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Original Article

Combination of cetuximab with met inhibitor in control of cetuximab-resistant oral squamous cell carcinoma

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Abstract: *Objective:* To investigate the underlying molecular mechanisms contributing to oral squamous cell carcinoma (OSCC) cell resistance to the epidermal growth factor receptor (EGFR) inhibitor. *Materials and methods:* OSCC cell lines HSC-2 and HSC-3 were assessed *in vitro* for drug treatment, cell viability, and gene expression and the online gene expression in OSCC tissues was analyzed for association with OSCC prognosis. *Results:* HSC-2 and HSC-3 cells expressed high EGFR levels, but hepatocyte growth factor (HGF) treatment induced cetuximab resistance, whereas the Met inhibitor PHA-665752 as well as Met siRNA was able to restore OSCC cell sensitivity to cetuximab. HGF treatment induced tumor cells to express p-Akt and p-ERK1/2. In contrast, the activity of Akt and ERK1/2 was suppressed by treatment with PHA-665752, Met siRNA, or their combination. Furthermore, Met was highly expressed in OSCC tissues and associated with a poor patient survival, while Met/HGF-activated Akt also was associated with a poor patient survival. *Conclusions:* This study demonstrates that Met/HGF expression results in OSCC resistance to cetuximab and tumor recurrence after cetuximab therapy; thus, inhibition of Met/HGF activity could restore OSCC sensitivity to cetuximab.

Keywords: Oral squamous cell carcinoma, cetuximab resistance, HGF, Met, Akt, ERK1/2

Introduction

Head and neck cancer, which occurs in the oral cavity, nose, throat, larynx, sinuses, salivary glands, nasopharynx, or hypopharynx, significantly affects the quality of life of patients [1]. Oral squamous cell carcinoma (OSCC) is a commonly diagnosed head and neck squamous cell carcinoma (HNSCC). The risk factors include tobacco smoking and alcohol consumption [2] as well as excessive consumption of processed meats and red meat [3], human papillomavirus infection [4], and Frequently chewing betel nuts [5]. OSCC management is usually surgery, chemoradiotherapy, targeted therapy, or photodynamic therapy [6, 7]. Early-stage OSCC has a favorable prognosis after treatment, but drug resistance, disease recurrence, and metastasis result in overall 5-year survival rates ≤ 50% [8]. Targeted therapy is a hot topic for the treatment of OSCC, and clinical drugs used frequently include epidermal growth factor receptor (EGFR) inhibitors, such as cetuximab, bevacizumab, and erlotinib, which have shown improvement of OSCC patient survival [9, 10]. Indeed, EGFR is a transmembrane receptor tyrosine kinase that is highly overexpressed in head and neck, lung, and breast cancers [11, 12]. EGFR promotes cancer development by increasing cell growth, migration, and survival [13]. Cetuximab is a chimeric monoclonal antibody that can bind to EGFR and in turn inhibit EGFR tyrosine kinase activity to suppress EGFR-positive cancer progression [14]. In the treatment of HNSCC, cetuximab has gained much attention as a novel therapeutic strategy and was approved by the European Medicines Agency in 2004 and the US Food and Drug

Administration (FDA) in 2006 [15]. Unfortunately, long-term cetuximab treatment results in HNSCC drug resistance, even in tumors with high EGFR expression [16, 17]. Thus, a better understanding of the underlying molecular events of this drug resistance is critical to restore the sensitivity of cancer cells to cetuximab [18-20]. To date, there is increasing evidence implicating that receptor tyrosine kinases play a pivotal role in regulating cetuximab resistance in colon and lung cancers [21, 22], and the combined treatment with receptor tyrosine kinase inhibitors may overcome cetuximab resistance [22].

Hepatocyte growth factor (HGF) plays a crucial role in cell motility, growth, and morphogenesis through binding to hepatocyte growth factor receptor (also known as Met) to activate the receptor tyrosine kinase cascade, which is related to cancer development [23-25]. HGF is highly expressed in OSCC, and the HGF/Met signaling pathway has been shown to induce OSCC cell migration, invasion, and metastasis through lamellipodia and filopodia formation [26] and the destruction of E-cadherin [27]. The cross-talk of Met with other signaling proteins, like EGFR, vascular endothelial growth factor receptor, and Wnt, also has been revealed in various cell lines [28-30], indicating the role of HGF/Met in cancer development. In addition, it has been shown that Met activation during cetuximab treatment of recurrent and metastatic HNSCC is associated with poor outcomes [31].

In this study, we investigated the underlying molecular events contributing to OSCC cell resistance to cetuximab using an *in vitro* OSCC cell line model. Our data provide novel insight into a therapeutic strategy to restore cetuximab sensitivity of OSCC.

Materials and methods

Cell culture

The human OSCC cell lines HSC-2 and HSC-3 (Immuno-Biological Laboratories Co. Shanghai, China) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco) in a humidified incubator with 5% CO_2 at 37°C.

Cell viability assay

Cell viability was measured by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. In brief, 2×10^3 tumor cells/well were seeded into 96-well plates and grown for 24 h and then treated with various concentrations of cetuximab (Merck, Germany), PHA-665752 (Selleck Chemicals, TX, USA), HGF (R&D Systems, Minneapolis, MN, USA), and a goat anti-human HGF neutralizing antibody, normal goat IgG (R&D Systems) for 72 h. At the end of each experiment, 50 μL of MTT solution (2 mg/mL; Sigma Chemicals, St. Louis, MO, USA) was added to each well of the cell culture plate, and the cells were incubated for an additional 2 h. The culture medium was replaced with 100 μL of dimethyl sulfoxide. The absorbance rate was measured with a microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 490 nm. The percentage of cell viability was calculated by comparison to untreated controls.

Western blot

A western blot assay was performed, as described previously [32]. Briefly, cells after treatment were lysed in cell lysis buffer, and the protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Biotechnology, Shanghai, China). Each total protein sample (30 μg per lane) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After that, the membranes were blocked by 5% skimmed dry milk solution in phosphate-buffered saline (PBS) at room temperature for 1 h and then incubated with a primary antibody at 4°C overnight (**Table 1**). On the following day, the membranes were washed briefly three times with PBS-Tween 20 and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The target protein bands were visualized by ECL plus western blotting detection reagent and exposed to X-ray films.

RNA interference (RNAi) assay

For the RNAi assay, Duplexed Stealth RNAi targeting ErbB3 and Met or Stealth RNAi Negative Control Low GC Duplex #3 were purchased from Invitrogen (Carlsbad, CA, USA). OSCC cells

Table 1. Antibodies used for Western blot

Antibody	Cat #	Source	Dilution
Anti-EGFR	R&D Systems, AF231	Rabbit	1 μg/mL
Anti-phospho-EGFR	Abcam, ab32578	Rabbit	1:1000
Anti-Met	CST, 3127	Mouse	1:1000
Anti-phospho-Met	Abcam, ab47402	Rabbit	1:1000
Anti-Akt	CST, 9272	Rabbit	1:1000
Anti-phospho-Akt	CST, 4060	Rabbit	1:1000
Anti-ErbB3	CST, 4754	Rabbit	1:1000
Anti-phospho-ErbB3	CST, 4791	Rabbit	1:1000
Anti-ERK1/ERK2	R&D Systems, AF1576	Rabbit	0.2 μg/mL
Anti-phospho-ERK1/ERK2	R&D Systems, AF1018	Rabbit	0.1 μg/mL

CST, Cell Signaling Technology (Danvers, MA, USA); Abcam, Cambridge, MA, USA.

were seeded into 24-well plates at a density of 2×10^4 per well in 400 μL of antibiotic-free DMEM and grown overnight. On the next day, the cells were transfected with siRNA (50 μmol) using Lipofectamine 2000 (Invitrogen) for 24 h, according to the manufacturer's instructions, then washed with ice-cold PBS and reseeded into 96-well plates for treatment with cetuximab (1 μg/mL) and/or recombinant human HGF (20 ng/μL) for 72 h, and finally subjected to the MTT assay or another assay. The siRNA sequences targeting ErbB3 and Met were 5'-GGCCAUGAAUGAAUUCUCUACUCUA-3' and 5'-UCCAGAAGAUCAGUUUCCUAAUUCA-3', respectively.

Online gene expression database and survival analysis

Transcriptome data of OSCC tissues were retrieved from the NCBI-GEO database with the series ID of GSE42743 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42743). R statistical software v3.3.3 (https://www.rproject.org/) was used for data analysis. In brief, we utilized the Robust Multichip Average algorithm in the "oligo" package [33] to normalize the raw data on gene expression and generated the normalized expression matrix. The gene annotation and integration of the expression matrix was conducted by using a custom-designed Python code, according to a previous study [34]. We then removed probes without any gene annotations or those that matched multiple gene symbols. After that, we calculated the average expression value for each gene when there were multiple probe IDs that matched one official gene symbol and made this value to represent the expression intensity of the corresponding gene symbol. Differentially expressed genes were then obtained by using the empirical Bayes method in the "limma" package [35]. The upregulated genes were considered to be those with logarithmic transformed fold-change (log2FC) ≥ 1, and the downregulated genes were considered to be log2FC ≤ -1. A false discovery rate-adjusted P value ≤ 0.05 indicated statistical significance. All survival analyses were conducted using the "survival" package in R. Kaplan-Meier survival curves were used to show the prognostic differences between two groups.

Statistical analysis

All data were expressed as the mean ± standard deviation of triplicate experiments. The two-tailed unpaired Student's t test or the one-way analysis of variance test was used to determine the p values; $P < 0.05$ was considered as a significant difference.

Results

HGF induction of OSCC cell resistance to cetuximab by an increase in akt and ERK1/2 phosphorylation

First, we detected the levels of EGFR and c-Met expression in the OSCC cell lines HSC-2 and HSC-3 using western blot. We found that both cell lines significantly expressed EGFR and MET (**Figure 1A**). We then assessed the effect of cetuximab on OSCC viability using the MTT assay and found that HSC-2 and HSC-3 cell growth was moderately inhibited by cetuximab in a dose-dependent manner (**Figure 2B**). To obtain cetuximab resistance, we cotreated HSC-2 and HSC-3 cells with cetuximab (5 μg/mL) and HGF (20 μg/mL); however, we did not find a significant change in cell viability (**Figure 1B**), indicating that both OSCC cell lines became resistant to cetuximab after HGF treatment. At the protein level, HGF treatment induced Met activity (**Figure 1C**), which could be partially blocked by cetuximab treatment. Specifically, cetuximab treatment downregulated the phosphorylation of Akt and ERK1/2, but it recovered back to the previous levels after the addition of

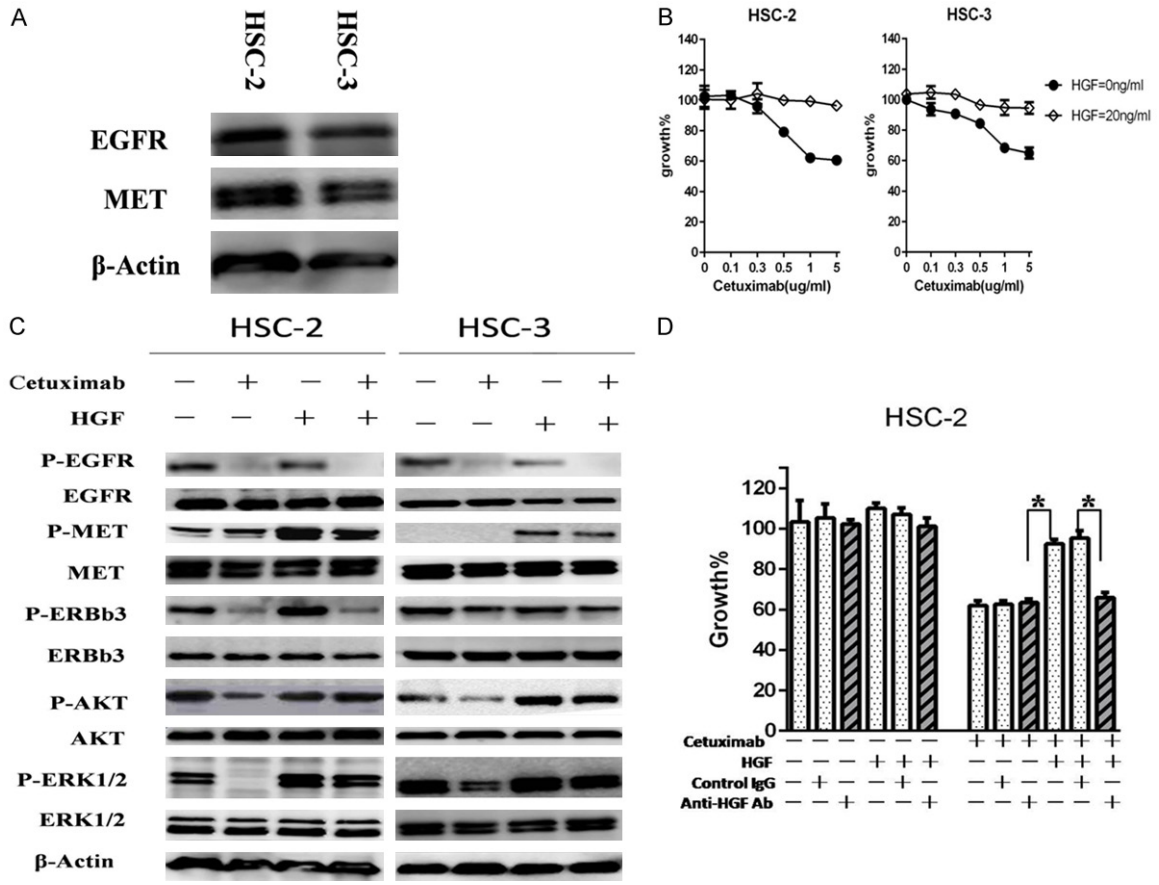

Figure 1. HGF induction of OSCC resistance to cetuximab. A. Western blot. HSC-2 and HSC-3 cells were subjected to protein extraction and showed high protein expression levels of EGFR and MET by western blot. B. The MTT assay. HSC-2 and HSC-3 cells were treated with different doses of cetuximab (0-5 μg/mL) and HGF (20 ng/mL) for 72 h, and the cell proliferation levels were shown by the MTT assay. C. Western blot. HSC-2 and HSC-3 cells were pretreated with control, HGF (20 ng/mL), cetuximab (1 μg/mL), or their combination. D. The MTT assay. HSC-2 cells treated with HGF (20 ng/mL) and the HGF neutralizing antibody or cetuximab (1 μg/mL) for 72 h showed different proliferation abilities by the MTT assay. *$P < 0.05$.

HGF (**Figure 1C**). Under these conditions, p-ErbB3 was dramatically inhibited with cetuximab, even after the addition of HGF, indicating that p-ErbB3 is the target of cetuximab but not involved in in the HGF-induced cetuximab resistance of OSCC cells; whereas the Met, PI3K/Akt, and MAPK pathways may be involved in the HGF-induced cetuximab resistance of OSCC cells. For further confirmation, we pretreated these cells with the HGF neutralizing antibody to block HGF signaling and found that HSC-2 cells regained their sensitivity to cetuximab treatment (**Figure 1D**).

Met inhibitor restoration of cetuximab sensitivity of HGF-treated OSCC cells by decreasing akt and ERK1/2 phosphorylation

The biological effects of HGF are through binding to its receptor, Met; thus, we treated the cells with the Met inhibitor PHA-665752 in the same setting. Our MTT assay data showed that PHA-665752 significantly reduced the tumor cell viability in a dose-dependent manner (**Figure 2A**). Without the stimulus of HGF, PHA-665752 and cetuximab together strongly induced inhibition of cell proliferation, even at a low dose of PHA-665752 (< 1 μM). After stimulus with HGF, the OSCC cells showed cetuximab resistance in low-dose PHA-665752-treated cells, but the cell viability was dramatically reduced after the PHA-665752 dose reached 1 μM, which was similar to that without the HGF stimulus.

To evaluate the mechanism by which HGF rescues OSCC cancer cells from cetuximab-induced inhibition of proliferation, we determined activation of the downstream signaling molecules and found that cetuximab not only

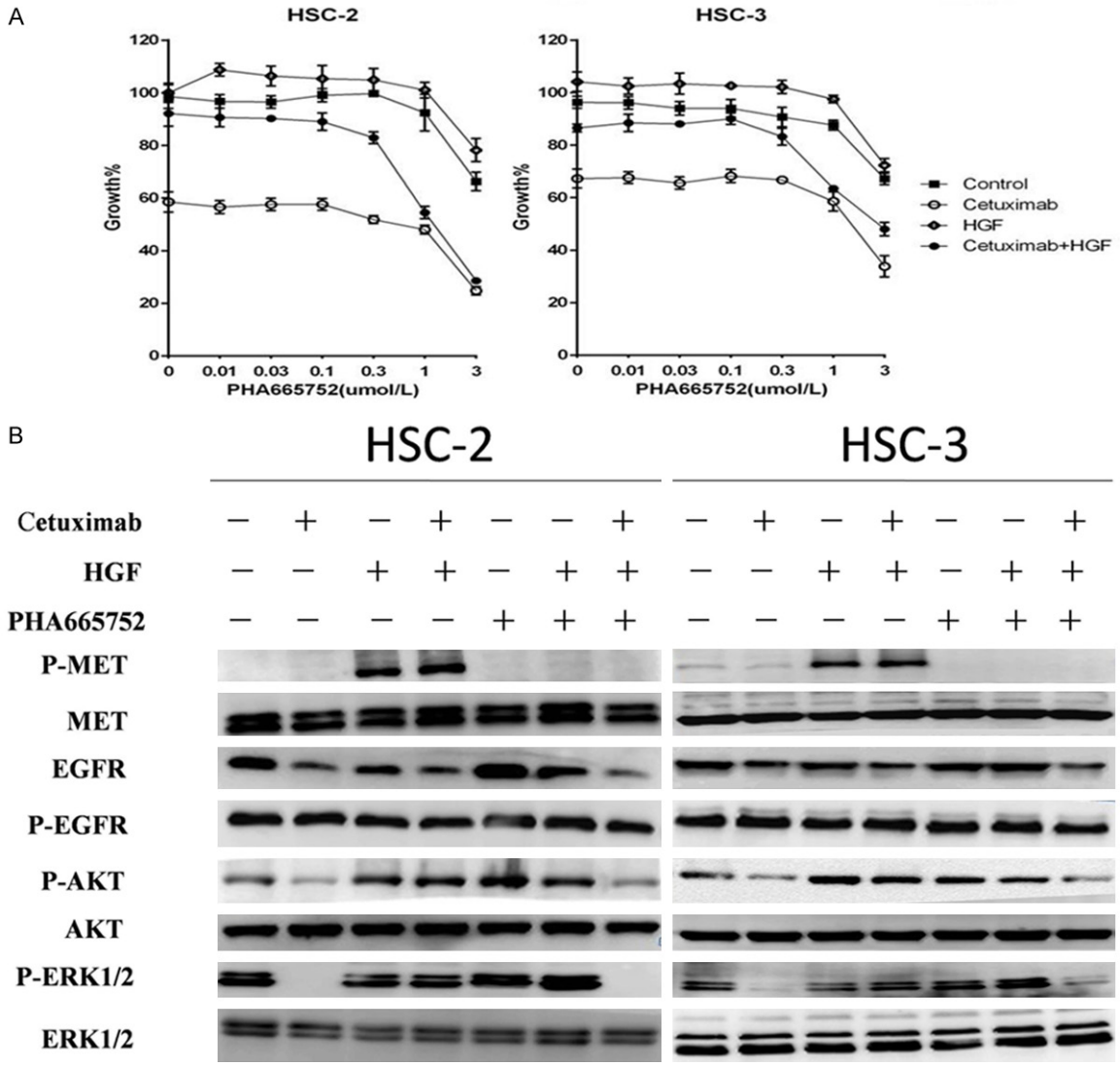

Figure 2. Treatment with the Met inhibitor restored OSCC cell sensitivity to cetuximab in HGF-induced cetuximab-resistant OSCC cells and decreased Akt and ERK1/2 phosphorylation. (A) The MTT assay. HSC-2 and HSC-3 cells were grown and treated with different doses of PHA-665752 or pretreated with or without HGF (20 ng/mL) and cetuximab (1 μg/mL). (B) Western blot. The cells described in (A) were subjected to western blotting.

inhibited EGFR phosphorylation, but also AKT and ERK1/2 activation in HSC-2 and HSC-3 cells. Treating the cells with a combination of cetuximab with HGF led to MET phosphorylation and reactivation of AKT and ERK1/2. However, when treating the cells with a combination of cetuximab with PHA-665752, inhibition of EGFR and MET phosphorylation led to sustained inhibition of the AKT and MAPK pathways in the presence of HGF (**Figure 2B**).

Met siRNA restoration of cetuximab sensitivity of HGF-treated OSCC cells

Next, we assessed whether Met siRNA possesses the same effects as PHA-665752 on HSC-2 cells; it is because cetuximab had even better inhibitory effects on the tumor cells and ErbB3 siRNA was used as a negative control since ErbB3 is not involved in this effect. The western blot data showed that Met siRNA was able to knock down Met expression in OSCC cells, compared to scrambled and ErbB3 siRNA transfection (**Figure 3A**). Moreover, Met siRNA reduced the OSCC cell viability and reversed the cetuximab resistance induced by HGF treatment, while ErbB3 siRNA did not show these effects (**Figure 3B**). At the protein level, p-Akt and p-ERK1/2 expression was blocked by cetuximab treatment and was restored partially by HGF; however, the expression of these pro-

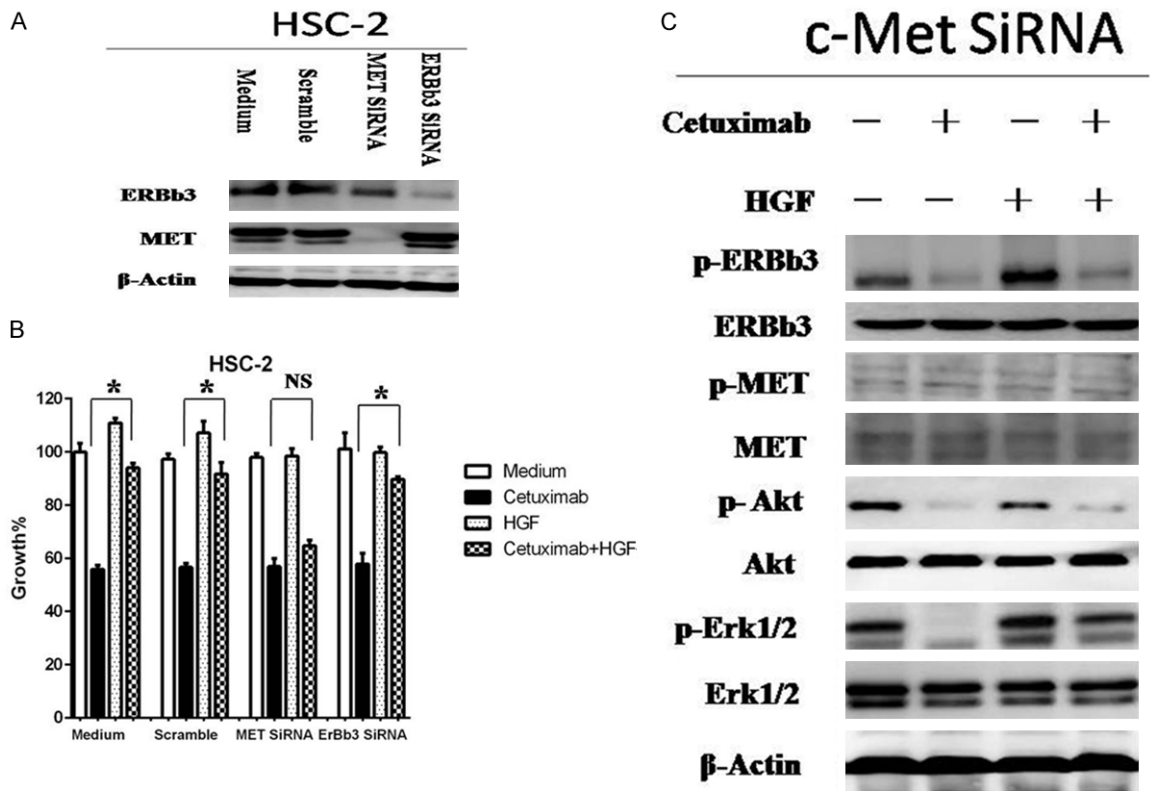

Figure 3. Met siRNA restoration of OSCC cell sensitivity to cetuximab in HGF-induced cetuximab-resistant OSCC cells. A. Western blot. HSC-2 cells were grown and transiently transfected with Met, negative control, or ErbB3 siRNA for 48 h and then subjected to western blotting. B. The MTT assay. HSC-2 cells were grown and transiently transfected with Met, negative control, or ErbB3 siRNA for 48 h and then subjected to the MTT assay. C. Western blot. HSC-2 cells were grown and transiently transfected with Met siRNA for 48 h and then treated with cetuximab (1 μg/mL) for 72 h before being subjected to western blotting.

teins was almost completely blocked by Met siRNA transfection (**Figure 3C**).

Association of met and akt expression with poor OSCC outcomes

To relate our current data clinically, we assessed and retrieved data on the differentially expressed genes in OSCC tissue samples from the NCBI-GEO database and then identified the expression of HGF, Met, ERK1 (also called MAPK3 in **Figure 4D**), ERK2 (also called MAPK1 in **Figure 4E**), and Akt in OSCC tissues. The Kaplan-Meier curves were plotted against the expression of these genes and then statistically analyzed by using the log rank test. We found that the expression of Met and Akt was associated with a poor survival of OSCC patients (P = 0.021 and 0.011, respectively; **Figure 4A** and **4F**), whereas HGF expression was associated with a better patient survival (P = 0.026; **Figure 4B**). Moreover, interactive analysis of HGF and Met expression did not yield any statistical significance for patient survival (**Figure 4C**). In addition, the expression of ERK1 and ERK2 also did not show any significant association with patient survival (P = 0.20 and 0.51; **Figure 4D** and **4E**).

Discussion

Cetuximab is a monoclonal antibody used to treat various human cancers by blockage of EGFR activation to inhibit the downstream pathways in tumor cell growth and invasion [36]. However, primary and acquired cetuximab resistance occurs in most cancer cases, even if the tumor cells express a high level of EGFR [37]. The underlying molecular mechanisms of drug resistance are complicated. For example, in colon cancer, resistance to anti-EGFR antibodies is mainly through the irregular activation of the EGFR downstream proteins by other proteins, like RAS [38], STAT [39], PTEN [40], or

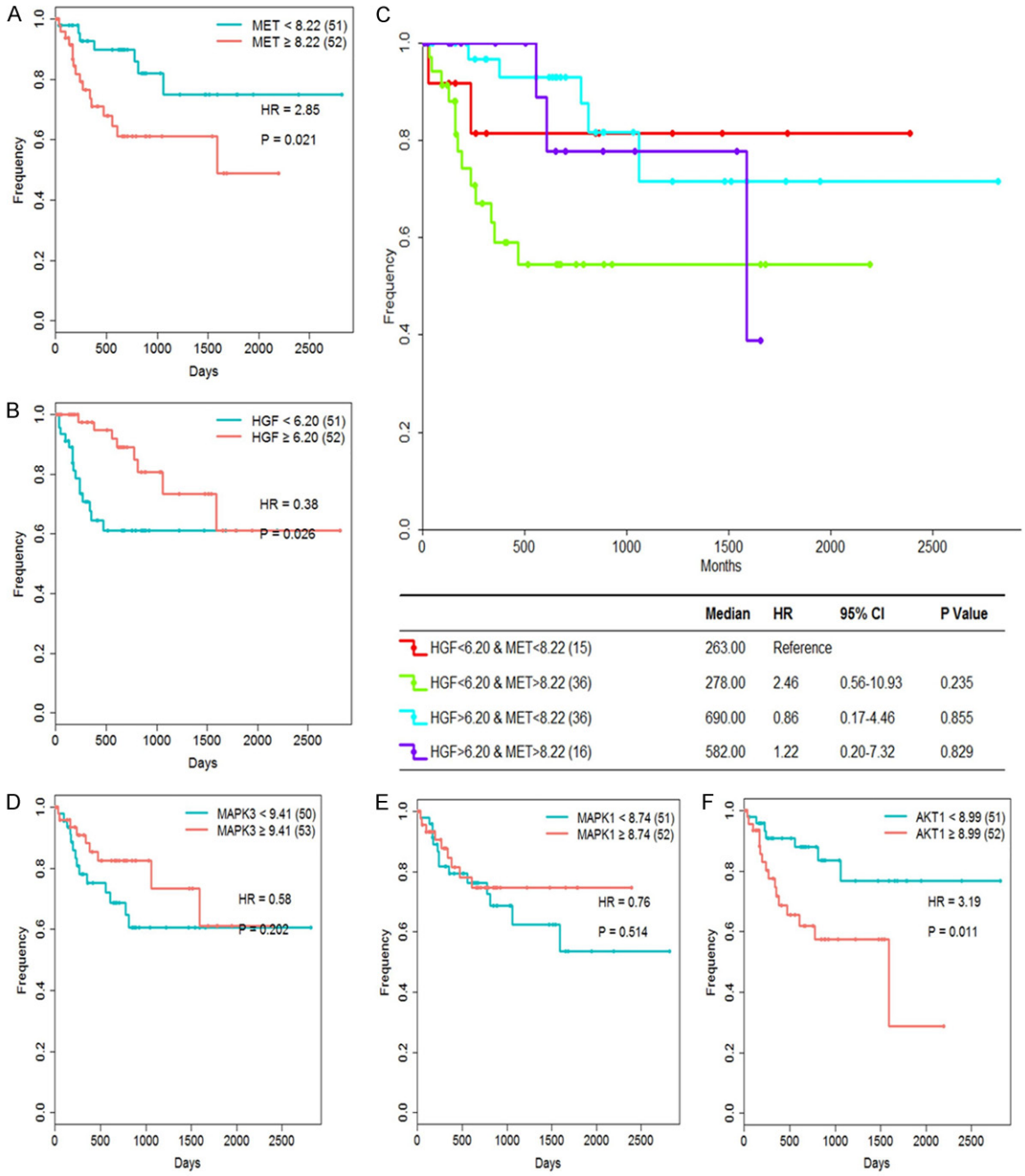

Figure 4. Association of gene expression with the survival of OSCC patients. The online database data were retrieved and statistically analyzed. A. Association of Met expression with the overall survival (OS) of OSCC patients. High Met expression predicted a poor OS (P = 0.021). B. Association of HGF expression with the OS of OSCC patients. A high HGF level was associated with a better OS (P = 0.026). C. Association of Met/HGF expression with the OS of OSCC patients. The combined expression of Met and HGF failed to predict the OS of OSCC patients. D and E. Association of ERK1 and ERK2 expression with the OS of OSCC patients. ERK1 and ERK2 expression failed to predict the OS of OSCC patients. F. Association of Akt expression with the OS of OSCC patients. A high Akt expression predicted a poor OS of OSCC patients (P = 0.011).

Met [41]. A single nucleotide polymorphism of the EGFR gene has been reported to occur in more than 40% of HNSCC patients, a change that could affect the affinity of cetuximab to bind to EGFR protein [42]. In another previous study, Smad4 expression was found to be criti-

cal in mediating cetuximab sensitivity and resistance [43]. In addition, in cetuximab-sensitive HNSCC A431 cells, cetuximab downregulated p-ERK and p-Akt expression, whereas Akt phosphorylation was unchanged in cetuximab-resistant cells [44]. In our current study, we found that HGF-induced cetuximab resistance occurred in both HSC-2 and HSC-3 cells with high levels of p-Akt and p-ERK1/2 when they were cotreated with cetuximab, indicating that Akt and ERK1/2 were responsible for mediating cetuximab resistance in OSCC.

Met protein is frequently highly expressed in various human cancers. It is activated by its ligand HGF to trigger activation of the downstream signaling pathways and multiple cellular events [45], like cancer development and metastasis. However, it has been shown in lung cancer and hepatocellular carcinoma that aberrant HGF-independent Met activation occurs and then in turn triggers the ErbB3/PI3K/Akt signaling pathway, leading to cancer progression [46, 47]. In HNSCC, a previous study has revealed that ErbB3 is required to activate EGFR and drug resistance [48]. However, in our current study, we did not provide any evidence showing the importance of ErbB3 in HGF-induced cetuximab resistance in OSCC cells. Furthermore, in non-small cell lung cancer, it has been demonstrated that there is a synergistic effect of HGF and EGF on cancer cell growth and that the cross-talk between Met and HGF, at the molecular level, is crucial for tumor cell growth [49]. In colorectal cancer, HGF induction of Met activation resulted in cetuximab resistance [50]. Furthermore, Met siRNA was able to reduce HNSCC cell viability and migration *in vitro* and tumor xenograft growth *in vivo* [51]. In addition, in human OSCC tissues, Met expression was significantly upregulated compared with that of the adjacent normal tissues [35]. Met inhibitors exhibited a strong effect on inhibition of OSCC cell growth and induction of OSCC cell apoptosis by suppression of Akt and ERK1/2 activities [52]. In our current study, HGF induced Akt and ERK1/2 phosphorylation, leading to cetuximab-resistant OSCC cells. With the combination of the Met inhibitor and Met siRNA, OSCC cell viability was significantly reduced and Akt and ERK1/2 phosphorylation was dramatically decreased. Taken together, we speculated that Met and EGFR cross-talk could synergistically promote cancer progression and that anti-Met therapy could be effective even in OSCC cells resistant to anti-EGFR therapy. In this context, the combination of cetuximab and Met inhibitor could be a better option for the treatment of OSCC patients with primary or acquired cetuximab resistance.

Indeed, different Met inhibitors are under clinical trials, and crizotinib and cabozantinib were approved by the US FDA to treat NSCLC and medullary thyroid cancer, respectively [53]. Another Met inhibitor, PHA-665752, is a compound used to inhibit Met phosphorylation and the downstream signaling cascades [54]. In ovarian cancer, PHA-665752 and Met siRNA have been shown to inhibit tumor cell growth and overcome cisplatin resistance [55]. While in colorectal cancer, the combination of PHA-665752 and cetuximab significantly decreased tumor cell proliferation compared with that of either agent alone [56]. In our current study, PHA-665752 was shown to resensitize OSCC cells to cetuximab resistance, and its combination with cetuximab had a better antitumor efficacy. At the molecular level, our current data showed that both HGF and cetuximab were able to regulate Akt and ERK1/2 activities. Indeed, Yu *et al.* have demonstrated that the PI3K/Akt/mTOR pathway is an ideal target for controlling OSCC because the combination of PI3K/Akt inhibitor with radiation improved the radiation efficacy for the treatment of OSCC [57]. Targeting of the PI3K/Akt/mTOR pathway also improved the effects of doxorubicin on its antitumor activity in OSCC [58]. Furthermore, sulfasalazine (SSZ), an anti-inflammatory drug, has been demonstrated to have a potential therapeutic ability in the treatment of OSCC by promoting autophagy-induced tumor cell death and inhibiting the PI3K/Akt and MAPK pathways [59]. Another previous study has revealed that activation of the ERK, Akt, and p38 pathways is involved in HGF- and EGF-induced OSCC cell migration [60]. Taken together, the PI3K/Akt and MAPK pathways could be involved in OSCC development and progression, and these signaling pathways are activated by different upstream factors, e.g. EGFR and Met. When OSCC cells became sensitive to cetuximab treatment, the Akt and ERK activities were significantly suppressed in OSCC cells, leading to a decrease in OSCC progression; whereas in cetuximab-resistant cells, the cetuximab-EGFR-

Akt/ERK axis was aberrant, but there was an increase in HGF-induced Met activity to induce Akt/ERK phosphorylation, leading to OSCC cell proliferation. However, blockage of these two signaling pathways using the combination of cetuximab and Met inhibitor or Met siRNA inhibited Akt and ERK phosphorylation, resulting in cell growth inhibition. Thus, our current data provide insightful information regarding the effects of the combined treatment of cetuximab and Met inhibitor as a novel therapeutic strategy in the control of OSCC.

In addition, our *ex vivo* data analysis showed that the expression of the cell growth signaling proteins Akt and Met was associated with a poor survival of OSCC patients. However, due to the small sample size, the expression of Met with HGF did not show statistically significant data that predict OSCC patient survival. Future studies with a large sample size should be performed to confirm our current findings. However, our current study does have some limitations; for instance, all the experiments were performed *in vitro*, and *in vivo* experiments are needed to assess and validate the effects of the combined treatment of cetuximab and Met inhibitor on the control of OSCC in the future. Furthermore, our *ex vivo* data lacked a sufficient sample size as well as cetuximab or Met inhibitor treatment data for OSCC patients.

Conclusions

Our current study revealed that HGF activated Met, subsequently increasing the phosphorylation of the downstream PI3K/Akt and MAPK pathways, which were responsible for the cetuximab resistance of OSCC, and that inhibition of Met expression or activity restored OSCC sensitivity to cetuximab treatment. Future studies are needed to confirm the effects of the combination treatment of cetuximab with Met inhibitor in the control of OSCC clinically.

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Disclosure of conflict of interest

None.

Abbreviations

Akt, Protein kinase B; c-Met, hepatocyte growth factor receptor; EGFR, epidermal growth factor receptor; ERK1/2, Extracellular signal-regulated kinase; HGF, Hepatocyte growth factor; OSCC, Oral squamous cell carcinoma.

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