Polymorphisms of Promoter Region of TNF-α Gene in Iranian Azeri Turkish Patients with Behçet’s Disease

Reza Abdolmohammadi1,2 and Mortaza Bonyadi1,3

1Liver & Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; 2Department of Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran; 3Department of Molecular-Medical Genetics, Center of Excellence for Biodiversity, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran

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Address for Correspondence:
Mortaza Bonyadi
Department of Molecular-Medical Genetics, Center of Excellence for Biodiversity, Faculty of Natural Sciences, University of Tabriz, Tabriz 51368, Iran
E-mail: jabbarpour@tabrizu.ac.ir

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INTRODUCTION

Behçet’s disease (BD) is a complex chronic relapsing inflammatory disorder which is more common along the Mediterranean and Eastern Asia, than in the other areas (1). The mean age of onset of BD is in the early 30s, and the male-to-female ratio varies with ethnicity (2). BD is a neutrophilic perivasculitis with a wide range of cardiovascular abnormalities namely; aneurysms, pseudoaneurysms, vascular ruptures, intra-cardiac and venous thromboses. The low grade of inflammation in course of BD could lead to thromboses and vascular dysfunction (3). In addition, the involvement of thrombosis factors in occurrence of BD has been reported in some studies (4,5). BD is mainly characterized by recurrent oral and genital ulcers, uveitis, and skin lesions. The disease can involve joints, central nervous system, and the gastrointestinal tract (6).

The etiopathogenesis of BD is not clearly established. However, immune malfunction, characterized by activation in neutrophils and T cells and release of Th1-type pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), has been considered as the major pathogenetic factor of the disease (7-9). The association of BD and promoter region of TNF-α gene was firstly reported in Japanese population (10). TNF-α protein, a multifunctional and pro-inflammatory cytokine with confirmed role in activation of macrophages and apoptosis, plays an important role in the regulation of immune system. Moreover, it appears to be in charge of the recurrent inflammatory reactions encountered in BD patients (11). The TNF-α gene lies on the short arm of chromosome 6 (6p21), encoded in the class III region of human leukocyte antigen (HLA) complex, adjacent to HLA-B (12). A number of single nucleotide polymorphisms (SNPs) in TNF-α promoter region have been identified, of which we chose to genotype TNF-α -857T/C and TNF-α -238A/G polymorphisms. It has been shown that the -857C and -238A alleles cause an increase in TNF-α production (13,14). The implication of these polymorphisms in susceptibility to several autoimmune diseases, such as rheumatoid arthritis, ankylosing spondylitis, and systemic lupus erythematosus, has been assessed (15-17).

Iran is among the countries in which BD is rather common (18) and this study was designed to determine the frequency of TNF-α -857T/C and TNF-α -238G/A polymorphisms among Iranian Azeri Turkish BD patients.

MATERIALS AND METHODS

Subjects
A total of 161 Iranian Azeri Turkish subjects were included in this study, all the participants were single ethnic Iranian sub-
DNA Extraction and Genotyping
Genomic DNA was extracted from 2 mL of peripheral venous blood by a modified “salting out” technique (20), precipitated with ethanol and resuspended in sterile distilled water and DNA concentrations were determined with a UV spectrophotometer at 260 nm (Technne, Stone, United Kingdom).

The TNF promoter polymorphism (-238G>A & -857C>T) was evaluated by polymerase chain reaction (PCR) amplifications using sequence specific primers. Briefly, for polymorphism at -238G>A amplification with primer sense 5’-GAA ACC CCC CTC GGA ATC G/A-3’, antisense 5’-GGC TGG GTG TGC CAA CAA C-3’ and for polymorphism at -857C>T, antisense 5’-TCA CAT GGC CCT GTC TTC G-3’ or 5’-CTC ACA TGG CCC TGT CTT C-3’, sense 5’-AAG ATA AGG GCT CAG AGA G-3’. For PCR amplification, a total volume of 25 µL, containing 250 ng genomic DNA, 20 pmol of each primers, 300 µmol dNTPs mix (CinnaGen, Tehran, Iran), 1,500 µmol MgCl₂, 2.5 µL 10 × PCR buffer (500 mM KCl and 200 mM Tris-HCl; pH 8.4), and 2 units Taq DNA polymerase (CinnaGen) were used.

PCR conditions were as following: denaturation at 94°C for 5 minutes, 35 cycles at 94°C for 35 seconds, 60°C for 20 seconds, and 72°C for 30 seconds, followed by one cycle of final extension at 72°C for 5 minutes. The PCR products were then electrophoresed on 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light (87 bp for TNF -238G>A and 290 bp TNF -857C>T). Primer connx30 was added to each reaction as an internal control (400 bp). Distilled water was used as negative controls. In order to confirm the methodology, 30 samples that were screened by ARMS-PCR were subjected to automated sequence analysis.

Statistical analysis
Statistical analysis was carried out with the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were evaluated by standard χ² or Fisher exact tests, Allele and genotype frequencies were calculated and compared with non-parametric tests followed by Fisher’s exact analysis using STATA version 8 (StataCorp LP, College Station, TX, USA). A P value of < 0.05 was considered significant.

Table 1. TNF-α genotypes and allele frequencies at position -857 in patients with BD and in healthy controls

| Genotypes | BD, No. (%) | Control, No. (%) | P value | OR | 95% CI |
|-----------|-------------|-----------------|---------|----|--------|
| C/C       | 51 (78.5)   | 52 (54.2)       | 0.001   | 3.0824 | 1.592–6.013 |
| C/T       | 14 (21.5)   | 42 (43.7)       | 0.003   | 0.353 | 0.181–0.658 |
| T/T       | 0 (0.0)     | 2 (2.1)         | -       | -    | -      |
| ̲C  Allele | 116 (89.23) | 146 (76.0)      | 0.002   | 2.616 | 1.368–4.980 |
| ̲T  Allele | 14 (10.77)  | 46 (24.0)       | 0.0028  | 0.383 | 0.2008–0.73 |

TNF = tumor necrosis factor, BD = Behçet’s disease, OR = odds ratio, CI = confidence interval.
**Table 2.** Haplotypes of TNF-α promoter region (-238 & -857) in Iranian Azeri Turkish patients with BD and healthy controls

| Haplotypes | Patients (%) | Controls (%) | P-value | OR (95% CI) |
|------------|-------------|--------------|---------|-------------|
| GC         | 51.0        | 49.1         | 0.444   | 1.083 (0.599–1.960) |
| AC         | 47.1        | 44.0         | 0.388   | 1.129 (0.622–2.048) |
| GT         | 1.9         | 5.2          | 0.056   | 0.353 (0.044–2.148) |
| AT         | 0.0         | 1.7          | 0.750   | 0.784 (0.464–1.324) |

TNF = tumor necrosis factor, BD = Behçet’s disease, OR = odds ratio, CI = confidence intervals.

**Table 3.** Haplotypes of TNF-α promoter region (-857 & -1031) in Iranian Azeri Turkish patients with BD and healthy controls

| Haplotypes | No. of patients | No. of controls | P-value | OR (95% CI) |
|------------|-----------------|-----------------|---------|-------------|
| CT         | 57              | 110             | 0.362   | 0.784 (0.464–1.324) |
| CC         | 33              | 23              | 0.001   | 3.363 (1.825–6.196) |
| TT         | 4               | 33              | 0.001   | 1.791 (0.061–0.523) |
| TC         | 0               | 0               | -       | -            |

TNF = tumor necrosis factor, BD = Behçet’s disease, OR = odds ratio, CI = confidence intervals.

**Table 4.** Genotype and allele distribution of BD TNF-α -857C/T polymorphism between IBD patients and clinical characteristics

| Features | Genotypes and allele distribution |
|----------|-----------------------------------|
|          | C/T                               |
|          | C/C                               |
|          | T/T                               |

**BD** = Behçet’s disease, **TNF** = tumor necrosis factor, **IBD** = inflammatory bowel disease, **OR** = odds ratio.

TNF-α -857T-238G, TNF-α -857T-238A haplotypes were 0.51, 0.471, 0.019, and 0 in BD patients; and 0.491, 0.44, 0.052, 0.017 in healthy controls, respectively (Table 2). Among four haplotypes, TNF-α -857C-238G was the most common haplotype in Iranian Azeri Turkish population. Although distribution of these haplotypes showed no significant association with our patients and control group, haplotype of TNF-α -857C (from this study) and TNF-α -1031C (from the previous study) showed statistically a significant association (P = 0.001) with BD in our cohort (Table 3).

We also analyzed the allele and genotype frequencies according to the clinical features in patients with described clinical characters and those without the clinical characters.

The TNF-α -238G/A polymorphism did not show any association with clinical findings (data not shown). The associations between the frequencies of TNF-α -857C/T genotypes and clinical findings of BD are shown in Table 4. TNF-α -857C/T polymorphisms in BD patients with Posterior uveitis, anterior uveitis, skin lesions, pseudo folliculitis and sided were not significantly different from BD patients without the manifestations (data not shown). However, the TNF-α -857T-allele frequency was significantly higher in patients with arthritis, sacroiliitis, gastrointestinal involvement and neurological symptoms compared with those of patients without the manifestations. Conversely, the TNF-α -857T-allele frequency was significantly lower in BD patients with Genital ulcers than BD patients without this clinical character (Table 3).

**DISCUSSION**

BD is an inflammatory multi-systemic disorder with unknown etiology and it seems that both genetic and environmental factors trigger the development and severity of this disease. Although, the association of HLA-B51 with BD (22), peculiar geographical distribution of this disease along the old silk route and familial aggregation of BD patients (23) strongly support the contribution of genetic factors to the pathogenesis of BD, disease-susceptible genes yet remain to be determined. In view of...
the TNF genes’ biological properties and roles of these cytokines in inflammatory disorders, it has been postulated that polymorphic TNF genes could play an important role in the BD (24). The increased levels of circulating TNF-α and enhanced TNF-α mRNA expression in BD has been reported (11). Alterations of the TNF expression related to the polymorphic alleles of TNF genes may implicate a pathogenetic role in the increased activity of this cytokine in BD. To study the influence of TNF-α gene in susceptibility to BD we have already shown a significant association of BD with TNF-α -1031C allele (21). To have a comprehensive study, the distribution of TNF-α promoter -238G>A and -857C>T polymorphisms were investigated in both BD patients and sex-age-ethnic matched healthy controls. These results demonstrated that the frequency of TNF-α -857C allele was significantly higher in Behçet’s patients than in healthy controls and it was strongly associated with BD (P = 0.001; OR = 2.616; 95% CI = 1.129–6.160), whereas the frequency of TNF-α -238A allele was similar in the two compared groups and was not associated with susceptibility to BD in this ethnic group. This is the first report of assessment of TNF-α -857C/T allele promoter polymorphism and its significant associations with BD in patients from Iranian Azeri Turk ethnic group. The results of this study are consistent with results reported in Turkish (25,26) and Korean (27) populations. In the aforementioned populations no association was found between TNF-α -238G>A polymorphism and susceptibility to BD. Conversely, the TNF-α association with BD in Caucasian population Ahmad et al. (28), showed the higher frequency of TNF-α -238A allele in BD patients in comparing to that of healthy controls. In a meta-analysis, Touma et al. (29) reported that TNF-α -1031C (OR = 1.35; 95% CI = 1.09–1.68), -238A (OR = 1.51; 95% CI = 1.12–2.04) and -857T (OR = 0.76; 95% CI = 0.58–0.98) had a significant association with BD. The ethnic variation might be responsible for these discordant results in the frequency of -857 and -238 gene polymorphisms between the present study and the other studies (28,29).

Haplotype analysis of TNF-α promoter polymorphisms (at positions -857, -1031) in our cohort showed a strong association with BD disease (P = 0.001; OR = 3.36; 95% CI = 1.825–6.196) (Table 4), whereas this association was not seen in haplotype analysis of the other polymorphisms in TNF-α promoter region (at positions -238, -857, -308) (Table 2). These findings are in consistent with a previous report (28) but it differs with some other studies (27,30). On the base of our findings the TNF-α -857T-1031T haplotype is associated with resistance to BD (P = 0.001; OR = 0.17; 95% CI = 0.061–0.523) (Table 4).

In order to study the association of BD clinical parameters with different genotypes (or alleles), our results showed the significant association of arthritis and sacrolitis with TNF-α -857T allele. Conversely, patients with a positive genital ulcer have a lower frequency of TNF-α -857T allele (Table 3). These findings suggest that TNF-α -857C/T gene polymorphism could have an effect on the development of clinical features of BD in this ethnic group.

In conclusion, the TNF-α -857C, -1031C alleles and their haplotype are significantly associated with BD. These alleles may contribute to the enhanced inflammatory reactivity observed in patients with BD. By considering the effect of geographical variations on occurrence of this disease, the association of TNF-α gene cluster polymorphisms with BD in other ethnic groups needs to be further studied.

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DISCLOSURE

The authors have no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTION

Conception and design: Bonyadi M. Genotyping, Acquisition of data and analysis of data: Abdolmohammadi R. Revision and approval of manuscript: all authors.

ORCID

Reza Abdolmohammadi http://orcid.org/0000-0002-6670-6781
Mortaza Bonyadi http://orcid.org/0000-0003-3216-2947

REFERENCES

1. Sakane T, Takeno M, Suzuki N, Inaba G. Behçet’s disease. N Engl J Med 1999; 341: 1284-91.
2. Bang DS, Oh SH, Lee KH, Lee ES, Lee SN. Influence of sex on patients with Behçet’s disease in Korea. J Korean Med Sci 2003; 18: 231-5.
3. Aksu K, Donmez A, Keser G. Inflammation-induced thrombosis: mechanisms, disease associations and management. Curr Pharm Des 2012; 18: 1478-93.
4. La Regina M, Gasparyan AV, Orlandini F, Prisco D. Behçet’s disease as a model of venous thrombosis. Open Cardiovasc Med J 2010; 4: 71-7.
5. Yang SS, Park KM, Park YI, Kim YW, Do YS, Park HS, Park KB, Kim DI. Peripheral arterial involvement in Behçet’s disease: an analysis of the results from a Korean referral center. Rheumatol Int 2013; 33: 2101-8.
6. Toutou I, Koné-Paut I. Autoinflammatory diseases. Best Pract Res Clin Rheumatol 2008; 22: 811-29.
7. Lehner T. Immunopathogenesis of Behçet’s disease. Ann Med Interne (Paris) 1999; 150: 483-7.
8. Gill A. Behçet’s disease: an update on the pathogenesis. Clin Exp Rheumatol 2001; 19: S6-12.
9. Direskeneli H. Behçet’s disease: infectious aetiology, new autoantigens, and HLA-B51. Ann Rheum Dis 2001; 60: 996–1002.
10. Verity DH, Wallace GR, Vaughan RW, Stanford MR. Behçet’s disease: from
Hippocrates to the third millennium. *Br J Ophthalmol* 2003; 87: 1175-83.

11. Oztas MO, Önder M, Gurer MA, Bukan N, Sancak B. Serum interleukin 18 and tumor necrosis factor-alpha levels are increased in Behçet's disease. *Clin Exp Dermatol* 2005; 30: 61-3.

12. Sayinalp N, Ozcebe OI, Ozdemir O, Haznedaroğlu IC, Dündar S, Kirazlı S. Cytokines in Behçet's disease. *J Rheumatol* 1996; 23: 321-2.

13. Higuchi T, Seki N, Kamizono S, Yamada A, Kimura A, Kato H, Itoh K. Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. *Tissue Antigens* 1998; 51: 605-12.

14. Grove J, Daly AK, Bassendine MF, Day CP. Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis. *Hepatology* 1997; 26: 143-6.

15. Rudwaleit M, Tilký M, Khamashta M, Gibson K, Klinke J, Hughes G, Wordsworth P. Interethnic differences in the association of tumor necrosis factor promoter polymorphisms with systemic lupus erythematosus. *J Rheumatol* 1996; 23: 1725-8.

16. Fraile A, Nieto A, Beraín Y, Vinasco J, Matarán L, Martín J. Tumor necrosis factor gene polymorphisms in ankylosing spondylitis. *Tissue Antigens* 1998; 51: 386-90.

17. Kaijzel EL, van Krugten MV, Brinkman BM, Huizinga TW, van der Straaten T, Hazes JM, Ö. Functional analysis of a human tumor necrosis factor alpha (TNF-alpha) promoter polymorphism related to joint damage in rheumatoid arthritis. *Mol Med* 1998; 4: 724-33.

18. Shahram F, Nadji A, Jamshidi AR, Chams H, Chams C, Shafaie N, Akbarian M, Gharibdoost F, Davatchi F. Behçet disease in Iran, analysis of 5,059 cases. *Arch Iran Med* 2004; 7: 9-14.

19. International Study Group for Behçet’s Disease. Criteria for diagnosis of Behçet’s disease. *Lancet* 1990; 335: 1078-80.

20. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16: 1215-20.

21. Bonyadi M, Jahanfrooz Z, Esmaeili M, Kolahi S, Khabazi A, Ebrahimi AA, Hajialilo M, Dastgiri S. TNF-α gene polymorphisms in Iranian Azeri Turkish patients with Behçet’s disease. *Rheumatol Int* 2009; 30: 265-9.

22. Verity MR, Marr JE, Ohno S, Wallace GR, Stanford MR. Behçet's disease, the Silk Road and HLA-B51: historical and geographical perspectives. *Tissue Antigens* 1999; 54: 213-20.

23. Gül A, Inanç M, Ocal L, Aral O, Konıçe M. Familial aggregation of Behçet’s disease in Turkey. *Ann Rheum Dis* 2000; 59: 622-5.

24. Verjans GM, Messer G, Weiss EH, van der Linden SM, Kijlstra A. Polymorphism of the tumor necrosis factor region in relation to disease: an overview. *Rheum Dis Clin North Am* 1992; 18: 177-86.

25. Ateş A, Kinikli G, Düzgün N, Duman M. Lack of association of tumor necrosis factor-alpha gene polymorphisms with disease susceptibility and severity in Behçet’s disease. *Rheumatol Int* 2006; 26: 348-53.

26. Storz K, Löffler J, Koch S, Vonthurn R, Zouboulis CC, Fresko I, Yazici H, Kötter I. IL-6 receptor, IL-8 receptor and TNF-alpha238 (G/A) polymorphisms are not associated with Behçet’s disease in patients of German or Turkish origin. *Clin Exp Rheumatol* 2008; 26: S103-6.

27. Park K, Kim N, Nam J, Bang D, Lee ES. Association of TNFA promoter region haplotype in Behçet’s disease. *J Korean Med Sci* 2006; 21: 596-601.

28. Ahmad T, Wallace GR, James T, Neville M, Bunce M, Mulcahy-Hawes K, Armuzzi A, Crawford J, Fortune E, Walton R, et al. Mapping the HLA association in Behçet’s disease: a role for tumor necrosis factor polymorphisms? *Arthritis Rheum* 2003; 48: 807-13.

29. Touma Z, Farra C, Hamdan A, Shamsedddeen W, Uthman I, Hourani H, Arayssi T. TNF polymorphisms in patients with Behçet disease: a meta-analysis. *Arch Med Res* 2010; 41: 142-6.

30. Amirzargar A, Shahram F, Nikoopour E, Rezaei N, Saeedifar K, Ziaei N, Davatchi F. Proinflammatory cytokine gene polymorphisms in Behçet’s disease. *Eur Cytokine Netw* 2010; 21: 292-6.