Vascular endothelial growth factor gene +405 C/G polymorphism is associated with susceptibility to advanced stage endometriosis

Sung Hoon Kim1, Young Min Choi2,3,4, Seon Ha Choung2, Jong Kwan Jun2, Jung Gu Kim2 and Shin Yong Moon2,3

1Department of Obstetrics and Gynecology, College of Medicine, University of Ulsan, Asan Medical Center and 2Department of Obstetrics and Gynecology and 3The Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University College of Medicine, Korea

4To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, The Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University College of Medicine, 28 Yungun-dong, Chongno-ku, Seoul 110–744, Korea. E-mail: ymchoi@snu.ac.kr

BACKGROUND: Vascular endothelial growth factor (VEGF) is known to play a pivotal role in the development of endometriosis. This study was performed to investigate whether the VEGF gene 5'-untranslated region polymorphism is associated with susceptibility to advanced stage endometriosis. METHODS: This study comprised 215 women with advanced stage endometriosis, 219 control women without endometriosis, and 70 fertile women. Following extraction of genomic DNA, genotyping of the −460 C/T and +405 C/G polymorphisms of the VEGF gene were performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. RESULTS: The distribution of genotypes and allele frequencies of the −460 C/T polymorphism in the endometriosis group did not differ from those in the control group and the fertile women group. However, genotype distribution of the +405 C/G polymorphism was significantly different between patients with and without endometriosis (P = 0.01) and between patients with endometriosis and the fertile women (P = 0.02). Patients with endometriosis showed a higher incidence of the +405 CC genotype compared with the controls and the fertile women (P = 0.007 and 0.016 respectively).

CONCLUSIONS: These findings suggest that the VEGF +405 C/G polymorphism may be associated with the risk of advanced stage endometriosis in the Korean population.

Key words: angiogenesis/endometriosis/polymorphism/VEGF

Introduction

Endometriosis is defined as the presence of endometrial tissue outside the uterus, causing diverse diseases, including infertility, pelvic pain, and dysmenorrhea. The prevalence of endometriosis has been found to range from 2 to 18% among women who seek tubal ligations and from 5 to 50% among infertile women (Missmer and Cramer, 2003). As retrograde menstruation has been demonstrated in up to 90% of menstruating women with patent Fallopian tubes (Halme et al., 1984), it remains unclear why endometriosis affects only a certain group of women. Using the analogy of tumour metastasis, some investigators have postulated that the angiogenic potential may predict the likelihood that endometriotic lesions will become established (Taylor et al., 1997; Donnez et al., 1998). Indeed, the peritoneal environment of women with endometriosis is highly angiogenic with increased angiogenic activity in peritoneal fluid and increased amounts of angiogenic factors (McLaren, 2000). Endometriotic implants often are surrounded by vascularity, and extrapelvic endometriosis typically occurs in well-vascularized organs (Taylor et al., 2002).

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a heparin-binding glycoprotein with potent angiogenic and endothelial cell-specific mitogenic activities. Some investigators have demonstrated higher peritoneal concentrations of VEGF in women with advanced stage endometriosis (McLaren et al., 1996; Shifren et al., 1996), and others have shown increased VEGF mRNA and protein expression in the eutopic endometrium from subjects with endometriosis (Donnez et al., 1998; Tan et al., 2002). Recently, it has been demonstrated that use of antihuman VEGF effectively interferes with the maintenance and growth of endometriosis by inhibiting angiogenesis in a nude mouse model (Nap et al., 2004).

Based upon the genetic predisposition (Campbell and Thomas, 2001; Zondervan et al., 2001; Bischoff and Simpson, 2004) and the possible pivotal role of VEGF in the pathogenesis of endometriosis, it is necessary to investigate whether the VEGF gene polymorphisms are associated with susceptibility to endometriosis. Hsieh et al. (2004) have shown that individuals...
with T/T homozygosity and the T allele of the VEGF-460 gene have a higher risk of advanced endometriosis in a Taiwanese population. However, there have been no other reports demonstrating or supporting the hypothesis that the VEGF gene polymorphism is associated with susceptibility to endometriosis.

The present study was designed to explore the association between the VEGF gene polymorphism and the risk of advanced endometriosis in a Korean population. We investigated the frequency of 5′-untranslated region –460 and +405 polymorphisms in patients with and without advanced endometriosis.

Materials and methods

Subjects

Peripheral blood was obtained from a total of 434 patients who had undergone diagnostic laparoscopy, pelviscopic surgery, exploratory laparotomy, or transabdominal hysterectomy. All subjects were of Korean origin, which is made up of a single ethnic group. A total of 215 patients had surgical and histological evidence of advanced endometriosis, while 219 patients without the disease served as controls. In addition, we recruited a total of 70 fertile women visiting for regular check-up without history of any gynaecological problems and operations, to evaluate whether the genotype distribution of the control group in the present study is different from the general population. Patients having rheumatoid arthritis, giant cell arteritis, diabetic retinopathy, psoriasis, or Behçet’s disease were initially excluded from this study due to the possible association with a VEGF gene polymorphism (Awata et al., 2002; Boiardi et al., 2003; Han et al., 2004; Salvaresi et al., 2004; Young et al., 2004). All patients in the endometriosis group had ovarian endometrioma, and the extent of the disease was staged according to the guidelines of the American Society for Reproductive Medicine (1997). Sixty-five patients were diagnosed as having stage III and 150 patients had stage IV endometriosis.

None of the subjects had received hormone therapy during the previous 12 months. All of the patients who had the diagnosis of minimal or mild endometriosis were excluded in both the case and control groups. The indications for surgery amongst the endometriosis group were dysmenorrhea (n = 65), pelvic pain (n = 57), adnexal mass (n = 49), infertility (n = 32), and others (n = 12). Pelviscopic surgery, exploratory laparotomy and transabdominal hysterectomy were performed in 141 (65.6%), 47 (21.8%) and 27 (12.6%) patients respectively in the endometriosis group.

Patients with leiomyoma, adenomyosis, invasive carcinoma of the uterine cervix or ovarian cancer were excluded from the control group. The indications for surgery or diagnostic laparoscopy in the control group were benign ovarian cyst (n = 76), infertility (n = 60), pelvic pain or dysmenorrhea (n = 56) and carcinoma in situ of the uterine cervix (n = 27). Pelviscopic surgery, exploratory laparotomy, transabdominal hysterectomy and diagnostic laparoscopy were performed in 87 (39.7%), 38 (17.3%), 34 (15.6%) and 60 (27.1%) patients respectively in the control group. The review board for human research of Seoul National University Hospital approved this project, and informed written consent was obtained from each woman. Ages ranged from 18 to 50 years (31.0 ± 7.2, mean ± SD) in the endometriosis group, from 20 to 54 years (43.4 ± 10.1) in the control group, and from 40 to 49 years (43.3 ± 2.6) in the fertile women group.

Genomic DNA analysis

Peripheral blood was drawn from each patient and collected in an EDTA-containing tube. Genomic DNA was extracted from the peripheral blood with the Wizard DNA Purification Kit (Promega, Madison, WI, USA).

Genotyping of each polymorphism was carried out by PCR–RFLP analysis, as previously described by Watson et al. (2000) for the −460 C/T polymorphism and as described by Awata et al. (2002) for the +405 C/G polymorphism, with minor modifications. The PCR primers for the −460 C/T and the +405 C/G polymorphisms were 5′-TGTGCGTGTGGGTGAGCG-3′ (forward) and 5′-TACGTGGGACAGGGCCCTGA-3′ (reverse) and 5′-TTGCTTGCCATCCCACCTTGA-3′ (forward) and 5′-CCGAGCCA-GAACAGCCCCAGA-3′ (reverse), respectively. Following an initial denaturation step (5 min at 94°C), samples were subjected to 30 rounds of PCR at 94°C for 30 s (−460 C/T) or 1 min (+405 C/G), 60°C (−460 C/T) or 62°C (+405 C/G) for 30 s (−460 C/T) or 1 min (+405 C/G), and 72°C for 60 s with a final extension time of 5 min at 72°C. The PCR products were digested with either 2 IU of restriction enzyme BstU1 (New England Biolabs, USA) at 60°C overnight for the −460 C/T polymorphism or with BsmFI (New England Biolabs) at 65°C overnight for the +405 C/G polymorphism, separated by 3% (−460 C/T) or 2% (+405 C/G) agarose gel electrophoresis, and identified using ethidium bromide staining. The −460C allele was cut into two fragments of 155 and 20 bp, while the −460T allele remained uncut (175 bp). The +405G allele was cut into two fragments of 273 and 196 bp, while the +405C allele remained uncut (469 bp).

Statistical analysis

The numbers for the endometriosis and control groups were initially chosen to detect a difference between the two groups assuming that odds ratio is 2.0 with a power of 0.8 and 5% type I error. Genotype distributions were examined for significant departure from Hardy–Weinberg equilibrium by a goodness-of-fit χ2-test. χ2-Analysis was used to evaluate differences in the proportions of the genotypes between the endometriosis and control groups. Haplotype frequencies, the disequilibrium coefficient (r2) and the standardized disequilibrium coefficient (D′) were evaluated utilizing Haplovview program (Version 3.2, available at http://www.broad.mit.edu/mpg/haploview/index.php) and haplotype association was analysed by univariate logistic regression. P < 0.05 was considered significant.

Results

Genotyping of the −460 C/T and +405 C/G polymorphisms was successfully achieved for all subjects. Genotypic distributions of the −460 C/T and +405 C/G polymorphisms were in Hardy–Weinberg equilibrium in all three groups. The distribution of genotypes and allele frequencies of the −460 C/T polymorphism in the endometriosis group did not differ from those in the control and the fertile women group (Table I). However, genotype distribution of the +405 C/G polymorphism was significantly different between patients with and without endometriosis (P = 0.01) and between patients with endometriosis and the fertile women (P = 0.02) (Table II). Patients with endometriosis showed a higher incidence of the −460C allele (23.3 versus 13.2 and 10.0%), with a higher risk of advanced endometriosis in a Taiwanese population. None of the patients had received hormone therapy during the previous 12 months. All of the patients who had the diagnosis of minimal or mild endometriosis were excluded in both the case and control groups. The indications for surgery amongst the endometriosis group were dysmenorrhea (n = 65), pelvic pain (n = 57), adnexal mass (n = 49), infertility (n = 32), and others (n = 12). Pelviscopic surgery, exploratory laparotomy and transabdominal hysterectomy were performed in 141 (65.6%), 47 (21.8%) and 27 (12.6%) patients respectively in the endometriosis group.

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Genomic DNA analysis

Peripheral blood was drawn from each patient and collected in an EDTA-containing tube. Genomic DNA was extracted from the peripheral blood with the Wizard DNA Purification Kit (Promega, Madison, WI, USA).
Distribution of haplotypes at the –460 and +405 polymorphisms in the VEGF gene in endometriosis patients and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>–460 C/T genotypes</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC n (%)</td>
<td>CT n (%)</td>
</tr>
<tr>
<td>Endometriosis (n = 215)</td>
<td>19 (8.8)</td>
<td>83 (38.6)</td>
</tr>
<tr>
<td>Control (n = 219)</td>
<td>16 (7.3)</td>
<td>83 (37.9)</td>
</tr>
<tr>
<td>Fertile women (n = 70)</td>
<td>6 (2.7)</td>
<td>27 (12.9)</td>
</tr>
</tbody>
</table>

ab: χ2-Test in comparison with the control group.
bc: χ2-Test in comparison with the fertile women group.

Table II. Distribution of +405 C/G polymorphism in the VEGF gene in endometriosis patients and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>+405 C/G genotypes</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC n (%)</td>
<td>CG n (%)</td>
</tr>
<tr>
<td>Endometriosis (n = 215)</td>
<td>50 (23.3)</td>
<td>89 (41.4)</td>
</tr>
<tr>
<td>Control (n = 219)</td>
<td>29 (13.2)</td>
<td>116 (53.0)</td>
</tr>
<tr>
<td>Fertile women (n = 70)</td>
<td>7 (10.0)</td>
<td>41 (58.6)</td>
</tr>
</tbody>
</table>

ab: χ2-Test in comparison with the control group.
bc: χ2-Test in comparison with the fertile women group.

Table III. Distribution of haplotypes at the –460 and +405 polymorphisms in the VEGF gene

<table>
<thead>
<tr>
<th>–460/+405 haplotype</th>
<th>Endometriosis n (%)</th>
<th>Control n (%)</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>119 (27.7)</td>
<td>114 (26.0)</td>
<td>–</td>
<td>Reference</td>
</tr>
<tr>
<td>TC</td>
<td>187 (43.5)</td>
<td>173 (39.5)</td>
<td>0.16</td>
<td>1.28 (0.90–1.82)</td>
</tr>
<tr>
<td>TG</td>
<td>122 (28.4)</td>
<td>150 (34.2)</td>
<td>0.84</td>
<td>0.97 (0.69–1.34)</td>
</tr>
<tr>
<td>CC</td>
<td>2 (0.5)</td>
<td>1 (0.2)</td>
<td>0.60</td>
<td>0.52 (0.05–5.84)</td>
</tr>
</tbody>
</table>

OR = odds ratio; CI = confidence interval.

Discussion

Recent studies have demonstrated a possible association between the VEGF gene polymorphism and various diseases in which angiogenesis may be critical in disease development. Analysing seven common polymorphisms in the VEGF gene, Awata et al. (2002) demonstrated that genotype distribution of the +405 C/G polymorphism (−634 polymorphism in their report) is significantly different between diabetic patients with and without retinopathy, and diabetic patients with the +405 CC genotype have about a three times higher risk of retinopathy than those with the +405 GG genotype. Young et al. (2004) have shown that patients with early onset psoriasis have a significantly increased frequency of the +405 CC genotype and the C allele, compared to healthy controls. A genetic study in a Korean population has revealed that the frequency of the +936 T allele is significantly increased in patients with rheumatoid arthritis, compared with controls (Han et al., 2004). In addition, the association between the VEGF gene polymorphism and susceptibility to Behçet’s disease (Salvarani et al., 2004) and giant cell arteritis (Boiardi et al., 2003) has also been demonstrated.

Analysing the –460 C/T and +405 C/G polymorphisms of the VEGF gene, we found that genotype distribution of the +405 C/G polymorphism was significantly different between
patients with and without endometriosis, and women with
the +405 CC genotype had a significantly increased risk of
endometriosis compared with those without the genotype.
The findings of the present study are not consistent with the
previous report by Hsieh et al. (2004), which demonstrated a
significant association between the −460 C/T polymorphism
and susceptibility to endometriosis. The discrepancy might
be due to ethnic composition differences between the two
studies. However, the previous report by Hsieh et al. (2004)
included no subjects with the −460 CC genotype and the fre-
quency of the −460 CC, −460 CT and −460 TT genotypes of
the VEGF gene polymorphism in the control group were not
in Hardy–Weinberg equilibrium ($P = 0.00006$). The results
of the present study may be more reliable, since the distribu-
tion of the genotypes of the control subjects used in an asso-
ciation study should not show a significant deviation from
Hardy–Weinberg equilibrium. Considering that all the
women except two with +405 CC genotype in the endometri-
osis and control groups of the present study had −460 TT
genotype, it is also possible that the original reported associ-
ation with the −460TT genotype resulted from skewed distri-
bution of genotype in the endometriosis patients in their
report, in which most of the −460 TT genotype might have
had +405 CC genotype.

We tried to recruit pre-menopausal women aged 40–50
years as control subjects, and the mean age of the control sub-
jects was higher than that of the endometriosis patients. As
described by Hadfield et al. (2001), recruiting women from
this age group has the merit of maximizing the probability that
they were unaffected by endometriosis, i.e. to avoid including
younger women who might develop the disease in later life. As
of April, 2005, we have no other data about the allele frequen-
cies for +405 C/G or the frequency of +405 CC genotype in the
Korean population except ours and Han et al.’s (2004). The
frequency of +405 CC genotype in healthy individuals of the
previous report by Han et al. (2004) was similar to the fre-
quency reported for the endometriosis patients and higher than
that of the control group in the present study. However, the fre-
quency of +405 CC genotype of the control group (13.2%) in
the present study is slightly higher than that in the control
group of the previous study by Awata et al. (2002) (10.3%) in a
Japanese population. Despite few data among the general
population in Korea, the control subjects in the present study
do not seem to have a different frequency of the +405 CC gen-
otype from the general population, as the frequency of +405 CC
 genotype in the 70 women with proven fertility without any
gynaecological disease was 10.0%.

Although it is unclear how the polymorphisms in the untra-
slated region of the VEGF gene influence its protein pro-
duction, several reports have demonstrated that single-nucleotide
polymorphisms of the VEGF gene are associated with VEGF
synthesis. Watson et al. (2000) have demonstrated that the
genotype for the +405 polymorphism in the VEGF gene is
significantly correlated with VEGF production from stimulated
peripheral blood mononuclear cells. Awata et al. (2002) have
shown the association of the +405 CC genotype with a higher
serum VEGF concentration in a normal Japanese population.
A recent study suggested that genetic polymorphisms including
+405 region are correlated with VEGF protein expression in
cancer cells and tumour angiogenic activity (Koukourakis
et al., 2004). Transfection analysis of the human VEGF
promoter revealed that estrogen has a direct transcriptional
impact on VEGF gene expression and that estrogen-regulated
transcription requires a variant estrogen response element
(Mueller et al., 2000). Considering that the +405 site is located
adjacent to the +410 estrogen response element and that
the −460/+405 polymorphism significantly alters
VEGF promoter activity and responsiveness (Stevens et al.,
2003), it may be suggested that the +405 polymorphism itself
has an influence on the transcriptional activity by possible
alteration of response to estrogen.

Individuals with a specific single nucleotide polymorphism
of the VEGF gene may have a higher risk of developing
endometriosis through increased expression of VEGF in various
cells. An elevated concentration of VEGF in the peritoneal
fluid due to increased production of VEGF by activated peri-
toneal macrophages can be a critical process in the pathogenesis
of endometriosis. Alternatively, increased expression of VEGF
in eutopic endometrial cells may lead to implantation and
proliferation of endometrial cells at ectopic sites through retro-
grade menstruation. Based on the findings of the previous
report by Awata et al. (2002), it is also possible that individuals
with the +405 CC genotype have an increased risk of endome-
triosis due to increased serum levels of VEGF, although
contradictory results have been reported as to whether circulat-
ing VEGF is modulated in endometriosis (Gagné et al., 2003;
Mataliotakis et al., 2003).

There is accumulating evidence to support the hypothesis
that angiogenesis is of pivotal importance in the development
of endometriosis. The present study has shown that the sub-
jects with a specific genotype in the VEGF gene polymorphism
had a significantly increased risk of endometriosis than those
without the genotype. These findings strongly suggest that
endometriotic lesions could become established in certain
groups of women in whom angiogenic activity is enhanced due
to the presence of specific genotypes. Further studies on the
functional relevance of the VEGF polymorphism in endometri-
osis are necessary to confirm these observations.

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PG6-01GN13-0002).

References
American Society for Reproductive Medicine (1997) Revised American Society
for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril,
67, 817–821.
Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, Imukai K, Inoue I and
Katayama S (2002) A common polymorphism in the 5′-untranslated region
of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes.
Diabetes 51,1635–1639.
Acad Sci 1034,284–299.
Boiardi L, Casali B, Nicolli D, Farnetti E, Chen Q, Macchioni P, Catanozo MG,
Pulsatelli L, Meliconi R and Salvarani C (2003) Vascular endothelial
growth factor gene polymorphisms in giant cell arteritis. J Rheumatol
30,2160–2164.


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