Abstract. Background/Aims: Endometriosis is regarded as a complex disease, 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 genotype frequencies among the cases 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, r²=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.

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 anticoagulated 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’-ATTATTATTGTGTTGCATT-3’ and 5’-GTGCTGCTTCTGCTCGTCA-3’, respectively. Cleavage of the 304 bp amplified product by the BsmFI 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’-TGTGCCGTGTGG GTTTGAGCG-3’ and 5’-TAGTGGCGGACA 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.

**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 –460 polymorphisms was assessed using D’ and r² 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 –460 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, χ²=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 the endometriosis. The frequency of the +405 GC genotype in

<table>
<thead>
<tr>
<th>Genotype/ Alleles</th>
<th>Controls (n=60)</th>
<th>Patients (n=52)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF 405</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>21</td>
<td>29</td>
<td>0.02</td>
</tr>
<tr>
<td>GC</td>
<td>30</td>
<td>16</td>
<td>0.03</td>
</tr>
<tr>
<td>GG</td>
<td>9</td>
<td>7</td>
<td>0.81</td>
</tr>
<tr>
<td>C</td>
<td>72</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>48</td>
<td>30</td>
<td>0.08</td>
</tr>
<tr>
<td>VEGF 460</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>30</td>
<td>27</td>
<td>0.83</td>
</tr>
<tr>
<td>CT</td>
<td>11</td>
<td>14</td>
<td>0.27</td>
</tr>
<tr>
<td>CC</td>
<td>19</td>
<td>11</td>
<td>0.21</td>
</tr>
<tr>
<td>T</td>
<td>71</td>
<td>68</td>
<td>0.33</td>
</tr>
<tr>
<td>C</td>
<td>49</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>
the control group was higher than in the endometriosis group (50.0% versus 30.8%). The difference was statistically significant ($p=0.039$, $\chi^2=4.25$, 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 ($p=0.08 \chi^2=3.05$).

The $\text{VEGF} –460 \ C/T$ 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 $\text{VEGF} –460 \ C/T$ 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 $\text{VEGF} –460 \ C/T$ 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 $\text{VEGF} –460 \ C/T$ and +405 G/C polymorphisms ($D'=0.197$, $r^2=0.013$). Haplotype analysis confirmed the association of $\text{VEGF} –460 \ C/T$ and +405 G/C gene variants with endometriosis and revealed that the frequency of only one haplotype, $\text{VEGF} 460T/405C$, 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 $\text{VEGF} –460 \ C/T$ and +405 G/C genotypes according to the stages of the endometriosis. We did not find any association between the distribution of $\text{VEGF} –460 \ C/T$ and +405 G/C genotypes and endometriosis stage ($p>0.05$) (Table III).

### Discussion

Several reports have demonstrated that SNPs of the $\text{VEGF}$ gene are associated with VEGF synthesis. However, it is unclear how the polymorphisms in the untranslated region of the $\text{VEGF}$ gene influence its protein production (16-26). Individuals with a specific SNP of $\text{VEGF}$ may have a higher risk of developing endometriosis as a result of increased expression of $\text{VEGF}$ in various cells. High concentrations of $\text{VEGF}$ 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 $\text{VEGF} –460 \ C/T$ and +405 G/C polymorphisms in women with and without endometriosis in a Turkish population. We found that genotype distribution of the $\text{VEGF} +405 \ C/G$ polymorphism was significantly different between patients with and without endometriosis. While women with the $\text{VEGF} +405 \ CC$ genotype had a significantly increased risk of endometriosis compared with those without this genotype, women with the $\text{VEGF} +405 \ G$ allele had a protective effect against endometriosis. We observed that there was linkage disequilibrium between $\text{VEGF} –460 \ C/T$ and +405 G/C polymorphisms. We found that the $\text{VEGF} 460T/405C$ haplotype frequency in 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 association 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

VEGF Polymorphism in Endometriosis