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*Rsa*I polymorphism of the *ERβ* gene in women with endometriosis

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ABSTRACT. We examined the frequency of *Rsa*I polymorphism of the *ERβ* gene in 54 patients diagnosed with endometriosis and 46 controls. Peripheral blood was collected from women undergoing laparoscopy with a confirmed diagnosis of endometriosis. Polymorphisms of the *ERβ* gene and *p53* were assessed by PCR and analyzed on 2% agarose gel stained with ethidium bromide. The AG polymorphism genotype frequency in patients with endometriosis was 59.3%, with 40.7% GG. In the control group, the frequency of AG was 6.5%, with 93.5% GG. The frequency of heterozygous AG was nine times higher in patients with endometriosis than in the control group ($P < 0.0001$).

Key words: Endometriosis; Polymorphism and Infertility; Estrogen receptor

INTRODUCTION

Endometriosis is an estrogen-dependent disease. This hormone is involved in growth, differentiation and functioning of reproductive tissues, including ovaries, uterus, mammary glands, and vagina (Zhao et al., 2000). Its action is mediated by intracellular receptors, which, with the binding of ligands, are translocated to the nucleus, where it activates gene transcription. There are two subtypes of estrogen receptors (*ERα* and *ERβ*); they exhibit distinct cell and tissue differentiation distributions (Sneige et al., 2006).

The *ERβ* gene has been mapped and is located in the long arm of chromosome 14 at locus 2 among subloci 23 and 24 (14q22-24) (Enmark et al., 1997). The first studies were conducted on this gene after the initial characterization performed by Tsukamoto et al. (1998) of highly polymorphic dinucleotide repeats in exon 5 of the human *ERβ* gene in the Japanese population. Five different sequences of variants, including two mutations and three polymorphisms, were detected by systematic mutation screening. The first was silent transition T1421C in exon 7; the second was silent transition G1082A in the binding domain of exon 5, and the third was single nucleotide polymorphism (SNP) A1730G in the 3'-untranslated region of exon 8 (Nakata et al., 2004).

More recently, five new polymorphisms were identified in an African population. Three of them (C143T in exon 1, A566T in exon 2, and T1100G in exon 5) are silent polymorphisms, while the other two exchanged the amino acid sequence of *ERβ*. These include A105G SNP in exon 1, changing the isoleucine to valine and a T1057G SNP in exon 5 requires the substitution of valine for glycine at position 320 in helix 4 in the binding domain (Galliano, 2009).

The study of estrogen receptors and their correlation with endometriosis could help explain the genetic etiology, collaborating in diagnosis and treatment. To this end, we examined the frequency of the *Rsa*I polymorphism of the *ERβ* gene in patients with endometriosis and without symptoms.

MATERIAL AND METHODS

We collected 100 samples of peripheral blood from: 1) 54 women with endometriosis with a mean age of 32.5 years, and 2) 46 women without clinical disease with a mean age of 37.4 years. All patients had the diagnosis confirmed by laparoscopy and were classified according to the American Fertility Society - 1985, and revised in 1996 by the American Society for Reproductive Medicine as Grade I (minimum), Grade II (mild), Grade III (moderate), and Grade IV (severe). The patients answered a questionnaire about personal data and social habits.

DNA extraction from peripheral blood was performed in accordance with the manufacturer of the GFX™ kit (Amersham Pharmacia Biotech, USA) protocol for whole blood, following the guidelines of the technique described by Miller (1988). The DNA integrity was certified by electrophoresis on 2% agarose gel stained with ethidium bromide (0.5 mg/mL) and visualized with a Video Documentation System VDS® (Amersham Biosciences, USA).

Polymerase chain reaction (PCR)

We performed allele-specific PCRs to detect variants of the *Rsa*I polymorphism of the *ERβ* gene, using specific primers for the polymorphic variant "A" and the wild-type "G"; each reaction was carried out with control primers. The primers and expected size of the frag-

ments were as suggested by Aschim et al. (2005). The control had 409 bp and the variants A and G had 127 bp: *Rsa*I Fw 5' ACT TGC CAT TCT GTC TCT ACA 3', *Rsa*I Control Rev 5' CAC AGG ACC CTG AAT CTC 3', *Rsa*I A Rev 5' AGC TCT CCA AGA GCC GT 3', Rev G *Rsa*I 5' AGC TCT CCA AGA GCC GC 3'. The PCR conditions were established to generate both a control fragment and a shorter one, allele-specific band in the presence of the variant, and only the control fragment in the absence of the variant. The possible outcomes of the genotypes of *Rsa*I polymorphism of the *ERβ* gene using the primers proposed by Aschim et al. (2005) are AA, AG or GG (Figure 1). The PCR product was separated by electrophoresis on 2% agarose gel stained with ethidium bromide (10 mg/mL) in Tris-borate EDTA (TBE) at 1X solution. The gel was subjected to a constant electric field of 10 V/cm for 1 h and 30 min. Then, the gels were stained with ethidium bromide at 5 g/mL for 20 min. The visual record of the gel was made with the aid of a video-documentation system (Image Master® VDS - Amersham Pharmacia Biotech, USA). The reactions were run in duplicate.

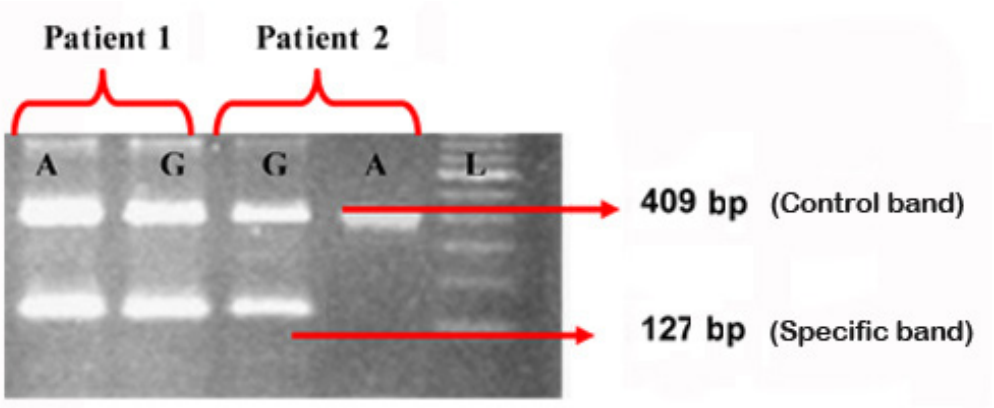

Figure 1. Ethidium-bromide-stained 2% agarose gel, showing the bands for each primer used in the analysis of the *Rsa*I polymorphism of the *ERβ* gene. Patient 1: Heterozygous AG; Patient 2: Homozygous wild-type GG. L: molecular weight marker 100-bp (Invitrogen).

RESULTS

The genotype frequencies found in patients with endometriosis (N = 54) were 0% of the AA genotype, 59.3% of the AG genotype and 40.7% of the GG genotype. Among the control patients (N = 46) the frequencies were 0% of the AA genotype, 6.5% of the AG genotype and 93.5% of the GG genotype. The frequency of heterozygous genotype AG of the *Rsa*I polymorphism of the *ERβ* gene in patients with endometriosis was approximately nine times higher than in control patients ($P < 0.001$; Table 1).

The group of patients with endometriosis was divided into two subgroups of fertile and infertile. The distribution of allelic frequency of fertile patients (N = 25) was 17 AG and 8 GG. Among the infertile patients (N = 27), there were 15 AG and 12 GG. The AA genotype frequency was not found in any group. The AG allele frequency in the fertile patients with endometriosis was approximately 10.5 times higher than in the control group, and the allelic frequency of the AG genotype in the infertile women was 8.5 times higher compared to controls ($P < 0.0001$ for both; Table 2).

Table 1. Distribution of allelic frequency of the *Rsa*I polymorphism of the *ERβ* gene of patients with endometriosis and controls.

Group/genotype	AA		AG		GG		P
	N	%	N	%	N	%	
Endometriosis (N = 54)	0	0	32	59.3	22	40.7	<0.0001
Control (N = 46)	0	0	3	6.5	43	93.5	

The P value was calculated by the χ^2 test.

Table 2. Distribution of allele frequencies in samples from patients with endometriosis in subgroups of fertile and infertile patients and controls.

Group/genotype	AA		AG		GG		P
	N	%	N	%	N	%	
Endometriosis							
Fertiles (N = 25)	0	0	17	68.0	8	32.0	
Infertiles (N = 27)	0	0	15	55.6	12	44.4	<0.0001
Controls (N = 46)	0	0	3	6.5	43	93.5	

The P value was calculated by the χ^2 test.

The genotypic frequency of p53 and *Rsa*I polymorphisms of the *ERβ* gene in patients with endometriosis was 15/20 AG genotype of *Rsa*I polymorphism of the *ERβ* gene with Arg/Arg and 5/20 GG genotype for the same allele. The genotypes Arg/Pro + Pro/Pro were 17/33 AG and 16/33 GG. The frequency of the AG genotype of the *Rsa*I polymorphism of the *ERβ* gene in the group with endometriosis was three times higher for the allele Arg/Arg than the GG genotype for the same allele. In the control group (N = 40), the genotype Arg/Arg was 1/23 AG and 22/23 GG. The Arg/Pro + Pro/Pro was 1/17 AG and 16/17 GG. We obtained about 8.5 times more genotype Arg/Pro + Pro/Pro in the group with endometriosis than in the control group ($P < 0.0001$; Table 3).

Table 3. Genotypic frequency of p53 and *Rsa*I polymorphisms of the *ERβ* gene of pacients with endometriosis and controls.

*Rsa*I	AG		GG		P
	N	%	N	%	
Endometriosis					
Arg/Arg	15	75.0	5	25.0	
Arg/Pro + Pro/Pro	17	51.5	16	48.5	
Total	32		21		
Controls					<0.0001
Arg/Arg	1	4.3	22	95.7	
Arg/Pro + Pro/Pro	1	6.0	16	94.0	
Total	2		38		

The P value was calculated by the χ^2 test.

DISCUSSION

We found the allelic frequency of *Rsa*I polymorphism of the *ERβ* gene in AG to be about nine times higher in patients with endometriosis compared to controls, similar to what

was found by Sundarrajan et al. (2001). They found that in Chinese women with ovulatory dysfunction, the allele frequency of *Rsa*I polymorphism of the *ERβ* gene was significantly higher than in controls. However, they found the homozygous AA genotype in patients with ovulatory dysfunction and not in the control group, different from our findings.

Renner et al. (2006) reported in their studies that endometriosis is an estrogen-dependent pathology; it is possible that genetic variation in the mediation of the estrogen pathway allows for potentiation of estrogen, facilitating the initiation and growth of endometriosis. We found that the frequency of the *Rsa*I polymorphism of the *ERβ* gene was higher in fertile women with endometriosis, indicating more of this hormone.

However, when we compared fertile and infertile subgroups, we found no significant differences. Hapangama et al. (2008) claimed that endometriosis is associated with decreased expression of endometrial *ERβ* and is associated with cell proliferation and up-regulation of telomerase. They indicated that telomerase specifically correlates with cell proliferation and that many cancers express high levels of telomerase. Tempfer et al. (2009) reported that the *ERβ* gene is associated with increased risk of stage IV endometriosis in Japanese women, while we found polymorphism in all stages of classification of endometriosis of the patients.

Hsieh and Lin (2006) concluded that there is an association between endometriosis and p53 polymorphisms. The arginine allele in homozygosis at codon 72 is associated with low susceptibility to developing endometriosis. The proline allele in double dose (Pro/Pro) or only one allele (Pro/Arg) is related to higher susceptibility. In a study by Chang et al. (2002), the proline allele was found to be related to a two to three times higher incidence of endometriosis. We also found that the genotype frequency of p53 polymorphism is correlated with the *Rsa*I polymorphism of gene *ERβ* and that there is a higher frequency in the genotypes Arg/Pro + Pro/Pro and AG in patients with endometriosis than in the control group. Endometriosis is enigmatic because its etiology is not fully understood. We examined the molecular basis of endometriosis and its correlation with the *Rsa*I polymorphism of the estrogen receptor beta gene. Further prospective studies should examine the molecular events in gene *ERβ* in endometriosis, exploring various methodologies such as endometrial biopsies, as well as quantitative analysis of mRNA, with emphasis in the cyclical hormonal levels, correlating with different degrees of classification of endometriosis.

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