

Inflammation-related cytokine gene polymorphisms in Behçet's disease

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Abstract: Behçet's disease (BD) is a complex, multisystemic inflammatory disorder of unclear etiology. Single nucleotide polymorphisms in tumor necrosis factor (TNF) and interleukin (IL)-10 genes have been implicated in susceptibility to BD with inconsistent results in several ethnic populations. The aim of this case-control study was to evaluate the association of TNF- α (-308G/A), TNF- β (+252A/G), and IL-10 (-1082G/A, -819C/T, and -592 C/A) polymorphisms with susceptibility of BD in Saudi patients. Molecular genotyping of TNF- α , TNF- β , and IL-10 gene polymorphisms was performed to analyze the alleles and genotypes distribution in 272 Saudi subjects, including BD patients (61) and healthy controls (211). The frequencies of allele A and genotype GA of TNF- α (-308G/A) were significantly higher, whereas those of allele G and genotypes GG were significantly lower in BD patients than controls, indicating that A allele and GA genotype are susceptible, while G allele and GG genotype may be refractory to BD. The distribution of frequencies of alleles and genotype of TNF- β (+252A/G) promoter polymorphism was not significantly different between BD patients and healthy controls. Genotypes 1082GG, -819TT, and 592AA of IL-10 polymorphisms are significantly associated with susceptibility risk of BD, while genotypes 1082AA, 1082GA, 819CC, 819CT, 592CC, and 592CA are resistant to BD. This study indicates that TNF- α (-308G/A) and IL-10 (-1082G/A, -819C/T, and -592C/A) polymorphisms are associated with risk of BD susceptibility in Saudi patients. However, larger scale studies in Saudi population as well as in other ethnicities are needed to confirm this association.

Keywords: tumor necrosis factor, interleukin-10, polymorphism, Saudis, Behçet's disease

Background

Behçet's disease (BD; MIM 109650) is a multisystemic inflammatory disorder of unclear etiology, characterized by recurrent oral aphthous ulcers, ocular symptoms, skin lesions, and genital ulcerations. This systemic vasculitis is more prevalent in countries along the ancient Silk Route, mainly in the Far East, the Mediterranean, and the Middle Eastern countries.¹⁻³ Its prevalence is highest in Turkey (80-420 cases per 100,000), with 7.6, 15, 17, 20, 22, 30.2, 80, 110, 146.4 cases per 100,000 in Egypt, Morocco, Iraq, Saudi Arabia, Japan, Korea, Iran, People's Republic of China, and Israel, respectively.¹⁻⁴

BD is a complex disease and its pathogenesis/etiology is not well established; however, environmental factors, genetic determinants, and diverse immunological responses have been implicated and studied extensively.^{5,6} Inflammation, a characteristic feature in BD, is thought to be mediated by cytokines.^{7,8} Decreased levels of interleukin (IL)-10 and increased levels of tumor necrosis factor (TNF)- α have been observed in the serum and active lesions of BD patients and has been indicated to play

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a significant role in the immune response, pathogenesis, and activity in BD.⁹⁻¹⁵

IL-10 and TNF- α production may be regulated at the transcriptional level, and several single nucleotide polymorphisms (SNPs) at the promoter region of *IL-10* and *TNF- α* gene have been shown to be associated with changes in the expression levels of IL-10 and TNF- α production.^{16,17} On the other hand, numerous recent studies have demonstrated an association between BD and SNPs of IL10¹⁸⁻²² and TNF- α ²²⁻²⁵ in different ethnic groups.

Three polymorphisms -1082 A/G (rs1800896), -819 T/C(rs1800871), and -592 A/C (rs1800872) in the promoter region of the *IL-10* gene are correlated to the expression level of IL-10. Similarly, several polymorphisms within the promoter region of TNF- α and the intron 1 polymorphism of TNF- β , in particular, have been associated with altered levels of circulating TNF- α .^{26,27} TNF- α (-308G/A) polymorphism (rs1800629) affects a consensus sequence for the binding site of transcription factor AP-2 and leads to a less common allele-A (allele 2), which has been associated with increased TNF- α production in vitro²⁸ and higher rate of TNF- α transcription than wild-type allele G (allele 1). Allele A has been shown to produce six- to sevenfold higher levels of TNF- α transcription.^{17,29,30}

TNF- β , which is closely linked to TNF- α , has also been shown to contribute to the susceptibility of several inflammatory/autoimmune diseases.³¹⁻³⁵ Higher level of TNF- β is produced by the $\gamma\delta$ T-cells of BD patients than by those of healthy controls.^{36,37} A polymorphism TNF- β (+252A/G) (rs909253) has been reported to consist of a guanine (TNF- β +252G) on one allele and an adenine (TNF- β +252A) on the alternate allele. The presence of G at this position defines the mutant allele known as TNF- β *1 (allele-1) which is the less frequent allele in white subjects and is associated with higher TNF- α and TNF- β production.^{38,39}

The candidate gene approach has been useful in identifying susceptibility and severity genes in BD.⁴⁰ Besides HLA-B51 molecules, SNPs in *TNF* and *IL-10* genes have been implicated in susceptibility to BD.^{24,41-43} However, the results are inconsistent and variations are found among several ethnic populations. So in this study, the association of five polymorphisms in *TNF- α* , *- β* , and *IL-10* genes with BD susceptibility risk in Saudi patients was evaluated.

Methods

Subjects

A total of 272 Saudi subjects visiting Prince Sultan Military Medical City (PSMMC), Riyadh, Saudi Arabia, were recruited

for this study. Sixty-one unrelated patients with BD, ranging from 20 to 64 years, and 211 unrelated healthy matched voluntary Saudi blood donors, age ranging from 20 to 60 years, were studied for polymorphisms in *TNF- α* , *- β* , and *IL-10* genes. Patients with any other inflammatory/autoimmune diseases were excluded from the study. Power was calculated online (<http://www.stat.ubc.ca/~rollin/stats/ssize/caco.html>).

This study was approved by the research and ethical committee of PSMMC, and written informed consent was obtained from each subject before recruitment. All patients were diagnosed according to the diagnostic criteria prepared by the international study group for BD.⁴⁴

Detailed information including demographics, disease duration, treatment duration, and clinical features were obtained from a review of medical records and an interview at the time of enrollment. We evaluated the clinical features such as oral ulcers, genital ulcers, skin lesions (including papulopustular and erythema nodosum-like), pathergy response, ocular inflammation, gastrointestinal lesions, articular manifestations, vascular lesions (including deep vein thrombosis, superficial thrombophlebitis, and aneurysm), central nervous system involvement, and epididymitis. Active or inactive form of BD was determined by clinical parameters.⁴⁴

PCR amplification

Genomic DNA was extracted from the peripheral blood of BD patients and controls using QIAamp[®] DNA mini kit (Qiagen, Valencia, CA, USA). *TNF- α* , *TNF- β* , and *IL-10* genes were amplified using amplification refractory mutation systems (ARMS)-polymerase chain reaction (PCR) methodology⁴⁵ to detect polymorphisms at position -308 of TNF- α , +252 in Intron1 of TNF- β , and at positions -1082, -819, and -592 of *IL-10* genes. PCR amplification was carried out using Ready-To-Go[™] PCR Beads (GE Healthcare UK Ltd, Little Chalfont, UK). PCR consisted of ten temperature cycles of denaturation for 15 seconds at 94°C, annealing for 50 seconds at 65°C, and extension for 40 seconds at 72°C. This was followed by 25 cycles of denaturation for 20 seconds at 94°C, annealing for 50 seconds at 59°C, and extension for 50 seconds at 72°C. Final extension was performed at 72°C for 7 minutes. A positive control was included in the PCR assay by amplification of the human growth hormone gene. The amplified product for various samples were separated on a 1.5% agarose gel, stained with ethidium bromide, and then photographed. For quality control, 25% of the random blind samples were repeated for genotyping and positive and negative controls were also used in the PCR.

Statistical analysis

The differences in allele/genotype frequencies between patients and controls were analyzed by the Fisher's exact test. *P*-values ≤ 0.05 were considered significant. The strength of the association of disease with respect to a particular allele/genotype is expressed by odds ratio, interpreted as relative risk (RR) following the Woolf's method as outlined by Schallreuter et al.⁴⁶ It was calculated only for those alleles/genotypes which were increased or decreased in BD patients as compared to control group. The RR was calculated for all the subjects using the formula given below:

$$RR = \frac{a \times d}{b \times c} \quad (1)$$

where, *a* = number of patients with expression of allele or genotype, *b* = number of patients without expression of allele or genotype, *c* = number of controls with expression of allele or genotype, and *d* = number of controls without expression of allele or genotype.

Etiologic fraction (EF) indicates the hypothetical genetic component of the disease. The values 0.0–0.99 are of significance. EF was calculated for positive association only, where $RR > 1$ using the following formula:⁴⁷

$$EF = \frac{(RR - 1)f}{RR}, \quad \text{where } f = \frac{a}{a + c} \quad (2)$$

Preventive fraction (PF) indicates the hypothetical protective effect of one specific allele/genotype for the disease. PF was calculated for negative association only, where $RR < 1$ using the following formula:⁴⁷

$$PF = \frac{(1 - RR)f}{RR(1 - f) + f}, \quad \text{where } f = \frac{a}{a + c} \quad (3)$$

Values < 1.0 indicate the protective effect of the allele/genotype against the manifestation of disease.

Results

Demographic data and clinical characteristics of patients with BD are presented in Tables 1 and 2. The number of controls per case was 3.4, which yielded a power of 90%. The most frequently found clinical symptoms in BD patients were oral, genital, ocular, musculoskeletal, and cutaneous followed by gastrointestinal, nervous, renal, vascular, and pulmonary symptoms, whereas cardiovascular symptoms were absent in our BD patients. Among BD patients, the male to female ratio was 43:18 (2.4:1). There was no significant difference in clinical manifestation or prognosis comparing men to women in our study.

Table 1 Demographic features of Saudi patients with Behçet's disease and controls from same ethnic population

Features	Data
Number of patients	61
Mean body weight (kg)	70.50±10.25
Mean BMI (kg/m ²)	25.4±5.30
Sex, M:F	43:18 (2.4:1)
Active disease (35), M:F	24:11
Age of patients (range)	21–64 years
Age of patients (mean)	37.87±12.5 years
Mean duration of disease	9.99 years
Inactive (26), M:F	18:8
Age of patients (mean)	37.75±10.5
Age of patients (range)	20–61 years
Mean duration of disease	7.74
Number of controls	211
Age (mean)	36±10 years
Age (range)	20–60 years
Sex, M:F	150:61 (2.4:1)
Control: cases	211:61=3.4 (power =90%)

Abbreviations: BMI, body mass index; M, male; F, female.

The genotype and allele frequencies of TNF- α (–308G/A) and promoter polymorphism are presented in Table 3. For quality control, 25% of the random blind samples were repeated and genotyping results were compared with 100% success rate.

The frequency of heterozygous genotype GA was significantly higher in BD patients than controls ($P < 0.01$), whereas the frequency distribution of homozygous genotypes GG and AA was significantly lower in BD than controls ($P < 0.05$). The frequency of allele A was significantly higher in BD patients than control subjects ($P < 0.01$). On the other hand, allele G was significantly lower in BD patients as compared to controls ($P < 0.01$).

The distribution of frequencies of alleles and genotype of TNF- β (+252A/G) promoter polymorphism did not differ

Table 2 Clinical manifestations in patients with Bahcet's disease

Organ/ organ system involvement	At any time		At the time of study					
	Total (61)		Total (61)	Active (35)	Inactive (26)			
	N	%	N	%	N	%		
Oral	61	100	38	62.29	31	90.32	7	26.92
Genitals	49	80.32	18	29.09	16	45.71	2	7.69
Ocular	43	70.49	10	16.39	9	25.71	1	3.84
Musculoskeletal	41	67.21	12	19.67	8	22.85	4	15.38
Cutaneous	37	60.65	8	13.11	8	22.85	0	0
Gastrointestinal	22	36.06	2	3.27	1	2.85	1	4.17
Nervous system	14	22.95	1	1.64	1	2.85	0	0
Renal	2	3.27	1	1.64	1	2.85	0	0
Vascular	1	1.64	0	0	0	0	0	0
Pulmonary	1	1.64	0	0	0	0	0	0
Cardiovascular	0	0	0	0	0	0	0	0

Table 3 Genotype and allele frequencies of TNF- α and TNF- β variants in BD patients and matched controls

Genotype/ allele	BD (N=61)		Control (N=211)		P-value	RR	EF ^a /PF
	N	%	N	%			
-308G/A							
GG	2	3.28	116	54.98	<0.01 ^b	0.03	0.84
GA	59	96.72	80	37.91	<0.01 ^b	43.24	0.52 ^a
AA	0	0	15	7.11	0.04 ^b	–	–
G allele	63	51.64	312	73.93	<0.01 ^b	0.37	0.61
A allele	59	48.36	110	26.07	<0.01 ^b	2.65	0.21 ^a
+252A/G							
GG	4	6.56	29	13.75	0.17	0.48	0.43
GA	49	80.33	156	73.93	0.39	1.26	0.04 ^a
AA	8	13.11	26	12.32	0.84	1.25	0.06 ^a
G allele	57	46.72	214	50.71	0.41	0.83	0.37
A allele	65	53.28	208	49.29	0.41	1.20	0.05 ^a

Notes: ^aData for EF; ^bstatistically significant using Fisher's exact test.

Abbreviations: BD, Behçet's disease; N, number of subjects; RR, relative risk; EF, etiologic fraction; PF, preventive fraction; TNF, tumor necrosis factor.

significantly between BD patients and healthy controls (Table 3). The frequencies of allele A and genotypes GA and AA were slightly higher, whereas those of allele G and genotype GG were lower in BD patients than control subjects.

The frequency of –1082GG genotype was found to be significantly higher ($P=0.04$) in BD patients as compared to controls subjects. On the contrary, the frequency of heterozygous genotype GA was significantly lower ($P=0.03$) in patients as compared to control subjects. The frequency of homozygous AA genotype was higher in patients compared to controls, but the difference was not statistically significant (Table 4).

The frequencies of –819CC and –819CT genotypes do not differ significantly between BD patients and controls, while the frequency of –819TT genotype was significantly higher in BD patients as compared to controls ($P=0.04$). Similarly, the frequency of homozygous –592AA genotype was significantly higher in BD patients as compared to controls ($P=0.04$), while CC and CA genotypes frequencies varied insignificantly in patient and control groups (Table 4).

Discussion

Clinical manifestations in our BD patients were almost similar to those reported earlier from Saudi BD patients by Al-Dalaan et al.⁴⁸ Men were more affected than women. The male preponderance has also been reported in the Middle Eastern countries earlier by Kaklamani et al.⁴⁹ Influence of sex on BD is well-known, and several reports have suggested a more severe course of disease among males.⁵⁰ However, recently Davatchi et al⁵¹ found no strong association between

Table 4 Genotype and allele frequencies of IL-10 variants in BD patients and matched controls

Genotype/ allele	BD (N=61)		Control (N=211)		P-value	RR	EF ^a /PF
	N	%	N	%			
-1082G/A							
GG	10	16.39	16	7.58	0.04 ^b	2.08	0.20 ^a
GA	37	60.66	159	75.36	0.03 ^b	0.50	0.15
AA	14	22.95	36	17.06	0.34	1.45	0.08 ^a
G allele	57	46.72	191	45.26	0.83	1.06	0.01 ^a
A allele	65	53.28	231	54.74	0.83	0.94	0.01
-819C/T							
CC	27	44.26	88	41.71	0.76	1.11	0.02 ^a
CT	22	36.07	102	48.34	0.11	0.60	0.01
TT	12	19.67	21	9.95	0.04 ^b	4.06	0.27 ^a
C allele	76	62.30	278	65.88	0.51	0.85	0.34
T allele	46	37.70	144	34.12	0.51	1.16	0.34 ^a
-592C/A							
CC	27	44.26	88	41.71	0.76	1.11	0.02 ^a
CA	22	36.07	102	48.34	0.11	0.60	0.01
AA	12	19.67	21	9.95	0.04 ^b	4.06	0.27 ^a
C allele	76	62.30	278	65.88	0.51	0.85	0.34
A allele	46	37.70	144	34.12	0.51	1.16	0.34 ^a

Notes: ^aData for EF; ^bstatistically significant Fisher's exact test.

Abbreviations: BD, Behçet's disease; IL-10, interleukin 10; N, number of subjects; RR, relative risk; EF, etiologic fraction; PF, preventive fraction.

the male sex and major organ involvement, except for vascular lesions. In contrast, females were more affected with BD than males in Japan and Korea.⁵² In our study, although no significant difference in clinical manifestation or prognosis between men and women was noticed, however, results should be interpreted with caution as the number of male and female patients is very low.

Significantly higher frequencies of allele A and genotype GA of TNF- α (308) polymorphism in patient group than controls ($P<0.001$) indicated that allele A and genotype GA are associated with the BD susceptibility risk in Saudi patients. The RR in GA carriers for the disease was very high (RR =43.236). On the other hand, the homozygous genotypes GG and AA might be protective for BD as their frequencies were significantly lower in patient groups than controls ($P<0.001$ and $P=0.04$, respectively). Our results are in agreement with a recent meta-analysis which showed higher frequency of allele A in Asian BD population.⁴²

Our results clearly suggested that A allele and GA genotype confer susceptibility to BD and might be responsible for higher levels of TNF- α in BD patients as TNF- α (308) allele A has been associated with increased TNF- α expression.⁵³ Moreover, patients with ocular manifestation have statistically significant higher serum levels of TNF- α than those without ocular manifestations.

Significantly higher TNF- α level has been reported in BD patients with active disease compared to those with inactive disease.^{14,54} Contrary to these, the G allele and GG genotype of TNF- α (308) polymorphism have been associated with increased risk of BD in Koreans,⁵⁵ while no association of TNF- α (308) polymorphism with BD was reported in Turkish,^{22,56,57} Korean,⁵⁸ Tunisian,²³ Lebanese,⁵⁹ Iranian,⁶⁰ and Moroccan population.²⁵

Though several individual studies from various geographical regions or ethnicities showed no association between TNF- α (308) polymorphism and BD, when pooled together in meta-analysis, a significant association of TNF- α polymorphism with BD was observed.^{42,43} Zhang et al⁴³ indicated that the TNF- α (-308G/A) and TNF- α (-857T/C) polymorphisms are linked with BD only in Asians, while the TNF- α (-238A/G) and TNF- α (-1031C/T) polymorphisms are associated with BD in Caucasians. The other meta-analysis of 12 studies by Liang et al⁴² using the fixed-effect model also showed an association of the TNF- α (-308G/A) polymorphism with the susceptibility to BD. The discrepancy between the individual studies and meta-analysis has been explained by the facts that the sample size of individual studies is relatively small and that the allelic frequencies of genes often differ substantially in different ethnic groups as shown in various reports.^{42,43}

In our study, the frequency of TNF- α (308) AA genotype was zero in Saudi BD patients. These results are in agreement with several other findings which also reported the absence of AA genotype in Lebanese, Iranian, and Moroccan BD patients.^{25,59,60} Absence of AA genotype in BD patients indicates that AA genotype might be protective for BD. Similarly, lower frequency of GG genotype in Saudi BD suggests its protective role in the Saudi cohort.

Our results of TNF- β (+252A/G) polymorphism showed no significant association with BD in Saudi patients. Other available reports also indicated that there is no association of TNF- β polymorphism with BD in Korean and Tunisian populations.^{23,58} In contrast, Verity et al⁶¹ reported a higher TNF- β *2 allele (A allele) frequency among BD cases compared to controls in Palestinian and Jordanian populations. They found strong linkage disequilibrium between HLA-B*51 and allele A of TNF- β (+252A/G) and suggested that both the alleles contribute to disease risk and that their coexpression leads to severe blinding in BD patients. Mizuki et al⁶² also reported significantly lower frequency of TNF- β 1 (G allele) homozygote in Japanese ocular BD patients than controls. These variations in the association between TNF- β (+252) polymorphism and BD may reflect the heterogeneity in the genetic susceptibility to this disorder.

Our results indicated that genotype-1082GG of IL-10 (-1082G/A) is susceptible to BD, while genotype AA and GA are resistant to BD. Similar to our results, Talaat et al⁶³ also reported increased frequency of genotype-1082GG and decreased frequency of genotype GA in Egyptian BD patients as compared to controls. However, Dilek et al⁶⁴ found lower frequency of AA genotype in Turkish BD patients compared to the control group, but a higher frequency of -1082GA genotype. Contrary to this, Wallace et al²⁰ reported from two populations (UK and Middle Eastern cohort) that the -1082AA genotype was weakly associated with BD when all patients were analyzed as a group, but not in the UK or Middle Eastern cohorts of patients alone compared to local controls.

Our results also showed that -819TT and 592 AA genotypes of IL-10 are significantly associated with susceptibility risk of BD in Saudi patients. IL-10 (-819T/A) polymorphism has been indicated to be significantly associated with BD.⁴² Wu et al⁶⁵ reported that -819 T/A polymorphism is associated with BD in Chinese Han population. An association with -819T was also reported in UK and Middle Eastern BD patients, and this was because of an association in the UK but not Middle Eastern patients.²⁰ Another meta-analysis with results from Turkish and Korean cohorts also showed genome-wide significant associations with -819T of IL10.¹⁹ A meta-analysis showed that IL-10 (-1082G/A, -819C/T, -592C/A) polymorphisms are significantly associated with BD,⁴² while Ates et al²² found no significant associations between IL10-1082G/A, -819C/T, -592C/A polymorphisms and Turkish BD.

Recently, emphasis has been given to address and clarify the functional relevance of the different genes found to be associated with BD susceptibility and the potential interactions between genes located within and outside the major histocompatibility complex region.⁶⁶ Our results, suggesting an association of allele A and genotype GA of TNF- α (308) with BD in Saudi patients, indicate the functional relevance of the allele A as it is linked with higher levels of TNF- α reported in active BD patients as compared to controls.^{14,54} The proinflammatory cytokines, including TNF- α , are potent inducers of inflammation, and elevated levels of these cytokines are frequently associated with the activation of macrophages, thereby influencing the severity of inflammatory responses. These cytokines induce local inflammatory responses and also exert systemic effects. The overexpression of these cytokines may be responsible for the pathogenesis of recurrent BD.⁵⁵ TNF- α has been suggested to be directly responsible for activation of T-cells and neutrophils, the main pathogenic changes in BD.⁶⁷ Moreover, low producers

(1082GG genotype) of IL-10, an anti-inflammatory cytokine, may not suppress the activity of TNF- α , resulting in increased inflammatory responses in BD patients. IL-10 limits the secretion of proinflammatory cytokines, such as TNF- α and IL-12.⁶⁸ It is possible that the pathophysiology of a deficiency in the IL-10 involves undue and prolonged activation of mononuclear cells, resulting in an augmented efflux of inflammatory cytokines including TNF- α . IL-10 is a multifunctional cytokine with profound involvement in diverse areas of the human immune system.⁶⁹

These results might have prognostic value for future clinical observations. Further, TNF- α (-308) polymorphism may serve as a guideline in determining the response to anti-TNF- α therapy, as patients with GG genotype are better responders to anti-TNF- α treatment than those with AA or GA.^{70,71} However, such associations need further validation and investigation in more patients with BD, as they may have implications for the development of novel therapies as suggested by Xavier et al.⁷² The main weakness of the study is the small sample size, while the strengths of the study lies in the fact that it is the first report from Saudi patients with BD, showing association of five polymorphisms in *IL-10* and *TNF- α* and *- β* genes. The errors in genotyping both cases or controls have been avoided carefully by using a standard protocol with positive and negative controls. Statistical analysis was performed to get *P*-values, RR, etiologic fraction and preventive fraction, and power.

Conclusion

This study, though based on a small number of cases, clearly shows that TNF- α (-308) and IL-10 (-1082, -819, and -592) polymorphisms are significantly associated with BD susceptibility. This is the first report showing association of TNF- α and IL-10 polymorphisms with BD in Saudi Arabian patients.

Acknowledgments

The authors thank PSMC for providing all facilities and S Sadaf Rizvi and Mohammad Al-Asmari for their help with laboratory work.

Disclosure

The authors report no conflicts of interest in this work.

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