Abstract. Mesenchymal-epithelial transition factor (c-Met) is the only high affinity receptor for hepatocyte growth factor (HGF), and is frequently activated in many human cancers. However, little is known about the role of the HGF/c-Met signaling pathway in the progression of human oral squamous cell carcinoma (OSCC). This study evaluated the role of the HGF/c-Met signaling pathway in the progression of human OSCC. We found that the expression of c-Met was significantly increased in human OSCC tissues than in normal mucosa adjacent to the tumor (P<0.05), but was not correlated with clinicopathological parameters. Additionally, the selective c-Met inhibitor JNJ was found to inhibit cell viability and migration and promote apoptosis in OSCC cell lines, and also blocked the activation AKT, ERK1/2, and NF-κB p65; thus, suggesting that HGF/c-Met signaling may play an important role in the tumorigenic properties of OSCC cells. We found that the expression of c-Met was significantly increased in human OSCC tissues than in normal mucosa adjacent to the tumor (P<0.05), but was not correlated with clinicopathological parameters. Additionally, the selective c-Met inhibitor JNJ was found to inhibit cell viability and migration and promote apoptosis in OSCC cell lines, and also blocked the activation AKT, ERK1/2, and NF-κB pathways. Collectively, these results indicated that HGF/c-Met signaling may serve essential roles in the progression of human OSCC, and may thus be a basis for the development of novel therapeutic approaches in the treatment of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is among the most prevalent malignant neoplasms worldwide, and frequently occurs in developing countries, including those of Southeast Asia (1,2). Despite recent advances in surgical and radio-therapeutic strategies, a high proportion of patients with OSCC still have poor prognosis, which has remained relatively unchanged over the past 20 years (3,4). Therefore, it is necessary to explore novel molecular targets for the development of more effective therapeutic strategies for patients with OSCC.

Mesenchymal-epithelial transition factor (c-Met) is the only known high-affinity receptor for hepatocyte growth factor (HGF). The HGF/c-Met signaling pathway can stimulate the growth of hepatocytes, and may also promote proliferation, migration, survival, angiogenesis, and invasion in a broad range of human solid tumors, including ovarian, stomach, lung, breast, liver, and brain tumors (5-12). Moreover, high expression of c-Met was found in pancreatic cancer stem cells, and knockdown of c-Met or treatment with a c-Met inhibitor blocked the formation of tumor spheres in a population of pancreatic cancer cells with stem cell like properties (13,14). Furthermore, inhibition of the c-Met signaling pathway has been reported to increase mitochondrial release of cytochrome c and the Bax/Bcl-2 ratio (15). These data regarding the aberrant expression and activity of c-Met revealed that it may play an important role in the progression of human cancers, and may be an important target in cancer therapy. However, little is known about the biological functions of the HGF/c-Met signaling pathway in the progression of human OSCC.

To elucidate the underlying functions of the HGF/c-Met signaling pathway in the progression of OSCC, the present study first examined c-Met expression in 40 human OSCC tissues, 20 human normal oral mucosa and two OSCC cell lines. Subsequently, we evaluated the effects of a c-Met inhibitor on the viability, migration, and apoptosis of the OSCC cell lines. Finally, we preliminarily investigated the potential molecular mechanisms underlying the activity of the HGF/c-Met signaling pathway in the progression of OSCC.

Materials and methods

Cell lines and cell culture conditions. The human HIOEC, HN30 and CAL-27 cell lines were obtained from the Laboratory of Oral Oncology, Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China. The three cell lines were cultured in DMEM and medium
supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and of 1% penicillin/streptomycin (Gibco). Cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

**Reagents and antibodies.** Human recombinant HGF was purchased from GenScript (Nanjing, China). The c-Met kinase inhibitors JNJ38877605 (JNJ) was obtained from Selleckchem (Houston, TX, USA). Antibodies used included: Met mAb (#8198), phosphor-Met (Tyr1234/1235) mAb (#3077), AKT mAb (#9272), phosphor-AKT (Ser473) mAb (#4060), p44/42 MAP kinase mAb (#4696), phosphor-p44/42 MAP kinase (Thr202/Tyr204) mAb (#4376), NF-kB p65 mAb (#6131), GAPDH mAb (#2118), MMP-9 mAb (#3852), HRP-linked anti-mouse IgG antibody (#7076), and HRP-linked anti-rabbit IgG antibody (#7074). The PE-labeled anti-rabbit IgG antibody was used as a secondary antibody. All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). VEGF mAb (#19003) was purchased from Proteintech (Chicago, IL, USA).

**Immunohistochemistry (IHC).** IHC was performed as previously described (16). Forty human OSCC specimens were collected from patients who had undergone surgery between September 2009 and September 2010 at the Department of Oral and Maxillofacial Surgery, Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University. All experimental procedures received ethics approval from the independent Ethics Committee of the Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine (no. 200926). The pathological characterization of the OSCC patients included in this study is summarized in Table I. For immunohistochemical examination, tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of the samples were blocked with 10% goat serum in PBS and incubated overnight at 4°C with anti-c-Met, anti-VEGF-A or anti-MMP-9 antibodies. After 3 washes with PBS, the sections were incubated with the peroxidase-conjugated goat anti-rabbit antibody for 1 h, following by incubation with 3,3'-diaminobenzidine (DAB) substrate for 3 min. Counter-staining was performed with hematoxylin, and dehydration was then performed with ethanol and dimethyl benzene. The IHC results in tissues were scored by two independent investigators based on the level of staining intensity as follows: none (-), 0% of stained cells; weak (+), 1-25% of stained cells; moderate (++), 26-50% of stained cells; strong (+++), >50% of stained cells.

**RNA isolation and RT-PCR.** Total RNA was isolated and RT-PCR was performed as previously described (16). The primer pairs were as follows: c-Met, forward 5'-TTC-ACC-GCG-GAA-ACA-CCC-ATC-3', and reverse 5'-GTC-TTC-CAG-CCA-GGC-CCA-3'; GAPDH, forward 5'-CAT-CTC-TGC-CCC-CTC-TGC-TGA-3', and reverse 5'-GGA-TGA-CCT-TTG-GCA-CCA-CAG-CCT-3'.

**Western blotting.** Western blot analysis was performed as previously described (16). The cells were lysed with M-PER mammalian protein extraction (Pierce, Rockford, IL, USA). Proteins were quantified by the BCA Protein Assay kit (Pierce) according to the manufacturer's instructions. Samples containing a total of 50 µg protein were incubated at 100°C for 5 min, separated by SDS-polyacrylamide gel electrophoresis, and subsequently electrotransferred onto a polyvinylidene difluoride membrane. Essential component detection in the cells was performed using an antibody with overnight incubation at 4°C, and then an HRP-conjugated secondary antibody (1:5,000 dilution) was added for 1 h at room temperature, followed by the development of reactions in a chemiluminescent detection system.

**Viability and apoptosis assays.** Cells were seeded in a 96-well plate at 1x10⁴ cells/well and were grown in the presence of JNJ for 2 h. Then, they were treated with HGF. After 3 days, 20 µl MTS (Sango, Shanghai, China) was added to each sample and incubated for 4 h. The absorbance of the solution was recorded at 490 nm with a Thermo microplate reader. The results of the MTS assay reflected the cell viability.

Apoptotic cells were assessed by flow cytometry as follows: cells were harvested and washed with PBS, resuspended in pre-diluted binding buffer, and stained with Annexin V-FITC (BD Biosciences, San Diego, CA, USA) for 30 min at room temperature. After being washed and resuspended in PI binding buffer, the cells were immediately subjected to apoptosis analyses by flow cytometry using Cell Quest Software.

**In vitro scratch wound healing migration assays.** HN30 or CAL-27 cells were plated in a 6-well plate. After overnight incubation, a sterile 10 µl pipette tip was used to make a wound across a cell culture monolayer. Cells were incubated in DMEM-0% FBS in the presence of JNJ for 2 h, and then were treated with HGF (100 ng/ml). Multiple images of the wound were taken immediately after wounding at 0 and 24 h under a phase-contrast microscope. The efficiency of the wound healing process was determined by calculating the area of the cell gap at the indicated time-points (0 and 24 h) using ImageJ software. Three images were used for each wound at each experimental point. The results are expressed as percentage of healing at 24 h with respect to time-point zero.

**Immunofluorescence analysis.** Tumor cells deposited on glass slides were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 20 min. The cells were further permeabilized with 0.1% Triton X in PBS for 8 min, washed and blocked with 5% bovine serum albumin in PBS for 30 min, and then treated with a monoclonal mouse anti-p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibody overnight. PE-labeled (1:100) anti-rabbit IgG served as the secondary antibody. The sections were then mounted in a medium containing DAPI for 5 min to visualize cell nuclei. The slides were evaluated with fluorescence microscope TCS SP2 (Leica, Wetzlar, Germany).

**Tumor xenograft study.** Approximately 5- to 6-week-old male athymic nude mice (approximately 20 g) were obtained from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were kept in a specific pathogen-free (SPF) facility. CAL-27 cells (5x10⁴) were subcutaneously injected into the right flank region of mice. After 6 days, allowing the tumors to grow to approximately 50 mm³, the mice were randomized into control and treatment groups. For the CAL-27 xenograft, the mice were treated as follows: JNJ (10 mg/kg) oral administration drugs once a day for 4 weeks. Each group consisted of 5-6 mice. Concomitantly, the body weight and tumor size...
were assessed using an electronic balance and a vernier caliper. The tumor volume was calculated using the formula: volume = (length x width²) x 1/2. Following the endpoint (28 days after the implantation), mice were euthanized when moribund for the collection of tumors. This study was performed via protocols approved by the Institutional Animal Care and Use Committee of Fudan University (Shanghai, China).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD) of at least three separate experiments. One-way ANOVA was performed with post hoc SNK for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of c-Met in human OSCC tissues and cell lines. To investigate whether c-Met was expressed in human OSCC tissues, 40 human OSCC specimens and 20 normal oral tissue samples adjacent to the tumor were assessed by immunohistochemistry. A high level of c-Met expression was detected in 60% (24/40) of the carcinoma samples, while only in 25% (5/20) of the normal oral epithelial tissues. In the tumor samples, c-Met was found to be localized in the cell membrane and cytoplasm (Fig. 1A). The expression of c-Met in situ was not correlated with patient clinicopathological characteristics, including tobacco use, alcohol consumption, sex, tumor site, tumor stage, nodal status or pathological differentiation grade (Table I). Additionally, we detected upregulated c-Met in HIOEC and the OSCC cell lines (HN30 and CAL-27) by RT-PCR and western blot analysis, as shown in Fig. 1B. Thus, the increased expression of c-Met in OSCC tissues and cell lines revealed that c-Met may be functionally important in the progression of human OSCC.

JNJ inhibits the effects of HGF on cancer cell viability, migration, and anti-apoptosis. Since the HGF/c-Met signaling pathway can mediate tumor growth, migration,
invasion, and survival (6, 7), we first sought to assess the effect of JNJ on tumor cell viability. JNJ is a small-molecule ATP-competitive inhibitor of the catalytic activity of c-Met, which exhibited ~600-fold selectivity for c-Met compared...
with a panel of ~250 diverse tyrosine and serine-threonine kinases. It was also found to potently inhibit HGF-stimulated and constitutively activated c-Met phosphorylation (17). HN30 and CAL-27 cells were treated with or without JNJ for 2 h prior to treatment with HGF, and an MTS cell viability assay was performed subsequently. The results revealed that JNJ could significantly reverse the stimulatory effect of HGF on cancer cell viability (Fig. 2A). Next, the effect of JNJ on the migration of HN30 and CAL-27 cells was characterized. In a wound healing assay, it was observed that JNJ (10 µM) could significantly suppress HGF-induced migration of the tumor cells (Fig. 2B).

We also evaluated the effect of JNJ on c-Met signaling in the regulation of cancer cell death. HN30 and CAL-27 cells were treated with JNJ for 2 h, and then with combinations of HGF and RAPA. After treatment for 48 h, the cells were analyzed by flow cytometry to evaluate the apoptotic index. The results revealed that HGF could significantly rescue RAPA-induced HN30 cell apoptosis when compared with the control group. Notably, the percentage of RAPA-induced apoptotic cells was decreased from 32.87 to 17.59% following HGF stimulation of the cancer cells. In addition, pretreatment with JNJ could markedly suppress the effect of HGF. Thus, following JNJ pretreatment, the percentage of apoptotic cells increased from 17.59 to 26.91% for the RAPA-induced + HGF-treated HN30 cells (Fig. 2C). The same phenomenon was observed in CAL-27 cells. Altogether, our results indicated that JNJ may play an important role in inhibiting the pro-proliferation, pro-migration, and anti-apoptotic effects of HGF in human OSCC cell lines.

**JNJ inhibits tumor development in vivo.** JNJ displayed excellent oral bioavailability approaching 100% in all examined species and JNJ in a single dose was observed to inhibit Met phosphorylation in tumor xenografts for up to 16 h (18). Finally, the therapeutic efficacy of JNJ was explored in mice in vivo. Following CAL-27 cell xenografts, the mice were treated with or without JNJ (10 mg/kg). As shown in Fig. 3A and B, JNJ treatment significantly reduced the tumor size when compared with the control group (P<0.001). We also found that JNJ could reduce the expression of VEGF-A and MMP-9, two key proteins related to angiogenesis and migration, which indicated its ability to inhibit cancer cell angiogenesis, migration, and invasion in vivo (Fig. 3C). These data collectively indicated the potency of JNJ in inhibiting tumor growth, angiogenesis, and migration in vivo.

**JNJ inhibits HGF-mediated upregulation of c-Met downstream molecules.** The PI3K/AKT and ERK signaling pathways act downstream of c-Met signaling, and thus we examined the effects of JNJ on these downstream pathways in HN30 and CAL-27 cells. Western blot analysis revealed that the HGF-mediated upregulation of p-c-Met in the cancer cells was abolished by JNJ treatment (Fig. 4A). In addition, upregulation of p-AKT was also observed in the cancer cells.
in response to HGF stimulation, which was also inhibited by treatment with JNJ. Similarly, HGF-mediated upregulation of p-ERK1/2 was inhibited by JNJ. Meanwhile, in CAL-27 cells immunofluorescence staining indicated that there was a significant increase in the nuclear translocation of the NF-κB subunit p65 after HGF stimulation (Fig. 4B), which could also be significantly inhibited by JNJ treatment (4 and 10 μM). These results revealed that the AKT, ERK1/2, and NF-κB p65 pathways play essential roles in the growth, migration, and apoptosis of OSCC cells, possibly mediating the effects of JNJ on the inhibition of OSCC progression after targeting c-Met.

Cigarette smoking is considered to be among the major risk factors for OSCC (19). Additionally, cigarette smokers have been reported to have a 2-5 times greater risk of developing oral cancer than non-smokers. Furthermore, the risk of oral cancer increases with the number of cigarettes smoked and the duration of smoking (20). In cigarette smokers, overexpression of c-Met has been found to be induced in microvessels of oral lichen planus, and the majority of smoker samples exhibit c-Met-positive expression (21). Additionally, it was reported that nicotine can induce HGF overexpression in lung cancer tissues, as well as in type II normal pneumocytes, while overexpression of c-Met was frequently detected in adenocarcinoma cells, and nicotine can enhance the pro-migratory effect of HGF on lung cancer cells, thus possibly contributing to lung cancer progression (22,23).

A previous study on head and neck squamous cell carcinoma (HNSCC) demonstrated that c-Met-positive cells had cancer stem cell properties and could resist the effect of cisplatin (24). c-Met was upregulated and functional in 90% of HNSCC cell lines and 84% of patient samples (25,26). It has also been suggested that the HGF/c-Met signaling pathway is associated with the progression and invasive behavior of OSCC (27-29). The length of survival was significantly reduced in oral tongue carcinoma patients with c-Met expression compared to those without c-Met expression (30). Activation of HGF/c-Met was
critical for enhanced proliferation, invasion, and metastasis in HNSCC, which was correlated with decreased survival, increased recurrence rates and poor patient prognosis (31-33). c-Met knockdown in OSCC cell lines reduced cervical lymph node spread and increased survival of mice in an orthotopic animal model (34). However, the relationship between the HGF/c-Met signaling pathway and the progression of OSCC is not fully understood. It is essential to understand the molecular mechanism so as to aid clinical intervention.

In the present study, we examined the degree of c-Met expression in OSCC tissues and its potential correlation with clinicopathological parameters. We found that 60.0% (24/40) of the OSCC tissues exhibited a high level of c-Met expression, whereas only 25% (5/20) of the normal oral epithelial tissues expressed c-Met. This result revealed that the c-Met protein was produced in the majority of OSCC cases. However, we found that there were no significant correlations between c-Met expression and clinicopathological variables, such as tobacco use, alcohol consumption, sex, tumor site, tumor stage, nodal status or pathological differentiation grade (Table I). Nevertheless, the aberrant expression of c-Met in OSCC samples compared with normal tissues revealed that it may play an important role in the progression of human OSCC. Cigarette smoking can induce overexpression of c-Met, which may in turn result in OSCC. However, there was no significant correlation between c-Met expression and cigarette smoking. This may be a limitation of the present study, as only 40 specimens of OSCC were investigated, and thus a larger number of specimens should be investigated in future studies.

Overexpression of c-Met has been reported in many types of cancer, and potentially leads to aberrant signaling associated with cancer development and progression (35,36). Activation of the HGF/c-Met signaling pathway promotes tumor cell proliferation, migration, and invasion and tumor angiogenesis, and was associated with poor prognosis (37-39). HGF can promote tumor angiogenesis and reverse suspension-induced apoptosis (anokies), which in turn, can increase not only tumor growth, but also tumor invasion and metastasis (40-44). To further investigate the role of the HGF/c-Met signaling pathway in the progression of OSCC, in vitro experiments were carried out in the present study. We found that c-Met was overexpressed in two OSCC cell lines (Fig. 1B), indicating that OSCC cell lines may obtain a growth advantage by upregulating c-Met. Exposure to a selective c-Met inhibitor, JNJ, had substantial effects on cell viability, migration, and upregulating c-Met. Exposure to a selective c-Met inhibitor, that OSCC cell lines may obtain a growth advantage by inhibiting apoptosis. Our findings demonstrated that OSCC cell lines can be stimulated to grow by HGF stimulation, and thus c-Met may be aberrantly activated in the progression of OSCC.

HGF can induce c-Met phosphorylation, which in turn activates multiple downstream pathways, including the PI3K/AKT and MAPK/ERK signaling pathways (6,40). c-Met inhibitors have demonstrated antitumor efficacy in preclinical studies and are currently being evaluated in human cancer clinical trials (45,46). In the present study, the selective c-Met inhibitor JNJ exerted antitumor effects on OSCC cell growth potentially by blocking activation of AKT, ERK, and NF-xB p65 (Fig. 4A and B). These data indicated that activation of the HGF/c-Met system may stimulate cancer cell survival and growth through the ERK, PI3K/AKT, and NF-xB signaling pathways.

In summary, our results ascertained that JNJ can inhibit HGF-stimulated cell viability and migration, and further confirmed the potent opposing activity of JNJ against the anti-apoptotic effect of HGF. These findings indicated the potential role of c-Met in the progression of OSCC. The HGF/c-Met system functions as a potent pro-growth signal that exacerbates the malignant progression of OSCC. Thus, c-Met inhibition is considered to exert a potent pro-apoptotic effect as part of its direct impact on OSCC. Accordingly, our data revealed that JNJ inhibited HGF-induced survival of the OSCC cell lines in vitro. In particular, JNJ markedly inhibited the pro-proliferative, pro-migration, and anti-apoptotic effects of HGF by inhibiting the c-Met pathway. JNJ also inhibited tumor growth, angiogenesis, and migration in OSCC xenografts (Fig. 3A-C). The expression of Ki-67 related to tumor proliferation was also analyzed. However, for some reason, there were no differences between the control and JNJ-treated group. In addition, immunohistochemical staining of human OSCC tissues revealed high expression of c-Met. Therefore, we have not only demonstrated the pro-proliferative, pro-promigratory, and anti-apoptotic activity of HGF/c-Met signaling in OSCC cells, but also provided solid data on the possible molecular mechanisms mediating such activity, which may serve important roles in the progression of OSCC. Therefore, c-Met may be an important target for the development of new therapeutic approaches in the treatment of OSCC.

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References


