Abstract. Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis. Several studies have reported that genomic VEGF polymorphisms may influence VEGF synthesis. To evaluate the role of VEGF single nucleotide polymorphisms (SNPs), we examined the expression of several angiogenesis-related proteins [VEGF, hypoxia-inducible factor-1α (HIF-1α) and delta-like ligand 4 (Dll4)] and the spread of microvessels in resected non-small cell lung cancer (NSCLC). Blood and tumor tissue from 83 patients with NSCLC were examined for VEGF -460T/C (rs833061) and VEGF +405G/C (rs2010963) SNPs using the SNaPshot method. Immunohistochemical staining was performed to measure protein expression and microvessel density (MVD). VEGF -460T/C and +405G/C SNPs showed no association with VEGF or HIF-1α expression and MVD. Patients with VEGF -460TT and the TC genotype had significantly higher MVD compared to those with the CC genotypes. Furthermore, patients with the VEGF -460TT genotype had significantly higher Dll4 expression compared to those with the TC or CC genotypes, while the VEGF +405G/C SNP displayed no association with Dll4 expression and MVD. These findings indicate that VEGF SNPs may have a functional influence on tumor angiogenesis in NSCLC. We hypothesize that VEGF SNPs may influence angiogenesis through Dll4.

Introduction

Angiogenesis plays an important role in tumor progression and metastasis, and vascular endothelial growth factor (VEGF) is a key component. Several studies have demonstrated that VEGF mRNA and protein overexpression are associated with tumor progression and prognosis in non-small cell lung cancer (NSCLC) (1-3). Several VEGF single nucleotide polymorphisms (SNPs) have been recently described (4). VEGF is located on chromosome 6p21.3 and is organized into eight exons and seven introns (5,6). The VEGF -460T/C SNP (rs833061) is located in the promoter region and may influence promoter activity (7). Furthermore, the VEGF +405G/C SNP (rs2010963) is located within the 5'-untranslated region and may affect transcription factor binding affinity (7,8). These two SNPs have been investigated in different types of cancers, and the association of various VEGF SNPs with risk or prognosis of several cancers has been examined (9-12). Recently, VEGF +405 and -460 SNPs have been found to be significantly associated with risk and survival in NSCLC (13-15). However, the influence of VEGF SNPs on tumor angiogenesis remains unclear. In this study, we examined whether VEGF -460 and +405 SNPs may influence VEGF expression and microvessel density (MVD) in NSCLC.

Tumor angiogenesis is influenced by a number of proteins. Hypoxia occurs early in tumor development and results in stable binding of hypoxia-inducible factor-1α (HIF-1α) to DNA and the activation of other angiogenic genes, such as VEGF (16,17). Delta-like ligand 4 (Dll4) is a ligand for Notch proteins that is expressed by endothelial cells (18,19) and may be induced by VEGF and HIF-1α (20). It plays an important role in tumor vessel maturation and remodeling (21,22). Therefore, we studied whether these VEGF SNPs were associated with the expression of the angiogenesis-related proteins HIF1α and Dll4.

Patients and methods

Study population. Blood and tumor samples were obtained from 83 patients with NCSLC who underwent surgical resection at the Kawasaki Medical School Hospital between October, 2008 and December, 2010. The patients did not receive radio- or chemotherapy before surgery. This study was approved by the Ethics Committee of the Kawasaki Medical School, and informed consent was obtained from all patients for the use of their tissue specimens.
Analysis of VEGF-A -460T/C and +405G/C polymorphisms. Blood samples were collected from all subjects before surgery. Genomic DNA was isolated from peripheral whole blood using the QIAamp™ DNA Blood Mini kit (Qiagen, Hilden, Germany). Genomic regions containing the VEGF -460T/C and +405G/C SNPs were amplified by polymerase chain reaction using the following primers: -460T/C, 5’-CGAGAGTGGACGTGTGTG-3' (forward) and 5’-ATTGGAATCCTGAGTGACC-3' (reverse); +405G/C, 5’-GAGAGACGGGGTCAGAGA-3' (forward) and 5’-CCCAAAAGCAGGTCCTCA-3' (reverse). The VEGF SNPs were genotyped by a single-base primer extension assay using the SNaPshot™ Multiplex kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The following primers were used: -460T/C, 5’-ttttttttCTTCTCCCCGCTCAGTAC-3'; +405G/C, 5’-tttttttttttttGTGCGAGCAGCGAAGTAC-3'.

DNA sequencing. Polymorphism analysis was performed using the ABI PRISM® 310 Genetic Analyzer, and results were evaluated using GeneMapper® software, ver. 4.1 (all were from Applied Biosystems).

Immunohistochemical staining. VEGF, HIF-1α, Dll4 and CD31 (to measure MVD) expression was analyzed using resected, paraffin-embedded lung cancer tissue. After microtome sectioning (4-µm thick), tissue slides were processed on an automated immunostainer (NexES; Ventana Medical Systems, Tucson, AZ, USA) or manual methods. Streptavidin-biotin-peroxidase detection was performed with diaminobenzidine as the chromogen. The following primary antibodies were used according to the manufacturer's instructions: VEGF (rabbit polyclonal; sc-152; 1:300 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), HIF-1α (mouse monoclonal; ESEE122; 1:1,000 dilution; Novus, Littleton, CO, USA), Dll4 (rabbit polyclonal; ab17280; 1:50 dilution; Abcam, Cambridge, MA, USA), and CD31 (mouse monoclonal; 1:50 dilution; Dako, Carpinteria, CA, USA). The slides were examined by two investigators blinded to the corresponding clinicopathological data. The expression of each protein marker was examined and evaluated according to previously reported protocols (1,23-26).

VEGF staining and scoring. To evaluate VEGF expression, the percentage of positively stained cells and staining intensity were scored as follows: grade 0, negative; grade 1, weak; grade 2, moderate; grade 3, high; and grade 4, very high (23). Grade 0 indicated staining intensity equal to the negative control, grade 3 indicated intensity equal to the positive control, and grade 4 indicated intensity higher than the positive control. Stain intensity in the cell cytoplasm was similarly scored (23). To determine the percentage of cells with the various staining intensities, the number of immunoreactive cells at each intensity was divided by the total number of tumor cells in three fields at x200 magnification (Fig. 1A). The overall VEGF staining score was calculated as follows: VEGF score = 1 x percentage of grade 1 cells + 2 x percentage of grade 2 cells + 3 x percentage of grade 3 cells + 4 x percentage of grade 4 cells. The score was analyzed as a continuous and a dichotomous variable.

HIF-1α staining and scoring. Tumor cells were scored on the intensity and extent of staining as follows: score 1, tumor cells with absent or weak cytoplasmic reactivity and no nuclear reactivity; score 2, tumor cells with moderate/strong cytoplasmic reactivity with a percentage of tumor cells less than their mean percentage and no nuclear reactivity; score 3, tumor cells with moderate/strong cytoplasmic reactivity with...
a percentage of tumor cells more than their mean percentage; score 4, tumor cells with clear nuclear reactivity (with or without cytoplasmic reactivity regardless of the intensity) (Fig. 1B). Tumors with scores of 1 and 2 were considered to exhibit low HIF-1α expression, whereas those with scores of 2 and 3 were considered to exhibit high HIF-1α expression (24).

**Dll4 staining and scoring.** Dll4 expression was considered only in endothelial cells, although recent reports have demonstrated its wide cellular distribution beyond vessels (25,26). To evaluate Dll4 staining in tumor cells (Fig. 1C and D), the intensity of expression was scored on a semiquantitative scale in three x200 magnification fields. Negative cores were scored as 0, cores with weak expression were scored as 1 and those with moderate/strong expression were scored as 2. High Dll4 expression was defined as a score greater than 1.5 (26).

**Microvessel staining and counting.** MVD was assessed by counting the number of microvessels stained for CD31. Vessels with a clearly defined lumen or well-defined linear vessel shape and no single endothelial cells were selected for counting. Microvessels were counted in the three x200 magnification fields with the highest density (Fig. 1E), and the mean MVD was calculated (1).

**Statistical analysis.** Vascular scores were presented as the means ± standard deviation and the difference between the groups was analyzed using the unpaired Student’s t-test. The association of VEGF SNPs with clinicopathological parameters and immunostaining results was examined using Chi-squared and Fisher’s exact tests, respectively. The level of significance was set at P<0.05. All analyses were performed using SPSS software (version 17.0; SPSS, Chicago, IL, USA).

### Results

**Clinical characteristics.** Characteristics of the patients with NSCLC are summarized in Table I. Patients ranged in age from 49 to 89 years (median, 72 years), with 52 men and 31 women. Fifty-six (67.5%) patients were former/current smokers. There were 40 (48.2%) stage IA, 17 (20.5%) stage IB, 11 (13.3%) stage IIA, 9 (10.8%) stage IIB, 6 (7.2%) stage III. Fifty-two (62.7%) patients had adenocarcinoma, 19 (22.9%) had squamous cell carcinoma, and 12 (14.4%) had other histological malignancies.

**Immunohistochemistry of angiogenesis-related proteins.** Forty-two patients (50.6%) exhibited a marked increase in VEGF immunoreactivity of tumor cells. The mean VEGF staining score was 2.79±0.67, and the median score of 2.90 was used to distinguish between low and high VEGF staining. VEGF expression was correlated with HIF1α expression (P=0.003), but not with Dll4 expression (P=0.446) (Table II).

**VEGF SNPs and clinicopathological characteristics.** For the VEGF +405G/C SNP, 50.6% of patients had the GC genotype, 25.3% had CC and 24.1% had GG. For the VEGF -460T/C SNP, 50.6% had the TT genotype, 38.6% had TC and 10.8% had CC. No significant association was observed between VEGF SNPs and clinicopathological characteristics such as gender, pathological stage, lymphatic invasion, vascular invasion, histological type, and smoking status (Table III).

**VEGF SNPs and angiogenesis-related proteins.** Both SNPs displayed no association with VEGF or HIF-1α expression; however, Dll4 expression was significantly higher in patients with the VEGF -460TT genotype (P=0.031) (Table IV).

### Table I. Characteristics of the patients with NSCLC.

| Characteristic       | No. of patients | %     |
|----------------------|-----------------|-------|
| Age (years)          |                 |       |
| Median               | 72              | 48.2  |
| Range                | 49-89           | 20.5  |
| Gender               |                 |       |
| Male                 | 52              | 62.7  |
| Female               | 31              | 37.3  |
| Smoking              |                 |       |
| Never                | 27              | 67.5  |
| Former/Current       | 56              | 32.5  |
| Stage                |                 |       |
| IA                   | 40              | 62.7  |
| IB                   | 17              | 22.9  |
| IIA                  | 11              | 14.4  |
| IIB                  | 9               | 7.2   |
| III                  | 6               | 7.2   |
| Histology            |                 |       |
| Adenocarcinoma       | 52              | 62.7  |
| SCC                  | 19              | 22.9  |
| Other types          | 12              | 14.4  |

SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer.

### Table II. Relationships between angiogenesis related protein expression as determined by immunohistochemistry.

| Variable | VEGF  | HIF-1α |
|----------|-------|--------|
|          | High  | Low    | High  | Low    |
| HIF-1α   |       |        |       |        |
| High     | 29    | 15     |       |        |
| Low      | 13    | 26     |       |        |
| P-value  |       | P=0.003|       |        |
| DLL4 (T) |       |        |       |        |
| High     | 27    | 23     | 34    | 16     |
| Low      | 15    | 18     | 10    | 23     |
| P-value  |       | P=0.446|       | P<0.001|

VEGF, vascular endothelial growth factor; DLL4, delta-like ligand 4; HIF-1α, hypoxia-inducible factor-1α; T, tumor cells.
Angiogenesis-related proteins and MVD. MVD ranged from 2.0 to 80.0, with a mean value of 29.9±15.9 and a median score of 29. High MVD was significantly associated with high VEGF (P<0.001) and Dll4 (P=0.026) expression, but not with HIF-1α expression (P=0.235) (Table V).

VEGF SNPs and MVD. Patients with the VEGF -460TT and TC genotypes had significantly greater MVD compared to those with the CC genotype (TT/TC vs. CC; P=0.027) (Table VIA). Moreover, in a group of tumors with high VEGF expression, patients with the VEGF -460TT genotype...
had significantly higher MVD compared to those with the CC genotypes (P=0.033) (Table VIB).

**Discussion**

Angiogenesis is important for tumor progression and utilizes several factors, with VEGF being the key factor. Recently, several VEGF SNPs have been identified, and their effect has attracted a great deal of attention. An *in vivo* study by Stevens *et al* (7) discovered that VEGF -460/+405 SNPs significantly altered VEGF promoter activity in response to phorbol esters. Recent literature has reported the association of VEGF SNPs with risk or prognosis of various types of cancers (9-12). A large case-control study in Caucasians demonstrated that male patients with NSCLC and the VEGF +405CC+CG genotype had a higher risk of lung adenocarcinoma, while those with the -460T/+405G/936C haplotype had a reduced risk. (14). The C allele of the VEGF +405G/C SNP significantly improved survival in early-stage NSCLC (13), whereas the -460CC genotype decreased overall survival in advanced-stage NSCLC (15). Other studies have suggested a lower survival rate for the VEGF +405CC genotype in gastric and ovarian cancers (27,28). The reason for these conflicting results is currently unclear, and the influence of VEGF SNPs remains uncertain and controversial.

However to date, few studies have focused on the association between VEGF SNPs and VEGF expression. Therefore, we conducted a study with NSCLC patients to examine the functional activity of VEGF SNPs and their possible role in VEGF expression and angiogenesis.

Table VI. VEGF SNPs and MVD.

| Genotype | MVD | P-value |
|----------|-----|---------|
| VEGF +405 |     |         |
| CC | 27.3±17.0 | CC/GC vs. GG 0.426 |
| GC | 31.9±16.4 | GG/GC vs. CC 0.961 |
| GG | 28.8±14.0 | |
| VEGF -460 |     |         |
| TT | 31.9±18.1 | TC/CC vs. TT 0.550 |
| TC | 31.4±16.0 | TT/TC vs. CC 0.027 |
| CC | 23.9±7.8 | |

**Table V. Angiogenesis-related protein expression and MVD.**

| Protein marker expression | MVD | P-value |
|---------------------------|-----|---------|
| VEGF                      |     |         |
| High | 37.2±18.0 | <0.001 |
| Low | 24.3±11.7 | |
| DLL4 (T)                  |     |         |
| High | 33.9±17.4 | 0.026 |
| Low | 26.1±13.9 | |
| HIF-1α                    |     |         |
| High | 32.9±16.5 | 0.235 |
| Low | 28.5±16.3 | |

VEGF, vascular endothelial growth factor; DLL4, delta-like ligand 4; HIF-1α, hypoxia-inducible factor-1α; MVD, microvessel density; SD, standard deviation; T, tumor cells.

From these results, we concluded that DLL4, induced by the VEGF -460TT genotype, influenced the spread of microvessel density; SD, standard deviation; VEGF, vascular endothelial growth factor.

**Table V. Angiogenesis-related protein expression and MVD.**

| Protein marker expression | MVD | P-value |
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| Low | 26.1±13.9 | |
| HIF-1α                    |     |         |
| High | 32.9±16.5 | 0.235 |
| Low | 28.5±16.3 | |

MVD, microvessel density; SD, standard deviation; VEGF, vascular endothelial growth factor.

We discovered that patients with the VEGF -460TT and TC genotype had significantly higher MVD compared to those with CC genotypes. In general, as in our study, high VEGF expression is associated with high vascular density. However, there was no association between the VEGF -460TT/C SNP and the VEGF expression in tumors. Furthermore, even in high VEGF expression cases, the -460TT genotype was associated with significantly higher MVD compared to CC genotype. This result suggested that high MVD in -460TT genotype was not caused by VEGF expression. The VEGF -460TT genotype was associated with significantly higher DLL4 protein expression, which demonstrated a significant association with high MVD. From these results, we concluded that DLL4, induced by the VEGF -460TT genotype, influenced the spread of microvessel density; SD, standard deviation; VEGF, vascular endothelial growth factor.
sels. Dll4 is generally upregulated by VEGF, which in turn acts as a negative feedback regulator of VEGF. Our results suggest that VEGF SNPs may influence VEGF downstream signaling to Dll4, although potential mechanisms have not been examined in this study. Dll4 is associated with tumor vessel maturation and remodeling (21,22). Thus, high Dll4 expression should theoretically lead to fewer but larger vessels, and Dll4 overexpression or inhibition may consequently impair tumor angiogenesis. However, further study of this visceral function is warranted.

In conclusion, the VEGF -460T/C SNP may have a functional influence on tumor angiogenesis in NSCLC. Although VEGF SNPs were not associated with VEGF expression in tumor cells, they are considered to regulate the response to Dll4 signaling through functional changes in VEGF.

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