**Polymorphism of the estrogen receptor β gene is related to infertility and infertility-associated endometriosis**

**ABSTRACT**

Objective: To determine the frequency of the estrogen receptor b gene (ERβ) +1730 G/A polymorphism in infertile women with and without endometriosis and controls. Subjects and methods: Case-control study that included 136 women with endometriosis, 69 women without endometriosis and 209 fertile women as controls. The ERβ gene + 1730 G/A polymorphism was identified by RFLP-PCR (Restriction Fragment Length Polymorphism – Polymerase Chain Reaction). Results: Genotypes GG, GA and AA of the ERβ gene presented frequencies of 60.3%, 38.2% and 1.5%, respectively, in the women with endometriosis (p < 0.0022). Of the infertile women without endometriosis, 63.8% presented the normal homozygous genotype GG, 30.4% the GA heterozygous genotype, and 5.8% the homozygous mutated genotype AA (p < 0.0275). In the control group, 77.5% presented the normal homozygous genotype GG, 21.1% the heterozygous genotype GA, and 1.4% the homozygous mutated genotype AA. Conclusion: The data suggest that the estrogen receptor β gene (ERβ) +1730 G/A polymorphism can be associated with risk of infertility and endometriosis-associated infertility. Arq Bras Endocrinol Metab. 2010;54(6):567-71

**Keywords**

Endometriosis; estrogen receptor β; estrogen; infertility; polymorphism

**RESUMO**

Objetivo: Determinar a frequência do polimorfismo +1730 G/A do gene do receptor beta de estrógeno (ERβ) em mulheres inférteis com e sem endometriose e controles. Sujeitos e métodos: Estudo caso-controle que incluiu 136 mulheres com endometriose, 69 mulheres sem endometriose e 209 mulheres férteis como controles. O polimorfismo ERβ + 1730 G/A foi identificado por RFLP-PCR (Restriction Fragment Length Polymorphism - Polymerase Chain Reaction). Resultados: Os genótipos GG, GA e AA do polimorfismo ERβ + 1730 G/A apresentaram frequência de 60,3%, 38,2% e 1,5%, respectivamente, nas mulheres com endometriose (p = 0,0022). Das mulheres inférteis sem endometriose, 63,8% apresentaram o genótipo homozigoto normal GG, 30,4% o genótipo heterozigoto GA e 5,8% o genótipo homozigoto mutado AA (p = 0,0275). No grupo controle, os genótipos GG, GA e AA apresentaram frequência de 77,5%, 21,1% e 1,4%. Conclusão: Os dados sugerem que o polimorfismo ERβ +1730G/A pode estar associado ao risco de infertilidade e infertilidade associada à endometriose. Arq Bras Endocrinol Metab. 2010;54(6):567-71

**Descritores**

Endometriose; receptor β de estrógeno; estrógeno; infertilidade; polimorfismo

**INTRODUCTION**

Endometriosis is a steroid-dependent condition in which a tissue that is histologically similar to the endometrium with glands and stroma grows outside the uterine cavity and becomes implanted in tissues and organs such as the Fallopian tubes, ovaries, peritoneum,
ERβ Polymorphism in Infertility

colon, the retrovaginal region and the bladder (1), being able to cause pelvic pain, dysmenorrhea and infertility (2). Endometriosis affects 3%-10% of women in their reproductive years and 20%-50% of women with infertility (3). Susceptibility to endometriosis depends on a complex interaction of immunologic, genetic and hormonal factors (4). Many aspects of the female reproductive function are strongly influenced by genetic factors, and numerous studies have attempted to identify susceptibility genes for disorders affecting female fertility such as endometriosis (5).

The estrogen receptor (ER) plays an important role in mediating estrogen action on target tissues. There are two isoforms of estrogen receptors, ERα and ERβ, which are encoded by different genes (6). Estrogen receptor α has a higher affinity for estrogen and is the predominant form in normal endometrium. Because large amounts of ERβ messenger ribonucleic acid (mRNA) are found in ovaries and granulosa cells, ERβ is likely to play a role in the ovulatory function (7). Previous studies have demonstrated that both ERα and ERβ are expressed in human endometriotic tissues (8), but the distribution of the isoforms is different between eutopic endometrium and ovarian endometriotic tissues (9).

Bianco and cols. (10) studied infertile women with endometriosis and controls for the +1730 G/A polymorphism in the ERβ gene (rs4986938) and found that this polymorphism was associated with an increased risk of developing endometriosis, regardless of the stage of the disease. However, all patients with endometriosis studied were infertile.

Thus, the objective of the present study was to determine whether the ERβ gene +1730 G/A polymorphism is actually associated with endometriosis and/or infertility.

**SUBJECTS AND METHODS**

One hundred and thirty six infertile women with endometriosis (mean age: 33.7 ± 4.01 yr.) from the Endometriosis Outpatient Clinic of the ABC School of Medicine (FMABC) were studied. Women with endometriosis diagnosed by laparoscopy were selected and classified by histological criteria, according to the American Society for Reproductive Medicine (11). In the endometriosis group, 52.2% of the patients (71/136) had minimal or mild (stage I/II) and 47.8% (65/136) had moderate or severe (stage III/IV) endometriosis. Sixty-nine infertile women without endometriosis (mean age: 34.8 ± 5.1 yr.) were screened at the Human Reproduction Service of FMABC. All women who had a partner with any infertility factor were excluded from the study. For the control group, 210 women (mean age: 39.8 ± 4.5 yr.) who had been submitted to tubal ligation, which allowed confirming the absence of endometriosis, were selected from the Family Planning Outpatient Clinic of FMABC.

The cause of infertility was investigated according to the minimum propedeutic procedures for infertile couples: hormone and chemistry profile, serum testing, testing for sexually transmitted diseases, imaging examinations, investigation of genetic and/or immunologic abnormalities, hysterosalpingography, hysteroscopy, laparoscopy (laparoscopy was performed on all women up to 36 years old and also in patients over 36 whenever there were symptoms or abnormalities on imaging examinations), and semen analysis. Patients with endometriosis who did not achieve pregnancy after at least six natural or induced cycles following laparoscopy were considered infertile. All women whose partners had any male factors associated with infertility were excluded from the study.

Clinical data and peripheral blood samples were collected only after explaining the objectives of the study and obtaining signed informed consent, as approved by the Research Ethics Committee of the ABC School of Medicine.

Peripheral blood was collected from each patient and control in an EDTA-containing tube. Genomic DNA was extracted from peripheral blood lymphocytes using an Illustra blood genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, USA), according to the manufacturer’s instructions.

Molecular analysis of the ERβ gene (MIM 601663/Genbank ID 2100) +1730 G/A polymorphism (rs4986938) was performed according to the protocol of Lee and cols. (2007) (12), with modifications. The primers used were: 5′-TTTTTTGTCCCCATAGTACACA-3′ (forward) and 5′-AATGAGGACCACAGCA-3′ (reverse). PCR reaction was carried out in a final volume of 25 μL, containing 1X buffer, 2.5 mM of MgCl₂, 0.1 mM of each dNTP, 50 nM of each primer, 1U Taq Polymerase (Invitrogen), and 200 ng of DNA. Amplification was performed with an initial denaturation step at 95°C for 7 minutes, followed by 35 cycles of: denaturation at 95°C for 45 seconds, annealing at 53°C for 1 minute, extension at 72°C for 1 minute, and a final extension step at 72°C for 7 minutes. PCR products were analyzed for restriction fragment length polymorphism (RFLP) by using 5U of AluI restriction
enzyme at 37°C overnight and visualized in 2% agarose gel stained with ethidium bromide under UV light.

A G/A exchange at nucleotide 1730 in exon 8 introduces a recognition site for AluI. Digestion by AluI produces one band of 307 bp in the normal ERβ sequence (GG); three separate bands of 307, 240, and 67 bp, respectively, in the heterozygous polymorphism (GA); and two separate bands of 240 and 67 bp, respectively in the homozygous polymorphism (AA).

A random subset (~20% of samples) was repeated by qPCR to verify the results. Detection of the ERβ +1730 G/A polymorphism (rs4986938) was made by TaqMan real-time PCR, using the Rotor-Gene Q 6plex Platform (QIAGEN, Valencia, CA, USA). Commercially available Taqman primers and probes for ERβ +1730 G/A polymorphism were used (C__11462726_10, Applied Biosystems®, Foster City, CA, EUA). Assays were performed with Taqman Universal Master Mix (Applied Biosystems®, Foster City, CA, EUA), with 50 ng of DNA per reaction. PCR conditions were as recommended by the manufacturer: initial denaturation at 95°C (15 min), followed by 40 denaturation cycles at 95°C (15 sec), and a final annealing/extension cycle at 60°C (1 min).

The chi-square test was used to compare allele and genotype frequencies between groups and to estimate the Hardy-Weinberg equilibrium. Statistical tests of significance and χ² analysis were carried out using SPSS for Windows 8.0 (SPSS, Inc., Chicago, IL). All p-values were two-tailed and 95% confidence intervals (CIs) were calculated. A p-value < 0.05 was considered statistically significant.

RESULTS

In the women with endometriosis, the frequencies of genotypes GG, GA and AA of the ERβ gene +1730 G/A polymorphism were 60.3% (82/136), 38.2% (52/136) and 1.5% (2/136), showing a significant difference in the genotype distribution related to control group (p = 0.0022). Among the infertile women without endometriosis, 63.8% (44/69) presented the normal homozygous genotype GG, 30.4% (21/69) the heterozygous genotype GA, and 5.8% (4/69) the mutated homozygous genotype AA, also showing a statistical difference in genotype distribution (p = 0.0275). In the control group, 77.5% (162/209) presented the normal homozygous genotype GG, 21.1% (44/209) the heterozygous genotype GA, and 1.4% (3/209) the homozygous mutated genotype AA (Table 1).

Regarding the alleles, allele G was present in 79.4% of the patients with endometriosis, in 79.0% of the infertile women without endometriosis and in 88.0% of the controls, whereas allele A was present in 20.6%, 21.0% and 12.0%, respectively, of the patients with endometriosis (p = 0.003), infertile women without endometriosis (p = 0.0124) and controls (Table 1).

The control group and infertile patients without endometriosis were in Hardy-Weinberg equilibrium (HWE). The group of infertile patients with endometriosis was not in HWE.

DISCUSSION

Estrogen produced in the ovaries controls the secretion of pituitary gonadotropins and is a key intravarian modulator of ovarian activity, mainly affecting the function of granulosa cells. It contributes to oocyte maturation, fertilization, and embryo quality. Moreover, estrogen plays a crucial role in embryonic and fetal development, influencing secondary sexual characteristics, the reproductive cycle, fertility and maintenance of pregnancy. Additionally, it also plays a regulatory role in endometrial cell growth and differentiation (13).

Studies of the receptors’ tissue distribution and expression pattern indicate that ERα has a broad expression pattern, whereas ERβ has a more focused pattern with high levels in the ovary, prostate, epididymis, lung, and hypothalamus (14). However, the exact physiological responses attributable to each receptor are unknown.

ERα is found in all human reproductive tissues. Its role in reproduction has been elucidated by studies on male and female ERα knockout mice that showed com-

<table>
<thead>
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<th>Population studied</th>
<th>n</th>
<th>Distribution of genotypes</th>
<th>p-value</th>
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<th>Alleles</th>
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<td></td>
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<td>52 (38.2)</td>
<td>2 (1.5)</td>
<td>0.0022</td>
<td>0.1496</td>
<td>216 (79.4)</td>
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<tr>
<td>Infertile women without endometriosis</td>
<td>69</td>
<td>44 (63.8)</td>
<td>21 (30.4)</td>
<td>4 (5.8)</td>
<td>0.0275</td>
<td>109 (79.0)</td>
<td>29 (21.0)</td>
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<tr>
<td>Control group</td>
<td>209</td>
<td>162 (77.5)</td>
<td>44 (21.1)</td>
<td>3 (1.4)</td>
<td>368 (88.0)</td>
<td>50 (12.0)</td>
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* versus controls; ** versus infertile women without endometriosis; OR: odds ratio; CI: confidence interval.
complete infertility (15). The female mice were infertile because they were anovulatory, had altered pituitary gonadotropin concentrations and impaired uterine response to estrogen (16). Recently, polymorphisms in ERβ have been associated with male and female infertility (17).

A study performed in female ERβ knockout mice has confirmed that ERβ is essential for normal ovulation efficiency but not for sexual differentiation, fertility, or lactation (18), and several reports have demonstrated that ERβ proteins can be detected in multiple cell types throughout the female reproductive system (13). In fact, ERβ proteins are detected in human placenta (19) and in multiple cell nuclei within the glands and stroma of the human endometrium, where the intensity of immunostaining varies depending upon the stage of the menstrual cycle (20). One cell type that might represent a target for estrogen action mediated exclusively by ERβ is represented by the endothelial cells of the endometrium. These cells are ERβ-positive and synthesize low (21) or even undetectable (22) amounts of ERα. Studies on endothelial cells isolated from human endometrium have shown that exposure to E2 stimulates endothelial cell proliferation (21). The human endometrium also contains a subset of uterine-specific natural killer (uNK) cells that plays an important role(s) in decidualization, menstruation and implantation.

The ERβ gene is located on chromosome 14q22-24 (23). Systematic mutation screening of the coding region and part of the 50 and 30 regions of the ERβ gene revealed two common single nucleotide polymorphisms: G/A exchange at nucleotide 1730 in the 30 untranslated region in exon 8, and a silent 1082 G/A transition in exon 5 (24). The functional significance of the +1730 G/A polymorphism remains to be clarified. Although +1730 G/A polymorphisms in the ERβ gene do not lead to amino acid changes in the ERβ protein, it is possible that these polymorphisms are in linkage disequilibrium with other regulatory sequence variations that may affect gene expression or function (25). Furthermore, it has been recently reported that genes containing SNPs can cause different structural folds of mRNA (26). These mRNA variants may possess different biological functions that interact with other cellular components.

In the present study, there was a statistically significant difference between the groups of infertile women with and without endometriosis compared to the control group (p = 0.0022 and p = 0.0124, respectively) with regard to the ERβ +1730 G/A polymorphism, suggesting that this polymorphism might be related to infertility, particularly to endometriosis-associated infertility. The presence of only one copy of the polymorphic allele A leads to an increased risk of 1.91 times (OR = 1.91, 95% CI = 1.26 – 2.89) to develop endometriosis and 1.96 (OR = 1.96, 95% CI = 1.18 – 3.24) to develop infertility in relation to the control group. When we compared infertile groups with and without endometriosis, in order to detect if the polymorphism was linked to endometriosis or infertility, there were no statistically significant differences related to the studied polymorphism frequency, suggesting that maybe endometriosis is only a coincidental finding along with infertility.

The Hardy-Weinberg equilibrium showed a light deviation for the group of infertile patients with endometriosis. HWE is an approximation, because these specific assumptions are rarely perfectly met in human populations plus a large sample is usually required to conform to the ‘infinity population’ requirement. Deviation from HWE tests may indicate failure in one or more assumptions such as population stratification, selection bias or genotyping error (27,28). The ERβ gene +1730 G/A polymorphism was identified by RFLP-PCR and a random subset (~20% of samples) was repeated by qPCR to verify the results. However, only the genotype distribution of the patient group showed deviation from HWE, providing additional support for an association of the marker locus with endometriosis (29).

It is important to keep in mind that the study was performed in a special group of patients, who were operated on by videolaparoscopy and, after surgery, were exposed for at least twelve months to the possibility of pregnancy, had no male factor involved in the causes of infertility and, nevertheless, did not achieve pregnancy.

Human fertility is a complex feature determined by interactions between genetic and environmental factors. A recent series of twin studies provided evidence that genetic factors represent a significant component of human fertility measured as waiting time to pregnancy, completed family size and age at first conception (30-32). In addition, behavioral genetics studies have documented genetic influences on fertility precursors such as onset of puberty, sexual behavior and desire to have children. It would be of great interest to characterize the actual relationship between this mutation and endometriosis and/or infertility in a large number of cases. Endometriosis is found in up to 20%-50% of infertile women (3), but the reason why women with endometriosis have impaired fertility is uncertain and remains an area of active investigation.
In conclusion, we found significant differences between infertile women both with and without endometriosis and controls regarding the frequencies of the ER\(\beta\) gene +1730 G/A polymorphism. Thus, our data suggest that an ER\(\beta\) gene polymorphism may be associated with infertility and infertility-associated endometriosis, although endometriosis might be only a coincidental finding along with infertility. However, further studies with much larger sample sizes are needed to evaluate the true role of the ER\(\beta\) gene in infertility and endometriosis.

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