RESEARCH ARTICLE

Overexpression of RUNX3 Inhibits Malignant Behaviour of Eca109 Cells *in Vitro* and *Vivo*

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Abstract

Runt-related transcription factor 3 (RUNX3) is a tumor suppressor gene whose reduced expression may play an important role in the development and progression of esophageal squamous cell cancer (ESCC). The aim of this study was to investigate the clinical relevance of RUNX3 in ESCC patients and effects of overexpression on biological behaviour of Eca109 cells *in vitro* and *in vivo*. Immunohistochemistry was performed to detect the clinical relevance of RUNX3 and lymph node metastasis in 80 ESCC tissues and 40 non-cancerous tissues using the SP method. RT-PCR and Western blotting were applied to assess the RUNX3 level and verify the Eca109 cell line with stable overexpression. Localization of RUNX3 proteins was performed by cell immunofluorescence. CCK-8 and Scrape motility assays were used to determine proliferation and migration and the TUNEL assay to analyze cell apoptosis. Invasive potential was assessed in cell transwell invasion experiments. In nude mice, tumorigenesis *in vivo* was determined. Results showed decreased expression of RUNX3 in esophageal tissue to be significantly related to lymph node metastasis (LNM) ($P<0.01$). In addition, construction of a recombinant lentiviral vector and transfection into the human ESCC cell line Eca109 demonstrated that overexpression could inhibit cell proliferation, migration and invasion, and induce apoptosis. The *in vivo* experiments in mice showed tumorigenicity and invasiveness to be significantly reduced. Taken together, our studies indicate that underexpression of RUNX3 in human ESCC tissue is significantly correlated with progression. Restoration of RUNX3 expression significantly inhibits ESCC cells proliferation, migration, invasion and tumorigenesis.

Keywords: Esophageal squamous cancer - RUNX3 - lentivirus - overexpression - proliferation - apoptosis

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Introduction

Esophageal squamous cell cancer (ESCC) is a malignancy that arises from esophageal epithelial cells, it is a potentially fatal disease with high incidence worldwide, particularly in China (Sun et al., 2011). The treatment for ESCC includes surgery, radiotherapy and chemotherapy. Despite technical progress for ESCC, the effect of treatment is still unsatisfactory. ESCC is still a leading cause of cancer-related death (Dresner and Griffin., 2000). Consequently, it is of great clinical value to find new genes involved in ESCC tumorigenesis and progression, in order to develop early diagnosis and improved disease outcome predication following treatment of this dangerous disease (Subramaniam et al., 2009).

Multiple genes participate in the occurrence and development of ESCC. Runt-related transcription factor 3 (RUNX3) is one of the runt-domain family of transcription factors which has been first reported in gastric cancer. The RUNX3 gene is regarded as a tumor suppressor gene in many human tumors, and its inactivation is believed to be related with the occurrence and development of tumor (Ito et al., 2008). Recently it was reported that RUNX3 gene expression is downregulated in various solid tumors. Previous study demonstrated that the down-regulation of Runx3 in ESCC tissues is associated with poor prognosis (Soong et al., 2009).

Lymph node metastasis (LNM) is a significant factor for determining the prognosis of patients with esophageal cancer. However, little data is available on the relationship between RUNX3 gene expression in esophageal cancer tissues and LNM (Nevadunsky et al., 2009). It is also less known about the effect of its overexpression in esophageal squamous cell cancer (ESCC), the previous studies were restricted to cancer lesions specimen of clinical patients, few papers are reported on the effect of the biological behaviour of ESCC cell line by up-regulation of RUNX3 expression, especially using lentiviral vector for transfection of gene (Li et al., 2002).

In the present study, we focus on the correlation between RUNX3 expression and LNM, detection the inhibit effects of RUNX3 overexpression in ESCC cells biological behaviour and tumorigenesis, aimed to identify a new therapeutic target and provide a foundation for gene therapy in esophageal cancer.

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Materials and Methods

Tissue collection

120 esophageal squamous tissue samples (40 normal esophageal tissues, 40 esophageal cancer tissues with LNM, 40 esophageal cancer tissues without LNM) during resective surgery were collected for this study from Department of Thoracic Surgery, Provincial Hospital Affiliated to Shandong University. Postoperative staging was based on the tumor-node-metastasis (TNM) TNM classification of the International Union against Cancer (UICC) in 2009, esophageal cancer without LNM patients had stageIB (pT1, 2 N0M0) disease, esophageal cancer with LNM patients had stageIIB (pT1, 2 N1M0) disease. The clinicopathologic variables such as gender, age, the histologic type and the status of the resection margin were retrospectively reviewed on the basis of the medical records. All patients were not pretreated with radiotherapy or chemotherapy prior to surgery. This study was approved by Ethical Committee of Provincial Hospital Affiliated to Shandong University, and written informed consent was obtained from all patients.

Cell culture

Human ESCC Eca109 cell line, which was first cultured and purchased from Academia Sinica (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 enriched with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). Cell culture plates were maintained in humidified incubators at 37°C in a 5% CO_2 incubator.

Lentiviral vector construction

For the transfection of the tumor cell lines, lentiviral vectors harboring RUNX3 gene (NM-004350) were constructed (GeneChem, China). The RUNX3 plasmid or empty vector plasmid and its packaging plasmid were both extracted using a plasmid extraction kit and were cotransfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). After 48 hours, supernatants were collected from these cells and passed through a 0.45 μm filter (Millipore), aliquoted, and stored in small aliquots at -80°C. The lentivirus carried GFP, therefore, viral titer was determined by the method of end point dilution through counting the numbers of infected green cells at ×100 magnification under fluorescence microscope (Olympus, Tokyo, Japan). Briefly, the Eca109 cells were transfected according to the manufacturer's instruction. The same amount of negative control vector was also transfected. 24 h later, the virus-containing medium was replaced with fresh complete medium. GFP was observed by fluorescence microscopy after 72 h.

Tissue immunohistochemistry

Sections (4 μm thick) were heat fixed, deparaffinized, and rehydrated by standard methods. After the formalin-fixed, paraffin-embedded tissues were deparaffinized and antigen retrieved, tissue sections were incubated overnight at 4°C with the monoclonal mouse antihuman RUNX3 antibody (1: 50, Santa Cruz, USA). After washing with PBS, the slides were incubated with biotionylated second antibody (1: 100, Zhongshan Biotech, China) for 30 min at 37°C, followed by streptavidin-peroxidase incubation at 37°C for 30 min. Finally, sections were colored with diaminobenzidine tetrahydrochloride (DAB) for 2 min. For negative controls, we replaced the primary antibody with phosphatebuffered saline. All sections were examined by two independent pathologists who were blinded to the clinical data. The expression of RUNX3 was graded as positive when 5% of tumor cells showed immunopositivity. Biopsies with 5% tumor cells showing immunostaining were considered negative.

RNA extraction and real time-PCR

Total RNA was extracted from cells by RNAiso Plus (Takara, JPN), according to the manufacturer's protocol. All RNA samples were treated with RNase-free (Takara, JPN) and stored at -80°C. Two micrograms of RNA was subjected to reverse transcription, using reverse transcriptase reagent Kit with gDNA Eraser (Takara, JPN) to obtain cDNA. β-actin was used as an internal control, the PCR primers (Takara, JPN) used were as follows:
RUNX3 forward: 5'- ACCTGTCACAACGGCCAGAAC-3';
RUNX3 reverse: 5'- TTCCAGTGAGGACAGGCCAAG -3;
β-actin forward: 5'- GGCGGCACCACCATGTACCCT -3';
β-actin reverse: 5'- AGGGGCCGGACTCGTCATACT -3'.

RUNX3 mRNA expression was evaluated by LightCycler480 sequence detection system (Roche Diagnostics GmbH, USA) according to the supplied protocol. ACTB (β-actin) was used as an internal control. Amplification conditions were as follows: Reverse-transcription reaction: 42°C, 30 minutes per cycle. PCR cycling conditions were as follows: Enzyme activation 95°C 15 minutes per cycle, denaturation 95°C at 15 seconds per 40 cycles and Annealing/Extension at 60°C for 60 seconds. Measurements between samples were compared by the threshold cycle of amplification (C_T). The fold change in expression levels was determined by a comparative C_T method using the formula:$2^{-\Delta\Delta Ct}$, $\Delta\Delta CT = (C_T RUNX3\text{-}C_T \beta\text{-actin})sample\text{-} (C_T RUNX3\text{-}C_T \beta\text{-actin})$ Eca109.

Total protein extraction and Western blot assay

Cells were collected by trypsinization. Total protein was extracted using the RIPA (Reusable Industrial Packaging Association, ZSGB-BIO) plus PMSF (phenylmethylsulfonyl fluoride, ZSGB-BIO) according to manufacturer's in instructions. The total protein extracted from cells was diluted in 4×loading buffer, applied to a 10% polyacrylamide gel, underwent electrophoresis, using PageRuler Prestained Potein Ladder (Fermentas) as size markers. The proteins were transferred to Hybond-P polyvinylidene difluoride membranes (Amersham) in trans-buffer containing 25mM Tris and 185mM glycine, PH8.3 together with 20% methanol. After transfer, the membranes were blocked for 1h in blocking buffer (TBS containing 0.1% Tween-20, TBST), supplement with 5% non-fat dry milk) and then the membranes were incubated overnight at 4°C with mouse anti-human RUNX3 and GAPDH monoclonal antibodies in blocking buffer. After being washed three times with TBST, the membranes were incubated with HRP-conjugated goat anti-mouse

secondary antibodies (1:10000, Zhongshan Biotech, China) for 30 min, then the immunoblots were exposed to X-ray film. Western blotting densitometric analysis was performed using Image Reader LAS-4000 analysis software. The band densities were normalized to the densities of GAPDH. The electrophoretic results were scanned into images. Data were collected by Multi Gauge software and the ratio of strip density to GAPDH density served as index for statistical analysis.

Cell immunofluorescence

Cell immunofluorescence staining was used to examine the expression of RUNX3. After treatment, Eca109 cells in the three groups on slips were washed with PBS three times and blocked by incubation in 3%H2O2 for 10min at 37°C. The slips were fixed with 4% paraformaldhyde (PH=7.0) for 30min following with trutonX-100 for 30min at 37°C. Each slip was incubation with mouse anti-human RUNX3 (1: 50, Santa Cruz, USA) as primary antibodies overnight at 4°C. After incubation, the slips were rinsed with PBS and then incubation with an HRP-conjugated goat anti-mouse IgG (1:50, Zhongshan Biotech, China) as the second antibody. HRP activity was detected using diaminobenzidine tetrahydrochloride (DAB) as substrate for 2min in accordance with the manufacturer's instructions. Images of cells were acquired by using a LeicaTM fluorescence microscope equipped with a LeicaTM camera.

Cell proliferative analysis

Cell Counting kit (CCK)-8 (Zhongshan Biotech, China) is the routine checks of the cell growth was performed to assess the proliferation of the transduced cells. Cells were seeded at an initial density of 5×10^4 cells/ml in 96-well plates (three wells per group, total 5 plates) for 12, 24, 48, 72, 120h post-transfection respectively.CCK-8 was added to the wells (10 μL per well) at the experimental period. After 2 hours of incubation at 37°C in 5% CO_2, Optical density (OD) was determined by a Tecan Infinite M200 Multimode microplate reader at the absorbance of each well at 450 nm wavelengths. Triplicate wells were used foreach data point. All experiments were performed in triplicate, and the average of the results was calculated.

Scrape motility assay

Eca109 cells from different groups were plated at a density of 1×10^5 cells/ml in 6-well plates in 1640 medium with 10% FBS. After overnight incubation, the tip of a plastic pipette was drawn across the center of the well to produce a scraped area. The culture cells were washed twice with PBS, followed by incubation in 1640 medium. Immediately after scraping (0h), 72 h incubation, the two-dimensional Eca109 migration into the cell-free area at the center of the well was photographed with OLYMPUS IX70 inverted microscope (Olympus Corporation, Tokyo, Japan) and printed. Cells that migrated into the scraped area after 72 h were counted by an observer blinded to the study design.

Cell invasion assay

To assess the role of RUNX3 overexpression on invasion, Eca109 cells from different groups were seeded on a fibronectin-coated polycarbonate membrane insert (6.5 mm in diameter with 8.0 μm pores) in a transwell apparatus (Merck Millipore Bioscience, Germany). For invasion assay, lower chambers of matrigel coated invasion plates were coated with 10 mg/ml fibronectin overnight at 4°C and cells invading through matrigel were fixed and stained after 48 hours. Each upper well was loaded with 2.5x105cells in a total volume of 200 μl of serum-free medium. The lower wells of the chamber were loaded with 600 μl of 1640 medium with 10% FBS. At the end of the experimental time period, any cells remaining on top of the insert were removed by scraping. Cells that migrated to the bottom surface of the insert were fixed with ethanol-based crystal violet solution for 10min. Cells were counted based on12 random high power fields (×200) under a light microscope (LEICA DM4000B, Leica, Wetzlar, Germany). The invasion of three groups Eca109 cells were expressed as mean number of cells per high power field.

Apoptosis assay

To quantify the relative numbers of Eca109 cells with DNA fragmentation, a TUNEL assay was performed for all groups, using TUNEL assay Roche kit (Roche, USA) according to the manufacturer's protocol. Apoptosis was evaluated by counting the positive cells as well as the total number of cells in 10 arbitrarily selected fields at×400 magnification under optical microscope (Olympus) in a double-blinded manner. The apoptotic index (per×400 microscopic fields) was calculated as number of apoptotic cells×100/total number of cells. Green-stained nuclei immediately at the edge of a tissue section were excluded from cell counts to minimize false positives.

BALB/c Nude Mice Transplantation

Six-week-old BALB/c Nude Mice were purchased from Vital river co. [Certificate of Quality No.: SCXK (Beijing) 2012-0001] and maintained in specific pathogen-free facilities at the Experimental Animal Center of Provincial Hospital Affiliated to Shandong University. Mice were provided with free access to food, water and bedding at all time and were housed with a 12h light/dark cycle in filter top cages containing a maximum of five mice per cage. The mice were randomly separated into 3 groups of 6 each. 1. Eca109 as the Eca109 group; 2.Lentivirus-CMV-GFP /Eca109 as the EV group; 3.Lentivirus-CMV-GFP/RUNX3/Eca109 as the RUNX3 group. Under aseptic conditions, the cells were digested and respectively implanted into t the skin of the right shoulder of the mice in a total volume of 100 μl (1×10^6) cells resuspended by PBS without FBS. Mice in the third group received no treatment. Mice were sacrificed by cervical dislocation, and growth of the tumor was evaluated up to day 28 after cell inoculation. The tumor-bearing mice were sacrificed 4 weeks after inoculation, and the tumors were removed and weighed. Tumor volume was calculated using the formula: Tumor volume = long diameter × short diameter^2/2.

Statistical analysis

SPSS 19.0 software was used for all statistical analysis.

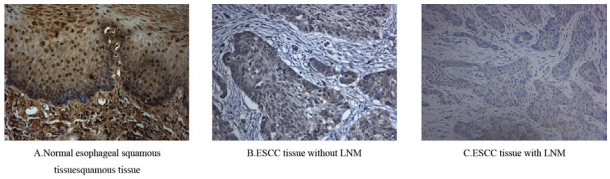

Figure 1. Immunohistochemical Staining of RUNX3. in ESCC Tissues and Paired Non-cancerous Tissues. RUNX3 immunoreactivity was localized in both the nucleus and cytoplasm, positive RUNX3 staining in nucleus of the normal esophageal squamous tissue, and negative RUNX3 staining in the nucleus of ESCC tissue (×400)

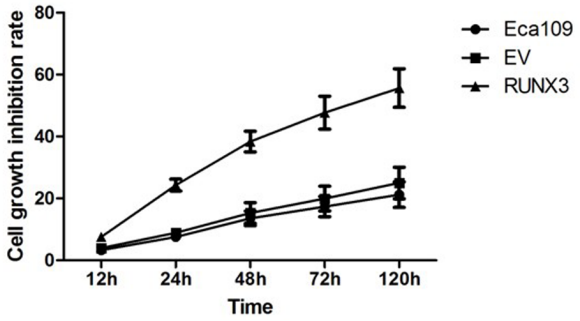

Figure 3. RUNX3 Overexpression Inhibited Eca109 Cell Proliferation. Cell growth inhibition rate of each Eca109 cell group. Cell proliferation was determined at 12, 24, 48, 72 and 120 h, respectively. Absorbance from the plates was read at 490 nm. Eca109: non-infected control, EV: lenti-empty-vector, RUNX3: lenti-RUNX3-vector

significance was assessed by comparing mean values (Mean±SD) using the Student's t-test for independent groups. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Lower expression of RUNX3 in ESCC

By immunohistochemical staining, the positive expression of RUNX3 protein showed as yellow or brownish yellow stain in the cytoplasm and/or nucleus of tumor cells. It was determined that reduced RUNX3 expression in the nucleus in two types of human ESCC through immunohistochemical analysis. RUNX3 expression in the nucleus was not detected in 85.0% (34/40) ESCC patients with LNM. In contrast, negative expression of RUNX3 was observed 62.5% (25/40) in ESCC patients without LNM and 25.0% (10/40) in the normal esophageal squamous tissue. Analysis indicated that positive expression of RUNX3 in ESCC and normal esophageal squamous tissue was significantly different ($p<0.01$). The result confirms that RUNX3 was commonly expressed in esophageal squamous tissue cells but decreased or absent in ESCC cells. and RUNX3 protein expression varied significantly with LNM (Figure 1).

RUNX3 overexpression inhibits ESCC cell biological behaviour ***in vitro***

To examine the effect of RUNX3 on cell biological behaviour, Eca109 cells cells were infected with either lentivirus containing the RUNX3 gene (lenti-RUNX3-vector) or empty virus (lenti-empty-vector). The cells were randomly divided into three groups: the Eca109

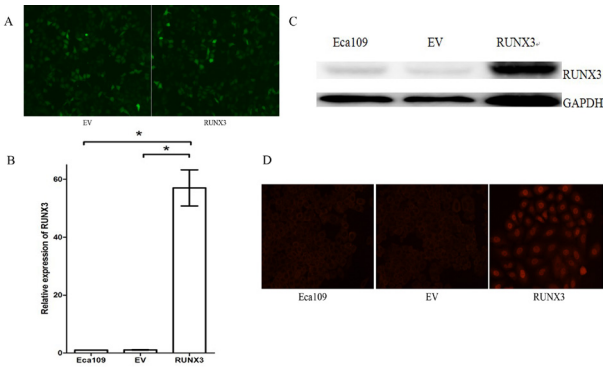

Figure 2. Targeted Depletion of RUNX3 Through Lentivirus-mediated RUNX3. (A) Representetive graphs of Eca109 cells infected with indicated lentivirus at MOI of 50 were shown (×200). (B) Following infection of cells with indicated lentivirus for 120h, RUNX3 mRNA levels were measured with real-time PCR, (C) and protein levels were detected by Western blot analysis. (D) RUNX3 expression in three Eca109 group by immunofluorescence and confocal microscope in 72h after virus infection (×200). *$p<0.01$, compared to control cells. Eca109: non-infected control, EV: lenti-empty-vector, RUNX3: lenti-RUNX3-vector

group (control group), EV group (lenti-vector group) and RUNX3 group (lenti-RUNX3). Effeciency of lentivirus infection was more than 95% as evidenced by GFP expression 72 h after infection (Figure 2A). RUNX3 mRNA expression was then measured with real-time PCR. As shown in Figure 2B, exogenous RUNX3 mRNA was obviously increased in RUNX3 group. Compared with that in the Eca109 group, The ΔΔCt in EV group was 1.058±0.0921, and in RUNX3 group was 57.05±6.224 ($p<0.01$). We further determined the suppression effect by measuring RUNX3 protein levels using Western blot assay. It was demonstrated that the relative optical density values of the RUNX3 protein expressions in Eca109 cells of RUNX3 group (0.9761±0.0225) was significantly up-regulated than that in Eca109 group (0.1604±0.0248) and EV group (0.1553±0.0365) ($p<0.01$) (Figure 2C). There was no statistical significance in Eca109 group and EV group ($p>0.05$). It was demonstrated that transfection of Eca109 cells with RUNX3 lentiviral vector resulted in a significantly increased expression of RUNX3 at both mRNA and protein levels ($p<0.01$). Immunocytochemistry microscopy showed that the positive expression rate of RUNX3 expression in three cell groups did not have obviously change. In RUNX3 group, a higher level of HLA-G is expressed in nucleus than other two groups (Figure 2D).

To elucidate the role of RUNX3 in ESCC proliferation, each group cells were first examined by CCK-8 assay. The result showed that the cell growth inhibition rate for RUN3 group was significantly higher compared to those of control cells ($p<0.01$). The rate of cell growth inhibition was similar in the two control groups. Along with the extension of the time, the inhibition rate was increased. There was a significant increase in the cell number in RUNX3 group compared to the cell number in control and scramble negative controls at 24, 48, 72, 96, 120, and 144 h. The inhibition of Eca109 proliferation in the RUNX3 group was most evident at 48 h (Figure 3).

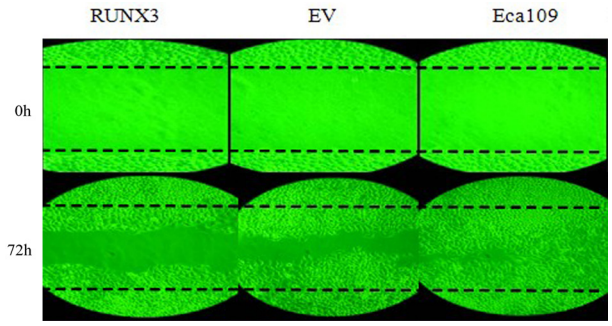

Figure 4. RUNX3 Overexpression Suppresses Eca109 Cell Migration. Representative images of scrape motility assay in monolayer culture are shown for Eca109 cells 0hand 72h after the scratch. Eca109: non-infected control, EV: lenti-empty-vector, RUNX3: lenti-RUNX3-vector

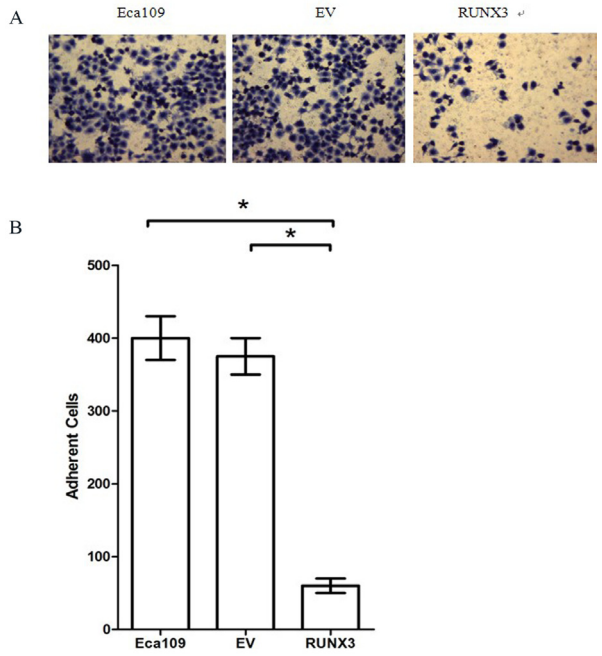

Figure 5. RUNX3 Overexpression Inhibits Invasion of Eca109 Cells. (A) Representative photos of Eca109 invasion assay after cultivation for 48 h (×200). (B) The cells on the lower surface of the membrane were counted in 12 randomly selected fields. The value represents the expressed as the mean ± SD. *p<0.01,Eca109: non-infected control, EV: lenti-empty-vector, RUNX3: lenti-RUNX3-vector

In the ESCC, the migrated of ESCC cells into the normal esophageal squamous tissue is considered important for ESCC development. Therefore, by using scrape motility assay in monolayer culture, it showed that the number of surviving colonies of RUNX3 overexpression cells was markedly decreased compared with those of control cells that migrated to the scraped area (Figure 4). The result investigated that RUNX3 could directly alter the migratory behavior of these cells,

On the other hand, we performed an invasion assay in a modifed Boyden chamber to examine the effect of RUNX3 overexpression on the invasive potency of the Eca109 cells *in vitro*. Movement of cells through Matrigel-coated Boyden chambers mimics the early steps of tumor invasion. After cultivation for 48h, the mean ± SD of cells attached to the lower surface of the membrane of different groups, as indicated previously, were as follows:

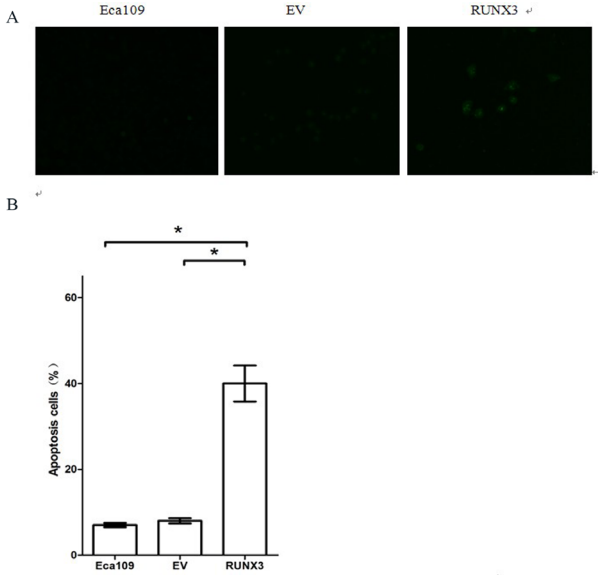

Figure 6. RUNX3 Overexpression Stimulates Apoptosis in Eca109 Cell. (A) TUNEL staining in 48 Hours after lenti-vector Treated, green fluorescence is due to FITC staining and observed under blue filter (×400). (B) The apoptosis rates in three groups cells value represents as the Mean ± SD. *p<0.01, Eca109: non-infected control, EV: lenti-empty-vector, RUNX3: lenti-RUNX3-vector

Eca109 group, 409.35±30.16; EV group, 374.69±25.43; and RUNX3 group, 61.02±9.73 (Figure 5A, 5B). The cells in RUNX3 group exhibited significantly reduced invasive tendencies compared with those of control cells (p < 0.01 5). Therefore, disruption of exogenous RUNX3 overexpression resulted in inhibition of cell invasion in ESCC cells.

Furthermore, apoptotic cells were visualized in situ through labeling of fragmented DNA by TUNEL in each Eca109 cells. The results indicated that apoptosis indices of Eca109 cells were elevated in RUNX3 group (41.0±7.3%) in comparison to Eca109 group (6.8±0. 7%) and EV group (7.1±0. 9%) for 48 h resulted in apoptosis (Figure 6A, 6B). respectively. Differences between the RUNX3 group and other control groups were statistically significant (p <0.01).

RUNX3 overexpression repressed the tumorigenicity of ESCC cells ***in vivo***

Next, *in vivo* subcutaneous tumor formative assay was adopted to examine the tumorigenesis of Eca109 cells in nude mice. In-vivo experiment showed that the tumor mass could be touched in the mice 7 days after inoculation, the tumor formation rate being 100%. After 4 weeks after inoculation, Compared to control cells, the injection of RUNX3 group cells led to a significantly decrease in tumor volume and weight (Figure 7). The average tumor volume in RUNX3 group (0.610±0.093 cm^3) was significantly lower than that in Eca109 group (1.340±0.351 cm^3)and EV group (1.412±0.233 cm^3) (p<0.01). The average tumor weight in RUNX3 group (0.66 ± 0.18 g) was lower than that in Eca109 group (1.45±0.26g)and EV group (1.57±0.31 g) too (p<0.01). No obvious difference was found in body weight of mice in the treated and control groups.

Discussion

RUNX3 is located on human chromosome region 1p36 and plays an important role in the transforming growth factor (TGF)-β signaling pathway (Bangsow et al., 2001). Being a downstream transcription factors of TGF-β. Growing interest is currently focused on the role of RUNX3 in the regulation of cell survival and death (Sakakura et al., 2007). RUNX3 has previously been reported to be expressed at low levels in esophageal carcinoma tissues compared with the adjacent non-cancerous tissues (Tonomoto et al., 2007). The research *in vivo* of Hiramatsu found that in 144 ESCC cases RUNX3 expression was significantly higher in the 19 well-differentiated SCCs than in the 56 moderately or 69 poorly differentiated SCCs. The 3-year survival rate was significantly lower in the 29 patients with lower RUNX3 expression (6.9%) than in the 37 patients with higher expression (35.1%) (Hiramatsu et al., 2005). Lower level of gene expression in the ESCC cells was associated with patients' prolonged survival, the lower the RUNX3 expression, the poorer the prognosis (Miyazono et al., 2003). *In vitro* trials, Torquati demonstrated that ESCC cell line SEG-1 was lack of RUNX3 protein expression, while RUNX3 is highly expressed in normal human gastric and esophageal epithelium cells (Torquati et al., 2004). In the present study, we found that 85% of ESCC tissues (34/40) showed RUNX3 gene downregulation, whereas it was downregulated in only 25% of normal ESCC tissues (10/40) by tissue immunohistochemistry. These results strongly suggest that the expression of RUNX3 in esophageal tissue is significantly related to LNM in patients with ESCC (Chen et al., 2009).

At present, gene alteration is known to be a key factor for a cancer cell to regulate its biological behavior (Lau et al., 2006). The ideal viral vector should provide efficient gene transfers, stable long-term gene expression and good biological safety (Nakagawa et al., 2004). The lentivirus vectors offer significant advantages over retroviral vectors in the process of gene delivery. HIV-1 is the best studied lentivirus and most of the currently used lentiviral vectors (LVs) which can stably integrate their genetic information into target cells (Sugiura et al., 2008). It can transfect most range of cells and integrate into the host genome in dividing and post-mitotic cells, resulting in long-term expression of the transgene *in vitro* and *in vivo*.

In this research, a lentivirus co-expressing GFP and RUNX3 genes was first constructed. After transfected, the results indicated that the stable mRNA and protein levels of RUNX3 in the Eca109 cells were higher than the levels in the Eca109 cells. A continuous CCK-8 assay and scrape motility assay demonstrated that cellular proliferative capacity was suppressed after overexpression of RUNX3 protein, suggesting a role of RUNX3 in inhibiting tumor cell growth. TUNEL staining shown that after the transfection, overexpression RUNX3 could contribute to cell apoptosis. Out of control growth and malignant metastasis is one of the major causes of mortality in ESCC patients. Tumor growth and metastasis follow and promote each other all the time (Ito et al., 2005). Migration and invasion ability of the cancer cell itself are important influence factors of alternative of surgery modality and prognosis (Kim et al., 2008). Scrape motility assay and cell transwell invasion assay shown that the numbers of migrated cells in overexpresssion RUNX3 groups was found to be significantly fewer than those in the two control groups. Our finding suggests that overexpression RUNX3 in ESCC cells dramatically inhibit migration and invasion ability (Won et al., 2003). The result of cell immunofluorescence found in the RUNX3 group, the RUNX3 increase significantly in nucleus than other two control groups, but RUNX3 in cytoplasmic hasn't changed remarkably. It proves that the overexpression RUNX3 also transfers to the cytoplasm of cells to produces its effect. These results suggest that RUNX3 was present in the nucleus of cells in control tumors (Subramaniam et al., 2009). And the cytoplasmic RUNX3 protein does not elicit tumor suppressive activity, and that it is therefore a novel mechanism of RUNX3 inactivation. The presence of RUNX3 in the cytoplasm of cells suggests at least two possibilities: RUNX3 has an as yet unknown function in the cytoplasm and/or it is retained in the cytoplasm in an inert form until it is mobilized to the nucleus under appropriate conditions (Smith et al., 2008). These results imply that besides promoter region hypermethylation and mutation (Wang et al., 20138), mislocalization of active nuclear RUNX3 protein to the cytoplasm may be one of the main reasons for the RUNX3 inactivation in ESCC cells.

Furthermore, to validate effectiveness of up regulation of RUNX3 expression, We employed a nude mouse model of xenotransplantation, the average volume of the tumor in the RUNX3 group were significantly lower than those in the control group when RUNX3 mRNA and protein were by lentivirus vector. This agreed with our vitro research. Our *in vivo* experiments showed that the lentivirus-delivered overexpression RUNX3 inhibited the tumorigenicity of Eca109 cells and suppressed tumor growth significantly.

In conclusion, our results provide strong evidences that RUNX3 overexpression in ESCC cell and RUNX3 plays an oncogenic role in tumorigenesis progression of ESCC. Lentivirus-mediated RUNX3 overexpression has effectively up regulated the expression of RUNX3 gene on mRNA and protein levels. Moreover, overexpression of RUNX3 has led to the significant decrease in proliferation, migration and invasion, inhibition of migration of Eca109 cells *in vitro*, slow down the tumor growth *in vivo*. Therefore, these results suggest that RUNX3 is down-regulated during ESCC progression and LNM is a regulatory factor of RUNX3 expression clinically. In addition, it indicates the potential value to apply lentiviral-based RUNX3 overexpression as gene transfervector for ESCC gene therapy.

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