Original Article

Study of association between promoter tumor necrosing factor alpha gene polymorphisms in -850T/C, -863 A/C, and endometriosis

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Abstract

Background: The purpose of this study was to determine whether variability in gene encoding for promoter of tumor necrosis factor participates to women differences in susceptibility to endometriosis.

Materials and Methods: The study involved 130 women; 65 endometriotic and 65 healthy control women. The blood samples were genotyped for -850 T/C and -863 C/A polymorphisms in TNF alpha gene promoter. Chi-square, odd ratio, and confidence interval 95% were used to evaluate genotypes and allele frequency differences between two groups.

Results: No significant differences in genotypes distribution of -850 T/C ($P = 0.32$) and 863 C/A ($P = 0.34$) polymorphisms were obtained between two groups.

Conclusion: According to this study, these two polymorphisms have no risk or protective factor to develop endometriosis.

Key Words: Endometriosis, polymorphism, tumor necrosis factor

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INTRODUCTION

Endometriosis is a common and complicated problem in women.[1,2] Presence and growth of endometrial cells outside the uterus cavity, is called endometriosis which impair fertility.[3] The incidence varies from 109 to 247 per 100,000.[3]

However, other epidemiological features of the disease are not clear, because of the need for surgical procedure to define and confirm the problem.[3] This disease is usually limited to pelvis.[1] The clinical manifestation of the disease includes pain, menstrual disorder, infertility, and mass. Some studies show changes in immunological status of patients with endometriosis.[4] Pathogenesis of this disease is not well understood, but there have been three hypotheses; First one retrograde menstruation or menstrual blood reflux through the fallopian tubes into the pelvic cavity, second one immunological factors (including

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reduced cellular immunity, decreased NK cells activity, and increased concentrations of leukocytes and macrophages and ultimately increased IL-6 and 8 and TNF in peritoneal fluid), and the last one genetic factors.[5,6]

Many genes, including detoxification enzyme genes, estrogen and progesterone receptors genes have been studied previously in association with endometriosis.[3,7]

TNF α is a cytokine that is produced by activated macrophages, it performs its function through the TNF receptor, including TNFR1 and TNFR2. The system of TNF and its receptors plays an important role in inflammation, angiogenesis, proliferation, and apoptosis. These are the key components of the pathophysiology of endometriosis. TNF is higher in the peritoneal fluid of patients with endometriosis in comparison to normal women.[7,8]

Among the three types of TNF, the active form in endometriosis is TNF-α. In human beings, the place of TNF-α gene is at position 31 and 21 6P, which is one of the 20 genes of the HLA system.[5,9]

Special single nucleotide polymorphism (SNP) such as -863 C/A and -850 T/C, which are located in promoter of TNF-α transcription site, have direct effect on TNF alpha production of this cytokine.

Polymorphisms affect the quality of the TNF-α regulation. Therefore, it can explain the susceptibility of patients to some diseases such as endometriosis.[3,10]

The aim of this study was to investigate the possible association of polymorphism of -863C/A and -850 C/T with endometriosis. This polymorphism is located in TNF-α transcription site.

MATERIALS AND METHODS

This was a case-control study which was carried out in AL Zahra and Beheshti hospitals which are affiliated to ISFAHAN university of medical sciences, Iran, in 2012.

This study was reviewed and approved by the ethical committee, and all subjects filled out informed consent form.

The study's population consisted of a group of women with endometriosis and a control group of women who were admitted at these centers for other reasons.

To be eligible for the study, women had to have endometriosis which was confirmed by laparoscopy and be in the stage II, III, or IV of endometriosis classification. Women with Myoma or any benign or malignant mass were excluded from the study. The specimens that were not enough, to confirm the disease were excluded.

The control group was women who were hospitalized to deliver a baby or the women had also undergone laparoscopy but the gynecologist and pathologist have not diagnosis for endometriosis or endometriosis was not confirmed for them. The case and control groups were matched for age (in case group 30.53 ± 7.18, in control group 29.04 ± 7.25 and $P = 0.145$), and sample size was calculated using the proportion comparison formula, with considering the confidence coefficient of 95%, statistical power of 80%. The estimated prevalence of polymorphisms in the -850 C/T was about 1%, and effect size 0.1; therefore, 65 subjects per group were enrolled in the study.

-850 T/C

DNA was isolated from peripheral white blood cells by using the PrimePrep Genomic DNA Isolation kit from Blood (GeNet Bio; Korea). Genotyping was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. PCR amplification was performed in a volume of 250 μL for each sample. PCR reaction was done using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 10 p.M each primer (forward 5'-AAGTCGAGTATGGGGACCCCCCGTTAA -3' and reverse 5'- CCCCAGTGTGGCCATATCTTCTT -3'), one unit Taq polymerase, and 50 ng of genomic DNA. PCR conditions included a denaturation at 96˚C for 5 minutes followed by 35 cycles of 94˚C for 30 seconds, 55˚C for 1 minute, and 72˚C for 2 minutes, and a final extension at 72˚C for 2 minutes. PCR products digested using 1 μL of *HincII* restriction enzyme for each sample in a volume of 10 μL. The TNFalpha -850 T/C polymorphism was detected after separation of enzyme-treated PCR products on a 2% agarose gel and followed by GelRed™ staining. Samples with C allele at position -850 HincII digestion produce 105 and 23 bp fragments. And if the 128 bp frgment remains undigested, there was the location of T allele.

-863C/A

DNA was isolated from peripheral white blood cells by using the PrimePrep Genomic DNA Isolation kit from Blood (GeNet Bio; Korea). Genotyping was performed by PCR-restriction fragment length polymorphism analysis. PCR amplification was performed in a volume of 25 μL for each sample. PCR reaction was done using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 10 p.M each primer (forward 5'- GGCTCTGAGGAATGGGTTAC and reverse 5'-

CCTCTACATGGCCCTGTCTTCACTAAG -3'), one unit Taq polymerase, and 50 ng of genomic DNA. PCR conditions included a denaturation at 96˚C for 5 minutes followed by 35 cycles of 94˚C for 30 seconds, 59˚C for 1 minute, and 72˚C for 2 minutes and a final extension at 72˚C for 2 minutes. PCR products were digested using 1 μL of *StyI* restriction enzyme for each sample in a volume of 10 μL. The TNF alpha -863C/A polymorphism was detected after separation of enzyme-treated PCR products on a 2% agarose gel and followed by GelRed™ staining. Samples with C allele at position -863 StyI digestion produce 108 and 25 bp fragments. And if the 133 bp fragment remains undigested, there was the location of A allele.

Data analysis was performed using SPSS (20) statistical package, descriptive statistics, X^2 was used and, OR and CI 95% was estimated. Two-tailed *P* values less than 0.05 were considered significant.

RESULT

In a case-control study, 130 subjects were compared in terms of polymorphism of - 850T/C and -863C/A.

29 (22.3%), 31 (23.8%), and 5 (3.8%) of the patients were in the second, third, and fourth stage of the disease, respectively [Table 1].

Totally in both case and control groups, the homozygous TT genotype was seen in 5 (3.8%), TC in 2 (1.5%), and CC in 123 (94. %) in blood samples in – 850 T/C [Table 1].

The -850 CC genotype was more frequent in the endometriosis group in comparison to the control group (92.3% *vs* 96.9%), but the TT and TC - 850 genotypes were more prevalent in the control group.

There was not any observation of the TC -850 genotype in endometriosis group.

Statistically, there was not any difference between two groups based on -850 genotypes. [x^2 = 2.27, df = 2, *P* = 0.32].

In terms of the -863 genotype, 13 (10.1%) had AA, 97 (74.6%) had CA, and 20 (15.4%) had CC genotypes.

The difference between case and control group was not significant. [x^2 = 2.1, DF = 2, *P* = 0.34].

The frequency of different genotypes according to the stage of the disease is demonstrated in Table 2.

The risk of endometriosis was estimated with considering the different genotypes. The results are shown in Table 3.

DISCUSSION

TNFα (Tumor necrotizing factor) is a pro-inflammatory cytokine that is involved in many infectious and inflammatory processes. Two forms of TNF have been recognized, TNFα and TNF β.

TNFα is produced during inflammatory processes.[11]

Several studies have suggested that TNFα plays an important role in the pathogenesis of inflammatory diseases, for example SLE, Crohn, Diabetes.[12,13]

Inflammatory nature of the endometriosis is characterized by different investigations.[2]

Also, some studies had shown the association between TNF and endometriosis.[14]

Table 1: Genotype distribution of single nucleotide polymorphism in the case and control groups

Genotype	Group (%)	
	Control	Case
Genotype 863		
AA	7 (10.8)	6 (9.2)
CA	51 (78.5)	46 (70.8)
CC	7 (10.8)	13 (20)
Genotype 850		
TT	3 (4.6)	2 (3.1)
TC	2 (3.2)	0 (0)
CC	60 (92.3)	63 (96.9)

Table 2: Genotype distribution of TNF single nucleotide polymorphisms based on endometriosis stages

Genotype	Stage IV (10)	Stage III (31)	Stage II (29)	Control (65)	Total (130)	Test
Genotype 850						
TT	0	2	0	3	5	X^2=2.2
CC	5	29	29	60	123	Df=2
TC	0	0	0	2	2	Pv=0.32
Genotype 863						
AA	1	2	3	7	13	X^2=4.08
CA	4	20	22	51	97	Df=4
CC	0	9	4	7	20	Pv=0.3

TFN: Tumor Necrosis factor alpha, Df: Degree of freedom, Pv: *P* value

Table 3: Odds ratio and 95% confidence interval for endometriosis with regards to genotypes

	OR	CI 95%
-863 A/C AA/CA	1.05	0.3-3.3
AA/CC	2.1	0.5-9
-850 T/C CT/CC	2	0.7-5
TT/CC	1.5	0.25-0.97

CI: Confidence interval, OR: Odd ratio

Our study evaluates the promoter TNFα, -C850T and -C863T polymorphisms in a number of Iranian women with endometriosis.

Our data showed an increase in the frequency of the Genotype -850 (CC) and Genotype -863 (CA) in the patients with endometriosis which was statistically non-significant. Also, there were not any association between the stage of endometriosis and different types of genotypes. None of the genotypes and alleles was a risk factor for developing endometriosis.

Other similar studies in different countries did not show any association between TNFα polymorphism and endometriosis.[15]

But in a study which was performed on Indian women, significant increase of endometriosis in women which was carrying -850 TT genotype was seen. The TT genotype increases the risk of endometriosis by fourfold.[16]

In Japanese population, carrying TNF α –T1031C polymorphism was associated to decreased risk of endometriosis.[16] But when the endometriosis group was divided into a subgroup of women with stage IV, the frequency of -1031C allele was significantly lower than control in this subgroup.[17]

In Korean women, association between TNF α gene polymorphisms with advanced.

Stage endometriosis was seen.[14] This study demonstrated that the TNF: g.- 1031CC homozygote may be associated with advanced stage endometriosis and that the TNF: g.-863CC homozygote has a protective role in the pathogenesis of endometriosis.[14]

Our assumption, according to Hardy-Weinberge equilibrium was that allele and genotype frequencies in each population, ideally, are constant among generations in the absence of other evolutionary influences. These influences include *non-random* mating, mutation, selection, genetic drift, gene flow, and meiotic drive. Because one or more of these influences are typically present in real populations, the Hardy-Weinberg principle describes an ideal condition.

The seven assumptions underlying Hardy-Weinberg equilibrium are as follows: Organisms are diploid, only sexual reproduction occurs, generations are non overlapping, mating is random, population size is infinitely large, allele frequencies are equal in the sexes, and there is no migration, mutation, or selection.

Violations of the Hardy-Weinberg assumptions can cause deviations from expectation. How this affects the population depends on the assumptions that are violated or the disease is not genetic dependent.[18]

In our study, the genotype distribution in endometriotic group and control groups have no significant deviation from Hardy- Weinberg principle (-850 X2 = 2.3 df = 2, $P = 0.101$, -863 X2 = 2.4 df = 3 $P = 0.105$). And this was not our expectation from a disease- associated gene.

In conclusion, this study demonstrates that the polymorphisms in -850 T/C and -863 TNFα may not be associated with endometriosis.

Additional studies should be implemented on a larger population to elucidate the actual value of the polymorphic markers of the different positions of TNF promoter region in determining the genetic susceptibility to endometriosis more.

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