Involvement of the TGF-β1 pathway in caveolin-1-associated regulation of head and neck tumor cell metastasis

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent malignancy with a 5-year survival rate of 54%. Therefore, disease management improvement is required. The present study aimed to assess the role of caveolin-1 (Cav-1) in the metastasis of head and neck tumor cells. Short hairpin RNA was used to silence Cav-1 expression in Tu686 cells. Proliferation, migration, invasion, morphology and the levels of effector proteins were assessed in cells. Upon Cav-1 silencing, E-cadherin levels were decreased, while vimentin levels were significantly increased. Cell migration, quantified by wound healing and Transwell assays, was significantly increased. Meanwhile, Cav-1 and transforming growth factor β1 (TGF-β1) receptor were identified to be co-localized. In addition, Cav-1 knockdown resulted in increased phosphorylation of SMAD family member 2 (P<0.05), a downstream effector of TGF-β signaling. In addition, there was a mutual regulation, with increasing TGF-β1 levels leading to a dose-dependent decrease of Cav-1 expression levels (P<0.05). These findings indicate that Cav-1 inhibits cell metastasis in HNSCC, suggesting the involvement of the TGF-β signaling pathway.

Introduction

It has been reported that >90% of head and neck malignant tumor cases are squamous cell carcinoma (1-3). Head and neck squamous cell carcinoma (HNSCC) mainly arises from the epithelium of the oral cavity, oropharynx and larynx (4). Currently, treatment for HNSCC involves surgery, radiotherapy and adjuvant chemotherapy. Despite continuous improvements and updates in treatment, the five-year survival rate of patients with HNSCC has not improved (5). This is partly due to the occurrence of early tumor metastasis (6,7). Lymph node metastasis has been reported in 50%, with distant metastasis observed in 20-30% of patients with HNSCC in the two years following therapy (8,9). However, almost two thirds of patients with HNSCC, who receive combined treatment of chemo- and radiotherapy succumb within three years; therefore, further improvements in the treatment and management of HNSCC are required (10). Although a number of genes and pathways are aberrantly expressed in HNSCC, including tumor protein 53, cyclin dependent kinase inhibitor 2A, Ras, nuclear factor κB and epidermal growth factor receptor, an understanding of the functional underlying mechanisms by which they regulate metastasis and recurrence remains undetermined (11-15). Therefore, it is crucial to identify proteins that regulate metastasis and understand their functional mechanisms, which in turn would promote the development of novel prognostic biomarkers and therapeutic approaches for the disease.

Epithelial-to-mesenchymal transition (EMT) is a crucial process in metastasis, as cells acquire mesenchymal and fibroblast-like properties and indicate increased motility and reduced adhesion (16-18). A number of pathways have been reported to induce EMT, including transforming growth factor β1 (TGF-β1)-mediated signaling (19-21). Although TGF-β1 activates multiple signaling pathways, the main one for EMT regulation is as follows; TGF-β1 induces its own receptor, leading to the activation of downstream signaling molecules, which reduces SMAD family member 2 (Smad2)/Smad3 phosphorylation, followed by Smad2/Smad3 forming heterodimers with Smad4 (22). This complex enters the nucleus, upregulates nuclear transcription factors and inhibits the epithelial cell marker E-cadherin, ultimately leading to EMT and tumor cell invasion and metastasis (23). Therefore, the aim of the present study was to identify a key regulator of TGF-β1-mediated cell signaling to prevent EMT and metastasis.

Caveolin-1 (Cav-1), a 21-24 kDa integral membrane protein and a principal structural component of caveolae, serves a
critical role in TGF-β-associated transactivation of epidermal growth factor (EGF) receptor signaling in hepatocytes; in addition, caveolin was reported to suppress TGF-β signaling, with TGF-β receptors known to be endocytosed in a caveolin-dependent manner (24-26). Previous studies have reported that high Cav-1 expression levels are associated with tumor progression and metastasis in prostate and pancreatic cancer, while inhibiting metastasis and tumor progression (27-29). Furthermore, Tu686 and 686LN cell lines, established from pharyngeal squamous cell carcinoma and its metastatic lymph nodes, respectively, were assessed, and it was reported that Cav-1 expression was reduced in 686LN cells compared with Tu686 cells, with greater metastatic and invasive abilities exhibited in 686LN cells (30). However, a comprehensive understanding of the role of caveolin in cancer metastasis is required.

In the present study, the role of Cav-1 in the regulation of HNSCC metastasis was examined, and the mechanism by which Cav-1 regulates TGF-β signaling in metastasis and EMT was investigated by silencing Cav-1 using short hairpin (sh)RNA technology.

Materials and methods

Cell lines. The HNSCC Tu686 cell line, provided by the Winship Cancer Institute of Emory University (Atlanta, GA, USA) and established from a primary base of tongue tumor (31), was used in the present study. The cells were maintained as monolayer cultures in DMEM/F12 (1:1) (Gibco/Brl; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco/Brl; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂.

Construction of Cav-1 shRNA and the cDNA plasmid vector. shRNA expressing vector pLKO.1-puro was purchased from Addgene, Inc. (Cambridge, MA). Three putative candidate sequences were designed using the Oligoengine software (version 2; www.oligoengine.com), with their specificities confirmed by nucleotide BLAST searches. The two putative candidate sequences and a scramble sequence were as follows: Sequence-1, sense 5’-CCGGTTACATCCATTATAAGCTGC TCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’ and antisense 5’-AAATTCAAAAAGTACATCCATTATAAGC TGCTCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’; Sequence-2, sense 5’-CCGGGAGCGCGCAGACTTCTCCCTCCTCG AGGGGAGAGTCGTCTCGCCCGCTTTTTTTG-3’ and anti-sense 5’-AAATTCAAAAAGTACATCCATTATAAGC TGCTCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’; and Sequence-3, sense 5’-CCGGGAGCGCGCAGACTTCTCCCTCCTCG AGGGGAGAGTCGTCTCGCCCGCTTTTTTTG-3’ and anti-sense 5’-AAATTCAAAAAGTACATCCATTATAAGC TGCTCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’.

Construction of Cav-1 RNAi and the cDNA plasmid vector. Cav-1 RNAi expressing vector pLKO.1-puro was purchased from Addgene, Inc. (Cambridge, MA). Three putative candidate sequences and a scramble sequence were as follows: Sequence-1, sense 5’-CCGGTTACATCCATTATAAGCTGC TCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’ and antisense 5’-AAATTCAAAAAGTACATCCATTATAAGC TGCTCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’; Sequence-2, sense 5’-CCGGGAGCGCGCAGACTTCTCCCTCCTCG AGGGGAGAGTCGTCTCGCCCGCTTTTTTTG-3’ and anti-sense 5’-AAATTCAAAAAGTACATCCATTATAAGC TGCTCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’; and Sequence-3, sense 5’-CCGGGAGCGCGCAGACTTCTCCCTCCTCG AGGGGAGAGTCGTCTCGCCCGCTTTTTTTG-3’ and anti-sense 5’-AAATTCAAAAAGTACATCCATTATAAGC TGCTCGAGCAGCTTTATAATGGATGATCCTTTTTTGG-3’.

A total of 4.0x10⁴ cells were inoculated per well in a 24-well plate for 24 h, and three uniformly distributed wells were selected at 60% confluency. First, 500 µl cell culture containing polybrene at a final concentration of 5 µg/ml was added to the three wells. Subsequently, the two experimental groups (Cav-1 shRNA lentiviral particle suspension, sequence-1 or sequence-2; 80 µl), the negative control group (negative control lentiviral particle suspension; 80 µl) and the green fluorescent protein (GFP) group (control group lentiviral particle suspension containing the fluorescent GFP marker to observe the transfection efficiency; 80 µl) were incubated at 37°C with 5% CO₂ overnight. The medium (DMEM; Gibco/Brl; Thermo Fisher Scientific, Inc.) containing viral particles was changed with fresh medium every 2 days. The selection medium containing puromycin (5 µg/ml) was added to screen positive clones from the third day. After four weeks, a positive clone cell line (Tu686 cav-1RNAi) was selected, cultured and expanded.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), for protein extraction. Equal amounts of total protein (100 µg), measured by the bicinechonic acid method, were separated by 12% SDS-PAGE electrophoresis and transferred onto polyvinylidene fluoride membranes. The membrane was placed in a blocking buffer (cat. no. P0023B; Beyotime Institute of Biotechnology) for 1 h. The membrane was incubated with primary antibody (rabbit anti-human Cav-1 polyclonal antibody; cat. no. PA1-064; 1:800; Santa Cruz Biotechnology, Inc, Dallas, CA, USA) at room temperature for 2 h followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat antibody (Beyotime Institute of Biotechnology, Haiman, China; 1:1,000) at room temperature for 2 h. Other primary antibodies included mouse anti-human E-cadherin polyclonal (Cell Signaling Technology, Inc.; cat. no. sc-8426; 1:400), mouse anti-human vimentin polyclonal (Cell Signaling Technology, Inc.; cat. no. sc-32322; 1:200), mouse anti-human Smad2 monoclonal (Cell Signaling Technology Inc.; cat. no. 3103s; 1:800), rabbit anti-human p-Smad2 polyclonal (Cell Signaling Technology, Inc.; cat. no. ser465/467; 1:800) and mouse anti-human β-actin monoclonal (Beyotime Institute of Biotechnology; cat. no. AF0003; 1:1,000) antibodies. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Beyotime Institute of Biotechnology; cat. nos. A0562 and A0568; 1:1,000) were incubated for 2 h at 37°C. The membrane was subsequently washed and treated with Pierce enhanced chemiluminescence (ECL) western blotting substrate (Thermo Fisher Scientific, Inc.). Immunoreactive bands were visualized by ECL on a LAS4000 imager and densitometric analysis was performed using Image Quant LAS 500 (GE Healthcare, Chicago, IL, USA).

CCK-8 assay. CCK-8 assay was used to evaluate proliferation. Following transduction of control shRNA sequence and the experimental sequence for 24 h, respectively, Tu686 cells were seeded in 96-well plates at 5,000 cells/well. After 1-7 days of culture at 37°C with 5% CO₂, CCK-8 (Beyotime Institute of Biotechnology) was added to the cells (10 µl/well), which were further incubated for 1 h at 37°C. Absorbance was measured at a wavelength of 450 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were performed in triplicate. Semi logarithmic curves for cell survival were drawn by Microsoft Excel 2000 software (Microsoft Corporation, Redmond, WA, USA).

Wound healing assay. Tu686 cells (1x10⁴) were seeded in 6-cm plates coated with 10 mg/ml type I collagen (Advanced Biomatrix). Following incubation for 24 h, the monolayer was disrupted with a cell scraper (1.2 mm width), and micrographs...
were acquired at 0 and 24 h on a phase contrast microscope (magnification, x50; Olympus Corporation, Tokyo, Japan). Experiments were performed in triplicate, and four fields of view in each data point were recorded in a blinded manner.

**Transwell assay.** For invasion assay, the upper chambers of Matrigel pre-coated Transwell inserts (cat. no. ECM550; Chemicon International, USA) prior to addition of Tu686 cells (2x10⁴). The lower chambers were filled with 0.8 ml conditioned DMEM supplemented with 10% fetal bovine serum (Gibco/Brl; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to induce chemotaxis in Tu686 cells. After 24 h of incubation at 37°C, the cells were fixed in 4% methanol at room temperature (25°C) for 10 min and stained with hematoxylin and eosin at room temperature (25°C) for 30 min. Cells that invaded through the pores to the lower side of the filter were counted under an inverted light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan). Three migration chambers were used for each condition and the mean number of cells was calculated.

**Activation of the TGF-β₁ signaling pathway in TU686 cells.** Logarithmic growth was observed in Tu686 cells, using serum for 12 h prior to withdrawal and culture for 24 h at 37°C, maintaining parameters, including cell confluence of 80-90%, 0.25% trypsin + 0.02% EDTA digestion and density adjustment to 2.5x10⁴ cells/ml. The cells were maintained in 50 ml disposable cell suspension culture bottles at 37°C and 5% CO₂ for 24 h with serum-free DMEM/F12 for 24 h. Subsequently, five groups were set up with TGF-β₁ (cat. no. 100-21/100-21C; PeproTech US, Rocky Hill, NJ, USA) at the final concentrations of 5, 10, 15, 20 and 25 ng/ml, diluted with serum-free DMEM/F12 to 4 ml, and incubated at 37°C with 5% CO₂ for 48 h. The cells were subsequently collected for further experiments.

**Immunofluorescence.** For the immunofluorescence staining, Tu686 cells (4x10⁴) grown on coverslips were fixed with warm PHEMO buffer (68 mM PIPES, 25 mM HEPES, pH 6.9, 15 mM EGTA, 3 mM MgCl₂), and 10% (v/v) DMSO containing 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100 for 10 min at room temperature (25°C). Cells were blocked with 5% BSA (cat. no. sw3015; Beijing Solarbio Science and Technology Co., Ltd.) for 1 h at room temperature (25°C) and incubated with primary at 37°C for 1 h or at 4°C overnight and subsequently with fluorophore-conjugated secondary antibodies at 37°C for 1 h, including mouse anti-human Cav-1 polyclonal antibody (cat. no. 610493, BD Biosciences, San Jose, CA, USA; 1:800), mouse anti-human transforming growth factor β₁ (cat. no. sc-130348; 1:1,000; Santa Cruz Biotechnology, Inc.), and fluorescein isothiocyanate- and rhodamine B isothiocyanate-conjugated IgG antibodies (cat. no. zf-0311; 1:100; OriGene Technologies, Inc., Beijing, China). Images were acquired on a Zeiss LSM 510 META confocal microscope (magnification, x630).

**Statistical analysis.** Statistical analysis was performed with the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Quantitative data are expressed as the mean ± standard deviation. Statistical significance was estimated by two-tailed Student's t-test or one-way analysis of variance followed by the Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Silencing of Cav-1 expression by shRNA in Tu686 cells.** In the present study, shRNA was used to inhibit Cav-1 expression. Candidate sequence 1 significantly inhibited the protein expression of Cav-1 by >60% and candidate sequence 2 inhibited the expression by 30-40%. Control sequences caused no detectable changes in Cav-1 expression (Fig. 1A). These results indicated that candidate sequence 1 was more efficient compared with candidate sequence 2. A change in cell morphology was also detected following
transfection under an inverted microscope (magnification, x100; Olympus Corporation, Tokyo, Japan), where cells with silenced Cav-1 displayed a spindle shape and pseudopodia, similar to fibroblasts (Fig. 1B). Control cells are presented in Fig. 1C.

*Cav-1 promotes migration but not proliferation in Tu686 cells.* Taking into consideration that upregulation of Cav-1 expression is associated with HNSCC metastasis (30), we hypothesized that Cav-1 may be involved in the invasive phenotype of HNSCC by promoting cell migration. To test
this possibility, the effect of Cav-1 on the metastasis of Tu686 cells was assessed. Wound healing and Transwell assays were employed to determine the migration and invasion abilities of Tu686 cells, respectively. Tu686 Cav-1RNAi+ cells exhibited a 15% increase in wound closure compared with Tu686 Cav-1RNAi− cells (P<0.05) in the wound healing assay. In the Transwell assay, a significantly higher number of Tu686 Cav-1RNAi+ cells passed through the Matrigel compared with Tu686 Cav-1RNAi− cells, suggesting a role for Cav-1 in the migration of Tu686 cells (P<0.05; Fig. 2).

A CCK-8 assay was employed to assess the growth and proliferation ability of Tu686, Tu686 Cav-1RNAi+ and Tu686 Cav-1RNAi− cells. No statistical differences were identified between the Tu686 and Tu686 Cav-1RNAi− groups, Tu686 and Tu686 Cav-1RNAi− groups, and Tu686 Cav-1RNAi− and Tu686 Cav-1RNAi+ groups (P>0.05; Fig. 3).

Cav-1 silencing upregulates vimentin and reduces E-cadherin expression. EMT is marked by two crucial events: Loss of E-cadherin, a transmembrane protein involved in cell-cell adhesion, and an increase in the expression of vimentin, an intermediate filament protein (32). Therefore, the present study assessed the expression levels of these two proteins following Cav-1 silencing. It was indicated that Cav-1 shRNA significantly reduced the expression of E-cadherin and increased that of vimentin in Tu686 cells (Tu686 Cav-1RNAi+ vs. Tu686 cells and Tu686 Cav-1RNAi−; P<0.05; Fig. 4).

Cav-1 silencing induces TGF-β1 signaling. Confocal microscopy was used to determine the in vivo localizations of Cav-1 and TGF-β1 receptors. As indicated in Fig. 5A, TGF-β1R and Cav-1 were co-localized inside the cells.

It was also identified that stable silencing of Cav-1 in Tu686 cells led to an increased expression of phosphorylated Smad2, indicating an increased activation of the TGF-β pathway (Fig. 5B). A dose-dependent decrease in Cav-1 expression level was observed with gradually increasing TGF-β1 levels. These findings suggest a mutual regulation of TGF-β1 signaling and Cav-1 (Fig. 5C).

Discussion
The aim of the present study was to examine the molecular regulation of HNSCC metastasis. The role of Cav-1 in regulating TGF-β-induced EMT was assessed. Silencing of Cav-1 expression led to reduced E-cadherin expression, cell migration and increased vimentin expression, all key markers of EMT (33). It was also indicated that Cav-1 downregulation resulted in increased TGF-β signaling, reflected by increased phosphorylation of Smad2. Therefore, the present study proposes that Cav-1 expression inhibits EMT by regulation of TGF-β signaling.

In order to gain novel insights into the role of Cav-1 in HNSCC, shRNA was employed to knockdown Cav-1 expression in Tu686 cells. Cav-1 depletion resulted in increased cell migration, with no significant effects on proliferation. In addition, changes in cell morphology were observed, as cells were spindle-shaped and a number of them presented with pseudopodia, similar to fibroblasts. These findings of morphological changes are in accordance with previous studies on EMT-induced shape changes (34-36). Therefore, downregulation of Cav-1 expression resulted in phenotypic changes similar to EMT in Tu686 cells.

A number of studies have assessed the role of Cav-1 expression during EMT and cancer metastasis. Lu et al (37) demonstrated that EGF-stimulating factor in tumor cells significantly reduced Cav-1 expression, activated the β-catenin-T-cell factor/lymphoid enhancer-binding factor transcription factor, decreased the levels of the epithelial cell marker E-cadherin, blunted cell-cell contact, and enhanced the cellular phenotypes of EMT and metastasis. Bailey et al (38) indicated that Cav-1 regulation during EMT is mediated by focal adhesion kinase.

Abnormalities in the TGF-β signaling pathway are closely associated with tumor cell invasion and metastasis (39). In breast cancer, activated TGF-β signaling pathway can cause hypermethylation and subsequent loss of expression of E-cadherin, cingulin, claudin 4 and kallikrein related peptidase 10, resulting in EMT and breast cancer metastasis (40).
Araki et al (41) reported that activated TGF-β1 signaling in breast cancer cells increases the expression of E3 ubiquitin ligase human murine double minute, leading to P53 gene destabilization and the EMT phenotype in cells.

By using confocal microscopy, the present study observed that Cav-1 and TGF-β1 receptors were co-localized on the plasma membrane, indicating that the effects of Cav-1 on EMT may involve TGF-β1 signaling. Schwartz et al (42) reported that TGF-β receptor (TGF-βR) I and II co-localize with Cav-1 and nitric oxide synthase 3 (eNOS) in human umbilical vein epithelial cells. They further indicated that TGF-β1 interacts and regulates eNOS activity in the caveolae.

In the present study, Cav-1 could inhibit TGF-β signaling by regulating the phosphorylation levels of its downstream effector SMAD2. In addition, Cav-1 silencing significantly increased the expression of phosphorylated Smad2, indicating an induction of TGF-β1R II signaling. The present study also indicated a dose-dependent decrease in Cav-1 levels with increasing levels of TGF-β1, which further indicates the mutual inhibitory regulation between the TGF-β1 signaling pathway and Cav-1.

A limitation of the current study should be mentioned. Although EMT is a complex event involving transcription factors, cytoskeletal proteins and extracellular matrix components among others (43), E-cadherin and vimentin were only assessed in this experiment; therefore, further comprehensive studies should examine the effects of Cav-1 on other factors affecting EMT.

In conclusion, the present study indicated that Cav-1 may regulate EMT by influencing cell invasion and migration in HNSCC cells. It was further demonstrated that the aforementioned regulation may involve the TGF-β1 signaling pathway. The current study also provided a basis for a mutual association between Cav-1 and TGF-β1 signaling for metastasis regulation in HNSCC.

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Authors' contributions
JS conceived and coordinated the study, designed and performed the experiments, and wrote the manuscript. YL, CY, TX, GN, BM and XZ performed data collection, data analysis and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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