

Association between susceptibility to advanced stage endometriosis and the genetic polymorphisms of aryl hydrocarbon receptor repressor and glutathione-S-transferase T1 genes

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BACKGROUND: This study was performed to determine whether genetic polymorphisms of aryl hydrocarbon receptor repressor (*AhRR*), glutathione-S-transferase M1 (*GSTM1*) and glutathione-S-transferase T1 (*GSTT1*) are associated with susceptibility to advanced stage endometriosis in a Korean population. **METHODS:** This study comprised 316 women with advanced stage endometriosis and 256 control women without endometriosis. Genotyping of the *AhRR* codon 185 was performed by real-time polymerase chain reaction (PCR) analysis. *GSTM1* and *GSTT1* genotyping for gene deletions were carried out by multiplex PCR analysis. **RESULTS:** G allele frequency at codon 185 of *AhRR* was increased in patients with endometriosis ($P = 0.047$), and there was a trend for an association of C/G + G/G genotypes with risk of endometriosis ($P = 0.06$). The proportion of null mutation at *GSTT1* also tended to increase ($P = 0.06$) in patients with endometriosis, whereas there was no difference in the genotype distribution of *GSTM1* genes. Analyzing *AhRR* and *GSTT1* together, we found that patients with high-risk genotypes at both loci have increased risk of endometriosis, compared with patients without high-risk genotypes ($P = 0.015$). **CONCLUSIONS:** These findings suggest that the *AhRR* codon 185 and *GSTT1* polymorphisms are associated with the risk of advanced stage endometriosis.

Keywords: aryl hydrocarbon receptor repressor; glutathione-S-transferase M1; glutathione-S-transferase T1; endometriosis; polymorphism

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% within infertile women (Missmer and Cramer, 2003). Although retrograde menstruation is widely accepted as a major contributing factor in the pathogenesis of endometriosis, it is also a common phenomenon occurring in up to 90% of menstruating women with patent fallopian tubes (Halme *et al.*, 1984). Therefore, it has yet to be determined why endometriosis affects only a certain group of women.

Endometriosis shows heritable tendencies, with 5–8-folds increased risks for first-degree relatives, indicating that

polygenic and multifactorial etiology is far more likely to be the cause than Mendelian inheritance (Simpson and Bischoff, 2002). There is also growing evidence that exposure to environmental contaminants could contribute to the pathogenesis of endometriosis. Dioxin is a typical environmental contaminant exerting adverse estrogen-related effects (Bock, 1994), and has been shown to induce a dose-dependent increase in severity of endometriosis in non-human primates (Rier *et al.*, 1993). Extrapolation to women was initially thought to be epidemiologically plausible, as Belgium, with the highest dioxin pollution in the world, has the highest incidence of endometriosis as well as the highest prevalence of severe endometriosis (Giudice and Kao, 2004). However, Guo *et al.* (2004) pointed out that the statistical analysis in the Rier *et al.* (1993) report was not sound, and there has been no human

epidemiological study definitely linking dioxin to the risk of endometriosis.

Based upon the possible contribution of dioxin to the pathogenesis of endometriosis along with its genetic predisposition, it has been postulated that variants in the genes involved in xenobiotic detoxification process may be associated with the risk of endometriosis. Indeed, several investigators have reported possible involvement of some detoxification enzyme gene polymorphisms in the development of endometriosis (Baranova *et al.*, 1997, 1999; Arvanitis *et al.*, 2003; Lin *et al.*, 2003; Hsieh *et al.*, 2004). However, a meta-analysis (Guo, 2005) and a systematic review (Guo, 2006) do not support their findings and suggest that there is no strong indication that any specific detoxification enzyme gene polymorphism is consistently associated with susceptibility to endometriosis. Recently, Tsuchiya *et al.* (2005) revealed that a genetic polymorphism of aryl hydrocarbon receptor repressor (*AhRR*), a down-regulator of the genes regulated by *AhR*, is associated with susceptibility to endometriosis in a Japanese population. They have shown that the C/G and the G/G genotypes at codon 185 of *AhRR* are associated with the risk of endometriosis. However, it is necessary to clarify whether *AhRR* polymorphism is associated with endometriosis in another ethnic group with adequate sample number, since their report included only a small number of patients and inconsistent results have been reported in the same population (Watanabe *et al.*, 2001).

The primary goal of the present study was to explore the association between *AhRR* polymorphism and the risk of advanced endometriosis in a Korean population. We also tried to determine whether the genetic polymorphisms of glutathione-S-transferase M1 (*GSTM1*) and glutathione-S-transferase T1 (*GSTT1*) are associated with susceptibility to endometriosis in the same population.

Materials and Methods

Subjects

Peripheral blood was obtained from the patients who had undergone diagnostic laparoscopy, pelviscopic surgery, exploratory laparotomy or transabdominal hysterectomy from October 2000 to September 2005. All subjects were of Korean origin, which is made up of a single ethnic group. A total of 316 patients had surgical and histological evidence of advanced endometriosis, whereas 256 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). Sixty-four patients were diagnosed as having stage III and 252 patients had stage IV endometriosis. None of the subjects had lived in the vicinity of any potentially higher risk environments, such as chemical factories or farms, had specific dietary habits, such as vegetarianism or nearly pure meat or fish diets, or 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 or the diagnostic laparoscopy among the endometriosis group were dysmenorrhea ($n = 95$), pelvic pain ($n = 74$), infertility ($n = 74$), adnexal mass ($n = 63$) and others ($n = 10$). Patients with leiomyoma, adenomyosis, invasive carcinoma of the

uterine cervix or ovarian cancer were excluded from the control group. The indications for surgery or the diagnostic laparoscopy among the control group were benign ovarian cyst ($n = 155$), pelvic pain or dysmenorrhea ($n = 24$), tubal ligation ($n = 23$), carcinoma *in situ* of the uterine cervix ($n = 20$), tubal reanastomosis ($n = 12$) and others ($n = 22$). The review board for human research of Seoul National University Hospital approved this project, and informed written consent was obtained from each woman. Underlying infertility rates were 1.6% in the control group and 23.4% in the endometriosis group. Ages ranged from 19 to 40 years (29.6 ± 4.3 , mean \pm SD) in the endometriosis group and from 16 to 55 years (36.9 ± 9.2) in the control group.

Genomic DNA analysis

Peripheral blood was drawn from each patient and collected in an EDTA-containing tube, and genomic DNA was extracted with the Wizard DNA Purification Kit (Promega, Madison, WI, USA). The *AhRR* polymorphism was genotyped by real-time PCR analysis on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using fluorescent labeled probes. Each 20 μ l PCR reaction contained 10 pmol of forward primer 5'-AGACGG ATGTTATGCACCAGAA-3', 10 pmol of reverse primer 5'-AG AGGCAGCGATGTGTATGG-3', 4 pmol of C-allele probe 5'-(FAM)-TGGGCAGCCCCCGCC-(TAMRA)-3', 4 pmol of G-allele probe 5'-(VIC)-TGGGCAGCCCCCGCC-(TAMRA)-3', 10 μ l of 2X TaqMan Universal PCR Master Mix (Applied Biosystems) and 25 ng DNA. The PCR cycling conditions consisted of one 2 min cycle at 50°C and one 10 min cycle at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Distilled water was used as a negative PCR control in each amplification.

GSTM1 and *GSTT1* genotyping for gene deletions were carried out by multiplex PCR analysis. The PCR primers for the *GSTM1* and the *GSTT1* polymorphisms were 5'-GAACTCCCTGAAAAGCTAAAGC-3' (forward) and 5'-GTTGGGCTCAAATATACGGTGG-3' (reverse) (Bioneer, Seoul, Korea), which produced a 219 bp product, and 5'-TTCCTACTGGTCTCACATCTC-3' (forward) and 5'-TCACCGGATCATGGCCAGCA-3' (reverse) (Bioneer), which produced a 459 bp product, respectively. Amplification of β -globulin gene with the primers 5'-CAACTTCATCCACGTTCCACC-3' and 5'-GAA GAGCCA AGGACAGGTAC-3' (Bioneer) was used as an internal control and produced a 268 bp product. A 25 ng sample of genomic DNA was added to a PCR mixture containing 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM each primer and 1.0 U of *Taq* polymerase. The PCR cycling conditions were as follows: an initial denaturation step at 94°C for 5 min, amplification for 40 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The amplified product was visualized in an ethidium bromide stained 3% agarose gel. If the study subject is null for the gene, no PCR product is present, and the β -globulin gene fragment acts as a positive PCR control (Fig. 1).

Statistical analysis

Power calculation based on the numbers of patients and controls in the present study revealed that the power to detect a significant difference of genotype frequency similar to the report by Tsuchiya *et al.* (2005) (25.3% versus 45.8% for CC genotype) was 1.0. Genotype distributions were examined for significant departure from Hardy-Weinberg equilibrium by a goodness of fit chi-square test. Chi-square analysis and univariate logistic regression were used to evaluate differences in the proportions of the genotypes between the endometriosis and control groups. Distribution of two genotypes at two loci was analyzed by univariate logistic regression. $P < 0.05$ was considered significant.

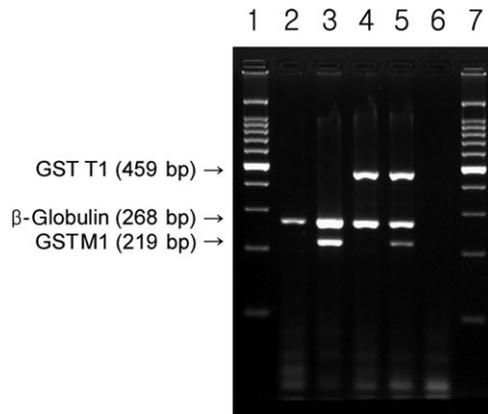


Figure 1: Patterns of multiplex PCR for glutathione-*S*-transferase M1 (*GSTM1*) and glutathione-*S*-transferase T1 (*GSTT1*) genes. β -Globulin gene was used as an internal positive control. Lane 1 and Lane 7 are the 100 bp DNA ladder and Lane 6 is negative control. Lane 2 represents null genotypes for both *GSTM1* and *GSTT1* genes. Lane 3 is the wild genotype for the *GSTM1* gene and the null genotype for the *GSTT1* gene, lane 4 is the null genotype for the *GSTM1* and the wild genotype for the *GSTT1* gene and lane 5 represents wild genotypes for both *GSTM1* and *GSTT1* genes.

Results

Genotyping of the three polymorphic loci was successfully achieved for all subjects. Genotypic distributions of the codon 185 of *AhRR* genes were in Hardy–Weinberg equilibrium in both groups. G allele frequency at codon 185 of *AhRR* genes was increased in patients with endometriosis [38% versus 32.2%, $P = 0.047$, odds ratio (OR): 1.29, 95% confidence interval (CI): 1.01–1.65] (Table 1). There was a trend for patients with C/G + G/G genotypes to have an increased risk of endometriosis compared with those with C/C genotypes (61.4% versus 53.5%, $P = 0.058$, OR: 1.38, 95% CI: 0.99–1.33) (Table 1).

The proportion of null mutation at *GSTT1* also tended to increase in patients with endometriosis (56.3% versus 48.4%, $P = 0.060$, OR: 1.37, 95% CI: 0.99–1.91), whereas there was no difference in the genotype distribution of *GSTM1* genes (57.9% versus 57.0%, $P = 0.865$, OR: 0.97, 95% CI: 0.69–1.35) (Table 2). Analyzing *AhRR* and *GSTT1* together, we found that patients with high-risk genotypes at both loci have increased risk of endometriosis, compared with patients without any high-risk genotypes ($P = 0.015$, OR: 1.86, 95% CI: 1.13–3.06) (Table 3).

Discussion

Despite its physiological role in the detoxification of polycyclic aromatic compounds, the activity of cytochrome P450 1A1 (*CYP1A1*) enzyme can be deleterious, since it generates mutagenic metabolites and active oxygen (Nebert and Gonzalez, 1987; Nebert *et al.*, 2000). The induction of *CYP1A1* gene is regulated by AhR that is present in the cytosol in a complex with heat shock protein (Hsp) 90 in the absence of ligand (Mimura and Fujii-Kuriyama, 2003). Upon binding to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the AhR complex translocates into the nucleus and the AhR dissociates from Hsp90 complex to form a heterodimer with its partner molecule, AhR nuclear translocator (*ARNT*). The AhR/*ARNT* heterodimer recognizes a DNA enhancer element sequence, designated xenobiotic responsive element (XRE), located in the promoter region of *CYP1A1* gene, resulting in the enhanced expression of the gene (Mimura and Fujii-Kuriyama, 2003). Mimura *et al.* (1999) have identified AhRR and demonstrated that AhRR localizes in the nuclei and forms a heterodimer with Arnt constitutively. They have shown that the AhRR/*ARNT* heterodimer also recognizes the XRE, but functions as a transcriptional repressor, indicating that AhRR functions as a negative regulator of AhR by competing with AhR for forming a heterodimer with Arnt and binding to the XRE sequence.

Extensive studies on the AhR function using AhR-deficient mice have shown that AhR is responsible for most of toxic effects caused by TCDD, with induction of *CYP1A1* gene and other dioxin-inducible genes (Mimura *et al.*, 1997; Shimizu *et al.*, 2000). Accordingly, loss or reduction of *AhRR* expression in cells can lead to increased susceptibility to toxic effects of TCDD by impeding the negative regulatory effects of AhRR. Based on the expectation that polymorphisms of *AhRR* genes could be relevant to the individual's susceptibility to dioxins, some investigators have explored the possible association of these polymorphisms with susceptibility to specific diseases for which environmental contaminants could contribute to the pathogenesis. Evaluating the genotype frequency of the *AhRR* codon 185 polymorphism in a Japanese population, Fujita *et al.* (2002) revealed that there is a significant difference between subjects with and without micropenis, suggesting that subjects with a specific genotype of the *AhRR* codon 185 polymorphism might have increased susceptibility to the undermasculinizing effects of dioxin exposure in utero,

Table 1: Distribution of codon 185 polymorphism in the *AhRR* gene in endometriosis patients ($n = 316$) and controls ($n = 256$)

Genotype/ allele	Endometriosis n (%)	Control n (%)	P -value	OR (95% CI)
CC	122 (38.6)	119 (46.5)		Reference
CG	148 (46.8)	109 (42.6)	0.12 ^a	1.32 ^a (0.93–1.89)
GG	46 (14.6)	28 (10.9)	0.08 ^a	1.60 ^a (0.94–2.73)
CC	122 (38.6)	119 (46.5)	0.058 ^b	1.38 ^b (0.99–1.33)
Non-CC	194 (61.4)	137 (53.5)		
C allele	392 (62)	347 (67.8)	0.047 ^b	
G allele	240 (38)	165 (32.2)	0.047 ^b	1.29 (1.01–1.65)

^aEvaluated by logistic regression analysis in comparison with the control group.

^bEvaluated by chi-square test in comparison with the control group.

Table 2: Prevalence of homozygosity for the *GSTM1* and *GSTT1* null mutations in endometriosis patients and controls

Group	<i>GSTM1</i>				<i>GSTT1</i>			
	Present n (%)	Null n (%)	P-value	OR (95% CI)	Present n (%)	Null n (%)	P-value	OR (95% CI)
Endometriosis (n = 316)	133 (42.1)	183 (57.9)	0.865 ^a	0.97 ^a (0.69–1.35)	138 (43.7)	178 (56.3)	0.060 ^a	1.37 ^a (0.99–1.91)
Control (n = 256)	110 (43.0)	146 (57.0)			132 (51.6)	124 (48.4)		

^aEvaluated by chi-square test in comparison with the control group.

presumably through the diminished inhibition of AHR-mediated signaling. These findings are consistent with genetic studies performed in other Japanese populations (Watanabe *et al.*, 2004; Soneda *et al.*, 2005).

Recently, Tsuchiya *et al.* (2005) have shown that the C/G and the G/G genotypes at codon 185 of *AhRR* are associated with the risk of endometriosis in a Japanese population. However, they suggested that their findings may have limitations due to the small number of subjects recruited, and that further studies are necessary in other ethnic groups with larger sample size. Recruiting a total of 572 patients who had been confirmed surgically, the present study demonstrated that G allele frequency at codon 185 of *AhRR* was increased in patients with endometriosis ($P = 0.047$), but the C/G + G/G genotypes showed only a trend toward association with the risk of endometriosis. Since the proportion of null mutation at *GSTT1* also showed this trend in patients with endometriosis, we analyzed the risk of the disease according to the number of putative high-risk genotype at the two loci. Compared with patients without any high-risk genotypes, those with two high-risk genotype(s) at the two loci had significantly increased risk of endometriosis.

Based on the findings of the present study, it may be speculated that a single genetic polymorphism involving *AhRR* or *GSTT1* exhibits only a marginal effect, but can confer a significant increase in the risk of endometriosis if the putative high-risk genotypes at the two loci are combined. Performing a meta-analysis, Guo (2005) has shown that there is no evidence that women with *GSTM1* null genotype have increased risk of endometriosis, but reported that the risk associated with *GSTT1* null genotype is higher than other genotypes, although the

estimate could easily lose its significance, if there is a realistic 69–80% publication probability. Guo suggested that it is possible that *GSTM1* or *GSTT1* alone does not exhibit an appreciable effect on the risk of endometriosis but interacts with other genes to confer, jointly, increased risk of endometriosis. Indeed, testing for association of endometriosis with *GSTM1*, *GSTT1* and *CYP1A1 MspI* polymorphisms, Hadfield *et al.* (2001) have shown that the combination of the *GSTM1* null genotype and the *CYP1A1 MspI* polymorphism was associated with increased risk of endometriosis, whereas no significant differences were found between cases and controls in the frequencies of the *GSTM1*, *GSTT1* and *CYP1A1 MspI* polymorphisms. It might be suggested that any single genetic polymorphism in the aryl hydrocarbon gene battery does not lead to a serious increase in the risk of developing endometriosis due to the possible genetic redundancy existing in the detoxification pathways. However, if an individual has high-risk genotypes at multiple loci that are involved in the detoxification pathways, it is possible that she could have significantly increased risk of endometriosis.

The mean age of the control subjects in the present study was higher than the endometriosis patients. However, as described by Hadfield *et al.* (2001), recruiting women from this age group has a 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. The control group in the present study may not be representative of the general population, since they all had undergone diagnostic laparoscopy, pelviscopic surgery, exploratory laparotomy or transabdominal hysterectomy. However, our study had the advantage of using a completely disease-free control

Table 3: Risk of advanced stage endometriosis according to the number of possible high risk *AhRR* and *GSTT1* genotypes

Number of high risk genotypes	Genotypes group				P-value and OR			
	<i>AhRR</i>	<i>GSTT1</i>	Endometriosis n (%)	Control n (%)	Crude P-value	Crude OR (95% CI)	Age-adjusted P-value	Age-adjusted OR (95% CI)
0	CC	Present	54 (17.1)	52 (20.3)				
1	CC CG or GG	Null Present	152 (48.1)	147 (57.4)	0.985 ^a	1.00 (0.64–1.55)	0.594 ^a	0.87 (0.53–1.45)
2	CG or GG	Null	110 (34.8)	57 (22.3)	0.015 ^a	1.86 (1.13–3.06)	0.048 ^a	1.77 (1.01–3.13)

^aVersus zero high risk genotypes.

group, and thus compared the genotypes between individuals with and without advanced endometriosis more completely.

In summary, the present study has shown that a single genetic polymorphism involving *AhRR* or *GSTT1* exhibits a marginal effect, but can confer a significant increase in the risk of endometriosis if the putative high-risk genotypes at the two loci are combined. Our data suggest that combined analysis of genetic polymorphisms in the several candidate genes involved in xenobiotic detoxification process is necessary to elucidate the possible genetic etiologic factors in endometriosis.

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