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HGF/c-Met induces cell migration of oral squamous cell carcinoma via lamellipodin

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Cell migration potency is often regulated by extracellular stimuli. We previously reported that the migration potency of the oral squamous cell carcinoma (OSCC) cell lines SAS and HSC3 have high epidermal growth factor receptor (EGFR) inhibitor sensitivity, while the migration of HSC4 is not affected. Hepatocyte growth factor (HGF)/c-Met is known as one of the signaling pathways for cell migration in some cancer cells. In this study, we demonstrated the importance of the c-Met signaling pathway for filopodia and lamellipodia formation in cell migration using HSC4. The cell migration of HSC4 used the HGF/c-Met pathway. Additionally, the EGF/EGFR pathway is not related to lamellipodia formation. The HGF/c-Met pathway is important for the formation of filopodia and lamellipodia in HSC4. Furthermore, signaling from c-Met increases the amount of lamellipodin protein, which is necessary for formation of lamellipodia. Our results suggested that signaling from the HGF/c-Met pathway increases the quantity of lamellipodin protein, and induces cell migratory capacity by inducing lamellipodia forming potency in HSC4. (J Osaka Dent Univ 2017 ; 51 : 1-8)

Key words : c-Met ; HGF ; AG1478 ; SU11274 ; Lamellipodin ; Cell migration

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the most common cancer in the head and neck region. The overall five-year survival rate of HNSCC, including oral squamous cell carcinoma (OSCC), has not improved markedly in recent years. OSCC has high local invasiveness,[1, 2] its metastases correlate with clinical stage,[3] and it has a poor prognosis. During tumor invasion, cell migration and the interaction between the tumor and stroma are necessary at the invasive front, and stimulation from growth factors and the extracellular matrix are important.[4-8]

Cell migration potency, which plays an important role in the metastasis of tumors, is driven by the epidermal growth factor receptor (EGFR) pathway in HNSCC. Furthermore, because overexpression is found in approximately 90% of HNSCC, a high expression of EGFR correlates with the clinical stage, and is a factor in poor prognosis.[9, 10] Previously, we examined the influence of the EGFR pathway on the growth and migration of the OSCC cell lines HSC4, SAS and HSC3. Although the growth of HSC4 and HSC3 were sensitive to the EGFR inhibitor, the growth of SAS was not. On the other hand, while the migration potency of SAS and HSC3 are sensitive to EGFR inhibitor, and migration of HSC4 is not affected.[11] Our results suggested that the EGFR pathway is used for proliferation and regulation of cell migration in a cell-type dependent manner.

The c-Met pathway has been reported to be important in some forms of lung and breast cancer.[12, 13] c-Met, along with EGFR, is a receptor tyrosine kinase (RTK). It is expressed in a variety of cells including epithelial cells and vascular endothelial cells. Hepatocyte growth factor (HGF, scatter factor) is a ligand of c-Met, and is made from stromal and some of tumor cells.[14, 15] When c-Met binds

to HGF, it is activated and induces cell proliferation, cell migration, invasion, metastasis, angiogenesis, wound healing, and tissue regeneration through several signaling pathways. Therefore, the c-Met pathway, which can promote metastasis, is thought to be important for the regulation of some cancers, including breast cancer.[14]

When cells migrate, the formation of lamellipodia and filopodia by the remodeling of the actin cytoskeleton is observed at the leading edge of the moving side.[13, 16] The activation of the Rho family members, Rac 1 and Cdc 42, by the PI3K/Akt or MEK/ERK pathway promotes the formation of lamellipodia or filopodia in prostate and breast cancer.[17-19] Further, lamellipodin, which is a protein necessary for formation of lamellipodia, and the interaction between the Ena/vasodilator-stimulated phosphoprotein (VASP) and the Scar/WAVE complex have been reported to be important in cell migration.[20] However, although Wnt signaling has been reported to promote formation of pseudopodia by stimulation of Cdc 42 and RhoA in OSCC,[21] other protein level and signal pathways necessary for the formation of pseudopodia have not yet been clarified. We examined the importance of the c-Met signaling pathway through filopodia and lamellipodia formation for cell migration in the HSC4 cell line.

MATERIALS AND METHODS

Cell culture and reagents

The OSCC cell line HSC4 was purchased from RIKEN Bioresource Center (Ibaraki, Japan). The 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-lamellipodin (Cell Signaling Technology Japan, Tokyo, Japan), anti-cortactin (Santa Cruz Biotechnology, Dallas, USA), and anti-α-tubulin (Sigma-Aldrich, Tokyo, Japan). SU11274 was obtained from Sigma-Aldrich, and AG1478 was from Calbiochem® (Merk Millipore, Tokyo, Japan).

Cell proliferation assay

Proliferation of AG1478 transfected cells after incubation for 6 days in 96-well plates was assessed using the MTT assay (CellTitler 96® Non-Radioactive Cell Proliferation Assay ; Promega, Tokyo, Japan) according to the manufacturer's instructions. The absorbance at 570 nm was measured using a 96-well plate reader (Labsystems Multiskan MS-UV ; Labsystems, Helsinki, Finland).

Wound healing assay

Cell migration was also assessed using an *in vitro* scratch wound healing assay. HSC4 was cultured with DMEM supplemented with 10% FBS until semi-confluence in 12-well plates. The cells were then treated with 10 μg/mL of Mitomycin-C for 4 h to block proliferation and subsequently a scratch was 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 of AG1478 and SU11274. When we determined the effect of ligands, one group of cells was cultured in DMEM as a control. Other groups were treated with 10 ng/mL of EGF, and 20 ng/mL of HGF. After the incubations, 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) Nonidate 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 determined 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).

Immunofluorescence staining

Cultured cells were fixed in 3.5% (w/v) formaldehyde, permeabilized in 0.2% (v/v) Triton X-100, and blocked in 2% (w/v) bovine serum albumin (BSA). The primary antibodies were incubated at 4°C overnight. Alexa Fluor® 594-conjugated IgG (Thermo Fisher Scientific) was used as the secondary antibody. After incubation with the antibodies, cells were incubated in 100 nM of Acti-stain™ 488 phalloidin (Cytoskeleton, Denver, USA). The specimens were observed using fluorescence microscopy.

Statistical analysis

All data are represented as mean and standard deviation from three independent experiments unless stated otherwise. The unpaired Student's t test was used to assess the difference between the treated and control samples. Values of $p<0.05$, $p<0.01$ and $p<0.001$ were considered significant.

RESULTS

Migration of HSC4 cells induced by serum or HGF/c-Met signaling

Previously, we showed that HSC4 uses the EGFR pathway for physiological reaction of cells, and that EGFR is phosphorylated in the OSCC cell line HSC4.[22] Therefore, we investigated the effect of the EGFR pathway for cell proliferation and migration. First, we investigated the effect of AG1478 treatment for the proliferation activity of HSC4 by MTT assay. We observed that the cells treated with AG 1478 had a significantly decreased proliferation rate compared with the control cells (Fig. 1 A). Next, to investigate the effect of the EGFR pathway on cell migration, we performed the wound healing assay by treating HSC4 with the addition of AG1478 and EGF. We found that the treatments with AG1478 (Fig. 1 B) and EGF (Fig. 1 C) did not affect the cell migration of HSC4 compared with control cells, respectively. These results suggest that HSC4 used the EGF/EGFR pathway for cell proliferation, but not for cell migration.

Further, we investigated how the serum components related to the migration activity of HSC4. Ad-

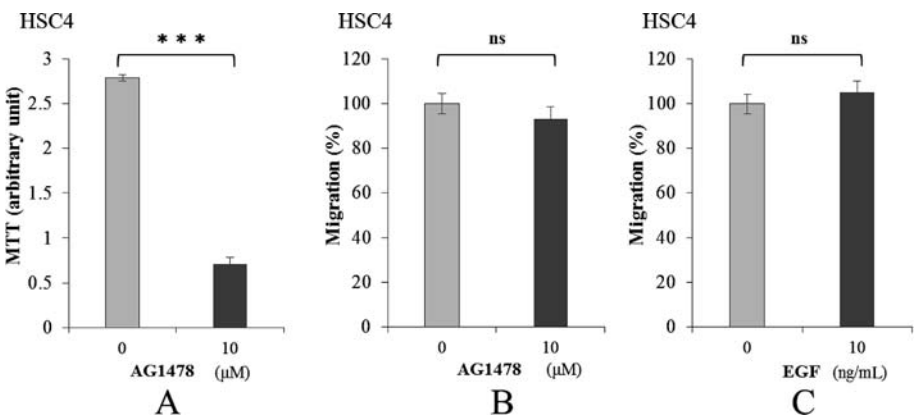

Fig. 1 HSC4 utilization of the EGF/EGFR pathway for cell proliferation, but not for cell migration.
(A) This shows the effect of AG1478 on the growth of HSC4, measured using the MTT assay. (B and C) This shows the wound healing assays in HSC4 treated with AG1478 and EGF, respectively. The width of the scratches were measured at 0 h and after 12 h of culture using ImageJ software. The relative distance was calculated as the mean width of the cell scratch. The effect of AG1478 and EGF treatment on cell migration were investigated by comparing the width of the treated and non-treated cells. The non-treated width set at 100%. Error bars indicate the mean and standard deviation using Student's t test (***$p<0.001$, $n=3$, ns : Not significant).

dition of serum significantly induced migration activity compared with the serum-free medium (Fig. 2 A). These results showed that the migration of HSC4 is important to serum components other than EGF. We then focused on c-Met which plays a role as a tyrosine kinase receptor for cell migration along with EGFR.[23, 24] Therefore, we investigated the effect on cell migration potency of c-Met signaling using wound healing assay in HSC4. Treatment with 10 μM SU11274, which is a c-Met inhibitor, significantly reduced the migratory potency in HSC4 (Fig. 2 B). Further, addition of 20 ng/mL of HGF to the serum-free medium significantly induced migration activity of HSC4 (Fig. 2 C). These results suggest that the cell migration of HSC4 used the HGF/c-Met pathway rather than the EGF/EGFR pathway.

Filopodia and lamellipodia formation are not induced by the EGF/EGFR pathway in HSC4 cells

Cells promote actin polymerization in the direction they move, and migrate by extruding the cell membrane from the inside. At this time, filopodia and lamellipodia are formed on the leading edge of the

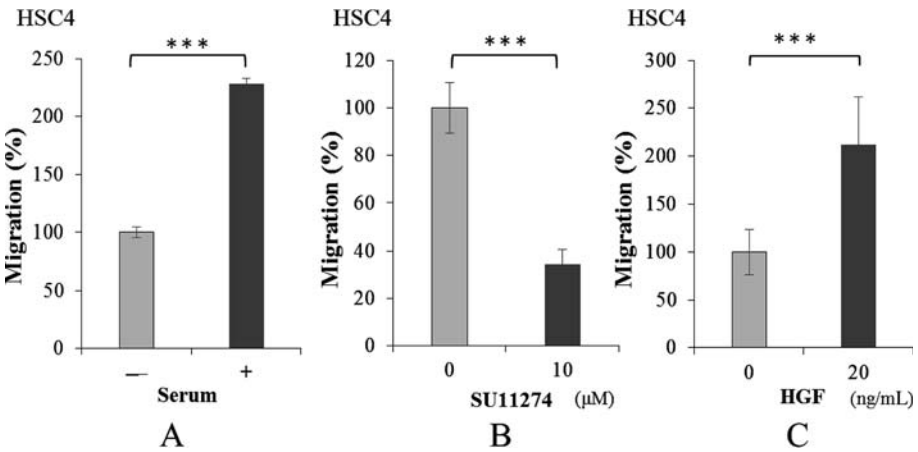

Fig. 2 Migration potency of HSC4 cells induced by activation of c-Met via serum or HGF. The width of the scratches were measured at 0 h and after 12 h of culture using ImageJ software. The relative distance was calculated as the mean width of the cell scratch. The effect of serum, SU11274 and HGF treatment on cell migration was investigated by comparing the width of treated and non-treated cells. The non-treated width set at 100%. The error bars indicate mean and standard deviation with the Student's t test (*p＜0.05, **p＜0.01, ***p＜0.001, n＝3).

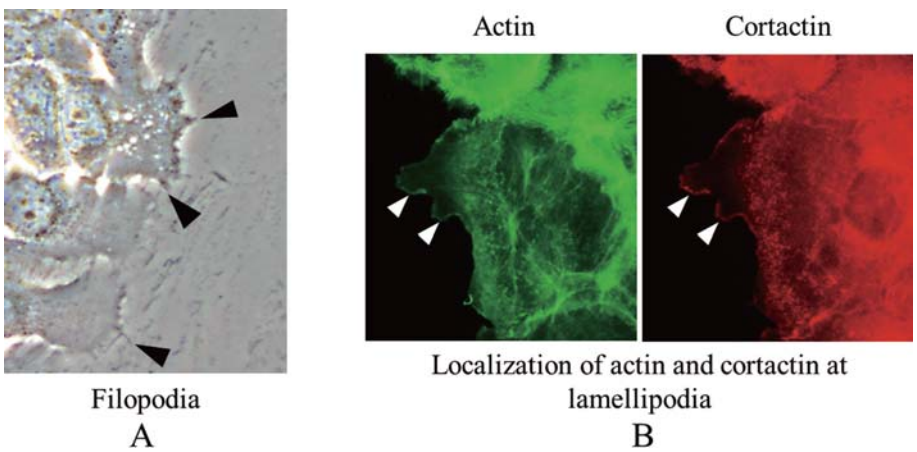

Fig. 3 Typical images of filopodia and lamellipodia.
(A) Arrow heads indicate filopodia which are thin cytoplasmic projections that extend beyond the leading edge in migrating cells. (B) It is known that actin and cortactin co-localize at lamellipodia. These images show immunofluorescence staining of actin and cortactin in HSC4 cells. Arrow heads indicate lamellipodia.

cells.[13, 16] To confirm the formation of lamellipodia in HSC4 cells, we used an index that determines the formation of lamellipodia as co-localization of actin and cortactin by immunocytochemistry.[25] Filopodia are spindle-like cell processes that extend from the leading edge of migrating cells (Fig. 3 A) and lamellipodia are sheet-like membrane processes found at the leading edge of motile cells (Fig. 3 B). We then examined the relationship of the formation of filopodia and lamellipodia with the EGFR pathway in HSC4.

At first, we investigated the effect of filopodia formation in HSC4 cells facing a scratch wound by treatment with the EGFR inhibitor AG1478. After 12 hours of AG1478 treatment, the formation of filopodia was observed in all cells, including nontreated control cells (Fig. 4 A). These results showed that the EGFR pathway does not affect the formation of filopodia in HSC4.

Next, we examined the effects of filopodia formation on EGF stimulation. EGF is one of the ligands of EGFR. Formation of filopodia were observed in

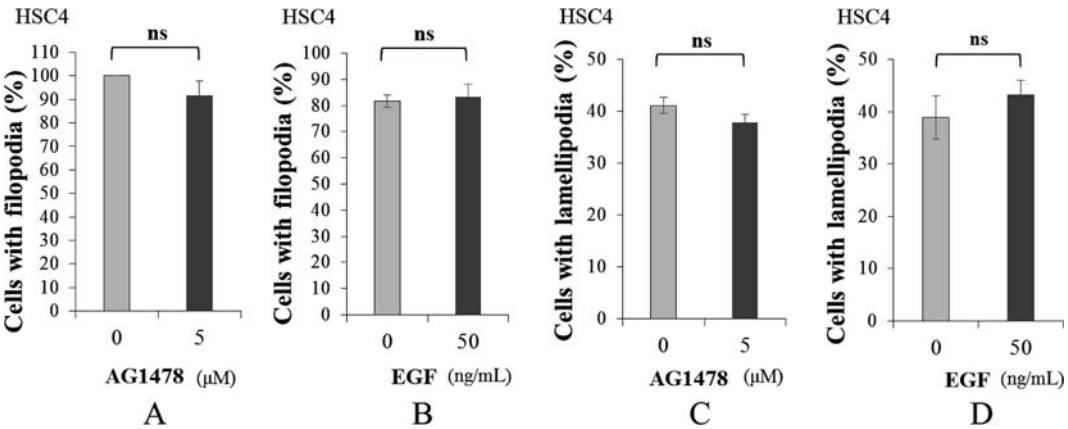

Fig. 4 Filopodia and lamellipodia formation are not induced by EGF/EGFR signaling in OSCC HSC4 cells. AG1478 (A) and EGF (B) did not inhibit filopodia formation in HSC4 cells. AG1478 (C) and EGF (D) did not inhibit lamellipodia formation in HSC4 cells. (A-D) HSC4 cells were grown on 96-well plates overnight, then treated and subjected to scratch wound healing assay. The percentage of cells forming filopodia was determined by visual assessment of confocal images from the center fields in each well. Statistical analysis is explained in Materials and Methods (n＝3).

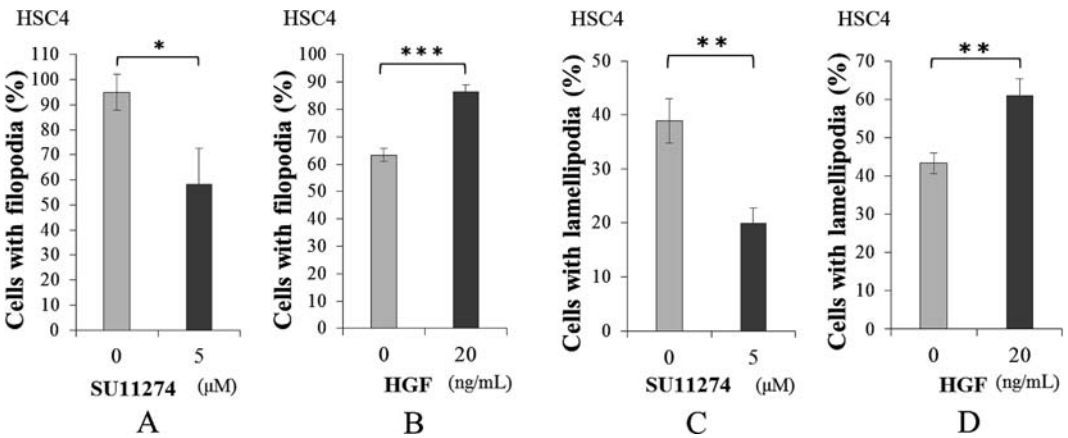

Fig. 5 Formation of filopodia and lamellipodia is induced by HGF/c-Met signaling in HSC4 cells. The c-Met inhibitor SU11274 inhibited filopodia (A) and lamellipodia (C) formation in HSC4 cells, while HGF promoted filopodia (B) and lamellipodia (D) formation in HSC4 cells. The percentage of cells forming filopodia and lamellipodia was determined by visual assessment of confocal images from center fields in each well. Statistical analysis is shown in Materials and Methods (*p＜0.05, **p＜0.01, ***p＜0.001, n＝3).

most of the cells with or without serum after 12 hours (Fig. 4 B). Namely, we found that there is no influence of EGF-stimulation on the formation of filopodia in HSC4.

In addition, the effect of lamellipodia formation by AG1478 treatment could not be observed in HSC4 (Fig. 4 C). The 50 ng/mL EGF stimulation also did not increase the number of cells with lamellipodia compared with serum-free medium after 12 hours in HSC4 (Fig. 4 D). These results suggest that the EGF-EGFR pathway is also not related to lamellipodia formation in HSC4.

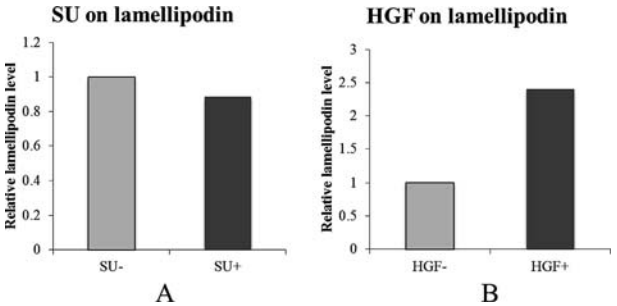

Fig. 6 Lamellipodin is regulated by the HGF/c-Met pathway in HSC4 cells. Cells were treated with SU11274 and HGF, and the level of lamellipodin was determined by immunoblotting for lamellipodin. α-Tubulin was used as a loading control.

Filopodia and lamellipodia formation are induced by the HGF/c-Met pathway in HSC4 cells

We evaluated the effect on filopodia formation and lamellipodia formation of c-Met which is related to the migration potency of HSC4. The cell ratio of filopodia was significantly reduced by SU11274 compared with non-treatment after 12 hours (Fig. 5 A). Since it has been shown that formation of filopodia is decreased by inhibition of the c-Met pathway, we examined the effects of HGF, which is the ligand of c-Met. After 12 hours of HGF treatment, the proportion of filopodia cells were increased compared with the serum-free medium (Fig. 5 B).

In addition, the effect of SU11274 treatment on the proportion of cells that formed lamellipodia was significantly reduced after 12 hours treatment as compared with the non-treatment cells (Fig. 5 C). Further, the effect of HGF stimulation on the formation of lamellipodia was significantly increased compared with the non-treatment cells after 12 hours (Fig. 5 D). These results suggest that the HGF/c-Met pathway is important for the formation of filopodia and lamellipodia of HSC4. Furthermore, these results are in agreement with the results of HSC4 cell migration, and suggest that there is a correlation between cell migration and the formation of filopodia and lamellipodia.

Lamellipodin regulation in HSC4 cells by the HGF/c-Met pathway, but not by the EGF/EGFR pathway

In order to examine whether the HGF/c-Met pathway is related to the formation of lamellipodia, we focused on the amount of lamellipodin protein known to be related to the formation of lamellipodia which localized at the leading edge with Ena/VASP proteins.[26] At first, we confirmed that the amount of lamellipodin protein in HSC4 was decreased by SU 11274 treatment (Fig. 6 A). We also found that addition of HGF increased the amount of lamellipodin protein (Fig. 6 B). These results suggest that the HGF/c-Met pathway regulates the formation of lamellipodia by regulating the amount of lamellipodin, resulting in cell migration in HSC4.

DISCUSSION

The mechanism of EGFR activation has been described as ligand bindings inducing the dimerization and activation of the cytoplasmic kinase domains, and regulating cell growth and migration. Until now, it has been reported that the pathway through EGFR is important for cell migration of cancer cells.[27, 28] We previously reported that an OSCC cell line, SAS, needs an EGFR pathway for cell migration.[22] However, cell migration of HSC4 was not affected by the EGFR pathway because it was under the influence of the c-Met pathway (Fig. 1).

It has been reported that activation of c-Met by stimulation of HGF promotes not only angiogenesis and tumorigenesis, but also cell migration in some normal tissues and cancer cell lines.[22, 29-32] In this report, the migration potency of the OSCC cell line HSC4 was promoted by HGF stimulation and was decreased by treatment with SU11274 (Fig. 2).

These results suggest that the HGF/c-Met pathway also has an important role for OSCC cell migration. Therefore, targeting the HGF/c-Met pathway is necessary in order to inhibit cancer cell migration.

In this study we showed that the HGF/c-Met pathway is effective not only on the migration of the OSCC cell line, but is also related to the formation of lamellipodia and filopodia (Figs. 2, 4 and 5). It has previously been reported that HGF/c-Met signaling promotes the formation of lamellipodia and consequently enhances cell migration in vascular endothelial cells.[12] Therefore, c-Met signaling also induces cell migration through promotion of the formation of lamellipodia and filopodia in OSCC.

Lamellipodin is a protein necessary for the formation of lamellipodia.[16] However, although the protein of lamellipodin, which interacts with the Ena/VASP and Scar/WAVE complex, has been reported to be important for cell migration,[20] the signal that regulates its protein is not known.

In this report, we showed that the protein level of lamellipodin is upregulated by HGF in HSC4 (Fig. 6). It has also been shown that upregulation of lamellipodin protein levels dramatically induces cell migration. Because it is important to regulate cell migration in clinical cancer treatment, it is necessary to make a more detailed analysis of the signaling pathway.

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