RESEARCH COMMUNICATION

RNA Interference of Caveolin-1 Via Lentiviral Vector Inhibits Growth of Hypopharyngeal Squamous Cell Carcinoma FaDu Cells *In Vitro* and *In Vivo*

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Abstract

Objective: To investigate the effects of caveolin-1 (CAV1) on the growth of hypopharyngeal squamous cell carcinoma (HSCC) FaDu cells *in vitro* and *in vivo*. Methods: A CAV1-RNAi-lentivirus construct was transfected into FaDu cells and expression of caveolin-1 was tested by RT-PCR and western blotting analysis. Cell apoptosis was analyzed by transferase-medisated dUTP nick-end labeling (TUNEL) assay. Tumor inhibition effects were investigated by injecting rCAV1-RNAi-lentivirus construct into tumors created with FaDu cells in the HSCC mouse model, with the empty-vector lentivirus as a control. CAV1 expression in xenografts was tested by RT-PCR and immunohistochemistry. Results: RT-PCR and western blot analysis demonstrated successful construction of the CAV1-RNAi-lentivirus construct producing small hairpin RNA. The average weights and volumes of tumor in mice treated with CAV1-RNAi-lentivirus were lower than in mice with control treatment ($P<0.05$). RT-PCR revealed weak positive expression of CAV1 in CAV1-construct–treated xenografts and immunohistochemistry confirmed lower CAV1 expression than in controls.($P<0.05$). In addition, downregulation of CAV1 increased cell apoptosis *in vitro*. Conclusion: The growth of HSCCs could be inhibited by recombinant CAV1-RNAi-lentivirus *in vitro* and *in vivo*.

Keywords: Caveolin-1 - Lentivirus - RNA interference - Hypopharyngeal carcinoma cells

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Introduction

Caveolae are 50- to 100-nm vesicular membrane structures found in plasma membranes of many cell types(Anderson, 1998) (Yamada, 1955). Caveolin-1 (CAV1) is a principal component of caveolae; it is a multifunctional scaffolding protein with multiple binding partners that is associated with regulation of lipid raft domains andnumerous physiological and pathophysiological processes (Rothberg et al., 1992). It has functions in signal transduction(Smart et al., 1999), cholesterol homeostasis(Pike et al., 2002), and vesicular transport(Orlandi and Fishman, 1998), as well as tumor suppression and oncogenesis. Thus, CAV1 could be a key molecule in the development of cancer.

Downexpression has been found in lung, breast, and ovarian cancers, and in sarcomas by experimental studies (Belanger et al., 2004; Chen et al., 2004; Park et al., 2005a; Wiechen et al., 2001a; Wiechen et al., 2001b). The exogenous expression of CAV1 inhibited cell growth and tumorigenesis in oncogenically transformed cells and cancer cells (Bender et al., 2000; Engelman et al., 1997; Lee et al., 1998). However, overexpression of CAV1 has been observed in prostate and pancreatic cancer (Suzuoki et al., 2002; Yang et al., 1999). Thus, the function may depend on the tissue. The role in hypopharyngeal squamous carcinoma (HSCC) is unclear.

RNAi or silencing small double-stranded RNAs induce homology-dependent degradation of mRNA (Montgomery et al., 1998; Nishikura, 2001; Sharp, 2001). Recently, small interfering RNAs (siRNAs) have been efficacious in silencing target genes. Since siRNAs are small nucleic acid reagents, they can be easily manipulated and delivered by lentiviral vectors to target cells. Lentiviral vectors are probably the best choice currently for delivering and stably expressing shRNAs or siRNAs in target cells. Lentiviral vectors expressing siRNAs represent a potential gene therapeutic approach. In this study, we constructed the CAV1-RNAi-lentivirus and examined the biologic effects of CAV1 in human HSCC FaDu cells in vitro and in vivo.

Materials and Methods

Recombinant CAV1-RNAi-Lentivirus Construct

The lentiviral vector system with the marker green

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fluorescent protein (GFP) pGCL-GFP was from Ji Kai Genechem Co. (Shanghai). The SuperScript III cDNA reverse transcription kit was from Invitrogen (Carlsbad, CA, USA). The plasmid extraction kit was from Promega(Madison, WI, USA).

Induced silent sites with a ratio of G to C of 30% to 50% were selected from the CAV1 gene with use of the Ambion small hairpin RNA analysis software (http://www.invitrogen.com/rnai), and BLAST analysis of the NCBI database (http://www.pubmed.gov) suggested that it had no homology with other genes. Two complementary oligonucleotide strands were designed on the basis of the following sites: top strand, 5'-AAAAGGGA CATCTCTACACCGTTCCTTCGGGAACGGT GTAGAGATGTCCC-3'. In total, 5 ml of each complementary oligonucleotide strand (200 μM) and 2 μl of 10×denaturation buffer solution was mixed, and sterilized deionizated H_2O was added to a final volume of 20 μl. The reaction mixture was denaturated at 95 ºC for 4 min, then annealed at room temperature for 10 min until it formed double-stranded oligonucleotides (ds-oligo). T4 DNA ligase was used to clone the ds-oligo into the linear vector pGCL-GFP, and the reaction system was built following the manufacturer's instructions. TOPO 10 competent cells of Escherichia coli were transformed at room temperature for 5 min, on ice for 30 min, and spread by Luria Broth solid medium with kanamycin. Monoclonal colonies were selected 1 d later, and sequenced by TaKaRa (Dalian, China).

293T cells (1×10^5 cells) were transfected with the CAV1-RNAi-pGCL-GFP by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The lentiviral vector was concentrated by low centrifugation at 6000 ×g for 16 h and resuspended in 1 ml RPMI-1640 medium after culture for 48 h. The lentivirus titer was detected.

Cell Culture and Generation of Stably Transfected Cells

The FaDu cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Beijing). The cells were cultured at 37°C, 5% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells at 50%-60% confluence were transfected with the construct in six-well culture plates according to the Recombinant Lentivirus manufacturer's instructions (Cyagen Biosciences, China) and viewed under a fluorescent microscope (Leica, Germany).

Analysis of CAV1 Gene by Semi-quantitative RT-PCR

Total RNA was isolated from cultured cells by use of TRIzol reagent (Sangon, Shanghai). To synthesize first-strand cDNA, 1 μg RNA was reverse transcribed with use of 100 U PrimerScript Reverse Transcriptase (TaKaRa Biotechnology) and 50μM Oligo dt Primer (Takara Biotechnology) according to the manufacturer's protocol.

The sequences of gene-specific primers for CAV1 were forward, 5'-ACA TCT CTA CAC CGT TCC CAT-3', and reverse, 5'-TGT GTG TCC CTT CTG GTT CTG-3' (Genbank accession number BC009685; 206 bp) and for β-actin, forward, 5'-AAT GGG TCA GAA GGA CTC-3', and reverse, 5'-AGA GCA ACA TAG CAC AGC-3' (538 bp).

PCR was carried out in a final volume of 10 μl containing 10× LA-PCR buffer (TaKaRa Biotechnology), 2μM of each primer, 500μM of each dNTP, 50 ng of template cDNA, and 0.1 U of TaKaRa LA Taq (TaKaRa Biotechnology). Before amplification, samples were incubated at 95°C for 5 min. Each amplification cycle consisted of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C-62°C, and extension for 30 sec at 72°C. The number of PCR cycles was optimized from 26 to 32 for each gene-specific primer to ensure that amplification was in the linear range. The PCR product (10 μl) was electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide.

Western blot analysis

Cells were cultured to 80%-90% confluence, then lysed in ice-cold lysis buffer (50 mmol/L NaCl, 0.01 mmol/L Tris-Cl, 5 μmol/L EDTA, 0.5% NP-40, 1 mmol/L phenylmethysulfonyl fluoride, 1 μg/mL Aprotinin, 1 μg/mL Leupeptin, pH 8.0). Proteins were quantified according to the instructions of a BCA protein assay kit (Beyotime Biotechnology, China). Briefly, equal amounts of proteins were separated by 16% SDS-PAGE; separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were probed with rabbit anti-CAV1 polyclonal antibody(Santa Cruz Biotechnology,USA) at 4°C overnight. Membranes were washed in TBST and incubated with IRDye 800-conjugated goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) for 2 hr at 37°C. Protein bands were visualized with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE), and fluorescence intensity was calculated by use of the Odyssey software.

TUNEL Assay

Fragmented DNA indicating cell apoptosis was observed in cells by use of transferase-mediated dUTP nick-end labeling (TUNEL). After being fixed in 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS, pH 7.4), cells were stained with an in situ apoptosis detection kit (Boster Biological Technology, Wuhan, China) according to the manufacturer's instructions.

Briefly, slides were incubated with 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase activity, then with a mixture of TdT and DIG-dUTP solutions in a humidified chamber at 37°C for 2 h. Subsequently, the sections were incubated with blocking reagent in the humidified chamber at room temperature for 30 min and then incubated with anti-DIG-biotin (1:100) at 37°C for 30 min. After the streptavidin-biotin complex (SABC, 1:100) was applied to the slides at 37°C for 30 min, immunoreactivity was visualized with use of a standard ABC method (DAB kit, Boster Biological Technology). The negative control was replacing the TdT solution with distilled water. The presence of clear nuclear staining was indicative of apoptotic cells. At

least 500 cell nuclei were examined in the most evenly and distinctly labelled areas. The number of TUNEL-positive tumor cell nuclei was counted, and the apoptotic index was the percentage of apoptotic cells in the tumor.

Tumorigenicity Assay in Nude Mice

We evaluated the effect of CAV1 on FaDu cells growing in nude male BALB/c mice (4-5 weeks old, 18-22 g) obtained from the Bayerd Biotechnology Co.(Suzhou). The mice were inoculated subcutaneously in the right posterior flank with 1×10^7 FaDu cells in 200 μl PBS. A mass formed at the injection site about 10 days later. The mice were randomized into 3 groups (n=8 mice each). Tumors were injected with recombinant CAV1-RNAi-lentivirus once a week for four weeks, the empty vector GFP-lentivirus as a control, or no injection as a blank control. The mice were monitored every 4 days. Perpendicular tumor diameters were measured by use of vernier calipers, and tumor volume was estimated by use of the formula for ellipsoid volume, $V = a\times b^2/2$, where "a" is the longest tumor axis and "b" is the shortest tumor axis. After 6 weeks of tumor growth, mice were killed, and tumors were weighed and excised for immunocytochemical analysis and RT-PCR.

Immunohistochemistry

The immunohistochemistry kit was from Zhongshan GoldenBridge Biotechnology Co.(China).All specimens were fixed in 10% buffer formalin, dehydrated and embedded in paraffin blocks. Sections were cut to 4 μm thick and placed on poly-L-lysine-coated glass slides. After deparaffinization in xylene and graded alcohols, epitope retrieval was performed. Target retrieval for activated CAV1 was performed in 0.01 M sodium citrate buffer (pH 6.0) for 20 min in a microwave at 92-95°C. Endogenous peroxidase was blocked by immersing the sections in 3% H_2O_2 for 30 min at room temperature. Sections were blocked in 10% goat serum and incubated with the primary antibody (anti-CAV1; 1:100) at 4°C overnight. The peroxidase-labeled polymer conjugated to goat anti-rabbit antibody was used to detect antigen-antibody reaction. Sections were then visualized with 3,3-diaminobenzidine as a chromogen for 5 min and counterstained with Mayer's hematoxylin for 7 min. CAV1 expression was evaluated by immunohistochemistry with rabbit polyclonal antibody against CAV1(Santa Cruz Biotechnology, USA). Immunoreactivity was visualized with use of a standard ABC method (DAB kit, Boster Biological Technology).

Results

Transfection efficiency of CAV1-RNAi- and GFP-lentiviruses in FaDu cells

The CAV1-RNAi-lentivirus was constructed successfully, and the titer was about 1×10^8/ml. The CAV1-RNAi-lentivirus and GFP-lentivirus were transfected into FaDu cells successfully as seen on fluorescence microscopy. We obtained stably transfected cells at a multiplicity of infection of 10 (Figure 1). To confirm the expression of CAV1 in FaDu cells, we performed

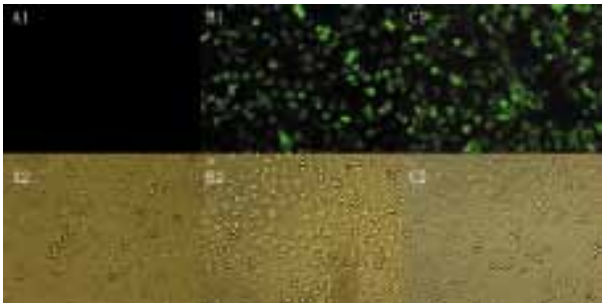

Figure 1. Transfection Efficiency Caveolin1 (CAV1)-RNAi-lentivirus and green fluorescent protein (GFP)-lentivirus in hypopharyngeal squamous cell carcinoma FaDu cells under fluorescence microscope (×200). A1, A2 FaDu cells in blank control group. B1, B2 CAV1-RNAi-lentivirus in FaDu cells. C1, C2 GFP-lentivirus in FaDu cells

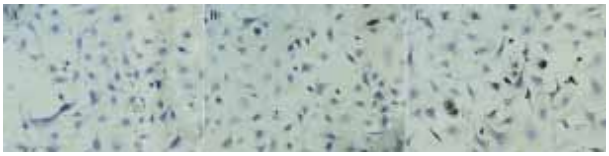

Figure 2. TUNEL-Positive Cells. A. Blank control group; B. GFP-lentivirus control; C. CAV1-RNAi-lentivirus (×400)

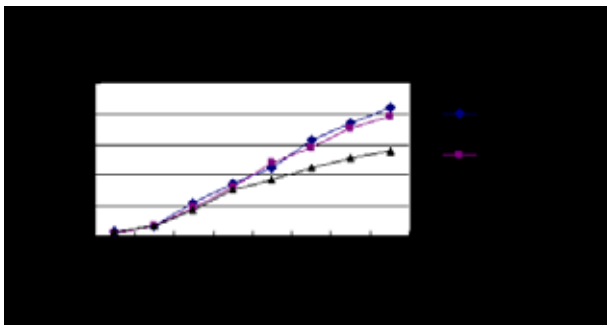

Figure 3. Tumor Volumes. Calculated from bidimensional measurements on the indicated days after injection, and the growth curves of tumors

semiquantitative RT-PCR analysis and western blot analysis. The results showed weak positive expression of CAV1 mRNA and weak positive expression of CAV1 protein, which confirmed the downregulation of CAV1.

TUNEL Assay

Few TUNEL-positive cells were detected in the blank control and GFP-lentivirus groups, with no significant difference between the groups. However, the number of TUNEL-positive cells was significantly higher in FaDu cells infected with CAV1-RNAi-lentivirus than that in control group cells (Figure 2).

Downregulation of CAV1 in FaDu Cells Reduced Tumor Formation in Nude Mice

The average tumor weight in CAV1-RNAi-lentivirus group was significantly lower than that in blank and GFP-lentivirus groups (1.23±0.42 vs 1.88±0.54 and 1.79±0.42, respectively, both $P<0.05$). The average tumor volume in CAV1-RNAi-lentivirus group was 1.40±0.49 cm^3, which was significantly lower than that in blank and GFP-lentivirus group (2.10±0.55 cm^3 and 1.97±0.45, respectively, $P< 0.05$) (Figure3). The tumor inhibitive rate was 34%.

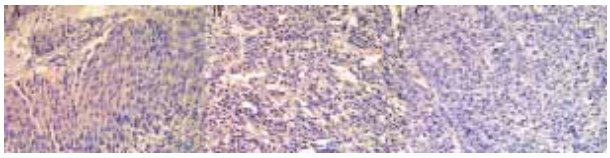

Figure 4. Immunohistochemical Staining for Caveolin-1 in Xenografts. A Blank control; B GFP-lentivirus control; C. CAV1-RNAi-lentivirus (×400)

RT-PCR showed weaker expression of CAV1 mRNA in the CAV1-RNAi-lentivirus-treated group than in the 2 control groups. Immunoreactivity of CAV1 was observed at the cell membrane and in cytoplasm, as evidenced by the presence of stained granular immunoreaction products (Figure 4). Overexpression of CAV1 was discovered in vascular endothelial cells. CAV1 immunostaining was semiquantitatively estimated on the basis of the proportion (percentage of positive cells) and intensity, as described(Sinicrope et al., 1995). Immunohistochemistry showed proliferating cell nuclear antigen index significantly lower in the CAV1-RNAi-lentivirus-treated group than in the control groups ($P < 0.05$).

Discussion

Caveolae are a subdomain of lipid rafts that are defined as "small (10–200 nm) heterogeneous membrane domains enriched in sterol and sphingolipids that are involved in the compartmentalization of various cellular processes" (Pike, 2006). CAV1 is a 21- to 24-kDa molecule that is an essential structural component of caveolae membranes in vivo and participates in vesicular trafficking events and transduction processes. It plays a regulatory role in several signaling pathways, such as Src family tyrosine kinase, G-proteins, epidermal growth factor receptor, protein kinase C, and endothelial nitric oxide synthase, which may be involved in the development of human cancer. The human CAV1 gene was found localized to chromosome 7q31.1.

The different expression of CAV1 in relation to the neoplastic histotype is not a novel finding. CAV1 downregulation has been found in certain tumors (Wiechen et al., 2001a; 2001b ; Belanger et al., 2004; Chen et al., 2004; Park et al., 2005b), but its overexpression has been documented in others (Yang et al., 1999; Kato et al., 2002; Suzuoki et al., 2002), thus indicating a tissue-dependent behavior of CAV1 in carcinogenesis. Indeed, absence of CAV1 expression characterizes pulmonary adenocarcinoma, whereas CAV1 overexpression has been demonstrated in squamous cell lung carcinoma (Kato et al., 2004).

In the present study, we evaluated the effect of CAV1 expression transfected by an RNAi-lentiviral construct on the growth of FaDu cells in vitro and in vivo to verify whether CAV1 expression may be related to HSCC biological aggressiveness, similar to other tumors(Engelman et al., 1997; Lee et al., 1998; Yang et al., 1999; Bender et al., 2000; Wiechen et al., 2001a; 2001b; Belanger et al., 2004; Chen et al., 2004; Park et al., 2005b; Guo et al., 2010). We stably transfected the CAV1-RNAi-lentivirus construct into FaDu cells and detected the CAV1 expression by western blot analysis and RT-PCR. We evaluated the effect of CAV1 in vitro on FaDu cells and in vivo on FaDu cells growing in BALB/c nude mice. TUNEL assay revealed increased apoptosis of cells with downregulated CAV1 expression. Ten days after the nude HSCC mouse model was established, recombinant CAV1-RNAi-lentivirus was injected into tumors, with an empty vector lentivirus injected as a control. With CAV1-RNAi-lentivirus treatment, tumor growth was slower than that with control treatment. Furthermore, the final tumor weight was significantly lower with CAV1-RNAi-lentivirus than that with control treatment. On immunohistochemistry, the protein expression of CAV1 in tumors was lower than that with control treatment, which confirmed RT-PCR findings of CAV1 mRNA expression.

The current results suggest that overexpression of CAV1 may be a marker of HSCC. However, the precise mechanism by which increased levels of CAV1 in FaDu cells promote carcinoma formation is not clear. In a model of tumor cell injection in caveolin-deficient mice (Cav–/–), angiogenesis was markedly lower than that in wild-type animals.(Woodman et al., 2003) Sonveaux et al(Sonveaux et al., 2004) documented that defects in activation of endothelial nitric oxide (NO) synthase (eNOS) with vascular endothelial growth factor (VEGF) stimulation were involved in the inability of Cav–/– mice to stimulate angiogenesis, which was further validated in endothelial cells (ECs) from these mice: the ability of Cav–/– ECs to form tubes on Matrigel with VEGF stimulation was significantly lower than that of Cav+/+ ECs. VEGF-induced NO production was greatly inhibited in Cav–/– ECs and VEGF-induced phosphorylation of eNOS on Ser1177 and dephosphorylation on Thr495, both considered hallmarks of eNOS activation, were abrogated in Cav–/– ECs but not in Cav+/+ ECs. We also found overexpression of CAV1 in vascular ECs. Thus, the overexpression of CAV1 may be related to tumorigenesis of HSCC by positive regulation of angiogenesis. Nonetheless, whether this overexpression is the critical factor requires further studies.

Moreover, the incidence of HSCC is higher in men than women, and the influence of sex hormones in this disease has been reported: epidermal growth factor receptor and nuclear estrogen receptor α levels were significantly increased in head and neck SCC tumors(Egloff et al., 2009). Still, overexpression of CAV1 potentiates ligand-dependent androgen receptor activation(Lu et al., 2001). In prostate carcinoma, which is also androgen dependent, CAV1 mediates hormone-stimulated survival or clonal growth of cells and promotes metastatic activity(Li et al., 2001). Similar mechanisms may be at work in HSCC.

In summary, we found that treatment with the recombinant CAV1-RNAi-lentivirus could effectively downregulate CAV1 mRNA and protein expression and inhibit the growth of FaDu cells in vitro and in vivo in mice. Such treatment may have potential for human cancer and may be a new approach for HSCC gene therapy. The positive regulation of the angiogenesis

pathway and inhibition of apoptosis and the sex hormone may be critical for understanding the mechanism of the role of CAV1 in tumor promotion. Nonetheless, further studies are needed to deeply demonstrate the mechanisms.

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