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Interleukin-33 Gene expression and rs1342326 Polymorphism in Behçet's Disease

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Highlights

- 44 BD cases and 61 healthy controls were enrolled in the case-control study.

- The rs1342326 polymorphism was associated with BD and IL-33 expression significantly increased in patients with BD compared to healthy controls.
- No significant correlation was observed between rs1342326 polymorphism and mRNA expression level of IL-33 with clinical manifestations and BD activity.

Abstract

Objective: Behçet's disease (BD) is a chronic multi-factorial inflammatory disease with the important role of genetic in activation of its inflammatory response. Interleukin (IL)-33 is a member of the IL-1 family of cytokines that affects innate and adaptive immune systems to promote inflammatory responses. In the current study, we investigated the association of IL-33 gene rs1342326 polymorphism and expression levels of this gene in peripheral blood mononuclear cells (PBMCs) with the susceptibility to BD in Azari population of Iran.

Methods: We recruited 44 patients with BD and 61 age and sex-matched healthy controls in this cross-sectional study. The existence of rs1342326 T/G IL-33 gene single nucleotide polymorphism was investigated using Tetra-Amplification Refractory Mutation System (Tetra-ARMS)-PCR. Allele and genotype distributions were evaluated among groups using chi-square or Fisher's test. Moreover, the mRNA levels of IL-33 in PBMCs were assessed through the Real-time PCR.

Results: Patients with BD exhibited a significantly higher prevalence of the T/G genotype of rs1342326 polymorphism compared with the control group. Moreover, the expression level of IL-33 in PBMCs was significantly higher in the BD group compared to the healthy controls. Interestingly, the rs1342326 T/G polymorphism was associated with higher IL-33 expression in patients with BD. There was no association between the clinical manifestation of BD and disease activity with rs1342326 polymorphism and IL-33 expression.

Conclusions: Our study implies that rs1342326 T/G polymorphism of the IL-33 gene may contribute to the genetic susceptibility to BD in part through regulation of the IL-33 expression.

Keywords: IL-33, Behçet Disease, Gene Polymorphism

1. Introduction

Behçet's disease (BD) is a chronic multisystem inflammatory disease characterized by recurrent oral and genital aphthous ulcers, skin lesions, and uveitis. BD is prevalent in countries along the Old Silk Route such as Japan, China, Iran, and Turkey [1, 2]. The etiology of BD is unknown; however, an inflammatory response triggered by environmental factors in a genetically susceptible individual has been proposed as a causative [3]. Microbial agents and vitamin D deficiency are the most important environmental factors [4, 5]. HLA-B51 is has been confirmed as the strongest genetic risk factor for BD in various populations [6, 7]. In addition, several non-HLA genes, such as vitamin D receptor (VDR) [8] , signal transducer and activator of transcription-4 (STAT4) [9] , fork head box P3 (Foxp3) [10], interleukin (IL)-2, IL-4, transforming growth factor (TGF)-beta [11] , IL-27 [12, 13] , IL-23R [14] , tumor necrosis factor (TNF)-alpha [15] , small ubiquitin-like modifier 4 (SUMO4) [16], and Mediterranean fever gene (MEFV) [17] are associated with BD.

Innate and acquired immune components, particularly neutrophil hyperactivity and endothelial damage participate in the inflammatory response of BD. Several cytokines are implied in the immunopathogenesis of BD in part through abnormal inflammatory response. IL-33 is a member of the IL-1 family of cytokines that affect innate and adaptive immune responses [18]. Several immune and non-immune cells such as small airway epithelial and endothelial cells, bronchial smooth muscle cells, macrophages, and dermal fibroblasts can produce IL-33 [19]. Injury to these cells can lead to necrosis and release of IL-33. This cytokine exerts its biological functions by interacting with the ST2 receptor on a variety of immune cells and then triggering intracellular nuclear factor-κβ (NF-κβ) and mitogen activated protein (MAP) kinases. In a study of Hamzaoui K *et al.*, IL-33 has been shown to act as a dual role, both as a traditional cytokine and as a nuclear transcription factor. In addition, it has been shown that this cytokine acts as an "alarmin", which seeks to release the cell necrosis and alert the immune system to tissue damage [20].

IL-33 stimulates IL-2 production by dendritic cells (DCs) and generation of T helper 2 (Th2) cytokines (e.g. IL-4, IL-5, IL-6, and IL-13). It can also activate CD8+ T lymphocytes and $ST2^{+}$ Treg (regulatory T) cells expansion [21, 22]. IL-33 is also an activator of the innate immune system; ST2 receptor expression on the mast cells, basophils, and NK cells makes them responsive

to IL-33. It has been demonstrated that administration of IL-33 to mice leads to an intense eosinophilia [23] and production of superoxide anion and IL-8 [24]. The results of López-Mejías R *et al*. study showed that IL-33 polymorphism in the T allele could have a protective role in the development of atherosclerosis in patients with rheumatoid arthritis [25]. Also, the results of Latiano A *et al.* study indicated that there is a significant association between genotype and allele with IL-33 rs3939286 polymorphism in patients with Crohn's disease and ulcerative colitis [26]. These results may indicate that IL-33 polymorphisms can play a role in the risk of diseases.

In a study conducted by Koca SS *et al.* in 2015, although the results showed that serum IL-33 levels were lower in active BD patients than inactive BD patients, there was no significant difference in terms of genotype and phenotype of the desired polymorphisms (rs1157505 and rs1929992) [27]. While the results of the study, Çerçi P *et al*., were the opposite, the serum level of IL-33 in active BD individuals was higher than inactive BD individuals [21]. Also, the results of Kim DJ *et al.* showed that serum IL-33 levels were higher in BD patients than in healthy subjects [22]. Considering the importance of rs1342326 polymorphism in various studies, we have tried to investigate the effect of different alleles on IL-33 expression and its effect on the disease process. In most studies, the proposed rs1342326 is effective in inflammation and inflammatory diseases [28, 29]. In another study, rs1342326 C allele was associated with an increase in the level of interleukin-33 expression in children [30].

Although several polymorphisms in multiple immunoregulatory genes recognized as a risk factor for developing BD, many other gene polymorphisms remain elusive to resolve the specific genetic susceptibility to BD. Despite the evidences implying the role of IL-33 in the pathogenesis and clinical features of T-cell mediated disorders such as rheumatoid arthritis and inflammatory bowel disease [26, 29], little is known about the relation between IL-33 gene polymorphisms and BD in different populations. This study had been designed to investigate the association of IL-33 gene rs1342326 polymorphism, and also IL-33 expression by peripheral blood mononuclear cells (PBMCs) with susceptibility to BD in Azari population of Iran.

2. Materials and methods

2.1. Samples

The present study was approved by the Ethical Committee of the University of Medical Sciences Tabriz, Iran. A total of 44 patients with BD (17 females, 27 males) and 61 (24 females, 37 males) age and sex-matched healthy controls were recruited in this cross-sectional study from June 2015 to May 2016. This study was BD diagnosis was performed by the International Criteria for BD, at the Connective Tissue Diseases Research Center (CTDRC) of Tabriz University of Medical Sciences (TUOMS), approved by the local ethic committee of TUOMS which was in compliance with the Helsinki declaration. All patients signed the written informed consent. The activity of BD was assessed by the Iranian BD Dynamic Activity Measure (IBDDAM) and Total Inflammatory Activity Index (TIAI) [31, 32]. Patients with pan-uveitis, pan-ophthalmitis, vasculitis, and central nervous system involvement grouped as severe BD.

2.2. Primer design

IL-33 gene sequence and data about rs1342326 single nucleotide polymorphisms (SNPs) were achieved from the National Center for Biotechnology Information (NCBI) and Ensembl (http://asia.ensembl.org/) databases. The primer pairs for IL-33 mRNA sequence 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 including the polymorphic nucleotides in their 3' ends were designated for rs1342326. Primer sequences and specifications are presented in Table 1.

Table 1. PCR primers and product size used in this study

Primers		**Primer sequence(5′–3′)**	**Amplicon size**
Tetra ARMS primers for Rs1342326 G/T genotyping	Forward outer primer	AATCAAGTGTCCATTTACTCAATA	429 bp
	Reverse inner primer (G allele)	TATAAATAAGAATAAGAGGTCATAC	
	Forward inner primer (T allele)	ATCTTTTCTCATGAAGACACCCT	684 bp
	Reverse outer primer	AACCCTTACTTAGTGACAGCCT	

Internal Control Primer	AATCAAGTGTCCATTTACTCAATA AACCCTTACTTAGTGACAGCCT	1066 bp
IL-33 (relative expression)	GTGACGGTGTTGATGGTAAGA CTCCACAGAGTGTTCCTTGTT	92 bp
β-actin (Normalizer for qRT-PCR)	GGTGAAGGTGACAGCAGT TGGGGTGGCTTTTAGGAT	154 bp

bp: base pair, quantitative real-time PCR

2.3. DNA and RNA extraction and RT-PCR

PBMCs were prepared from EDTA treated blood tubes by Ficoll (Lymphodex, Inno -Train, Germany) density-gradient centrifugation and stored at −80 °C until use. Genomic DNA samples of patients with BD and healthy controls were extracted using the standard salting-out method. Total RNA was extracted from the PBMCs using TRIzol (Invitrogen, San Diego, CA). Purity and concentration of total RNA were then assessed by Nanodrop ND1000 and at 260-280 nm. The entirety of total RNA was shown by gel electrophoresis of the individual samples on a 1% agarose gel. cDNA synthesis was performed by reverse transcription reagent kit (Thermo Fisher Scientific, USA).

2.4. Genotyping of IL-33 –T/G SNP (rs1342326) by Tetra-ARMS-PCR

The rs1342326 SNP was assessed by Tetra-ARMS-PCR. Primer sequences are shown in Table 1. The T and G alleles were amplified using different Tetra-ARMS PCR method. The outer primers were used for amplification of a 1066-bp internal control fragment (Table 1). PCR reaction was carried out in a total volume of 25 µL, using the following conditions: initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 35s, annealing temperature was 60°C (40 seconds), extension at 72°C for 30s, and one final extension step at 72°C for five minutes. Electrophoresis was performed on a 2% agarose gel and the resulting banding pattern was visualized using safe stain (SinaClon). The frequency of a given genotype was evaluated by direct counting.

2.5. Real-time PCR

The expression of IL-33 was measured by Rotor-gene 6000 real-time instrument (Corbett, Foster City, CA, USA) and was determined using the SYBR Premix Ex Tag II kit (Takara Bio Inc, Japan)

and normalized to β-actin mRNA. Relative expression levels were evaluated using the $2^{-\Delta\Delta CT}$ method. The following sequences of the sense and antisense primers of truncated IL-33 were used: forward 5′-GTGACGGTGTTGATGGTAAGA-3′ and reverse 5′-CTCCACAGAGTGTTCCTTGTT-3 ′. For the internal reference gene, the relative expression level of IL-33 mRNA was normalized by the expression of β-actin mRNA in each sample. Its expression was detected by the following primers (Table 1).

2.6. Statistical analysis

Statistical analysis was performed using SPSS software version 17 (SPSS, Chicago, IL, USA). The association between the genotypes of IL-33 rs1342326 T/G polymorphisms and risk of BD was assessed by calculating the odds ratio (OR) and the 95% confidence intervals (CI). Normal distributions were tested with the Kolmogorov–Smirnov test with Lilliefors correction. Quantitative data were presented as the mean ± standard deviation (SD) or median (minimum-maximum). The association between the genotypes of IL-33 polymorphisms rs1342326 T/G and risk for BD were tested for consistency with the Hardy-Weinberg equilibrium. Allelic and genotypic associations of SNPs were performed by Pearson's x2 test (or Fisher's when appropriate) followed by odds ratio and 95% CI. P-values of less than 0.05 were considered significant.

3. Results

We studied IL-33 rs1342326 polymorphism in accordance with its expression levels in PBMC samples derived from 44 patients with BD and 61 healthy controls. The demographic and clinical features of the patients and controls are summarized in Table 2.

Table 2**.** Demographic and clinical features of patients and controls

	BD (n = 44)	Controls (n = 61)	P value
Age (years)	38.1 ± 10.3	37.4 ± 8.5	NS
Male (%)	27 (61.4)	37 (60.6)	NS
Female (%)	17 (38.6)	24 (39.4)	NS
HLA-B5 (%)	31 (70.4)	-	-
HLA-B51 (%)	25 (56.8)	-	-

HLA-B27 (%)	4 (9.09)	-	-
Oral ulcer (%)	42 (95.4)	-	-
Genital ulcer (%)	23 (52.2)	-	-
Eye involvement (%)	35 (79.5)	-	-
Pseudofolliculitis (%)	11 (25)	-	-
Arthritis (%)	9 (20.4)	-	-
Erythema nodosum (%)	7 (15.9)	-	-
Vasculitis (%)	5 (11.3)	-	-
Severe disease (%)	30 (68.1)	-	-
IBDDAM	2.4 ± 1.1	-	-
TIAI	11.4 ± 8.3	-	-

Data illustrated as mean ± SD. IBDDAM; Iranian Behçet's disease Dynamic Activity Measure, TIAI; Total Inflammatory Activity Index

3.1. Associations between the rs1342326 T/G polymorphism of the IL-33 gene and BD

The genotype distribution of the examined SNP in the IL-33 gene was in Hardy-Weinberg equilibrium in both the BD and control groups. There was no statistically significant difference in the amounts of missing genotype data between patient and control groups ($p>0.05$). There were significant differences between patients and control subjects regarding the frequencies of rs1342326 SNP. Patients with BD exhibited a significantly higher prevalence of the TG genotype of rs1342326 polymorphism compared with the control group (Table 3).

Table 3. The distribution of allele and genotype frequencies of rs1342326 polymorphism of IL-33 gene in BD patients and healthy controls under Co-Dominant, Recessive and Dominant models.

dbSNP	**Frequency**							
		BD (%)	**Controls (%)**		**BD (%)**	**Controls (%)**	**Genotypic**	**Allelic**
rs1342326 [G/T]	**Genotype**	**N=44**	**N=61**	**Allele**	**N=44**	**N=61**	**P-values**	**P-values**
	TT	18 (40.9)	42 (68.9)	G (MAF)	28 (31.8)	20 (16.4)	0.016*	0.012*
	GT	24 (54.6)	18 (29.5)	T	60 (68.2)	102 (83.6)		
	GG	2 (4.5)	1 (1.6)	GG+TT *vs.* GT	OR (95%CI) : 2.86 (1.27- 6.43)		0.015*	
				GT+TT *vs.* GG	OR (95%CI) : 2.85 (0.251-32.53)		0.398	
				GT+GG *vs.* TT	OR (95%CI) : 0.313 (0.139-0.703)		0.005*	
HWE P-values		0.088	0.55	**OR (95%CI) : 2.38 (1.23-4.58)**				

MAF: minor allele frequency, HWE: Hardy Weinberg Equilibrium, BD: Behçet's disease, OR: odds ratio, CI: confidence interval, dbSNP: database of single nucleotide polymorphisms, *P<0.05.

3.2. Expression of IL-33 in the PBMCs

The expression level of IL-33 by PBMCs was significantly higher in the BD group compared to the healthy controls (Fig 1). There was no significant difference in the mRNA expression levels of IL-33 in the patients with severe and ophthalmic BD compared to mild and non-ophthalmic BD, respectively ($p>0.05$). The effect of the genotype on the IL-33 mRNA expression was tested in the BD and control groups. As shown in Fig 2, rs1342326 T/G polymorphism was associated with higher IL-33 expression in patients with BD. The expression of IL-33 showed no significant difference between TT and TG/GG carriers of rs1342326 in healthy controls.

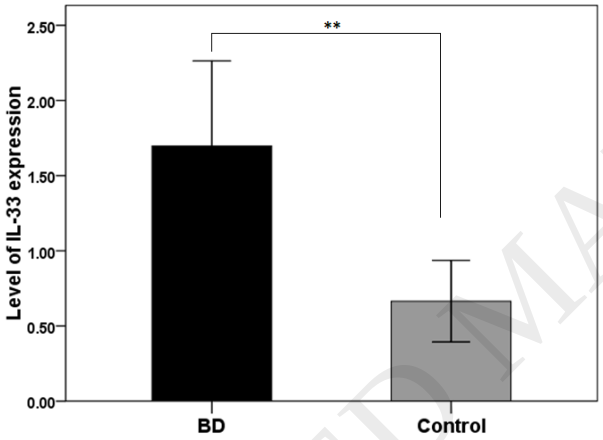

Figure 1. IL-33 expression in PBMCs derived from BD was significantly higher than in healthy individuals. Data are shown as mean ± SD. $P < 0.01$.

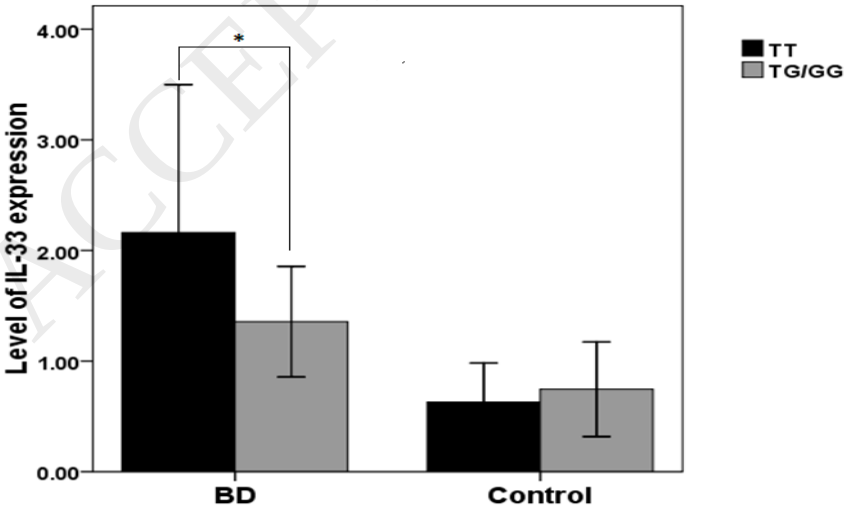

Figure 2. Effect of genotype on IL-33 mRNA expression of rs1342326 T/G. BD samples showed an increased IL-33 expression compared with control samples. Data are shown as mean± SD. P < 0.01; P < 0.05.

3.3.The relationship between demographic characteristics and genotypes

This analysis was performed on patients with BD. The results showed that there were no significant differences in the level of IL-33 expression among individuals for different genotypes (TT, TG, GG) for male, female, HLA-B51, oral pest, ulcer Genital, severe BD, EN subgroups (P>0.05). If the level of IL-33 expression was significantly different in the subjects for severe eye involvement and HLA-B5 subgroups with different genotypes (TT, TG, and GG). In the TG genotype, the level of IL-33 expression was lower than that of the TT genotype.

Table 4. The relationship between demographic characteristics and genotypes

Characteristics and Clinical features expression	Frequency	Change fold of IL-33 expression (mean ± SD)	P-value
Male			
TT	11(39.3%)	1.98±1.88	0.112
TG	16(57.1%)	1.05±0.85	
GG	1(3.6%)	---	
female			
TT	7(43.8%)	2.44±3.79	0.309
TG	8(50%)	1.61±1.07	
GG	1(6.3%)	---	
HLA-B5-			
TT	9(33.3%)	3.71±4.03	**0.048**
TG	18(60%)	1.09±0.63	
GG	1(6.7%)	---	
HLA-B51			
TT	7(25%)	7.08±5.17	0.072
TG	17(62.5%)	1.18±0.57	
GG	1(12.5%)	---	
Oral aphtha			
TT	17(40.5%)	2.28±2.71	0.698
TG	23(54.8%)	1.27±0.95	
GG	2(4.8%)	2.73±3.67	
Genital ulcer			
TT	10(43.5%)	2.67±3.17	0.232
TG	11(47.8%)	0.86±0.67	
GG	2(8.7%)	2.73±3.67	
Folliculitis			
TT	3(27.3%)	1.85±2.6	0.999

TG	8(72.7%)	0.81±0.72	
GG	0	---	
Sever B.D			
TT	9(33.3%)	2.64±3.36	0.186
TG	17(63%)	1.24±1.05	
GG	1(3.7%)	---	
Severe eye involvement			
TT	2(25%)	3.82±1.44	**0.046**
TG	6(75%)	1.16±0.94	
GG	0	---	
EN			
TT	4(57.1%)	3.55±4.83	0.999
TG	3(42.9%)	1.26±0.25	
GG	0	---	

4. Discussion

In the present study, IL-33 gene rs1342326 polymorphism and IL-33 mRNA expression levels were evaluated in the Iranian Azari patients with BD. Our study showed that IL-33 rs1342326 polymorphism was associated with BD and IL-33 expression significantly increased in patients with BD compared to healthy controls. We found no significant correlation between rs1342326 polymorphism and mRNA expression level of IL-33 with clinical manifestations and BD activity.

In accordance with our data, previous studies in other ethnic groups have shown an association between BD and IL-33. For instance, Kim et al. showed that IL-33 and soluble ST2 (sST2) are highly expressed in Korean patients with BD and further demonstrated that sST2, but not Il-33 was associated with BD activity [22]. In their study, serum sST2 but not IL-33 was associated with BD activity. They also found high expression of IL-33 and sST2 in the skin of patients with BD. Moreover, there was a correlation between IL-33 and sST2 with thrombosis and gastrointestinal system involvement. Hamzaoui *et al.* in their study on 46 patients with BD found higher IL-33 level in patients with active disease compared to patients with inactive disease and healthy controls. Patients with retinal vasculitis had the highest serum levels of IL-33 [33]. They also found a higher IL-33 level and IL-33 mRNA expression in cerebrospinal fluid of patients with neuro BD compared with patients who had the non-inflammatory neurological disease and patients with headache attributed to BD in another study [34]. In the cohort study on Turkish patients with BD, there were rs7044343 and rs11792633 polymorphisms of IL-33 gene, however, there was no significant difference in genotypic and allelic distributions of rs1157505 and rs1929992

polymorphisms between the BD and control groups [27]. In contrast to the previously mentioned studies, there was no significant difference in the serum level of IL-33 in BD and control groups. Interestingly serum level of IL-33 in patients with active BD was lower than patients with inactive BD and normal subjects.

Our study was the first study about the IL-33 SNPs in the Azari population of Iran. Evaluation of serum levels of IL-33 could confirm mRNA results. Moreover, investigation of other SNPs of the IL-33 gene could help to high-resolution clarification on the effect of genetic factors on susceptibility to BD. In conclusion, our study showed that rs1342326 T/G polymorphism of the IL-33 gene may contribute to the genetic susceptibility to BD by regulating the expression of IL-33.

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Conflict of interest

The authors confirm that this article content has no conflict of interest.

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