Polymorphisms for Interleukin-4 (IL-4) –590 Promoter, IL-4 Intron3, and Tumor Necrosis Factor Alpha –308 Promoter: Non-Association With Endometriosis

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Interleukin-4 (IL-4) is a cytokine with anti-inflammatory properties. Tumor necrosis factor alpha (TNF- α), a pluripotent proinflammatory cytokine, plays an important role in the process of numerous inflammatory and autoimmune diseases. We aimed to investigate whether gene polymorphisms for the IL-4 –590 promoter, IL-4 intron3 and TNF α –308 promoter could be used as markers of susceptibility in endometriosis. The subjects, 226 premenopausal Taiwan Chinese women with surgically diagnosed endometriosis and nonendometriosis, were divided into two groups: 1) endometriosis (n = 120) and 2) nonendometriosis (n = 106). Polymorphisms for the IL-4 –590 promoter, IL-4 intron3, and TNF α –308 G-to-A promoter were detected by polymerase chain reaction (PCR). Genotypes and allelic frequencies for these gene polymorphisms in both groups were compared. We observed no significant differences in genotype distri-

bution and allele frequency of the IL-4 –590 promoter, IL-4 intron3, and TNF α gene polymorphism between both groups. The proportions of the –590* C homozygote/heterozygote/–590* T homozygote for the IL-4 promoter in both groups were 1.6/31.6/66.6% in group 1, and 5.8/33.0/61.2% in group 2. The proportions of the RP1 homozygote/heterozygote/RP2 homozygote for IL-4 intron3 in both groups were 62.5/34.1/2.5% (group 1), and 64.1/32.0/3.9% (group 2). The proportions of –308* A homozygote/heterozygote/–308* G homozygote for the TNF α promoter in both groups were 7.5/20.8/71.7% (group 1), and 7.5/17/75.5% (group 2). We concluded that there is no association between endometriosis and the IL-4 and TNF α gene polymorphisms. The IL-4 –590 promoter, IL-4 intron3, and TNF α –308 G-to-A polymorphisms are not useful markers for predicting susceptibility to endometriosis. *J. Clin. Lab. Anal.* 16:121–126, 2002. © 2002 Wiley-Liss, Inc.

Key words: endometriosis; interleukin-4; IL-4; polymorphism; tumor necrosis factor alpha; TNF- α

INTRODUCTION

Endometriosis, a complex disease, is associated with immunologic and genetic changes. Numerous cytokines are associated with endometriosis, including interleukin-1 (IL-1), IL-6, IL-8, and IL-4 (1), and tumor necrosis factor alpha (TNF α) (2). IL-4 is a cytokine with anti-inflammatory properties. IL-4 inhibits the production of proinflammatory cytokines such as IL-1, IL-6, and TNF (3). TNF- α , a pluripotent proinflammatory cytokine, plays an important role in the process of numerous inflammatory and autoimmune diseases, including diabetes (4), systemic lupus erythematosus (SLE) (5), systemic sclerosis (6), and ankylosing spondylitis (7). TNF α of peritoneal fluid has important proinflammatory and angiogenic activities in the complex mechanisms of peritoneal endometriosis (8).

Two polymorphisms for IL-4 have been demonstrated, including the IL-4 –590 promoter and IL-4 intron 3. The single

nucleotide polymorphism (SNP) of the IL-4 –590 promoter appears as a C-to-T substitution at position –590 of the promoter region (9). The polymorphism of IL-4 intron 3 is composed of a variable number of tandem repeats (VNTRs) of a 70-bp sequence in intron 3 (10). Cantagrel et al. (11) demonstrated the association between rheumatoid arthritis and IL-4 intron3. The RP1 allele in intron 3 was shown to have a higher frequency in rheumatoid arthritis patients. They also observed a non-association between rheumatoid arthritis and the IL-4

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–590 promoter. In a previous study (Shi et al., unpublished data), we observed that the IL-4 –590 promoter and IL-4 intron3 could not be used as a genetic marker for susceptibility to Kawasaki disease.

The TNF α gene encodes a pleiotropic cytokine that plays a critical role in basic immunologic processes (12). A biallelic G-to-A polymorphism 308 nucleotides upstream from the transcription initiation site in the TNF promoter is associated with elevated TNF α levels, disease susceptibility, and poor prognosis in several diseases (13). There are no studies in the literature concerning the association between endometriosis and the TNF α polymorphism. In the present work, we evaluate whether these three polymorphisms are useful markers for predicting susceptibility to endometriosis.

SUBJECTS AND METHODS

This study included 226 premenopausal Taiwan Chinese women with surgically diagnosed endometriosis and nonendometriosis. The patients were divided into two groups: 1) endometriosis (n = 120) and 2) nonendometriosis (n = 106). The study was approved by the ethics committee and institutional review board of the China Medical College Hospital. Informed consents were signed by all women who donated their blood. There were nonsignificant differences between both groups in age, weight, and height. All women accepted the peripheral blood sampling for genotype analyses.

Genomic DNA was isolated from peripheral blood using the DNA extractor Genomaker DNA extraction kit (Blossom, Taiwan). About 50 ng of genomic DNA was mixed with 20 pmole of each PCR primer in a total volume of 25 μ l containing 10 mM Tris-HCL, pH 8.3, 50 mM KCL, 2.0 mM MgCL₂, 0.2 mM deoxyribonucleotide triphosphate, and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer, Applied Biosystems, Foster City, CA). The primer sequences and PCR conditions for the IL-4 and TNF α polymorphisms were as previously described (11,14). A single base pair (bp) polymorphism at the –590 position in the promoter of IL-4 was analyzed by the PCR-restriction fragment length polymorphism (RFLP) method. The polymorphisms were studied after restriction digestion with *Bsm* FI for IL-4 –590. Biallelic base polymorphisms in the IL-4 –590 gene were detected. IL-4 intron3 contains a 70-bp length of VNTRs. Its loci involve a G-to-A transition at position 308 in the 5'-flanking promoter/enhancer region of the TNF α gene.

Gene polymorphism was analyzed by PCR amplification followed by NcoI restriction analysis (9). PCR products were analyzed by electrophoresis on agarose gel. Each allele was recognized according to its size. Allelic sizes (bp) after enzyme digestion were as follows: IL-4 –590*C (192 + 60 bp), IL-4 –590*T (252 bp); RP0 (113 bp), RP1 (RP0 + 70 bp), and RP2 (RP0 + 140 bp). Homozygote A for the TNF –308 promoter showed two fragments of 97 bp and 20 bp. Homozygote G was undigested and resulted in a single band of

117 bp. Heterozygote A/G was detected by the presence of all three fragments.

Allelic frequencies are expressed as a percentage of the total number of alleles. The genotypes and allelic frequencies for the IL-4 –590 promoter, IL-4 intron3, and TNF α polymorphisms in both groups were compared. The SAS system (Version 8.1, SAS Institute, Inc., Cary, NC) with χ^2 , and Fisher's exact test were utilized for statistical analyses. A *P*-value < 0.05 was considered statistically significant.

RESULTS

The genotype proportions of the IL-4 promoter and IL-4 intron3 polymorphisms in both groups did not differ significantly (Table 1). The most common genotypes for the IL-4 promoter and IL-4 intron3 in both groups were the –590*T and RP1 homozygotes. The proportions of the –590*C homozygote/heterozygote/–590*T homozygote for the IL-4 promoter in groups 1 and 2 were 1.6/31.6/66.6% and 5.8/33.0/61.2%, respectively. The proportions of RP1 homozygote/heterozygote/RP2 homozygote for the IL-4 intron3 were 62.5/34.1/2.5% and 64.1/32.0/3.9%, respectively. Only one endometriosis case (0.8%) presented the RP1/RP2 heterozygote for the IL-4 intron3 (Table 1).

There were no significant differences in allele frequencies for the IL-4 promoter and IL-4 intron3 between the groups (Table 2). The most common alleles for the IL-4 promoter and IL-4 intron3 in both groups were alleles T and RP1. The proportions of alleles C and T for the IL-4 promoter in groups 1 and 2 were 17.5/82.5% and 22.3/77.7%, respectively. The proportions of RP1 and 2 for the IL-4 intron3 were 79.5/20.0% and 80.1/19.9%, respectively.

There were no significant differences in genotype distribution and allele frequency of the TNF α gene polymorphism between the groups (Table 3). The predominant genotype for the TNF α promoter gene in both groups was the G homozygote. The A^{–308} allele for the TNF α promoter is not associated with a higher susceptibility to endometriosis. Proportions of the –308*A homozygote/heterozygote/–308*G homozygote for the TNF α promoter in groups 1 and 2 were 7.5/20.8/71.7% and 7.5/17/75.5%, respectively (Table 3). The distributions

TABLE 1. Genotypes for IL-4 promoter and intron 3 polymorphisms in women with and without endometriosis

Genotype	Endometriosis n = 120 (%)	Non-endometriosis n = 103 (%)	<i>P</i> -value ^a
IL-4 promoter			0.236
–590*C/–590*C	2 (1.6)	6 (5.8)	
–590*C/–590*T	38 (31.6)	34 (33.0)	
–590*T/–590*T	80 (66.6)	63 (61.2)	
IL-4 intron3			0.870
RP1/RP1	75 (62.5)	66 (64.1)	
RP1/RP2	41 (34.1)	33 (32.0)	
RP2/RP2	3 (2.5)	4 (3.9)	
RP0/RP2	1 (0.8)	0	

^a*P*-values were calculated by Fisher's exact test.

TABLE 2. Allelic frequencies for IL-4 promoter and intron 3 polymorphisms in women with and without endometriosis

Allele frequencies	Endometriosis n = 120 (%)	Non-endometriosis n = 103 (%)	P-value
IL-4 promoter			0.201 ^a
Allele C	42 (17.5)	46 (22.3)	
Allele T	198 (82.5)	160 (77.7)	
IL-4 intron3			1.000 ^b
RP1	191 (79.5)	165 (80.1)	
RP2	48 (20.0)	41 (19.9)	
RP0	1 (0.4)	0	

^aP-values were calculated by χ^2 tests.^bP-values were calculated by Fisher's exact test.

of different alleles in both groups also did not differ significantly. The predominant allele for the TNF α gene in both groups was the G allele. The proportions of allele A/G in groups 1 and 2 were 17.9/82.1% and 16/84%, respectively (Table 1).

DISCUSSION

Endometriosis involves a complex interaction between the immunoinflammatory process, cytokines activation, and genetic factors (15). Cytokines are proteins that play a role in the communication link between the immunological system and endometriosis tissues. IL-4, a 20-kd cytokine, acts as an autocrine growth factor for differentiated T-helper cells (16). T-cell-mediated cytotoxicity is suppressed by IL-4 (17). Elevated IL-4 expression was previously noted in women with endometriosis (17,18). The TNF α gene is involved in tumor immunity and cancer pathogenesis (19). TNF α has been shown to stimulate proliferation of endometriotic stromal cells through induction of the IL-8 gene and protein expression (20). The SNP marker provides a new way to identify complex gene-associated diseases such as endometriosis. In a previous study (Hsieh et al., unpublished data), we observed a non-association between endometriosis and IL-1 polymorphisms (IL-1 β -511 promoter, IL-1 β exon 5).

The gene for IL-4 has been mapped to the 5q23-33 (21). IL-4 polymorphisms have been associated with various diseases, or susceptibility to them, including rheumatoid arthritis (11); asthma, rhinitis, and atopic dermatitis (22); Graves'

disease (23); and multiple sclerosis (24). The RP2 allele for IL-4 intron3 is a protective factor for joint destruction in patients with rheumatoid arthritis (25). However, there have been few reports concerning the role of IL-4 in endometriosis. Odukoya et al. (26) demonstrated that IL-4 mRNA was not expressed in the ovarian endometrioma. In contrast, Nakayama et al. (27) demonstrated that IL-4-589T increases IL-4 production. In this study, we observed that the genotype and allelic frequencies of the IL-4 promoter and IL-4 intron3 polymorphisms were not candidate genetic markers for susceptibility to endometriosis in Taiwan Chinese women. The T allele for the IL-4 promoter and RP1 allele in intron 3 were not associated with an increased risk of endometriosis. Furthermore, there was no association between the IL-4 promoter and the IL-4 intron3 polymorphisms.

In a previous study (9), heterozygotes (C/T) for the IL-4 -590 promoter were related to higher IL-4 activity and IgE secretion in asthma patients. The T allele for the IL-4 gene promoter has been associated with increased risk of atopic dermatitis as compared to the C allele (28). A reduced frequency of the T allele in the IL-4 -590 promoter has been associated with Graves' disease (23). In contrast, several investigators have indicated a non-association between the IL-4 polymorphism and individual diseases. A non-association between the IL-4 polymorphism (interferon regulatory factor 1 (29), C/T alleles for IL-4 -590 promoter (30)) and asthma has been demonstrated. IL-4 polymorphisms do not appear to be associated with susceptibility to other illnesses, including multiple sclerosis (31), myasthenia gravis (32), and minimal change nephropathy (33).

The A⁻³⁰⁸ allele of the TNF α promoter was associated with increased susceptibility to, or severity of numerous diseases, including rheumatoid arthritis (34), diabetes (4), Kawasaki disease (35), cervical intraepithelial neoplasia and human papillomaviruses type 16 infection (36), preterm premature rupture of the fetal membranes (37), malignant tumors (38), obesity (4), sepsis and septic shock (39), atopy (40), sinusitis (41), IgA nephropathy (42), idiopathic dilated cardiomyopathy (43), coronary artery disease (44), psoriasis (45), SLE (5), systemic sclerosis (6), and ankylosing spondylitis (7). The TNF α polymorphism may be a marker for the prediction of disease severity rather than susceptibility (46). The A⁻³⁰⁸ allele was associated with anti-Ro and anti-La antibody production in patients with SLE (47). In contrast, several investigators indicated a non-association between the TNF α polymorphism and individual diseases, including diabetes (48); atopy, asthma, and rhinitis (22); chronic obstructive pulmonary disease (COPD) (49); bronchiectasis (50); IGA deficiency (51); and rheumatoid arthritis (52). These differences may be due to different illness classifications, and racial and disease variations. It has been demonstrated that the TNF α -308 promoter polymorphism is associated with insulin resistance in Caucasians (53). In contrast, Hayakawa et al. (54) demonstrated that this polymorphism is not a major contrib-

TABLE 3. Genotypes and allelic frequencies for TNF α -308 promoter in women with and without endometriosis

Genotype	Endometriosis n = 120 (%)	Non-endometriosis n = 103 (%)	P-value ^a
A/A	9 (7.5)	8 (7.5)	0.759
A/G	25 (20.8)	18 (17)	
G/G	86 (71.7)	80 (75.5)	
Allelic frequency			0.596
Allele A	43 (17.9)	34 (16)	
Allele G	197 (82.1)	178 (84)	

^aP-values were calculated by χ^2 test.

uting gene to insulin resistance in Japanese. In the present study, the genotype and allelic frequencies of the TNF α -308 polymorphism were not statistically different between the two groups. This suggests that this polymorphism is not a candidate genetic marker for susceptibility to endometriosis.

The A⁻³⁰⁸ allele for the TNF α promoter affects the binding of transcription factors and higher transcriptional promoter activity, which may further alter TNF production, immune response, and susceptibility to certain autoimmune, infectious, and malignant diseases (55). Agarwal et al. (56) demonstrated that the presence of a G-to-A polymorphism at position -308 of the TNF α promoter gene can increase transcription six- to sevenfold. The polymorphic variation lies within the promoter region of the TNF α locus, making it a likely candidate for playing a regulatory role in disease pathogenesis. The A⁻³⁰⁸ allele may inhibit a repressor of transcription (13). The A/G heterozygote phenotype showed increased TNF α levels compared to that in G homozygous subjects (57).

The TNF α gene is located within the class III region of the major histocompatibility complex (MHC) region on chromosome 6p21.3 (58). Some of these polymorphisms form extended haplotypes with the human leucocyte antigen (HLA) class I and II alleles. In a previous study, the TNF α allele A⁻³⁰⁸ was strongly associated with the HLA A1, B8, and DR3 alleles, while allele G⁻³⁰⁸ was associated with DR4 and DR6 (59). TNF α promoter polymorphism may be functionally silent and have some functional and selectable effect only when its linkage with selectable HLA alleles (60). Administration of corticosteroid may inhibit TNF α transcription (61).

In conclusion, the results of this study indicate that there is no association between endometriosis and the IL-4 and TNF α gene polymorphisms. The genotype and allele frequencies of the IL-4 -590 promoter, IL-4 intron3, and TNF α -308 promoter polymorphisms are not useful markers for the predicting susceptibility to endometriosis. This work could provide a database for further surveys of the IL-4 and TNF α polymorphisms. However, the real role of the IL-4 and TNF α polymorphisms in endometriosis remains to be clarified. Furthermore, the effects of other cytokine polymorphisms on endometriosis development merit further study.

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