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SHORT COMMUNICATION

Failure to detect significant association between estrogen receptor-alpha gene polymorphisms and endometriosis in Japanese women

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Abstract
Objectives The aim of the study was to test whether estrogen receptor 1 (*ESR1*) gene polymorphisms are correlated with the risk of the development of endometriosis in Japanese women, as a preliminary study.
Methods To compare allelic frequencies and genotype distributions, a case-control study of 100 affected women and 143 women with no evidence of disease was performed using 10 microsatellite repeat markers and 66 single-nucleotide polymorphisms (SNPs) in the *ESR1* gene region.
Results Although our results might be insufficient to detect genetic susceptibility, owing to the small sample size and low genetic power, statistical analysis of the differences in allelic frequency between the cases and controls at each microsatellite locus demonstrated that no microsatellite locus in the *ESR1* gene displayed a significant association with the disease when multiple testing was taken into account. Also, there were no statistically significant differences in the SNP allele frequencies and genotypes between the cases and controls when multiple testing was taken into account.
Conclusion The findings in our pilot study suggest that *ESR1* polymorphisms do not contribute to endometriosis susceptibility.

Keywords Endometriosis · Estrogen receptor-alpha gene · Endocrinology · Association study

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Introduction

Endometriosis (MIM131200) is a common gynecological disorder and reportedly affects 6–10% of women of reproductive age [1], with substantial annual health costs [2] and health burdens for individuals [3]. The condition, in which endometrial cells are found in sites outside the uterine cavity and which is defined as the occurrence of ectopic steroid hormone-dependent endometrium-like tissue consisting of glands and stroma [4, 5]. While the exact etiology and pathogenesis of endometriosis is unclear, the susceptibility to this disease depends on a complex interaction of environmental and genetic factors. As for genetic factor(s) of susceptibility to endometriosis, the incidence of the disease is approximately seven times higher in relatives of women with endometriosis than in those without a family history [6].

A relationship between exposure to dioxin and the risk of developing endometriosis was previously reported in

rhesus monkeys. That study indicated that dioxin could induce the development of endometriosis, with the incidence and severity of the condition being proportional to the dioxin dose [7]. Moreover, Ohtake et al. showed that dioxin-activated arylhydrocarbon receptors (*AhRs*) and estrogen receptors (*ESRs*) interacted functionally and the binding of dioxins to *AhRs* controlled the transcriptional activation of estrogen-responsive elements and estrogenic effects [8]. There is also growing evidence that exposure to environmental contaminants could contribute to the pathogenesis of endometriosis [9, 10].

Estradiol-17β (E2) stimulates uterine and vaginal epithelial proliferation in vivo [11]. The predominant biological effects of estrogen are mediated through two distinct intracellular receptors, *ESR* α and -β, that are encoded by the *ESR1* and *ESR2* genes, respectively, which are both expressed in normal endometrium and in endometriotic lesions [12]. E2 elicits its effects via the *ESR*, which functions as a ligand-activated transcription factor to turn on target genes in E2-responsive tissues. Both the *ESR1* and *ESR2* genes have been previously disrupted by targeted mutagenesis [13], which resulted in pleiotropic effects with distinct phenotype.

The *ESR1* gene, which consists of eight exons that are transcribed into a 6.8-kb mRNA, spans a genomic sequence of over 27 kb located on chromosome 6 at position 6q25.1. *ESR1s* are members of the nuclear receptor superfamily and act as ligand-regulated transcription factors [14]. Some previous studies have reported on the existence of a positive association between endometriosis and *ESR1* polymorphisms and splicing variants [15–17]. However, the results of other studies are inconsistent, cannot be replicated, and may vary because of differences between cohorts and populations, diagnostic errors, differences in sample sizes and in choice and type of genetic markers, and errors in laboratory methodology [5, 18, 19].

In order to gain a better understanding of the association between *ESR1* polymorphisms and endometriosis, we investigated and genotyped Japanese females with endometriosis (cases) and controls, using microsatellite and single-nucleotide polymorphism (SNP) markers within the *ESR1* DNA sequence.

Subjects, materials, and methods

Subjects

All patients and controls were females and we obtained their written informed consent to participate in this study. The patients with endometriosis were diagnosed by laparoscopy and histological examination and the severity of the disease was staged according to the revised classification of the American Society of Reproductive Medicine (r-ASRM) [20]. Of the 100 endometriosis cases (age 38 $\pm$ 4.8 years), 27, 10, 31, and 32 cases were classified as stage I, II, III, and IV disease, respectively [21]. The 143 healthy controls (age 43 $\pm$ 12.3 years) were patients with unexplained infertility who had received diagnostic laparoscopy and had no evidence of endometriosis (Table 1). Patients with apparent disease(s) were excluded from the control group. Ethics approval for this study was obtained from the Institutional Review Board at Tokai University School of Medicine.

Table 1 Characteristics of endometriosis cases and controls

Cohort	Cases	Controls
No. of samples	100	143
Age (years)	38 $\pm$ 4.8	43 $\pm$ 12.3
Endometriosis stage		
Stage I	27 (27.0%)	
Stage II	10 (10.0%)	
Stage III	31 (31.0%)	
Stage IV	32 (32.0%)	

DNA extraction

Genomic DNA samples were isolated from peripheral blood leukocytes in blood drawn from each subject into tubes containing heparin. The genomic DNA samples were isolated by using a Qiagen DNA extraction Kit (Qiagen, Tokyo, Japan) after lysis with proteinase K and 0.5% sodium dodecyl sulfate (SDS) at 37°C for 1 h.

Analysis of microsatellite polymorphism in the *ESR1* gene region

To determine the number of repeat units of the microsatellite loci exhibiting polymorphisms in the *ESR* gene, unilateral primers were synthesized by labeling at the 5′ end with the fluorescent reagent, 6-FAM (PE Biosystems, Foster City, CA, USA). Thirteen polymerase chain reaction (PCR) primer sets were used for the amplification of 13 microsatellite loci in the *ESR*α gene region, respectively (Supplementary Table 1). PCR amplification and GenScan (Applied Biosystems, Tokyo, Japan) detection of these microsatellites were carried out as previously described [22].

Genotyping

To examine the distribution of alleles and the genotype frequency of each SNP, we performed direct sequencing analysis of the *ESR1* gene using 43 pairs of oligonucleotide PCR primers (Supplementary Table 2). The reaction mixture (20 µl) contained 2 µl of dNTP mixture (each 2.5 mM of dATP, dCTP, dGTP, and dTTP), genomic DNA (5 µl,

2 ng/μl), 2 μl of 10× buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 20 pmol of forward and reverse primers, and 0.5 U of r*Taq* (TaKARa Bio. Inc., Shiga, Japan). PCR amplification was performed in a GeneAmp PCR system 9700 automated thermal cycler (Applied Biosystems, Tokyo, Japan). PCR reaction conditions consisted of initial denaturation for 5 min at 96°C, followed by 30 amplification cycles of 45 s at 96°C, 45 s at various temperatures depending on the primers used (Supplementary Table 2) for annealing, and 2 min at 72°C with a final extension of 7 min at 72°C. Each PCR product was purified by exonuclease I and then sequenced using an ABI 3100 automated sequencer (Applied Biosystems, Tokyo, Japan).

Statistical analyses

Allele and genotype frequencies were determined by direct counting. The significance of differences in the distribution of alleles and genotypes between the patients and controls was tested, using a case-control design, by Fisher's exact probability test (*P* value test). A finding of $P_c < 0.05$; that is, a significant correction for multiple testing, using the Bonferroni method, was considered as statistically significant. The odds ratio (OR) and 95% confidence interval (CI) were also calculated for all SNPs. The Haploview program (Dr. Mark Daly's lab, MIT/Harvard Broad Institute) was used to estimate pairwise linkage disequilibrium (LD) and haplotype frequency [23]. The CaTS algorithm (MIT/Harvard Broad Institute, Massachusetts, USA) was used to calculate the genetic power for an association study (http://www.sph.umich.edu/csg/abecasis/CaTS/).

Results

To investigate genetic variations in the *ESR1* gene associated with endometriosis, a total of 100 Japanese patients with endometriosis and 143 healthy controls were enrolled for the association analysis, which was carried out using 13 microsatellites found in the region of the *ESR1* gene. Three microsatellites with the dinucleotide repeats $(CA)_n$, $(AAAC)_n$, and $(GTTT)_n$, located in introns 4and 6 and the 3′ flanking region, respectively, were not polymorphic (data not shown). As shown in Table 2, none of the microsatellites in the *ESR1* gene were significantly associated with endometriosis after the *P* values were corrected (P_c) for multiple testing (10 tests).

A total of 66 SNPs in the *ESR1* gene region were genotyped in the 100 patients with endometriosis and 143 controls. None of the SNPs in the *ESR1* gene were significantly ($P_c > 0.05$) associated with endometriosis (Supplementary Table 3) after the *P* values were corrected for 66 tests. The SNP site (C/G) in exon 4 was located at the nucleotide position 186,086 of the gene and was not involved in an amino acid change, i.e., Pro (rs1801132). The prevalences of the T/T, C/C, and C/C genotypes of the rs1884049, rs1884053, and rs1884054 polymorphisms were significantly higher in the patients than in the control subjects ($P = 0.022$, 0.014, and 0.028, Table 3) without correction for multiple testing. This significance was lost ($P > 0.05$) after correcting for 66 statistical tests.

We evaluated the LD extension of approximately 280 kb of the *ESR1* genomic region with 64 SNPs. For the LD block evaluation, we included SNPs with a minor allele frequency of >0.2, genotype success rate of >0.8, and $P > 0.001$ in the Hardy–Weinberg equilibrium test. Of these 64 SNPs, 58 met the criteria, and the pairwise LD index (Δ) was calculated and plotted (Supplementary Fig. 1). We identified five LD blocks with a threshold of $\Delta = 0.6$. We numbered these blocks 1–5 from ATG to the stop codon in the *ESR1* gene. Blocks 1–5 spanned roughly 49.2, 3.0, 87.7, 33.8, and 79,7 kb, respectively. We further analyzed the haplotype constitution with the three SNPs, rs1884049, rs1884053, and rs1884054. Four haplotypes

Table 2 Results of association tests for ten microsatellite markers in the *ESR1* gene

Location	Repeat unit	No. of alleles	Patients *n* (%)	Controls *n* (%)	Odds ratio (90% confidence interval [CI])	*P* value	P_c value
Promoter	$(TA)_n$	15	34 (19.4)	38 (14.3)	1.45 (0.87–2.40)	0.153	1.000
Intron 3	$(TC)_n$	5	30 (16.3)	31 (11.1)	1.56 (0.91–2.68)	0.103	1.000
Intron 4	$(GT)_n$	6	45 (24.3)	58 (20.2)	1.27 (0.82–1.97)	0.291	1.000
Intron 4	$(TTG)_n$	3	5 (2.9)	2 (0.7)	3.92 (0.85–18.21)	0.081	0.808
Intron 4	$(GT)_n$	9	27 (15.4)	26 (9.5)	1.75 (0.99–3.09)	0.055	0.553
Intron 4	$(GT)_n$	8	11 (5.9)	5 (1.8)	3.41 (1.23–9.41)	0.025	0.246
Intron 5	$(AAAT)_n$	4	18 (9.5)	16 (5.6)	1.75 (0.88–3.50)	0.112	1.000
Intron 5	$(CA)_n$	12	31 (15.4)	23 (8.1)	2.08 (1.18–2.08)	0.011	0.111
Intron 5	$(CT)_n$	2	49 (28.2)	66 (24.9)	1.18 (0.77–1.82)	0.448	1.000
3′ Flanking region	$(GT)_n$	4	135 (93.8)	181 (92.3)	1.24 (0.53–2.92)	0.618	1.000

P_c value was corrected for 10 tests

Table 3 Genotype frequencies of the human *ESR1* gene and the significance of their association with endometriosis

dbSNP Accession no.	Location	Genotype[a]	Genotype frequency		OR (90% CI)	*P* value	P_c value
			Patients (%)	Controls (%)			
rs1884049	Intron 4	C/C	14 (14.0)	31 (22.0)			
		C/T	49 (49.0)	77 (54.6)			
		T/T	37 (37.0)	33 (23.4)	1.92 (1.10–3.36)	0.022	1.000
rs1884053	Intron 4	T/T	9 (9.0)	14 (9.9)			
		C/T	37 (7.0)	74 (52.1)			
		C/C	54 (54.0)	54 (38.0)	1.91 (1.14–3.21)	0.014	0.924
rs1884054	Intron 4	A/A	8 (8.0)	14 (9.8)			
		A/C	40 (40.0)	75 (52.5)			
		C/C	52 (52.0)	54 (37.8)	1.79 (1.07–2.99)	0.028	1.000

P_c value was corrected for 66 tests

[a] Each genotype is represented by the nucleotide sequence of the sense strand of each gene

Table 4 Association between *ESR1* gene haplotypes and endometriosis

No.	Haplotype[a]	Patients (%)	Controls (%)	*P* value	P_c value
Hap1	TCC	0.596	0.489	0.021	0.084
Hap2	CTA	0.266	0.343	0.073	0.292
Hap3	CCC	0.115	0.151	0.267	1.000
Hap4	TTA	0.012	0.017	0.687	1.000

Haplotypes with frequencies of <0.01 are not included in the Table. Hap1–Hap5 covers 99.5% of existing haplotypes. P_c value was corrected for 4 tests

[a] Order of SNPs consisted the haplotype are rs1884049, rs1884053, rs1884054

were estimated to have a frequency of >0.01, using Haploview. Haplotype 1 (TCC) was more frequently observed and over-represented in the patients, with a significant difference in frequency between the case and the control group ($P_c = 0.084$, Table 4).

Discussion

Endometriosis is considered to be an estrogen-dependent disorder in women, with estrogen and *ESR* playing major roles in its pathogenesis, although genetic defects, immunity, and environmental and other hormonal factors might also contribute to the disease [4, 7]. Some studies observed a positive association between endometriosis and *ESR1* polymorphisms and splicing variants [15–17], whereas others reported equivocal or negative results [10, 19]. Polymorphisms and ER-related genotypes may also determine the function of the sex-steroid system both at the receptor level and at the level of hormone synthesis [24, 25]. Therefore, to elucidate these questions about the risk of endometriosis, we performed an association analysis to determine the relationship between polymorphisms within the *ESR1* gene region and endometriosis in Japanese women, using 10 microsatellite repeats and 66 SNPs distributed from the promoter region to the 3′ flanking region. Statistical analysis of the differences in allelic frequencies between the cases and controls at each microsatellite locus demonstrated that two microsatellite loci in introns 4 and 5 of the *ESR1* gene displayed a significant association with the disease ($P = 0.025$ and 0.011, respectively, Table 1). There were also statistically significant differences (uncorrected for multiple testing) between the cases and controls for nine allele frequencies and three genotypes for the SNP markers in intron 4 of the *ESR1* gene (Supplementary Table 3). However, when we corrected our results by Bonferroni's method for multiple testing, we did not obtain any positive markers at a significance level of $P_c < 0.05$. Therefore, we conclude that the microsatellite or SNP allelic genotypic markers that we investigated in the *ESR1* gene region are not significantly associated with endometriosis.

Despite our conclusion on the absence of an association between the *ESR1* gene polymorphisms and endometriosis, it remains problematic that the statistical corrections using the Bonferroni test may be too stringent for association studies and that we may have missed a biologically important or genetically interesting positive association by creating false-negatives or a type II error with the corrections [26, 27].

For multifactorial or complex diseases, including endometriosis, association studies with a high density of genetic markers have identified many statistically valid associations for the susceptibility genes, as well as several quantitative traits based on the 'common disease, common variant hypothesis', i.e., that the etiology of a common disease is mediated by commonly occurring genomic variants in a population. These studies can be divided into two types, that is direct and indirect approach. The former is an association study to analyze a particular functional variant that alters amino acids in coding regions or

expression levels in regulatory regions of candidate genes for a disease. On the other hand, in an indirect study, a particular set of genetic markers is investigated to expect the disease locus by LD analysis between disease allele and genetic marker. Of interest, in a multifactorial disease such as endometriosis, it is important to determine the level of the population-attributable risk (PAR) in order to detect the susceptibility genes. As an example, if a causal variant is rare and the disease is common, the PAR and consequently the OR will be low, and the genetic power would be decreased. In a model proposed by Madsen and Browning [28], using 1,000 cases and 1,000 controls, and PAR values of 0.02, 0.1, and 0.25, the power of the test at these PARs was estimated to be 0, 0.68, and 1, respectively. Thus, the detection of susceptibility genes by the indirect approach is dependent not only on the degree of linkage disequilibrium (LD) between the disease variant and the genetic marker but also on the genotype relative risk, whose factors influence the genetic power for an association study. However, it is widely known that allele frequencies and LD strengths vary considerably in different populations and chromosome regions [29]. As for the number of genetic markers used in an association study, a large number of genetic markers with various allele frequencies is needed, because the frequency of the disease variant is unknown. However, if a large number of markers were to be used for an association study, there would be a possibility that the *P* value may not show a significant association due to the Bonferroni adjustment.

Also, the calculation of the power and sample size required for association studies is important to ensure that the study is sufficiently powered to detect the subtle genetic effects that contribute to most complex diseases. The factors that affect statistical power in the detection of genetic associations are the disease prevalence, effect size (genotype relative risk), allele frequency, and genotype errors [30, 31]. Therefore, we calculated the power in the present association study using the following factors: disease prevalence 0.06–0.10, disease allele frequency 0.2–0.5, and effect size 1.10–1.50. The calculations for the power showed values between 0.07 and 0.68 (Supplementary Table 4). However, in a recent report of a genome-wide association study of endometriosis, the OR for the identified SNP was approximately 1.20 [32]. Given the results of that report, the power in the present study would be 0.14–0.20 (Supplementary Table 4). To increase the power to 0.90, this study would need 1,000 cases and 1,000 controls. In this regard, future follow-up studies using greater sample numbers might help to determine whether the significant differences ($P < 0.05$) in the frequencies of the microsatellite or SNP markers between the control and disease groups that we identified without the statistical corrections could still be replicated and recognized as genetically significant. Also, it was reported that body habitus, personal habits, and menstrual characteristics were significantly associated with the development of endometriosis [33]. Therefore, for genetic analysis, it might be important to identify the causative factors for endometriosis.

In conclusion, our results suggest that *ESR1* gene polymorphisms are not significantly associated with the development of endometriosis in Japanese women. However, this lack of association might be due to our study's small sample size and low genetic power to detect disease susceptibility. The mechanism by which these polymorphisms might affect the function of the estrogen receptor still needs to be clarified in order to better understand the pathogenesis of endometriosis and other estrogen-dependent diseases.

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Conflict of interest The authors declare that they have no conflict of interest.

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