



## Polymorphism for Interleukin-4 (-590 C/T) Promoter: Non-Association with Endometriosis

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### Article Info

#### Article history:

Accepted February, 2022

Revised February, 2022

Received 24 Feb 2022

#### Keywords:

Polymorphism

Interleukin-4 gene (-590T/C)

endometriosis.

### ABSTRACT

Endometriosis is a benign gynecological disorder that often occurs in women of reproductive age, usually characterized by endometrial epithelium and stroma outside their usual location. It is estimated that this disease affects 56% of women worldwide. Various types of cytokines are associated with endometriosis, including interleukin IL-4, which inhibits the production of proinflammatory cytokines that stimulate active B cells and T cell proliferation, the differentiation of B cells into plasma cells. IL-4 promoter region polymorphism (-590C/T) is involved in genetic susceptibility to endometriosis. This study aims to determine whether Gen IL-4 (-590C/T) polymorphism indicates endometriosis. This study is an analytical observational study conducted at the Laboratory of Molecular Biology in Palembang City in August 2021, with the approach of Case-Control study. There were 70 samples divided into two groups, namely 35 cases and 35 controls. Determination of genotypes and alleles using PCR-RFLP and chi-square analyzed data to determine the relationship. The results showed no significant difference. The genomorphism of the Interleukin-4(-590C/T) gene cannot be used to indicate endometriosis susceptibility.

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## 1. INTRODUCTION

Endometriosis, a complex disease, is associated with immunological and genetic changes. Many cytokines are associated with endometriosis, including interleukin IL-4(1). IL-4 is a cytokine with anti-inflammatory properties that inhibits the production of proinflammatory cytokines such as IL-1, IL-6, and TNF(2). Some of the causative factors that endometriosis occurs are in women with a long and long history of menstruation, women who experience menarche at an early age, women with Muller duct disorder (3,4). Pathological endometriosis can cause infertility up to 6-10%. It is estimated that the disease affects 176 million women worldwide( 4). The incidence of endometriosis is often found in Indonesian society. It is a progressive gynecology disease, but its prevalence and incidence in the general population are unknown.

At the molecular level, endometriosis tends to decrease the inhibitory activity of the cell cycle (5,6)." Some evidence also supports the role of cellular and humoral immunity in the pathogenesis of endometriosis and endometriosis-related infertility on the ability of T cells and cytotoxic. Natural killer (NK) cells recognize and lyse endometrial cells essential for the pathogenesis of endometriosis. NK cells recognize particular HLA class 1 molecules that inhibit killing the function of these cells (6-8).

Immune factors, along with endocrine factors, are involved in the development of endometriosis(5). Cytokines are messenger proteins between cells in the immune system to communicate; one cytokinin is the secreted IL-4 of cell Th2, a subset of T helper IL-4 receptor lymphocytes. The experiment results obtained the presence of IL-4 natural stromal endometrial cells(5). The Th2 cells present in endometriosis tissue in significantly higher amounts than in eutopic endometrial tissue. IL-4 stimulates the proliferation of endometrial stromal cells and the secretion of eotaxin from endometrial stromal cells (2,9,10).

## 2. RESEARCH METHOD

This study is an analytical observational study with a case-control design that aims to determine whether genetic risk factors influence the occurrence of endometriosis. The study used blood samples of patients diagnosed with endometriosis and those who were not. The study sample of 70 women was divided into 35 case groups of 35 control groups, met by consecutive sampling techniques.

**DNA extraction**, takes 200 µl of blood inserted into a sterile 1.5 ml tube, Washed with a PBS pH of 7.4 as much as 1000 µL, then dysentery at a speed of 5,000 rpm for 5 minutes, the supernatant discarded. This stage is repeated 2-3 times. The supernatant is discarded, then added 500 µL of saponins 0.5%, mixed well-using vortex. Incubation for 24 hours in the refrigerator -20oC. Then the vortex returns to melt immediately, then centrifuge at a speed of 12,000 rpm for 10 minutes. Supernatant discarded, Add PBS 1000 µL, Centrifuge at a rate of 5000 rpm for 10 minutes, discard the supernatant repeated 2x until the supernatant clear. The supernatant is discarded, plus 50 µl Chelex and 100 µl ddH<sub>2</sub>O, Incewed in boiling water (using a heat-lock tool) for 5 minutes in the vortex. Centrifuge at 1000 rpm for 1 minute, Inc increased in boiling water for 10 minutes, Centrifuge at 12000 rpm for 10 minutes. DNA will be in the supernatant (DNA containing water). Then this part is moved in a sterile tube and stored at a temperature of -200C.

**Polymerase Chain Reaction (PCR)**, The working principle of PCR goes through 3 stages, namely denaturation, annealing, and extension. In this study, interleukin gene polymorphisms -4 (-590C/T) with primary forward: **5'-TAA ACT TGG GAG AAC ATG GT-3'** and reverse primary: **5'- TGG GGA AAG ATA GAG TAA TA-3'**Mix can ddH<sub>2</sub>O 9µl, primary forward and primary reverse by 0.5 µl, Go Tag Green 10µl, and DNA samples 5µl. Amplification with PCR method is carried out on DNA Thermal cycle brand Icyler BIO-RAD Laboratories GB that has been programmed, namely: Denaturation of the initial step at 94°C for 5 minutes 36 amplification cycles at 94°C for 1 minute, 48°C for 1 minute and 72°C for 1 minute Last step extension at 72°C for 5 minutes (Kitawi, 2004).

**RFLP**, The Interleukin-4(-590C/T) gene polymorphism is determined by analysis of RFLP analysis using enzymes: The RFLP process uses a mixture of Ava II 0.5 µl, 1 µl buffer and 3.5 µl ddH<sub>2</sub>O into a drop tube containing 10 µl of PCR (DNA Teamplet) products, then a spin-down of a few seconds. Inc increased in a water bath at 37°C for 2 hours 30 minutes. After visualizing the enzyme Ava II, the PCR product is electrophoresis at 2% Agarose gel for 25 minutes.

**Electrophoresis**, Four grams of agarose are weighed and put in an Erlenmeyer glass. Added 100 ml of the buffer. Mixed and heated in the microwave for 1 minute 30 seconds. Then add 7 µl ethidium bromide to refrigerate in a mold for 30 minutes. 4 µl loading dye and 0.7 µl of DNA leader are mixed and used as markers. PCR 15 µl products and tags are inserted into the agarose well and then inserted into the electrophoresis device. The device is set at a voltage of 100mV, 400 amperes, for the next 25 minutes is visualized using Gel-Doc equipment made by BIO-RAD Laboratories USA connected to the computer using Quantity One.

The study involved 70 premenopausal women diagnosed with endometriosis and non-endometriosis. Patients are divided into two groups: 1) endometriosis (n = 35) and 2) non-endometriosis (n = 35). The ethics committee approved this study of the Palembang Ministry of Health No. 1270/KEPK/Adm2/VI/2021 Date 1 june 2021. Informed consent is signed by all women who donate their blood. There are 100 differences between the two groups: marital status and family history of endometriosis. All women access peripheral blood sampling for genotype analysis. This data was analyzed to determine the distribution, frequency of genotype, and alleles of the Interleukin-4 (-950C/T) gene in the endometriosis group and the non-endometriosis group the polymorphic distribution and relationship of interleukin-4 (-590C/T) genes with endometriosis events using the Chi-Square test.

## 3. RESULTS AND ANALYSIS

**Table 4.1. Characteristics Women**

Characteristics	Group	
	Endometriosis Case n = 35 (%)	Non endometriosis Control n=35(%)
<b>Married Status</b>		
<b>Marry</b>	<b>33 (94,2)</b>	<b>35 (100)</b>



<b>Not married</b>	2 (5,8)	0 (0)
<b>History of endometriosis in the family</b>		
<b>Exist</b>	2 (5,8)	0 (0)
<b>None</b>	33 (94,2)	35(100)
<b>History of Use of Hormonal Contraceptives</b>		
<b>Exist</b>	6 (17,1)	6 (17,1)
<b>None</b>	29 (82,9)	29 (82,9)

In table 4.1 above, it can see that in the endometriosis group there are 94.2% of respondents with a married status greater than those who are not married as many as 5.8%. The table also shows that in the endometriosis group of respondents who have a family history of endometriosis, as much as 5.8% smaller than respondents who have no family history of endometriosis as much as 94.2%. From table above, it can be seen that in the endometriosis group of respondents who have an account of hormonal contraceptive use, 17.1% smaller than respondents who have no history of hormonal contraceptive use as much as 82.9%.

### 3.1 Polymorphism of the Interleukin-4(-590C/T) gene

PCR products, in the form of amplicons, are evaluated to prove the success of DNA extraction that has been done. The evaluation process is carried out using electrophoresis through a 2% agarose gel medium containing ethidium bromide. The PCR cycle at a temperature at 94°C for 5 minutes 36 amplification cycles at 94°C for 1 minute, 48°C for 1 minute and 72°C for 1 minute Last step extension at 72°C for 5 minutes AMPLIFICATION PCR using primary: 5'-TAA ACT TGG GAG AAC ATG GT-3' and reverse primary: 5'- TGG GGA AAG ATA GAG TAA TA-3' Ava II enzyme mixture 0.5 µl, 1 µl buffer and 3.5 µl ddH<sub>2</sub>O into a *drop* tube containing 10 µl PCR products (DNA template), then spin down a few seconds. Inc increased in *the water bath* at 37°C for 2 hours 30 minutes. After inceased Interleukin-4 590 products C/T performed electrophoresis to scope 2% agarose containing ethidium bromide, genetic IL-4 -590 C/T produced pieces 195, 175, 20 and Homozygous T/T made fragments of 195 C/C did not divide and had fragments 172 and 20 whereas the heterozygous combination of all elements (195, 175, 20).

The results of electrophoresis visualization of interleukin-4 (-590C/T) gene PCR visualization results are seen in the 195 bp band position as in the figure below:

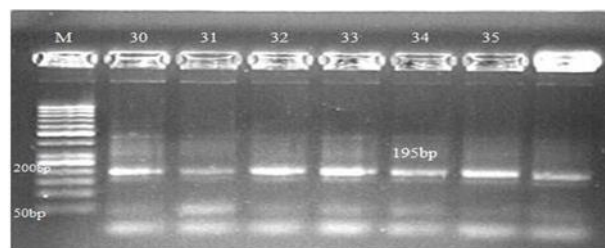


Figure 4.1 Electrophoresis visualization of the PCR results of the Interleukin-4 gene(-590C/T) in the case group is seen at the position of 195bp

Factors Interleukin-4 gene polymorphisms (-590C/T) will be visualized using ultraviolet light into three variations of genotypes, namely Figure 1 band, which is 195 bp, which means that the genotype is homozygous TT. Figure 3 bands, namely 195 bp, 175 bp, and 20 bp, show heterozygous CT genotype. Figure 2 band is 175bp, and 20 bp means it offers the CC genotype (Wild Type).

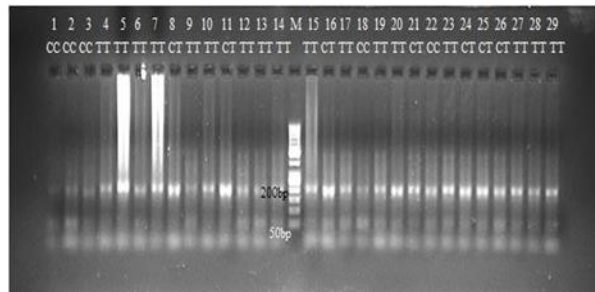


Figure 4.2 Polymorphism of the Interleukin -4(-590C/T) gene with the enzyme *Ava II* after RFLP, M= Marker DNA marker, case group. Genotype TT (195 bp), Genotype CT(195 bp, 175 bp, and 20 bp), Genotype CC (175 bp, and 20 bp).

**II-4 Genotype Distribution (-590C/T)**, All research subjects have undergone a DNA isolation process, PCR,RFLP, so that there will be a genotype distribution of il-4 -590 C/T C/G as shown in the table below:

**Table 4.2. Distribution Of Genotypes In The Endometriosis**

Genotype	Endometriosis	Not Endometriosis
	n %	n %
CC	7 (20)	10 (28,6)
CT	10 (28,6)	17 (48,6)
TT	18 (51,4)	8 (22,8)
<b>Total</b>	<b>35 (100)</b>	<b>35 (100)</b>

From Table 4.2, the distribution of genotypes in the endometriosis group was CC 7 (20%), CT genotype was 10 (28.6%), and TT genotype was 18 (51.4%). Meanwhile, for the non-endometriosis group, there were 10 CC genotypes (28.6%), 17 CT genotypes (48.6%), TT genotypes 8 (22.8%).

**Table 4.3 Frequency Distribution of II-4 Gene Alleles (-90C/T)**

Allele	Endometriosis		Not Endometriosis	
	n %	n %	n %	n %
C	24 (34,3)	16 (22,9)	49 (35)	
T	46 (65,7)	54 (77,1)	91 (65)	
<b>Total</b>	<b>70 (100)</b>	<b>70 (100)</b>	<b>140 (100)</b>	

The result of the frequency distribution of Table 4.3 obtained the number in the Endometriosis group there is an Allele C frequency of 24 (34.3%), Allele T as much as 46 (65.7%). As for the non-endometriosis group, allele C was 37 (52.9%), and Allele T by 33 (47.1%).

**Table 4.4 Analysis of Genotype relationships of Interleukin-4Gene Polymorphism (-590 C/T)**

Genotype Gen	Endometr iosis	Nonendometriosis	<i>p value</i>
	n %	n %	
CT/TT	28 (80)	25 (71,4)	0,403
CC	7 (20)	10 (28,6)	
<b>Total</b>	<b>35 (100)</b>	<b>35 (100)</b>	<b>Total</b>



The results of the frequency distribution of Table 4.4 obtained the number in the Endometriosis group there is a genotype frequency of CT / TT 28 (80%), CC genotype as much as 7 (20%). As for the non-endometriosis group, Genotype CT / TT results were as much as 25 (71.4%) genotype CC as much as 10 (28.6%). Analysis of the relationship of Genotype polymorphism CT / TT and CC Gen Interleukin-4 (-590C / T) with the incidence of Endometriosis using the *Chi-Square test* obtained a value of  $p = 0.403$  which means there is no relationship between polymorphism of the Interleukin-4 gene (-590C / T) with the incidence of Endometriosis.

### 3.2 Analysis

Endometriosis involves complex interactions between immuno-inflammatory processes, cytokine activation, and genetic factors(2). Cytokines are proteins that play a role in linking the immunological system and endometriosis tissue. IL-4, a 20-kd cytokine, acts as an autocrine growth factor for different T-helper cells(11). T-cell-mediated cytotoxicity is suppressed by IL-4(11). The increased IL-4 expression is previously noted in women with endometriosis (12,13). Previous studies found no link between endometriosis and IL-4 polymorphism. IL-4 polymorphism has been linked to various diseases or susceptibility, including rheumatoid arthritis (14), asthma, rhinitis, and atopic dermatitis: multiple sclerosis(15). The RP2 allele for IL-4 is a protective factor for joint destruction in patients with rheumatoid arthritis (2).

However, there have been some reports regarding the role of IL-4 in endometriosis. Odukoya et al. (15) showed that IL-4 mRNA is not expressed in ovarian endometriomas. In contrast, Nakayama et al. (16) indicated that the IL-4-589T increased IL-4 production. This study observed that the genotype and allelic frequency of promoter IL-4 and IL-4 polymorphism are not genetic risk factors that contribute to a woman's susceptibility to endometriosis. The T allele for IL-4 promoters was not associated with an increased risk of endometriosis. In previous studies, heterozygous (C/T) for promoters of IL-4-590 was associated with higher IL-4 activity and IgE secretion in asthma patients. Allele T for supporters of the IL-4 gene has been associated with an increased risk of atopic dermatitis compared to allele C. Reduced frequency of T alleles in IL-4-590 promoters has been associated with Graves' disease(2).

In contrast, other researchers have shown no association between IL-4 polymorphism and individual diseases, such as asthma, multiple sclerosis, myasthenia gravis, and minimally changeable nephropathy Roberts AK, Monzon-Bordonaba F, Van Deerlin PG, Holder J, MaconesGA, Morgan MA, Strauss 3rd JF, Parry S. Association of polymorphism within the promoter of the tumor necrosis factor-alpha gene with increased risk of preterm premature rupture of the fetal membranes. *Am J Obstet Gynecol* (17–19).

Some studies get endometriosis in many teenagers (20) age, and this will affect their quality of life. Factors that are at risk for endometriosis need to be carefully focused such as the linkage of activity factors associated with gadget addiction (21), drugs (22), and stressing factors caused by association such as bullying (23). Family as a support system must be the vanguard to detect this problem and provide information and take important steps if it happens(24).

### 4. CONCLUSION

In conclusion, the results of this study showed that there was no association between endometriosis and IL-4 polymorphism genes. The genotype and frequency allele promoter IL-4-590, promoters of polymorphism, are not helpful determinants for predicting endometriosis susceptibility. This stinginess can provide a database for further surveys of other cytokinins that determine a disease. However, the proper role of IL-4 polymorphism in endometriosis remains to be clarified. In addition, the effects of other cytokine polymorphisms on the development of endometriosis deserve further study.

### ACKNOWLEDGEMENTS (10 PT)

Our thanks to the participants, hospital, public health center, and the management of Politeknik Kesehatan Palembang, who have supported the implementation of this research.

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