

mRNA Expression Level of Interleukin Genes in the Determining Phases of Behçet's Disease

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Background: Behçet's disease (BD), first described in 1937 as a triadic complex of symptoms (oral aphthae, genital ulcers, and hypopyon uveitis), is a chronic, relapsing, multisystemic idiopathic inflammatory disease. **Objective:** The objective of this study was to investigate the usability of messenger RNA (mRNA) expression of cytokine genes for following up patients with BD and also assess polymorphisms in these genes as to how they influence mRNA expression. **Methods:** This study investigated the role of the *IL1A* –889(C/T), *IL1B* –511(C/T), and *IL2* –330(T/G) polymorphisms by polymerase chain reaction (PCR)-restriction fragment length polymorphisms and the expression levels of the genes by real-time PCR in BD. **Results:** The frequency of the *IL2* –330 G allele was found to be significantly higher in patients with BD. A decreased level of *IL1A* gene expression was found in the patient group with clinically active BD compared to controls. Increased *IL1B* gene expression levels were found in patient groups with active, inactive, or ocular BD ($p < 0.001$). *IL2* gene expression level manifested no significant change compared to controls in the patient group with clinically active BD; it increased in the groups with clinically inactive BD or ocular involvement. **Conclusion:** *IL1A*, *IL1B*, and *IL2* gene expression, and *IL2* promoter polymorphisms, may be valuable markers for predicting risk in the development of BD. We believe that the results reveal the importance of achieving a better understanding of BD and the

prospects of developing future therapeutic strategies. (Ann Dermatol 27(3) 291 ~ 297, 2015)

-Keywords-

Behçet's disease, Cytokine, Inflammation, mRNA expression, Promoter polymorphism

INTRODUCTION

Behçet's disease (BD), first described in 1937 as a triadic complex of symptoms (oral aphthae, genital ulcers, and hypopyon uveitis), is a rare, chronic, relapsing, multisystemic idiopathic inflammatory disease. BD manifests as oral and genital ulcerations, skin lesions, and uveitis; there is also vascular, central nervous system, and gastrointestinal involvement. Vasculitis is the main histopathological finding; this is widespread, in arteries and veins of all sizes¹. As to geographic distribution, BD is more prevalent along the Mediterranean coast and in Eastern Asia, with Turkey having the highest reported prevalence of between 110 and 420 per 100,000 inhabitants. In Portugal, the National Study Group for BD reported a prevalence of 2.5 per 100,000 inhabitants². The prevalence of BD was estimated as 17 cases per 10,000 population in Kayseri, Turkey; furthermore, this previous study in Turkey reported the world's highest prevalence of BD³. Although the specific etiology remains unknown, present dogma suggests that BD is caused by unknown environmental triggering factors, ranging from infectious agents to pollution, and against a background of genetic susceptibility. The *HLA-B51* gene variant is the strongest predisposition factor defined for BD so far, while other major histocompatibility complex-associated genes, such as *MICA* and *TNF*, might contribute to the disease because of their linkage disequilibrium with *HLA-B51*².

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Since a specific laboratory test for BD is not available, its diagnosis is based exclusively on clinical assessment of patients. Several diagnostic classifications were proposed for BD, but a consensus was reached only in 1990 with the creation of the International Study Group Classification⁴. There have been several reports suggesting that various host genetic factors play significant roles in susceptibility to BD. Therefore, identification of the host genes responsible for susceptibility and resistance to BD should provide a significant contribution towards understanding its pathogenesis, and may lead to the development of new prophylactic and treatment strategies⁵.

The main approaches to treating patients with BD are symptom management, inflammation suppression at an early stage, and damage control for specific organs. Cytokines, which are crucially involved in the regulation of normal human immune responses, are important in this regard. Deregulation of cytokine expression has been shown to play a role in the pathogenesis of autoimmune diseases. Analyzing the expression of cytokines has enabled a better understanding of the pathogenesis of various diseases⁶.

The activation of inflammatory mechanisms in BD is considered as being governed by soluble factors, namely proinflammatory cytokines such as interleukin (IL)1, IL8, and tumor necrosis factor (TNF)- α , whose serum levels are known to increase in BD. These cytokines play a pivotal role in the activity of BD. Previous studies have shown an increase in inflammatory cytokines and chemokines, which are secreted from mononuclear phagocytes and neutrophils, in patients with BD, such as interferon- γ , TNF- α , IL1, IL2, IL6, IL8, IL12, and IL18⁷. It was also demonstrated that the serum of patients with BD induces classical (proinflammatory) activation of human peripheral blood macrophages. Moreover, the important cytokine, IL1, also causes an increase in the serum levels of some other cytokines (such as IL8) that are reliable markers for gauging the activity of BD⁷.

In this study, we aimed to investigate the usability of messenger RNA (mRNA) expression of cytokine genes for following up patients with BD and also assess polymorphisms in these genes as to how they influence mRNA expression.

MATERIALS AND METHODS

Study design

In total, 30 patients, who were referred to the Behçet Clinic of the Erciyes University Dermatology Department and diagnosed with BD according to the International Study Group Criteria for Behçet's Disease, were included

in this study⁴. This study was approved by the Erciyes University Ethics Committee (decision number: 2008/626). The patients with BD were divided into three groups, in order to detect any changes in the mRNA expression of *IL1A*, *IL1B* and *IL2* genes in relation to pathogenesis. The first or "active" group consisted of 10 patients with active lesions, who suffered from papulopustular skin lesions, and oral and/or genital aphthae, at the time their blood was sampled; patients with oral or genital aphthous ulcers and a high level of C-reactive protein (CRP) were evaluated as active (Table 1)⁴. The second, "inactive," group again consisted of 10 patients but with no presently active lesions; these patients did previously have skin lesions but displayed no dermatological findings at the time their blood was sampled. A further 10 patients, who suffered posterior uveitis or panuveitis attacks at the present or any past stage of the disease, were designated as the third group, having eye involvement, the "ocular" group⁴. Patients with BD who also had another systemic disorder were not included in the study.

Patients in the inactive group were only receiving colchicine during the inactive phase of their disease. Patients in the active and ocular groups were receiving steroids, salazopyrin, azathioprine, methotrexate, cyclophosphamide, interferon, and anti-TNF drugs, in addition to colchicine;

Table 1. Clinical data of patients with Behçet's disease

Clinical characteristic	Data	Treatment	p-value
Active group (n=10)			
Age (yr)	35.44±6.93	Colchicine	0.772
Female	5 (50.0)		1.000
Male	5 (50.0)		
Oral aphthae	3 (30.0)		
Oral aphthae/genital lesions	4 (30.0)		
Papulopustular skin	3 (30.0)		
Inactive group (n=10)			
Age (yr)	36.21±9.83	Colchicine	0.772
Female	5 (50.0)		1.000
Male	5 (50.0)		
No presently active lesion	10 (100.0)		
Ocular group (n=10)			
Age (yr)	38.82±7.66	Colchicine	0.772
Female	5 (50.0)		1.000
Male	5 (50.0)		
Posterior uveitis	6 (60.0)		
Panuveitis	4 (40.0)		
Control group (n=10)			
Age (yr)	37.90±7.20		0.772
Female	5 (50.0)		1.000
Male	5 (50.0)		

Values are presented as mean±standard deviation or number (%).

each patient was receiving one or more drug in combination therapy. For the active and ocular groups, patients' blood samples had been collected before they started their medical therapy.

Another group of 10 healthy subjects was included in the study as controls. Controls were evaluated by the same procedure as the cases, and were selected if they were negative for BD and any other rheumatologic or autoimmune disorder. Controls were selected from healthy subjects matched by age, sex, and ethnicity. All study subjects (patients and controls) were ethnically Turkish and from the same geographic area.

Individuals excluded from both the patient and control groups were as follows: (i) those with any metabolic or hormonal abnormality, (ii) postmenopausal women, (iii) men older than 50 years, (iv) pregnant women, (v) nursing women, and (vi) those taking medications such as estrogens, phenothiazine, cimetidine, antidepressants, sulpiride, verapamil, metoclopramide, or opiates.

There was no statistically significant difference between the patients and the controls in terms of mean age and gender. It needs be noted that, although BD is more frequent in Turkey and among the populations of countries the Silk Road used to course through (www.orpha.net), its prevalence is nevertheless quite low (1~9 per 100,000; data from www.orpha.net). This demographic impediment perforce limited the pool of patients who could be included in the study, as well as the number of controls, somewhat obscuring the level of reliability in absolutely ascertaining what role proinflammatory cytokine expression plays in the pathogenesis of BD. Nevertheless, the results can be considered as indicative where any doubt may fall on their confirmative capacity.

Determination of cytokine gene polymorphisms

Venous blood samples were taken from patients and controls for the analysis of gene polymorphisms and gene expression. DNA was isolated using the MagNA Pure LC2.0 automated isolation system (Roche, Mannheim, Germany). *IL1A* -889(C/T) and *IL1B* -511(C/T) genotypes and allele frequencies were determined by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified DNA fragments (PCR-RFLP)⁷.

The *IL2* -330(T/G) polymorphism was genotyped using PCR with confronting two-pair primers. PCR products were electrophoresed in 2% agarose gels containing ethidium bromide stain⁸.

RNA isolation, cDNA synthesis, and mRNA expression

Total RNA was isolated using TRIzol reagent (Roche) from

the cells of venous blood samples. RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples were stored at -80°C until used. Total RNA (1 μg) served as a template for first-strand cDNA synthesis in a 20 μl reaction using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The cDNA was amplified for 30 min at 55°C and then heated for 5 min at 85°C . To ensure the fidelity of mRNA extraction and reverse transcription, all samples were normalized by PCR amplification with oligonucleotide primers specific for the constitutively expressed gene encoding β -actin. Quantitative real-time PCR (qPCR) was performed using the Roche 480 Light Cycler for the *IL1A*, *IL1B*, and *IL2* genes. All samples were analyzed in duplicate. The qPCR data was analyzed using the delta cycle threshold method. The cycle threshold for each sample was calculated and relative mRNA abundance determined based on that of the β -actin control.

Statistical analysis

The relationship between BD and cytokine gene promoter polymorphisms was analyzed using a chi-square test. Logarithmic transformation (log base 2) was applied to mRNA expression data. Shapiro-Wilk's test was used to check data normality and Levene's test was used to ascertain variance homogeneity. Values are expressed as geometric means with 95% bootstrap confidence intervals obtained from 1,000 bootstrap samples. One-way analysis of variance was used to compare the difference between the groups and Tamhane's T^2 test was used for pairwise comparisons. Analysis was conducted using the IBM SPSS Statistics ver. 20.0 (IBM Co., Armonk, NY, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Clinical data and cytokine gene polymorphisms

Among the patients with BD included in the study within the active group, three had oral aphthous ulcers, four had oral aphthous ulcers/genital lesions, while three had papulopustular skin lesions and CRP levels higher than normal. None of the patients in the inactive group had active lesions. Six patients of the 10 included in the ocular group had posterior uveitis, whereas four were suffering from panuveitis. The clinical data of the patients included in this study are presented in Table 1.

The distribution of the *IL1A* -889(C/T), *IL1B* -511(C/T), and *IL2* -330(T/G) alleles in active, inactive, and ocular patients with BD and in controls are summarized in Table 2, and the distribution of these alleles in all patients with BD compared to controls are summarized in Table 3.

Table 2. Distribution of *IL1A* –889(C/T), *IL1B* –511(C/T), and *IL2* –330(T/G) genotypes and allele frequencies between the active, ocular, and inactive Behçet's disease and control groups

Gene polymorphism	Group				p-value*	
	Active (n=10)	Ocular (n=10)	Inactive (n=10)	Control (n=10)		
<i>IL1A</i> gene –889(C/T)	CC	4 (40)	7 (70)	5 (50)	5 (50)	0.30
	CT	4 (40)	3 (30)	5 (50)	5 (50)	
	TT	2 (20)	0 (0)	0 (0)	0 (0)	
Allele frequency	C allele	12 (60)	17 (85)	15 (75)	15 (75)	
	T allele	8 (40)	3 (15)	5 (25)	5 (25)	
<i>IL1B</i> gene –511(C/T)	CC	1 (10)	1 (10)	4 (40)	4 (40)	0.088
	CT	5 (50)	8 (80)	6 (60)	3 (30)	
	TT	4 (40)	1 (10)	0 (0)	3 (30)	
Allele frequency	C allele	7 (35)	10 (50)	14 (60)	11 (55)	
	T allele	13 (65)	10 (50)	6 (30)	9 (45)	
<i>IL2</i> gene –330(T/G)	TT	4 (40)	2 (20)	2 (20)	8 (80)	0.006
	TG	3 (30)	8 (80)	7 (70)	2 (20)	
	GG	3 (30)	0 (0)	1 (10)	0 (0)	
Allele frequency	T allele	11 (55)	12 (60)	11 (55)	18 (90)	
	G allele	9 (45)	8 (40)	9 (45)	2 (10)	

Values are presented as number (%).

*Comparing the frequency of the same genotype between the control and Behçet disease groups.

Table 3. Distribution of *IL1A* –889(C/T), *IL1B* –511(C/T), and *IL2* –330(T/G) genotypes and allele frequencies in patients with Behçet's disease (BD) and controls

Gene polymorphism		Patients with BD (n=30)	Controls (n=10)	p-value*
<i>IL1A</i> gene –889(C/T) genotype	CC	16 (53.3)	5 (50)	0.30
	CT	12 (40)	5 (50)	
	TT	2 (6.7)	0 (0)	
Allele frequency	C allele	44 (73)	15 (75)	
	T allele	16 (27)	5 (25)	
<i>IL1B</i> gene –511(C/T) genotype	CC	6 (20)	4 (40)	0.088
	CT	19 (63.3)	3 (30)	
	TT	5 (16.7)	3 (30)	
Allele frequency	C allele	31 (52)	11 (55)	
	T allele	29 (48)	9 (45)	
<i>IL2</i> gene –330(T/G) genotype	TT	8 (26.7)	8 (80)	0.006
	TG	18 (60)	2 (20)	
	GG	4 (13.3)	0 (0)	
Allele frequency	T allele	34 (57)	18 (90)	
	G allele	26 (43)	2 (10)	

Values are presented as number (%).

*Comparing the frequency of the same genotype between patients with BD and controls.

A chi-square test was applied in order to compare the *IL1A* –889(C/T), *IL1B* –511(C/T), and *IL2* –330(T/G) polymorphisms among the study groups, and no significant differences were recorded for the *IL1A* –889(C/T) and *IL1B* –511(C/T) polymorphisms. A higher frequency of the *IL2* –330 G allele was found to be statistically significant in BD patients compared to controls ($p=0.006$, Table 2; $p=0.011$, Table 3).

mRNA expression of the *IL1A*, *IL1B*, and *IL2* genes

Significant differences in *IL1A* gene expression level were observed between the patient groups. A decreased *IL1A* gene expression level was found in the active patient group compared to controls ($p<0.001$; Table 4), whereas, an increased expression level was found in the inactive and ocular groups compared to the control group. Among the patient groups, *IL1 α* gene expression levels were

Table 4. Comparison of messenger RNA (mRNA) expression levels of the *IL1A*, *IL1B*, and *IL2* genes in the active, inactive, and ocular Behçet's disease and control groups

mRNA expression	Group				p-value
	Active (n=10)	Inactive (n=10)	Ocular (n=10)	Control (n=10)	
<i>IL1A</i>	2.84 × 10 ⁻³⁵ (2.33 × 10 ⁻³⁹ ~ 8.26 × 10 ⁻³¹)*	7.91 × 10 ⁻² (3.04 × 10 ⁻² ~ 2.20 × 10 ⁻¹) [†]	1.60 × 10 ⁻⁵ (1.09 × 10 ⁻⁶ ~ 1.71 × 10 ⁻⁴) [‡]	6.70 × 10 ⁻⁸ (4.20 × 10 ⁻⁸ ~ 1.01 × 10 ⁻⁷) [§]	<0.001
<i>IL1B</i>	1.40 × 10 ⁻⁴ (2.35 × 10 ⁻⁵ ~ 8.69 × 10 ⁻⁴)*	8.21 × 10 ⁻³ (2.76 × 10 ⁻³ ~ 2.44 × 10 ⁻²) [†]	1.52 × 10 ⁻³ (9.21 × 10 ⁻⁵ ~ 2.81 × 10 ⁻²)* [‡]	9.37 × 10 ⁻⁵ (3.84 × 10 ⁻⁵ ~ 2.07 × 10 ⁻⁴)*	0.005
<i>IL2</i>	2.68 × 10 ⁻⁶ (7.30 × 10 ⁻⁸ ~ 8.78 × 10 ⁻⁵)*	2.98 × 10 ⁻² (1.86 × 10 ⁻² ~ 5.26 × 10 ⁻²) [†]	1.37 × 10 ⁻¹ (1.55 × 10 ⁻² ~ 9.90 × 10 ⁻¹) [‡]	5.55 × 10 ⁻⁴ (2.39 × 10 ⁻⁴ ~ 1.73 × 10 ⁻³)*	<0.001

Values are presented as the geometric mean (95% bootstrap confidence interval) obtained from 1,000 bootstrap samples.

Different superscripts in a row indicate statistically significant difference, as follows: *with respect to the active group, [†]with respect to the inactive group, [‡]with respect to the ocular group, [§]with respect to the control group.

highest in the inactive group while they were recorded at remarkably low levels in the active group (Table 4). *IL1B* gene expression displayed significant differences between the groups; increased *IL1B* gene expression levels were found in the active, inactive, and ocular groups compared to controls (Table 4). *IL2* mRNA expression levels also manifested no significant change in the active group in comparison with the control group, but increased in the inactive and ocular groups.

DISCUSSION

Current evidence suggests that genetic, environmental, and immunologic factors may play a role in the development of BD^{9,10}.

We examined promoter polymorphisms of the *IL1A*, *IL1B*, and *IL2* genes and their mRNA expression in patient groups with BD in the active, inactive, or ocular phase, and in a control group. No significant difference was found in the genotype distribution or allele frequency of the *IL1A* -889(C/T) polymorphism between patients and controls in our study. Previous studies demonstrated that the presence of the *IL1A* -889C allele was significantly associated with BD^{10,12}, although Coskun et al.⁷ did not observe any such relationship.

There are studies that support a functional polymorphism in the *IL1B* gene possibly playing a role in BD etiology, as IL1 β plays an important role in the initiation of the pro-inflammatory process in BD¹⁰. No significant difference was found in the genotype distribution or allele frequency of the *IL1B* -511(C/T) polymorphism between patients with BD and controls in our study.

In the present study, mRNA expression of the *IL1A*, *IL1B*, and *IL2* genes in BD was also examined. Some studies reported elevated serum levels of the IL1 β protein in patients with BD but no correlation with disease activity^{13,14}.

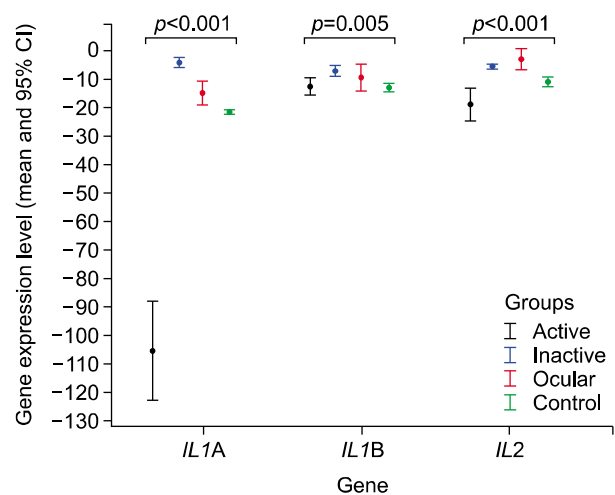


Fig. 1. Error bars indicating the expression distributions of *IL1A*, *IL1B* and *IL2* genes among active, inactive, ocular and control groups.

Furthermore, an increased expression of synovial IL1 β in BD patients was observed in previous studies^{13,15,16}. Evereklioglu et al.¹⁷ did not detect any difference between patients with active or inactive BD and controls in respect of their serum levels of IL1 β . In our study, the *IL1B* gene expression level was significantly increased in the active, inactive, and ocular groups compared to the control group (Fig. 1).

We also found statistically significant differences in mRNA expression level of the *IL1A* gene in the active, inactive, and ocular groups compared to the control group (Fig. 1); it was increased in the inactive and ocular groups compared to the controls, and decreased in the active group in comparison with the other groups (Table 4; Fig. 1). The change in the expression level of the *IL1A* gene in different patient groups strongly indicates that gene analysis could be useful in the prognosis and treatment of BD.

IL2 plays an important role in the pathogenesis of BD and the *IL2* gene therefore attracts much attention as a candidate locus for BD. IL2 is produced and secreted by T cells and is crucial for the stimulation of T cell proliferation¹⁸. Secreted IL2 stimulates autocrine and paracrine IL2 receptor (IL2R) signaling¹⁹. Inadequate production of IL2 may cause an increase in the number of activated autoreactive cells^{19,20}.

The importance of the IL2/IL2R system for T cell homeostasis, at the levels of repertoire selection, generation of suppressive regulatory T cells, T cell homing, and clonal contraction via activation-induced cell death, indicates its relevance to the development of autoimmune disease²⁰. Deficient production of IL2 may cause an increase in the number of activated autoreactive cells^{19,20}.

We examined the *IL2* -330(T/G) polymorphism and *IL2* mRNA expression in this study; statistically significant increases were found in the frequencies of the *IL2* -330 GG and TG genotypes in patients with BD compared to controls (Table 3). Alayli et al.¹¹ found no significant difference between patients with BD and a control group in terms of the *IL2* -330(G/T) polymorphism. However, in 2011, Shahram et al.²¹ reported that BD was associated with the GG genotype of the *IL2* -330(T/G) polymorphism.

Furthermore, we also examined mRNA expression of the *IL2* gene. In our study, we found statistically significant reduced mRNA expression in the active group compared to the control group. The results obtained in this study, increased frequency of the *IL2* -330 G allele along with reduced mRNA expression of the *IL2* gene in the active group, led us to believe that the *IL2* gene -330 G allele may function to reduce mRNA expression of the *IL2* gene, and explain the lower *IL2* mRNA levels in the active group compared to the controls. Information concerning the mRNA expression of the *IL2* gene in BD is not available in the literature. Other studies investigated the IL2 protein and reported an increase in serum levels of IL2 in patients with BD compared to a control group²². Significant differences in IL2 serum levels were not found between patients with BD and controls in two other studies conducted in Turkey^{17,23}. Differences between the serum IL2 protein and *IL2* mRNA expression data maybe related to regulation occurring at the transcriptional level.

BD is a complex disease, different patients will manifest different symptoms, and there is a clear geographical distribution to its prevalence. The candidate-gene approach has been useful in identifying genes affecting the susceptibility to and severity of BD. Recent scientific discoveries pertaining to the pathogenesis of BD have demonstrated the important roles of genetic and immunologic factors. BD is characterized by a divergence in the production

profile of cytokines, in which several factors are required for optimal induction of T helper (Th)1 activity^{10,11,19}. There is evidence that an imbalance in the polarization of Th1/Th2 cells in favor of Th1 cells may be the key to the pathogenic mechanism of chronic inflammatory diseases, as well as organ-specific autoimmune diseases like BD. An overproduction of proinflammatory cytokines from several cellular sources appears to be responsible for the inflammatory reaction in BD, with levels of interferon- γ , TNF- α , IL6, IL8, and IL12 being higher in patients with BD^{22,23}.

The expression and promoter polymorphisms of genes have been determined using the same patient and control groups, so as to record how polymorphisms have had an impact on the expression of genes, because promoter sequences are potential sites of polymorphisms affecting gene expression. Promoters are involved in initiating transcription and are therefore among the many important *cis*-acting elements that regulate gene expression and that might harbor functionally relevant polymorphisms²⁴.

The relatively small sample size imposes limitations on the statistical strength of this study. Admittedly, the inclusion of a few further samples may have had an effect upon the range of expression for genes reported here as, most importantly, the number of patients with BD was not sufficient for such a study. However, the results are sufficient to justify our conviction that further studies along the same lines, with larger and diversified population groups, need to be performed to test our findings. Our study reasonably demonstrates that this approach is capable of opening a diagnostic and prognostic vista for assaying the progression of BD; the decrease, at the incipient phase of BD, in the mRNA expression levels of the *IL1A* and *IL2* genes within the active group compared to the control group, as the *IL2* and *IL1A* mRNA expression levels increase in the inactive and ocular groups relative to the control group, certainly seems to point in that direction.

In the light of its findings, and despite its limitations, our study demonstrates that promoter polymorphisms and mRNA expression of the *IL1A*, *IL1B*, and *IL2* genes indicate susceptibility to BD. We believe that the results reveal the importance of achieving a better understanding of BD and the prospects of developing therapeutic strategies for the future.

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