Original Article

CD26 expression in oral squamous cell carcinoma and its potential role in modulating metastasis

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Abstract: Previous studies on CD26 in different types of cancer, including ovarian, prostate, and thyroid cancer among others, have yielded varying results. However, the role of CD26 in oral squamous cell carcinoma (OSCC) is not fully understood. Thus, the aim of our study was to investigate the effect of CD26 expression on lymph node metastasis in OSCC and to explore its potential mechanism. The expression of CD26 in OSCC tissues was evaluated by immunohistochemistry. CD26 was down-regulated by RNA interference. The protein and mRNA levels of CD26 were detected by western blot and RT-PCR, respectively. High content screening was used to determine tumor cell motility and invasion potential. Our results showed that 57.8% of tissue samples from primary carcinoma with lymph node metastasis stained positively for CD26 expression compared with tissues without lymph node metastasis. In addition, expression of CD26 was significantly higher in highly metastatic cell lines than in cell lines without metastatic ability. In vitro suppression of endogenous CD26 expression by siRNA in the highly metastatic cell lines significantly reduced cell motility and invasion. Furthermore, siRNA-mediated inhibition of CD26 also led to up-regulation of E-cadherin and down-regulation of MMP-9 expression in the highly metastatic cell lines. These findings indicated that CD26 was expressed at a significantly higher level in OSCC tissues with lymph node metastasis than in OSCC tissues without lymph node metastasis. Additionally, CD26 modulates the expression of E-cadherin and MMP-9, and this may be the mechanism by which it regulates cell migration and metastasis in OSCC.

Keywords: Oral squamous cell carcinoma, CD26, E-cadherin, MMP-9, neoplasm metastasis

Introduction

Oral squamous cell carcinoma (OSCC) ranks sixth worldwide for cancer-related mortality, with an estimated 500,000 new cases diagnosed yearly [1]. Although recent molecular studies have advanced our understanding of the disease and provided a rationale for the development of novel therapeutic strategies, OSCC is still associated with severe mortality. The 5-year survival rate for OSCC has not improved in more than 30 years [2]. The major circumstances that contribute to low survival include local regional relapse, lymph node or distant metastatic spread of the primary tumor, and other factors.

Tumor progression to the invasive and metastatic stage represents the most ominous challenge in the management of OSCC. OSCC metastasis is a complex process associated with multiple biochemical and genetic changes. Increased cell motility and invasive growth are considered to be important events during the metastatic cascade. Degradation of the extracellular matrix and tumor angiogenesis may also contribute to OSCC metastasis. Several proteins have been studied in OSCC metastatic disease, such as BMP-2/4 [3], MDC1 [4], S4S8-RPA [5], and NRP1 [6], HMGA2 [7]. However, the actual mechanisms driving OSCC metastasis remain unclear.

CD26 is a 110 kDa cell surface glycoprotein with intrinsic dipeptidyl peptidase IV (DPP IV) activity and is expressed on a variety of cell types, including T lymphocytes, endothelial cells, and epithelial cells. CD26 is known to have multiple biological functions [8, 9]. Recent work suggested that CD26 plays a significant role in tumor pathogenesis and progression [10], although previous studies on CD26 have yielded varying
results in different cancers. Preclinical studies showed that increased CD26 expression inhibited metastasis in ovarian cancer [11], whereas suppression of CD26 promoted metastasis in prostate cancer [12]. On the other hand, inhibition of CD26 in renal cell carcinoma decreased tumor growth and reduced the binding of the cancer cells to fibronectin and collagen [13]. Moreover, clinical studies in thyroid cancer and gastrointestinal stromal tumors suggested that CD26 expression was associated with distant metastasis or recurrence after resection [14, 15]. The pleiotrophic effects of CD26 may account for its varied roles in different cancers [9]. In colorectal cancer, significantly higher levels of circulating CD26 have been observed in patients with metastatic disease [16]. Recent studies suggested that down-regulation of CD26 reduced the migration and invasion capacities of CD26+ cells as well as their binding to fibronectin and collagen, which appeared to be mediated at least in part through the dephosphorylation of integrin β1 [17]. These data suggest that CD26 may play a functional role in the invasive capacity of the CD26+ cancer stem cells (CSCs) [18]. The detection of circulating CD26+ cells in the portal veins of mice after cecal wall injection demonstrated that CD26+ cells were capable of invasion via the ECM and intravasation into the circulation. Furthermore, clinical studies showed metastatic growth in the liver after intraportal vein injection of CD26+ cells, suggesting that CD26+ cells are capable of initiating growth of metastasis in distant organs.

These data further support the metastatic capacity of CD26+ CSCs, however, the function of CD26 in oral squamous cell carcinoma (OSCC) has not been fully understood. Thus, we hypothesized that CD26 may be involved in OSCC metastasis. We first studied the expression level of CD26 in both metastatic and primary OSCC cells and then correlated the expression levels of CD26 with cell motility and invasion. siRNA was also used to knock down CD26 expression in metastatic OSCC cells to determine its function in metastasis.

Materials and methods

Patients and specimens

All tumor tissue surgical specimens were collected from NO. 9 Hospital of Shanghai Jiao Tong University, Shanghai, China, between 2010 and 2013. A total of 147 OSCC patients, including 90 men and 57 women (aged from 37 to 78 years, mean age 58.6 years) were enrolled in this study. Pathologists examined and confirmed the diagnoses of all tumor tissues. The TNM stage was assessed according to the American Joint Committee on Cancer TNM classification of oral carcinoma (7th ed., 2010). The severity of lymph node metastasis was divided into strong positive (N0), weak positive (N1) and negative (N2-N3). None of the patients received chemotherapy or radiotherapy before surgery. All patients consented to the use of their tumor tissue for clinical research, and this study was approved by the Ethical Committee of NO. 9 Hospital. All patients were followed-up twice a year until July 31, 2013.

Immunohistochemical staining

All samples were fixed with 4% formaldehyde, embedded in paraffin, and sectioned at 5 μm thickness. The sections were deparaffinized in xylene and dehydrated with alcohol. Endogenous peroxidase activity was blocked for 15 min with 3% hydrogen peroxide in phosphate-buffered saline (PBS). Antigen retrieval was performed with 0.05% saponin for 10 min in a microwave oven. Nonspecific binding was prevented by 5% bovine serum albumin for 30 min at room temperature. Rabbit monoclonal anti-CD26 antibody (1:300, Abcam, USA) was used as primary antibody overnight at 4°C. The sections were then incubated with anti-rabbit IgG-horseradish peroxidase (1:2,000, Zhongshan, China) for 1 h at 37°C. Binding sites were visualized using standard DAB reagents, and the cell nuclei were counterstained with hematoxylin. After staining, the slides were observed via microscopy by three different researchers. The intensity of CD26 expression in immunohistochemical staining slides was scored as 0 (colorless), 1 (light yellow), 2 (brown yellow) or 3 (brown) based on the staining intensity and slides color, while the proportion of positive cells was ranked as 0 (0%), 1 (0-30%), 2 (30-60%) or 3 (> 60%). The two scores were added to obtain the final results as follows: negative, 0; weak, 1-2; moderate, 3-4; and strong, 5-6.

Cell lines and culture

The highly metastatic human OSCC cell lines SCL138-P and CAL27-P and the non-metastatic cell lines SCL138-N and CAL27-N were genetically constructed. The cells were mainatin-
ed in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin in a humidified atmosphere of 5% CO₂ at 37°C, with the media replaced every 3 days. The cells were used for experiments after 14 days of culture. During cell culture, cell density was monitored to prevent excessive growth and was maintained 70% or less confluence.

**Western blot analysis**

Proteins were extracted from total cell lysates using cell lysis buffer (Sigma). The total proteins were separated on 8% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk at room temperature for 1 h and then incubated overnight at 4°C with the primary antibody against CD26 (1:300 dilution, Abcam), E-cadherin (1:100, Santa Cruz Biotechnology), MMP-9 (1:200, Cell Signaling Technology), MMP-2 (1:200, Cell Signaling Technology) and β-actin (1:2,000 dilution Sigma). After several washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies (1:3,000 Sigma) for 1 h at room temperature. Immunoblots were detected with an enhanced chemiluminescence system (Amersham) according to the manufacturers’ protocol.

**Small interfering RNA construction and transfection**

Lentivirus-mediated small interfering RNA against CD26 and control siRNA were constructed by Shanghai Gene Chem Co, Ltd. The CD26 siRNA sequences used were sense 5'-CGTGCAACATGACAAGtt-3' and antisense 5'-CCGGAACTCGTCACTTCCAGAGGtt-3', and those of the non-silencing control were according to a previous publication. For transfection, SCL138-P and CAL27-P cells were plated into 24-well plates. When the cells were 40% confluent, the CD26-siRNA-lentivirus and control-siRNA were transfected into cells using transfection reagents. Cells containing the siRNA constructs were named SCL138-P-si, CAL27-P-si, SCL138-P-control and CAL27-P-control. These cells were used for western blot analysis, RT-PCR and transwell experiments. To assess the metastasis ability of these cell lines, 1 x 10⁶ cells of SCL138-P-si or SCL138-P-control and CAL27-P-si or CAL27-P-control were injected into immune-compromised nude mice (ten mice for each group) through the tail vein. After five weeks of injection, the mice were sacrificed. The lung and liver tissues were obtained for macroscopic observation for the number of metastatic tumor formation, and serial sections of the tissues were prepared for pathological assessment of metastasis under an Olympus light microscope.

**RNA isolation and real time RT-PCR**

Total RNA was extracted from the differentiated cells with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA), and cDNA was synthesized with a First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. Then, RT-PCR was performed in triplicate on a sequence detection system (ABI Prism 7000; Life Technologies/Applied Biosystems, Inc., Foster City, CA, USA). The CD26 and GAPDH primers were synthesized by Takara. The sequences were as follows: CD26 forward: 5'-CGCTGGCCACCCTGCAAGATA-3' and CD26 reverse: 5'-CCGGAACTCTCGATGTGGTA-3'; GAPDH forward: 5'-GCCGGCAAGGCTGAGAAC-3' and GAPDH reverse: 5'-TGTTGAAGACGCCAGTGGA-3'. Quantitative real-time PCR was performed using the SYBR premix Ex Taq (Takara) on the CFX96 Touch TM PCR system (Bio-Rad). The experiment was performed in triplicate.

**High content screening for cell motility**

High content screening was used to evaluate cell motility. The GFP-labeled cells, which were transfected with CD26-siRNA-lentivirus or control-siRNA, were seeded into a 96-well plate. Each cell type was seeded into a total of ten wells. After the cells adhered to the wells, they were incubated in a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific) in a humidified atmosphere of 5% CO₂ at 37°C. The cells were cultured in a medium containing the aforementioned concentrations of OMT, TMZ, or 1% DMSO for 10 hours. Cell motility was then measured using the ArrayScan HCS Reader (Thermo Fisher Scientific In, USA) by following the manufacturer’s protocol. Images were collected on the Cellomics HCS Reader and analyzed using the Cell Motility BioApplication. The
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The SPSS18.0 software program (Chicago, IL, USA) was used for statistical analysis. Values of \( P < 0.05 \) were considered statistically significant.

**Results**

**CD26 is highly expressed in OSCC and correlates with some clinical features**

CD26 expression in primary OSCC tissues from 147 patients was determined by immunohistochemistry. A strong staining pattern for CD26 was observed in primary carcinoma tissues with lymph node metastasis, whereas CD26 expression was weak in the tissues without lymph node metastasis. CD26 was mainly expressed in the cytoplasm of OSCC cells from patients with lymph node metastasis (Figure 1). Among the 147 cases, 85 primary tumors (57.8%) stained positive for CD26, and of these, 43 were strongly positive, 30 were moderately positive and 12 were weakly positive (Figure 1). Next, we used univariate analysis to evaluate the relationship between the level of CD26 expression and patient clinical features, including age, gender, size, histological cell type, location, UICC stage, postoperative T stage and lymph node metastases. As shown in Table 1, there was no significant association between CD26 and these clinical features except for lymph node metastases and UICC stage \( (P < 0.05) \). Approximately 53.7% (79/147) of the patients were in stage II; therefore, the predictive value of CD26 expression for the LN metastases in stage II patients is critical for individual therapy. The entry conditions of Cox multivariate analysis were related to factors with \( p \) value \( \leq 0.10 \) of univariate analysis, and our multivariate Cox regression analysis showed that the patients with higher CD26 expression had a greater risk of developing lymph node metast-
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<td>≥ 50</td>
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Table 1. Relationship between CD26 expression and clinical features of OSCC patients

tases (N1-3 vs N0; p < 0.05; hazard ratio, 2.469; 95% confidence interval, 1.176-7.406).

Relationship between the level of CD26 expression and survival rate

To determine whether the survival of OSCC patients was associated with CD26 expression, we analyzed level of CD26 expression and its relationship with patient survival length. As shown in Figure 1E, the survival rate of patients with weak, moderate or strong CD26 expres-
sion was lower than that of patients who were negative for CD26 expression. The log-rank test revealed that the survival lengths of patients with negative, weak, moderate or strong CD26 expression were 34.23 ± 1.98, 27.54 ± 2.78, 22.64 ± 1.94 and 18.07 ± 2.62 months, respectively (P = 0.008).

Expression of CD26 in highly invasive and non-invasive cell lines

Highly invasive and non-invasive OSCC cell lines were established using a repeat trans-well assay. The highly metastatic cell lines were SCL138-P and CAL27-P, and the non-metastatic cell lines were SCL138-N and CAL27-N. CD26 expression in these highly metastatic and non-metastatic cell lines was determined by western blot and RT-PCR. Compared with the non-metastatic cell lines, SCL138-P and CAL27-P both had increased protein levels of CD26. Expression of CD26 mRNA in the highly metastatic cell lines SCL138-P and CAL27-P was 2.41-fold and 1.67-fold of that in SCL138-N and CAL27-N, respectively, as determined by RT-PCR (Figure 2). These findings suggested that CD26 may contribute to tumor metastasis.

Suppression of CD26 resulted in inhibition of in vitro tumor invasion

Cell motility is often used to determine cancer cell metastatic ability. In the present study, a high content screening assay was used to quantitatively track the length of each cell as an indicator of cell motility. To better understand the potential function of CD26 in tumor cell migration and invasion, the highly metastatic cell lines SCL138-P and CAL27-P were infected with lentivirus containing either a siRNA targeting CD26 or a non-specific scramble siRNA and were specified as SCL138-P-si, CAL27-P-si and SCL138-P-control and CAL27-P-control, respectively. As shown in Figure 3A-D, CD26 protein and mRNA expression in SCL138-P-si and CAL27-P-si were dramatically suppressed by siRNA as demonstrated by western blot and RT-PCR. Furthermore, suppression of CD26 by siRNA in SCL138-P-si or CAL27-P-si led to a significant decline in cellular motility when compared with the cells transfected with control siRNA (P < 0.001), which showed that suppression of CD26 resulted in inhibition of in vitro tumor invasion (Figure 3E, 3F). Besi-
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Figure 2. Expression of CD26 in OSCC cell lines. A, B. CD26 protein levels in highly metastatic cell lines (SCL138-P and CAL27-P) as well as non-metastatic cell lines (SCL138-N and CAL27-N). C, D. Level of CD26 mRNA expression in highly and non-metastatic cell lines by RT-PCR. *P < 0.05, **P < 0.01.

des, mice injected with SCL138-P-control and CAL27-P-control had a significantly higher number of metastatic tumor formations in the lung than the mice injected with SCL138-P-si or CAL27-P-si as observed macroscopically as well as microscopically (Figure 4).

The potential mechanism of CD26 association with metastasis

CD26 is known to modulate expression of genes that may contribute to cancer metastasis. Therefore, to further explore the CD26-mediated mechanism OSCC metastasis, the levels of E-cadherin, MMP-2 and MMP-9 were determined by immunoblot in the highly metastatic cell lines SCL138-P and CAL27-P following siRNA-mediated suppression of CD26. As shown in Figure 5, the expression levels of E-cadherin and MMP-9, but not that of MMP-2, were significantly altered in the SCL138-P-si and CAL27-P-si when compared with SCL138-P-control and CAL27-P-si, respectively.

Discussion

Distant metastasis is one of the leading causes for poor clinical outcome in patients, and thus, it is one of the biggest challenges for cancer treatment of primary tumors. It has been reported that distant metastases are frequently present in patients with oral squamous cell carcinoma (OSCC), but the mechanisms underlying this process remain undefined [19]. Biomarker identification is therefore necessary to predict patient prognosis and guide clinical therapy [20]. In the present study, we demonstrated that 85 out of 147 primary OSCCs (total 57.8%) stained positively for CD26 and that CD26 expression was inversely correlated with patient survival. Furthermore, we found that highly invasive cells contain higher levels of CD26 and that suppression of CD26 by siRNA results in loss of invasive cellular features by in vitro assay. Suppression of CD26 also leads to significant alterations in E-cadherin and MMP-9 expression in the highly invasive cells.
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To the best of our knowledge, this report is the first study to demonstrate CD26 expression in oral squamous cell carcinoma and its potential role in modulating metastasis.

Augoff et al. reported that CD26 was an independent prognostic factors along with distant lymph node metastasis in esophageal squamous cell carcinoma [21]. Consistent with this report, we also previously identified genes associated with lymph node metastasis by gene expression profiling assays and showed that CD26 expression was much higher in the tumors with lymph node metastasis than in those without lymph node metastasis. In the present study, we further demonstrated by immunohistochemistry that CD26 was over-expressed in OSCC tissues with lymph node metastasis compared with the tissues without lymph node metastasis. In addition, CD26 was more strongly expressed in the highly metastatic cell lines EC190-P and CAL27-P than in the non-metastatic cell lines SCL138-N and CAL27-N. An in vitro high content screening assay demonstrated that the highly metastatic cell lines EC190-P and CAL27-P partially lost their invasion and motility abilities when the cells were transfect-
Epithelial-mesenchymal transition (EMT) is a phenotypic alteration acquired by cancer cells in which cells lose cell-cell adhesion and polarity and become migratory and invasive [22]. E-cadherin, which is an epithelium biomarker, is remarkably decreased during the EMT process. CD26 may regulate the expression of E-cadherin and may enhance the motility and invasive ability of cancer cells [23]. Degradation of the extracellular matrix by MMPs is also involved in cell migration and invasion processes and is believed to play a critical role in initiating distant metastasis. Of the MMP family members, MMP-2 and MMP-9 are expressed in OSCC, and their expression correlates with OSCC invasion [24, 25]. Additionally, it has been reported that CD26 is involved in the regulation of MMPs in ovarian carcinoma cells [26]. The role of CD26 in modulating MMPs and E-cadherin in OSCC, however, has not been reported. Therefore, the effect of CD26 on the expression of E-cadherin, MMP-2 and MMP-9 in OSCC cell lines was investigated. Consistent with previous reports of other cancer cell types, E-cadherin was weakly expressed in the highly metastatic OSCC cell lines and was profoundly up-regulated when CD26 was suppressed by siRNA in these cells. In contrast, MMP-9 was highly expressed in the highly metastatic OSCC cell lines and dramatically down-regulated in the cells lacking CD26. MMP-2 expression, however, was not altered by CD26-siRNA. Although the mechanism of CD26 regulation on E-cadherin and MMP-9 remains to be defined, our findings indicate that CD26 modulates OSCC migration and invasion through modulating expression of E-cadherin and MMP-9, which are two important proteins that trigger cancer cell migration.

Figure 4. Representative photos of lung parenchymal tissues with or without metastasis. Lung tissue slides were prepared and stained with hematoxylin and eosin (H&E). A. Lung tissue obtained from mice injected with SCL138-P-si, showing no metastasis. B. Representative image of lung dissected from mice injected with SCL138-P, showing a metastatic nodule in lung parenchyma (× 10).

Figure 5. Expression of E-cadherin, MMP-9 and MMP-2 in highly metastatic cell lines transfected with CD26-siRNA or control siRNA. A. Cells were transfected with lentivirus containing scramble siRNA or a siRNA targeting CD26 as described in the “Materials and Methods” section. Total cell lysates were immunoblotted for E-cadherin, MMP-9, MMP-2 and β-actin as loading control. SCL138-P-control or CAL27-P-control: cells were transfected with scramble siRNA; SCL138-P-si or CAL27-P-si: cells were transfected with a siRNA targeting CD26. B and C. Level of E-cadherin, MMP-9 and MMP-2 expression in highly metastatic cell lines transfected with CD26-siRNA or control siRNA. *P < 0.05.
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This mechanism is consistent with the roles of E-cadherin and MMP-9 in the metastasis of adenocarcinoma such as hepatic carcinoma and colorectal cancer [27]. Therefore, we hypothesize that level of E-cadherin, MMP-2 and MMP-9 be determined by immunoblot in the highly metastatic cell lines SCL138-P and CAL27-P following suppression of CD26 by siRNA. The expression levels of E-cadherin and MMP-9, but not MMP-2, were significantly altered in SCL138-P-si and CAL27-P-si compared with the SCL138-P-control and CAL27-P-si, respectively.

**Conclusion**

The present study demonstrated that CD26 was positively detected in more than half of the OSCC patients enrolled in this study, and level of CD26 expression was strongly correlated with lymph node metastasis (TNM stage). Expression of CD26 was inversely correlated with patient survival. Highly metastatic OSCC cell lines strongly expressed CD26, and suppression of CD26 by siRNA resulted in partial blockade of cell motility and invasive ability in the in vitro study. Suppression of CD26 by siRNA also led to alterations in E-cadherin and MMP-9 expression in the highly metastatic OSCC cell lines. These findings strongly suggest that CD26 may contribute to distant lymph node metastasis of OSCC, and thus, CD26 might be a prognostic biomarker predicting survival as well as state of distant lymph node metastasis in patients with OSCC.

**Disclosure of conflict of interest**

None.

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