The vascular endothelial growth factor (VEGF) +405G>C 5′-untranslated region polymorphism and increased risk of endometriosis in South Indian women: a case control study

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BACKGROUND: Vascular endothelial growth factor (VEGF), a major mediator of angiogenesis and vascular permeability, is known to play a key role in the pathophysiology of endometriosis. METHODS AND RESULTS: The single nucleotide polymorphisms, −460C>T and +405G>C, in the 5′-untranslated region of the VEGF gene were tested for association in a case–control study of 215 affected women and 210 women with no evidence of disease. All the women were of South Indian origin and ascertained from the same infertility clinic. The genotype and allele frequencies of the −460C>T polymorphism did not differ significantly between cases and controls. In contrast, the genotype (P = 0.002) and allele (P = 0.001) frequencies of the +405G>C polymorphism showed a significant difference between cases and controls. The +405 GG genotype was found more often in patients with an endometrioma >3 cm compared to controls. The frequency of the −460T/+405C haplotype (P = 0.016) was significantly lower in affected women compared to controls. CONCLUSIONS: The −460T/+405C haplotype in the VEGF gene, which is associated with lower promoter activity, was significantly less common in women with endometriosis than in controls. These data suggest that the +405G allele may influence the likelihood of a woman developing the disease.

Key words: endometriosis/polymorphism/VEGF

Introduction

Endometriosis is a chronic gynaecological disease characterized by the growth of hormonally responsive, endometrial-like tissue outside the uterine cavity. Up to 10% of women of reproductive age may be affected (Eskenazi and Warner, 1997). The clinical presentation varies, although affected women usually have one or more pain symptoms and/or difficulty conceiving. Although Sampson’s theory of the transplantation of endometrial tissue onto the pelvic peritoneum via retrograde menstruation is one of the most widely accepted explanations for the development of the disease (Sampson, 1927), the actual cellular and molecular mechanisms responsible are unclear. However, the cause is almost certainly multifactorial involving environmental, immunological, endocrine and genetic processes (Olive and Schwartz, 1993). Association studies have identified mutations and single nucleotide polymorphisms (SNPs) in a number of genes that might confer susceptibility to endometriosis, but their precise role remains to be determined.

Histologically, endometriosis is a benign disease but it can behave like a malignancy in terms of growing, infiltrating and adhering to the surrounding tissues (Varma et al., 2004). To survive and develop into an endometriotic lesion, the ectopic tissue must induce new vessel formation to connect to the vascular system (Nisolle and Donnez, 1997; Taylor et al., 2002). A key mediator in neoangiogenesis is vascular endothelial growth factor (VEGF) as it stimulates endothelial cell proliferation and migration, and increases vascular permeability (Risau, 1997; Ferrara, 1999); it is therefore regarded as a marker of tumour invasion and metastasis (Carmeliet and Jain, 2000). SNPs within the VEGF gene have been identified, some of which have functional significance. For example, the +405G>C polymorphism in the 5′-untranslated region has a significant effect on VEGF protein production (Brogan et al., 1999; Watson et al., 2000). Association has been reported in case–control studies between VEGF polymorphisms and diseases such as diabetic retinopathy (Ray et al., 2004), prostate cancer (Lin et al., 2003) and breast cancer (Krippl et al., 2003).

VEGF may have a pivotal role in the development and progression of endometriosis because: (a) it is expressed in human uterine epithelial and stromal cells and regulated by...
VEGF polymorphism and endometriosis

Materials and methods

Subjects

All the subjects were non-smokers and of South Indian origin. Two hundred and fifteen unrelated women with moderate–severe (III–IV) endometriosis staged using the revised American Fertility Society classification system (rAFS) were recruited at the Infertility Institute and Research Centre (IIRC), Hyderabad, India. All women had a trans-vaginal ultrasound scan (TVS) at screening followed by laparoscopy to confirm the diagnosis (rAFS III–IV). The controls consisted of 141 (67%) women with no evidence of endometriosis on TVS and laparoscopy who therefore did not subsequently have a laparoscopy. Their mean age (range 20–40) years.

Two hundred and ten women were recruited from the same clinic population and had an equal opportunity to be identified as cases, thereby meeting the criteria for appropriate controls set by Zondervan et al. (2002a). The controls consisted of 141 (67%) women with no evidence of endometriosis on TVS and laparoscopy and 69 (33%) women with no evidence of an ovarian endometrioma on TVS (and no clinical symptoms of endometriosis) who therefore did not subsequently have a laparoscopy. Their mean age ± SD was 27.5 ± 4.4 (range 20–40) years. All the patients complained of dysmenorrhea (mild = 47%; moderate = 30%; severe = 23%) and 75% had dyspareunia. Most women (97.7%) were infertile (primary = 78%; secondary = 22%).

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Determination of the VEGF genotype

DNA extraction

Genomic DNA was extracted from 1 ml of EDTA anti-coagulated whole blood by the salting out method (Miller et al., 1988).

Primers and polymerase chain reaction

Genotyping of the −460C>T and +405G>C polymorphisms in the 5’ UTR of the VEGF gene was determined by PCR and sequencing analysis. PCRs were carried out in a total volume of 25 μl contai
and \( G = 81.7\% \) (cases) and 72.7\% (controls). Allele frequencies were significantly different between cases and controls \( (P = 0.001) \), which resulted from an increased proportion of homozygote GG genotype carriers (but not heterozygote GC carriers) among endometriosis patients as compared to controls \( (P = 0.002) \). We have also analysed the data considering only 141 laparoscopically tested controls and we did not find any variation in the association status with both the SNPs (data not shown).

Genotype frequencies for the VEGF \(-460\text{C} \rightarrow \text{T}\) and \(+405\text{G} \rightarrow \text{C}\) SNPs were further analysed based on the size of the largest endometrioma present (Table II). For the \(+405\) polymorphism, statistically significant differences in the frequencies of GG, GC and CC genotypes were observed in patients with an endometrioma \( >3\text{ cm} \) \((4–5\text{ cm}, P = 0.04; >6\text{ cm}, P = 0.003)\) compared to controls. A higher frequency of GG homozygotes but not GC heterozygotes was found in cases compared to controls (Table II). However, for the \(-460\text{C} \rightarrow \text{T}\) polymorphism, no significant differences in the frequencies of the CC, TC and TT genotypes were observed.

Haplotype frequencies were estimated by the Expectation–Maximization algorithm implemented in the Alrequin 2000 software (Table III). There was strong linkage disequilibrium between the \(-460\) and \(+405\) alleles with a standardized disequilibrium coefficient \( (D') \) of 1.000 between the two loci. The frequencies of haplotype CG, CC, TG and TC in control subjects were 44.2\%, 2.4\%, 28.3\% and 25.0\%, respectively. There was a significant deficit in the frequency of haplotype

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Stage of endometriosis</th>
<th>Cases (215)</th>
<th>Controls (210)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-460\text{T} \rightarrow \text{C})</td>
<td>III ((n = 80))</td>
<td>IV ((n = 135))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>23 (28.8)</td>
<td>24 (17.8)</td>
<td>47 (21.7)</td>
<td>42 (20.0)</td>
</tr>
<tr>
<td>TC</td>
<td>37 (46.2)</td>
<td>75 (55.6)</td>
<td>112 (52.1)</td>
<td>112 (53.3)</td>
</tr>
<tr>
<td>TT</td>
<td>20 (25.0)</td>
<td>36 (26.7)</td>
<td>56 (26.1)</td>
<td>56 (26.7)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.52</td>
<td>0.46</td>
<td>0.48</td>
<td>0.466</td>
</tr>
<tr>
<td>T</td>
<td>0.482</td>
<td>0.545</td>
<td>0.521</td>
<td>0.534</td>
</tr>
<tr>
<td>(+405\text{G} \rightarrow \text{C})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1 (1.3)</td>
<td>3 (2.2)</td>
<td>4 (1.9)</td>
<td>18 (8.6)</td>
</tr>
<tr>
<td>GC</td>
<td>26 (32.5)</td>
<td>45 (33.3)</td>
<td>71 (33.0)</td>
<td>79 (37.6)</td>
</tr>
<tr>
<td>GG</td>
<td>53 (66.2)</td>
<td>87 (64.5)</td>
<td>140 (65.1)</td>
<td>113 (53.8)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.175</td>
<td>0.188</td>
<td>0.183</td>
<td>0.273</td>
</tr>
<tr>
<td>G</td>
<td>0.825</td>
<td>0.812</td>
<td>0.817</td>
<td>0.727</td>
</tr>
</tbody>
</table>

*Fisher’s exact test \( (3 \times 2 \text{ table at } 2 \text{ df}) \) \( P < 0.05 \).

**Fisher’s exact test \( (2 \times 2 \text{ table at } 1 \text{ df}) \) \( P < 0.05 \).
Table II. Genotype frequencies of VEGF polymorphisms in endometriosis patients (n = 198) based on size of the endometrioma

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Endometriotic cyst (cm)</th>
<th>Controls (210)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;3</td>
<td>4–5</td>
</tr>
<tr>
<td>−460T&gt;C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>15  (7.6)</td>
<td>62  (31.3)</td>
</tr>
<tr>
<td>TC</td>
<td>4   (26.7)</td>
<td>17  (27.4)</td>
</tr>
<tr>
<td>TT</td>
<td>8   (53.3)</td>
<td>37  (59.7)</td>
</tr>
<tr>
<td>P-value*</td>
<td>0.76</td>
<td>0.08</td>
</tr>
<tr>
<td>+405G&gt;C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0   (0.0)</td>
<td>2   (3.2)</td>
</tr>
<tr>
<td>GC</td>
<td>3   (20.0)</td>
<td>16  (25.8)</td>
</tr>
<tr>
<td>GG</td>
<td>12  (80.0)</td>
<td>44  (71.0)</td>
</tr>
<tr>
<td>P-value*</td>
<td>0.1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*All the patients studied had endometriotic cysts but the details of the size were available for only 198 patients.
*Fisher’s exact test (3 × 2 table at 2 df) P < 0.05.

Discussion

Association has been reported between endometriosis and a number of functional candidate genes involved in detoxification, galactose metabolism, steroid hormone production and inflammation (see up-to-date review maintained on the genetic epidemiology website http://www.well.ox.ac.uk/~krinaz/genepi_endo.htm) (Zondervan et al., 2002b). In the present study, two SNPs in the region of the VEGF gene were investigated to ascertain whether the polymorphisms are associated with endometriosis susceptibility in South Indian women.

The VEGF gene is located on chromosome 6p21.3 (Vincenti et al., 1996) and consists of eight exons exhibiting alternate splicing to 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 (Akiri et al., 1998). Polymorphisms within the 5′-untranslated region lead to differences in VEGF expression between individuals and could influence the etiology of a variety of pathological conditions with which VEGF has been associated. Thus, Watson et al. (2000) reported that a G allele at position +405, which probably lies within the myeloid zinc finger protein (MZF1) binding site, 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. Furthermore, the −460C/+405G haplotype has been associated with higher promoter activity, and therefore higher VEGF expression, than the −460T/+405C haplotype (Stevens et al., 2003).

In the present study, the frequency of the +405G allele was significantly higher in women with rAFS Stage III–IV endometriosis than unaffected controls drawn from the same South Indian population, but the −460C>T polymorphism was not associated with the disease. Recently, Hsieh et al. (2004) showed an association between −460C>T polymorphism and endometriosis which is not in agreement with the present result. Possible reasons for this discrepancy could be: first, ethnic variation observed in the SNPs analysed. Indeed drastic difference in T allele distribution was observed in the two populations studied. The frequencies of mutant T allele in their controls and cases were 68.3% and 77.9% respectively compared to 53.4% and 52% in the present study (Table I). Second, the sample size in their study (cases, 122; controls, 131) is comparatively less than the sample size of the present study (cases, 215; controls, 210).

We also observed that the −460T/+405C haplotype, which is associated with lower promoter activity, was significantly more common in controls than in affected women (P = 0.016). The data are consistent with the previously reported finding of higher VEGF expression in women with endometriosis (Shifren et al., 1996; Donnez et al., 1998; Tan et al., 2002). Carrying the +405G allele may increase VEGF promoter activity leading to higher protein production and possibly a greater risk of developing the disease, whereas the +405C allele may be protective.

VEGF transcription is regulated by estrogen in uterine endometrium (Hyder et al., 1996). Estrogens directly regulate VEGF transcription in target tissue and tumour via a complex interplay of cis- and trans-acting elements. Recently, Kawai et al. (2002) reported that BRCA-1 and the estrogen receptor α (ERα) also modulate VEGF transcription in breast cancer cells. In endometrial cells, estradiol-induced VEGF gene transcription is also estrogen receptor dependent and is activated through a variant estrogen response element (ERE) localized −1.2 kb upstream from the VEGF transcription start site (Hyder et al., 2000). The +405 site is located close to the ERE and AP-1 transcription factor binding sites in

Table III. Haplotype frequencies of two VEGF biallelic polymorphisms

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Controls n (%)</th>
<th>Endometriosis n (%)</th>
<th>P-value*</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−460</td>
<td>405</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>G</td>
<td>186 (44.3)</td>
<td>196 (45.6)</td>
<td>−</td>
</tr>
<tr>
<td>CC</td>
<td>C</td>
<td>10 (2.4)</td>
<td>10 (2.3)</td>
<td>0.91</td>
</tr>
<tr>
<td>TC</td>
<td>G</td>
<td>119 (28.3)</td>
<td>153 (35.6)</td>
<td>0.21</td>
</tr>
<tr>
<td>TT</td>
<td>C</td>
<td>105 (25.0)</td>
<td>71 (16.5)</td>
<td>0.016</td>
</tr>
</tbody>
</table>

*Univariate logistic regression was used for haplotype association analysis.
*Observed haplotype frequencies were estimated by Expectation–Maximization method using Arlequin software v2000.
*From χ2-test between control and endometriosis cases.
the VEGF promoter region (Stevens et al., 2003), which indicates a possible role for this polymorphism in the regulation of VEGF by estrogen.

In endometriosis, neovascularization is essential for the implantation of endometrial cells in ectopic sites to be successful (Taylor et al., 2002). VEGF, a potent angiogenic factor, is involved in neovascularization, which may explain why its expression is up-regulated in the glandular epithelium of endometriosis lesions (Donnez et al., 1998). Peritoneal fluid concentrations of VEGF were significantly higher in women with rAFS Stage III–IV endometriosis (the phenotype in the present study) than in those with rAFS Stage I–II disease. The increase was attributed to the ability of endometriotic lesions to produce VEGF (Shifren et al., 1998). The increase was attributed to the ability of endometriotic lesions to produce VEGF (Shifren et al., 1998). How-

ever, McLaren et al. (1996) demonstrated that activated macrophages are a major source of VEGF in endometriosis. They also showed that expression of this factor was regulated by steroid hormones like estradiol and progesterone. More recently, Tan et al. (2002) reported high levels of VEGF mRNA and low levels of TSP-1 (thrombospondin-1, an anti-

angiogenic factor) mRNA in red peritoneal endometriotic lesions.

It is well established that endometriosis is an estrogen-

dependent disease. In normal women, aromatase expression is absent in eutopic endometrium, but in endometriosis there is aberrant expression of steriodogenic factor (SF)-1, a tran-

scription factor which increases aromatase expression and, in turn, is involved in the establishment of a positive feedback loop in favour of continuous local biosynthesis of estrogen (Kitawaki et al., 2002; Bulun et al., 2004). Aromatase promoter II activity is regulated by cyclic AMP (cAMP) and requires both CREB and SF-1 (Simpson, 2000). It is possible therefore that VEGF plays a central role in the positive feedback loop of estrogen production, and thereby in the etiology of endometriosis, by enhancing aromatase production via PI3k-Akt-CREB signalling cascade and testing of this hypothesis is going to be the focus of our future activity in this area. In conclusion we report that the +405G allele in the 5'-untranslated region of VEGF gene may influence the likeliness of a woman developing endometriosis.

Acknowledgements

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