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ORIGINAL ARTICLE

Analysis of aromatase (*CYP19*) gene in Iranian women with endometriosis

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Abstract Endometriosis is a chronic, inflammatory, estrogen dependent disease that affects up to 10% of all women of fertile age. It is characterized by the presence and proliferation of functional endometrial glands and stroma outside the uterine cavity. The aim of this study was to assess whether intron 4 (TTTA)n repeat and TCT deletion/insertion polymorphisms of *CYP19* gene are associated with endometriosis in northern Iran. This study involved 110 patients with endometriosis and 200 healthy controls, who were genotyped for (TTTA) repeats in the fourth intron of the *CYP19* gene. Genomic DNA from patients and controls was genotyped by polymerase chain reaction (PCR). A total of eight alleles were observed in our study population, ranging from 7 repeats to 13 repeats. (TTTA) repeat lengths of $\leqslant 9$ were classified as short (S), and those $\geqslant 10$ were classified as long (L). Compared to women who possessed the S/S genotype, those who carried L/L (OR, 5.56; 95% CI, 3.33–9.29) had significantly increased risk of endometriosis. There was a significant trend between L/L genotype and higher stage of endometriosis ($P < 0.001$). In conclusion, a significant association was identified between endometriosis and the *CYP19* gene polymorphism, with endometriosis having longer *CYP19* repeat lengths than control subjects. The strong association of *CYP19* gene polymorphism with high-stage endometriosis suggests that *CYP19* may have a prognostic implication.

1. Introduction

Endometriosis is characterized by the presence of uterine tissues (endometrial glands and stroma) in areas other than the uterus, such as the pelvic floor or around the fallopian tubes and ovaries [1]. The prevalence in women without symptoms is 2–50%, depending on the diagnostic criteria used and the populations studied [2]. The incidence is 40–60% in women with dysmenorrhea and 20–30% in women with subfertility. The severity of symptoms and the probability of diagnosis increase with age. Endometriosis is associated with increased

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overall cancer risk, with particular elevation of ovarian cancer risk [3]. Two principal explanations for the development of endometriosis are retrograde menstruation and coelomic metaplasia hypothesis. The most common theory is retrograde menstruation, which consists of the reflux of menstrual fluid through the Fallopian tubes to the abdominal cavity [4].

It was widely accepted that both genetic and environmental factors may be involved in the etiology of endometriosis. Candidate genes specifically studied for association or linkage with endometriosis includes galactose-1-phosphate uridyl transferase [5], phase I and II detoxification genes [6], adhesion *ICAM-1* [7] and *VEGF* [8,9].

The *CYP19* gene encodes aromatase that is the key enzyme for the terminal step of estrogen biosynthesis by converting 19-carbon steroids (testosterone and androstenedione) to 18-carbon estrogen (estradiol and estrone). Aromatase is expressed in ovarian, placental, testicular, adipose, bone and brain tissues [10].The *CYP19* gene is located in the chromosome 15q21.2 region and is comprised of a 30 kb coding region and a 93 kb regulatory region. Tissue specificity is regulated by the use of nine alternate untranslated first exons located in the large 93 kb gene regulatory unit. It is reported that several single nucleotide polymorphisms (SNPs) of the *CYP19* gene were associated with variations in serum androgen concentrations among women, both within and between racial/ethnic groups. The *CYP19* gene has a tetranucleotide repeat polymorphism $(TTTA)_{n=7\text{–}13}$ in intron 4, about 80-bp downstream of intron 4, with the 7 and 11 repeats being most common [11]. There is also a 3-bp deletion 50-bp upstream of the repeat [12]. The deletion is found in those with 7 repeats, generating 2 alleles: 7 repeats with the 3-bp deletion; and 7 repeats without the deletion. This polymorphism has been associated with the hyperandrogenic phenotype of polycystic ovary syndrome [13]. It has also been related to increased risk for the development of various estrogen dependent diseases in women such as breast and lung cancers and osteoporosis [14–18]. However, the molecular mechanisms responsible for changes in aromatase activity and susceptibility in estrogen-dependent diseases are unclear.

Endometriosis is an estrogen-dependent disease. We hypothesized that the longer alleles of *CYP19* would be more frequent and preferentially more active among the patients with endometriosis than the controls.

2. Subjects and methods

2.1. Characteristics of subjects

All subjects were Iranian, unrelated, and residents of the Guilan province in northern Iran. 110 patients with endometriosis diagnosed by laparoscopy and classified by histological criteria according to the Revised American Society for Reproductive Medicine were selected. For the control group, 200 fertile women who had undergone tubal ligation were included in this study. Clinical information on patients was collected from clinical notes, including lesion size, location, stage of disease, drug treatment and fertility. The control patient was confirmed to have no endometriotic or other pathological lesions in the pelvic cavity. Written consent of the patients was obtained according to the Declaration of Helsinki. After cases and controls were identified, whole blood samples of 1 ml were collected from each subject in heparin-containing tubes. The samples were stored at 4 °C and centrifuged at 2800 rpm for at least 10 min within the next 24 h. The three independent fractions were isolated and stored at −70 °C until analysis. Laboratory personnel blinded to the case-control status of the samples performed all genotyping, and each plate included blinded replicate samples for quality control purposes. The replicate samples were 100% concordant for all genotypes.

2.2. DNA isolation

Genomic DNA was isolated from peripheral leukocytes by DNG ™-Plus Kit (Cinnagen, Iran). DNA was dissolved in TE buffer [10 mM Tris (PH 7.8), 1 mM EDTA].The DNA integrity was certified by electrophoresis on 2% agarose gel stained by ethidium bromide (0.5 mg/ml) and visualized with a Gel Documentation System (BioRad). The final preparation was stored at- 20 °C and used as a template for polymerase chain reaction (PCR).

2.3. $(TTTA)_n$ repeat length determinations

The *CYP19* (TTTA)n repeat was typed by PCR amplification of genomic DNA in the presence of a forward primer; 5'-GCAGGTACTTAGTTAGCTAC-3' and reverse primer; 5'-TTACAGTGAGCCAAGGTCGT-3'. The primers were designed in our laboratory using Oligo7 software. The PCR reaction contained 1 mM of each primer, 0.5 U Taq polymerase, 200 mM dNTP mixture, and 2 mM $MgCl_2$ in addition to test DNA, made upto a final volume of 25 μl.

PCRs were performed in the MJ Mini™ Gradient Thermal Cycler (Bio-Rad), which was programed as follows: initial denaturing at 94 °C for 7 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. The PCR products were visualized on 6% polyacrylamide gel by silver staining. The size of PCR fragment sizes was assigned by comparison to a sequence-verified fragment ladder by two independent readers. The products were 168–195-bp in respect of the number of TTTA repeats.

2.4. Statistical analysis

Statistical analysis was performed using the χ^2 test and the Med Calc version 9.3. Strength of association between endometriosis and alleles of the TTTA repeat and TCT deletion/insertion polymorphisms of *CYP19* were estimated using odds ratios (OR) and 95% confidence intervals (CI). Statistical significance was defined as $P \leqslant 0.05$.

3. Results

The age of the patients ranged from 21 to 36 years. There was no significant difference in terms of distribution of age between the cases and controls (P = 0.02). All patients were infertile [primary infertility in 87 (79%) and secondary infertility in 23 (21%)], with 94 (85%) of 110 complaining of chronic pelvic pain, 65 (59.1%) having dyspareunia. Eighty-seven (79.1%) women had dysmenorrhea (Table 1). Significant differences

Table 1 General characteristics of the study population.

No. of patients	110
Median age in years	32
Symptoms	
Chronic pelvic pain	94 (85%)
Dysmenorrhea	87 (79.1%)
Dyspaurnia	65 (59.1%)
Primary infertility	87 (79%)
Secondary infertility	23 (21%)
Stage of disease[a]	
Stage I–II	43
Stage III–IV	67

[a] According to the Revised American Fertility Society staging system.

were observed between the groups in the prevalence of all symptoms ($P < 0.001$).

3.1. *CYP19 (TTTA)n polymorphism*

In the present study, eight different TTTA alleles were identified by size. These alleles contained a sequence ranging from 7 (168-bp) to 13 TTTA repeats (195-bp). The global allele frequencies of the *CYP19* (TTTA) repeat polymorphism in the healthy control women and patients with endometriosis are illustrated in Fig. 1. The shortest allele of 168-bp carried seven TTTA repeats and a 3-bp deletion 50-bp upstream of the repetitive sequence, and we identified it as (TTTA)7 + delTCT; the allele of 171-bp had the same number of TTTA repeats but no TCT deletion, so we defined it as (TTTA)7. The next most-frequent allele of 187-bp had 11 (TTTA) repeats and a TCT insertion and we defined it as (TTTA)11. TTTA repeat lengths of ⩽9 were classified as short (S), and those ⩾10 were classified as long (L). Proportions of the *CYP19* $(TTTA)_n$ alleles in both groups were significantly different (Table 2 and Fig. 1).

Because women have two *CYP19* gene alleles, homozygosity was defined as two repeats of the same length. Different lengths for two repeats indicated heterozygosity. Patients and control subjects were separated into subgroups comprising those with two short alleles (S/S), those with one short and one long allele (S/L), and those with both long alleles (L/L). Compared to women who possessed the TTTA S/S genotype, those who carried L/L genotype (OR, 5.56; 95% CI, 3.33–9.29) had significantly increased risk of endometriosis (Table 2).

3.2. *Association between CYP19 (TTTA) n repeat polymorphism and stages of endometriosis*

To evaluate whether the TTTA polymorphism in *CYP19* is associated with the severity of endometriosis, participants were categorized into three groups according to the revised American Society for Reproductive Medicine: controls, stage I–II and stage III–IV (Table 3). Among 110 women with endometriosis, 43 and 67 women were classified as stage I–II and stage III–IV, respectively. A significant difference in the distribution of genotypes for the *CYP19* polymorphism and stages of disease were found. There was also a significant trend between the L/L genotype and stage of endometriosis ($P < 0.001$).

4. Discussion

Endometriosis is thought to be estrogen-dependent *in vivo* for several reasons. Endometriosis does not occur before menarche and symptoms abate after menopause [19]. Estrogen agonists worsen lesions and antagonists are used to treat them [20]. Markers of increased serum estrogen levels (high body fat, low waist:hip ratio) are linked to increased disease risk, while anti-estrogenic influences (smoking, vigorous exercise) are associated with decreased risk [21]. Endometriosis implants synthesize large quantities of estradiol (E2) locally during the secretary phase than the dose endometrium from women without the disease [22]. Aromatase, which catalyzes the final step in estrogen (E1) and E2 biosynthesis, is expressed in endometriotic stroma but not in the normal endometrium [23].

Changes in aromatase biosynthesis are usually preceded by changes in its gene transcription and mRNA level. Gene variability could contribute to the level of the aromatase biosynthesis. A polymorphic tetranucleotide repeat (TTTA)n has been identified in intron 4 about 80 nucleotides downstream of exon 4 in the *CYP19* gene near the intron/exon border. This close proximity to the intron/exon suggests a possible role for these tetranucleotide repeats in the determination of splicing sites [24]. Longer repeats (which various studies define as between 7 and 10 repeats) have been associated with higher levels of circulating estrogen levels in older men [16] and women [17]. Studies of hormone-related cancers report conflicting findings with regard to this polymorphism and cancer risk, with

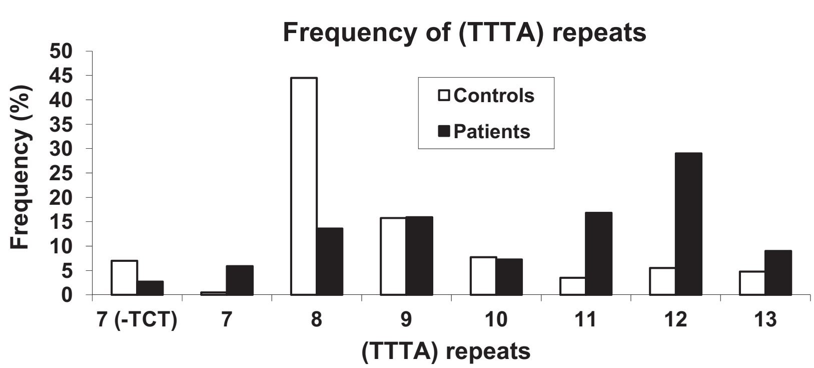

Figure 1 Frequency of (TTTA) repeats in the studied population.

Table 2 Frequency of *CYP19* (TTTA)n alleles and genotypes in the studied populations.

Allele/genotype	Endometriosis n (%)	Controls n (%)	OR (95% CI)	*P* value
Short	84 (38.2)	314 (78.5)	1.00 (reference)	
Long	136 (61.8)	86 (21.5)	5.91 (4.11–8.48)	< 0.0001***
S/S	18 (16.4)	82 (41)	0.28 (0.15–0.50) [a]	< 0.001**
S/L	28 (25.4)	78 (39)	0.53 (0.31–0.89) [b]	< 0.01*
L/L	64 (58.2)	40 (20)	5.56 (3.33–9.29) [c]	< 0.0001***

[a] Calculation was performed following a dominant genotype model for S/S versus S/L and L/L.
[b] Calculation was performed for S/L versus S/S and S/L.
[c] Calculation was performed following a recessive genotype model for L/L versus S/L and S/S.
* $P < 0.01$.
** $P < 0.001$.
*** $P < 0.0001$.

Table 3 *CYP19*(TTTA)n genotype frequencies in different stages of endometriosis.

Genotype	Stage I–II n (%)	Stage III–IV n (%)
S/S	13 (11.8)	5 (4.5)
L/S	9 (8.2)	11 (10)
L/L	21 (19.1)	51 (46.4)

$\chi^2 = 11.57$, $P = 0.003$.

inconsistent findings reported for breast [25], prostate [26] and endometrial cancer [18].

It has been demonstrated that vascular endothelial growth factor (VEGF), GSTM1 and intercellular adhesion molecule-1 (ICAM-1) gene polymorphism are associated with the risk of endometriosis [27–29]. Berstein et al. noted an increased estrogen biosynthesis (the highest aromatase activity) in endometrial tumors from (TTTA)11/(TTTA)11 and (TTTA)11/(TTTA)12 genotype carriers [15]. The results are compatible with those reported by Gennari et al. in skin fibroblasts [16]. So far, there are only few reports concerning the relationship of *CYP19* genetic polymorphism with endometriosis. To the best of our knowledge, this is the first study on Iranian women to examine the association of *CYP19* polymorphism with endometriosis. In this study, we found that the alleles containing 11 and 12 TTTA repeats were over-represented in the patients with endometriosis. Our results support an association between long *CYP19* alleles and an increased risk for endometriosis in Iranian women. When comparing patients with different stages of endometriosis, we found a trend toward higher frequency of longer alleles in patients with stages III and IV (19.1 vs 46.4%; $P = 0.003$). Arvanitis et al. found that the allele 10 TTTA repeats were more prevalent in patients with endometriosis (OR, 4.99; 95% 95% CI, 1.351 to 18.436) [30]. However, in a study by Kado et al., the authors found no association between the allele frequency of the TTTA repeat polymorphism and endometriosis [31]. A study of Korean women reported that the frequency of the higher risk alleles of the CYP19 gene was not higher in endometriosis patients than in controls. They also found that the risk of endometriosis also did not increase significantly with the number of higher risk alleles of the *CYP19* gene [32]. The lack of consistent association of the *CYP19* (TTTA) polymorphism with endometriosis risk may be due to differences of allele frequencies between ethnic groups, genetic heterogeneity in the pathogenesis of endometriosis, and different environmental factors.

In conclusion, this pilot study carried out in Iran focused on 110 women with endometriosis. A strong association between long *CYP19* alleles and endometriosis was confirmed overall. Our findings also suggest that *CYP19* (TTTA)n genetic polymorphism is associated with advanced-stage endometriosis in Iranian women. Further studies in larger populations are required to confirm the implication of the (TTTA)n repeat polymorphism in the intron 4 of the *CYP19* gene in the pathogenesis of endometriosis.

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