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Journal	Oncology reports, 20(6): 1353-1357
URL	http://hdl.handle.net/10130/1119
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Inhibition of syndecan-1 expression and function in oral cancer cells

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Received April 16, 2008; Accepted July 31, 2008

DOI: 10.3892/or_00000151

Abstract. Syndecan-1 has been shown to be a prognostic factor in various types of tumors, suggesting its correlation with malignancy and metastasis. In the present study, we examined the expression of syndecan-1 in oral cancer cell lines and tested whether transfection of an siRNA against human syndecan-1 affected the malignant potential of these cells. Seven different human oral cancer cell lines (HSC2, HSC3, HSC4, Ca9-22, SAS, KB and BSC-OF) were used. To evaluate the mRNA expression of syndecan-1 in these cell lines, quantitative real-time RT-PCR (QRT-PCR) was carried out. In order to examine syndecan-1 function, siRNA was transfected into the cells, after which the cell growth rate and invasive ability were evaluated. As a negative control, a random sequence siRNA was used. QRT-PCR showed that syndecan-1 was expressed in Ca9-22 cells and that it was significantly higher (>10-fold) than in the other oral cancer cell lines. Transfection of syndecan-1 siRNA was carried out on Ca9-22 cells, which increased their growth rate 1.4-fold above controls. The invasive ability of Ca9-22 cells treated with syndecan-1 siRNA was significantly higher (2-fold; n=5) than the controls. These results suggest that Ca9-22 oral cancer cells are a useful model to study syndecan-1 function and they show that syndecan-1 directly contributes to the growth and invasive ability of these cells.

Introduction

Oral cancer is the fifth most common type of cancer in the world. Despite modern intervention, the 5-year survival rate for this disease has improved only marginally over the past

decade (1) and recurrent disease is observed in 50% of patients (2,3). Survival curves of oral cancer patients have plateaued over the past 2 decades and remain among the worst of all cancer sites. Therefore, recent studies in this field have focused on the development of biomarkers for early detection, disease monitoring and determining the prognosis of patients with oral cancer (4,5).

Cell surface adhesion receptors bind cells to the extracellular matrix (ECM) and couple such interactions with intercellular signaling mechanisms (6). It is apparent that alterations in cell adhesion can influence almost every stage of cellular transformation. The development of malignant epithelial neoplasms is associated with the disruption of cell-to-cell and cell-to-matrix adhesion (7,8). Syndecans are a family of heparan sulphate proteoglycan receptors that participate both in cell-to-cell and in cell-to-matrix adhesion (9). The syndecan family is composed of four closely related proteins (syndecans-1 to 4) which are encoded by four different genes (9). Syndecan-1 binds cells via its heparan sulphate chains to a variety of components of the interstitial matrix, including types I, III and V collagen, fibrillar collagen (10), fibronectin (11) and tenascin (11). Earlier studies noted that syndecan-1 levels correlate with malignancy in various tissues including the uterine cervix (12,13) and the esophagus (14). Studies on syndecan-1 expression in head and neck carcinomas have suggested that reduced expression of syndecan-1 is associated with the prognosis of these neoplasms (15,16). Ro *et al* (17) reported that a reduction of syndecan-1 expression correlated with tumor size and invasion in squamous cell carcinomas of the tongue. However, expression levels and function(s) of syndecan-1 in oral cancers remain unknown. In the present study, we examined syndecan-1 expression in a number of oral cancer cell lines and used the siRNA approach to characterize the function(s) of syndecan-1 in these cells.

Materials and methods

Cell cultures. Human oral squamous cell carcinoma (SCC) cell lines, HSC2, HSC3, HSC4, SAS and KB, were obtained from

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Key words: oral cancer, invasion, syndecan-1, squamous cell carcinoma

Table I. Sequences of primers used in this study.

	Primer sequences	Product size
Syndecan-1	F: 5'-AGGACGAAGGCAGCTACT CCT-3' R: 5'-TTTGGTGGGCTTCTGGTAGG-3'	71 bp
GAPDH	F: 5'-GTCATGGGTGTGAACCATGAGAA-3' R: 5'-TGGTCATGAGTCCTTCCACGAT-3'	136 bp

the Japanese Cancer Research Resources Bank (JCRB). BSC-OF was established from an oral basaloid squamous cell carcinoma by Abiko *et al* (18). All cell lines were grown in Dulbecco's modified Eagle medium (DMEM) or in DMEM/F-12 (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and penicillin-streptomycin (100 U/ml, Invitrogen) at 37°C in a humidified incubator containing 5% CO₂.

RNA isolation and quantitative real-time RT-PCR (QRT-PCR). Total RNA was extracted using the acid guanidinium-thiocyanate-phenol-chloroform (AGPC) method with TRIzol (Invitrogen) and cDNA synthesis was performed as described in our previous study (19). QRT-PCR was carried out using SYBR-Green and AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) according to our previous study (19). Primers for human syndecan-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used; the sequences of the primers are shown in Table I. Amplification was then performed in duplicate using the primer sets in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems) with denaturation for 10 min at 95°C, followed by 40 PCR cycles of denaturation at 95°C for 15 sec and annealing or extension at 60°C for 1 min. The threshold cycle (Ct) value for each reaction was calculated and the relative quantitation ($\Delta\Delta$ Ct method) was carried out as described in our previous studies (19,20). The amplified products were analyzed by 1.7% agarose gel electrophoresis and were visualized by ultraviolet illumination after staining with ethidium bromide.

Immunofluorescence. Cells were cultured on cover glasses and then were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The specimens were washed in phosphate-buffered saline (PBS) and were blocked in 10% normal goat serum. They were then incubated with anti-human syndecan-1 antibody (1:500, Dako, Copenhagen, Denmark) at room temperature for 60 min. After washing in PBS, Alexa488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA) was used as a secondary antibody and the specimens were observed using a fluorescence microscope (Axiophot2, Zeiss, Oberkochen, Germany).

siRNA transfection. In order to knock down syndecan-1 function in the cells, an siRNA insert of syndecan-1 was designed using a website named siRNA Target Finder (Ambion, Austin, TX, USA). Since the siRNA target sequence of syndecan-1 was 5'-AACTTCTCCGGCTCAGG TGCA-3', synthetic top strand oligonucleotide templates of

the insert for syndecan-1 siRNA: 5'-CTTCTCCGGCTCAG GTGCATTCAAGAGATGCACCTGAGCCGGAGAAGTT TTTT-3' and the bottom strand oligonucleotide template: 5'-AATTAATAAACTTCTCCGGCTCAGGTGCATCTCT TGAATGCACCTGAGCCGGAGAAGGGCC-3' were incubated with annealing buffer (TEN buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.6 and 100 mM NaCl) at 95°C for 5 min, then cooled to 37°C and incubated for 1 h at 37°C. Synthetic oligonucleotides of syndecan-1 were inserted into the *Apal* and *EcoRI* sites of the pSilencer™ 1.0-U6 vector (Ambion). The plasmid and pSilencer 1.0 Negative Control (Ambion) were transfected into the cells using the lipofection method (Effecten™, Qiagen, Hilden, Germany) according to the manufacturer's protocol. Decreased expression of syndecan-1 in the cells was confirmed by RT-PCR.

Cell growth rate. The cells were seeded at 1.0x10⁵ cells/ml into 35-mm plates. The cells were detached using 0.25% trypsin every 24 h and were counted using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA). Results were analyzed and growth curves were plotted.

Invasion assay. The invasion assay was carried out according to our previous studies (19,21). Aliquots of 1x10⁵ cells were plated onto 6-well BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences, San Jose, CA, USA) and were cultured for 24 h. Cells digested the Matrigel, enabling them to pass through the micro-pores in the gel, after which they were fixed and stained. The numbers of invasive cells were counted using a light microscope per 1 mm² in the central area of the Matrigel. The assays were repeated three times and results are shown as mean \pm SD.

Results

Expression of syndecan-1 mRNA in human oral carcinoma cell lines. To validate the high expression levels of syndecan-1 in human oral cancer cell lines, QRT-PCR was carried out. Based on the $\Delta\Delta$ Ct relative to KB cells, the relative expression levels of syndecan-1 mRNA in oral carcinoma cell lines were calculated. Several cell lines showed expression of syndecan-1 at high levels. In particular, syndecan-1 was expressed in Ca9-22 cells at a higher level (13.2-fold) than in KB cells (Fig. 1A). Moreover, relatively higher expression levels of syndecan-1 were seen in BSC-OF and in HSC3 cells (7.6-fold).

Immunofluorescence analysis showed that positive reactions for syndecan-1 were observed strongly at the cell membrane of Ca9-22 cells (Fig. 1B) while diffuse faint dot reactions were seen in KB cells (Fig. 1C). Based on those

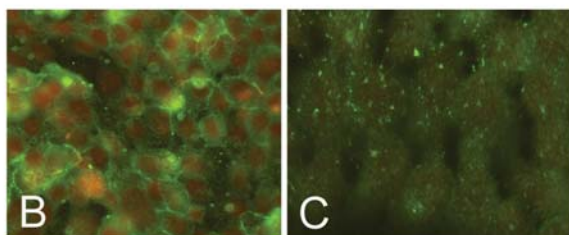
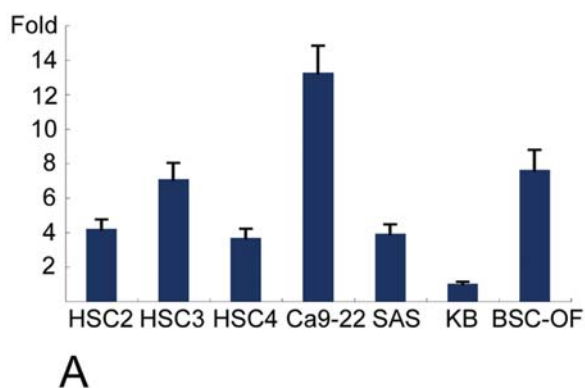


Figure 1. Expression of human syndecan-1 in oral carcinoma cells. (A) QRT-PCR analysis shows that syndecan-1 is expressed in Ca9-22 cells at a higher level (13.2-fold) than in KB cells. Moreover, a relatively higher expression level of syndecan-1 is seen in BSC-OF cells and in HSC3 cells (7.6-fold). The $\Delta\Delta C_t$ relative to KB cells is considered as 1. (B and C) Immunofluorescence analysis shows that positive reactions for syndecan-1 are strongly observed at the cell membrane of Ca9-22 cells (B) while diffuse faint dot reactions are seen in KB cells (C).

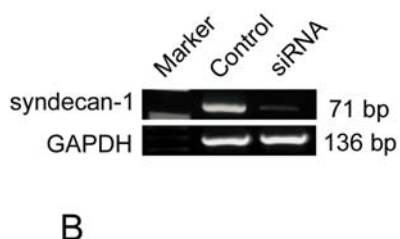
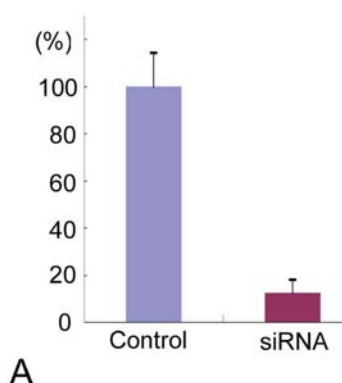


Figure 2. Efficiency of human syndecan-1 siRNA. (A) In order to characterize the effects of syndecan-1 siRNA, QRT-PCR analysis was performed on mRNAs from non-transfected (control) and from siRNA-transfected Ca9-22 cells. After 48 h incorporation, decreased expression (1/10-fold) of syndecan-1 was seen in the siRNA-transfected cells, suggesting that siRNA blocks syndecan-1 expression very efficiently in Ca9-22 cells. (B) Electrophoresis analysis confirming a decline in syndecan-1 in siRNA-transfected Ca9-22 cells.

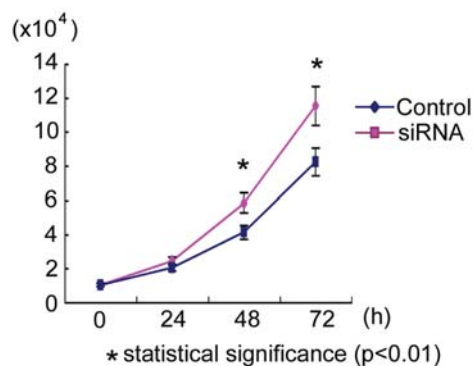


Figure 3. Cell growth rates. Ca9-22 cells transfected with syndecan-1 siRNA had increased growth. The growth rate of control cells was lower than the siRNA-treated cells at 48 and at 72 h. The growth rates were significantly different between the siRNA and the control groups at 48 and at 72 h ($p < 0.01$).

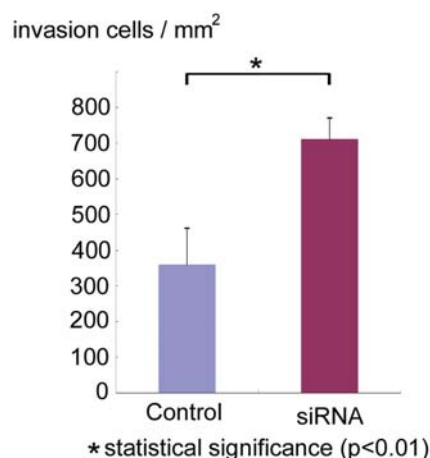


Figure 4. Invasion assay. The Matrigel assay was used to investigate the effects of syndecan-1 on the invasive ability of Ca9-22 cells. The number of invading cells was significantly higher in the siRNA-transfected group ($p < 0.01$).

QRT-PCR and immunofluorescence results, we used Ca9-22 cells as a model for high expression of syndecan-1 in this study.

Effects of human syndecan-1 siRNA. In order to characterize the effects of syndecan-1 siRNA, we carried out QRT-PCR analysis using mRNAs from non-transfected (control) and from transfected Ca9-22 cells. After 48 h of incorporation, decreased expression (1/10-fold) of syndecan-1 was seen in the siRNA-transfected cells, suggesting that siRNA blocked syndecan-1 expression very efficiently in Ca9-22 cells (Fig. 2A). Electrophoretic analysis confirmed that a decline of syndecan-1 was observed in transfected Ca9-22 cells (Fig. 2B).

Cell growth. To examine whether syndecan-1 is associated with cell growth, the growth of siRNA-transfected cells and control cells was measured. Ca9-22 cells had increased growth after syndecan-1 siRNA transfection. The numbers of siRNA-transfected Ca9-22 cells and control cells at 48 h were $6.0 \pm 0.4 \times 10^5$ and $4.1 \pm 0.3 \times 10^5$ (average \pm SD), respectively and at 72 h were $11.8 \pm 1.4 \times 10^5$ and $8.1 \pm 0.5 \times 10^5$,

respectively. The cell growth rate of control cells was lower than the siRNA-transfected cells at 48 and at 72 h (Fig. 3) and were significantly different at both time points ($p < 0.01$).

Invasion assay. To examine the effect of syndecan-1 on the invasive ability of Ca9-22 cells, a Matrigel assay was used. The numbers of invasive siRNA-transfected and control Ca9-22 cells were 718.4 ± 64.7 per mm^2 and 378.8 ± 102.1 per mm^2 (average \pm SD), respectively, which was significantly higher ($p < 0.01$) (Fig. 4).

Discussion

Syndecan-1 has been reported to be a prognostic factor for tumor progression and survival in various types of malignant tumors, which suggests a close correlation of syndecan-1 expression with malignancy and metastasis (16,22). In general, transformed cells are often characterized by an abundant secretion of syndecan-1, which results in metastasis formation (23,24). Earlier studies associated syndecan-1 levels with prognosis and have suggested syndecan-1 as a candidate biomarker for the malignant potential of head and neck tumors (16). Our earlier study showed that syndecan-1 contributed to their malignant behavior including changes in growth and invasive ability (17). However, expression levels and function(s) of syndecan-1 in oral cancers have not been clarified, and therefore, we examined that in this study using siRNA to block syndecan-1 functions. Our results show that three oral cancer cell lines, Ca9-22, BSC-OF and HSC3, express syndecan-1 at high levels compared to KB cells. Ca9-22 cells are therefore a useful model for elucidating the function of syndecan-1 in oral cancers. Furthermore, our results show that the siRNA used in this study is specific and is effective for blocking syndecan-1 expression and thus will be helpful for analyzing the function(s) of syndecan-1 in oral cancer cells.

Our previous study showed that the reduction of immunoreactivity for syndecan-1 in oral squamous cell carcinoma cells was associated with tumor size, suggesting that syndecan-1 contributes to malignant behavior including changes in growth and invasive ability. Recent studies have shown that a reduction of syndecan-1 expression was associated with proliferative activity (Ki-67 expression) (25). Furthermore, Su *et al* reported that shedding of syndecan-1 by stromal fibroblasts stimulated the proliferation of human breast cancer cells via activation of FGF2 (26). Our results show that the reduction of syndecan-1 function by siRNA leads to higher levels of cell proliferation, which suggests that syndecan-1 is directly associated with cell proliferation.

Cell migration has been reported to influence invasiveness and to be an important factor in the incidence of metastasis. Moreover, the invasive ability of tumors is closely related to the incidence of metastasis and the prognosis of the disease. As shown in previous studies, reduced expression of syndecan-1 correlates with metastasis of various tumors. There have been only a few studies that showed a correlation between syndecan-1 and invasion in oral cancers, but there has been no previous functional study of syndecan-1 using an oral cancer cell line. Therefore, we examined whether syndecan-1

was associated with the invasive ability of Ca9-22 cells. Our results show that invasiveness increased when syndecan-1 function was blocked in siRNA-transfected Ca9-22 cells. The expression of syndecan-1 is known to suppress the level of matrix metalloproteinase (MMP)-9 and to inhibit cell invasion into type I collagen (27,28). Moreover, syndecan-1 can be degraded by heparanase (29), and invasion is associated with MMPs (30) and heparanase activities (31). The syndecan-1 siRNA may induce MMPs and heparanase activity and thus reduce the expression of syndecan-1 in Ca9-22 cells.

Acknowledgements

This study was supported, in part, by the Japanese Ministry of Education, Culture, Sports, Science and Technology, Grant-in-Aid No. 14657489.

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