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ORIGINAL ARTICLE

A Single Nucleotide Polymorphism in the FOXP3 Gene Associated with Behçet's Disease in an Iranian Population

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SUMMARY

Background: **Behçet's Disease (BD) is a rare autoimmune disease that involves the dysfunction of regulatory T cells. FOXP3 is a key transcription factor in the development and function of T_{reg} cells. Recent studies have shown SNPs in the FOXP3 contribute to the susceptibility to some autoimmune disorders.**
Methods: **To clarify the association between the FOXP3 gene and the risk of BD, 50 patients diagnosed with BD and 50 healthy controls from north-western Iran were genotyped by PCR-RFLP (*Mun I* and *Pst I*) for two SNPs including rs3761547 (-3499T/C) and rs3761548 (-3279 C/A) in the promoter region of the FOXP3 gene. In addition, a 506 bp nucleotide sequence of FOXP3 promoter was analyzed.**
Results: **The allele -3279 C/A was significantly associated with BD [p = 0.002; odds ratio (OR) = 3.841; 95% confidence interval (CI) 1.610 - 9.161]; whereas, there was no contribution of the FOXP3 polymorphism -3499T/C to BD [(p = 0.084); (OR = 0.348, 95% CI = 0.101 - 1.195)]. Meanwhile, sequence analysis showed 100% similarity in both controls and BD patient groups.**
Conclusions: **Therefore, the SNP rs3761548 in the FOXP3 gene appears to contribute to the risk of Behçet's disease among the north-western Iranian population.**
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KEY WORDS

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INTRODUCTION

Relapsing genital ulcers, mucocutaneous lesions, uveitis, and the manifestations including neurological, pulmonary, and gastrointestinal involvement characterize Behçet's diseases (BD), a multi-system auto-inflammatory syndrome [1,2]. It can involve any organs and results in notable morbidity and increased mortality [2]. BD has worldwide distribution but it has a distinct geographical distribution along the "Silk Road" that extends from the Mediterranean to far East Asia in Eurasian and Asian populations [3].

The prevalence of BD is high in countries such as Iran, Turkey, Japan, Korea, Tunisia, China, the Mediterranean and the Middle East countries [4-6]. Although the exact cause of BD remains unknown, the epidemiological findings suggest that the disease may be triggered by an infection or environmental factors in patients with a background of genetic susceptibility [4,7].

Different studies have reported that regulatory T cells (T_{regs}), a sub-population of T cells, are paradoxically expanded in BD [8,9]. T_{regs} play a central role in regulating of immune response and are essential for maintaining self-tolerance [10,11]. The FoxP family of transcription factors plays an important role in development [12] and mutation in FOXP3 is linked to autoimmune disease [13]. The transcription factor Foxp3 is an acetylated and oligomeric protein that has a key role in the T_{reg} cell development and function [13,14].

Mutations in the human FOXP3 gene cause disease immune dysregulation, polyendocrinopathy, enteropathy, and x-linked (IPEX) syndrome [15,16]. Many researchers have reported that there are 20 mutations in FOXP3 in patients with IPEX syndrome [17,18]. Van der Vliet and Nieuwenhuis (2007) concluded that mutations in FOXP3 results in IPEX [19]. In the current study, we investigate the hypothesis that mutation in the FOXP3 gene may be involved in Behçet Disease. Therefore, we chose two SNPs in BD patients and assessed their association among BD patients and normal controls. Furthermore, we analyzed FOXP3 promoter sequences in BD patients and control groups. Further knowledge of the molecular biology of regulatory T cells will likely result in new insights for Behçet Disease control and treatment.

MATERIALS AND METHODS

Patient demographics and DNA extraction

In the current study, 100 Iranian native Azeri [50 patients with Behçet's Disease (29 male and 21 female; mean age 34.02 ± 7.39 years) and 50 normal healthy controls (26 male and 24 female; mean age 34.42 ± 8.27 years) were analyzed. All patients were diagnosed with BD by at least two independent dermatologists. The Ethical Committee of Tabriz University of Medical Sciences (TUMS) approved the protocol of the study, which complied with the Helsinki Declaration. Written agreement was obtained from all participating individuals. Genomic DNA was extracted from whole blood by using standard salting out method. The DNA samples were each re-suspended in 30 µL of sterile TE buffer and stored at -20°C until use.

Designing specific primers and PCR amplification

Specific primers were designed for amplification of the region containing rs3761547 T/C and rs3761548 C/A SNPs using Oligo 5 software. BDF47 (5'- CAA TCC TCC TCT CGC ACA CA-3') and BDF48 (5'- CCT CTC CGT GCT CAG TGT AG -3') were used as forward primers; and BDR47 (5'- CAC AGC CTG ACT GAC TGA CAT -3') and BDF48 (5'-CAC AGC CTG ACT GAC TGA CAT-3') used as reverse primers. PCR amplification was carried out in a final volume of 25 µL using a Peqlab thermal cycler. Each reaction contained 20 ng of DNA, 0.5 µM of each primer, and 12.5 µL of 2 x master mix Amplicon^{TM} (Amplicon, Herlev, Denmark). PCR conditions included an initial denaturation step at 95°C for 4 minutes, which was followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 59°C for 50 seconds, and extension at 72°C for 80 seconds and a final extension step of 72°C for 5 minutes. PCR products were detected by 2% (w/v) agarose gel electrophoresis in TAE buffer stained with SYBR Green (DNA safe stain, Tehran, Iran).

Genotyping of the SNPs in the FOXP3 gene

PCR-RFLP for genotyping the rs3761547 (-3499T/C) and rs3761548 (-3279 C/A) polymorphisms were performed and PCR products were digested by *Mun* I and *Pst* I enzymes, respectively. Each reaction contained 7 µL of purified PCR product, 2 µL of 10 x enzyme buffer, 5 units of restriction enzyme and nuclease free water up to a final volume of 20 µL. The reactions were incubated overnight at 37°C and digested products were visualized by UV trans-illumination after electrophoresis on 2% agarose gels and again stained with SYBR Green (DNA safe stains, Tehran, Iran).

FOXP3 promoter sequencing

The PCR products were subjected to sequencing in an ABI373 automatic sequencer, using the amplification primer to obtain the sequence.

Sequence analysis

The acquired sequences were double-checked with Chromas software version 2.31 and annotated following comparison with GenBank data including Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/blast/) and Mantel et al. (2006) [20]. The sequences related to control and BD patients were aligned and compared using Clustal W [21].

Table 1. Comparisons of allele and genotype frequency for the C/A and T/C, FOXP3.

SNP	Genotype frequency			χ2 (p-value)	Allele frequency		χ2 (p-value)	OR (95% CI)
-3279C/A	C/C	C/A	A/A		C	A		
BD	22%	6%	72%	0.003	25%	75%	0.002	3.841 (1.610 - 9.161)
Control	52%	24%	24%		64%	36%		
-3499T/C	T/T	T/C	C/C	χ2 (p-value)	T	C	χ2 (p-value)	OR (95% CI)
BD	90%	6%	4%	0.108	93%	7%	0.084	0.348 (0.101-1.195)
Control	76%	10%	14%		81%	19%		

Between Behçet's patient cases and healthy controls.

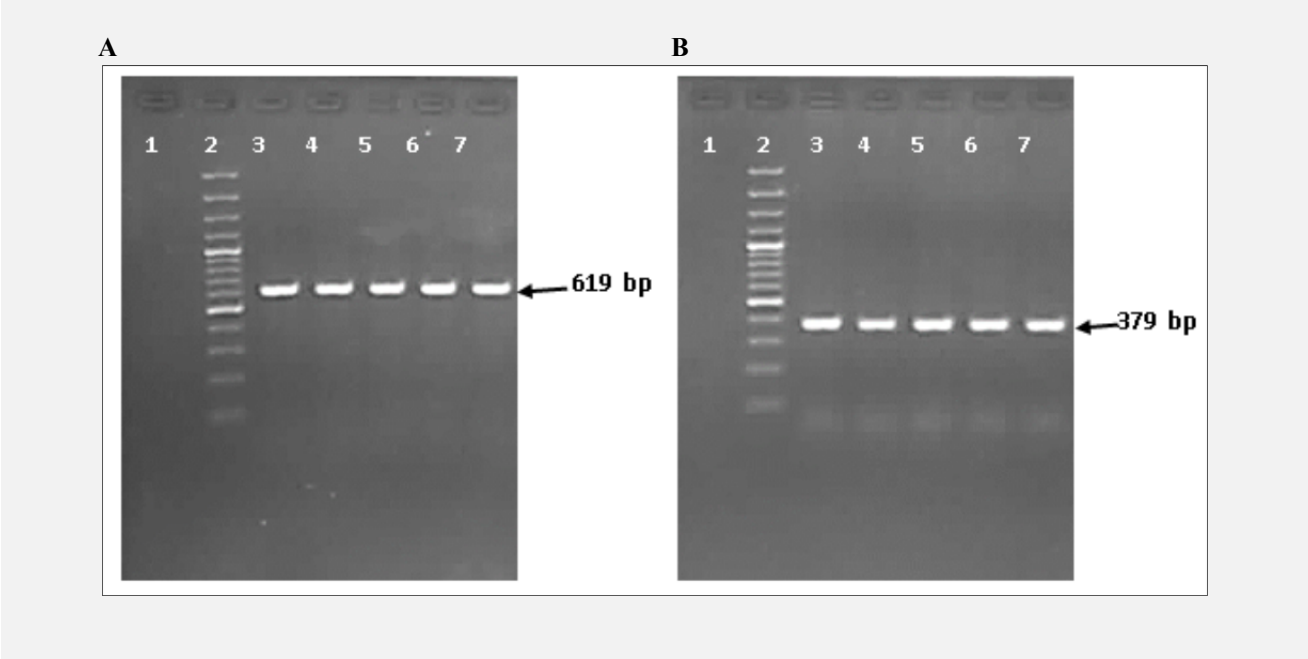


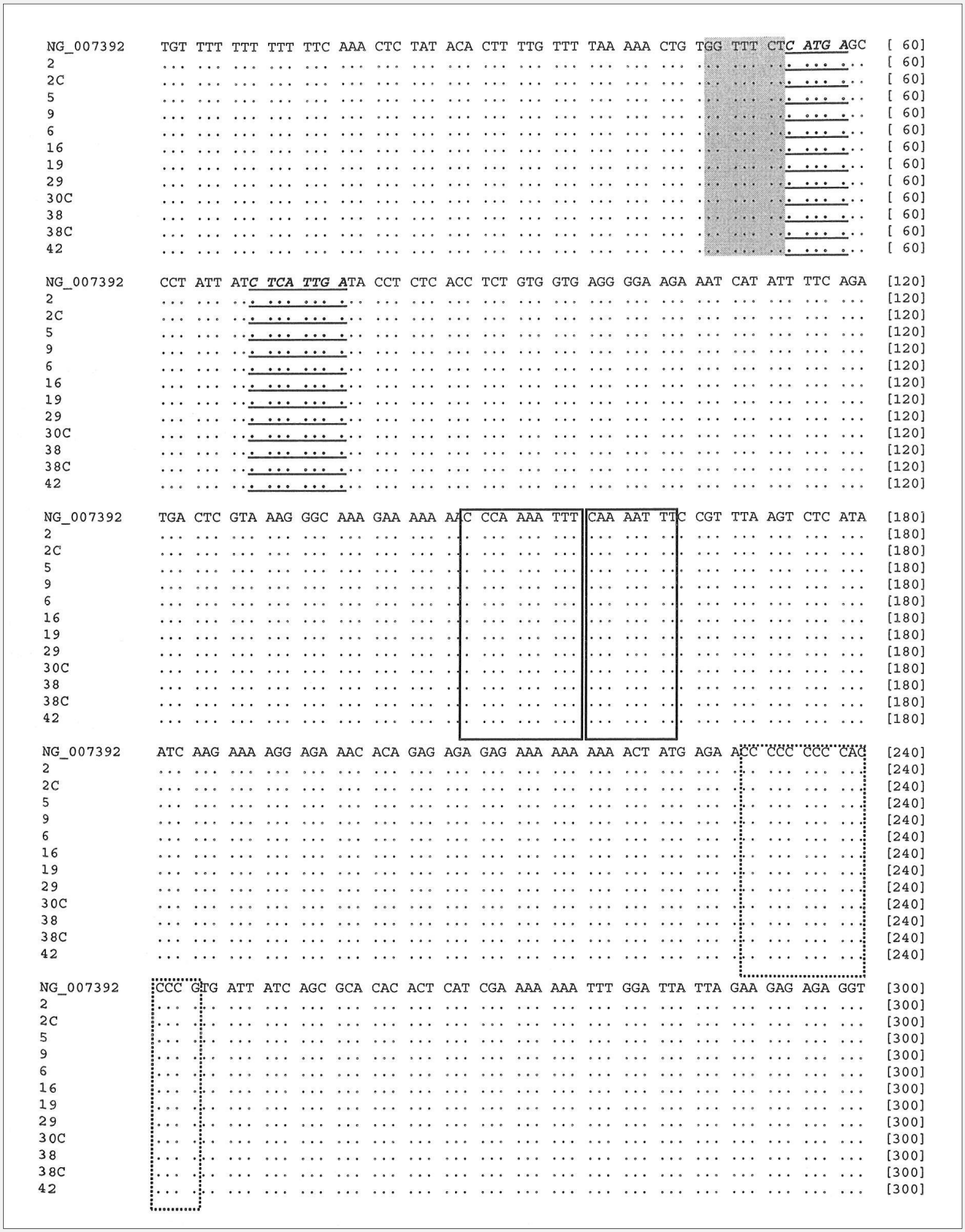
Figure 1. PCR amplification of rs3761547 (A) and rs3761548 (B).

RESULTS

Genomic DNA from 50 BD patients and 50 controls was extracted and amplified by specific primers. These primers amplified 619 bp and 379 bp fragments (Figure 1). -3279C/A genotype frequencies for the BD and control groups were CC:CA:AA = 22%:6%:72% and 52%:24%:24%, respectively (Table 1). The AA genotype was significantly associated with BD. In addition, the A allele frequency of 75% was present in BD patients compared to 36% of controls and was significantly associated with BD [(p = 0.002); (OR = 3.841, 95% CI = 1.610 - 9.161)]. Therefore, the C/A polymorphism in FOXP3 was associated with susceptibility to BD.

There was no contribution of the FOXP3 polymorphism -3499T/C to BD [(p = 0.084); (OR = 0.348, 95% CI = 0.101 - 1.195)]. -3499T/C genotype frequencies for the BD and control groups were TT:TC:CC = 90%:6%:4% and 76%:10%:14%, respectively (p = 0.108), whereas the frequency of T and C alleles in patients and controls was 93%:7% and 81%:19%, respectively (Table 1).

A 693 bp of FOXP3 sequences was amplified in both control and BD patient groups, while 506 bp was sequenced successfully in all samples. BLAST analysis showed 100% similarity among current study sequences and GenBank data (NG_007392). The similarity within and between the control and BD patient was 100%. Several common features of the basal promoter including a GC and a TATA box are conserved in all sequences (Figure 2).


NG_007392 TGT TTT TTT TTT TTC AAA CTC TAT ACA CTT TTG TTT TAA AAA CTG TGG TTT CTC ATG AGC [60]
NG_007392 CCT ATT ATC TCA TTG ATA CCT CTC ACC TCT GTG GTG AGG GGA AGA AAT CAT ATT TTC AGA [120]
NG_007392 TGA CTC GTA AAG GGC AAA GAA AAA AAC CCA AAA TTT CAA AAT TTC CGT TTA AGT CTC ATA [180]
NG_007392 ATC AAG AAA AGG AGA AAC ACA GAG AGA GAG AAA AAA AAA ACT ATG AGA ACC CCC CCC CAC [240]
NG_007392 CCC GTG ATT ATC AGC GCA CAC ACT CAT CGA AAA AAA TTT GGA TTA TTA GAA GAG AGA GGT [300]

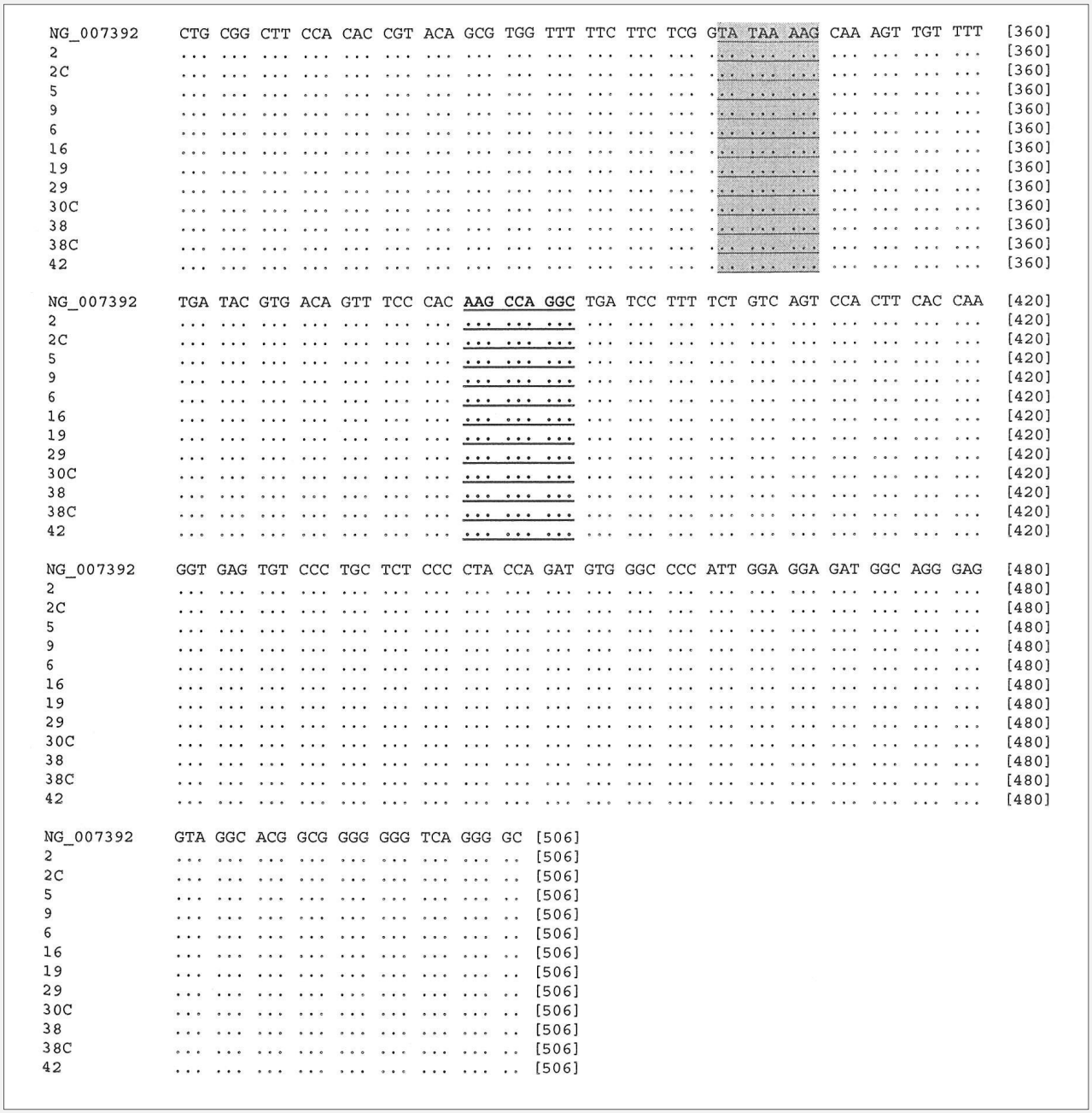


Figure 2. Multiple sequence alignment of human FOXP3 promoter in several control and BD patient groups. Highlighted region in grey colour is NFAT, bold, italic and underline is AP-1, Rectangular is CAAT box, Dotted rectangle is GC box, grey and underline is TATA box, bold and underlined regions indicate TSS regions.

DISCUSSION

The etiopathogenesis of BD is not defined; however, it has been shown that genetic factors such as the HLA-B51 locus and the TNF-α gene [22,23] may be implicated in the susceptibility to the disease [24,25]. Recently, the links between some single nucleotide polymorphisms (SNPs) in KIAA1529, CPVL, LOC100129342, UBASH3B, and UBAC2 genes and particular manifestations of BD has been shown [26]. In the current study,

the association between BD and rs3761547 and rs3761548 SNPs in the FOXP3 candidate gene in the Iranian population was analyzed. This is the first report demonstrating that the rs3761547 and rs3761548 alleles were associated with BD.

The correlation of rs3761547 (-3499T/C) in Celiac and Graves' diseases and Hashimoto's thyroiditis has been analyzed [27,28]. Polymorphism in -3499T/C FOXP3 was not related to Celiac disease, whereas Wawrusiewicz-Kurylonek et al. (2012) suggested that the -3279G/T polymorphism in the FOXP3 gene could have a protective role in predisposition to Hashimoto's thyroiditis [27,28]. A study on the association of the FOXP3 polymorphism with allergic rhinitis in a Chinese population has shown that the heterozygous allele in rs3761547 appeared significant [29]. Based on the results of the current study, it may suggest that -3279G/T polymorphism in the FOXP3 gene could have a protective role in Iranian population in Behçet's disease.

Regarding the loss of self-tolerance leading to autoimmune diseases (ADs), a meta-analysis has investigated the FOXP3 -3279 A/C polymorphism for AD susceptibility [30]. They concluded that FOXP3 -3279 A/C polymorphism may influence AD risk, and the A allele variant carriers of FOXP3 -3279 A/C polymorphism definitively are associated with AD susceptibility [30]. However, the contribution of FOXP3 in allergic rhinitis (AR) has been studied in Hungarian female population [31]. Their results showed that females homozygous for the rare FOXP3 rs3761548 allele (A/A) were protected against AR; however, females who were either wild types (C/C) or heterozygote carriers (C/A) of the rare allele were more susceptible to AR [31]. In addition, the potential role of FOXP3 rs37161548 as a polymorphic marker for tumor progression in premenopausal breast cancer patients in Indian women was studied [32]. The results of the current study support the previous studies and indicate that FOXP3-3279 C/A polymorphism (rs3761548) may be associated with susceptibility to Behçet's disease in the Iranian population.

The chromatin accessibility is essential for many genes during T cell differentiation, like IL-4 and IFN-γ [33, 34]. However, the chromatin structure may be an important aspect of FOXP3 regulation [35]. Also, Mantel et al. (2006) showed that 245 bp upstream of TSS containing the core promoter of FOXP3 and the specific mutation of the TATA, the GC and CAAT boxes reduce activity of the core promoter [20]. Therefore, conserved sequences of these boxes in control and BD patients in the current study indicate that BD could be associated with mutation(s) outside of these boxes.

CONCLUSION

The findings of the current study provide evidence for an association between the FOXP3 gene and BD. More extensive studies with more samples are still necessary to increase our knowledge base for the FOXP3 gene and its role in BD. Such advances might also enable more appropriate exploitation of FOXP3 for the prevention, diagnosis, treatment, and monitoring of BD.

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Declaration of Interest:
There is no conflict of interest.

References:

1. Kaya TI. Genetics of Behcet's Disease. Patholog Res Int 2012; 91.1-6.
2. Mendes D, Correia M, Barbedo M, et al. Behcet's disease a contemporary review. J Autoimmun 2009;32:178-88.
3. Galeone M, Colucci R, D'Erme AM, Moretti S, Lotti T. Potential Infectious Etiology of Behçet's Disease. Pathol Res Inter 2012;1-4.
4. Cho SB, Cho S, Bang D: New Insights in the Clinical Understanding of Behçet's Disease. Yonsei Med J 2012;53:35-42.
5. Kaneko F, Togashi A, Saito S, et al. Behçet's disease (Adamantiades-Behçet's disease). Clin Dev Immunol 2011;68:195-199.
6. Mendoza-Pinto C, Garcia-Carrasco M, Jimenez-Hernandez M, et al. Etiopathogenesis of Behcet's disease. Autoimmun Rev 2010; 9:241-245.
7. Kaneko F, Oyama N, Yanagihori H, Isogai E, Yokota K, Oguma K. The role of streptococcal hypersensitivity in the pathogenesis of Behcet's Disease. Eur J Dermatol 2008;18:489-498.
8. Shimizu J, Yoshikawa H, Takada E, Hirotsu C, Suzuki N. Unbalanced helper T cell function in Behcet's disease. Inflammation and Regeneration 2011;31:296-301.
9. Hamzaoui K, Houman H, Hamzaoui A. Regulatory T cells in cerebrospinal fluid from Behçet's disease with neurological manifestations. J Neuroimmunol 2007;187:201-204.
10. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005;22:329-341.
11. Janson PCJ, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 Promoter Demethylation Reveals the Committed Treg Population in Humans. PLoS One 2008;3:e1612.
12. Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. Dev Biol 2002;250:1-23.
13. Ziegler SF. FOXP3. Of mice and men. Annu Rev Immunol 2006; 24:209-226.
14. Curotto de Lafaille MA, Lafaille JJ. Natural and Adaptive Foxp3+ Regulatory T Cells: More of the Same or a Division of Labor? Immunity 2009;30:626-635.

15. Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. PLoS One 2008;3:e1612.

16. Gambineri E, Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. Curr Opin Rheumatol 2003;15:430-435.

17. Myers AK, Perroni L, Costigan C, Reardon W. Clinical and molecular findings in IPEX syndrome. Arch Dis Child 2006;91:63.

18. Le Bras S, Geha RS. IPEX and the role of FOXP3 in the development and function of human Tregs. J Clin Invest 2006;116:1473.

19. van der Vliet HJJ, Nieuwenhuis EE. IPEX as a Result of Mutations in FOXP3. Clin Dev Immunol 2007;1-5.

20. Mantel PY, Ouaked N, Ruckert B, et al. Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol 2006; 176:3593-3602.

21. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-4680.

22. Mizuki N, Meguro A, Ota M, et al. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behcet's disease susceptibility loci. Nat Genet 2010;42:703-706.

23. Bonyadi M, Jahanafrooz Z, Esmaeili M, et al. TNF-alpha gene polymorphisms in Iranian Azeri Turkish patients with Behcet's disease. Rheumatol Int 2009;23:285-289.

24. Radouane A, Oudghiri M, Chakib A, Bennani S, Touitou I, Barat-Houari M. SNPs in the TNF-alpha gene promoter associated with Behcet's disease in Moroccan patients. Rheumatology 2012;51: 1595-1599.

25. Khaib Dit Naib O, Aribi M, Idder A, et al. Association Analysis of IL10, TNF-alpha, and IL23R-IL12RB2 SNPs with Behcet's Disease Risk in Western Algeria. Front Immunol 2013;4:342-349.

26. Fei Y, Webb R, Cobb BL, Direskeneli H, Saruhan-Direskeneli G, Sawalha AH. Identification of novel genetic susceptibility loci for Behcet's disease using a genome-wide association study. Arthritis Res Ther 2009;11:R66.

27. Wawrusiewicz-Kurylonek N, Bossowski A, Kretowski A, et al. Analysis of selected FOXP3 gene polymorphisms in children and adolescents with Graves' disease and Hashimoto's thyroiditis. Endocrine Abstracts 2012;29:1305-1312.

28. Serena G, Sturgeon C, Fasano A. Su2075 Rs3761547 FOXP3 Polymorphism is Not Related to Celiac Disease. Gastroenterology 2012;142(5):S-562.

29. Zhang L, Zhang Y, Desrosiers M, Wang C, Zhao Y, Han D. Genetic association study of FOXP3 polymorphisms in allergic rhinitis in a Chinese population. Hum Immunol 2009;70:930-934.

30. He Y, Na H, Li Y, Qiu Z, Li W. FoxP3 rs3761548 polymorphism predicts autoimmune disease susceptibility: a meta-analysis. Hum Immunol 2013;74:1665-1671.

31. Fodor E, Garaczi E, Polyanka H, Koreck A, Kemeny L, Szell M. The rs3761548 polymorphism of FOXP3 is a protective genetic factor against allergic rhinitis in the Hungarian female population. Hum Immunol 2011;72:926-929.

32. Jahan P, Cheruvu R, Tippisetty S, Komaravalli PL, Valluri V, Ishaq M. Association of FOXP3 (rs3761548) promoter polymorphism with nondermatomal vitiligo: A study from India. J Am Acad Dermatol 2013;69:262-266.

33. Messi M, Giacchetto I, Nagata K, Lanzavecchia A, Natoli G, Sallusto F. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. Nat Immunol 2003;4:78-86.

34. Agarwal S, Rao A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 1998;9:765-775.

35. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-336.