulic acid is partly related to the reduction of Hsp60 and Hsp90 levels and the induction of nitric oxide in the A375 melanoma cell model, while no changes were observed in Hsp70 expression after 2-methoxyestradiol and ferulic acid treatment separately or in combination. This is especially important in case of chemoresistance mechanisms because the accumulation of Hsp70 reduces induction of cancer cell death, thus decreasing antitumour efficacy.

1. Introduction

Melanoma is an agressive type of skin cancer with one of the highest mortality rates, while its incidence in the last few decades has increased faster than any other cancer [1]. Although there has been tremendous progress in the treatment of melanoma patients in recent years, and in over the last 7 years the US Food and Drug Administration (FDA) has authorized many antimelanoma drugs, the ideal treatment is still not clearly defined and remains the subject of great debates [2].

While being an integral evaluation criterion of graduation of melanoma for many years, Clark's level is no longer recommended as it is not an independent prognostic factor. Histological features such as tumour thickness, but also rate of mitosis, are crucial for prognosis and determination of the stage of melanoma [3]. The evidence-based analysis that led to the development of recommendations for the assessment of melanoma progression was based on the updated database of the US Cancer Staging Manual (AJCC). The Melanoma Evaluation Committee recommended that the mitotic rate should be determined by the "hotspot" method and expressed as the number of mitoses per square millimeter of the primary tumour [4]. The Melanoma Evaluation Committee recommended that the mitotic rate should replace Clark's level as the main criterion for determining T1b

melanoma [5]. Moreover, it is enormously vital to constantly strive to explore knowledge about substances that can increase the effectiveness of cancer therapies. A larger understanding of the molecular mechanisms of potential drugs can lead to creating new or developing existing therapies that take into account the individual physiological profile of the patient.

Anticancer agent that may be effective in treatment of melanoma is 2-methoxyestradiol (2-ME), which is a natural compound, a metabolite of 17β-estradiol, and a hormone of both women and men [6]. 2-ME is a monomethyl ether of 2-hydroxyestradiol formed in the reaction catalyzed by catechol-O-methyltransferase (COMT). Its physiological level in the blood serum ranges from 30 pM up to 30 nM during pregnancy [6], while pharmacological relevant concentrations involve micromolar concentrations [7]. Induction of nitro-oxidative stress is involved in antitumour activity of 2-ME against various cancer cellular models. In our previous studies, we evidenced that 2-ME, at both physiological and pharmacological relevant concentrations, increases the nuclear fraction of neuronal nitric oxide synthase (nNOS) in osteosarcoma 143B cells. Thus, we suggested nNOS as a molecular messenger of 2-ME. Induction of nNOS via 2-ME increased production of nitric oxide leading to DNA strand breaks and eventually cell death [8]. Another anticancer mechanism of 2-ME revealed by our team is regulation of mitochondrial biogenesis and inhibition of the activity of succinate dehydrogenase complex in osteosarcoma 143B cells [9].

The effectiveness of 2-ME has been demonstrated *in vitro* in many cancers, including lung cancer, breast cancer, colorectal cancer, and pancreatic cancer [10–16]. Currently, 2-ME trade name PANZEM is in the second phase of clinical trials in the treatment of kidney, prostate, ovarian, and carcinoid tumours with high metastatic potential [7, 17–26]. Notably, 2-ME seems to be cytotoxic towards melanoma cells in both *in vitro* and *in vivo* models [12, 27–29]. It is further hypothesized that 2-ME specifically kills cancer cells without affecting normal cells [30].

In the current study, we combined 2-ME with a natural compound, ferulic acid (FA). FA belongs to the group of hydroxycinnamic acids found in plant tissues [31] (Figure 1). FA is a phenolic compound that possesses three characteristic structural domains that may contribute to the ability to reduce free radicals [32]. The antioxidant properties of FA depend on its chemical structure [33]. FA, due to the phenolic structure and the unsaturated side chain, may easily form a resonant-stabilized phenoxyl radical, which is responsible for its strong antioxidant activity [34]. The health benefits of using phenolic compounds, such as FA, attract the attention of many researchers due to their antioxidant potential. Antitumor activity of polyphenols includes antiproliferative and proapoptotic effects in tumour cells [35]. Phenolic acids of plant origin, like FA with strong antioxidant activity, have received special attention as potential tumour inhibitors [36].

Notably, major heat shock proteins, such as Hsp90, Hsp70, and Hsp60, may be considered as biomarkers for cancer diagnosis and prognosis, as well as efficacy of anticancer therapies [37–42]. These Hsps are also implicated in cancerogenesis and further progression of melanoma [43–45]. Therefore, herein we address the question about the role of major Hsps in efficacy of supportive anticancer treatment of FA separately and in combination with a potent, anticancer agent, 2-ME, in the A375 melanoma cellular model.

2. Materials and Methods

2.1. Cell Culture. Human melanoma A375 cells (CRL-1619) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37°C.

2.2. Experimental Design: Cell Treatment. In the study we used the A375 human melanoma cell model. First of all, the A375 cells were seeded in the standard medium at appropriate densities on the plates according to the specific experimental design 24 h before the treatment. The treatments were performed in DMEM containing 1% charcoal-stripped FBS and 1% antibiotic cocktail (Sigma-Aldrich, Poland). Charcoal-stripped FBS is used to elucidate the effects of hormones in various *in vitro* systems.

Subsequently, the A375 cells were treated with 2-ME separately or in combination with FA for 24 hours or 8 hours according to the experimental design. Based on previous research, 10 μM 2-ME was used [8, 46]. While, based on MTT results, we chose 1 mM FA for further studies. Following the incubation, the cells underwent procedure according to the specific experimental design described below.

In order to avoid the impact of the solvents, for the further studies, control cells were treated with an equal volume of the solvent used to prepare 2-ME and FA solutions. The final concentration of solvents in the incubation medium was less than 0.1%.

2.3. Cell Viability/Cell Proliferation Assay (MTT Assay). A375 melanoma cells were seeded into a 96-well plate at a density of 10,000 cells per well. After 24 hours, the cell culture medium was removed and the cells were treated with serial dilutions of FA within the concentration range between 1 mM and 31.25 μM. Based on the results, for further studies 1 mM FA was chosen. Consequently, the cells were treated with 1 mM FA and 10 μM 2-ME separately or in combination for 24 hours. Solvent-treated A375 melanoma cells were considered as the control (100% of cell viability).

After the appropriate incubation time, 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added (Sigma-Aldrich, Poland). The plates were incubated at 37°C for 4 hours, and the supernatant was removed after centrifugation (700 $\times g$ for 10 min). Finally, 100 μl of DMSO (Sigma-Aldrich, Poland) was added to dissolve the formazan crystals. Absorbance at 570 nm was

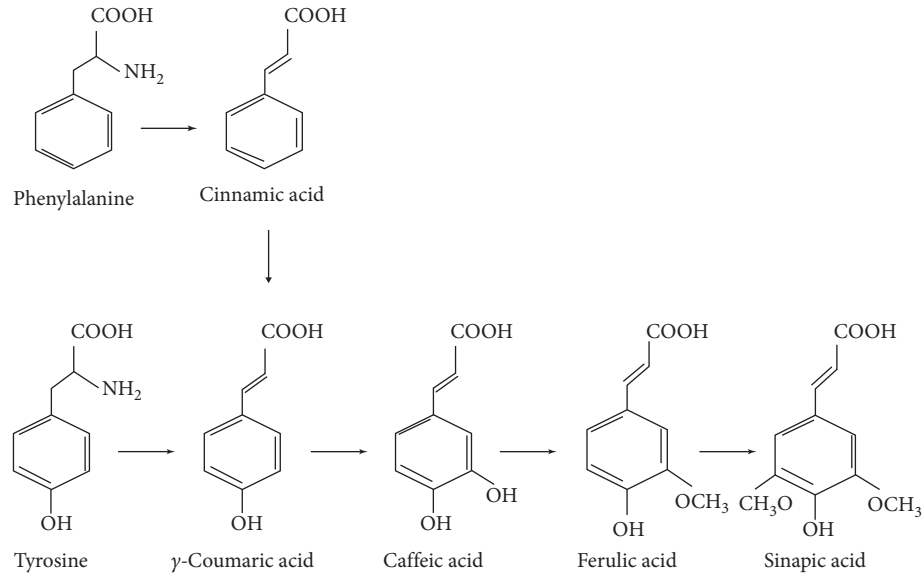

Figure 1: The synthesis pathway of hydroxycinnamic acids in plants (Castelluccio i wsp., 1995).

read using a microplate reader (BioTek Instruments, Inc., USA). The data are presented as a percent of control. Each experiment was carried out at least three times.

2.4. Determination of the Nitro-Oxidative Stress Pool by Flow Cytometry. A375 cells were seeded into 6-well plates at the density of 300,000 cells per well. Subsequently, A375 cells were treated with 1 mM FA and 10 μM 2-ME separately or in combination for 8 hours. Eight hours incubation time for nitro-oxidative stress was based on our previous results [8, 46].

The level of oxidative stress was determined by the fluorescence intensity of 2,7-dichlorofluorescein diacetate (DCF-DA), and DCF was added at 10 μM final concentration 30 minutes before the end of incubation time. DCF does not show fluorescent properties until the oxidation reaction is carried out in presence of free radicals.

The cells were detached from the plates with trypsin, collected, and centrifuged (1200g for 5 minutes). Washed twice with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, and 4.3 mM Na2HPO4, pH 7.4), suspended in PBS, and then analyzed by flow cytometry. The nitric oxide level was determined using a 4,5-diaminofluorescein diacetate (DAF-DA) detector. A LSR II flow cytometer (Becton Dickinson, USA) equipped with FACSDiva software was used. The entire procedure was carried out on ice. Then 30,000 cells were counted and analyzed by flow cytometry (BD FACScan) with a dye spectrum filter (excitation $l = 495$ and $l = 530$). The results were analyzed using Cyflogic software, version 1.2.1. The procedure was repeated at least 3 times to ensure repeatability of results.

2.5. Analysis of Apoptosis and Necrosis by Flow Cytometry. Analysis of the level of apoptosis and necrosis was performed by means of flow cytometry. Briefly, A375 cells were seeded in 6-well plates at a density of 300,000 cells per well. After 24 hours, the cells were treated with 1 mM FA and 10 μM 2-ME separately or in combination for 24 hours. The cells were then trypsinased and then harvested by centrifugation at 1200g for 7 minutes. The samples were washed 3 times with ice-cold PBS. The cells were then incubated with annexin V and PI for 15 minutes at room temperature. The whole procedure except incubation with annexin V and PI was carried out on ice. The cells were then counted at 30,000, and the fluorescence signals of annexin V and PI conjugate were detected in fluorescence intensity channels FL1 and FL3 (BD FACScan). The results were analyzed using Cyflogic software, version 1.2.1. The procedure was repeated at least 3 times to ensure repeatability of results.

2.6. Analysis of Hsp 70, 60, and 90 Protein Levels by Western Blot Technique. The level of Hsp 70, 60, and 90 proteins and β-actin were determined by western blot technique. After 24 hours, the cells were treated with 1 mM FA and 10 μM 2-ME separately or in combination for 24 hours. Then, the cells were harvested and centrifuged. The pellets were washed 3 times with PBS (Sigma-Aldrich, Poland) and then suspended in RIPA buffer (Sigma-Aldrich, Poland) and a cocktail of protease inhibitors (Calbiochem, Germany). Protein concentration was determined using the Bradford reagent [Bradford, 1976]. Afterwards, samples containing 100 μg of protein were mixed with Laemmli loading buffer (Sigma-Aldrich, Poland) and incubated at 95°C for 10 min. The proteins were separated on a 7–20% gradient of polyacrylamide gel (GE Healthcare, Poland) by electrophoresis. The separated proteins were transferred to a methanol-activated PVDF membrane in TBE buffer (90 mM Tris, 90 mM boric acid, and 1 mM EDTA, pH 8) using a semi-dry transfer device (250 mA, 63 V, and 45 minutes) (GE Healthcare, Poland). Then, after 1 hour of blocking in 5% nonfat milk in TBS-T (0.5% Tween20, 20 mM Tris-HCl, pH 7.4, and 0.5 M NaCl), the membranes were incubated with

primary antibodies overnight at 4°C. The Hsp90 beta antibody (catalog number ab80159) and Hsp70 antibody [EP1007Y] purchased from Abcam (catalog number ab45133), and Hsp60 antibody (H-1) (catalog number sc-13115) and beta-actin (catalog number sc-47778) purchased from Santa Cruz Biotechnology were used in the study. After incubation time, the membranes were washed 3 times for 5 minutes in TBS-T and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1 : 50,000 dilution in TBS-T) for 1 hour at room temperature. Visualization was performed using chemiluminescence enhanced with a luminol reagent (chemiluminescence blotting, GE Healthcare, Poland) according to the manufacturer's protocol. The signal was read using ImageQuant LAS 500 (GE Healthcare, Poland). Protein levels were quantified using densitometry analysis by the Quantity One program. The results were normalized to β-actin. Each experiment was carried out at least three times.

2.7. Statistical Analysis of the Obtained Results. The results are represented by the mean ± SD of at least three independent experiments. Differences between control and treated samples were assessed by means of one-way analysis of variance (ANOVA) with a post hoc test using Tukey's multiple comparison test. A p value less than 0.01 was considered to be equivalent to statistical significance. Data were analyzed using GraphPad Prism (GraphPad Software, Inc., version 6, USA).

3. Results

3.1. Antiproliferative Effect of FA and 2-ME in the Melanoma A375 Cellular Model. First of all, we addressed the question about antiproliferative efficacy of FA in A375 cells by means of MTT assay. The antiproliferative potential of FA was evaluated by 24-hour treatment of A375 cells with serial dilutions of FA within the concentration range between 1 mM and 31.25 μM (Figure 2(a)). The percentage of viable cells in samples was calculated in comparison to control A375 cells, which viability was assumed to be 100%. Based on the survival curves obtained by the GraphPad Prism Software, the calculated EC50 (50% decrease in the viability of the treated cells) concentration was equal to 701.9 μM.

Therefore, for further studies, a representative concentration of 1 mM FA was chosen. The next goal of the study was to determine the efficacy of combined treatment of FA with a potent anticancer agent, 2-ME, in the melanoma A375 cellular model. The concentration of 10 μM of 2-ME was chosen as representative, corresponding to the pharmacological concentration range, based on previous studies [8, 46]. As presented in Figure 2(b), we did observe statistical significant correlation between combined treatment of FA and 2-ME as compared to separate treatments.

3.2. Effect of Combined and Separate Treatment with FA and 2-ME on Induction of Melanoma A375 Cell Death. In order to further explore the anticancer efficacy and interaction between 2-ME and FA in A375 cells, we next determined the impact of the compounds on the induction of cell death.

As demonstrated in Figure 3, 24-hour treatment with 10 μM 2-ME or 1 mM FA did not significantly increase the number of early and late apoptotic cells, while increased the number of necrosis up to 9% and 4%, respectively. Notably, combined treatment with 2-ME and FA induced both apoptosis and necrosis in A375-treated cells. We observed approximately 10% apoptotic cells and 25% necrotic cells after 24-hour combined treatment with 10 μM 2-ME and 1 mM FA as compared with control cells (0.6% apoptotic cells, 3% necrotic cells, respectively) (Figure 3).

3.3. Nitro-Oxidative Stress Is Involved in Anticancer Mechanisms of 2-ME and FA in the Melanoma A375 Cellular Model. Due to the fact that both 2-ME and FA may regulate the level of reactive oxygen (ROS) and nitrogen species (RNS) in cancer cells [47–50], we evaluated the effect of the compounds on pool of nitro-oxidative stress in the melanoma A375 cellular model by means of flow cytometry. First of all, we performed the experiments using DCF-DA staining to determine the level of reactive oxygen species [51].

As demonstrated in Figure 4(a), 8-hour treatment with 1 mM FA reduced the level of DCF-DA-stained cells which confirms its antioxidant properties. On the other hand, separate 8-hour treatment with 10 μM 2-ME increased the level of oxidative stress in melanoma A375 cells. Notably, FA scavenged the 2-ME-generated oxidative stress in our experimental model (Figure 4(a)).

We have previously evidenced that one of the anticancer modes of 2-ME is associated with a selective increase in the nitric oxide level [8, 9, 46, 47]. Therefore, in the next part of the study, we aimed to determine the impact of compounds on changes within the level of nitric oxide in melanoma A375 cells via DAF-DA staining [8, 52, 53]. Notably, herein, we evidenced that induction of nitric oxide after 8-hour treatment with 10 μM 2-ME can be also extended to the melanoma A375 cellular model (Figure 4(b)). We further evaluated that separate 8-hour treatment with 1 mM FA either increased the level of nitric oxide in the established experimental model. Interestingly, combined 8-hour treatment with 2-ME and FA significantly increases the level of free radical as compared to separate treatment with both compounds (Figure 4(b)). This result may suggest an observed synergistic effect between 2-ME and FA in melanoma cells.

3.4. Effect of 2-ME and FA on the Level of Major Hsps: Hsp60, Hsp70, and Hsp90 in the Melanoma A375 Cellular Model. Due to the fact that Hsps may be considered as the indicators and biomarkers of nitro-oxidative stress, we determined the impact of both 2-ME and FA on the level of Hsp60, Hsp70, and Hsp90 by means of western blotting analyses.

At the outset, we evaluated the influence of the 24-hour treatment with 10 μM 2-ME and 1 mM FA, separately or in combination, on the Hsp60 protein level. As presented in Figure 5(a), western blot analyses of Hsp60 indicate a decrease in Hsp60 protein level by 35% and 65% relative to the

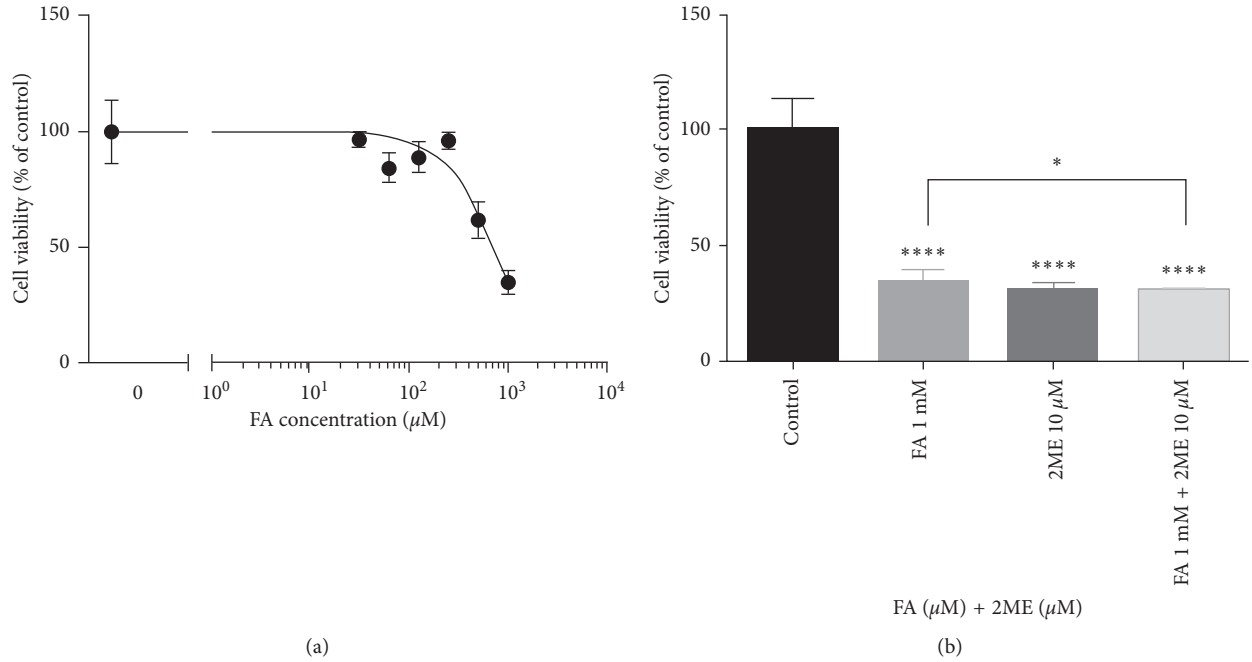

Figure 2: (a) The A375 cell viability graph after incubation with FA within the concentration range between 1 mM and 31.25 μM for 24 hours. (b) The viability of melanoma A375 cells is inhibited after treatment with 10 μM 2-ME, 1 mM FA, and combination of both for 24 hours. The cell viability was determined by MTT assay. Values are the mean ± SE of six independent experiments ($N = 6$ repeats). $^{*}p < 0.01$ and $^{****}p < 0.00001$ vs. control. Statistical significance was determined by a one-way ANOVA analyses followed by Tukey's multiple comparison test and unpaired t test.

control, after separate treatment with 10 μM 2-ME and after combined treatment with 10 μM 2 ME and 1 mM FA in melanoma A375 cells, respectively. The Hsp60 protein level was not significantly changed after combined treatment with separate treatment with 1 mM FA.

Subsequently, the changes within Hsp70 protein level were investigated in the melanoma A375 cellular model (Figure 5(b)). Notably, the obtained results indicate no changes in Hsp70 protein level as compared to the control after 24-hour treatment with both 10 μM 2-ME and 1 mM FA separately or in combination.

Furthermore, our western blot analyses indicate a decrease in Hsp90 protein expression in the A375 cell line by 13%, 29%, and 69% relative to the control, after 24-hour treatment with 1 mM FA and 10 μM 2 ME separately and in combination, respectively (Figure 5(c)).

4. Discussion

In the current study, we presented the anticancer potential of 2-ME in the melanoma cellular model. Previously, the efficacy of 2-ME towards melanoma cells was investigated in both *in vitro* and *in vivo* studies [12, 27, 28, 54]. 2-ME has pleiotropic activity in cancer cells. Interestingly, 2-ME suppresses the glycolytic state of melanoma 435R cells [27]. Moreover, 2-ME treatment decreases pRb and cyclin B1 expression, increases p21/Cip1 expression, and induces G2/M cell cycle arrest in both 2D and 3D melanoma cellular models [12].

Notably, employed in our studies a natural compound, FA, has anticancer potential and even enhanced anticancer activity of 2-ME in melanoma cells. It is suggested that phenolic compounds generally maintain normal homeostasis by inducing apoptosis in various tumour cells [55]. Many studies investigated cytotoxic and proapoptotic effects of polyphenols in various cancers [36, 50, 56, 57]. In consistency with our outcomes, Park et al. established the anticancer potential of FA in the mouse B16F10 melanoma cells [58]. Furthermore, Khanduja et al. proved that phenolic compounds, such as FA, significantly reduce apoptosis in normal peripheral blood mononuclear cells, which suggests limited cytotoxicity of FA [59]. Even more importantly, the significant role of FA in the prevention of skin cancer was also proved [57].

The cytotoxic activity of both compounds seems to be strictly associated with induction of nitro-oxidative stress. In our previous studies, we evidenced that 2-ME selectively upregulates neuronal nitric oxide synthase which results in generation of nitric oxide in cancer cells [8]. Herein, indeed we observed increased level of nitric oxide both after treatment with FA and 2-ME. This effect was even enhanced after combined treatment with the compounds. The mechanism of induction of nitric oxide by FA in cancer cells still needs to be evaluated. Nonetheless, FA was reported to generate nitric oxide through upregulation of argininosuccinate synthase in inflammatory human endothelial cells [60]. On the other hand, FA inhibits nitric oxide production and inducible nitric oxide synthase expression in rat primary astrocytes [61].

Notably, in contrast to altered nitric oxide induction, FA scavenged ROS in our melanoma experimental model. The compounds were also able to reverse 2-ME induction of

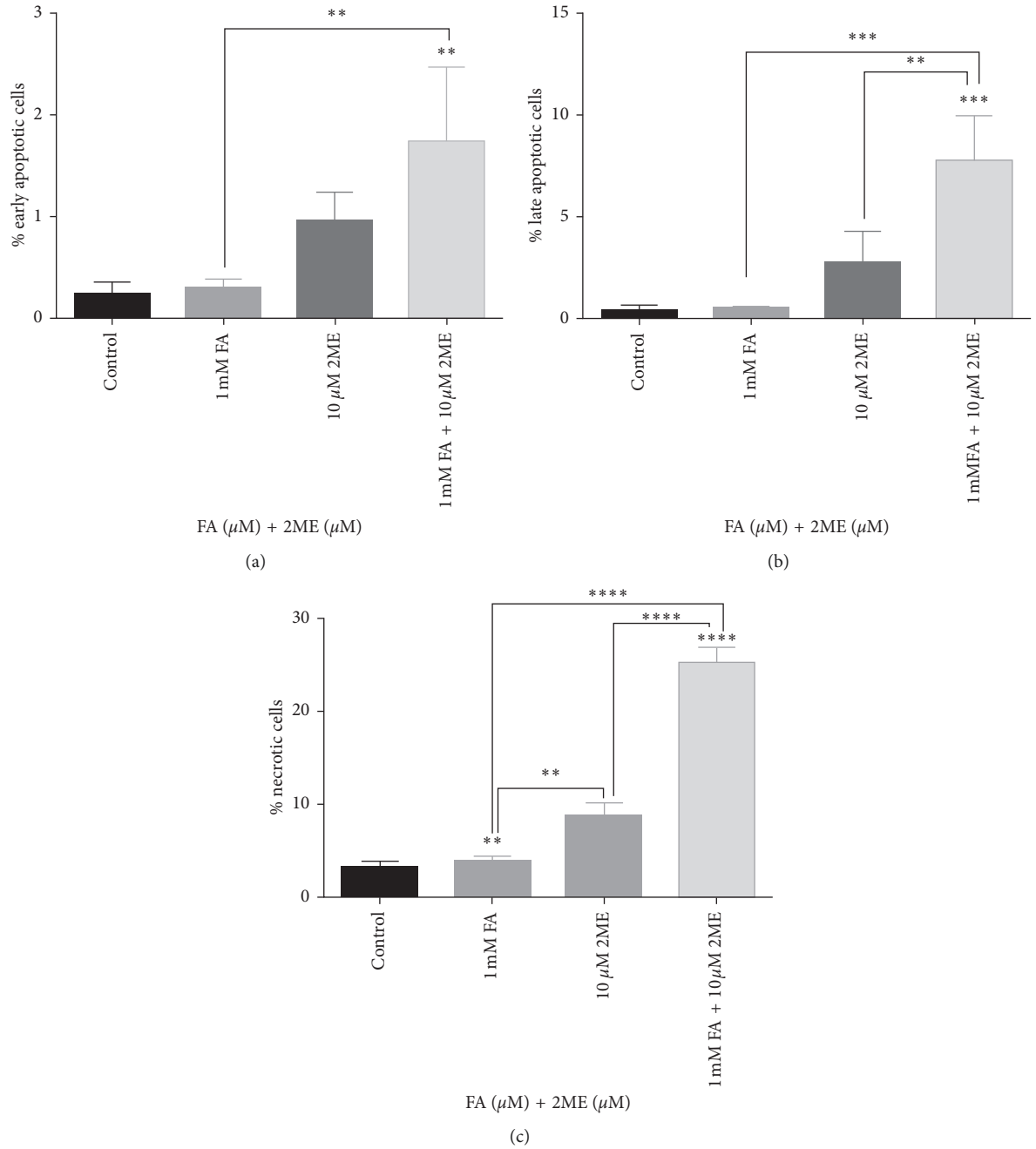

Figure 3: (a) Total cell levels in the early apoptosis phase after 24 hours incubation of A375 line cells with 10 μM 2-ME and 1 mM FA separately or in combination. (b) Total cell level in the late phase of apoptosis after 24 hours incubation of A375 line cells with 10 μM 2-ME and 1 mM FA separately or in combination. (c) Total cell level in the necrotic phase after 24 hours incubation of A375 with 10 μM 2-ME and 1 mM FA separately or in combination. Values are the mean ± SE from three independent experiments. No error bar means the thickness of the line is greater than the error. $^{*}p < 0.01$ compared with the vehicle. The data were analyzed using GraphPad Prism Software version 6.02, performing one-way ANOVA analyses followed by Tukey's multiple comparison test. $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$, and $^{****}p < 0.00001$ vs. control.

ROS. These results confirm antioxidant properties of FA. However, the observed contradictory effect of 2-ME and FA on ROS may result in protective role of FA against cytotoxicity of 2-ME. Indeed, the protective role of FA against cisplatin-induced ototoxicity was previously demonstrated [62]. FA was also reported to protect against methotrexate nephrotoxicity [63].

Herein, we presented the involvement of major Hsps namely Hsp60, Hsp70, and Hsp90 in the modes of action of 2-ME and FA in the melanoma A375 cellular model. Notably, these Hsps seem to be also responsible for the mechanism of interaction between both compounds. To this date, there are only a few studies considering the role of Hsps in anticancer mechanism of action of 2-ME [64–67], while

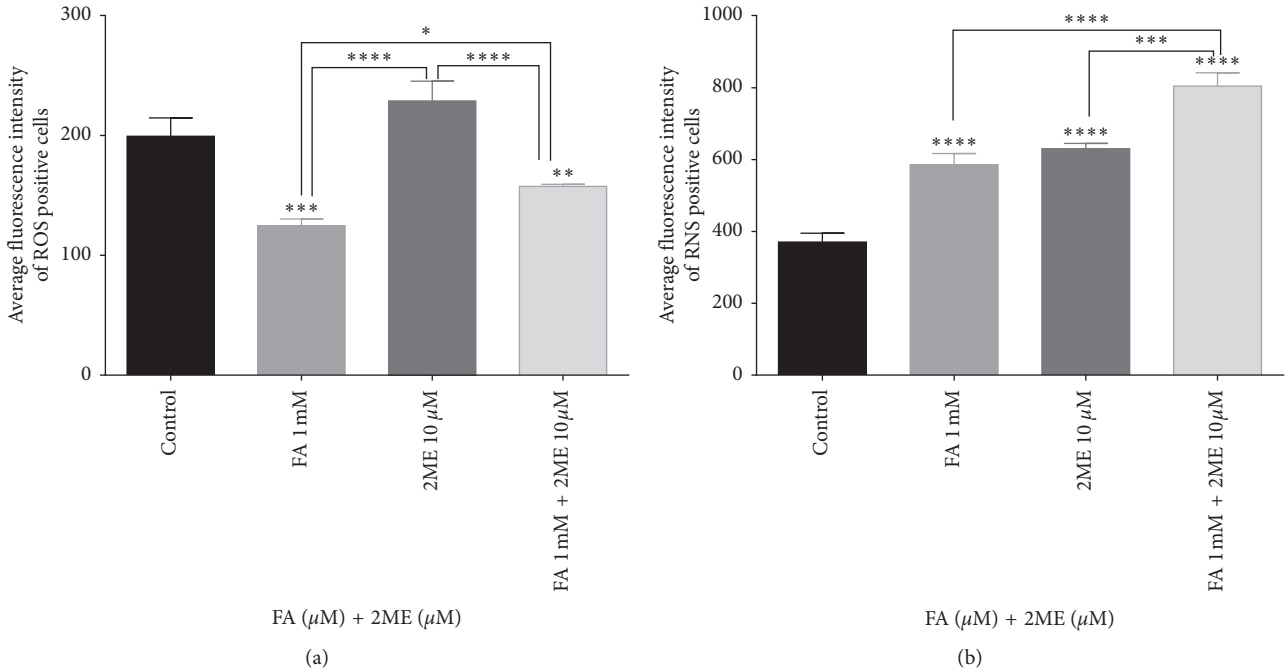

Figure 4: (a) Mean fluorescence intensity of ROS positive cells after 8 hours incubation of A375 line cells with 10 μM 2-ME and 1 mM FA separately or in combination. (b) Mean fluorescence intensity of RNS after 8 hours incubation of A375 line cells with 10 μM 2-ME and 1 mM FA separately or in combination. Values are the mean ± SE from three independent experiments. No error bar means the thickness of the line is greater than the error. $^{*}p < 0.01$ compared with the vehicle. The data were analyzed using GraphPad Prism Software version 6.02, performing one-way ANOVA analyses followed by Tukey's multiple comparison test. $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$, and $^{****}p < 0.00001$ vs. Control.

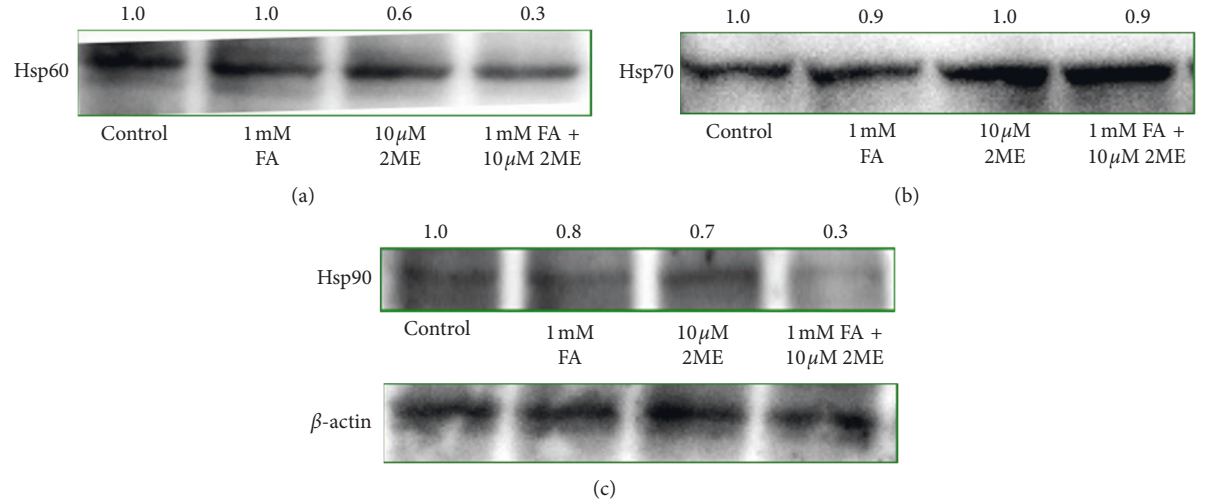

Figure 5: (a) Impact of separate and combined 24-hour treatments with 10 μM 2-ME and 1 mM FA on Hsp60 protein expression in A375 cells evaluated by western blotting. (b) Impact of separate and combined 24-hour treatments with 10 μM 2-ME and 1 mM FA on Hsp70 protein expression in A375 cells evaluated by western blotting. (c) Impact of separate and combined 24-hour treatments with 10 μM 2-ME and 1 mM FA on Hsp90 protein expression in A375 cells evaluated by western blotting. Densitometric analysis of HSP/beta-actin was performed using Quantity One 4.5.2 software. The representative images are shown.

no one conducted on the melanoma experimental model. Similarly, there are only limited data investigating Hsps in FA mechanism [68, 69]. Depending on their localization and expression Hsps may have a dichotomal effect in cancer biology. The 60 kDa heat shock protein (Hsp60) is classically known as a mitochondrial chaperonin protein. However, accumulating data support that it is localized in extra-mitochondrial compartments as well [70–75]. As a primary mitochondrial chaperone, Hsp60 is essential for mitochondrial protein homeostasis [76]. However, it is also

implicated in the cell survival and apoptosis signaling pathways [41]. Increased protein level of Hsp60 has been detected in various malignant cells including colon [77], cervix [78], prostate [79], or melanoma [80]. In many of the cases examined, higher expression was correlated with poorer prognosis [81–83].

In consistency with these studies, our obtained outcomes indicate that 2-ME decreased Hsp90 protein level in melanoma cells. To this date, there are no data considering the role of FA in regulation of Hsp60 protein level. Although FA itself does not affect the Hsp60 protein level in melanoma cells, it enhances the activity of 2-ME to decrease Hsp60 expression. These data indeed are strictly associated with the level of apoptotic and necrotic cells as well as concentration of nitric oxide in melanoma 2-ME and FA-treated cells. On the other hand, 2-ME was reported to increase Hsp60 protein level in estrogen-positive breast adenocarcinoma MCF-7 cells [66]. Thus, the role of expression of Hsp60 seems not to be clear and to depend on an experimental model, i.e., type of cancer cells. Indeed, higher expression of Hsp60 was observed in early-stage ovarian cancer than advanced-stage in one other report [84]. It was further investigated that increased expression of Hsp60 is correlated with higher susceptibility of melanoma cells to immune chemotherapy [85].

Targeting Hsp70, beyond Hsp60, is a new therapeutic approach. Most compounds are active Hsp90/Hsp70 inhibitors and induce cancer cell death [86]. Hsp70 directly or indirectly modulates the intrinsic and extrinsic apoptotic pathways. Inhibition or knockdown of Hsp70 increases sensitivity of cells to apoptosis [87, 88]. Human cells produce high levels of Hsp70, constitutively expressed as Hsc70, mitochondrial Hsp75, and GRP78, which are found in the endoplasmic reticulum [86]. Under nonstressed conditions, cells express constitutive levels of Hsp70. However, their enhanced expression, a feature of cancerous or stressed cells, increases survival of these cells. Clinical studies indicate that increased expression of Hsp70 is associated with tumorigenesis, poor prognosis, and chemoresistance of numerous malignancies, including melanoma [86, 89, 90]. Notably, in our melanoma experimental model, changes in Hsp70 were not observed after treatment with 2-ME and FA separately or in combination. It is important, especially for mechanisms of chemoresistance, as accumulation of Hsp70 reduces the induction of cancer cell death, thus decreasing the antitumour efficacy [86].

Due to the fact that Hsp90 forms a chaperone machinery with Hsp70, we have established the impact of 2-ME and FA on this protein. Hsp90 is an interesting target for cancer therapy because it is involved in folding and stabilization of numerous proteins, including those that contribute to the development of cancer. In mammals, Hsp90 chaperones include Hsp90 alpha and Hsp90 beta, GRP94 (94 kDa glucose-regulated protein), and TRAP-1 (tumour necrosis factor receptor-associated protein 1) localized in the cytoplasm, ER, and mitochondria, respectively [91]. Hsp90 is implicated in the pathogenesis of numerous diseases, including cancer. Several cancer proteins depend on Hsp90 machinery and chaperones for their folding and maturation, i.e., steroid hormone receptors and transcription factors [91]. Therefore, pronounced expression of Hsp90 has been detected in almost all types of cancers, including melanoma [44, 92, 93]. Hsp90 expression is higher in metastatic melanoma and associated with malignant features as Clarke's level in cutaneous melanoma and larger tumour size in uveal melanoma [44]. Herein, we evidenced that both FA and 2-ME downregulate the Hsp90 expression, this effect is even enhanced after combined treatment with the compounds. These data are consistent with increased anticancer efficacy of combination of compounds in relation to separate treatments. Up to date, there are only few data about the role of Hsp90 in anticancer mode of action of 2-ME. Chauhan and coworkers evidenced that downregulation of Hsp90 gene expression via 2-ME is a mechanism of overcoming the chemoresistance [94]. On the other side, Kim et al. established upregulation of Hsp90 alpha in breast cancer MCF-7 adenocarcinoma cells [66]. To this date, there are no studies about the role of Hsp90 in anticancer mode of action of FA. Nonetheless, it was hypothesized that antidepressant-like effect of FA is associated with activation of MAPK kinases pathway and Hsp90 [95, 96]. These contradictory results may be explained by different experimental models (cancer and nontransformed cells) as well as experimental conditions, i.e., time of incubation.

5. Conclusions

Herein, we presented a synergism between a potent anticancer compound, 2-ME, and a naturally occurring polyphenol, FA. The molecular mechanism of observed interaction is at least partially associated with downregulation of Hsp60 and Hsp90 and induction of nitric oxide in the melanoma A375 cellular model. Furthermore, scavenging of 2-ME-induced ROS by FA may be a protective mechanism against enhanced toxicity of 2-ME. Therefore, further investigation of sources of nitro-oxidative stress in 2-ME and FA-treated cells is still needed. Nonetheless, the obtained data strongly support the anticancer effect of 2-ME and FA and their potential role in adjuvant chemotherapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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