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SHORT COMMUNICATION

TNF-α gene polymorphisms in Iranian Azeri Turkish patients with Behcet's Disease

Mortaza Bonyadi · Zohreh Jahanafrooz · Mohsen Esmaeili · Susan Kolahi · Alireza Khabazi · Ali Asghar Ebrahimi · Mehrzad Hajialilo · Saeed Dastgiri

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Abstract Genetic factors that predispose individuals to Behcet's disease (BD) are considered to play an important role in the development of the disease. The serum level of tumor necrosis factor (TNF) is elevated in patients with BD, and a dramatic response to anti-TNF-α antibody treatment further supports the role of TNF in BD. We investigated the distribution of TNF-α promoter −1031T/C and −308G/A polymorphisms in 53 BD patients of Iranian Azeri Turks and 79 matched healthy controls, via the PCR–RFLP technique. The frequency of the TNF-α −1031C allele was significantly higher in Behcet's patients than in healthy controls ($p < 0.0001$, OR = 3.08; 95% CI = 1.73–5.47), whereas the frequency of the TNF-α −308A allele was similar in the two compared groups. The frequency of CG haplotype was significantly higher ($p < 0.0001$, OR = 3.42; 95% CI = 1.89–6.18), and that of the TA haplotype was significantly lower in BD patients than in healthy controls. These results suggest that TNF-α is a susceptibility gene for BD in patients from Iranian Azeri Turk ethnic group.

Keywords Behcet's disease · TNF-α · Polymorphism · Iranian Azeri Turks · Genetic susceptibility

Abbreviations

BD	Behcet's disease
TNF-α	Tumor necrosis factor-alpha
HLA	Human leukocyte antigen
PCR–RFLP	Polymerase chain reaction–restriction fragment length polymorphism

Introduction

Behcet's disease (BD) is a systemic vasculitis characterized by recurrent oral and genital ulcers, skin lesions, and uveitis. Other manifestations may include arthritis, central nervous system disease, and gastrointestinal tract (GIT) disease with diarrhea and abdominal pain. Patients with BD may manifest all or only some of these clinical features depending on environmental factors and their genetic background [1–3]. Behcet's disease has been classified as an autoinflammatory disease (AID) because of the observed enhanced inflammatory response [4]. Although BD does not have the features of a classical autoimmune disorder, an antigen-driven immune response is seen in BD that possibly develops on the background of enhanced innate immune reactivity [4]. Familial aggregation of BD patients [5], association of HLA-B51 with BD, and peculiar geographical distribution of this disease along the old silk route (running from the Mediterranean, through the Middle East, and to Asia including countries such as Turkey and Iran) [6] strongly support the contribution of genetic factors to

M. Bonyadi (✉) · Z. Jahanafrooz
Medical-Molecular Genetics,
Center of Excellence for Biodiversity,
Faculty of Natural Sciences, University of Tabriz,
Tabriz, Iran
e-mail: jabbarpour@tabrizu.ac.ir

M. Bonyadi · Z. Jahanafrooz · M. Esmaeili
Liver and Gastrointestinal Disease Research Center,
Tabriz University of Medical Sciences, Tabriz, Iran

S. Kolahi · A. Khabazi · A. A. Ebrahimi · M. Hajialilo
Imam Reza Hospital, Rheumatology Ward,
Faculty of Medicine, Medical University of Tabriz, Tabriz, Iran

S. Dastgiri
Department of Community and Family Medicine,
School of Medicine, National Public Health Management Center,
Tabriz University of Medical Sciences, Tabriz, Iran

the pathogenesis of BD. Extrinsic triggering factors such as bacterial and viral infections are suggested to be important in the pathogenesis of BD [7]. Tumor necrosis factor-alpha (TNF-α) is a multifunctional, pro-inflammatory cytokine, which plays an important role in the regulation of the immune response as part of the cytokine network, including activation of macrophages and apoptosis, which appears to be responsible for the recurrent inflammatory reactions encountered in BD patients. Increased levels of circulating TNF in Behcet's disease have been reported [8]. TNF-α is encoded in the class III region of the HLA complex, adjacent to HLA-B, and many studies showed that the relative levels of cytokine production may be important in the determination of disease onset, progression, and outcome, thus implicating it as both a positional and functional candidate gene in the pathogenesis of BD [9]. A number of single nucleotide polymorphisms (SNPs) in the TNF-α promoter region have been identified, of which we choose to genotype TNF-α −1031T/C and TNF-α −308G/A polymorphisms. It has been shown that the −1031C and −308G alleles causes an increase in TNF-α production [10, 11]. The implication of these polymorphisms in susceptibility of several autoimmune diseases like systemic lupus erythematosus, insulin-dependent diabetes, and inflammatory bowel disease has been assessed [12–14]. The objective of the current study was to investigate the possible relation between TNF-α −1031T/C and TNF-α −308G/A polymorphisms and susceptibility to Behcet's disease in Iranian Azeri Turkish Patients.

Materials and methods

Subjects

We studied 53 Iranian Azeri Turkish patients (31 males, 22 females; mean ± SD age, 34.25 ± 8.33) from 17 to 50 years of age with BD. All these patients were from Azeri Turk ethnic group referred to Molecular-Medical Genetic Center of Tabriz by specialists. All patients were diagnosed according to the diagnostic criteria proposed by the international study group for BD [15]. Characteristics of the patients are summarized in Table 1. We also studied 79 ethnically sex-matched healthy controls (44 male and 35 female) without BD or other inflammatory diseases and unrelated to each other or to the patients. The genotype frequencies of BD patients and healthy controls conformed to the Hardy–Weinberg equilibrium ($p = 0.95$ for BD patients and $p = 0.95$ for healthy controls).

Cytokine gene polymorphisms

Each participant was informed about the study and consent was obtained from them. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols [16]. Polymorphisms in the promoter region of the TNF-α gene at positions −1031T/C and −308G/A were detected by a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assay. The 270-bp region of the TNF-α gene, encompassing the −1031T/C polymorphism site, was amplified via polymerase chain reaction (PCR) using the sense (5′-GGGGA GAACAAAAGGATAAG) and antisense (5′-CCCCATA CTCGACTTTCATA) primer pair. Initially, the PCR reaction was subjected to denaturation for 5 min at 95°C, followed by 30 cycles of amplification (30 s at 95°C, 30 s at 55°C and 30 s at 72°C). A final elongation step (5 min at 72°C) was applied at the end of the 30 cycles. The PCR is followed by an overnight digestion with the restriction enzyme BbsI (C allele, 159 and 111 bp; T allele, 270 bp) at 37°C. Digested PCR fragments were separated by an 8% polyacrylamid gel electrophoresis and visualized by ethidium bromide staining. Primers (5′-AGGCAATA GGTTTTGAGGGCCAT) and (5′-TCCTCCCTGCTCCG ATT CCG) were used to amplify the 107-bp DNA fragment of the TNF-α −308A/G polymorphism. PCR conditions were 5 min for initial denaturation at 95°C; 35 cycles at 95°C for 1 min for denaturation, 30 s at 65°C for annealing and 30 s at 72°C for extension, followed by 5 min at 72°C for final extension. After amplification, PCR products were digested (at 37°C) by restriction endonuclease NcoI (G allele, 87 and 20 bp; A allele, 107 bp) for 16 h. Digested PCR products were electrophoresed in an 8% polyacrylamide gel and visualized by ethidium bromide staining.

Table 1 The clinical features of patients with BD

Clinical characteristics	Number of patients (%)
Oral ulcer	52 (98.1)
Genital ulcer	35 (66.0)
Ocular involvement	
Anterior uveitis	21 (39.6)
Posterior uveitis	26 (49.1)
Retinal vasculitis	23 (43.4)
Pathergy test	23 (43.4)
Skin lesions	
Pseudofolliculitis	17 (32.1)
Erythema nodosum	11 (20.8)
Arthritis	4 (7.5)
Neurological involvement	3 (5.6)
Gastrointestinal involvement	2 (3.7)
Epididymitis	2 (3.7)

BD Behcet's disease

Statistical analysis

Comparison of alleles and genotypes frequencies between patients and healthy controls was carried out using chi-square test with Yates' correction or Fisher's exact test, where appropriate. Probability values of 0.05 or less were regarded as statistically significant. Differences in haplotypes frequencies of TNF-α promoter region between patients and healthy controls were also analyzed using chi-square test. The odds ratio (OR) and the 95% confidence intervals (CI) were calculated whenever applicable.

Results

Comparison of the allele and genotype frequencies of TNF-α −308G/A polymorphism showed no significant difference between BD patients and healthy controls in this ethnic (Table 2). The frequencies of the TNF-α −308 G/G, G/A, and A/A genotypes were 0.906, 0.094, and 0 in BD patients; and 0.798, 0.177, 0.025 in healthy controls, respectively, ($p = 0.2340$ by Fisher's exact test), and the allele frequencies of TNF-α −308G were 0.953 in BD patients and 0.886 in healthy controls ($p = 0.0966$, OR = 0.39; 95% CI = 0.14–1.07).

Allele and genotype distributions of TNF-α −1031T/C polymorphism was significantly different between BD patients and healthy controls. It was seen that, in this ethnic group, the TNF-α −1031C allele frequency was significantly higher in patients compared with healthy controls (Table 2). The frequencies of the TNF-α −1031 T/T, T/C, and C/C genotypes were 0.377, 0.491, and 0.132 in BD patients; and 0.696, 0.279, 0.025 in healthy controls, respectively, ($p = 0.0006$ by χ^2 test), and the allele frequencies of TNF-α −1031T were 0.623 in BD patients and 0.835 in healthy controls ($p < 0.0001$, OR = 3.08; 95% CI = 1.73–5.47). The frequencies of the TNF-α −1031T −308G, TNF-α −1031T −308A, TNF-α −1031C −308G, and TNF-α −1031C −308A haplotypes were 0.584 (62/106), 0.038 (4/106), 0.368 (39/106), and 0.010 (1/106) in BD patients; and 0.741 (117/158), 0.095 (15/158), 0.145 (23/158), 0.019 (3/158) in healthy controls, respectively. Among four haplotypes, TNF-α −1031T −308G was the most common haplotype in the Iranian Azeri Turkish population. The TNF-α −1031C −308G haplotype containing the TNF-α −1031C and TNF-α −308G alleles was found to be significantly associated with BD ($p < 0.0001$, OR = 3.42; 95% CI = 1.89–6.18). Conversely, the TNF-α −1031T −308A haplotype, which dose not harbor these allelic polymorphisms, was associated with a reduced susceptibility to BD (Table 3). We also analyzed the data according to the presence of genital ulcer, ocular involvements, and skin lesions, TNF-α −1031T/C, −308G/A polymorphisms, and haplotypes in patients with these clinical characters were not significantly different from those without these clinical characters (data not shown).

Discussion

This is the first report assessment of TNF-α gene promoter polymorphisms and their associations with BD in patients

Table 2 TNF-α polymorphisms in Iranian Azeri Turkish patients with Behcet's disease and healthy controls

SNP	Genotype frequency (%)			χ^2 (p value)	Allele frequency (%)		χ^2 (p value)	OR (95% CI)
−1031T/C	T/T	T/C	C/C		T	C		
Patients	20 (37.7)	26 (49.1)	7 (13.2)	14.9 (0.0006)	66 (62.3)	40 (37.7)	15.32 (<0.0001)	3.08 (1.73–5.47)
Controls	55 (69.6)	22 (27.9)	2 (2.5)		132 (83.5)	26 (16.5)		
−308G/A	G/G	G/A	A/A		G	A		
Patients	48 (90.6)	5 (9.4)	0(0.0)	– (0.2340)	101 (95.3)	5 (4.7)	2.76 (0.0966)	0.39 (0.14–1.07)
Controls	63 (79.8)	14 (17.7)	2 (2.5)		140 (88.6)	18 (11.4)		

TNF tumor necrosis factor, *SNP* single nucleotide polymorphism, *OR* odds ratio, *CI* confidence intervals

Table 3 Haplotypes of TNF-α promoter region in Iranian Azeri Turkish patients with Behcet's disease and healthy controls

Haplotype	Patients	Controls	χ^2	p value	OR (95% CI)
GT	62 (58.4%)	117 (74.1%)	7.04	0.0080	0.49 (0.29–0.83)
GC	39 (36.8%)	23 (14.5%)	17.45	<0.0001	3.42 (1.89–6.18)
AT	4 (3.8%)	15 (9.5%)	2.31	0.1285	0.37 (0.12–1.16)
AC	1 (1.0%)	3 (1.9%)		0.6511	0.49 (0.05–4.80)

TNF tumor necrosis factor, *OR* odds ratio, *CI* confidence intervals

from Azeri Turks living in northwestern Iran. This ethnic group, constituting 25% of the Iranian population, is ethnically identical to Azeris and closely related to Turks. The etiology of BD as yet remains to be discovered, but both genetic and environmental factors appear to trigger the disease. HLA-B51 is found commonly in patients from the old silk route [17]. However, a study on Tau-a microsatellite polymorphism suggested that the pathogenic gene is not HLA-B51 itself but other gene(s) located near HLA-B51 [18]. Single nucleotide polymorphisms (SNPs) of multiple genetic factors on chromosome 6 or other chromosomes [9, 17, 19–26] and also some gene mutations including familial Mediterranean fever (FMF) gene (MEFV) and autoinflammatory gene mutations [27, 28] have been investigated in development of Behcet's disease. Overexpression of proinflammatory cytokines such as TNF-α in this disease through an unknown mechanism is a prominent feature compatible with the findings in other AIDs [29]. In this study, we have investigated the association of Behcet's disease with two single nucleotide polymorphisms, TNF-α −1031T/C and TNF-α −308G/A in Iranian Azeri Turkish patients with Behcet's disease by using PCR–RFLP technique. The TNF-α −1031C allele was strongly associated with Behcet's disease in Iranian Azeri Turkish patients ($p < 0.0001$, OR = 3.08; 95% CI = 1.73–5.47). In the case of another polymorphism, TNF-α −308G/A, no significant difference was observed in the allele and genotype frequencies between BD patients and healthy controls ($p = 0.0966$, OR = 0.39; 95% CI = 0.14–1.07). Our results are consistent with previous results reported in the Turkish [9, 21], UK [17], Korean [20], and Tunisian [22] populations. In the aforementioned populations the TNF-α −1031C allele was associated with BD but no association was found between TNF-α −308G/A polymorphism and susceptibility to Behcet's disease. Using in vitro techniques, Higuchi et al. have shown that the TNF-α −1031CC caused an increase in TNF-α production in response to specific external stimuli [11] which might be one reason for high TNF-α levels in patients with BD. This could justify strong association of TNF-α −1031C allele with BD patients in our cohort. To our knowledge, TNF-α −1031C allele shows positive association with BD in all reports [17, 19–22]. Therefore, this study should be conducted in other ethnic groups to investigate the mechanism of TNF-α gene contribution in BD susceptibility. Haplotypes analysis of the TNF-α promoter in our cohort showed that the TNF-α −1031C −308G haplotype, which harbors the −1031C and −308G alleles, was significantly associated with BD ($p < 0.0001$, OR = 3.42; 95% CI = 1.89–6.18). Conversely, the TNF-α −1031T −308A which did not have these allelic polymorphisms was associated with a reduced susceptibility to BD ($p = 0.1285$, OR = 0.37; 95% CI = 0.12–1.16). This result is consistent with previous reports from UK and Korea [17, 19].

We did not find significant association between these two polymorphisms (or haplotypes) and clinical parameters in BD patients which is also consistent with some previous reports [9, 20, 22]. For this study, 53 BD patients and 79 matched healthy controls were identified with the sample size determination formula for case–control studies using alpha = 0.05 and beta = 0.71 [30]. In the meantime, this was the maximum available number of BD patients which was available in the study setting over the study period. Further studies are required to investigate other possible associations between TNF-α gene polymorphisms with BD in patients from this ethnic group in order to elucidate the effects of TNF-α gene on the development of BD. The results of the present study suggest that the TNF-α is a susceptibility gene to BD in Iranian Azeri Turkish patients. However, the sample size of this study could not be sufficient to confirm (100%) the association.

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References

1. Wallace GR, Kondeatis E, Vaughan RW, Verity DH, Chen Y, Fortune F et al (2007) IL-10 genotype analysis in patients with Behcet's disease. Hum Immunol 68:122–127
2. Harmouche H, Maamar M, Sahnoune I, Tazi-Mezalek Z, Aouni M, Maaouni A (2007) Fever revealing Behcet's disease: two new cases. Eur J Intern Med 18:146–147
3. Schwartz T, Langevitz P, Zemer D, Gazit E, Pras M, Livneh A (2000) Behcet's disease in familial Mediterranean fever: characterization of the association between the two diseases. Semin Arthritis Rheum 29:286–295
4. Touitou I, Kone-Paut I (2008) Autoinflammatory diseases. Best Pract Res Clin Rheumatol 22:811–829
5. Gul A, Inanc M, Ocal L, Aral O, Konice M (2000) Familial aggregation of Behcet's disease in Turkey. Ann Rheum Dis 59:622–625
6. Verity DH, Marr JE, Ohno S, Wallace GR, Stanford MR (1999) Behcet's disease, the silk road and HLA-B51: historical and geographical perspectives. Tissue Antigens 54:213–220
7. Durrani K, Papaliodis GN (2008) The genetics of Adamantiades–Behcet's disease. Semin Ophthalmol 23:73–79
8. Oztas MO, Onder M, Gurer MA, Bukan N, Sancak B (2005) Serum interleukin 18 and tumor necrosis factor-alpha levels are increased in Behcet's disease. Clin Exp Dermatol 30:61–63
9. Ates A, Kinikli G, Duzgun N, Duman M (2006) Lack of association of tumor necrosis factor-alpha gene polymorphisms with disease susceptibility and severity in Behcet's disease. Rheumatol Int 26:348–353
10. Higuchi T, Seki N, Kamizono S, Yamada A, Kimura A, Kato H et al (1998) Polymorphism of the 5′-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. Tissue Antigens 51:605–612
11. Wilson AG, Symons JA, Mcdowell TL, Mcdevitt HO, Duff GW (1997) Effects of a polymorphism in the human tumor necrosis factor α promoter on transcriptional activation. Immunology 94:3195–3199
12. van Heel DA, Udalova IA, De Silva AP, Mc Govern DP, Kinouchi Y, Hull J et al (2002) Inflammatory bowel disease is associated

with a TNF polymorphism that affects an interaction between the OCT1 and NF-κB transcription factors. Hum Mol Genet 11:1281–1289
13. Christen U, Wolfe T, Mohrle U, Hughes AC, Rodrigo E, Green EA et al (2001) A dual role for TNF-α in type 1 diabetes: islet-specific expression abrogates the ongoing autoimmune process when induced late but not early during pathogenesis. J Immunol 166:7023–7032
14. Zuniga J, Vargas-Alarcon G, Hernandez-Pacheco G, Portal-Celhay C, Yamamoto-Furusho JK, Granados J (2001) Tumor necrosis factor-α promoter polymorphisms in Mexican patients with systemic lupus erythematosus (SLE). Genes Immun 2:363–366
15. International Study Group for Behcet's Disease (1990) Criteria for diagnosis of Behcet's disease. Lancet 335:1078–1080
16. Miller SA, Dynes DD, Polesky F (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
17. Ahmad T, Wallace GR, James T, Neville M, Bunce M, Mulcahy-Hawes K et al (2003) Mapping the HLA association in Behcet's disease: a role for tumor necrosis factor polymorphisms? Arthritis Rheum 48:807–813
18. Mizuki N, Ohno S, Sato T, Ishihara M, Miyata S, Nakamura S, Naruse T, Mizuki H, Tsuji K, Inoko H (1995) Microsatellite polymorphism located between the TNF and HLA-B genes in Behcet's disease. Hum Immunol 43:129–135
19. Park K, Kim N, Nam J, Bang D, Lee ES (2006) Association of TNFA promoter region haplotype in Behcet's disease. J Korean Med Sci 21:596–601
20. Lee EB, Kim JY, Lee YJ, Park MH, Song YW (2003) TNF and TNF receptor polymorphisms in Korean Behcet's disease patients. Hum Immunol 64:614–620
21. Akman A, Sallakci N, Coskun M, Bacanli A, Yavuzer U, Alpsoy E et al (2006) TNF-a gene 1031 T/C polymorphism in Turkish patients with Behcet's disease. Br J Dermatol 155:350–356
22. Kamoun M, Chelbi H, Houman MH, Lacheb J, Hamzaoui K (2007) Tumor necrosis factor gene polymorphisms in Tunisian patients with Behcet's disease. Hum Immunol 68:201–205
23. Ben Dhifallah I, Houman H, Khanfir M, Hamzaoui K (2008) Endothelial nitric oxide synthase gene polymorphism is associated with Behcet's disease in Tunisian population. Hum Immunol 69:661–665
24. Lee YJ, Kang SW, Song JK, Baek HJ, Choi HJ, Bae YD et al (2007) Associations between interferon regulatory factor-1 polymorphisms and Behcet's disease. Hum Immunol 68:770–778
25. Sahin N, Bicakcigil M, Atagunduz P, Direskeneli H, Saruhan-Direskeneli G (2007) PTPN22 gene polymorphism in Behcet's disease. Tissue Antigens 70:432–434
26. Baranathan V, Stanford MR, Vaughan RW, Kondeatis E, Graham E, Fortune F et al (2007) The association of the PTPN22 620 W polymorphism with Behcet's disease. Ann Rheum Dis 66:1531–1533
27. Touitou I, Magne X, Molinari N, Navarro A, Le Quellec A, Picco P et al (2000) MEFV mutations in Behcet's disease. Hum Mutat 16:271–272
28. Kone-Paut I, Sanchez E, Le Quellec A, Manna R, Touitou I (2007) Autoinflammatory gene mutations in Behcet's disease. Ann Rheum Dis 66:832–834
29. Gul A (2005) Behcet's disease as an autoinflammatory disorder. Curr Drug Targets Inflamm Allergy 4:81–83
30. Schlesselman J (1982) Case–control studies: design, conduct, analysis, equation 6.6 and 6.7: sample size determination with an unequal case–control ratio. Oxford, New York