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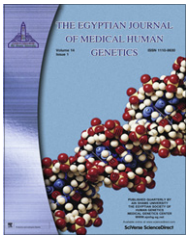

ORIGINAL ARTICLE

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

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KEYWORDS

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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 $(TTTA)_{n=7–13}$ 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