Vascular endothelial growth factor C is an indicator of lymph node metastasis in thoracic esophageal squamous cell carcinomas and its role in long-term survival after surgery

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Keywords
Esophageal cancer; lymph node metastasis; real-time polymerase chain reaction; survival; VEGF-C.

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Abstract
Background: To define the role of vascular endothelial growth factor C (VEGF-C) on lymph node (LN) metastasis of human esophageal squamous cell carcinoma (ESCC), and to investigate its impact on overall survival.

Methods: Real-time polymerase chain reaction was introduced to quantify the expression of VEGF-CmRNA. One hundred and eight samples (59 tumor tissue and 59 paired normal tissue) were analyzed.

Results: VEGF-CmRNA expression was significantly higher in tumor tissues than in normal mucosa (P = 0.02). VEGF-CmRNA expression was significantly higher in LN (+) patients than in LN (-) patients (P = 0.04). VEGF-CmRNA expression was related to a positive LN number (P = 0.06) and a positive LN station number (P = 0.04). VEGF-CmRNA expression was significantly higher in stage III and IV patients than in stage I and II patients (P = 0.03). A logistic regression model showed that VEGF-CmRNA and T status were independent risk factors for LN metastasis (P < 0.05). In univariate analysis, survival tended to be poorer in the VEGF-CmRNA high expression group (22.0 months vs. 44.0 months, P = 0.08). A Cox regression model revealed that a positive LN station number was the only independent risk factor for overall survival (P < 0.01).

Conclusion: VEGF-C was a useful indicator for LN metastasis in human ESCC, and it might have some influence on long-term survival by affecting LN metastasis.

Introduction
Esophageal cancer is one of the common cancers leading to an unfavorable outcome. Lymph node (LN) metastasis strongly influences the prognosis of esophageal squamous cell cancer (ESCC). Many patients who have undergone R(0) esophagectomy still lose their lives as a result of LN recurrence. However, the mechanism of LN metastasis still remains unknown.

Until recently, histological examination was still the golden standard for identifying LN status. However, a LN overlooked during surgery might mislead adjuvant therapy, causing an unfavorable outcome. Therefore, it is necessary to find a biological factor that could reflect the LN status and help in guiding postoperative treatment. Vascular endothelial growth factor C (VEGF-C), a member of the VEGF gene family, is a relatively specific lymphangiogenic growth factor.

The VEGF-C gene is located on chromosome 4q34, and it is initially isolated from the PC-3 prostatic adenocarcinoma cell. VEGF-C can induce hyperplasia of lymphatic vasculature, increase the permeability of lymphatic vessels, and create an advanced condition to the lymphatic diffusion of cancer cells. In the transgenic mice model, overexpression of VEGF-C can induce lymphangiogenesis, rather than blood vessel angiogenesis. VEGFR3 (Flt-4) is a VEGF-C receptor and it expresses selectively on the lymphatic endothelial cell. Through binding VEGFR3, VEGF-C can regulate lymphangiogenesis by the paracrine route.

There has been previous research on VEGF-C expression in breast, prostate, gastric, colon, and lung cancer, where it was reported to be associated with lymphangiogenesis and LN metastasis. A similar tendency has been reported for esophageal cancer. Our research further analyzes the relationship between VEGF-C expression and the extent of LN metastasis.
metastasis in human ESCC. Our results help to strengthen the importance of VEGF-C in the progress of LN metastasis and suggest it as a potential indicator. We also attempt to discern the impact of VEGF-C on long-term survival of esophageal cancer, after a McKeown procedure.

Methods

Patients

Fifty-nine patients with thoracic ESCC who underwent surgery at Shanghai Chest Hospital from July 2003 to April 2005 were included in the study. None of the patients received neoadjuvant radiotherapy or chemotherapy. Patients who underwent incomplete resection were excluded from this study. Tumor specimens and paired adjacent pathologically confirmed normal mucosa (at least 5 cm away from the tumor margin) were collected from the same patient undergoing unified surgical operation (McKeown procedure with systemic LN dissection). Tumor stage was determined according to the 7th edition of the Tumor Node Metastasis (TNM) classification of the Union for International Cancer Control. Adjuvant radiotherapy was suggested for T3-4 patients, while adjuvant chemotherapy was suggested for patients with LN metastasis.

RNA extraction

The total RNA was extracted from the tissue specimens by the TRI Reagent method (Molecular Research Center, Inc.) according to the suggested protocol. Homogenization: 50 mg tissue samples were homogenized in 1 ml TRI Reagent, then incubated for five minutes at room temperature. RNA Extraction: 0.2 ml chloroform was added and mixed well, and then the samples were incubated at room temperature for five to 15 minutes. The samples were centrifuged at 12 000 × g for 10–15 minutes at 4°C, then 400 ul of the aqueous phase was transferred to a fresh tube. RNA Precipitation and Wash: 400 ul isopropanol was added, vortex occurred for five to 10 seconds, then the samples were incubated at room temperature for five to 10 minutes. They were then centrifuged at 12 000 × g for eight minutes at 4°C. Four hundred ul of 75% ethanol was added, and the samples were centrifuged at 7500 × g for five minutes. The ethanol was then removed and the RNA pellet briefly air-dried. To determine RNA solubilisation, RNA was dissolved in the 50 ul DEPC-H2O. Spectrophotometry was used to assess RNA yield and quality, and A260/A280 was recorded.

cDNA synthesis

A cDNA synthesis kit was purchased from MBI Fermentas. Five ug RNA, and 1 ul Oligo(dT)18 primer with adjusted DEPC-H2O was added to the final volume of 12 ul then heated in 70°C water bath for five minutes. One ul RNase inhibitor, 2 ul 10 mM dNTP, 1 ul Promega M-Mulv RT, and 4 ul 5 x reaction buffer with premix were then added. Heat activated reverse transcriptase was then performed: 37°C for five minutes, 42°C for 60 minutes and 70°C for 10 minutes.

Real-time quantitative

Polymerase chain reaction (PCR) multiplex mixture (Oligonucleotide primers, Taqman probes, Taqman enzyme) and standard preparation (VEGF-C and β-actin) were purchased from Shanghai Cima Cancer Biological Hi-tech Co., Ltd. The β-actin gene was used as an endogenous control to normalize the expression of VEGF-C. Quantitative real time PCR was then performed using a 96-well optic tray on the ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) sequence detection system. The standard preparation was diluted (1:10:100:1000:10000), then 2 ul of diluted standard preparation and 2 ul of DEPC-H2O were added to the first five optical reaction plates. This process was performed to determine the standard curve and quantification of each sample. Two ul of each sample cDNA was added to the remaining reaction plate, followed by 23 ul multiplex mixture in each plate. The real-time PCR procedure was then run. The PCR thermal cycle condition is specified in Table 1. When the correlation coefficients of the standard curve reached ≥0.98, the data was qualified for quantification. VEGF-C/(1000*β-actin) was used for calculation.

Statistical analysis

Statistical analyses were performed with SPSS software, version 13.0. A rank sums Chi-square and T-test were used to compare variables and P < 0.05 was considered to be statistically significant. Survival was examined using the Kaplan-Meier method, and survival times were compared by the Breslow test. Logistic regression and Cox’s proportional hazards models were used to investigate multivariate associations.

Results

The demographics of the patients are listed in Table 2.
The relationship between vascular endothelial growth factor (VEGF)-CmRNA and clinicopathological features

The expression of VEGF-CmRNA was significantly higher in the tumor tissues than in normal mucosa (6.30 vs. 2.81, \( P = 0.02 \)). Among 59 ESCC patients, 28 cases had LN metastasis. VEGF-CmRNA expression was related to LN metastasis (10.11 vs. 4.15, \( P = 0.04 \)) and TMN stage (10.11 vs. 3.45, \( P = 0.03 \)), but was not related to any other clinical pathological parameters (Table 3). Furthermore, it was revealed that VEGF-CmRNA expression was related to a positive LN number (16.09 vs. 5.93, \( P = 0.06 \), Table 4) and a positive LN station number (18.98 vs. 4.92, \( P = 0.04 \), Table 4).

The relationship between lymph node status and clinicopathological features

In univariate analysis, it was shown that LN status was related to T status (\( P = 0.03 \)) and VEGF-CmRNA (\( P = 0.04 \)). However, LN was not related to age (\( P = 0.29 \)), gender (\( P = 1.0 \)), location (\( P = 0.20 \)), differentiation (\( P = 0.11 \)), or length (\( P = 0.32 \)). The cut off value for discriminating high from low levels was 6.30, which was the median expression of VEGF-CmRNA. When all of these variables were included in the logistic regression model, VEGF-CmRNA and T status were independent risk factors for LN metastasis (\( P < 0.05 \)).

Survival analysis

The median survival time was 35 months (5–117 months). By the end of the study, 49 patients had died. The overall five-year survival after surgery was 32%. In univariate survival analysis, LN status (\( P < 0.01 \)), positive LN number (\( P < 0.01 \)), and positive LN station number (\( P < 0.01 \)) were shown to be related to overall survival (OS). Although statistical significance was not reached, survival tended to be poorer for the VEGF-CmRNA high expression group (22.0 months vs. 44.0 months, \( P = 0.08 \), Fig 1). T status (\( P = 0.27 \)), tumor location (\( P = 0.56 \)), length (\( P = 0.20 \)), differentiation (\( P = 0.21 \)), gender (\( P = 0.47 \)), smoking history (\( P = 0.30 \)), and alcohol history (\( P = 0.68 \)) were not related to OS.

Table 2 Demographics of esophageal squamous cell carcinoma patients

| Clinicopathological parameters | Number | Percent (%) |
|--------------------------------|--------|-------------|
| Age                            |        |             |
| ≥60                            | 25     | 42.4        |
| <60                            | 34     | 57.6        |
| Gender                         |        |             |
| Male                           | 50     | 84.7        |
| Female                         | 9      | 15.3        |
| Smoking history                |        |             |
| Yes                            | 27     | 45.8        |
| No                             | 32     | 54.2        |
| Alcohol history                |        |             |
| Yes                            | 26     | 44.1        |
| No                             | 33     | 55.9        |
| Location                       |        |             |
| Upper                          | 2      | 3.4         |
| Middle                         | 42     | 71.2        |
| Lower                          | 15     | 25.4        |
| Length                         |        |             |
| <50 mm                         | 32     | 54.2        |
| ≥50 mm                         | 27     | 45.8        |
| Differentiation                |        |             |
| Well                           | 7      | 11.9        |
| Moderate                       | 35     | 59.3        |
| Poor                           | 17     | 28.8        |
| T status                       |        |             |
| T1                             | 4      | 6.8         |
| T2                             | 11     | 18.6        |
| T3                             | 41     | 69.5        |
| T4                             | 3      | 5.1         |
| PN                             |        |             |
| N0                             | 31     | 52.5        |
| N1                             | 14     | 23.75       |
| N2                             | 14     | 23.75       |
| TMN                            |        |             |
| I + II                         | 30     | 50.8        |
| III + IV                       | 29     | 49.2        |

PN, pathological node; TMN, tumor node metastasis.

Table 3 Relationship between vascular endothelial growth factor-CmRNA and clinical pathological index

| Clinical pathological index | Cases | VEGF-CmRNA expression (median value) | P-value |
|-----------------------------|-------|--------------------------------------|---------|
| Gender                      |       | Male 50 7.11                         | 0.76    |
| Age (years)                 |       | ≥60 25 4.92                          | 0.02    |
| Location                    |       | Upper 2 4.16                         | 0.13    |
| Differentiation             |       | Poor 17 6.13                         | 0.60    |
| T status                    |       | T1 + T2 15 5.24                      | 0.36    |
| Length                      |       | <50 mm 32 5.24                       | 0.32    |
| Lymph node status           |       | Negative 31 4.25                     | 0.04    |
| TMN stage                   |       | I + II 30 3.45                       | 0.03    |

TMN, tumor node metastasis; VEGF, vascular endothelial growth factor.

Table 4 Analysis of vascular endothelial growth factor-CmRNA in lymph node (+) patients

| Clinical pathological index | Cases | VEGF-CmRNA expression (median value) | P-value |
|-----------------------------|-------|--------------------------------------|---------|
| Positive LN number          |       | 1–2 14 5.93                          | 0.06    |
| ≥3                          | 14    | 16.09                                | 0.06    |
| Positive LN station number  |       | 1–2 15 4.92                          | 0.04    |
| ≥3                          | 13    | 18.98                                | 0.04    |

LN, lymph node; VEGF, vascular endothelial growth factor.
When all of these variables were included in the Cox multivariate analysis, a positive LN station number was the only independent risk factor \( (P < 0.01, \text{Fig 2}) \).

**Discussion**

Our study contained three distinctive characters compared to former research. The first important character was that it was an absolute quantification of VEGF-C mRNA in human ESCC. Real-time reverse transcription (RT)-PCR was introduced in our study and showed significant merit,\(^{15,16}\) as the process was easy and rapid to perform, it was capable of high throughput, and could combine high sensitivity with reliable specificity. Secondly, all of the cases used in this study were ESCC. As tumors with different cell types have different biological behavior and different prognoses, using only thoracic ESCCs and the same cell type helped to clarify the real impact of VEGF-C on LN metastasis and prognosis. Thirdly, all surgical procedures performed were the McKeown procedure with systemic LN dissection. Using one type of surgical procedure had two advantages. Poster-lateral thoracotomy enhanced the LN dissection of the upper and posterior mediastinum, while systemic LN dissection strengthened dissection of the cervical and abdominal regions, ensuring that we could gather more information about LN metastasis and TMN classification, and that it was more accurate. The other advantage was that it could eliminate the prognosis bias generated by various surgical procedures.

In the present study, VEGF-C mRNA expression was significantly higher in tumor tissues. This revealed that VEGF-C plays an important role in tumor progression. In some of the previous studies, benign tumors or normal tissue from other cases was selected as the control group. However, tissues from different individuals may have different background expression of VEGF-C, therefore potentially disturbing the comparison between tumor expression and normal controls. In this study, normal and tumor tissues were obtained from the same patient, and the normal control was over 5 cm away from the tumor margin. Paired-sample analysis could control factors regulating VEGF-C expression that differed from patient to patient, such as platelet derived growth factor, epidermal growth factor, and TGF-\( \beta \).\(^{17}\)

Our study also showed that VEGF-C mRNA expression was related to LN metastasis with TMN stage. These results were similar to previous studies on immunohistochemistry, RT-PCR, and enzyme-linked immunosorbent assay methods.\(^{18-20}\) In the present study, VEGF-C mRNA was found to be related to a positive LN number and a positive LN station number. This is the first report describing the relationship between VEGF-C and the different extent of LN metastasis in thoracic ESCC. Multivariate analysis showed that VEGF-C mRNA and T status were independent risk factors for LN metastasis. This strongly suggested that VEGF-C plays an important role in the LN metastasis of human ESCC and it might be a promising indicator of LN metastasis.

Whether VEGF-C can influence the prognosis of human cancer remains controversial. Liu et al.\(^{18}\) reported that median OS in a VEGF-C negative group was significantly higher than in a VEGF-C positive group (28.5 vs. 10.3 months, \( P = 0.003 \)). Tanaka et al.\(^{21}\) used the quantitative RT-PCR technique to measure the expression of VEGF-C mRNA, and found that VEGF-C mRNA expression was related to postoperative survival. However, multivariate
analysis showed that LN status and the extent of the primary tumor were independent risk factors. In our univariate survival analysis, although it did not reach statistical significance, the median OS of VEGF-C high expression patients tended to be poorer (22.0 months vs. 44.0 months, \( P = 0.08 \)). Therefore, we believe that VEGF-C might have an influence on the prognosis of ESCC by affecting LN metastasis. In multivariate analysis, a positive LN station number was the only independent risk factor. We considered that a positive LN station number could represent the metastatic region better than a positive LN number, which would better reflect the extent of disease progression.

Based on the investigation that VEGF-C may play an important role in tumor lymphangiogenesis and LN metastasis, some researchers attempt to blockade tumor development by targeting VEGF-C/VEGFR3. Ye et al.\(^\text{25}\) found that miR-27b could repress colorectal cancer cell proliferation, colony formation, and tumor growth in vitro and in vivo. They reported that VEGF-C was the target for the anti-tumor role. Liu et al.\(^\text{26}\) reversed the cell proliferation and migration of esophageal squamous cell carcinoma, which was induced by VEGF-C through silencing of CNTN-1. They also found that nude mice inoculated with VEGF-C shRNA-transfected cells exhibited a significantly decreased tumor size in vivo via reducing VEGFR-2 and VEGFR-3 phosphorylation and microvesSEL formation. Although there have only been a few papers, preliminary investigation suggests that VEGF-C may carry a promising target gene of tumor suppression in esophageal cancer. Our next step would be to focus on tumor inhibition by targeting VEGF-C/VEGFR3.

**Conclusion**

In conclusion, our study has shown that VEGF-C is an indicator of LN metastasis in human ESCC and may influence long term survival by affecting LN metastasis.

**Disclosure**

No authors report any conflict of interest.

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