

Original Article

The effect of high Sox3 expression on lymphangiogenesis and lymph node metastasis in esophageal squamous cell carcinoma

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Abstract: Objective: This study aimed to investigate the effect of Sox3 expression on biological behaviors of esophageal squamous cell carcinoma (ESCC) and explore its possible mechanism. Methods: ESCC cell lines that highly expressed Sox3 were selected and transfected with lentivirus carrying sox3 siRNA to establish ESCC cell lines which expressed Sox3 of different levels. Using *in vitro* experiments including cell invasion, cell scratch, cell proliferation and tube formation of lymphatic endothelial cells, as well as an *in vivo* experiment of axillary lymph node metastasis in a nude mouse model of a xenotransplanted tumor, the effect of Sox3 expression variation on lymphangiogenesis and lymph node metastasis in ESCC cells was investigated. In addition, ELISA, Western blot and immunohistochemical methods were used to study the regulatory effects of Sox3 on relevant molecules such as VEGF-C/D and to explore the potential mechanisms that affected lymphatic metastasis. Results: The high expression of Sox3 in ESCC cells *in vitro* could significantly promote the proliferation, invasion, migration and tube formation of lymphatic endothelial cells. High expression of Sox3 *in vivo* could significantly promote lymph node metastasis of ESCC cells, and we have demonstrated that the upregulation of Sox-3 expression could promote the expression and secretion of VEGF-C and VEGF-D both *in vivo* and *in vitro*. After blocking the VEGFR-3 receptors on lymphatic endothelial cells, the effect of Sox3 on promoting lymphangiogenesis has decreased significantly, confirming that Sox3 acts through VEGF-C/D to promote lymphangiogenesis. Conclusions: It is suggested that Sox3 possibly induces lymphangiogenesis by increasing the expression of VEGF-C/D in ESCC cells, thereby promoting the lymph node metastasis of the tumor. Thus, Sox-3 may become a new prognostic marker and therapeutic target in ESCC.

Keywords: Vascular endothelial growth factor C/D, Sox3, esophageal squamous cell carcinoma

Introduction

The incidence of esophageal cancer ranks the 8th among the most common malignancies worldwide, and in the cancer related deaths, esophageal cancer ranks the 5th and 8th in men and women, respectively [1]. Because most patients suffering esophageal cancer have been already in the locally advanced stage or have suffered distant metastasis when they visit the hospital, the 5-year survival rate of esophageal cancer patients is as low as 19% [2]. Esophageal cancer is divided into two main pathological types: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). EAC is the main type in the United States and Europe, while in Asia most patients are of ESCC. China is a country with a high inci-

dence of esophageal cancer and 95% of the tumors are ESCC affecting the thoracic region, with esophageal cancer as the 4th leading cause of cancer related deaths [3]. The prognosis of ESCC is much different compared to the EAC, wherein the 10-year survival rate of EAC is over 40% compared to about 20% of ESCC [4]. Even the 5-year survival rate of early diagnosed ESCC is significantly worse than that of the adenocarcinoma in the same early stage [5]. The reasons for the different prognosis in EAC and ESCC are unclear at this moment, and most researchers believe the reasons are due to the different types of lymphatic metastasis in the two types of tumor [6].

The Sox (Sry-like HMG box) protein family belongs to the HMGB (high-mobility group box)

Table 1. Primers for fluorescence quantitative PCR

Primers	Sequence (5'-3')	Products (bp)
Sox3 F	5'-GCAAGCTGGCAAAGTTGATCT-3'	106
Sox3 R	5'-GCTGCTCAGTTCACCGATG-3'	
Actin F	5'-CTGCCTGACGGCCAGG-3'	89
Actin R	5'-GATTCCATACCCAAGAAGGAAGG-3'	

protein superfamily and is a type of DNA-binding protein [7]. In this protein family, Sox3 has been shown to be a proto-oncogene containing a retroviral insertion site, and it could lead to lymphoma upon binding onto a retroviral DNA [8]. A recent study has shown that the serum auto-antibody level of Sox3 in small cell lung cancer (SCLC) patients is significantly higher [9], therefore Sox3 may be related to tumor occurrence and development. In our department, a preliminary study on the expression of Sox3 in ESCC was carried out, and it was found that the expression of Sox3 in ESCC was increased and closely related to lymph node metastasis in the tumor region as well as TNM staging; the increase in Sox3 expression was related to poor prognosis in ESCC patients, and Sox3 was an independent prognostic factor of ESCC [10]. Our research showed that SOX3 may play important roles in biological behaviors of ESCC such as proliferation, apoptosis, invasion and particularly lymph node metastasis. To further investigate the function and molecular mechanism of Sox3 in lymphatic metastasis, ESCC cell lines were used in this study as the model, and ESCC cell lines expressing different levels of Sox3 were constructed by using adenovirus vectors carrying Sox3 siRNA. *In vivo* studies in nude mice and *in vitro* experiments were conducted to investigate the effect of Sox3 expression variation on lymphatic metastasis of ESCC cells. Using different methods including the tube formation experiments of lymphatic endothelial cells, ELISA, Western blot and immunohistochemical assays, it was found that Sox3 may promote lymphatic metastasis of the tumor by upregulating the expression of VEGF-C/D in ESCC cells and inducing the lymphangiogenesis. The results showed that Sox3 plays an important role in esophageal and lymphatic metastasis of the tumor, and may become a new prognostic marker and therapeutic target for new ESCC treatment.

Materials and methods

Materials

Human ESCC cell lines TE-1, TE-10, TE-11, EC109, EC9706 (Shanghai cell bank of the China cell line repository, China); human lymphatic endothelial cells (Cell bank of Guangzhou Jiniou Biotechnology, China); RPMI 1640 medium (Hyclone, USA), DMEM medium (Hyclone, USA); fetal bovine serum (Gibco, USA), penicillin (North China pharmaceutical factory, China); streptomycin (North China Pharmaceutical Factory, China); SAR131675 (Selleck, China), Matrigel gel (BD, USA); human vascular endothelial growth factor C (VEGF-C) ELISA kit (Shanghai Sangon, China); vascular endothelial growth factor D (VEGF-D) ELISA kit (Shanghai Sangon, China); HRP secondary antibody (GenScript, USA); internal control primary antibody (GenScript, USA); VEGF-C primary antibody (Abcam, USA); VEGF-D primary antibody (Abcam, USA); sox3 antibody (Abcam, USA); sox3-RNAi lentivirus (Genechem, China).

Western blot

After digestion, the cells were collected and added in an appropriate amount of RIPA lysis buffer. The tissues were first cut into fragments of 1 mm³, added into the RIPA lysis buffer and homogenized. After lysis, the cells or tissue fragments were centrifuged at 4°C and 12,000 g for 5 min, and the supernatant was collected for SDS-PAGE and Western blot. After scanning of the exposed images, the UVP gel image analysis system Labworks4.6 software was used to analyze the gray value of the bands.

RT-PCR

After cell lysis and collection, total RNA was extracted for random primer reverse transcription reaction and SYBR green I fluorescence quantitative PCR (Eppendorf, USA), and then RNA levels of sox3 were measured. The primer sequences are shown in **Table 1**, and the PCR conditions were as follows: 94°C for 4 min; and 35 cycles of 94°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec. Each sample was examined in triplicate.

Transwell invasion assay

TE-10 cells were digested and collected by centrifugation, washed once with PBS, resuspend-

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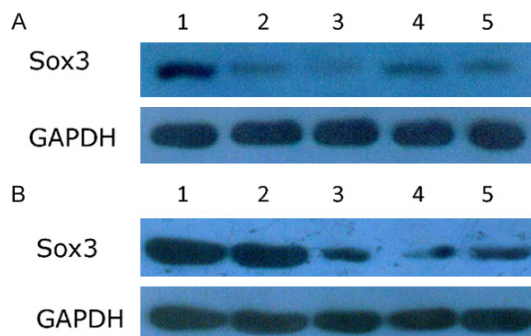


Figure 1. Expression of Sox 3 in ESCC cell models. A: In esophageal squamous cell carcinoma cell line. 1: TE-10; 2: TE-11; 3: TE-1; 4: EC109; 5: EC9706 cell. B: In TE-10 cell after si-RNAi interference. 1: Control group; 2: Empty virus group; 3: si-RNAi 1 group; 4: si-RNAi 2 group; 5: si-RNAi 3 group.

ed in serum-free medium, and adjusted to a concentration of 1×10^5 cells/ml. During the experiment, 600 μ l of complete medium was added into each well of a 24-well plate, and the TE-10 cells were seeded in 8 μ m transwell chambers (Corning, USA) at a concentration of 2×10^4 cells/well. After culturing for another 48 h, the small chambers were removed, and the medium was aspirated. 800 μ l of methanol were added into each chamber, and the chambers were fixed for 30 min at room temperature; after discarding the fixative solution, 800 μ l of Giemsa dye was added and the chambers were stained at room temperature for 15-30 min; after washed several times with running water, the base film was peeled off to dry; for observation, the film was transferred onto a slide after mounted with a neutral gum.

Scratch test

After digestion and adjustment of the cell concentration, TE-10 cells were seeded in 6-well plates at a concentration of 5×10^5 cells/well and incubated overnight until the confluency reached over than 90%. Vertical lines were made on cell membrane using a pipette tip along a ruler. Cell culture medium was disposed and the cells were washed three times with PBS. The serum-free medium was added, and the incubation and observation were continued.

Cell proliferation assay (MTT)

After digestion, TE-10 cells were seeded in 96-well culture plates at a density of 1×10^4

cells/well, and five replicates were set for each cell line. The cells were cultured for another 24 h. In each well, 20 μ l of 5 mg/ml MTT (Sigma, USA) reagents were added, and the cells were cultured for an additional 4 h. The medium was then discarded, 150 μ l DMSO was added into each well, and the plates were put on a shaker for 10-minute low-speed oscillation at room temperature. The absorbance at the 490 nm wavelength was measured using a microplate reader, with DMSO blank wells used.

Tube formation test of lymphatic endothelial cells before and after VEGFR-3 inhibition

After the Matrigel (BD, USA) was placed for 12 h at 4°C to achieve liquefaction, 200 μ l of Matrigel was added into a 24-well plate, and cultured for 30 min in an incubator. The lymphatic endothelial cells (5×10^4 cells) in 200 μ l of conditioned medium with or without anti-VEGFR-3 antibody (SAR131675, 20 nM) were added to each well and cultured for another 24 h. Subsequently, the plate was placed under an inverted microscope to observe the formation of closed lumen.

ELISA to measure the concentration of VEGF-C/D in cell culture supernatant

Using an ELISA assay kit based on the double-antibody sandwich method, the content of VEGF-C or VEGF-D in the supernatant of TE-10 cell culture was measured following the kit's instructions, and the levels of VEGF-C or VEGF-D in each sample were calculated according to the standard curve.

Tumorigenesis and axillary lymph node metastasis in nude mice

BALB/C mice aged 4-6 weeks were purchased from the Center for Experimental Animals, Third Military Medical University, without sex limitation. The mice were randomly divided into two groups, empty vectors group and vectors carrying the interfering siRNA group, with 6 mice in each group. TE-10 cells were digested and washed with PBS twice, and the cell density was adjusted to 1×10^7 cells/ml. At the axillary region of each nude mouse, 1×10^6 cells were inoculated subcutaneously. At 3 days after the inoculation, the mice were sacrificed by spinal cord dislocation and the size of lymph nodes was measured. The tissues in tumor formation were removed for subsequent experiments.

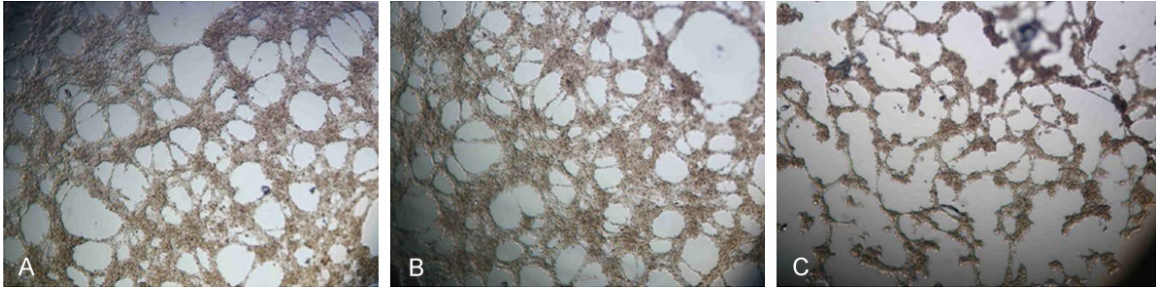


Figure 2. Results of tube test and the number of lumens comparison in each group. A: Control group; B: Empty virus group; C: si-RNAi group.

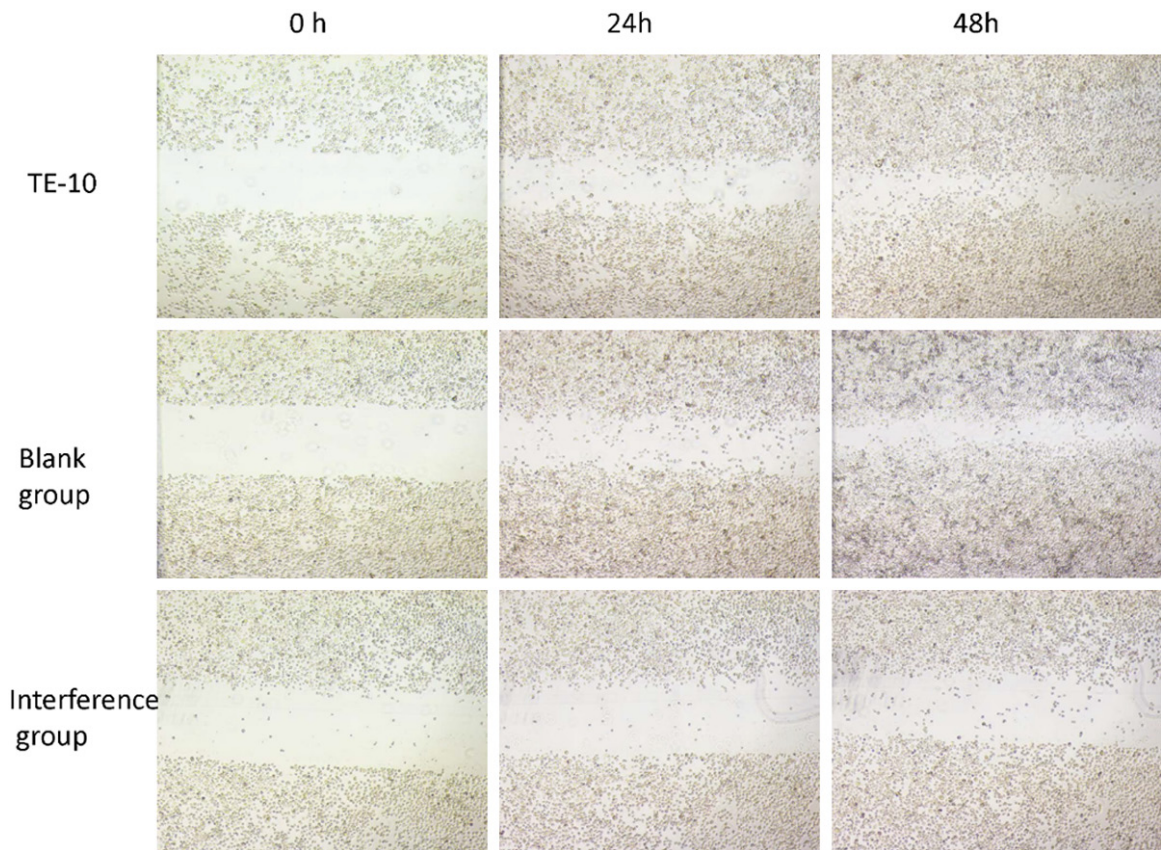


Figure 3. The migration ability of TE-10 cells comparison between each group and between each time point by scratch test. The results suggested that after the interference with si-RNAi2 recombinant lentivirus, the migration ability of TE-10 cells decreased.

Immunohistochemical examination of VEGF-C/D expression in tumor tissues

Tumor tissue sections were fixed with 4% paraformaldehyde and were made transparent using PBS containing 0.5% Triton X-100. After using 3% H₂O₂ to eliminate endogenous peroxidase activity and sealing in 6% goat serum, the VEGF-C or VEGF-D primary antibodies at 1:200 dilution were added onto each slide and incubated overnight at 4°C; biotin-labeled second-

ary antibodies (BD, USA) at 1:200 dilution were then added onto each slide and incubated at 37°C for 30 min; the slide was subsequently incubated in a streptavidin working solution labeled with HRP at 37°C for 30 min; the slide was developed in DAB substrate for 10 min in the dark; after re-staining with hematoxylin for 1 min and washed with water, each slide underwent dehydration using gradient alcohol and was made transparent by xylene, which was mounted with neutral gum.

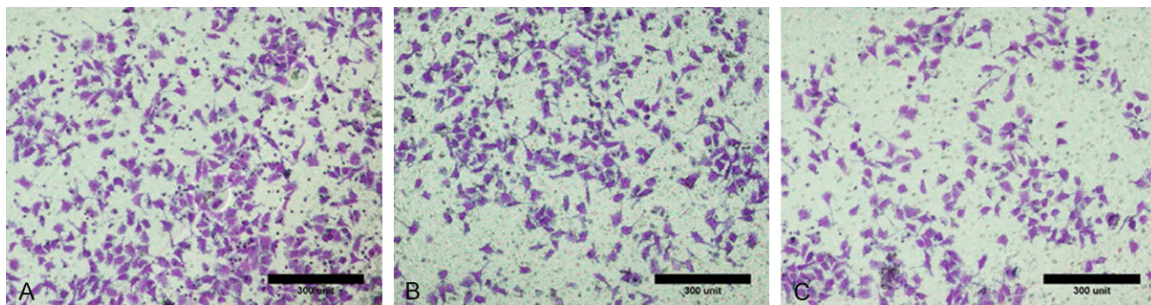


Figure 4. The invasion ability comparison among each groups. The results showed that after the interference with si-RNAi2 recombinant lentivirus, the invasion ability of TE-10 cells were attenuated significantly. A: TE-10; B: Empty virus group; C: si-RNAi group.

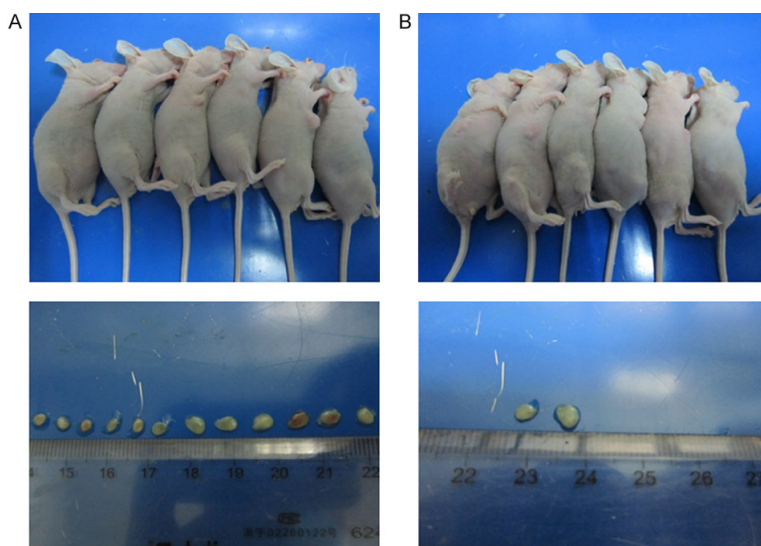


Figure 5. Tumour formation comparison in mice between groups (A: Empty virus group; B: si-RNAi group). The results showed in the interfering lentivirus group, only two mice showed the metastasis to form a solid tumor.

was the highest in TE-10 cells (**Figure 1A**). TE-10 cells were then selected and screened for the interference effects of si-RNAi1, si-RNAi2 and si-RNAi3 recombinant lentiviruses. The Western and RT-PCR results showed that the si-RNAi2 recombinant lentivirus achieved the best interference effect on the sox3 expression in TE-10 cells (**Figure 1B**), indicating that by using the recombinant lentivirus to deliver RNA interference, ESCC cell models with different expression levels of sox3 were successfully constructed.

Sox3 could promote lymphangiogenesis in vitro

Statistical methods

The data were analyzed using the SPSS 12.0 statistical software. All measurement data were presented as mean + standard deviation. The mean comparison between two groups was done using the t-test, and the comparison among multiple groups was done using ANOVA (One-way ANOVA). $P < 0.05$ indicated statistical significance.

Results

Successfully constructed ESCC cell models with different sox3 expression

Western and RT-PCR results on sox3 in human ESCC cell lines TE-10, TE-11, TE-1, EC109 and EC9706 showed that the expression of sox3

Normal lymphatic endothelial cells gradually elongated in cell culture process and connected with each other to form cord-like and network structures during the formation of different sized and shaped lumen structures (**Figure 2**). Lumen count results showed that the number of lumens in the control group (48.2 ± 4.6) was slightly higher than that in the empty vector group (46.4 ± 3.0) ($P > 0.05$), but was significantly higher than that in the siRNAi lentiviral group (22.6 ± 2.6) ($P < 0.05$), indicating that the high expression of sox3 *in vitro* improved the tube formation of lymphatic endothelial cells.

Sox3 promotes tumor cell proliferation, migration and invasion in vitro

The results from MTT arrays are indicated that wherein as compared with the normal TE-10

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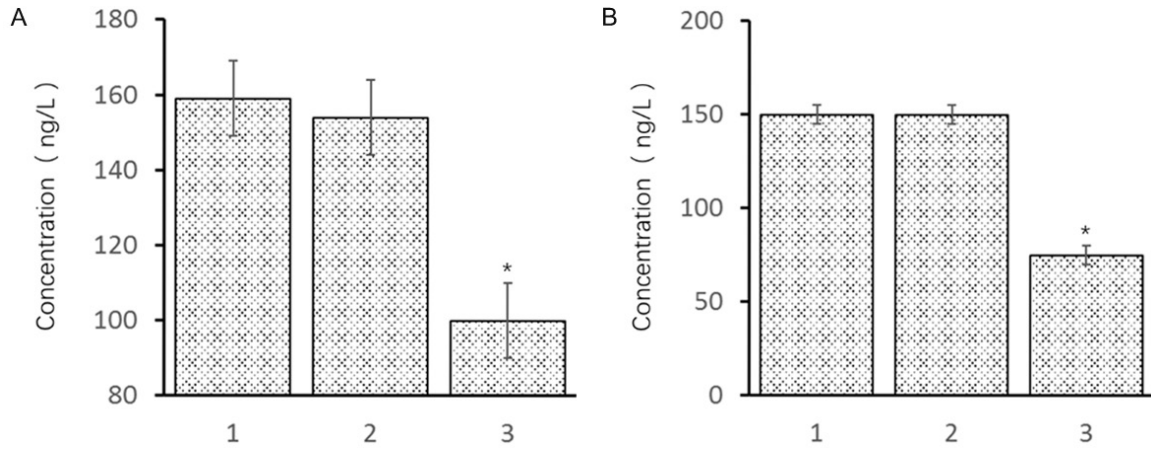


Figure 6. VEGF-C (A) and VEGF-D (B) concentration comparison among three groups in cell culture supernatant by ELAS detection. 1: TE-10; 2: Empty virus group; 3: si-RNAi group. Compared to group 1 or group 2, *P<0.005.

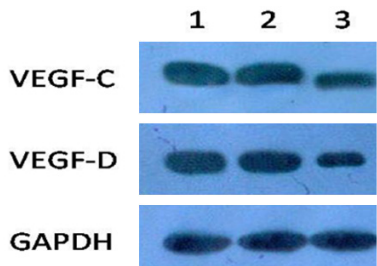


Figure 7. VEGF-C and VEGF-D protein expression comparison among three groups in cell culture supernatant by western blot. 1: TE-10; 2: Empty virus group; 3: si-RNAi group. Compared to group 1 or group 2, *P<0.005.

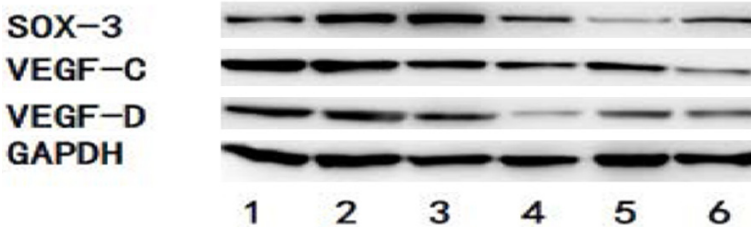
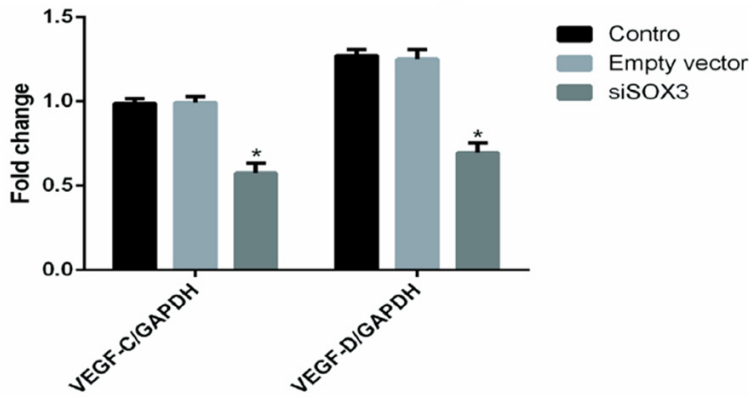


Figure 8. The expression comparison of VEGF-C and VEGF-D protein in mice tumor between empty group and si-RNAi group by Western blot detection. 1, 2, 3: Empty virus group; 4, 5, 6: si-RNAi group.

cells, the proliferation of the cells infected with empty lentivirus vectors did not change signifi-

cantly ($P > 0.05$), but the proliferation in the cells infected with si-RNAi2 recombinant lentivirus decreased ($P < 0.05$). Scratch test results showed that after the interference with si-RNAi2 recombinant lentivirus, the migration ability of TE-10 cells decreased (Figure 3). The invasion assay results further showed that after the interference with si-RNAi2 recombinant lentivirus, the invasion ability of TE-10 cells was significantly attenuated (Figure 4). These results suggested that the high expression of *sox3* *in vitro* could promote the proliferation, migration and invasion of ESCC cells.

Sox3 promotes lymph node metastasis of the tumor in vivo

The tumorigenesis results in nude mice showed that in the 6 mice of the empty lentivirus vector group, evident axillary lymph nodes appeared at the side of inoculation in all mice, indicating lymph node metastasis. However, in the interfering lentivirus group, only two mice showed the metastasis to form a solid tumor (Figure 5), suggesting that the high *sox3*

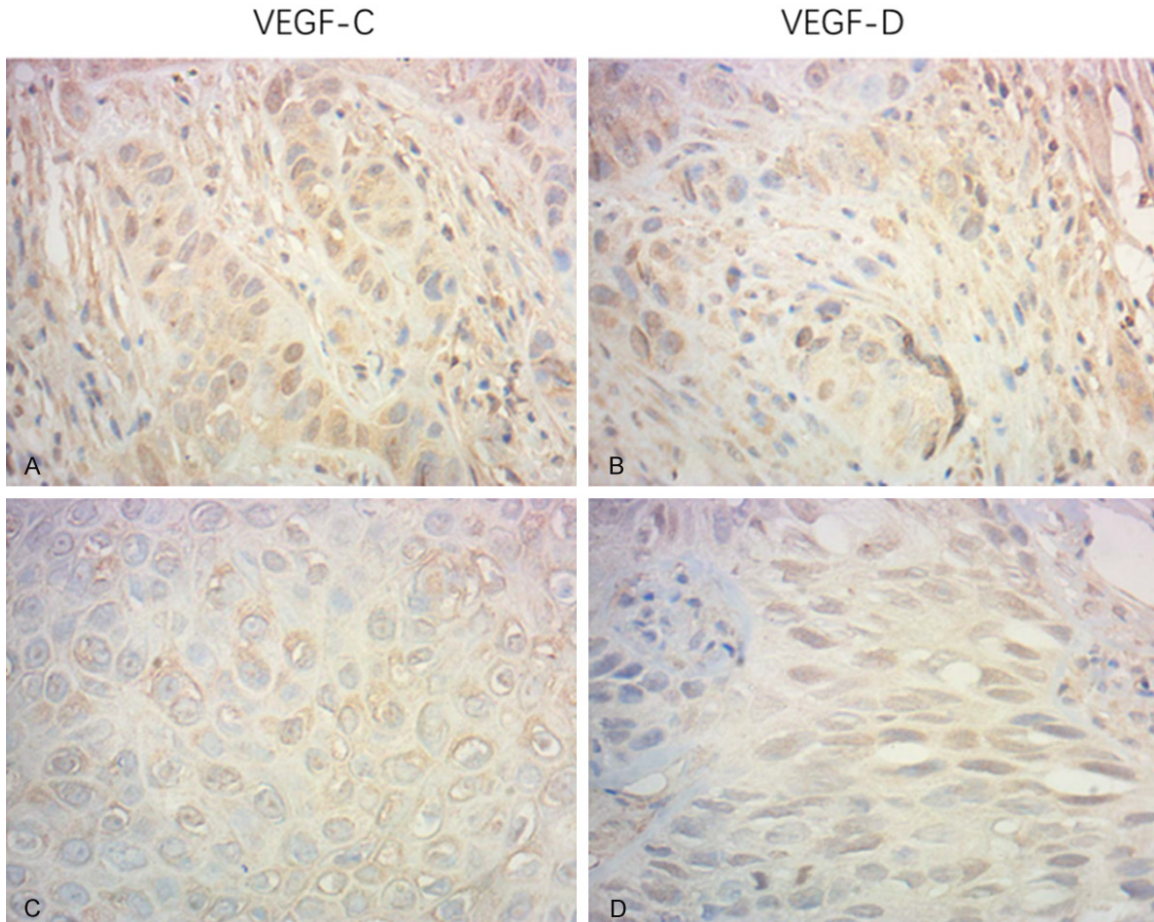


Figure 9. The expression comparison of VEGF-C and VEGF-D protein in mice tumor between empty group and si-RNAi group by immunohistochemistry detection. A and C: Empty virus group; B and D: si-RNAi group.

expression *in vivo* could promote lymph node metastasis of ESCC cells.

Sox3 could increase the VEGF-C/D expression in ESCC cells both in vivo and in vitro

ELISA of VEGF-C or VEGF-D in cell culture supernatant showed that after interference with the si-RNAi2 recombinant lentivirus, the expression of these two cytokines in the supernatant of TE-10 cell culture were significantly reduced (**Figure 6**), which was further confirmed by the Western Blotting (**Figure 7**). In Western Blotting (**Figure 8**) and immunohistochemistry (**Figure 9**) of VEGF-C or VEGF-D expression in mouse tumor tissues, it was revealed that after the interference by si-RNAi2 recombinant lentivirus, the expression levels of the two cytokines in the tumor tissues were significantly reduced. These results suggested that *sox3* could promote the expression of VEGF-C/D in ESCC cells both *in vitro* and *in vivo*.

VEGF-C/D is required during the promotion of lymphangiogenesis by Sox3

After VEGFR-3 was blocked, it was found that the tube formation ability in the empty vector group and the interfering lentivirus group was significantly decreased (**Figure 10**), suggesting that VEGF-C/D was required when lymphangiogenesis was promoted by *sox3*.

Discussion

As one of the malignant tumors with stronger invasive ability and higher mortality, the prognosis of esophageal cancer is heavily dependent on lymph node metastasis [11, 12]. Different from the fact that lymphatic vessels first appear in the submucosa layer at other parts of the digestive tract, lymphatic capillaries start to appear in the lamina propria and mucosal muscle at the deep surface of the esophageal mucosa basement membrane.

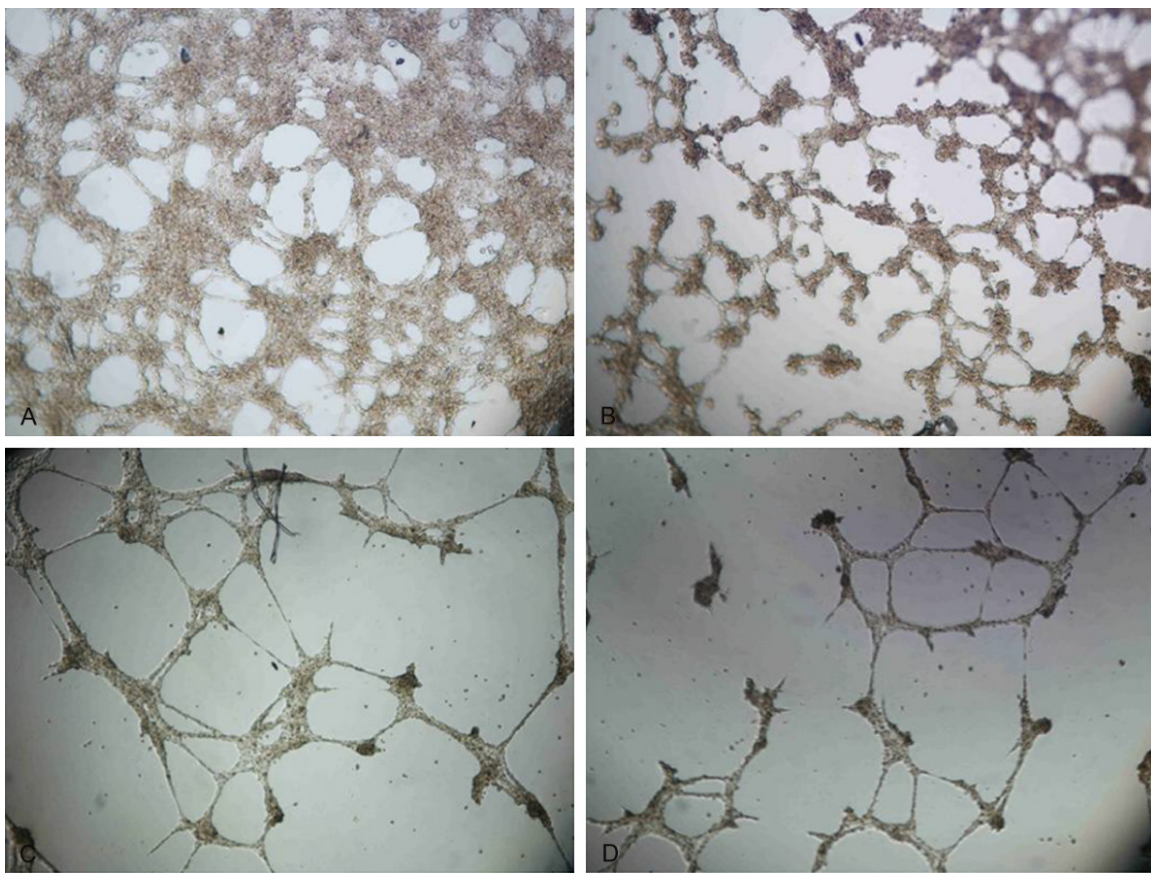


Figure 10. Tube formation ability comparison between empty vector group (C and D) and interfering lentivirus group (A and B). The results showed that after VEGFR-3 was blocked, the ability of tube formation in the empty vector group was decreased significantly.

Therefore, as long as the esophageal tumor invades the lamina propria, lymphatic metastasis may occur [13]. It is especially true that for ESCC in which lymph node metastasis starts early in a wider region, it is difficult to accurately predict lymph node metastasis according to the location of the primary tumor [14-16]. Therefore, the exploration and discovery of the molecular markers and predictors for lymph node metastasis in ESCC may be of great importance for its therapy selection, prognosis prediction and the development of targeted drugs against lymph node metastasis.

The Sox protein family is highly conserved among different species and participates in a variety of physiological processes involved in the development, including neurogenesis and the formation of cartilage and testis [17]. Human Sox3 is a member of the SoxB1 sub-groups and a transcription factor, which is located on chromosome Xq27.1. During the

development, the exogenous expression of Sox3 gradually decreases and transforms the cells from a proliferative type into a differentiated type. An increase in the exogenous expression of Sox3 induces the transformation of chicken embryo fibroblasts to tumor cells *in vitro* [18].

In our previous study, it was found that the expression of sox3 was related to lymph node metastasis and prognosis of ESCC patients, but its mechanism was not clear. In this study, by constructing ESCC cell models with different levels of sox3 expression, it was found that a high expression of sox3 *in vivo* could promote the lymph node metastasis in the xenograft ESCC tumor in nude mice, and promote tumor cell proliferation, invasion, migration and lymphangiogenesis *in vitro*.

In recent years, with the discovery of lymphatic endothelial cell-specific markers such as

VEGFR-3, Prox-1, Podoplanin and LYVE-1, as well as the identification of the lymphatic endothelial cell-specific growth factor VEGF-C/D-VEGFR-3 signaling pathway, a significant progress has been made in studying tumor lymphangiogenesis and its role in lymphatic metastasis [19, 20].

A large number of previous studies have shown that in a variety of human tumors including ESCC, high expression of VEGF-C or VEGF-D is found in all types of tumor cells and related to the density of lymphatic vessels inside/around the tumor, lymph node metastasis and poor prognosis. The lymphatic endothelial cell growth factor secreted by tumor cells could promote lymphangiogenesis and increase the density of lymphatic vessels, while it may induce the dilation of lymphatic vessels, thereby promoting the tumor cell metastasis to lymph nodes or distant sites [19-24]. The inhibition of tumor-induced lymphangiogenesis, on the other hand, effectively reduces the rate of lymph node metastasis in tumors including ESCC, thereby improving the prognosis of patients [19, 20, 25]. We found that both *in vivo* and *in vitro*, the expression of VEGF-C and VEGF-D was increased after upregulating the expression of Sox3 in ESCC, and blocking the function of VEGF-C/D using a VEGFR-3 inhibitor SAR131675, and the ability of Sox3 promoting lymphangiogenesis in ESCC cells decreased significantly *in vitro*, indicating Sox3 promoted lymphangiogenesis through VEGF-C/D.

Lymph node metastasis in ESCC is a complex biological process involving multiple steps and numerous molecular pathways. There has been a lot of research trying to elucidate the molecular mechanisms from different aspects and to provide a possible target for treating ESCC. Our study suggested that high expression of sox3 upregulated the expression of VEGF-C and VEGF-D in ESCC and promoted its lymph node metastasis. And sox3 may act through inducing the lymphangiogenesis. Combined with other studies, it is believed that the over-expression of sox-3 plays an important role in the development and lymph node metastasis of ESCC. Therefore, sox-3 may become a new prognostic marker and therapeutic target of ESCC, which requires further studies.

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Disclosure of conflict of interest

None.

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