VCAM1-targeted RNA interference inhibits the proliferation of human oral squamous carcinoma HN12 cells

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Abstract. In the present study, RNA interference (RNAi) was used to investigate the effect of vascular cell adhesion molecule 1 (VCAM1) silencing on the proliferation of human oral squamous carcinoma HN12 cells. HN12 cells were divided into three groups: The untreated blank control cell group (CK), the negative control group transfected with non-homologous vector (NC) and the positive group transfected with the target sequence VCAM1 small hairpin RNA (KD). Reverse-transcription polymerase chain reaction and western blot analysis were used to examine the effects of VCAM1-knockdown on the mRNA expression of VCAM1 and subsequent protein expression. Furthermore, the HN12 cell growth inhibition rate was detected using the cell counting kit -8 method. The VCAM1-targeted lentiviral vector RNAi significantly inhibited VCAM1 mRNA, and subsequent protein, expression. Compared with the NC group, the VCAM1 gene knockdown efficiency was ~85%, while the expression level of VCAM1 protein was reduced by ~74% in KD group cells. In addition, cell growth was significantly inhibited in the KD group, with a growth inhibition rate of ~34%. Therefore, this targeted lentiviral vector RNAi effectively inhibited VCAM1 gene, and subsequent protein, expression, as well as the proliferation of oral squamous carcinoma cells. These results may provide an experimental reference for the diagnosis and treatment of oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma, characterized by its poor overall prognosis, complex pathogenesis and high mortality rate, is the sixth most common type of malignant cancer globally (1). Approximately 300,000 oral squamous cell carcinoma cases are reported annually (1), with the likelihood of severe progression partnered with a high risk of nodal metastasis and locoregional invasion (2). In addition, the combined actions of various factors, including genetic factors and tumor microenvironment are implicated in the complex pathogenesis of oral squamous cell carcinoma (3,4). Despite recent advances in surgery, chemotherapy and radiotherapy, the 5-year survival rate of oral squamous cell carcinoma has remained at ~50% for the past 10 years (5). Therefore, to potentially identify targets that may aid therapeutic intervention, further research is required to investigate the underlying mechanisms implicated during progression of oral squamous cell carcinoma.

Vascular cell adhesion molecule 1 (VCAM1) is a member of the immunoglobulin superfamily that binds integrin receptors α4β1 and α4β7 (6). The VCAM1 gene is ~25 kb long and is located in the lp31-32 human chromosomal region (6). The VCAM1 protein may potentially be released from the cell membrane and function in a soluble form in response to environmental cues (7,8). As an immunoglobulin-like adhesion molecule, VCAM1 serves a significant role in various pathophysiological tissues (9,10). Aberrant expression of VCAM1 frequently occurs in various types of cancer (10). For example, abnormal expression of VCAM1 is associated with the metastasis of gastric carcinoma (11,12). Furthermore, VCAM1 is implicated in the preferential attachment of highly metastatic melanoma cells to microvesicles within the tumor microenvironment (13). VCAM1 is also key metastasis of breast cancer to the lung and, thus, may present a potential therapeutic target (10). Functioning as an environmental sensor that regulates adult neurogenesis, VCAM1 expression is induced in the neural stem cell niche of the subventricular zone (14). Therefore, a potentially useful approach for assessing prognosis in patients with oral tongue squamous cell carcinoma may be monitoring changes in VCAM1 expression in lymphatic vessels (15).

RNA interference (RNAi) refers to the gene-silencing phenomenon induced by small molecules of double-stranded RNA (dsRNA) (16), in which RNA molecules inhibit gene
expression or translation, by neutralizing targeted mRNA molecules (17,18). RNAi is controlled by the RNA-induced silencing complex (RISC), and initiated in the cell cytoplasm by short double-stranded RNA molecules that interact with the catalytic RISC component Argonaute (16). The synthetic dsRNA, which is then introduced into cells, has the ability to induce the suppression of target genes (16). Recently, RNAi has become a key technology for identifying the components within particular cell processes.

VCAM1 expression has been demonstrated to be induced in oral tongue squamous cell carcinoma (15). In addition, a previous study demonstrated that overexpression of the VCAM1 gene serves a potential role in oral squamous cell carcinoma development, which was closely associated with lymph node metastasis and angiogenesis (19). However, there remain few studies on the effect of VCAM1 silencing on the proliferation of human oral squamous carcinoma (15). Therefore, to provide an experimental basis for the diagnosis and colorectal cancer treatment, the present study aimed to investigate the effect of VCAM1 silencing on the proliferation of human oral squamous carcinoma HN12 cells.

Materials and methods

Construction of VCAM1 silencing HN12 cell lines. HN12 human oral squamous carcinoma cells (Michigan State University, East Lansing, MI, USA) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated at 37˚C with 5% CO2. To create the VCAM1 short hairpin RNA (shRNA)-silenced sub-cell lines, the following shRNA sequence was designed against the VCAM1 gene: 5'-GGCTTGGAGATAGACTTTAC TTTCAAGAAAGTAGTCATATTCTCCGACGCTTTTTTA CGCGTG-3'. The VCAM1 RNAi was purchased from the Daan Gene Co., Ltd. (Guangzhou, China), and the HN12 cells were transfected with the plasmids, psPAX2 and pMD2.G using Lipofectamine (20000 transfaction reagent according to the manufacturer’s protocol (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, HN12 Cells were divided into three groups: The untreated blank control cell group (CK), the negative control group transfected with non-homologous vector (NC) and the positive group transfected with the VCAM1-kncodown shRNA sequence (KD).

Infection efficiency measurement. For cell infection, the HN12 cells (2.5x105 cells/well) were seeded in 24-well plates and cultured in an incubator for 12 h. Following this, 1, 1.0, 5.0 or 10.0 µl of blank lentiviruses with green fluorescence protein (GFP) [1x106 transducing units (TU)/ml] were added to the wells. For each concentration of lentiviruses, three wells were used. After 12 h, lentiviruses were washed with PBS and the cells were further cultured in DMEM containing 10% fetal bovine serum in an incubator at 37˚C with 5% CO2. The infection efficiency was observed after 48 h, and the multiplicity of infection (MOI) was evaluated using a fluorescence microscope to analyze the expression level of GFP.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In 6-well plates, 3x105 HN12 cells were cultured for 3 days at 37˚C with 5% CO2, and cell culture was continued for an additional 2 days at 37˚C with 5% CO2 when the MOI was >50%. Following digestion and centrifugation (400 x g) for 5 min at 37˚C, total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocol. RNA was reverse-transcribed using the FastQuant RT kit (Tiangen Biotech Co., Ltd., Beijing, China) at 37˚C. Next, SYBR Green I-labeled PCR product (Takara Bio, Inc., Otsu, Japan) was used according to the manufacturer’s protocol for fluorescent qPCR to obtain quantification cycle (Cq) values. The following thermocycling conditions were maintained: 95˚C for 3 min; 95˚C for 10 sec and 60˚C for 30 sec for 39 cycles; and melting curve analysis using increase from 65.0 to 95.0˚C in 0.5˚C increments for 5 sec. Finally, the 2-ΔΔCq method (20) was used to analyze differences in relative gene expression in each sample, using β-actin as the internal reference gene. Primer sequences are listed in Table I.

Western blot analysis. The HN12 cells were collected 7 days after lentivirus infection and lysed in SDS sample buffer [100 mM Tris-HCl (pH 6.8), 10 mM ethylenediaminetetraacetic acid, 4% SDS, and 10% glycerine]. Next, the protein content was evaluated using the Lowry method (21). To detect target proteins, equal amounts of protein samples were separated by SDS-PAGE (12% gel) and transferred to polyvinylidene difluoride membranes. Next, the membranes were incubated with TBST [25 mM Tris (pH 7.4) 150 mM NaCl, 0.1% Tween-20] containing 5% non-fat dry milk at room temperature for 1 h. Following washing with TBST for 3 times, the membranes were probed with the primary antibody anti-VCAM1 rabbit mAb (dilution, 1:500; cat. no. ab106777; Abcam, Cambridge, UK) and anti-β-actin rabbit mAb (dilution, 1:500; cat. no. ab6272; Abcam) at 4˚C overnight, followed by incubation with a goat anti-rabbit IgG horseradish peroxidase-linked antibody (dilution, 1:1,500; cat. no. 7074 CST Biological Reagents Co., Ltd., Shanghai, China) for 1 h at room temperature. Finally, the blots were detected with an enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.) following the manufacturer’s protocol. β-actin was used as the reference control. The relative expression level of VCAM1 was acquired based on the gray values, and analyzed with Quantity One 1-D analysis software v4.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell Counting Kit-8 (CCK-8) assay. The lentivirus transfected HN12 cells were seeded into 96-well plates at a density of 2x103 cells per well and incubated for 12 h at 37˚C. Then cells were treated with 20 ml Lipofectamine®-small interfering RNA (siRNA) complexes, which contained 2 nM siRNA. Cells treated with scrambled siRNA were used as negative controls. Cell proliferation rate was measured 5 days following transfection, by adding 10 ml CCK-8 solution (Takara Bio, Inc.) to each well, followed by incubation at 37˚C for 2 h. Absorbance was evaluated at 450 nm by spectrophotometry using a SpectraMax 190 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). In each assay, six parallel wells were included, and the results were collected to evaluate the mean of three independent experiments.
Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as the mean ± standard deviation. One-way analysis of variance was performed to analyze the effect of VCAM1 silencing on the proliferation of HN12 cells. P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection efficiency of lentivirus. Fluorescence microscopy was used to detect the expression level of GFP. Following this, the transfection efficiency of viruses was evaluated by analyzing the MOI of HN12 cells. It was observed that the infection rate was <10% when the MOI was 1.0 (Fig. 1A). Furthermore, the infection rate was 10‑20% at an MOI of 10.0 (Fig. 1B). At a MOI of 50, the toxic side effects of the virus were negligible and cell density was not significantly altered (Fig. 1C). At this MOI, the transfection efficiency was ~95% (Fig. 1C). However, a cytotoxic effect was observed with a MOI of 100, and the infection rate was >95% (Fig. 1D). Therefore, an optimum MOI of 50 was selected for use in the present study. Compared with the NC group, the knockdown efficiency of the VCAM1 gene was ~85%, and the expression level of VCAM1 protein was reduced by ~74% in the KD group.

VCAM1 gene expression in HN12 cells. As the lentiviral vector system was efficiently transduced into HN12 cells, the level of VCAM1 gene expression was further analyzed. RT-qPCR results indicated that there was no statistical difference in VCAM1 gene expression in HN12 cells between the NC group and the CK group (P>0.05; Fig. 2). However, compared with the NC group, VCAM1 gene expression was significantly reduced in the KD group (P<0.05), and the knockdown efficiency of VCAM1 was ~85% (Fig. 2).

VCAM1 protein expression in HN12 cells. Western blot analysis was applied to detect the expression level of VCAM1 protein in the HN12 cells infected by lentiviral RNAi vectors. No significant difference in VCAM1 protein expression level was observed between the CK group and the NC group (P>0.05) (Fig. 3). Compared with the NC group, the expression level of VCAM1 was significantly decreased, by ~74%, in the KD cell group (P<0.05; Fig. 3).

Cell growth inhibition rate as evaluated by a CCK-8 assay. To investigate the effect of VCAM1 silencing on cell proliferation, the HN12 cell growth inhibition rate was assayed using the CCK-8 method. Compared with the NC group, cell growth was significantly inhibited in the KD group (P<0.05), with a

Table I. Primers for ACTB and VCAM1.

| Gene    | Accession number |
|---------|------------------|
| ACTB    | NM_001101.3      |
| VCAM1   | NM_001078.3      |

| Gene    | Accession numbera |
|---------|--------------------|
| ACTB    | F: TGTTACAGGAAGTCCCTTGCCATC      |
|         | R: CTGTGTGGGACTTGAGGAAGGAC        |
| VCAM1   | F: TTCTGTGCCCACAGTAAGG           |
|         | R: GCAGCCTTGTGGAGATGATTC         |

aAccession numbers from the National Center for Biotechnology Information. VCAM1, vascular cell adhesion molecule 1; ACTB, β-actin; F, forward; R, reverse.

Figure 1. The expression of green fluorescent protein in HN12 cells transfected with lentiviral vectors, viewed with fluorescence microscopy. (A) MOI=1, (B) MOI=10, (C) MOI=50, (D) MOI=100. Scale bar, 100 µm. MOI, multiplicity of infection.
Discussion

In the present study, RNAi was used to investigate the effect of VCAM1 silencing on the proliferation of human oral squamous carcinoma HN12 cells. The results of RT-qPCR and western blotting demonstrated that RNAi using a VCAM1-targeted lentiviral vector significantly inhibited the expression of VCAM1. In addition, cell growth was significantly inhibited in the KD group cells, with a cell growth inhibition rate of ~33.97%. A previous study indicated that VCAM1 serves a significant role as a molecular switch in the various signal transduction pathways of tumor cells (6). VCAM1 not only induces the recruitment of tumor angiogenesis factors, but also mediates the adhesion of endothelial cell and matrix by sensing the surrounding environment, leading to further tumor invasion and metastasis (6). It has been observed that VCAM1 expression is induced in the vascular endothelium of oral squamous cell carcinoma (21). In addition, the abnormal expression of VCAM1 is associated with the metastasis of gastric carcinoma (11,12). In addition, the current study indicated that VCAM1-targeted RNAi is able to effectively inhibit the gene and protein expression of VCAM1 as well as the proliferation of oral squamous carcinoma cells, which may provide an experimental basis for the diagnosis and treatment of colorectal cancer.

Nevertheless, the occurrence and development of oral squamous cell carcinoma is associated with the aberrant expression of multiple genes, as well as a variety of in vitro and in vivo factors (3). Previous studies have indicated that succinobucol-loaded nanoparticles exhibit therapeutic efficacy in the metastasis of breast cancer to the lung by inhibiting VCAM1 expression (22). Furthermore, anti-VCAM1 treatment is able to significantly decrease cancer-endothelial adhesion and block fusion (21). Until recently, studies on gene therapy of oral squamous carcinoma primarily focused on single-target gene treatments (4). Despite this progress, treatments targeting multiple genes have not been investigated in vitro and in vivo. The results of the present study potentially provide a theoretical basis for future animal studies and multi-gene targeting therapies in oral squamous carcinoma.

In conclusion, VCAM1-targeted RNAi effectively inhibits the gene and protein expression of VCAM1, as well as the proliferation of oral squamous carcinoma cells. These results may provide an experimental reference for the diagnosis and treatment of colorectal cancer.
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