Functional analysis of Zyxin in cell migration and invasive potential of oral squamous cell carcinoma cells

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Abstract. Zyxin is an evolutionarily conserved protein that has been implicated in the regulation of actin assembly and is mainly located at focal adhesions. However, the biological roles of Zyxin in cancer cells are incompletely understood. We analyzed the functions of Zyxin in cell migration and the invasive potential of OSCC. Zyxin expression was examined using eight OSCC cell lines with two different cell morphologies (6 epithelial type and 2 fibroblastic type). To knockdown Zyxin expression, OSCC cells were transfected with Zyxin siRNA and control siRNA. The cell lines were studied by western blot analysis, immunocytochemical analysis and cell migration and invasion assay. Epithelial type OSCC cells showed a high level of E-cadherin expression and a low level of Zyxin expression. N-cadherin as well as Zyxin were strongly expressed in fibroblastic type OSCC cells. Expression levels of LPP and TRIP6, members of the human Zyxin family, did not differ between epithelial type and fibroblastic type. Knockdown of Zyxin expression by siRNA in fibroblastic type OSCC cells was associated with cell morphological changes from spindle (fibroblastic) to polygonal (epithelial) shape and significantly inhibited cell growth as well as cell migration and invasion. Expression levels of Rac1 and Cdc42 were weaker in Zyxin siRNA-treated fibroblastic type OSCC cells than in control siRNA-treated cells, but the expression of RhoA did not differ significantly. Treatment of fibroblastic type OSCC cells with Rac1 inhibitor decreased the expression of Zyxin mRNA and protein. Zyxin is suggested to promote growth, migration and invasiveness of fibroblastic type OSCC cells by upregulating Rac1 and Cdc42.

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Abbreviations: OSCC, oral squamous cell carcinoma; LPP, lipoma preferred partner; TRIP6, thyroid receptor-interacting protein 6

Key words: Zyxin, oral squamous cell carcinoma, cell migration, invasion, morphology, siRNA

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common neoplasm in the world, characterized by a poor prognosis and low survival rates (1,2). Worldwide, the GLOBOCAN 2002 database estimated that 274,000 new cases of oral cancer and 127,000 deaths from the disease occur annually [The GLOBOCAN 2002 database. International Agency for Cancer Research (IARC), http:// www-dep.iarc.fr/]. In patients with locally advanced disease, the main causes of treatment failure are locoregional recurrence and metastatic disease. Despite recent advances in diagnosis and surgical, radiotherapeutic, and chemotherapeutic management, cervical lymph node involvement is associated with an approximately 50% lower 5-year survival rate, as well as an increased risk of distant metastasis (3,4). Patients with highly invasive carcinomas have poor outcomes because tumor cells deeply invade surrounding fibrous tissues, metastasize more frequently to lymph nodes and are less sensitive to chemotherapeutic agents than lowly invasive carcinomas. Invasion and metastasis are thus the most crucial characteristics of malignant tumors.

OSCC cell lines are important preclinical models in the search for novel targeted therapies for oral cancer. Unlike many other types of cancer, a wide variety of primary and metastatic OSCC cell lines are available. In fact, more than 300 cell lines of head and neck cancer have been established, as compared with approximately 70, 60 and 10 cell lines derived from breast, colon and prostate cancers, respectively (5,6,7). Yokoyama *et al* (8) reported the establishment of subclonal OSCC cell lines that showed negative expression of E-cadherin and fibroblastic spindle shape and had higher invasive activity than the respective parental OSCC cell lines. Moreover, they demonstrated that OSCC cell lines contained two kinds of cells: one with an epithelial shape and the other with a spindle or fibroblastic shape in culture. The cells with epithelial shape expressed E-cadherin, whereas the cells with fibroblastic shape did not. Frixen *et al* (9) reported that carcinoma cell lines with epithelial phenotype were non-invasive and expressed E-cadherin, whereas carcinoma cell lines with fibroblastic phenotype were invasive and had lost E-cadherin expression. These findings indicate that OSCC is characterized by a heterogeneous cell population. Fibroblastic type OSCC cells have been acknowledged to result from epithelial-mesenchymal transition (EMT) in epithelial cell

lines, whereas the relation between cell morphology and tumor cell motility in OSCC is incompletely understood.

Zyxin is an evolutionary conserved protein that has been implicated in the regulation of actin assembly and is mainly localized at focal adhesions. Zyxin is a family of proteins that also includes lipoma preferred partner (LPP) and thyroid receptor-interacting protein-6 (TRIP-6) (10,11). Although Zyxin has been shown to be diffusely distributed in cytoplasm, it is likely that part of Zyxin enters the nucleus, binds to h-warts and leads to G2-cell cycle arrest and inhibition of proliferation, as observed after silencing of LASP-1, a transcriptional factor of Zyxin (12,13). Zyxin is characterized by a nuclear-cytoplasmic shuttling of focal contact proteins and has a potential mechanism for communication between sites of cell adhesion and the nucleus (14,15,16). In cancer cells, Zyxin is significantly upregulated in melanoma cells as compared with melanocytes, and Zyxin expression is directly related to cell spreading and proliferation and inversely related to differentiation (17). However, Sperry *et al* (18) found that expression of Zyxin activity is downregulated at cell-cell junctions during EMT. Therefore, the biological roles of Zyxin in cancer cells remain controversial. In this study, we investigated the functions of Zyxin using eight OSCC cell lines with two different cell morphologies (epithelial type and fibroblastic type) to clarify the biological roles of Zyxin in OSCC.

Materials and methods

Cell culture and cell lines. The eight oral squamous carcinoma cell lines [SCCKN (19), HSC-2, HSC-3 (20), OSC-19 (21), OSC-20 (22), HOC-313, TSU (23-25) and SCC25] were grown in DMEM containing 10% FBS as growth medium and subcultured. OSC-19, OSC-20, HOC-313 and TSU were kindly provided by Professor S. Kawashiri (Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan).

RNA extraction and real-time polymerase chain reaction (PCR). Total-RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA), and cDNAs were generated by using a First-Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) with 2 μg of total-RNA and oligo (dT) (GE Healthcare Japan, Tokyo, Japan). All reagents required for real-time PCR were from Applied Biosystems (Foster City, CA, USA). Oligo-nucleotide primers and fluorescent probes for Zyxin and GAPDH were designed using a primer design program (Primer Express, Applied Biosystems) and were obtained from Integrated DNA Technologies (Coralville, IA, USA).

Protein preparation and western blot analysis. Cell lysates were submitted to western blot analysis as described previously (26,27). The following primary antibodies used: rabbit polyclonal antibodies against Zyxin (Sigma-Aldrich Co., St. Louis, MO, USA), N-cadherin, RhoA, CDC42 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Rac1/2/3 (Cell Signaling Technology, Boston, MA, USA); mouse monoclonal antibodies against E-cadherin (Santa Cruz Biotechnology), LPP (Cell Signaling, Acc, Switzerland), and TRIP-6 (Abnoba, Taipei, Taiwan); and goat polyclonal antibodies against actin (Santa Cruz Biotechnology). The secondary antibodies used were anti-goat, anti-mouse or anti-rabbit IgGs conjugated with alkaline phosphatase (all products, Santa Cruz Biotechnology). Actin was used as an internal control.

Immunocytochemical analysis. The primary antibodies used in this study included rabbit anti-Zyxin (Sigma-Aldrich) and mouse polyclonal antibody against human actin (Santa Cruz Biotechnology). Cultured cells were washed with PBS (-) twice and fixed in 3.7% paraformaldehyde for 20 min at room temperature. After blocking with 2% bovine serum albumin, the cells were treated with a primary antibody at 4˚C overnight. The cells were washed and incubated with anti-rabbit fluorescein isothiocyanate or anti-mouse rhodamine phalloidin (Cytoskeleton, Denver, CO, USA), followed by counterstaining with 4,6-diamidino-2-phenylindole (DAPI). Finally, fluorescence images were obtained by using a confocal laser microscope, LSM 510 version 3.2 (Carl Zeiss Co. Ltd., Oberkochen, Germany).

Transfection of siRNAs. Cells were cultured in DMEM supplemented with 10% FBS for 24 h and then transfected with 5 μM of siRNA, using Thermo Scientific DharmaFECT Transfection Reagents (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. SMART pool siRNA targeting Zyxin (L-016734-00-0020) and control siRNA, On-Target plus GAPDH (D-001830-01-20), were purchased from Dharmacon Inc. (Lafayette, CO, USA).

Cell growth assay. Cells were plated at 2.5×10^4 cells/well in 1-ml volumes in 12-well plates and cultured in growth medium at 37˚C. At selected intervals, cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or by using a hemocytometer as described previously (26).

Measurement of apoptosis. Cells were plated at 3×10^4 cells/well in 100-μl volumes in 96-well plates and cultured in growth medium at 37˚C. Apoptosis induction was examined after 48 h using an ssDNA Apoptosis ELISA Kit (Chemicon International Inc., Temecula, CA, USA).

Flow cytometry. For cell-cycle analysis, cells were harvested 48 h after Zyxin siRNA transfection. The apoptosis index was measured using a Cell Cycle Phase Determination kit (Cayman Chemical, Ann Arbor, MI, USA).

Scratch assay. Cells were plated at 5×10^5 cells/dish in 60-mm dishes (Asahi Techno Glass Co., Tokyo, Japan) and treated with 25 nM Zyxin siRNA. Scratch assay was performed by scraping confluent cell monolayers with a sterile pipette tip after 24 h. Cell migration was examined by measuring the distance from edge to edge after 6-h incubation.

Invasion assay. Cell invasion assay was carried out using BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA, USA) consisting of transwell membrane filter inserts in a 24-well tissue culture plate. The transwell filter had an 8-μm pore size membrane coated with Matrigel. One hundred thousand cells were seeded in the upper chamber of the transwell with serum-free DMEM and treated with 25 nM Zyxin siRNA. DMEM containing 10% FBS was added to the

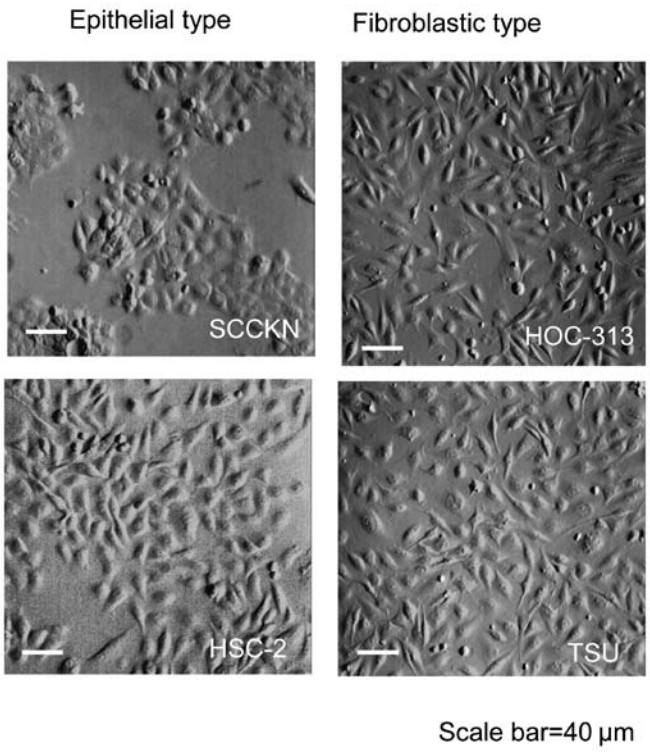

Figure 1. Phase contrast photographs of representative OSCC cell lines that showed epithelial or fibroblastic type morphology. Scale bars, 40 μm.

lower chamber after 24 h. Non-invading cells were removed by wiping the upper side of the membrane, and invading cells were fixed and stained with a Diff-Quick kit (Kokusaishiyaku Co., Kobe, Japan) after 24-h incubation. The number of invading cells per membrane was counted in triplicate under a light microscope at x200 magnification in four fields per membrane.

Expression of Zyxin after Rac1 inhibitor treatment. To examine the effect of Rac-1 inhibitor on expression of Zyxin, cells were treated with 50 nM of Rac1 inhibitor (EHT 1864, R&D Systems, IA, USA) for 4 h. Western blot assay and real-time PCR analysis of Zyxin protein and mRNA in HOC-313 cells were performed.

Statistical analysis. All values in the figures and text are expressed as means ± SD. The results were analyzed and individual group means were compared with the use of Student's t-test. A p-value of <0.05 was considered to indicate statistical significance.

Results

Expression of cadherins, Zyxin and Zyxin family proteins in OSCC cell lines in relation to cell morphology. To examine the relations between the morphology of OSCC cell lines and EMT markers, expressions of cadherins, Zyxin and Zyxin family proteins were examined using western blot analysis. All eight OSCC cell lines expressed EGFR, indicating that these cell lines were of epithelial origin and not mesenchymal origin. The OSCC cell lines showed two morphological types: epithelial type cells such as SCCKN and HSC-2; and fibroblastic type cells such as HOC-313 and TSU (Fig. 1). Western blot analysis revealed high expression levels of E-cadherin protein in 6 cell lines with epithelial type morphology, including SCCKN, HSC-2, OSC-20, HSC-3, OSC-19 and SCC25. These cell lines formed flatter colonies on the plastic dish surface. In contrast, HOC-313 and TSU showed fibroblastic morphology and were completely negative for E-cadherin. These cell lines formed disperse colonies and expressed N-cadherin, suggesting mesenchymal transition. To gain insight into the functions of Zyxin family proteins in OSCC cell lines, expressions of Zyxin and its family members LPP and TRIP-6 were examined. The cell lines with epithelial type morphology and high levels of E-cadherin

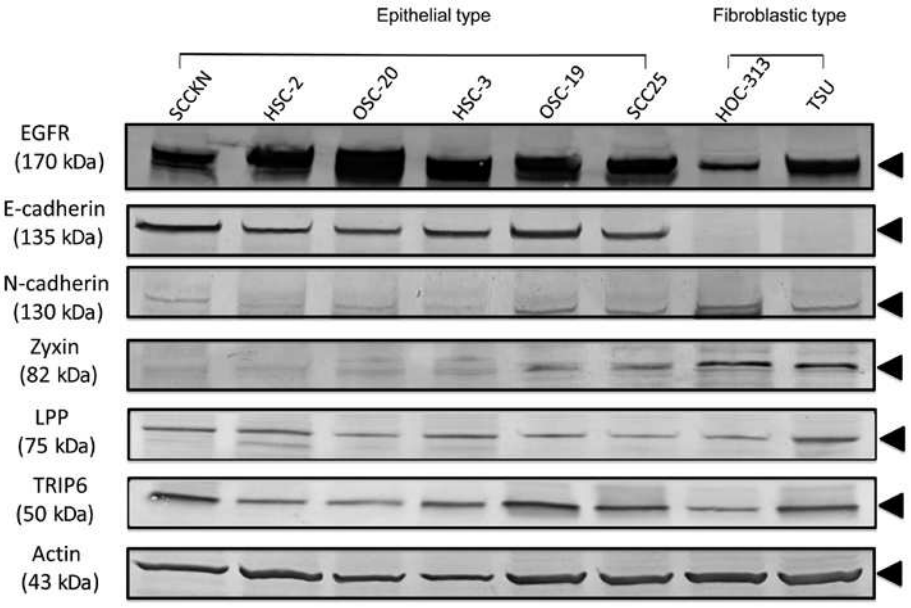

Figure 2. Results of western blot analysis of eight OSCC cell lines, showing expression of EMT markers and Zyxin family members. Decreased expression of E-cadherin and increased expressions of N-cadherin and Zyxin was found in parallel to morphological changes from epithelial type to fibroblastic type. Lipoma preferred partner (LPP) and thyroid receptor-interacting protein 6 (TRIP-6) are human Zyxin family members.

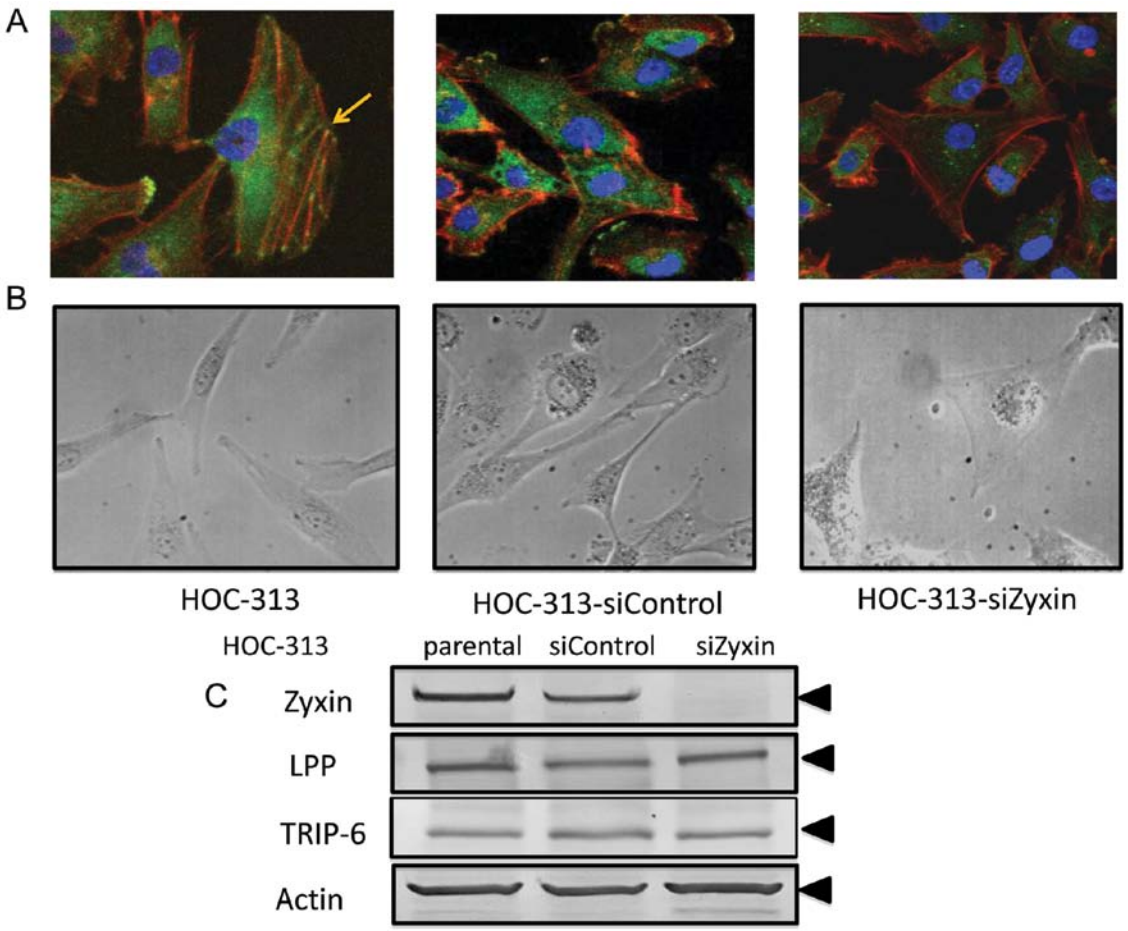

Figure 3. (A and B) Immunocytochemical analysis of cell morphological changes induced by Zyxin siRNA treatment. Blue, nuclear (DAPI); red, actin (phalloidin); green, Zyxin (FITC). Note that Zyxin was localized at adhesion plaques and some overlapped with actin stress fibers (arrow). Treatment with Zyxin siRNA resulted in reduced expression of Zyxin and morphological changes from spindle to polygonal shape. (C) Inhibition of Zyxin expression by Zyxin siRNA treatment did not alter LPP or TRIP-6 expression.

expression showed low levels of Zyxin expression. High levels of Zyxin and N-cadherin expression were detected in cell lines with fibroblastic type morphology, such as HOC-313 and TSU (Fig. 2). When expressions of Zyxin family members such as LPP and TRIP-6 were examined in OSCC cell lines, there were no differences between epithelial and fibroblastic type OSCC cell lines, and all cell lines showed similar expression. These expression patterns suggested that Zyxin family members did not compensate for each other. These results indicated that expression of Zyxin was suppressed in E-cadherin-expressing epithelial cell lines, while N-cadherin-expressing cell lines strongly expressed Zyxin.

Morphological changes of HOC-313 induced by treatment with Zyxin siRNA. Next, we examined the cellular localization of Zyxin in HOC-313, a fibroblastic type OSCC cell line maintaining an invasive character (28). Endogenous Zyxin was localized at adhesion plaques and some overlapped with actin stress fibers in HOC-313 (Fig. 3A). To clarify the function of Zyxin in highly invasive cells, knockdown experiments of Zyxin by siRNA were performed. On immunocytochemical analysis, a dramatic reduction in Zyxin expression was observed in Zyxin siRNA-treated cells as compared with control siRNA-treated cells (Fig. 3A). On western blot analysis, the protein level of Zyxin was also reduced in Zyxin siRNA-treated cells (Fig. 3C). These data indicated that Zyxin siRNA specifically targeted Zyxin expression. Moreover, Zyxin siRNA-treated HOC-313 showed morphological changes (Fig. 3B). Zyxin siRNA-treated HOC-313 showed tripolar or polygonal shape and a large projected cell area as compared with control siRNA-treated HOC-313. Therefore, Zyxin was suggested to play important roles in maintenance of cell morphology via actin re-arrangement. When expression of Zyxin family members including LPP and TRIP-6 in HOC-313 were examined after Zyxin siRNA treatment, their expression was found to be uninhibited by such treatment (Fig. 3C).

Inhibition of cell proliferation, cell migration and invasive potential in OSCC cell lines with fibroblastic type morphology after treatment with Zyxin siRNA. Since we found high levels of Zyxin expression in OSCC cell lines with fibroblastic type morphology as compared with cell lines with epithelial type morphology, the effects of knockdown of Zyxin on cell proliferation, migration, and invasive potential were examined in fibroblastic type of OSCC. Growth curves of HOC-313 and TSU treated with Zyxin siRNA and control siRNA are shown in Fig. 4. Cell proliferation was significantly inhibited in Zyxin siRNA-treated HOC-313 and TSU from day 4 onward, and

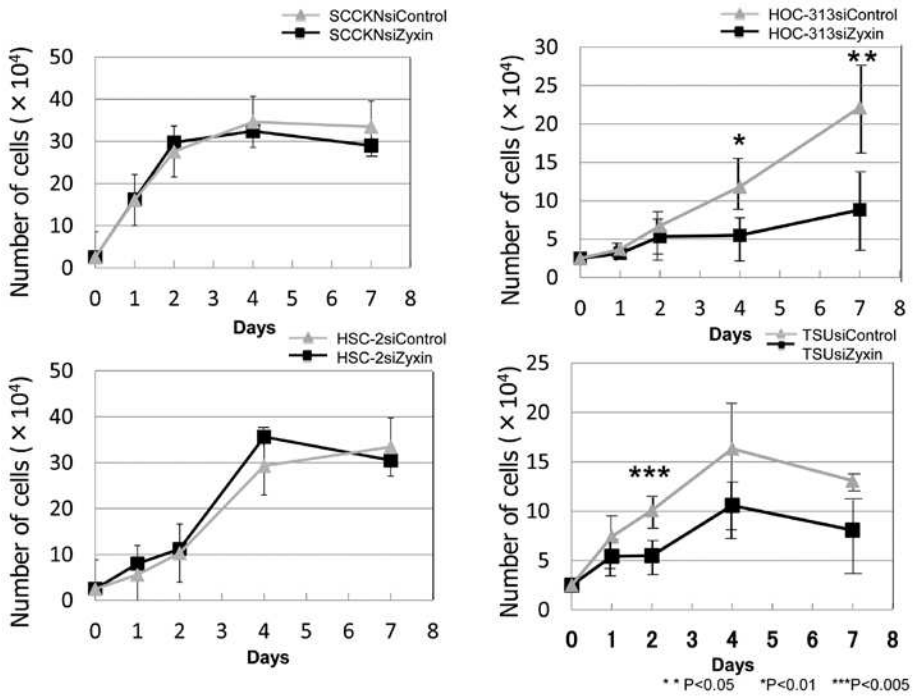

Figure 4. Inhibition of cell growth of fibroblastic type OSCC cell lines HOC-313 and TSU by Zyxin siRNA treatment. Cell growth did not differ until day 2 after Zyxin siRNA treatment, but was significantly inhibited from day 4 onward as compared with control siRNA treatment. Significant difference was observed only on day 2 in TSU.

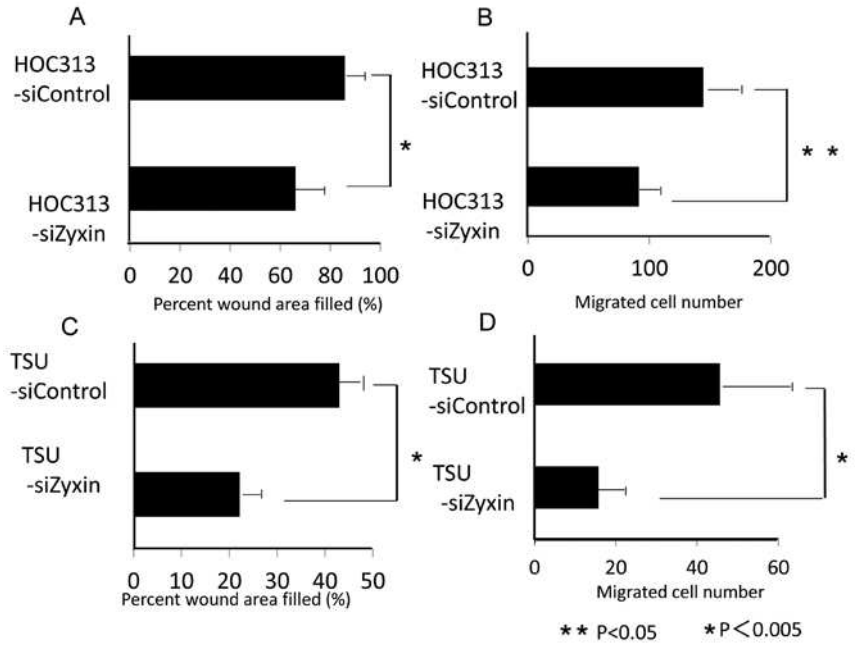

Figure 5. Inhibition of cell migration and invasiveness of fibroblastic type OSCC cell lines HOC-313 and TSU by Zyxin siRNA treatment. (A and C) Scratch assay: cell migration was determined by measuring the distance from edge to edge after 6-h incubation. Zyxin siRNA inhibited cell migration significantly as compared with control siRNA treatment. (B and D) Invasion assay: cell invasion assay was performed using Matrigel invasion chambers. HOC-313 and TSU cells were seeded and treated with Zyxin siRNA. After 24-h incubation, cells invading through Matrigel-coated membrane were counted. Zyxin siRNA significantly inhibited cell invasion.

the reduction rate on day 4 was 53.5 and 35.2%, respectively. Significant difference was observed only on day 2 in TSU. However, cell proliferation of SCCKN and HSC-2 lines that do not express Zyxin, was not inhibited by Zyxin siRNA treatment.

Zyxin siRNA treatment of HOC-313 also significantly inhibited cell migration and invasion. It inhibited cell migration by 19.6% and cell invasion by 36.7% as compared with control siRNA treatment (Fig. 5A and B). Zyxin siRNA treatment of TSU also

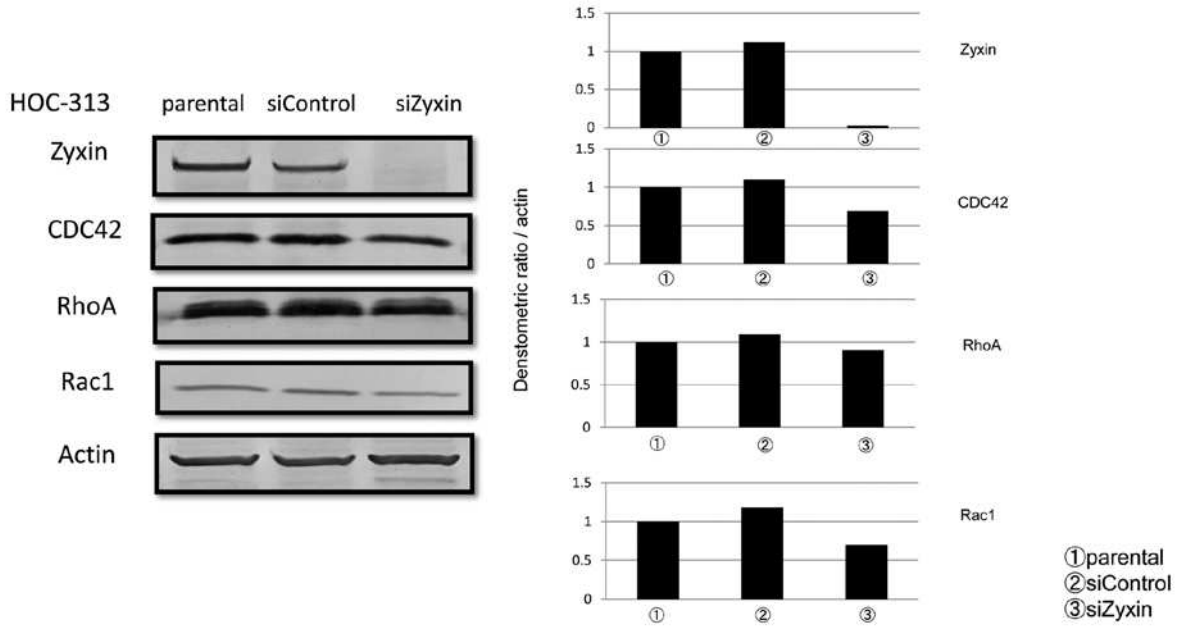

Figure 6. Analysis of Rho family proteins by western blotting. Rho family small GTPases involved in cell migration and invasion, such as RhoA, Rac1 and CDC42, were examined. There was no significant difference in expression of RhoA, but expressions of Rac1 and CDC42 were slightly reduced in Zyxin siRNA-treated cells.

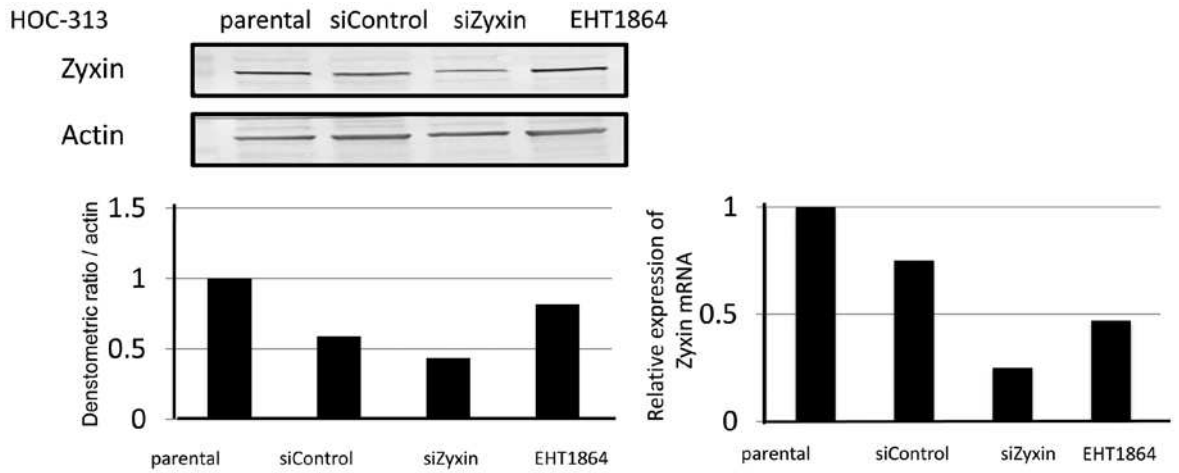

Figure 7. Effect of Rac1 inhibitor on expression of Zyxin in HOC-313. Cells were treated with 50 nM of Rac-1 inhibitor, EHT1864, for 4 h. Western blot analysis and real-time PCR analysis of Zyxin protein and mRNA in HOC-313 cells were performed. Expression of Zyxin in HOC-313 was partially inhibited by Rac1 inhibitor.

significantly inhibited cell migration by 20.9% and invasion by 65.7% as compared with control siRNA treatment (Fig. 5C and D). To investigate why Zyxin siRNA treatment inhibited proliferation of HOC-313, the apoptosis index and cell cycle distribution were examined. However, the apoptosis index did not increase, and the cell cycle distribution was unaffected by Zyxin siRNA treatment (data not shown).

Expression of Rho family proteins in Zyxin siRNA-treated HOC-313 cells. Because Rho family small GTPases such as RhoA, Rac1 and CDC42, are involved in cell migration and invasive potential, as well as in regulation of EMT (29), functional interactions between Rho family and Zyxin were examined in HOC-313. Although there was no significant difference in expression of RhoA between control siRNA- and siZyxin-treated cells, expression of CDC42 and Rac1 was reduced by 37.6 and 40.9%, respectively, on western blot analysis (Fig. 6). Since it is well known that CDC42 mainly forms filopodia that act as sensors of the cancer microenvironment and Rac1 forms lamellipodia that induce motogenic activity and resolve extracellular matrix, the effect of the Rac1 inhibitor EHT1864 on expression of Zyxin in HOC-313 was examined. When cells were treated with EHT1864, expression of Zyxin protein was reduced slightly, but Zyxin mRNA decreased by half on real-time PCR analysis (Fig. 7). These results indicated that interactions between Zyxin and Rac1 were one of the factors related to migration and invasiveness of fibroblastic type OSCC cells.

Discussion

An important hallmark of metastasis is increased cell motility accompanied by actin cytoskeletal re-arrangement. To gain insight into the relation between tumor cell morphology and motility in OSCC, we analyzed the roles of Zyxin in cell motility and cell morphology, using eight OSCC cell lines with different morphological phenotypes. We found loss of E-cadherin expression and increased N-cadherin expression on transition from epithelial type to fibroblastic type. Reduced E-cadherin expression in OSCC cells is associated with more aggressive tumor behavior and worse outcomes (30,31). Moreover, cadherin switching (i.e., the loss of E-cadherin expression and gain of N-cadherin expression) is a crucial event of EMT in human cancers, which correlates with histologic differentiation, invasion pattern and lymph node metastasis. Head and neck cancer cells also show cadherin switching associated with EMT features and EMT cancer cells show increased invasiveness (32). However, molecular relations among cell morphology, cell motility and EMT in OSCC cells are incompletely understood.

Zyxin, a focal adhesion-associated LIM protein family, harbors distinct actin polymerization activity (33) and is located primarily at focal adhesions and regulates actin cytoskeleton dynamics, cell movement, and signal transduction (34,35). When we reduced Zyxin expression levels in fibroblastic type OSCC cells by treatment with Zyxin siRNA, cell growth was inhibited significantly as compared with control siRNA-treated cells. To clarify why Zyxin siRNA treatment inhibited the growth of fibroblastic type OSCC cells, cell cycles and apoptosis induction were examined. However, cell cycles were unaffected, and the apoptosis index was not increased by Zyxin siRNA treatment. We therefore speculated that the involvement of cell growth factor was decreased by nutrient availability, hypoxia, heat shock, DNA damage and osmotic stress (36).

Cells with inhibited Zyxin expression display reduced adhesive properties, reduced migration, reduced capacity to build robust actin stress fibers in response to a chemical stimulus, and disturbed focal adhesion accumulation of actin regulators in the Zyxin family. Shinto *et al* (37) reported that stable expression of the oncoprotein associated with scirrhous gastric cancer cells resulted in decreased Zyxin levels and a corresponding increase in motility. Furthermore, Amsellem *et al* (38) reported that knockout of Zyxin in Ewing's sarcoma cells is associated with enhanced cell motility. However, knockdown of Zyxin by siRNA in SKOV-3 cells, a human ovarian cancer cell line, had no influence on cell migration (39). To date, these disparate effects have not been fully elucidated, but may result from specific cellular features.

When Rho family protein expression was examined in Zyxin knockdown cells, reduced expression of Rac1 and CDC42 was found. This finding was consistent with the results of Pratt *et al* (40), who suggested that the Ajuba/Zyxin family of LIM proteins leads to activation of Rac during cell migration. These results suggest that Zyxin is a potential EMT marker and that overexpression of Zyxin promotes cell growth and invasion via up-regulation of Rac1, CDC42 or both in OSCC cells. Although we have not yet found an inhibitor of CDC42, EHT1864 decreased Zyxin mRNA and protein in HOC-313. Our results suggest a mechanism by which reduced Zyxin expression might contribute to tumor regression by affecting cytoarchitecture and motility.

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