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Original Article

High expression of SOX3 in esophageal carcinoma cells affected lymphatic endothelial cell proliferation

Hui-Juan He

Department of Cancer Radiotherapy, Quzhou People's Hospital, 324000, Zhejiang, China

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Abstract: Objective: To investigate the relationship between Sox3 upregulation in esophageal carcinoma cells and lymphatic endothelial cell (LEC) proliferation. Method: Esophageal squamous cell carcinoma cells showing high Sox3 expression, lentivirus-infected esophageal carcinoma cells, and lentivirus-infected esophageal carcinoma cells carrying Sox3 interference vector were chosen to study the influence of Sox3 expression on LEC proliferation. Serum VEGF-C/D level in esophageal carcinoma cell culture was detected by ELISA. Expression of VEGF-C/D in esophageal carcinoma cell culture was detected by Western Blot. VEGFR-3 inhibitor was used to block the expression of VEGF-C/D to the influence on LEC proliferation through tube formation assay of LECs. Results: The ratio of VEGF-C to VEGF-D increased, which enhanced the tube-forming ability of LECs. Western Blot results indicated that VEGF-C/D was down regulated in Sox3 interference group, but the ratio of VEGF-C to VEGF-D increased. After the inhibition of VEGF-C/D expression, the ability of esophageal carcinoma cells to induce tube formation by LECs was enhanced. However, the inducing ability of Sox3 interference group was still higher than that of control group. Conclusion: Blocked expression of Sox3 reduced secretion of VEGF-C and VEGF-D. LEC proliferation was mainly affected by ratio of VEGF-C to VEGF-D.

Keywords: Sox3, esophageal carcinoma cells, lymphatic endothelial cells (LECs), VEGF-C/D ratio

Introduction

Sox (SRY-related high-mobility-group box) gene family has a HMG-box conservative domain which binds to DNA in a sequence-specific manner. The first member discovered is sex-determining gene SRY [1], and Sox3 is a member of Sox gene family that has the highest homology with SRY. Sox3 is the only member localized to X chromosome, and human Sox3 gene is located at Xq27 [2]. As a gene family related to development, Sox family consists of many genes such as PAX and HOX that are involved in malignancies [3, 4]. A large number of studies indicate that Sox family members play a direct or supplemental role in oncogenic transformation and conversion of tumor cells. Zhou et al. [5] found that Sox2 gene negatively regulated S3a expression and positively regulated ENO1 expression, which promoted the proliferation and migration of colorectal cancer cells. Chen et al. [6] detected two mutant loci in HMG box frame of Sox4 in non-small cell lung cancer, namely, 679 bp CGA→CAA and 692 bp CGG→CGC mutations. The former caused arginine to glutamine mutation. Schlierf B et al. [7] found that SOX13 was upregulated in oligodendroglioma. In vitro studies showed that abnormal expression of Sox3 induced oncogenic transformation of chicken embryonic myofibroblasts. This process was related to nucleus localization and transcriptional regulatory activity. The latter occurred in the form of HMG gene deletion or deactivation of transcriptional activation domain [8]. Vascular endothelial growth factor (VEGF) is the heparin-binding growth factor specific to blood vessels and LECs. VEGF-C is a secretory polypeptide that can be produced by blood vessels, LECs, tumor cells and interstitial cells and functions through autocrine secretion or paracrine secretion [9]. VEGF-D is an endothelial mitogen. By binding to VEGFR-3, VEGF-C and VEGF-D induce the formation of LECs and promote the migration, invasion and metastasis of tumor cells. Recent researches show that the abnormal expression of some members of Sox family is related to tumor development and lymph node metastasis [10-

12]. VEGF-C and VEGF-D are the lymphatic growth factors that induce the formation and expansion of lymphatic vessels inside and around tumors. Therefore, VEGF-C and VEGF-D are closely related to lymph node metastasis in malignant tumors. Han et al. found that Sox2 induced formation of lymphatic vessels in breast cancer by promoting VEGF-C secretion [13]. Ma et al. found that Sox18 mediated formation of lymphatic vessels by regulating VEGF-C expression, thus promoting lymph node metastasis in gastric cancer [14]. However, Sox3 as an important member of Sox gene family is rarely studied with respect to its role in the formation of lymph vessels. Moreover, the relationship between VEGF-C and VEGF-D is also insufficiently investigated. We used esophageal carcinoma cells and tumor-bearing nude mice to investigate the mechanism of influence of Sox3 up regulation on LEC proliferation.

Materials and methods

Materials

RPMI 1640 medium (Gibco, USA); fetal bovine serum (Invitrogen, USA); trypsin (Hyclone, USA); penicillin and streptomycin (North China Pharmaceutical Factory); SAR131675 and Matrigel matrix (BD Corporation, USA); VEGF-C ELISA kit and VEGF-D ELISA kit (Sangon Biotech Shanghai Co., Ltd.); 5XSDS loading buffer (Sangon Biotech Shanghai Co., Ltd.); 10% separation gel and 4% concentration gel (Amersham Pharmacia Biotech); electrophoresis running buffer and transfer buffer (Western Biotechnology); prestained protein maker (Fermentas, USA); HRP-labeled secondary antibody, primary antibody of internal reference (Gen Script, USA); ECL reagent (PERCIE, USA); TBST and TBS (Sinopharm Group Co., Ltd;); VEGF-C primary antibody and VEGF-D primary antibody (Sigma, USA).

Method

Tube formation assay: Esophageal carcinoma cell line with high Sox3 expression was used as control, while those infected by lentivirus and carrying Sox3 interference vector were the empty vector group and the interference group, respectively. After culture for 48 h under the same conditions, the culture medium was discarded and the cells were washed with PBS buffer. Then the cells were further incubated with serum-free medium for 24 h. The supernatant was collected, filtered to serve as conditioned medium and preserved at -20°C. Matrigel matrix was melted at 4°C overnight. Pipette tips and 24-well plate were freeze-stored. Into the 24-well plate 100 ul Matrigel was added into each well, and the Petri dish was placed in the incubator for 30 min to make Matrigel solidify. LECs reaching the logarithmic phase of growth were collected and digested with serum-free 1640 medium. The concentration of cell suspension was adjusted to 2×10^5 cell/ml, and inoculation was performed using 24-well plate coated with Matrigel at the density of 5×10^4 cells per well. The above three culture media (1 ml for each) were added to incubate the cells for 24 h, respectively, with 3 replicates for each group. The cells were observed under the inverted microscope.

ELISA detection of VEGF-C and VEGF-D expression in cell culture supernatant: The standard curves and standard equations of VEGF-C and VEGF-D concentrations VS. OD value at 450 nm were established according to the manufacturer's instructions of VEGF-C ELISA kit and VEGF-D ELISA kit (VEGF-C: $y = 0.0053x + 0.2755$, $R2 = 0.9962$; VEGF-D: $y = 0.0068x + 0.0415$, $R2 = 0.9958$, respectively). OD values of the samples were detected and substituted into the equation to calculate sample concentration, which was the actual concentration of the sample.

Western Blot detection of VEGF-C/D expression in esophageal carcinoma cells: Esophageal carcinoma cells were cultured in normal control group, empty vector group and Sox3 interference group. The culture medium was discarded and the cells were washed with PBS buffer. The cells were suspended and collected after centrifugation. For every 10^6 cells, 0.1 ml RIPA buffer was added. The full contact between the lysis buffer and the cells was facilitated by blowing and mixing. The cells were placed on ice for several minutes and gently blown with pipette tips so that the cells were fully lysed. The Petri dish was gently tilted to make the lysis products flow to one side or one corner of the dish. Then the cells were transferred to 1.5 ml centrifuge tube and violently oscillated for 30 s. Centrifugation was performed at 12,000 × g at 4°C for 5 min and the supernatant was collect-

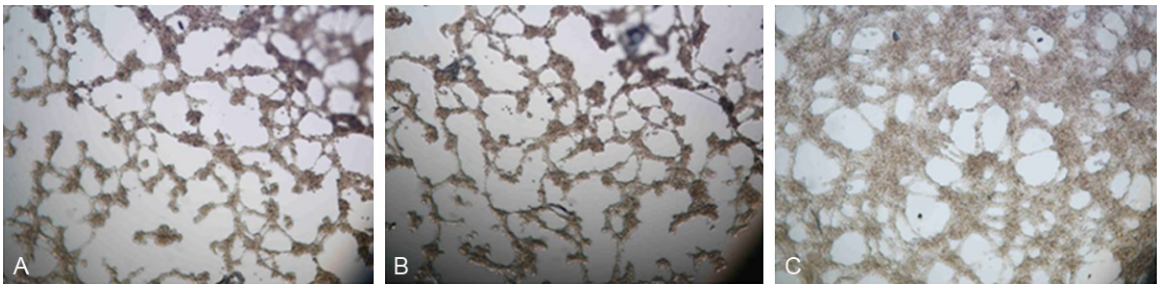

Figure 1. Images of tubes formed by LECs treated by different cell culture supernatants. A. Tubes formed by LECs treated by culture supernatant of esophageal carcinoma cells with high Sox3 expression (control); B. Tubes formed by LECs treated by culture supernatant of esophageal carcinoma cells infected by lentivirus (empty vector group); C. Tubes formed by LECs treated by culture supernatant of esophageal carcinoma cells infected by lentivirus and carrying Sox3 interference vector (interference group).

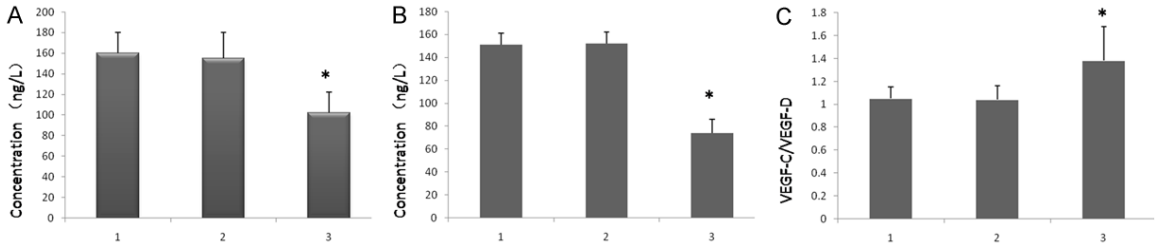

Figure 2. A. VEGF-C concentration in cell culture supernatants under different treatment conditions; B. VEGF-D concentration in cell culture supernatants under different treatment conditions; C. Ratio of VEGF-C/VEGF-D. 1. Normal negative control (control group); 2. Infection by lentivirus alone (empty vector group); 3. Infection by lentivirus and carrying Sox3 interference vector (interference group).

ed as protein sample. Next 5 × SDS loading buffer was added in the proportion of 4:1 and the protein was denatured by boiling in hot water for 5 min. Electrophoresis, membrane transfer, color development and fixing were performed successively. The films were scanned and processed by UVP gel imaging system and Labworks 4.6 software to calculate the gray values of the target bands.

Influence of VEGFR-3 inhibitor for blocking the expression of VEGF-C/D on LEC proliferation: Esophageal carcinoma cell line with high Sox3 expression was used as control and the cells infected by lentivirus and carrying Sox3 interference vector used as the interference group. After cell culture for 48 h, the culture medium was discarded, and the cells were washed with PBS buffer. Then serum-free medium was used and VEGFR3 inhibitor (SAR131675) was added to adjust the final concentration to 20 nm. The cells were further cultured for 24 h, and the culture supernatant was collected and filtered as conditioned medium, which was preserved at -20°C. Tube formation assay was implemented using the method in 2.2.1, and the results were observed under the inverted microscope.

Statistical analysis

All statistical analyses were performed using SAS 9.0 software, and the data were expressed as mean ± standard deviation. One-way ANOVA was employed and $P < 0.05$ indicated statistical significance.

Results

Esophageal carcinoma cells induced tube formation by LECs, and the inducing capacity was enhanced after interference of Sox3 expression

As shown in **Figure 1**, the cell culture supernatants of the control group, empty vector group and interference group could all induce tube formation by LECs. Compared with control group and empty vector group, the tubes formed by LECs under induction in the interference group were denser, with similar tubular structure.

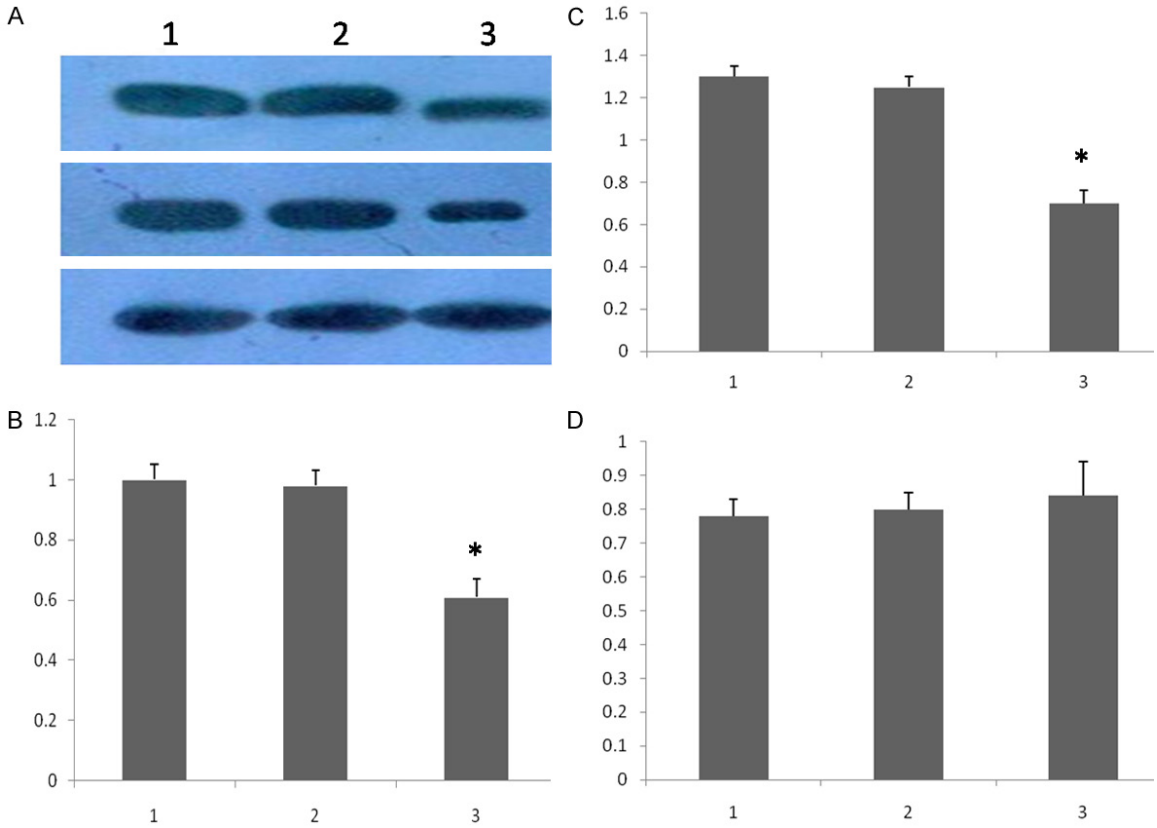

Figure 3. A. SDS-PAGE patterns of VEGF-C, VEGF-D and GAPDH + in esophageal carcinoma cells under different treatment conditions; B. VEGF-C/GAPDH + ratio in esophageal carcinoma cells under different treatment conditions; C. VEGF-C/GAPDH + ratio in esophageal carcinoma cells under different treatment conditions; D. VEGF-C/VEGF-D ratio in esophageal carcinoma cells under different treatment conditions. 1. Normal negative control (control group); 2. Infection by lentivirus alone(empty vector group); 3. Infection by lentivirus and carrying Sox3 interference vector (interference group).

VEGF-C/D expression decreased after interference of Sox3 expression

As seen in **Figure 2A**, **2B**, compared with control group and empty vector group, VEGF-C and VEGF-D contents in cell culture supernatants in the interference group decreased significantly. But no significant difference was observed between control group and empty vector group. It was shown in **Figure 2C** that VEGF-C/VEGF-D ratio in the interference group was obviously higher than that of control group and empty vector group. However, the difference between the latter two was not significant.

VEGF-C/D expressions decreased in esophageal carcinoma cells after interference of Sox3 expression

As shown in **Figure 3A**, compared with control group and empty vector group, VEGF-C/D bands in the interference group were weak. Gray value analysis (**Figure 3B**, **3C**) indicated that both VEGF-C/GAPDH + ratio and VEGF-D/GAPDH + ratio decreased significantly in interference group compared with control group and empty vector group. After the blocking of Sox3 expression, VEGF-C/D expression in esophageal carcinoma cells decreased. It was also shown in **Figure 3D** that although VEGF-C/D expressions decreased, the VEGF-C/D ratio increased.

VEGFR-3 inhibitor lowered the tube-forming ability of LECs induced by esophageal carcinoma cells

As shown in **Figure 4**, cell culture supernatants in control group and interference group with and without VEGFR-3 inhibitor both induced tube formation by LECs. Compared with control group, the tubes formed by LECs in interference group were denser; compared with the subgroup with VEGFR-3 inhibitor, the subgroup

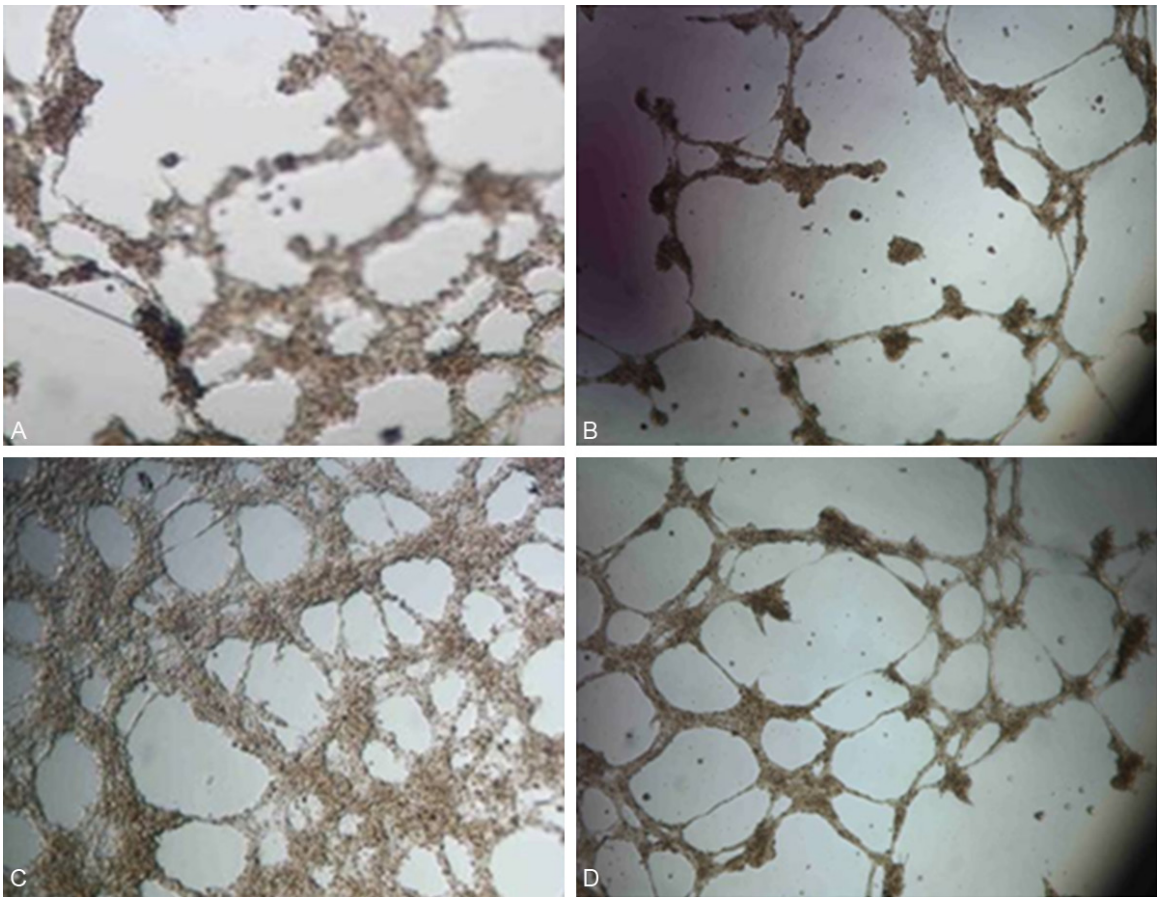

Figure 4. Images of tubes formed by LECs under induction by culture supernatant of different esophageal carcinoma cells. A. Tubes formed by LECs under induction by culture supernatant of esophageal carcinoma cells with high Sox3 expression; B. Tubes formed by LECs under induction by culture supernatant of esophageal carcinoma cells with high Sox3 expression using VEGFR-3 inhibitor; C. Tubes formed by LECs under induction by culture supernatant of esophageal carcinoma cells carrying Sox3 interference vector and infected by lentivirus; D. Tubes formed by LECs under induction by culture supernatant of esophageal carcinoma cells carrying Sox3 interference vector and infected by lentivirus using VEGFR-3 inhibitor.

without VEGFR-3 inhibitor showed denser tubes formed by LECs.

Discussion

VEGF-C is upregulated in many tumors compared with normal tissues, while VEGF-D is usually down regulated. For instance, VEGF-C is up regulated in colorectal cancer tissues compared with normal colonic tissues, whereas VEGF-D is down regulated [15, 16], leading to an increase of ratio of VEGF-C to VEGF-D. Similar findings were obtained from the studies on gastric cancer, head and neck squamous cell carcinoma, hepatic cancer, breast cancer, cervical cancer, pancreatic cancer, colorectal cancer and diffuse large B-cell lymphoma [17-22]. We did not set up control using normal esophageal epithelial cells. Although VEGF-C and VEGF-D contents in interference group decreased significantly both inside and outside esophageal carcinoma cells, the ratio of VEGF-C to VEGF-D increased. The corresponding cell culture supernatant also had an obviously enhanced induction of tube formation by LECs. This pointed to the important role of the ratio of VEGF-C to VEGF-D in the induction of tube formation by LECs. The higher the VEGF-C/VEGF-D ratio, the more enhanced the tube formation by LEC induced by esophageal carcinoma cells. After the blocking of VEGF-C/D expression in esophageal carcinoma cells by VEGFR-3 inhibitor, the tube formation by LECs under induction was weakened considerably. The inducing capacity of interference group was also stronger than that of control group. Therefore VEGF-C

and VEGF-D expressions and ratio of VEGF-C to VEGF-D played important roles in tube formation by LECs induced by esophageal carcinoma cells.

We chose esophageal carcinoma cell line with high Sox3 expression and that with interference of Sox3 expression as research objects. After the interference of Sox3 expressions, VEGF-C and VEGF-D contents inside and outside esophageal carcinoma cells decreased significantly. This implied that the blocking of Sox3 expression severely affected VEGF-C and VEGF-D expressions. VEGF-C and VEGF-D expressions and ratio of VEGF-C to VEGF-D are important regulatory signals in tube formation by LECs. It is obvious that Sox3 gene plays a crucial role in the proliferation and migration of LECs and tube formation induced by esophageal carcinoma cells.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hui-Juan He, Department of Cancer Radiotherapy, Quzhou People's Hospital, 324000, Zhejiang, China. Tel: +86-131-86707211; E-mail: 6824844@qq.com

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