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ORIGINAL ARTICLE

IL-27 Gene Polymorphisms in Iranian Patients with Behcet's Disease

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SUMMARY

Background: **Behcet's Disease (BD) is a chronic systemic inflammatory disease of unknown etiology, principally characterized by relapsing periods of a broad range of clinical symptoms. Cytokines play fundamental roles in the pathogenesis of BD. Polymorphisms within cytokine genes have been found to play a pathogenic role in the development of autoimmune/inflammatory disorders. Interleukin 27 (IL-27), a new pro-/anti-inflammatory cytokine, is a great candidate for chronic inflammatory disease studies. The purpose of this study was to investigate a possible association between polymorphisms in the IL-27 gene and susceptibility to BD.**

Methods: **Fifty Iranian patients with BD and one hundred healthy individuals were examined for rs153109A/G and rs181206T/C IL-27 gene single nucleotide polymorphisms using RFLP-PCR and ARMS-PCR, respectively. Allele and genotype distributions were compared between groups using chi-square or Fisher's exact test.**

Results: **Frequencies of the rs153109AA genotype and rs153109A allele were statistically higher in BD patients compared with the control group ($p = 0.034$ and $p = 0.011$, respectively). The genotype and allele frequencies of rs181206 T/C polymorphism in BD patients were not significantly different from those of healthy controls.**

Conclusions: **Present findings demonstrate for the first time that the IL-27 gene rs153109 A/G SNP may be involved in susceptibility to BD in the Iranian population.**

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KEY WORDS

Behcet's Disease, gene polymorphism, interleukin 27

INTRODUCTION

Behcet's disease (BD) is a chronic multisystemic inflammatory disorder characterized by uveitis, oral and genital ulcers, and skin lesions. Involvement of joints,

large vessels, gastrointestinal tract, and central nervous system may also occur in BD [1,2]. The etiology of BD has not been fully elucidated; however, an inflammatory response triggered by infectious or environmental factors in a genetically susceptible individual has been suggested as the cause of BD [3,4]. The high incidence of BD along the ancient Silk Road (running from Mediterranean countries including Turkey and Iran to the Far East) [5] and familial aggregation of BD patients [6] support the contribution of genetic predisposition as well as environmental factors to the pathogenesis of BD. Genetic factors are thought to play an important role in the evolution and severity of the disease and might be utilized as a prognostic tool in the treatment of BD [7]. The strong association between HLA-B51 and BD supports the direct involvement of certain genes in the pathogenesis of BD [4]. A complicated cytokine network is also closely involved in the onset and development of BD [8]. Thus, it is reasonable to speculate that genetic polymorphisms which influence the production of certain cytokines could be seen as significant determinants of susceptibility to BD.

The list of inflammatory mediators implicated in the pathogenesis of autoimmune/inflammatory disorders has been recently expanded by another cytokine- IL-27. The human IL-27 gene is located on chromosome 16p11 and is composed of five exons [9]. This cytokine which was discovered in 2002 [10] belongs to the IL-12 family and is composed of two subunits: the Epstein-Barr virus-induced gene 3 protein (EBI3) and p28, which noncovalently bind to each other to form a complete heterodimeric IL-27 cytokine [11,12]. IL-27 has been found to induce both pro- and anti-inflammatory immune responses [13,14]. As an initiator of pro inflammatory responses, it mediates naive T cell proliferation and is found to be a potent inducer of IFN-γ production, particularly in synergy with IL-12, which leads to generation of Th1-mediated immune responses [15]. Thus, it may play an important role in the development of immune-related diseases. Indeed, recent investigations have revealed the involvement of IL-27 in asthma [16], uveitis [17], and post-influenza pneumococcal pneumonia [18]. Up-regulation of IL-27 has been reported in inflammatory bowel disease [19], granulomatouse diseases [20], and in mouse models of multiple sclerosis and uveitis [21]. As a repressor of immune responses, it reduces the development of effectors and memory T cells and suppresses pro-inflammatory cytokine production, while it up-regulates IL-10 production. IL-27 has been found to suppress Th1, Th2, and Th17 immune responses in different models of infection and autoimmunity [22-24]. This immunosuppressive property of IL-27 may be important to inhibit excessive inflammation, which leads to organ damage or consequent autoimmunity.

Although the available evidence indicates that human IL-27 is one of the marvelous candidate genes implicated in the pathogenesis and clinical features of T-cell mediated disorders, a relation between IL-27 gene polymorphisms and development of human diseases has been explored in a few reports; however, none of these had studied the Behcet's Disease patient population. The IL-27 polymorphisms have been found to be associated with susceptibility to rheumatoid arthritis [25], allergic rhinitis [26], asthma [27], inflammatory bowel disease [19], and chronic obstructive pulmonary disease [28], but not with type 1 diabetes [29] and various types of cancer [30-32]. Considering the importance of IL-27 as a critical mediator of pro- and anti-inflammatory immune responses, we decided to evaluate two polymorphisms located in this gene in correlation to Behcet's Disease.

MATERIALS AND METHODS

Study population

The study group consisted of 50 Iranian patients with BD [29 (58%) men and 21 (42%) women, range 27-41 years] and one hundred healthy individuals. All patients fulfilled the international study group criteria for BD. Patients with BD were recruited at the Connective Tissue Research Diseases Center of Tabriz University of Medical Sciences. Characteristics of the patients are shown in Table 1. The control group composed of 100 age, gender, and ethnically matched healthy individuals (54% men versus 46% women) without any clinical or laboratory signs of autoimmune or inflammatory diseases. The study protocol was approved by the Ethic Committee of Tabriz University of Medical Sciences and written informed consent was received from participants.

Primer design

IL-27 gene sequence and information about rs153109 and rs181206 SNPs were acquired from The National Center for Biotechnology Information (NCBI) and Ensembl (http://asia.ensembl.org/) databases. For rs153109 SNP, the primer pairs were designed using OLIGO7 Software, (Molecular Biology Insights, Inc., Cascade, CO., USA). In the same way, one common forward primer, and two discriminative reverse primers comprising the polymorphic nucleotides in their 3' ends were designated for rs181206. Primer sequences and specifications are presented in Table 2.

DNA extraction

Genomic DNA was extracted from the whole blood collected in tubes containing EDTA using the standard salting-out method.

Genotyping of IL-27 -964A/G SNP (rs153109)

The rs153109 polymorphism in IL-27 gene was analyzed by the Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) method, utilizing the specific primers. PCR reaction was carried out in a total volume of 25 µL, using the following conditions: initial denaturation at 94°C for four minutes,

then forty cycles at 94°C (40 seconds), 62°C (40 seconds), 72°C (25 seconds), and one final extension at 72°C for five minutes The PCR products (937 bp) were digested with XhoI restriction enzyme at 37°C for 30 minutes. Electrophoresis was performed on a 2% agarose gel and the resulting banding pattern was visualized using safe stain (SinaClon).

Genotyping of IL-27 4730T/C SNP (rs181206)

The IL-27 rs181206 polymorphism was genotyped using Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method. The T and C alleles were amplified using different reverse primers in distinct reactions. A common primer was used as a forward primer in both reactions. These reactions amplify allele-specific sequences of 247 bases of the exon 4 of the IL-27 gene. Additional primers (amplifying a 379-bp fragment from intron 1 of the FOXP3 gene) were included into both reactions to amplify an internal control band. PCR reactions were performed according to the following cycling conditions: 4 minutes at 94°C, followed by forty cycles of 94°C for 40 seconds, 62°C for 40 seconds, 72°C for 25 seconds, and final extension at 72°C for five minutes. PCR products were loaded onto 2% agarose gels before electrophoresis.

The results of both PCR methods were validated by performing a direct sequencing analysis of 25 randomly selected specimens.

Statistical analysis

Statistical analysis was carried out using SPSS statistical software Version 22 (SPSS Inc., Chicago, USA). The association between the genotypes of IL-27 polymorphisms (rs153109 and rs181206) and risk for BD was assessed by calculating the odds ratio (OR) and the 95% confidence intervals (CI). Comparison of alleles and genotypes frequencies between patient and normal groups was performed by chi-square test or Fisher's exact test where appropriate. Differences were regarded as significant in p-value < 0.05 ranges.

RESULTS

To determine whether there is an association between IL-27 SNPs and susceptibility to BD, the genotypes and allele frequencies of the IL-27 polymorphisms were assessed between the BD patients and the healthy individuals. These polymorphisms were genotyped in 100 unrelated healthy controls and 50 unrelated patients suffering from BD (Table 3). The genotype distributions of the two polymorphisms in both patients and normal groups were in agreement with Hardy-Weinberg equilibrium according to χ^2 tests.

Enzymatic digestion of rs153109 (A/G) polymorphism led to a full length PCR 937 bp product for allele A and 570 and 367 bp fragments for allele G. Comparison of the genotype frequencies of IL-27 rs153109 (A/G) polymorphism revealed statistically significant differences between BD patients and normal groups. Similarly, allelic frequencies also exhibited significant changes in the case-control distribution. Our results showed significantly higher frequency of AA genotype (40% versus 22%; p = 0.034) and lower frequencies of GG and AG genotypes (respectively: 12% versus 25% and 48% versus 53%) in BD patients as compared to the healthy individuals. To sum up, our data showed that having the rs153109 A allele was associated with a significantly higher risk of Behcet's Disease as compared to the G allele (p = 0.011).

No significant differences were observed in allele and genotype frequencies of the rs181206 SNP between BD patients and controls. This data indicates that the assessed polymorphism is not associated with susceptibility to BD in the investigated Iranian population.

Furthermore, the genotypes obtained with sequencing analysis and PCR techniques were identical.

DISCUSSION

Recent years brought fundamental growth to our knowledge on BD etiopathogenesis; however, it still remains mostly unknown. Although some genes have been suggested as susceptibility factors of the disease [7], there is no determined hereditary profile that might verify this association. In this study, we investigated the IL-27 gene polymorphisms in BD and demonstrated a novel association between rs153109 polymorphism in the IL-27p28 gene and BD in an Iranian population. The genotype and allele frequencies of rs153109 polymorphism A/A and A were significantly enhanced in BD patients compared with healthy controls, indicating that this genotypic variant may confer the susceptibility to BD in the Iranian population. However, no statistically significant differences were found in the genotype and allele frequencies of rs181206 polymorphism between patients and control groups (Table 3).

IL-27 is a novel IL-12 cytokine family member which acts as a mediator between the innate and adaptive immune systems [33]. IL-27 induces the rapid expansion of antigen-specific naive CD4+ T cells by binding to a heterodimeric receptor composed of WSX-1 and gp-130, promoting the expression of the transcription factor T-bet, which leads to the induction of Th1 type T cells [34]. In addition, IL-27 has been shown to synergize with IL-12 to promote the IFN-γ production and Th1 polarization of naive CD4+ T cells [10]. It can also increase the proliferation and antibody production of B cell subsets [35]. In response to IL-27, mast cells and eosinophils enhance their pro-inflammatory responses by increasing cytokine secretion of IL-1, IL-6, and TNF-α [36]. Thus, it is reasonable to describe a role for IL-27 in promoting inflammation in autoimmune conditions which has been evidenced both *in vitro* and *in vivo*. On the other hand, IL-27 has been found to exert profound anti-inflammatory effects through suppressing

Table 1. The clinical features of BD patients and demographic characteristics of control and patient groups.

Characteristics	BD patients (n = 50)	Controls (n = 100)
Age (mean ± SD) years	34.02 ± 7.39	34.21 ± 8.45
Gender (male:female) (%)	58:42	54:46
HLA-B51-Positive	16 (32%)	-
HLA-B27-Positive	4 (8%)	-
Family history of BD	-	-
Clinical Manifestations		
Major symptoms		
Oral ulcer	47 (94%)	-
Genital ulcer	22 (44%)	-
Skin manifestations		
Erythema nodosum	5 (10%)	-
Pseudofolliculitis	11 (22%)	-
Ocular involvement		
Uveitis (anterior)	27 (54%)	-
Uveitis (posterior)	31 (62%)	-
Cataract	14 (28%)	-
Retinal vasculitis	5 (10%)	-
Minor symptoms		
Arthritis	6 (12%)	-
Central nervous system involvement	1 (2%)	-
Vascular involvement	6 (12%)	-
Skin pathergy test	14 (28%)	-

Table 2. The primer sequences used for amplifying flanking regions of two SNPs studied.

Reference SNP ID	Primer sequence	Product size
rs153109 (A/G)	(F): 5′CAGGTCCGAGGCCATATCTG3′	937 bp
	(R): 5′ATGGAGAGAGCCGAAGCGAG3′	
rs181206 (T/C)	Common forward primer (F): 5′CAGACAGTGGAGATGGAAGGAATG3′	247 bp
	Primer C (R): 5′TCCCCAGCCCTCCCAGCG3′	
	Primer T (R): 5′TCCCCAGCCCTCCCAGCA3′	
Internal Control (IC) Primer (FOXP 3 gene)	(F): 5′CCTCTCCGTGCTCAGTGTAG3′	379 bp
	(R): 5′CACAGCCTGACTGACTGACAT3′	

the production of pro-inflammatory cytokines such as IL-6, TNF-α, IFN-γ, and IL-17 and increasing IL-10 production [22]. IL-27 can also inhibit the responsiveness of macrophages to cytokines such as IL-1 and TNF-α via reducing expression of their related receptors [37]. In addition, it inhibits the differentiation of Th17 cells which leads to suppression of inflammation [34]. Altogether, these findings reflect IL-27 as a crucial candidate gene involved in the pathogenesis of inflammatory based disorders. However, little is known about the impact of SNPs of IL-27. Therefore, we concentrated on association between SNPs of IL-27 and Behcet's Dis-

Table 3. The genotypes and alleles frequencies of IL-27 polymorphisms in BD patients and controls.

SNPs	BD n (%)	Control n (%)	χ^2	p-value	OR (95% CI)
rs153109 (A/G)					
Genotypes					
AA	**20 (40)**	**22 (22)**	**6.745**	**0.034**	
AG	**24 (48)**	**53 (53)**			
GG	**6 (12)**	**25 (25)**			
Alleles					
A	**64 (64)**	**97 (48.5)**	**6.441**	**0.011**	**1.888 (1.152 - 3.092)**
G	**36 (36)**	**103 (51.5)**			
rs181206 (T/C)					
Genotypes					
TT	**25 (50)**	**50 (50)**	**3.261**	**0.196**	
TC	**21 (42)**	**48 (48)**			
CC	**4 (8)**	**2 (2)**			
Alleles					
T	**71 (71)**	**148 (74)**	**0.304**	**0.581**	**0.86 (0.504 - 1.469)**
C	**29 (29)**	**52 (26)**			

χ^2 - chi-square, OR - odds ratio, CI - confidence interval, and p-value is significant < 0.05.

ease as one of the auto inflammatory diseases and explored whether IL-27 could be a prognostic marker for these patients.

The rs153109 (A/G) polymorphism is located in the promoter region of IL-27 gene. Since the promoter is known to be a transcription initiator, the polymorphisms within the promoter region might play a considerable role in modulating transcription and protein expression processes. Numerous reports have demonstrated an association between some genetic related diseases and variations in promoters. In our study, there were significant differences between BD patients and healthy controls in genotype distribution and allele frequencies for the rs153109 polymorphism. As the polymorphic allele rs153109 A and in consequence the rs153109 AA genotype were more commonly observed in patients than in healthy subjects, we can assume that the IL-27 rs153109 polymorphism may be regarded as a genetic risk factor predisposing to BD development in the Iranian population. Consistent with our study, Li [19] and Gorycka et al. [25] performed similar research in Korean and Polish Caucasian populations reporting significant associations between IL-27 gene rs153109 polymorphism and risk of inflammatory bowel disease and rheumatoid arthritis, as examples for autoimmune diseases with strong inflammatory background in which IL-27 is expected to contribute. Likewise, IL-27 rs153109 polymorphism has been found to be correlated with susceptibility to asthma [27], allergic rhinitis [26], and chronic obstructive pulmonary disease [28] in Asians. In contrast to former reports and our current study, some reports indicated that genotype distributions and allele frequencies of rs153109 polymorphism in various types of cancers and T1D were not significantly different from those in normal subjects [29-32]. Discrepancies between findings from reports may be due to limited sample size, heterogeneity of the explored diseases as well as distinct ethnicities. We propose that IL-27 rs153109 A/G SNP existence, by altering either the structure of the transcription factors binding region or transcription initiation site may lead to reduced IL-27 expression level or disruption of IL-27/IL-27R interaction, which causes the initiation of Th1 responses. Additionally, as for the IL-27 gene rs181206 T/C SNP, a missense polymorphism, alteration of T allele to C allele results in the amino acid change from leucine to glutamine. Our findings, similar to previous reports [25,28-30,32], indicated that the IL-27p28 rs181206 polymorphism is not associated with susceptibility to auto-inflammatory disorder, in particular to BD in our population.

There are some limitations to our study, which may contribute to the false negative or positive outcomes. First, our sample size was comparatively small, so this is just a preliminary study. Additional studies on larger patient cohorts would be required to reach an adequate statistical power for these consequences. Second, we did not assess the protein levels of IL-27 in the peripheral blood. Hence, we cannot describe a certain conclusion about the impact of these gene polymorphisms on

the cytokine levels. However, to the best of our knowledge, our study was the first evaluation of the association of IL-27 gene polymorphisms with susceptibility to BD.

CONCLUSION

Taken as a whole, our data demonstrate that the IL-27 gene rs153109 polymorphism might be associated with sensitivity to BD. The polymorphisms in the IL-27 gene have been found to be correlated with other autoimmune diseases. However, there is no consensus on the role of cytokine gene variability. Furthermore, there is no data about genetic susceptibility to BD driven by the IL-27 polymorphisms. Since the exact mechanism through which the IL-27 gene acts on the sensitivity to inflammatory responses is not obvious, it would be attractive to look into the role of this cytokine in the immune response and inflammatory process. The consequences of this research somewhat fill the gap, which might provide insights into our knowledge about the mechanisms governing the pathogenesis of BD and lead to the search for new therapeutic methods.

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Declaration of Interest:
The authors declare no conflict of interest.

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