Vascular Endothelial Growth Factor Gene Polymorphisms and Risk of Primary Lung Cancer

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Abstract

Angiogenesis is an essential process in the development, growth, and metastasis of malignant tumors including lung cancer. DNA sequence variations in the *vascular endothelial growth factor* (*VEGF*) gene may lead to altered VEGF production and/or activity, thereby causing interindividual differences in the susceptibility to lung cancer via their actions on the pathways of tumor angiogenesis. To test this hypothesis, we investigated the potential association between three VEGF polymorphisms (−460T > C, +405C > G, and 936C > T)/haplotypes and the risk of lung cancer in a Korean population. *VEGF* genotypes were determined in 432 lung cancer patients and 432 healthy controls that were frequency matched for age and sex. *VEGF* haplotypes were predicted using Bayesian algorithm in the phase program. Compared with the combined +405 CC and CG genotype, the +405 GG genotype found associated with a significantly decreased risk of small cell carcinoma [SCC; adjusted odds ratio (OR), 0.36; 95% confidence interval (95% CI), 0.17-0.78]. The 936 CT genotype and the combined 936 CT and TT genotype were also associated with a significantly decreased risk of SCC compared with the 936 CC genotype (adjusted OR, 0.47; 95% CI, 0.26-0.85 and adjusted OR, 0.44; 95% CI, 0.24-0.80, respectively). Haplotype CGT was associated with a significantly decreased risk of SCC (adjusted OR, 0.39; 95% CI, 0.18-0.87), whereas haplotype TCC conferred a significantly increased risk of SCC (adjusted OR, 1.63; 95% CI, 1.14-2.33). None of the *VEGF* polymorphisms studied significantly influenced the susceptibility to lung cancer except SCC. However, haplotypes TCT and TGT were significantly associated with the risk of overall lung cancer, respectively (adjusted OR, 0.38; 95% CI, 0.25-0.60 and adjusted OR, 3.94; 95% CI, 2.00-7.76, respectively). These effects of haplotypes TCT and TGT on lung cancer risk were observed in three major histologic types of lung cancer. These results suggest that the *VEGF* gene may be contribute to an inherited predisposition to lung cancer. (Cancer Epidemiol Biomarkers Prev 2005;14(3):571–5)

Introduction

Although cigarette smoking is the major cause of lung cancer, only a small fraction of smokers develop this disease, which suggests that genetic factors contribute to lung cancer risk. This genetic susceptibility may result from a combination of low-penetrance gene polymorphisms (1, 2).

Angiogenesis is an essential process in the development, growth and metastasis of malignant tumors (3-5). Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis, and several studies have shown that its increased expression is associated with the grade of angiogenesis and with a poor prognosis in various human cancers, including lung cancer (6-8).

The *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons that exhibit alternate splicing to form a family of proteins (9, 10). Several polymorphisms have been described in the *VEFG* gene. Some of these variants [−460T > C, −116G > A and +405C > G (transcription start site counted as +1) in the promoter or 5′-untranslated region and 936C > T in the 3′-untranslated region] have been associated with variations in VEGF protein production (11-13) and have reported to be involved in several disorders in which angiogenesis is critical in the development of disease (14-17).

In spite of the importance of the *VEGF* gene in lung cancer carcinogenesis, no investigation of the role of *VEGF* polymorphisms in relation to lung cancer has been undertaken. In the present study, we conducted a case-control study to evaluate the association between three *VEGF* polymorphisms [−460T > C, +405C > G (−634 from translation start site), and 936C > T] and lung cancer. The −116G > A (−1154 from translation start site) polymorphism was rare in the preliminary study consisting of 50 cases and 50 controls (the frequencies of polymorphic allele, 2% and 3%, respectively) and was not analyzed further.

Materials and Methods

Study Population. This case-control study included 432 lung cancer patients and 432 healthy controls. The method used for subject enrollment was same as used in our previous study (18). Eligible cases included all patients newly diagnosed with primary lung cancer between January 2001 and February 2002 at Kyungpook National University Hospital, Daegu, Korea. There were no age, sex, histologic, or stage restrictions, but patients with a prior history of cancer were excluded. The cases included 210 (48.6%) squamous cell carcinomas, 141 (32.6%) adenocarcinomas, 73 (16.9%) small cell carcinomas (SCC), and 8 (1.9%) large cell carcinomas. The demographics and clinical characteristics of the cases were consistent with those of a nationwide lung cancer survey conducted by the Korean Academy of Tuberculosis and Respiratory Disease in 1998 (19). Controls were randomly selected from a pool of healthy volunteers who visited the general health check-up center at Kyungpook National University Hospital during the

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same period. Controls were frequency matched (1:1) to cases based on sex and age (±5 years). All cases and controls were ethnic Koreans and resided in Daegu City or in the surrounding regions. A detailed questionnaire was completed for each patient and control by a trained interviewer. The questionnaire included information on the average number of cigarettes smoked daily and the number of years the subjects had been smoking. For smoking status, a person who had smoked at least once a day for >1 year in his or her lifetime was regarded as a smoker. A former smoker was defined as one who had stopped smoking at least 1 year before diagnosis in the case of patients and 1 year before study commencement in the case of controls. Cumulative cigarette dose (pack-years) was calculated using the following formula: pack-years = (packs per day) × (years smoked).

VEGF Genotyping. Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and phenol/chloroform extraction. The *VEGF* −460T > C, +405C > G, and 936C > T genotypes were determined using a PCR-RFLP assay. PCR primers were designed based on the Genbank reference sequence (accession no. NT_007592). The PCR primers used for −460T > C, +405C > G, and 936C > T polymorphisms were 5′-CCTCTTTAGCCAGAGCCGGGG-3′ (forward) and 5′-TGGCCTTCTCCCCGCTCCGAC-3′ (reverse); 5′-CGACGGCTTGGGGAGATTGC-3′ (forward) and 5′-GGGCGGTGTCTGTCTGTCTG-3′ (reverse); and 5′-AGGGTTCGGGAACCAGATC-3′ (forward) and 5′-CTCGGTGATTTAGCAGCAAG-3′ (reverse), respectively. PCR reactions were done in a 20-μL reaction volume containing 100 ng genomic DNA, 25 pmol/L each primer, 0.2 mmol/L deoxynucleotide triphosphates, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 1 unit of Taq polymerase (Takara Shuzo Co., Otsu, Shiga, Japan). PCR cycle conditions consisted of an initial denaturation step at 94°C for 5 minutes followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 62°C; 30 seconds at 72°C, and a final elongation at 72°C for 10 minutes. The PCR products were digested overnight with the appropriate restriction enzymes (New England BioLabs, Beverly, MA). The restriction enzymes for the −460T > C, +405C > G, and 936C > T genotypes were *Bsa*HI, *Bsm*FI, and *Nla*III, respectively. The digested PCR products were resolved on 6% acrylamide gel and stained with ethidium bromide for visualization under UV light. For quality control, genotyping analysis was done blind with respect to case/control status and was repeated twice for all subjects. To confirm the genotyping results, selected PCR-amplified DNA samples (n = 2, for each genotype) were examined by DNA sequencing.

Statistical Analysis. Cases and controls were compared using the Student's t test for continuous variables and the χ^2 test for categorical variables. Hardy-Weinberg equilibrium of alleles at individual loci was tested using a goodness-of-fit χ^2 test with one degree of freedom to compare the observed genotype frequencies with the expected genotype frequencies among the subjects. Haplotypes and their frequencies were estimated using Bayesian algorithm in the phase program (20), which is available at http://www.stat.washington.edu/stephens/phase.html.. Unconditional logistic regression analysis was used to calculate odds ratio (OR) and 95% confidence interval (95% CI), with adjustment for possible confounders (sex, as a nominal variable and age and pack-years, as continuous variables). A referent (to the homozygotes of wild-type allele) and 3 alternative models (codominant, dominant, and recessive for the variant allele) were used in the analyses. When multiple comparisons are made, corrected Ps (P_c values) were also calculated for multiple testing using Bonferroni's inequality method. All analyses were done using Statistical Analysis Software for Windows, version 8.12 (SAS Institute, Cary, NC).

Results

The demographics of the cases and controls enrolled in this study are shown in Table 1. There were no significant differences between the cases and controls in mean age or sex distribution, suggesting that the matching based on these two variables was adequate. Cases had a higher prevalence of current smokers than controls ($P < 0.001$), and the number of pack-years in smokers was significantly higher in cases than in controls (39.9 ± 17.9 versus 34.4 ± 17.6 pack-years; $P < 0.001$). These differences were controlled for later by multivariate analyses.

The genotype and polymorphic allele frequencies of the three *VEGF* polymorphisms (−460T > C, +405C > G, and 936C > T) among controls and cases are shown in Table 2. A total of 861 [99.6%: cases, 429 of 432 (99.3%) and controls, 432 of 432 (100%)] of the enrolled subjects were successfully genotyped for all *VEGF* polymorphisms. The genotype distributions of the three polymorphisms among the controls were in Hardy-Weinberg equilibrium. When overall lung cancer cases were compared with the controls, no significant difference was found in the distributions of genotypes for any polymorphisms studied. When the lung cancer cases were categorized by tumor histology, however, the distributions of the +405C > G and 936C > T genotypes in SCC cases differed from those of the controls, respectively (P = 0.02 and P_c = 0.06, both), and the frequency of the variant allele of the 936C > T polymorphism in SCC differed significantly from that of the controls (0.110 versus 0.206, P = 0.006 and P_c = 0.018).

Table 3 shows the lung cancer risk related to the *VEGF* polymorphisms. No significant association was found between the *VEGF* −460T > C polymorphism and the risk of lung cancer, and the +405C > G genotypes were not associated with lung cancer risk according to the reference model. When the combined +405 CC + CG genotype was used as the reference genotype (a recessive model for the variant G allele), however, the +405 GG genotype was associated with a significantly reduced risk of SCC (adjusted OR, 0.36; 95% CI, 0.17-0.78; P = 0.01 and P_c = 0.03). The 936 C > T genotypes were not associated with an altered risk of overall lung cancer, squamous cell carcinomas, and adenocarcinomas. However, the 936 CT genotype was associated with a significantly reduced risk of SCC as compared with the 936 CC genotype (adjusted OR, 0.47; 95% CI, 0.26-0.85; P = 0.01 and P_c = 0.03), and the combined 936 CT + TT genotype was also associated with a significantly decreased risk of SCC compared with the 936 CC genotype (a dominant model for the variant T allele; adjusted OR, 0.44; 95% CI, 0.24-0.80; P = 0.007 and P_c = 0.021).

VEGF −460T > C and +405C > G polymorphisms were in strong linkage disequilibrium, and seven haplotypes were estimated of the eight (2^3) possible haplotypes. Table 4 shows

Table 1. Characteristics of the study population

Variable	Cases (n = 432)	Controls (n = 432)
Age (y)	61.6 ± 9.0	60.9 ± 9.3
Sex		
Male	352 (81.5)*	352 (81.5)
Female	80 (18.5)	80 (18.5)
Smoking status†		
Current	317 (73.4)	229 (53.0)
Former	39 (9.0)	98 (22.7)
Never	76 (17.6)	105 (24.3)
Pack-years‡	39.9 ± 17.9	34.4 ± 17.6

*Numbers in parenthesis, percentage.
† P = 0.001.
‡ In current and former smokers, $P < 0.001$.

Table 2. Genotype frequencies of *VEGF* polymorphisms in lung cancer cases and controls

Polymorphism	Variables	Genotype*			Polymorphic allele frequency	*n*
		1/1	1/2	2/2		
−460T > C	Controls	237 (54.9)	168 (38.9)	27 (6.2)	0.257	432
	All cases	228 (53.0)	184 (42.8)	18 (4.2)	0.256	430
	Squamous cell carcinoma	107 (51.0)	95 (45.2)	8 (3.8)	0.264	210
	Adenocarcinoma	73 (51.8)	62 (44.0)	6 (4.2)	0.262	141
	Large cell carcinoma	1 (16.7)	5 (83.3)	0 (0.0)	0.417	6
	Small cell carcinoma	47 (64.4)	22 (30.1)	4 (5.5)	0.205	73
+405C > G	Controls	92 (21.3)	232 (53.7)	108 (25.0)	0.519	432
	All cases	76 (17.6)	247 (57.3)	108 (25.1)	0.537	431
	Squamous cell carcinoma	34 (16.3)	117 (56.0)	58 (27.8)	0.557	209
	Adenocarcinoma	28 (20.0)	75 (53.2)	38 (27.0)	0.535	141
	Large cell carcinoma	0 (0.0)	4 (50.0)	4 (50.0)	0.750	8
	Small cell carcinoma	14 (19.2)	51 (69.9)	8 (11.0)†	0.459	73
936C > T	Controls	266 (61.6)	154 (35.6)	12 (2.8)	0.206	432
	All cases	281 (65.1)	137 (31.7)	14 (3.2)	0.191	432
	Squamous cell carcinoma	134 (63.8)	69 (32.8)	7 (3.3)	0.198	210
	Adenoca.	86 (61.0)	49 (34.7)	6 (4.3)	0.216	141
	Large cell carcinoma	4 (50.0)	3 (37.5)	1 (12.5)	0.312	8
	Small cell carcinoma	57 (78.1)	16 (21.9)	0 (0.0)†	0.110‡	73

NOTE: Difference from controls.
*Wild-type allele is denoted by 1 and the polymorphic allele by 2.
† $P = 0.02$ and $P_c = 0.06$ (Bonferroni corrected P).
‡ $P = 0.006$ and $P_c = 0.018$.

an inferred haplotype distribution for cases and controls, and the lung cancer risk related to the haplotypes. Haplotype TCT was associated with a significantly decreased risk of overall lung cancer as compared with other haplotypes (adjusted OR, 0.38; 95% CI, 0.25-0.60; $P = 0.0001$ and $P_c = 0.0007$), whereas haplotype TGT had a significantly increased risk of overall lung cancer (adjusted OR, 3.94; 95% CI, 2.00-7.76; $P = 0.0001$ and $P_c = 0.0007$). These effects of haplotypes TCT and TGT on lung cancer risk were observed in three major histologic types (squamous cell carcinoma, adenocarcinoma, and SCC) of lung cancer. The five other haplotypes were not associated with a risk of overall lung cancer, squamous cell carcinoma, and adenicarcinoma. However, haplotype TCC was associated with a significantly increased risk of SCC (adjusted OR, 1.63; 95% CI, 1.14-2.33; $P = 0.007$ and $P_c = 0.049$) compared with other haplotypes. Haplotype CGT was associated with a significantly decreased risk of SCC (adjusted OR, 0.39; 95% CI, 0.18-0.87; $P = 0.022$ and $P_c = 0.154$), although this was statistically not significant after the Bonferroni correction for multiple comparison had been taken into consideration.

Discussion

DNA sequence variations in the *VEGF* gene may alter VEGF production and/or activity, thereby causing interindividual differences in susceptibility to lung cancer due to their effects on the pathways of tumor angiogenesis. To test this hypothesis, we evaluated the potential association of three *VEGF* polymorphisms (−460T > C, +405C > G, and 936C > T) with lung cancer risk. In addition, we inferred *VEGF* −460/+405/936 haplotypes and compared their frequency distributions in lung cancer cases and controls. This is the first case-control study of *VEGF* polymorphisms and haplotypes in relation to lung cancer.

In agreement with previous reports (11, 13), the −460T > C and +405C > G polymorphisms in the 5′ region of the *VEGF* promoter were in linkage disequilibrium. However, the frequencies of *−460C* and *+405G* alleles among healthy Koreans were 0.257 and 0.519, respectively, which differed from those (0.474 and 0.291, respectively) of 115 mixed race healthy individuals in previous study (11) done in the United Kingdom. The frequencies of haplotype −460T/+405C, −460T/+405G, −460C/+405C, and −460C/+405G among the

Table 3. Adjusted ORs (95% CIs) for lung cancer associated VEGF genotypes

Polymorphism	Genotype	All cases	Squamous cell carcinoma	Adenocarcinoma	Small cell carcinoma
−460T > C	TT	1.0	1.0	1.0	1.0
	TC	1.15 (0.87-1.52)	1.27 (0.32-1.69)	1.18 (0.79-1.77)	0.66 (0.38-1.15)
	CC	0.75 (0.40-1.41)	0.73 (0.90-1.81)	0.74 (0.29-1.89)	0.80 (0.27-2.44)
+405C > G	CC	1.0	1.0	1.0	1.0
	CG	1.26 (0.88-1.80)	1.35 (0.85-2.15)	1.10 (0.66-1.84)	1.41 (0.74-2.69)
	GG	1.21 (0.80-1.83)	1.39 (0.82-2.34)	1.24 (0.70-2.21)	0.47 (0.19-1.18)
	CC + CG*	1.0	1.0	1.0	1.0
	GG	1.02 (0.75-1.40)	1.11 (0.75-1.63)	1.16 (0.74-1.80)	0.36 (0.17-0.78)†
937C > T	CC	1.0	1.0	1.0	1.0
	CT	0.84 (0.63-1.12)	0.87 (0.60-1.25)	0.96 (0.63-1.45)	0.47 (0.26-0.85)†
	TT	1.11 (0.50-2.46)	1.35 (0.50-3.63)	1.47 (0.52-4.19)	—
	CC‡	1.0	1.0	1.0	1.0
	CT + TT	0.86 (0.65-1.14)	0.90 (0.63-1.28)	0.99 (0.67-1.48)	0.44 (0.24-0.80)§

NOTE: Adjusted for age, sex, smoking status, and pack-years of smoking.
*Recessive model for the variant allele.
† $P = 0.01$ and $P_c = 0.03$ (Bonferroni corrected P).
‡ Dominant model for the variant allele.
§ $P = 0.007$ and $P_c = 0.021$.

Table 4. Distribution of *VEGF* haplotypes predicted by Bayesian algorithm in controls and cases

Haplotype*	Controls (n = 864), n (%)	All cases (n = 864)		Histological type of lung cancer †					
				Squamous cell carcinoma (n = 420)		Adenocarcinoma (n = 282)		Small cell carcinoma (n = 146)	
		n (%)	OR‡ (95% CI)	n (%)	OR (95% CI)	n (%)	OR (95% CI)	n (%)	OR (95% CI)
T C C	327 (37.8)	361 (41.8)	1.17 (0.97-1.43)	167 (39.8)	1.10 (0.86-1.41)	118 (41.8)	1.15 (0.87-1.52)	73 (50.0)	1.63 (1.14-2.33)§
T C T	73 (8.4)	29 (3.3)	0.38 (0.25-0.60)‖	15 (3.6)	0.42 (0.24-0.75)¶	10 (3.5)	0.39 (0.20-0.78)§	3 (2.1)	0.25 (0.08-0.79)**
T G C	231 (26.7)	211 (24.4)	0.87 (0.70-1.09)	107 (25.5)	0.89 (0.67-1.17)	64 (22.7)	0.83 (0.60-1.15)	34 (23.3)	0.81 (0.53-1.23)
T G T	11 (1.3)	43 (5.0)	3.94 (2.00-7.76)‖	20 (4.8)	3.66 (1.69-7.91)††	16 (5.7)	5.00 (2.24-11.15)‡	6 (4.1)	3.09 (1.10-8.70)‡‡
C C C	16 (1.9)	10 (1.2)	0.59 (0.26-1.34)	4 (0.9)	0.48 (0.15-1.50)	3 (1.1)	0.58 (0.17-2.06)	3 (2.1)	1.19 (0.34-4.17)
C G C	112 (13.0)	117 (13.5)	1.10 (0.83-1.46)	59 (14.0)	1.17 (0.82-1.66)	36 (12.8)	1.02 (0.68-1.55)	20 (13.7)	1.13 (0.67-1.90)
C G T	94 (10.9)	93 (10.8)	0.98 (0.72-1.34)	48 (11.4)	1.05 (0.72-1.54)	35 (12.4)	1.10 (0.72-1.69)	7 (4.8)	0.39 (0.18-0.87)§§

*The order of the polymorphisms is as follows: −460T > C, +405C > G, 936C > T.
† Eight large cell carcinoma cases were excluded from analysis.
‡ OR and 95% CI for each haplotype compared with all the other haplotypes combined are shown. Adjusted for age, sex, smoking status and pack-years of smoking.
§ P = 0.007 and P_c = 0.049 (Bonferroni corrected P).
‖ P = 0.0001 and P_c = 0.0007.
¶ P = 0.004 and P_c = 0.028.
** P = 0.019 and P_c = 0.133.
†† P = 0.001 and Pc = 0.007.
‡‡ P = 0.033 and P_c = 0.231.
§§ P = 0.022 and P_c = 0.154.

controls in the present study were 0.462, 0.280, 0.019, and 0.239, respectively, which also differed from those (0.287, 0.187, 0.004, and 0.522, respectively) of the previous study (11). In the present study, the frequency of 936C > T polymorphism among controls was 0.206, which was lower than that (0.294) found in healthy Austrians (17), and was higher than those of Japanese (0.148-0.158; ref. 21, 22) and Caucasians (0.12-0.13; refs. 14, 23).

A few studies have reported that these three *VEGF* polymorphisms are associated with VEGF production, but the results are inconsistent. Awata et al. (21) reported that individuals with the +405 CC genotype had a higher fasting serum VEGF level than those with other genotypes, and that they carried an increased risk of diabetic retinopathy. On the other hand, Watson et al. (11) reported that the *+405G* allele is associated with higher lipopolysaccharide-stimulated VEGF production by peripheral blood mononuclear cells than the +405C allele. Stevens et al. (13) also reported that haplotype −460C/+405G has a higher promoter activity than haplotype −460T/+405C. For the 936C > T polymorphism, Renner et al. (12) reported that the 936T allele is associated with lower VEGF plasma levels. Krippl et al. (17) also reported that the 936T allele is associated with low VEGF plasma levels and a decreased risk of breast cancer. In contrast, in a Japanese study, no relation was found between this polymorphism and VEGF serum levels (21). In the present study, the +405C > G and 936C>T polymorphisms were associated with a significantly reduced risk of SCC. Consistent with these results, by further haplotype analysis, haplotype CGT containing 78% of +405G/936T in the study population showed a similar protective effect on the risk of SCC, whereas haplotype TCC containing 96% of +405C/936C in the population showed an increased SCC risk. Our findings that the +405GG genotype and the combined 936 CT + TT genotype are associated with a significantly reduced risk for SCC are in agreement with some reports (12, 17, 21) but are in disagreement with others (11, 13). Different results in different populations may be due to the different genetic backgrounds.

In the current study, none of the *VEGF* polymorphisms studied (-460T > C, +405C > G, and 936C > T) significantly influenced susceptibility to lung cancer except SCC. However, haplotypes TCT and TGT were significantly associated with the risk of lung cancer, overall and for each histologic type. This finding may be because each polymorphism alone is insufficient to influence the susceptibility to lung cancer, but that the set of the three polymorphisms (haplotype) effect on lung cancer risk due to a combined effect on gene function. Another possible explanation is that the effect of the *VEGF* haplotypes on lung cancer risk may be due to linkage disequilibrium with other functional variants in the *VEGF* gene (13, 15, 24). However, it is possible that such a finding is attributable to chance because the number of TCT and TGT haplotypes was small. Thus, additional studies with more subjects will be needed to confirm this finding.

In conclusion, we found that the *VEGF* haplotypes of three polymorphisms (−460T > C, +405C > G, and 936C > T) are associated with the risk of lung cancer, especially SCC. These results suggest that the *VEGF* gene may contribute to an inherited predisposition to lung cancer, although additional studies with larger sample sizes are required to confirm our findings. Future studies of other *VEGF* sequence variants and on their biological functions are also needed to understand the role of the *VEGF* polymorphisms and haplotypes in determining the risk of lung cancer. Moreover, since genetic polymorphisms often vary between ethnic groups, further studies are needed to clarify the association between the *VEGF* polymorphism and lung cancer in diverse ethnic populations.

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