RNAi-mediated down-regulation of α-actinin-4 decreases invasion potential in oral squamous cell carcinoma


Abstract. α-actinin-4, originally identified as an actin-binding protein associated with cell motility, invasion, and metastasis of cancer cells, appears to be overexpressed in various human epithelial carcinomas, including colorectal, breast, esophageal, ovarian, and non-small cell lung carcinomas. The authors evaluated whether α-actinin-4 might be appropriate as a molecular target for cancer gene therapy. In 64 primary oral squamous cell carcinomas (OSCCs) and 10 normal oral mucosal specimens, and in seven human OSCC cell lines, α-actinin-4 expression was evaluated immunologically and correlations with clinicopathologic factors were examined. Overexpression of α-actinin-4 was detected in 38 of 64 oral squamous cell carcinomas (70%); significantly more frequently than in normal oral mucosa. The expression of α-actinin-4 was significantly associated with invasion potential defined by the Matrigel invasion assay. Cancer cell lines with higher α-actinin-4 expression had greater invasive potential. An RNAi-mediated decrease in α-actinin-4 expression reduced the invasion potential. These results indicated that the overexpression of α-actinin-4 was associated with an aggressive phenotype of OSCC. The study indicated that α-actinin-4 could be a potential molecular target for gene therapy by RNAi targeting for OSCC.

Keywords: α-actinin-4; invasion; metastasis; RNA interferenoe; oral squamous cell carcinoma.

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Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck region and accounts for more than 90% of cancers of the oral cavity. The primary therapeutic modality for OSCC is surgery. Recent advances in surgical techniques and anticancer agents have improved tumor regression and survival rate, but wide surgical resection of OSCC causes oral dysfunction so new treatment strategies are required.

Neck lymph node metastasis is strongly related to a poor prognosis in SCC of the head and neck. Alterations in the expression of adhesion-related molecules are associated with poor prognosis in OSCC patients. α-actinin-4, an actin-binding and cross-linking protein, is thought to play important roles in forming stress fibers, promoting cell adhesion and regulating cell shape and motility. Of the 4 isoforms of α-actinin, the expression of α-actinin-2 and α-actinin-3 is limited to skeletal and cardiac muscle sarcomeres. In contrast, α-actinin-1 and α-actinin-4, which are non-muscular α-actins, are widely expressed. The latter 2 isoforms share a high degree of similarity (87% amino acid homology) but
have different subcellular localizations\(^5\). \(\alpha\)-actinin-1 is localized within the end of actin stress fibers and adherens junctions, and associates them with the cell membranes\(^15\). \(\alpha\)-actinin-4 is present in the cytoplasm and nucleus and colocalizes with actin stress fibers\(^12\). The expression of \(\alpha\)-actinin-4 is highly concentrated at the leading edge of motile cells and in the cytoplasm of sharp cell extensions\(^15\).

Recent reports have suggested that the increased expression of \(\alpha\)-actinin-4 in the cytoplasm of various malignant neoplasms is correlated with poor prognosis, enhanced cell motility, advanced tumor stage, and lymph node metastasis\(^5,8,13,15,17,22,27\). The relationship between \(\alpha\)-actinin-4 expression and invasiveness or metastatic ability in OSCC and the mechanisms underlying any such relationship remain unknown.

In this study, the authors immunohistochemically examined \(\alpha\)-actinin-4 expression in OSCC. They determined the clinicopathological significance of \(\alpha\)-actinin-4 expression in relation to various parameters such as patient characteristics and histopathological findings. siRNA analysis strongly suggested that \(\alpha\)-actinin-4 would be a potent molecular target for cancer gene therapy in OSCC.

Materials and methods

Patients

Paraffin-embedded sections were obtained from biopsy specimens of 64 patients with OSCC who underwent radical surgery. Tumor stage was classified according to the TNM classification of the International Union Against Cancer, histological differentiation was defined according to the WHO classification, and invasion pattern was determined according to Braye's classification\(^5\). As controls, samples of normal oral epithelium were obtained after informed consent from 10 patients undergoing routine surgical removal of their third molars.

Cell lines

The following human OSCC cell lines were obtained from the Human Science Research Resource Bank (Osaka, Japan): SAS, SCC25, OSC20, HSC-2, HSC-3, HSC-4, Ca9-22, and the human keratinocyte cell line, HEKa, as a control. All cells were cultured under conditions recommended by their depositors.

Immunohistochemical staining and evaluation

Serial 4-\(\mu\)m thick specimens were taken from the tissue blocks. Sections were deparaffinized in xylene, soaked in target retrieval solution buffer (Dako, Glostrup, Denmark) and placed in an autoclave at 121°C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% \(\text{H}_2\text{O}_2\) in methanol for 30 min. Immunohistochemical staining was performed using the Envision system (Envision\(^+\), Dako, Carpinteria, CA). The primary antibody used was directed against \(\alpha\)-actinin-4 (4D10, Abnova, Taipei, Taiwan). The sections were incubated with the monoclonal antibody overnight at 4°C. Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and the samples were counterstained with Meyer's hematoxylin and mounted. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline. \(\alpha\)-actinin-4 expression was defined as the presence of specific staining in the cytoplasm and nuclei of tumor cells. The immunoreactivity of \(\alpha\)-actinin-4 was scored by staining intensity and immunoreactive cell percentage as follows\(^8\): staining index 0, tissue with no staining; 1, tissue with faint staining or moderate staining in less than 25% of tumor cells; 2, tissue with moderate staining or strong staining in less than 25% of tumor cells; 3, tissue with strong staining in more than 25% of tumor cells. Overexpression of \(\alpha\)-actinin-4 was defined as staining index \(\geq 2\).

RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA) and first-strand cDNA was synthesized from 1 \(\mu\)g total RNA using Oligo d (T) primer (Invitrogen) and ReverTra Ace (Toyobo, Osaka, Japan). For PCR analysis, cDNA was amplified by Taq DNA polymerase (Takara, Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Each PCR program involved a 3-min initial denaturation step at 94°C, followed by 28 cycles (for \(\alpha\)-actinin-4), or 18 cycles (for GAPDH) at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, on a PCR Thermal Cycler MP (Takara). Primer sequences were as follows: AGTATGGACGCAGCTCGTG for \(\alpha\)-actinin-4(F); GGTGGAGATCGTTTCTCCA for \(\alpha\)-actinin-4(R); ATGTGCCTGGAGCGTACTGGC for GAPDH(F); and TGACCTTGCCCAAGCCTG for GAPDH(R). The amplified products were separated by electrophoresis on ethidium bromide-stained 2% agarose gels. Band intensity was quantified by Image J software.

Invasion assay

The BioCoat Matrigel invasion chamber (Becton Dickinson, Bedford, MA) was used. This contains an internal chamber with an 8-\(\mu\)m porous membrane bottom that was coated with Matrigel. Six-well cell culture inserts and a six-well multi-well companion plate were used for the experiment. The membranes were rehydrated with warm serum-free medium for 2 h. The internal chamber was filled with 1.25 \(\times\) 10\(^5\) cells in medium containing 10% fetal bovine serum (FBS) as a chemoattractant. Cells were incubated for 72 h at 37°C in a 5% \(\text{CO}_2\) atmosphere. After incubation, non-invading cells were removed from the top of the wells with a cotton swab, and cells that transferred to the reverse surface of the membrane were subjected to Diff-Quick staining. Cells were counted under a microscope at 100X magnification. For the control cell count, cells that passed through a control chamber without Matrigel were counted. All experiments were performed in triplicate, and cell numbers at least in 4 fields/ well were counted. The ratio of the cell count that passed through the Matrigel chamber to the control cell count was defined as the invasion index, expressed as a percentage.

RNA interference (RNAi)

All siRNAs were purchased from Takara Bio Inc. (Otsu, Japan). Cells were transfected with double-strand RNA using TransIT-siQUEST\(^\circ\) transfection reagent (Mirus, Madison, USA) according to the manufacturer's protocol. The OSCC tongue cancer cell line was used for this experiment. Briefly, 1.0 \(\times\) 10\(^5\) OSCC cells were plated in each well of six-well plates and allowed to grow for 24 h, till they reached 50% confluence. Cells were then transfected with siRNA at a concentration of 200 nM using the transfection reagent and serum-free medium. Following 24 h of incubation, serum-rich medium was added. The \(\alpha\)-actinin-4 siRNA sequences were 5'-GGCAGAUGUUCACUGCAUTT-3' and 5'-TTTGCUAAGGCGACGGAUAUA-3'. The scrambled control siRNA sequences were 5'-UCAGGAGCUACCUUAUU-3' and 5'-TTGACUAAGGCGACGGAUAUA-3'. All sequences were submitted to the National Institutes of Health Blast program to ensure gene specificity.
Western blot analysis

Cells were harvested by trypsinization, washed, and precipitated by centrifugation. The Mammalian Cell Extraction Kit (BioVision Research Products, Mountain View, CA) was used for the extraction of proteins. All subsequent manipulations were performed on ice. The cells were incubated in extraction buffer mix. The lysed cells were centrifuged at 15,000 rpm for 3 min and the supernatant was collected as the cytoplasmic fraction. The protein concentration of each sample was measured with micro-BCA protein assay reagent (Pierce Chemical Co. Rockford, USA). Samples were denatured in sodium dodecylsulfate (SDS) sample buffer and loaded onto 12.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane and immunoblotted with anti-α-actinin-4 (4D10, Abnova, Taipei, Taiwan) or anti-β-actin (9F2, Abcam, Cambridge, UK; 0.02 μg/ml). Signals were detected using a horseradish peroxidase-conjugated secondary antibody (ECL antimouse IgG, Amersham Biosciences, Piscataway, NJ; 0.01 μg/ml), and visualized using an ECL Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical analysis

Statistical analysis was performed using StatMate® (ATMS Co., Tokyo, Japan). The associations between α-actinin-4 expression and clinicopathologic features were assessed by Fisher’s exact test. To determine significant prognostic factors related to survival, multivariate analysis was performed using the Cox proportional hazards regression model. Continuous data are given as mean ± standard deviation. Data sets were examined by one-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc test. The correlation between α-actinin-4 mRNA expression and invasion index was determined using Pearson’s correlation coefficient. P values less than 0.05 were considered significant.

Results

Correlation between α-actinin-4 overexpression and clinicopathologic features

Immunohistochemistry with an anti-α-actinin-4-specific monoclonal antibody

<table>
<thead>
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<th>α-actinin-4 overexpression</th>
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<tr>
<td>Normal epithelium</td>
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Fig. 2. (A) Representative Western blot analysis for α-actinin-4 in SAS, SCC25, OSC20, HSC-2, HSC-3, HSC-4, Ca9-22, and HEKa cells. The α-actinin-4/β-actin intensities are the mean ± SD of triplicate experiments. (B) Invasion index (%) was expressed as the ratio of mean number of cells that passed through the Matrigel chamber to mean number of cells that passed through the control chamber, according to the manufacturer’s recommendations. Data are presented as means of three separate experiments, each performed in triplicate; bars, SD. (C) Correlation between α-actinin-4 protein expression and invasion index (%). α-actinin-4 protein expression was highly correlated with invasion index (Pearson’s correlation, r = 0.755; P < 0.05). ●, SAS cells; ▲, SCC25 cells; ○, OSC20 cells; ■, HSC-2 cells; △, HSC-3 cells; ●, HSC-4 cells; □, Ca9-22 cells.
was performed on a series of 64 patients with OSCC. Representative immunohistochemical staining is shown in Fig. 1. Overexpression of α-actinin-4 was undetectable in the normal epithelium (Fig. 1A). In the SCC cells, strong α-actinin-4 staining was apparent at the invasive front and the diffuse invasive area. α-actinin-4 overexpression was detected significantly more frequently in OSCC (38 of 64; 70%) than in normal oral epithelium (0 of 10; 0%; P < 0.001, Table 1). α-actinin-4 overexpression was significantly more frequent in higher grade cancers (grades 3/4 vs. grades 1/2; P = 0.004, Table 1). Cox regression analysis was performed with the parameter of α-actinin-4 overexpression. There was no significant independent predictor of survival (Hazard ratio: 1.50, 95% CI: 0.769–2.94, P value: 0.233).

**α-actinin-4 is associated with OSCC invasion pattern**

The α-actinin-4 protein expression of OSCC cell lines SAS, SCC25, OSC20, HSC-2, HSC-3, HSC-4, and Ca9-22 was analyzed by Western blot analysis. α-actinin-4 protein was detectable in all OSCC cell lines. The α-actinin-4/β-actin intensity ratio varied from 0.151 to 0.289 (Fig. 2A). To characterize the invasion potential of OSCC cells in vitro, Matrigel invasion chambers were used. The highest invasion index was noted in HSC-3, followed by SAS and OSC20 with similar indices; the index was lowest in HSC-2 (Fig. 2B). The α-actinin-4 protein expression level was significantly correlated with.

![Image](image_url)

**Fig. 3.** (A) Representative RT-PCR for the suppression of α-actinin-4 in OSC20 cells. Cells were transfected with scrambled siRNA (control) or α-actinin-4 siRNA. After 72 h, isolated total RNA was analyzed using RT-PCR. (B) Western blot analysis for the suppression of α-actinin-4 in OSC20 cells. Western blot analyses show α-actinin-4 and β-actin expression in the whole cell lysate proteins. (C) The graph shows a significant decrease in the invasion index in OSC20 cells treated with α-actinin-4 siRNA (P < 0.01).
the invasion index of the OSCC cells (Fig. 2C).

Effect of decreasing α-actinin-4 expression on the invasion potential of OSCC20 cells

To determine the effect of decreasing α-actinin-4 expression on invasion potential, OSCC20 cells were transfected with α-actinin-4 siRNA (Fig. 3A,B) and the Matrigel invasion assay carried out. Transfection with α-actinin-4 siRNA significantly decreased the mRNA and protein levels of α-actinin-4, compared with those in non-transfected cells and cells transfected with scrambled siRNA (Fig. 3A, B). Concomitantly, the invasion index of the OSCC20 cells decreased significantly from 77% (in cells treated with vehicle alone) and 84% (in cells transfected with scrambled siRNA) to 39% in cells transfected with α-actinin-4 siRNA (Fig. 3C).

Discussion

The α-actins, which are members of the spectrin gene superfamily, are rod-shaped proteins that bind and crosslink actin12. There are two isoforms of α-actinin: muscular and non-muscular types3. In non-muscle cells, in addition to being involved in actin stress fiber formation, the α-actins play an important role in promoting cell adhesion and in regulating cell shape and motility5,8,12. α-actinin-4 has been proposed as a new marker of motility in non-neoplastic cells and tumor cells, and has also been associated with the metastatic potential of malignant cells1,3. The authors examined the overexpression of α-actinin-4 in biopsy specimens of human OSCC patients using immunohistochemistry. Overexpression of α-actinin-4 is recognized in various cancers. In breast cancer and non-small cell lung cancer, the overexpression and subcellular localization of α-actinin-4 is significantly correlated with poorer prognosis5,12. Increased expression of α-actinin-4 can enhance cell motility and promote lymph node metastasis in colorectal cancer7 and is significantly correlated with advanced tumor stage as well as lymph node metastasis in esophageal cancer5. In ovarian carcinoma, high expression of α-actinin-4, resulting from its cytoplasmic accumulation, is strongly associated with higher histological grade, advanced clinical stage, greater residual tumor, and poor prognosis21. The down-regulation of α-actinin-4 can decrease the invasion potential in pancreatic cancer13. Decreased expression of α-actinin-4 is reported in neuroblastoma and prostate carcinoma2,22. The clinicopathological significance of α-actinin-4 overexpression is controversial. In agreement with previous reports, the present data showed that, from the clinicopathological standpoint, increased expression of α-actinin-4 is significantly correlated with invasion pattern in OSCC. These findings support the conclusion that α-actinin-4 affects the cell motility and invasion of OSCC. As reported in studies finding similar clinicopathological impacts of α-actinin-4 in other carcinomas, α-actinin-4 may be ubiquitously expressed in various malignant tumors and involved in invasion and metastasis. In previous reports, overexpression of α-actinin-4 is correlated with poor prognosis9,15,27. In this study, since there was no correlation between survival date and overexpression of α-actinin-4, α-actinin-4 expression was not a prognostic factor in OSCC patients.

To evaluate the effect of α-actinin-4 siRNA on invasive potential, chemically synthesized α-actinin-4 siRNA was used to achieve direct homology-dependent post transcriptional gene silencing. When the time course experiment of α-actinin-4 siRNA was assessed by RT-PCR, the potential of α-actinin-4 mRNA inhibition peaked at 72 h post transfection, and the obvious effect of α-actinin-4 inhibition was maintained for 5 days at least (data not shown). Since this anti-α-actinin-4 mAb specificity reacts α-actinin-4 on immunoblotting analysis (Abcam, Cambridge), the slightly lower inhibition of α-actinin-4 protein expression compared with mRNA expression might be due to the longevity of the protein rather than cross-reactivity to α-actinin-1. α-actinin-4 siRNA significantly inhibited the invasiveness of OSCC20 cells. The invasion of malignant tumors is mediated by interactions between epithelial cancer cells and the extracellular matrix. The behavior of malignant tumor cells infiltrating the stroma may be affected by interactions with the extracellular matrix24. Dynamic regulation of the actin cytoskeleton by the various classes of actin-binding proteins plays a crucial role in cell movement28. In colorectal cancer, immunofluorescence microscopy revealed that α-actinin-4 and β-catenin were colocalized in bleb-like membrane protrusions on the free surface of tumor cells13. E-cadherin is reported to regulate this association between α-actinin-4 and β-catenin, and the dynamic shift of β-catenin from the cell adhesion complex into a complex containing α-actinin-4 may evoke cell movement and mediate cancer invasion and metastasis13. In the present study, the down-regulation of α-actinin-4 significantly reduced the invasion potential of OSCC20 cells in vitro. The authors did not directly demonstrate the association between α-actinin-4 and the extracellular matrix proteins, such as E-cadherin and β-catenin, but the disruption of such interactions may be involved in enhancing the invasion potential of OSCC20 cells.

RNAi technology is a specific and powerful tool to turn off the expression of oncogenic target genes26. In oral cancer, the possibility of RNA-mediated gene therapy has been reported19,28. The authors successfully applied RNA silencing to inhibit the expression of α-actinin-4, thereby decreasing the invasion potential of OSCC. They propose that RNAi-mediated gene silencing of α-actinin-4 might be a useful modality for OSCC treatment.

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Competing interests
None declared.

Ethical approval
Not required.

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