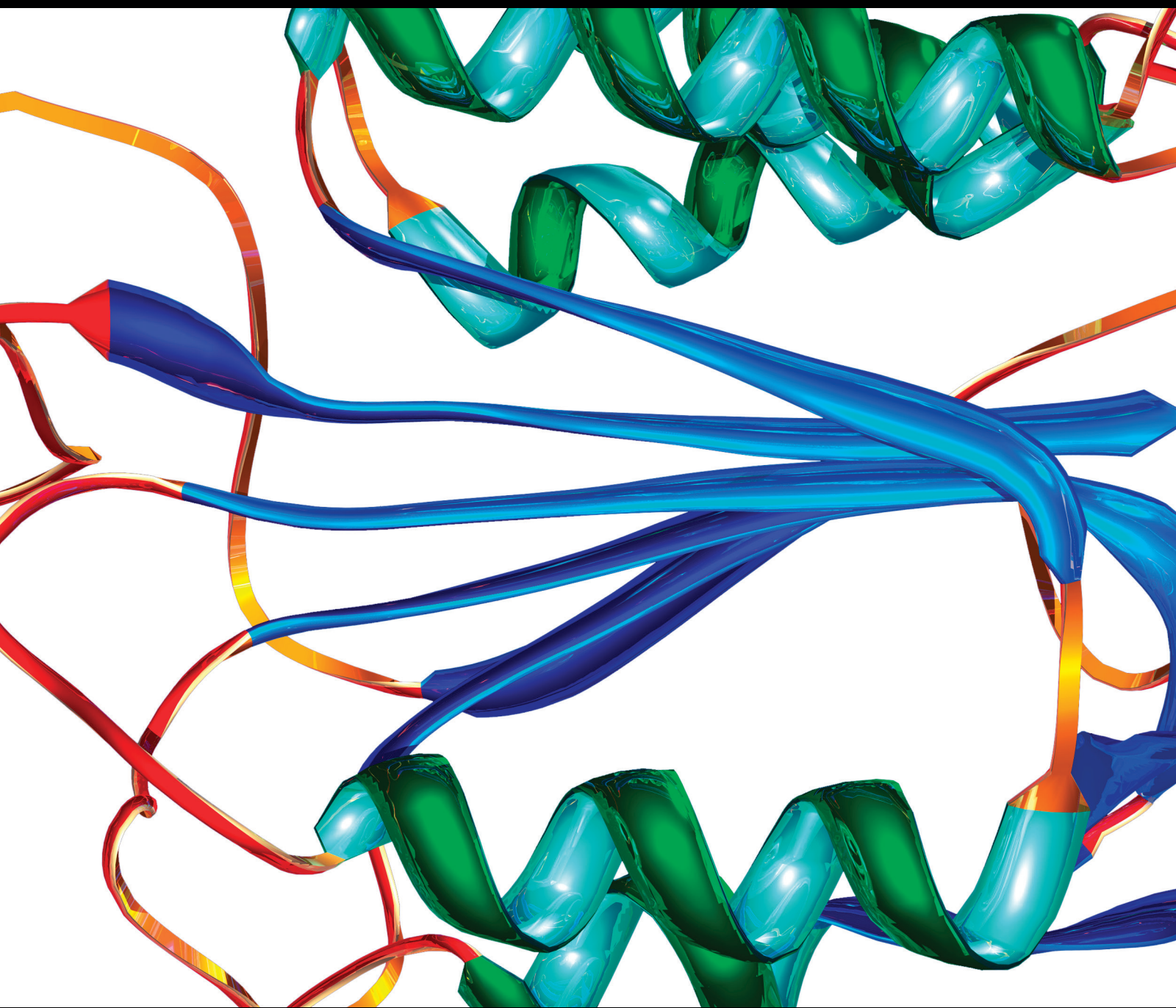
Disease Markers

Emerging Biomarkers in the Era of Personalized Cancer Medicine

Lead Guest Editor: Biagio Ricciuti
Guest Editors: Giulia C. Leonardi and Marta Brambilla



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Editorial Board

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Editorial

Emerging Biomarkers in the Era of Personalized Cancer Medicine

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Personalized oncology is an evidence-based, patient's tailored approach that is aimed at identifying and treating each cancer patient based on its genetic makeup and molecular features. Biomarkers are a key player in personalized cancer medicine, and biomarker discovery and development represent an area of active research and specific challenges. Currently, predictive and prognostic biomarkers that can guide the therapeutic decision-making process are already available in the clinical practice. Patients with solid tumors and hematological malignancies derive great clinical benefit and access to specific treatment upon specific biomarker assessment. First, the advent of molecular diagnostics with single/multi-, gene/protein variant detection enabled the identification of patients with exquisite sensitivity to targeted therapies or immunotherapy. More recently, the advent of high-throughput genomic and molecular profiling and "omics" techniques has led to the discovery of a wide spectrum of potentially relevant biomarkers that will hopefully provide a deeper understanding of cancer biology and host interaction, raising the bar of personalized cancer medicine. This special issue includes selected articles focusing on emerging biomarkers and their potential clinical application in different solid tumors.

T. Shen et al. showed that kinesin family member 20A (KIF20A) promotes the growth of the bladder tumors *in vivo*, and its overexpression associates with a poor prognosis in patients with bladder cancer. This study suggests that KIF20A may become an independent prognostic factor in patients with bladder cancer and a potential therapeutic target as selective KIF20A inhibitors are in development.

C.-Y. Huang and colleagues demonstrated that GRP94 silencing may increase the resistance of osteosarcoma cell lines (MG63 and 143B) to paclitaxel, gemcitabine, and epirubicin treatments by inhibiting the induction of apoptosis, suggesting that GRP94 may be a key biomarker for the chemotherapeutic response of osteosarcoma.

By using gynecologic cancer cell lines with known TP53 mutational status, X. Meng et al. demonstrated that proteasome inhibition induced cell death in cells with two recurrent gain of function (GOF) TP53 mutations (R175H and R248Q) and that the addition of a histone deacetylase inhibitor (HDACi) enhanced this effect. This study provides preliminary evidence for a novel therapeutic strategy for tumors with GOF TP53 mutations using drugs that are already being advanced in clinical trials.

C. Mecca and colleagues analyzed the rationale of targeting mTOR in GBM and the available preclinical and clinical evidences supporting the choice of this therapeutic approach, highlighting the different roles of mTORC1 and mTORC2 in GBM biology.

G. Cervino and colleagues provided an overview of the emerging diagnostic and prognostic biomarkers in oral cancer, which still represents one of the leading causes of death in developing countries.

In conclusion, this special issue wants to emphasize the central role of biomarker identification and implementation as the cornerstone of personalized cancer medicine and highlights how the bench to bedside translational science has a great impact on the clinical practice and patient's quality of life.

Conflicts of Interest

The editors have no conflict of interest to disclose.

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Giulia C. Leonardi
Marta Brambilla

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Research Article

KIF20A Affects the Prognosis of Bladder Cancer by Promoting the Proliferation and Metastasis of Bladder Cancer Cells

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Guest Editor: Marta Brambilla

Objective. To investigate the expression of kinesin family member 20A (KIF20A) in bladder cancer, the effect of KIF20A on the proliferation and metastasis of bladder cancer cells, and the effect of KIF20A expression on the prognosis of bladder cancer patients. *Methods*. Bladder cancer tissue and its adjacent tissues were collected from tumour patients. The mRNA and protein expression levels of KIF20A in the tissue samples were detected by qRT-PCR and western blot. Immunohistochemical (IHC) staining was used to identify the expression and distribution of KIF20A proteins in the tissue samples. The relationship between the KIF20A expression and the clinical pathology of bladder cancer was analysed. The effect of the differential expression of KIF20A on the prognosis of patients with bladder cancer was analysed by the TCGA database. The plasmid was transfected into the bladder cell lines T24 and 5637 to construct two stable cell lines with knocked down KIF20A. The effect of KIF20A expression on the proliferation and invasion of T24 and 5637 bladder cells was explored in vitro using the abovementioned stable cell lines. The effect of the KIF20A expression on the proliferation of bladder cancer cells was evaluated by a mouse xenograft model. *Results*. The expression of KIF20A was significantly higher in the bladder cancer tissues than in the adjacent control tissues. The expression of KIF20A was significantly associated with the degree of pathological differentiation of bladder cancer. Patients with a higher expression of KIF20A had a higher tumour grade and a more advanced stage. The mean survival of patients with a high KIF20A expression was significantly lower than the mean survival of patients with a low KIF20A expression. The in vitro experiments demonstrated that the knockdown of KIF20A significantly inhibited T24 and 5637 cell proliferation and invasion. The in vivo experiments showed that the knockdown of KIF20A significantly inhibited the proliferation of the bladder tumours. *Conclusion*. KIF20A promotes the proliferation and metastasis of bladder cancer cells. Bladder cancer patients with a high KIF20A expression have a worse tumour differentiation and a poor prognosis. KIF20A may become an independent factor that affects the prognosis of bladder cancer patients and a therapeutic target for bladder cancer.

1. Introduction

Bladder cancer is one of the most common malignant tumours in the urinary system. According to cancer statistics, the estimated number of new bladder cancer cases increased from 79030 in 2017 to 81190 in 2018 in the United States. The number of deaths also increased from 16870 to 17240 [1, 2]. Treating bladder cancer is often difficult and expensive [3]. In recent years, bladder cancer morbidity and mortality have increased in the Chinese population. The time of diagnosis plays a crucial role in achieving a good prognosis [4]. Current chemotherapy methods and surgery can effectively prolong the survival of patients with bladder cancer, and patients need to bear the high expenses and pain caused by surgical treatment. Therefore, finding molecular markers that are potential therapeutic targets and prognostic indicators of bladder cancer is critical for a clinically accurate diagnosis and treatment.

The KIF family of molecules possesses a highly conserved kinetic domain, and many of its family members have ATP activity and are able to move towards the positive pole of the microtubule [5–9]. These molecules are involved in various physiological functions, such as intracellular spindle formation, chromosome partitioning, and substance transport [5, 8, 9]. Kinesin family member 20A (KIF20A), also known as MKLP2 and RAB6KIFL, is located on chromosome 5q31.2 [5]. The encoded protein contains 890 amino acids and has a molecular weight of approximately 100 kDa [10]. KIF20A mainly accumulates in the central region of the mitotic cell spindle and participates in the process of cell mitosis [11]. Studies have found that KIF20A is highly expressed in many types of tumours, such as lung cancer [12, 13], breast cancer [14], gastric cancer [15], liver cancer [16], bladder cancer [17], and pancreatic cancer [18–20]. Taniuchi et al. found that the KIF20A levels are elevated in pancreatic cancer [21]. If the expression of KIF20A is downregulated, there is a significant reduction in the proliferation of pancreatic cancer cells. In recent years, some scholars have pointed out that the downregulation of KIF20A can induce gastric cancer cell mitosis (G2/M phase) arrest and enhance chemotherapy drug sensitivity [15]. At present, research on the effect of KIF20A on the proliferation, invasion, and migration of bladder cancer cells is still in the preliminary stage, and the specific regulation and mechanisms of KIF20A have yet to be studied.

In this study, we examined the expression of KIF20A in clinical specimens of bladder cancer and found that the expression of KIF20A in the bladder cancer tissues is higher than that in the adjacent tissues. We further analysed the relationship between the KIF20A expression and the clinical pathology of bladder cancer. Statistical results showed that patients with a higher expression of KIF20A had a higher tumour grade and a more advanced stage. The effect of the differential expression of KIF20A on the prognosis of patients with bladder cancer was analysed by the TCGA database. The effects of KIF20A on the proliferation and invasion of bladder cancer cells were detected in vitro and in vivo.

2. Materials and Methods

2.1. Antibodies. Antibodies for the following proteins were used in this study for western blot and immunohistochemistry: KIF20A (Abcam, ab104118, 1 : 1000 dilution for western blot and 1 : 200 dilution for IHC-P), PCNA (Abcam, ab92552, 1 : 1000 dilution for western blot), Ki67 (Abcam, ab16667, 1 : 1000 dilution for western blot), Bcl-2 (Abcam, ab32124, 1 : 1000 dilution for western blot), caspase-3 (Abcam, ab13847, 1 : 500 dilution for western blot), MMP-2 (Abcam, ab37150, 1 : 500 dilution for western blot), and GAPDH (Sungene Biotech, KM9002, 1 : 5000 dilution for western blot).

2.2. Cell Culture and Cell Lines. The cell lines involved in this experiment, including T24, 5637, EJ, BIU87, and SV-HUC-1, were purchased from ATCC. These cells were cultured in RPMI 1640 medium (Gibco, Waltham, MA, USA) containing 10% foetal bovine serum (Gibco, Waltham, MA, USA) with culture conditions of 37.0°C with 5% CO_2.

2.3. MTT Assay. The MTT powder was formulated into a solution at a concentration of 5 g/mL. The cells were seeded in a 96-well plate and incubated for 3-6 days at 37°C with 5% CO_2 in a cell culture incubator. Then, 50 μL of MTT solution was added to each well and incubated at 37°C for 4 hours. The supernatant was aspirated, and 150 μL of DMSO was added to each well. A microplate reader measured the optical density (OD) value of each well at a wavelength of 490 nm.

2.4. Western Blot. Total cellular protein was extracted. The prepared protein samples were added to the corresponding gel lane of the separation gel, and electrophoresis was carried out using a constant voltage. Protein transfer was performed using a PVDF membrane. After the completion of the electroporation, the PVDF membrane was sealed with skim milk for 60 minutes. After washing the gel with TBST, the primary antibody was added and the membrane was incubated overnight at 4°C. The primary antibody was washed away by TBST; the secondary antibody solution corresponding to the primary antibody was added, and the membrane was incubated at room temperature for 1 hour. After washing away the secondary antibody with TBST, the membrane was prepared for exposure. The exposure reagents A and B were mixed in equal proportion; the mixed solution was applied to the PVDF membrane, and the membrane was exposed.

2.5. Immunohistochemistry. The paraffinized tissue sections were dewaxed in water and subjected to antigen retrieval. Briefly, 3% H_2O_2 was added to the sections, and the sections were incubated for 15 minutes at room temperature. After washing with PBS, the primary antibody was added dropwise and the sections were incubated at 4°C for 18 hours. After washing with PBS, the secondary antibody was applied to the specimens and the specimens were incubated at 37°C for 1 hour. The sections were stained with DAB solution. After washing with tap water, haematoxylin was added to the specimen to counterstain the cell nuclei. After washing with tap water, the sections were dehydrated, a transparent coverslip was mounted, and the slides were sealed. The results were observed under a microscope, and the positive staining rate was counted.

2.6. Colony Formation Assay. The cells were seeded in a culture dish and cultured in the abovementioned manner. The culture was terminated when macroscopic colonies appeared in the culture dish. The supernatant was discarded, and the cells were washed 3 times with PBS. The cells were fixed for 15 minutes, and an appropriate amount of Giemsa staining solution was added for 10 to 30 minutes. The number of colonies was then counted.

2.7. Transwell Invasion Assay. Matrigel was stored at 4°C overnight. Matrigel was diluted with prechilled RPMI 1640 medium, and 60 μL of the diluted gel was added to each Transwell chamber of a 24-well plate for 2 hours. Cells were plated in each Transwell chamber. The serum-containing medium was added to the lower Transwell chamber surface. The cells were cultured for 24 hours with the abovementioned cell culture method. The Transwell

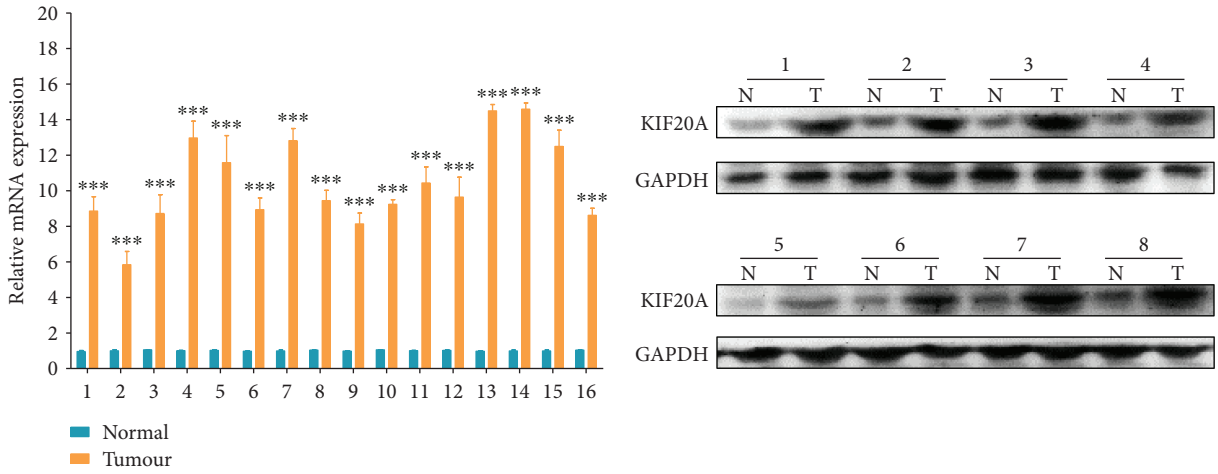

Figure 1: KIF20A is upregulated in bladder cancer tissues. (a) qRT-PCR was used to detect the expression level of KIF20A mRNA in surgical specimens of bladder cancer and its adjacent tissues from tumour patients. (b) Western blot was used to detect the protein expression level of KIF20A in the abovementioned specimens.

chambers were removed, and cell fixation, staining, and counting were performed.

2.8. In Vitro Transfection. The following shRNA plasmids were used in this study for in vitro transfection: KIF20A human shRNA plasmid (CAT#: TG311916, OriGene) and HuSH shRNA RFP cloning vector (CAT#: TR30014, OriGene). The cells were transfected with liposomes. The plasmid was mixed with the transfection reagent at a ratio of 1 : 1-1 : 4, and the mixture was added to Opti-MEM for 30 minutes. The above mixture was then added to the medium with the cells. The medium was replaced with new medium after 24-48 hours. The transfected cells were screened using G418. A stably transfected cell line was finally obtained.

2.9. RNA Isolation and Quantitative RT-PCR Analysis. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was reverse transcribed using a reverse transcription kit to obtain cDNA. The mRNA reverse transcription-PCR (RT-PCR) primers for KIF20A and GAPDH were purchased from Applied Biosystems. The primers were designed as follows: for KIF20A, forward primer, 5′-TGCTGTCCGATGACGA TGTC-3′, reverse primer, 5′-AGGTTCTTGCGTACCACA GAC-3′; and for GAPDH, forward primer, 5′-AGGTTC TTGCGTACCACAGAC-3′, reverse primer, 5′-GCCATCA CGCCACAGTTTC-3′. The expression of the mRNAs was determined in quantitative RT-PCR with an Applied Biosystems 7900 Real-Time PCR System (Thermo Scientific, Waltham, MA, USA). Small nucleolar RNA U6 was used as an internal reference for normalization.

2.10. Statistical Analysis. Statistical processes were performed with SPSS 20.0. Multiple groups were compared using one-way analysis of variance. The LSD test was used for comparisons between groups. Comparisons between the different treatment groups and control groups were performed using paired t-tests. Data analysis was performed with GraphPad Prism 5. $P < 0.05$ indicated a statistically significant difference in the results. $P < 0.05$ was marked as $*$, $P < 0.01$ was marked as $**$, $P < 0.01$ was marked as $***$, and no significant difference was expressed by "n.s."

3. Results

3.1. KIF20A Expression Is Upregulated in Bladder Cancer. To study the expression of KIF20A in gastric cancer, the research team collected 16 surgical specimens of bladder cancer and their adjacent tissues from tumour patients. We first used qRT-PCR to detect the mRNA expression level of KIF20A in the above samples (Figure 1(a)) and found that the mRNA expression level of KIF20A was higher in the bladder cancer tissues than in the adjacent tissues of 16 sample pairs. Subsequently, we randomly selected 8 pairs of the 16 pairs of samples to detect the protein expression level of KIF20A. Western blot results showed that the expression level of the KIF20A protein was higher in the tumour tissue than in the adjacent tissues (Figure 1(b)). The preliminary results indicated that both the transcriptional and translational KIF20A expression levels were increased in bladder cancer.

3.2. High Expression of KIF20A Suggests a High Degree of Malignancy and a Poor Prognosis in Bladder Cancer. To further explore the relationship between the expression of KIF20A and the malignancy of bladder cancer, the research team performed immunohistochemical staining on 108 pairs of paraffinized bladder cancer and adjacent tissue sections (Figure 2(a)). The results showed that KIF20A was mainly expressed in the cytoplasm and membranes. According to the results of the immunohistochemical scoring, the positive rate of the KIF20A expression in bladder cancer tissues was 67.6% (17.9% strong positive, 49.7% weak positive) (Figure 2(b)). The positive rate in the adjacent tissues

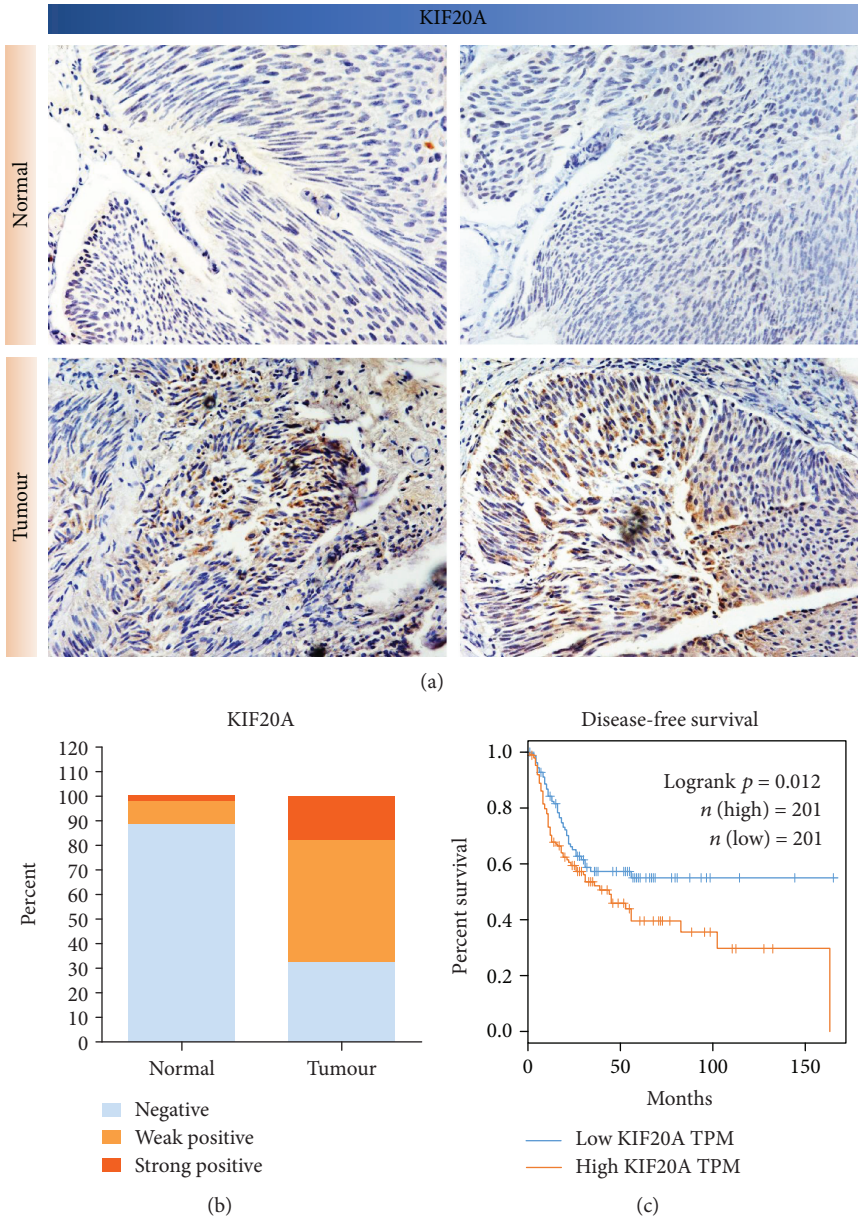

Figure 2: High KIF20A expression suggests a poor prognosis in bladder cancer. (a) Representative IHC staining images of KIF20A in bladder cancer and its adjacent tissues. (b) Chart of positive immunohistochemical rates and the associated statistics. (c) Survival curves for patients with bladder cancer ($n = 402$, $P = 0.012$) that were mapped with the website http://gepia.cancer-pku.cn/.

was 11.7% (2.2% strong positive, 9.5% weak positive) (Figure 2(b)). These results confirmed that the expression of KIF20A in the bladder cancer tissues was significantly higher than that in the adjacent tissues and the difference was statistically significant ($P < 0.05$). This result again confirms the conclusion from Figure 1. We collected essential and tumour status information from 108 patients with bladder cancer. Table 1 showed that patients with a higher expression of KIF20A had a higher tumour grade and a more advanced stage. Additionally, lymph node metastasis and vascular invasion of the tumours were also associated with a high KIF20A expression.

We used the GEPIA website (http://gepia.cancer-pku.cn/) [22] to examine the effect of the KIF20A expression on patient survival. These data are from The Cancer Genome Atlas (TCGA) database. The survival rate of patients with bladder cancer with a high KIF20A expression was significantly lower than that of patients with bladder cancer with a low KIF20A expression ($P = 0.012$) (Figure 2(c)). This suggests that the high KIF20A expression indicates a poor prognosis in patients with bladder cancer. KIF20A can be an independent factor that affects the prognosis of patients with bladder cancer.

3.3. Knockdown of KIF20A Inhibits the Proliferation and Invasion of Bladder Cancer Cells. We further explored the effect of KIF20A on the biological function of bladder cancer cells in vitro. First, we used western blot to detect the protein

Table 1: Relationships of KIF20A and the clinicopathological characteristics of 108 patients with bladder cancer.

Variables	All $n = 108$	KIF20A Low $n = 35$	KIF20A High $n = 73$	P value[#]
Age				
<65	56	17	39	0.64
≥65	52	18	34	
Sex				
Male	65	20	45	0.65
Female	43	15	28	
Tumour stage				
T2	54	23	31	0.02*
T3/T4	54	12	42	
Tumour grade				
Low	64	34	30	0.01*
High	54	11	43	
Lymph node metastasis				
No	45	20	25	0.02*
Yes	63	15	48	
Distant metastasis				
No	60	20	40	0.82
Yes	48	15	33	
Vascular invasion				
No	54	23	31	0.02*
Yes	54	12	42	

[#]P value was analysed by a chi-square test; * indicates $P < 0.05$ with statistical significance.

expression levels of KIF20A in the bladder cancer cell lines T24, BIU87, EJ, and 5637 and in the normal bladder cell line SV-HUC-1. The results showed that the protein levels of KIF20A in the bladder cancer cell lines were higher than those in the normal bladder cell line (Figure 3(a)). This is consistent with the results shown in Figure 1. Since the protein expression level of KIF20A was higher in T24 and 5637 cell lines than in the other bladder cancer cell lines, we chose these two cell lines for experimental studies. We transfected T24/5637 cells with the shKIF20A plasmid and screened them to obtain the stable T24/5637 knockdown cell lines T24shKIF20A/5637shKIF20A. The knockdown efficiency was again detected by western blot (Figures 3(b) and 3(c)). It has been reported that KIF20A is involved in cell proliferation, apoptosis, and even metastasis [13, 23, 24], so we tested the corresponding indicators in the abovementioned stable cell lines. We found that the expression levels of PCNA and Ki67 in T24shKIF20A/5637shKIF20A were lower than those in the control group (Figure 3(d)). That is, the proliferation of bladder cancer cells is inhibited after knocking down KIF20A. At the same time, the expression level of Bcl-2 in T24shKIF20A/5637shKIF20A was lower than that in the control group (Figure 3(d)), and the expression level of caspase-3 was higher in T24shKIF20A/5637shKIF20A than in the control group (Figure 3(d)). These data indicate that the knockdown of KIF20A effectively promoted apoptosis in the bladder cancer cell lines T24/5637. Moreover, the expression of MMP-2 in T24shKIF20A/5637shKIF20A was also lower than that in the control group (Figure 3(d)), indicating that the invasive ability of the cells was also inhibited compared with that of the control group. The MTT assay further tested the effect of knocking down KIF20A on the proliferation of the bladder cancer cell lines T24/5637. The results showed that the proliferation ability of T24shKIF20A/5637shKIF20A was weaker than that of the control group (Figure 3(e)). The results of the colony formation assay showed that the number of cell colonies in the T24shKIF20A/5637shKIF20A group was significantly lower than that in the control group (Figures 3(f) and 3(g)), which confirmed that the knockdown of KIF20A could effectively inhibit the proliferation of bladder cancer cells. Transwell invasion assays were performed to detect the effect of KIF20A on the invasion of bladder cancer cells, and the results showed that the number of invading cancer cells in the T24shKIF20A/5637shKIF20A group was significantly lower than that in the control group (Figures 3(h) and 3(i)). We believe that knocking down KIF20A can effectively reduce the invasion ability of bladder cancer cells.

3.4. KIF20A Promotes the Growth of Bladder Tumours In Vivo. The abovementioned two cell lines were used to establish xenograft tumour models. We divided 12 nude BALB/C mice into four groups on average, and each group was implanted with the following cell lines: T24, T24shKIF20A, 5637, and 5637shKIF20A. The size of the tumour was measured with the Vernier calipers two weeks after the inoculation and then measured once a week. The tumours were removed at week 5, and each tumour was weighed. Based on the tumour photograph (Figure 4(a)) and tumour growth curve (Figure 4(b)), the tumour volume of the shKIF20A group was significantly smaller than that of the shCON group. The tumour weight of the shKIF20A group was also lower than that of the shCON group (Figure 4(c)). We extracted total protein from the tumour tissues and examined the indicators for proliferation, apoptosis, and metastasis by western blot (Figure 4(d)). The results showed that the proliferation and metastatic ability of the tumours in the shKIF20A group were weaker than those of the tumours in the control group. The in vivo experiments confirmed that tumour growth was significantly inhibited after knocking down KIF20A.

4. Discussion

Because the diagnosis and treatment of bladder cancer is difficult, it is especially important to find a biomarker for the early diagnosis of bladder cancer and as a target for treatment. Based on our knowledge of the current scientific research, this is the first report on KIF20A in bladder cancer. We confirmed that KIF20A promotes the proliferation and metastasis of bladder cancer cells. Bladder cancer patients with a high KIF20A expression have a worse tumour differentiation and a poor prognosis. More importantly, KIF20A may become an independent factor that affects the prognosis

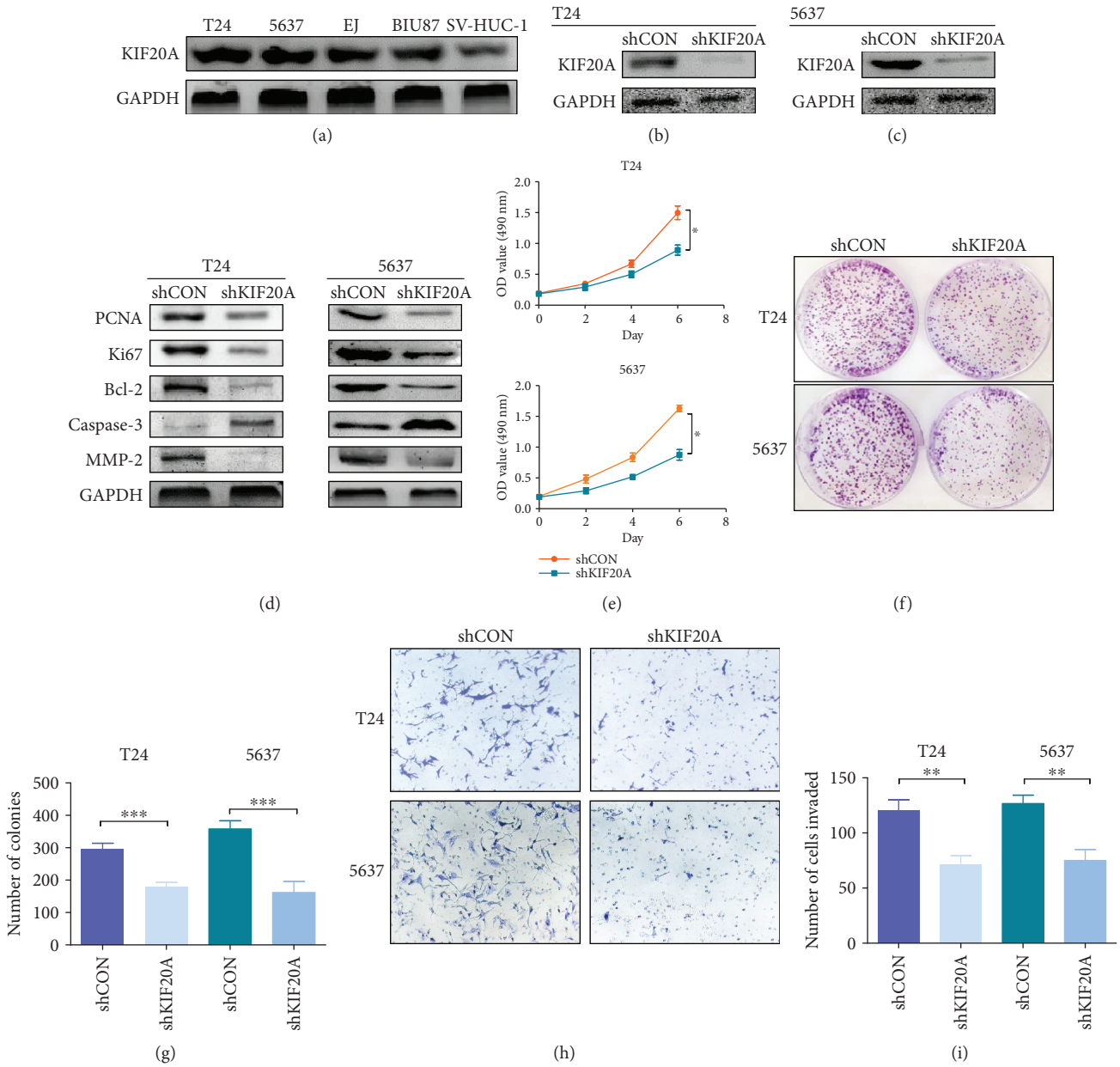

Figure 3: Knockdown of KIF20A inhibits the proliferation and invasion of bladder cancer cells. (a) Western blot was used to detect the protein expression levels of KIF20A in T24, BIU87, EJ, 5637, and SV-HUC-1 cells. (b, c) The shKIF20A plasmid was transfected into T24 and 5637 cells. The protein expression levels of KIF20A after transfection were detected by western blot. (d) Western blot was used to detect the expression levels of PCNA, Ki67, Bcl-2, caspase-3, and MMP-2 after the knockdown of KIF20A in T24 cells. (e) The MTT assay detected the growth of bladder cancer cells after the knockdown of KIF20A. The absorbance value was detected at a wavelength of 490 nm ($^{*}P < 0.05$). (f) A cloning formation assay detected the growth of bladder cancer cells after the knockdown of KIF20A. (g) The number of colonies in (g) was counted and plotted on a graph ($^{***}P < 0.001$). (h) Transwell invasion assays detected the invasiveness of the bladder cancer cells after the knockdown of KIF20A. (i) The number of invaded cells in (h) was counted and plotted on a graph ($^{**}P < 0.01$).

of bladder cancer patients and a therapeutic target for bladder cancer.

Kinesin family member 20A (KIF20A) is also known as mitotic kinesin-like protein 2 (MKLP2) [5]. As a member of the kinesin-6 subfamily, KIF20A is a microtubule-associated motor protein. KIF20A is involved in the transport of organelles or cell membranes, as well as in activities such as cell division [24–26]. KIF20A is also involved in the formation of the spindle [8, 13]. It has been reported that the mitosis of cells could be regulated by KIF20A [27, 28]. The abnormal expression of KIF20A may lead to abnormal cell division, which can then lead to chromosomal aneuploidy and genomic instability in cancer [29–31]. In 2005, scientists first discovered that KIF20A is overexpressed in pancreatic cancer and silenced KIF20A with siRNA to inhibit the growth of pancreatic cancer cells [21]. In a related study of liver cancer, Lu et al. reported that KIF20A might be an independent factor that predicts overall survival and

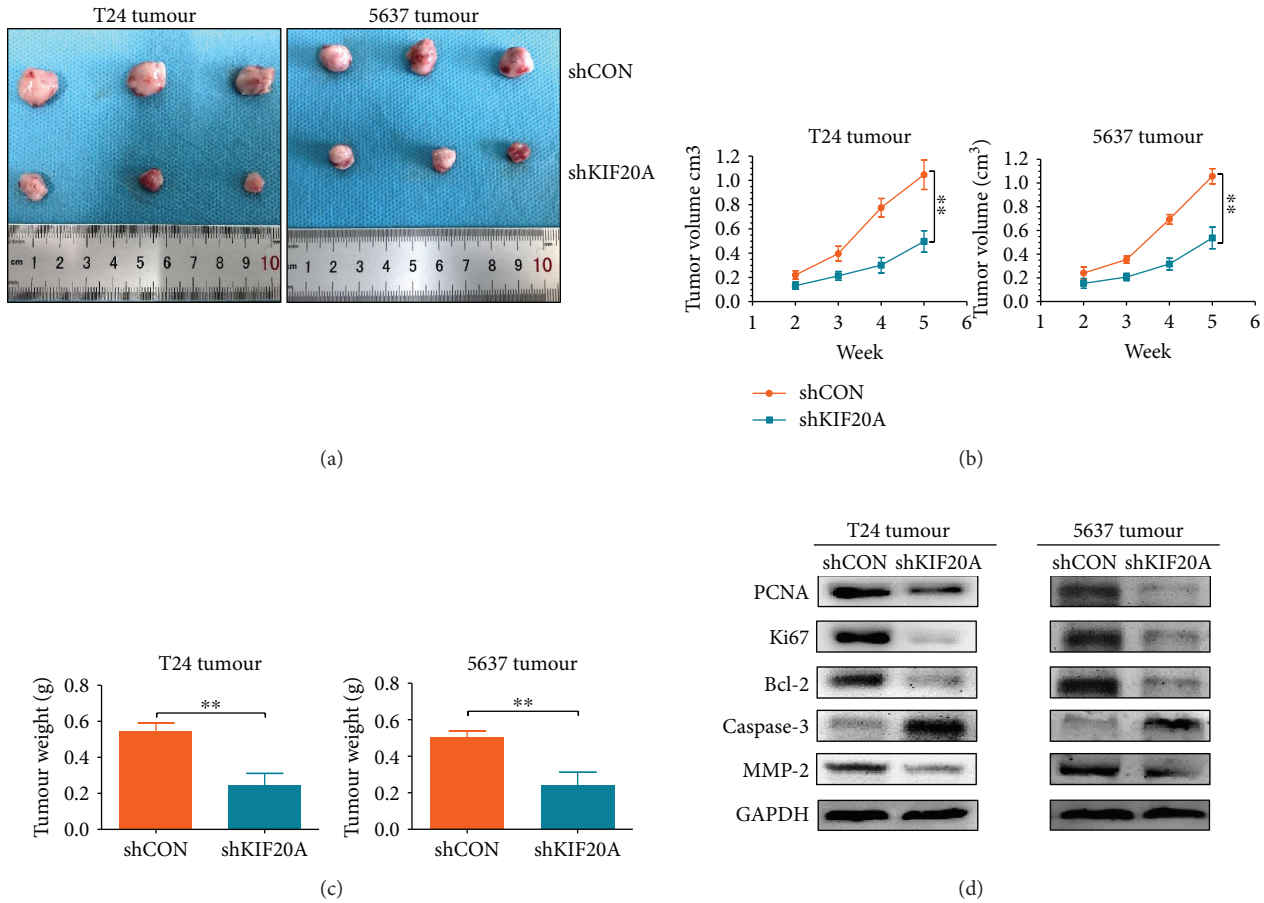

FIGURE 4: KIF20A promotes the growth of the bladder tumours in vivo. (a) The tumours were compared between the shKIF20A and shCON groups. (b) Subcutaneous T24 xenograft tumour volumes were compared between the shKIF20A and shCON groups ($^{**}P < 0.01$). (c) The tumour weights were compared between the shKIF20A and shCON groups ($^{**}P < 0.01$). (d) Western blot analysis of PCNA, Ki67, Bcl-2, caspase-3, and MMP-2 expression levels in the tumours of the shKIF20A and shCON groups.

recurrence-free survival in patients with hepatocellular carcinoma [32]. These conclusions are consistent with our experimental results. In addition, KIF20A is highly expressed in glioma cell lines and glioma tissues. Patients with gliomas and a high KIF20A expression have a poor prognosis [33]. Studies have even shown that KIF20A also plays an essential role in the resistance to traditional chemotherapy drugs, such as paclitaxel. The high expression of KIF20A leads to paclitaxel resistance in breast cancer cell lines [34]. The molecular mechanism of KIF20A in cancer is still unclear. It has been reported that FOXM1 can enhance the radioresistance of lung cancer by inducing the expression of KIF20A [35].

Many studies have found that the upregulation of KIF20A is associated with cancer, but the development and potential molecular mechanisms of KIF20A in bladder cancer are not well understood. We studied the relationship between the expression of KIF20A and the clinicopathological features and prognosis of bladder cancer. The final results showed that the tumour differentiation of patients with a high KIF20A expression was worse than that of patients with a low KIF20A expression. According to the analysis of TCGA data, the high KIF20A expression in bladder cancer patients leads to a decrease in disease-free survival. Because KIF20A is closely related to cell division, a significant feature of malignant tumours is uncontrolled cell growth. Based on the above information, we hypothesized that KIF20A could affect the proliferation of bladder cancer cells. To demonstrate this hypothesis, we confirmed in vitro and in vivo that the high expression levels of KIF20A indeed promote the proliferation of bladder cancer cells.

In summary, KIF20A is likely to be a potential target for cancer therapy in bladder cancer. This finding will help in the development of antibladder cancer drugs. Our research had certain limitations. Due to the lack of follow-up of clinical patients in our hospital, we were unable to perform survival analyses. In subsequent work, we will expand the sample size. The mechanism by which KIF20A promotes the proliferation and metastasis of bladder cancer cells has not been studied in depth. Our group will continue to explore the molecular mechanism of KIF20A in the development of bladder cancer.

Data Availability

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

Conflicts of Interest

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

Authors' Contributions

Tianyu Shen, Long Yang, and Zheng Zhang contributed equally to this work.

Acknowledgments

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Review Article

Molecular Biomarkers Related to Oral Carcinoma: Clinical Trial Outcome Evaluation in a Literature Review

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Backgrounds. The objective of the present research was to systematically revise the international literature about the genetic biomarkers related to oral cancer (OC) evaluating the recent findings in clinical studies. *Methods*. A comprehensive review of the current literature was conducted according to the PRISMA guidelines by accessing the NCBI PubMed database. The authors conducted the search of articles in the English language published from 2008 to 2018. The present systematic review included only papers with significant results about correlation between wound healing, genetic alteration, and OC. Prognostic capacity of genetic markers was not evaluated in vivo. *Results*. The first analysis with filters recorded about 1884 published papers. Beyond reading and consideration of suitability, only 20 and then 8 papers, with case report exclusion, were recorded for the revision. *Conclusion*. All the researches recorded the proteomic and genetic alterations in OC human biopsy cells. The gene modification level in the different studies, compared with samples of healthy tissues, has always been statistically significant, but it is not possible to associate publications with each other because each job is based on the measurement of different biomarkers and gene targets. Further investigations should be required in order to state scientific evidence about a clear advantage of using these biomarkers for diagnostic purpose.

1. Introduction

Oral cancer (OC) is today considered one of the principal causes of deaths with an increasing distribution located in the developing countries. The difficulty in performing a quick diagnosis and prompt management seems to be the reason for this high mortality and morbidity. Recently, several investigation methods and modern instruments have been analyzed in order to help clinicians in doing noninvasive analysis and fast recognition of this kind of oral pathological lesions [1–9].

OC is a highly relevant problem of global public health, especially for dental surgeons. It is among the top 10 most frequent cancers, and though current research in the field

discovered new therapies and treatment options, the survival still remains low representing a continuing challenge for the clinicians [7, 9–18].

A quick diagnosis is crucial in order to control a possible malignant transformation of oral premalignant diseases and for increasing the overall survival rate of the patients. Numerous techniques and methods like scraping the surface of the lesion analyzing the cytological characteristics of the oral premalignant lesions are essential for doing the right diagnosis. It is hard to state but clinicians should be able to recognize the features of the oral lesions just by doing a simple view and without touching the lesions avoiding possible modifications in the cells of the tissue [2–10, 16–24].

Nowadays, though the current standard of performing diagnosis in oral pathology is related to incisional biopsy with histology, this method is painful for patients and involves a delay in the diagnosis, although histology is fully done. A new technique for doing noninvasive analysis of a soft tissue lesion is the autofluorescence. It can be used as a helpful method useful to find oral precursor malignant lesions and the correct location for taking biopsies within the altered mucosa. However, the main limitation of this procedure is related to the possibility of frequently occurring false-positive results [1, 3, 18–20].

A novel issue in the OC diagnosis is connected to the molecular biology investigations. This procedure is able to highlight any modification at a molecular stage much before using a microscope and much before clinical changes happen.

Moreover, their molecular features can also classify oral lesions. So it is possible to predict malignant potential of oral lesions decreasing the incidence and to improve early diagnosis and treatment of OC [13, 21–29].

The progress into the understanding of human genome and the numerous possibilities of genetic and molecular researches can be used as diagnostic and prognostic tools for performing quick diagnosis and management of oral lesion by doing molecular investigation.

Molecular detection instruments can be classified into nucleic acid-based and protein-based markers. Nucleic acid-based modifications happen due to preceding epigenetic processes or existing genetic mutations, amplifications, and polymorphisms. These mechanisms lead to aberrant expressions of genes [30–36]. Unlike nucleic acid-based techniques, protein-based early detection tools detect posttranscriptional and posttranslational changes that may take place as a result of carcinogenesis. The reason of investigating the oral biomarkers available in the clinical study is related to the possibility of evaluating the soft tissue healing phases. In oral pathology, the wound healing physiological steps involve a complex interplay of cells, mediators, growth factors, and cytokines. The cascade of this inflammatory process starts with clotting and recruitment of inflammatory cells, and then, it proceeds to a highly proliferative state. At this time, fibroblasts are involved in the collagen matrix synthesis and remodelling. The keratinocytes spread across the wound to form a new epithelial layer, and angiogenesis occurs, regulating the tissue healing. A close correlation between specific OC biomarkers and wound healing should be significant in the whole health recovering inflammatory processes [1, 4, 7, 19, 36].

In this article, the authors will discuss genetic and molecular pathways as possible genesis of oral carcinoma. Clinical reports related to the soft tissue healing will be selected in order to determine useful prognostic and diagnostic factors for OC.

Moreover, the objective of the present revision is to overview the recent literature clinical trials based on diagnostic and prognostic possibilities of genetic and proteomic biomarkers of oral cancer.

2. Materials and Methods

2.1. Application Protocol and Website Recording Data. The inclusion parameters for the current research was collected in a protocol and then submitted in advance and documented in the CRD York website PROSPERO, an international prospective register of systematic reviews: application ID number: CRD 86658 (registration in progress).

The data of this systematic investigation observed the Preferred Reporting Items for Systematic Review accordingly with the PRISMA statement [37, 38].

2.2. Outcome Questions. The following next two questions were sentenced and structured according to the PICO study design:

(i) Are there some molecular biomarkers for oral carcinoma wound healing process?

(ii) What is the diagnosis method for oral carcinoma, and what biomarkers are they using on clinical trials?

2.3. Searches. The PubMed-Medline resource database was explored through advanced searches. The keywords and search inquiries used during the first selection stage were as follows: “oral cancer biomarker”, “oral cancer gene”, and “soft tissue wound healing”. Additional manually selected articles were included following the eligibility criteria. Figure 1 represents the flow diagram of the selected studies according to guidelines and following the criteria for the investigated papers choice.

2.4. Data Recorded from the Selected Manuscripts. The Medical Subject Headings (MeSH) was applied for finding the keywords used in the present revision. The selected keywords: “oral” OR “facial” AND “cancer” OR “tumor” AND “biomarkers” AND “gene” AND “clinical” AND “wound healing”, were recorded for collecting the data.

2.5. Selections of the Papers. Four independent reviewers of different Italian Universities (Messina, Foggia, Catania, and Naples) singularly investigated the obtained full-text papers in order to select inclusion and exclusion criteria as follows. Reviewers compared decisions and resolved differences through discussion and consulting a third party when consensus could not be reached. For the stage of reviewing of full-text articles, a complete independent dual review was undertaken.

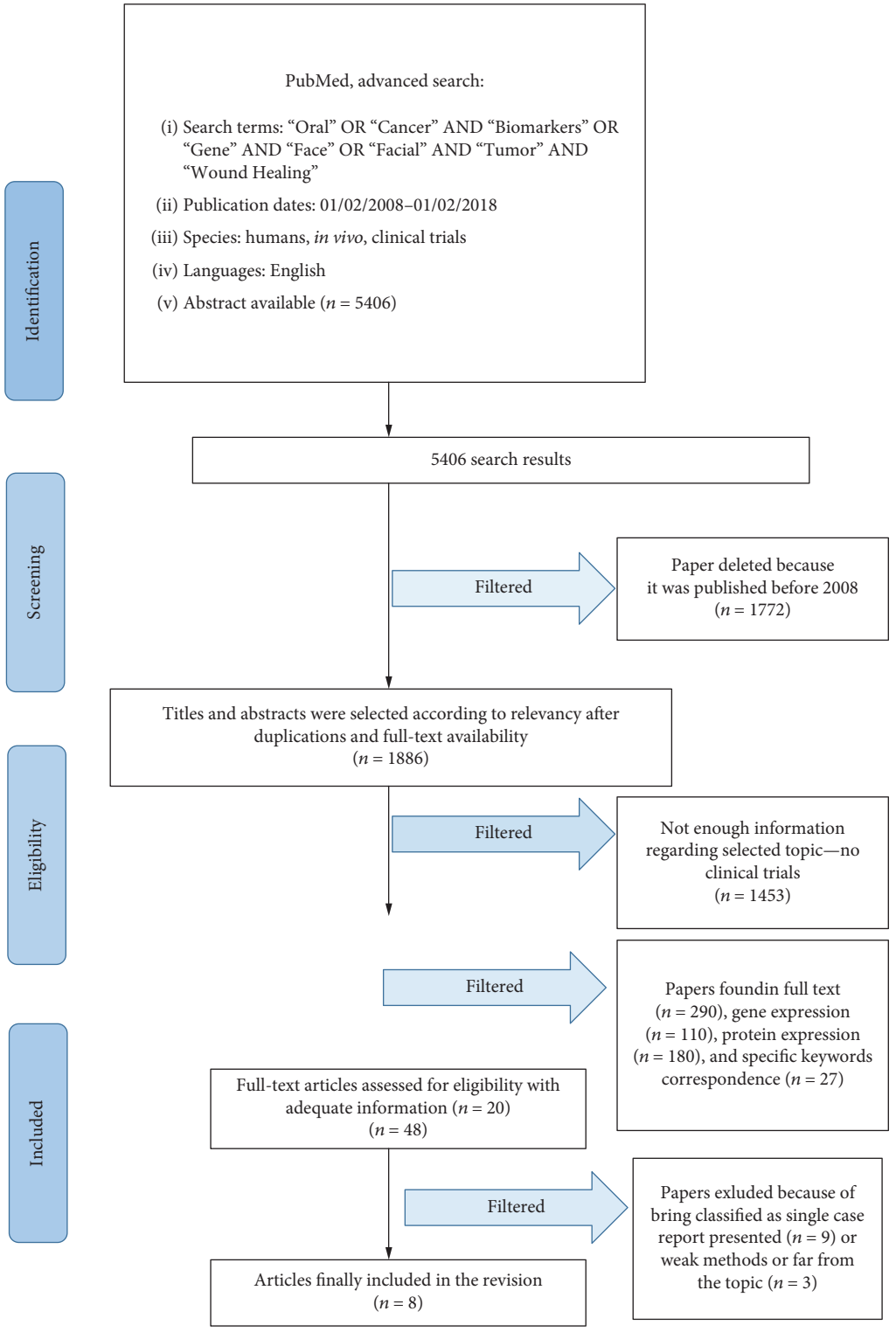

Figure 1: Prisma flow diagram.

The manuscripts selected in the present revision highlighted the clinical researches on humans published in the English language. Letters, editorials, case reports, animal studies, and PhD thesis were excluded.

2.6. Research Classifications. The method of classification included all human prospective and retrospective clinical studies, split mouth cohort studies, case-control papers, and case series manuscripts, published between December

2008 and January 2018, on biomarkers for oral cancer and wound healing.

2.7. Statement of the Problem. The sentence case of "oral cancer biomarkers clinical trials wound healing" was searched over each selected papers.

2.8. Exclusion and Inclusion Criteria. The applied inclusion criteria for the studies were created as follows:

(i) English language

(ii) Clinical human studies of oral cancer and molecular biomarkers

(iii) Last ten-year data of publishing

The following types of articles were excluded as follows:

(i) In vivo/in vitro studies

(ii) Studies of testing medication and/or new treatment methodologies

(iii) Studies of cancer in locations other than mentioned

(iv) Studies not relevant to our selected diagnostic methods

(v) Animal studies

(vi) Literature review articles published prior to February 1st, 2008

(vii) No access to the title and abstract in the English language

2.9. Strategy for Collecting Data. Following the initial literature search, all the article titles were screened in order to eliminate irrelevant publications, review articles, case reports, and animal studies. Next, studies were excluded based on data obtained from screening the abstracts. The final stage of screening involved reading the full texts confirming each study's eligibility based on the inclusion and exclusion criteria.

2.10. Data Extraction from the Collected Papers. The data and the results of the full-text manuscript screened were compared. The conclusions were used for assembling the data, according to the aims and themes of the present revision, as listed onwards.

The following key criteria were used as guidelines for agglomerating the data and then structured following the schemes:

(i) "Author (year)"—revealed the first author and the year of publication

(ii) "Type of study"—indicated the method of the research

(iii) "Sample origin"—describes the number of particular investigated samples in the study and its origin (e.g., BS: blood sample; SS: saliva sample; and TT: tumor tissue)

(iv) "Follow-up"—yes/no described the duration of the observed outcomes

(v) "Result"—indicates the parameters that were coherent with alterations of particular biomarkers in prognostic studies

2.11. Risk of Bias Assessment. The grade of bias risk was independently considered and in duplicate by the two independent reviewers at the moment of data extraction process.

The quality of all included studies was assessed during the data extraction process. The quality appraisal involved evaluating the methodological elements that might influence the outcomes of each study. According to Moher et al. and Higgins et al., this revision followed the Cochrane Collaboration's two-part tool for assessing risk of bias and PRISMA statement [37, 38].

Risk of bias (e.g., absence of information or selective reports on variables of interest) was assessed on a study level. The risks were indicated as lack of precise information of interest related to the keywords selected.

This method applied by the four reviewers was valuable for giving to each study a level of bias. Then, the selected papers were classified with low, moderate, high, and unclear risk.

3. Results

3.1. Manuscript Collection. Manuscript choice and analyzing data process followed the PRISMA flow diagram (Figure 1). The first electronic and hand search performed on PubMed-Medline and Dentistry and Oral Sciences Source resulted with a total of 5406 papers. 1772 papers were excluded because they were published prior to February 1st, 2008. Then, the other 1886 papers were not involved in the revision because they were not available in full text. Then, the other 1453 papers were not selected because they were not directly developed as clinical trials. At this point, 290 titles and abstracts were evaluated: then, the papers were classified into papers that revealed gene expression $n = 110$ and protein expression $n = 180$; 27 articles were selected as having significant data regarding "Oral Cancer Tumor Biomarkers Clinical Trials Wound Healing" topic. 20 articles were determined as full-text papers, 8 of which were incorporated in this work. Some researches were excluded because of being classified as a single case report presented ($n = 9$) or weak methods or far from the topic ($n = 3$).

3.2. Statistical Analysis. No meta-analyses could be performed due to the heterogeneity between the studies (different study designs, control groups, and observation periods).

3.3. Study Characteristics. After the manuscript selection, a new time for screening related to the kind of gene expression or protein expression has been performed:

(i) Gene expression ($n = 110$)

Table 1: Altered biomarkers in OC.

#	Year	Author	Subjects (n)	Sample origin*	Gene marker**	Result	P value
1	2010	Taoudi Benchekroun et al.	162	HB	EGFR (U)	An increased EGFR gene copy number increases the risk of OSCC	$P = 0.062$
2	2012	Jung et al.	17	TB	134 different miRNA (see image 1)	Keratinization and high miR-21 levels are important indicators of oral cancer patient prognosis	$P < 0.05$
3	2013	Minakawa et al.	106	TB	KIFGA (U)	Results showed that KIFGA is overexpressed in OC	$P < 0.05$
4	2015	Luo et al.	121	HB	OPN (osteopontin)	Tumor OPN plays an important role in tumor development particularly in tumor invasion and metastasis	$P = 0.002$
5	2014	Su et al.	7	HB	DEPDC1B (U)	DEPDC1B is highly expressed in oral cancer tissue, compared to adjacent tissue. The overexpression in cells promotes cell migration and induces cell invasion in cancer cell lines	/
6	2011	Cao et al.	76	TB	EZM2(D)	EZH2 expression is an independent predictor for OSCC. EZH2 may serve as a biomarker for oral cancer risk	$P = 0.05$
7	2009	Saintigny et al.	162	HB	deltaNp63 (U), EIC (U), podoplanin (U)	Hazard risk of OC with upregulated genes is augmented. Considering all three biomarkers, OC patient survival rate is strikingly higher compared with no, one, or two positive biomarkers	$P < 0.0001$
8	2011	Saintigny et al.	162	HB	Has-miR-101 (D), deltaNp63 (U), P63 (U), DNMT3B (U)	It demonstrated the value of gene expression profiles in predicting oral cancer development in OPL patients. The microRNA-based strategies might therefore be considered in future chemoprevention studies	/

*Type of sample: HU: human biopsy; TB: tissue bank sample. **Type of altered gene regulation: D: downregulation, diminution; U: upregulation, augmentation.

(ii) Protein expression ($n = 180$)

The final clinical papers in full text selected were numbered as 8.

3.4. Possible Bias of the Selected Studies. The possible risk of bias was evaluated for each selected papers. The final number of the selected papers was limited to eight papers. The inclusion criteria were really restrictive and for this reason also, the risk of bias was low. Seven studies were considered as having a low risk of bias [39–45]; another one was classified as moderate risk [46].

Current analysis of the data extracted from studies written in English only could introduce a publication bias. About possible bias, some of the selected papers did not specify the inclusion criteria of the patient selection. Another key parameter that can be assumed as bias is related to the evaluation of the clinical condition for selecting the patient. Some studies referred "patients with oral preneoplastic lesions," while another study wrote about "patients with neoplastic lesion" [39, 43]. The soft tissue healing after the surgical excision was not evaluated in all the selected studies. Moreover, data recorded from the eight studies pointed out the heterogeneity of the research methods, selections of the patients, and therapeutic options. One paper started the investigation not directly from the patient but from immortalized human OSCC-derived cell lines (HSC-2, HSC-3, HSC-4, Ca9-22, Sa3, HO-1-u-1, and KON) obtained from the Human Science Research Resources Bank (Osaka, Japan) or the RIKEN BRC (Ibaraki, Japan) through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, and this is another bias [46].

Tables 1 and 2 resume the studies selected and their results related to the altered biomarkers and to the biomarker measurements.

3.5. Genetic Alterations in Oral Cancer and Wound Healing. The chosen clinical papers evaluated the alterations in some gene expressions able to influence a predisposition by the patient on developing oral cancer and consequently the possibility on having a better healing. In the selected clinical studies, the oral cancer soft tissue biopsies have been recorded and then, the genetic expression of these biopsies was evaluated, highlighting any possible alterations. Alterations in the EGFR gene copy number, or alterations in miR-7, miR-21, mRNA-KIFGA, OPN, DEPDC1B, EZH2, deltaNp63, and DNMT3B were significant for early evaluation and correlation with oral cancer. It is fundamental to underline how sometimes the quick presumptive diagnosis of preoral cancer lesion and the stage of diagnosis remain the fundamental steps on recording positive oral cancer diagnosis. In the final 8 studies, the degree of significance of these data was never higher than $p < 0.05$. In one paper, the correlation between the patient's degree of survival and the

Table 2: Biomarker measurement.

#	Year	Author	Subjects (n)	Sample origin*	Gene marker**	Sample preparation	Method
1	2010	Taoudi Benchekroun et al.	162	HB	EGFR (U)	Human OC biopsy formalin fixed and paraffin-embedded	FISH
2	2012	Jung et al.	17	TB	134 different miRNAs (see image 1)	Cell culture and transfection of oral cancer cells and normal cell biopsy	mirVana™, microarray gene expression, qRT-PCR
3	2013	Minakawa et al.	106	TB	KIFGA (U)	Immortalized human OSCC-derived cell lines obtained from the tissue bank. Human biopsy fixed in 20% buffered formaldehyde solution	qRT-PCR
4	2015	Luo et al.	121	HB	OPN (osteopontin)	Human OC biopsy formalin fixed and paraffin-embedded	Western blot
5	2014	Su et al.	7	HB	DEPDC1B (U)	Human biopsy	Immunoprecipitation, Northern blot, Western blot
6	2011	Cao et al.	76	TB	EZM2(D)	Human biopsy sample paraffin included and sectioned. Colored with H&E	Western blot
7	2009	Saintigny et al.	162	HB	deltaNp63 (U), EIC (U), podoplanin (U)	Human OC biopsy formalin fixed and paraffin-embedded	Cell membrane immunoreactivity, microscope
8	2011	Saintigny	162	HB	Has-miR-101 (D), deltaNp63 (U), P63 (U), DNMT3B (U)	Whole biopsy including both the epithelial cells and the underlying stroma	Microarray gene expression

*Type of sample: HU: human biopsy; TB: tissue bank sample. **Type of altered gene regulation: D: downregulation, diminution; U: upregulation, augmentation.

expression of miR-21 is also considered. If the miR-21 values are high, the patient's chances of survival are lower. In one study, the degree of dysplasia is evaluated based on the expression of the EZH2 gene. Another study illustrated the possibility of evaluating the predisposition to the formation of OC by evaluating deltaNp63 and EIC, also using the expression of podoplanin [39–46].

In oncology, the tumor markers or tumor indicators are classified as substances that can be found in the blood or less often in the ascitic fluid, which show a significant increase in their concentration in some types of neoplasia. A high level of a tumor marker may indicate the presence of cancer, although other causes of raising those values may exist. Some markers are specific to certain tumors while others increase in many neoplasms. Tumor markers can be produced directly from tumor cells or from normal cells. The tumor markers, on the other hand, are more useful when they are used to monitor a possible recurrence of cancer after the treatment (surgical or medical) of the primary tumor. Many proteins are known to regulate programmed cell death (or apoptosis), and members of the Bcl-2 family are the most important example. This group includes at least 15 different proteins both with antiapoptotic function (Bcl-2, Bcl-X) and proapoptotic (Bax, Bak), and it represents the balance between these two activities determining cell fate. Regarding their role in the forms of OSCC, an increase in the levels of Bcl-2 and Bcl-X expression was observed, both in dysplastic oral lesions and in oral cancer [47]. p53 is a tumor suppressor involved in several mechanisms including cell cycle progression, differentiation, DNA repair, and apoptotic process regulation. p53, also known as tumor protein 53 (TP53 gene), is a transcription factor that regulates cell cycle and covers tumor suppressor function. It intervenes in many antitumor mechanisms, activates the repair of damaged DNA (if the DNA is repairable), and can initiate apoptosis, inducing the transcription of Noxa, in case DNA damage is irreparable; if the DNA is repaired, p53 is degraded and there is a recovery of the cell cycle. Some pathogens can instead directly affect the p53 protein. An example is the human papillomavirus (HPV), which encodes a protein which binds p53 inactivating it. This, in synergy with the inactivation of another cell cycle regulator, the p105RB, allows repeated cell divisions that occur in the clinical form of the wart. The introduction of p53 into cells with protein deficiency has shown to cause a rapid death of cancer cells or a block of cell division. This phenomenon reflects the possibility on having good therapeutic prognosis. For this reason, it is one of the most widely studied oral cavity biomarkers. The gene encoding is mutated in the 50% of the tumor forms, particularly in 25-69% of OSCC cases [48]. A high expression of p53 was observed in 40-67% of cases of carcinoma of the head and neck, and this variability is related to problems inherent in the method. Some authors [49, 50] have observed a direct relationship between overexpression of p53 and a poor prognosis in terms of survival. In other works, on the contrary, a correlation between p53 overexpression and survival did not clearly

emerge, while an important role of p53 in the carcinogenesis process was highlighted, as an early event of malignant transformation, and of the histological progression of the tumor [51, 52]. The expression of p53 above the basal layer is considered an early event of the oral carcinogenesis process. It is an indicator of the development of carcinoma, even before the definite morphological changes of the involved tissue. The inactivation of this protein or the alteration of the coding gene could therefore play an important role in the genesis of OC. This could certainly represent a parameter (biomarker) to be taken into consideration during the diagnostic or interceptive phase of the tumor. Inactivated p53 is not able to stop the reproduction of cells with damaged DNA. This could be a starting point for OC. The Rb (retinoblastoma) pathway also plays a key role in regulating cell cycle progression, and this activity can be inhibited by specific mutations. Although Rb mutations are rare in oral cancer, its loss of expression was seen in 66% of OSCC cases and in 64% of premalignant lesions [22]. Another possible marker of oral cancer is Survivin, an apoptotic process inhibitor, expressed in about 80% of the forms of squamous cell oral carcinoma and whose expression is related with an aggressive phenotype [53]. It has been shown that miRNAs can have specific expression profiles for developmental stages, tissues, and various pathologies. Studies on several forms of cancer, including oral cancer, have shown an altered expression of miRNA in tumor tissue compared to healthy tissue, suggesting the involvement of these molecules in carcinogenesis [54–56]. Human cells have a limited capacity for self-replication and, after numerous cell divisions, cease to grow and enter on senescence phase. Cells with carcinogenic characteristics need to be immortal in order to replicate infinitely and succeed in maintaining the length of their telomeres unaltered.

Since tumor growth is limited to 1-2 mm3 in the absence of adequate perfusion, solid tumors require substantial blood supply to be able to grow and metastasize [57]. The angiogenic phenomenon is the result of the opposing action of proangiogenic signals (vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and interleukin 8 (IL-8)) and antiangiogenic signals (interferons and proteolytic fragments such as angiostatin and endostatin). Oral squamous cell cancer has an important local invasive capacity and a high predisposition to metastasize in the cervical lymph nodes. The invasive and metastatic phenomena are the result of a series of processes involving cell adhesion, cytoskeletal rearrangement, cell migration and degradation of the basement membrane, passage and survival in the bloodstream, and the ability to escape from this and colonize distant sites with the formation of new vessels.

3.6. Proteomic Changes of Oral Cancer. A total of eight clinical studies, in which samples were analyzed, described protein biomarkers and evaluated the wound healing of the site after the surgery. In biology, a biomarker is a molecule that identifies the presence of a tissue. The marker can be of any nature, but substantially it is a protein, or otherwise polypeptide, since it is the proteins that are translated by DNA. For this reason, a marker is such: it is a molecule that is produced mainly by that type of cell. If the marker is used as a disease index, it should only be produced in the presence of this disease. Few markers however meet these needs. The major problem is given by tumor cell markers: as cells, however, are not completely extraneous to the body, neoplastic cells do not translate for molecules that make their dosage accurate method. From a molecular point of investigation, studies involved evaluated the aberrant expressions of candidate protein biomarkers and their quantitative yield in specimens. The protein modification is related to the genetic or epigenetic alterations. In some cases, the marker can be represented by high-density lipoprotein components, HDLs, and HDL-cholesterol, [16, 41–48] or even by genetic alterations such as those found in some solid tumors [50–54]. Proteins are fundamental for physiological cell functioning and life. Aberrant genetic expressions of potential proteins alter cell division, proliferation, immune response, tissue growth, and finally metastasis [48–55]. As for other kind OC cancers, typical patterns of protein expression or individual proteins with specific features have been recorded and classified as oral cancer biomarkers in order to perform diagnosis and therapy.

4. Discussion

The purpose of this review was to systematically overview published studies restricted to "clinical trials" concerning genetic and proteomic biomarkers for detection and prognosis of OC and their relation to wound healing.

Luo et al. [39] evaluated the role of osteopontin (OPN) in chemosensitivity in locally advanced oral squamous cell carcinoma (OSCC) in humans. Authors considered 121 patients and validated the role of OPN in cell proliferation. The recombinant human OPN was executed to SAS cells (human tongue carcinoma cell line) to investigate if the increased OPN protein could influence a proliferative advantage to SAS cells. The presence of OPN is related to bone resorption, wound repair, immune function, and angiogenesis. However, it is particularly strongly associated with tumorigenesis also. The authors demonstrated that the proliferation percentage was significantly increased in matricellular OPN in a dose-dependent manner in SAS cells. This result demonstrates that one of the major roles of OPN is to promote growth of OSCC cells. Moreover, it was concluded how OPN-mediated cisplatin resistance contributes to a poorer clinical outcome and local wound healing in patients with locally advanced inoperable OSCC treated with cisplatin-based IC and CCRT.

Taoudi Benchekroun et al. [40] performed a study investigating oral premalignant lesions. The authors obtained data indicating that an increased *EGFR* gene copy number is common. Therefore, it is associated with OSCC development in patients with oral premalignant lesions (OPLs) expressing high EGFR, particularly OSCC developing at the site of a high-expression OPL; the authors also suggested that EGFR inhibitors might prevent oral cancer in patients with OPLs having an increased *EGFR* gene copy number. Moreover, the authors also demonstrated that an increased *EGFR* gene copy number in OPLs is a precursor to *EGFR* gene amplification in HNSCC (as is chromosome 7 increased copy number)

and an important oncogenesis-driving effector in oral oncogenesis reducing the possibility of having healing at the surgical site and final good prognosis for the patient.

Jung et al. [41] identified deregulated miRNAs in oral cancer and further focus on specific miRNAs that were related to patient survival. Authors reported that miRNA expression profiling provided more precise information when oral squamous cell carcinomas were subcategorized on the basis of clinic pathological criteria. Data extracted from their research highlighted that the interpretation of miRNA expression patterns could be better resolved when one takes into consideration clinical pathological data of OSCC subtypes. Patient survival data demonstrated that the keratinization and the high miR-21 levels were significant factors of OC patient prognosis. Moreover, miR-7 and miR-21, two keratinization-associated miRNAs, could influence the modification of the tumor suppressor gene RECK in OC. Even if the 17 analyzed tumors clinically showed similar features, unique miRNA expression patterns were generated for specific subtypes of OSCCs. Finally, the recorded data underlined that different clinicopathological features and miRNA expression profiles could be used as specific signatures of individual subtypes of oral tumors with different final prognoses and healing possibilities.

Minakawa et al. [46] assumed that Kinesin family member 4 (KIF4A) is involved in oral squamous cell carcinomas (OSCCs) pathogenesis by the activation of the spindle assembly checkpoint (SAC). KIF4A is overexpressed frequently in OSCC, which suggests interference in the function of the spindle checkpoint proteins such as BUB1, MAD2, and CDC20. KIF4A expression was correlated with tumor size in KIF4A-positive cases, suggesting that SAC activation plays a significant role in cellular proliferation in OSCC. The authors concluded that KIF4A expression is likely to be a key regulator of carcinogenesis progression in OSCCs.

Su et al. [42] studied how the DEPDC1B (defined like guanine nucleotide exchange factor) induced both cell migration in a cultured embryonic fibroblast cell line. Moreover, it was recorded to favor anchorage-independent growth in oral cancer cells. It was demonstrated that DEPDC1B exerts a biological function by regulating Rac1. To determine whether DEPDC1B played a role in the induction of cell proliferation, contributing to faster wound healing, the authors evaluated the growth rate of cells expressing DEPDC1B and control cells founding no substantial difference between the growth rates of DEPDC1B-expressing cells and control cells.

However, the authors concluded that oral cancer samples overexpressed DEPDC1B proteins, compared with normal adjacent tissue, and so DEPDC1B plays a role in the development of oral cancer.

Cao et al. [43] investigated the role of the transcriptional repressor named Enhancer of Zeste Homolog 2 (EZH2) in oral carcinogenesis and its clinical implication as an OSCC risk predictor. The study revealed how at 5 years after diagnosis, the 80% of patients whose OLs expressed strong EZH2 developed OSCC. In Leuk-1 cells, EZH2 downregulation resulted in G1 arrest, decreased invasion capability, decreased anchorage independent growth, downregulation of cyclin D1, and upregulation of $p15^{INK4B}$. The recorded data suggested that EZH2 seems to have a fundamental role in OL malignant transformation and may be a biomarker in predicting OSCC development in patients with OLs. Moreover, classifying the EZH2 expression in three stages as weak, moderate, and strong, the authors correlated this situation with better or not clinical healing, patient survival, and final prognosis. Quick diagnosis results are fundamental in order to approach the right therapy and for long survival.

Saintigny et al. [44] considered deltaNp63 as homolog of the p53 tumor suppressor and frequently amplified and overexpressed in squamous cell carcinomas, including head and neck squamous cell carcinoma. The authors were able to determine, in a relatively large population from whom OPL samples had been collected in a prospective longitudinal manner, how the level of overexpression of deltaNp63 alone or in combination with other molecular and morphologic features can be associated with a high risk to develop oral cancer. This investigation referred only oral cancers that developed in the same site as the OPL; 25% of the patients positive for podoplanin developed cancer, compared with 4% of the patients negative for podoplanin; 24% of the patients positive for deltaNp63 developed cancer, compared with 7% of the patients negative for deltaNp63; and 40% of the patients positive for all the biomarkers developed oral cancer, compared with 9% of the patients with no, one, or two positive biomarkers. The authors concluded that because the measurement of the three biomarkers can be done in routine pathology laboratories, it can be useful for evaluating soft tissue healing after OC removal and then patient survival.

Saintigny et al. [45] in a next investigation tried to determine the value of gene expression profiling in predicting oral cancer development. Gene expression profile was measured in 86 of 162 OPL patients who were enrolled in a clinical chemoprevention trial that used the incidence of oral cancer development as a prespecified endpoint. The results showed that gene expression profiles might improve the prediction of oral cancer risk in OPL patients. Moreover, the significant genes identified may serve as potential targets for oral cancer chemoprevention. Tumor progression from normal mucosa to dysplastic mucosa and eventually cancer is the result of a series of gene modifications affecting the normal functions of genes such as protooncogenes and tumor suppressors. Such alterations can be partly inherited but most are mutations that develop ex novo and accumulate in precancerous and cancerous tissue. These mutations can cause alterations in cell cycle regulation, differentiation, proliferation, DNA repair mechanisms, and cellular immunity. Chromosomal aberrations such as deletions, amplifications, and structural rearrangements are common in neoplasms and therefore also in head and neck cancer.

All those clinical studies evaluated an alteration of genomic proteins leading a tissue transformation directed to the OC formation. The possibility of quickly knowing those entire factors such as oral premalignant lesions (OPLs) may help in quick diagnosis and management.

Unfortunately, among the studies taken into consideration, few of those evaluate the same markers; thus, the risk of bias of this review study is classified as "high." It is not possible to make a real report of the statistics of the different

studies, which, however, despite the small number of patients have satisfactory statistical results. It is very interesting to consider the possibility that these biomarkers represent a factor to intervene early in the pathology so as to make complex reconstructions more rare [57]. Specifically in those cases, clinicians should avoid the use of complex rehabilitations placing dental implant fixtures increasing chronic inflammatory process of the jaws and exposing the patient to a risk [58]. In this way, the risk of psychological complications can be reduced and it can affect the patients' oral health and quality of life [59]. It is interesting to highlight anomalies in the crevicular fluid associated with the inflammatory state of the mucosa therefore with precancerous lesions, benign lesions, or OC [60, 61].

5. Conclusions

It is estimated that in the world, the annual cases of squamous cell head/neck neoplasia are more than 640,000 (with 350,000 deaths). After the success in HER2-positive metastatic breast cancer, lapatinib (a small oral molecule that is the result of GSK research) has also opened an important path in the treatment of head and neck cancer. These results tell us that the use of a dual tyrosine kinase inhibitor such as lapatinib may be clinically important not only in breast cancer but also probably in other tumors such as the head and neck, where EGFR is overexpressed. Surely, the possibility of identifying markers for a diagnosis of a primary oral cavity tumor or a relapse, especially if early, can save the life of numerous patients. The possibility of having a set of biomarkers that represent a certain risk for OC and above all the ease of sampling may constitute real screening for all patients at risk (genetic predisposition or family history) or exposed to environmental risks (alcohol, smoking, etc.). The present systematic review of clinical studies discovered genes and proteins associated with OC and strictly related with the wound healing, the prognosis, and patients' long-term survival. Due to high heterogeneity of the researches, it was not possible to perform meta-analysis for comparing the data of the selected papers. Due to poor materials and several parameters recorded, it is not possible to establish biomarkers specific for oral cancer. The diagnostic capabilities are also not sufficiently developed and used to allow the use of these markers. However, the highlighted papers demonstrated how the high, low, or moderate marker expression might influence the clinical status and the final prognosis of the patients. At this stage, it seems not possible to define standard genetic patterns of tumor cells.

Conflicts of Interest

The authors report no conflicts of interest related to this study.

Authors' Contributions

G.C. is the author responsible for writing the paper. A.S.H., U.R., and G.L. are the chief reviewers for the collected data and responsible for the language proof and revision. L.F., S.C., and A.B. are responsible for the collection of data and tables. C.D. and R.D. are responsible for funding acquisition data. G.T., R.S., L.L., and G.L. are responsible for editing, original data, and text preparation. M.C. is responsible for the supervision.

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Research Article

Glucose-Regulated Protein 94 Modulates the Response of Osteosarcoma to Chemotherapy

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Background. Osteosarcoma (OS) is the most common and most aggressive primary solid malignant bone tumor in children and young adults and has high rates of recurrence and metastasis. The endoplasmic reticulum (ER) stress pathway is important in regulating the chemo-responsiveness of cancer. However, the role of glucose-regulated protein 94 (GRP94) in regulating the response of OS to chemotherapy has never been explored. *Methods*. In this study, two OS cell lines, MG63 and 143B cells, were used to evaluate the mechanism by which GRP94 modulates the response of osteosarcoma to chemotherapy. GRP94-knockdown (GRP94-KD) OS cells were generated using short hairpin RNAs, and the response to chemotherapy was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell apoptosis was quantified with propidium iodide (PI) staining and flow cytometry. *Results*. Silencing of GRP94 in MG63 and 143B cells did not influence the growth and migration of the cells, but reduced the colony formation. GRP94-KD OS cells were more resistant to paclitaxel, gemcitabine, and epirubicin treatments than cells transfected with the scrambled control, and more cells transfected with the scrambled control underwent apoptosis after paclitaxel, gemcitabine, and epirubicin treatments than GRP94-KD cells. *Conclusions*. Therefore, GRP94 silencing may increase the resistance of MG63 and 143B cells to paclitaxel, gemcitabine, and epirubicin treatments by inhibiting the induction of apoptosis. Thus, GRP94 may be a key biomarker for the chemotherapeutic response of OS.

1. Introduction

Osteosarcoma (OS) is the most common type of primary solid malignant bone tumor in children and young adults (nearly 5% of all cases of cancer in children), with 70–75% of cases occurring between the ages of 10 and 25 years [1]. OS forms at the ends of the long bones of the body, such as in the arms and in the legs, mainly near the knee [2]. OS is an aggressive disease with a high recurrence rate after treatment and is highly metastatic to lungs and bones, thus leading to a poor prognosis [1, 3]. Currently, the standard therapeutic strategy for OS is surgical resection

and chemotherapy. However, although several new drugs have been developed in the past decade, the efficacy of these treatments is not satisfactory [3]. Moreover, the lack of prognostic biomarkers for early diagnosis and therapeutic responses is a major issue in the management of OS.

Stress-related proteins such as glucose-related protein 94 (GRP94) play critical roles in tumor progression and therapeutic efficacy. GRP94 has been shown to aid cells in evading lethal stresses, such as ischemic injury, radiation exposure, and chemotoxicity [4–6]. As a member of the heat shock protein 90 (HSP90) family of molecular chaperones, GRP94 is located in the ER. GRP94 is overexpressed in cancer tissues, and this overexpression is associated with cancer aggressiveness, the metastatic potential, and chemotherapy responses [7, 8]. Strategies targeting GRP94 have been shown to enhance the degradation of GRP94 client proteins and to induce cell apoptosis in different cancers [9, 10]. According to previous clinical trials, a GRP94-targeting drug reduces metastasis and improves the responses of specific cancers to chemotherapy [11–13].

The roles of GRP94 in the progression and therapeutic response of OS are not clear. Therefore, we aim to explore the roles of GRP94 in OS to ultimately determine effective approaches for managing the disease.

2. Materials and Methods

2.1. Chemicals, Reagents, and Cell Culture. Human OS cells MG63 were purchased from ATCC and cultured in Eagle's minimum essential medium (MEM) (Gibco BRL, Grand Island, NY, USA) containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA), and 2% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin). The 143B cell line was provided by Dr. Pei-Ni Chen (Chung-Shan Medical University) and cultured in RPMI supplemented with 10% fetal bovine serum. Cells were incubated in a humidified incubator (37°C, 5% CO_2) and were either subcultured or used before they reached 80% confluence. Triton X-100, Tris-HCl, neomycin, trypan blue/EDTA, ribonuclease-A, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against GRP94 and GAPDH were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Caspase 3, caspase 7, and PARP antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States).

2.2. Generation of GRP94-Knockdown OS Cell Lines. The expression of GRP94 in MG63 cells and 143B cells was silenced using a small hairpin RNA (shRNA). A GRP94-specific shRNA was purchased from the National RNAi Core Facility, Academia Sinica, Taiwan, and was described in a previous study [14]. The target sequences for human GRP94 mRNA (NM_003299) and a nontarget shRNA were described in the same study. The GRP94-shRNA and control-shRNA plasmids were transfected into MG63 cells and 143B cells using a Neon® Transfection System (Life Technologies, Grand Island, NY). The stably transfected cells were selected using the antibiotic puromycin, as previously described [15, 16]. After 48 h, the expression of GRP94 was verified by quantitative real-time PCR and Western blotting.

2.3. Protein Extraction and Immunoblot Analysis. Protein abundance was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as previously described [17]. Cells were washed with cold PBS and lysed with cell lysis buffer containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN). Equal amounts of proteins were separated on a 10% SDS-PAGE gel under reducing conditions and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were subsequently blotted using antibodies against GRP94, caspase 3, caspase 7, PARP, or GAPDH and horseradish peroxidase-conjugated secondary antibodies, visualized using TOOLS Ultra ECL-HRP Substrate (BIO-TOOLS Co., Ltd., Taiwan), and then detected using a VersaDoc 5000 imaging system (Bio-Rad Laboratories).

2.4. Cell Viability Assay. Cells were plated in 24-well plates at a density of 2×10^4 cells/well and incubated overnight. Cells were incubated with different concentrations of paclitaxel (0–600 ng/mL), gemcitabine (0–40 μg/mL), or epirubicin (0–2 μg/mL) for various periods to determine the dose that resulted in 50% inhibition (IC_{50}). dH_2O was used as a vehicle control. The medium was aspirated at selected time points. The remaining cells were further incubated with 0.25 mg/mL MTT for 1 h and subsequently extracted with DMSO, and the color change in the extract was measured at 515 nm using a spectrophotometer (GE Healthcare).

2.5. DAPI Staining. Approximately 2×10^5 cells/well in a four-well chamber slide were incubated with paclitaxel (6 ng/mL), epirubicin (2 ng/mL), or gemcitabine (4 ng/mL) for 48 h. The cells were then fixed, stained with 4′,6-diamidino-2-phenylindole (DAPI), and imaged using a fluorescence microscope.

2.6. Propidium Iodide (PI) Staining for Determining Apoptosis. Cells (3×10^5) were seeded into six-well plates and allowed to adhere overnight. Cells were incubated with paclitaxel (6 ng/mL), gemcitabine (4 ng/mL), or epirubicin (2 ng/mL) for 48 h and then harvested and washed with PBS at different time intervals. Then, the cells were fixed with pure methanol, treated with RNase A at a final concentration of 40 μg/mL, and stained with propidium iodide (40 μg/mL) for 30 min at room temperature. The stained cells were analyzed using Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, United States), and the DNA content was quantified using the Modfit software (Verity Software House, Inc., Topsham, ME). The percentage of hypodiploid cells (sub-G_1) was used to quantify dead cells. The results were analyzed using FlowJo Software.

2.7. Transwell Migration Assay. In vitro cell migration was investigated using an 8 μm BD Falcon™ culture insert (BD Biosciences), as previously described [18]. Specifically, five hundred cells were suspended in 500 μL of serum-free media and then seeded into the upper compartment of the chamber. The lower compartment was filled with 1 mL of media

containing 10% FCS. After 24 h of incubation, the nonmigrating cells were scrubbed from the upper surface of the membrane. The migrated cells on the reverse side of the membrane were stained with 0.1% crystal violet, and the cells were counted under a microscope at 100-fold magnification.

2.8. Colony Formation Assay. Cells were seeded in a 6-well plate at a density of 10000 cells/well and cultivated for 2 weeks. Subsequently, cells were fixed and stained with crystal violet. Crystal violet staining was observed and quantified under a phase contract microscope.

2.9. Analysis of Caspase 3/7 Activity. Caspase 3/7 activity was measured using a SensorLyte® Homogeneous AMC Caspase 3/7 assay kit, according to the manufacturer's instructions (AnaSpec, Inc., Fremont, CA). Fluorescence intensity was measured using a Varioskan Flash (Thermo Fisher Scientific, Waltham, MA) at an excitation wavelength of 354 nm and an emission wavelength of 442 nm.

2.10. Statistical Analysis. All experiments were repeated a minimum of three times. All data reported are presented as means ± SD. The data presented in the figures were obtained from representative experiments and were quantitatively similar to the replicate experiments. Statistical significance of differences in data between two samples was determined using Student's *t*-test (two-tailed) with Microsoft Excel.

3. Results

3.1. GRP94 Silencing Did Not Influence the Proliferation or Migration but Reduced the Colony Formation Ability of OS Cells. GRP94 expression was knocked down with an shRNA, and stably transfected cells were selected using antibiotics to further dissect the role of GRP94 in OS. The knockdown efficiency was confirmed by Western blotting, and GRP94 expression in knockdown cells was reduced by greater than 80% at both the transcriptional and translational levels compared with that in cells transfected with the scrambled control (Figure 1(a)). The growth of GRP94-KD and scrambled control MG63 cells was determined using the MTT assay to analyze the biological effects of the downregulation of GRP94 expression on MG63 cells and in 143B cells. As shown in Figure 1(b), the growth of GRP94-KD cells and scrambled control OS cells was similar. Moreover, the transwell migration assay did not reveal differences in the number of migrating cells between the GRP94-KD and scrambled control MG63 and 143B cells (Figure 1(c)). Interestingly, the numbers of colonies were dramatically reduced in GRP94-KD cells compared with scrambled control cells (Figure 1(d)). These results obtained after GRP94 silencing suggest that GRP94 does not influence the growth or migration of MG63 and 143B cells but may mediate the ability of OS cells to form colonies.

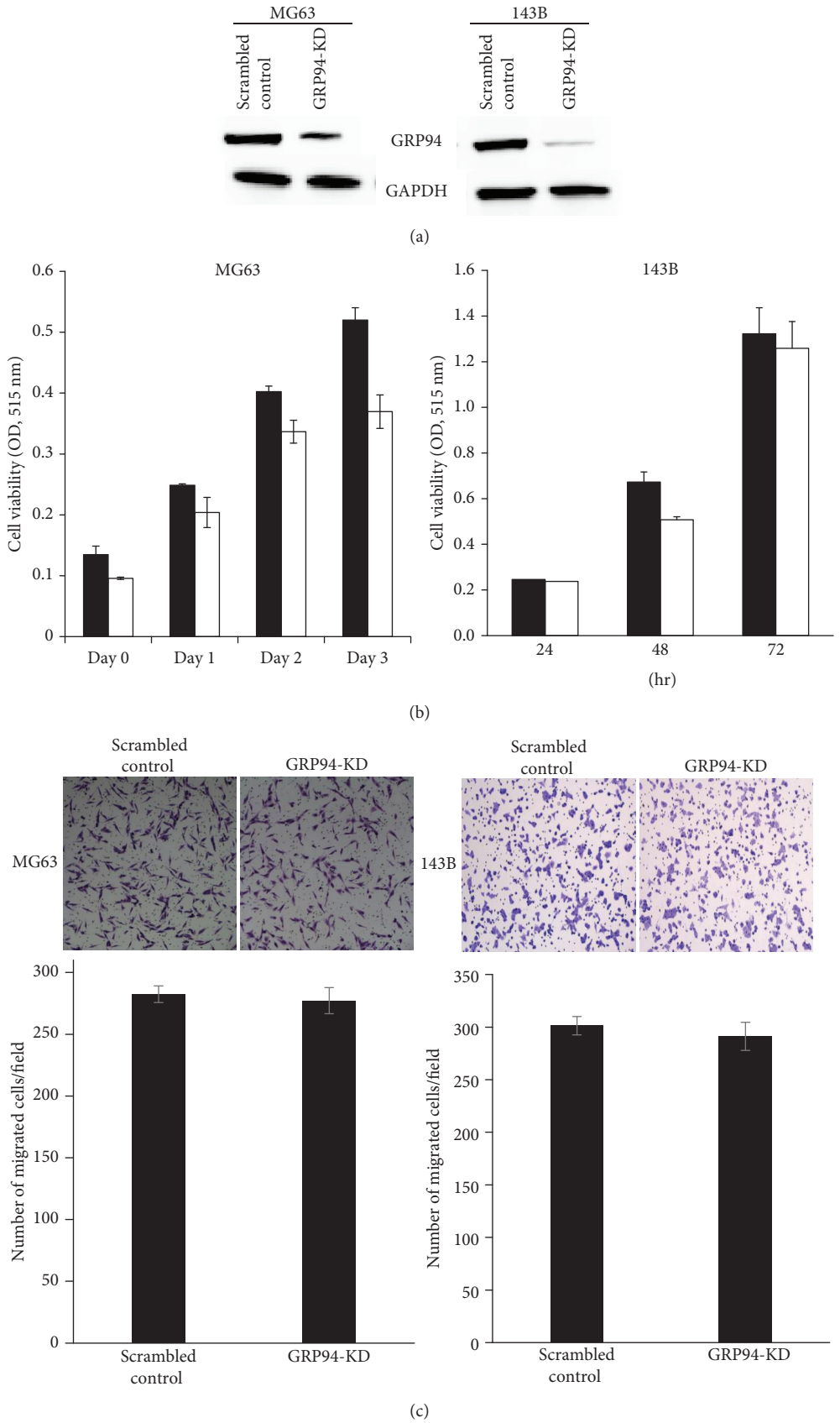
3.2. Knockdown of GRP94 Increased the Resistance of OS Cells to Chemotherapy. Scrambled control and GRP94-KD cells were treated with different doses of paclitaxel (0–600 ng/mL), gemcitabine (0–40 μg/mL), or epirubicin (0–2 μg/mL), and the 50% growth inhibition (IC_{50}) doses in the GRP94-KD and scrambled control MG63 and 143B cells were determined to identify the role in mediating the cellular response to chemotherapy. As shown in Figure 2, GRP94-KD cells exhibited increased IC_{50} values for paclitaxel, gemcitabine, and epirubicin compared with scrambled control cells. Based on these results, GRP94 silencing may increase the resistance of OS cells to paclitaxel, gemcitabine, and epirubicin treatments.

3.3. Knockdown of GRP94 Inhibited Chemotherapy-Induced Apoptosis in OS Cells. DAPI staining was performed to further explore how GRP94 expression influences the cytotoxic effects of paclitaxel, gemcitabine, and epirubicin on MG63 cells and 143B cells. Chemotherapy-induced apoptosis in a greater number of scrambled control cells than in GRP94-KD MG63 cells, as shown by the DAPI staining (Figure 3(a)). In scrambled control cells, it shows lower cell densities and higher apoptotic cell population upon exposure to paclitaxel, gemcitabine, and epirubicin. We further confirmed the cytotoxic effects of paclitaxel, gemcitabine, and epirubicin on scrambled control and GRP94-KD OS 143B cells using PI staining. As shown in Figure 3(b), the sub-G_1 population of scrambled control 143B cells was increased after paclitaxel, gemcitabine, and epirubicin exposure. However, the sub-G_1 population of GRP94-KD-treated cells was reduced compared with that of scrambled control-treated cells. Thus, GRP94 silencing may reduce the sensitivity of OS cells to paclitaxel, gemcitabine, and epirubicin treatments.

3.4. Determination of the Activity and Expression Levels of Caspases 3/7. The activities of caspase 3 and caspase 7, which are executor caspases that act together to facilitate apoptosis, were determined in MG63 cells using a fluorometric assay to further confirm these results. Briefly, in this assay, the hydrolysis of a specific substrate results in the generation of a fluorescent molecule, and thus, the fluorescence intensity reflects the activities of caspases 3/7. Paclitaxel, gemcitabine, and epirubicin treatment dramatically increased caspase 3/7 activities in scrambled control cells compared to GRP94-KD cells (Figure 4(a)). Next, we confirmed the levels of the caspase 3 and caspase 7 proteins by Western blotting. The levels of cleaved caspases 3 and 7 were dramatically increased in OS cells treated with paclitaxel, gemcitabine, and epirubicin (Figure 4(b)). The levels of cleaved caspase 3, caspase 7, and PARP were increased to a greater extent in scrambled control-treated cells than in GRP94-KD cells. Based on these data, GRP94 silencing induces chemotherapy resistance in OS cells due to the suppression of the caspase-mediated mitochondrial cell death pathway.

4. Discussion

OS is a highly aggressive malignant bone tumor, with approximately 20% of all patients presenting with metastasis at the initial visit [19]. Primary OS has been shown to be resistant to high-dose chemotherapy, and its 5-year overall survival rate has plateaued at 60–70% in the past two decades [20–22]. Therefore, the identification of prognostic



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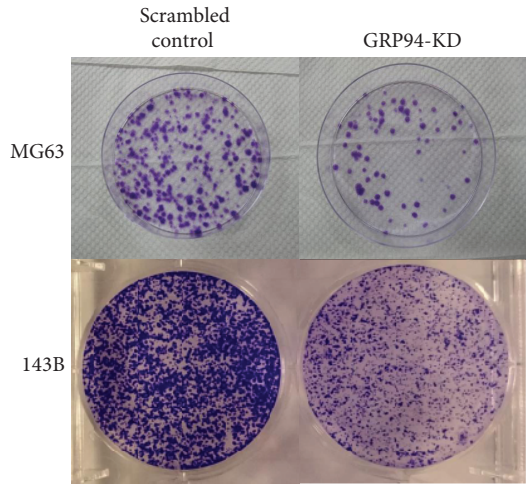

(d)

Figure 1: The effects of downregulating GRP94 expression on MG63 and 143B cells. (a) GRP94 was silenced using an shRNA. The levels of GRP94 in the control and GRP94-knockdown (GRP94-KD) MG63 and 143B cells were confirmed by Western blotting. (b) The growth of GRP94-KD and scrambled control MG63 and 143B cells was determined using the MTT assay. (c) Migration was assessed using transwell migration assays. (d) The colony formation assay was performed using scrambled control and GRP94-KD cells. The results reported were obtained from at least three independently repeated experiments ($^{**}p < 0.01$).

or therapeutic biomarkers that may enhance the therapeutic response of OS and improve the management approaches for this disease is critically needed. Higher levels of GRP94 are correlated with poor disease outcomes in different cancers [23, 24], but there is limited information of the roles of GRP94 in therapeutic response in OS. The current study has demonstrated that GRP94 silencing does not influence the proliferation or migration, but causes a reduction in colony formation in both MG63 cells and 143B cells. The finding that GRP94 may not be involved in the mechanism regulating the growth and migration is unique to OS compared with the findings from other cancers. In addition, GRP94-KD reduced the colony formation ability, indicating that GRP94 may be correlated with the malignant features of OS. GRP94-KD also decreased cells' sensitivity to chemotherapeutic drugs (paclitaxel, gemcitabine, and epirubicin).

GRP94 is well known for its therapeutic and prognostic roles in cancer. GRP94 is induced as a defense mechanism for the survival of cancer cells exposed to stressful conditions [10]. It is elevated as a response to the inhibition of glycosylation, Ca^{2+} pool depletion, and malfolded proteins and is regulated through antiapoptotic (BCL-2) target proteins [25, 26]. ER stress may induce apoptotic signaling pathways as cells mount the unfolded protein response (UPR) as a self-protective mechanism for ER function disruption [27, 28]. This leads to the accumulation of different unfolded or misfolded proteins in ER [28]. GRP78 and GRP94 expression are hallmarks of ER stress and UPR [29, 30]. Caspase-mediated apoptosis is said to be an important mechanism which regulates tumor progression [26]. McCormick et al. observed that mouse lymphoma cells that fail to mount GRP94 stress response are more susceptible to the inhibitor of Ca^{2+} uptake into the ER, thapsigargin (TG) [31]; interestingly, the inhibition of GRP94 stress response did not enhance the cytotoxicity of the inhibitor of *N*-linked glycosylation, tunicamycin (TN) [32]. This suggests that two pathways may be involved in the regulation of GRP94: glycosylation inhibition mediated and the one mediated by Ca^{2+} [31], where GRP94 expression promotes radiochemotolerance in cancer cells during the maintenance of cellular Ca^{2+} homeostasis when combating ER stress, after going through cleavage by calpain, simultaneously preventing apoptosis [9].

Fu et al. observed a similar phenomenon in multiple myeloma (MM) where cells expressing low GRP94 and GRP78 were resistant to bortezomib (BTZ). In this study, inducing ER stress with tunicamycin reversed drug resistance of MM cells by inhibiting the PI3K/Akt/mTOR signaling pathway [33]. The same phenomenon has also been observed in ovarian, breast, esophageal, and lung cancer cells treated with different agents and radiotherapy [34–38]. Our previous study also identified a novel pathway by which GRP94 regulates resistance, whereby GRP94 knockdown reduced the sensitivity to taxanes by suppressing the caspase-mediated mitochondrial cell death pathway and by altering the activation of apoptosis and associated proteins [39]. Agreeing with these findings, we propose that GRP94-KD OS cells were resistant to chemotherapy because of their failure to respond to ER stress, which lead to reduced apoptosis and therefore treatment response.

In addition to the above discussion, knockdown of GRP94 leads to AKT activation and the expansion of hematopoietic stem cells (HSCs), which correspond with the loss of surface expression of integrin $\beta 4$ and HSC niche attachment [40, 41]. The liver-specific knockout of GRP94 in mice disrupts cell adhesion, activates liver progenitor cells, and accelerates liver tumorigenesis [42]. These observations may partly explain our finding that GRP94-KD impaired

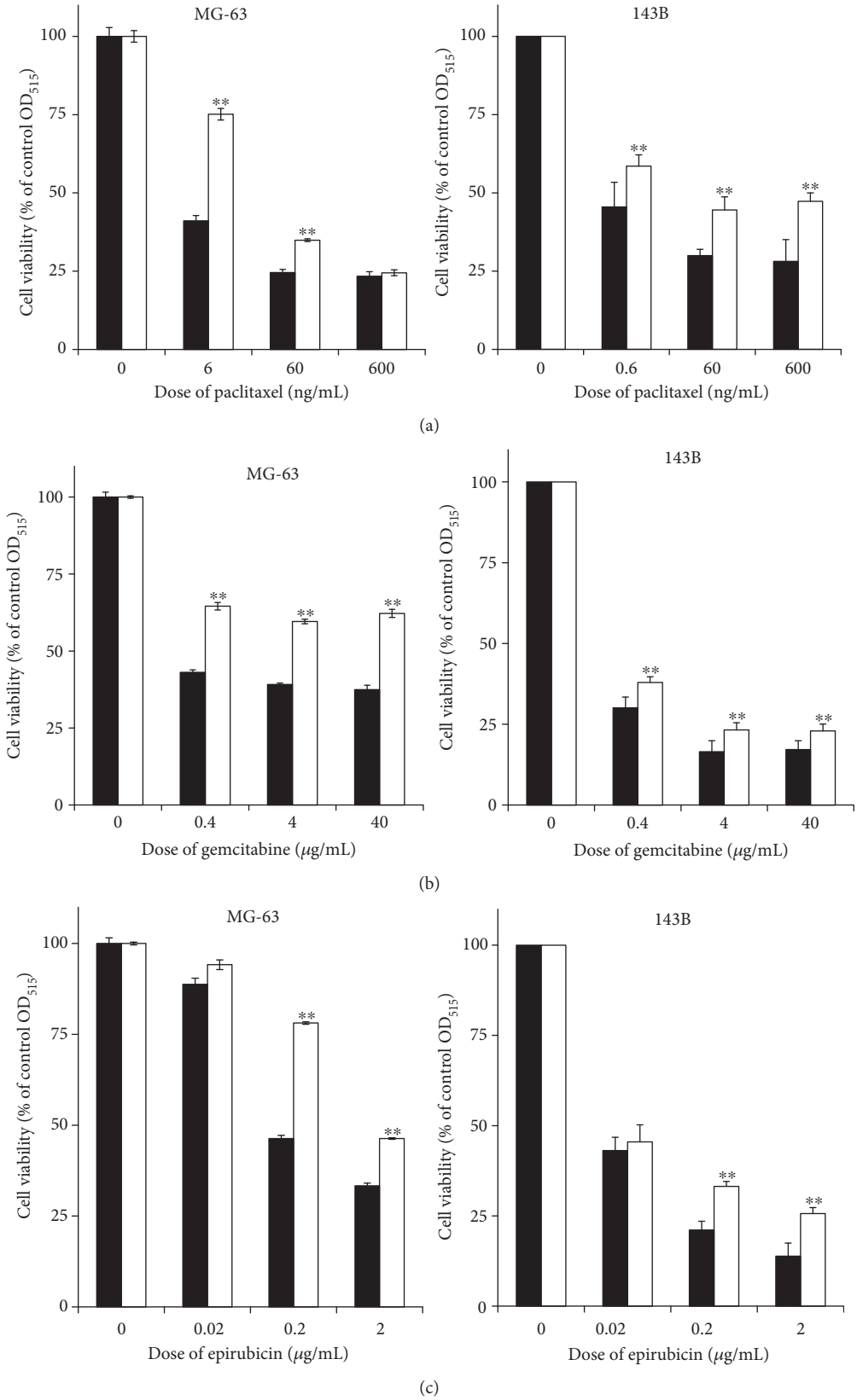

Figure 2: The role of GRP94 in chemotherapeutic resistance. The scrambled control and GRP94-KD OS (MG63 and 143B) cells were treated with different doses of (a) paclitaxel (0–600 ng/mL), (b) gemcitabine (0–40 μg/mL), or (c) epirubicin (0–2 μg/mL). The results reported were obtained from at least three independently repeated experiments ($^{**}p < 0.01$).

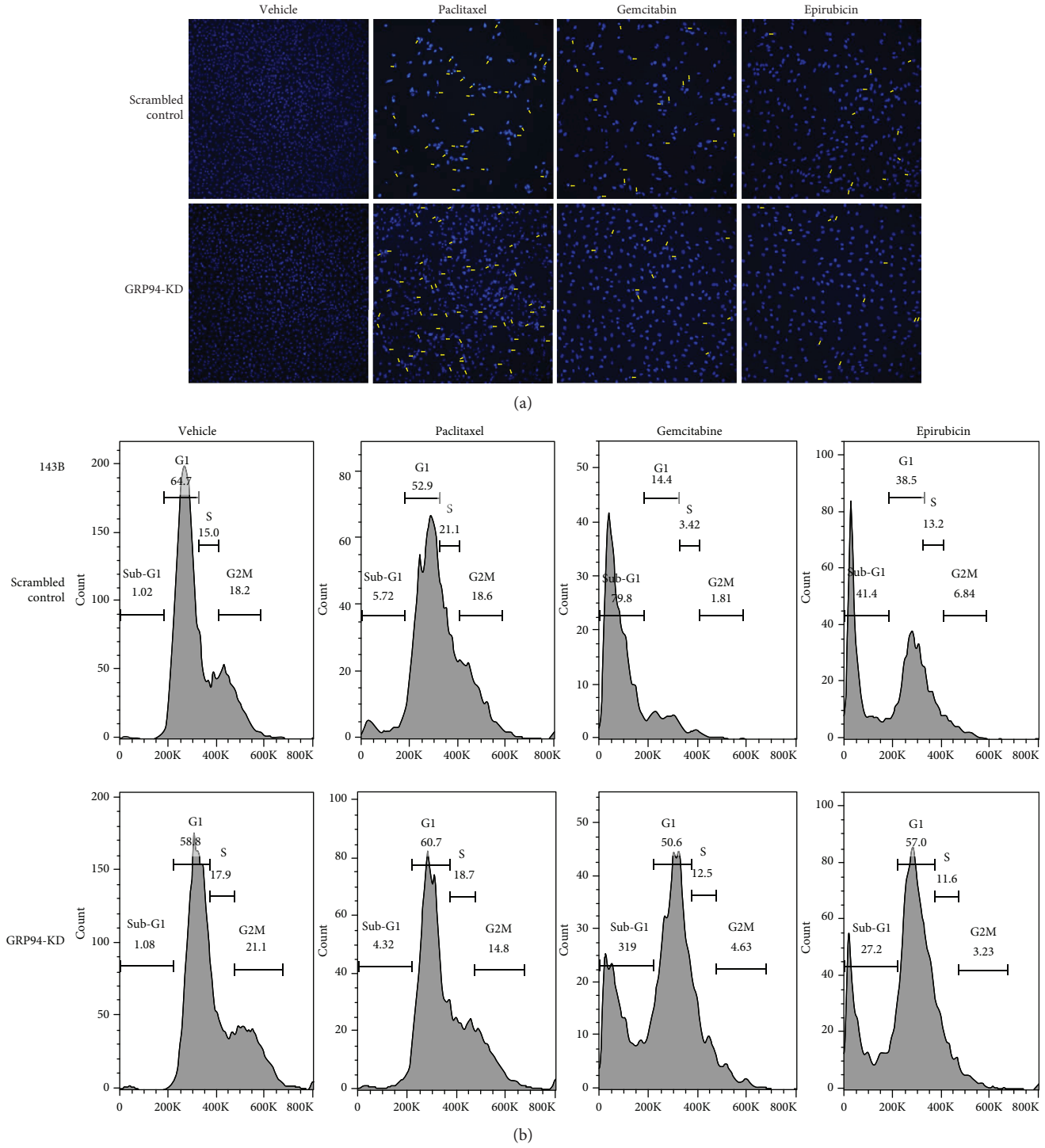

Figure 3: GRP94-KD inhibited chemotherapy-induced apoptosis in OS cells. (a) Representative DAPI staining of MG63 cells. Scrambled control and GRP94-KD MG63 cells were incubated with paclitaxel (6 ng/mL), gemcitabine (4 μg/mL), or epirubicin (2 μg/mL) for 48 hr and then stained with DAPI. Apoptotic cells were imaged using a fluorescence microscope. (b) The populations of scrambled control and GRP94-KD 143B cells in different phases of the cell cycle and sub-G_1 populations after chemotherapy treatment. The flow cytometry analysis of the cell cycle was performed by staining DNA with PI. The results reported were obtained from at least three independently repeated experiments.

anchorage-dependent colony formation. Therefore, GRP94 protects cells from the host defense systems and promotes tumor progression and therapeutic response through its pro-proliferation and antiapoptotic functions [43].

Although the mechanisms are not fully explored in the present study, it has provided important insights into the role of GRP94 in OS, which are crucial for the management of OS and the development of novel drug targets.

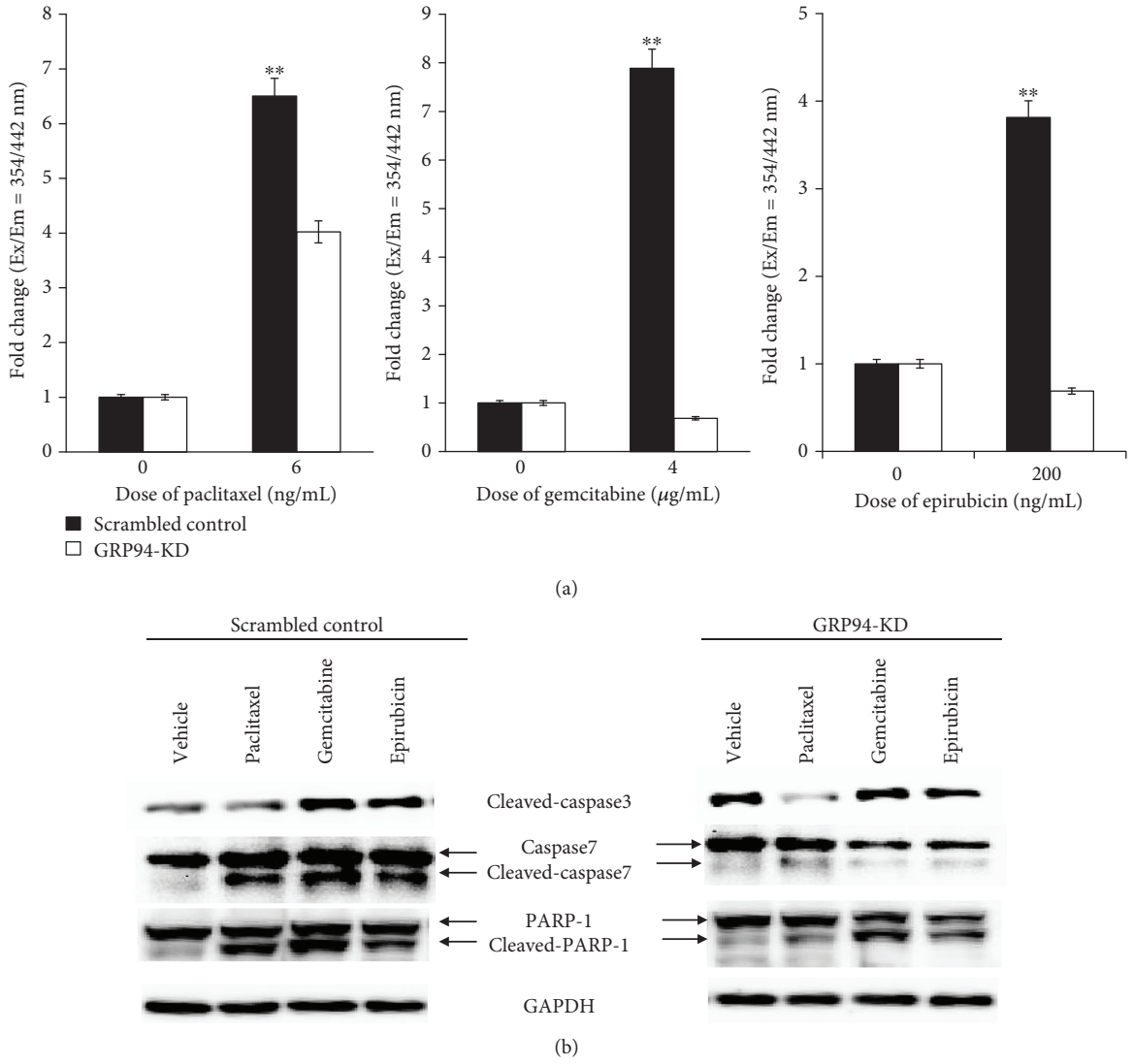

Figure 4: GRP94-KD decreased the chemotherapy-induced caspase 3/7 levels. (a) Caspase 3/7 activities in MG63 cells treated with paclitaxel, gemcitabine, and epirubicin. Fluorescence intensity indicated the level of activated caspases 3/7 in the scrambled control and GRP94-KD MG63 cells after exposure to chemotherapeutic drugs. (b) The levels of cleaved caspase 3, caspase 7, cleaved caspase 7, cleaved PARP-1, and GAPDH in scrambled control and GRP94-KD 143B cells were confirmed by Western blotting. The results reported were obtained from at least three independently repeated experiments ($^{**}p < 0.01$).

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Chien-Yu Huang and Po-Li Wei contributed equally to this work.

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Review Article

Targeting mTOR in Glioblastoma: Rationale and Preclinical/Clinical Evidence

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The mechanistic target of rapamycin (mTOR) drives several physiologic and pathologic cellular processes and is frequently deregulated in different types of tumors, including glioblastoma (GBM). Despite recent advancements in understanding the molecular mechanisms involved in GBM biology, the survival rates of this tumor are still disappointing, primarily due to the lack of efficacious treatments. The phosphatase and tensin homolog (PTEN)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mTOR pathway has emerged as a crucial player in GBM development and progression. However, to date, all the attempts to target this pathway with PI3K, AKT, or mTORC1 inhibitors failed to improve the outcome of patients with GBM. Despite these discouraging results, recent evidence pointed out that the blockade of mTORC2 might provide a useful therapeutic strategy for GBM, with the potential to overcome the limitations that mTORC1 inhibitors have shown so far. In this review, we analyzed the rationale of targeting mTOR in GBM and the available preclinical and clinical evidence supporting the choice of this therapeutic approach, highlighting the different roles of mTORC1 and mTORC2 in GBM biology.

1. Introduction

In the last decades, we witnessed important advancements in understanding the molecular mechanisms involved in GBM biology; however, GBM remains one of the deadliest types of tumor worldwide [1]. Indeed, the paradigm of medical treatment of chemotherapy plus radiation therapy has reached an efficacy plateau and several drugs designed to target one of the most deregulated pathways in GBM (PTEN/PI3K/AKT/mTOR) failed to improve the outcome of these patients [2–4]. More in detail, the limited blood-brain barrier penetration and the compensatory activation of collateral signaling pathways caused the failure of PI3K inhibitors [1, 4]. Additionally, the lack of mTORC2 inhibition that results in AKT activation led to disappointing results of mTORC1 inhibitors in the clinical trials conducted to date [5, 6]. On the other hand, emerging preclinical evidence suggests that targeting mTORC2 might provide an efficacious therapeutic strategy for GBM as it can overcome the limitations of mTORC1 inhibitors and pave the way for a personalized targeted treatment.

2. mTOR: A Brief Overview

2.1. mTORC1 Composition, Upstream Activators, and Downstream Targets. mTOR is a 289 kDa serine/threonine protein kinase localized in two structurally and functionally distinct multiprotein complexes known as mTORC1 and mTORC2 [7]. mTORC1 is composed of regulatory-associated protein of mTOR (RAPTOR), proline-rich AKT substrate 40 kDa (PRAS40), mammalian lethal with Sec-13 protein 8 (mLST8) and DEP domain TOR-binding protein (DEPTOR), and it is inhibited by rapamycin, a macrolide produced by the bacterium Streptomyces hygroscopicus (Figure 1(a)).

Rapamycin inhibits mTORC1 by binding the 12 kDa intracellular FK506-binding protein (FKBP12) that in turn directly interacts with mTORC1 but not with mTORC2 [7].

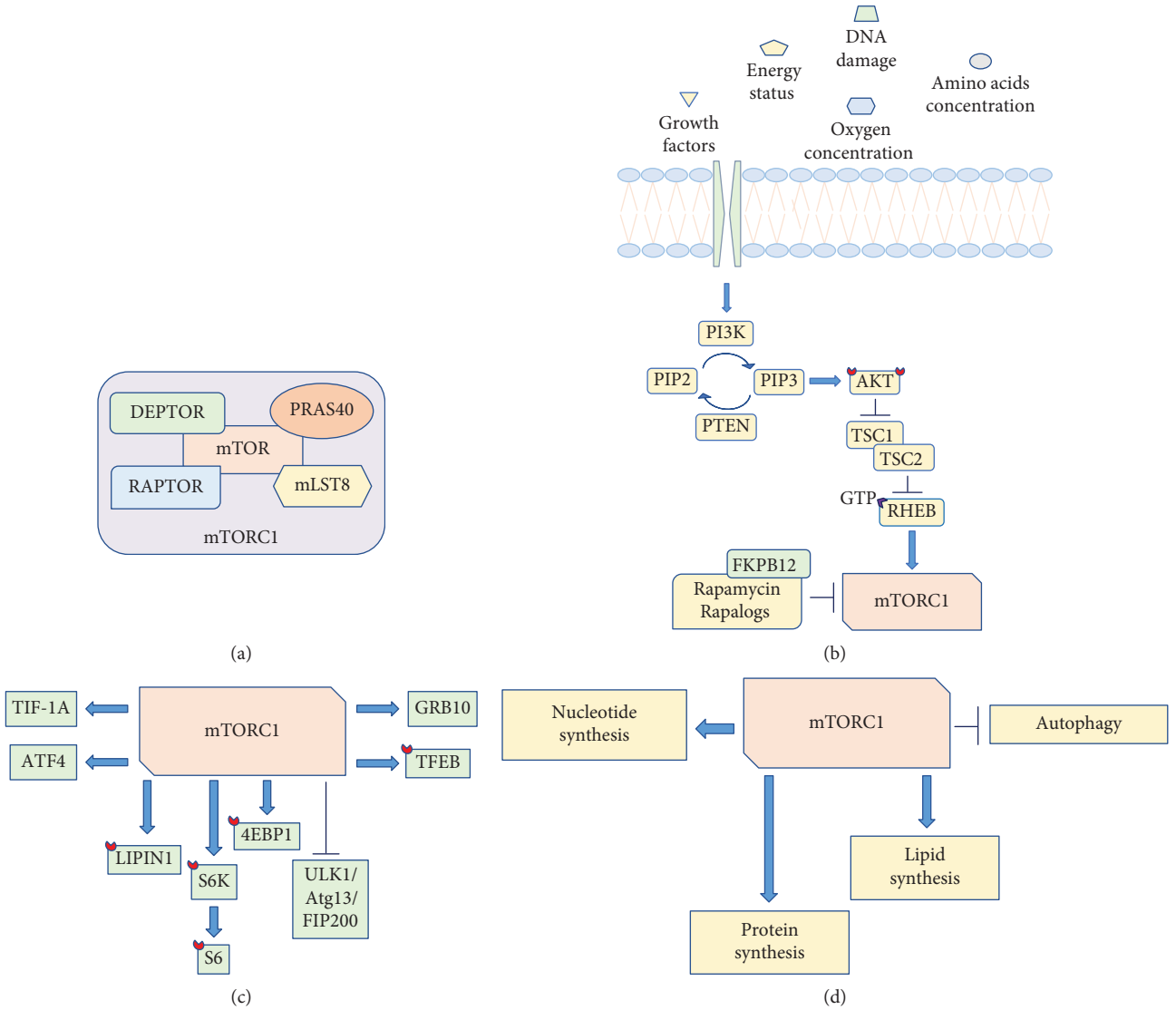

Figure 1: (a) mTORC1 protein composition; (b) mTORC1 upstream activators; (c) mTORC1 downstream targets; (d) mTORC1 cellular functions. Legend: DEPTOR: DEP domain TOR-binding protein; mTOR: mechanistic target of rapamycin; RAPTOR: regulatory-associated protein of mTOR; PRAS40: proline-rich AKT substrate 40 kDa; mLST8: mammalian lethal with Sec-13 protein 8; PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol (4,5)-bisphosphate; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; PTEN: phosphatase and tensin homolog; AKT: protein kinase B; TSC1/2: tuberous sclerosis complex 1/2; GTP: guanosine triphosphate; RHEB: RAS homolog enriched in brain; FKBP12: 12-kDa intracellular FK506-binding protein; rapalogs: rapamycin analogs; 4EBP1: eukaryotic initiation factor 4E (eIF4E)-binding protein; S6K: ribosomal S6 kinases; GRB10: growth factor receptor-bound protein 10; ULK1/Atg13/FIP200: unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa; ATF4: activating transcription factor 4; TFEB: transcription factor EB.

mTORC1 is activated by at least five cues: growth factors, stress, energy status, oxygen, and amino acid concentration. Growth factors, low energy status, low oxygen level, and DNA damage converge on the tuberous sclerosis complex 1/2 (TSC1/2) that acts as a GTPase-activating protein for the GTPase RAS homolog enriched in brain (RHEB), which in turn directly binds mTORC1 resulting into the stimulation of its kinase activity [7] (Figure 1(b)).

Upon activation, mTORC1 promotes cell growth by phosphorylating two downstream targets, namely, eukaryotic translation initiation factor 4E- (eiF4E-) binding protein 1 and ribosomal protein S6 kinase (S6K) that drive protein synthesis (Figures 1(c) and 1(d)). Indeed, S6K phosphorylates the 40S ribosomal subunit, thus triggering the translation of mRNA transcripts with 5′-terminal oligopolypyrimidine, and phosphorylates the eukaryotic translation initiation factor (eIF)4B on serine 422, ultimately promoting eIF4B association with eIF3 and eIF4F complex formation. On the other hand, mTORC1-mediated phosphorylation of 4EBP1 causes the release of eIF4E from 4EBP1, allowing eIF4E-eIF4G association and cap-dependent translation [7]. Furthermore, mTORC1 contributes to protein synthesis by activating the transcription intermediary factor 1-alpha (TIF-1A) that induces RNA polimerase to transcribe rRNA

genes and also by inhibiting a polimerase III repressor known as MAF1, thus enabling 5sRNA and tRNA transcription [8, 9] (Figure 1(c)). Another mTORC1 target is the growth factor receptor-bound protein 10 (GRB10), whose activation is responsible for the degradation of the insulin receptor substrate-1 (IRS-1) and the feedback inhibition of PI3K [10] (Figure 1(c)). In addition to its extensively investigated role in protein synthesis, mTORC1 also participates in lipid and nucleotide synthesis, whose rapid turnover is a hallmark of tumors, including GBM (Figure 1(d)). More in detail, mTORC1 phosphorylates LIPIN-1 and prevents it from entering the nucleus, resulting in the suppression of the sterol regulatory element-binding protein 1/2 (SREBP1/2), a transcription factor involved in fatty acid and cholesterol synthesis [11] (Figure 1(c)). Instead, mTORC1 contribution to purine synthesis occurs through the induction of the activating transcription factor 4 (ATF4) that in turn triggers methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase (MTHFD2) expression, a key component of the mitochondrial tetrahydrofolate cycle [12] (Figure 1(c)). Lastly, mTORC1 contributes to tumor cell growth by inhibiting autophagy, a catabolic pathway that degrades aged or damaged organelles (Figure 1(d)); mTORC1 suppresses autophagy directly by inhibiting the kinase complex unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa (ULK1/Atg13/FIP200) which is a key component required for the autophagy induction and indirectly by modulating the expression of death-associated protein 1 (DAP1), a novel substrate of mTORC1 that negatively regulates autophagy [13, 14] (Figure 1(c)). Along with the aforementioned mechanisms, mTORC1 also blocks autophagy induction through the negative regulation of lysosome biogenesis; indeed, mTORC1 can inhibit the expression of genes involved in lysosomal functions by phosphorylating the transcription factor EB (TFEB) and preventing its nuclear entry [15, 16] (Figure 1(c)).

2.2. mTORC2 Composition, Upstream Activators, and Downstream Targets. Differently, mTORC2 is composed of the rapamycin-insensitive companion of mTOR (RICTOR), DEPTOR, mLST8, stress-activated map kinase-interacting protein 1 (mSIN1), and protein observed with RICTOR (PROTOR) and is considered rapamycin insensitive because, as previously mentioned, the complex rapamycin-FKBP12 is not able to directly bind mTORC2 [7] (Figure 2(a)).

However, it has been demonstrated that prolonged treatment with rapamycin might inhibit mTORC2 assembly by sequestering mTOR in some cell cultures [17]. Differently from mTORC1, less is known about mTORC2 upstream activators; it is triggered by growth factors but does not respond to nutrients [7] (Figure 2(b)). Once activated, mTORC2 drives cell proliferation, motility, and survival primarily through the activation of different AGC protein kinases (Figure 2(c)). In fact, mTORC2 phosphorylates protein kinase C (PKC)δ, PKCζ, PCKγ, and PKCε that are involved in cytoskeleton assembly and cell migration, besides AKT on serine 473 [18–21] (Figure 2(c)). Intriguingly, it has recently been demonstrated that mTORC2 triggers the activation of the serum and glucocorticoid-regulated kinase 1 (SGK1), which is involved in ion transport and cell survival [22] (Figures 2(c) and 2(d)).

The direct involvement of mTORC2 in GBM biology clearly emerged in a Drosophila glioma model obtained by hyperactivating the epidermal growth factor receptor (EGFR), RAS and PI3K. In this model, it has been observed that RICTOR and mSIN1 loss of function prevented tumor formation [23]. Later on, Bashir et al. established that RICTOR overexpression alone was sufficient to promote multifocal infiltrating oligodendroglial tumors in the subventricular zone and lateral ventricles of mice that showed an increased mTORC2 activity that sustained cancer stem cell amplification [24]. In addition to the role in tumor induction, mTORC2 activation is also responsible for GBM growth and progression. Indeed, Gulati el al. observed that treatment of GBM cell lines with rapamycin not only resulted in a time-dependent decrease of S6K phosphorylation but also caused a paradoxical increase of AKT phosphorylation on serine 473 which is known to be responsible for cell proliferation. The same authors reported that this paradoxical increase of AKT phosphorylation can be reversed by RICTOR but not RAPTOR knockdown by siRNA [25].

mTORC2 is also involved in the induction of the Warburg effect, a metabolic process by which tumor cells metabolize glucose via the aerobic glycolysis also in the presence of sufficient oxygen levels to supply the macromolecular demand of rapidly growing cells (Figure 2(d)). Indeed, by stimulating AKT phosphorylation on serine 473, mTORC2 triggers the expression of the glucose transporter type 4 (GLUT4) and the activation of the glycolytic enzyme hexokinase 2 (HK2) and phosphofruttokinase-1 (PFK-1) [26–28] (Figure 2(c)). Moreover, mTORC2 phosphorylates and inactivates class IIa histone deacetylases (HDACs) that causes Forkhead box O (FOXO)1 and FOXO3 acetylation, resulting in c-MYC release from the suppressive miR-34-c [29] (Figure 2(c)). More recently, it has been shown that the acetyl coenzyme A (acetyl-CoA) derived from glucose and lactate metabolism is used by GBM cells to induce RICTOR acetylation that results in mTORC2 activation; this mechanism creates an autoactivation loop by which mTORC2 triggers cell proliferation and growth, bypassing growth factor-activated upstream signaling and rendering GBM cells resistant to receptor tyrosine kinase inhibitors [30]. Additionally, mTORC2 has recently been reported to confer resistance to the alkylating agent cisplatin via nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activation in an AKT-independent way [31] (Figures 2(c) and 2(d)). In this study, Tanaka et al. demonstrated that NF-κB signaling is upregulated in GBM cell lines and in GBM patients treated with rapamycin and that the inhibition of both mTORC1 and mTORC2 with the mTOR kinase inhibitor PP242 sensitizes *EGFRvIII*-mutant tumors to cisplatin-induced cell death, confirming the direct involvement of mTORC2 activation in chemotherapy resistance [31]. As previously mentioned with regard to mTORC1, mTORC2 also participates in lipid synthesis (Figure 2(d)). In GBM, the cleavage of SREBP1 that occurs on Golgi membranes and is triggered

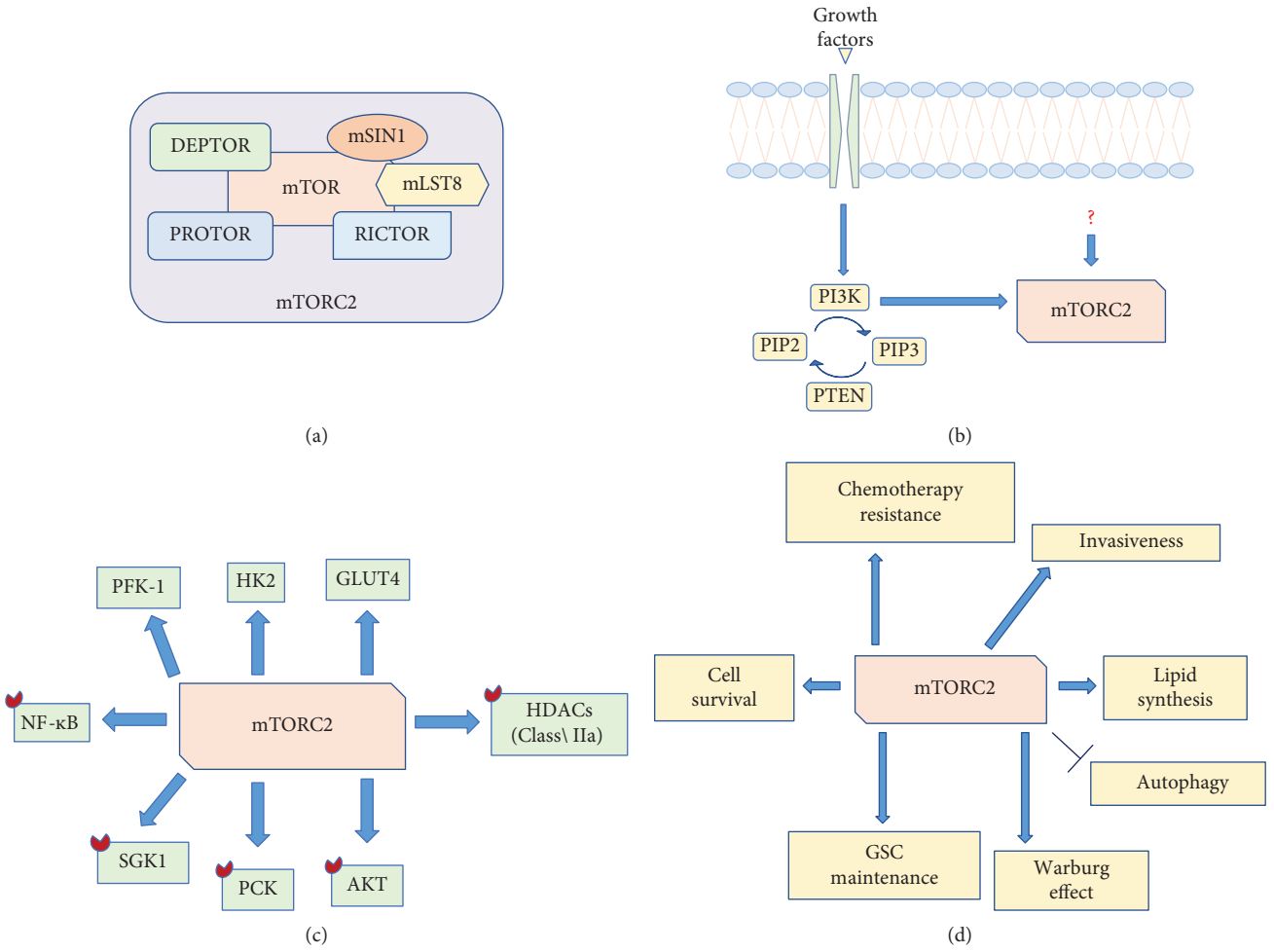

Figure 2: (a) mTORC2 protein composition; (b) mTORC2 upstream activators; (c) mTORC2 downstream targets; (d) mTORC2 cellular functions. Legend: mTOR: mechanistic target of rapamycin; RICTOR: rapamycin-insensitive companion of mTOR; mLST8: mammalian lethal with Sec-13 protein 8; DEPTOR: DEP domain TOR-binding protein; PROTOR: protein observed with RICTOR; mSIN1: stress-activated map kinase-interacting protein 1; PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol (4,5)-bisphosphate; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; PTEN: phosphatase and tensin homolog; AKT: protein kinase B; SGK1: serum and glucocorticoid-regulated kinase 1; PKC: protein kinase C; HDACs: histone deacetylases; PFK-1: phosphofruttokinase-1; HK2: hexokinase 2; GLUT4: glucose transporter type 4; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

by mutant *EGFR* has been demonstrated to be rapamycin insensitive. By contrast, mTORC2 induces SREBP1 cleavage through AKT-dependent and AKT-independent mechanisms, resulting in the expression of acetyl-CoA carboxylase, fatty acid synthase and acyl-CoA synthetase genes, which are all involved in fatty acid and cholesterol synthesis [32, 33].

3. Preclinical Data and Clinical Application of mTOR Kinase Inhibitors

Despite the pivotal role of mTOR in GBM which is now widely recognized, the first generation of mTORC1 inhibitors gave disappointing results in clinical trials. The magnitude of this failure is ascribable to the pharmacodynamic properties of these compounds, as they only target mTORC1, leading to an incomplete inhibition of mTORC1 downstream targets and to the deregulation of a negative feedback following mTORC1 inhibition that results in PI3K reactivation [10]. In addition, the lack of activity against mTORC2 represents another major clinical limitation to the efficacy of rapamycin analogs (rapalogs) [34]. To overcome these limitations, a new generation of ATP-competitive mTOR kinase inhibitors has been developed. These compounds include Torin1, PP242, PP30, Ku-0063794 (KuDOS Pharmaceuticals), WAY-600 (Wyeth), WYE-687 (Wyeth), WYE-354 (Wyeth), INK128 (Intellikine), CC214-1/2 (Celgene Corporation, San Diego, U.S.A.), AZD2014, AZD8855 (AstraZeneca), and OSI-027 (OSI Pharmaceuticals) and have been designed in order to target the mTOR kinase domain and irreversibly block both mTORC1 and mTORC2 activation [35]. Some of these compounds have been tested both *in vitro* and *in vivo* and confirmed the pivotal role of mTORC2 in GBM biology. Indeed, Gini et al. proved that the mTOR kinase inhibitors CC214-1and CC214-2 (orally available) (Celgene Corporation (San Diego, U.S.A.)) are able to overcome the limitations of rapamycin and rapalogs and to inhibit GBM growth by blocking mTORC2 activity

in vitro and *in vivo*, respectively [36, 37]; moreover, the same authors demonstrated that the sensitivity to CC214 compounds is significantly increased in the presence of EGFRvIII and PTEN loss and that the pharmacologic inhibition of autophagy induced by CC214 sensitizes GBM cells to cell death, preventing a cytostatic effect [37]. Furthermore, Kahn et al. demonstrated that AZD2014 enhances the radiosensitivity of glioblastoma stem cells (GSCs) *in vitro* and under *in vivo* orthotopic conditions by inhibiting mTORC1/2 [38]. Additionally, our group has recently demonstrated that the treatment of genetically different GBM cell lines with PP242 but not with rapamycin induces a dramatic and permanent reduction of AKT phosphorylation on serine 473 that not only counteracts tumor growth and invasiveness but also prevents GSC proliferation. Moreover, we also proved that mTORC2 activation is independent from PI3K, as the irreversible inhibition of PI3K with wortmannin is not able to prevent mTORC2 activation, which is evaluable analyzing mTOR phosphorylation on serine 2481 [39].

As preclinical studies confirmed the superiority of mTOR kinase inhibitors compared to rapalogs and have revealed the efficacy of mTORC2 inhibition in counteracting GBM growth, invasiveness, and GSC proliferation, the mTOR kinase inhibitors AZD8055 (AstraZeneca) and OSI-027 (OSI Pharmaceuticals) have already entered clinical trials [40] (Figure 3). A phase I study of AZD8055 (AstraZeneca) in advanced solid malignancies (NCT00973076) and in recurrent GBM (NCT01316809) has completed the recruitment, and results are eagerly awaited; a phase I study of OSI-027 (OSI Pharmaceuticals) in advanced solid tumors and lymphoma started in 2008 and is now completed (NCT00698243). As single agent, OSI-27 has shown to be well tolerated and evidence of activity has emerged [41].

4. Evaluation of mTOR Activation in GBM Patients

Despite PTEN/PI3K/AKT/mTOR pathway is considered a hallmark of GBM and the inhibition of this pathway represents to date an interesting strategy against this lethal tumor [1, 42], the direct evaluation of this pathway activation in GBM patients is not routinely performed or standardized and results are still controversial. In this context, the first analysis of PTEN/PI3K/AKT/mTOR pathway activation in patient specimens was carried out on 45 untreated primary GBM; immunohistochemistry analysis revealed that PTEN loss correlated with AKT activation and that in turn AKT phosphorylation significantly correlated with mTOR, FOXO1, FOXO3a, FOXO4, and S6 activation [43]. Moreover, a tight relation between EGFRvIII expression and the activation of PI3K downstream targets has been also observed in this study [43] (Table 1).

Later on, Chakravarti et al. analyzed the expression of total PI3K, AKT, and S6K in 92 gliomas with different malignancy degree by western blot and did not find any difference in the total expression of PI3K, AKT, and S6K between GBM and non-GBM tumors. However, despite the total expression of these protein kinases was unchanged between groups, the authors reported that PI3K, AKT, and S6K phosphorylation was significantly higher in GBM versus non-GBM tumors. Moreover, the levels of PI3K, AKT, and S6K phosphorylation were inversely related to the expression of the cleaved caspase 3 and correlated with radiation resistance and an adverse outcome [44] (Table 1). The activation status of AKT and in addition of NF-κB and STAT3 and their correlation with tumor grade has also been analyzed in 259 diffuse gliomas by Wang et al., by microarray and immunohistochemistry. These authors described consistent AKT and NF-κB activation in tumor samples but not in astrocytes or oligodendrocytes of normal brain cortex and cerebellum; moreover, these authors reported a positive correlation between AKT, NF-κB activation, and tumor grade but not between them and STAT3 [45] (Table 1). In another study, Riemenschneider et al. reported the colocalization of TSC2, mTOR, 4EBP1, S6K, S6, and STAT3 phosphorylation with AKT activation, although only TSC2, S6K, and S6 phosphorylation has been found to correlate with AKT activation [46] (Table 1).

Contrariwise, Fiano et al. did not find any correlation between AKT phosphorylation, cyclin D1, p27/Kip1, and *PTEN* or *EGFR* mutations in 65 GBM surgical samples [47]. Instead, Hlobilkova et al. found a strong relation between EGFR expression and tumor grade in 89 samples of glioma with different malignancy degree but they observed comparable levels of AKT phosphorylation between low- and high-grade gliomas [48] (Table 1).

Higher levels of AKT, mTOR, and S6K phosphorylation in high-grade glioma compared with low-grade glioma have been also reported by Li et al., who analyzed 87 tissue samples and found that the percentage of patients with high AKT, mTOR, and S6K phosphorylation, as detected by immunohistochemistry, was greater in grades III and IV than in grades I and II glioma [49] (Table 1). Similarly, Korkolopoulou et al. analyzed the expression of mTOR, S6K, and 4EBP1 phosphorylation in 111 tissue samples (grades II–IV) by immunohistochemistry and validated their analysis in 3 primary GBM cell cultures by western blotting analysis [50] (Table 1). They found that while normal tissues had no positivity for all the proteins considered, none of the tumor samples was negative for mTOR, S6K, or 4EBP1 phosphorylation. With the exception of one sample, tissues that showed mTOR positivity were also positive for S6K staining and all mTOR-positive specimens also showed 4EPB1 coexpression. Moreover, these authors demonstrated that 4EBP1 phosphorylation increased with the histological grade and that mTOR phosphorylation was higher in grade III/IV glioma compared with grade II. Of note, no significant difference in terms of S6K phosphorylation was reported according to tumor grades. They next sought to investigate the correlation between the level of mTOR/S6K/4EBP1 phosphorylation and survival and found that 4EBP1 expression was an independent adverse prognostic index in all the cohort analyzed, while the increased level of mTOR phosphorylation correlated with disease-free survival (DFS) [50] (Table 1). More recently, Machado et al. demonstrated that mTOR expression was significantly higher in wild-type *IDH1* primary GBM, when compared to healthy tissue, and was also higher compared with *R132H IDH1*-mutant GBM

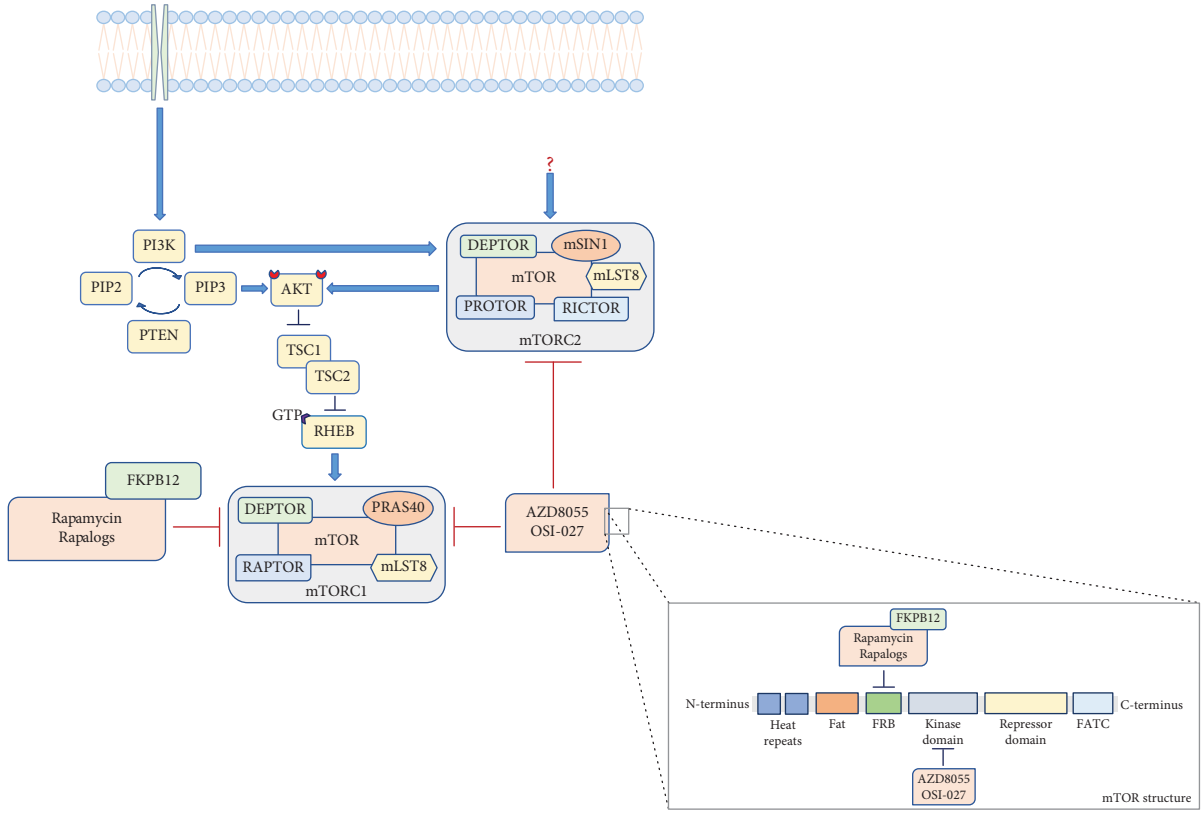

Figure 3: Schematic representation showing paths and points of action of the mTOR kinase domain inhibitors (AZD8055 (AstraZeneca) and OSI-027 (OSI Pharmaceuticals)) currently evaluated in clinical trials. Legend: PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol (4,5)-bisphosphate; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; PTEN: phosphatase and tensin homolog; AKT: protein kinase B; TSC1/2: tuberous sclerosis complex 1/2; GTP: guanosine triphosphate; RHEB: RAS homolog enriched in brain; mTOR: mechanistic target of rapamycin; RAPTOR: regulatory-associated protein of mTOR; PRAS40: proline-rich AKT substrate 40 kDa; mLST8: mammalian lethal with Sec-13 protein 8; DEPTOR: DEP domain TOR-binding protein; RICTOR: rapamycin-insensitive companion of mTOR; PROTOR: protein observed with RICTOR; mSIN1: stress-activated map kinase-interacting protein 1; FKBP12: 12-kDa intracellular FK506-binding protein; rapalogs: rapamycin analogs; FATC: FAT carboxyl terminal; FRB: FKBP-rapamycin-binding domain.

[51]. In addition, these same authors proved that mTOR phosphorylation on serine 2448 and S6 phosphorylation on serine 240 and 244 were increased in wild-type *IDH1* GBM compared with *R132H IDH1*-mutant GBM [51] (Table 1).

Compared with mTORC1 and its upstream and downstream targets, the evaluation of mTORC2 activation in GBM patients is further underestimated. The activation of mTORC2 has been evaluated in 5 GBM cell lines, in 31 tumor samples and 5 normal brain tissues by Masri et al. [52]. These authors observed that the expression of activated mTORC2 was quite undetectable in normal brain tissue while it was high in the tumor cell lines they analyzed. Consistently, they observed increased RICTOR expression and extent of AKT phosphorylation on serine 473 only in tumor cells. Of note, RICTOR overexpression appeared to be independent of *PTEN* status. In accordance with the *in vitro* data, these same authors found that the 86% of tumor samples had RICTOR overexpression and 70% of them showed high mTORC2 activity [52] (Table 1). More recently, Alvarenga et al. described RICTOR expression and AKT phosphorylation on serine 473 in 195 patients with brain tumors (38 grade I, 49 grade II, 15 grade III, and 93 grade IV astrocytoma) and correlated AKT activation with overall survival (OS) [53]. They did not find any differences in AKT phosphorylation on serine 473 between low-grade glioma and normal brain tissue but they observed a significant increase in AKT phosphorylation in GBM patients compared with normal brain tissue; moreover, the increased expression of activated AKT correlated with a reduced OS [46]. These same authors analyzed RICTOR expression, and although they did not find an increased expression of this mTORC2 component between normal brain tissue and all grade astrocytoma, they observed nuclear localization of RICTOR in GBM that might suggest a change of its binding partner and a possible implication in tumor progression [53] (Table 1).

5. Discussion and Future Perspective

As discussed, mTOR pathway is certainly one of the most compelling mechanisms driving GBM biology. However, to date, there are still some cruxes that need to be unraveled to translate the encouraging preclinical results reported in the clinical management of GBM patients.

First of all, the direct evaluation of mTOR pathway activation in GBM patients is not routinely performed and results are affected by discrepancies due to different

Table 1: Clinical evaluation of PTEN/PI3K/AKT/mTOR pathway activation in GBM patients.

No. of samples	Methods	Main findings	Reference
45	Immunohistochemistry	Correlation between PTEN loss and AKT activation, correlation between AKT phosphorylation and FOXO and S6 activation, and correlation between EGFRvIII expression and PI3K pathway activation	43
92	Western blot	No difference in PI3K AKT and S6K total expression between GBM and non-GBM tumors; increased PI3K, AKT, and S6K phosphorylation in GBM compared with non-GBM tumors; correlation between PI3K, AKT, and S6K phosphorylation and adverse outcome	44
259	Microarray and immunohistochemistry	AKT and NF-κB activation in tumor samples and not in normal brain and positive correlation between AKT and NF-κB activation and tumor grade	45
29	Immunohistochemistry	Correlation between AKT activation and TSC2, S6K, and S6 phosphorylation	46
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89	Immunohistochemistry	No difference of AKT phosphorylation between low- and high-grade glioma	48
87	Immunohistochemistry	Higher levels of AKT, mTOR, and S6K in high-grade glioma compared with low-grade glioma	49
111 + 3 primary GBM cell cultures	Immunohistochemistry and western blot	Absence of mTOR, S6K, and 4EBP1 positivity in normal brain tissues; increase of mTOR and 4EBP1 phosphorylation with histological grade; correlation between 4EBP1 expression and adverse prognosis; correlation between mTOR phosphorylation and disease-free survival	50
225	Immunohistochemistry	Higher mTOR expression in WT *IDH1* GBM compared with healthy tissues and *R132H IDH1*-mutant GBM and increased mTOR and S6 phosphorylation in WT *IDH1* GBM compared with *R132H IDH1*-mutant GBM	51
36 + 5 GBM cell cultures	Immunohistochemistry, western blot, real-time PCR, and cell proliferation and migration assays	Undetectable mTORC2 activation in normal cells and high expression in GBM cell cultures and correlation between RICTOR expression and mTORC2 activation	52
196	Immunohistochemistry and western blot	Increased AKT phosphorylation in GBM compared with normal brain tissues and correlation between increased AKT activation and reduced overall survival	53

methodologies of quantification applied by distinct laboratories, which makes it challenging to select those patients that, based on their molecular profile, might mainly benefit of treatment with mTOR kinase inhibitors. As it is now widely accepted that genetic background influences and predicts the outcome of targeted therapy, one of the future challenges for GBM treatment will be to improve the precision and reproducibility of molecular analysis of mTOR pathway activation and standardize this evaluation among laboratories.

Another concern regarding the use of mTOR kinase inhibitors in clinic is their potential immunosuppressive activity, as the impairment of the immune system is widely known to favor tumor growth and progression. However, assays of adaptive immune functions of 1–3 weeks in leukemia revealed that the anticancer properties of PP242 are dominant over its immunosuppressive activity compared with those of rapamycin and another mTOR kinase inhibitor known as PI103 but the outcome of prolonged treatment with this compound and other mTOR kinase inhibitors remains to be investigated [54]. Additionally, as most of the clinical trials evaluating mTOR kinase inhibitors in GBM are ongoing, data regarding the tolerability and the safety profile of these drugs are still not available and the results of these studies will further help to define the role of mTOR as therapeutic target, hopefully providing a new tool against GBM.

Abbreviations

mTOR: Mechanistic target of rapamycin
GBM: Glioblastoma
PTEN: Phosphatase and tensin homolog
PI3K: Phosphatidylinositol-3-kinase
AKT: Protein kinase B
RAPTOR: Regulatory-associated protein of mTOR
PRAS40: Proline-rich AKT substrate 40 kDa
mLST8: Mammalian lethal with Sec-13 protein 8
DEPTOR: DEP domain TOR-binding protein

FKBP12: 12 kDa intracellular FK506-binding protein
TSC1/2: Tuberous sclerosis complex 1/2
RHEB: RAS homolog enriched in brain
S6K: Ribosomal S6 kinase
4EBP1: Eukaryotic initiation factor 4E-(eIF4E-) binding protein
eIF: Eukaryotic translation initiation factor
TIF-1A: Transcription intermediary factor 1-alpha
GRB10: Growth factor receptor-bound protein 10
IRS-1: Insulin receptor substrate-1
SREBP1/2: Sterol regulatory element-binding protein 1/2
ATF4: Activating transcription factor 4
MTHFD2: Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase
ULK1/Atg13/FIP200: Unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa
DAP1: Death-associated protein 1
TFEB: Transcription factor EB
RICTOR: Rapamycin-insensitive companion of mTOR
mSIN1: Stress-activated map kinase-interacting protein 1
PROTOR: Protein observed with RICTOR
PKC: Protein kinase C
SGK1: Serum and glucocorticoid-regulated kinase 1
EGFR: Epidermal growth factor receptor
GLT4: Glucose transporter type 4
HK2: Hexokinase 2
PFK-1: Phosphofruttokinase-1
HDACs: Histone deacetylases
FOXO: Forkhead box O
Acetyl-CoA: Acetyl coenzyme A
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
Rapalogs: Rapamycin analogs
GSC: Glioblastoma stem cell
DFS: Disease-free survival
OS: Overall survival.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research Article

Combination of Proteasome and Histone Deacetylase Inhibitors Overcomes the Impact of Gain-of-Function p53 Mutations

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Mutations in the "guardian of the genome" *TP53* predominate in solid tumors. In addition to loss of tumor suppressor activity, a specific subset of missense mutations confers additional oncogenic properties. These "gain-of-function" (GOF) mutations portend poor prognosis across cancer types regardless of treatment. Our objective in this study was to identify novel therapeutic opportunities to overcome the deleterious effects of GOF *TP53* mutants. Using gynecologic cancer cell lines with known *TP53* mutational status, we established that treatment with a proteasome inhibitor induced cell death in cells with two recurrent GOF *TP53* mutations (R175H and R248Q), and addition of a histone deacetylase inhibitor (HDACi) enhanced this effect. By contrast, p53-null cancer cells were relatively resistant to the combination. Proteasome inhibition promoted apoptosis of cells with *TP53* GOF mutations, potentially through induction of the unfolded protein response. In line with the reported hyperstabilization of GOF p53 protein, cells treated with HDACi exhibited reduced levels of p53 protein. Together, these data form the basis for future clinical studies examining therapeutic efficacy in a preselected patient population with GOF *TP53* mutations.

1. Introduction

The Cancer Genome Atlas (TCGA) project has substantiated the long-held notion that the "guardian of the genome" *TP53* is the most mutated gene in tumors [1]. Certain tumor types have an exceptionally high preponderance of mutations in *TP53*: for example, mutations in *TP53* occur in 96% of all serous ovarian tumors [2], and nearly all serous and ~25% of high-grade endometrioid endometrial cancers have mutations in *TP53* [3]. The prevalence of *TP53* mutations is also particularly high in head and neck cancer and breast cancer [1, 4].

While it is appreciated that *TP53* mutations occur in a substantial number of tumors, it is critically important to note that varying types of p53 mutant proteins exist, with different implications for chemosensitivity. Some mutations are relatively inconsequential from the perspective of p53 function, and proteins of this type retain wild-type activity. Other mutations are loss of function (LOF) or p53-null in which single amino acid changes completely inactivate or destabilize the protein. Finally, an interesting category is the gain-of-function (GOF) or "oncogenic" *TP53* mutations that convert p53 from a tumor suppressor to an oncogene. The majority of LOF and GOF *TP53* mutations result in loss of DNA binding to canonical p53 targets. However, GOF mutants also have new protein: protein interactions and/or transcriptional targets that confer an additional "oncogenic" functions [5–8]. To date, eight missense mutations in human

TP53 have been established as GOF mutations and result in the following amino acid changes: P151S, Y163C, R175H, L194R, Y220C, R248Q, R248W, R273C, R273H, R273L, and R282W.

Substantial clinical and preclinical data from a wide range of cancers demonstrate that GOF *TP53* mutations predict for poor response to treatment. In a recently published work, we evaluated the relationship of the eight GOF *TP53* mutations with progression-free survival (PFS), risk of recurrence, and response to standard platinum and taxane chemotherapy in serous ovarian cancer [9]. We found that 21.2% of serous ovarian cancer patients in TCGA cohort have a GOF *TP53* mutation, whereas 18.9% have LOF mutations [9]. Ovarian cancer patients with GOF *TP53* mutations have worse clinical outcomes compared to patients with unclassified *TP53* mutations (i.e., variants of unknown significance), including a shorter PFS and a 60% greater risk of recurrence [9]. These findings have important potential implications for all cancers characterized by mutations in *TP53*.

Analysis of *TP53* mutational status is now included in many next-generation sequencing tests. An obvious question, therefore, is how to convert these deadly oncogenic mutations into actionable mutations. Herein, we identify the combination of a proteasome inhibitor with an epigenetic modulator (histone deacetylase inhibitor (HDACi)) as a potent therapeutic strategy to overcome the deleterious effects of *TP53* GOF mutations. These preclinical data serve as the proof of concept for future trials evaluating specific combinatorial therapies in patients whose tumors contain *TP53* GOF mutations.

2. Materials and Methods

2.1. Reagents. All antibodies were purchased from Cell Signaling. Bortezomib, LBH589 (panobinostat), and MLN2238 (ixazomib) were purchased from Selleck Chemicals and suspended in DMSO.

2.2. Cell Lines and Culture Conditions. All cell lines used in this study were purchased from ATCC, except for Hec50 endometrial cancer cells that were kindly provided by Dr. Erlio Gurpide (New York University) as previously described [10]. Hec50 cells expressing R175H *TP53* GOF have been previously described [10]. All cell lines have been authenticated using STR analysis by biosynthesis.

2.3. Cell Viability Assays. Beginning 24 h after plating equal numbers of cells, cells were treated for 72 h followed by assessment of cell viability using the WST-1 assay per manufacturer's instructions (Clontech). Data were quantitated relative to values obtained for control (untreated) cells, which were set at 100% viability.

2.4. Western Blot Analysis. As previously described [10], cells were plated in 100 mm dishes and were allowed to grow for 24 h prior to treatment. After treatment, cells were harvested, lysed with extraction buffer (1% Triton X-100, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 20 μg/ml aprotinin, 1 mM PMSF, and 2 mM Na_3VO_4), and subjected to three freeze/thaw cycles. Equal amounts of protein (determined by the method of Bradford, BioRad) were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes (BioScience). Membranes were probed with primary antibodies against cleaved caspase 3, Bip, α-tubulin, p53, p21, or β-actin followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibody. The signal was visualized by chemiluminescence using ECL western blotting detection reagents (Pierce).

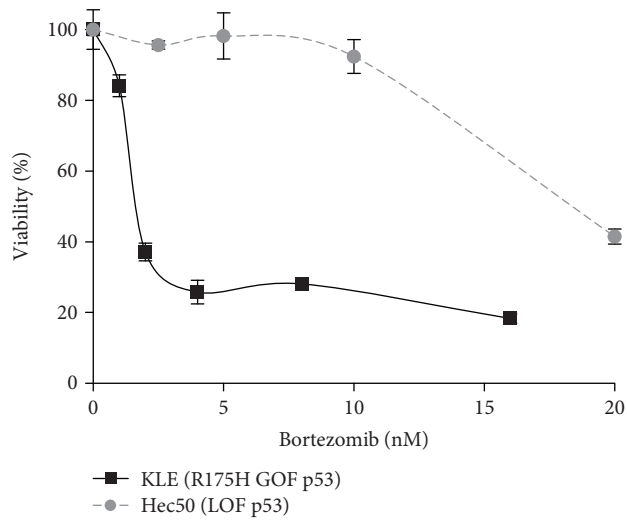

Figure 1: Proteasome inhibitor bortezomib induces massive cell killing in endometrial cancer cells with *TP53* GOF mutation R175H (KLE cells) but not LOF mutation (Hec50 cells). All experiments were performed three times. IC50: KLE cells, 2.1 ± 0.3 nM; Hec50 cells, 19.4 ± 1.0 nM; $^*P < 0.05$ by Student's *t*-test.

2.5. Statistical Analysis. All data were expressed as the mean ± SD. All statistical comparisons were performed using GraphPad Prism software. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Sensitivity of Cancer Cells with Known p53 Status to Proteasome Inhibitors. We first examined the sensitivity of two well-characterized endometrial cancer cell lines with known p53 mutational status to the proteasome inhibitor, bortezomib (Velcade®). We made the unexpected discovery that KLE cells with the R175H GOF mutation were highly sensitive to the proteasome inhibitor bortezomib, whereas Hec50 cells with LOF p53 mutation were relatively resistant to bortezomib (Figure 1).

3.2. Addition of HDACi Enhances Sensitivity to Proteasome Inhibitor Treatment in Cells with Endogenous TP53 GOF Mutations. Next, we examined the impact of the addition of a histone deacetylase inhibitor. The combination of bortezomib with the HDACi LBH589 (panobinostat) further increased cell killing in KLE cells (R175H GOF) as compared to bortezomib alone (Figure 2(a)). Studies were also performed in the OVCAR3 ovarian cancer cell line that contains a different GOF *TP53* mutation, R248Q. Consistent with our

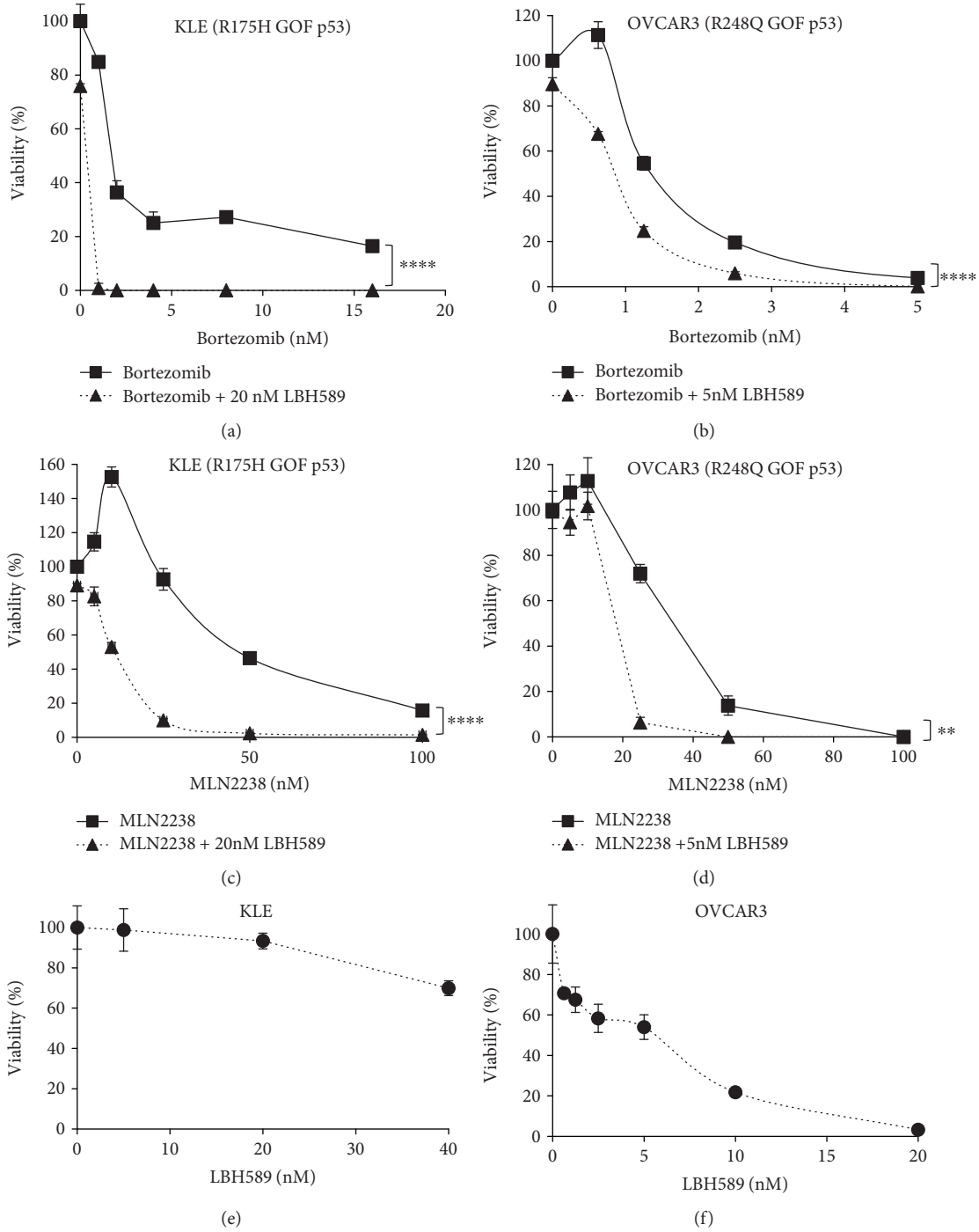

Figure 2: Gynecologic cancer cells with GOF *TP53* are highly sensitive to proteasome inhibitors alone or in combination with LBH589/panobinostat. Sensitivity (as measured by percent viability relative to untreated control) to bortezomib (a, b) or MLN2238/ixazomib (c, d) alone or in combination with LBH589/panobinostat was examined in KLE endometrial cancer cells with R175H GOF mutant (a, c) and OVCAR3 ovarian cancer cells with R248Q GOF mutant (b, d). The concentration of LBH589/panobinostat used in (a–d) was based on sensitivity to LBH589/panobinostat alone in KLE (e) and OVCAR3 (f) cells. All experiments were performed three times. $^{**}P < 0.01$; $^{****}P < 0.0001$ by two-way ANOVA with Sidak's multiple comparison test.

findings in endometrial cancer cells, OVCAR3 cells were highly sensitive to bortezomib alone or in combination with HDACi (Figure 2(b)). The specific dose of LBH589/panobinostat was determined by assessing the sensitivity of each cell line to treatment with LBH589/panobinostat alone (Figures 2(e) and 2(f)). Since OVCAR3 cells contain a different *TP53* GOF mutation than KLE cells, these data suggest that the sensitivity to proteasome inhibition is not restricted to the R175H mutation.

Ixazomib (MLN2238) is a next-generation proteasome inhibitor that has replaced bortezomib in the clinic for multiple myeloma due to its improved activity and other

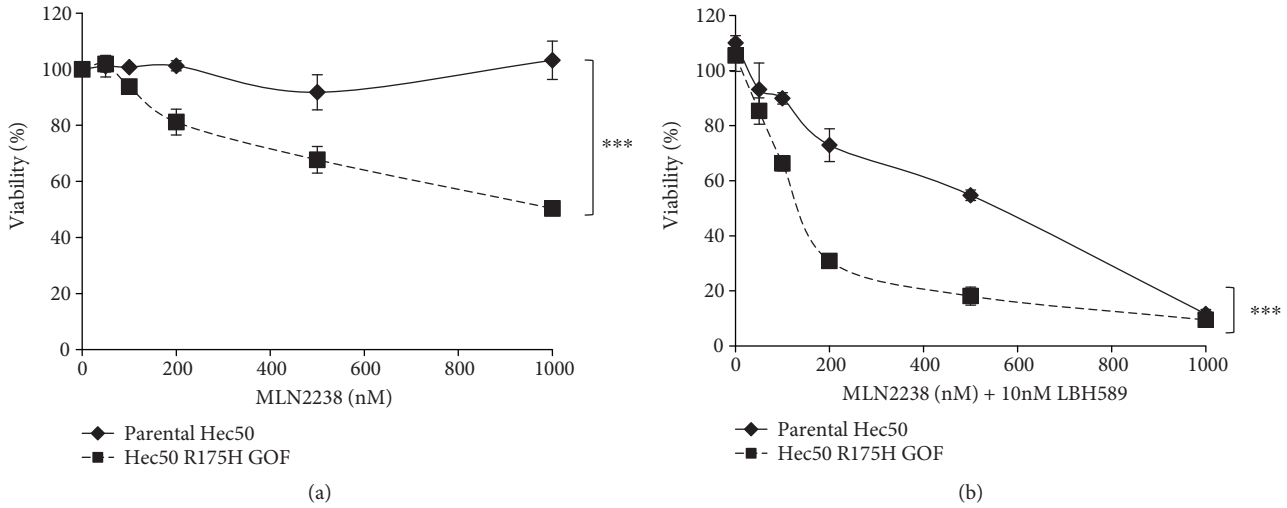

Figure 3: Sensitivity to MLN2283/ixazomib and LBH589/panobinostat combination treatment is dependent on the expression of GOF *TP53*. Sensitivity to MLN2238/ixazomib alone (a) or in combination with LBH589/panobinostat (b) was examined in parental Hec50 cells or Hec50 cells expressing the R175H GOF mutant. $^{***}P < 0.001$ by two-way ANOVA with Sidak's multiple comparison test.

characteristics, such as oral bioavailability [11, 12]. Therefore, we repeated the above experiments using ixazomib, either alone or in combination with the HDACi LBH589/panobinostat in KLE and OVCAR3 cells that express different *TP53* GOF mutations. Similar to the bortezomib studies, both KLE and OVCAR3 cells responded well to MLN2238/ixazomib (Figures 2(c) and 2(d)). Moreover, MLN2238/ixazomib was highly synergistic with the HDACi.

3.3. Exogenous Expression of GOF TP53 in p53-Null Cells Sensitizes Cells to Proteasome Inhibitor + HDACi Therapy. To further address the specific role of *TP53* GOF mutations in response to proteasome inhibitor + HDACi treatment, we introduced the p53 GOF mutant, R175H, in p53-null cells by exogenous expression [10]. As compared to parental cells, expression of p53R175H partially restored sensitivity to MLN2238/ixazomib (Figure 3(a)), and the addition of the HDACi LBH589 to the proteasome inhibitor backbone treatment substantially increased cell death.

An established mechanism of action of proteasome inhibitors is the induction of cell death via apoptosis [13]. In both KLE and OVCAR3 cells, treatment with MLN2238/ixazomib promoted cleavage of caspase 3, a marker for apoptosis (Figure 4). Others have shown that proteasome inhibitors induce apoptosis by activating the unfolded protein response (UPR) pathway, a homeostatic mechanism that is normally triggered by accumulation of misfolded proteins in the endoplasmic reticulum [13]. A hallmark of the UPR pathway is increased expression of Bip/GRP78, a chaperone protein that induces proper folding of misfolded proteins such as GOF p53. Immunoblotting revealed that treatment with MLN2238/ixazomib increased the expression of Bip (Figure 4).

Mutant p53 has also been shown to interact with histone deacetylases (e.g., HDAC2/6), which contributes to its stabilization and aberrant functions [14, 15]. Published evidence suggests that HDACi like LBH589/panobinostat may decrease the stability of mutant p53 [14, 15]. Consistent

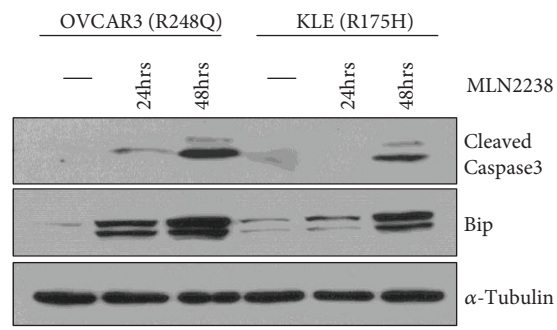

Figure 4: Treatment with MLN2238/ixazomib promotes apoptosis, potentially through the UPR pathway. Cells were treated for 0, 24, or 48 h with MLN2238/ixazomib and cell lysates analyzed by western blotting with the indicated antibodies (α-tubulin served as a loading control).

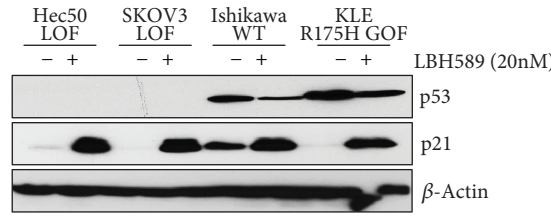

Figure 5: Treatment with LBH589/panobinostat reduces p53 protein levels. Cells lacking p53 (LOF) or expressing the indicated forms of p53 (WT or R175H GOF mutant) were treated with 20 nM panobinostat and levels of the indicated proteins measured by western blotting. p21 served as a positive control for HDACi activity. β-Actin served as a loading control.

with these results, we found that treatment with LBH589/panobinostat caused a marked decrease in the total protein levels of p53 in KLE cells with the R175H GOF mutant (Figure 5). As a control for drug activity, we also examined p21 levels, which are known to be increased following

treatment with HDACi regardless of p53 expression [16]. LBH589/panobinostat increased p21 in all cell lines examined (Figure 5).

4. Discussion

Despite clear data in multiple cancer types that *TP53* GOF mutations predict for poor outcomes, including resistance to therapy, to date no clinical trials have tested treatment strategies designed to specifically overcome the effects of *TP53* GOF mutations. In fact, *TP53* mutational status is widely ignored when making treatment decisions. Herein, we present a novel combinatorial strategy that effectively induces cell death specifically in cancer cells bearing GOF *TP53* mutations. Of note is that the combinatorial strategy of proteasome inhibitor plus HDACi was highly effective in cells with different recurrent *TP53* GOF mutations. We demonstrate this effect with two different proteasome inhibitors, bortezomib and ixazomib, indicating the potential generality of the approach. These data set the stage for future clinical studies in patients with GOF *TP53* mutations.

The cornerstone of personalized medicine is designing treatment strategies that overcome driver mutations in patients. However, mutations in *TP53* are not considered actionable in the traditional sense. One strategy to make *TP53* mutations druggable is based upon the principles of synthetic lethality, the term for a historical genetic observation that in the presence of certain single gene mutations, blocking or mutating a second gene leads to cell death, though neither mutation alone has a phenotype [17]. With respect to cancer therapy, synthetic lethality means capitalizing on the presence of a driver mutation to design novel treatments which block the compensatory survival pathways activated as a result of the mutation. To create therapeutic synthetic lethality, one must first know the driver mutation, understand the compensatory survival pathway that has been activated as a result of the mutation, and have an agent which can block this critical pathway. In a series of published studies, our group has established that treatment with a tyrosine kinase inhibitor (e.g., gefitinib, nintedanib, and cediranib) sensitizes p53-null cancer cells to paclitaxel-containing chemotherapy [10, 18, 19]. The mechanism is through abrogation of the G2/M cell cycle checkpoint. Enforcing the G2/M cell cycle checkpoint allows tumor cells to repair damaged DNA before entering mitosis, leading to chemoresistance [20–26]. Wild-type p53 normally maintains both the G1/S and G2/M checkpoints. However, emerging data suggest that p38MAPK can also maintain the G2/M checkpoint [27–29]. In cells with LOF p53, p38 is activated as an alternative means to maintain the G2/M checkpoint [25]. Therefore, treatment with an upstream agent that blocks p38 activation (e.g., tyrosine kinase inhibitors) sensitizes p53-null cells to paclitaxel, resulting in accumulation of cells in mitosis and massive cell death via mitotic catastrophe [10, 18].

Unfortunately, this same combinatorial strategy is not effective in cells with GOF p53. Specifically, our published data from cell models with endogenous and exogenous expression of GOF p53 mutants demonstrate that, in contrast to LOF p53, GOF forms of p53 constitutively enhance the G2/M checkpoint and are resistant to paclitaxel + tyrosine kinase inhibitors [10, 18]. Others have reported that p53 GOF mutants R175H, R273H, and R280K aberrantly induce p38 activation via transcriptional activation of MKK3 (an upstream kinase of p38), thereby maintaining the G2/M checkpoint [30]. Other established cancer therapeutics, such as temozolomide and tamoxifen, likewise are ineffective against tumor cells expressing *TP53* GOF mutants due to specific effects of mutant p53 on O6-methylguanine DNA-methyltransferase (MGMT) and estrogen receptor expression, respectively [31]. Therefore, alternative strategies are necessary to overcome the effects of GOF p53. One approach is to use small molecule drugs to restore the wild-type p53 conformation and thereby restore normal p53 anticancer function [31]. Our approach instead takes advantage of the unique properties of GOF p53 mutants, namely, aberrant folding and increased stability.

Here, we discovered that cells with GOF but not LOF *TP53* mutations are hypersensitive to proteasome inhibition, and addition of an HDACi (here, panobinostat) further enhanced cell killing. Both histone deacetylase inhibitors (vorinostat, panobinostat) and proteasome inhibitors (bortezomib, ixazomib) have been extensively studied in preclinical and clinical models of multiple cancer types [32]. Herein, we extend these prior findings to our cell models of ovarian and endometrial cancer, diseases for which new therapies are urgently needed.

Studies in multiple myeloma have provided significant mechanistic insight into why proteasome inhibitors are highly toxic to the cancer cells. For example, proteasome inhibition has been shown to promote apoptosis via terminal UPR [13]. Consistent with these data, we found that ixazomib treatment induced cleavage of caspase 3, a canonical marker of apoptosis, as well as expression of Bip/GPR78, a marker for ER stress. Since p53 GOF mutant protein is a misfolded protein, proteasome inhibition may induce cell death through excessive accumulation of misfolded proteins. Several studies have reported hyperstabilization of GOF p53 protein in cancer [33], which has been postulated to occur through more than one mechanism [14, 15, 34]. First, p53 GOF proteins are unable to bind the E3 ligase Mdm2, which negates the typical pathway of p53 ubiquitination and degradation via the proteasomal pathway [34]. Instead, p53 GOF protein is thought to be degraded by the lysosome in a process termed "chaperone-mediated autophagy" (CMA) [35]. Intriguingly, inhibition of the proteasome results in a compensatory induction of the activity of the CMA pathway [35]. Second, mutant p53 can be stabilized through interactions with heat shock proteins and histone deacetylases, and published evidence suggests that HDACi may decrease the stability of mutant p53 by disrupting its association with heat shock proteins [14, 15]. Therefore, one possibility is that HDACi potentiates the effects of the proteasome inhibitor by removing components of the chaperone complex, improving uptake in the lysosome, and leading to CMA-mediated p53 GOF degradation. Supporting this notion, we observed decreased total p53 protein levels upon treatment with panobinostat. In addition, histone deacetylase inhibitors have been shown to induce cell cycle arrest at the G1/S transition

via upregulation of p21 [16], which we also demonstrate in cells with both wild-type (Ishikawa cells) and p53 GOF mutants (KLE, R175H p53). Whereas early studies with vorinostat suggested that G1/S cell cycle arrest is accomplished through upregulation of p53 [36], others have established that HDACi treatment destabilizes mutant p53, resulting in a marked decreased in p53 levels [14]. Our data are in line with the latter findings whereby treatment with LBH589/panobinostat resulted in a 50% or greater decrease in both GOF p53 (KLE cells) and wild-type p53 (Ishikawa cells).

Multiple myeloma is typified by accumulation of high levels of immunoglobulin, and thus the cells are extremely dependent upon proteasomal pathways for survival [37]. We speculate that GOF p53 mutants create a similar scenario that also necessitates a functional proteasome to maintain cell survival. Indeed, it has been suggested in the literature that excessive accumulation of mutant p53 may be more sensitive to proteasome and/or histone deacetylase inhibition [38]. Inhibiting the proteasome pathway would create a reliance on the lysosomal pathway for degrading the mutant p53, which is recognized as a misfolded protein. In line with this concept, we observed increased expression of Bip, a marker of the misfolded protein response pathway.

It is possible that distinct GOF mutations may differentially affect sensitivity to the single or combinatorial treatment regimen presented in this manuscript. In contrast to the findings presented herein, other studies have provided evidence that bortezomib sensitivity is dependent on wild-type p53 expression, whereby apoptosis is induced through p53-mediated downregulation of the prosurvival factor survivin [39, 40]. Cells that express a mutant p53 or p53-null cells were found to be resistant to bortezomib-induced apoptosis through sustained expression of survivin [40]. However, the specific GOF p53 mutants included in a previous study were R280K (MDA-MD-231 breast cancer cells) and E285K (RPMI-8226 multiple myeloma cells). While our data show similar results using cells expressing the R175H (Hec50 endometrial cancer cells) and R248Q (OVCAR3 ovarian cancer cells) mutants, a comprehensive analysis of cells expressing other recurrent p53 GOF mutants is warranted.

5. Conclusions

In summary, we present a novel therapeutic strategy for tumors with GOF *TP53* mutations using drugs that are already being advanced in clinical trials. These data suggest that p53 mutational status can be used as the foundation for defining personalized treatments.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

Conflicts of Interest

KWT is a cofounder of Immortagen Inc. All other authors declare no conflict of interest.

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