

The Interleukin-6 –174G/C Promoter Polymorphism Is Not Associated With Endometriosis in South Indian Women

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OBJECTIVE: To investigate the association of the –174 G/C promoter polymorphism of the interleukin-6 (IL-6) gene with endometriosis in South Indian women.

METHODS: The genotype frequencies of the common IL-6 –174 G/C polymorphism were compared in infertility patients with (n = 232) and without (n = 210) endometriosis using polymerase chain reaction (PCR) and sequencing analysis.

RESULTS: The genotype frequencies among the cases and controls were G/G 62.9% and 71.9%, G/C 34.1% and 25.2%, and C/C 3.0% and 2.9%. The G and C allele frequencies were 80% and 84.6%, and 20% and 15.4%, respectively. There were no statistically significant differences in the genotype distributions or allele frequencies between the cases and controls (P = .12).

CONCLUSIONS: The present study demonstrates no significant association between the IL-6 –174 G/C promoter polymorphism and endometriosis in South Indian women. (*J Soc Gynecol Investig* 2005; 12:365–9) Copyright © 2005 by the Society for Gynecologic Investigation.

KEY WORDS: Endometriosis, IL-6 promoter, polymorphism.

Immune dysregulation and inflammation are the hallmarks of endometriosis, a common gynecologic disease^{1,2} characterized by the presence of endometrial tissue in ectopic sites. The adhesion of viable endometrial cells to the peritoneal surface can cause a local inflammatory reaction,³ which involves activation of macrophages. Activated macrophages in turn produce a number of cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF- α), which may stimulate the growth of ectopic endometrial cells in the early stages of disease development.⁴ These factors may also influence the way in which endometriosis presents clinically, by causing pain and/or subfertility.

IL-6 is a pleiotropic cytokine implicated in several physiologic and pathologic processes.⁵ It plays a pivotal role in reproductive physiology, including the regulation of ovarian steroid production, folliculogenesis, and early events related to implantation.⁶ It is produced by a variety of cell types, including lymphocytes, monocytes, and endothelial cells.⁷ Recent studies indicate that IL-6 is also produced by both eutopic and

ectopic endometrium.⁸ It has been suggested that IL-6 production may be modulated by factors such as ovarian steroids and inflammation-associated cytokines such as IL-1 β , interferon gamma (IFN- γ), and TNF- α .⁹ Increased IL-6 levels have been reported in the peritoneal fluid and serum of endometriosis patients.^{10–12} These levels were correlated with the disease stage.¹³ More recently, Bedaiwy and Falcone¹⁴ have suggested that IL-6 could serve as a diagnostic marker for endometriosis.

A common G→C single-nucleotide polymorphism (SNP) in the IL-6 gene promoter at position –174 influences IL-6 expression,^{15,16} although its allelic frequency varies with ethnicity.^{15,17} The SNP has been implicated in breast and ovarian cancer, polycystic ovarian syndrome, and a variety of other diseases^{18–20} but not in endometriosis.^{21,22}

Considering the important role that IL-6 plays in the pathophysiology of endometriosis and the variations in the frequency of the –174G/C promoter polymorphism of IL-6 in different ethnic groups, we chose to analyze whether the SNP is associated with the disease in South Indian women, which has not previously been attempted.

PATIENTS AND METHODS

Patients

All the women were of South Indian origin and nonsmokers. Two hundred thirty-two unrelated women with moderate to severe (III–IV) endometriosis staged using the revised American Fertility Society (rAFS) classification system²³ were re-

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cruited at the Infertility Institute and Research Centre (IIRC), Hyderabad, India. All women had a trans-vaginal ultrasound scan (TVS) at screening followed by a laparoscopy to confirm the diagnosis (rAFS III = 76; IV = 156). Women with other ovarian cysts, adenomyosis, ovarian cancer, fibroids, or stage I and II endometriosis were excluded. Their mean age \pm SD was 27.5 ± 4.4 (range 20–40) years. They all complained of dysmenorrhoea (mild = 43%; moderate = 32%; severe = 25%) and 70% had dyspareunia; 70% had primary and 30% had secondary infertility.

Two hundred ten women were recruited from the same clinic population and had an equal opportunity to be identified as cases, thereby meeting the criteria for appropriate controls set by Zondervan et al.²⁴ These controls consisted of 141 (67%) women with no evidence of endometriosis on TVS and laparoscopy and 69 (33%) women with no evidence of an ovarian endometrioma on TVS and no clinical symptoms of endometriosis who therefore did not subsequently have a laparoscopy. Their mean age \pm SD was 26.7 ± 3.9 (range 22–39) years. Among the controls, 29% complained of mild dysmenorrhoea and 20% had dyspareunia; 78% had primary and 22% had secondary infertility. Written, informed consent was obtained from all participants. The Institutional Review Board of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, approved the study.

Determination of the *IL-6* Genotype

DNA EXTRACTION. Genomic DNA was extracted from 1 mL of EDTA anticoagulated whole blood by the salting out method.²⁵

PRIMERS AND POLYMERASE CHAIN REACTION. Genotyping of the –174 G/C SNP in the promoter region of the *IL-6* gene was determined by polymerase chain reaction (PCR) and sequencing analysis. PCR was performed in a total volume of 25 μ L containing 50 ng genomic DNA, 2 to 6 pmol of each primer, IX Taq polymerase buffer (1.5 mM MgCl₂), and 0.25 U of Amplitaq DNA polymerase (Perkin Elmer, Foster City, CA). The primers used were 5'-TGACTTCAGCTTTACTCTTGT-3' (forward), and 5'-CTGATTGGAAACCTTATT AAG-3' (reverse). PCR amplification was performed in a programmable thermal cycler gradient PCR system (Eppendorf AG, Hamburg, Germany). The PCR amplification was performed for 35 cycles (denaturation at 94C for 1 minute, annealing at 55C for 1 minute, extension at 72C for 1 minute, and final extension for 10 minutes at 72C). The PCR product of 191 bp was analyzed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining and ultraviolet visualization and then sequenced with a Taq-Dye deoxy-terminator cycle sequencing kit (Applied BioSystems) using an automated ABI 3770 sequencer (Applied BioSystems).

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package (V 11.0; SPSS, Inc, Chicago, IL). The genotypic

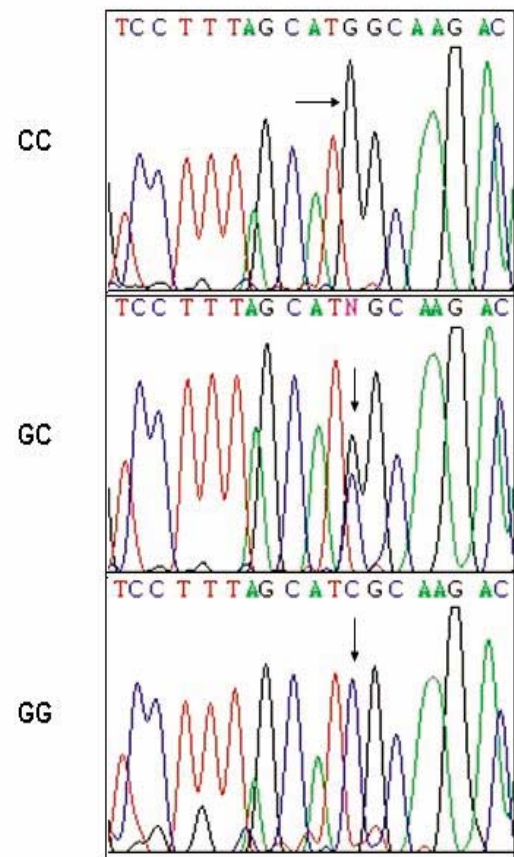


Figure 1. Genotyping of the *IL-6* –174 G/C polymorphism. The three different genotypes are indicated by the sequence analysis of the PCR-amplified product using a reverse primer.

distribution amongst subjects was tested for Hardy-Weinberg equilibrium using the Arlequin program (Version 2.000; Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland).²⁶ The allele ratios and genotype distributions in the cases and controls were analyzed with Pearson χ^2 test. All *P* values were two-tailed and 95% confidence intervals (CIs) are given. The sample size of this study had a power of 80% to detect a difference of 10–14% in allele frequency between cases and controls, for estimated allele frequencies among controls that varied from 0.10 to 0.40.

RESULTS

Among subjects, the genotypic distributions of the individual SNPs, as well as the *IL-6* allele system, were all in Hardy-Weinberg equilibrium. Sequence analyses of the 191-bp product of the *IL-6* –174 promoter region SNP are shown in Figure 1. G/G and C/C homozygotes manifest as a single peak, whereas the heterozygote G/C is seen as a double peak. Allele and genotype frequencies for the SNP are summarized in Table 1. The genotype frequencies among the 232 cases and 210 controls were G/G 62.9% and 71.9%, G/C 34.1% and

Table 1. Genotype and Allele Frequencies (%) of *IL-6* -174 G/C Polymorphism in Endometriosis Patients and Controls

Polymorphism	Stage of endometriosis		Cases (n = 232)	Controls (n = 210)	P value
	III (n = 76)	IV (n = 156)			
-174 G/C Genotype					
C/C	1 (1.3)	6 (3.8)	7 (3.0)	6 (2.9)	.122*
G/C	24 (31.6)	55 (35.3)	79 (34.1)	53 (25.2)	
G/G	51 (67.1)	95 (60.9)	146 (62.9)	151 (71.9)	
Allele					
C	26 (17.1)	67 (21.4)	93 (20.0)	65 (15.4)	.077†
G	126 (82.9)	245 (78.6)	371 (80.0)	355 (84.6)	

* Fisher's exact test (3×2 table at 2 *df*), $P < .05$.

† Fisher's exact test (2×2 table at 1 *df*), $P < .05$.

25.2%, and C/C 3.0% and 2.9%. The G and C allele frequencies were 80% and 84.6%, and 20% and 15.4%, respectively. There were no statistically significant differences in the genotype distributions or allele frequencies of the *IL-6* -174 SNP between the cases and controls ($P = .12$).

Genotype frequencies for the *IL-6* -174 SNP were further analyzed based on the size of the largest endometrioma present (Table 2). Compared to controls, statistically significant differences in the frequencies of G/G, G/C, and C/C genotypes were observed in patients with an endometrioma ≤ 3 cm ($P = .01$) but not in those with larger endometriomas.

DISCUSSION

Endometriosis is considered to be a polygenically inherited disease with multifactorial pathogenesis.²⁷ The disease is characterized by an inflammatory peritoneal environment. Activated macrophages and endometriotic implants, specifically stromal cells, are implicated as causes of elevated concentrations of several cytokines such as IL-6, TNF- α , and IL-1.^{28,29} IL-6 almost certainly plays a critical role in the establishment of endometriosis by decreasing the phagocytic function of macrophages in the peritoneal fluid via up-regulation of haptoglobin (endometriosis protein-I).³⁰ Recent studies emphasize the role of IL-6 in impairing sperm motility, which may explain its significance in endometriosis-associated infertility.³¹ IL-6 is involved in a positive feed back loop of estrogen biosynthesis through up-regulation of the 1.4 promoter of the aromatase

Table 2. Genotype Frequencies (%) of *IL-6* -174 Polymorphism in Endometriosis Patients ($n = 203$) Based on Size of the Endometrioma

Polymorphism	Endometriotic cyst (cm)			Controls (n = 210)
	≤ 3	4 to 5	≥ 6	
-174 G/C				
C/C	1 (5.0)	1 (1.7)	2 (1.6)	6 (2.9)
G/C	11 (55.0)	15 (25.4)	40 (32.3)	53 (25.2)
G/G	8 (40.0)	43 (72.9)	82 (66.1)	151 (71.9)
P value*	.012	.884	.323	

All of the patients studied had endometriotic cysts but precise measurements were only available for 203/232 (88%) patients.

* Fisher's exact test (3×2 table at 2 *df*), $P < .05$.

gene via the JAK-STAT cascade, which is important in endometriosis given that it is an estrogen-dependent disease.³²⁻³⁴ Lastly, IL-6 production in endometriotic cells decreases in response to gonadotropin-releasing hormone (GnRH) agonist treatment.³⁵

The *IL-6* gene has been mapped to chromosomal region 7p21³⁶ and a G/C SNP at position -174 of the promoter is known to influence *IL-6* expression. In response to stimuli such as lipopolysaccharide and IL-1, the -174G reporter construct showed much higher expression compared to -174C in transient transfection assays.^{15,37} A variety of transcription factors, including cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), nuclear factor kappaB (NF- κ B), and activator protein-1 (AP-1), contribute to the regulation of *IL-6* in vivo.³⁸ The -174 SNP maps to a negative regulatory domain (-225 to -164) very close to the cAMP-responsive element (CRE). Interestingly, this SNP is contained within a sequence, bearing partial nucleotide homology with the Smad4 binding element and the presence of the C allele may bind Smad4 more effectively and thereby inhibit *IL-6* transcription.³⁹ This site is known to be under estrogen regulation, although the -174C variant has been shown to be estrogen-insensitive.⁴⁰⁻⁴²

Previous studies showed no association between the *IL-6* -174 G/C promoter polymorphism and endometriosis in Korean and middle European, white women.^{21,22} Our results are entirely consistent with the previously published data even though the proportion of controls with the *IL-6* -174 SNP was not comparable. For example, the "C" allele frequency was very low in South Korean women²¹ and quite high in middle European, white women,²² compared to the frequency in our study (Table 3). We did find a statistically significant difference in the frequency of genotypes in patients with an endometrioma ≤ 3 cm compared to controls ($P = .01$). However, this observation is difficult to explain in the absence of an effect in women with larger endometriomas, and it has to be interpreted in the light of the small number of patients in that category ($n = 20$).

Although we did not measure the levels of *IL-6* in the present study, it is noteworthy that some studies have reported that the homozygous CC genotype is associated with higher

Table 3. Comparison of the *IL-6* -174 G/C Genotype and Allele Frequencies in the Present Study and Previously Published Studies

Polymorphism	Present study		Lee et al ²¹		Wieser et al ²²	
	Endometriosis, n = 232 (%)	Controls, n = 210 (%)	Endometriosis, n = 70 (%)	Controls, n = 202 (%)	Endometriosis, n = 94 (%)	Controls, n = 70 (%)
-174 G/C Genotype						
C/C	7 (3.0)	6 (2.9)	0 (0.0)	0 (0.0)	16 (17.0)	13 (18.6)
G/C	79 (34.1)	53 (25.2)	1 (1.4)	0 (0.0)	44 (46.8)	37 (52.9)
G/G	146 (62.9)	151 (71.9)	69 (98.6)	202 (100)	34 (36.2)	20 (28.6)
Allele						
C	93 (20.0)	65 (15.4)	1 (0.01)	0 (0.0)	76 (40.4)	63 (45.0)
G	371 (80.0)	355 (84.6)	139 (99.99)	404 (100)	112 (59.6)	77 (55.0)

serum IL-6 levels,^{43,44} in contrast to studies showing higher levels for the -174 GG genotype.^{15,37} It appears that transcription control of *IL-6* is complex and it is determined by a combination of base changes at several sites rather than by an isolated SNP.¹⁶ In endometriosis, conflicting results are reported for IL-6 levels: some studies show elevated levels¹⁰⁻¹² and some no variation.^{45,46} However, it is unclear whether the variation in cytokine levels is a cause or a consequence of the disease. In conclusion, the present study demonstrates no significant association between the *IL-6* -174 G/C promoter polymorphism and endometriosis in South Indian women.

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