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mRNA Expression of Vascular Endothelial Growth Factor-C and -D in Esophageal Squamous Cell Carcinoma

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Summary. **Lymph node metastasis is a major prognostic factor for esophageal cancer patients. However, the molecular mechanisms underlying node metastasis remain unclear. Vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D), which are ligands for VEGFR-3, have been reported to be capable of stimulating lymphangiogenesis under *in vivo* experimental conditions. The aim of the present study was to measure VEGF-C and/or VEGF-D mRNA expression in clinical specimens of esophageal squamous cell carcinoma, and to examine the correlation between VEGF-C or VEGF-D messenger ribonucleic acid (mRNA) expression and conventional clinicopathological parameters, especially the lymphatic involvement of esophageal squamous cell carcinoma. As a previous study has demonstrated that an expression pattern of high VEGF-C and low VEGF-D is correlated with both lymph node metastasis and lymphatic invasion of cancer cells, we also investigated the VEGF-C/D ratio. Total RNAs were isolated from 51 surgical specimens of esophageal carcinoma tissue and 42 normal esophageal mucosae. The relative mRNA abundance of VEGF-C and VEGF-D was measured by real-time quantitative reverse transcription polymerase chain reaction (PCR) analysis, and mRNA expression of VEGF-C and VEGF-D was standardized against glceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. VEGF-C mRNA was expressed similarly in esophageal carcinoma tissue and normal mucosal tissue; however, VEGF-D mRNA expression was significantly lower in carcinoma tissue compared with normal mucosal tissue, and therefore the VEGF-C/VEGF-D ratio was significantly increased in tumors compared with normal mucosae. However, neither mRNA expression of VEGF-C or VEGF-D nor the VEGF-C/VEGF-D ratio correlated with any clinicopathological factors, including lymphatic invasion, venous invasion, lymph node status, and tumor stage. VEGF-C and VEGF-D gene expression do not appear to be involved in the lymphatic progression of esophageal carcinoma.**

Key words — VEGF-C, VEGF-D, mRNA, esophageal carcinoma, RT-PCR.

INTRODUCTION

The dissemination of malignant cells from the primary tumor to local tissue or to distant organs via the lymphatic system or the blood stream is a characteristic of cancer progression[1]. It has been well established that angiogenesis, the formation of new blood vessels, is necessary for tumor progression because it allows oxygenation and nutrient perfusion of the tumor. In the absence of neovascularization, a solid tumor cannot develop into a large mass[2,3]. However, it is unclear whether tumors can stimulate lymphangiogenesis or whether activation of the lymphatic system may affect

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Abbreviations–GAPDH, glyceraldehyde-3-phosphatedehydrogenase; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; VEGF-C, vascular endothelial growth factor-C; VEGF-D, vascular endothelial growth factor-D.

tumor progression and metastasis.

Recently, several new members of the vascular endothelial growth factor (VEGF) family have been identified, including vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D). VEGF-C was first identified in 1996 as a factor able to stimulate tyrosine phosphorylation of a receptor tyrosine kinase, Flt-4 (later referred to as VEGFR-3), and was then isolated and its cDNA was cloned. Subsequently, the molecule was identified as a novel homologue of VEGF and was denoted VEGF-C[4,5]. VEGF-D was first isolated as a partial cDNA from a differential display screening of murine genes expressed in fibroblasts from normal mice, but not expressed in fibroblasts from mice with targeted inactivation of the c-fos gene[6]. The identified protein was first denoted as c-fos-induced growth factor, but was later renamed as VEGF-D to indicate that it was a novel VEGF homologue which binds to and activates VEGFR-3[7].

VEGFR-3 is initially expressed in all embryonic endothelia, but its expression in the blood vessel endothelium decreases during development, and it becomes largely restricted to the lymphatic endothelium in adult tissues[8]. Therefore, it has been suggested that VEGF-C and VEGF-D are involved in promoting tumor lymphangiogenesis and angiogenesis. Experimental evidence in animal models has shown that VEGF-C and VEGF-D overexpression in tumor cells leads to spreading of the tumor to lymph nodes[9,10], and several studies have demonstrated that VEGF-C and/or VEGF-D are expressed in many types of human cancers, and that their expression correlates with lymphatic invasion and lymph node metastasis[11-16].

It is well known that esophageal carcinoma is one of the most lethal malignant tumors in the gastrointestinal carcinoma family. The aggressive behavior of this tumor has often been associated with systemic spread of the disease at diagnosis. In particular, lymph node status is the most important prognostic factor in esophageal carcinoma patients[17].

The involvement of VEGF-C and/or VEGF-D in esophageal carcinoma and their relationship with clinicopathological parameters have not been clearly demonstrated. Therefore, in this study, we quantified the expression of VEGF-C and VEGF-D mRNA in esophageal carcinoma specimens obtained during surgery, using a real-time quantitative reverse transcription PCR, and investigated whether the levels of VEGF-C and VEGF-D mRNA correlated with conventional clinicopathological parameters for esophageal carcinoma, with particular reference to the lymphatic involvement.

PATIENTS AND METHODS

Patients

The protocol for the following studies was approved by the Ethical Committee of the Niigata University Medical Hospital Trust, Japan.

We studied 51 patients who underwent an esophagectomy for esophageal squamous cell carcinoma in the Department of Digestive and General Surgery, Niigata University Medical Hospital and at an affiliated hospital between July 1998 and July 2003. The clinical and pathological characteristics of the patients are summarized in Table 1.

Assessment of the stage of the disease was based on the Sixth Edition of the *AJCC Cancer Staging Manual*. The patients comprised 47 males and four females, and had a median age of 64.0 years old (range, 50-83 years old). No patients presented with detectable metastasis

Table 1. Clinicopathological features of patients with esophageal squamous cell carcinoma

Characteristics	Number of patients (N = 51)
Gender	
Male	47
Female	4
Primary tumor (T)	
T1	7
T2	9
T3	34
T4	1
Regional lymph nodes (N)	
N0	16
N1	15
M1a	0
M1b	20
Distant metastasis (M1)	
M0	51
M1	0
Pathological stage	
I	4
IIA	11
IIB	5
III	11
IV	0
IVA	0
IVB	20
Histological grade (G)	
G1	15
G2	24
G3	12
G4	0

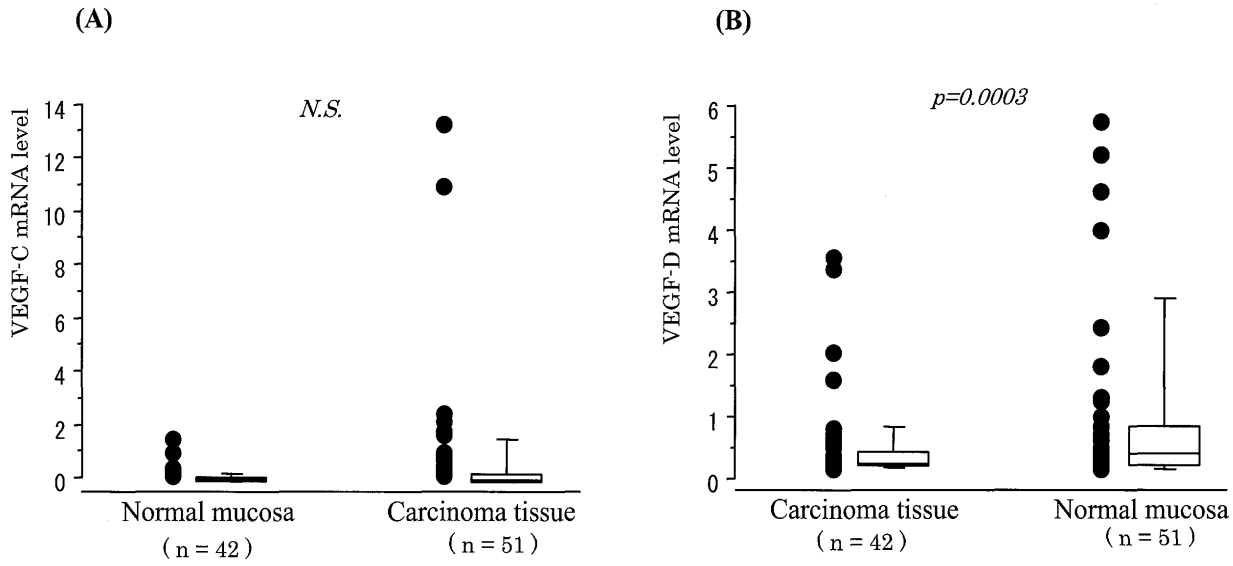

Fig. 1. The vascular endothelial growth factor-C (VEGF-C) mRNA expression level was similar in carcinoma tissue and normal mucosal tissue (**A**), whereas vascular endothelial growth factor-D (VEGF-D) mRNA expression was significantly lower in carcinoma tissue compared with normal mucosal tissue (**B**).N.S., not significant.

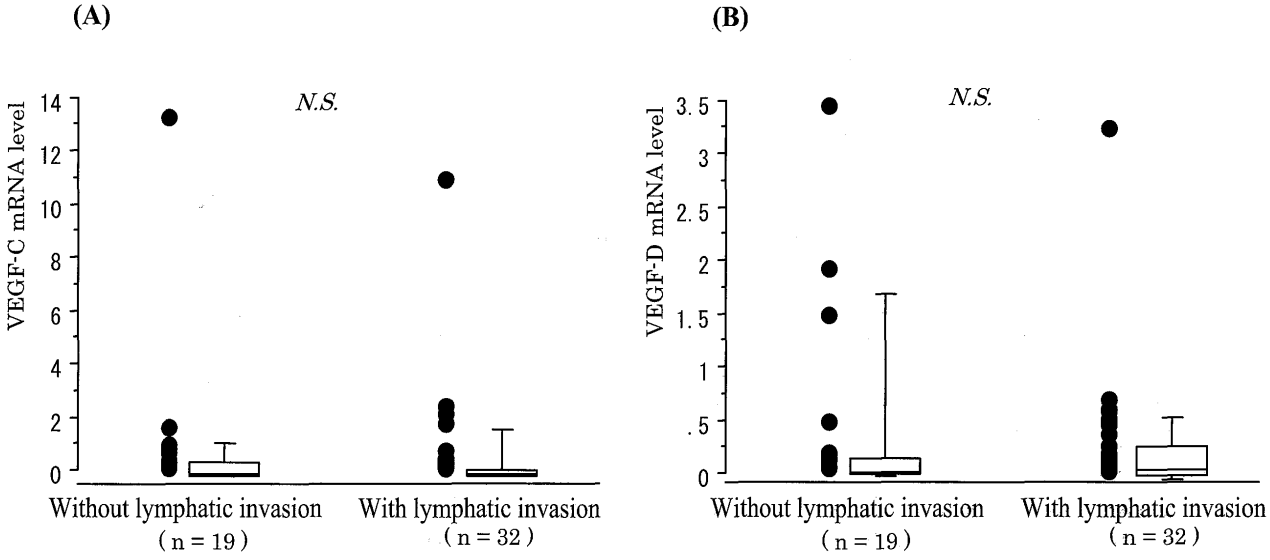

Fig. 2. The VEGF-C mRNA expression level was similar in the lymphatic invasion-positive and lymphatic invasion-negative groups (**A**). The VEGF-D mRNA expression level was also similar for the two groups (**B**). N.S., not significant.

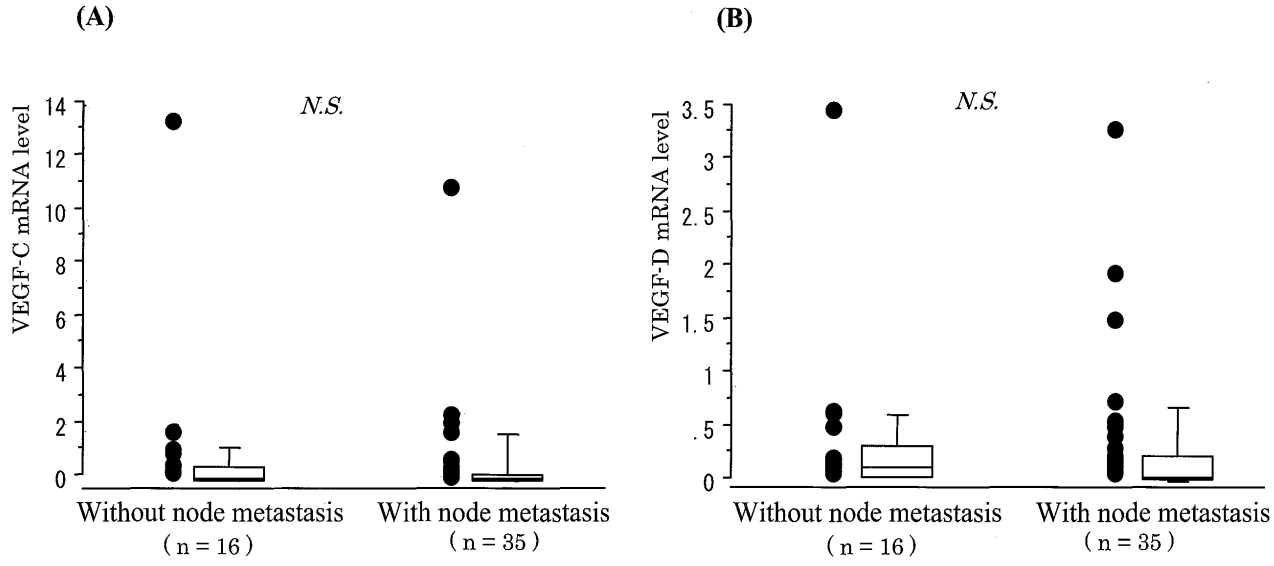

Fig. 3. VEGF-C mRNA was expressed at similar levels in the carcinoma tissue of patients with positive and negative lymph node metastasis (**A**). The VEGF-D mRNA expression level was also similar for the two groups (**B**). N.S., not significant.

in distant organs at that time of surgery. Ten patients previously received preoperative chemotherapy, and adjuvant treatment was given after radical surgery in appropriate cases, following the hospital protocol.

Tissue samples and total RNA isolation

Fresh esophageal carcinoma tissue samples were obtained from each of the 51 patients. In each case, a portion of the tumor was resected near the advancing edge of the tumor, thus avoiding the necrotic center. Macroscopically normal mucosae resected at a distance of 5 cm from the tumor area were obtained in 42 cases and used as controls. The sites from which the carcinoma and normal tissue samples were obtained and checked microscopically after resection. In total, RNAs were isolated from 51 surgical specimens of esophageal carcinoma tissue and from 42 normal esophageal mucosae, using a modified acid guanidium thiocyanate phenol-chloroform extraction method.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as reported previously[18]. In detail, total RNA (1 μg) from each sample was reverse-transcribed at 42 °C for one h using an oligo (dT) primer and Superscript II reverse transcriptase (Gibco BRL) in a volume of 20 μL. Each reverse-transcript was used as a template in a real-time quantitative polymerase chain reaction (PCR) assay in order to separately quantify the levels of VEGF-C, VEGF-D, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression, by means of an ABI PRISM 7700 Sequence Detection Instrument.

The VEGF-C specific primer sequences were 5'-GC CCAAACCAGTAACAATCA-3' and 5'-TGCCTGA CACTGTGGTAGTGTTG-3', and the corresponding fluorogenic probe sequence was 5'-CTTCCTGCCGA TGCATGTCTAAACTGGA-3'. The VEGF-D specific primer sequences were 5'-TGCAGGAGGAAAATCCA CT-3' and 5'-ATCATGTGTGGCCCACAGAG-3', and

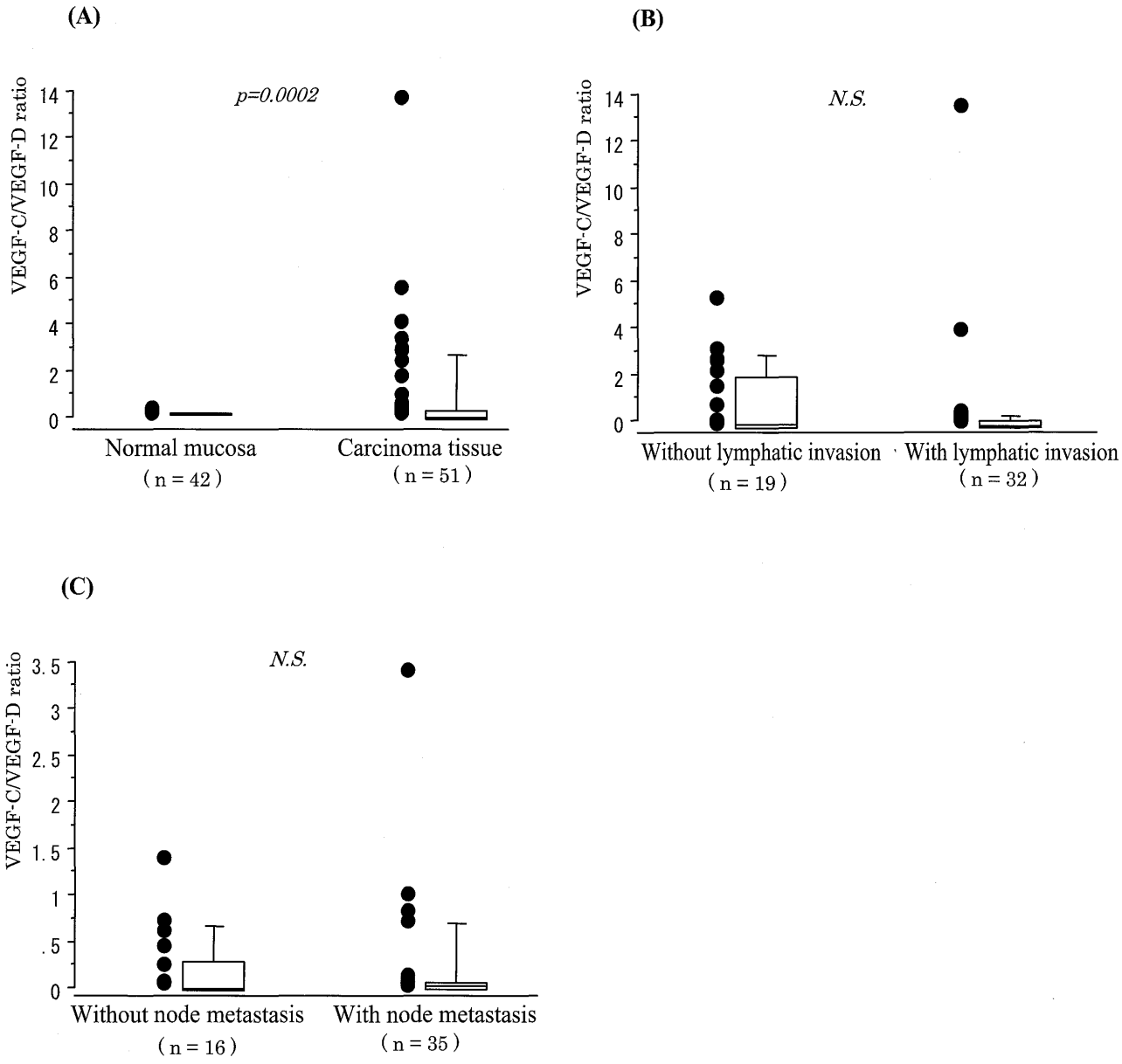

Fig. 4. The VEGF-C/D ratio was higher in carcinoma tissues compared with normal mucosae (**A**), but this ratio was not correlated with either lymphatic invasion (**B**) or lymph node metastasis (**C**).N.S., not significant.

the corresponding fluorogenic probe sequence was 5'-ACAGAAGACCACTCTCATCTCCAGGAACCA-3'. The TaqMan® GAPDH Control Reagent was used for amplifying human GAPDH as an internal control.

For quantification of VEGF-C or VEGF-D mRNA, the amplification reaction solution (50 μL) contained the reverse-transcript (1 μL), 1 × TaqMan Universal PCR Master Mix, 300 nmol/L of each of the VEGF-C or VEGF-D primers, and 100 nmol/L of the corresponding VEGF-C or VEGF-D fluorogenic probe. For GAPDH amplification, the reaction solutions (50 μL) contained the reverse-transcript (1 μL), 1 × TaqMan Universal PCR Master Mix, 200 nmol/L of human GAPDH primer, and 100 nmol/L of human GAPDH fluorogenic

probe. All quantitative two-step PCR reactions were performed according to the manufacturer's instructions, using the following thermocycler conditions: 50 °C for two min., followed by 95 °C for 15 sec and 60 °C for one min. Template-negative controls were run on each PCR plate. A calibrator reverse-transcript sample was amplified in parallel on all plates in order to allow comparisons of samples which were run at different times.

Statistical analysis

The data were analyzed using Sequence Detection Software and represented as the mean ± SE of the VEGF-C or VEGF-D amplicon/GAPDH amplicon ratio. The data were also analyzed and represented as the mean ± SE of the VEGF-C/VEGF-D ratio (VEGF-C amplicon/VEGF-D amplicon). The VEGF-C and VEGF-D mRNA expression levels and the VEGF-C/VEGF-D ratio were compared with lymphatic invasion, lymph node involvement, and other clinicopathological factors. All statistical calculations were carried out using StatView 5.0 software (SAS Institute, NC, USA). A Mann-Whitney U test was used to compare the levels of mRNA expression between two given groups. A P value of < 0.05 was considered to be statistically significant.

RESULTS

VEGF-C and VEGF-D mRNA expression in normal mucosa and esophageal squamous cell carcinomas

VEGF-C and VEGF-D mRNA expression was examined in normal esophageal mucosae and esophageal squamous cell carcinoma tissues. VEGF-C mRNA was expressed in both tissues, and the mRNA expression levels were similar in the two tissue (Fig.1A). VEGF-D mRNA expression was also detected in both normal mucosa and carcinoma tissue; however, the VEGF-D mRNA expression level was significantly lower in carcinoma tissue compared with that in normal esophageal mucosal tissue ($P = 0.0003$; Fig. 1B).

Relationship between VEGF-C and VEGF-D mRNA levels and lymphatic invasion

Lymphatic invasion was observed in 32 of the 51 patients by conventional histological examination. The VEGF-C mRNA expression level was similar in the lymphatic invasion-positive and lymphatic invasion-negative groups (Fig. 2A), and the VEGF-D mRNA expression level was also similar in the two groups (Fig. 2B).Thus, there was no significant difference between the lymphatic invasion-positive and -negative groups in terms of VEGF-C and VEGF-D mRNA levels.

Relationship between VEGF-C and VEGF-D mRNA levels and nodal status

Lymph node metastasis was observed in 35 of 51 patients by conventional histological examination, and both VEGF-C and VEGF-D mRNA expression was examined in the esophageal carcinoma tissue of patients with and without lymph node metastasis. VEGF-C mRNA was expressed at similar levels in the carcinoma tissue of both groups of patients (Fig. 3A), and the VEGF-D mRNA expression level was also similar in the two groups (Fig. 3B).Thus, there was no significant difference between the lymph node metastasis-positive and -negative groups in terms of VEGF-C and VEGF-D mRNA levels.

VEGF-C/D ratios in normal mucosa and esophageal squamous cell carcinoma tissue, and the relationship of VEGF-C/D ratio with node status and lymphatic invasion

The VEGF-C/D ratio was higher in carcinoma tissue than in normal mucosa (Fig. 4A), however, the ratio was not correlated with either lymph node metastasis or lymphatic invasion (Figs. 4B and C).

DISCUSSION

The presence of lymph node metastasis has a strong impact on patient survival, even after extended radical esophagectomy[19,20], and it is therefore important to investigate the molecular mechanisms underlying the spread of cancer via the lymphatic system. Evidence that VEGF-C and VEGF-D both bind to VEGFR-3, a tyrosine kinase receptor that is restricted to the lymphatic endothelium in normal adult tissues, has suggested that these two ligands play an important role in lymphangiogenesis[4,7,21]. Although subsequent clinical studies have reported a correlation between VEGF-C, VEGF-D expression, and lymphatic spread in various carcinomas[11-16,22-25], limited and conflicting evidence exists for the role of VEGF-C in esophageal carcinoma, and few data are available concerning VEGF-D[26,27]. Therefore, in the present study, we investigated whether the expresssion of VEGF-C mRNA and VEGF-D mRNA is associated with lymphatic involvement in esophageal carcinoma.

Our main hypothesis was that VEGF-C and/or VEGF-D may contribute to lymphatic involvement in

cancer progression. Kitadai et al. have demonstrated that VEGF-C mRNA is detected only in tumor tissues and not in normal mucosae, and that VEGF-C expression is correlated with lymphatic invasion and lymph node metastasis, based on immunostaining[26]. Given these data, our real-time RT-PCR results showing that VEGF-C mRNA was expressed in both normal mucosae and carcinoma tissues were unexpected. Furthermore, the mRNA expression levels were similar in the normal mucosal tissue and the carcinoma tissue, and there was no significant correlation between VEGF-C expression and clinicopathological parameters involved in lymphatic spread. These discrepancies may be related to the different sensitivities of staining and RT-PCR, particularly since RT-PCR with fluorescence detection is even more sensitive and specific than ordinary RT-PCR visualized with gel imaging. It should be noted that an association of VEGF-C with lymphatic metastasis is not universally seen in all tumor types; previous studies have been reported that no difference in VEGF-C mRNA expression relative to lymph node status in breast cancer[28] and colorectal cancer[29].

With respect to VEGF-D, the present study is the first report of VEGF-D mRNA expression in esophageal squamous cell carcinoma. We found that VEGF-D mRNA levels were much higher in normal tissue than in tumor tissue; however, there was no significant relationship between VEGF-D expression and clinicopathological parameters, including lymph node metastasis and lymphatic invasion. VEGF-D mRNA is strongly expressed in normal tissue such as skeletal muscle, the heart, lung, and intestinal mucosa[7,30], whereas an early study of VEGF-D expression in tumor cell lines and tumor biopsy samples found the expression to be either weak or absent[30]. Other studies on different tumor types also support our observation of decreased VEGF-D mRNA expression in cancer tissue[13,14,15].

We also examined the significance of the VEGF-C/VEGF-D mRNA ratio. Both VEGF-C and VEGF-D bind to common receptors, VEGFR-2 and VEGFR-3, and we therefore considered it worthwhile to evaluate the expression of both mRNAs as a ratio. The study has also indicated the usefulness of the ratio of VEGF-C to VEGF-D in lung adenocarcinoma patients[13]. Our results showed that the VEGF-C/D ratio is increased in carcinoma tissues – compared to normal mucosae, but the VEGF-C/D ratio was not correlated with either lymph node metastasis or lymphatic invasion. The increase in the VEGF-C/VEGF-D ratio in cancer tissues can clearly occur due to increased VEGF-C mRNA expression and/or decreased VEGF-D mRNA expression, and the latter appears to be the situation in esophageal squamous cell carcinoma.

The role of VEGF-D in tumors is largely unknown, but if VEGF-D acts as a competitive agonist for VEGF-C, the fall in VEGF-D levels may allow VEGF-C, a more potent lymphangiogenic cytokine, to have increased access to the two receptors. There are considerable discrepancies between the results of immunohistochemical studies and mRNA expression studies regarding the levels of VEGF-C and VEGF-D, and it remains unclear whether these discrepancies are related to methodological differences. Most immunohistochemical studies have reported a correlation between the overexpression of VEGF-C or VEGF-D in cancer patients with lymphatic invasion, lymph node metastasis, and a poor prognosis[11,12,23-25]. We have also tried to perform immunohistochemistry using paraffin-embedded clinical samples; however, we failed to obtain positive staining for VEGF-C or VEGF-D (unpublished data). Immunohistochemical staining can be influenced by factors such as the duration of the fixation or diaminobenzidine reaction. In contrast, it is not difficult to obtain a consistent RT-PCR result if the same primers and PCR conditions are used-although it is difficult to determine the localization of the target cells. For this reason, in the present study we analyzed both cancer cells and stromal cells collectively as carcinoma tissue. Further investigation using tissue microdissection might address the conflicting reports regarding the precise involvement of VEGF-C and VEGF-D in lymphatic invasion and lymph node metastasis, and it will be important to establish appropriate and consistent sampling and measurement methodologies in future studies.

CONCLUSIONS

Our results suggest that no VEGF-C and VEGF-D gene expression appear to be involved in the progression of esophageal carcinoma.

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