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# Study of RsaI polymorphism of the ERβ gene in Greek women with endometriosis

**Elli Anagnostou, Agathi Theodoropoulou, Despina Mavrogianni, Athanasios Protopapas, Peter Drakakis, Dimitrios Loutradis**

1st Department of Obstetrics and Gynecology, Division of Human Reproduction, IVF Unit, Alexandra Hospital, Medical School of National Kapodistrian University of Athens, Athens, Greece

**Corresponding Author**

Elli Anagnostou, Division of Human Reproduction, IVF Unit, 1st Department of Obstetrics and Gynecology, Alexandra Hospital, Athens University Medical School, Athens, Greece
Tel.: +30 210 3613241, e-mail: elli.anagnostou@gmail.com

## Abstract

Estrogens and estrogen receptors (ERs) play an important role in the pathogenesis of endometriosis. The aim of this study was to investigate the presence of gene polymorphism RsaI in the gene of the estrogen receptor ERβ in the Greek female population, and its distribution in women suffering from endometriosis and in a control group. We included 67 consecutive infertile women of Caucasian origin who were operated laparoscopically in our Gynecological Endoscopy Unit for endometriosis, and 96 women participated as control group. Patients were genotyped for RsaI (G/A, rs1256049) polymorphism in ESR2 exon 5, using real-time PCR. The patients' genotype distribution did not differ from the control group. There were no women homozygous for the polymorphic allele in neither group. The different genotypes of ESR2 could not be associated with the stage of endometriosis. The data of this study point that in Greek population who had proven endometriosis the determination of RsaI polymorphism of ESR2 gene doesn't offer any information for the progression of endometriosis, regarding the genetic profile of this particular gene.

**Key words:** ERβ, SNP, gene polymorphism, endometriosis

## Introduction

Endometriosis is an estrogen dependent inflammatory condition in which the endometrium develops in a region outside the uterus, as in the organs of the abdomen and pelvis, such as the ovaries, peritoneum or vagina, or rarely in distant organs such as the lungs[1].

Estrogens and estrogen receptors (ERs) play an important role in the pathogenesis of endometriosis. Estradiol is involved in the growth, differentiation and function of reproductive tissues, while endometriosis is an estrogen-dependent disease. Genetic abnormalities and environmental factors including dietary, hormonal and non-hormonal conditions could contribute to the development of endometriosis[2].

The pathogenesis of endometriosis is not known. There are several theories that interpret it partially but not completely. Many studies have shown that in some women there is a predisposing hereditary factor, which in combination with other immunological and biochemical factors results in the formation of endometrial foci outside the uterus[3]. Environmental parameters also seem to enhance the development of endometriosis. The most well-known theory of endometriosis development is through the migration of endometrial cells during menstruation, through the fallopian tubes, into the abdomen.

The clinical picture depends on the location and extent of the lesions, although the severity of the symptoms is not necessarily compatible with the spread and severity of the condition. Endometriosis can result to the impairment of the woman's reproductive function. One in three women with infertility problems has endometriosis. It has been observed that women with endometriosis have difficulty in conceiving[4].

Genetic polymorphism is defined as the occurrence of multiple alleles in a genetic locus, where at least two alleles occur with a frequency greater than 1% of the population. A polymorphic genetic site can be found anywhere in the genome and not necessarily in a gene. The importance of polymorphisms lies in the fact that in many cases these variants modify the structure or function or expression of a protein, and this can have consequences for the function of enzymes, carriers, receptors or other possible target molecules involved in mediation of the action of drugs. This, in turn, explains in part the observed - among individuals - differences in clinical phenotypes and pharmacological responses[5].

Estrogens mediate their actions through two subtypes of nuclear receptors, ERa and ERb, which are encoded by *ESR1* and *ESR2* genes, present on distinct chromosomes (locus 6q25.1 and locus 14q23-24.1, respectively). Both forms of the receptor have been identified in the human ovary[6].

### *ERa and ESR1 gene*

ESR1 gene contains some well-studied single nucleotide polymorphisms (SNPs). The most widely-studied polymorphisms of restriction fragments length (RFLPs) are PvuII (T397C) and Xbal (A351G) in intron I, and the (TA) variable number of tandem repeat (VNTR) region within the promoter of the genomic oxide. In various studies, these polymorphisms have been associated with a number of pathological conditions: breast cancer and prostate cancer, osteoporosis, Alzheimer's disease, and cardiovascular disease.

Georgiou *et al* in 1999 investigated in the Greek population the relationship between the PvuII polymorphism and the (TA) variable number of the estrogen receptor with endometriosis[7]. There was a statistically significant difference between the patients and the controls in the frequency of the two-allele Pvu II polymorphism (0.72 vs. 0.49) and in the median repeats of the (TA)n multiallele polymorphism (15 vs. 20 repeats). In both groups, linkage was found between the fewer (TA)n repeats (range, 12–19) and the positive Pvu II polymorphism. The study concluded that the gene diversity of the estrogen receptor (ER) is likely to contribute to the pathogenesis of endometriosis.

Hsieh *et al* in 2007 observed that the distributions of frequencies of the genotypes Xba I A/G and PvuII T/C of ERa was significantly different between individuals with and without endometriosis[8]. Specific allele variants for the ERa correlated with more severe endometriosis: XbaI GG genotype is associated with moderate association with endometriosis.

The role of PvuII polymorphism of the ESR1 gene reportedly correlates with the severity of endometriosis[7-8] as well as with the ovarian stimulation during IVF[9] and the outcome of the pregnancy after IVF[10]. Morever, the repeat polymorphisms dinucleotide (TA) of the promoter of the ESR1 gene has been proposed to increase the risk of premature ovarian failure[11] and of endometriosis[7].

A recently conducted study included twenty consecutive infertile Greek women who were operated laparoscopically in our Gynecological Endoscopy Unit for advanced endometriosis and 48 parous women as controls, with a history of at least one successful pregnancy, no history of spontaneous abortion, and no known history of endometriosis (unpublished data). Real time PCR was applied in all samples to detect the distribution of the PvuII polymorphism of ERa. The presence of the PvuII polymorphism in both alleles (CC genotype) was found in only 6.7% of patients with endometriosis, compared with 35.4% of controls (p=0.033). In this study the *ESR1* polymorphism was found less commonly in a homozygous condition in patients with endometriosis in comparison with controls, a finding which is not of accordance with previous studies that have demonstrated a positive correlation between endometriosis and *ESR1* polymorphisms[12-13], whereas others reported no significant association[14].

### *ERb and ESR2 gene*

The *ESR2* gene coding for ERβ has been mapped and is located on the long arm of chromosome 14. The first studies that were performed on this gene concerned the highly polymorphic recurrent dinucleotide in exon 5 in a Japanese population[15]. Polymorphisms involve the simultaneous appearance of a population of two or more alternative genotypes, each of which has a higher frequency than it could be justified by repeated mutations alone. Five different sequences of variations, including two mutations and three polymorphisms, were detected. More recently, five new polymorphisms have been identified in the African population. Three of them (C143T on exon 1, A566T on exon 2 and T1100G on exon 5) are silent polymorphisms, while the other two changed the sequence of ERβ amino acids.

Hapangama *et al* support that endometriosis is associated with decreased ERβ endometrial expression and is associated with cell proliferation and ascending telomerase regulation[16]. Silva *et al* explored whether estrogen receptors are associated with endometriosis and whether they could offer a genetic explanation of the disease etiology, contributing to its diagnosis and treatment[17]. For this purpose, the frequency of RsaI polymorphism of the ERβ gene in patients with endometriosis and in asymptomatic patients was examined. The genotypic frequencies found in patients with endometriosis (N = 54) were: 0% with AA gene genotype, 59.3% with AG genotype and 40.7% with GG genotype. Among the control group of patients (N = 46) the frequencies were 0% with AA gene genotype, 6.5 % with AG genotype and 93.5% with GG genotype. The frequency of the heterozygous AG genotype of RsaI polymorphism in patients with endometriosis was about nine times higher than in patients in the control group (P <0.001).

Sundarrajan *et al* suggested that the RsaI (G/A) locus in the *ESR2* gene is associated with ovarian failure of unknown cause[18]. Others studies did not find any effect of the polymorphism RsaI G/A and AluI G/A of the *ESR2* gene on the COS outcome[19-20]. The results suggest that these polymorphisms may be associated with ovulatory abnormalities in some patients, especially in couples with unknown causes of infertility.

The aim of this study was to investigate the presence of gene polymorphism RsaI in the gene of the estrogen receptor ERβ in the Greek female population, and its distribution in women suffering from endometriosis and in a control group. The presence of RsaI gene polymorphism was also evaluated regarding the stage of endometriosis, the age of the diagnosis, menarche, and the pregnancy outcome as well.

## Materials and Methods

This study was conducted in the 1st Department

of Obstetrics and Gynecology of the National and Kapodistrian Univesity of Athens, Greece. We included 67 consecutive infertile women of Caucasian origin who were operated laparoscopically in our Gynecological Endoscopy Unit for endometriosis. In all cases disease was confirmed histologically. Written informed consent was read and signed by all participants. The study has been approved by the Review Board of our Institution. As control group, 96 women participated: they were all parous women with a history of at least one successful pregnancy, no history of spontaneous abortion, and no known history of endometriosis.

### *Molecular analysis*

Peripheral blood was collected from all women, in both groups. Genomic DNA was obtained from peripheral blood leucocytes with the QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Patients were genotyped for RsaI (G/A, rs1256049) polymorphism in ESR2 exon 5, using real-time PCR (Light Cycler 480 II, Roche Diagnostics, Germany). The conditions of the real-time PCR for the ESR2 RsaI polymorphism are described elsewhere[21].

### *Statistical Analysis*

Statistical analysis was performed with Statistics Package for Social Sciences (SPSS), version 15, Minitab 12, while the Sasieni algorithm (1997) and Hardy-Weinberg equilibrium were performed with the on line calculator which is available on http://ihg.gsf.de. The statistical methods used for the control of statistical hypothesis were: two independent samples t-test, 2-proportion test (normal approximation) and parametric one way Analysis of Variance. For qualitative data used the chisquare test (Fisher exact test and Monte Carlo procedure). The non parametric tests Mann-Whitney U and Kruskal-Wallis test were used when needed.

A *P-value* less than 0.05 were regarded as statistically significant. Values are presented as mean±SD, unless otherwise stated.

## Results

This study included 67 women with endometriosis and 96 women as controls.

In all cases, ESR2 genotyping was performed and the women in both groups were categorized as homozygous for the wild type WT allele (GG genotype), heterozygous (GA genotype), and homozygous for the polymorphic allele (AA genotype), according to PCR results.

The distribution of women according to the genotype for RsaI gene polymorphism is shown in Table 1. In the control group, 91/96 (94.8%) women were homozygous for the wild type (GG), 5/96 (5.2%) were heterozygous (GA) and no woman (0%) was homozygous for the polymorphism (AA). In the endometriosis group, 65/67 (97%) were homozygous for the wild type and 2/67 (3.0%) were heterozygous, and no woman (0%) was homozygous for the polymorphism respectively. The patients' genotype distribution did not differ from the control group (p-value 0.490).

Further on, the different genotypes of ESR2 are associated with the stage of endometriosis, which reflects the severity of the disease (Table 2). The presence of wild type genotype in stages I, II was 27.1%, in stage III 44.1% and in stage IV 28.8%.

Table 1. Genotype distribution of RsaI ESR2 gene polymorphism in the control group and the endometriosis group of women (p-value 0.490).

| GENOTYPES OF RSAI SNP ESR2 GENE | CONTROL GROUP (N=96) | ENDOMETIOSIS GROUP (N=67) |
|---|---|---|
| GG | 91 (94.8%) | 65 (5.2%) |
| GA | 5 (97%) | 2 (3%) |
| AA | 0 (0%) | 0 (0%) |

Table 2. Genotype association of RsaI *ESR2* gene polymorphism with the stage of endometriosis (p value = 1.0).

| GENOTYPES OF RSAI ESR2 GENE | STAGE I-II (N=16) | STAGE III (N=27) | STAGE IV (N=18) |
|---|---|---|---|
| GG | 16 | 26 | 17 |
| GA | 0 | 1 | 1 |

Concerning the heterozygous for the polymorphism patients (n=2), one patient presented stage III endometriosis and the other stage IV endometriosis (p value = 1.0).

All parameters regarding the age at the diagnosis of endometriosis, menarche, pregnancies and deliveries, were not statistically different, except the age of the diagnosis of the endometriosis. Of course, the statistical analysis is weak, because in the study population the prevalence of the homozygous for the polymorphism genotype of ESR2 is 0% and concerning the heterozygous it ranged between 3-5.2%, in both groups. Taking into account that this gene is very rare in Greek population, it is difficult to draw distinct conclusions regarding the significance of this genotype and the relation with endometriosis in Greek population.

## Discussion

In this study we examined the association of RsaI ESR2 polymorphism in endometriosis, and the impact of this genotype in the features of endometriosis in Greek population.

It is already know from several studies in different ethnicities that the estrogen receptors are associated with endometriosis and there is a genetic etiology, which may contribute to its diagnosis and treatment regarding this disease.

Silva *et al* examined the estrogen receptors in association with endometriosis in order to highlight a genetic connection[17]. The frequency of RsaI polymorphism of the ERβ in patients with endometriosis and in asymptomatic patients was examined. The genotype distribution found in patients with endometriosis (N = 54) was 0% for AA gene genotype, 59.3% for AG genotype and 40.7% for GG genotype. Among the control group patients (N = 46) the distribution was 0% for AA gene genotype, 6.5 % for AG genotype and 93.5% for GG genotype. The incidence of heterozygous genotypes AG of RsaI polymorphism of the ER β gene in patients with endometriosis was about nine times higher than in patients in the control group (P <0.001). Our data did not reveal any difference in the genotype distribution profile in the endometriosis group and the control group in Greek population. More specifically, the wild type distribution was >90% in both endometriosis and control group. Thus, our results suggest that the RsaI gene polymorphism is not associated with endometriosis and that there is no over-presentation of a certain genotype in endometriosis in the Greek population. This design of this study did not permit to drawn any other conclusion, because 97% of both populations carried the wild type genotype (GG).

The positive correlation between endometriosis and *ESR1* polymorphisms is well known. The study of Hsieh *et al* (2007) observed that the distributions of frequencies of the genotypes XbaI A/G and PvuII T/C of ERa were significantly different between individuals with and without endometriosis[8], while a recent study in a Greek population (unpublished data) did not associate the presence of the PvuII polymorphism of *ESR1* gene in patients with endometriosis, compared to controls. -

To date, the studies in Greek population regarding the *ESR1* gene imply that it is related to endometriosis, while *ESR2* gene polymorphism doesn't seem to be implicated with the presence of endometriosis and this polymorphism in particular is poorly represented. This result is also confirmed by the study of Georgiou *et al*, which showed that ERb

polymorphisms are rare in Greek patients enrolled in an IVF attempt[9].

In this study we also examined the association of the genotypes of ESR2 with the stage of endometriosis, which reflects the severity of the disease. The presence of wild type genotype in stages I, II was 27.1%, in stage III 44.1% and in stage IV 28.8%. Concerning the heterozygous for the polymorphism patients (n=2), one patient presented stage III endometriosis and the other stage IV endometriosis. Thus, these data do not allow us to draw conclusions about the implication of RsaI SNP in the severity of endometriosis. It would be of interest to see if the populations that express in greater percentage the polymorphism, whether it is associated with severe endometriosis.

Furthermore, our data regarding the age at the diagnosis of endometriosis, menarche, pregnancies and deliveries, were not statistically different, except the age of the diagnosis of the endometriosis.

Many epidemiological studies have suggested an association between estrogen receptor-beta (ER-β) polymorphisms with endometriosis risk. However, the results of these studies have been inconsistent. The meta-analysis that conducted Guo in 2014 suggested that the ER-β rs4986938 and rs1256049 polymorphisms are not associated with endometriosis risk in overall population, a result which was consistent with the results of a previous meta-analysis[22].

If we consider the contemporary meta-analysis in this field, there is insufficient evidence for implication of the ER-β polymorphism in the etiology of endometriosis- associated infertility for population testing. The observed increase in risk of endometriosis-associated infertility may be due to bias because of the inclusion of small-scale studies. It is critical that larger and well-designed multicenter studies should be performed to re-evaluate potential associations.

Finally, an Italian study conducted by Pagliardini, confirmed that the polymorphisms of the Wnt4, CDKN2BAS and FN1 genes as genetic sites involved in endometriosis pathology[23]. Also, another study conducted in Brazilian population by Barbosa, showed that the combined examination of the genotypes of the FCRL3 and FOXP3 polymorphisms present an interaction of the FOXP3CT / FCRL3-TT or TC or CC genotypes with an accumulated profile which drive to the evolutionary effect of endometriosis[24].

## Conclusion

In conclusion, the data of this study point that in Greek population who had proven endometriosis the determination of RsaI polymorphism of *ESR2* gene doesn't offer any information for the progression of endometriosis, regarding the genetic profile of this particular gene.

Overall, the impact of endometriosis and its staging on assisted reproduction outcome is quite important, as recent meta-analysis underlines[25], thus studies with larger populations would be of greater value, in order to define the genetic mechanisms behind the disease and the role of the specific gene polymorphisms that are involved in the pathology of endometriosis.

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