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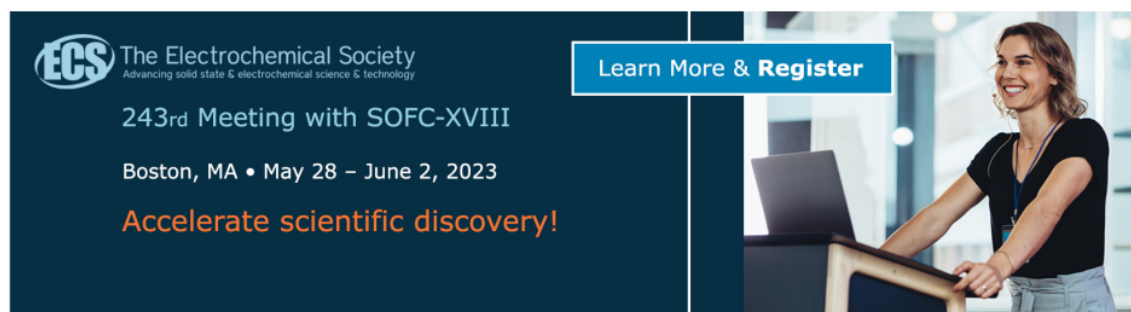
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Estrogen Receptor Alpha (ERα) *Pvu* II 397 T/C Related Genotypes and Alleles are Associated with Higher Susceptibilities of Endometriosis

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Abstract. Endometriosis is an estrogen dependent disease that is proved by its development in reproductive age and the decrease after menopause or ovariectomy. Endometriosis characterized by the growth of endometrial outside the uterine cavity, which is found in women with subfertility and pelvic pain problems. Effect of the estrogen hormone depends on the binding to its receptor. Estrogen receptor alpha (ERα) polymorphisms which admit by *Pvu II* enzyme restriction has associate to some women's disease like endometriosis. This Study aimed to determine the correlation between ERα 397 T/C with the risk of endometriosis. There were 70 samples which were divided into 2 groups: 35 cases (endometriosis group) and 35 controls (non-endometriosis group). The determination of genotypes and allotypes used PCR-RFLP method and the data analysis by *chi* square test $\alpha < 0,05$. The results showed that genotype frequencies of case group are 11,4% TT, 54,3% TC and 12% CC while genotype of control group are 34,3% TT, 51,4% TC and 14,3% CC with p value 0,032. Allele frequencies of case group are 38,6% T and 61,4% C while control group are 60% T and 40% C with p value 0,011. Conclusion: there is a significant correlation between the genotype and allele polymorphism of ERα gene 397 T/C with the risk of endometriosis.

Key Word: Polymorphism, ERα, *Pvu II*, Endometriosis

1. Introduction

Endometriosis is an endocrine disease affecting an estimated 89 million women and girls worldwide, as young as eight years to post menopause [1]. Endometriosis is a chronic, benign, oestrogen-dependent inflammatory disease affecting approximately 10% of reproductive age women and 35–50% of women with pelvic pain and infertility and causing 20% of all operation in gynaecology [2, 3]. The disease has been defined as misplaced endometrial tissue, the terms endometriotic tissue and endometriosis refer to the pathological state in which ectopic endometrium-like tissues grow within the pelvic peritoneum or ovaries [1, 4].

The presence of endometriosis is caused of multifactor such as anatomic, immunologic, hormonal and genetic factors [5]. Endometriosis is an estrogen-dependent disease [4]. Estrogen plays a key role

in its development and progression [6]. Estrogen is mainly produced in the ovaries and regulates the growth of endometrial tissue, basically by stimulating proliferation [7].

Estrogens play a fundamental role in the physiology of the reproductive system [8]. The principle is, 17β-estradiol (E2), synthesized by testosterone aromatization in the ovary and in other tissues, plays a central role in the control of sexual behavior and reproductive functions. E2 regulates human physiology *via* diffusion through the plasma membrane of target cells and signaling through intracellular hormone specific estrogen receptors (ERs) [9].

Estrogen receptor is a member of the nuclear receptor super family of ligand-activated transcription factors, which mediates estrogen actions in target tissues [10]. ERα resides on chromosome 6q25 and is comprised of 8 exons and 7 introns with a total size of 140 kilo bases [11].

Polymorphisms involved in steroid hormone biosynthesis and signaling may be useful genetic biomarkers for hormone-related diseases. Molecular geneticist are developing the third-generation human genome map with single nucleotide polymorphisms (SNPs) [10]. SNPs in promoter regions can result in reduced or increased gene expression, whereas SNPs in introns can result in defective splicing or a change in transcription rate if a regulatory element is mutated [12]. One of the best characterized SNPs of ERα is the c454-397T/C site polymorphism, which located in the first intron. This polymorphisms is 397bp upstream of exon 2 and have been described by the name of detecting restriction enzyme, *Pvu II* or its reference ID numbers rs2234693 [13].

2. Method

The population in this study was female patients who had come to Obstetrics and Gynecology Polyclinic and who were hospitalized in Obstetrics and Gynecology of Dr. Mohammad Hoesin Hospital Palembang in 2014, which the samples patient's blood has been collected from the laboratory of Microbiology of Dr. Mohammad Hoesin Hospital, Palembang. The samples were 35 cases and 35 controls. The samples were the patients who meet the inclusion criteria.
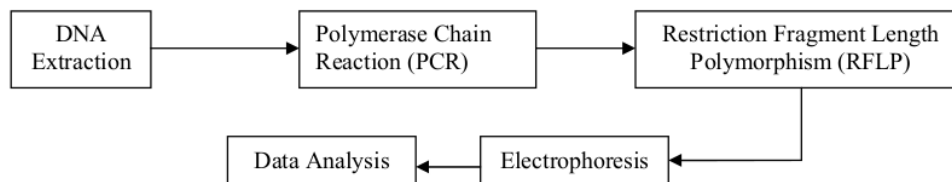
Figure 1. Method

Amount of 200 µl of blood were inserted into a 1.5 ml sterile tube, dissolved with 1000 µl of PBS pH 7.4, then centrifuged at 5000 rpm for 5 min, repeated 2-3 times. Furthermore, the supernatant was removed, and then added 500 µl saponin 0.5%, mix it using the vortex tools and then it was incubated in the -20°C at refrigerator for 24 hours. Repeat the vortex process to melt immediately and then centrifuged at 12000 rpm for 10 minutes, afterwards the supernatant is removed. Add 1000µL of PBS, centrifuged it at 5000 rpm for 10 minutes, and then supernatant removed, repeated it twice until the supernatant becomes clear. The supernatant are removed and add 50 µl of *Chelex* and add 100µL of ddH$_2$O, incubate it for 5 minutes then do the vortex process again. Centrifuged at 1000 rpm for 1 minute and then incubate again in boiling water for 10 minutes. Centrifuged at 12000 rpm for 10 minutes and the DNA will be in the supernatant section (DNA Containing water). Then this part/section is moved in a sterile tube and stored at -20^{0}C.

PCR methods with 3 stages are: denaturation, annealing, and extension. In this research, the assessment of polymorphism of ERα 397 T/C gene with forward primer: 5'-CTGCCACCCTATCTG TATCTTTTCCTATTCTCC-3' and reverse primer 5'-TCTTTCTCTGCCACCCTGGCGTCGATT ATCTGA-3'. For PCR stage, mix 9 µl of ddH2O, 10 µl of Green go Taq, 0.5 µl of Primer Forward, 0.5 µl of Reverse Primer and 5 µl of DNA. Amplify by PCR method, which was performed on the DNA

Thermal Cycle with brand BIO-RAD *Icycler* laboratories GB, started with the initial denaturation stage at 95°C for 5 minutes, then amplification for 30 cycles at 94°C for 1 minute, annealing at 62°C (replaced it to be 60°C according to yield Optimization of T annealing) for 1 minute and extension at 72°C for 1 minute then followed by final extension at 72°C for 6 minutes

Polymorphism of ERα 397 T/C gene was analyzed by RFLP method using *Pvu II* enzyme. The RFLP process uses mixing of 2.6 µl of ddH2O, 1.2 µl of Buffer, 0.2 µ of *Pvu II* enzyme, and 8 µl of amplicon. Hereinafter done the vortex process for a few second, and incubated in a waterbath at 37°C for 1 hour. After being digested by the *Pvu II* enzyme, the product was electrophoresed on 2% of agarose gel and can be observed with Ethidium Bromide (EtBr) staining.

Amount of 1 gram of agarose are weighted and put into a glass Erlenmeyer. Add 50 ml of TAE buffer. Mixed and heated it in microwave for 1 minute 30 seconds. Then add 3.5 µl of ethidium bromide, and chill it in mold for 30 minutes. The mixture of loading dye and DNA ladder is used as a marker. The 12 µl RFLP product and the marker were inserted into the agarose wells then inserted them into electrophoresis tool. The tool is set to 80 Volt and 400 ampere for 25 minutes and then visualized using Gel-Doc, manufactured by BIO-RAD Laboratories, USA, which is connected to the computer using "Quantity One" software program.

All data were analyzed using SPSS 24.00 program for windows to assess the distribution of genotype frequency and allele of ERα 397 T/C gene in case group and control group. To determine the relationship of ERα 397 T/C gene polymorphism with endometriosis that using Chi-Square test. Odd Ratio (OR) with 95% confidence limits and $\alpha = 0.05$.

3. Result and Discussion

3.1. Characteristic

Figure 2 showed that the mean age in the endometriosis group was 34.46 ± 5.57 and the mean age in the non-endometriosis group was 30.29 ± 5.64.

Figure 2. Age

From the data of respondent that based on the characteristic of age, there was a slight difference in the mean age of the endometriosis group and the non-endometriosis group, but it can be concluded that both of groups were at the same age because of their reproductive age (active menstrual cycle phase) and no menopausal respondents in both of groups. Endometriosis occurs almost exclusively in menstruating women of reproductive age [14]. This desease often begins in adolescence, but is most often recognized after years of dysmenorrhea [15].

In figure 3, the history for using of hormonal contraception, in the endometriosis group there were 9 respondents (25.7%) that using the hormonal contraception and those who did not use the hormonal contraception were 26 respondents (74.3%). While in the non-endometriosis group, there were 6 respondents (12.1%) that using the hormonal contraception and who did not use the hormonal contraception were 29 respondents (82.9%).
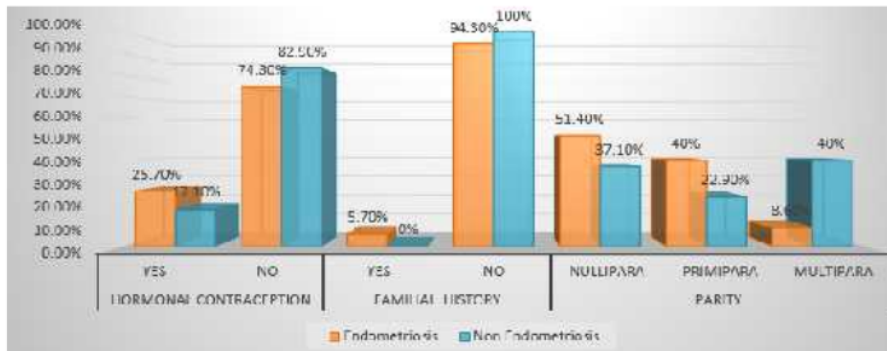
Figure 3. Characteristic of hormonal contraception, familial history and parity

Based on the characteristics of hormonal contraceptive use, most respondents (78.6%) did not use hormonal contraceptives. The use of combination of oral contraceptives estrogen and progestin is considered to be a preliminary treatment for pelvic pain associated with endometriosis. Study by Harada et al. randomly assigned 100 women with chronic pelvic pain secondary to endometriosis to therapy with a low-dose oral contraceptive or placebo cyclically for 4 cycles. There was significant relief of dysmenorrhoea with the oral contraceptives compared with placebo but no difference in relief of non-menstrual pelvic pain [16]. Suppression of the estrogen level by danazol or gonadotrophine releasing-hormone (GnRH) agonists provides the regression of endometriotic lesion [17]. Estrogen can stimulate the growth of endometriosis, but in oral contraceptives it contains estrogen and progestin, the progestin itself has been used to manage the reduction of chronic pain in patients with endometriosis. In exacerbate endometriosis case, medical interventions that inhibit estrogen production or action, i.e. down-regulating doses of gonadotropin-releasing hormone (GnRH) analogues, progestins, androgens and anti-estrogens ameliorate its symptoms [18].

Characteristics based on family history, in the endometriosis group was found in 2 respondents (5.7%) who had a family history/relatives who also suffered from endometriosis and 33 respondents (94.3%) had no family history of endometriosis. In the non-endometriosis group, there are no one (0%) of respondents who have a family history of endometriosis also means that all (100%) had no family history of endometriosis.

Observed from a family history of endometriosis, only 5.7% of patients had a family history of endometriosis. In contrast to the research of Renner et al., at 2006, where show a tendency of genetic predisposition in patients with endometriosis [12]. Although the analysis of this study is not related, this study still shows a family history of endometriosis will increase the risk 2.061 fold for exposed to endometriosis. A genetic basis for the development of endometriosis is suggested by the reports of familial aggregation, the high risk of endometriosis in those with an affected first degree relative, and the observations of concordance of endometriosis in twins [2].

Based on the parity status, the frequency in the endometriosis group was 18 nulliparous (51.4%) or had never had children, 14 primiparous (40%) or newly had 1 child and 3 multiparous (8.6%) or had more than one child. While in the non-endometriosis group there were 13 nulliparous (37.1%), 8 primiparous (22.9%) and 14 multiparous (40%).

Parity status of respondents showed a significant association with the incidence of endometriosis. This is in line with the study by Uimari (2018) which states that endometriosis and fibroids proved to be independent factors associated with nulliparity [19]. Increased parity and prolonged or irregular menses decrease the likelihood of the disease, whereas nulliparity, subfertility, and prolonged intervals since pregnancy are all associated with an increased risk of endometriosis [16].

3.2. *ERα 397 T/C Gene Polymorphism*

The result of PCR product, in the form of amplicon, was evaluated to prove the successful of DNA extraction that has been done. The evaluation process was carried out by electrophoresis and showed an ERα gene of 397 T/C at 1374 bp. Polymorphism of ERα 397 T/C gene was analyzed by RFLP method using *Pvu II* enzyme. The ERα 397 T/C gene polymorphism was visualized using ultraviolet light, visualized into 3 genotype variations i.e. TT genotype yielded 1 band of 1374 bp, TC genotype yielded 3 bands (1374 bp, 937 bp and 437 bp) and CC genotype yielded 2 cuttings of bands (937 bp and 437 bp) as shown in figure 4.
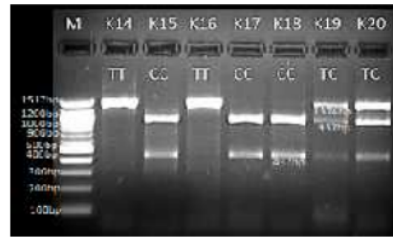
Figure 4. Genotype visualization for the result of PCR-RFLP result of ERα 397 T/C gene

3.3. *Genotype Distribution of ERα 397 T/C Gene*

All research subjects had undergone DNA isolation, PCR, RFLP, and it would get the distribution of ERα 397 T/C gene genotypes. From figure 5 the genotype distribution in the endometriosis cases group given TT (wild type) were 4 respondents (11.4%), TC or heterozygote mutant were 19 respondents (54.3%) and mutant homozygous CC were 12 respondents (34.3%), While for control group, TT genotype were 12 respondents (34.3%), TC were 18 respondents (51.4%) and CC were 5 respondents (14.3%).
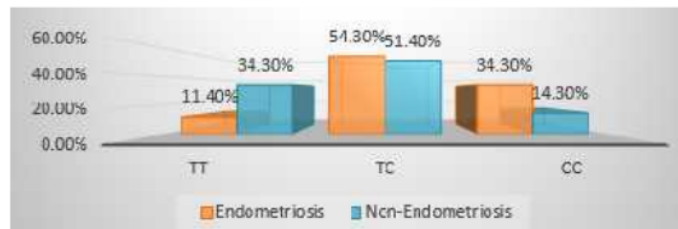
Figure 5. Genotype Distribution ERα 397 T/C gene

Based on the Chi-Square analysis test in table 1, the results obtained *p* value were 0.023, Odds Ratio (OR) were 4.043 and 95% Confidence Interval with upper border at 14.164 and lower border at 1.154, which means there are any relationship between ERα 397 T/C gene polymorphism with the incidence of endometriosis.
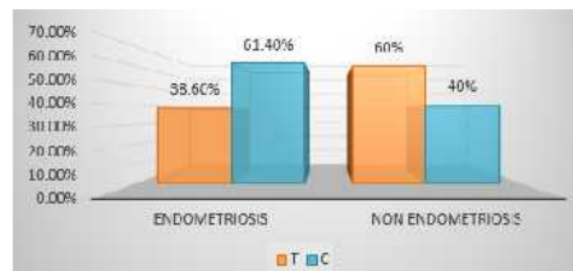
Table 1. Correlation Analysis of ERα 397 T/C Genotype Gene Polymorphism

Genotype gene	Endometriosis %	Non endometriosis %	OR (95%CI)	p value
TC+CC	31(88.6)	23(65.7)	4.043	
TT	4(11.4)	12(34.3)	(1.154-14.164)	0.023[a]
Total	35(100)	35(100)		

[a]*Chi*-Square test p<0.05

Based on odd ratio values, it shows that individuals who have mutations in their genotype have 4,034 times greater risk of endometriosis than individuals who do not have mutations. However, a fairly wide CI range (1,154-14,164) suggests that the distribution of samples is still inaccurate, this may be due to differences in variation or interactions between genes or there are other factors that influence, because endometriosis is also a multifactorial disease.

As shown in figure 6, frequency of allele distribution showed that in endometriosis group occurred 27 T allele (38.6%) and 43 C allele (61.4%). And in non-endometriosis group occurred 42 T allele (60%) and 28 C allele (40%).

Figure 6. Allele Distribution of ERα 397 T/C Gene

In table 2, based on the Chi-Square analysis test, the results obtained p value were 0.011, Odd Ratio (OR) were 2.389 and 95% Confidence Interval with upper border at 4.708 and lower border at 1.212, which means there are any relationship between the ERα 397 T/C gene polymorphism with the incidence of endometriosis.

Table 2. Correlation Analysis of ERα 397 T/C Allotype Gene Polymorphism

Allele Gene	Endometriosis %	Non Endometriosis %	OR (95% CI)	p value
C	43(61.4)	28(40)	2.389	
T	27(38.6)	42(60)	1.212-4.708	0.011[a]
Total	70(100)	70(100)		

Chi-Square test p<0.05

Based on the odd ratio value, it shows that the mutant allele (C) has a risk factor of 2.389 times greater than the wild type allele (T) for endometriosis.

This research shows that most of the ERα genes were undergoing heterozygous and homozygous mutations, and only a few the ERα genes are not mutated (wild type). While on the control group, many of genes are non-mutated (wild type), and many mutated of heterozygote mutants and fewer mutated of homozygous mutants. Based on the statistical calculation with *chi square* test, it was concluded that the polymorphism of ERα *Pvu II* 397 T/C gene had a significant influence on endometriosis disease. This is in line with Georgiou's research, et al (1999) in Greece, Kitawaki, et al (2001) in Japan, Hsieh, et al (2007) in Taiwan and Govindan, et al (2009) in India but not in line with Wang's Et al., research (2004) in Japan and Renner, et al., (2009) in Germany [10, 12, 17, 21, 22]. The relationship of the ERα gene polymorphism with the occurrence of endometriosis still shows controversial results that are influenced by various factors such as the number and selection of control groups, but most occur in women in Asia who may be affected by ethnic or racial factors.

4. Conclusion

From this experiment, we can conclude that there are relationship between genotypes and alleles polymorphism of estrogen receptor alpha (ERα) *Pvu II* 397 T/C gene with higher susceptibilities of endometriosis, with p = 0.032 and p = 0.011, respectively.

Ackowledgement

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