in vivo *24*: 297-302 (2010)

Genetic Variants of Vascular Endothelial Growth Factor and Risk for the Development of Endometriosis

RUKSET ATTAR[1], BEDIA AGACHAN[2], SIBEL BULGURCUOGLU KURAN[3], BAHAR TOPTAS[2], ILHAN YAYLIM ERALTAN[2], ERKUT ATTAR[3] and TURGAY ISBIR[2]

[1]*Department of Obstetrics and Gynecology, Yeditepe University Hospital, Istanbul, Turkey;*
[2]*Department of Molecular Medicine, Institute for Experimental Medicine, Istanbul University, Istanbul, Turkey;*
[3]*Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility, Istanbul University, Istanbul Medical School, Istanbul, Turkey*

Abstract. *Backround/Aims: Endometriosis is regarded as a complex disese, in which genetic and environmental factors contribute to the disease phenotype. Whether vascular endothelial growth factor (VEGF) –460 C/T and +405 G/C polymorphisms are associated with susceptibility to endometriosis was investigated. Patients and Methods: Diagnosis of endometriosis was made on the basis of laparoscopic findings. Stage of endometriosis was determined according to the Revised American Fertility Society classification. Sixty out of the 112 women enrolled had no endometriosis, 11 had mild or early-stage endometriosis and 41 had severe endometriosis. Polymerase chain reaction (PCR), restriction fragment length polymorphism and agarose gel electrophoresis techniques were used to determine the –460 C/T and +405 G/C genotypes. Results: The VEGF +405 G/C genotype frequencies among the cases and controls were CC 55.8% and 35%; GC 30.8% and 50.0%; GG 13.5% and 15.0%, respectively. The allelic frequencies were C 71.15% (cases) and 60.0% (controls) and G 28.8% (cases) and 40% (controls). Patients with endometriosis had a higher incidence of the VEGF +405 CC genotype compared with the controls (p=0.027). Women with VEGF +405 CC genotype had 2.3-fold higher risk for endometriosis. VEGF +405 GC genotype and G allele in the control group was higher than the endometriosis group (p=0.039, p=0.027 respectively). The VEGF –460 C/T genotype frequencies among the cases were CC 21.2%, CT 26.9% and TT 51.9%; the C and T allelic frequencies were 34.6% and 65.3%, respectively. The VEGF –460 genotype frequencies among the controls were CC 31.70%, CT 18.3% and TT 50.0%; the C and T allelic frequencies were 40.8% and 59.1%, respectively (p>0.05). There was linkage disequilibrium between VEGF –460 C/T and +405 G/C polymorphisms (D': 0.197, r^2=0.013). We observed that the VEGF 460T/405C haplotype frequency was significantly higher in patients compared to controls (p=0.011). Conclusion: Our data suggest that the CC genotype of VEGF +405 and 460T/405C haplotypes of VEGF may be associated with the risk of endometriosis, but the G allele of VEGF +405 appears to be protective against endometriosis.*

Correspondence to: Associated Professor Bedia Agachan, Institute for Experimental Medicine (DETAE), Department of Molecular Medicine, Capa 34390 Istanbul, Turkey. Tel: +90 2126351959, Fax: +90 2126351959, e-mail: agachanb@yahoo.com

Key Words: VEGF, polymorphism, endometriosis, RFLP.

Endometriosis is characterized by the presence of endometrial tissue outside the uterus, more commonly found in the form of implants in viscera and in the peritoneum of the pelvic cavity (1). It is a multifactorial and polygenic disease in which angiogenesis may be implicated (2-6). Angiogenesis is under the control of numerous inducers, including those of the vascular endothelial growth factor (*VEGF*) family (4). *VEGF*, also known as vascular permeability factor, is a key mediator in neoangiogenesis. It stimulates endothelial cell proliferation and migration, and increases vascular permeability (7, 8). It is expressed in human uterine epithelial and stromal cells and is regulated by estrogen (9, 10). Therefore it may have a pivotal role in the development and progression of endometriosis. *VEGF* is localized in the epithelium of endometriotic implants, particularly in red coloured lesions of endometriosis (3, 4). Several studies have reported an increase in VEGF levels in the peritoneal fluid and serum of endometriosis patients and it has been suggested that *VEGF* plays a role in the progression of the disease (3, 6, 11-13).

The *VEGF* gene is located on chromosome 6p21.3 (14) and consists of eight exons exhibiting alternate splicing which form a family of proteins. Several transcription factor-binding sites are found in the *VEGF* 5'-untranslated region

and transcriptional regulation of the gene is complex (15). Polymorphisms within the 5'-untranslated region lead to differences in *VEGF* expression between individuals and may influence the aetiology of a variety of pathological conditions with which *VEGF* has been associated (16).

Several polymorphisms have been described for the *VEGF* gene. Among them *VEGF* –460 C/T, +405 G/C, and 936 C/T polymorphisms have been associated with variation in VEGF protein production (17, 18). They also have been related to several diseases in which angiogenesis is involved (19-21).

Researchers have investigated the association between endometriosis and *VEGF* –460 C/T and +405 G/C polymorphisms in South Indian, Korean, Japanese, Chinese and Caucasian women (16, 22-25). However, the analysis of genotype and allelic frequencies demonstrated a clear discordance among these reports compared with women without endometriosis.

Bhanoori *et al*. reported that patients with endometriosis had a higher frequency of the +405G allele (16), However Kim *et al*. and Gentini *et al*. reported that patients with endometriosis had a higher frequency of the +405C allele (21, 25) and Ikuhashi *et al*. reported similar frequencies in their study (23). In the majority of the studies, no association was found between the -460C/T polymorphism and endometriosis (16, 22,23). However, Hsieh *et al*. (24) reported that the –460T allele was associated with higher susceptibility to endometriosis. Furthermore, a positive association was found between the *VEGF* 936T allele and severe endometriosis in a Japanese population (23), but not in a Korean population (26).

The aim of the present study was to evaluate *VEGF* +405 G/C and –460 C/T polymorphisms in a group of women with and without endometriosis in a Turkish population.

Patients and Methods

One hundred and twelve women of reproductive age undergoing laparoscopy at the Obstetrics and Gynecology Department of I.U. Istanbul Medical School were included in the study. Sixty women did not have endometriosis. Eleven women had mild or early stage endometriosis and forty-one women had severe endometriosis. Indications for laparoscopy were unexplained infertility, pelvic pain, ultrasonographically identified adnexal mass, or tubal ligation. Diagnosis of endometriosis was made on the basis of laparoscopic findings. The revised American Fertility Society staging system (also known as the Revised American Society for Reproductive Medicine Classification of Endometriosis, 1996) was used for staging (1997) (27).

Information on medical, gynaecological and obstetric history, and sociodemographic parameters was obtained from each participant. All participants signed an informed consent form before enrollment and Institutional Ethical Committee approval was obtained for the study.

Genotyping. Genomic DNA was extracted from 10 ml of EDTA anti-coagulated whole blood by the salting-out method (28). Individuals were genotyped for both *VEGF* polymorphisms as described previously (17). Briefly, for the *VEGF* +405 (G/C) polymorphism, the sense and antisense primers were 5'-ATTTATTTTTGCTTGCCATT-3' and 5'-GTCTGTCTGTCTGTCCGTCA-3', respectively. Cleavage of the 304 bp amplified product by the *Bsm*FI restriction enzyme in 193 and 111 bp confirms the presence of the G allele. For the *VEGF* –460 (C/T) polymorphism, the sense and antisense primers were 5'-TGTGCGTGTGG GGTTGAGCG-3' and 5'-TACGTGCGGACA GGGCCTGA-3'. 175-bp fragment containing the polymorphic site and a cleavage site for the *BsrUI* restriction enzyme was amplified. Digestion produced fragments of 155 bp and 20 bp in the presence of the C allele.

Table I. *Distribution of VEGF –460 C/T and +405 G/C polymorphisms in endometriosis patients and controls*.

Genotype/ Alleles	Controls (n=60)		Patients (n=52)		*p*-Value
	n	%	n	%	
VEGF 405					
CC	21	35.0	29	55.8	0.02
GC	30	50.0	16	30.8	0.03
GG	9	15.0	7	13.5	0.81
C	72	60.0	74	71.1	
G	48	40.0	30	28.8	0.08
VEGF 460					
TT	30	50.0	27	51.9	0.83
CT	11	18.3	14	26.9	0.27
CC	19	31.7	11	21.2	0.21
T	71	59.1	68	65.3	
C	49	40.8	36	34.6	0.33

Statistical analysis. Statistical analyses were performed with SPSS (8.0) for windows. Distributions of genotypes and haplotypes were compared using the Chi-square test. Linkage disequilibrium among *VEGF* +405 and –406 polymorphisms was assessed using D' and r^2 values obtained through the Haploview program (http://www.broad.mit.edu/mpg/haploview/documentation.php). The ratios, genotypic and allelic distribution among participants were analyzed with Chi-square and Fisher's exact tests. Statistical significance was accepted at $p<0.05$.

Results

The frequencies of *VEGF* +405 and –406 alleles and genotypes are summarized in Table I. The *VEGF* +405 genotype frequencies among the cases and controls were CC 55.8% and 35%; GC 30.8% and 50.0%; GG 13.5% and 15.0%, respectively. The allele frequencies were C 71.15% (cases) and 60% (controls) and G 28.84% (cases) and 40% (controls). Patients with endometriosis showed a higher incidence of the +405 CC genotype compared with the controls (55.8% *versus* 35%). The difference was statistically significant ($p=0.027$, $\chi^2=4.86$, odds ratio, OR=2.34, 95% confidence interval, CI=1.09–5.01). Women with *VEGF* +405 CC genotype had 2.3-fold higher risk for endometriosis. The frequency of the +405 GC genotype in

Table II. *The frequencies of VEGF –460 C/T and +405 G/C haplotypes in patients and controls.*

Number of haplotype	Haplotype associations	Frequency			Chi-squared	*p*-Value
		Overall	All patients	Controls		
1	T:C	0.378	0.466	0.302	6.388	0.0115
2	C:C	0.273	0.245	0.298	0.779	0.3775
3	T:G	0.242	0.187	0.289	3.163	0.0753
4	C:G	0.106	0.101	0.111	0.053	0.8182

The order of haplotypes is VEGF –460 C/T and VEGF +405 G/C.

the control group was higher than in the endometriosis group (50.0% *versus* 30.8%). The difference was statistically significant (p=0.039, χ^2=4.25, OR=0.44, 95% CI=0.20-0.096). Women with the +405 GC genotype had 2.25-fold lower risk for endometriosis. The frequency of the G allele in the control group was significantly higher than the endometriosis group (p=0.08 χ^2=3.05).

The *VEGF* –460 C/T genotype frequencies among the cases were CC 21.2%, CT 26.9% and TT 51.9%; the C and T allelic frequencies were 34.6% and 65.3%, respectively. The *VEGF* –460 C/T genotype frequencies amongst the controls were CC 31.70%, CT 18.3% and TT 50.0%; the C and T allele frequencies were 40.8% and 59.1%, respectively. There was no statistically significant difference in the genotype distributions or allelic frequencies of *VEGF* –460 C/T between the cases and controls.

In addition to SNP analyses, haplotypes were evaluated for association with endometriosis (Table II). There was a linkage disequilibrium between *VEGF* –460 C/T and +405 G/C polymorphisms (D': 0.197, r^2=0.013). Haplotype analysis confirmed the association of *VEGF* –460 C/T and +405 G/C gene variants with endometriosis and revealed that the frequency of only one haplotype, *VEGF* 460T/405C, in the endometriosis group was significantly higher than the control group (p=0.0115). The frequency of the 460T/ 405G in the endometriosis group was lower than in the control group, but the difference was not significant.

We also investigated the distribution of *VEGF* –460 C/T and +405 G/C genotypes according to the stages of the endometriosis. We did not find any association between the distribution of *VEGF* –460 C/T and +405 G/C genotypes and endometriosis stage (p>0.05) (Table III).

Table III. *Distribution of VEGF –460 C/T and +405 G/C polymorphism according to the severity of endometriosis.*

Genotype/ Alleles	Early stage		Late stage		*p*-Value
	N	%	N	%	
VEGF +405					
CC	7	63.6	22	53.7	
GC	2	18.2	14	34.1	
GG	2	28.2	5	12.2	0.57
VEGF –460					
TT	6	54.5	21	51.2	
CT	2	18.2	12	29.3	
CC	3	27.3	8	19.5	0.71

Discussion

Several reports have demonstrated that SNPs of the *VEGF* gene are associated with VEGF synthesis. However, it is unclear how the polymorphisms in the untranslated region of the *VEGF* gene influence its protein production (16-26). Individuals with a specific SNP of *VEGF* may have a higher risk of developing endometriosis as a result of increased expression of *VEGF* in various cells. High concentrations of VEGF in the peritoneal fluid due to an increase in VEGF production by activated peritoneal macrophages may be a critical process in the pathogenesis of endometriosis (18, 26). An increase in VEGF expression in eutopic endometrial cells may also lead to implantation and proliferation of endometrial cells at ectopic sites through retrograde menstruation (3, 5, 22).

In the present study, we investigated *VEGF* –460 C/T and +405 G/C polymorphisms in women with and without endometriosis in a Turkish population. We found that genotype distribution of the *VEGF* +405 C/G polymorphism was significantly different between patients with and without endometriosis. While women with the *VEGF* +405 CC genotype had a significantly increased risk of endometriosis compared with those without this genotype, women with the *VEGF* +405 G allele had a protective effect against endometriosis. We observed that there was linkage disequilibrium between *VEGF* –460 C/T and +405 G/C polymorphisms. We found that the *VEGF* 460T/405C haplotype frequency in the the endometriosis group was significantly higher than the control group. This result is in discrepancy with the previous report by Stevens *et al*. which

demonstrated that the +460C/–405G haplotype was associated with higher promoter activity and higher *VEGF* expression than the +460T/–405C haplotype (29). The discrepancy may be due to racial differences.

Our findings are consistent with Kim *et al*. and Gentilini *et al*. who demonstrated a significant assocation between the *VEGF* +405 C/G polymorphism and susceptibility to endometriosis (22, 25). They analysed the genotype distribution of the *VEGF* +405 C/G polymorphism and confirmed a statistically significant association between endometriosis and the +405 C/G polymorphism. They stated that the risk of endometriosis was significantly higher in women carrying the C allele.

VEGF +405 G/C polymorphism is known to affect VEGF production *in vivo* and *in vitro*, and this effect seems to be dependent on the cellular population involved (17, 30). However, it is still unclear whether the C variant has a dominant effect on *VEGF* gene expression as different studies report conflicting results (17, 30, 31). Watson *et al*. demonstrated that the genotype for the +405 polymorphism in the *VEGF* gene is significantly correlated with VEGF production from stimulated peripheral blood mononuclear cells (17). They reported that a G allele at position +405 affects transcriptional activity and increases *VEGF* production in peripheral blood mononuclear cells in response to lipopolysaccharide. They also showed a dose-dependent effect of the G allele: highest VEGF protein production was recorded for the GG genotype, intermediate for GC and the lowest for the CC genotype. Stevens *et al*. suggested that the –460C/+405G haplotype was associated with higher promoter activity, and therefore higher *VEGF* expression, than the –460T/+405C haplotype (29).

In contrast, Awata *et al*. demonstrated the association of the +405 CC genotype with a higher serum VEGF concentration in a normal Japanese population (30). Mueller *et al*. showed that transfection analysis of the human *VEGF* promoter revealed that estrogen has a direct transcriptional effect on *VEGF* gene expression and that estrogen-regulated transcription requires a variant estrogen response element (10). The *VEGF* +405 site is located adjacent to the +410 estrogen response element and carriage of the –460/+405 polymorphism significantly alters *VEGF* promoter activity and responsiveness (29). Therefore Stevens *et al*. suggested that the +405 polymorphism itself has an influence on the transcriptional activity by possible alteration of the response to estrogen. The discrepancy between the studies might be due to racial differences, as all of the studies were carried out in different ethnic groups.

We also investigated the *VEGF* –460 C/T polymorphism. An association between the *VEGF* –460 C/T polymorphism and endometriosis was not established. This result is in accordance with most of the previous reports (16, 22, 23). +405 G/C polymorphisms and the severity of endometriosis were also examined. No significant association between the presence of the C allele and the severity of the disease was found. The presence of the C allele could represent a risk factor for the implantation of endometrial fragment refluxed in the peritoneal cavity while having no effect on the development of an invasive form of the disease (22). However, our sample size was calculated based on the primary aim of the study, which was to compare the frequency of the polymorphism in women with and without endometriosis in general. Therefore, our sample size might be insufficient to draw firm conclusions regarding specific subgroups.

In conclusion, the results from the present study suggest that *VEGF* +405 CC genotype and 460T/405C haplotypes are associated with an increased susceptibility to endometriosis. On the other hand, the G allele of *VEGF* +405 may be protective against endometriosis.

To the best of our knowledge, this is the first study to demonstrate a relationship between *VEGF* +405G/C polymorphism and susceptibility to endometriosis in a Turkish population. Further larger sample size studies are required to confirm our findings.

References

1 D'Amora P, Sato H, Girão MJ, Silva ID and Schor E: Polymorphisms in exons 1B and 1C of the type I interleukin-1 receptor gene in patients with endometriosis. Am J Reprod Immunol *56*: 178-184, 2006.

2 Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB and Taylor RN: Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab *81*: 3112-3118, 1996.

3 Donnez J, Smoes P, Gillerot S, Casanas-Roux F and Nisolle M: Vascular endothelial growth factor (*VEGF*) in endometriosis. Hum Reprod *13*: 1686-1690, 1998.

4 McLaren J: Vascular endothelial growth factor and endometriotic angiogenesis. Hum Reprod Update *6*: 45-55, 2000.

5 Tan XJ, Lang JH, Liu DY, Shen K, Leng JH and Zhu L: Expression of vascular endothelial growth factor and thrombospondin-1 mRNA in patients with endometriosis. Fertil Steril *78*: 148-153, 2002.

6 Gilabert-Estellés J, Ramón LA, España F, Gilabert J, Vila V, Réganon E, Castelló R, Chirivella M and Estellés A: Expression of angiogenic factors in endometriosis: relationship to fibrinolytic and metalloproteinase systems. Hum Reprod *22*: 2120-2127, 2007.

7 Risau W: Mechanisms of angiogenesis. Nature *386*: 671-674, 1997.

8 Ferrara N: Molecular and biological properties of vascular endothelial growth factor. J Mol Med *77*: 527-543, 1999.

9 Hyder SM, Stancel GM, Chiappetta C, Murthy L, Boettger-Tong HL and Makela S: Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. Cancer Res *56*: 3954-3960, 1996.

10 Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC and Taylor RN: Regulation of vascular endothelial growth factor (*VEGF*) gene transcription by estrogen receptors alpha and beta. Proc Natl Acad Sci USA *97*: 10972-10977, 2000.

11 Fasciani A, D'Ambrogio G, Bocci G, Monti M, Genazzani AR and Artini PG: High concentrations of the vascular endothelial growth factor and interleukin-8 in ovarian endometriomata. Mol Hum Reprod *6*: 50-54, 2000.
12 Takehara M, Ueda M, Yamashita Y, Terai Y, Hung YC and Ueki M: Vascular endothelial growth factor A and C gene expression in endometriosis. Hum Pathol *35*: 1369-1375, 2004.
13 Mahnke JL, Dawood MY and Huang JC: Vascular endothelial growth factor and interleukin-6 in peritoneal fluid of women with endometriosis. Fertil Steril *73*: 166-170, 2000.
14 Vincenti V, Cassano C, Rocchi M and Persico G: Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation *93*: 1493-1495, 1996.
15 Akiri G, Nahari D, Finkelstein Y, Le SY, Elroy-Stein O and Levi BZ: Regulation of vascular endothelial growth factor (*VEGF*) expression is mediated by internal initiation of translation and alternative initiation of transcription. Oncogene *17*: 227-236, 1998.
16 Bhanoori M, Arvind Babu K, Pavankumar Reddy NG, Lakshmi Rao K, Zondervan K, Deenadayal M, Kennedy S and Shivaji S: The vascular endothelial growth factor (*VEGF*) 405G>C 50-untranslated region polymorphism and increased risk of endometriosis in South Indian women: a case-control study. Hum Reprod *20*: 1844-1849, 2005.
17 Watson CJ, Webb NJ, Bottomley MJ and Brenchley PE: Identification of polymorphisms within the vascular endothelial growth factor (*VEGF*) gene: correlation with variation in *VEGF* protein production. Cytokine *12*: 1232-1235, 2000.
18 Renner W, Kotschan S, Hoffmann C, Obermayer-Pietsch B and Pilger E: A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels. J Vasc Res *37*: 443-448, 2000.
19 Lin CC, Wu HC, Tsai FJ, Chen HY and Chen WC: Vascular endothelial growth factor gene –460 C/T polymorphism is a biomarker for prostate cancer. Urology *62*: 374-377, 2003.
20 Krippl P, Langsenlehner U, Renner W, Yazdani-Biuki B, Wolf G, Wascher TC, Paulweber B, Haas J and Samonigg H: A common 936 C/T gene polymorphism of vascular endothelial growth factor is associated with decreased breast cancer risk. Int J Cancer *106*: 468-471, 2003.
21 Lee SJ, Lee SY, Jeon HS, Park SH, Jang JS, Lee GY, Son JW, Kim CH, Lee WK, Kam S, Park RW, Park TI, Kang YM, Kim IS, Jung TH and Park JY: Vascular endothelial growth factor gene polymorphisms and risk of primary lung cancer. Cancer Epidemiol Biomarkers Prev *14*: 571-575, 2005.
22 Kim SH, Choi YM, Choung SH, Jun JK, Kim JG and Moon SY: Vascular endothelial growth factor gene 405 C/G polymorphism is associated with susceptibility to advanced-stage endometriosis. Hum Reprod *20*: 2904-2908, 2005.
23 Ikuhashi Y, Yoshida S, Kennedy S, Zondervan K, Takemura N, Deguchi M, Ohara N and Maruo T: Vascular endothelial growth factor 936 C/T polymorphism is associated with an increased risk of endometriosis in a Japanese population. Acta Obstet Gynecol Scand *86*: 1352-1358, 2007.
24 Hsieh YY, Chang CC, Tsai FJ, Yeh LS, Lin CC and Peng CT: T allele for *VEGF* gene –460 polymorphism at the 50-untranslated region: association with a higher susceptibility to endometriosis. J Reprod Med *49*: 468-472, 2004.
25 Gentilini D, Somigliana E, Vigano P, Vignali M, Busacca M and Di Blasio AM: The vascular endothelial growth factor 405G>C polymorphism in endometriosis. Hum Reprod *23*: 211-215, 2008.
26 Kim JG, Kim JY, Jee BC, Suh CS, Kim SH and Choi YM: Association between endometriosis and polymorphisms in endostatin and vascular endothelial growth factor and their serum levels in Korean women. Fertil Steril *89*: 243-245, 2008.
27 Revised American Society for Reproductive Medicine classification of endometriosis: Fertil Steril *67*: 817-821, 1996.
28 Miller SA, Dykes DD and Polesky HF: A simple salting-out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res *16*: 1215, 1988.
29 Stevens A, Soden J, Brenchley PE, Ralph S and Ray DW: Haplotype analysis of the polymorphic human vascular endothelial growth factor gene promoter. Cancer Res *63*: 812-816, 2003.
30 Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, Inukai K, Inoue I and Katayama S: A common polymorphism in the 50-untranslated region of the *VEGF* gene is associated with diabetic retinopathy in type 2 diabetes. Diabetes *51*: 1635-1639, 2002.
31 Koukourakis MI, Papazoglou D, Giatromanolaki A, Bougioukas G, Maltezos E and Sivridis E: *VEGF* gene sequence variation defines *VEGF* gene expression status and angiogenic activity in non-small cell lung cancer. Lung Cancer *46*: 293-298, 2004.

Received November 2, 2009
Revised April 8, 2010
Accepted April 16, 2010