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Prognostic Value of Vascular Endothelial Growth Factor Expression in Primary Lung Carcinoma

HaiYun SHENG, Motoi AOE, Hiroyoshi DOIHARA, Akio ANDOU and Nobuyoshi SHIMIZU*

Department of Surgery II, Okayama University Medical School, Okayama 700-8558, Japan

To investigate whether an association exists between vascular endothelial growth factor (VEGF) expression and tumor prognosis in primary lung carcinoma, we used immunohistochemical techniques to analyze microvessel density and VEGF expression in lung carcinoma tissue from 98 patients. Tissue had been fresh-frozen at the time of operation and preserved for more than 5 years. The results indicated that VEGF expression was positive for 50 of the 98 patients (51.0%), with 27 (27.6%) being weakly positive and 23 (23.5%) being strongly positive. The microvessel density in tissue showing weakly positive and strongly positive VEGF expression was significantly higher than that in VEGF-negative tumor tissue ($P < 0.05$: negative *vs.* weakly positive, $P < 0.01$: negative *vs.* strongly positive), we showed demonstrating that VEGF expression was significantly associated with intratumoral microvessel density. The 5-year survival rates were 8.7% for strongly VEGF-positive patients, 43.9% for weakly VEGF-positive patients and 79.2% for VEGF-negative patients, respectively ($P < 0.01$: negative *vs.* weakly positive or strongly positive). Furthermore, multivariate analysis employing multiple regression analysis indicated that VEGF expression correlates highly with the overall survival rates of patients with primary lung carcinoma. Two variables, N status and VEGF expression, were found to be significant prognostic factors ($P < 0.01$). The results of this study suggest that VEGF expression is associated with intratumoral microvessel density. VEGF expression may constitute important independent prognostic evidence that can help us in predicting the outcomes of patients with primary lung carcinomas.

Key words: prognostic value, vascular endothelial growth factor, neoangiogenesis, multivariate analysis, primary lung carcinoma

In Japan, lung cancer has become the most common cause of cancer-related death in humans, the 5-year survival rate of primary lung carcinoma being 10% to 30% (1). Although surgical resection is considered the best treatment for lung cancer, the prognoses of patients with lung carcinoma are still poor.

Recently, many studies have shown that neoangiogenesis plays an important role in the growth, progression and metastasis of solid tumors (2, 3). Among angiogenic factors, vascular endothelial growth factor (VEGF) is known to be a powerful endothelial cell-specific mitogen involved in tumor neovarscularization (4). Some researchers have asserted that the correlation between VEGF expression and microvessel count in gastric cancer (3, 5) is one of the more important factors influencing the survival of surgically treated breast cancer patients (2, 6). However, there have also been few studies on the association of the expression of angiogenic factors and prognosis of patients with malignant tumors (7, 8).

In the present investigation, we examined the expression of VEGF and microvessel density (MVD) using immunohistochemical procedures, and attempted to analysis the association of VEGF expression with neoangiogenesis and clinicopathologic factors. In addition, the prognostic value of using VEGF expression associated with neoangiogenesis is analyzed in primary lung carcinoma.

* To whom correspondence should be addressed.

Materials and Methods

Clinical characteristics of the patients. Our study was done on 98 patients who underwent operations for the purpose of resecting primary lung carcinomas. The surgery was performed at the Department of Surgery II, Okayama University Medical School in Japan, between February 1989 and December 1991. Follow-up of least 5 years duration was available for all patients. The patient group consisted of 68 males and 30 females with a mean age of 63.8 years (range 24 to 84). According to the International System for Staging Lung Cancer (9), these cases comprised 29 patients with pathologic Stage IA, 19 patients with pathologic Stage IB, 2 patients with pathologic Stage IIA, 8 patients with pathologic Stage IIB, 27 patients had pathologic Stage IIIA, 5 patients with pathologic Stage IIIB, and 8 patients with pathologic Stage IV (Table 1). No patient received chemotherapy or radiation therapy before surgery. After surgery, 39 patients had not received chemotherapy or radiation therapy, 46 patients had received chemotherapy, 6 patients had received radiation therapy, and 7 patients had received chemotherapy and radiation therapy.

Tumor specimens. The tumor specimens were obtained at the time of operation and frozen in liquid nitrogen with tissue-Tek compound (Sakura Finetek Europe B.V, Zoeterwoude, Netherlands). Frozen tissue specimens were preserved at -80 °C.

VEGF immunostaining. Four-μm frozen tissue sections were made on a cryostat (Leica CH1850, Nussloch, Germany) and fixed with acetone (concentration 99.5%) for 1 min at room temperature. They were then immersed for in protein blocking agent (PBA) at room temperature for 5 min, followed by incubation for 16 h with anti-VEGF antibody (Santa Cruz Biotechnology, Sata Cruz, CA, USA) at a dilution of 1:40 at 4 °C. They were then washed 3 times with phosphate-buffered saline (PBS). The sections were treated with the secondary antibody (Shandon Lipshaw Co, Pittsburgh, PA, USA) for 2 h at 4 °C and then washed with PBS 3 times. Diaminobenzidine (Shandon Lipshaw Co.) was used as a chromogen. Counterstaining was performed using hematoxylin for 2 min at room temperature. Negative controls were incubated without the primary antibody.

Factor VIII immunostaining. The sections were immunostained with anti-Factor VIII antibody (Santa Cruz Biotechnology) at a 1:150 dilution for 10 min at room temperature and then washed 3 times with PBS. They were then treated with the secondary antibody (Shandon Lipshaw Co.) for 10 min at room temperature and washed 3 times with PBS. Finally, they were treated with Diaminobenzidine for 25 min at room temperature. Counterstaining was performed using hematoxylin for 2 min at room temperature. Negative controls were incubated without the primary antibody.

VEGF determination and microvessel count. VEGF expression was evaluated as a percentage of positive cells in a total of at least 1,000 tumor cells at 200-times magnification (10 × objective lens and 20 × ocular lens). The sections of VEGF expression were classified as strongly positive if 51–100% of the tumors cells were positively stained, weakly positive if 5–50% were positively stained, or negative if 0–4% were positively stained.

Table I Clinical information of patients studied

Parameter	Number
Gender	
Male	68
Female	30
Age (years)	
Mean	63.8
Range	24–84
Histological type	
Adenocarcinoma	57
Squamous cell carcinoma	31
Others	10
Cell differentiation	
Well	41
Moderate	40
Poor	17
Lymph node metastasis	
Negative	52
Positive	46
Distant metastasis	
Negative	92
Positive	6
Stage grouping	
IA	29
IB	19
IIA	2
IIB	8
IIIA	27
IIIB	5
IV	8
Tumor size (cm)	
≤ 3	40
>3	58

For microvessel count, the sections with Factor VIII staining were used. In each section, brown-stained vessel lumen structures or cell clusters were counted as microvessels. The slides were screened under low magnification to identify the vascular areas of the highest density within the tumor, and 5 areas were selected for examination at 200-times magnification as above. All classifications and counting were performed blind. The mean number of microvessels in 5 view fields at 200-times magnification was defined as MVD.

Statistical analysis. The relationship between VEGF expression, MVD and various clinicopathologic factors were statistically examined using the χ^2 test. The Kaplan-Meier method was used to estimate the probability of the overall survival rates of the groups and these were compared with each other using the log-rank test. Clinicopathologic factors were determined as independent or dependent using multivariate analysis with multiple regression analysis. Since the prognostic factors of a patient's status and tumor's clinicopathologic characters were not homogeneous, as indicated by the performance status and advanced stage status of primary lung carcinoma, the degrees of freedom analysis values were calculated according to conventional analysis procedure. Only those variables that were significantly associated with prognosis as determined by multivariate analysis were inserted in a multiple regression model in order to evaluate their joint effect. The contribution of each factor was evaluated using this model. A P-value less than 0.05 was considered statistically significant.

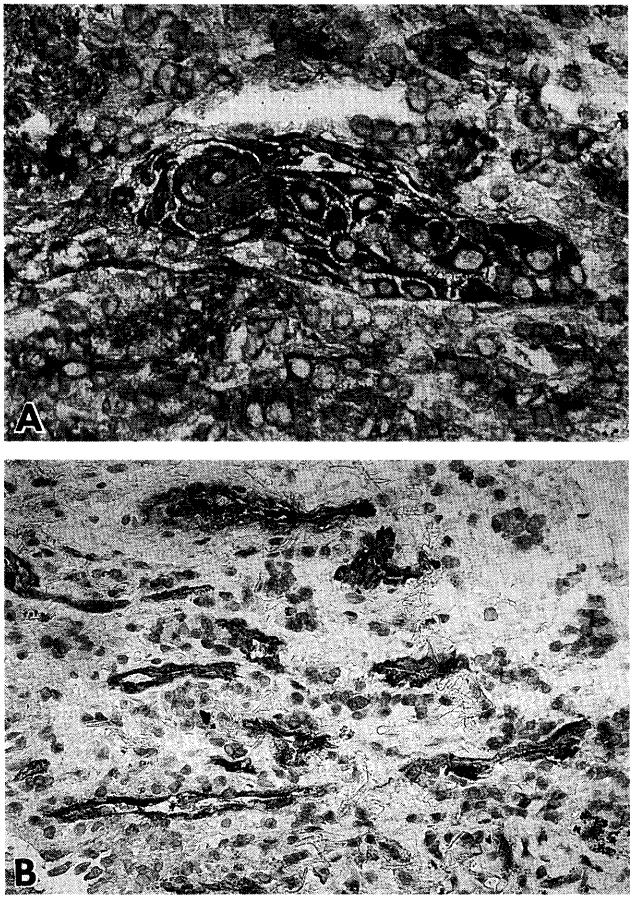

Fig. 1 Immunohistochemical staining for vascular endothelial growth factor (VEGF) expression and Factor VIII in primary lung carcinoma (hematoxylin counterstaining, original magnification × 200). VEGF expression is observed primarily in the cytoplasm portion of the cancer cell within the tumor tissue (Photo A). Anti- Factor VIII antibody immunoreactivity was observed in the cytoplasm of endothelial cells (Photo B).

Results

VEGF expression. Ninety-eight patients having primary lung carcinomas with antibodies to VEGF were analyzed immunohistochemically. Positive staining for VEGF was found in 50 of 98 cases (51.0%) (Fig. 1A), of which, 27 (27.6%) showed weakly positive expression and 23 (23.5%) showed strongly positive VEGF expression. VEGF expression was identified mainly in the cytoplasm of the tumor cell. Among the 98 patients investigated, 46 had lymph node metastasis, of which 14 (30.4%) showed weakly positive VEGF expression and 17 (37.0%) showed strongly positive VEGF expression. In contrast, 13 (25.0%) of the 52 patients with no lymph node metastasis were found to have weakly positive expression and 6 (11.5%) were found to have strongly positive expression. Among the 29 patients in Stage IA, 7 (24.1%) showed weakly positive VEGF expression and 2 (6.9%) showed strongly positive VEGF expression. Out of the 8 patients in Stage IB, 5 (26.3%) showed weakly positive VEGF expression and 2 (10.5%) showed strongly positive VEGF expression. The 2 patients in Stage IIA were VEGF negative. Of the 8 patients in Stage IIB, 2 (25.0%) showed weakly positive VEGF expression and 3 (37.5%) showed strongly positive VEGF expression. Among the 27 patients in Stage IIIA, 8 (29.6%) showed weakly positive VEGF expression and 12 (44.4%) showed strongly positive VEGF expression. Of the 5 patients in Stage IIIB, 3 (60.0%) showed weakly positive VEGF expression and one (20.0%)

showed strongly positive VEGF expression. Among the 8 patients in Stage IV, 2 (25.0%) showed weakly positive VEGF expression and 3 (37.5%) showed strongly positive VEGF expression (Table 2).

Microvessel density. Staining for Factor VIII-antibodies was easy to interpret because of the specificity of the staining with minimal background staining. High vascularity was invariably observed as lung cancer tissue. Since the median microvessel density was 7.1, tumors were classified into the hypervascular group (MVD > 7.1), and the hypovascular group (MVD ≦ 7.1). There were no significant statistical effects regarding tumor size, T classification, histology or distant metastasis on microvessel density. The microvessel density showed statistical significance in patients with lymph node metastasis and in the advanced stage groups ($P < 0.01$) (Table 3).

Relationship between VEGF expression and MVD. The relationship between VEGF expression and MVD in the tumors was also analyzed. The areas of high vascularization occur most frequently at the margins of carcinomas. The mean MVD value in tumors weakly positive for VEGF and in those strongly positive for VEGF was significantly higher than that in VEGF-negative tumors. MVDs were 8.7 ± 2.3, 10.9 ± 1.8 and 4.6 ± 1.8 for weakly positive VEGF, strongly positive VEGF and VEGF negative tumors, respectively. (The corresponding P-value was < 0.05 for weakly positive VEGF versus VEGF negative tumors, and was < 0.01 for strongly positive VEGF versus VEGF-negative (Fig. 2)).

Table 2 Relationship between clinicopathologic features and VEGF expression in primary lung carcinoma of 98 patients

Variable	Total	VEGF expression			
		Negative	Weakly positive	Strongly positive	*P*-value
No. of patients	98	48	27	23	
Tumor size (cm)					NS
≦3	40	25	10	5	
>3	58	23	17	18	
T classification					NS
T1 T2	84	45	22	17	
T3 T4	14	3	5	6	
Lymph node metastasis					< 0.01
Negative	52	33	13	6	
Positive	46	15	14	17	
Distant metastasis					NS
Negative	92	46	25	21	
Positive	6	2	2	2	
Stage grouping					< 0.05
IA	29	20	7	2	
IB	19	12	5	2	
IIA	2	2	0	0	
IIB	8	3	2	3	
IIIA	27	7	8	12	
IIIB	5	1	3	1	
IV	8	3	2	3	
Histological type					NS
Ad	57	29	15	13	
Sq	31	12	11	8	
Others	10	7	1	2	

Ad, adenocarcinoma; Sq, squamous cell carcinoma; Other, miscellaneous carcinoma; NS, not significant; VEGF, vascular endothelial growth factor.

Table 3 Relationship between clinicopathologic features and MVD in primary lung carcinoma of 98 patients

Variable	Total	MVD		
		Low (≦ Median)	High (> Median)	*P*-value
No. of patients	98	50	48	
Tumor size (cm)				NS
≦3	40	24	16	
>3	58	26	32	
T classification				NS
T1 T2	84	45	39	
T3 T4	14	4	10	
Lymph node metastasis				< 0.01
Negative	52	35	17	
Positive	46	15	31	
Distast metastasis				NS
Negative	92	48	44	
Positive	6	2	4	
Stage grouping				< 0.01
IA	29	19	10	
IB	19	14	5	
IIA	2	2	0	
IIB	8	4	4	
IIIA	27	7	20	
IIIB	5	1	4	
IV	8	3	5	
Histological type				NS
Ad	57	29	28	
Sq	31	17	14	
Others	10	6	4	

MVD, microvessel density.
Ad, Sq, Other, NS, see legend to Table 2.

Relationship between VEGF expression/ MVD and clinicopathologic factors. VEGF expression and degree of MVD were measured in all 98 patients. There were no statistically significant relationships between VEGF expression and clinicopathologic factors such as T classification, tumor size, distant metastasis, and histological types. Similarly, we found no statistically significant effects of these clinicopathologic factors on MVD. However, we did find that VEGF expression correlated significantly with lymph node metastasis ($P < 0.01$) and advanced stage groups ($P < 0.05$). We also found that MVD correlated significantly with lymph node metastasis ($P < 0.01$) and advanced stage groups ($P < 0.01$) (Tables 2 and 3).

Relationship between VEGF expression and overall survival. The overall survival rate of all 98 patients was compared according to VEGF expression status. The patients showing negative VEGF expression had significantly more favorable prognoses than those showing VEGF-positive expression. The 5-year survival rates were 79.2% for the 48 patients with VEGF-negative tumors; 43.9% for the 27 patients with weakly positive tumors, and 8.7% for the 23 patients with strongly positive tumors. There were significant differences between the negative, weakly positive, and strongly positive groups ($P < 0.01$) (Fig. 3). Since the number of adenocarcinoma or squamous cell carcinoma cases was too small to analyze statistically for VEGF expression between the 3 sub-groups, all cases were divided into 2 groups (VEGF-positive and VEGF-negative) and evaluated as to whether VEGF expression affects the survival for adenocarcinomas or squamous cell carcinomas. The 5-years survival rate of the VEGF-negative group of adenocarcinoma was 89.7% and that of the VEGF-positive group was 28.6%, showing a significantly better survival rate for the VEGF-negative group ($P < 0.01$) (Fig. 4). The survival rate of the VEGF-negative group of squamous cell carcinoma was 75.0% and that of the VEGF-positive group was 33.3%.

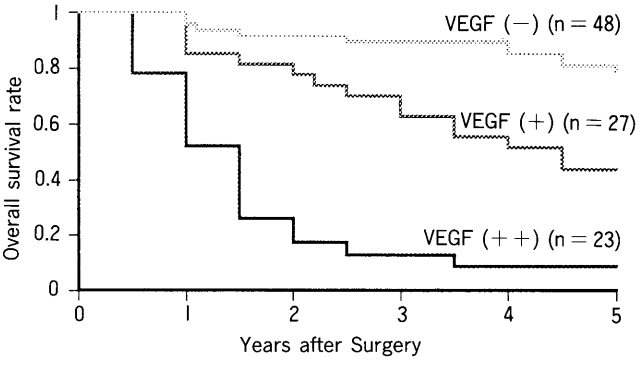

Fig. 3 Overall postoperative survival rate according to VEGF expression in 98 patients with primary lung carcinoma. Five-year survival rates: strongly positive; 8.7%, weakly positive; 43.9%, negative; 78.2%. The patients with weakly positive and strongly positive expression had a significantly more favorable prognoses than those with negative expression (weakly and strongly positive *vs.* negative: $P < 0.01$).
VEGF, see legend to Table 2.

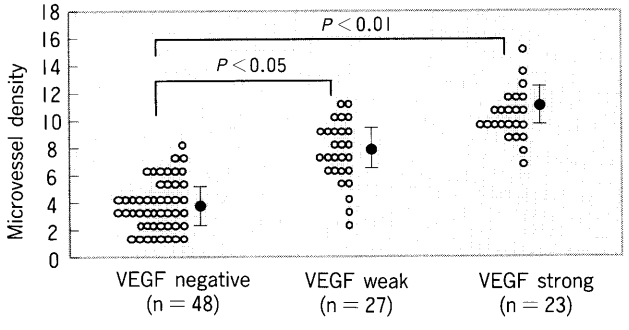

Fig. 2 Correlation between microvessel density and expression of vascular endothelial growth factor. The plot of microvessel density is shown according to the status of vascular endothelial growth factor expression. Closed circles (●) and horizontal bars indicate mean ± standard deviation.

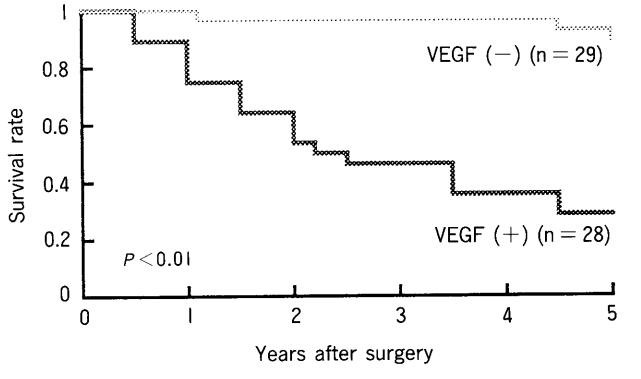

Fig. 4 Overall survival rate of 57 patients with adenocarcinoma. Five-year survival rates: positive; 28.6%, negative; 89.7%. The patients showing positive VEGF expression had significantly more favorable prognoses than did those with negative VEGF-expression (positive *vs.* negative: $P < 0.01$).
VEGF, see legend to Table 2.

Again, a significantly higher survival rate was shown for the VEGF-negative group ($P < 0.05$) (Fig. 5).

Prognostic value of VEGF expression. The results of multivariate regression analysis are shown in Table 4. The estimated prognostic value of each variable in relation to overall survival among the 98 patients studied is expressed as a P value. N status and VEGF expression were found to be significant prognostic factors ($P < 0.01$). Other variables such as age, sex, histology, T classification and tumor size were found to have no significant effects on the overall survival of the 98 patients with primary lung carcinoma.

Discussion

The independent prognostic influence of VEGF expression has been widely reported in gastric carcinoma and breast carcinoma (2, 3, 5, 6). In our study, we attempted to determine whether VEGF expression is associated with neoangiogenesis, and whether it can provide additional prognostic information for predicting the survival of patients with primary lung carcinoma.

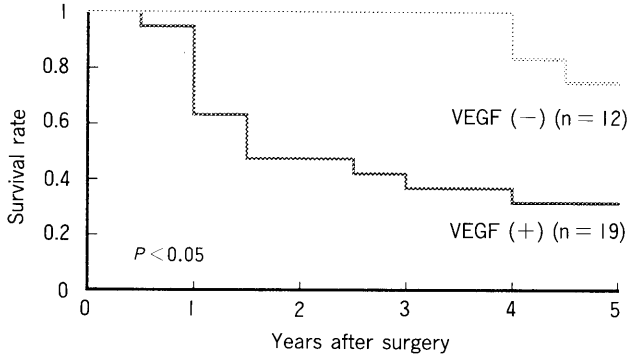

Fig. 5 Five-year survival rates of 31 patients with squamous cell carcinoma, positive; 33.3%, negative; 75.0%. This result in overall survival analysis was also significant (positive *vs.* negative: $P < 0.05$).

Neoangiogenesis and prognosis. Angiogenesis, the development and formation of new blood vessels, is important in a variety of physiological processes such as growth, differentiation, ovulation, wound healing, and neoplasia (5). Neoangiogenesis, however, is also indispensable to tumor growth, invasion, and metastasis. Neoangiogenesis is required to supply nutrients for the tumor cells shed into circulation. As well, newly formed capillaries of the leak because of fragmented basement membranes, making them more accessible to errant tumor cells. Recent evidence suggests that tumor angiogenesis is associated with patient outcome in a number of malignancies and is an independent prognostic marker (10). Many studies have shown that there is a relationship between angiogenesis and prognosis in human cancers such as mammary (11), head and neck (12), prostate (13), ovarian (14), colorectal (15), testicular (16), urethra (17), cutaneous (18), nerve (19), bronchial carcinoma (20) and non-small cell lung carcinoma (7). The above studies, especially those concerned with breast cancers, have shown that intratumoral neoangiogenesis has an independent prognostic significance when compared with traditional prognostic markers in multivariate analysis (11). In our study, increasing intratumoral microvessel density indicated a more advanced stage group, as well as lymph node metastasis ($P < 0.01$) (Table 3). Usually, patients in this stage of disease advancement have poor prognoses. The biological foundation of the

Table 4 Multivariate regression analysis of clinical pathological factors on the survival of 98 patients

Variable	β	SE	95%CI	t value	P value
Age (years) ($\leqq 63$ *vs.* > 63)	−0.1	0.084	−.267–0.067	−1.192	0.2364
Gender (male *vs.* female)	0.06	0.098	−.134–0.255	0.616	0.5396
Size (cm) ($\leqq 3$ *vs.* > 3)	0.133	0.104	−.075–0.340	1.269	0.2077
T status (T1, T2 *vs.* T3, T4)	0.091	0.069	−.047–0.229	1.313	0.1924
N status (negative *vs.* positive)	0.167	0.048	0.072–0.262	3.504	0.0007
Histology (Ad, Sq *vs.* others)	0.128	0.066	−.003–0.259	1.947	0.0546
VEGF expression (negative *vs.* weakly, strongly positive)	−0.331	0.086	−.502–0.161	−3.867	0.0002

Ad, adenocarcinoma; Sq, squamous cell carcinoma; Other, miscellaneous carcinoma; SE, standard error; β, regressive coefficient; CI, confidence interval; VEGF, vascular endothelial growth factor.

prognostic potential of tumor microvessel count is that the formation of new capillaries possibly advances the development of metastasis (6).

Relationship between MVD and VEGF. Solid tumors require angiogenesis for growth and metastasis, and tumor angiogenesis may be regulated by angiogenic factors that are secreted by tumor cells. Recently, several angiogenic factors have been identified, and VEGF is thought to be one such factor (21). Four VEGF types (VEGF121, VEGF165, VEGF189 and VEGF206) have been described in humans, VEGF165 being the most abundant (5). The anti-VEGF antibody used in our study can be applied generally and reacts with VEGF165, VEGF189 and VEGF121 amino acid splice variants of VEGF. VEGF plays a crucial role in the control of angiogenesis both in physiological and pathological situations, including tumor development and progression. VEGF is a selective mitogen for endothelial cells and may directly stimulate the growth of new blood vessels. It can also increase vascular permeability (10), resulting in the extravasation of plasma proteins, including fibrinogen, into the extravascular space. Extravasated fibrinogen clots, and other proteins such as fibronectin, may be incorporated into the fibrin clot. This clot provides a provisional matrix which is eventually transformed into vascularized connective tissue (22) by fibroblast, endothelial cells, and other migrating cells (5). Maeda *et al.* (3) analyzed 129 patients with gastric carcinoma and found that the expression of VEGF was closely associated with the increment of the vessel count. In our study, we also found that the microvessel density, in weakly VEGF-positive and strongly VEGF-positive tumors was significantly higher than in VEGF-negative tumors. VEGF expression was significantly associated with new vessel formation (Fig. 2). Moreover, newly formed capillaries have fragmented basement membranes and are leaky, making them more penetrable by tumor cells than are mature vessels. Therefore, intratumoral vessels are induced by VEGF and the metastatic process may be enhanced by the "leaky" nature of newly formed blood vessels, which in facilitating vascular invasion can cause poor prognosis.

VEGF expression and prognosis. Recently, in several studies the relationship between VEGF expression and prognosis has been identified, such as in breast cancer and gastric cancer, *etc.* Toi *et al.* (23) analyzed 103 patients with breast cancer and found that the relapse-free survival rate of patients with VEGF-positive tumors was significantly poorer than that of patients with VEGF-negative tumors. A multivariate analysis showed that VEGF expression is an independent prognostic indicator. Maeda *et al.* (3) analyzed 129 patients with gastric carcinoma who underwent curative resection. The recurrence rate in patients with VEGF-positive tumors was 41.2%, while in those with VEGF-negative tumors it was 19.7%, and a significantly shorter survival time was observed in patients with VEGF-positive tumors than in those with VEGF-negative tumors. Using the Cox model, serosal invasion, lymph node metastasis, and VEGF expression emerged as independent prognostic factors. Fontanini *et al.* (24) analyzed 42 patients with non-small-cell lung carcinoma, and 3 kinds of amplified cDNAs (VEGF-121, VEGF-165 and VEGF-189) were detected. VEGF-mRNA-165 expression significantly affected overall survival, in contrast to the other 2 kinds. These studies as well as our own indicate that VEGF expression may be an effective prognostic indicator. In our study, we found that patients with positive VEGF expression had been predicted to have a poorer overall survival rate. The 5-year survival rate was 43.9% for patients with weakly positive VEGF expression, and 8.9% with strongly positive VEGF expression. For patients with adenocarcinomas and squamous cell carcinomas, similar patterns of survival rates were also clearly observed. The 5-year survival rates were 28.6% and 89.7% for adenocarcinoma patients having positive and negative VEGF expression, respectively (positive versus negative: $P < 0.01$). Similarly, the 5-year survival rates were 33.3 % and 75.0% for squamous cell carcinoma showing VEGF-positive and VEGF-negative expression, respectively (positive versus negative: $P < 0.05$).

Moreover, in this study we noted that immunohistochemically, VEGF expression is associated with the neoangiogenesis status of primary lung carcinoma. Therefore, it can also serve as a highly independent prognostic factor for neoangiogenesis status of primary lung carcinoma. In fact, this data clearly supports the role of VEGF expression in predicting the overall survival rate for patients with primary lung carcinoma, and indicates the close relationship between the prognostic value of VEGF expression and the neoangiogenesis status of primary lung carcinoma. Therefore, immunohistochemical VEGF expression status in primary lung carcinoma may be influenced by postrecurrent therapy in postoperative patients. Our finding that VEGF expression status, as well as the state of lymph node involve-

ment, has a significantly high prognostic value, suggests that VEGF expression is a highly independent prognostic factor for us to consider, especially in the postoperative treatment of patients without lymph node involvement. Moreover, considering that immunohistochemistry may be routinely and easily performed in comparison with the other diagnostic methods, it seems to us that identifying immunohistochemical VEGF expression, which is highly associated with the neoangiogenesis status of primary lung carcinoma, is a practical tool for clinical diagnosis and treatment.

In conclusion, our study shows that VEGF expression is associated with intratumoral microvessel density. VEGF expression may provide a significantly accurate and independent prognostic evidence, and can help us in predicting the outcomes of patients with primary lung carcinoma.

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