Alternative splicing variant of vascular endothelial growth factor-A is a critical prognostic factor in non-small cell lung cancer

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Abstract. Lung cancer is one of the most common malignant diseases in the world, and its prognosis is generally poor. Cancer and metastasis involve numerous biological steps, including angiogenesis in both the primary and metastatic sites. Although various molecules that are involved in both tumor neovascularization (angiogenesis) and invasion have been identified, little is known about how these molecules interact in cancerous microenvironments. We previously reported that the gene expressions of some factors associated with vascularization correlated with the prognosis of non-small cell lung cancer (NSCLC). In this study, we performed multivariate analysis of the mRNA levels of 10 selected genes [VEGF-A, VEGF121, VEGF165, VEGF189, S100A4, E-cadherin, Thrombospondin (TSP)-1, TSP-2, matrix metalloproteinase (MMP)-2, and MMP-9] in 130 NSCLC specimens using the real-time quantitative reverse transcription-polymerase chain reaction. Spearman's rank correlation test was used to determine the co-expression patterns. The analysis demonstrated highly significant co-expressions (P<0.0001) among the VEGF isoforms (VEGF-A, VEGF121, VEGF165, and VEGF189). We also analyzed the correlations among the prognosis, gene expressions, clinical factors (age and gender), and pathological features (histological types, TNM status, stages, lymphatic involvement, and venous involvement) using the Cox proportional hazards model. Multivariate analyses showed that only VEGF189 expression was an independent prognostic indicator (P=0.0252). The alternative splicing variant VEGF189, the cell binding isoform, plays a leading role in the progression of NSCLC.

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Introduction

Lung cancer is one of the most common malignant diseases in the world, and its prognosis is generally poor. Surgical resection is currently the only potentially curative treatment; however, more than 50% of all such patients who undergo complete resection show recurrence. Cancer growth and metastasis involve numerous biological steps, including tumor cell invasion, motility, extracellular proteolysis, and angiogenesis in both primary and metastatic sites. Although various molecules that are involved in both tumor invasion and neovascularization have been identified, little is known about how these molecules interact in cancerous microenvironments. We previously reported that several gene expressions correlated with the prognosis of non-small cell lung cancer (NSCLC) or adenocarcinoma (1-7).

Vascular endothelial growth factor (VEGF-A) is one of the well-known molecules associated with neovascularization in the cancerous stroma. Molecular cloning has revealed at least six different isoforms (splicing variants of VEGF-A: VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206) which are generated from a single mRNA by alternative splicing, and which have significantly different biochemical features and biological effects (8-13). We have reported that the expression profiles of VEGF-A isoforms are closely associated with the progression of various malignant neoplasms, including non-small cell lung cancer (NSCLC) (5,14-16). In addition, the progression of pulmonary squamous cell carcinoma or pulmonary adenocarcnoma was proven to be closely associated with the expression of VEGF189 mRNA (2,5).

Table I. Sequences of PCR primers and probes specific for VEGF-A isoforms and TSP-2.

	Sequence (5'-3')
VEGF-A-F	CTT GCC TTG CTG CTC TAC CT
VEGF-A-R	GAT TCT GCC CTC CTC CTT CTG
VEGF-A-probe	CAT GCC AAG TGG TCC C
VEGF189-F	GCG CAA GAA ATC CCG GTA TAA GT
VEGF189-R	GCT TTC TCC GCT CTG AGC AA
VEGF189-probe	CCT GGA GCG TTC CCT G
VEGF165-F	ACA ACA AAT GTG AAT GCA GAC CAA A
VEGF165-R	GCT TTC TCC GCT CTG AGC AA
VEGF165-probe	CCA CAG GGA TTT TCT
VEGF121-F	ACA ACA AAT GTG AAT GCA GAC CAA A
VEGF121-R	CTG AGG GAG GCT CCT TCC T
VEGF121-probe	CAA GAA AAA TGT GAC AAG CCG
TSP-2-F	GCT GGT TCA GAC AGC CAA CTC
TSP-2-R	TAA CCA AAG ACG AAG CCA GCA T
TSP-2-probe	TGC CAC TGA AGT CCA CAG ACC CAA ACT

VEGF, vascular endothelial growth factor; TSP, thrombospondin; F, forward; R, reverse.

Thrombospondin (TSP) is a family of glycoproteins with at least five subtypes encoded by independent genes, in which TSP-1 and TSP-2 contain three properdin-like type-1 repeats, unlike other TSPs (17-19). Only TSP-1 and TSP-2 function as endogenous negative regulators of angiogenesis in tumorigenesis, because their anti-angiogenic activity has been mapped to the type-1 repeats (4,20,21). Angiogenesis is partially regulated by the balance between the angiogenic inducer VEGF and the angiogenic inhibitor TSP (22,23). However, TSP mediation of the angioinhibitory effect is facilitated not only by VEGF but also by some matrix proteases (24-27).

Extracellular proteolysis for degradation of the extracellular matrix is very important for angiogenesis and cancerous cell invasion. This allows endothelial and tumor cells to migrate through the tissue stroma and subsequently to enter and spread via the blood and/or lymphatic systems. The matrix metalloproteinases (MMPs), including MMP-2 and MMP-9 (gelatinase-A and -B, respectively), are amongst the most extensively studied proteases in this respect.

E-cadherin is the main cell-to-cell adhesion molecule that participates in homophilic, calcium-dependent interactions to form the epithelial adherence junction (28,29). Thus, E-cadherin is assumed to act as the main invasion-suppressor molecule (30,31). The S100 family of calcium-binding proteins is associated with a variety of physiological functions, such as cellular proliferation, adhesion, and motility (32). In pulmonary adenocarinoma cases, those expressing combined lower E-cadherin and higher S-100A4, a small member of the S100 family which is associated with an increased metastatic capacity of cancer cells, levels showed a significantly poorer prognosis, although each of E-cadherin and S100A4 gene expressions did not independently contribute to the prognosis (3,33,34).

The aim of our study was to identify which genes are co-expressed in tumors in which angiogenesis and/or lymphangiogenesis are prominent. We performed multivariate analysis of the mRNA levels of the above-described 10 genes (VEGF-A, VEGF121, VEGF165, VEGF189, S100A4, E-cadherin, TSP-1, TSP-2, MMP-2, and MMP-9) in 130 NSCLC specimens using the real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). We also evaluated the clinical prognosis based on long-term observation periods of 5 to 15 years.

Materials and methods

Patients. One hundred and thirty NSCLC specimens were obtained from surgical specimens with the patients' informed consent from October 1985 to November 1995. All cases underwent complete resection (lobectomy or pneumonectomy and dissection of mediastinal lymph nodes) of NSCLC. Tissues were immediately frozen and stored at -80˚C until analysis. Surgical specimens were also processed for routine histopathological analysis. The pathological features of the samples were classified according to the WHO histological criteria (35).

The patients consisted of 82 men and 48 women, with a mean age of 62.60±9.16 years. The pathological classifications were 98 adenocarcinomas, 27 squamous cell carcinomas, 2 adenosquamous carcinomas, and 3 large cell carcinomas. The tumor status was T1 in 48 patients, T2 in 63, T3 in 15, and T4 in 4. The lymph node status was N0 in 76 patients, N1 in 15, N2 in 38, and N3 in 1. The pathological stages were as follows: stage I, 69 patients; stage II, 18; stage III, 43; stage IV, 0. Lymphatic involvement was noted in 66 cases (50.8%), with venous involvement in 68 (52.3%).

RNA extraction and preparation of cDNA. Total cellular RNA was prepared from the frozen specimens by standard acid guanidine isothiocyanate-phenol-chloroform extraction procedures. After the heat-denaturation of total RNA specimens (1 μg), reverse transcription was performed [10 mM DTT (Invitrogen Corp., Carlsbad, CA, USA), 0.2 mM dNTPs (Toyobo Co., Osaka, Japan), 100 pmol of Primer, Random

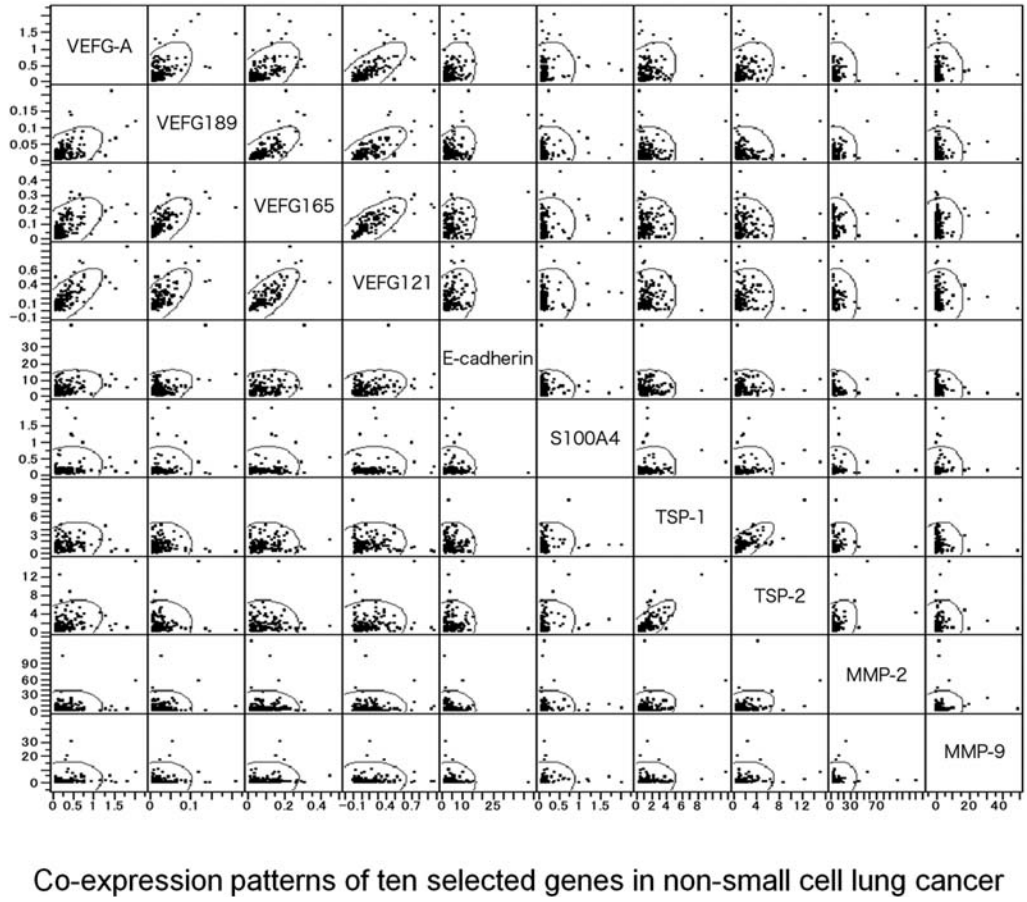

Figure 1. Co-expression patterns of ten selected genes in non-small cell lung cancer. VEGF, vascular endothelial growth factor; TSP, thrombospondin; MMP, matrix metalloproteinase.

PD (N6) (Roche Diagonostics Co., Indianapolis, IN, USA), and 200 Units of Superscript™ II RNase H-Reverse Transcriptase (Invitrogen) at 42˚C, 60 min] (2,7).

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR for VEGF-A, VEGF121, VEGF165, VEGF189, S100A4, E-cadherin, TSP-1, TSP-2, MMP-2, MMP-9, and ß-actin mRNA was performed according to the manufacturer's recommendations. The primers used for TSP-2 and VEGF-A are shown in Table I. The primers for TSP-1 (Hs00170236), S100A4 (Hs00243201), E-cadherin (Hs00170423), MMP-2 (Hs00234422), and MMP-9 (Hs00234579) were purchased from TaqMan® Gene Expression Assays (PE Applied Biosystems, Foster City, CA, USA). We used TaqMan® Universal PCR Master Mix (PE Applied Biosystems) for the real-time PCR. For the internal controls, ß-actin-probe-primer mixture for ß-actin mRNA was used (human ACTB, 4310881E, PE Applied Biosystems). Real-time PCR assays were duplicated using an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems) with the following protocol: after initial denaturation: 2 min at 50˚C and 10 min at 95˚C, amplification: 50 cycles of 15 sec at 95˚C and 60 sec at 60˚C. We determined the threshold cycle (Ct), which was defined as the PCR cycle number at which point the fluorescence intensity exceeded the threshold from the Ct of the sample and the RNA standard curve. Then, the relationship between the Ct and initial standard copy number was expressed as a logarithmic formula. The obtained copy of each gene was then standardized with the ß-actin mRNA quantity as the endogenous control using the following equation: Result = log (RNA copy number of each gene in sample/ß-actin RNA copy number in sample) x ($6.1x10^9$).

Statistical analysis. Data are shown as the mean ± standard deviation (SD). Spearman's rank correlation test was used to compare the co-expression patterns. On multivariate analyses, the Cox proportional hazards model was used. These analyses were performed using the JMP version 6 software (SAS Institute Inc., Cary, NC, USA). P-values <0.05 were considered significant.

Results

Gene expressions of NSCLC. All NSCLC cases showed significant gene expressions by real-time PCR. The gene expression level of VEGF-A was 0.3314±0.3389, and those of isoforms were as follows: VEGF189, 0.02811±0.02904; VEGF165, 0.09372±0.07272; VEGF121, 0.1850±0.1769. The gene expression level of E-cadherin was 5.356±4.554, S100A4 was 0.1935±0.2770, TSP-1 was 1.578±1.425, TSP-2 was 1.884±2.077, MMP-2 was 8.549±15.250, and MMP-9 was 2.327±5.451.

Gene co-expression patterns in NSCLC. Fig. 1 shows the gene co-expression pattern of 10 selected cancer-related molecules. Spearman's rank correlation test disclosed many

Table II. Spearman's correlation matrix to assess gene co-expressions (with rho and P-values) in non-small cell lung cancer.

	VEGF-A	VEGF189	VEGF165	VEGF121	E-cadherin	S100A4	TSP-1	TSP-2	MMP-2	MMP-9
VEGF-A										
rho	1	0.423	0.609	0.706	0.270	0.188	0.177	0.317	-0.031	0.220
P-value		**<0.0001**	**<0.0001**	**<0.0001**	**0.001**	**0.028**	**0.037**	**<0.001**	0.714	**0.009**
VEGF189										
rho	0.423	1	0.798	0.676	0.141	-0.118	-0.158	-0.173	-0.025	-0.020
P-value	**<0.0001**		**<0.0001**	**<0.0001**	0.101	0.169	0.064	**0.042**	0.766	0.812
VEGF165										
rho	0.609	0.798	1	0.859	0.164	-0.109	0.017	0.010	-0.008	0.115
P-value	**<0.0001**	**<0.0001**		**<0.0001**	0.056	0.205	0.843	0.910	0.921	0.176
VEGF121										
rho	0.706	0.676	0.859	1	0.282	-0.047	0.033	0.103	0.040	0.150
P-value	**<0.0001**	**<0.0001**	**<0.000**1		**<0.001**	0.586	0.703	0.226	0.638	0.078
E-cadherin										
rho	0.270	0.141	0.164	0.282	1	-0.010	-0.068	0.062	-0.162	-0.247
P-value	**0.001**	0.101	0.056	**<0.001**		0.910	0.435	0.481	0.060	**0.004**
S100A4										
rho	0.188	-0.118	-0.109	-0.047	-0.010	1	0.160	0.121	0.142	0.368
P-value	**0.028**	0.169	0.205	0.586	0.910		0.065	0.162	0.100	**<0.0001**
TSP-1										
rho	0.177	-0.158	0.017	0.033	-0.068	0.160	1	0.668	0.211	0.147
P-value	**0.037**	0.064	0.843	0.703	0.435	0.065		**<0.0001**	**0.013**	0.085
TSP-2										
rho	0.317	-0.173	0.010	0.103	0.062	0.121	0.668	1	0.350	0.312
P-value	**<0.001**	**0.042**	0.910	0.226	0.481	0.162	**<0.0001**		**<0.0001**	**<0.001**
MMP-2										
rho	-0.031	-0.025	-0.008	0.040	-0.162	0.142	0.211	0.350	1	0.180
P-value	0.714	0.766	0.921	0.638	0.060	0.100	**0.013**	**<0.0001**		**0.033**
MMP-9										
rho	0.220	-0.020	0.115	0.150	-0.247	0.368	0.147	0.312	0.180	1
P-value	**0.009**	0.812	0.176	0.078	**0.004**	**<0.0001**	0.085	**<0.001**	**0.033**	

VEGF, vascular endothelial growth factor; TSP, thrombospondin; MMP, matrix metalloproteinase. Values in bold type indicate significant differences.

significant relations (Table II). The analysis demonstrated highly significant co-expressions (P<0.0001) among the VEGF isoforms (VEGF-A, VEGF121, VEGF165, and VEGF189). Moreover, the following significant co-expression patterns were also observed: VEGF-A and E-cadherin (P=0.001), VEGF-A and S100A4 (P=0.028), VEGF-A and TSP-1 (P=0.037), VEGF-A and TSP-2 (P<0.001), VEGF-A and MMP-9 (P=0.009), VEGF121 and E-cadherin (P<0.001), VEGF189 and TSP-2 (P=0.042), TSP-1 and TSP-2 (P<0.0001), TSP-1 and MMP-2 (P=0.013), TSP-2 and MMP-2 (P<0.0001), MMP-9 and E-cadherin (P=0.004), MMP-9 and S100A4 (P<0.0001), MMP-9 and TSP-2 (P<0.001), and MMP-9 and MMP-2 (P=0.033).

Gene expression and clinicopathological features. We also analyzed correlations among the prognosis, gene expressions, clinical factors (age and gender), and pathological features (histological types, TNM status, stages, lymphatic involvement, and venous involvement) using the Cox proportional hazards model. Multivariate analyses showed that only VEGF189 expression was an independent prognostic indicator (P=0.025, Table III).

Discussion

In this study, we identified many co-expression patterns centering on VEGF isoforms. There were strong interactions between VEGF-A and its isofoms (VEGF121, VEGF165, and VEGF189). Table I shows two clusters of gene co-expression: one is the aforementioned group centering on VEGF isoforms, and the other is a group consisting of TSP-1, -2, MMP-2, and -9. However, few significant relationships were observed among VEGF-A splice variants and MMPs. Only between MMP-9 and VEGF-A there was a significant

Table III. Multivariate analyses using the Cox proportional hazards model.

Variable	Strata	P-value
Age	62.60±9.16	0.562
Gender	Male, Female	0.355
Histological types	Ad, Sq, AdSq, La	0.109
T status	T1, T2, T3, T4	0.433
N status	N0, N1, N2, N3	0.535
Stages	I, II, III	0.198
Lymphatic involvement	ly(+), ly(-)	0.068
Venous involvement	v(+), v(-)	0.781
VEGF-A	0.3314±0.3389	0.376
VEGF189	0.02811±0.02904	**0.025***
VEGF165	0.09372±0.07272	0.700
VEGF121	0.1850±0.1769	0.149
E-cadherin	5.356±4.554	0.135
S100A4	0.1935±0.2770	0.311
TSP-1	1.578±1.425	0.472
TSP-2	1.884±2.077	0.081
MMP-2	8.549±15.250	0.237
MMP-9	2.327±5.451	0.416

Ad, adenocarcinoma; Sq, squamous cell carcinoma; AdSq, adenosquamous carcinoma; La, large cell carcinoma; VEGF, vascular endothelial growth factor; TSP, thrombospondin; MMP, matrix metalloproteinase. *P<0.05.

co-expression pattern. Regarding co-expressions among VEGF-A isoforms, correlations between VEGF121 and VEGF165 in NSCLC and among VEGF-C, the lymphangiogenic factor, as well as VEGF-A isoforms in cervical cancer were reported (36,37). There are many reports that TSPs interact with MMPs but not VEGFs in various malignant neoplasms (1,23,38-40). In the Cox proportional hazards model, the gene expression of VEGF189 was proven to be the only independent prognostic indicator of NSCLC.

Some studies reported that VEGF-A gene expression was related to the clinical prognosis in NSCLC (5,36,41). Previously, we demonstrated that the expression of VEGF189 was associated with a poor prognosis in squamous cell carcinoma using the RT-PCR assay (5). In another study using real-time PCR, we clearly demonstrated that VEGF189 overexpression was quantitatively related to vascularization, the postoperative relapse time, and prognosis based on long-term observation periods in pulmonary adenocarcinoma (2). Yuan *et al* reported that VEGF189 expression showed a stronger correlation with tumor angiogenesis and survival than other VEGF-A isoforms in NSCLC by RT-PCR (36). We reported that patients with pulmonary adenocarcinoma overexpressing VEGF189 showed a significantly higher incidence of intrapulmonary, bone, and hepatic metastasis (2). We also reported that VEGF189 was seen in the cytoplasm of tumor cells of pulmonary adenocarcinoma overexpressing the VEGF189 gene by immunohistochemistry (2). The results suggest that the cellular binding isoform VEGF189 works in the microenvironment of the primary cancer lesion. Many reports employing immunohistochemical analysis showed that VEGF-A expression was related to angiogenesis in NSCLC, and that not only VEGF-A expression but also other factors affected the clinical prognosis (42-46).

The larger isoforms VEGF189 and VEGF206 are cell-associated and show heparin-binding activity, while the smaller isoforms VEGF121 and VEGF165 are secretory proteins (8). VEGF189 is more strongly associated with the cell membrane than VEGF121 or VEGF165. The lung cancer cell lines that specifically suppressed VEGF189 expression showed decreased growth properties *in vivo* (6). The *in vivo* tumor growth of VEGF189 transfectants was faster than that of VEGF121 transfectants (47). The protein levels of VEGF-A were not increased in the supernatant of VEGF189 transfectant culture. The *in vivo* growth enhancement of VEGF189 transfectants was due to the unique features of VEGF189 molecules in the cancer microenvironment.

TSP-2 was a prognostic indicator following VEGF189 in gene expressions, however, non-significant (P=0.081). Angiogenesis is partially regulated by the balance between the angiogenic inducer VEGF and angiogenic inhibitor TSP-2 (22). The mediation by TSP-2 of the angioinhibitory effect might be induced not only by VEGF but also by some matrix proteases (24,25). MMPs play active roles during matrix remodeling and other tissue regeneration processes, including the modulation of angiogenesis.

In multivariate analysis of clinicopathological features and the survival rate, VEGF expression in esophageal squamous cell carcinoma and VEGF189 expression in NSCLC were reported to be independent prognostic factors (36,48). However, these studies did not analyze gene expressions except for VEGF isoforms. Multivariate analysis of various kinds of gene expression measured quantitatively by real-time RT-PCR and prognosis have yet to be published. In this study, we identified many associations between VEGF isoforms and other cancer molecules. It is concluded that the alternative splicing variant VEGF189, the cell binding isoform, plays a leading role in the progression of NSCLC.

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