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Association of Vascular Endothelial Growth Factor Gene Polymorphisms with Susceptibility and Clinicopathologic Characteristics of Colorectal Cancer

Since vascular endothelial growth factor (VEGF) is known to be a potent pro-angiogenic factor, we evaluated the potential association of two *VEGF* gene polymorphisms (-634G>C and 936C>T) with the susceptibility and the clinicopathologic characteristics of colorectal cancer (CRC). The *VEGF* genotypes were determined using fresh colorectal tissue from 465 patients who had undergone a surgical resection and peripheral blood lymphocytes from 413 healthy controls by PCR/DHPLC assay. For the -634G>C polymorphism, the -634 GC or CC genotype was associated with a decreased risk of CRC (odds ratio [OR], 0.62; p=0.001) as a dominant model of C allele, whereas the 936 TT genotype correlated with advanced stage/metastasis, a high serum level of CA19-9, and an higher grade in patients with CRC. In the haplotype analyses, haplotype -634C/936C and -634G/936T were associated with a decreased susceptibility of CRC (OR, 0.53 and 0.56; p<0.001, respectively). These observations imply that the *VEGF* gene polymorphisms may be associated with the susceptibility or clinicopathologic features of CRC. However, further studies of other *VEGF* sequence variants and their biological functions are needed to understand the role of the *VEGF* gene polymorphisms in the development and progression of CRC.

Key Words : *Angiogenesis; Colorectal Neoplasms; Vascular Endothelial Growth Factors; Polymorphism, Genetic*

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the U.S.A. and Europe (1). During the last few years, many attempts have been made to define the biological profile of CRC in order to improve early diagnosis and the prognostic stratification, and eventually find a cure (2, 3). Although many biological factors have been implicated in the development of CRC, a clinical relevance has not yet been reached for most of them.

Angiogenesis, the formation of a new blood vessel from endothelial precursors, is a prerequisite for the development, growth, and progression of solid malignancies (4). The vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a critical role in angiogenesis. VEGF specifically binds to VEGF receptor tyrosine kinase on endothelial cells to initiate intracellular signal transduction pathways that mediate angiogenesis and vascular permeability. In addition to stimulating angiogenesis, VEGF may also have autocrine functions, acting as a survival factor for tumor cells by protecting them from various forms of stress and apoptosis (5-8).

The *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons that exhibit alternative splicing to form a family of proteins (9, 10). Several polymorphisms have been associated with variations in VEGF protein production and reported to be involved in the development of several tumors (11, 12) and autoimmune diseases (13, 14). Given these results, it is also possible that a functional genetic variation in the *VEGF* gene may contribute to the development and progression of CRC. However, there have been no studies in the literature that have investigated the single nucleotide polymorphisms (SNPs) of the *VEGF* gene and their relationship to the susceptibility of CRC. Accordingly, the present study examined 2 *VEGF* gene polymorphisms (-634G>C and 936C>T) previously reported to be related with VEGF production, and evaluated the potential association of three *VEGF* gene polymorphisms with the susceptibility and clinicopathologic characteristics of CRC in the Korean population.

MATERIALS AND METHODS

Study populations

All the tissues investigated in this study were obtained from 465 native Korean patients with CRC who had undergone a surgical resection between January 2003 and August 2006 at Kyungpook National University Hospital (Daegu, Korea). Based on a health-screening questionnaire, 413 unrelated, healthy Korean individuals without known medical problems were also enrolled as the control group. The study was approved by the Institutional Research Board of Kyungpook National University Hospital, and all individuals gave written informed consent for study participation.

The diagnosis and staging of the colorectal adenocarcinoma were assessed according to the WHO classification (15) and TNM classification set out by the American Joint Committee on Cancer (AJCC-UICC) (16).

Genotyping of *VEGF* gene polymorphisms

The extraction of genomic DNA from the peripheral blood lymphocytes of the control group was performed using proteinase-K digestion and phenol/chloroform extraction. For the patient group, the genomic DNA was extracted from fresh colorectal tumor tissue at the time of surgery using a Wizard genomic DNA purification kit (Promega, Madison, WI, U.S.A.). The *VEGF* -634G>C and 936C>T genotypes were determined using a polymerase chain reaction/denaturing high-performance liquid chromatography (PCR/DHPLC) assay as described in the previous studies (17, 18). PCR primers were designed based on a Genbank reference sequence (accession no. NT_007592). The PCR primers used for the -634G>C and 936C>T polymorphisms were 5′-CGACGGCTTGGGGAGATTGC-3′ (forward) and 5′-GGGCGGTGTCTGTCTGTCTG-3′ (reverse); and 5′-AGGGTTTCGGGAACCAGATC-3′ (forward) and 5′-CTCGGTGATTTAGCAGCAAG-3′(reverse), respectively. The PCR reactions were performed in a 50-μL reaction volume containing 50 ng genomic DNA, 50 pM/L each primer, 10 mM/L dNTP, 5 ×Q- solution, 10×PCR buffer (Tris-HCl , KCl, 15 mM/L $MgCl_2$, $[NH_2]_2SO_4$; pH8.7) and 2.5 units of HotStarTaq polymerase (QIAGEN, Hilden, Germany). The PCR cycle conditions consisted of an initial denaturation step at 94℃ for 15 min, followed by 40 cycles of 45 sec at 94℃, 45 sec at 57℃, 45 sec at 72℃, and a final elongation at 72℃ for 10 min. The PCR products were denatured at 94℃ for 10 min, and hybridized for 45 min, and screened for a heterozygous polymorphism based on a DHPLC analysis using a gradient solution of 0.1 M TEAA (pH 7.0), 0.1 M TEAA, 25% acetonitrile, a washing solution with 8% acetonitrile (syringe washing solution), and 75% acetonitrile (DNASep® Cartridge UltraClean and Storage Solution, Transgenomic, Omaha, NE, U.S.A.), Column: alkylated nonporous poly (styrene-divinylbenzene) DNASep® Cartridge (Transgenomic, Omaha, NE, U.S.A.), flow rate: 0.9 mL/min, oven temperature: 64℃, and UV: 260 nm.

The remaining samples showing a single peak on the DHPLC were mixed with the PCR products of a known homozygous genotype (homozygous A), and hybridized to run the DHPLC again, as described above. Another type of homozygous genotype (homozygous B) was confirmed when a double peak appeared on the DHPLC. Several samples with three different patterns on the DHPLC were directly sequenced to reconfirm the accuracy of the DHPLC (Fig. 1).

Statistical analyses

The Hardy-Weinberg equilibrium for each polymorphism was analyzed using the χ^2 test, which was also used to examine the statistical significance of the differences in the allele frequency and genotype distribution between the groups. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using an unconditional logistic regression analysis. For the patient group, the χ^2-test or ANOVA was used to evaluate the relation between each polymorphism and the tumor characteristics. The software PHASE (version 2.1), which uses a Bayesian statistical method, was used to reconstruct the haplotypes for the *VEGF* gene (19). The *p* value was generated using a 2-sided test. The analyses were conducted using SPSS version 12.0 (SPSS, Chicago, IL, U.S.A.) and SAS Genetic software (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Patient characteristics

The clinical and pathologic characteristics of patients are summarized in Table 1. The median age of the patients was

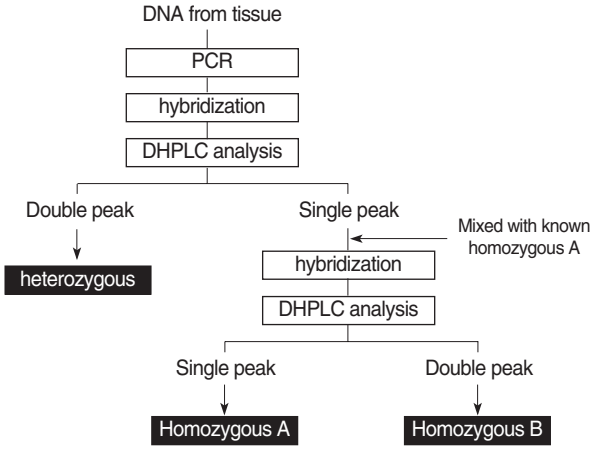

Fig. 1. Algorithm for genotyping of *VEGF* gene polymorphisms using a polymerase chain reaction/denaturing high-performance liquid chromatography (PCR/DHPLC) assay.

64 (range, 21-89) yr, and 248 patients (51.8%) were male. There was no difference in age between the patient and control groups, while there was a male predominance in the control group. The pathologic stages after the surgical resection were as follows: stage I (n=80, 17.2%), stage II (n=157, 33.8%), stage III (n=157, 33.8%), and stage IV (n=71, 15.3%).

Table 1. Characteristics of patients with colorectal cancer and controls

Characteristics	Patients (n=465)	Controls (n=413)
Age, median (range)	64 (21-89)	61 (34-86)
Sex (%)		
Male	241 (51.8)	333 (80.6)
Female	224 (48.2)	80 (19.4)
Location of primary tumor (%)		
Colon cancer	264 (56.7)	
Rectal cancer	201 (43.3)	
Stage (%)		
I	80 (17.2)	
II	157 (33.8)	
III	157 (33.8)	
IV	71 (15.3)	
Tumor marker (%)*		
CEA, elevated	117 (25.3)	
CA19-9, elevated	79 (17.1)	
Differentiation (%)		
Well	67 (14.4)	
Moderate	357 (76.9)	
Poor	21 (4.5)	
Mucinous	18 (3.9)	
Signet ring cell	2 (0.4)	

*, tested in 462 patients. CEA, carcinoembryonic antigen.

Genotype frequencies

The *VEGF* genotypes were successfully identified in all 878 enrolled subjects, and the frequencies of the genotypes and alleles are listed in Table 2. The genotype distributions of the two polymorphisms among the patients and controls followed the Hardy-Weinberg equilibrium, and the frequencies of the -634C and 936T alleles among the healthy Koreans were 0.473 and 0.209, respectively. The incidence of each genotype with the -634G>C polymorphism differed between the two groups (p=0.001), where the combined GC and CC genotype was significantly associated with a decreased risk of CRC (OR, 0.62; 95% CI, 0.47-0.83; p=0.001) compared with the GG genotype as the dominant model for the C allele. For the 936C>T polymorphism, there was no difference in the genotype or allele distribution between the patient and control groups (p=0.64 and 0.71, respectively).

Association of genotypes with clinicopathologic features

For the patient group, the TT genotype of 936C>T polymorphism was significantly associated with advanced stage (OR, 3.83; 95% CI, 1.22-12.03; p=0.02), distant metastasis (OR, 8.38; 95% CI, 3.00-23.51; p<0.001), high serum level of CA19-9 (OR, 2.96; 95% CI, 1.04-8.40; p=0.04), and

Table 2. Genotype distributions (%) and allele frequencies in patients with colorectal cancer and controls

Polymorphism	Total (n=878)	Patient (n=465)	Control (n=413)	*p* value	Odds ratio (95% CI)
-634G>C					
Genotype				<0.001*	
GG	272 (31.0)	166 (35.7)	106 (25.7)		Reference
GC	416 (47.4)	193 (41.5)	223 (54.0)	<0.001	0.55 (0.41-0.75)
CC	190 (21.6)	106 (22.8)	84 (20.3)	0.26	0.81 (0.55-1.18)
Dominant model of C allele					
GG	272 (31.0)	166 (35.7)	106 (25.7)		Reference
GC+CC	606 (69.0)	299 (64.3)	307 (74.3)	0.001	0.62 (0.47-0.83)
Allele					
G	960 (54.7)	525 (56.5)	435 (52.7)		Reference
C	796 (45.3)	405 (43.5)	391 (47.3)	0.11	0.86 (0.71-1.04)
936C>T					
Genotype				0.64*	
CC	545 (62.1)	293 (63.0)	252 (61.0)		Reference
CT	305 (34.7)	156 (33.5)	149 (36.1)	0.43	0.89 (0.67-1.18)
TT	28 (3.2)	16 (3.4)	12 (2.9)	0.84	1.19 (0.50-2.35)
Dominant model of T allele					
CC	545 (62.1)	293 (63.0)	252 (61.0)		Reference
CT+TT	333 (37.9)	172 (37.0)	161 (39.0)	0.54	0.92 (0.70-1.21)
Allele					
C	1,395 (79.4)	742 (79.8)	653 (79.1)		Reference
T	361 (20.6)	188 (20.2)	173 (20.9)	0.71	0.96 (0.76-1.21)

*, p value was estimated using χ^2-test for distribution of three genotypes.
CI, confidence interval.

Table 3. Association of *VEGF* gene polymorphisms with clinical characteristics in patients with colorectal cancer (n=465)

	-634G>C			936C>T		
	GG	GC	CC	CC	CT	TT
Sex						
Male	86	99	56	152	77	12
Female	79	94	51	140	79	5
p value		0.92	0.88		0.59	0.15
OR (95% CI)	1	1.02 (0.67-1.55)	0.96 (0.59-1.57)	1	1.11 (0.76-1.64)	0.45 (0.16-1.32)
Age (yr)						
≤60	65	74	42	121	56	4
>60	100	119	65	171	100	13
p value		0.80	0.90		0.25	0.15
OR (95% CI)	1	1.06 (0.69-1.62)	1.03 (0.63-1.70)	1	1.26 (0.85-1.89)	2.30 (0.73-7.22)
CEA*						
Normal	125	146	74	224	110	11
Elevated	39	47	31	65	46	6
p value		0.90	0.30		0.11	0.23
OR (95% CI)	1	1.03 (0.63-1.68)	1.34 (0.77-2.33)	1	1.44 (0.93-2.24)	1.88 (0.67-5.28)
CA19-9*						
Normal	135	160	88	244	128	11
Elevated	29	33	17	45	28	6
p value		0.88	0.75		0.52	0.04
OR (95% CI)	1	0.96 (0.56-1.66)	0.90 (0.47-1.73)	1	1.19 (0.71-1.99)	2.96 (1.04-8.40)
Stage						
I+II	74	111	52	159	74	4
III+IV	92	81	55	134	81	13
p value		0.009	0.40		0.20	0.02
OR (95% CI)	1	0.57 (0.36-0.87)	0.81 (0.50-1.32)	1	1.29 (0.87-1.91)	3.83 (1.22-12.03)
LN involvement						
Negative	76	117	59	165	81	6
Positive	87	75	49	127	73	11
p value		0.007	0.20		0.43	0.10
OR (95% CI)	1	0.56 (0.37-0.86)	0.73 (0.45-1.18)	1	1.17 (0.79-1.73)	2.38 (0.86-6.61)
Distant metastasis						
Negative	137	173	85	265	121	9
Positive	28	19	23	28	34	8
p value		0.05	0.38		<0.001	<0.001
OR (95% CI)	1	0.55 (0.29-1.00)	1.31 (0.71-2.43)	1	2.65 (1.54-4.57)	8.38 (3.00-23.45)

*, tested in 462 patients.
VEGF, vascular endothelial growth factor; OR, odds ratio; CI, confidence interval; LN, lymph node; CEA, carcinoembryonic antigen.

higher grade (p=0.002). However, no association of -634G>C polymorphism with clinicopathologic features was observed (Table 3, 4).

Haplotype analyses

The *VEGF* -634G>C and 936C>T polymorphisms exhibited an intermediate linkage disequilibrium (D´=0.50), and Table 5 shows the frequencies and ORs of the 4 reconstructed haplotypes for the two polymorphisms of the *VEGF* gene, as predicted by a Bayesian algorithm. Haplotype GC was the most frequent type in the patient group (41.5%), whereas haplotype CC was most frequent in the control group (43.1%). When compared with wild type (GC), the haplotype CC (OR, 0.53; 95% CI, 0.43-0.66; p<0.001) and GT (OR, 0.56; 95% CI, 0.42-0.74; p<0.001) were significantly associated with a decreased susceptibility of CRC. However, no significant associations were observed between any of the haplotypes and the clinical features of CRC (data not shown).

DISCUSSION

The present study investigated the potential impact of 2 *VEGF* gene polymorphisms on the susceptibility and clinicopathologic features of colorectal adenocarcinoma in quite a large population of Korean patients. As a result, the fre-

Table 4. Association of *VEGF* gene polymorphisms with pathologic characteristics in patients with colorectal cancer

	-634G>C			936C>T		
	GG	GC	CC	CC	CT	TT
Histolotic grade (n=444)						
Grade 1	21	27	18	42	24	0
Grade 2	125	150	82	228	118	11
Grade 3	9	7	5	11	6	4
p value		0.70	0.71		0.96	0.002
Lymphatic invasion (n=464)						
Negative	70	75	44	119	62	8
Positive	93	118	64	173	93	9
p value		0.46	0.72		0.88	0.61
OR (95% CI)	1	1.18 (0.78-1.81)	1.10 (0.67-1.79)	1	1.03 (0.29-1.54)	0.77 (0.29-2.06)
Vascular invasion (n=463)						
Negative	154	184	103	279	145	17
Positive	9	8	5	12	10	0
p value		0.55	0.75		0.28	1.00
OR (95% CI)	1	0.74 (0.28-1.98)	0.83 (0.27-2.55)	1	1.60 (0.68-3.80)	-
Neural invasion (n=464)						
Negative	97	106	54	172	78	7
Positive	66	87	54	120	77	10
p value		0.38	0.12		0.08	0.16
OR (95% CI)	1	1.21 (0.79-1.84)	1.47 (0.90-2.40)	1	1.42 (0.96-2.09)	2.05 (0.76-5.53)

VEGF, vascular endothelial growth factor; OR, odds ratio; CI, confidence interval; LN, lymph node.

Table 5. Distribution of *VEGF* haplotypes predicted by Bayesian algorithm in patients with colorectal cancer and controls

-634G>C/ 936C>T haplotype	Patient (%)	Control (%)	*p* value	Odds ratio (95% CI)
GC	454 (41.5)	297 (36.0)		1 (Reference)
GT	117 (14.4)	138 (16.7)	<0.001	0.56 (0.42-0.74)
CC	288 (38.3)	356 (43.1)	<0.001	0.53 (0.43-0.66)
CT	71 (5.9)	35 (4.2)	0.20	1.20 (0.86-2.04)

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

quencies of -634C and 936T alleles were 0.473 and 0.209, respectively, which differ from those of Japanese (0.353 and 0.150, respectively) as well as those of Caucasians (0.335 and 0.150, respectively) (20, 21). Moreover, it was observed that the combined GC and CC genotypes of the -634G>C polymorphism, and haplotype -634C/936C and -634G/936T were associated with a decreased susceptibility, while 936 TT genotype with an adverse clinicopathologic feature of CRC. Given the homogenous ethnic background of the Korean patients, any potential confounding effect due to ethnicity is likely to be small in the present study. However, these results might be caused by somatic alteration in the process of colorectal carcinogenesis because tumor tissue was used for DNA source of the patient group in the present study, although data on the difference between germline and somatic SNPs of *VEGF* gene have not been reported. Furthermore, the effect of the *VEGF* gene polymorphisms on the susceptibility of CRC may be due to linkage disequilibrium with other functional variants in the *VEGF* gene or other cytokine gene (22, 23).

Since VEGF or its family plays a critical role in tumor-related angiogenesis, several functional polymorphisms in the *VEGF* gene have already been reported to be associated with a *VEGF* gene expression or an increased risk of solid tumors (11, 12, 23, 24), making them potential predictive markers for clinical outcomes (22-24). For example, the -634C and 936T alleles as a dominant model or CGT haplotype (-1498T >C, -634G>C, and 936C>T) among the VEGF gene polymorphisms have been associated with a significantly decreased risk of small cell lung cancer (SCLC), while the TCC haplotype conferred a significantly increased risk of SCLC in a previous study by the present authors (25). Plus, a strong association between the -634 CC or 936 TT genotypes and a larger tumor size was observed, while the -634 CC genotypes was strongly correlated with poor differentiation and advanced stage of disease in gastric cancer (24).

One possible explanation for these results is that the DNA sequence variations in the *VEGF* gene may alter VEGF production and/or activity, thereby causing inter-individual differences in the development and progression of tumors. A few studies have already reported that *VEGF* gene polymorphisms are associated with VEGF production, yet the results are inconsistent. Awata et al. (26) reported an association between the -634 CC genotype and a higher serum VEGF concentration in the normal Japanese population. Koukourakis et al. (27) also reported that genetic polymorphisms including the -634 region were correlated with VEGF pro-

tein expression in cancer cells and angiogenesis in tumor tissue. Renner et al. (28) demonstrated that VEGF plasma levels were significantly lower in carriers of the 936T allele, whereas Krippl et al. (23) reported that the 936T allele was associated with a lower VEGF plasma level and decreased risk of breast cancer (OR, 0.51; 95% CI, 0.38-0.70). Meanwhile, Watson et al. (10) documented that the GG genotype for the -634G>C polymorphism was significantly correlated with higher VEGF production from stimulated peripheral blood mononuclear cells. Stevens et al. (29) also reported that haplotype -460C/+405G had a higher promoter activity than haplotype -460T/+405C.

In summary, the -634G>C and 936C>T polymorphisms in the *VEGF* gene was found to be associated with susceptibility or adverse clinicopathologic features of CRC in the current study. However, further studies of other *VEGF* sequence variants and their biological functions are needed to understand the role of the *VEGF* polymorphisms and haplotypes in the development and progression of CRC. Moreover, since genetic polymorphisms often vary between ethnic groups, more studies are also warranted to clarify the association between the *VEGF* polymorphisms and CRC in diverse ethnic populations.

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