Journal of Oral Science, Vol. 54, No. 3, 241-250, 2012

Original

Podoplanin promotes cell migration via the EGF-Src-Cas pathway in oral squamous cell carcinoma cell lines

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(Received 8 June and accepted 10 July 2012)

Abstract: Human podoplanin is a type-1 transmembrane sialomucin-like glycoprotein that is involved in cell migration, tumor cell invasion and metastasis. Our recent study of oral squamous cell carcinoma (OSCC) demonstrated that the degree of immunohistochemical expression of podoplanin was correlated with the severity of epithelial dysplasia and significantly associated with a poor pathologic grade of differentiation. Furthermore, it has been reported that Src directly associates with the epidermal growth factor receptor (EGFR) in OSCC cells upon stimulation with EGF and phosphorylates Crk-associated substrate (Cas), podoplanin acting downstream of Src and Cas to promote cell migration. However, the molecular function of podoplanin remains unclear. In this study we performed real-time RT-PCR, Western blotting and scratch assay using OSCC cell lines in order to clarify the molecular biological function of podoplanin expression associated with various growth factors including EGF and with the Src-Cas signaling pathway. Podoplanin was found to have a marked influence on cancer cell migration and the expression of matrix metalloprotease-9 (MMP-9) in the oral cavity upon stimulation with EGF. Podoplanin promotes oral cancer cell migration, and the EGF-Src-Cas pathway is one of the possible mechanisms responsible for progression of cancer in the oral cavity. (J Oral Sci 54, 241-250, 2012)

Keywords: oral squamous cell carcinoma; podoplanin; EGF; MMPs; EGF-Src-Cas pathway.

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Introduction

Human podoplanin is a type I transmembrane sialomucin-like glycoprotein consisting of 162 amino acids (1). The protein was originally detected as a 38-kDa mucoprotein on the surface of podocytes in rats with puromycin-induced nephrosis, and was linked to the flattening of foot processes (2). Podoplanin may play a role in cellular contractility and cytoskeletal reorganization.

Several studies have suggested that overexpression of podoplanin is associated with a poor clinical outcome in oral squamous cell carcinoma (OSCC) (3-5). Our previous study of OSCC also showed that the intensity

of immunohistochemical expression of podoplanin was correlated with the severity of epithelial dysplasia (P = 0.016) and significantly associated with a poor pathologic grade of differentiation (P = 0.020). These findings suggested that podoplanin is associated with tumor development via the oral dysplasia-carcinoma sequence (6).

A developmental regulatory program, referred to as the "epithelial-mesenchymal transition" (EMT), has become prominently implicated in the ability of transformed epithelial cells to disseminate and acquire invasiveness and resistance to apoptosis (7-9). Two previous studies of the association of EMT with podoplanin expression have produced contrasting findings. One suggested that podoplanin plays a role in tumor invasion by binding to proteins such as ezrin, radixin and moesin to activate RhoA, thereby remodeling the actin cytoskeleton of tumor cells and contributing to their increased motility (10). The other study suggested that podoplanin shifts the pattern of invasion from that of single cells involving EMT to that involving large cell sheets in the absence of EMT (11). In our previous study we found that 62% of podoplanin-positive primary OSCCs with pathological node metastasis (pN^+) showed EMT, whereas the remainder lacked EMT. These findings suggest that podoplanin expression in tumor cells does not interfere with the occurrence of EMT in OSCCs (6).

Src belongs to a family of 11 non-receptor tyrosine kinases known as the Src family kinases (SFKs) (12). SFKs play critical roles in a variety of cellular signal transduction pathways, regulating diverse processes including cell division, motility, adhesion, angiogenesis, and survival (13). SFKs are capable of inducing malignant transformation in a variety of cell types, and are frequently overexpressed in various kinds of epithelial and non-epithelial malignant neoplasms. The extent of increased Src family activity often correlates with malignant potential. Src acts as a signal transducer from cell surface receptors through sequential phosphorylation of tyrosine residues on substrates (14). Binding to activator molecules such as receptor kinase adaptors including EGF, platelet-derived growth factor (PDGF), fibrous growth factor-basic (bFGF), and hepatocyte growth factor (HGF), or the activated receptor itself, or dephosphorylation of the C-terminal tyrosine by certain tyrosine phosphatases, converts SFKs to functionally active forms, resulting in phosphorylation of downstream effectors and leading to cellular responses (15).

p130Cas is a 130-kDa scaffolding protein that is recruited to focal adhesions upon ligation to integrin (16). The protein was so named because of its association with, and phosphorylation by, Crk-associated substrate (17). p130Cas contains an SH3 domain, which regulates localization to the focal contact through interaction with focal adhesion kinase (FAK). The poly-proline-rich regions of p130Cas, located between the substrate domain and the C-terminal, mediate interaction with SFKs (18). In addition, in CHO cells, p130Cas acts downstream of FAK to promote cell migration (19), and in human pancreatic carcinoma cells interaction between p130Cas and Crk activates Rac, which is necessary for cell migration. Therefore, the FAK/p130Cas interaction has been considered a "molecular switch" for cell migration (20). With regard to the association between the Src-Cas signaling pathway and podoplanin expression, Yongquan et al. suggested that podoplanin acts downstream of Src and Cas, and that Src utilizes Cas to induce podoplanin expression, thus promoting tumor cell migration and leading to cancer invasion and metastasis (21).

The aim of the present study was to clarify the molecular biological role of podoplanin in tumor cell migration and invasion upon stimulation with cancer microenvironmental growth factors such as bFGF, transforming growth factor-β1 (TGF-β1), HGF, PDGF, and EGF in OSCC cell lines, and to examine subsequent Src-Cas signaling activity.

Materials and Methods

Cell lines and cell culture

The human oral squamous cell carcinoma-derived cell lines Ca9-22, HSC-2, -3, and -4 were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Each cell line was routinely grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/mL penicillin-streptomycin (GIBCO Invitrogen, Carlsbad, CA, USA), 10 U/mL fungizone (GIBCO Invitrogen), and 10% fetal bovine serum (GIBCO Invitrogen) in a humidified atmosphere of 5% CO_2 at 37°C.

Real-time quantitative RT-PCR

Total RNAs were extracted using a Protein and RNA Extraction Kit for mammalian cells (PARExt; Takara, Tokyo, Japan) in accordance with the manufacturer's instructions after each cell line had been treated with 100 ng/mL bFGF, TGF-b1, HGF, PDGF, or EGF for 24 h or 48 h. Real-time RT-PCR was performed using a Thermal Cycler Dice Real Time System (Takara) in accordance with the manufacturer's instructions. A one-step SYBR primeScript RT-PCR Kit II (Takara) was used for the reaction. The primers, based on sequences for podoplanin, MMP-2, -7, -9 and glyceraldehyde 3

Table 1 Base sequences of the primers

Product name	Sequences (5'→3')	
Podoplanin	Forward	TGACTCCAGGAACCAGCGAAG
	Reverse	GCGAATGCCTGTTACACTGTTGA
MMP-2	Forward	TACTGGATCTACTCAGCCAGCA
	Reverse	CTTCAGGTAATAGGCACCCTTG
MMP-7	Forward	GAGTGCCAGATGTTGCAGAA
	Reverse	AAATGCAGGGGGATCTCTTT
MMP-9	Forward	CCGAGCTGACTCGACGGTGATGG
	Reverse	GAGGTGCCGGATGCCATTCACGTC
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA

Table 2 Primary antibodies used in the study

Antibodies	Dilution	Source
β-actin	1:200	Santa Cruz Biotechnology, CA, USA
GAPDH	1:500	Santa Cruz Biotechnology, CA, USA
podoplanin	1:50	Santa Cruz Biotechnology, CA, USA
p130Cas	1:100	Santa Cruz Biotechnology, CA, USA
Src Rabbit mAb	1:1000	Cell Signaling Technology, Denvers, USA
Non-phospho-Src (Ty527) Antibody	1:1000	Cell Signaling Technology, Denvers, USA
Phospho-Src (Ty527) Antibody	1:1000	Cell Signaling Technology, Denvers, USA

dehydrogenase (GAPDH), as an internal normalization control, are shown in Table 1. Each PCR mixture (final reaction volume, 20 μL) contained 10.0 μL of One Step SYBR RT-PCR Buffer 4, 0.8 μL of PrimeScript 1step Enzyme Mix 2, 0.8 μL of forward primer (100 pmol/μL), 0.8 μL of reverse primer (100 pmol/μL), and 7.6 μL of the RNA and sterile water. The PCR conditions were: 42°C / 5 min; 95°C / 10 s; 45 cycles of 95°C / 5 s, 60°C / 30 s. Dissociation was performed according to a melting program. Briefly, the RQ value for each sample was based on duplicate determination and compared to with the average value for GAPDH.

siRNA transfection

siRNAs for podoplanin (Santa Cruz Biotechnology, CA, USA), c-Src siRNA (Santa Cruz Biotechnology) and p130Cas (Santa Cruz Biotechnology) were diluted 1:1 with FuGENE transfection reagent (Roche Diagnostics, Mannheim, Germany), and mixed with RPMI1640 medium.

PP2, an inhibitor of Src

PP2 was purchased from Enzo Life Science Ltd. (Exeter, UK). A stock solution of this compound was prepared in DMSO (25 mg/mL, Sigma-Aldrich) and stored at -80°C.

Western blotting

Cellular proteins were extracted using PAREx (Takara) after treatment with 100 ng/mL bFGF, TGF-b1, HGF, PDGF and EGF for 24 h and 48 h. For inhibition assay, the mixture of podoplanin siRNA (10 nM, for 24 h), c-src siRNA (50 nM and 100 nM, for 24 h) and p130Cas siRNA (50 nM and 100 nM, for 24 h) with PP2-DMSO solution (20 μM and 40 μM, for 15 min) was added, followed by treatment with 100 ng/mL EGF for 24 h and 48 h. Cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatants were recovered for determination of protein content. Aliquots of proteins (30 μg/well) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), which were blocked in 1% (*w/v*) bovine serum albumin (BSA). Specific proteins on the membranes were detected by incubation with a specific primary antibody overnight at 4ºC, followed by a species-specific secondary antibody such as anti-mouse or anti-rabbit conjugated with peroxidase and 3,3'-diaminobenzidine tetrahydrochloride (DAB)/H_2O_2 solution for 5-10 min. Primary antibodies used in this study are shown in Table 2.

Cell migration assay (scratch assay)

Cell lines were seeded in 12-well tissue culture slides

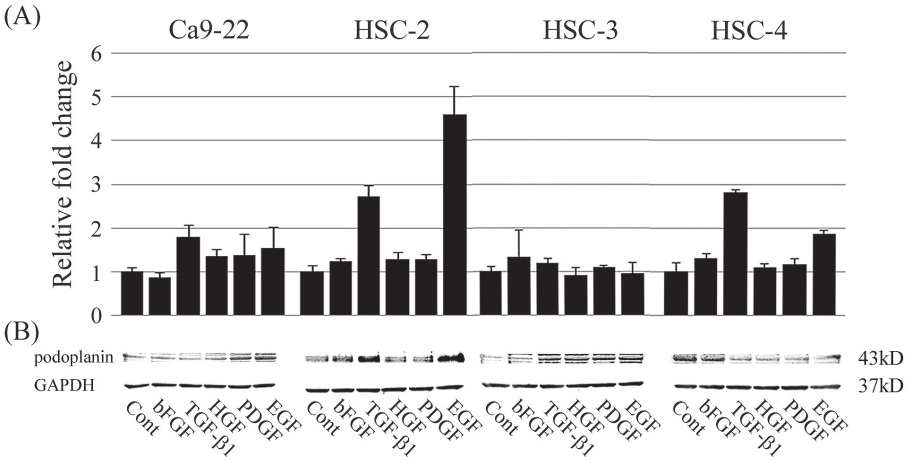

Fig. 1 Effects of bFGF, TGF-β1, HGF, PDGF, and EGF on expression of podoplanin in four OSCC cell lines.
(A) The expression of podoplanin mRNA was detected by real-time RT-PCR. Expression levels were normalized to that of control RNA. Total RNAs were prepared from Ca9-22, HSC-2, -3, and -4 cells treated with 100 ng/mL bFGF, TGF-β1, HGF, PDGF, and EGF for 24 h. Each bar represents the mean ± SD of three separate experiments with duplicate samples. (B) The expression of podoplanin and GAPDH was examined by Western blot analysis after treatment with 100 ng/mL bFGF, TGF-β1, HGF, PDGF, and EGF for 24 h.

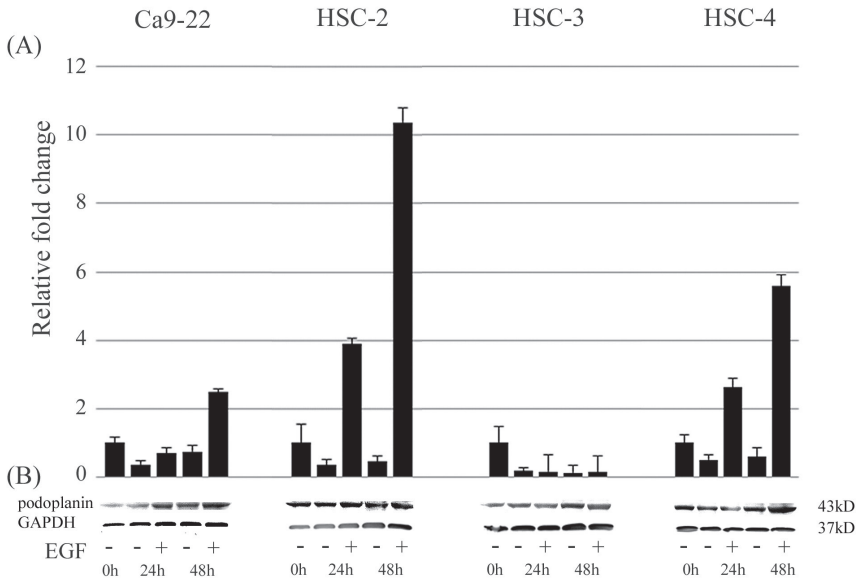

Fig. 2 Effects of EGF on expression of podoplanin in four OSCC cell lines.
(A) The expression of podoplanin mRNA was detected by real-time RT-PCR. Expression levels were normalized to that of control RNA. Total RNAs were prepared from Ca9-22, HSC-2, -3, and -4 cells treated with 100 ng/mL EGF for 24 h or 48 h. Each bar represents the mean ± SD of three separate experiments with duplicate samples. (B) The expression of podoplanin and GAPDH was examined by Western blot analysis after treatment with 100 ng/mL EGF for 24 h or 48 h.

at 5×10^5 cells/well and incubated at 37°C for 24 h. One microliter of mixture (final concentration of siRNA for podoplanin, 10 nM) was applied to each well of a 12-well tissue culture slide and incubated for 24 h. A scratch through the central axis of the plate was gently made using a pipette tip, and the cells were then incubated with EGF (100 ng/mL). Migration of the cells into the scratch was observed, and the migration distance was measured at 0 h and 27 h.

The study protocol was reviewed and approved by the Research Ethics Committee of Meikai University School of Dentistry (A0832).

Results

Effects of bFGF, TGF-β1, HGF, PDGF and EGF on podoplanin expression

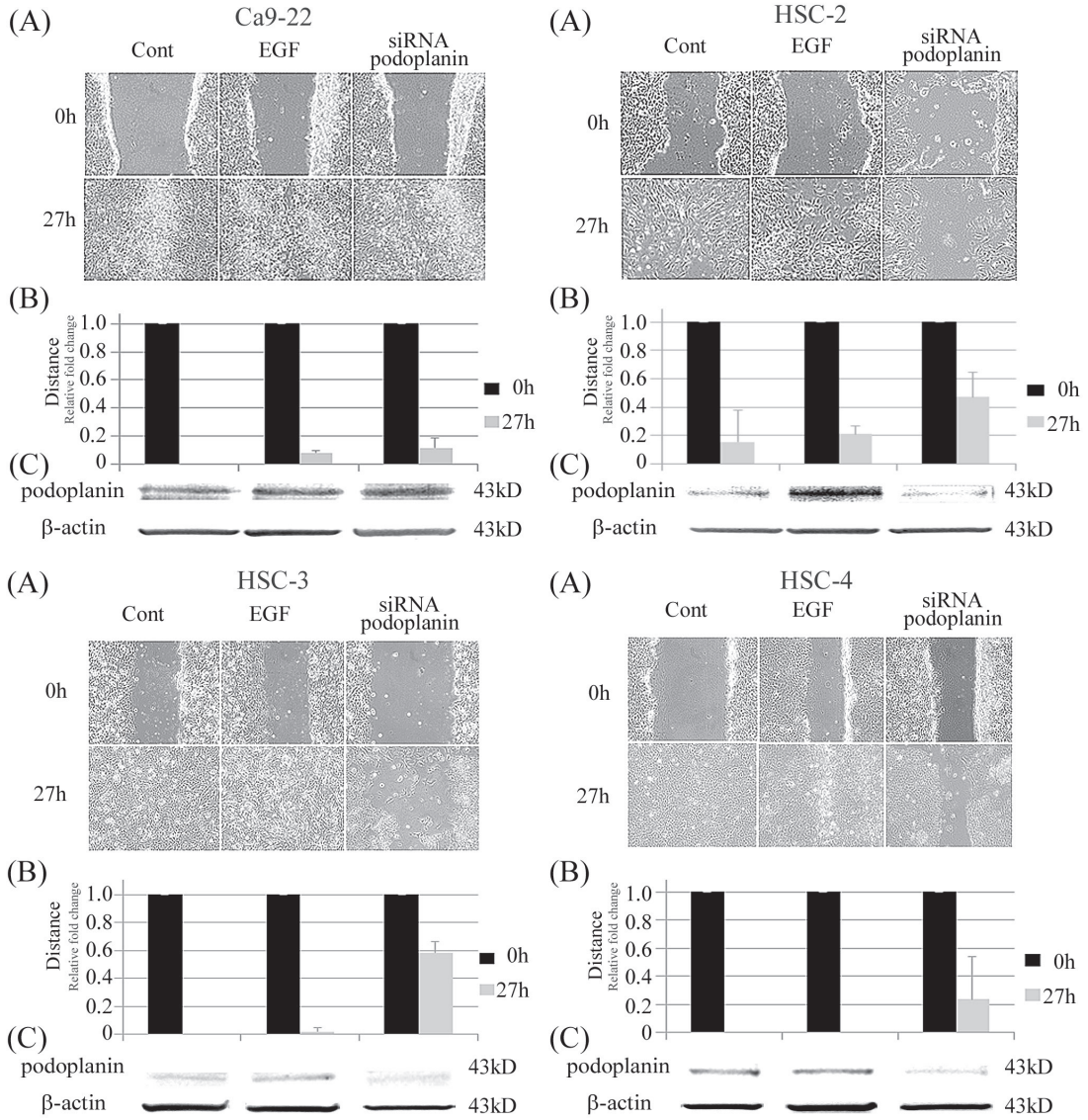

Fig. 3 Effects of EGF on oral cancer cell migration induced by podoplanin and podoplanin-specific siRNA.
(A) The scratch assay was performed to examine the effects of EGF on cancer cell migration. The Ca9-22, HSC-2, -3, and -4 cell lines were incubated in 12-well tissue culture dishes with 100 ng/mL EGF or with 10 nM siRNA for podoplanin. Pictures were taken at the beginning of the experiment (0 h) and after 27 h of incubation (27 h). (B) Bars indicate the relative distance migrated by the cells at 0 h and 27 h. Each bar represents the mean ± SD of three separate experiments. (C) The expression of podoplanin and β-actin was examined by Western blot analysis using the samples collected from the scratch assay.

To investigate which growth factor induces podoplanin expression, we used bFGF, TGF-β1, HGF, PDGF, and EGF in Ca9-22, HSC-2, HSC-3, and HSC-4 cells. In all OSCC cell lines except for HSC-3, EGF and TGF-β1 enhanced the expression of both podoplanin mRNA and protein (Figs. 1A and B). In HSC-3, no significant effect on podoplanin expression by any growth factors was observed. EGF pretreatment for 48 h predominantly increased the expression of podoplanin in all of the cell lines except HSC-3 (Figs. 2A and B).

Effects of EGF on oral cancer cell migration induced by podoplanin and podoplanin-specific siRNA

We assayed the loss of function using siRNA specific for podoplanin. Bars indicate the relative distance migrated by the cells at 0 h and 27 h. Each bar represents the mean ± SD of three separate experiments. Podoplanin-specific siRNA suppressed cell migration in HSC-2, -3 and -4 cells after the scratch (Figs. 3A and B), in which podoplanin protein expression was also being inhibited (Fig. 3C).

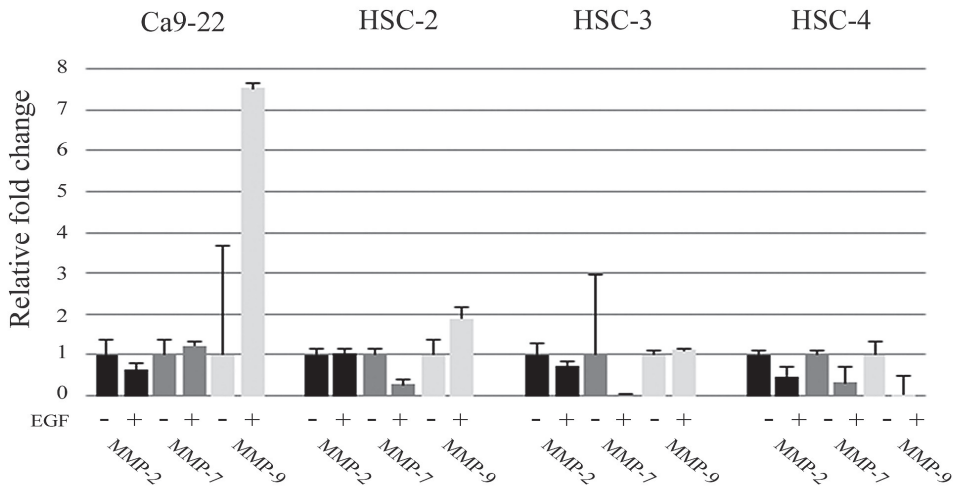

Fig. 4 Effects of EGF on expression of mRNA for MMPs.
The expression of MMP-2, -7, and -9 was detected by real-time RT-PCR. Expression levels were normalized to that of control RNA. Total RNAs were prepared from Ca9-22, HSC-2, -3, and -4 cells treated with 100 ng/mL EGF for 24 h. Each bar represents the mean ± SD of three separate experiments with duplicate samples.

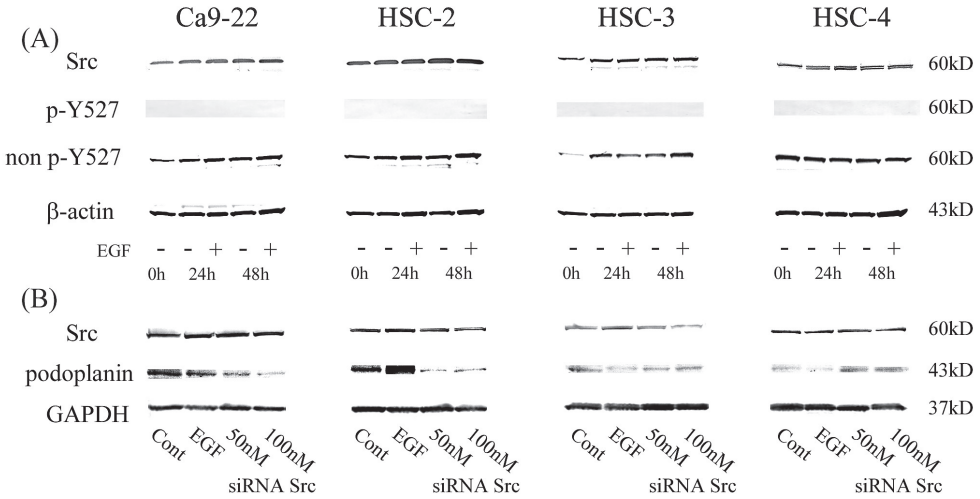

Fig. 5 Src activity in OSCC cell lines pretreated with EGF and Src-specific siRNA, and its effect on podoplanin expression.
(A) The expression of Src, p-SrcY527, non-p-SrcY-527, and β-actin was examined by Western blot analysis after treatment with 100 ng/mL EGF for 24 h or 48 h in 4 OSCC cell lines. (B) The expression of Src, podoplanin, and GAPDH was also examined after treatment with 100 ng/mL EGF or with siRNA for Src (50 nM, 100 nM) for 48 h.

Effects of EGF on expression of mRNA for MMPs

EGF treatment enhanced the expression of MMP-9 mRNA in Ca9-22 and HSC-2 cells, but did not alter that of MMP-2 and -7 mRNA, or even decreased it, in HSC-2, 3, and 4 (Fig. 4).

Src activity in OSCC cell lines pretreated with EGF and Src-specific siRNA, and its effect on podoplanin expression

As Src induces podoplanin expression to promote cell migration, we next investigated the association between Src and podoplanin expression in OSCC cell lines. Src was ubiquitously expressed in all OSCC cell lines, and was enhanced upon EGF stimulation (Fig. 5A). Western blotting assay also revealed that Src was activated by non-phosphorylation of Y527. siRNA specific for Src inhibited podoplanin expression especially in Ca9-22 and HSC-2 cells (Fig. 5B).

p130Cas activity in OSCC cell lines pretreated with EGF and p130Cas-specific siRNA, and

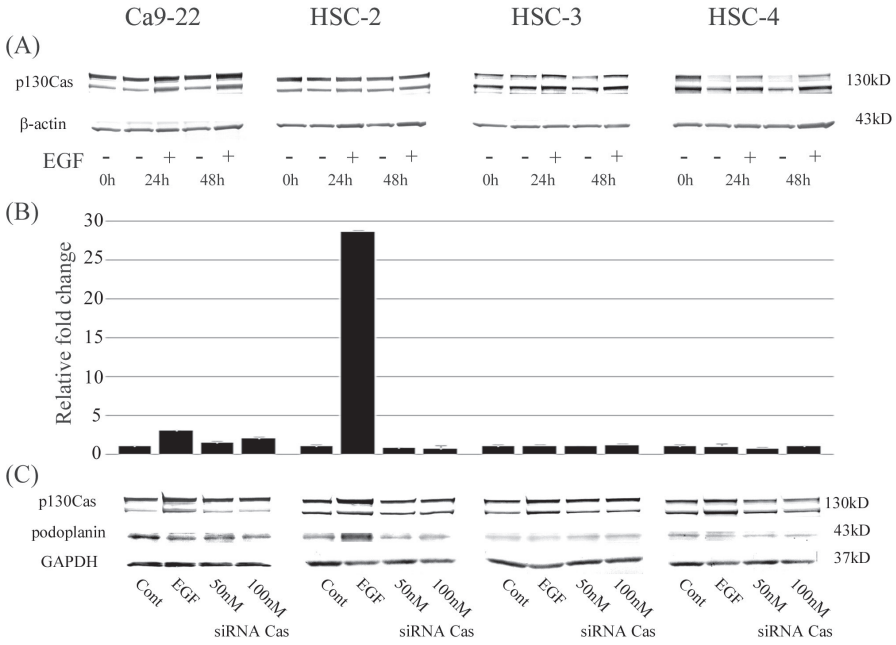

Fig. 6 p130Cas activity in OSCC cell lines pretreated with EGF and p130Cas-specific siRNA, and its effect on podoplanin expression.
(A) The expression of p130Cas and β-actin was examined by Western blot analysis after treatment with 100 ng/mL EGF for 24 h or 48 h in 4 OSCC cell lines. (B) The expression of podoplanin mRNA was detected by real-time RT-PCR. Expression levels were normalized to that of control RNA. Total RNAs were prepared from Ca9-22, HSC-2, -3, and -4 cells treated with 100 ng/mL EGF or with siRNA for p130Cas (50 nM and 100 nM) for 48 h. Each bar represents the mean ± SD of three separate experiments with duplicate samples. (C) The expression of p130Cas, podoplanin, and GAPDH was examined by Western blot analysis after treatment with 100 ng/mL EGF and siRNA for p130Cas (50 nM and 100 nM) for 48 h.

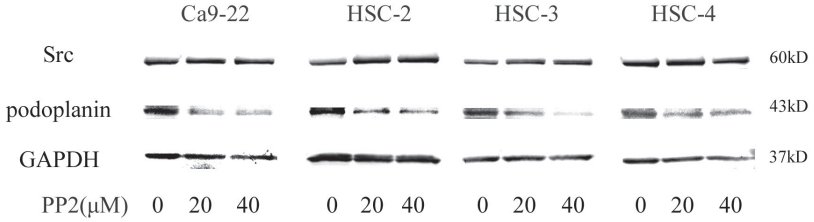

Fig. 7 Effects of PP2 on podoplanin expression.
The expression of Src, podoplanin, and GAPDH was examined by Western blot analysis after treatment with PP2, a known inhibitor of Src (0 μM, 20 μM, and 40 μM) for 48 h in 4 OSCC cell lines.

its effect on podoplanin expression

p130Cas downstream of Src and podoplanin was investigated in OSCC. p130Cas was ubiquitously expressed in all OSCC cell lines, and enhanced upon EGF stimulation (Fig. 6A). siRNA specific for p130Cas inhibited the expression of both podoplanin mRNA and protein, especially in HSC-2 cells (Figs. 6B and C).

Effects of PP2 on podoplanin expression

PP2, known to be a Src inhibitor, did not affect the expression of Src itself. Podoplanin expression was inhibited in a dose-dependent manner in all of the OSCC cell lines (Fig. 7).

Discussion

Among the growth factors related to receptor tyrosine kinases, few studies have examined the association between HGF or PDGF and OSCC, in comparison with that of EGF. HGF acts mainly as a paracrine factor in head and neck SCC (HNSCC) cells, the HGF/c-Met pathway being frequently up-regulated and functional in HNSCC (22). c-Src and c-Met interactions are distinct

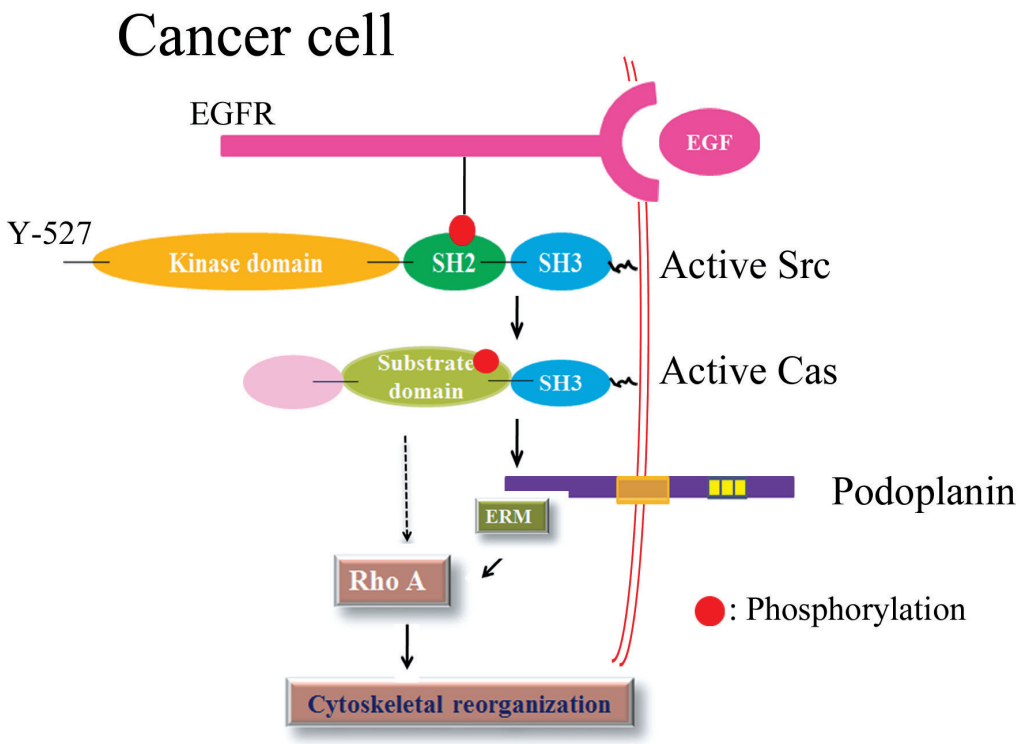

Fig. 8 The EGF-Src-Cas pathway in OSCC cell lines.
The EGF-Src-Cas pathway in OSCC cell lines is related to cytoskeletal reorganization, resulting in enhancement of cell migration. EGF: epidermal growth factors, EGFR: epidermal growth factor receptor, Y-527: the 527th tyrosine, SH2: Src homology 2, SH3: Src homology 3, ERM: ERM proteins such as ezrin, radixin and moesin.

in HNSCC (23). HGF is mitogenic and motogenic for human HNSCC cell lines (24). Incubation of HNSCC cells in the presence of HGF leads to a rapid increase in phosphorylation of both FAK and the growth-promoting kinase Erk. ERK MAPKs, one of the major three subfamilies of mitogen-activated protein kinases (MAPKs), are located downstream from the growth factors EGF, NGF and PDGF (25). Nevertheless, our present study demonstrated that among the growth factors related to SFK receptors, only EGF markedly enhanced the expression of podoplanin, especially at 48 h after the start of treatment. In addition, it is noteworthy that TGF-β1, which is not one of the SFKs and is one of the most extensively investigated cytokines known to induce EMT (26), also showed an increase in its expression. This finding indicates that there may be a podoplanin-dependent signaling pathway via TGF-β1 in OSCC. A recent study has indicated that TGF-β1 and STAT-3 induce podoplanin expression in keratinocytes, which might be significantly involved in the process of wound healing (27).

The scratch assay and examination of MMP mRNA expression demonstrated obvious podoplanin-dependent cell migration in OSCC. MMPs, which are secreted from both tumor and/or stroma cells, play a role in tumor invasion and metastasis by degrading the extracellular matrix (ECM) surrounding the tumor, especially the basement membranes (28). Emerging evidence indicates that MMPs can stimulate processes associated with EMT to enhance tumor cell invasion and metastasis potential (29). Most reports have suggested that predominant expression of MMP-2, -3 and -9 proteins is correlated with a poorer prognosis (30). MMP-9, known as type IV collagenase, is particularly abundant in basement membranes and the metastatic potential of carcinoma might correlate with the degree of enzymatic degradation of basement membrane type IV collagen (31,32). In the present study, the expression of MMP-9 was enhanced by EGF treatment in the Ca9-22 and HSC-2 cell lines, which was consistent with a previous study of HNSCC indicating that activation of EGFR promotes cell migration and invasion through production of MMP-9, followed by degradation of E-cadherin (33). Although emerging evidence suggests that SFKs may mediate MMP activity (34), further studies to clarify the correlation between Src and MMP activities in OSCC cell lines are required.

Podoplanin might facilitate tumor invasion through its ability to promote actin cytoskeleton remodeling of tumor cells, contributing to their increased motility

(35). The association between podoplanin and the actin cytoskeleton seems to be mediated by ezrin, which is markedly phosphorylated in the presence of podoplanin overexpression (11,36,37). Our series of studies on the EGF-Src-Cas-podoplanin signaling pathway have revealed that oral cancer cells express Src and p130Cas ubiquitously. Activation of both Src and p130Cas is important for inducing podoplanin expression. Therefore, podoplanin utilizes Src and p130Cas to reorganize the cancer cell cytoskeleton and may be associated with tumor cell invasion in the oral cavity.

The biological function of podoplanin may vary among different types of cancer. It has been reported that podoplanin can induce Rho A activation and EMT in MDCK cells (37), whereas it attenuates Rho A activity and does not induce EMT in a breast carcinoma cell line (11). These contradictory findings suggest that the functions of podoplanin are cell type-specific (38). The results of our experimental studies suggest that the expression of podoplanin promotes cell migration, and that the EGF-Src-Cas pathway (Fig. 8) is one of the mechanisms involved in cancer progression in the oral cavity.

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