Research Article

Polymorphisms of Estrogen Receptor- α and Estrogen Receptor- β Genes and its Expression in Endometriosis

Östrojen Reseptör- α ve Östrojen Reseptör- β Genlerinin Polimorfizmi ve Endometriozis İfadesi

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ABSTRACT

Objectives: Endometriosis is a common gynecological disorder, characterized by the presence of endometrial-like tissue in extrauterine location. Increases of estradiol concentration can influence the risk of endometriosis and activity of estrogen receptors. Polymorphism in estrogen receptors (ER) cause the alteration of gene expression and influence the interaction hormone-receptors. This research aimed to determine the role of estrogen and genetic aspect of ER in the pathogenesis of endometriosis.

Materials and Methods: This research is a case-control study comparing 83 women with endometriosis and 76 women without endometriosis for polymorphisms, but for RE gene expression analysis and estrogen level measurement using samples obtained from 18 women with endometriosis and 18 women without endometriosis. Polymorphism was determined with PCR-RFLP. Data were analyzed by Chi-square, Mann-Whitney, Spearman rho, t-independent, a two-tailed.

Results: Association between the allele ER α rs9340799 A/G and endometriosis was significant ($p=0.012$). In contrast, rs2234693 T/C polymorphism showed no association with endometriosis. Association between the genotypes frequencies and alleles ER β rs4986938 G/A with endometriosis was found significantly different ($p=0,015$; $p=0.034$).

Conclusions: The increases of estradiol level and ER- β expression, polymorphism genotypes and alleles of ER β rs4986938 G/A gene and alleles frequency of ER α rs9340799 A/G gene have a role for endometriosis.

Key words: Estradiol (E₂), estrogen receptor (ER α and ER β), endometriosis

ÖZ

Amaç:

Endometriozis, ekstrauterin lokasyonda endometrial benzeri dokunun varlığı ile karakterize yaygın bir jinekolojik hastalıktır. Östradiol konsantrasyonunun artması, endometriozis riskini ve östrojen reseptörlerinin aktivitesini etkileyebilir. Östrojen reseptörlerinde (ER) polimorfizm, gen ekspresyonunun değişmesine neden olur ve etkileşim hormon reseptörlerini etkiler. Bu araştırma ER'nin östrojen ve genetik yönünün endometriozis patogenezindeki rolünü belirlemeyi amaçlamıştır.

Gereç ve Yöntemler: Bu araştırma, endometriozisli 83 kadını ve polimorfizmler için endometriozisi olmayan 76 kadını, endometriozisi olan 18 kadından ve endometriozisi olmayan 18 kadından elde edilen numuneleri kullanarak RE gen ekspresyonu analizi ve östrojen seviyesi ölçümünü karşılaştıran bir vaka kontrol çalışmasıdır. Polimorfizm, PCR-

RFLP ile belirlenmiştir. Veriler Ki-kare, Mann Mann-Whitney, Spearman rho, t-bağımsız ve iki kuyruklu testleri kullanılarak analiz edildi.

Bulgular: Allel ER α rs9340799 A / G ile endometriozis arasındaki ilişki anlamlıydı (p = 0.012). Aksine, rs2234693 T / C polimorfizmi, endometriozis ile bir ilişki göstermedi. Genotip ve allel ER β 4986938 G / A frekansları ile endometriozis arasındaki ilişki anlamlı bulundu (p = 0,015; p = 0,034).

Sonuç: Östradiol düzeyi ve ER- β ekspresyonu, polimorfizm genotipleri ve ER β 4986938 G / A geninin alelleri ve ER α rs9340799 A / G geninin allel sıklığı endometriozis için rol oynamaktadır.

Anahtar kelimeler: Estradiol (E2), östrojen reseptörü (ER α ve ER β), endometriozis
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INTRODUCTION

Endometriosis is a disease or chronic gynecological disorder that is manifested by the presence of endometrium-like tissue that grows outside the cavity uterus.^{1,2} The incidence of endometriosis is estimated at 10-15%, and clinical data from various hospitals in Indonesia reached 13.6 to 69.5 % of group infertile.^{2,3,4} Until now, the etiology and pathophysiology of endometriosis is not fully understood. It is because endometriosis is a multifactorial disease that is caused by the interaction of various factors such as the environment, immunology, and genetics.

Endometriosis is closely related to estrogen levels. Generally, patients with endometriosis have higher levels of estrogen than normal women, which can stimulate growth of endometrial tissue and cell stroma outside the uterine cavity, causing pain and inflammation. Based on the women's normal menstrual cycle, estrogen levels in the serum had twice the highest peak of the proliferative phase (before ovulation) and the luteal phase. While it is known from previous studies that high estradiol concentrations in the tissues of endometriosis, but it is not known how the level of serum estradiol (E2) peripheral highest in patients with endometriosis, especially the proliferative phase.

The biological activity of the estrogen receptor is mediated by estrogen receptors (ER) so that changes in the quantity and the affinity of these receptors can cause a pathological condition. There are two subtypes of estrogen receptors, namely RE α and RE β encoded by two different genes and chromosomes. RE α and RE β located in chromosomes six locus q25.1 and chromosome 14 locus q22-24.^{8,9} Studies show that RE gene is a candidate gene for the disease endometriosis. RE α rs2234693 gene polymorphism contributes to an increased risk of endometriosis, especially in the Caucasian group and RE α gene rs9340799 with GG genotype (mutant) to increased endometriosis risk by 4 times (OR = 4.67, IK95% = 1.84 to 11.83) with values p = 0.001 and 3 times the risk of failure of IVF with p = 0.018 (OR = 3.33, IK95% = 1.38 to 8.03).¹⁰ Likewise RE β gene polymorphism rs4986938 showed the risk of endometriosis and infertility in Brazil.¹¹ RE gene polymorphism showed inconsistent results between countries/ethnicity and in Indonesia had not done the research on the characteristics of the ER gene, especially in patients with endometriosis.

The presence of this polymorphism in the RE gene thought to be contributing to elevated or low levels of expression of the genes. Results of previous studies reported that there is an increased expression in endometriosis tissue RE β 100 times compared to normal endometrial

tissue and the expression RE β suppress RE α expression in endometriosis tissue, but the mechanism is not understood.¹² Therefore required further research on ER gene polymorphism in patients with endometriosis and can be used as the latest investigations on the endometriosis.

MATERIALS AND METHODS

Materials

The research samples are blood sample derived from 83 women suffering endometriosis by laparoscopy and 76 normal women who do not suffer from endometriosis. All the samples were obtained from the Polyclinic immuno-endocrinology, Department of Obstetrics and Gynecology, RSCM-FKUI, RS Budhi Jaya and RS Sammeri. RE α intron one gene polymorphisms rs9340799 and rs2234693 and rs4986938 RE β exon 8.

Isolation of DNA from peripheral blood

Peripheral blood 1.5 ml is mixed into 4.5 mL Red Blood Cell (RBC) 1x (199 mM EDTA, 100 mM KHCO₃, 1.45 NH₄ Cl), then inverted and incubated for 10 min at room temperature. Samples were centrifuged in centrifuge machine (NF 400 / NF400R Bench-Top Centrifuge) at a speed of 1500 rotation per minute (rpm) for 10 minutes at room temperature. The supernatant was discarded slowly leaving the rest of the sediment in the form of leukocytes in the next tube. 300 mL solution of Cell Lysis Solution (CLS) containing 10 mM Tris-HCl, 0.25 mM EDTA and Sodium Dodecyl Sulfate (SDS) 20% included into the tube and then pipetting until a homogeneous solution and then incubated in a water bath at 37 ° C for 30-60 minutes. Followed by the addition of 300 mL solution Precipitation Protein (PP) containing 5 M ammonium acetate and then homogenize with Stuart Scientific Autovortex SA6 engine for 10-20 seconds until you see the brown granules. Samples were centrifuged back at a speed of 3,000 rpm for 15 min at 4°C. Results centrifugation a brown pellet form (protein) and the supernatant containing DNA. The supernatant was transferred into a new tube which already contains 2.3 ml of solution isopropanol cold, then the tube inverted approximately 25-30 times until DNA looks like a collection of threads. Samples were then centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was discarded, and the pellet was washed with 1.3 mL ethanol 70% sterile, then centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet (DNA) in the dry in the open air by turning the tube for approximately 2 hours at room temperature or 1 hour at 37°C. DNA rehydrated with 300 mL of TE solution (10 mM Tris-HCl, 0.25 EDTA) and then incubated for 2 h at 37°C. Then DNA was transferred to a 1.5 mL sterile tube. The concentration and purity of DNA in the case and control group measured using a NanoDrop (Maestro). The solution was then stored at -20 ° C until further examination.

Amplification DNA fragments of RE genes

Amplification of DNA fragments using PCR method and using primers specific to the target gene based software primer 3. The primer sequences used for amplification RE α gene intron 1 is 5'-CAG-GGT-TAT-GTG-GCA-AC-ATG-3' (forward) and 5'-TAC-CTA-TAA-TGA-CAA-AAA-AA-3' (reverse), while the primer sequences for genes RE β exon 8 is 5'-CGG-CAG-AGG-ACA-GTA-AAA -GC-3' (forward) and 5'-AGG-CCA-TTG-AGT-GTG-GAA-AC-3' (reverse). The volume of each reagent is 25 μ L consisting of 5 μ L of genomic DNA, 12.5 μ L of master mix (Kappa from Bioline), 0.5 μ L of forward primer, reverse primer 0.5 μ L and 6.5 μ L of aquabidest (ddH₂O).

DNA samples were amplified for 35 cycles at each gene. PCR conditions used for gene RE α on the region intron 1 begins with an initial denaturation at a temperature of 94°C for 6 minutes, then get into a cycle consisting of denaturation at a temperature of 94°C for 60 seconds, annealing at a temperature of 56°C for 40 seconds and elongation at a temperature of 72°C for 90 seconds. At the end of the cycle is elongation at a temperature of 72°C for 5

minutes. RE β gene amplification stages in the area of exon 8 PCR gene RE α almost the same, but the annealing temperature used was 54°C. The results of amplification are separated by electrophoresis on 2% agarose gel containing ethidium bromide (0.5 mg/mL) in 1X TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA pH 8.0) at a 90V voltage for 40 minutes. Amplicons size of RE α gene is 255bp, and RE β is 293 bp.

RE gene polymorphism analysis with Restriction Fragment Length Polymorphism (RFLP)

RE α gene polymorphism in intron 1 rs9340799 known area by using enzymes XbaI, rs2234693 with PvuII enzyme and gene RE β in exon 8 rs4986938 area with AluI enzyme. Cutting is done by adding the enzyme XbaI, PvuII or AluI (New England Biolabs) of 0.5 μ L (10U / μ L) into tubes already containing 10.5 μ L DNA fragment amplification product, 1 mL of buffer solution RE 10X and 9 mL ddH₂O, total volume is 21 μ L. Furthermore incubated in a water bath at a temperature of 37°C for 4 hours. DNA fragments were analyzed on a 3% agarose gel in 1x TAE at a voltage of 90 volts for 1 hour. Visualization fragment electrophoresis was observed with UV on UV illuminator long liveTM Filter spectroline^R and photographed with a digital camera.

Statistical Analysis

Genotype distribution and allele frequencies of polymorphisms tested using Thesias 3.0 software program with genetics packages and continued with SPSS version 21 at $\alpha = 0.05$. Data were analyzed by using Chi-square, Mann-Whitney, Spearman rho, t-independent, and a two tailed tests.

RESULT

RE α gene polymorphism rs9340799 A/G, RE α gene rs2234693 T/C and RE β gene rs4986938 G/A

The PCR products of RE α genes intron 1 rs9340799 were restricted with the enzymes XbaI showed changes in nucleotide A/G (Figure 1), resulting in a DNA band size of 255 bp (homozygous mutant/GG), two bands of DNA size of 142 bp and 113 bp (homozygous wild-type/AA) and three DNA with a size of 255 bp, 142 bp and 113 bp (heterozygous/GA).

Figure 1. Results of RFLP PCR gene intron 1 rs 9340799 RE α using enzyme XbaI. M = marker. Well no 1 and 3 = homozygous mutant (GG), the well no 2,6,7 = heterozygous (AG), the well no 4,5 = homozygous wild type (AA)

The PCR products of genes RE α intron 1 rs2234693 were restricted with enzyme PvuII showed nucleotide changes T/C (Figure 2), generating a DNA band size of 255 bp (homozygous wild-type/TT), two bands of DNA size of 158 bp and 97 bp (homozygous mutant/CC) and three DNA with a size of 255 bp, 158 bp and 97 bp (heterozygous/TC).

Figure 2. The results of PCR-RFLP gene intron 1 rs 2234693 RE α using PvuII enzyme. M = marker, well no 3 = wild type genotype (TT), well no 2,4,6 = homozygous mutant genotype (CC), well no 1,5 = heterozygous genotype (TC)

The PCR products of genes RE β exon 8 rs4986938 were restricted with the enzymes AluI showed nucleotide changes G/A (Figure 3), resulted in a DNA band size of 293 bp (homozygous wild-type/AA), two bands of DNA size of 200 bp and 92 bp (homozygous mutant/GG) and three DNA with a size of 293 bp, 200 bp and 92 bp (heterozygous/AG).

Figure 3. The results of PCR-RFLP RE β exon 8 rs4986938 using enzymes AluI. M = markers, wells No. 1 and 5 = heterozygous genotypes (GA), well no 2,3,4,6,7 = Wild-type homozygous genotype (GG). In this image does not look the mutant genotype (AA)

The genotype distribution and allele frequency Gen RE α rs9340799 A / G, Gen RE α rs2234693 T / C and Gen RE β rs4986938 G / A on Cases and Controls Group

Comparison of genotype distribution and allele frequency of RE α gene rs9340799 with XbaI enzyme shows in the Table 1.

Table 1. Genotype and Allele Frequency of ER α rs9340799 Gene with XbaI Restrictal Enzyme in Intron 1 Area, A/G on Endometriosis Case and Control Groups

Genotype			X ²	p	Allele Frequency		X ²	p	OR
AA	AG	GG			A	G			(IK=9 5%)

Control	32 (42,1%)	28 (36,8 %)	16 (21,1 %)			92 (60,5 %)	60 (39,5 %)			
Endometriosis Case	22 (26,5%)	33 (39,8 %)	28 (33,7 %)	5,23 6	0,0 73	77 (46,4 %)	89 (53,6 %)	6,3 72	0,01 2	2,54
Total	54	61	44			167	151			

From the Table 1 shows that GG genotypes (normal) were higher in the endometriosis case group compared to the control group. Heterozygous genotype GA was also higher in the endometriosis case group compared to the control group, while the AA genotype (wild type) was lower in the endometriosis case group compared to the control group. G allele frequency was higher in the endometriosis case group compared to controls, whereas the frequency of allele A was lower in the endometriosis case group compared to control. The results of Pearson Chi-square test showed genotype frequencies in the endometriosis case and control groups was not significant, whereas the allele frequencies in endometriosis case and control groups showed no significant differences with $p = 0.012$ and the control group is G allele (mutant).

Comparison of genotype distribution and allele frequencies of REα gene with PvuII enzyme shows on the Table 2.

Table 2. Genotype and Allele Frequency of REα rs9340799 Gene with PvuII Restriction Enzyme on Endometriosis Case and Control Groups

	Genotype			X ²	p	Allele Frequency		X ²	p
	TT	TC	CC			C	T		
Control	18 (23,7 %)	46 (60,5 %)	12 (15,8 %)			70 (46,1 %)	82 (53,9 %)		
Endometriosis Case	17 (20,5 %)	46 (55,4 %)	20 (24,1 %)	1,724	0,422	86 (51,8 %)	80 (48,2 %)	1,05 1	0,305
Total	35	92	32			156	162		

Table 2 shows the normal genotype (TT) was lower in the endometriosis case group than in control, heterozygous genotype (CT) was lower in the endometriosis case group than in control, while homozygous mutant genotype (CC) was found higher in the endometriosis case group than in control. T allele frequency in endometriosis case group was lower than in control group, while the C allele was higher in the endometriosis case group compared to control. The results of the Pearson Chi-square test demonstrated that the distribution of genotypes at endometriosis and control groups showed no significant difference ($p = 0.422$). Likewise with T and C allele frequency, also showed no significant difference ($p = 0.305$). The comparison of genotype distribution and allele frequency REβ with restriction enzyme AluI were shown on Table 3.

Table 3. Genotype and Allele Frequency of RE β Gene with AluI Restriction Enzyme on Cases and Control Group.

	Genotype			X ²	P OR	Allele Frequency		X ²	P	OR (IK=9 5%)
	GG	GA	AA			G	A			
Control	67 (88, 2%)	8 (10, 5%)	1 (1,3 %)			142 (93,4 %)	10 (6,6 %)			
Endometri- osis Case	60 (72, 3%)	23 (27, 7%)	0 (0,0 %)	8,35	0,015 0,31	143 (86,1 %)	23 (13, 9%)	4, 5 1 7	0,0 34	0,44
Total	127	31	1			287	31			

Table 3 shows that the normal genotype (GG) was lower in the endometriosis case group than in controls, heterozygous genotype (GA) was higher in the endometriosis case group than in control, whereas homozygous mutant genotype (AA) was not found in the group of endometriosis case but it found one in the control group. G allele frequency in endometriosis case group was lower than in control group, while the A allele frequency was higher in the endometriosis case group compared to control group.

Pearson Chi-square test showed the distribution of genotypes in endometriosis case and control groups was significantly different ($p = 0.015$), as well as the frequency of allele G and A ($p = 0.034$).

DISCUSSION

Gene Polymorphism Analysis RE α rs9340799 A / G, Gen RE α rs2234693 T / C, and Gen RE β rs4986938 G / A

Amplification of the RE α gene rs9340799 changes in nucleotide A to G using restriction enzymes XbaI in region intron 1 were reported by Paskulin *et al.*¹¹ Study from NCBI showed that individuals with genotype AA normal is an individual with a lower risk of cases of endometriosis, whereas individuals with genotype GG has 10 times higher against the risk of endometriosis. The results in this experiment found the proportion of genotype GA in the case of endometriosis was more dominant than the other genotypes. This study revealed that the genotype frequencies in control and endometriosis groups showed no difference.

Amplification of the RE α gene region intron 1 SNP rs2234693 T nucleotide changes into C using the restriction enzyme PvuII as reported by Paskulin *et al.*¹¹ The second difference in allele frequency in both groups (endometriosis and control) conducted chi-square test. It can be concluded that the genotype frequency in endometriosis and control groups were no difference, as well as statistical tests that appeared no difference in allele frequency between the two groups of the endometriosis and the control with $p = 0.422$ ($p > 0.05$). Hardy-Weinberg balance test, also showed that there were no differences with p values > 0.05 . It showed the genotype frequencies no difference between endometriosis and control groups.

The other study reported by Li *et al.*. They found that RE α gene rs2234693 with PvuII enzyme associated with a decreased risk of male infertility, a change in the function of the estrogen receptor (ER) in the subject of polymorphism, in general, has not comprehensible.¹³

Amplification of RE β gene region of exon 8 rs4986938 nucleotide changes G / A with restriction enzymes AluI, were done by Paskulin *et al.*¹¹ Result of statistical test that the frequency of genotypes at RE β showed significant differences in case of endometriosis and control groups, with $p = 0.015$ ($p < 0.05$).

The distribution of alleles in RE β showed no significant differences between the normal allele G and mutant allele A in the endometriosis case compared to control group. It means that normal allele G can reduce the risk by 0.44 times against cases of endometriosis. Distribution of normal allele G seemed to dominate than others allele. This is similar to a study conducted by Zulli et al.¹⁴ The presence of a significant difference genotype frequency and distribution of alleles in this study can be concluded that the gene polymorphism RE β with rs9340799 region of exon 8 was possibly contribute to endometriosis, due to mutations in this area can affect the composition changes of amino acids which allows no finding of a stop codon. Thus, the transcription keep working that causing the increased gene expression. The possible mutations are deletions, which may cause frameshift errors.

CONCLUSION

Polymorphism genotyping and gene alotip RE β rs4986938 G/A and allele frequencies RE α gene rs9340799 A/G may contribute to the risk of endometriosis occurrence.

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