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Gender-specific Association between Polymorphism of Vascular Endothelial Growth Factor (VEGF 936C > T) Gene and Patients with Stomach Cancer

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Purpose: Angiogenesis plays an important role in the growth, progression, and metastasis of tumors. Vascular endothelial growth factor (VEGF) overexpression has been associated with advanced stage and poor survival in several cancers. We investigated the present case-control study to determine whether there is an association between the VEGF 936C > T polymorphism and stomach cancer. **Patients and Methods:** The association of functional single nucleotide polymorphisms (SNPs) of the VEGF gene with stomach cancer development was evaluated in a case-control study of 154 Korean stomach cancer patients. Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. **Results:** Our results revealed significant association of T allele-bearing genotypes with increased risk for stomach cancer development. Genotype frequencies of the VEGF 936C > T polymorphisms were significantly different between patient and control groups (CT, AOR: 2.007, 95% CI: 1.277 - 3.156, TT, AOR: 4.790, 95% CI: 1.174 - 19.539, CT + TT, AOR: 2.147, 95% CI: 1.382 - 3.337). When stratified by gender and age, genotype frequencies were significantly different for stomach cancer in women and in patients younger than 55 years (in women, CT, OR: 3.049, 95% CI: 1.568 - 5.930, CT+TT, OR: 3.132, 95% CI: 1.638 - 5.990; in < 55 years, CT, OR: 3.306, 95% CI: 1.413 - 7.732, CT + TT, OR: 3.967, 95% CI: 1.729 - 9.104). In addition, this association partially remained in cases with intestinal and diffuse-type stomach cancer. **Conclusion:** Our present study suggests that the VEGF 936C > T polymorphism is a susceptibility factor for stomach cancer, at least in Korean. These observations, however, require further confirmation by a larger multi-ethnic study.

Key Words: Stomach cancer, polymorphism, vascular endothelial growth factor, Korean

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INTRODUCTION

As one of the most common human malignant tumors, stomach cancer ranks as the first leading cause of gastrointestinal cancer-related mortality worldwide.[1] Over the past few years, many attempts have been made to better define the biological profile of stomach cancer in order to improve early diagnosis and prognostic stratification, and to find an eventual cure.[2-4]

Angiogenesis is an essential step for tumor growth, playing a critical role in invasion and metastasis.[5] It is regulated by various growth factors, among which VEGF plays a central role. A number of studies have confirmed that VEGF expression is closely associated with the extent of vascularization and prognosis in many solid tumors, and is predictive of resistance to radiotherapy, chemotherapy, and endocrine therapy.[6-8] Furthermore, *in vitro* and *in vivo* experiments have shown that increased VEGF expression is associated with tumor growth and metastasis, whereas inhibition of VEGF expression results in suppression of tumor growth and tumor-induced neoangiogenesis.[9] Clinical observations have revealed that high levels of VEGF expression and increased microvessel density in tumors are associated with advanced-stage disease and worse

prognosis for various types of tumors, including stomach cancer.[6,7,10] Many studies have found a correlation between VEGF expression and several malignancies, such as stomach cancer and breast, gastrointestinal, urinary tract, and ovarian tumors.[11-15] In stomach cancer, VEGF has been correlated with vascular involvement, lymph node and liver metastasis.[16]

Several SNPs in the VEGF gene have been shown to affect the expression of the gene.[17] One of these, the 936C > T polymorphism in the 3-untranslated region (UTR) of the VEGF gene, was shown to affect VEGF plasma levels, and carriers of the VEGF 936T allele had significantly reduced VEGF plasma levels.[18] Although some association studies have been performed, the effect of VEGF polymorphisms on the risk of stomach cancer has not yet been evaluated.

Since VEGF is significant in the angiogenesis of various types of tumors, it is reasonable to hypothesize that VEGF is a good candidate for determining the risk of developing stomach cancer. To test this hypothesis, we investigated genetic variations at the 936C > T polymorphic site in the 3′-UTR of the VEGF gene in a Korean population who have had stomach cancer.

PATIENTS AND METHODS

Subjects

A total of 154 patients (mean age ± SD, 58.05 ± 12.74 years) with stomach cancer diagnosed at Bundang CHA Hospital, Pochon CHA University, from July 1999 to June 2004, were enrolled in this study. The diagnosis and staging of stomach cancer were assessed according to the Lauren classification.[19] Among stomach cancer patients, there were 91 men (age 57.76 ± 12.31 years; range, 30 - 88 years) and 63 women (age 58.46 ± 13.42 years; range, 28 - 81 years). Ninety-nine patients (age 59.85 ± 11.45 years; range, 28 - 88 years) had intestinal subtype stomach cancer and 55 patients (age 54.80 ± 14.32 years; range, 29 - 81 years) had diffuse-subtype stomach cancer. The control group consisted of 229 individuals (age 59.57 ± 11.80 years; range, 31 - 91 years) who were randomly selected through health screening to exclude those with a history of thrombotic diseases or cancer. This study was approved by the Institutional Review Board of Pochon CHA University, Korea.

VEGF genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and phenol/chloroform extraction. The VEGF 936C > T (rs3025039) genotype polymorphisms were determined using the PCR-RFLP method. The PCR primers used to detect the VEGF 936C > T polymorphism were 5′-AGG AAG AGG GAC TCT GCG CAG AGC-3′ (forward) and 5′-TAA ATG TAT GTA TGT GGG TGG GTG TGT CTA CAG G-3′ (reverse). The PCR product was digested overnight with NlaIII, the appropriate restriction enzyme (New England BioLabs, Beverly, MA, USA) for VEGF 936C > T genotyping. The VEGF 936T allele was cut into 2 fragments of 122 and 86 base pairs, whereas the VEGF 936C allele remained uncut with a length of 208 base pairs.

Data analysis

Genotype frequencies of patients and healthy control subjects were in Hardy-Weinberg equilibrium, as tested by χ^2 test. Allele and genotype frequencies between the case and control groups were compared using χ^2 test and Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used as the measure of the strength of association between the VEGF genotypes and stomach cancer. Stratification analysis was used to study subgroups by age and gender. StatsDirect Statistical Software Version 2.4.4 (StatsDirect Ltd., Altrincham, UK) was used to calculate the adjusted odds ratio (AOR) and 95% CI.

RESULTS

The median patient age was 60 years (range, 28 - 88 years), and 91 patients (59.1%) were male. There was no statistically significant difference in age ($p = 0.230$) or gender distribution ($p = 0.060$) between patients and controls (Table 1). The histopathological classification after surgical resection or biopsy included at least 2 main subtypes:

Table 1. Baseline Characteristics in Patients with Stomach Cancer and Controls

	Controls (n = 229)	Stomach cancer (n = 154)	*p* value
Age (yrs, mean ± SD)	59.57 ± 11.80	58.05 ± 12.74	0.230
Male (%)	112 (48.9)	9 1(59.1)	0.060
Stage (%)			
I	-	60 (39.7)	-
II	-	28 (18.5)	-
III	-	29 (19.2)	-
IV	-	34 (22.5)	0.250
Smoking (%)	21/199 (10.6)	70/85 (82.4)	< 0.0001

Table 2. Comparison of Genotype Frequencies of the Vascular Endothelial Growth Factor 936C > T Polymorphism in Patients with Stomach Cancer and Controls

Genotype	Controls (%)	Cases (%)	OR (95% CI)	AOR* (95% CI)
CC	169 (73.8)	89 (57.8)	1.0 (-)	1.0 (-)
CT	57 (24.9)	58 (37.7)	1.932 (1.236 - 3.021)	2.007 (1.277 - 3.156)
TT	3 (1.3)	7 (4.5)	4.431 (1.118 - 17.56)	4.790 (1.174 - 19.539)
CT+TT	60 (26.2)	65 (42.2)	2.057 (1.332 - 3.178)	2.147 (1.382 -3.337)
T allele	0.138	0.234	-	-

OR, odds ratio; AOR, adjusted odds ratio.
*Adjusted by age and gender.

intestinal type (n = 99, 64.3%) and diffuse type (n = 55, 35.7%).

Tables 2 and 3 compare genotype frequencies of the VEGF 936C > T polymorphism according to the study group as a whole or by gender and age between case and control groups. Genotypes were in Hardy-Weinberg equilibrium in both the study and control groups ($p = 0.241$). The distribution of genotypes with the T allele at the VEGF 936C > T polymorphism was significantly different between the control and case groups (936CT, AOR, 2.007; 95% CI, 1.277 - 3.156; 936TT, AOR, 4.790; 95% CI, 1.174 - 19.539; 936CT + TT, AOR, 2.147; 95% CI, 1.382 - 3.337; Table 2). Frequencies of the 936CT and 936CT+TT genotypes in patients were associated with increased risk for stomach cancer in females (OR, 3.049; 95% CI, 1.568 - 5.930; $p = 0.001$ and OR, 3.132; 95% CI, 1.638 - 5.990; $p = 0.001$; Table 3) when stratified by gender. When the data were stratified by age, the association remained in patients who were less than 55 years old (936CT, OR, 3.306; 95% CI, 1.413 - 7.732; $p = 0.006$, 936CT + TT, OR, 3.967; 95% CI, 1.729 - 9.104; $p = 0.001$).

Moreover, in females with diffuse-subtype stomach cancer, the 936CT and 936CT + TT genotypes were associated with increased risk for stomach cancer (936CT, OR, 4.889; 95% CI, 1.970 - 12.14; $p = 0.001$, 936CT + TT, OR, 5.462; 95% CI, 2.266 - 13.17; $p = 0.000$) (Table 5). For intestinal-subtype stomach cancer, however, there was no significant difference in the genotype frequencies between the 2 groups (Table 4).

DISCUSSION

Angiogenesis is essential for tumor growth and plays a critical role in the invasion and metastasis of tumor cells. It is regulated by many growth

Table 3. Comparison of Subtype Frequencies of the Vascular Endothelial Growth Factor 936C > T Polymorphism in Patients with Stomach Cancer and Controls

Genotype	Controls (%)	Cases (%)	OR (95% CI)	*p* value
Male				
CC	81 (72.3)	58 (63.7)	1.0 (-)	-
CT	30 (26.8)	29 (31.9)	1.350 (0.732 - 2.489)	0.352
TT	1 (0.9)	4 (4.4)	5.586 (0.608 - 51.31)	0.165
CT + TT	31 (27.7)	33 (36.3)	1.487 (0.8200 - 2.696)	0.225
T allele	0.143	0.204	-	-
Female				
CC	88 (75.2)	31 (49.2)	1.0 (-)	-
CT	27 (23.1)	29 (46.0)	3.049 (1.568 - 5.930)	0.001
TT	2 (1.7)	3 (4.8)	4.258 (0.679 - 26.70)	0.126
CT + TT	29 (24.8)	32 (50.8)	3.132 (1.638 - 5.990)	0.001
T allele	0.132	0.278	-	-
≥ 55 yrs				
CC	109 (69.0)	56 (57.7)	1.0 (-)	-
CT	46 (29.1)	38 (39.2)	1.608 (0.940 - 2.751)	0.097
TT	3 (1.9)	3 (3.1)	1.946 (0.380 - 9.962)	0.417
CT + TT	49 (31.0)	41 (42.3)	1.629 (0.963 - 2.754)	0.080
T allele	0.165	0.227	-	-
< 55 yrs				
CC	60 (84.5)	33 (57.9)	1.0 (-)	-
CT	11 (15.5)	20 (35.1)	3.306 (1.413 - 7.732)	0.006
TT	0 (0.0)	4 (7.0)	-	-
CT + TT	11 (15.5)	24 (42.1)	3.967 (1.729 - 9.104)	0.001
T allele	0.077	0.246	-	-

OR, odds ratio.

factors, among which VEGF plays a central role and serves as an important prognostic factor in a variety of tumors, including stomach cancer. The role of genetic polymorphisms, which are important determinants of endogenous causes of cancer, in the risk of stomach cancer has attracted increasing interest due to advances in DNA analysis technologies and knowledge of the human genome. VEGF and its family play a critical role in tumor-related angiogenesis, and several functional polymorphisms in the VEGF gene have already been reported to be associated with VEGF gene expression or an increased risk of solid tumors, making them potential predictive markers for clinical outcome.[7,20-24] For example, the less common T allele of the 936C > T polymorphism in the 3′-untranslated region of the VEGF gene is correlated with lower VEGF levels.[20] The contribution of this polymorphism to oncogenesis has been investigated in several types of cancer but

Table 4. Comparison of Genotype Frequencies of the Vascular Endothelial Growth Factor 936C > T Polymorphism in Patients with Intestinal-type Stomach Cancer and Controls

Genotype	Controls (%)	Cases (%)	OR (95% CI)	*p* value
CC	169 (73.8)	65 (65.7)	1.0 (-)	-
CT	57 (24.9)	33 (33.3)	1.505 (0.899 - 2.521)	0.138
TT	3 (1.3)	1 (1.0)	0.867 (0.088 - 8.488)	1.000
CT + TT	60 (26.2)	34 (34.3)	1.473 (0.886 - 2.451)	0.145
T allele	0.138	0.177		
Male				
CC	81 (72.3)	44 (68.8)	1.0 (-)	-
CT	30 (26.8)	19 (29.7)	1.166 (0.590 - 2.306)	0.727
TT	1 (0.9)	1 (1.6)	1.841 (0.112 - 30.17)	1.000
CT + TT	31 (27.7)	20 (31.3)	1.188 (0.607 - 2.325)	0.610
T allele	0.143	0.165		
Female				
CC	88 (75.2)	21 (60.0)	1.0 (-)	-
CT	27 (23.1)	14 (40.0)	2.173 (0.974 - 4.847)	0.082
TT	2 (1.7)	0 (0.0)	-	-
CT + TT	29 (24.8)	14 (40.0)	2.023 (0.913 - 4.485)	0.090
T allele	0.132	0.200		
≥ 55 yrs				
CC	109 (69.0)	44 (64.7)	1.0 (-)	-
CT	46 (29.1)	23 (33.8)	1.239 (0.672 - 2.282)	0.529
TT	3 (1.9)	1 (1.5)	0.826 (0.084 - 8.160)	1.000
CT + TT	49 (31.0)	24 (35.3)	1.213 (0.665 - 2.213)	0.538
T allele	0.165	0.184		
< 55 yrs				
CC	60 (84.5)	21 (67.7)	1.0 (-)	-
CT	11 (15.5)	10 (32.3)	2.597 (0.965 - 6.993)	0.066
CT + TT	11 (15.5)	10 (32.3)	2.597 (0.965 - 6.993)	0.066
T allele	0.077	0.162		

OR, odds ratio.

not in stomach cancer until now to the our best knowledge.[6,10,20,25-28]

Based on the involvement of VEGF in the risk of advanced-stage cancer through tumor growth and metastasis of several types of cancer, including stomach cancer, we evaluated the relationship between the VEGF 936C > T polymorphism and stomach cancer in a Korean patient case-control study. In the present study, the VEGF 936T allele was found to be associated with

Table 5. Comparison of Genotype Frequencies of the Vascular Endothelial Growth Factor 936C > T Polymorphism in Patients with Diffuse-type Stomach Cancer and Controls

Genotype	Controls (%)	Cases (%)	OR (95% CI)	*p* value
CC	169 (73.8)	24 (43.6)	1.0 (-)	-
CT	57 (24.9)	25 (45.5)	3.088 (1.636 - 5.832)	0.001
TT	3 (1.3)	6 (10.9)	14.08 (3.301 - 60.08)	0.000
CT + TT	60 (26.2)	31 (56.4)	3.638 (1.979 - 6.689)	0.000
T allele	0.138	0.337		
Male				
CC	81 (72.3)	14 (51.9)	1.0 (-)	-
CT	30 (26.8)	10 (37.0)	1.929 (0.774 - 4.808)	0.217
TT	1 (0.9)	3 (11.1)	17.36 (1.682 - 179.1)	0.015
CT + TT	31 (27.7)	13 (48.1)	2.426 (1.026 - 5.740)	0.063
T allele	0.143	0.296		
Female				
CC	88 (75.2)	10 (35.7)	1.0 (-)	-
CT	27 (23.1)	15 (53.6)	4.889 (1.970 - 12.14)	0.001
TT	2 (1.7)	3 (10.7)	13.20 (1.964 - 88.74)	0.014
CT + TT	29 (24.8)	18 (64.3)	5.462 (2.266 - 13.17)	0.000
T allele	0.132	0.375		
≥ 55 yrs				
CC	109 (69.0)	12 (41.4)	1.0 (-)	-
CT	46 (29.1)	15 (51.7)	2.962 (1.287 - 6.819)	0.014
TT	3 (1.9)	2 (6.9)	6.056 (0.918 - 39.94)	0.095
CT + TT	49 (31.0)	17 (58.6)	3.151 (1.398 - 7.101)	0.006
T allele	0.165	0.328		
< 55 yrs				
CC	60 (84.5)	12 (46.2)	1.0 (-)	-
CT	11 (15.5)	10 (38.5)	4.545 (1.579 - 13.09)	0.007
TT	0 (0.0)	4 (15.4)	-	-
CT + TT	11 (15.5)	14 (53.8)	6.364 (2.331 - 17.37)	0.000
T allele	0.077	0.347		

OR, odds ratio.

increased risk for stomach cancer not only overall but also in female subjects and subjects less than 55 years old when stratified by gender and age.

Several attempts to classify stomach cancer have been made in the past decades. The most successful and widely used classification is by

Lauren, who distinguished 2 main cancer pathogeneses, diffuse and intestinal subtypes, by microscopic morphology alone. These 2 subtypes clearly appear as clinical and epidemiological by dissimilar entities. Here, we analyzed samples from each patient according to the main differences in epidemiology, histopathology, and molecular pathology of the 2 main subtypes of stomach cancer based on the Lauren classification.[29] There was no significant difference in the genotype frequencies with intestinal-subtype stomach cancer between the control and patient groups. However, despite the relatively small number of individuals in this study, highly significant differences were noted with diffuse-subtype stomach cancer between patients and controls.

Surprisingly, stomach cancer in women is associated with some genotypes of the VEGF 936C > T polymorphism in this study. These trends remained in women with diffuse-subtype stomach cancer, even when the data were stratified by the Lauren classification. That is, the association was gender-specific, suggesting that VEGF production according to VEGF 936C > T genotype might differ between men and women. In addition, the association was also specific for the histological subtype of the stomach tumor, indicating that VEGF production depends on the histological subtype of the stomach tumor.

Our results strongly indicate that the T allele is linked to an increased risk for stomach cancer, despite being associated with lower circulating levels of VEGF. It may, therefore, be assumed that high VEGF levels are not a prerequisite for stomach cancer susceptibility. Similarly, in gliomas, low-expression VEGF genotypes coexist with high VEGF levels in patients but not in healthy controls.[30] High VEGF expression is attributed to independent cancer and tumor stroma production.[30]

The findings of this study clearly demonstrate that the low-expression T allele is associated with an increased stomach cancer risk, however, the underlying mechanism might not involve angiogenesis but rather other VEGF-related functions such as thrombosis. As previously mentioned, in accordance with our results, a significant increase in the T allele frequency has been found in cancer patients with thrombotic complications compared to healthy controls and non-thrombotic cancer patients.[31] This association has been reported in many cases, while the 936TT genotype is associated with larger tumors and presence of metastases.[24] This study clearly implicates the low-expression T allele with an increased risk of stomach cancer.

In cancer patients, additional risk factors for thrombotic complications raise close relationships between tumors and activation of blood coagulation. Tumor and tumor-associated endothelial cells are able to produce and release some molecules,[32] and one of the molecules is VEGF, which is a the well-characterized regulator of angiogenesis. VEGF also affects hemostasis of endothelial cells. VEGF has a prothrombotic effect on endothelial cells, thus encouraging tissue factor expression and additionally platelet activation.[33] Polymorphisms of the VEGF gene have been shown to correlate with variation in VEGF protein production.[34] Other oral and colon cancer studies have reported that low VEGF production of the 936C > T polymorphism is strongly associated with increased cancer risk.[35,36]

We were interested in studying VEGF 936C > T polymorphisms in the Korean population because it has a relatively homogeneous ethnic origin in contrast to the more heterogeneous characteristics of ethnic groups examined in previous studies.[36] The literature survey indicates that the frequency of VEGF 936T allele among healthy controls was 0.138 in Koreans and 0.157 in Japanese, indicating that the allele ratio was similar among Asian populations.[36,37] This study revealed that allele frequencies of the VEGF 936C > T polymorphism in our study control group were not significantly different from other Asian populations. On the other hand, there was a racial difference in the frequency of the T allele: 0.167 in Canadians, 0.161 in Polish (only female group), 0.140 in Americans, and 0.091 in Germans.[6,26,38,39] Earlier differences in the literature might have been results may be due to different genetic backgrounds.

Functional polymorphisms, which affect the regulation of gene expression, can contribute to differences in susceptibility to and severity of a disease between individuals. The effect may be seen with polymorphism alone or in combination

with other polymorphisms. These functional polymorphisms may result in altered transcription factor recognition sites, which subsequently affect transcriptional activation and alter protein production.[37] Haplotype analysis, which is currently the focus of intense genetic research efforts, will make risk estimates more specific than single locus analyses.[40] Identification of associations between candidate genes and disease will be one of the main objectives in the development of personalized and interactive medicine.

In conclusion, our present findings indicate that the VEGF 936C > T polymorphism may be a useful indicator of susceptibility to stomach cancer. Large-scale genetic studies including haplotype analyses may be needed to improve statistical power. In addition, studies of other VEGF sequence variants and their biological functions are also needed to determine the risk of stomach cancer. Since genetic polymorphisms often vary between ethnic groups, further studies are needed to clarify the association between VEGF polymorphisms and stomach cancer in diverse ethnic populations.

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