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The Relationship between Vascular Endothelial Growth Factor -634G>C Polymorphism and Ovarian, Cervical and Endometrial Cancers

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ABSTRACT

Tumor growth and metastasis require the formation of new blood vessels, a process known as angiogenesis. One of the most important regulators employed in this process is the vascular endothelial growth factor (VEGF). The phenotypic effects of single nucleotide polymorphisms (SNPs) are based on direct genetic effects, gene-gene and gene-environment interactions. DNA sequence variations in VEGF gene might yield changes both in the production outcomes and in the activities of the gene. In this study, we aimed to determine how the changes in the VEGF -634G>C - a risk factor in the progress and metastasis of cancer - affect the gynecologic cancer patients in the Turkish population. A total of 100 gynecologic cancer patients (47 ovarian, 32 cervical and 21 endometrial) and 106 healthy controls were studied during the research. DNA extraction techniques were used to extract the genomic DNA from the whole blood. DNA analyses were carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). There were no significant differences between any of the three types of gynecologic cancer patients and controls in terms of the distribution of VEGF -634 genotypes and alleles (p>0.05). Odds ratios (ORs) were calculated by logistic regression analysis in comparison with the most common homozygote genotype observed in studied population. Although, a statistically significant difference was found for genotypic frequencies of the -634G>C polymorphism between endometrial cancer patients with GC genotype and those with GG genotype (OR=0.27; 95% Confidence Interval (CI)=0.09-0.81; p=0.015), this result was not clinically significant due to its negligible OR. A multivariable logistic regression analysis performed on the covariates - family history concerning gynecologic and/or other cancer types, stages of tumor, smoking habits and existence of other diseases - did not change these results. This study is the first report demonstrating that the investigated VEGF -634 gene polymorphism is not associated with any of the three types of gynecologic cancer.

Key words: angiogenesis, gynecologic cancer, polymorphism, vascular endothelial growth factor.

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INTRODUCTION

The determination of single nucleotide polymorphisms (SNPs) is a new method to study the etiology of polygenetic disorders with complex inheritance patterns, such as cancer (Saaristo et al., 2000). Tumor growth and metastasis, which employ a number of regulators such as the vascular endothelial growth factor (VEGF), require the formation of new blood vessels.

VEGF, a mitogen that promotes vascular endothelial cell proliferation and angiogenesis, plays a crucial role in physiological vasculogenesis and vascular permeability (Ferrara et al., 2003). The gene encoding VEGF is located on chromosome 6 (6p21.3) and comprises a 14 kb (kilobase) coding region with 8 exons and 7 introns (Vincenti et al., 1996). It is a secreted homodimeric glycoprotein of 45kDa (kiloDalton) with several protein variants resulting from alternative mRNA splicing (Ferrara et al., 2003). The importance of VEGF in the angiogenic process of human malignancies is well established (Ferrara and Davis-Smyth, 1997). A 28-base pair element within the human VEGF promoter believed to contain a consensus binding site for hypoxia-inducible factor-1 (HIF-1) is necessary to increase transcription of a CAT reporter gene in endothelial cells cultured under hypoxic conditions (Liu et al., 1995).

SNPs of the VEGF gene are associated with production of VEGF protein and are reported to be involved in susceptibility to several disorders in which angiogenesis might be critical in pathogenesis (Krippl et al., 2003; Howell et al., 2002). Different VEGF gene polymorphisms have been shown to result in different VEGF gene responsiveness to various stimuli and different capacity for VEGF protein production. (Watson et al., 2000; Renner et al., 2000; Awata et al., 2002). SNPs have been described within the VEGF promoter and 5′untranslated regions (UTR) (Brogan et al, 1999; Watson et al, 2000, Krippl 2003; Cheng-Chieh 2003; Han et al., 2004; Papazoglou et al., 2004), which are interestingly known to regulate VEGF expression at the post-transcriptional level (Brogan et al, 1999; Awata et al., 2002). Although it is unclear how the polymorphisms in the UTR region of the VEGF gene influence its protein production, several reports have demonstrated that SNPs of the VEGF gene are associated with VEGF synthesis. Ther are studies on the subject of -634G>C polymorphism in the 5′-UTR of the VEGF gene (Awata et al., 2002; Boiardi et al., 2003; Papazoglou et al., 2004; Cooke et al., 2004; Salvarani et al., 2004; Kariyazono et al., 2004; Koukorakis et al., 2004; Jin et al., 2005; Howell et al., 2005; Seo et al., 2005; Rueda et al., 2005a; Rueda et al., 2005b; Nam et al., 2005; Rueda et al., 2006). Some of these studies have demonstrated an association between the production of VEGF protein and the -634G>C polymorphism and reported susceptibility to several disorders (Awata et al., 2002; Boiardi et al., 2003; Cooke et al., 2004; Salvarani et al., 2004; Kariyazono et al., 2004; Koukorakis et al., 2004; Seo et al., 2005; Rueda et al., 2005b; Rueda et al., 2006).

Ovarian, cervical and endometrial cancers are the most common types of gynecologic cancer. As the female life expectancy increases, the likelihood of encountering those cancer types also increases (Repetto et al., 1998). Angiogenesis of gynecologic cancer is regulated by VEGF (Rasila et al., 2005). In studies of ovarian cancer, VEGF expression and microvessel density (MVD) have been correlated with poor survival (Raspollini et al., 2004). Further, evidence of increased neovascularization might be a prognostic indicator in many solid tumors, including cervical cancer (Ozalp et al., 2003). Another study suggests that the increase of proliferating cell nuclear antigen (PCNA) index and MVD may enhance development of endometrial carcinoma (Xiao and Tang, 2005). On the basis of these facts, VEGF might contribute to the development and progression of ovarian, cervical and endometrial cancers. However, no studies were published so far as regards to the relationship between VEGF -634G>C polymorphism and the mentioned cancer types, on any population. The purpose of the present study is to reveal this relationship, if any, in the Turkish population.

MATERIALS AND METHODS

Subjects

We studied a total of 100 gynecologic cancer patients who were selected from patients admitted to the Departments of Obstetrics and Gynecology, Faculty of Medicine, Gazi University and the Ankara Oncology Education and Research Hospital. A total of 106 subjects were enrolled as healthy controls (mean ages of menarche: 13.0±0.18 and mean age: 47±0.5 years, range 25-65). The study was approved by the Committee of Ethics of the Gazi University and informed consent from all subjects was obtained. All the cases and controls were of Turkish origin and were included in this study

Table 1. Demographic characteristics of the patients.

	Ovarian Cancer	Cervical Cancer	Endometrial Cancer
Number of cases	47 (47)	32 (32)	21 (21)
Mean ages of menarche (years)	13±0.23	13±0.18	13±0.14
Mean ages of first diagnosis (years)	48±0.51	49±0.46	50±0.52
Range (years)	16-73	25-68	39-81
1[a]	8 (17.0)	7 (21.8)	9 (42.8)
2[b]			
I	1 (2.1)	5 (15.6)	10 (47.6)
II	5 (10.7)	16 (50.0)	5 (23.8)
III	40 (85.1)	10 (31.3)	5 (23.8)
IV	1 (2.1)	1 (3.1)	1 (4.8)
3[c]	8 (17.0)	4 (12.5)	1 (4.76)
4[d]	11(23.4)	5 (15.6)	5 (23.8)

Values in parentheses are percentages.

[a]Family history concerning gynecologic and/or other cancer types.

[b]Stages of tumor (Staging was performed according to the current International Federation of Gynecology and Obstetrics (FIGO) classification)

[c]Smoking habits.

[d]Existence of other diseases.

after submitting their consent in written. Our study conforms to a great extent to the standards presented by Hegele, which outlined desirable attributes for genetic case-control studies (Hegele, 2002).

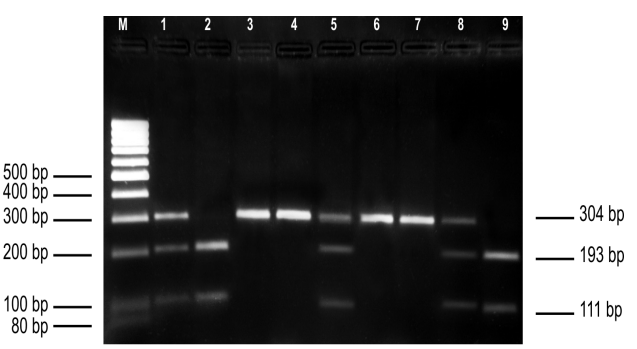


Figure 1. PCR-based restriction analysis of VEGF gene -634G>C polymorphism shown on 2% agarose electrophoresis. Polymorphic region was amplified by PCR, resulting in heterozygotes (GC) in lanes 1, 5, 8; digestible fragments in lanes 2 and 9 (GG homozygote); undigestible fragments in lanes 3, 4, 6 (CC homozygote). The left lane (marker) contains a 100 base pair (bp) ladder and lane 7 contains a 304 bp PCR product (uncut) of the gene.

In each case, age of menarche and first diagnosis, family history concerning gynecologic and/or other cancer types, stages (I-IV) of tumor and its histopathological classification, information on smoking habits and existence of other diseases such as rheumatoid arthritis, diabetic retinopathy, psoriasis and cardiovascular diseases (for which angiogenesis may be critical) were obtained from the patient files. None of the control individuals had family history concerning gynecologic or other cancers, smoking habits and existence of other diseases.

Forty-seven out of 100 patients had epithelial ovarian cancer, a total of 32 women had cervical cancer and the remaining 21 women had endometrial cancer. Demographic characteristics of the patients were shown in Table 1.

Genotyping

Genomic DNA was isolated from peripheral blood by using a DNA extraction kit (Heliosis®, Metis Biotechnology, Turkey) according to the manufacturer's instructions. Amplifications of the region of the VEGF gene were carried out by placing in a Mastercycler gradient (Eppendorf, Germany) thermal cycler, a total volume of 50 μL PCR mixture containing 50 ng genomic DNA, 2.5 mM $MgCl_2$, 100 μM dNTP, 50 pmol/μl of each primer and 1.0 U/μl Taq DNA polymerase. For the -634 G>C, the following pair of primers produced a PCR product of 304 base pair (bp): Forward 5′-ATTTATTTTTGCTTGCCA TT-3′, Reverse 5′-GTCTGTCTGTCTGTCCGTCA-3′ (Gene bank accession No. AF095785; nucleotides 2578-2881). We set the PCR cycling conditions for the VEGF gene as explained by Papazoglou (Papazoglou, 2004). The VEGF polymorphism was analyzed by digestion of the PCR products with restriction endonuclease, *Faq*I for -634G>C (Fermantas, USA). The -634G allele was cut into two fragments of 193 bp and 111 bp while the -634C allele remained uncut (304 bp). The PCR products and restriction fragments of VEGF -634G>C was loaded directly onto 2% agarose gel (containing 0.5% ethidium bromide). The products were separated by electrophoresis and visualized by Gel Logic 100 gel image system (Kodak, USA). PCR-based restriction analysis of VEGF gene -634G>C polymorphism was shown in Figure 1.

Statistical analysis

Tests for Hardy-Weinberg equilibrium, which revealed normal distribution of the population, were performed by

Table 2. Genotype and allele frequencies (%) of the VEGF -634G>C polymorphism in ovarian, cervical and endometrial cancer patients and controls.

Cancer Types	Patients (n)	Controls (n=106)	p*	OR (95% CI)**	p**
Ovarian	**n=47**				
Genotypes			0.634		
GG	16 (34)	29 (27)		1	
GC	22 (47)	58 (55)		0.68 (0.31-1.50)	0.347
CC	9 (19)	19 (18)		0.86 (0.32-2.33)	0.765
Alleles			0.658		
G	54 (57)	116 (55)		1	
C	40 (43)	96 (45)		0.90 (0.55-1.46)	0.658
Cervical	**n=32**				
Genotypes			0.886		
GG	10 (31.2)	29 (27)		1	
GC	16 (50)	58 (55)		0.80 (0.32-1.98)	0.629
CC	6 (18.8)	19 (18)		0.92 (0.29-2.94)	0.882
Alleles			0.829		
G	36 (56.3)	116 (55)		1	
C	28 (43.7)	96 (45)		0.94 (0.54-1.65)	0.829
Endometrial	**n=21**				
Genotypes			0.06		
GG	11 (52.4)	29 (27)		1	
GC	6 (28.6)	58 (55)		0.27 (0.09-0.81)	**0.015**
CC	4 (19.0)	19 (18)		0.56 (0.15-2.00)	0.364
Alleles			0.153		
G	28 (66.7)	116 (55)		1	
C	14 (33.3)	96 (45)		0.60 (0.30-1.21)	0.153

OR, Odds ratio; CI, confidence interval.

P value < 0.05 is shown in bold.

Values in parentheses are percentages.

* Results of ovarian, cervical and endometrial cancer groups compared with control group.

** Calculations were performed GG vs. GC and CC for VEGF -634.

the chi-square (x^2) test. Then, the differences in genotype distribution and allele frequency among the groups were examined for statistical significance using the Pearson's two-way chi-square (x^2) test. The relationships between genotypes and/or alleles and gynecologic cancer risks were determined by obtaining the odds ratios (ORs) through a logistic regression method [OR, 95% Confidence Interval (CI)]. Adjusted ORs for the other epidemiological covariant factors such as, family history concerning gynecologic and/or other cancer types, stages of tumor, smoking habits and existence of other diseases were determined by using a multivariable logistic regression method. *p* values smaller than 0.05 were considered statistically significant. The whole analysis was conducted by using SPSS V.11.5.

RESULTS

There were no significant deviations from the Hardy-Weinberg equilibrium ($p>0.05$) in the control group and the cancer patients. No significant differences in genotype (ovarian: $p=0.634$; cervical: $p=0.886$; endometrial: $p=0.06$) and allele (ovarian: $p=0.658$; cervical: $p=0.829$; endometrial: $p=0.153$) frequencies for VEGF -634G>C polymorphism were observed between ovarian, cervical and endometrial cancer patients and controls. ORs were calculated by logistic regression analysis in comparison with the most common homozygote genotype observed in study population. A statistically significant difference was found for genotypic frequencies of the -634G>C polymorphism between endometrial cancer patients with GC genotype and those

Table 3. Odds ratios (ORs), in all three types of gynecologic cancer patients, adjusted[a,b,c,d] by multivariable logistic regression analysis of VEGF -634G>C polymorphism.

Cancer Types	OR[a,**] (95% CI)	$p^{a,**}$	OR[b,**] (95% CI)	$p^{b,**}$	OR[c,**] (95% CI)	$p^{c,**}$	OR[d,**] (95% CI)	$p^{d,**}$
Ovarian								
Genotypes								
GG	1		1		1		1	
GC	3.33 (0.34-33.11)	0.374	1.43 (0.18-11.38)	1.000	5.67 (0.62-52.09)	0.208	0.88 (0.20-3.99)	1.000
CC	7.5 (0.65-87.19)	0.116	0.50 (0.06-4.34)	0.602	9.72 (0.92-103.04)	0.054	0.86 (0.12-5.94)	1.000
Cervical								
Genotypes								
GG	1		1		1		1	
GC	0.29 (0.04-2.32)	0.335	2.25 (0.23-22.14)	0.584	0.79 (0.06-10.38)	1.000	0.24 (0.02-2.79)	0.333
CC	0.14 (0.01-1.55)	0.158	4.50 (0.37-54.16)	0.516	0.43 (0.04-5.06)	0.619	0.23 (0.02-2.59)	0.333
Endometrial								
Genotypes								
GG	1		1		1		1	
GC	0.14 (0.02-0.99)	0.069	7.00 (0.71-69.12)	0.099	0.57 (0.07-4.88)	0.625	2.08 (0.19-23.30)	1.000
CC	0.13 (0.01-1.40)	0.141	9.00 (0.66-122.79)	0.118	1.10 (0.08-15.15)	1.000	1.80 (0.09-35.42)	1.000

OR, Odds ratio; CI, confidence interval.

** Calculations were performed GG vs. GC and CC for VEGF -634.

[a] OR adjusted for family history concerning gynecologic and/or other cancer types.

[b] OR adjusted for stages of tumor.

[c] OR adjusted for smoking habits.

[d] OR adjusted for existence of other diseases.

with GG genotype (OR=0.27; 95% CI=0.09-0.81; p=0.015). However, a similar relationship was not found between the GG and CC genotypes in the endometrial cancer patients (OR=0.56; 95% CI=0.15-2.00; p=0.364). The OR per copy of the "C" allele was 0.60 (95% CI=0.30-1.21, p=0.153). In other words, the allelic frequency of the gene was not significantly associated with the endometrial cancer patients.

The distribution of allelic and genotypic frequencies of VEGF -634G>C polymorphism with estimation of ORs in all three types of gynecologic cancer patients and controls are presented in Table 2.

Multivariable logistic regression analysis demonstrated that the occurrences of ovarian, cervical and endometrial cancers were not associated with family history concerning gynecologic and/or other cancer types, stages of tumor, smoking habits and existence of other diseases (p>0.05; Table 3).

DISCUSSION

Neovascularization is important for the progression of cancer which promotes angiogenesis by producing mediators including VEGF. The role of VEGF gene sequence variations in tumor development and progression have not yet been clearly explained. Polymorphisms of the gene may lead to differences in their expression levels among individuals and could potentially contribute to a variety of pathological processes. The -634 G>C polymorphism seems to affect the activity of an internal ribosomal entry site B involved in VEGF translation (Lambrechts et al., 2003). In view of these facts, we aimed to investigate the role of VEGF -634G>C polymorphism in the development of ovarian, cervical and endometrial cancers in the Turkish population.

The functionality of the VEGF -634G>C polymorphism has not been clearly defined. Watson et al. (2000) reported that the polymorphism was significantly correlated with lipopolysaccharide-stimulated VEGF production where highest production was observed for the GG genotype and the polymorphism was probably included in a potential binding site of the myeloid zinc finger (MZF1) transcription factor. VEGF -634GG genotype was found to be associated with the increased VEGF production, leading to abnormal neovascularization in the immature preterm eye (Cooke et al., 2004). On the contrary, significantly higher VEGF levels were found in control

subjects with the CC genotype rather than in those with the other genotypes (Awata et al., 2002). It was also revealed in the same study that genotype distribution of the -634G>C polymorphism differed significantly between patients without retinopathy and with any retinopathy and the C allele was significantly associated with the presence of retinopathy. -634G>C polymorphism in the 5′UTR of the VEGF gene was defined by Awata et al. as a novel genetic risk factor for diabetic retinopathy. In addition, Rueda et al. (2005b) demonstrated that VEGF -634G allele was significantly more frequent among giant cell arteritis (GCA) patients with severe ischemic complications compared with GCA patients not affected by ischemic events or with controls. Therefore, they suggested that there was a potential implication of the VEGF -634G>C polymorphism in the development of severe ischemic manifestations of GCA. Boiardi et al. (2003) also reported that carriers of 634C and 18 bp insertion alleles were associated with susceptibility to developing GCA. It was reported that polymorphisms of the VEGF gene may contribute to disease severity in ankylosing spondylitis (Seo et al., 2005). As the VEGF gene is very highly polymorphic, there is the possibility that if the impact of a single polymorphism is not overwhelming, the net effect of multiple polymorphisms may determine the production of VEGF, thus affecting the clinical manifestations. In Japanese Kawasaki Disease (KD) patients with coronary artery lesion (CAL) showed significantly higher frequencies of the G allele in VEGF -634G>C polymorphism and of the A1 allele in kinase insert domain receptor (KDR) +4422 (AC) 11-14 dinucleotide repeat polymorphism in intron 2 (Kariyazono et al., 2004). This finding suggested that VEGF and its receptor (KDR) genes contributed to the development of CAL in KD patients. Furthermore, it was showed that Behcet's disease (BD) occurred more frequently in the carriers of -634C and 18 bp insertion (I) alleles, which was associated with a higher production of the VEGF protein (Salvarani et al., 2004). On the other hand, the polymorphism is not associated with BD in the Korean population, but the CC genotype at -634 polymorphism revealed slight tendency to be protective against ocular inflammation in BD (Nam et al., 2005). The analyzed VEGF -634 polymorphism may not play a relevant role in Rheumatoid Arthritis (RA) pathogenesis in Spanish Caucasian population (Rueda et al., 2005a) and Korean population (Han et al., 2004). Another study showed that there was no significant association between spontaneous preterm delivery and -634 genotypes (Papazoglou et al., 2004).

VEGF polymophisms likely contribute to the pathogenesis of endometriosis and may become useful markers for predicting endometriosis susceptibility (Hsieh et al., 2004). However, the distribution of genotype and allele frequencies of the VEGF -460C>T polymorphism, in the 5′UTR, in the endometriosis groups was not found statistically significant in the Korean and South Indian populations (Bhanoori et al., 2005; Kim et al., 2005). The polymorphism of the VEGF gene selected in our study is the -634 gene region and some studies (Koukourakis et al., 2004; Jin et al., 2005) have analyzed this polymorphism in relation to different types of cancer. Analysis of VEGF polymorphism at -634 gene region revealed that the -634GG was linked with low VEGF expression and poor vascular density compared to the -634GC in non-small cell lung cancer (Koukourakis et al., 2004). In our study, we found that the most common genotype in Turkish patients who had ovarian and cervical cancer was GC heterozygote (47% and 50% of the patients, respectively). In the endometrial cancer group, GG homozygote was the most common genotype (52.4% of the patients). We found no statistically significant difference in the genotype and allele frequencies between the patients and controls (p>0.05). Allelic and genotypic frequencies were in close agreement with those previously published for other Caucasian populations studied (Howell et al., 2002; Lambrechts et al., 2003; Rueda et al., 2005a). A statistically significant difference was observed for genotypic frequencies of the -634G>C polymorphism between patients with GC genotype and those with GG genotype (p= 0.015), although this argument is not clinically significant due to the negligible OR (0.27). The -634CC genotype was found to be significantly associated with high tumor aggressiveness in breast cancer (Jin et al., 2005). However, we did not find a relationship between any of the three types of gynecologic cancer and tumor stages. The discrepancy between the findings of our study and those of Jin et al. (2005) might have stemmed from the fact that the mechanism by which the -634G>C polymorphism affects serum levels of VEGF, could be different during the progression of breast cancer than during that of gynecologic cancers. Another possible explanation of the absence of association between this SNP and the tumor stages of gynecologic cancers could be linkage disequilibrium between this SNP and another

functional variant elsewhere in the VEGF sequence. Furthermore, we did not find any statistically significant differences between family history concerning gynecologic and/or other cancer types, smoking habits, existence of other diseases and genotypes in any of the three types of gynecologic cancer (p>0.05). We were unable to compare our results with those of similar studies conducted on other ethnic populations, since the relationship between the VEGF -634G>C polymorphism and ovarian, cervical and endometrial cancers has not been a subject of a previous investigation.

In conclusion, the results of this study as regards to the ovarian, cervical and endometrial cancers observed in the Turkish population suggest that the -634G>C VEGF genetic variants do not play a relevant role in their pathogenesis. Our results are expected to contribute to the efforts to better understand the phenotypic effects of SNPs, which are based on direct genetic effects, gene-gene and gene-environment interactions. In addition, it may be worthwhile to investigate the possible association of VEGF gene receptor(s) polymorphisms with gynecologic cancers and measure protein levels in relation to genotyping. Our data did not point to a significant role of the VEGF -634G>C polymorphism in the pathogenesis of ovarian, cervical and endometrial cancers. Nevertheless, this study is the first to demonstrate the relationship between them. Further studies to be conducted on other populations by employing larger sample sizes should be expected to shed more light on the issue.

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