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Molecular Analysis of Interleukin-10 Gene Polymorphisms in Patients with Behçet's disease

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Abstract:

Background: Interleukin 10 (IL-10) is a cytokine with potent anti-inflammatory properties that play a fundamental role in restrictive host immune response to pathogens, by means of that is a crucial importance for chronic inflammatory disease studies. Therefore, the goal of this study was to measure the correlation of the IL-10 gene polymorphisms with the susceptibility to Behçet's disease compared with the control group in the Azeri population and to determine the expression of this gene in the two groups. Also, real-time PCR was performed for evaluate the IL-10 mRNA expression of the associated polymorphisms.

Methods: In this study, blood samples from 47 (1 missed) patients and 58 (3 missed) healthy control were taken, and then mononuclear cells isolated with ficoll protocol. The DNA and RNA were subsequently extracted. They were examined for -592A/C (rs1800872) of IL-10 gene single nucleotide polymorphism (SNP) using RFLP-PCR. Allele and genotype distributions were evaluated among groups using chi-square or Fisher's test. Following this, the extracted RNA was converted to cDNA using the RT-PCR method, after that expression of IL-10 evaluated by Real-

time PCR. Serum levels of IL-10 were measured using Enzyme-linked immunosorbent assay (ELISA).

Results: Rates of the rs1800872 A allele was statistically lower in the control group compared with BD patients (p = 0.0315 and OR = 1.90 (1.05-3.42)). Also, as we expected, the expression level of the IL-10 gene was seen to significantly decrease in the patient group compared to the control.

Conclusions:

Our study showed that the rs1800872 A allele of the IL-10 gene may contribute to the genetic susceptibility of BD by regulating the expression of IL-10. Also as we expected, the expression level of this gene was seen to significantly decrease in the patient group compared to the control.

Key word: rs1800872 SNP, Behçet's disease, IL-10, Azeri population

1. Introduction:

Behçet's disease (BD) is one of the chronic multi-systemic inflammatory disorders of complex pathologic process characterized by oral and genital ulcers, skin lesions, and severe ocular inflammation. It may also affect digestive, cardiovascular, nervous systems and involvement of joints and large vessels[1, 2]. The etiology of BD is largely mysterious; however, an inflammatory response triggered by genetic, immune, and unknown environmental factors in a genetically susceptible individual has been proposed as the cause of BD. Also the pathologic causes of BD remains unclear, it is currently thought a genetic factor coupled with a triggering event may play a crucial role in its progress [3, 4]. Among all genetic factors, HLA-B51 has been confirmed as the strongest risk factor for BD, which was verified in various populations [5]. The high prevalence of BD along the Old Silk Route such as Japan, China, Iran, Turkey, and the Mediterranean region [4, 6].

More recently studies have disclosed the association of many non-HLA genes with the BD, such as endoplasmic reticulum amino peptidase 1 (ERAP1), chemokine receptor 1 (CCR1) [7], Vitamin D Receptor (VDR) [8], signal transducer and activator of transcription-4 (STAT4) [9], fork head box P3 (Foxp3) [10], IL-2 , IL-4 , transforming growth factor (TGF)-beta [11], IL-27 [12], IL23R [13], tumor necrosis factor (TNF)-alpha [14], the small ubiquitin-like modifier 4 (SUMO4) [15], and Mediterranean fever gene (MEFV) [16]. Also more studies have identified

polymorphisms in multiple immunoregulatory genes as a risk predisposition for developing BD. Thus, it is sensible to theorize about that genetic polymorphisms which effect the production of clear cytokines could be seen as significant factors of susceptibility and severity to BD[17].

Interleukin-10 (IL-10) is a Type II cytokine and the member of a family of cytokines that include IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29[2, 18]. IL-10 is an anti-inflammatory cytokine that has been associated in several immune-mediated inflammatory diseases and it is an immune-inhibitory cytokine that plays a significant role in innate and adaptive immune systems [19, 20]. The human IL-10 gene is located on chromosome 1q21–32 and comprises 5 exons that are separated by four introns. IL-10 is greatly secreted in four major cells which they are T-helper type 2 (Th2) cells, subsets of regulatory T cells designated Tr1, Th1, and Th17 cells. [21] Also it is secreted by macrophages, dendritic cells, cytotoxic T cells, B lymphocytes, monocytes and mast cells [22].

This immunosuppressive characteristic of IL-10 may be essential to restrain extreme inflammation, which leads to organ impairment or subsequent autoimmunity[23].

IL-10 has been recognized pathologically in the active lesions of BD and as of stimulated T cells and serum from patients with the disease. IL-10 might act in the primary phase of bacterial or viral infection or at sites of tissue injury, as a strong stimulator of NK cells and of macrophage recruitment. Disappointment of an adequate innate response at this phase may lead to a disaster to perfect an infective emphasis resulting in lengthy immunologic motivation. As the adaptive immune response progresses, IL-10 suppresses the pro-inflammatory function of macrophages by antagonizing expression of costimulatory molecules and the release of proinflammatory cytokines consequently restrictive the motivation of Th1 cells. A failure to down-regulate this part of adaptive immunity accurately would cause to a strong Th 1 profile, exposed to be current in the disease [24].

Although the abundant evidence demonstrates that human IL-10 is one of the marvelous candidate genes implicated in the pathogenesis and clinical features of T-cell mediated disorders, and a relation between IL-10 gene polymorphisms and expansion of human diseases has been investigated in a few papers; however, none of these had considered the Behçet's disease patient population. Thus, the aim of the present study was to detect the serum IL-10 level and the potential association of IL-10 gene polymorphisms on the susceptibility, in BD.

2. Methods and materials:

2.1. Patients and healthy controls study population:

All subjects presented their written informed agreement for this study, and the study protocol was permitted by the ethics committee in Tabriz University of Medical Sciences, Tabriz, Iran (Permit Number: TBZMED.REC.1395.1320). The study group consisted of 47 Iranian patients with BD [29 (61.7%) men and 18 (38.3%) women, range 16-60 years] and 61 healthy candidates. The analysis of BD was based on the international study group criteria for BD. [25] Features of the patients were evaluated at the time of diagnosis and are summarized in **Table 1**. Patients with BD were employed at the Connective Tissue Research Diseases Center of Tabriz University of Medical Sciences. The control group composed of 61 age, gender, and ethnically matched healthy individuals (59% men versus 41% women) without any clinical or laboratory signs of autoimmune or inflammatory diseases.

Also, we measured BD activity by the Iranian Behçet's disease Dynamic Activity Measure (IBDDAM) and Total Inflammatory Activity Index (TIAI) [26]. In order to calculate IBDDAM, points were given to the non-ophthalmic manifestations of BD according to its severity and duration. Total scores are between 0 and 32. Total Inflammatory Activity Index (TIAI) is a tool for measuring ophthalmic disease activity. In order to calculate TIAI, a questionnaire was filled by an ophthalmologist. Eye lesions of BD (in every segment of eye) are graded from 0 (normal) to 4 (maximum inflammation). Total scores are between 0 and168. Patients with panuveitis, panophthalmitis, vasculitis, and central nervous system involvement grouped as severe BD [26, 27].

2.2. DNA, RNA extraction and RT-PCR:

Peripheral blood mononuclear cells (PBMCs) were prepared from EDTA blood tubes by Ficoll (Lymphodex, Inno -Train, Germany) density-gradient centrifugation and immediately stored at −80 °C until use. Genomic DNA samples of BD and healthy controls were extracted by using the rapid genomic DNA extraction (RGDE) method from the peripheral blood collected in tubes containing EDTA. Total RNA was extracted from the PBMCs according to the protocol of TRIzol (Invitrogen, San Diego, CA), followed by reverse transcription using the reverse transcription reagent kit (Thermo Fisher scientific, USA). Then, purity and concentration of total

RNA were estimated by nanodrop ND1000 and at 260-280 nm purity of RNAs were assessed. The entirety of total RNA was showed by gel electrophoresis of the individual samples on a 1.5% agarose gel.

2.3. Genotyping for IL-10 gene polymorphisms:

IL-10 (-592A/C) SNP was analyzed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay. The forward and reverse primers for IL-10 -592A/C rs1800872 were 5′-ACGGTAGGGGTCATGGTGAG-3′ and 5′-CAATGGGATTGAGAAATAATTGG-3′ respectively. The restriction enzyme for PCR product (384 bp) was *Rsa*I for IL-10 -592A/C at 37°C for 30 minutes. PCR reactions were carried out in a total volume of 25 µL, with an initial denaturation step of 5 minutes at 94°C, followed by 40 cycles at 94°C for 35 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 5 minute. 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 (**Figure 1**).

2.4. Primer design:

IL-10 gene sequence and data about promoter were picked up from the National Center for Biotechnology Information (NCBI), eukaryotic promoter database (EPD) and Ensembl (http://asia.ensembl.org/) databases. For IL-10 mRNA sequence, the primer pairs were designed using OLIGO7 Software, (Molecular Biology Insights, Inc., Cascade, CO., USA). In the same way, one pair primer including the polymorphic nucleotides in their 3’ ends was designated for rs1800872. Primer sequences and specifications are presented in **Table 2**.

2.5. Real-time PCR:

Peripheral blood mononuclear cells (PBMCs) were prepared from EDTA blood tubes by Ficoll (Lymphodex, Inno -Train, Germany) density-gradient centrifugation. Total RNA was extracted from the PBMCs using TRIzol (Invitrogen , San Diego, CA), followed by reverse transcription using a the reverse transcription reagent kit (Takara Biotechnology Co., Ltd., Ostu, Japan) and then the expression of IL-10 was measured by rotor gene 6000 real-time instrument (corbett, Foster City, CA, USA). The following sequences of the sense and antisense primers of IL-10 were used: forward 5′ – GTTGAGCTGTTTTCCCTGA-3′ and reverse 5′-

TGAAGTGGTTGGGGAATGAG-3′. For the internal reference gene, b-actin was chosen and its expression was detected by the following primers: forward 5′- GGTGAAGGTGACAGCAGT-3′ and reverse 5′ - TGGGGTGGCTTTTAGGAT -3′. Relative expression levels of IL-10 were calculated using the ΔΔCt method.

2.6. Enzyme-linked immunosorbent assay (ELISA):

Serum samples were individually collected from all patients and controls, plasma separated by centrifugation at 1500 rpm for 15 min at 4 °C, aliquotted, and stored at −80 °C until cytokine analysis. Serum levels of IL-10 were measured with ELISA kit that is specifically designed for these IL-10 cytokine (Catalog No.EK0416, BOSTER), according to the manufacturer's instructions. The intensity of the developed color was measured by reading optical absorbance at 450 nm using a microplate reader (SunriseTM, Tecan Group Ltd. Männedorf, Switzerland)). The ELISA reader-controlling software (Softmax) managed the digital data of raw absorbance values into a standard curve, from which the IL-10 concentration of the samples was derived. Results were expressed as picogram of cytokine per milliliter plasma (pg/ml).

2.7. Statistical analysis:

Statistical analysis was performed using SPSS software version 22.0 (SPSS, Chicago, IL, USA). The association between the genotypes of polymorphism -592A/C (rs1800872) and risk for 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 mean ± standard deviation (SD) or median (minimum–maximum). The association between the genotypes of IL-10 polymorphism (rs1800872) 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:

3.1. Subject Characteristics

Demographic and clinical characteristics of contributors are existed in **Table 1**. The patient group consisted of 29 males and 18 females, with a mean age of 38.02 ± 10.25 years. The control

subjects included 37 males and 24 females and had a mean age of 37.4 ± 8.5 years. No significant difference was observed in age between patients with BD and controls ($P > 0.05$).

3.2. Associations between the rs1800872 A/C of the IL-10 gene and BD

The genotype distributions of the one examined SNPs in the IL-10 gene were in Hardy-Weinberg equilibrium in both the BD and control groups. There were no statistically significant differences in the amounts of missing genotype data between cases and controls (p>0.05). The results of genotypic and allelic frequency analysis are shown in **Table 3**. There were significant differences between BD patients and controls concerning the frequencies of SNPs rs1800872. BD patients indicated a significantly lower prevalence of the AC genotype (pc=0.001; OR=0.435, 95% CI=0.313-0.604) and A allele (pc=0.0315; OR=1.90, 95% CI=1.05-3.42) of rs1800872, compared with the controls. The results of genotypic and allelic frequency analysis are shown in Table 3.

3.3. Associations between the rs1800872 A/C and expression and serum levels of the IL-10

In this section, we used Chi-square test to investigate the relationship between different genotypes of polymorphism and gene expression as well as serum IL-10 levels. We first calculated the level of gene expression and serum levels of IL-10 in terms of patient group and healthy control group (**Table 4**). In general, regardless of the group, there was a positive correlation between the expression levels and the serum levels of IL-10, and it was 88% (p-value <0.001). Also, if we compare this correlation in two groups, there is no significant correlation.

3.4. Real-time Quantitative PCR for IL-10 gene:

In order to compare the level of IL-10 gene expression in two groups of BD and healthy subjects, an independent T-test was adopted, taking into consideration the data obtained by employing kolmogorov smirnov test, was normal (p-value>0.05). The obtained results were indicative of significant difference between two groups (p-value<0.05). As we expected, the level of gene expression showed reduction in BD individuals group in comparison with healthy group (**Figure 2**).

3.5. Plasma levels of IL-10 in BD patients:

A significant decrease ($P < 0.0001$) in IL-10 in BD patients compared to healthy controls ($3.26 \pm .36$ versus $5.4 \pm .48$) was revealed. The disease was significantly associated with reduction of IL-10 ($P < 0.01$) secretion level.

As shown in **Table 5**, the mean plasma concentration of IL-10 -592 A/C was decreased significantly (P-value<0.0001) in BD patients with AA and AC genotypes; respectively. These data are compatible with those formerly reported.

4. Discussion:

Prior studies have clearly indicated the importance of cytokines in the pathophysiology of BD. Behçet's disease, as an auto-inflammatory disease, mainly characterized by uveitis, oral and genital ulcers, and skin lesions. In spite of wide study, the pathogenesis of BD remains not completely understood. It has been proposed that several genetic factors and others are involved in the progress of BD.

In our study, we evaluated the association between gene polymorphisms and gene expression of IL-10 in BD patients then compared with healthy controls. It is well known that IL-10 gene is an important cytokine for inhibition of inflammation through target Th1-type immune responses [28]. Also, promoter polymorphisms of genes encoding crucial molecules in the disease improvement and development are likely to exhibit vital factors in the amplification of intrinsic biological differences, resulting in clinically distinct outcomes. It has been described that IL-10 SNPs may effect immune role through controlling the activities of NK, T cells (Th1/Th2/Th17) and macrophages and thus alter the disease progression [20].

IL-10 was revealed to be an effective cytokine-regulating auto-inflammatory response [29, 30]. IL-10 regulates T cell function by controlling the gene expressions of proinflammatory cytokines, such as TNFα, IL-12, IL-1, IL-12, IL-8 and IL-33 [31]. Polymorphisms of IL10 gene were related with some immune-related diseases, including Crohn's disease [32], rheumatoid

arthritis [33], systemic sclerosis [34], and Graves' disease [35]. Several studies from Turkey, Japan and Iran have also recognized IL-10 as a risk factor for BD [36-38].

Our outcomes displayed that allelic frequencies and genotypic distributions at the IL-10 (-592 A/C) polymorphisms did differ between BD patients and the control group. Also, gene expression and plasma level of IL-10 are differing at groups as the level of IL-10 expression decreased in BD patients compared with healthy groups. BD patients indicated a significantly lower prevalence of the AC genotype (P-value<0.001; OR=0.435, 95% CI=0.313-0.604) and A allele (pc=0.0315; OR=1.90, 95% CI=1.05-3.42) of rs1800872, compared with the controls. It has been shown that the -592 AA causes a decrease in IL-10 production. This genotype is efficient of producing less IL-10 and also may explain the high IL-10 serum levels in patients with BD. This is suitable with the idea that the IL-10 cytokine has an essential genetic and/or functional association in the pathogenesis of BD in Iranian patients.

Polymorphism of IL-10 gene is as a candidate because of its polymorphisms were based on the relations reported for most autoimmune diseases [32, 35]. The other SNPs, including rs1800871, rs1800872, and rs1554286, showing a relationship in other study were in a strong linkage disequilibrium with each other [39]. Therefore, we focused on rs1800872 in one part of our study. One of the most important SNPs (- 592 C/A) in the promoter region of the IL10 gene have been indicated to change IL10 mRNA and plasma protein levels [40]. In conclusion, our study demonstrated rs1800872 of IL-10 was possibly related with BD. However, it is imprecise whether or how this SNP plays a role in BD. To explain this question, further studies should be carried out.

Conflict of Interest:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure Captions

Figure 1: IL-10 (-592A/C) PCR products of three samples. Lane (1*) 100 bp ladder, Sample 1 in lane (2, 4, 5 and 7) AC genotype, sample 2 in lane (6, 8 and 9) AA genotype and sample 3 in lane (3) CC genotype.

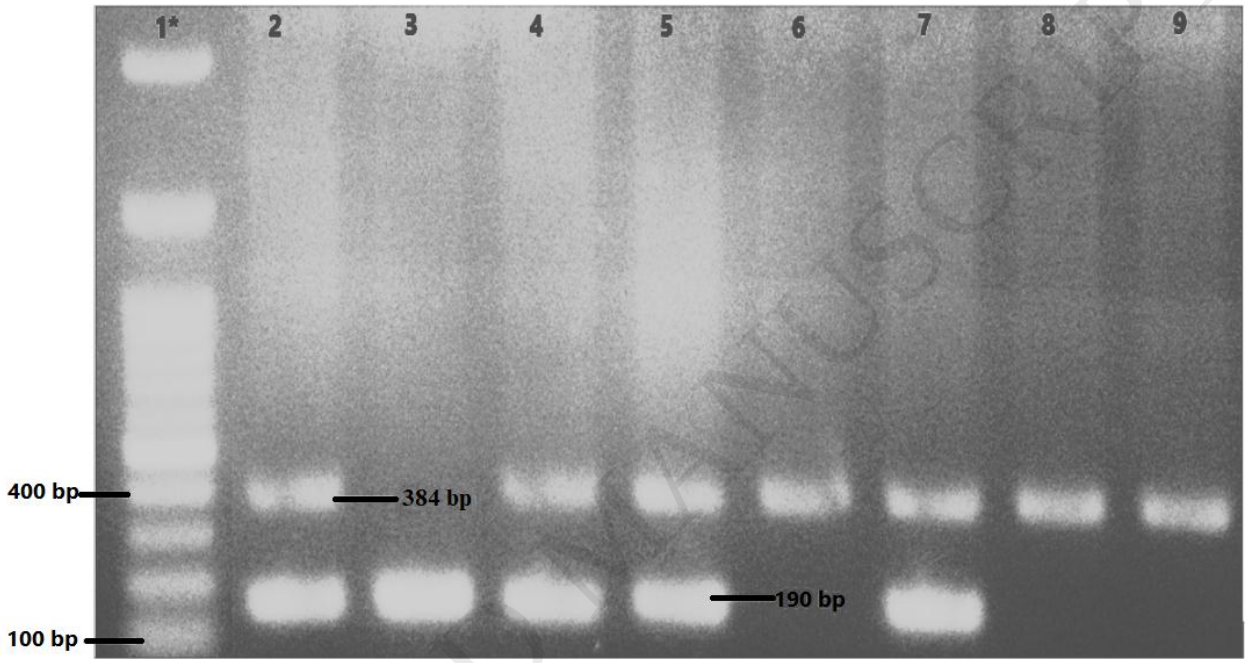
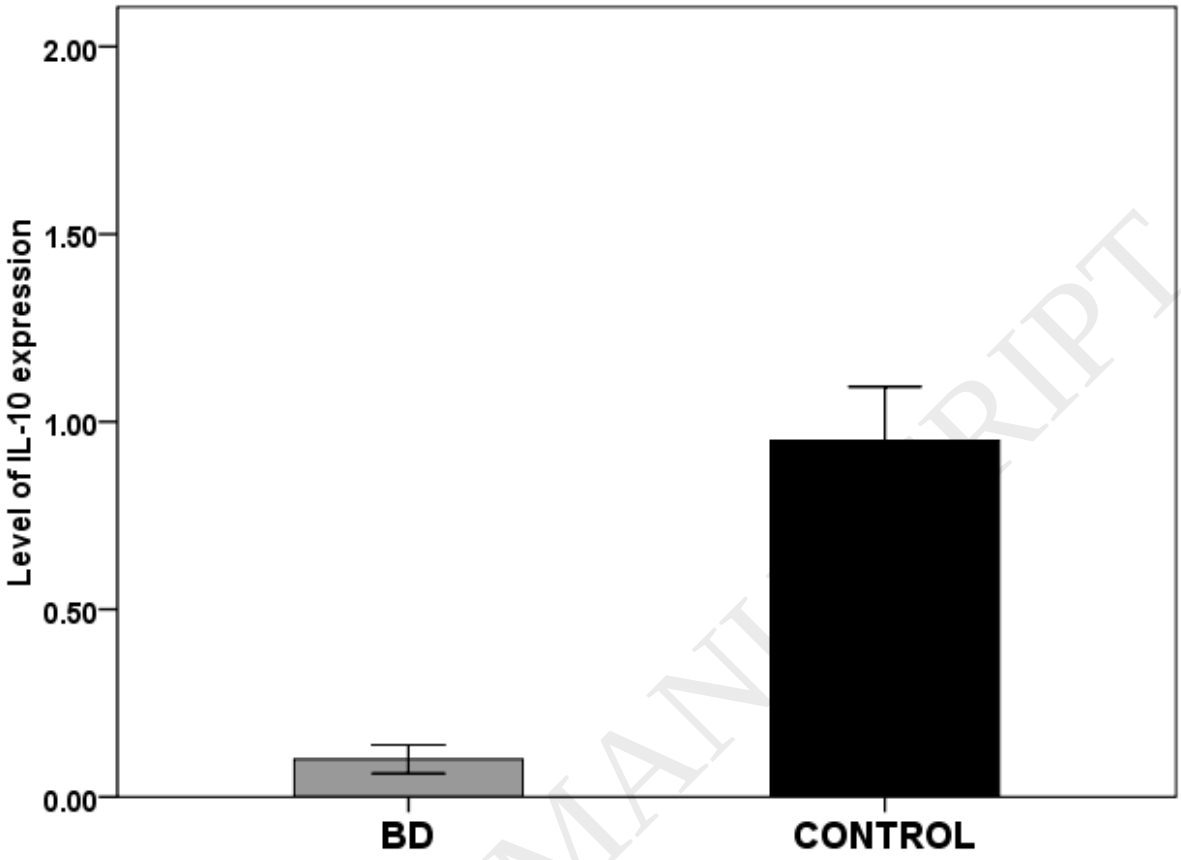


Figure 2. Change fold of IL-10 expression. Regarding the average changes in the expression of the IL-10 gene in the patients and the healthy groups, the amount of it is comparable to that of the healthy group in the patient group, which indicates that IL-10 gene expression was reduced among the patients in the patient group



Tables

Table 1- Demographic and clinical features of participants

Characteristics and Clinical features expression	Frequency	Change fold of IL-10 expression (mean ± SD)	P-value	Serum levels of IL-10 (mean ± SD)	P-value
Age					
<45	31 (63%)	0.18±0.04	0.15	3.03±0.35	0.18
≥45	15 (28%	0.16±0.03		2.86±0.38	
Gender					
Male	(29) 61.7%	0.17±0.04	0.77	2.9±0.37	0.73
female	(18) 38.3%	0.18±0.05		3.04±0.35	
HLA-B5-					
Positive	17 (33%)	0.16±0.03	0.07	3.02±0.36	0.42
Negative	9 (21%)	0.19±0.03		2.8±0.38	
HLA-B51					
Positive	8 (15%)	0.15±0.02	0.09	3.1±0.38	0.17
Negative	7 (13%)	0.19±0.03		2.9±0.4	
HLA-B27					
Positive	3 (6%)	0.17±0.005	0.79	2.5±0.13	0.18
Negative	23 (44%)	0.17±0.04		3.01±0.37	
Oral aphtha					
Positive	45 (96%)	0.17±0.04	0.77	2.9±0.37	0.49
Negative	2 (4%)	0.15±0.06		3.2±0.0	
Genital ulcer					
Positive	24 (51%)	0.17±0.03	0.94	2.8±0.35	0.16
Negative	23 (49%)	0.18±0.05		3.05±0.37	
Arthritis					
Positive	9 (19%)	0.17±0.04	0.46	2.9±0.37	0.3
Negative	38 (81%)	0.71±0.03		3.05±0.32	
Sever B.D					
Positive	30 (64%)	0.17±0.03	0.92	2.9±0.33	0.95
Negative	17 (36%)	0.18±0.05		2.9±0.41	
Severe eye involvement					
Positive	10 (22%)	0.15±0.03	0.2	3.1±0.33	**0.02**
Negative	36 (78%)	0.18±0.04		2.9±0.35	
Phlebitis					
Positive	6 (11%)	0.16±0.04	0.9	2.6±0.36	0.08
Negative	41 (89%)	0.17±0.04		3±0.34	

Cataract					
Positive	9 (19%)	0.15±0.03	0.08	3.03±0.39	0.44
Negative	36 (74%)	0.17±0.04		2.9±0.35	
Vision loss					
One eye	6 (13%)	0.160.04	0.83	3.1v0.38	0.12
No eye	38 (77%)	0.17±0.04		2.9±0.36	

As shown in the table, items that have a statistically significant difference are shown as **Bold**. IL-6: interleukin-6, SD: standard deviation, SD: standard deviation, HLA: Human leukocyte antigen, BD: Behçet's disease, EN: Erythema Nodosum.

Table 2- *PCR Primers AND Product size*

rs1800872	ACGGTAGGGGTCATGGTGAG CAATGGGATTGAGAAATAATTGG	
IL-10 for gene expression	GTTGAGCTGTTTTCCCTGA TGAAGTGGTTGGGGAATGAG	169
b-actin	GGTGAAGGTGACAGCAGT TGGGGTGGCTTTTAGGAT	154

Table 3- Frequencies of alleles and genotypes of IL-10 polymorphisms in BD patients (1 missed, n=46) and controls (n=61).

SNPs	Genotype	BD n (%)	control	P value	OR (95%)
rs1800872	AA	24 (52.2%)	16 (26.2%)	0.001	0.52 (0.39-0.68)
	AC	20 (43.5%)	41 (67.2%)	<0.001	0.435 (0.313-
	CC	2 (4.3%)	4 (6.6%)	0.62	0.604)
	AC/CC	22 (47.8%)	45 (73.8%)		0.64 (0.113-3.7)
	A	68 (68.2)	73 (83.6)	0.0315	
	C	24 (31.8)	49 (16.4)		1.90 (1.05-3.42)

Table 4- Associations between the rs1800872 A/C and expression and serum levels of the IL-10

SNPs		expression		P value	Serum levels		P value
rs1800872	**Genotype**	**BD (mean±SD)**	**Control (mean±SD)**		**BD (mean±SD)**	**Control (mean±SD)**	
	AA	0.17±0.03	1.03±0.14	<0.001	3.2±0.15	5.5±0.23	<0.001

	AC	0.18±0.05	0.99±0.14	<0.001	2.62±0.23	4.86±0.24	<0.001
	CC	0.11±0.01	1.09±0.34	0.011	3.38±0.27	4.24±0.21	0.013
	AC/CC	0.17±0.05	1.01±0.17	<0.001	2.69±0.32	4.8±0.29	<0.001

Table 5: mean plasma concentrations of IL-10 according to gene polymorphism -592 A/C in healthy and BD groups

Genotype	BD (mean±SD)	Control (mean±SD)	P value
IL-10 (592A/C) **A/A** **A/C** **C/C**	 3.2 ± .36 2.6 ± .72 3.1 ± .47	 5.4 ± .48 4.8 ± .64 4.2 ± .59	 P < 0.0001 P < 0.0001 0.0840