内分泌撹乱およびその他の化学物質の職業性曝露に関連する男性リプロダクティブ・ヘルス－疫学的知見

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要　旨：職業性曝露に関連するヒト，特に男性生殖系影響に関する研究の趨勢は，1990年代初頭頃に内分泌撹乱化学物質(endocrine disrupting chemicals, EDC)の概念が普及して以来分岐している．それ以前の研究の過半は限られた範囲の化学物質を対象に実施され，単一かつ比較的高濃度の曝露による生殖毒性の評価という枠組みを有していた(従来の枠組み)．この範疇の研究は現在も認められるが，EDCの概念の普及以来男性生殖系に関する研究については，内分泌撹乱作用の可能性をもった広範な化学物質の探索および化学物質の複合微量曝露の影響評価という新しい枠組みが与えられている．また両方の枠組みをもった研究もある．新しい枠組みをもった近年の研究では，男性生殖機能を評価するための標準的検査手法やより鋭敏な検出力をもった研究計画が採用されるようになった．その結果，職業環境中に内分泌撹乱化学物質の存在が示唆されている．しかしながら，職業性曝露を有する集団の同定や曝露評価に伴う困難から，疫学的知見の解釈は制約を受ける．今後は，知見の統合とともに慎重なプロトコールに立脚し，職業曝露集団に関する疫学研究の範囲を拡大する必要がある．

キーワード：内分泌撹乱，職業性曝露，疫学，生殖毒性．
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Association between endometriosis and genetic polymorphisms of the estradiol-synthesizing enzyme genes *HSD17B1* and *CYP19*

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BACKGROUND: Endometriosis, an estrogen-dependent disease, is believed to be influenced by multiple genetic and environmental factors. Here, we evaluated whether the risk and severity of endometriosis are associated with polymorphisms in estradiol-synthesizing enzyme genes: the Ser312Gly polymorphism in 17-beta-hydroxysteroid dehydrogenase type 1 (*HSD17B1*) and the Arg264Cys polymorphism in cytochrome P450, subfamily XIX (*CYP19*). METHODS: All participants underwent diagnostic laparoscopy, and the stage of endometriosis was determined according to the Revised American Fertility Society classification. Of the 138 women enrolled, 59 had no endometriosis, 21 had stage I, 10 had stage II, 23 had stage III and 25 had stage IV. SNPs were discriminated by allele-specific oligonucleotide hybridization. RESULTS: Individuals having at least one A-allele (A/G or A/A genotype) of *HSD17B1* showed a significantly increased risk of endometriosis (A/G genotype: adjusted OR, 3.06; 95%CI 1.21–7.74; A/A genotype: adjusted OR, 3.02; 95%CI 1.08–8.43). There was a significant trend associating A/G + A/A genotypes with severity of endometriosis (*P* for trend <0.01). No statistically significant association was found for the CYP19 polymorphism. CONCLUSIONS: Evidence for association between the Ser312Gly polymorphism in *HSD17B1* and endometriosis was found in a Japanese population. The A-allele of *HSD17B1* appears to confer higher risk for endometriosis.

Key words: CYP19/endometriosis/estrogen synthesis/genetic polymorphism/HSD17B1

Introduction

Endometriosis, one of the most common causes of female infertility and chronic pelvic pain, is defined as the presence of endometrial tissue outside the uterus. Three predominant theories have been proposed for the etiology of this disease: Mullerian remnants, metaplasia and direct implantation of endometrial cells (El-Mahgoub and Yaseen, 1980; Murphy *et al.*, 1986; Fujii *et al.*, 1991). Although the exact prevalence is still not known, endometriosis affects up to 5–10% of women of reproductive age (Wheeler *et al.*, 1989). The prevalence of endometriosis is as high as 20–50% in infertile women (Strathy *et al.*, 1982; Rawson *et al.*, 1991).

Endometriosis is regarded as a complex trait, in which genetic and environmental factors contribute to the disease phenotype (Kennedy *et al.*, 1998). A variety of factors affect the development of endometriosis, including hormonal status and genetic factors. For example, women with shorter intervals between menstruation and longer duration of menses are at higher risk for endometriosis (Vercellini *et al.*, 1997). The risk of endometriosis is seven times higher if a first-degree relative has been affected by endometriosis (Simpson *et al.*, 1980). However, the interaction between genetic susceptibility and environmental factors is not yet adequately understood.

The development of endometriosis is estrogen-dependent. Endometrial implants contain estrogen and progesterone receptors (Lessey *et al.*, 1989) and respond to ovarian hormonal changes, causing local bleeding, inflammation and formation of adhesions. The three main estrogens are estradiol, estrone and estriol. Estradiol, the most active form, is produced either from estrone via 17-β-hydroxysteroid dehydrogenase type 1 (HSD17B1) or from testosterone via cytochrome P450, subfamily XIX (CYP19, aromatase) (Mitrunen and Hirvonen, 2003).

Current evidence indicates that polymorphisms in genes of drug-metabolizing enzymes can affect phenotypic metabolic

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variations. The *HSD17B1* gene is located in chromosome 17q12 and has a polymorphism consisting of an A to G substitution in exon 6, resulting in an amino acid change of Ser312Gly (Puranen *et al.*, 1994). The *CYP19* gene, located in chromosome 15q21, has a polymorphism consisting of C to T substitution in exon 7, resulting in an amino acid change of Arg264Cys (Toda *et al.*, 1990).

Genetic polymorphisms involved in estrogen synthesis and metabolism may play an important role in the variation of endometriosis among individuals by altering local estrogen production or circulating levels of estrogen. Here, we evaluate whether the Ser312Gly polymorphism in *HSD17B1* and Arg264Cys polymorphism in *CYP19* are associated with the risk and severity of endometriosis. A case-control study was conducted on these two polymorphisms in patients with different stages of endometriosis and controls.

Materials and methods

The protocol for the study was approved by the Institutional Review Board of University of Miyazaki, The Jikei University School of Medicine and National Cancer Center. All subjects gave their written informed consent before the laparoscopic examination.

Study participants

This study was a part of a case–control study of endometriosis. During the years 1999–2000, 139 women were recruited at the Department of Obstetrics and Gynecology, The Jikei University School of Medicine Hospital. Participants were patients between the ages of 20 and 45 who had complained of infertility and attended the hospital. The study protocol excluded all women from the study who had ever given birth or lactated. Of the 139 women recruited, only one was excluded from subsequent analysis because a DNA sample was not available.

All participants underwent diagnostic laparoscopy, and the stage of endometriosis was determined according to the Revised American Fertility Society classification (r-AFS) (American Fertility Society, 1985). Of the 138 women enrolled, 59 women had no endometriosis, 21 had stage I, 10 had stage II, 23 had stage III and 25 had stage IV.

Cases and controls were similar in several confounding factors. Risk factors for endometriosis include age, shorter menstrual cycles and longer duration of menstrual flow (Vessey *et al.*, 1993; Eskenazi and Warner, 1997). The mean age of the cases was 32.4 years and 33.1 in the controls ($P = 0.35$). No significant difference was observed in the duration of menstruation. However, there was a significant trend towards cases having shorter menstrual cycles compared to the controls (28.8 days for cases and 30.7 for controls, $P = 0.03$).

Genotyping

Blood samples were obtained before the laparoscopic examination. Genomic DNA samples were extracted from peripheral white blood cells by using a DNA Extractor WB Kit (Wako, Osaka, Japan).

A 67 bp fragment in *HSD17B1*, including an SNP site located at 27 bases from the 5′ end, was amplified using sense (5′-CTGGGGCAGAGGACGAGG) and biotin-labeled antisense (5′-GCGGCCGGAGGATCG) primers. A 56 bp fragment of *CYP19*, including an SNP site located at 31 bases from the 5′ end, was amplified using biotin-labeled sense (5′-GCCATAGAAGTTCTGATAGCAG) and antisense (5′-AGTTTCTCTTCTGTGGAAATCCT) primers. PCR amplifications were performed using a TPC-200 thermal cycler (MJ Research Inc., Watertown, MA) in a total reaction volume of 25 μl containing 20 ng of DNA sample, 0.6 U AmpliTaq DNA polymerase (Applied Biosystems), 0.25 mM dNTPs, 0.2 μM primers and PCR buffer [1 × GC buffer II (Takara Bio Inc., Otsu, Japan) for *HSD17B1* and 1 × GeneAmp PCR buffer (Applied Biosystems) for *CYP19*]. The amplification protocol comprised initial denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 15 s and annealing at 55 °C for 30 s.

SNP discriminations were conducted in a manner similar to that described previously (Maruyama *et al.*, 2004) with details as follows, based on allele-specific oligonucleotide hybridization using bio-nano magnetite particles (Takeyama *et al.*, 2000; Matsunaga *et al.*, 2001). Cy3- and Cy5-labeled detection probes were designed for each SNP as follows: Cy3-labeled *HSD17B1* A-allele detection probe (5′-CCGGGCGCAGTGCGGTG), Cy5-labeled *HSD17B1* G-allele detection probe (5′-CCGGGCGCGGTGCGGTG), Cy3-labeled *CYP19* T-allele detection probe (5′-AATCCTGCATCTTTTTT) and Cy5-labeled *CYP19* C-allele detection probe (5′-AAATCCTGCGTCTTTTTT). All the following experiments were performed using the semi-automated SNP detection processor (Tanaka *et al.*, 2003).

Biotinylated PCR product (12.5 μl) and streptavidin-immobilized bio-nano magnetic particles (25 μg/12.5 μl), which were prepared according to the method described by Yoshino *et al.* (2002), were mixed and incubated for 15 min at room temperature for capturing the PCR products on the magnetic particles. PCR products were denatured into single strands by alkali treatment (10 mM NaOH). After neutralization of the mixture by neutralization buffer (100 mM Tris–HCl pH 7.5, 3 mM EDTA, 0.1% BSA), 25 μl of hybridization buffer (1 M tetramethylammonium chloride, 37.5 mM Tris–HCl pH7.5 and 3 mM EDTA) containing 12.5 pmoles of Cy3-labeled and Cy5-labeled detection probes was added to the PCR products captured on magnetic particles.

The mixture was rapidly heated up to 70 °C, and then cooled slowly to 25 °C over 10 min to allow hybridization of the detection probes and biotinylated PCR products. The optimum temperature for dissociating single-base mismatched probes was determined by analysis of dissociation curves. The mixture was heated to the optimum temperature: 56 °C for *HSD17B1* or 54 °C for CYP19, and the detection probes dissociated were removed at this temperature using an automated SNP detection processor (Maruyama *et al.*, 2004). Finally, the mixture was heated to 80 °C to liberate the hybridized detection probe into the supernatant.

The fluorescence intensity of the liberated detection probe was measured at Ex: 540 nm, Em: 570 nm for Cy3 and Ex: 645 nm, Em: 675 nm for Cy5 by a microplate reader (BMG Labtech, Offenburg, Germany), respectively. The samples were classified into three distinct categories according to the signal ratio of Cy5/Cy3: (i) those with values >2, representing samples with the homozygous G/G genotype in *HSD17B1* or the homozygous C/C genotype in *CYP19*; (ii) values <0.5, representing samples with the homozygous A/A genotype in *HSD17B1* or the homozygous T/T genotype in *CYP19*; (iii) intermediate values, representing samples with the heterozygous genotype.

Statistical analysis

To estimate the risk of endometriosis, crude odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for subgroups with the different genotypes with respect to the Ser312Gly polymorphism in *HSD17B1* and the Arg264Cys polymorphism in *CYP19*. ORs were adjusted for three endometriosis risk factors: age, menstrual cycle and duration of menstruation, using multiple logistic

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regression analysis by SPSS for Windows software (version 11.0.1J, SPSS Japan, Tokyo, Japan).

The Wilcoxon rank-sum test for trend was also used to estimate the associations between the two polymorphisms and stage of endometriosis (Goodman *et al.*, 1954). All statistical tests were based on two-tailed probability. $P < 0.05$ was considered statistically significant.

Results

Of the 138 DNA samples, 10 could not be reliably genotyped and were excluded from subsequent statistical analysis (six subjects for *HSD17B1*, four subjects for *CYP19*). The observed genotype distributions were in Hardy–Weinberg equilibrium in the control subjects.

Test for association with risk of endometriosis

Table I shows the genotypic and allelic distributions of *HSD17B1* and *CYP19* polymorphisms. Individuals with at least one *HSD17B1* A-allele (A/G or A/A genotype) showed a significantly increased risk of endometriosis (A/G genotype: adjusted OR, 3.06; 95%CI 1.21–7.74; A/A genotype: adjusted OR, 3.02; 95%CI 1.08–8.43). A similar result was obtained in comparison of the combined A/A + A/G genotypes with the G/G genotype (adjusted OR, 3.05; 95%CI 1.30–7.14). No significant association was observed between the Arg264Cys polymorphism in *CYP19* and risk of endometriosis (C/T genotype: adjusted OR, 1.35; 95%CI 0.62–2.95; T/T genotype: adjusted OR, 0.41; 95%CI 0.09–1.85).

Test for association with severity of endometriosis

To evaluate whether the Ser312Gly polymorphism in *HSD17B1* and the Arg264Cys polymorphism in *CYP19* are associated with the severity of endometriosis, participants were categorized into three groups according to r-AFS classification: controls, stage I–II and stage III–IV (Table II). There was a significant trend between the combined A/G + A/A genotypes and stage of endometriosis (P for trend <0.01). No statistically significant association was found between the Arg264Cys polymorphism in *CYP19* and stage of endometriosis (data not shown).

Discussion

In the present study, we evaluated whether two polymorphisms in estradiol-synthesizing enzyme genes are associated with the risk and severity of endometriosis. The results of this study suggested that the Ser312Gly polymorphism in *HSD17B1* is associated with both risk and severity of endometriosis, while no associations were found for the Arg264Cys polymorphism in *CYP19*.

In this study, we applied strict clinical criteria for the definition of cases and controls. One third of women with endometriosis are asymptomatic, and laparoscopy or laparotomy is indispensable for the diagnosis of endometriosis. All participants in the present study underwent diagnostic laparoscopy and were diagnosed according to the r-AFS classification. In addition, bias from confounding variables was minimized by adjusting ORs for the risk and stage of

Table I. Genotypic and allelic distribution of HSD17B1 and CYP19 polymorphisms

Polymorphisms	Cases *n* (%)		Controls *n* (%)		Crude OR (95%CI)	Adjusted OR[a] (95%CI)
	Genotype	Allele	Genotype	Allele		
HSD17B1 Ser312Gly						
G/G	13 (17.3)	G allele: 66 (44.0)	22 (38.6)	G allele: 65 (57.0)	1	1
A/G	40 (53.3)		21 (36.8)		**3.22** (1.36–7.66)**	**3.06* (1.21–7.74)**
A/A	22 (29.3)	A allele: 84 (56.0)	14 (24.6)	A allele: 49 (43.0)	**2.66* (1.02–6.94)**	**3.02* (1.08–8.43)**
A/G, A/A	62 (82.7)		35 (61.4)		**3.00** (1.35–6.68)**	**3.05** (1.30–7.14)**
CYP19 Arg264Cys						
C/C	35 (46.0)	C allele: 107 (70.4)	29 (50.0)	C allele: 81 (69.8)	1	1
C/T	37 (48.7)		23 (39.7)		1.33 (0.65–2.73)	1.35 (0.62–2.95)
T/T	4 (5.3)	T allele: 45 (29.6)	6 (10.3)	T allele: 35 (30.2)	0.56 (0.14–2.15)	0.41 (0.09–1.85)
C/T, T/T	41 (54.0)		29 (50.0)		1.17 (0.59–2.32)	1.13 (0.54–2.36)

[a]ORs adjusted for age and menstrual characteristics.
*$P < 0.05$.
**$P < 0.01$.

Table II. Severity of endometriosis associated with HSD17B1 polymorphism

Endometriosis	G/G genotype	A/G, A/A genotype	Crude OR (95%CI)	Adjusted OR[a] (95%CI)
Controls				
n (%)	22 (39.7)	35 (60.3)	1	1
Stage?-?				
n (%)	7 (23.3)	23 (76.7)	2.07 (0.76–5.62)	2.07 (0.69–6.20)
Stage ?-?				
n (%)	6 (13.3)	39 (86.7)	**4.08** (1.49–11.22)**	**3.99** (1.41–11.26)**

[a]ORs adjusted for age and menstrual characteristics.
P for trend <0.01.
**$P < 0.01$.

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endometriosis for variables known to affect endometriosis: age, menstrual cycle and duration of menstrual flow. Detailed questionnaires were designed to determine patients' menstrual cycle and duration of menstruation. The questionnaires were administered by a trained interviewer before the laparoscopic examination to minimize recall bias.

Retrograde menstruation is thought to be one of the main causes of endometriosis. However, retrograde menstruation is a common phenomenon (Kruitwagen *et al.*, 1991), and not all the women of reproductive age are affected by endometriosis. In short, women with endometriosis can be considered to have defects in the regulation of endometrial proliferation: (i) a tendency to proliferate endometrial tissue, and (ii) impaired clearance of abnormal endometrial tissue (Vinatier *et al.*, 2001).

Development of endometriosis is estrogen-dependent, and several features of this disease can be explained on the basis of overproduction of estrogen. Current therapy consists of hormone treatments aimed at lowering circulating estrogen. Genetic polymorphisms in the estrogen-synthesis or estrogen-metabolizing enzymes may cause inter-individual variation of levels and activity of circulating estrogen.

Estradiol, the most physiologically active form of estrogen, stimulates the proliferation of the endometrium during the ovulatory phase of the menstrual cycle. The *HSD17B1* genotypes found to be associated with endometriosis may confer increased activity or expression of HSD17B1 enzyme, causing increased exposure to estradiol. Our results suggest that possessing at least one A-allele of the Ser312Gly polymorphism in *HSD17B1* increases the risk of endometriosis. Furthermore, AG + AA genotypes showed a significantly increased risk for severe endometriosis (P for trend <0.01). These findings support the idea that the Ser312Gly polymorphism in *HSD17B1* is associated with the risk and progression of endometriosis, especially in terms of the inter-individual variation of estradiol synthesis.

Breast and endometrial cancer, like endometriosis, are considered to be estrogen-dependent diseases. The results of this study are consistent with those of a previous study that examined the Ser312Gly polymorphism in *HSD17B1* and breast cancer risk. When the *HSD17B1* A-allele and *CYP17* A2-allele were considered as the high-risk alleles, the risk of advanced breast cancer among women carrying four high-risk alleles (*HSD17B1* AA and *CYP17* A2A2) was 2.21 compared with that of women who carried none (Feigelson *et al.*, 2001).

The *CYP19* gene, encoding aromatase, plays a crucial role in estradiol synthesis. However, we could not find any association between the Arg264Cys polymorphism in *CYP19* and endometriosis. A possible explanation for this negative result might be a lack of a functional effect for this polymorphism. A previous study reported that aromatase activity was not affected by the Arg264Cys polymorphism (Watanabe *et al.*, 1997).

Three major limitations must be considered when evaluating the results of this study. First is the relatively small number of subjects. Although we found statistically significant differences, the 95% confidence intervals were relatively wide, reflecting the small number of cases. The sample size was sufficient to detect odds ratios of three or larger with 80% power at the 5% level of significance. The lack of a significant association with the *CYP19* Arg264Cys polymorphism means only that we failed to detect a difference. It remains unclear whether this polymorphism affects endometriosis.

Secondly, the distribution of *HSD17B1* alleles in the control group deviated considerably from the expected Hardy–Weinberg equilibrium, a difference that was not significant ($P = 0.06$), but was on the edge of being so. The discrepancy may result from the small number of subjects or from the characteristics of the control group. The setting of this study is a hospital, and the control group is women complaining of infertility. Because of the requirement for a surgical diagnosis, the selection of a control group in case–control studies of endometriosis has been particularly difficult (Zondervan *et al.*, 2002), and it is difficult to exclude a selection bias completely. Development of non-invasive methods for diagnosis or a prospective randomized trial will minimize any sampling bias.

Lastly, endogenous estrogen levels were not measured in this study. *HSD17B1* is not expressed in normal endometrium or endometrial hyperplasia (Utsunomiya *et al.*, 2001). One possible explanation of the apparent influence of *HSD17B1* is that the *HSD17B1* A-allele increases the level of circulating estradiol. Although an *in vitro* study failed to demonstrate any change in HSD17B1 catalytic activity produced by the Ser312Gly polymorphism (Puranen *et al.*, 1994), a recent molecular epidemiological study showed that *HSD17B1* A/A genotype was associated with higher estradiol levels in lean women (Setiawan *et al.*, 2004). The functional effects of the Ser312Gly polymorphism in *HSD17B1* and the Arg264Cys polymorphism in *CYP19* have not yet been clearly established. Further information on functional changes and more epidemiologic studies will help clarify the association between these polymorphisms and endometriosis.

Many alleles have been reported to vary in frequency among different ethnic or geographic populations. In the present study, allelic frequencies of *HSD17B1* Ser312Gly polymorphism in control individuals were similar to previously reported distributions in Chinese and American populations: A 0.43 and G 0.57 in Japanese and Chinese populations, and A 0.51 and G 0.49 in an American population (Wu *et al.*, 2003; Setiawan *et al.*, 2004). On the other hand, large variations were found between Japanese and Caucasians in the *CYP19* Arg264Cys polymorphism: C 0.70 and T 0.30 in a Japanese population, versus C 0.96 and T 0.04 in a Caucasian population (Hefler *et al.*, 2004).

In summary, we demonstrated that the Ser312Gly polymorphism in *HSD17B1* was associated with the risk and severity of endometriosis in a Japanese population. The A-allele of the *HSD17B1* gene is considered to be a high-risk allele for endometriosis. However, no association was found between the Arg264Cys polymorphism in *CYP19* and endometriosis. The results of this study are not conclusive and further investigation is warranted. Endometriosis is a complex trait. The many factors contributing to the disease

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phenotype make its pathophysiology very difficult to understand. Progress in the genetics of the disease, including understanding of genetic polymorphisms, will facilitate research on endometriosis.

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Running Title: AhRR polymorphism and endometriosis

Analysis of the AhR, ARNT and AhRR gene polymorphisms: Genetic contribution to susceptibility to and severity of endometriosis

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CAPSULE

A polymorphism in the aryl hydrocarbon receptor repressor (*AhRR*) gene was found to be associated with risk and severity of endometriosis. Polymorphisms in *AhR* and *ARNT* showed no such association.

ABSTRACT

Objective: To explore whether polymorphisms in AhR, ARNT and AhRR contribute to the susceptibility to and severity of endometriosis.

Design: Case-control study.

Setting: Hospital.

Patients: 138 women with or without endometriosis diagnosed endoscopically. Women who had ever given birth or lactated were not eligible. All women were staged into two groups: cases, with r-AFS stage I - IV endometriosis, and controls without endometriosis.

Main Outcome Measures: AhR, ARNT and AhRR polymorphisms were genotyped using real-time PCR analysis. Odds ratios and 95% confidence intervals were calculated for AhR, ARNT and AhRR genotypes to evaluate the risk of endometriosis. Associations between these polymorphisms and stage of endometriosis were also examined.

Results: The C/G + G/G genotypes at codon 185 of AhRR showed a significant association with risk of endometriosis (adjusted odds ratio, 2.53; 95% confidence interval, 1.16-5.55). Furthermore, there was a significant trend associating the C/G + G/G genotypes with the clinical stage of endometriosis (P for trend: 0.02). No

statistically significant association was observed between AhR codon 554 or ARNT codon 189 polymorphisms and endometriosis.

Conclusion: AhRR codon 185 polymorphism was associated with susceptibility to and severity of endometriosis in Japanese women.

Key Words: Endometriosis, genetic polymorphisms, AhR, ARNT, AhRR

INTRODUCTION

Endometriosis is a gynecologic condition that occurs in approximately 10% of women in the general population (1) and in 40% in infertile women (2). The most common symptoms associated with pelvic endometriosis are dysmenorrhea, chronic pelvic pain and infertility.

Endometriosis is regarded as a complex trait, in which genetic and environmental factors contribute to the disease phenotype (3). Genetic understanding of endometriosis has recently begun to progress rapidly, particularly through analysis of genetic polymorphisms. Genetic polymorphisms associated with endometriosis include drug metabolizing enzymes, growth factors and hormone receptor genes (4-7).

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that regulates cell differentiation and the induction of the phase I and II drug metabolizing enzymes (8-9). The AhR signaling pathway regulates induction of CYP1A1 and CYP1B1, representative phase I drug metabolizing enzymes (10-11). These isoforms catalyze the conversion of 17 β -estradiol to 2- or 4-hydroxyestradiol. Alterations in the AhR signaling pathway could affect the risk of endometriosis through altered expression of CYP1A1 and CYP1B1 or increased proliferation of endometrial cells.

The most well-known AhR ligands are polycyclic aromatic hydrocarbons, including

2,3,7,8-tetrachlorodibenzo-p-dioxin (12). Recently, Ohtake et al. reported functional cross-talk between dioxin-activated AhR and estrogen receptors (13). Exposure to dioxins has been suggested as a risk factor for endometriosis (14). However, several studies reached different conclusions, and the issue remains controversial (15-16).

AhR function is regulated by the AhR nuclear translocator (ARNT) and the AhR repressor (AhRR). Ligand-bound AhR translocates to the nucleus, where it heterodimerizes with ARNT. The AhR-ARNT heterodimer binds to xenobiotic response element sequences and facilitates activation of target genes (17). AhRR competes with AhR in heterodimer formation, thus down-regulating the genes regulated by AhR (18). Both AhR and ARNT are expressed in the female reproductive tract, and changes in their expression have been reported in specific pathological conditions (19).

Polymorphic sites have been identified in the coding regions of the AhR, ARNT and AhRR genes, including *AhR* codon 554 in exon 10 (AGA to AAA, Arg to Lys), *ARNT* codon 189 in exon 7 (GTG to GTC, silent mutation) and *AhRR* codon 185 in exon 5 (CCC to GCC, Pro to Ala) (20-22).

Altered AhR-mediated signaling caused by polymorphisms in AhR, ARNT and/or AhRR may account for individual differences in susceptibility to endometriosis. However, one previous study found no association between *AhR*, *ARNT* and *AhRR*

polymorphisms and endometriosis (23). In the present study, we explored whether these polymorphisms contribute to the susceptibility to and severity of endometriosis. A case-control study was conducted in patients with different stages of endometriosis and controls.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the University of Miyazaki, The Jikei University School of Medicine and National Cancer Center. All subjects gave their written informed consent before the laparoscopic examination.

Participants

During 1999-2000, 139 women were recruited at the Department of Obstetrics and Gynecology, Jikei University School of Medicine Hospital. Participants were patients between the ages of 20-45 who had complained of infertility and attended the hospital. The mean ages of cases and controls were similar (32 years for cases; 33 years for controls). No women ever had prior empiric therapy with either progestins or GnRH analogues before laparoscopic examination. Women who had ever given birth or lactated were not eligible for this study. One woman was excluded because a DNA sample was not available, leaving 138 women for the subsequent analysis.

All women underwent diagnostic laparoscopy as part of the infertility work-up. Women were classified into two groups according to the revised American Fertility Society (r-AFS) classification (24): endometriosis = r-AFS stage I - IV; controls = without endometriosis. For the most part, diagnosis was made by a single, trained

gynecologist. The diagnosis of endometriosis was established by visual criteria during laparoscopic examination, and histological confirmation was not always obtained. Of the 138 women enrolled, 59 had no endometriosis, 21 had stage I endometriosis, 10 had stage II, 23 had stage III and 25 had stage IV disease.

Genotyping

Blood samples were obtained before the laparoscopic examination. Genomic DNA was extracted from peripheral leucocytes using a DNA Extractor WB Kit (Wako, Osaka, Japan). Genotyping was performed blinded to case-control status, minimizing measurement bias.

AhR, ARNT and AhRR polymorphisms were genotyped by real-time PCR analysis on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Japan Ltd) using fluorescently labeled probes (25). All the primers and probes were designed appropriately with Primer Express 2.0 software (Applied Biosystems, Japan Ltd). Two differentially-labeled TaqMan probes and forward and reverse primers were prepared for each reaction. Primers and probes are listed in Table 1.

Reactions were performed with 200 nM of each probe, 900 nM each of forward primer and reverse primer, 1X TaqMan Universal PCR Master Mix (Applied

Biosystems), and 20 ng DNA. PCR cycling conditions consisted of one 2-minute cycle at 50°C, one 10-minute cycle at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. HPLC water was used as a negative PCR control in each amplification.

Statistical Analysis

Crude odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for *AhR*, *ARNT* and *AhRR* genotypes to evaluate association with endometriosis. Since endogenous estrogen exposure was thought to be correlated with the risk of endometriosis, ORs were adjusted for risk factors that might affect endogenous estrogen exposure: age (<35, ≥35 years), menstrual cycle (<26, 26-30, ≥31 days) and duration of menstruation (<6, 6-8, ≥9 days) using multiple logistic regression analysis by SPSS for Windows software (version 11.0.1J, SPSS JAPAN, Tokyo, Japan) (26-27). The Wilcoxon rank-sum test for trend was also used to examine the associations between *AhR*, *ARNT* and *AhRR* polymorphisms and stage of endometriosis (28). All statistical tests were based on two-tailed probability. P values <0.05 were considered statistically significant.

RESULTS

The genotype and allele frequencies of each polymorphism are shown in Table 2. The distributions of genotypes among controls were in Hardy-Weinberg equilibrium.

The C/G + G/G genotypes at codon 185 of *AhRR* showed a significant association with risk of endometriosis compared to the C/C genotype (adjusted OR: 2.53; 95% CI, 1.16-5.55). No statistically significant association was observed between the *AhR* codon 554 and *ARNT* codon 189 polymorphisms and risk of endometriosis (Table 3).

Furthermore, we evaluated whether these polymorphisms were associated with the stage of endometriosis. There was a significant trend between C/G + G/G genotypes and the stage of endometriosis (P for trend, 0.02; Table 4). The *AhR* codon 554 and *ARNT* codon 189 polymorphisms were not associated with a higher risk of advanced clinical stage (data not shown).

DISCUSSION

In the present study, we evaluated whether polymorphisms at *AhR* codon 554, *ARNT* codon 189 and *AhRR* codon 185 contribute to the susceptibility to and severity of endometriosis. Our results indicate that the *AhRR* codon 185 polymorphism is associated with both susceptibility and severity. The genotypic distribution of this polymorphism was significantly different in endometriosis and controls. The risk of endometriosis was approximately 2.5 times higher with the *AhRR* C/G + G/G genotype. In addition, the *AhRR* C/G + G/G genotype was more frequently observed in patients with advanced stage endometriosis. No such associations were found with the *AhR* codon 554 and *ARNT* codon 189 polymorphisms. We should not, however, definitively conclude that no association exists between *AhR* codon 554 or *ARNT* codon 189 polymorphisms and susceptibility to and severity of endometriosis, because sample size was too small to allow detection of subtle differences.

In a previous study, Watanabe et al. failed to find a relation between the *AhRR* codon 185 polymorphism and endometriosis (23). There are several reasons that might account for the discrepancy. First and most important is the definition of endometriosis. Because endometriosis is sometimes asymptomatic, it can be diagnosed only by laparoscopy or laparotomy. In the present study, all participants underwent laparoscopy, and

endometriosis and control groups were strictly defined according to the r-AFS classification. By this means, we could apply the best definition of cases and controls. In the previous study, control groups consisted primarily of asymptomatic volunteers. Without surgical diagnosis, the main concern is that the control population could contain a substantial number of women with undiagnosed endometriosis, thereby diluting the risk factor effects (29). Secondly, these studies differed in the study participants. It is known that pregnancy and lactation can cause disease regression and improvements in symptoms of endometriosis (30-31). To reflect the authentic phenotype of endometriosis at the time of surgical diagnosis, our study excluded women who had ever given birth or lactated. Lastly, considerable intraobserver and interobserver variability is reported in r-AFS classification (32). Although this variability is a concern in any case-control study of endometriosis, it does not significantly decrease the validity or reliability of the association found.

This study provides evidence for an association between the *AhRR* codon 185 polymorphism and endometriosis, although the exact mechanisms for this effect are still unknown. One possible explanation of this association is alteration of AhR-mediated signaling by the polymorphism. The *AhRR* C/G + G/G genotype may facilitate proliferation of endometrial cells through the diminished down-regulation of