Endothelial nitric oxide synthase gene Glu298Asp polymorphism is associated with advanced stage endometriosis

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BACKGROUND: Altered expression of endothelial nitric oxide synthase (eNOS) has been reported to be involved in the development of endometriosis. The genotypes of eNOS gene (NOS3) may be responsible for variation in its enzymatic activity as well as plasma concentration of nitric oxide. The Glu298Asp polymorphism of the NOS3 may modulate angiogenesis and influence individual susceptibility to endometriosis. This study was designed to evaluate the association between the Glu298Asp polymorphism of the NOS3 and advanced stage endometriosis.

METHODS: This study consisted of 299 women with advanced stage endometriosis and 459 control women without endometriosis in a Korean population. Genotyping results of the Glu298Asp polymorphism of the NOS3 were analyzed between the endometriosis and control group.

RESULTS: The genotypic frequencies were significantly different between women with and without endometriosis. The frequency of the non-GG genotype (GT + TT) was higher in the endometriosis group than in the control group (P = 0.001).

CONCLUSION: These findings suggest that the T allele, encoding aspartic acid, of the Glu298Asp polymorphism of the NOS3 may be associated with advanced stage endometriosis in the Korean population.

Key words: endometriosis / polymorphism / eNOS / NOS3

Introduction

Endometriosis, characterized by the presence and growth of ectopic endometrial tissue outside the endometrial cavity, can cause infertility, pelvic pain and dysmenorrhea. The estimated prevalence of endometriosis among women of reproductive age is as high as 18% and affects 5–50% of all infertile women (Missmer and Cramer, 2003). Although it is widely accepted that retrograde menstruation into the peritoneal cavity can lead to the development of the endometriosis, the definite etiology and pathogenesis of endometriosis remains unclear. It remains to be determined why the disease develops in only a certain group of women although retrograde menstruation occurs in most women. Endometriosis has shown heritable tendencies with recurrence risks of 5–7% for first-degree relatives, and familial and epidemiologic studies suggest polygenic and multifactorial inheritance (Bischoff and Simpson, 2004). Recently, we demonstrated that the vascular endothelial growth factor (VEGF) +405 C/G polymorphism may be associated with the risk of advanced stage endometriosis in the Korean population (Kim et al., 2005), in addition to many other genes.

Nitric oxide (NO), which is converted from L-arginine and molecular oxygen by a family of NO synthases (NOSs) (Moncada and Higgs, 1993), is known to play important roles in physiologic and pathologic pathways of almost every biological system. The three tissue-specific subtypes of this enzyme, neuronal (n), endothelial (e) and induced (i) NOS, are responsible for NO biosynthesis in various tissues (Moncada and Higgs, 1993). These not only induce cellular injury, altered vascular resistance and signal transduction (Knowles and Moncada, 1994; Duda et al., 2004) but also affect many angiogenic...
factors (Duda et al., 2004). In a mouse model, the endothelial nitric oxide synthase (eNOS) mediates VEGF-induced vascular permeability, vessel tone and angiogenesis (Fukumura et al., 2001). Thus, eNOS may play a role in the development of endometriosis via angiogenetic enhancement since angiogenesis is essential for the survival of ectopic endometrial tissue outside the uterus as well as the progression of endometriosis (Donnez et al., 1998).

In endometrial cells, NO is synthesized by eNOS (Marsden et al., 1993) and the expression of eNOS in endometrium has been shown to be phase-dependent changes during normal menstrual cycles (Ota et al., 1998). The expression of eNOS in endometrium is increased throughout the menstrual cycle in patients with endometriosis compared with women without endometriosis (Ota et al., 1998), and higher levels of NO were found in ectopic endometrium compared with eutopic endometrium (Wu et al., 2003). Furthermore, Khorram and Lessey (2002) demonstrated that the expression of eNOS is significantly increased in the glandular and luminal epithelium in patients with endometriosis. Taken together, the roles for eNOS have been implicated in the development of endometriosis.

The eNOS gene (NOS3) has numerous polymorphisms including a variable-number tandem repeats in intron 4, the T-786C polymorphism in the promoter region, and the Glu298Asp (G894T, rs1799983) polymorphism in exon 7. Among these reported polymorphisms of the NOS3, the Glu298Asp polymorphism leads to an amino acid substitution in the mature protein (Casas et al., 2006; http://pga.gs.washington.edu) that may cause a change in its enzymatic activity.

With regard to the association between the NOS3 Glu298Asp polymorphism and endometriosis, there are only two reports with conflicting results in Greek and Indian women (Zervou et al., 2003; Bhanour et al., 2008). Therefore, it is necessary to evaluate whether the NOS3 Glu298Asp polymorphism is associated with the risk of endometriosis in other ethnic groups including larger number of samples. For this purpose, we sought to investigate the association of the NOS3 Glu298Asp polymorphism with the risk of advanced stage endometriosis in the Korean population.

**Materials and Methods**

**Subjects**

Peripheral blood was obtained from patients who had undergone diagnostic laparoscopy, pelviscopic surgery, exploratory laparotomy or transabdominal hystectomy from October 2000 to September 2005. All subjects were of Korean origin, which is made up of a single ethnic group. A total of 299 patients exhibited surgical and histological evidence of advanced endometriosis, whereas 459 patients without the disease served as controls. 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). Ninety-five patients were diagnosed as having stage III and 204 patients had stage IV endometriosis. None of the subjects had received hormone therapy during the previous 12 months.

All patients with a diagnosis of minimal or mild endometriosis were excluded from both the case and control groups. The indications for surgery or diagnostic laparoscopy among the endometriosis group included dysmenorrhea, pelvic pain, infertility and adnexal mass. 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 among the control group were benign ovarian cyst, pelvic pain or dysmenorrhea, tubal ligation, carcinoma in situ of the uterine cervix and tubal reanastomosis. Subject ages ranged from 19 to 44 years (29.7 ± 5.3, mean ± SD) in the endometriosis group and from 19 to 55 years (41.9 ± 9.4) in the control group (P < 0.001). The review board for human research of Seoul National University Hospital approved this project, and informed written consent was obtained from each woman.

**Genomic DNA analysis**

Peripheral blood was drawn from each patient and collected in EDTA-containing tubes, and genomic DNA was extracted with the Wizard DNA Purification Kit (Promega, Madison, WI, USA). Genotyping of the Glu298Asp polymorphism was carried out by PCR–RFLP analysis, as previously described by Hingorani et al. (1999), with minor modifications. The PCR primers for the Glu298Asp polymorphism were 5’-CATGAGGCTCAGCCCCAGAAC-3’ (forward) and 5’-AGTCAATCCCTTTGGTGC TCAC-3’ (reverse). A total of 25 ng of genomic DNA was added to a PCR mixture containing 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 μM of each primer and 1.25 U of Taq polymerase. The PCR cycling conditions were as follows: an initial denaturation step at 94°C for 5 min, amplification for 30 rounds of PCR at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min followed by a final extension time of 10 min at 72°C. The PCR products were digested with 2 units of restriction enzyme MboI (New England BioLabs, Beverly, MA, USA) at 37°C overnight, separated by 2% agarose gel electrophoresis, and identified using ethidium bromide staining. The G allele remained uncut (206 bp), whereas the T allele was cut into 119 and 87 bp.

Prior to analyzing samples of subjects, we performed a preliminary experiment using the samples of other 20 subjects (they were not our study subjects). Namely, we did the experimental PCR–RFLP to get the external reference samples corresponding to each genotype (GG and GT). External reference samples were verified by direct sequencing, and were included in each experiment to prove the fidelity of each PCR–RFLP. Furthermore, PCR–RFLP for the analyses of this polymorphism was reconfirmed by real-time PCR using TaqMan assay in all endometriosis subjects and one-sixth of the controls.

**Statistical analysis**

Genotype distributions were compared with the frequencies expected under the Hardy–Weinberg equilibrium by χ2-test. A linear by linear association and χ2-test were used to assess any relationships between the genotype and allele groups. To evaluate the effects of the T allele, genotypes were grouped as GG and non-GG (GT + TT). SPSS for Windows version 12.0 (SPSS Inc, Chicago, Illinois, USA) was used for statistical analysis and P < 0.05 was considered to be statistically significant.

**Results**

The genotyping of the Glu298Asp polymorphism of the NOS3 was successfully performed in all subjects. The genotype distribution of the Glu298Asp polymorphism in patients with endometriosis and the control group was in the Hardy–Weinberg equilibrium.

The prevalence of the T allele was 6.9% in the control group, which was comparable with previous reports in Koreans (9.2%, Park et al., 2004; 6.9%, Yoon et al., 2005). The genotype distribution of the Glu298Asp polymorphism was significantly different between women with and without endometriosis (P = 0.001) (Table I). The frequency of the non-GG genotype (GT + TT) was higher in the
endometriosis group than in the control group (22.4% versus 13.3%, \( P = 0.001, \text{OR: 1.88, 95\% CI: 1.29–2.76}\)). Further analyses between Stage III and Stage IV endometriosis and between unilateral or bilateral endometriomas did not demonstrate any significant differences in genotype frequencies (data not shown).

**Discussion**

Our study demonstrated that the Glu298Asp polymorphism in the NOS3 is associated with an increased risk for advanced stage endometriosis, at least in the Korean population. Our results imply that the T allele of the Glu298Asp polymorphism may exert an important role in the progression of endometriosis.

An earlier study has shown the association between the Glu298Asp polymorphism of the NOS3 and endometriosis with a 10-fold increased risk of developing endometriosis in the presence of the T allele (Zervou et al., 2003). However, the control group of this study deviated from the Hardy–Weinberg equilibrium and it is uncertain whether a histologic diagnosis was made in that study. In contrast to our results (\( n = 758 \)) and those of Zervou et al. (2003) (\( n = 154 \)), Bhanoori et al. (2008) did not observe an association between the Glu298Asp polymorphism of the NOS3 and endometriosis in south Indian women (\( n = 442 \)). This inconsistency might have resulted from the different ethnicities of the subjects. In addition, Bhanoori et al. (2008) recruited infertile women as controls and laparoscopic diagnosis was not made in one-third of control group.

Minimal or mild endometriosis patients were not included in our study. Because of the high frequency with which minimal/mild endometriosis is found in asymptomatic women, and current theories of these disease stages representing a normal physiologic process, it appears logical for genetic association study to limit case definition to more severe stages (Zondervan et al., 2002). Although we could not compare between all four stages of endometriosis, no significant difference was observed in the frequency of the Glu298Asp polymorphism between Stage III and Stage IV endometriosis, which has not been described by any previous reports.

The frequency of the T allele in the controls (6.9%) of the present study was somewhat lower than that of other studies in Caucasians and south Indians [31.2% (Hingorani et al., 1999); 22.5% (Zervou et al., 2003); 19.1% (Bhanoori et al., 2008)]. Nevertheless, the prevalence of the T allele in our controls was comparable to those in other previous reports on Koreans [9% (Park et al., 2004); 6.9% (Yoon et al., 2005)], Japanese [7.6% (Kishimoto et al., 2004)] and Chinese [10.5% (Leung et al., 2005); 5.8% (Tso et al., 2006)]. Therefore, this discrepancy is likely to represent the difference in the frequency of the Glu298Asp polymorphism of the NOS3 in different ethnicities as documented previously (Casas et al., 2006).

To date, the effect of the Glu298Asp polymorphism on NO concentration remains unclear. Nonetheless, the Glu298Asp polymorphism has been suggested to influence the enzymatic activity of eNOS and NO production. It was documented that the T allele generates protein products with different susceptibility to cleavage, suggesting that this polymorphism has a functional effect on the eNOS protein (Tesauro et al., 2000). Additionally, Yoon et al. (2000) reported that the T allele is associated with an increased plasma concentration of NO product in healthy controls. Furthermore, Wang et al. (2000)

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**Table I**

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotypes n (%)</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GT</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>232 (77.6)</td>
<td>64 (21.4)</td>
</tr>
<tr>
<td>Control</td>
<td>398 (86.7)</td>
<td>59 (12.9)</td>
</tr>
</tbody>
</table>

**OR (95\% CI)**

<table>
<thead>
<tr>
<th>Group</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometriosis</td>
<td>1.88 (1.29–2.76)</td>
</tr>
<tr>
<td>Control</td>
<td>1.80 (1.26–2.77)</td>
</tr>
</tbody>
</table>

**OR** = odds ratio; CI = confidence interval.

\( P \) = linear association in comparison with the control group.

\( \chi^2 \) = Test in comparison with the control group.
have reported an association between the NOS3 polymorphism and protein levels of eNOS.

In conclusion, this study showed that the Glu298Asp polymorphism of the NOS3 is associated with the risk of advanced stage endometriosis in the Korean population. Although the mechanism underlying this association remains unclear so far, our findings suggest an important role of the NOS3 polymorphism in the pathogenesis of endometriosis as well as the ethnic differences in the frequency of the NOS3 polymorphism contributing to the increased risk of endometriosis.

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