Expression of vascular endothelial growth factor-C and angiogenesis in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of vascular endothelial growth factor-c (VEGF-C) mRNA and microvessel density (MVD) in human esophageal squamous cell carcinoma (ESCC) and its relationship with clinical significance.

METHODS: Specimens obtained from 43 patients undergoing surgical resection for ESCC were used in this study. The expression of VEGF-C mRNA was examined by in situ hybridization. Tumor MVD was determined immunohistochemically with anti-CD31 antibody and estimated by image analysis. Ten sections of adjacent normal mucosa were also examined.

RESULTS: VEGF-C mRNA expression was detected in cytoplasm of carcinoma cells. Of the 43 ESCC patients studied, 18 cases (41.9%) were positive for VEGF-C mRNA. No VEGF-C mRNA expression was observed in normal esophageal mucosa. VEGF-C mRNA expression correlated significantly with lymph node metastasis, TNM stage and depth of invasion (P < 0.05). Furthermore, histological grade (differentiation) tended to correlate with VEGF-C mRNA expression, but was not statistically significant (P > 0.05). In tumor lesions, the MVD was significantly greater than that in normal esophageal mucosa. MVD correlated significantly with lymph node metastasis, TNM stage and depth of invasion (P < 0.05), but not with histological grade (differentiation) (P > 0.05). Lesions with VEGF-C mRNA expression had a significantly higher MVD than that of those without VEGF-C mRNA expression (P < 0.05).

CONCLUSION: VEGF-C plays a role in lymphatic metastasis via lymphangiogenesis and angiogenesis in ESCC. VEGF-C is one of the important predictors of the biological behavior in ESCC.

INTRODUCTION

Lymph node metastasis is an important prognostic factor for human esophageal squamous cell carcinoma (ESCC)\[1,2,4\]. Angiogenesis induced by a variety of growth factors such as vascular endothelial growth factor (VEGF), has been recently considered necessary for the growth, invasion and metastasis of solid tumors\[3\]. Thus, measurement of vascular growth may be clinically important in esophageal cancer specimens. To assess angiogenesis, several markers of blood vessel endothelium have been developed for microscopic estimation of microvessel density (MVD) by immunohistochemistry, including CD31/PECAM-1, CD34, and factor VIII-related antigen (von Willebrand factor or vWF)\[4\]. VEGF-C, a member of the VEGF family, stimulates the proliferation of both vascular and lymphatic endothelial cells via VEGF receptor (VEGFR)-2 and VEGFR-3 in many physiological and pathological processes\[5,6\]. Previous reports have shown that VEGF-C expression in cancer tissues has a positive correlation with the risk of lymphatic metastasis in a variety of cancers\[7\], including prostatic\[8\], gastric\[9\], oral\[10\], lung\[11\], colorectal\[12\] and bladder carcinoma\[13\]. A similar tendency has been reported for esophageal cancers, although a significant correlation between VEGF-C expression and the frequency of nodal metastasis is not always found\[14\]. Hence, the precise role of VEGF-C in ESCC has not been clearly understood\[11\]. Therefore, in the present study, VEGF-C mRNA expression in biopsy specimens of ESCC was examined by in situ hybridization (ISH) and the association between VEGF-C mRNA expression and clinicopathological factors, including the intratumoral microvessel density (MVD) as a measure of tumor angiogenesis in ESCC was investegated.

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MATERIALS AND METHODS

**Patients and tumor specimens**
Tumor specimens from 43 ESCC patients (32 males and 11 females, mean age 62.5 years, range 47-76 years) undergoing surgical treatment in Jinhua People’s Hospital from 1997 to 2002 were included in the study. Specimens were classified according to the TNM classification system by UICC and 10 normal esophageal tissues were collected as control. None of the patients received any radiotherapy or chemotherapy prior to the study. Histological grades, depth of invasion and lymph node metastasis are shown in Table 1.

**Methods**

*In situ* mRNA hybridization (ISH) analysis was performed as described previously with minor modifications.[10] VEGF-C specific oligonucleotide probes were labeled with digoxin at the 5'-end and synthesized by Beijing Dingguo Co. Ltd (Beijing, China). The DNA oligonucleotide sequences used were 5'-TGTACAAGT GTGACTATGG-3' and 5'-CCACATCTAATACACCTCC-3'. Tissue sections (4 µm-thick) were incubated with 50 mg/L proteinase K, and then hybridization was performed in a moist chamber for 16 h at 45°C, using a probe concentration of 100 ng/L. The sections were then incubated with a staining solution containing NBT/BCIP in a dark box for 1-2 h. Negative controls were performed in all cases by omitting the probes.

A mouse CD31 mAb (JC/70A, DAKO Corporation, Denmark) was used as a vascular endothelial marker.[3,17] Immunohistochemical examination for CD31 was performed with EnVision kits to access MVD. In brief, after deparaffinization and rehydration, the sections were denatured for 3 min in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0) for antigen retrieval. Endogenous peroxide activity was quenched with hydrogen peroxide, and the sections were incubated with goat serum for 20 min at room temperature to block nonspecific binding of anti-CD31 mAb (JC/70A, 1:50 dilution) and then for 16 h at 4°C with CD 31 expression. Bound antibodies were detected by the EnVision system (DAKO Corporation, Denmark) and using 3, 3'-diamindoenzidine as the chromogen while the sections were incubated with a staining solution containing NBT/BCIP in a dark box for 1-2 h. Negative controls were performed in all cases by omitting the probes.

The results of ISH and MVD were examined by two of the authors with no prior knowledge of the identification of each section, using Leica Qwin image system (Leica Corporation, Germany). The expression of VEGF-C mRNA in the tissue was defined as positive if distinct staining was observed in at least 10% of tumor cells.[18] For determination of MVD, the number of microvessels with positive reaction to JC/70A was counted in five randomly selected microscopy fields on the vascular areas within a section (× 200, 0.7386 mm²/field) and the average was then calculated.

**Statistical analysis**
The data were evaluated by *t* test, Fisher’s exact probability test or chi square test. *P* < 0.05 was considered statistically significant for all tests. All statistical analyses were performed using the SPSS 10.0 statistical package (SPSS, Inc, Chicago, IL).

## RESULTS

### Expression of VEGF-C mRNA in ESCC tissues

Of the 43 patients studied, 18 cases (41.9%) were positive for VEGF-C mRNA expression in cytoplasm. The specimens were divided into two categories by the staining pattern of VEGF-C mRNA, diffuse or focal staining of carcinoma cells (Figures 1A and 1B). In contrast, no VEGF-C mRNA in specimens of adjacent normal esophageal mucosa was stained.

### CD 31 expression and MVD in ESCC tissues

Immunohistochemical expression of CD31 was detected in cytoplasm of vascular endothelial cells. Brown capillaries or small clusters standing out sharply from other tissues were found to be microvessels (Figures 2A and 2B). At the deepest invasive site of tumors, the MVD ranging 43-144/mm² (76.4 ± 20.3/mm²) was significantly higher than that in normal esophageal mucosa (*P* < 0.01).

### Correlation between VEGF-C mRNA expression and MVD and clinicopathological factors

VEGF-C mRNA expression in ESCC tissues was associated with the depth of tumor invasion, TNM stage and lymph node metastasis (*P* < 0.05), but not with histological grade (*P* > 0.05). Furthermore, MVD also correlated with TNM stage and lymph node metastasis in ESCC tissue (*P* < 0.05). No association was found between MVD and depth of tumor invasion and histological grade (*P* > 0.05). The patients were divided into VEGF-C mRNA positive and negative groups. The VEGF-C mRNA positive cases showed a higher MVD (79.5 ± 30.9/mm²) than the VEGF-C mRNA negative cases (57.5 ± 18.4/mm²) (*P* < 0.05 (Table 1).

| Clinicopathological factors | n | VEGF-C mRNA (n (%)) | MVD/mm² (mean ± SD) |
|-----------------------------|---|---------------------|----------------------|
| **TNM stage**               |   |                     |                      |
| I, II                      | 33 | 11 (33.3)           | 60.14 ± 20.2         |
| III, IV                    | 10 | 7 (70.0)*           | 83.68 ± 33.6*       |
| **Histological grade**     |   |                     |                      |
| GI                         | 12 | 4 (33.3)            | 60.5 ± 31.7          |
| GI, II                     | 20 | 8 (45.0)            | 66.30 ± 21.6         |
| GI, III                    | 11 | 6 (54.6)            | 71.66 ± 30.8         |
| **Depth of invasion**      |   |                     |                      |
| T1, T2                     | 23 | 5 (21.7)            | 64.24 ± 19.3         |
| T3, T4                     | 20 | 13 (65.0)*          | 68.86 ± 31.7         |
| **Lymph node metastasis**  |   |                     |                      |
| Negative                   | 31 | 10 (32.3)           | 58.66 ± 18.8         |
| Positive                   | 12 | 8 (66.7)*           | 85.30 ± 32.7*       |

*P* < 0.05 vs negative lymph node metastasis and TNM stages III and IV; *P* < 0.01 vs depth of tumor invasion of T1 and T2.
DISCUSSION

Recent studies have shown a significant relation between MVD, VEGF expression and prognosis in a variety of tumors including esophageal carcinoma[10]. Among the VEGF family members, VEGF-C appears to affect the lymphatic system as a ligand for VEGFR-3, which is specifically expressed on lymphatic vessels, and VEGF-C is suspected to play an important role in lymphangiogenesis[11]. But little is known about the mechanisms of lymphangiogenesis and lymphatic metastasis, while the effects of VEGF-C in ESCC remain controversial[11]. In this study, we investigated the clinicopathological significance of VEGF-C mRNA expression in relation to the angiogenesis in ESCC. The results showed that 41.9% of patients with ESCC had expression of VEGF-C mRNA, which correlated significantly with lymph node metastasis, depth of tumor invasion and TMN stage, suggesting that VEGF-C plays a role in ESCC. It was reported that VEGF-C can be detected using immunohistochemical staining and RT-PCR, and VEGF-C expression in ESCC may play a key role in tumor progression and lymphatic metastasis[12-14]. It was also reported that only histological grade, but not parameters involved in lymphatic spread, correlates with VEGF-C expression in ESCC[15]. These contradictory findings can be explained by difference in analytic method. Even in immunohistochemical analysis, the result may differ depending on the selected site for assessment.

More interestingly, we found that VEGF-C mRNA expression was significantly correlated with MVD, indicating the grade of angiogenesis in ESCC. Tsursusaki et al[16] and Akagi et al[18] have failed to find any remarkable differences in blood vessel density between VEGF-C positive and negative prostatic and colorectal carcinoma specimens. Also, VEGF-C expression is not correlated with MVD in human gallbladder cancer[19]. Whereas other studies reported that VEGF-C is associated with angiogenesis in breast cancer[20], colorectal carcinoma[21-23] and bladder transitional cell carcinoma[24], and plays an important role in angiogenesis and lymphangiogenesis in lung cancer[25] and malignant mesothelioma[26]. It has been reported that VEGF-C can bind to both VEGFR-2 and VEGFR-3[27]. The specificity of VEGF-C for its 2 receptor is known to be regulated by proteolytic processing.

Accordingly, only partially processed VEGF-C activates both VEGFR-2 and VEGFR-3, whereas the partially processed precursors act only through VEGFR-3[28]. Activation of VEGFR-2 results in mitogenesis of vascular endothelial cells, whereas VEGFR-3 activation by VEGF-C is considered to induce proliferation of lymphatic endothelial cells. Furthermore, recent studies have revealed that VEGFR-3 is expressed in angiogenic blood vessels in certain pathological conditions. For example, VEGFR-3 expression can detected in intratumor blood vessels of human breast cancer[21], cutaneous melanoma[27], head and neck squamous cell carcinoma[28], gliomas as well as in vascular endothelial cells activated during formation of granulation tissue in skin[29]. Thus, angiogenesis and lymphangiogenesis responses to VEGF-C have been found to depend on the expression of its receptors in blood and lymphatic endothelial cells of the target tissue.

In our present study, patients with VEGF-C mRNA expression had a significantly higher MVD that those without VEGF-C mRNA expression, suggesting that VEGF-C secreted from tumor cells may stimulate not only lymphangiogenesis but also angiogenesis, which are significantly correlated with lymph node metastasis in ESCC. As a rule, once cancer cells enter blood microvessels, they can reach regional lymph nodes through lymphatic vessels from blood vessels, via blood vessel-lymph vessel junctions. In fact, the lymphatic and vascular systems have numerous connections allowing disseminating tumor cells to pass rapidly from one system to the other. Furthermore, metastatic tumor cells expressing VEGF-C could grow in regional lymph nodes that they reached.

In conclusion, VEGF-C mRNA is expressed heterogeneously in tumor cells and plays an important role in angiogenesis and lymphangiogenesis, as well as growth and metastasis of ESCC. The combined examination of VEGF-C expression and MVD in biopsy specimens from ESCC patients can predict lymph node metastasis and select appropriate treatments.

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