GENERAL THORACIC SURGERY

VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN NON-SMALL-CELL LUNG CANCER: PROGNOSTIC SIGNIFICANCE IN SQUAMOUS CELL CARCINOMA

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Background: Recently, some studies have focused on the tumor angiogenesis and its prognostic value. We studied the expression of vascular endothelial growth factor, microvessel counts, and serum concentrations of vascular endothelial growth factor to investigate their association with clinicopathologic factors and prognosis in non-small-cell lung cancer. **Methods:** The expression of vascular endothelial growth factor was determined by an immunohistochemical analysis from 91 paraffin specimens of completely resected non-small-cell lung cancers using anti-growth factor polyclonal antibody. Microvessel staining was performed by immunohistochemical analysis with anti-factor VIII-related antigen polyclonal antibody. Measurement of the serum concentrations of vascular endothelial growth factor used the sandwich enzyme-linked immunosorbent assay technique. **Results:** Expression of vascular endothelial growth factor was detected in 48 of the 91 tumors. The positive ratio was significantly higher in patients with adenocarcinoma than in those with squamous cell carcinoma. The microvessel counts were significantly higher in the patients with nodal metastasis than in those without nodal metastasis. The serum concentrations of vascular endothelial growth factor were also significantly higher in the patients with T3-4 disease than in those with T1-2 disease. The microvessel counts were closely associated with expression of vascular endothelial growth factor. The prognosis of patients with a positive growth factor ratio was significantly worse than that of the patients with a negative ratio ($p = 0.002$), especially in squamous cell carcinoma. According to a multivariate analysis, only nodal status and expression of vascular endothelial growth factor were found to be independent prognostic factors. **Conclusions:** The expression of vascular endothelial growth factor was one of the most important prognostic factors in completely resected non-small-cell lung cancer, especially in squamous cell carcinoma. (J Thorac Cardiovasc Surg 1998;115:1007-14)

Angiogenesis is required for the growth and metastasis of solid tumors.^{1,2} Tumor angiogenesis is also closely associated with prognosis³⁻⁶ and may be

regulated by angiogenic factors, which are produced by the tumor cells.⁷⁻¹⁰ Recently, several angiogenic factors, such as fibroblast growth factor,⁸ hepatocyte growth factor,⁹ and platelet-derived growth factor,¹⁰ have been identified. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is one such factor that is a homodimeric 34 to 42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic, and vascular permeability-enhancing activities specific for endothelial cells.¹¹⁻¹³ The gene for human VEGF is organized into eight exons. As a result of alternative splicing, four transcripts encoding mature monomeric VEGF containing 121, 165, 189, and 206 amino acid residues (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆)

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Fig. 1. Immunohistochemical staining of VEGF. **A,** Positive VEGF immunostaining of squamous cell carcinoma. VEGF was mainly localized in the cytoplasm of the tumor cells (original magnification $\times 400$). **B,** Negative VEGF immunostaining of squamous cell carcinoma (original magnification $\times 400$).

have also been detected. VEGF₁₂₁ and VEGF₁₆₅ are diffusible proteins that are secreted into the medium. On the other hand, VEGF₁₈₉ and VEGF₂₀₆ have a high affinity for heparin and are mostly bound to heparin-containing proteoglycans in the extracellular matrix.¹¹⁻¹³ VEGF has been identified in some malignant tumors, for example, ovarian cancer,¹⁴ melanoma,¹⁵ and gastric cancer.¹⁶ It has also been identified in lung cancer¹⁷; however, there has been no report on VEGF expression as a prognostic factor.

Recently, the postoperative prognosis has improved in non-small-cell lung cancer (NSCLC),¹⁸ but this prognosis still remains worse than for other carcinomas. In this study, we examined the VEGF expression in tumor tissue to investigate the correlation with clinicopathologic factors and prognosis in patients with completely resected NSCLC. In addition, we also examined the microvessel counts that closely reflect angiogenesis in tumor tissue.

Thanks to recent advances in medical technology, a serum assay of VEGF concentrations can now be easily performed. We also examined the serum VEGF concentrations to investigate both their clinical value and correlation with VEGF expression.

Materials and methods

Samples. Resected specimens from 91 consecutive patients with lung adenocarcinoma or squamous cell carcinoma who could be followed completely and who underwent a complete surgical resection at our institution from September 1991 to March 1995 were studied. The median follow-up period was 725 days. The patients ranged in age from 38 to 80 years (average, 65.6 ± 9.7 years); 69 were men and 22 were women. All patients underwent diagnostic procedures before the operation, including brain computed tomography, body computed tomography, and bone scintiscan without mediastinoscopy. The histologic types included 57 adenocarcinomas and 34 squamous cell carcinomas. The pathologic stage was classified according to the International Staging System for Lung Cancer¹⁹ after a complete mediastinal lymph node dissection was carried

Table I. Correlation between the VEGF expression in tumor tissue and the clinicopathologic factors in 91 patients

Factors	No. of patients (%)			p Value
	Total	VEGF-positive	VEGF-negative	
Total patients	91	48 (52.7)	43	
Age (yr)				
<65	40	23 (57.5)	17	0.42
65≤	51	25 (49.0)	26	
Sex				
Male	69	38 (55.1)	31	0.43
Female	22	10 (45.5)	12	
Histologic type				
Adenocarcinoma	57	35 (61.4)	22	0.03
Squamous cell carcinoma	34	13 (38.2)	21	
T stage				
T1-2	54	27 (50.0)	27	0.52
T3-4	37	21 (56.8)	16	
Nodal status				
Positive	48	25 (52.1)	23	0.89*
N1	18	6 (33.3)	12	
N2	28	17 (60.1)	11	
N3	2	2 (100)	0	
Negative	43	23 (53.5)	20	
Pathologic stage				
I	32	19 (59.4)	13	0.50†
II	7	0 (0.0)	7	
IIIA	32	16 (50.0)	16	
IIIB	20	13 (65.0)	7	

VEGF, Vascular endothelial growth factor.

The p value was calculated between NO and N1-3* and between stage I-II and stage III.†

out in all cases: stage I in 32 patients, stage II in 7 patients, stage IIIA in 32 patients, and stage IIIB in 20 patients. Of the 20 patients with stage IIIB disease, 18 had T4 N0-2 disease and 2 had T1-3 N3 disease. A complete resection was defined as no gross and microscopic residual tumor remaining at the bronchial or vascular stump. Even if microscopic metastasis were present in the highest mediastinal lymph node, it was still considered a complete resection. The slides were reviewed independently by two observers (H.I. and T.O.) who had no knowledge of the patients' clinical data.

Immunohistochemical staining of VEGF. Formalin-fixed, paraffin-embedded tissue specimens were prepared by conventional techniques. Immunohistochemical studies were performed with the labeled streptavidin-biotin method with anti-VEGF rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, Calif.). This antibody recognizes the 121, 165, and 189 isoforms of VEGF. Five-micrometer-thick sections were dewaxed in xylene, taken through ethanol, and then incubated with 0.3% hydrogen peroxide in methanol for 30 minutes to block any endogenous peroxidase activity. Then the sections were reacted with VEGF antibody (negative controls with nonspecific rabbit serum) in a 1:200 dilution at room

Table II. Correlation between the microvessel counts, serum VEGF concentrations, and various prognostic factors

Factors	Microvessel counts		Serum VEGF concentrations	
	Counts/200×*	p Value	Pg/ml*	p Value
Total	85.9 ± 71.8		525 ± 442	
Histologic type				
Adenocarcinoma	96.8 ± 84.1	0.058	589 ± 493	0.13
Squamous cell carcinoma	67.4 ± 38.7		398 ± 289	
T stage				
T1-2	76.9 ± 62.5	0.15	393 ± 255	0.004
T3-4	98.9 ± 82.6		733 ± 583	
Nodal status				
Positive	100.1 ± 84.3	0.04	596 ± 499	0.20
Negative	70.6 ± 52.0		443 ± 358	
Pathologic stage				
I-II	67.3 ± 52.8	0.03	365 ± 224	0.02
III	99.8 ± 80.9		644 ± 524	

VEGF, Vascular endothelial growth factor.

*Mean ± standard deviation.

temperature for 2 hours. The Labeled Streptavidin Biotin kit (Dako Corp., Carpinteria, Calif.) provided the secondary reagents. After these treatments were completed, the diaminobenzidine method was next used to visualize peroxidase with hematoxylin as a counterstain.

The degree of polyclonal antibody reactivity with individual tissue sections was considered to be positive if unequivocal staining of the membrane or cytoplasm was seen in more than 5% of the tumor cells in the slide of the largest section of the tumor, as reported by Maeda and associates.¹⁶

Microvessel staining and counting. Microvessels in the tumor tissue were highlighted by immunohistochemical staining with anti-factor VIII-related antigen polyclonal antibody A0082 (Dako) in a 1:200 dilution at room temperature for 2 hours. The other method was the same as VEGF immunohistochemical staining. As described in another report,⁵ any single brown-stained cells or clusters of endothelial cells that were clearly separate from the adjacent microvessels, tumor cells, and other connective tissue elements were considered as a vessel. The vessels were counted in the five areas of highest vascular density at 200× magnification, as described in another report.⁵ The microvessel counts were expressed as the mean number of vessels in these areas.

Enzyme-linked immunosorbent assay (ELISA) of serum VEGF. After informed consent had been obtained from all patients, the serum from 57 of the 91 patients was stocked before the operation and frozen at -80° C. The sera were then melted at room temperature within 15 minutes before being subjected to an assay. The serum VEGF assay used the quantitative sandwich ELISA technique with the VEGF ELISA kit (R&D Systems, Inc., Minneapolis, Minn.) according to the manufacturer's instructions. Two antibodies in this assay reacted mainly to

Fig. 2. Survival curves of the 91 patients according to the VEGF expression.

VEGF₁₆₅. The optical density was determined within 30 minutes after these treatments were performed with a microtiter plate reader set to 450 nm. After averaging the duplication of this treatment for each sample, the concentrations of serum VEGF were calculated with a standard curve. The standard curve of VEGF was linear in a plot at concentrations from 0 to 2000 pg/ml ($r = 0.998$, $p = 0.0001$).

Statistical analysis. The microvessel counts and serum VEGF concentrations were expressed as the mean $\pm$ standard deviation. The relationship between the VEGF expression and various clinicopathologic factors were examined with the χ^2 test. The relationship between the microvessel counts, serum VEGF concentrations, and clinicopathologic factors were examined with the unpaired Student's t test. The relationship between the microvessel counts and serum VEGF concentrations was examined with the Spearman rank test. The survival curves were calculated with the Kaplan-Meier method and were then analyzed by the log rank test. A univariate and a multivariate analysis of various prognostic factors were assessed by the Cox's proportional-hazards regression model.²⁰

Results

The immunohistochemical detection of VEGF expression. VEGF was mainly localized in the cytoplasm of the tumor cells (Fig. 1). Tumor cells that stained strongly for VEGF were observed more often in the invasive front than in the center of the tumor, especially in squamous cell carcinoma. On the other hand, VEGF stained relatively homogeneously in adenocarcinoma.

VEGF expression was detected in 48 of the 91 tumors (52.7%). Table I shows the correlation between the VEGF expression and various clinicopathologic factors. A significant difference was noted with histologic types. The VEGF-positive rate was significantly higher in patients with adenocarci-

Fig. 3. Survival curves of the 34 patients with squamous cell carcinoma according to the VEGF expression.

noma than in those with squamous cell carcinoma ($p = 0.03$). However, there was no significant association between the VEGF expression and the age at operation, sex, T stage, nodal status, or pathologic stage.

Microvessel counts and serum VEGF concentrations. Table II shows the correlation between microvessel counts, serum VEGF concentrations, and various clinicopathologic factors. Microvessel counts ranged from 7 to 321 counts/200 $\times$ with a mean of 85.9 ± 71.8 counts/200 $\times$, and serum VEGF concentrations ranged from 0 to 2014 pg/ml with a mean of 525 ± 442 pg/ml. Microvessel counts and serum VEGF concentrations were not significantly associated with the histologic types. The microvessel counts and serum VEGF concentrations in patients with stage III disease were significantly higher than in those patients with stage I-II disease ($p = 0.03$ and $p = 0.02$, respectively). The microvessel counts in the tumors with nodal metastasis were also significantly higher than in those without nodal metastasis ($p = 0.04$). However, the T stage was not significantly associated with the microvessel counts. On the other hand, the serum VEGF concentrations were significantly associated with the T stage. The serum VEGF concentrations were significantly higher in patients with T3-4 disease than in those with T1-2 disease ($p = 0.004$). However, the nodal status was not significantly associated with the serum VEGF concentrations.

Relationship between VEGF expression and microvessel counts or serum VEGF concentrations. There was a significant association between the VEGF expression and the microvessel counts in the tumor tissue. The microvessel counts were signifi-

Table III. Correlation between VEGF expression in tumor tissue, the microvessel counts, and serum VEGF concentrations

Factors	Microvessel counts		Serum VEGF concentrations	
	Counts/200×*	p Value	Pg/ml*	p Value
VEGF expression				
Positive	102.9 ± 83.3	0.01	606 ± 552	0.20
Negative	66.8 ± 50.6		451 ± 300	

VEGF, Vascular endothelial growth factor.

*Mean ± standard deviation.

cantly higher in patients with VEGF-positive tumor (102.9 ± 83.3) than in patients with VEGF-negative tumor (66.8 ± 50.6) ($p = 0.01$). On the other hand, there was no significant association between the VEGF expression and the serum VEGF concentrations (Table III). There was no significant association between the serum VEGF concentrations and the microvessel counts ($p = 0.78$).

Survival analysis. The prognosis of the 48 patients with VEGF-positive tumor was significantly worse than the 43 patients with VEGF-negative tumor ($p = 0.003$) (Fig. 2). Table IV shows a univariate analysis of the various prognostic factors. T stage, nodal status, and VEGF expression were all statistically significant factors ($p = 0.004$, $p = 0.0001$, and $p = 0.003$, respectively). The microvessel counts might appear to be a prognostic factor but not reach statistical significance ($p = 0.083$); however, the serum VEGF concentrations were not an important prognostic factor ($p = 0.34$). A multivariate analysis with Cox's proportional-hazards regression model was performed to investigate the independent prognostic factors among the T stage, nodal status, VEGF expression, and microvessel counts. As a result, only the nodal status ($p = 0.0001$) and VEGF expression ($p = 0.004$) were identified to be independent prognostic factors (Table V). Because the incidence of VEGF expression differed significantly between the patients with squamous cell carcinoma and adenocarcinoma, univariate and multivariate subset survival analysis were performed on each histologic type. Especially in the 34 patients with squamous cell carcinoma, the prognosis of the patients with VEGF-positive tumor was significantly worse than for patients with VEGF-negative tumor ($p = 0.0001$) (Fig. 3). The same tendency was also observed in patients with adenocarcinoma, but it did not reach statistical significance ($p = 0.19$). According to multivariate analysis on each histologic type, VEGF expression was also an independent prognostic factor in patients with squamous cell carcinoma

($p = 0.03$); however, it did not reach statistical significant in patients with adenocarcinoma ($p = 0.24$).

Discussion

In this study, we detected the VEGF-positive expression in 48 of 91 patients (52.7%) with a completely resected stage I-III NSCLC. We observed no significant correlation between the VEGF expression and age, sex, T stage, nodal status, or pathologic stage. A significant relationship was only seen in the VEGF expression regarding histologic types. The positive ratio of VEGF expression in adenocarcinoma was higher than that in squamous cell carcinoma ($p = 0.03$). We also found a tendency that microvessel counts were higher in patients with adenocarcinoma than in those with squamous cell carcinoma. These findings mean that adenocarcinoma has higher angiogenic potential than squamous cell carcinoma. Recently, some reports^{21, 22} on genetic studies have shown a difference in the incidence of certain genetic changes in histologic type of lung cancer. For example, *ras* mutations are found predominantly in adenocarcinoma,²¹ whereas p53 gene mutations are more prevalent in squamous cell carcinoma than in adenocarcinoma.²² Such differences in the histologic type in lung cancer may be identified in both angiogenic factors and genetic changes.

Weidner and associates²³ reported that the microvessel counts were closely associated with lymph nodal metastasis in invasive breast cancer; other authors have reported them to be correlated with systemic metastasis in NSCLC.^{24, 25} In this study, the microvessel counts were closely associated with lymph nodal metastasis; moreover, the microvessel counts in VEGF-positive tumors were also significantly higher than in VEGF-negative tumors. It is therefore thought that VEGF might reflect the metastatic potential through the angiogenesis of the tumor.

Table IV. Univariate analysis of the various prognostic factors

Factors	Characteristics		Risk ratio	95% CI	p Value
	Unfavorable	Favorable			
Age	65≤	<65	0.899	0.500-1.615	0.72
Sex	Male	Female	0.882	0.455-1.710	0.71
Histologic types	Adenocarcinoma	Squamous cell carcinoma	0.885	0.487-1.609	0.69
T stage	T3-4	T1-2	2.358	1.307-4.255	0.004
Nodal status	Positive	Negative	4.743	2.381-9.451	0.0001
VEGF expression	Positive	Negative	2.588	1.373-4.878	0.003
Microvessel counts	High	Low*	1.697	0.933-3.089	0.083
Serum VEGF concentrations	High	Low†	0.695	0.328-1.473	0.34

CI, Confidence interval; VEGF, vascular endothelial growth factor.

*The cut off value represents the mean of all microvessel counts (85.9 counts/200×).

†The cut off value represents the mean of serum VEGF concentrations (525 pg/ml).

Table V. Multivariate analysis of various prognostic factors

Factors	Characteristics		Risk ratio	95% CI	p Value
	Unfavorable	Favorable			
T stage	T3-4	T1-2	0.663	0.352-1.249	0.20
Nodal status	Positive	Negative	4.245	2.029-8.878	0.0001
VEGF expression	Positive	Negative	2.590	1.349-4.973	0.004
Microvessel counts	High	Low*	1.282	0.694-2.369	0.42

CI, Confidence interval; VEGF, vascular endothelial growth factor.

*The cut off value represents the mean of all microvessel counts (85.9 counts/200×).

In this study, we also studied serum VEGF concentrations using ELISA. The serum VEGF was found to be associated with T stage, but no association was seen with nodal status. We also studied serum VEGF concentrations of 61 healthy persons (mean age: 63 years; male/female: 47/14). The serum VEGF concentrations of healthy persons was 181 ± 165 pg/ml. This was lower than that of patients with NSCLC. Moreover, we studied serum VEGF concentrations after surgery. We obtained the sample at 1 to 3 months after surgery because wound healing might increase the serum VEGF level through angiogenic response immediately after surgery. The serum VEGF was decreased after surgical resection from 525 ± 442 pg/ml to 243 ± 214 pg/ml (detailed data not shown). It appeared that the serum VEGF increased with growth of the tumor and also might reflect the tumor burden. A serum assay can be easily and frequently performed because of its minimal invasiveness compared with surgical examination of resected tissue material. Dirix and associates²⁶ reported that the serum VEGF might prove a useful tool in the quantification of angiogenesis and might be of valuable information in the decision process of initiating palliative

chemotherapy in colorectal cancer. Moreover, we think that an evaluation of serum VEGF is useful for both a serologic diagnosis and the monitoring of the tumor burden. We have not yet checked serum VEGF in patients with recurrence, and we should examine the values for a longer period. In this study, there was no significant association between the VEGF expression in tumor tissue and the serum VEGF concentrations. This discrepancy may be due to differences in the use of antibodies for each assay, such as anti-VEGF₁₂₁ for IHC and anti-VEGF₁₆₅ for ELISA. In addition, the mechanism of VEGF shedding from the tumor cell to the systemic circulation has not yet been clearly elucidated and may also be one of the reasons for this discrepancy.

The most interesting finding in this study was the prognostic impact of VEGF expression in patients with completely resected NSCLC. Toi and associates^{27,28} reported the VEGF expression to be a significant prognostic factor in patients with breast cancer. However, there has been no report that shows the VEGF expression to be associated with prognosis for patients with NSCLC. We found that the prognosis for patients with VEGF-positive tumor was significantly worse than that for patients

with VEGF-negative NSCLC. Harpole and associates²⁹ have reported that angiogenesis was the most significant prognostic factor in stage I lung cancer. In 32 patients with stage I in this study, the VEGF expression was not significant prognostic factor ($p = 0.12$). However, only one of 13 patients with VEGF-negative tumor died. On the other hand, 6 of 19 patients with VEGF-positive tumor died. Thus VEGF expression may be one of the most important prognostic factors in early stage lung cancer. According to a multivariate analysis using Cox's proportional-hazards regression model, the VEGF expression was found to be an independent significant prognostic factor along with nodal status. Because the incidence of VEGF expression differed significantly between the patients with squamous cell carcinoma and adenocarcinoma, univariate and multivariate survival analyses were performed on each histologic type. As a result, especially in patients with squamous cell carcinoma, the expression of VEGF was found to be closely associated with the prognosis and to be an independent prognostic factor.

In conclusion, the VEGF expression is one of the most important prognostic factors; the evaluation of the VEGF expression may be effective when adjuvant therapy is considered for patients with completely resected NSCLC, including antiangiogenic therapy.³⁰

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