要な役割を果たしているAhRR遺伝子多型において子宮内膜症との関連を報告している。さらに環境要因と遺伝要因の交互作用に焦点を当てた解析を行い、ゲニステイン曝露とERβRsa I 遺伝子多型、ダイオキシン類曝露とCYP1A1 Ile462Val遺伝子多型の間に交互作用を示唆する結果が得られている。

尿中BPAとの関連を検討したが、少なくとも5%有意水準下では、単調に直線的傾向を持った用量反応関係は見られず、尿中BPA濃度と子宮内膜症リスクとの間に関連はみられなかった。本研究で観察した尿中BPA濃度は平均一日経口BPA摂取量に換算すると2.8-192 ng/kg/dayであった。ちなみに動物実験ではラット経口投与200 mg/kg/day以上で子宮重量増加としてエストロゲン様作用が観察されている。

本研究は、グルクロン酸抱合体のみならず硫酸抱合体BPAの存在を考慮している点、交差反応が心配されるELISA法ではなく精確な機器分析法で測定している点、また、腹腔鏡検査によって症例・対照を確実に同定している点、断面調査(Consecutive sampling)によって選択バイアスを回避している点など、先行研究とは異なる研究手法上の利点が上げられる。一方、小標本ゆえに小さな効果は検出できない点、尿中BPA排泄量は短期曝露を反映し(14,15)、個人内変動が大きいことが知られている点(16)、曝露評価の測定誤差が相応の誤分類(nondifferential) を引き起こしていることが考えられ、それがひいてはnull resultにつながった可能性がある点などを考慮すると、結果の解釈には注意が必要である。また研究対象者が不妊外来受診者である点は、結果の一般化についても注意が必要であることを示唆している。

カドミウムは実験研究によりエストロゲン様作用をもつことが示唆されているが、子宮内膜症との関連を検討した疫学研究は少なく、これまでのところ2件とも関連なしという報告である。一方、乳がんリスクと関連ありという報告もあり、結果は一致していない。そこで本研究では、長期の曝露を反映する尿中カドミウム濃度を曝露指標とし子宮内膜症との関連を検討したが、先行研究と同様に有意なリスク上昇は観察されなかった。

その理由の1つには本研究が比較的低い曝露量の範囲の集団を観察していたために低用量域ではメタロチオネインによる解毒が飽和していなかった可能性がある。本研究では尿中カドミウム濃度とカドミウムの既知の曝露源である喫煙やコメ摂取頻度との間に関連が見られなかった。このように、今後の研究はカドミウム曝露量の範囲がより幅広い集団を対象とすることや、メタロチオネイン未結合の遊離体濃度と子宮内膜症の関連を検討することが考えられる。

McElroy et al. (17)は子宮内膜症同様にエストロゲン関連疾患である乳がんリスクとの間に有意な関連を見出した唯一の研究である。McElroy et al.の対象者は56歳(対照群中央値)であり本研究の対象者(同32歳)に比べてだいぶ高齢であるが、対照群の尿中カドミウム濃度中央値はMcElroy et al.では0.4 μg/g creatinine と本研究(同 0.54 μg/g creatinine)と同じか低い水準であった。蓄積性物質であるカドミウムの尿中排泄量は高齢であるほど高いことから、McElroy et al.の対象集団のカドミウム生涯曝露量は本研究の対象集団のそれよりもずっと低いことが考えられ、また、子宮内膜症と乳がんは両者ともエストロゲン依存性であるが発生機序に対してカドミウムの作用が異なる可能性が示唆された。

近年、フタル酸エステル類のエストロゲン様作用が疑われており、エストロゲンに敏感な疾患である子宮内膜症との関連が調べられてきた。インド人女性を対象とした症例対照研究では、子宮内膜症進行度が高いほど、ある種の血中フタル酸エステル類濃度が高

いことを示した。しかし、血中ではフタル酸ジ-2-エチルヘキシルの半減期は28~32分と報告されており、血中のジエステル体は曝露指標として適していないため、観察された関連には疑問がある。2000年以降、尿中の安定な代謝物(モノエステル体)が曝露指標として開発され、多くのフタル酸エステルの疫学研究が採用している。本研究では尿中の代謝物フタル酸モノエステル体を測定して、子宮内膜症とフタル酸エステル類曝露の関連を検討したところ、先行研究とは異なり、子宮内膜症との間に有意な関連は観察されなかった。

本研究はフタル酸エステル類曝露と子宮内膜症の関連を、より適切な曝露指標である尿中代謝物を指標として調べた初めての研究である。尿中フタル酸モノエステル濃度の曝露指標としての信頼性については、同一対象者から尿を複数の日に採取して個人内変動を検討した研究があり、級内相関係数0.53 (MBzP) ~ 0.80 (MEHP) との再現性を報告している(18)。それに加え、本研究の曝露指標測定における分析の精確さは先行研究以上であり、さらに信頼性の高い結果が得られていると推測される。一方、関連が検出されなかったのは小標本に起因する検出力不足のためかもしれない。本研究のサンプルサイズでは、曝露割合が今回と同様で$\alpha = 0.05, \beta = 0.20$のとき、四分位の最低濃度群を参照カテゴリとした場合の最高濃度群の粗オッズ比OR = 2.09 を検出するのに必要な最小標本サイズは今回の本研究の3.4倍である。本研究で観察された子宮内膜症との関連は血中濃度を指標とした先行研究結果と一致しておらず、今後さらに検討が必要と思われる。

2004年1月から2005年12月の期間に出版された、内分泌かく乱化学物質のヒト健康影響に関する疫学論文のうち、がんと子宮内膜症に関する論文26件のレビューを行った。特徴的な知見について考察する。

乳がんについては10件の研究が追加された。血清や脂肪組織中の内分泌かく乱化学物質を測定し、乳がん症例と対照で比較する症例対照研究が7件と最も多かった。多数の化合物が測定されているため、結果を概括するのは必ずしも容易ではない。とはいえ全体として、有意なリスク上昇を示す結果は少なく、反対に有意のリスク低下を示す結果も散見された。「内分泌かく乱化学物質の現状と今後の取組－内分泌かく乱化学物質の健康影響に関する検討会中間報告書追補その2－」では、「有機塩素系化合物に関しては明確なリスク上昇についての一貫した証拠は見出せなかった」と結論している(p98)。今回の検討では、この結論に変更を要するような新たな知見は見出せなかった。

前立腺がんに関する研究の中で、Van Maele-Fabry(2004)らは、農薬使用に関連する職業と前立腺がんに関する疫学研究のメタ分析を行った。対象は、1966-2003年に英語で出版された22件だった(コホート研究が15件、症例対照研究が7件)。Pooled rate ratio(95%CI)は、全22件では1.24(1.06-1.45)と有意なリスク上昇を認めた。15件のコホート研究では1.27(1.06-1.52)、7件の症例対照研究では1.15(0.77-1.72)と、コホート研究の方が高かった。したがって本研究は、職業性の農薬曝露による前立腺がんリスクの上昇が示唆している。とはいえ、本研究では職業性曝露を検討しているのみであるので、特定の化合物の影響についての評価は明らかではない。特定の化合物に関する研究はいまだ件数も少ない現状が確認された。

子宮内膜症については6件の研究が追加された。このうち2件は日本からの報告だったが、total TEQで負の用量反応関係の傾向を認めるもの(Tsukino, 2005)や、ビスフェノールAとの負の関連を示すもの(Hiroi, 2005)であり、内分泌かく乱化学物質によるリ

スク上昇を認めなかった。今後さらに日本人での検証が必要である。

次に2006年1月から2007年11月の期間に出版された、内分泌かく乱化学物質のヒト健康影響に関する疫学論文のうち、がんと子宮内膜症に関する論文26件のレビューを行った。特徴的な知見について考察する。

乳がんについては8件の研究が追加された。2件のコホート研究のうち、胎児期のジエチルベストロール(DES)曝露の影響を調べた米国の研究では、対象者全体では有意なリスク上昇を認めなかったが、40歳以上の対象者に限定すると有意なリスク上昇を認めた。また、有機溶剤を使用する電子機器工場の労働者を対象とする台湾の研究では、対象者全体では乳がんの標準化罹患率比の有意な上昇を認めなかったが、10年以上の従業者に限定すると有意なリスク上昇を認めた。症例対照研究の中では、血液中の各種の内分泌かく乱化学物質を測定した米国の研究が2件あったが、いずれも有意なリスク上昇を認めなかった。

前立腺がんについては10件の研究が追加された。前向きコホート研究が6件を占めたが、このうち5件は農薬取扱男性を対象とする同一のコホート集団を用いて、異なる農薬の影響を調べた報告であった。この5件の研究では、全般に有意なリスク上昇は認めなかったが、家族歴を有する男性に限りFonofosによる有意なリスク上昇を認めた。3件の症例対照研究では、職業記録で確認したtrichloroethilene曝露や、電話問診で確認した農薬使用歴による有意なリスク上昇を認めた。

子宮内膜症については7件の研究が追加された。PCBによるリスクの上昇傾向を示す報告が散見された。ラパロスコピー検査を施行した20-45歳の日本人女性138人を対象とする報告では、対象者全体では血清中のダイオキシンとPCBによる有意なリスク上昇を認めなかったものの、CYP1B1 Leu432Val多型による相互作用の可能性が示唆された。

喫煙の健康影響に関する2004年米国公衆衛生総監報告書と内分泌かく乱化学物質の健康影響に関する2002年IPCS報告書における、因果関係の判定規準の適用状況を、Parascancolaらの方法を援用して検討した。その結果、1964年報告書と類似の判定規準が二つの報告書でも採用されているが、個別疾患における因果関係の判断に際しては、これらの判定規準は必ずしも系統的に使用されていなかった。すなわち、因果関係の方法論と実際の適用状況との間に、乖離が認められることが明らかになった。さらに、2004年報告書における喫煙と胃がんの事例のように、判定規準と矛盾する知見が支配的であるにもかかわらず因果関係が肯定される場合もあることが明らかになった。

内分泌かく乱化学物質や喫煙のような曝露要因と健康障害との因果関係を適切に判断するためには、①方法論の明示と、②明示された方法論の系統的な適用という二つの要素が満たされる必要がある。二つの報告書を見ると、このうち①については(判定規準そのものの妥当性は別にして)概ね満足されていたものの、②については十分満たされてはいなかった。内分泌かく乱化学物質のヒト健康影響を明らかにするためには、個別の疫学研究の積み重ねに加えて、因果関係評価の方法論を整備し、その方法論を系統的に適用することの重要性が示唆された。

E. 結論

有機塩素系化合物などの化学物質曝露とホルモン関連腫瘍の関連を検討するために、乳がんと前立腺がんをエンドポイントとしたコホート内症例対照研究および症例対照研究を行っている。乳がんのコホート内症例対照研究は、血漿中イソフラボンと乳がんリ

スクの関連を検討し、血漿中ゲニステイン濃度の高い女性において乳がんリスクの低下が示唆された。また前立腺がんのコホート内症例対照研究は、内因性ホルモンおよびイソフラボン類の分析を終え、有機塩素系農薬類とPCB類の分析を開始した。乳がんの症例対照研究では、有機塩素系農薬類とPCB類について乳がんリスクとの関連を検討したところ、いずれも血清中濃度の高い群における乳がんリスクの上昇は観察されなかった。またイソフラボン摂取と乳がんリスクの間には有意な負の関連が見られた。さらにエストロゲン受容体β遺伝子多型、17β-HSD1遺伝子多型およびSHBG遺伝子多型との間に交互作用を示唆する結果が得られた。一方、前立腺がんの症例対照研究は、対象者の収集中である。子宮内膜症の症例対照研究では、尿中ビスフェノールA、尿中カドミウム、尿中フタル酸モノエステル類との関連を検討したが、いずれも統計学的に有意なリスク上昇は観察されなかった。疫学研究の文献的検討では、がんについての日本人での報告は存在せず、日本人における実証的検討をさらに進めるとともに、国際的な研究成果を踏まえて情報提供を行うことの重要性が示唆された。

F. 健康危険情報

なし

G. 研究発表

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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

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′-CTGGGGC-AGAGGACGAGG) and biotin-labeled antisense (5′-GCGGCCGG-AGGATCG) 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′-AATCCTGCATCTT-TTTT) and Cy5-labeled *CYP19* C-allele detection probe (5′-AAAT-CCTGCGTCTTTTTT). 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

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 I-II				
n (%)	7 (23.3)	23 (76.7)	2.07 (0.76–5.62)	2.07 (0.69–6.20)
Stage III-IV				
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.
$^{**}P < 0.01$.

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

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

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Objective: To explore whether polymorphisms in *AhR*, *ARNT*, and *AhRR* contribute to endometriosis susceptibility and severity.
Design: Case control study.
Setting: Hospital.
Patient(s): One hundred thirty-eight Japanese women with or without endometriosis, diagnosed endoscopically.
Intervention(s): Endoscopic laparoscopy, with blood samples for genotyping obtained before the laparoscopic examination for genomic DNA extraction from peripheral leukocytes.
Main Outcome Measure(s): *AhR*, *ARNT*, and *AhRR* polymorphisms were genotyped using real-time polymerase chain reaction (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.
Result(s): The C/G + G/G genotypes at codon 185 of *AhRR* showed a statistically significant association with risk of endometriosis (adjusted odds ratio, 2.53; 95% confidence interval, 1.16–5.55). Furthermore, a statistically significant trend associated the C/G + G/G genotypes with the clinical stage of endometriosis. No statistically significant association was observed between *AhR* codon 554 or *ARNT* codon 189 polymorphisms and endometriosis.
Conclusion(s): *AhRR* codon 185 polymorphism was associated with susceptibility to and severity of endometriosis in Japanese women. (Fertil Steril® 2005;84:454–8. ©2005 by American Society for Reproductive Medicine.)

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

Endometriosis is a gynecologic condition that occurs in approximately 10% of women in the general population (1) and 40% of 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 17β-estradiol to 2-hydroxyestradiol 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. (13) reported functional cross-talk between dioxin-activated *AhR* and estrogen receptors. Exposure to dioxins has been suggested as a risk factor for endometriosis (14), but several studies have reached different conclusions, and the issue remains controversial (15, 16).

The *AhR* nuclear translocator (*ARNT*) and the *AhR* repressor (*AhRR*) regulate *AhR* function. 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). In heterodimer formation, *AhRR* competes with *AhR*, 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 pathologic conditions (19).

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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 our 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 the National Cancer Center. All participants gave their written informed consent before the laparoscopic examination.

Participants

From 1999 to 2000, 139 women were recruited at the Department of Obstetrics and Gynecology, Jikei University School of Medicine Hospital. The participants were patients between the ages of 20 and 45 who had presented with infertility and attended the hospital. The mean ages of cases and controls were similar (32 years for cases; 33 years for controls). None of the women had had prior empiric therapy with either progestins or gonadotropin-releasing hormone (GnRH) analogues before the laparoscopic examination. Women who had given birth or lactated were not eligible for this study. One woman was excluded because no DNA sample was available, leaving 138 women for the subsequent analysis.

All of the women underwent diagnostic laparoscopy as part of the infertility work-up. Women were classified into two groups according to the revised American Fertility Society (AFS) classification (24): endometriosis (stage I to IV) and controls (no 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 histologic 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.

Genotyping

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

TABLE 1

Primers and probes used for real-time PCR analysis.

Primers	Sequence
AhR codon 554	AGA to AAA, Arg to Lys
Forward primer	5′-AAAAACAGTGACTTGTACAGCATAATGA-3′
Reverse primer	5′-CTGAAGTCAACCTCACCAGAAAAAT-3′
Probe: G allele	5′-FAM-TGAAGACATCAGACACAT-MGB-3′
Probe: A allele	5′-VIC-AGACATCAAACACATGC-MGB-3′
ARNT codon 189	GTG to GTC, silent mutation
Forward primer	5′-TGCTGCCAAACCATTCAGACT-3′
Reverse primer	5′-GGAACTGAAACATTTGATCTTGGA-3′
Probe: G allele	5′-VIC-CGGAGTCAGACACATA-MGB-3′
Probe: C allele	5′-FAM-ACGGAGTCAGAGACAT-MGB-3′
AhRR codon 185	CCC to GCC, Pro to Ala
Forward primer	5′-AGACGGATGTAATGCACCAGA A-3′
Reverse primer	5′-AGAGGCAGCGATGTGTTATGG-3′
Probe: C allele	5′-FAM-TGGGCAGCCCCCCGCC-TAMRA-3′
Probe: G allele	5′-VIC-TGGGCAGGCCCCGCC-TAMRA-3′

Note: *AhR* = aryl hydrocarbon receptor; *ARNT* = *AhR* nuclear translocator; *AhRR* = *AhR* repressor; PCR = polymerase chain reaction.

Tsuchiya. AhRR polymorphism and endometriosis. Fertil Steril 2005.

The *AhR*, *ARNT*, and *AhRR* polymorphisms were genotyped by real-time polymerase chain reaction (PCR) analysis on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Japan Ltd, Tokyo, Japan) using fluorescent-labeled probes (25). All the primers and probes were designed appropriately with Primer Express 2.0 software (Applied Biosystems). 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 primers, 1X TaqMan Universal PCR Master Mix (Applied Biosystems), and 20 ng DNA. The PCR cycling conditions consisted of one 2-minute cycle at 50°C, and 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 (OR) and 95% confidence intervals (95% CI) were calculated for *AhR*, *ARNT*, and *AhRR* genotypes to evaluate the association with endometriosis. Because endogenous estrogen exposure was thought to be correlated with the risk of endometriosis, the OR was adjusted for risk factors that might affect endogenous estrogen exposure: age (<35, ≥35 years), menstrual cycle (<26, 26 to 30, ≥31 days), and duration of menstruation (<7, ≥7 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 association between the *AhR*, *ARNT*, and *AhRR* polymorphisms and the stage of endometriosis (28). All statistical tests were based on two-tailed probability. $P<.05$ was 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 statistically significant association with risk of endometriosis compared with 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 or *ARNT* codon 189 polymorphism and the risk of endometriosis (Table 3).

Furthermore, we evaluated whether these polymorphisms were associated with the stage of endometriosis. There was

TABLE 2

Genotype and allele frequencies of *AhR*, *ARNT*, and *AhRR* polymorphisms.

			Genotype frequencies		Allele frequencies	
Polymorphisms	Codons	Amino acids	Endometriosis n (%)	Controls n (%)	Endometriosis n (%)	Controls n (%)
AhR codon 554						
G/G	AGA/AGA	Arg/Arg	24 (30.4)	22 (37.3)		
					G: 83 (52.5)	G: 73 (61.9)
A/G	AAA/AGA	Arg/Lys	35 (44.3)	29 (49.1)		
					A: 75 (47.5)	A: 45 (38.1)
A/A	AAA/AAA	Lys/Lys	20 (25.3)	8 (13.6)		
ARNT codon 189						
G/G	GTG/GTG	Val/Val	26 (32.9)	19 (32.2)		
					G: 82 (58.2)	G: 64 (54.2)
C/G	GTC/GTG	Val/Val	40 (50.6)	28 (44.1)		
					C: 68 (41.8)	C: 54 (45.8)
C/C	GTC/GTC	Val/Val	13 (18.5)	14 (23.7)		
AhRR codon 185						
C/C	CCC/CCC	Pro/Pro	20 (25.3)	27 (45.8)		
					C: 87 (55.1)	C: 81 (88.6)
C/G	CCC/GCC	Pro/Ala	47 (59.5)	27 (45.8)		
					C: 71 (44.9)	C: 37 (31.4)
G/G	GCC/GCC	Ala/Ala	12 (15.2)	5 (8.4)		

Note: *AhR* = aryl hydrocarbon receptor; *ARNT* = *AhR* nuclear translocator; *AhRR* = *AhR* repressor.

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TABLE 3

***AhR, ARNT,* and *AhRR* polymorphisms and risk of endometriosis.**

Polymorphism	Endometriosis n (%)	Controls n (%)	Crude OR (95% CI)	Adjusted OR[a] (95% CI)
AhR codon 554				
G/G	24 (30.4)	22 (37.3)	1	1
A/G + A/A	55 (69.6)	37 (62.7)	1.36 (0.67–2.78)	1.65 (0.76–3.61)
ARNT codon 189				
G/G	26 (32.9)	19 (32.2)	1	1
C/G + C/C	53 (67.1)	40 (67.8)	0.97 (0.47–1.99)	0.86 (0.39–1.87)
AhRR codon 185				
C/C	20 (25.3)	27 (45.8)	1	1
C/G + G/G	59 (74.7)	32 (54.2)	2.49[b](1.21–5.12)	2.53[b](1.16–5.55)

[a] OR adjusted for age and menstrual characteristics.
[b] $P<.05$.

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a statistically significant trend between C/G + G/G genotypes and the stage of endometriosis (P for trend, .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 statistically significantly different in women with 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 an advanced stage endometriosis. No such association was found with the *AhR* codon 554 or *ARNT* codon 189 polymorphism. We should not, however, definitively conclude that no association exists between the *AhR* codon 554 or *ARNT* codon 189 polymorphism and susceptibility to and severity of endometriosis because our sample size was too small to allow for the detection of subtle differences.

In a previous study, Watanabe et al. (23) failed to find a relation between the *AhRR* codon 185 polymorphism and endometriosis. There may be several reasons 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 the endometriosis and control groups were strictly defined according to the revised 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 control population could contain a substantial number of women with undiagnosed endometriosis, thereby diluting the risk factor effects (29). Second,

TABLE 4

***AhRR* codon 185 polymorphism and severity of endometriosis.**

Clinical stage	*AhRR* C/C genotype	*AhRR* C/G + G/G genotype	Crude OR (95% CI)	Adjusted OR[a] (95% CI)
Controls, n (%)	27 (45.8)	32 (54.2)	1	1
Stage I–II, n (%)	8 (25.8)	23 (74.2)	2.43 (0.94–6.30)	1.78 (0.64–4.98)
Stage III–IV, n (%)	12 (25.0)	36 (75.0)	2.53[b](1.10–5.81)	3.17[b](1.27–7.91)

[a] OR adjusted for age and menstrual characteristics.
[b] $P<.05$.
P for trend: .02.

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