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Effect of HGF/c-Met pathway in oral squamous cell carcinoma on EMT and metastatic potential

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Oral squamous cell carcinoma (OSCC) is characterized by highly invasive tumor cells. Hepatocyte growth factor (HGF) signaling plays an important role in the induction of epithelial-mesenchymal transition (EMT), a process that converts immotile epithelial cells into motile mesenchymal cells and is involved in cancer metastasis by promoting tumor cell scattering. The c-Met, which is a transmembrane tyrosine kinase receptor that may be activated by HGF, regulates the associated downstream gene expression. This process is important to cell migration in normal and cancerous conditions. This study explored the potential effects of c-Met on HGF-induced EMT in an OSCC cell line.

We investigated the function of c-Met in the process of EMT, and its molecular mechanism in oral cancer. OSCC cell line, HSC3 cells, were treated with HGF for varying durations. EMT-associated proteins, including E-cadherin and vimentin, were examined by western blot analysis. The role of c-Met in the mediation of EMT-like changes was investigated using western blot analysis and knockdown by c-Met inhibitor. Moreover, we carried out investigations using immunohistochemical and immunofluorescence staining.

We found that treatment with HGF induced EMT-like changes and enhanced the migrative potential of HSC3 cells. Furthermore, HGF-mediated EMT-like changes were associated with c-Met activation, and these changes could be blocked by c-Met knockdown. In particular, immunohistochemical and immunofluorescence staining of c-Met strongry expressed in the invasion front of the tumor cells. This study clearly demonstrated a crucial function for c-Met in EMT development in oral cancer. c-Met-targeted treatment may be an effective therapy for oral cancer. We believe that our data demonstrated that c-Met inhibitor could reduce HGF-induced EMT and cell motility via c-Met blockade and down-regulation of the pro-survival extracellular signal-regulated kinases pathway. (J Osaka Dent Univ 2020 ; 54 : 135-144)

Key words : HGF ; c-Met ; Oral squamous cell carcinoma ; SU11274

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is locally invasive[1, 2] and its rate of metastasis correlates with the clinical stage,[3] which is a factor related to its low survival rates. Thus, novel therapeutic strategies are needed. As most oral cancers are squamous cell carcinomas (SCCs), one feature is progressive local invasion.[1, 2] Therefore, it is necessary to improve treatments for OSCC by elucidating its invasion mechanisms. The processes of tumor invasion include cell migration, interaction between the tumor and stroma at the invasive front, and the involvement of growth factors and external substances, all of which influence the invading cells.[4-8]

Hepatocyte growth factor (HGF) has been identi-

fied as one of the molecules that induces tumor angiogenesis, and is a potent hepatotrophic factor responsible for regeneration of the liver.[9, 10] HGF is now recognized as a pluripotent cytokine that mediates tumor-stromal interaction with mitogenic, motogenic, and morphogenic activities.[11, 12] Moreover, HGF induces potent angiogenic activity in vascular endothelial cells.[13] HGF is a ligand for the c-Met proto-oncogene of the receptor tyrosine kinase.[12, 14] HGF and c-Met are upregulated in several types of cancers, such as colorectal, gastric, esophageal, breast, and lung cancers.[15-17] The upregulation of c-Met has been correlated with mortality in cancer patients.[18-20]

HGF induces degradative adhesion of epithelial cancer cells in a process called epithelial-mesenchymal transition (EMT), thereby increasing migration and invasiveness. EMT is important in cancer metastasis because it promotes the release of cancer cells from the primary tumor, and induces vasculature and lymphatic vessel invasion as well as secondary tumors in distant organs.[21] Although EMT induced by HGF has been investigated in different cancers,[22, 23] the tumorigenic association between EMT and c-Met remains unclear in oral cancer. The present study investigated the effects of the HGF/c-Met signaling pathway on EMT and the metastasis potential of OSCC.

MATERIALS AND METHODS

Clinical and pathological data (Table 1)

We used 50 OSCC samples (39 well-differentiated, 5 moderately-differentiated, and 6 poorly-differentiated) obtained from biopsies at Osaka Dental University Hospital. The subjects were 31 males, aged 18 to 86 (mean 63) years, and 19 females, aged 38 to 85 (mean 64) years, at the time of diagnosis. The primary OSCC location was the tongue. Sixteen of the cases were T1, 19 were T2, 10 were T3, and 5 were T4 ; the ages ranged from 38 to 85 (mean 62) years for T1, from 39 to 74 (mean 61) years for T2, from 18 to 82 (mean 68) years for T3, and from 43 to 86 (mean 71) years for T4. Twenty of the 50 cases had cervical lymph node metastasis (N(＋)), which was treated by neck dissection. Metastasis was confirmed by histopathology.

Table 1 Patient characteristics at primary surgery

Characteristic	Patients
Sex	
Male	31
Female	19
Median age in years (range)	
Male	63 (18-86)
Female	64 (38-85)
T stage	
T1	16
T2	19
T3	10
T4	5
N stage	
N0	30
N1	9
N2	11
C Stage	
Ⅰ	8
Ⅱ	13
Ⅲ	16
Ⅳ	13
Differentiation	
Well	39
Moderate	5
Poor	6

On the other hand, 30 of the cases had no cervical lymph node metastasis (N(−)). Thirteen were in males and 7 in females in the N(＋) group, whereas 18 were in males and 12 in females in the N(−) group. The ages ranged from 38 to 86 (mean 66) years in N(＋), and from 18 to 85 (mean 62) years in N(−). Eight cases were clinical stage I, 13 were stage II, 16 were stage III, and 13 were stage IV ; the ages ranged from 42 to 85 (mean 58) years for stage I, from 39 to 74 (mean 60) years for stage II, from 18 to 82 (mean 63) years for stage III, and from 60 to 86 (mean 71) years for stage IV. This study was approved by the ethics committee of Osaka Dental University (Approval No.110985).

Cell culture and reagents

The OSCC cell line HSC3 was purchased from RIKEN Bioresource Center (Ibaraki, Japan). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified atmosphere of

5% CO 2. DMEM and FBS were purchased from Gibco® (Life Technologies, Tokyo, Japan). Antibodies used included anti-c-Met (Cell Signaling Technology Japan, Tokyo, Japan), anti-phospho-c-Met (Tyr 1234/1235) (Cell Signaling Technology Japan), and anti-α-tubulin (Sigma-Aldrich, Tokyo, Japan). SU11274 was obtained from Sigma-Aldrich.

Wound healing assay

Cell migration was also assessed using an *in vitro* scratch wound healing assay. HSC3 cells were cultured with DMEM supplemented with 10% FBS until semi-confluence in 12-well plates. Cells were then treated with 10 μg/mL of mitomycin-C for 4 h to block proliferation and a scratch was subsequently made using a sterile 200-μl pipette tip to generate a cell-free gap approximately 1 mm in width. Cells were then washed with PBS and photographed to record the wound width at 0 h. Next, one group of cells was cultured in DMEM with 10% FBS as a control. Other groups were treated with 10 μM SU 11274. When we assessed the effects of ligands, one group of cells was cultured in DMEM as a control. Other groups were treated with 20 ng/mL of HGF. After incubation, photographs were taken to evaluate migration.

Western blotting

Cells were washed with phosphate-buffered saline (PBS) and then lysed with RIPA buffer consisting of 150 mM NaCl, 10 mM Tris HCl at pH 8.0, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) sodium dodecyl sulfate (SDS), 5 mM ethylenediaminetetraacetic acid (EDTA), 1 X Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Yokohama, Japan), and 1 X Halt™ Protein Phosphatase Inhibitor (Thermo Fisher Scientific). The protein concentration of the lysates was measured using a BCA™ Protein Assay Kit (Thermo Fisher Scientific) and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred onto PVDF membranes (GE Healthcare, Little Chalfont, UK). Non-specific binding was blocked by incubation in 5% (w/v) bovine serum albumin (BSA) in TBS/Tween-20 (TBS-T) for 1 h at room temperature. Membranes were probed with antibodies in TBS-T overnight at 4°C and then incubated with HRP-conjugated secondary antibody. Antibody-antigen complexes were detected by ECL plus Western blotting detection reagent (GE Healthcare).

Immunohistochemical staining of c-Met

The specimens were fixed in 10% formalin solution, dehydrated in a graded ethanol series, and embedded in paraffin. Sections of 5-μm thick paraffin embedded-tissues were cut and mounted on silane-coated glass slides. The sections were deparaffinized in Hemo-De and rehydrated through a graded ethanol series. Antigens were retrieved in 0.0 l M citrate buffer at pH 7.0 in a water bath at 98°C for 40 min, and endogenous peroxidase was blocked with 3% hydrogen peroxidase. The tissues were then incubated overnight at 4°C with a 1 : 200 dilution of anti-c-Met polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Samples were then incubated with peroxidase dextran polymer (Envision＋; DakoCytomation, Carpinteria, CA, USA) for 30 min at room temperature and visualized by 3,3'-diaminobenzidinetetrahydrochloride (DAB; DakoCytomation). All sections were counterstained using hematoxylin.

Immunofluorescence staining of c-Met

These new 5-μm thick sections were prepared from stored formalin-fixed, paraffin-embedded tissue blocks for hematoxylin-eosin staining and immunofluorescence staining of c-Met (Bioss Antibodies, Woburn, Massachusetts, USA). Immunofluorescence staining was performed using Envision technology. Antigen retrieval was performed by warming the deparaffinized sections in 0.1% sodium azide at pH 6 for 20 minutes, an then in hot water at 95°C for 30 minutes. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 20 minutes and the sections were soaked in 0.05 M Tris-buffered saline (TBS) before being soaked in blocking solution (DakoCytomation) for 20 minutes. They were then rinsed twice at room temperature

for 2 minutes. The tissue sections were next incubated for 60 minutes at room temperature with a rabbit anti-human c-Met polyclonal antibody (1 : 200) labeled with the optimally diluted primary antibody Alexafluor 488 Conjugate. Antigen sites were visualized using the above conjugate and counterstained with Mayer's hematoxylin. These specimens were observed with an LSM 700 laser scanning microscope (Carl Zeiss, Jana, Germany). The immunofluorescence signals were detected using confocal laser scanning microscopy with the same microscope. The immunofluorescence images were processed by the LSM 700 and the areas were calculated. The unpaired Mann-Whitney U test was used to evaluate the specific difference in the protein expression rate of c-Met from the control.

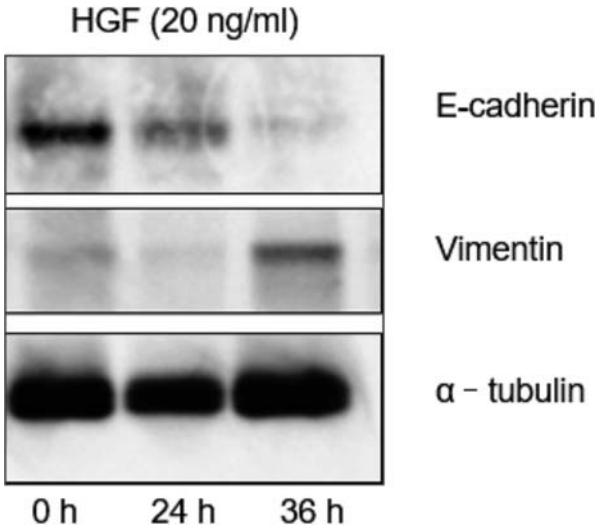

Fig. 1 HGF induced EMT-like changes in HSC3 cells. HGF (20 ng/mL) downregulated Ecadherin and upregulated vimentin in a timedependent manner compared with untreated HSC3 cells (control).

Statistical analysis

All data are represented as the mean and standard deviation from three independent experiments unless stated otherwise. Statistical analysis was performed by one-way analysis of variance (ANOVA), and the significant differences between the treated and control samples ($*p<0.05$, $**p<0.01$, and $***p<0.001$) were evaluated by the unpaired Student's t-test.

RESULTS

HGF induced EMT-like changes in HSC3 cells

Characteristic changes associated with EMT include the reduction of epithelial markers and induction of mesenchymal markers. These changes are associated with the growth of cancer cells, enabling cell-cell dissociation, migration, and motility.[24] In the present study, western blot analysis revealed that HGF treatment downregulated E-cadherin expression and upregulated vimentin expression in the HSC3 cells in a time-dependent manner. E-cadherin expression decreased slightly after 24 hours and almost disappeared after 36 hours. In comparison, although vimentin was not observed after 24 hours, it was clearly seen after 36 hours. Therefore, HSC3 cells acquired EMT-like changes following incubation with HGF (20 ng/mL) for 36 h (Fig. 1). These results suggest that HGF promotes altered expression of EMT markers in HSC3 cells. For this reason, HSC3 cells were treated with 20 ng/mL HGF for 36 h in all subsequent experiments.

c-Met expression was enhanced by HGF treatment

The expression level of the HGF receptor c-Met was measured to investigate the role of HGF in inducing EMT-like changes in HSC3 cells. c-Met is activated by being phosphorylated via HGF and activated c-Met is able to regulate several downstream target genes. Western blot analysis revealed that HGF treatment increased the expression of c-Met and phosphorylated-c-Met (Fig. 2). In particular, the expression of phosphorylated-c-Met was markedly increased.

HGF treatment increased the migration potential of HSC3 cells

We examined the effects of HGF treatment on the migration potential of HSC3 cells. The wound healing assay demonstrated that HGF increased cancer cell migration. HGF-treated HSC3 cells exhibited approximately twice the migration capacity as control HSC3 cells. This suggests that HGF increased

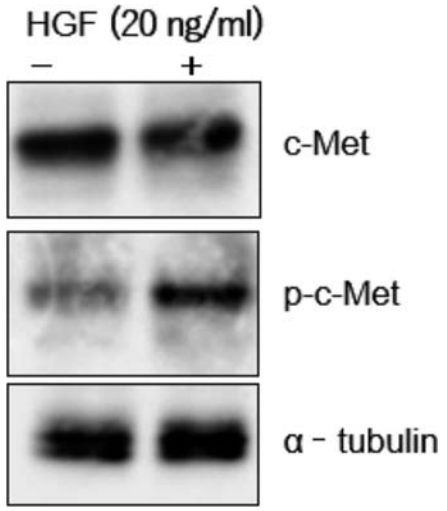

Fig. 2 Increase in c-Met expression by HGF. HSC3 cells were treated with 20 ng/mL HGF, and c-Met expression was measured at the protein level after treatment for 36 h (HGF : Hepatocyte growth factor ; p : Phosphorylated).

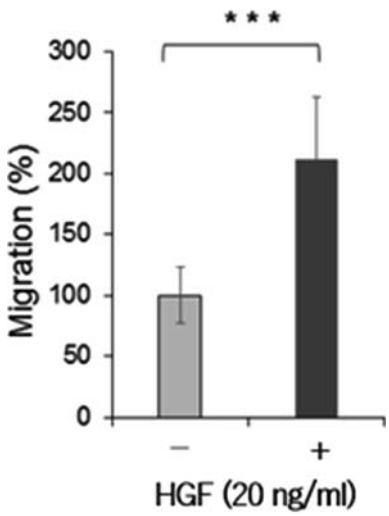

Fig. 3 Increase by HGF in the migration potential of HSC3 cells. HGF-treated cells demonstrated increased migration potential in the woundhealing assay (***$p<0.001$, n=3).

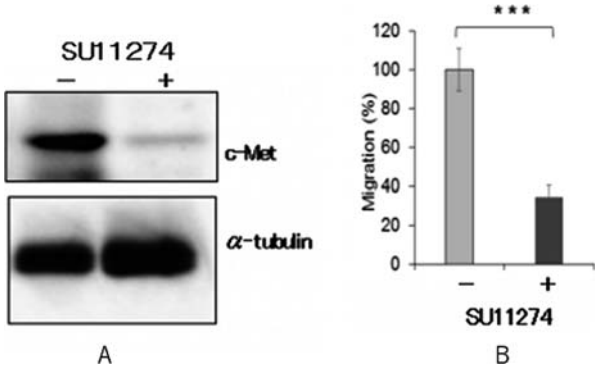

Fig. 4 Suppression of c-Met expression and reduction of the HGF/HSC3 migration ability by the c-Met inhibitor SU 11274. (A) c-Met inhibitor treatment suppressed c-Met expression. (B) The inhibitor reduced the migration ability compared with untreated cells (***$p<0.001$, n=3).

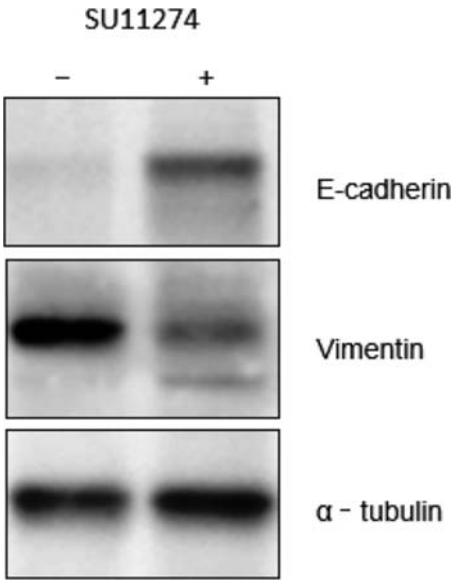

Fig. 5 Inhibition of HGF-induced EMT-like changes by the c -Met inhibitor SU11274. After incubation with HGF, untreated HSC3 cells exhibited the upregulation of E-cadherin and downregulation of vimentin compared with cells treated with SU11274.

the migration capacity of HSC3 that had undergone EMT-like changes (Fig. 3).

c-Met inhibitor SU11274 suppressed c-Met expression and reduced the HGF/HSC3 migration capacity

In order to confirm the involvement of the HGF/c-Met signaling pathway, we examined the effects of c-Met knockdown by SU11274. As shown in Fig. 4 A, the c-Met inhibitor suppressed c-Met expression. Furthermore, as shown in Fig. 4 B, the inhibitor treatment reduced the migration capacity compared with untreated cells. This suggests that HGF caused the EMT in HSC3 cells via the HGF/c-Met signaling pathway.

c-Met inhibitor SU11274 inhibited HGF-induced EMT-like changes

After HGF treatment, cells further treated with SU 11274 showed induced expression of E-cadherin

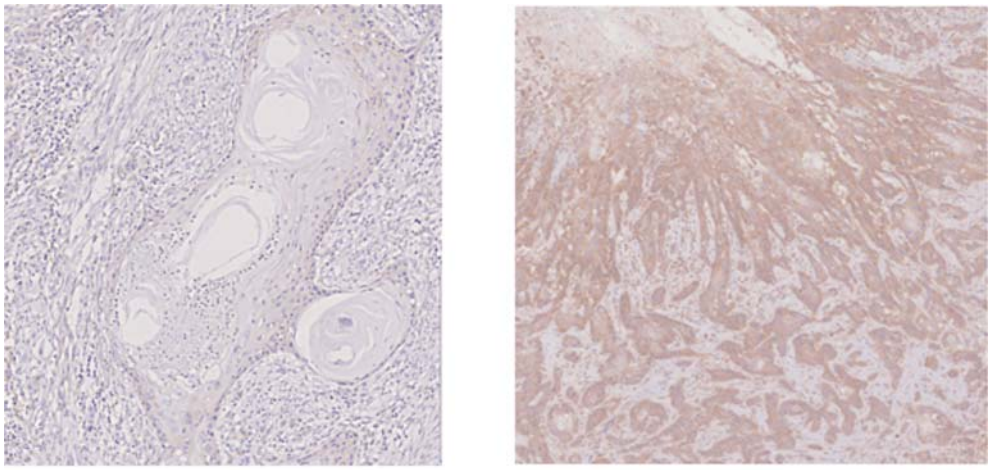

Fig. 6 Immunohistochemical evaluations of c-Met. (A) A well-differentiated OSCC showing c-Met staining in the nuclei of some tumor cells. The peripheral cells of the tumor nests were stained more densely than the central tumor cells of the tumor nests. (Original magnification × 10) (B) Tumor nests in the invasion front demonstrating dense cytoplasmic and nuclear staining in the tumor cells. Some of the fibroblasts, endothelial cells, and inflammatory cells were also positive for c-Met. (Original magnification × 5)

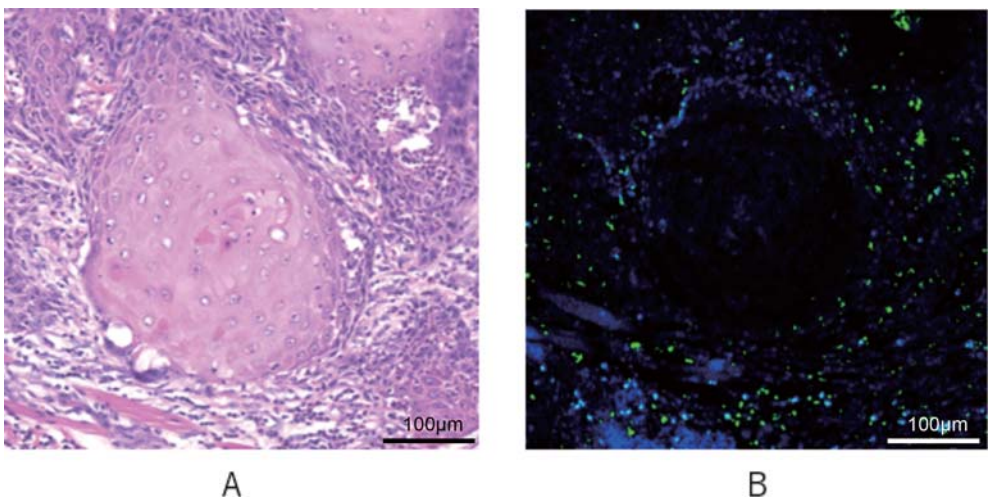

Fig. 7 Immunofluorescence staining of c-Met. c-Met signals were barely observed in well-differentiated tumor cells.

and downregulated expression of vimentin compared with untreated cells (Fig. 5). This suggests that the HGF-induced EMT was suppressed by the c-Met inhibitor and was converted from a mesenchymal to a native epithelial system.

Immunohistochemical evaluations of c-Met

Well-differentiated OSCC exhibits c-Met staining in the nuclei of some tumor cells. The peripheral cells of the tumor nests were stained more densely than the central tumor cells of the tumor nests (Fig. 6 A). In particular, in the invasion front of tumor nests, both the cytoplasm and nucleus had strong staining (Fig. 6 B). Significant differences between well-differentiated and poorly-differentiated SCC were observed. Moreover, the difference between the N (＋) and N(−) cases in c-Met expression was significant (data not shown). These results are similar to those in a previous report. Furthermore, there was a significant difference between N(＋) and N(−)

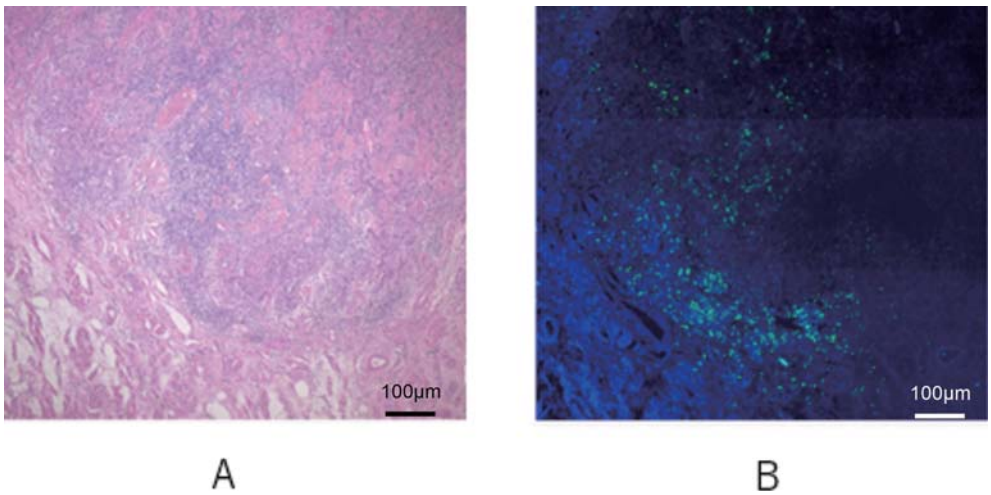

Fig. 8 Immunofluorescence staining of c-Met. Tumor nests at the invasion front demonstrated dense cytoplasmic and nuclear staining.

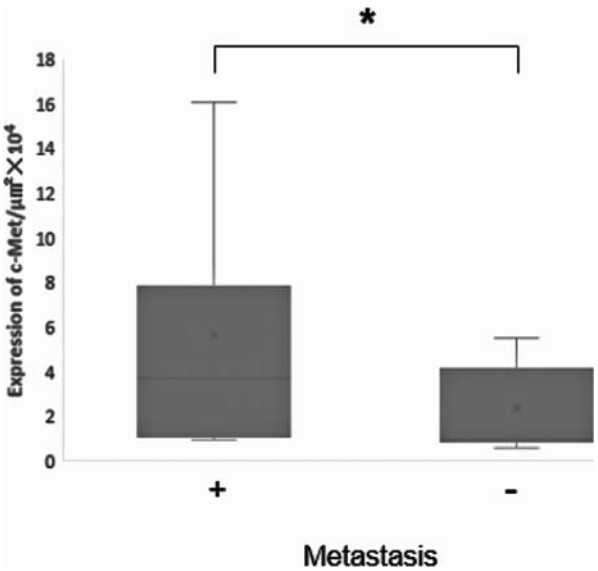

Fig. 9 Significant differences between N(+) and N(−) cases at the primary site shown by brightness analysis (*$p < 0.05$, n = 50).

cases at the primary site, which is a novel finding.

Immunofluorescence staining of c-Met

The results were similar to those of immunohistochemical staining. Positive c-Met signals were barely observed in well-differentiated tumor cells (Figs. 7 A and B). Tumor nests in the invasion front demonstrated dense cytoplasmic and nuclear staining (Figs. 8 A and B). Differences between N(+) and N(−) cases at the primary site were significant (Fig. 9).

DISCUSSION

The process of cell dedifferentiation by EMT is known as one of the important features of cancer.[25, 26] The EMT is a very important process in the early stage of tumor cell metastasis. In order for cancer cells to metastasize, they must first avoid anoikis. E-cadherin is typically present in epithelial cells and its decreased expression is an important feature of the EMT. In mesenchymal cells, these epithelial proteins are altered by mesenchymal-specific factors, including fibronectin, vimentin, and neural cadherin (N-cadherin).[27] The changes in the expression pattern of E-cadherin/N-cadherin are correlated with the avoidance of anoikis and an increase in cell invasiveness.[28] Furthermore, dysregulation of growth factor receptors may result in resistance to anoikis. Before cancer cells begin to migrate and metastasize, they must activate the genes that are necessary for cell differentiation, proliferation, and activation of anti-apoptotic pathways. Furthermore, alteration of cellular characteristics from the epithelial to the mesenchymal phenotype reduces the activity of adhesion molecules and proteases on the cell surface, which digest the components of the ECM.[29]

A number of factors that induce EMT have been identified, including transforming growth factor-β (TGF-β), HGF, epidermal growth factor (EGF), fi-

broblast growth factor (FGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), microRNA, hypoxia, and transcription factors.[30-32] Among these factors, EMT induction by the HGF/c-Met pathway is essential in certain types of cancer.[33-36] However, to the best of our knowledge, the detailed role of c-Met in the progression of oral cancer has not been reported. In this study, treatment of HSC3 cells with HGF induced EMT-like changes, as indicated by the downregulation of E-cadherin and upregulation of vimentin. Furthermore, HGF increased the migration potential of HSC3 cells.

c-Met overexpression has been identified in the majority of human cancers.[37, 38] In this study, c-Met expression was promoted by HGF-dependent transcriptional upregulation. In particular, there was a marked upregulation in phosphorylated-c-Met following HGF treatment. Therefore, HGF also activates c-Met in oral cancer cells. A similar study suggested that c-Met is activated via an HGF-independent signaling pathway, following transfection, in lung cancer.[39] In addition, c-Met and HGF may be transactivated by the mutant of epidermal growth factor receptor (EGFR), also termed EGFRvIII.[40] Furthermore, recent studies demonstrated that c-Met activation occurs in the absence of ligand binding. Integrin activation, plexins, CD 44, certain G protein-coupled receptors, and other receptor tyrosine kinases have all been implicated in c-Met activation without the requirement of HGF binding.[41] In addition, the activation of c-Met was found to activate the downstream signaling pathways PI 3K and ERK, which resulted in the downregulation of E-cadherin and upregulation of vimentin. E-cadherin downregulation is regarded as a characteristic change of the EMT.[42] As intercellular adhesions are essential for the maintenance of the epithelial phenotype, the downregulation of E-cadherin results in abnormal differentiation and the loss of cell polarity, which ultimately facilitates EMT.[43] In this study, the knockdown of c-Met by SU 11274 prevented HGF-induced EMT-like changes. This suggests that HGF induced the EMT in a c-Met-dependent manner in HSC3 cells.

Abnormal c-Met activation may occur in certain cancer types due to gene amplification, mutation, or transactivation.[15] However, c-Met overexpression as a result of upregulation at the transcriptional level is predominant in the majority of human malignancies.[44] The present study suggested the overexpression of c-Met signaling and the subsequent induction of EMT is a common phenomena in oral cancer cells. Furthermore, the EMT increased prostate cancer cell invasiveness *in vitro* and *in vivo.* Taken together, this study demonstrated the potential association among c-Met, EMT, and invasiveness in oral cancer. Similarly, in other solid human tumors, previous studies have revealed that c-Met-mediated signaling activation drives EMT, cancer cell migration, and metastasis.[45-47]

In this study, we also examined clinical samples. c-Met signals were almost never observed in well-differentiated tumor cells, and only the outer layer nests exhibited positive reactions. Moreover, relatively strong c-Met signals were observed in the infiltrating tumor tissue, the so-called invasion front. Tabuchi *et al.*[48] reported a significant correlation between c-Met and N(＋) in colon cancers. Regarding c-Met expression in OSCC, Endo *et al.*[49] and Chen *et al.*[50] reported the high expression of c-Met in cases of N(＋), and progressive T3, T4, stage lll, and stage IV cases. They also found a significant difference in c-Met expression among these cases. However, Muzio *et al.*[51] described a significant increase in c-MET expression in tumors larger than 1.5 cm, although the difference among clinical stages was not significant. In our study, expression was observed at the invasion front where invasion growth occurs. This was similar to the results of Chan *et al.*[50] This part of the tumor was thought to comprise metastatic cells undergoing EMT.

In conclusion, this study suggested that HGF directly promotes EMT and carcinogenic properties in oral cancer via the c-Met signaling pathway. Specific molecular targeting of this signaling pathway may be therapeutically beneficial for oral cancer patients. As it is important to regulate cell invasion, migration, and metastasis in clinical cancer treatment, more detailed analyses of the signaling path-

way are needed.

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