Vascular Endothelial Growth Factor Gene Polymorphisms Associated with Prognosis for Patients with Colorectal Cancer

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Abstract Purpose: Vascular endothelial growth factor (VEGF) or its family may be considered to play an important role in lymphangiogenesis and lymphatic tumor spread, thereby affecting prognosis of colorectal cancer. Accordingly, the present study analyzed VEGF gene polymorphisms and their effect on the prognosis for patients with colorectal cancer. Experimental Design: Four hundred and forty-five consecutive patients with surgically treated colorectal adenocarcinoma were enrolled in the present study. The genomic DNA was extracted from fresh colorectal tissue and three VEGF (-2578C/A, -634G/C, and +936C/T) gene polymorphisms were determined using a PCR/denaturing high-performance liquid chromatography assay. Results: Multivariate survival analysis showed that the survival for the patients with the -634 G/C genotype [overall survival (OS): hazard ratio (HR), 0.158; P < 0.001] or C/C genotype (OS: HR, 0.186; P < 0.001) were better than for the patients with the -634G/G genotype, whereas the +936 C/T genotype (OS: HR, 12.809; P < 0.001) or T/T genotype (OS: HR, 37.260; P < 0.001) was associated with a worse survival compared with the +936 C/C genotype. In haplotype analysis, the -2578A/-634G/+936T haplotype exhibited a significantly worse survival when compared with the wild -2578C/-634G/+936C haplotype (OS: HR, 3.866; P < 0.001). Conclusions: VEGF gene polymorphisms were found to be an independent prognostic marker for patients with colorectal cancer. Accordingly, the analysis of VEGF gene polymorphisms can help identify patient subgroups at high risk of a poor disease outcome.

Angiogenesis, the formation of new blood vessels from endothelial precursors, is a prerequisite for the growth and progression of solid malignancies, and the vascular endothelial growth factor (VEGF) superfamily of endothelial growth factors has been identified to critically influence tumor-related angiogenesis (1–3). Clinical studies have shown that an increased expression of VEGF or its family is associated with the grade of angiogenesis and the prognosis for various solid tumors (4–8). In particular, with colorectal cancer, the expression of VEGF or VEGF-C, which are intimately involved in the regulation of the lymphangiogenic process, has been reported to be correlated with a poor prognosis (9–11). Furthermore, White et al. (12) found that the expression of VEGF-D, but not that of its receptor VEGF receptor-3, was associated with lymphatic involvement, reduced patient survival, and poor prognosis in colorectal carcinoma. Given these results, VEGF or its family would seem to play an important role in lymphangiogenesis and lymphatic tumor spread, thereby affecting the prognosis of colorectal cancer.

The VEGF gene is assigned to chromosome 6p12-p21 and consists of eight exons separated by seven introns that exhibit alternative splicing to form a family of proteins (13, 14). Several polymorphisms have been described in the VEGF gene, and some of these variants [-2578C>A, -1154G>A, and -634G>C (translation start site counted as +1) in the promoter or 5′ untranslated region and +936C>T in the 3′-untranslated region] were found to be associated with variations in VEGF protein production (15–17). In clinical studies, these polymorphisms have been reported to be involved in the development of solid tumors, such as melanoma (18), lung (19), prostate (20), and breast cancer (21), where angiogenesis is critical in the pathogenesis of the disease. Moreover, recent studies have shown that genetic polymorphisms can be used to predict the clinical outcomes of gastric (22), breast (23, 24), ovary (25), and pancreatic cancer (26).

However, no study has yet been published that has investigated the single nucleotide polymorphisms of the VEGF gene and their relationship to the clinical outcomes...
of colorectal cancer. Therefore, the present study analyzed three VEGF gene polymorphisms and their effect on the prognosis for patients with colorectal cancer.

Materials and Methods

Study population. All the tissues investigated in this study were obtained from consecutive 445 Korean patients who had undergone a surgical resection between January 2003 and August 2006 at Kyungpook National University Hospital (Daegu, Korea). Written informed consent for gene expression analyses was received from all the patients before surgery, and the study was approved by the institutional research board at Kyungpook National University Hospital. The diagnosis and staging of colorectal cancer were assessed according to the WHO classifications (27) and TNM classifications set out by the American Joint Committee on Cancer (28).

Genotyping of VEGF gene polymorphisms. The genomic DNA was extracted from fresh colorectal tumor tissues at the time of surgery using a Wizard genomic DNA purification kit (Promega). The -2578C>A, -634G>C, and +936C>T genotypes were then determined using PCR/denaturing high-performance liquid chromatography assay (DHPLC). Based on the Genbank reference sequence (accession no. NT_007592), the PCR primers designed for -2578C>A, -634G>C, and +936C>T polymorphisms were 5'-GGGCTTAGGACCATACC-3' (forward) and 5'-CAGAGCTTCCCTATCC-3' (reverse); 5'-CAGGCTTGGAGGAGATTGC-3' (forward) and 5'-GGCCGCTGTCGTCGTCG-3' (reverse); and 5'-AGGGTTCGAAAGACAGTC-3' (forward) and 5'-CTGGTATTGACGACAGAG-3' (reverse), respectively. The PCR reactions were done in a 50 μL reaction volume containing 50 ng genomic DNA, 50 pmol/L of each primer, 10 mmol/L deoxynucleotide triphosphate, 5× Q-solution, 10× PCR buffer [Tris-HCl, KCl, 15 mmol/L MgCl2, (NH4)2SO4 (pH 8.7)], and 2.5 units of HotStarTaq polymerase (Qiagen). The PCR cycle conditions consisted of an initial denaturation step at 94°C for 15 min, followed by 40 cycles of 45 s at 94°C, 45 s at 57°C, 45 s at 72°C, and a final elongation at 72°C for 10 min. The PCR products were denatured at 94°C for 10 min, executed hybridization for 45 min, and screened for heterozygous polymorphism by DHPLC analysis using gradient solution 0.1 mol/L TEAA (pH 7.0), 0.1 mol/L TEA, 25% acetonitrile, washing solution with 8% acetonitrile (syringe washing solution), 75% acetonitrile (DNAsep Cartridge Ultraclean and Storage Solution). Column: alkylated nonporous poly(styrene-divinylbenzene) DNAsep Cartridge (Transgenic); flow rate: 0.9 mL/min; oven temperature: 64°C, UV 260 nm. Remaining samples showing a single peak on DHPLC were mixed with PCR products of known homozygous genotype (homozygous A) and hybridized to run DHPLC again as described above. We confirmed another type of homozygous genotype (homozygous B) when double peak appeared on DHPLC. Some of samples with three different patterns on DHPLC were directly sequenced to reconfirm accuracy of DHPLC.

Statistical analysis. The genotypes for each SNP were analyzed as a three-group categorical variable (reference model), and those were also grouped according to the dominant and recessive model. The haplotypes and their frequencies were estimated using the Bayesian algorithm in the phase program (29). The survival estimates were calculated using the Kaplan-Meier method. The differences in overall survival (OS) or progression-free survival (PFS) according to the three VEGF gene polymorphisms were compared using log-rank tests. Cox proportional hazard regression model was used for the multivariate survival analyses, and the analyses were always adjusted for age (<60 versus ≥60 years), sex (male versus female), site of disease (colon versus rectum), differentiation (well to poorly differentiation), stage (0 to IV), and adjuvant chemotherapy (i.e. versus oral versus not received). The hazard ratio (HR) and 95% confidence interval (95% CI) were also estimated. A cutoff P value of 0.05 was adopted for all the statistical analyses. The statistical data were obtained using an SPSS software package (SPSS 11.5, Inc.) or SAS Genetic software (SAS Institute).

Table 1. Multivariate survival analysis according to three VEGF gene polymorphisms

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (%)</th>
<th>OS HR (95% CI)</th>
<th>P</th>
<th>PFS HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2578C&gt;A polymorphism</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C/C</td>
<td>235 (52.8)</td>
<td>0.844</td>
<td>0.762</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/A</td>
<td>173 (38.9)</td>
<td>0.996 (0.547-1.816)</td>
<td>0.990</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>37 (8.3)</td>
<td>1.219 (0.535-2.775)</td>
<td>0.638</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-634G&gt;C polymorphism</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G/G</td>
<td>159 (35.7)</td>
<td>&lt;0.001</td>
<td></td>
<td>1.225 (0.645-2.326)</td>
<td>0.535</td>
</tr>
<tr>
<td>G/C</td>
<td>181 (40.7)</td>
<td>0.158 (0.079-0.318)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>105 (23.6)</td>
<td>0.188 (0.086-0.412)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+936C&gt;T polymorphism</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C/C</td>
<td>276 (62.0)</td>
<td>&lt;0.001</td>
<td></td>
<td>0.526 (0.304-0.911)</td>
<td>0.022</td>
</tr>
<tr>
<td>C/T</td>
<td>152 (34.2)</td>
<td>&lt;0.001</td>
<td></td>
<td>3.492 (2.344-5.201)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T/T</td>
<td>17 (3.8)</td>
<td>0.188 (0.086-0.412)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: P values correspond to multivariate Cox model adjusted for age, sex, site of disease, differentiation, stage, and adjuvant chemotherapy.
Results

Patient characteristics and survival analysis. The median age of the patients was 64 years (range, 22-89 years), and 231 (51.9%) patients were male. Two hundred fifty-three (56.9%) patients had colon cancer, and the others rectal cancer. Curative resections (R0) were done in 367 (82.5%) patients, whereas the others received a R1 (microscopic residual disease, \( n = 45 \)) or R2 (macroscopic residual disease, \( n = 33 \)) colectomy. The pathologic stages after the surgical resection were as follows: stage I (\( n = 77, 17.3\% \)), stage II (\( n = 152, 34.2\% \)), stage III (\( n = 148, 33.3\% \)), and stage IV (\( n = 67, 15.1\% \)). Among the 300 patients with stage II or III diseases, 273 (91.0%) patients received adjuvant chemotherapy with six cycles of 5-fluorouracil/leucovorin radiotherapy (\( n = 194 \)), eight cycles of capecitabine (\( n = 20 \)), or doxifluridine for 1 year (\( n = 59 \)). At the time of last analysis (January 2007), 124 patients had experienced a disease relapse and 84 patients had died as a result of colorectal cancer. However, the death of three patients was not related to colorectal cancer. At the median follow-up duration of 24.6 months (range, 0.7-50.8 months), the estimated 4-year OS and PFS for all the patients was 65.1\% and 63.0\%, respectively, plus survivals were different according to stage (\( P < 0.001 \); Fig. 1).

Genotype frequency and effects on survival. The three VEGF gene polymorphisms were successfully amplified in all cases. The frequencies of each genotype are shown in Table 1, which were conformed to Hardy-Weinberg equilibrium (\( P > 0.05 \)). The -2578C>A and +936C>T polymorphisms exhibited a relatively strong linkage disequilibrium (correlation coefficient, \( R = 0.42 \); Lewontin’s \( D' = 0.76 \)) in the study population, whereas the linkage of the -634G>C polymorphisms with the -2578C>A or +936C>T was weaker (correlation coefficient, \( R = 0.04 \); Lewontin’s \( D' = 0.01 \), or correlation coefficient, \( R = 0.16 \); Lewontin’s \( D' = 0.20 \)). In the univariate analysis, all three VEGF gene polymorphisms had significant effect on survival, whereas the multivariate survival analysis showed that -634G>C and +936C>T polymorphisms were independent prognostic factors. For the -634G>C polymorphism, the G/C genotype or C/C genotype exhibited a better OS and PFS compared with the G/G genotype (OS: HR, 0.158; \( P < 0.001 \); PFS: HR, 0.544, \( P = 0.009 \); C/G genotype: OS: HR, 0.188; PFS: HR, 0.526; \( P = 0.022 \); Table 1; Fig. 2). For the +936C>T polymorphisms, the survival for the patients with the +936 C/T or T/T genotype was worse than for the patients with the +936 C/C genotype (C/T genotype: OS: HR, 3.492; \( P < 0.001 \); PFS: HR, 3.221; \( P = 0.001 \); T/T genotype: OS: HR, 37.260; \( P < 0.001 \); PFS: HR, 3.221, \( P = 0.001 \); Table 1; Fig. 2).

Table 2. Multivariate survival analysis according to common haplotypes of three VEGF gene polymorphisms

<table>
<thead>
<tr>
<th>Haplotypes (-2578C&gt;A/-634G&gt;C/+936C&gt;T)</th>
<th>Haplotype frequency (%)</th>
<th>OS</th>
<th>PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGC 28.8</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCT 8.1</td>
<td>0.895 (0.468-1.713)</td>
<td>0.739</td>
<td></td>
</tr>
<tr>
<td>CCC 33.7</td>
<td>0.399 (0.198-0.580)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AGT 10.3</td>
<td>3.866 (2.493-5.995)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NOTE: \( P \) values correspond to multivariate Cox model adjusted for age, sex, site of disease, differentiation, stage, and adjuvant chemotherapy.
**Haplotype analysis.** The haplotype analyses were conducted to evaluate the combined effect of the three polymorphisms on colorectal cancer survival. Eight haplotypes (CGC, 28.8%; CCT, 8.1%; CCC, 33.7%; AGC, 15.5%; AGT, 10.3%; CGT, 1.7%; ACC, 1.1%; ACT, 0.8%) were estimated from the three polymorphisms in all cases. Because CGC, CCT, CCC, AGC, and AGT were the common haplotypes, the effect of these haplotypes on survival was analyzed (Table 2). The estimated 4-year OS rate for the patients with the CCC haplotype was 81.2 ± 6.9%, which was significantly superior to the rate for the patients with the CGC haplotype (wild-type; HR, 0.339; 95% CI, 0.198–0.580; P < 0.001; Table 2; Fig. 3). Meanwhile, OS for the patients with the AGT haplotype was worse than for the patients with the CGC haplotype (HR, 3.866; 95% CI, 2.493–5.995; P < 0.001; Table 2; Fig. 3). For the clinicopathologic variables, the differentiation, tumor-node-metastasis stage, and adjuvant chemotherapy were also significant prognostic factors in a Cox model for OS and PFS (P < 0.001).

**Discussion**

We have investigated the prognostic effect of three VEGF gene polymorphisms in quite a large population of patients with surgically resected colorectal adenocarcinoma. The current study showed that -634G>C, +936T>T polymorphisms, and their haplotypes had a significant effect on survival in these patients. Because VEGF or its family plays a critical role in tumor-related angiogenesis, the association of VEGF gene polymorphisms with the risk or prognosis of several solid tumors, such as melanoma (18), lung (19), prostate (20), and breast cancer (21, 23, 24), has already been shown. Recently, we also reported that -1498T>C polymorphism in VEGF gene was an independent prognostic factor in patients with early-stage gastric cancer (30). However, data on the relationship between the SNPs of VEGF gene and clinical outcomes of colorectal cancer have not been published yet.

In the present study, the AGT haplotype was associated with a significantly worse survival when compared with the CGC haplotype (OS: HR, 3.87; PFS: HR, 3.46), and the -634 G/G or +936 T/T genotype was a poor prognostic factor in patients with colorectal cancer. In a study by Lu et al. (23) that evaluated the effects of three VEGF gene polymorphisms (-1498T>C, -634G>C, and +936C>T) on survival in 1,193 Chinese patients with breast cancer, the survival was lower among the patients with the -1498 C/C and -634 G/G genotypes and higher among the patients with the -1498T/-634G/+936C haplotype. Recently, Hefler et al. (25) also reported that simultaneous carriage of the three homozygous genotypes (i.e., -2578C/C, -1154G/G, -634C/C), which is correlated with increased VEGF expression in an individual patient, was independently associated with a shortened OS in 563 patients with ovarian cancer. 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 interindividual differences in the lymphangiogenesis and lymphatic tumor spread. Given the homogenous ethnic background of Korean patients, any potential confounding effect due to ethnicity is likely to be small in the present study. The correlation between an elevated preoperative serum VEGF concentration and the poor survival in patients with colon cancer was already shown in a previous study (31). However, a few studies have reported that VEGF gene polymorphisms are associated with VEGF production. Nonetheless, the results are inconsistent. Awata et al. (32) reported that individuals with the -634 C/C genotype had a higher fasting serum VEGF level than those with other genotypes, and that they carried an increased risk of diabetic retinopathy. Meanwhile, Watson et al. (15) documented that the -634G allele is associated with higher lipopolysaccharide-stimulated VEGF production by peripheral blood mononuclear cells than the +405C allele. In a recent in vitro study (17), carrying a haplotype containing the −1498C/-634G polymorphisms was found to significantly increase basal VEGF promoter activity and phorbol ester–induced responsiveness compared with the presence of a haplotype containing the +1498T/-634C polymorphisms.

In conclusion, VEGF gene polymorphisms were found to be an independent prognostic marker for Korean patients with surgically resected colorectal adenocarcinoma. Consequently, in addition to the pathologic stage, the analysis of VEGF gene polymorphisms may help identify patient subgroups at high risk for a poor disease outcome, thereby helping to refine therapeutic decisions in colorectal cancer.

**References**


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