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Research Article

The rs2304256, a Non-Synonymous Polymorphism in Tyrosine Kinase 2 Gene is Associated with the Risk of Endometriosis

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Abstract

The objective of the study was to investigate whether the *TYK2* gene influences the risk of developing endometriosis in South Indian women. The non-synonymous SNP, rs2304256, in exon8 of the *TYK2* gene was tested for association in a case–control study of 150 affected women and 150 women with no evidence of disease. The genotype frequencies of the polymorphism were compared using polymerase chain reaction and restriction fragment length polymorphism. Immunohistochemistry was used to analyze the distribution and expression of TYK2 in the endometrium of women with and without endometriosis. According to codominant, dominant, and recessive genotype models, statistically significant differences were observed in the genotype distribution and allele frequency (P=0.0432) between the cases and controls. The distribution and expression of TYK2 did not vary in the endometrium of cases and controls. In the present study, we could establish an association between the *TYK2* rs2304256 non-synonymous polymorphism with the risk of endometriosis in South Indian women, indicating that this polymorphism may lead to significant disease susceptibility.

Keywords: Endometriosis; Tyrosine kinase 2; Polymorphism; South Indian women

Abbreviations: CI: Confidence Interval; χ^2: Chi Square; D': Disequilibrium Coefficient; LD: Linkage Disequilibrium; HWE: Hardy–Weinberg Equilibrium; OR: Odds Ratio; TYK2: Tyrosine Kinase 2; IL: Interleukin; STAT: Signal Transducer and Activator of Transcription; PCR: Polymerase Chain Reaction; SNPs: Single Nucleotide Polymorphisms

Introduction

Endometriosis is a chronic, endocrine gynecologic disease characterized by the implantation of functional endometrial tissue at ectopic positions. It is observed mainly in the pelvic area including the ovaries, peritoneal surfaces and ligaments including bowel and bladder [10]. It affects 10-15% of women in their reproductive age and is responsible for dysmenorrhea, dyspareunia, infertility and chronic pelvic pain. Retrograde menstrual invasion and implantation of endometrial stromal cells into the peritoneum are the widely accepted explanations for this condition [17]. Although retrograde menstruation is common in 70-90% of women, the much lower endometriosis prevalence suggests that there must be other variables that may contribute to endometriosis pathogenesis. We previously looked at the correlation between genetic variants in multiple candidate genes and endometriosis risk in Indian women [4,13-16,32,33], which suggested that the condition is polygenic and multifactorial [31].

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A complex network of cytokines mediates the immunomodulatory mechanisms leading to pathogenesis of endometriosis [39]. An altered secretion of Th1 and Th2 specific cytokines have been implicated in the pathogenesis of the disease. In the peritoneal fluid of affected women, there has been a shift in the balance of Th1/Th2 cytokines toward the Th2 response, contributing to the derangement of immunologic defense mechanism [28]. Elevated levels of Th2-specific cytokines such as interleukin *IL-4, IL-5, IL-10* and *IL-13* impairs T-cell cytotoxicity, enhancing the endometrial cell implantation and growth at the extra uterine sites [11]. Tyrosine Kinase 2 (*TYK2*) is located on chromosome 19p13.2 [9] and is implicated in signaling from Th2 cells, for example, through *IL-10* and *IL-13* receptors activating *STAT3* and *STAT6* signaling pathway [21,34]. *TYK2* is widely studied in the pathogenesis of several tumors because of its critical role in tumor immunosurveillance [20].

Earlier, few genetic association studies have been conducted on *TYK2* locus to study its impact on several autoimmune diseases, however, the identification has been duplicated in a large number of recent analyses and *TYK2* is now considered to be a molecular marker in a variety of autoimmune and inflammatory diseases [12]. A non-synonymous SNP of *TYK2*rs2304256, is widely studied for its association with diseases like systemic lupus erythematosus, Crohn's disease, rheumatoid arthritis, type 1 diabetes mellitus, ulcerative colitis, etc. [7,22,26]. This study was undertaken to investigate the association of *TYK2* rs2304256 polymorphism with the risk of endometriosis in South Indian women.

Materials and Methods

Study Population

The case-control study was carried out on three hundred women in their reproductive age, recruited at the Infertility Institute and Research Center (IIRC), Hyderabad, India. The study subjects were obtained as described earlier [33].

Tissue collection

The proliferative phase endometrial tissues were collected and fixed as per the method described by Bhanoori et al (2008) [4].

DNA Extraction

Salting out procedure was used to extract the genomic DNA from one milliliter of anticoagulated whole blood.

Molecular Analysis of *TYK2*

Genotyping of TYK2 gene polymorphism was carried out by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) and the results confirmed by Sanger sequencing method as described earlier [16]. The primers and PCR conditions are summarized in (Table 1). The PCR products of *TYK2* gene (385 bp) was digested with restriction enzyme (*BsmI* at 65°C) for 3 h and the DNA fragments were electrophoresed through a 2% agarose gel, stained with ethidium bromide. For the *TYK2BsmI* C/A SNP, the A allele was represented by DNA band of size 251 and 134bp, the C allele was represented by DNA bands of sizes 385 bp; whereas, the heterozygotes displayed a combination of both alleles (385, 251 and 134bp) (Figure 1).

Table 1: Primers and PCR conditions used for *TYK2* genotyping.

S.no	Gene	Primers	PCR conditions (35 cycles)	Amplicon Size
1	*TYK2*	F:5'TCACCAGGCACTTGTTGTCC 3' R:5'CGGCTTCCAGCATGTGTATG3'	5 min at 95˚C, 40 sec at 94˚C, 30 sec at **59˚C**,50 sec at 72˚C and 10min72˚C.	385

PCR*: Polymerase Chain Reaction

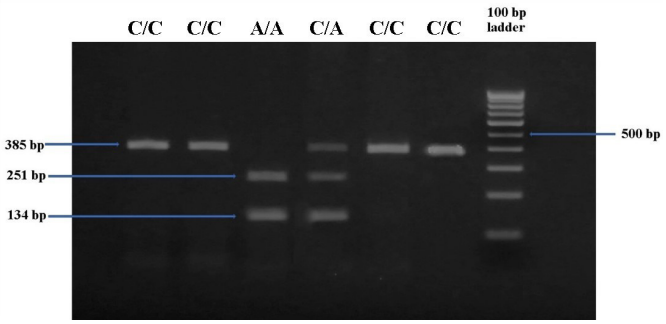

Figure 1: Restriction digestion of *TYK2* polymorphism rs2304256 (C/A) by *Bsm I*.

Immunohistochemical Analysis

Immunohistochemical analysis of TYK2 expression was performed on the proliferative phase endometrial tissue sections from endometriosis patients (n=5) and healthy controls (n=4), using the method described by Bhanoori et al. (2008) [4]. After the incubation of sections with rabbit polyclonal antibody against TYK2 (1:1000, Cell signaling technology, USA) and FITC-conjugated goat anti-rabbit secondary antibody (1:1000, Sigma–Aldrich, USA), they were washed and mounted with an anti-fade mounting medium (Vector Lab, USA) then visualized using an Axioplan 2 epifluorescence microscope (Carl Zeiss, Inc., USA). Negative controls were obtained by substituting *TYK2* antibody with the non-immune rabbit serum.

Statistical Analysis

Statistical analysis was performed as per the methods described in our previous study [33].

Results

All subjects (n=300) were successfully genotyped. The genotype distributions of individual SNPs, as well as allele system, were all in Hardy-Weinberg equilibrium (P<0.05) in both the cases and controls. The results were analyzed in a blinded fashion.

Genotyping of *TYK2* (rs2304256C/A) Polymorphism

Sequence analysis of *TYK2rs2304256* non-synonymous SNP is shown in Figure 2. The homozygotes *(C/C)* and *(A/A)* manifested as a single peak, whereas heterozygote (C/A) as double peaks. The genotype and allele (P=0.0432) distribution of *TYK2* rs2304256 C/A polymorphism showed significant difference between cases and controls according to codominant, dominant and recessive model (P< 0.05; Table2). The frequencies of allele '*A*' and genotype *AA* in patients with endometriosis were significantly higher than those in controls, suggesting that allele '*A*' and genotype *AA* are associated with endometriosis.

Immunohistochemistry of *TYK2*

Immunostaining of *TYK2* was observed in both glandular epithelial and stromal cells of endometrium. In the endometria of women with and without endometriosis, we observed no significant difference in the expression of TYK2 (Figure 3).

Table 2: Genotype and allele frequencies of *TYK2* gene polymorphism in endometriosis cases and controls.

Genotypes/ Alleles	Cases (n=150)	Controls (n = 150)	P-value	Odds ratio	95% CI
TYK2					
rs2304256/ Genotypes Codominant model					
CC	57	71	-	Reference	Reference
CA	80	75	0.2353	1.3287	0.8306-2.1256
AA ***Recessive model*** AA CA+CC	13 13 137	4 4 146	0.01328 0.02461	4.0482 3.4635	1.2519-13.0907 1.1025-10.8802
Dominant model CA+AA CC	93 57	79 71	0.10219	1.4664	0.9259-2.3225
Alleles					
C	194	217		Reference	Reference
A	106	83	0.0432	0.5901	0.3988-0.8731

CI: Confidence Interval.

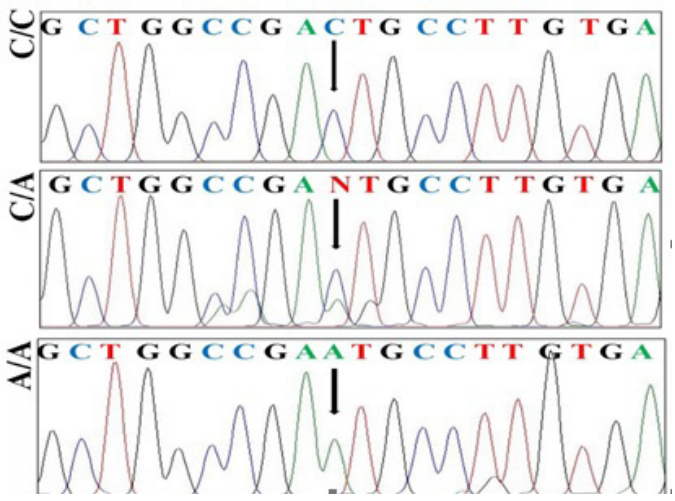

Figure 2: Genotyping of *TYK2* gene polymorphism rs2304256 by sequence analysis of the PCR amplified product using a forward primer.

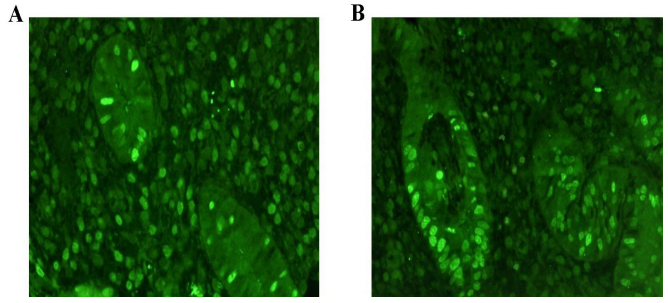

Figure 3: Immunohistochemical analysis of *TYK 2* expression in phase-matched (proliferative) endometrium from case (A) and control (B).

Discussion

Endometriosis is a polygenically inherited and multifactorial disease. A variety of genetic association studies have focused on the association between cytokine *(TNF-α, IL-2, IL-4, IL-6, IL-10, or IL-16)* gene polymorphisms and endometriosis risk [24,25,36,37,38]. However, the studies on the down-stream signaling molecules like *JAKs* and *STATs* are very few. It is evident from the literature that the Th2 immune response is associated with endometriosis. [1]. Interleukin *IL13*, a typical Th2 cytokine is the key regulator of inflammatory and immune responses, that is central to endometriosis and associated abnormalities [8]. Elevated mRNA and protein levels of *IL-13* was reported in the peritoneal fluid of endometriosis patients [35]. The same changes are observed in the ectopic endometrium of affected women which could be responsible for the defective immunosurveillance leading to endometriotic tissue overgrowth [8]. *TYK2* is required for mediating the biological function of *IL-13* in processes associated with Th2 immune response [5,21]. *IL-13/TYK2* signaling in B cell proliferation, Ig E and MHC class II expression and Th1-cytokines inhibition are well demonstrated in endometriosis [29]. An aberration in *TYK2* can lead to abnormal *STAT6* signaling, gaining resistance to apoptosis and escaping the immune surveillance [6,20]. Therefore, *TYK2*gene may have the crucial importance in regulating immune and/or inflammatory responses in endometriosis.

In the current study, SNP in *TYK2* rs2304256 was examined to ascertain whether the polymorphism is associated with endometriosis susceptibility in women from South Indian. The non-synonymous SNP rs2304256, a C to An alteration in exon 8 of TYK2, induces a change of valine to phenylalanine at position 362 in the JAK-homology 4 region of TYK2 [30]. Li et al showed that the allele 'A' disrupts a putative exonic splicing enhancer binding motif affecting the pre-mRNA processing of *TYK2*; thus, promoting the inclusion of exon 8 in the mRNA, which is essential for *TYK2* binding to cytokine receptor [23]. At the rs2304256 locus, we observed that the allele *'A'* and genotype *AA* occurred at significantly higher frequency in cases than in controls, indicating that the mutant allele 'A' could be a genetic risk factor for endometriosis. Of note, Peluso et al found no correlation between the SNP rs2304256 and endometriosis risk in the Brazilian population [27], which is inconsistent with the present finding. Possible reasons for this inconsistency could be: first, ethnic variation seen in the polymorphism analyzed. In point of fact, drastic difference was observed between the two populations in the distribution of the 'A' allele. In the Brazilian population, the mutant allele 'A' was found in 26.4 percent of cases and 23.6 percent of controls, whereas in the South Indian population, the frequencies were 35.3 percent and 27.6

percent in the current study. The second possible reason could be due to the difference in the sample size used in the study. However, larger population studies may require validating the exact role of this mutation in endometriosis.

The expression of TYK2 in the eutopic endometrium of women with and without endometriosis did not differ significantly in our study. Although there are conflicting results on TYK2 expression in various cancers, the majority of research found higher expression in tumor tissues when compared to controls [18,19]. Additionally, a strong correlation between TYK2 over-expression and late-stage tumors was observed, indicating that TYK2 over-expression contributes to the aggressiveness of tumors. However, we found no significant difference in TYK2 expression between eutopic endometria of women with and without endometriosis in the current study. To validate the role of TYK2 expression in the pathophysiology of endometriosis, more research with a bigger sample size is needed.

The minor allelic frequency for the SNP evaluated was compared with the mutation frequency data from populations of different ethnic origins, obtained from HapMap, 1000Genomes, Genome Aggregation Database (GnomAD) and EXAC database (dbSNP) (Supplementary Table 1). In the cases with the polymorphism under study, we observed that the minor allelic frequency was close to the values reported for Asian populations in the 1000 Genome Database. The frequency of 'A' allele is more common in Asians, Europeans, and Americans than it is in Africans for the TYK2 gene polymorphism rs2304256. Since Indians are a part of Asian ethnic group, the allelic frequency for the SNP rs2304256 found in the cases were very close with the Asians represented in 1000 Genomes, however, further confirmation requires studies with large sample sizes.

JAK-STAT signaling mediates immune regulatory processes that are central in endometriosis development and progression. We believe that the combined effect of various *JAK-STAT* mediator polymorphisms may disrupt immune homeostasis leading to the establishment and maintenance of endometrial cells at ectopic locations, as illustrated previously by our team considering *IL6* and *STAT6* [2,3]. The Discovery of how these genetic polymorphisms interact on endometriosis development may be a crucial step in understanding the pathophysiology of endometriosis.

In conclusion, our study shows that the *TYK2* gene polymorphism rs2304256 is significantly associated with endometriosis in South Indian women. Analysis of this polymorphism might help to identify patients at high risk for endometriosis development. Although further work is necessary to understand the molecular mechanism, our findings may lead to new insights into the disease pathogenesis.

Author Statements

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Conflict of Interests

The authors declare that there is no conflict of interests.

Ethical Approval

The study was approved by the ethical committee and review board of Centre of Cellular and Molecular biology (CCMB), Hyderabad. In the study all the participants were of South Indian origin (Dravidian linguistic group).

Informed Consent

Informed written consent form was obtained from all subjects prior to participation in this study.

Author's Contribution

KVV: execution of experiments, analysis and interpretation of data, statistical analysis and drafting of manuscript. MLM: Data collection. MD: acquisition of data. SS: analysis and interpretation of data, drafting of manuscript. MB: conception and design of study, analysis and interpretation of data, statistical analysis, drafting of manuscript. All authors will have seen and agreed to the 'Author Contribution' statement.

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