Involvement of vascular endothelial growth factor -460 C/T, +405 G/C and +936 C/T polymorphisms in the development of endometriosis

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Abstract. There are inconsistent data on the contribution of vascular endothelial growth factor (VEGF) -460 C/T (rs833061), +405 G/C (rs2010963) and +936 C/T (rs3025039) single-nucleotide polymorphisms (SNPs) to endometriosis in different ethnicities. Therefore, using high-resolution melting curve analysis, the present study examined the distribution of these SNPs in females with endometriosis-related infertility and a control group. None of the three VEGF SNPs were associated with endometriosis-related infertility in the dominant and recessive models. The lowest P-values of the trend were observed for the VEGF +936 C/T (rs3025039) SNP in endometriosis-related infertility (Pcorr=0.149). Similarly, haplotype analyses of VEGF SNPs did not demonstrate any SNP combination as a risk for endometriosis-related infertility, and the lowest overall P-values, P=0.141 and Pcorr=0.395, were observed for a haplotype (TGT) of the above SNPs. Taken together, these results did not demonstrate the contribution of VEGF C/T, +405 G/C and +936 C/T SNPs to endometriosis-related infertility.

Introduction

Endometriosis is common disorder of the female reproductive organs that is attributed to the existence of functional endometrial tissue outside the uterine cavity, most frequently within the pelvic or abdominal cavity (1). This disorder develops in 3-10% of females of the reproductive age and can be responsible for infertility in 30-50% of females with this condition (1-3). The development and progression of endometriosis may be supported by the abnormal expression of genes, such as those encoding immune components, proteins regulating estrogen and progesterin activity, cell growth factors and angiogenic proteins (4-9).

The vascular endothelial growth factor (VEGF) (10) is primarily involved in angiogenesis and is associated with various mechanisms encompassing action on endothelial cells, such as proliferation, survival and migration (11). VEGF was first discovered as a specific mitogen of endothelial cell (12), although it is biosynthesized by various cells, including keratinocytes, macrophages, platelets, mesangial cells in the kidney and malignant cells (11,13-16). As VEGF-induced angiogenesis is an integral step in the pathogenesis of endometriosis, the survival of endometrial implants is primarily dependent on a sufficient supply of blood (17-19). The early growth of endometrial implants is characterized by a pink-red appearance resulting from its increased vascular density (20,21). Furthermore, peritoneal fluid from females with endometriotic lesions shows increased levels of different angiogenic growth compounds and decreased concentrations of anti-angiogenic factors (22).

Although the association of various VEGF polymorphisms with the development of endometriosis in various ethnicities has been evaluated, the data are inconsistent between different studies (23-29). Therefore, the present study aimed to investigate the distribution of VEGF -460 C/T (rs833061), +405 G/C (rs2010963) and +936 C/T (rs3025039) single-nucleotide polymorphisms (SNPs) in females with endometriosis-related infertility and a control group.

Materials and methods

Study subjects. Peripheral blood samples from females with endometriosis and control females were collected from the Gynecologic and Obstetrical University Hospital, Division of Reproduction at Poznan University of Medical Sciences, Poland. The studied females included two groups: 154 were included in the infertile endometriosis group and 385 were used as the fertile control group (Table I). The stage of endometriosis was assessed according to the revised classification of the American Society for Reproductive Medicine (30). All the included patients with endometriosis and the controls had
a laparoscopic and histologically-confirmed diagnosis of endometriosis. The fertile females assigned to the control group exhibited chronic pelvic pain without any pelvic abnormalities determined by laparoscopy and were diagnosed as having varicose veins in the pelvic floor but no signs of past or present inflammation. The inclusion and exclusion criteria for the patients with endometriosis and the fertile control females were previously described in detail (31). The patients and controls were matched for age and were all Caucasians of Polish descent (Table I). Written informed consent was obtained from all the participating individuals. The study procedures were approved by the local Ethical Committees of Poznan University of Medical Sciences and were carried out in accordance with the code of ethics of the Declaration of Helsinki.

**VEGF polymorphism evaluation.** Genomic DNA was isolated from peripheral blood leukocytes by salt extraction. SNPs for genotyping were selected based on previous case-control studies (23-29). DNA samples were genotyped for three SNPs, -460 C/T (rs833061), +405 G/C (rs2010963) and +936 C/T (rs3025039), in VEGF. The genotyping was performed by a high-resolution melting curve analysis using the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) (Table II). The genotyping quality was evaluated by repeated genotyping of 10% randomly selected samples.

**Statistical analysis.** For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed by Pearson's goodness-of-fit $\chi^2$ statistic. Differences in the allele and genotype frequencies between the cases and controls were computed using Fisher's exact test. The SNPs were studied for associations with endometriosis using the Cochran-Armitage trend test. The odds ratio (OR) and associated 95% confidence intervals (95% CI) were also assessed. The data were analyzed under recessive and dominant inheritance models. Pair-wise linkage disequilibrium (LD) between the selected SNPs was computed as D' and $r^2$ values using HaploView 4.0 software (http://www.broadinstitute.org/scientific-community/software). HaploView 4.0 software was also used for a haplotype analysis. Significant P-values were corrected using the 1,000-fold permutation test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Association between the VEGF SNPs and endometriosis-related infertility.** The frequency of all the studied genotypes did not exhibit divergence from HWE between the studied groups (P>0.05). The number of genotypes, OR and 95% CI calculations for the three VEGF SNPs are listed in Table III. The lowest P-values of the trend test were observed for the VEGF +936 C/T (rs3025039) SNP with regards to endometriosis-related infertility ($P_{\text{rend}}=0.149$) (Table III). However, none of the three VEGF SNPs were associated with endometriosis-related infertility according to the dominant and recessive models (Table III). In addition, haplotype analyses of the VEGF SNPs did not reveal any SNP combination as a risk factor for endometriosis-related infertility (Table IV); the lowest overall P-values, $P=0.141$ and $P_{\text{rend}}=0.395$, were observed for a haplotype (TGT) of these SNPs (Table IV). The VEGF SNPs were in weak pairwise LD. The D' and $r^2$ values, as calculated from the control samples, had ranges of 0.007-0.964 (Table V).

<table>
<thead>
<tr>
<th>SNP</th>
<th>rs no.</th>
<th>Localization</th>
<th>SNP function</th>
<th>Alleles</th>
<th>MAF</th>
<th>Primers for PCR amplification (5'-3')</th>
<th>PCR product length, bp</th>
<th>Ann. temp., °C</th>
<th>Melt. temp., °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>-460 C/T</td>
<td>rs833061</td>
<td>nearGene-5</td>
<td>C/T</td>
<td>0.49</td>
<td></td>
<td>F: TCTTCGAGAGTGGAGCAGTG</td>
<td>108</td>
<td>61</td>
<td>80-95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ATTTGAAATCCTGGAGTGACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+405 G/C</td>
<td>rs2010963</td>
<td>chr6:43738350 UTR-5</td>
<td>G/C</td>
<td>0.30</td>
<td></td>
<td>F: GCTCCAGAGAACGTCAGGA</td>
<td>107</td>
<td>61</td>
<td>80-95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: CACCCCCAAAAAGCAGGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+936 C/T</td>
<td>rs3025039</td>
<td>chr6:43752536 UTR-3</td>
<td>C/T</td>
<td>0.12</td>
<td></td>
<td>F: CACACCATACCATCGACA</td>
<td>191</td>
<td>61</td>
<td>80-95</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: GCTCGGTGATTGCAGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*According to the Single Nucleotide Polymorphism database (dbSNP). Underlining denotes the minor allele in the control samples. MAF from 1000 Genomes project for EUR samples. All the polymorphisms were genotyped using high-resolution melting analysis. VEGF, vascular endothelial growth factor; SNP, single-nucleotide polymorphism; MAF, minor allele frequency; PCR, polymerase chain reaction; ann., annealing; temp, temperature; melt., melting.

Table II. Characteristics of the polymorphisms genotyped in the VEGF gene.
There are certain studies that report the increased production of VEGF in females with endometriosis, and increased VEGF levels have been demonstrated in the peritoneal tissue and blood plasma and peritoneal fluid of females with endometriosis (32-34). In addition to these observations, an in vitro study revealed that in the presence of peritoneal fluid from endometriotic females, endometrial cell cultures produce higher VEGF-A protein levels compared to the cultures from controls (35). There are also studies indicating an association between the VEGF levels in endometriosis and infertility. Lee and Ho (36) reported that VEGF in endometriotic females significantly inhibits sperm motility, acrosome reaction and sperm-oocyte interaction, which may result in endometriosis-associated subfertility/infertility.

There are also several animal model studies suggesting a role of VEGF overproduction, as well as anti-VEGF treatment in the regression of endometrial lesions. Vascular density and VEGF levels are also significantly increased in endometrial implants compared to eutopic endometrium in an experimental rat model of ectopic peritoneal endometriosis (37). Furthermore, a murine endometriosis implant model showed that VEGF-C is increased in the endometrium and promotes the development of experimental endometriosis (38). However, inhibitors of aromatase and tumor necrosis factor, and treatment with resveratrol and anti-VEGF monoclonal antibodies resulted in reduced VEGF levels, which were linked to the regression of endometriotic implants in a rat model of endometriosis (39-41). Recently, a role for miR-199a-5p in endometriosis development has been indicated in ectopic endometrial mesenchymal stem cells and targeting the 3'-untranslated region (UTR) of VEGF-A mRNA by miR-199a-5p in an animal model led to a decrease in the size of endometriotic lesions in vivo (42).

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Haplotypes</th>
<th>Frequency</th>
<th>Case/control ratios</th>
<th>( \chi^2 )</th>
<th>P-value</th>
<th>P(_{\text{corr}}) value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs833061_rs2010963</td>
<td>CG</td>
<td>0.507</td>
<td>0.525/0.500</td>
<td>0.562</td>
<td>0.454</td>
<td>0.815</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>0.267</td>
<td>0.252/0.273</td>
<td>0.483</td>
<td>0.487</td>
<td>0.844</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>0.220</td>
<td>0.215/0.222</td>
<td>0.062</td>
<td>0.804</td>
<td>1.000</td>
</tr>
<tr>
<td>rs2010963_rs3025039</td>
<td>GC</td>
<td>0.634</td>
<td>0.662/0.623</td>
<td>1.408</td>
<td>0.235</td>
<td>0.529</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0.207</td>
<td>0.204/0.208</td>
<td>0.018</td>
<td>0.894</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>0.093</td>
<td>0.079/0.099</td>
<td>1.107</td>
<td>0.293</td>
<td>0.626</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>0.066</td>
<td>0.056/0.070</td>
<td>0.727</td>
<td>0.394</td>
<td>0.750</td>
</tr>
<tr>
<td>rs833061_rs2010963_rs3025039</td>
<td>CGC</td>
<td>0.435</td>
<td>0.457/0.426</td>
<td>0.854</td>
<td>0.356</td>
<td>0.890</td>
</tr>
<tr>
<td></td>
<td>TCC</td>
<td>0.201</td>
<td>0.196/0.203</td>
<td>0.053</td>
<td>0.818</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>TGC</td>
<td>0.199</td>
<td>0.205/0.197</td>
<td>0.081</td>
<td>0.776</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>CGT</td>
<td>0.072</td>
<td>0.068/0.074</td>
<td>0.107</td>
<td>0.744</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>TCT</td>
<td>0.066</td>
<td>0.056/0.071</td>
<td>0.792</td>
<td>0.373</td>
<td>0.901</td>
</tr>
<tr>
<td></td>
<td>TGT</td>
<td>0.021</td>
<td>0.011/0.025</td>
<td>2.171</td>
<td>0.141</td>
<td>0.395</td>
</tr>
</tbody>
</table>

\(^a\)P-value calculated using permutation test and a total of 1,000 permutations. VEGF, vascular endothelial growth factor.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>rs833061</th>
<th>rs2010963</th>
<th>rs3025039</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs833061</td>
<td>-</td>
<td>0.964</td>
<td>0.179</td>
</tr>
<tr>
<td>rs2010963</td>
<td>0.365</td>
<td>-</td>
<td>0.182</td>
</tr>
<tr>
<td>rs3025039</td>
<td>0.007</td>
<td>0.017</td>
<td>-</td>
</tr>
</tbody>
</table>

D\(^a\) above diagonal; \( \chi^2 \) below diagonal. VEGF, vascular endothelial growth factor.

**Discussion**

There are certain studies that report the increased production of VEGF in females with endometriosis, and increased VEGF levels have been demonstrated in the peritoneal tissue and blood plasma and peritoneal fluid of females with endometriosis (32-34). In addition to these observations, there are studies indicating an association between the VEGF levels in endometriosis and infertility. There is evidence suggesting that VEGF in endometriotic females significantly inhibits sperm motility, acrosome reaction, and sperm-oocyte interaction, which may result in endometriosis-associated subfertility/infertility. There are also several animal model studies suggesting a role of VEGF overproduction, as well as anti-VEGF treatment in the regression of endometrial lesions. Vascular density and VEGF levels are also significantly increased in endometrial implants compared to eutopic endometrium in an experimental rat model of ectopic peritoneal endometriosis (37). Furthermore, a murine endometriosis implant model showed that VEGF-C is increased in the endometrium and promotes the development of experimental endometriosis (38). However, inhibitors of aromatase and tumor necrosis factor, and treatment with resveratrol and anti-VEGF monoclonal antibodies resulted in reduced VEGF levels, which were linked to the regression of endometriotic implants in a rat model of endometriosis (39-41). Recently, a role for miR-199a-5p in endometriosis development has been indicated in ectopic endometrial mesenchymal stem cells and targeting the 3'-untranslated region (UTR) of VEGF-A mRNA by miR-199a-5p in an animal model led to a decrease in the size of endometriotic lesions in vivo (42).
Altogether, these studies suggest that polymorphisms in the VEGF gene potentially modulate its expression and support the development of endometriotic lesions. However, in the present study, there was no association of VEGF polymorphism -460 C/T (rs833061), +405 G/C (rs2010963), +936 C/T (rs3025039) or SNP haplotypes with endometriosis in the presence of infertility.

Thus far, there are reports of no association of VEGF -460 C/T in Northern Iran and of VEGF +405 G/C in samples from all Iranian populations (23,24). Additionally, no contribution of the +936 C/T SNP with endometriosis in Korean females has been observed (25). However, the +405 G/C VEGF polymorphism has been associated with a higher susceptibility of endometriosis in Northern Iran, Turkish, South Indian, Italian and Korean females (23,26-29,43). Bhanooori et al (44) demonstrated that the -460T/+405C haplotype of VEGF was less frequently identified in females with endometriosis compared to the controls, and VEGF -460 T/T homozygotes and the T allele are associated with a higher risk of endometriosis in Chinese females. There are also studies that demonstrate the association of the VEGF +936 C/T polymorphism in Caucasian and Japanese females with endometriosis (45,46). In addition, several meta-analyses have demonstrated that the VEGF +936 C/T SNP can predispose to endometriosis (47-49).

A contribution of the VEGF -2578 A/C SNP to endometriosis was also observed in the Estonian population, as well as VEGF -460-1154-2578 TGC, CAA, TAA and TAC haplotypes to endometriosis in North Chinese females (50,51).

There are certain studies evaluating the functional role of -460 C/T, +405 G/C and +936 C/T SNPs on VEGF expression. The +405 G/C SNP in the 5'-UTR exhibits a strong effect on the production of the VEGF protein (52,53). Distinct SNPs located in the 5'-UTR may account for the binding of different transcription factors in modulating VEGF transcription levels (54). Watson et al (54) demonstrated a dose-dependent effect of the +405 G allele, whereby the highest VEGF protein biosynthesis was observed for the GG genotype, an intermediate level for GC and the lowest for CC. In addition to this finding, Stevens et al (55) reported increased promoter activity and VEGF expression for the -460C/+405G haplotype compared to the -460T/+405C haplotype. The study by Renner et al (56) observed that the +936 C/T SNP, which is situated in the 3'-UTR, is linked to VEGF production and blood plasma levels.

Despite the contribution of the VEGF -460 C/T, +405 G/C and +936 C/T SNPs to the development of endometriosis in several ethnicities, the present genetic investigation failed to confirm these selected SNPs as a risk factor or endometriosis. However, as the study was conducted using a relatively small sample, it should be replicated in larger groups from different populations.

Acknowledgements

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References


