Correlation Between Lymph Node Metastasis and the Expression of VEGF-C, VEGF-D and VEGFR-3 in T1 Lung Adenocarcinoma

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Abstract. *Vascular endothelial growth factor (VEGF)-C and VEGF-D influence lymphangiogenesis through the activation of the vascular endothelial growth factor receptor (VEGFR)-3. They have been implicated in lymphatic tumor spread, which is an important prognostic factor in patients with non-small cell lung carcinoma (NSCLC). Whether or not the expression of VEGF-C, -D, and VEGFR-3 correlates with clinico-pathological factors in patients with T1 lung adenocarcinoma was analysed. The tumor specimens were homogenized to determine the protein expression of VEGF-C, -D, and VEGFR-3 by enzyme-linked immunosorbent assay (ELISA). RNA fractions extracted from the tumor tissues were subjected to real-time reverse transcription-polymerase chain reaction (RT-PCR) to assess the mRNA levels of VEGF-C, -D, and VEGFR-3. The expression of VEGF-D protein and mRNA levels in patients without lymph node metastasis were significantly higher than those with metastasis ($p=0.013$, $p=0.0494$, respectively). However, the protein and mRNA levels of VEGF-C and VEGFR-3 were not significantly different in patients with or without metastasis. The 5-year survival rates of the patients with high VEGF-D levels were significantly higher than those of patients with low levels ($p=0.0221$). No significant difference in the survival rates was observed for VEGF-C and VEGFR-3. VEGF-D may be down-regulated in NSCLC tissues in comparison to adjacent normal tissue, resulting in lymph node metastasis and poor prognosis.*

Primary lung carcinoma is one of the leading causes of cancer death, and an improvement in the progress of such cases by early detection and treatment is awaited. The recently introduced computed tomography (CT) and positron emission tomography (PET) examinations now make it possible to discover smaller sized lung cancer. However, even if the size is smaller than 1 cm, the rate of lymph node metastasis is 10% (1). It is well known that lymph node metastasis is one of the most important prognostic factors in non-small cell lung carcinoma (NSCLC) (2). Therefore, the identification of molecular markers for the assessment of lymph node metastasis and prognosis in lung cancer patients is highly desirable.

The mechanisms of angiogenesis and lymphangiogenesis are complex. Angiogenesis, the formation of new blood vessels from endothelial cells, is a prerequisite for growth and progression of solid malignancies (3). Recently, four members of the vascular endothelial growth factor (VEGF) family have been identified, namely VEGF-A, -B, -C, and –D (4, 5). These growth factors exert their angiogenic and lymphangiogenic influences through the activation of three tyrosine kinase receptors, the vascular endothelial growth factor receptors, VEGFR-1 (flt-1), VEGFR-2 (flk-1/KDR), and VEGFR-3 (flt-4), which are expressed by endothelial cells, monocytes/ macrophages and haematopoietic precursors (6).

Lymphangiogenesis, the formation of new lymphatic vessels, has recently become a new research frontier in tumor metastasis due to the discovery that two major lymphangiogenic factors, VEGF-C and VEGF-D, have been linked to promotion of lymphangiogenesis in animal models (7, 8). Their receptor, VEGFR-3, is relatively specifically expressed on the surface of adult lymphatic endothelium, although it is also detected in other cell types (9). The binding of VEGFR-3 and VEGF-C or VEGF-D stimulates the proliferation and migration of lymphatic endothelial cells through the mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways (10). This phenomenon augments the assembly of new lymphatic vessels. In addition, the correlation of VEGF-C, -D and VEGFR-3 with lymph node metastasis, tissue invasion and poor prognosis has been reported in gastric

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Table I. *Real-time PCR assay variables.*

Gene and accession no.	5'-3' Forward/ reverse primer	Product size (bp)	Standard range*	Primer concentration	Product Tm**
VEGF-C X94216	GTTCCACCACCAAACATGCA/ CACTATATGAAAATCCTGGCTCACAA	83	120-0.12	900	77.4
VEGF-D D89630	TGGAACAGAAGACCACTCTCATCT/ GCAACGATCTTCGTCAAACATC	76	120-1.88	900	80.0
VEGFR-3 AY233382	AGGGAGACGCCCTTTCATG/ GAGGGCTCTTTGGTCAAGCA	80	120-0.08	900	78.1
β-2-microglobulin BC064910	TGACTTTGTCACAGCCCAAGATA/ AATCCAAATGCGGCATCTTC	85	120-0.06	900	80.5

*Standard curve range is the ng RNA equivalent of cDNA. **Tm: Melting temparature.

(11), colorectal (12, 13), ovarian (14), and breast carcinoma (15). Similarly, high expression levels of VEGF-C and VEGFR-3 have been found to correlate with a poor prognosis in NSCLC including adenocarcinoma (16, 17). However, in other studies, opposite relationships have also been found (18, 19).

The role and interaction of the VEGF-C, -D, and VEGFR-3 system in oncology is complex and may vary between malignancies or tissues. To our knowledge, there has been no study that has investigated the protein expression levels of VEGF-D and its relationship to the clinical outcome of lung adenocarcinoma. In the present study, the expression of VEGF-C, -D, and their receptor VEGFR-3 at both mRNA and protein levels in lung adenocarcinoma, especially the pathological T1 stage, and its relationship to lymph node metastasis and patient survival was studied.

Materials and Methods

Tissue samples and patients. From July 1993 to July 2003, 937 consecutive patients with a clinical diagnosis of non-small cell lung cancer underwent surgical resections at the Department of Thoracic Surgery, Fukuoka University Hospital, Japan. From these, tissue samples including adjacent normal lung tissues were randomly obtained from 55 patients with primary T1 lung adenocarcinoma. Tissue samples were used after approval of the study by the Human Ethics Review Committee of the Fukuoka University School of Medicine and signing of the informed consent by the patients. Freshly resected tissues were snap frozen in liquid nitrogen and stored at –80˚C until use. Lymph node status was determined by routine pathological examination of dissected pulmonary hilar and mediastinal lymph nodes, as well as intrapulmonary lymph nodes. The stage of the disease and pathological diagnosis were based on the Tumor-Node-Metastasis classification of the International Union Against Cancer, 1997 (20).

Enzyme-linked immunosorbent assay (ELISA) for VEGF-C, -D, and VEGFR-3. The protein levels of VEGF-C, VEGF-D, and VEGFR-3 in the tumor tissue were determined by ELISA for a total 55 cases of pathological T1 lung adenocarcinoma: 34 cases without lymph node metastasis and 21 cases with metastasis. Moreover, the protein levels of VEGF-C, VEGF-D, and VEGFR-3 in some adjacent normal lung tissues were randomly determined by ELISA.

During the analyses, extracts were made from the tissue samples and the protein levels of the extracts were analyzed. Our extraction method involved the following: cutting the lung adenocarcinoma tissue samples with a surgical knife to be about 3 mm cubed and homogenizing them in 1 ml of 3 mM tris-buffered 250 mM sucrose, pH 7.5, using an ultrasonic disintegrator for two minutes at 4˚C. After centrifugation (3,000 rpm for 20 minutes), supernatants were stored at –80˚C until they were analyzed (21).

The concentrations of VEGF-C, VEGF-D, and VEGFR-3 in the tissue extracts were determined using a human VEGF-C kit (IBL, Fujioka, Gunma, Japan), human VEGF-D Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) and human VEGFR-3 kit (R&D Systems), respectively. The limits of sensitivity of the VEGF-C, -D, and VEGFR-3 assays were 93.75 pg/ml, 62.50 pg/ml, and 156.25 pg/ml, respectively. The coefficient of variation was less than 8.0%. Protein concentrations were measured by the Lowry method.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from the tumor tissue of 20 out of the 55 cases, 10 cases without lymph node metastasis and 10 cases with lymph node metastasis, with an RNaesy Mini Kit (Qiagen, Valencia, CA, USA). After contaminating DNA was removed by digestion with DNase (Qiagen), total RNA was reverse transcribed to cDNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK). The purity and concentration of the RNA were determined by spectrophotometry at 260 and 280 nm. In confirmation of RNA quality, RT products were checked by PCR using a pair of primers specific for β2-microglobulin. No significant degradation was observed in any of the RNA samples.

The real-time RT-PCR was carried out with a QuantiTect SYBER Green PCR Kit (Qiagen) using the GeneAmp 5700

Table II. *Relationship between the clinicopathological findings and the expression levels of VEGF-C, -D and VEGFR-3.*

Clinicopathological findings		Tissue cytokine concentration (pg/mg protein)					
		VEGF-C		VEGF-D		VEGFR-3	
		median	*p*-value	median	*p*-value	median	*p*-value
Total	55	138.0		82.2		300.9	
Patient age							
≤65	26	128.0	0.441	89.6	0.601	284.7	0.501
>65	29	146.4		75.5		315.6	
Sex							
Male	29	123.4	0.192	42.3	0.009*	278.7	0.372
Female	26	154.4		126.7		325.8	
Histological grade							
Well-differentiated	25	126.5	0.140	129.5	0.022*	278.5	0.405
Moderately-differentiated	20	162.0		48.4		355.7	
Poorly-diffentiated	10	118.9		31.5		247.6	
Pleural involvement							
p0	32	144.4	0.526	80.8	0.922	322.0	0.258
p1	23	129.1		84.1		271.7	
N factor							
Negative	34	148.5	0.258	107.1	0.013*	329.9	0.090
Positive	21	121.0		41.8		254.0	
Stage							
I	34	148.5	0.465	107.1	0.045*	329.9	0.131
II	6	113.8		59.6		226.8	
III	15	123.9		34.7		264.9	

*Statistically significant.

Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA). The primer sequences and annealing temperatures for VEGF-C, VEGF-D, VEGFR-3 and β2-microglobulin are shown in Table I (22, 23). A cDNA pool serially diluted from 1:10 to 1:1,000 was used to generate standard curves. The data were normalized to the housekeeping β2-microgulobulin gene.

Statistical analysis. All statistical calculations were carried out using StatView statistical software, version 5.0 (SAS Institute, Inc., Cary, NC, USA). Statistical significance was determined by Student's *t*-test, Chi-square test or Mann-Whitney *U*-test. The survival rates were calculated starting from the day of surgery. Overall survival curves were drawn according to the Kaplan-Meier method, and differences were analyzed by the log-rank test. A Cox proportional hazards model was used to assess the effects of tumor variables on overall survival. Differences at $p<0.05$ were considered to be statistically significant.

Results

Correlation between lymph node metastasis and the expression of VEGF-C, VEGF-D, and VEGFR-3 at the protein level. Table II summarizes these results together with clinicopathological findings. The median VEGF-C, VEGF-D and VEGFR-3 concentrations patients without or with lymph node metastasis were 148.5 and 121.0, 107.1 and 41.8 and 329.9 and 254.0 pg/mg protein, respectively. The protein level of VEGF-D was significantly higher in the group without lymph node metastasis than in those with metastasis ($p=0.0129$, Figure 1 and Table II). Moreover, the protein levels of VEGF-D were statistically significantly different between

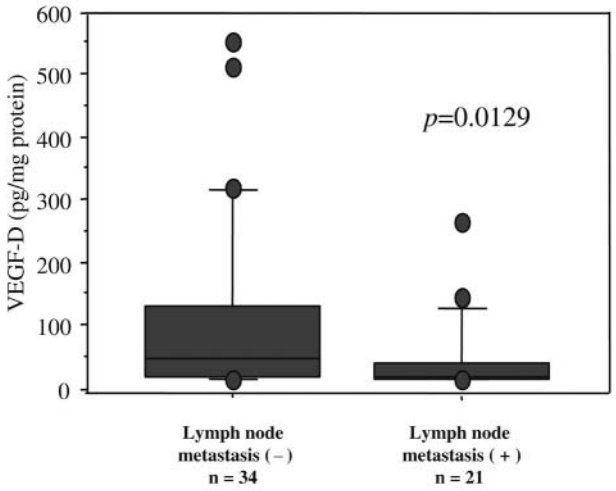

Figure 1. *VEGF-D levels in patients with lung cancer, as related to lymph node metastasis.*

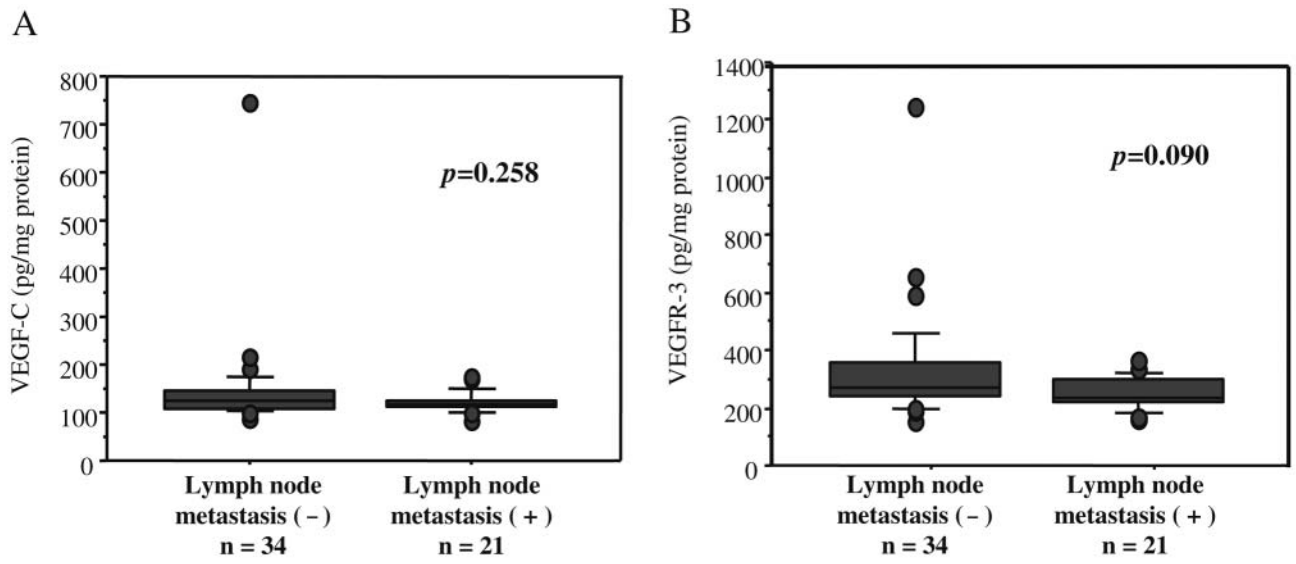

Figure 2. *A) VEGF-C levels in patients with lung cancer, as related to lymph node metastasis. B) VEGFR-3 levels in patients with lung cancer, as related to lymph node metastasis.*

disease stages ($p=0.045$, Table II). However, the protein levels of VEGF-C and VEGFR-3 were not significantly different between the groups without lymph node metastasis and those with metastasis ($p=0.258$, $p=0.090$, respectively, Figure 2A, B). The median levels of VEGF-D were statistically significantly different between the genders and histological grades ($p=0.009$, $p=0.022$, respectively), whereas no association was found between the VEGF-C or VEGFR-3 level and clinicopathological findings. The median VEGF-D concentration was 102.4 pg/mg protein in adjacent normal tissues. In comparison with the median level of VEGF-D in the tumor tissues, the median level of VEGF-D in the adjacent normal tissue was more likely to be high.

Correlation between lymph node metastasis and the expression of VEGF-C, VEGF-D, and VEGFR-3 at the mRNA level. The mRNA expression levels of *VEGF-C*, *VEGF-D* and *VEGFR-3* were quantitated using real-time RT-PCR. The median VEGF-C, VEGF-D and VEGFR-3 levels in patients without or with lymph node metastasis were 1.806 and 0.827, 4.826 and 1.396 and 0.753 and 0.044, respectively. In agreement with the result at the protein level, the mRNA expression levels of VEGF-C and VEGFR-3 showed no significant difference between the groups with and without lymph node metastasis ($p=0.3643$, $p=0.1509$, respectively, Table III), but the VEGF-D mRNA levels were significantly higher in the group without lymph node metastasis than in the group with metastasis ($p=0.0494$, Table III).

Table III. *Median and range of the relative expression levels of VEGF-C, -D and VEGFR-3 in the patients.*

Ligand or receptor	Lymph node metastasis (–) (n=10)		Lymph node metastasis (+) (n=10)		
	Median*	Range*	Median*	Range*	*p*-value
VEGF-C	1.806	0.147-19.209	0.827	0.267-8.088	0.3643
VEGF-D	4.826	0.664-83.926	1.396	0.026-153.699	0.0494
VEGFR-3	0.753	0.0-35.037	0.044	0.0- 84.557	0.1509

*Median and range of the relative expression levels were calculated relative to the standard curve and corrected for input cDNA based on the β2-microglobulin housekeeping gene.

Relation of VEGF-C, -D, and VEGFR-3 to survival analyses. The 55 patients were divided into two groups, patients with values higher than the mean and those with values lower than the mean. The 5-year survival rates of patients who showed a high level for VEGF-C and those with a low level of VEGF-C were 64.2% and 79.5%, respectively, and the survival rates of patients who showed a high level of VEGF-C were not significantly lower in comparison to those with a low level of VEGF-C ($p=0.2518$, data not shown). Figure 3 shows the Kaplan-Meier survival curves obtained by using the protein levels of VEGF-D. The 5-year survival rates for patients with a high level of VEGF-

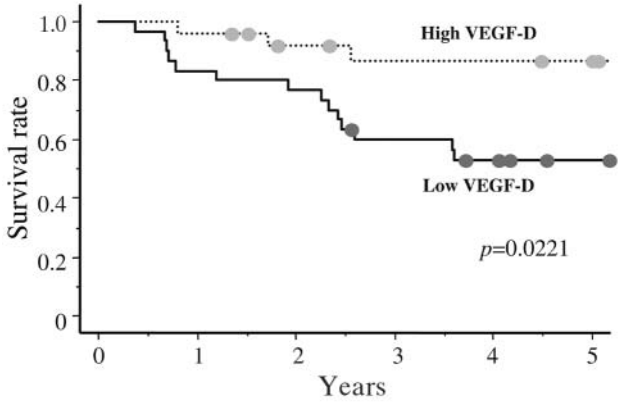

Figure 3. *Kaplan-Meier survival analysis. The overall survival of patients with VEGF-D. VEGF-D: Vascular endothelial growth factor-D.*

Table IV. *Univariate prognostic analysis of various factors in patients with T1 lung adenocarcinoma.*

Parameter	Relative risk ratio	95% Confidence limit	*p*-value
Age (≤65 years *vs.* >65 years)	0.490	0.184-1.307	0.1539
Gender (male *vs.* female)	0.889	0.350-2.258	0.8890
Histological grade (well- *vs.* moderately-, poorly-differentiated)	3.216	1.058-9.777	0.0349*
Pleural involvement (p0 *vs.* p1)	0.752	0.298-1.895	0.5452
N status (N0 *vs.* N1,N2)	0.085	0.024-0.294	<0.0001*
VEGF-C (high *vs.* low)	2.306	0.530-10.038	0.2655
VEGF-D (high *vs.* low)	3.398	1.116-10.343	0.0313*
VEGFR-3 (high *vs.* low)	0.371	0.049-2.793	0.3357

*Statistically significant.

D and those with a low level of VEGF-D were 86.8% and 52.8%, respectively, and the overall survival rates of patients with a low level of VEGF-D were significantly lower compared to those with a high level of VEGF-D ($p=0.0221$, Figure 3). The 5-year survival rates for patients with a high level of VEGFR-3 and those with a low level of VEGFR-3 were 64.8% and 87.5%, respectively, while the overall survival rates of patients with a low level of VEGFR-3 were not significantly lower than of patients with a high level of VEGFR-3 ($p=0.3161$, data not shown).

A univariate analysis of survival showed the histological grade, N status, and VEGF-D expression to be significant prognostic factors in patients with T1 lung adenocarcinoma (Table IV). However, a multivariate analysis of survival showed that only the N status was a significant prognostic factor in these patients.

Discussion

Contrary to our expectations, the expressions of VEGF-C and VEGFR-3 in lung adenocarcinoma were not significantly associated with lymph node metastasis or survival rates. However, interestingly, down-regulated VEGF-D expression was significantly associated with lymph node metastasis and poor prognosis.

In lung carcinoma, several studies have reported a positive association between the expression of VEGF-C and lymph node metastasis or survival rates (17, 24-27). Moreover, NSCLC patients with lymph node metastasis had higher serum VEGF-C levels than those without metastasis (17). In contrast, other studies have demonstrated a lack of any association between the expression of VEGF-C and lymph node metastasis or survival rates in non-small cell lung carcinoma (19). Interestingly, two studies reported that expression of VEGF-C may show a different clinical impact between lung adenocarcinoma and squamous cell carcinoma (26, 28).

VEGFR-3 expression has been reported, and significant correlations have been shown between up-regulation of the VEGFR-3 and lymph node metastasis in non-small cell lung carcinoma (16, 24, 27, 29, 30). Regarding survival, one study reported the expression of VEGFR-3 to be a significant prognostic factor in non-small cell lung carcinoma (30). However, in the present study, the expression of VEGFR-3 was not significantly associated with lymph node metastasis or survival.

Positive correlations between VEGF-D expression and lymph node metastasis or lymphatic vessel invasion have been reported in various malignancies (11-15). In contrast, several studies have shown that VEGF-D is down-regulated in tumor tissues in comparison to adjacent normal tissue (18, 19, 31), suggesting that VEGF-D has an antagonistic effect relative to VEGF or VEGF-C (18). These contradictory findings have not yet been explained. Al-Rawi and colleagues (32) documented that interleukin 7 (IL-7) up-regulated VEGF-D in breast cancer cells and induced lymphangiogenesis *in vivo*. Several factors might interact within the tumor microenvironment, thus eventually affecting tumor behavior such as lymphangiogenesis. It is well known, for example, that factors such as hypoxia and IL-8 can stimulate angiogenesis (33), and that IL-1β can up-regulate VEGF-C in colon cancer (34). However, in lung adenocarcinoma, it is possible that tumor proliferation-induced factors such as cytokines may inhibit VEGF-D production. As a result, there may be a possibility that lymph node metastases are promoted by down-regulated VEGF-D. In this study, the *VEGF-D* mRNA and protein expression levels were higher in cases of lung adenocarcinoma tissue without lymph node metastasis than in cases with lymph node metastasis. Furthermore, the expression levels of VEGF-D were statistically significantly

different between genders and histological grades. Previous studies have reported the survival rate for women to be better than for men (35, 36), and histological grade to be significantly associated with survival rate (37). Moreover, Wu and colleagues (38) reported that there was a statistically significant correlation between estrogen receptor β and gender, histological grade and the prognosis in NSCLC. Our results suggest the possibility that gender, histological grade and prognosis may also be associated with the down-regulation of VEGF-D.

In conclusion, the results suggest that VEGF-D is down-regulated in tumor tissues in comparison to adjacent normal tissue in adult lungs, and there may be the possibility that lymph node metastases are promoted by down-regulated VEGF-D. Further investigation is necessary to clarify and understand the role of VEGF-C, -D, and VEGFR-3 in patients with lung adenocarcinoma.

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