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Review Article

Effect of Caveolin-1 on human laryngeal cancer cell proliferation and EGFR signaling pathway

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Abstract: Primary laryngeal cancer refers to the cancer whose primary site is throat. Squamous cell carcinoma is the most common one. Caveolin-1 (CAV1) is an integral membrane protein, which is a main component of the microcapsules. It has been shown that CAV1 is closely linked with cancer. In this study, the effect of Caveolin-1 on human laryngeal cancer cell proliferation and EGFR signaling pathway was investigated. Seventy-eight patients diagnosed with laryngeal squamous cell carcinoma by our hospital from January 2012 to March 2015 were included in this study. Immunohistochemistry and RT-PCR were used to detect the expression level of CAV1 in both cancer tissues and adjacent tissues. CAV1 was overexpressed in Hep2 cells by transfection, MTT was used to measure the cells' proliferation and Western-Blot was used to detect the expression levels of p-EGFR and p-ERK1/2. Immunohistochemistry and RT-PCR results showed that positive expression rate of CAV1 in laryngeal carcinoma was significantly lower than that of adjacent tissues ($P < 0.05$), and the positive expression rate of CAV1 decreased along with the decrease of degree of tumor differentiation. Increase of CAV1 expression in Hep2 cells by transfection significantly decreased cell proliferation and lowered the expression levels of p-EGFR and p-ERK1/2 ($P < 0.05$). Compared with normal tissues, expression levels of CAV1 in laryngeal squamous carcinoma were low. Overexpression of CAV1 in laryngeal squamous carcinoma cells reduced cell proliferation possibly through inhibition of phosphorylation of EGFR and ERK1/2.

Keywords: Laryngeal cancer, Caveolin-1, cell proliferation, EGFR, ERK1/2

Introduction

Laryngeal cancer (LC) is malignant laryngeal epithelial tissue, accounting for 5.7%~7.6% of all malignancies, and squamous cell carcinoma is the most commonly one [1]. Etiology of LC has not yet clear, it is generally believed to be linked to smoking, drinking and other bad habits, environmental carcinogens, and virus infection [2, 3]. Currently, the main treatments for laryngeal cancer include surgery, chemotherapy and biological therapy, but there are still problems of recurrence [4]. Study of laryngeal carcinoma cells' behavior and their regulation mechanisms has great significance to improve the success rate of treatment of laryngeal cancer.

Caveolin-1 (CAV1) is a major component protein of caveolae [5]. Studies have shown that CAV1 participates in the regulation of cell proliferation and growth, which was mainly due to the binding and regulation of activation status of N-terminal amino acid sequence of CAV1 with a variety of important signaling molecules including G protein, EGF receptor, eNOS, and PKC etc. [6, 7]. With the in-depth study of malignant tumors, researchers found that CAV1 was abnormally expressed in malignant tumor including liver cancer, ovarian cancer, prostate cancer, and gastric adenocarcinoma, so it is speculated that CAV1 is closely linked with malignancies [8-11]. This study was to investigate the effect of CAV1 on human laryngeal cancer cell proliferation and EGFR signaling pathway.

Materials and methods

Subjects selection

Seventy-eight patients diagnosed with laryngeal squamous cell carcinoma by the third Xiangya hospital from January 2012 to March 2015

Table 1. Sequences of RT-PCR primer

Gene		Sequence	Amplicon
β-actin	F	5'-CGTGGACATCCGCAAAGACCT-3'	106 bp
	R	5'-AGCCAGAGCAGTGATCTCCTTC-3'	
CAV1	F	5'-TGAGCGAGAAGCAAGTGTACGA-3'	210 bp
	R	5'-GGGCAGACAGCAAGCGGTAA-3'	

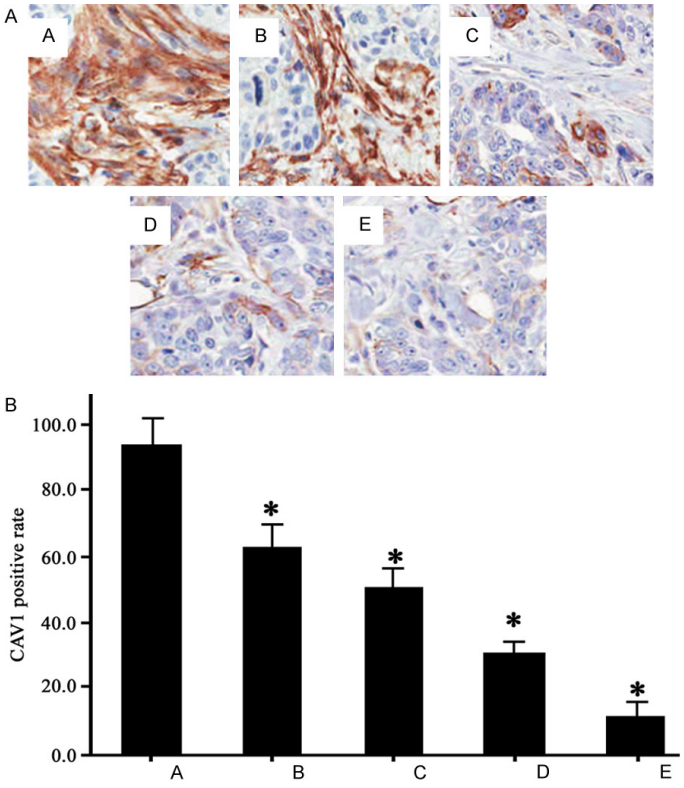

Figure 1. A: Immunohistochemical staining of CAV1 (× 400). B. CAV1 expression in different stages of laryngeal carcinoma. Note: A: Adjacent tissue; B: Stage I laryngeal carcinoma, C: Stage II laryngeal carcinoma, D: Stage III laryngeal carcinoma, E: Stage IV laryngeal carcinoma, *represents $P < 0.05$ when compared with group A.

were included in this study. There were 53 males and 25 females aged from 43 to 72 years with a mean of 57.1 ± 7.8. All patients were newly diagnosed and have not been treated with chemotherapy or surgery. According to staging reference of International Anti-Cancer Association (UICC) and clinical pathology, there were 6 of stage I cases, 10 of stage II cases, 26 of stage III cases, and 36 of IV cases. Cancerous tissues were removed from patients, frozen in liquid nitrogen or fixed in 10% neutral formalin, embedded in paraffin, and cut into sections of 5 μm for determination of the histological grades and immunohistochemistry. Adjacent tissues were determined to be normal epithelial tissues by pathological analysis. This study was approved by the Medical Ethics Committee of the third Xiangya hospital, all subjects were informed.

Immunohistochemistry

Immunohistochemistry was used to detect the expression levels of CAV1 in both laryngeal carcinoma and adjacent tissues. The main steps are as follows: sections were de-waxed with xylene, hydrated with graded alcohol and distilled water; peroxidase activity was blocked by 3% H_2O_2 followed by distilled water wash and PBS wash; sections were blocked with 5% BSA solution at room temperature; incubated with anti-CAV1 antibody working solution at 37°C for 2 h, washed with PBS to remove excess antibody; incubated with biotin-labeled secondary antibody at 37°C for 30 min, washed with PBS to remove excess antibody; incubated with streptavidin (horseradish peroxidase-labeled) at 37°C for 30 min, washed with PBS to remove unreacted streptavidin; incubated with DAB chromogenic reagent for 10 min then washed with distilled water; incubated with hematoxylin for 2 min then washed with distilled water; dehydrated, transparented, mounted, observed under a microscope. Five uniformly stained areas were randomly selected and observed (400 ×); the total numbers of cells and positively stained cells were counted [12].

For immunohistochemistry, if brown particles appear on the cell membrane, then the cell is designated positive cells, if the total number of positive cells were more than 40% of total cells, the case was designated CAV1 positive; otherwise, CAV1 negative [12].

RT-PCR

According to mRNA sequence of CAV1 (Genbank accession number: NM-001172896) PCR primers were designed for RT-PCR amplification (**Table 1**). β-actin was used as the reference gene to detect the relative expression levels of CAV1 in all samples. T total RNA was extracted from 20 mg of frozen tissue or adja-

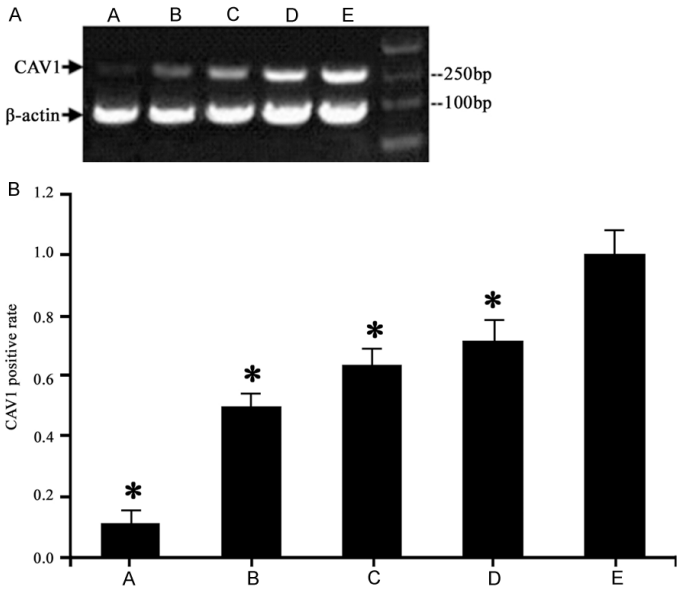

Figure 2. A. RT-PCR detection of CAV1. B. MRNA expression of CAV1 in laryngeal carcinoma tissues at different stages. Note: A: stage IV laryngeal carcinoma; B: Stage III laryngeal carcinoma, C: Stage II laryngeal carcinoma, D: Stage I laryngeal carcinoma, E: Adjacent tissue, *represents $P < 0.05$ when compared with group E.

cent tissues using RNA Rapid Extraction Kit (QIAGEN). RT-PCR kit (TianGen) was used to perform RT-PCR at 37°C for 2 h, and then cDNA obtained by RT-PCR was used as templates for PCR at 95°C/5 min, 95°C/1 min, 57°C/30 s, 72°C/1 min, 30 cycles. PCR products were resolved in 1% agarose gel, gel imaging system was used to calculate the relative expression of CAV1 [13].

Transfection and overexpression of CAV1

According to DNA sequences CAV1, two primers (CAV1-F: 5'-AGAGGCCTTT-GAAATTGT-3'; CAV1: 5'-AAATACTTTCAGGTCACGTC-3') were designed to amplify CAV1. Amplified CAV1 was inserted into a mammalian cell expression vector pBudCE4.1. The empty pBudCE4.1 vector was used as a negative control. INTERFERinTM transfection kit (Polyplus transfection) was used for transfection. Laryngeal cancer cell line HEp2 was purchased from the cell bank of Chinese Academy of Science, and cultured to logarithmic growth phase, trypsinized, counted, diluted with fresh medium, inoculated into 96-well plates for transfection according to the manufacturer instructions [14].

MTT assay

Successfully transfected HEp2 cells were grown to logarithmic growth phase and trypsinized, and centrifuged. The supernatant was discarded; cell pellet was re-suspended in cell culture medium. 6 μL of cell suspension was mixed with 6 μL of trypan blue solution for cell counting. The cell suspension was inoculated into 24-well plates and incubated at 37°C for 12 h. 70 μL of MTT solution was added to each well. Cells were incubated at 37°C for 3 h, DMSO was added. Absorbance was read with a microplate reader at A570 nm [15].

Western-Blot

HEp2 cells were collected, homogenized with 100 μL cell lysis buffer in a glass homogenizer, and centrifuged at 13000 g for 10 min. Supernatant was used for Western-Blot analysis. 15% gel was used for SDS-PAGE electrophoresis. Protein was transferred to PVDF membrane. Membranes were blocked with 5% skim milk for 1 h, washed with TBST, and incubated with mouse anti-human EGFR, ERK1/2, p-EGFR, p-ERK1/2 antibodies (1:2000) at 4°C overnight. Membranes were washed with TBST, incubated with horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (1:1000) at room temperature for 1 h, washed with TBST, incubated with freshly prepared DAB at dark for 10 min , washed with distilled water to stop color development. Gel imaging analysis system was used to analyze the Western-Blot image. β-actin was used as an internal reference.

Statistical analysis

Using SPSS17.0 statistical software was used for data analysis. All results were expressed as mean ± standard deviation. Count data was expressed as rate or percentage. T-test was use for two groups analysis, analysis of variance was used for comparison between multiple groups, $P < 0.05$ represents significant difference.

Results

Immunohistochemistry

Immunohistochemistry was used to analyze the expression of CAV1 in both laryngeal carcinoma tissues and adjacent tissues. The results

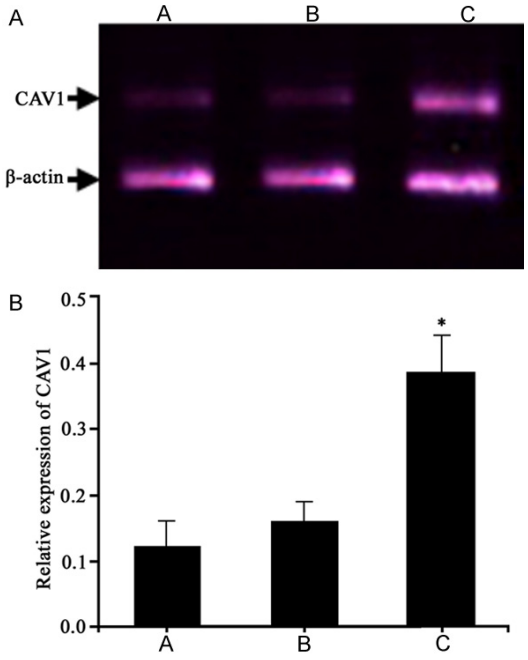

Figure 3. A. Overexpression of CAV1. B. CAV1 expression in Hep2 cells. Note: A: Untransfected Hep2 cells; B: Hep2 cells transfected with empty vector; C: Hep2 cells transfected with CAV1; *represents P < 0.05 when compared with group A.

showed that adjacent tissue was much darker that laryngeal carcinoma tissue (**Figure 1A**), indicating that the expression of CAV1 in laryngeal carcinoma tissues was down-regulated.

Five uniformly stained areas were observed under microscope to count the numbers of positive cells to calculate the positive rate. As shown in **Figure 1B**, CAV1 positive rate in the adjacent tissues was 93.6% (73/78); decreased along with the increase of histological grade (P < 0.05), CAV1 positive rate in stage IV laryngeal carcinoma was 11.1% (4/36). These results indicated that the expression of CAV1 was inhibited in laryngeal carcinoma.

RT-PCR

In order to investigate the expression changes of CAV1 at mRNA level in both laryngeal carcinoma and adjacent tissue, RT-PCR was performed. RT-PCR results showed that the mRNA expression level of CAV1 in laryngeal carcinoma was significantly lower than that of adjacent tissues (P < 0.05) (**Figure 2**), which was consistent with the immunohistochemical staining results.

Transfection and overexpression of CAV1

In order to study the role of CAV1 in the development of laryngeal carcinoma, CAV1 was overexpressed in laryngeal carcinoma cell line HEp2. The constructed CAV1 mammalian cell overexpressing vector was transfected into HEp2. RT-PCR was used to validate CAV1 expression levels in HEp2 cells (**Figure 3**). Results showed that compared with blank control and negative control, the expression level of CAV1 in transfected HEp2 cells was significantly increased (P < 0.05), suggesting that CAV1 was successfully overexpressed in transfected HEp2 cells.

MTT assay

MTT assay was used to measure the proliferation of HEp2 cells transfected with CAV1 at different time points, non-transfected HEp2 cells were used as control. Results showed that 4 days after inoculation, OD570 value of CAV1-transfected HEp2 cells was significantly lower than that of the control cells (P < 0.05) (**Figure 4**), indicating that overexpression of CAV1 significantly decreased HEp2 cells' proliferation capability.

Western-Blot analysis

Western-Blot was used to detection the expression of EGFR, ERK1/2, p-EGFR, p-ERK1/2 in HEp 2 cells (**Figure 5A**). The ratio of p-EGFR and total EGFR and the ratio of p-ERK1/2 and total ERK1/2 were calculated, respectively (**Figure 5B**). Results showed that overexpression of CAV1 had no effect on the expression of total EGFR and total ERK1/2. However, overexpression of CAV1 significantly decreased phosphorylated form of EGFR and phosphorylated form of ERK1/2 (P < 0.05).

Discussion

Laryngeal cancer is relatively common. It has higher mortality and a greater possibility of recurrence after surgery, so the pathogenesis and regulatory mechanisms of laryngeal carcinoma become a hot topic of health care workers and researchers [1]. In this study, immunohistochemistry and RTPCR were used to measure the expression levels of CAVE in laryngeal cancer tissues of different stages and results showed that the expression level of CAV1 in laryngeal carcinoma was significantly down-regulated, indicating that CAV1 might involve in the pathogenesis of laryngeal carcinoma.

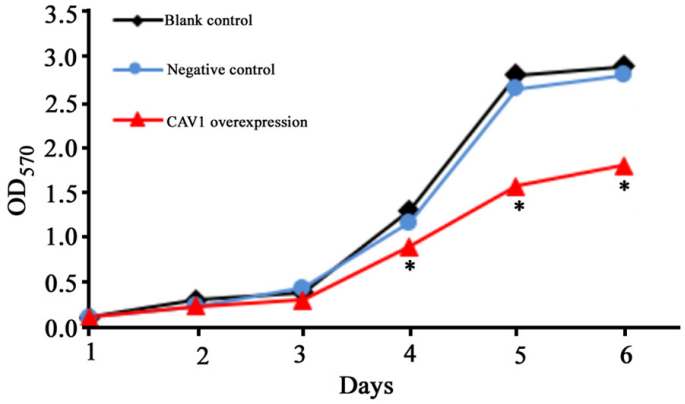

Figure 4. MTT assay of cell proliferation. *represents P < 0.05 when compared with blank control.

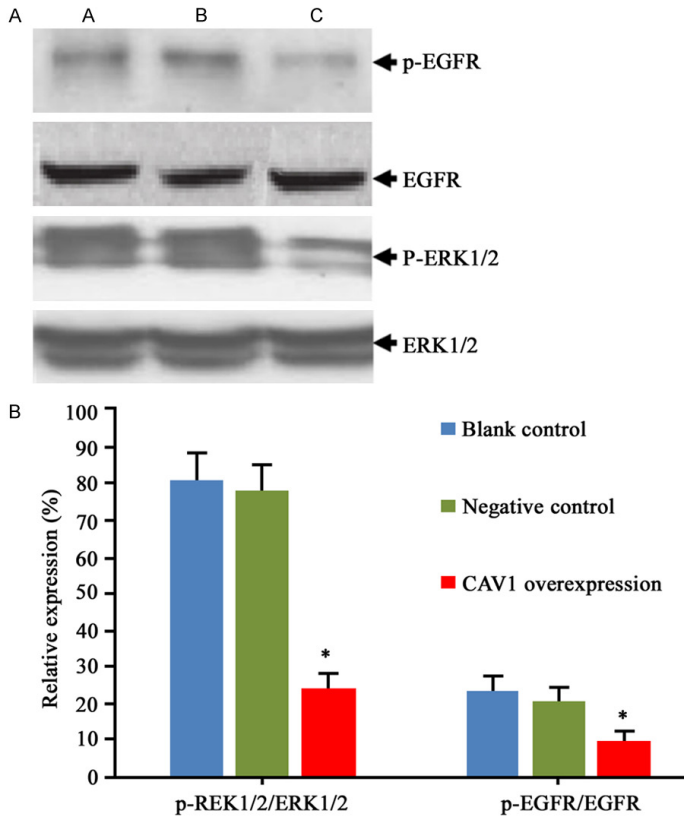

Figure 5. A. Western-Blot analysis. B. Ratios of p-EGFR, p-ERK1/2 and total EGFR and ERK1/2. Note: A: Untransfected Hep2 cells; B: Hep2 cells transfected with empty vector; C: Hep2 cells transfected with CAV1; *represents P < 0.05 when compared with group A.

CAV1 involved in the regulation of cell behavior is mainly due to its ability of binding a variety of important signaling molecules to control the activation status of these signaling molecules, thereby regulating a variety of cellular behavior [6, 7]. This suggests that CAV1 might be related to malignant cell behavior. Researchers have found the change of CAV1 expression levels in a variety of malignant tumor. Witkiewicz et al found that some breast cancer cells lost the expression of CAV1 and the loss of CAV1 was also correlated with poor prognosis in breast cancer patients [16]. Xu et al also found CAV1 expression levels was inhibited in ovarian cancer cells [17]. Yokomori et al showed that down-regulation of CAV1 in hepatocellular carcinoma was implemented by gene methylation [18].

To further investigate the effect of CAV1 on laryngeal cancer, CAV1 was overexpressed in laryngeal carcinoma cell line HEp2. It was found that overexpression of CAV1 in HEp2 cells decreased cell's proliferation capability. Western-Blot results showed that expression levels of phosphorylated EGFR and phosphorylated ERK1/2 were down-regulated, indicating that CAV1 may regulate cell behavior through regulation of phosphorylation of EGFR and ERK1/2.

As mentioned above, CAV1 can bind with a variety of signaling molecules and thereby regulate the activation status of these signaling molecules. This is mainly due to the polymerization of CAV1 facilitates the binding of its N-terminal with a variety of signaling molecules including EGFR, G protein, eNOS, PKC, H-Ras, and Src family of tyrosine kinases, so that these molecules have the opportunity to cross-react and regulate the activation state through the phosphorylation [19]. CAV1 can also inhibit the expression of CyclinD1 to inhibit ERK activation. EGFR and ERK are important proteins regulating cell growth and differentiation [20]. Phosphorylation of EGFR protein activates downstream signaling pathways including Ras-Raf-ERK1/2 pathway and PI3K/Akt pathway, which play important roles in the regulation of tumor cell proliferation, growth, invasion, and metastasis [21]. So, inhibition of CAV1 expression could

result in abnormally activation of downstream signaling pathways, leading to excessive cell proliferation and division.

Currently, the effect of CAV1 on the occurrence and development of laryngeal carcinoma has been reported. This study focused on the change of CAV1 expression in laryngeal carcinoma and its relationship with laryngeal carcinoma. This study also analyzed the effect of CAV1 on EGFR signaling pathway, providing a theoretical basis for the role of CAV1 on the future study and treatment of laryngeal cancer. However, how exactly CAV1 expression was inhibited in laryngeal carcinoma remains unclear and needs to be further studied.

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Disclosure of conflict of interest

None.

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