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# Association of *NFKB1 and NFKBIA* Polymorphisms in Relation to Susceptibility of Behçet's Disease

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## Abstract

Behçet's disease (BD) is a chronic inflammatory autoimmune disease. Although raised levels of proinflammatory cytokines in BD have been reported, the pathogenesis is still unknown. The aim of this study was to investigate the association of *NFKB1* and *NFKBIA* polymorphisms and their single and combined analysis effects on susceptibility of BD in Turkish population. We analysed the distribution of *NFKB1* -94 ins/del ATTG (rs28362491) and *NFKBIA* 3′ UTR A→G (rs696) polymorphisms using PCR-RFLP method in 89 patients with BD and 190 controls in this population. Statistical analysis of the results was performed by calculating OR, and 95% CI via $\chi^2$ test and using Bonferroni correction. According to the significant results of both single and combined genotype analysis, the frequencies of ins/ins genotype and ins allele of rs28362491 were significantly higher in patients with BD ($P_c$ = 0.003, 0.004, respectively). Also, higher frequencies of the rs696 variant containing AA genotype was found in patients with BD ($P_c$ = 0.0033), whereas no statistical significant differences in distribution of the alleles of rs696 polymorphism in patients and controls. In addition, according to the combined genotype analysis, the wild type of both rs28362491 and rs696 polymorphisms (ins/ins/AA genotype) was also significantly higher in BD cases ($P_c$ = 0.044). Our findings prove that both single and combined genotype analysis of rs28362491 and rs696 polymorphisms indicate that the wild genotypes of both two SNPs (ins/ins and AA genotypes) and ins/ins/AA combined genotype are strongly associated with enhanced risk of BD in a Turkish population.

## Introduction

Behçet's disease (BD) is a chronic inflammatory and systemic autoimmune disease characterized by recurrent oral ulcers, skin lesions, genital ulcers, vasculitis and ocular inflammation [1]. It follows a more severe course in patients with an early age of onset particularly in patients with eye and gastrointestinal involvement. The precise pathogenesis is still unknown as the immunological, genetic and environmental factors are claimed to contribute to BD development [2].

Several genes were shown to be associated with occurrence, progression and severity of BD disease [3] One of the split antigens of HLA-B5, HLA-B51, more typically seen along the Middle East to South Eastern Siberia, is the most probable candidate as a genetic marker for BD in different ethnic groups. Furthermore, according to GWAS, multiple non-HLA genes such as tumour necrosis factor [4], ERAP1 and CCR1 [5] are also involved in the susceptibility to BD. BD is known to be related to apoptosis resistance in T cell subsets and associated with the upregulation of antiapoptotic factors, and this relationship is based on the modulation the expression of antiapoptotic genes by the activation of a transcription factor, NF-κB [6]. Perazzio *et al.* [7] showed that NF-κB pathway of phagocytes is constitutively activated in patients via hyperactivity of neutrophils in BD.

Nuclear factor of kappa-B (NF-κB), one of the most important transcription factors, regulates the immune functions, and the inhibition of the gene provides a protection against the systemic or tissue inflammation. It has a key role in regulating the immune response to cell survival and proliferation, immunity, infection and differentiation [8]. Therefore, NF-κB has a vital role in inflammatory diseases and the development of autoimmunity [9, 10]. In mammals, NF-κB family has five members identified, NF-κB1 (p105/p50), NF-κ B2 (p100/p52), RelA, c-Rel and RelB [11]. *NFKB1* gene is located on

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chromosome 4q24 and consists of 24 exons [12]. One of the most prominent polymorphisms identified within the promoter of this gene is -94 ins/del ATTG (rs28362491) which is able to regulate the expression of *NFKB1*. A functional polymorphism(rs28362491) concludes as 4-base pair insertion/deletion in the promoter of the gene. Moreover, the decreased promoter activity with the deletion allele was determined in *in vitro* experiments [13].This polymorphism in the *NFKB1* gene which encodes the p50 subunit of NF-κB was investigated in many inflammatory and autoimmune diseases such as Hashimoto thyroiditis, rheumatoid arthritis, Graves's disease, multiple sclerosis and type 1 diabetes mellitus.

The IκBα (nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha), an inhibitory version of the NF-κB protein, binds to NF-κB in the cytoplasm and effects the transcriptional activity of NF*K*B1. IκBα is a member of cellular proteins and inhibits NF-κB by masking the nuclear localization signals of NF-κB and keeping NF-κB sequestered as inactive protein in the cytoplasm. Besides, IκBα prevents the ability of binding to DNA which is necessary for NF-κB's proper function [14].

IκBα, a classic form of the IκB family, is encoded by *NFKBIA* gene (nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha) that is located on chromosome 14q13 and contains six exons [15]. This gene probably plays an important role in inflammatory immunological diseases such as Crohn's disease or autoimmune diabetes mellitus [16, 17]. According to Goto *et al.* [18], the alterations in the expression of IκBα protein were related to the SNP rs696 in the 3′ UTR region of the *NFKBIA* gene and led to a change in NF-κB activity.

The aim of this study was to investigate the presence of any associations of two attractive candidate polymorphisms (rs28362491, rs696) in *NFKB1* and *NFKBIA* genes with BD disease risk and their combined effect with the susceptibility to BD in Turkish population as a case–control study.

## Patients and methods

*Study subjects*. A group of 89 patients with BD followed in the Department of Skin and Venereal diseases of Bezmialem University and a control group consisted of 190 healthy individuals were enrolled. Study subjects were patients with BD at 35.8 ± 7.2 years and healthy people at 36.4 ± 6.3 years. Studying patients with BD was all HLA-B51 positive and judged to be free of any history of autoimmune disease by questionnaires, medical history and clinical examination. The numbers of women and men were chosen near equally. There was no gender bias in the analysis (data not shown). All of the patients fulfilled the international classification criteria for BD. The presence of oral ulceration plus any of two of typical eye lesions, a positive skin pathergy test or typical skin lesions is required by the criteria [19]. All individuals of control group were carefully examined to exclude whether clinically having BD and other inflammatory disorders on their family stories. Exclusion criteria for participation to the study included pregnancy, smoking, alcohol usage or prescription drugs and having an autoimmune disease that might affect the results of this study. All study subjects were related to Turkish origin and provided signed informed consent prior to the sample and data collection, and study protocol was approved by Institutional Ethical Committee of Acibadem University. Although Turkey is an ethnically heterogeneous country, all patients and controls were inhabitants of Istanbul.

*Blood samples and DNA isolation*. Three millilitres of peripheral blood, which was taken from all subjects, was collected in EDTA tubes. Whole blood was stored immediately after collection at −20 °C until use. Genomic DNA was extracted from blood using Roche DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Concentration and purity of DNA measured by a NanoDrop™ spectrophotometer and determined by 260/280 nm OD ratio. DNA samples with 260/280 OD ratio of 1.8 ± 0.2 were included in the study.

*Genotyping*. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method is used to investigate *NFKB1* -94 ins/del ATTG (rs28362491) and *NFKBIA* 3′ UTR A-G(rs696) polymorphisms. A forward primer, TGG GCA CAA GTC GTT TAT G, and a reverse primer, CTG GAG CCG GTA GGG AAG, for rs28362491 and a forward primer, GGC TGA AAG AAC ATG GAC TTG, and a reverse primer, GTA CAC CAT TTA CAG GGA GGG, for rs696 were preferred. PCR was applied as follows: 95 °C for 1 min initial denaturation, followed by 35 cycles of 95 °C for 30 s denaturation, 60 °C (*NFKB1*) and 61 °C (*NFKBIA*) for 30 s annealing primers, 72 °C for 1 min extension and a final extension at 72 °C for 5 min.

PCR product was digested with 1 unit of restriction enzyme *PflMI* (10 U/μl, Fermentas) to detect two different alleles of rs28362491, the 281 bp (deletion wild-type allele) or 285 bp (insertion allele). The PCR product–enzyme mix was incubated overnight at 37 °C and subsequently run on electrophoresis for 30 min at 120V on 2% agarose gel. As deletion genotype did not contain *PflMI* (*Van91I*) restriction site, the PCR product of 281 bp remained undigested. On the other hand, the insertion variants were cleaved by *Pfl*MI (*Van91I*) restriction enzyme into two fragments of 240 and 45 bp. Heterozygotes showed all three bands (Fig. 1).

As for rs696, another restriction enzyme, *HaeIII* [10 U/ll,(Sigma,Saint Louis,Missouri,63103,USA)], was used for digestion. After overnight incubation at 37 °C, the new product was set to run on electrophoresis for 30 min at

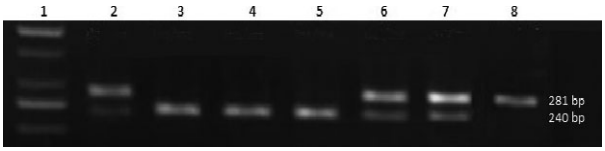


**Figure 1** The pattern of enzyme digestion. Lane 1 is 50-bp size marker; lanes 2, 6 and 7 are heterozygous ins/del ATTG; lanes 3, 4 and 5 are homozygous ins/ins ATTG; and Lane 8 is homozygous del/del ATTG genotype.

120 V on 2% agarose gel. The PCR product (424 bp) was cleaved into two fragments of 108 and 316 bp. Homozygosity of the common allele (represented by AA genotype) appeared as 424-bp bands, while the homozygosity of the variant allele (represented by GG genotype) appeared as bands, 316 and 108 bp, respectively (Fig. 2).

*Data processing and analysis*. Statistical analysis was performed using the Statistical Package for Social Sciences statistical software release 18 (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.) Genotype and allele frequencies and Hardy–Weinberg equilibrium (HWE) were determined for compatibility between patient and control groups using $\chi^2$(chi-squared) tests. Odds ratio (OR) and respective 95% confidence intervals (CIs) were reported to evaluate the effects of any difference between allelic and genotype distribution. A two-sided $P$ value <0.05 was considered statistically significant.

The Bonferroni correction was applied to the significant $P$ values ($P < 0.05$) to study groups to counteract the problem of multiple comparisons. Bonferroni multiple comparison correction method was employed in calculating the corrected value, as per: $Pc = 1 - (1 - P)^n$, where $n$ = number of comparisons.

## Results

### Distribution of polymorphisms in NFKB1(rs2836249) and NFKBIA(rs696) genes in BD

The distribution of the genotype and allele frequencies of all polymorphisms studied is shown in Table 1. All allele and genotype frequencies were within the range of Hardy–Weinberg equilibrium. The rs28362491 was successfully genotyped in 89 with BD and 190 control subjects. In single genotyping study, the overall frequencies of ins/ins, del/ins and del/del allele combinations were 48%, 43% and 9% in the patients' group and 27%, 59% and 14% in control group. The frequency of the ins/ins genotype of rs28362491 genotype was significantly higher in patients ($Pc = 0.003$, OR = 2.557, 95% CI = 1.477–4.428). According to our results, having ins/ins genotype of rs28362491 has 2.557 times risk factor in patients with BD. When the allele frequencies were compared, we found that having the ins allele increased the risk for BD by 1.8-fold ($Pc = 0.004$, OR = 1.800, 95% CI = 1.233–2.629). Recessive model ((ins/ins versus (ins/del+del/del)) ($Pc = 0.0006$, OR = 2.617, 95% CI = 1.546–4.431), also, is found to lead to increased risk when compared to dominant model (((ins/ins + ins/del) versus del/del)) ($Pc = 0.3014$, OR = 1.677, 95% CI = 0.7292–43.858).

We also detected that AA genotype of rs696 was significantly higher in the patients with BD than in the controls ($Pc = 0.033$, OR = 2.463, 95% CI = 1.216–4.988). No differences between patients and controls were detected at allele frequency of rs696. All these results are shown in Table 1.

### Combined genotype analysis of the polymorphisms in NFKB1 (rs28362491) and NFKBIA (rs696)

Table 2 summarizes the association study among the combined genotypes of the two SNPs and the overall risk for BD. The both SNPs rs28362491 and rs696 had significant differences among the combined genotypes of patients with BD and controls. When compared with ins/ins/AA (wild types of both SNPs) combined genotype, ins/ins/AG, del/ins/AG and del/ins/AG+GG combined genotypes' frequencies were significantly higher in the control group with respect to patient with BD group (OR: 5.923, 95% CI: 1.832–19.15, $Pc = 0.044$; OR: 5.33, 95% CI: 1.764–16.13, $Pc = 0.044$; OR: 5.111, 95% CI: 1.750–14.92, $Pc = 0.033$, respectively). After Bonferroni correction, ins/ins/AA combined genotype was still found to be a risk factor for BD disease ($Pc = 0.044$) when compared with the ins/ins/AG, del/ins/AG, del/ins/AG+GG combined genotypes as shown in Table 2.

## Discussion

Behçet's disease, characterized primarily by auto-inflammation of the blood vessels, is a systemic inflammatory disease. The exact cause of BD is still unknown, but there are some evidences that both genetic and environmental factors are involved. Combination of genetic and environmental factors in patients with BD might lead to pathogenic processes, which then probably affect the development of the disease and manage its course. A large

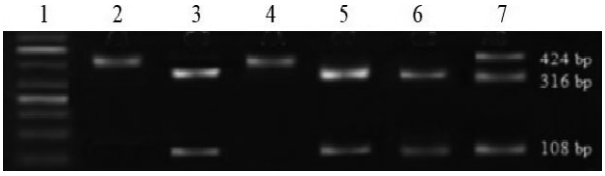


**Figure 2** The pattern of enzyme digestion. Lane 1 is 50-bp size marker; lanes 2 and 4 are homozygous AA; lanes 3, 5 and 6 are homozygous GG; and Lane 7 is heterozygous AG genotype.

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**Table 1** Distribution of genotype and allele frequencies of rs28362491 and rs696 polymorphisms in patients with BD and control subjects..

| Genotype/allele | Cases *n* (%) | Controls *n* (%) | OR (95% CI) | *P*c value |
|---|---|---|---|---|
| rs28362491 | 89 | 190 | | |
| ins/ins | 43 (48) | 50 (27) | 1 | |
| del/ins | 38 (43) | 113 (59) | 2.557 (1.477–4.428) | **0.003** |
| del/del | 8 (9) | 27 (14) | 2.903 (1.194–7.056) | 0.079 |
| Dominant model ((ins/ins + ins/del) versus del/del) | | | 1.677 (0.7292–3.858) | 0.3014 |
| Recessive model (ins/ins versus (ins/del+ del/del)) | | | 2.617 (1.546–4.431) | **0.0006** |
| ins allele frequency | 124 (70) | 213 (56) | 1 | |
| del allele frequency | 54 (30) | 167 (44) | 1.800 (1.233–2.629) | **0.004** |
| rs696 | 89 | 190 | | |
| AA | 18 (20) | 25 (14) | 1 | |
| AG | 38 (43) | 130 (68) | 2.463 (1.216–4.988) | **0.033** |
| GG | 33 (37) | 34 (18) | 0.742 (0.343–1.606) | 0.448 |
| A allele frequency | 72 (42) | 180 (48) | 1 | |
| G allele frequency | 104 (58) | 198 (52) | 0.783 (0.546–1.122) | 0.182 |

*P*c, corrected *P* value after Bonferroni correction. Statictically significant values are shown with bold characters.
The differences resulting from the genotypes were determined by proceeding $\chi^2$ test.

**Table 2** The distribution of rs28362491–rs696 combined genotypes..

| rs28362491–rs696 combined genotypes | Cases *n* (%) | Controls *n* (%) | OR (95% CI) | *P*c value |
|---|---|---|---|---|
| ins/ins/AA | 11 (13) | 6 (3) | 1 | |
| ins/ins/AG | 13 (15) | 42 (22) | 5.923 (1.832–19.15) | **0.044** |
| ins/ins/GG | 19 (22) | 8 (4) | 0.8148 (0.2226–2.983) | 0.981 |
| ins/ins/AG+GG | 32 (37) | 50 (26) | 2.865 (0.9636–8.515) | 0.094 |
| del/ins/AA | 5 (6) | 19 (10) | 6.967 (1.717–28.26) | 0.125 |
| del/ins/AG | 22 (25) | 64 (34) | 5.333 (1.764–16.13) | **0.044** |
| del/ins/GG | 11 (13) | 28 (15) | 4.667 (1.384–15.73) | 0.226 |
| del/ins/AG+GG | 33 (38) | 92 (49) | 5.111 (1.750–14.92) | **0.033** |
| del/del/AG | 3 (3) | 13 (7) | 7.944 (1.601–39.43) | 0.135 |
| del/del/GG | 3 (3) | 10 (5) | 6.111 (1.198–31.18) | 0.300 |
| del/del/AG+GG | 6 (6) | 23 (12) | 7.028 (1.839–26.86) | 0.075 |

Pc, corrected P value after Bonferroni correction. Statistically significant values are shown with bold characters.
del/del/AA combined genotypes were excluded in the table as none observed in the subjects or the size was not enough for the statistical analyse.

number of serological studies show a correlation between BD disease and HLA-B51 allele presence [20]. HLA-B51 is peculiarly more often found from the Middle East to South Eastern Siberia, and a 3-fold higher incidence of HLA-B51 than the normal population was seen in some BD studies. HLA-B51 is strongly associated with BD in Israeli and Korean patients and especially Turkish ones [21–23]. The presence of HLA-B51 alone is not sufficient to explain all the symptoms to develop the disease; however, BD can be related with many other genetic factors, and a network of immune mediators is known to have an important role in the inflammatory cascade of BD. NF-κB, one of the most searched transcription factor, produces inflammatory cytokines. It is also known that polymorphisms in the promoter regions of *NFKB1* lead to change of *NFKB1* expression, which then causes altered transcription of the inflammatory cytokines. Therefore, it can answer the overexpression of these cytokines in autoimmune inflammatory diseases.

In the last decade, many researchers have been underlined that the *NFKB1* and its inhibitor protein genes, *NFKBIA* variants, could potentially change the function of proteins so the process of inflammation. One of the most prominent polymorphisms known within the promoter of this gene is -94 ins/del ATTG rs28362491. A potential candidate for searching pathogenesis of inflammatory diseases, rs28362491, is shown to be associated with increased risk in rheumatoid arthritis [24], Graves's disease [25] and type 1 diabetes mellitus [26], whereas no associations with Hashimoto thyroiditis (HT)[27] and multiple sclerosis [28] have been yet shown. In addition, in American [13] and Dutch populations [29], rs28362491 in the *NFKB1* gene was associated with UC development but not in Spanish [30] and British populations [31]. Rogler *et al.* [32] determined that NF-κB activation was elevated in the intestinal mucosa of Crohn's disease (CD). Homozygous or heterozygous genotypes of rs28362491 were not associated with CD risk in German population [33].

However, heterozygote genotype of rs28362491 was found to be associated with decreased risk for SLE [34]. We have previously reported that the frequency of the del allele of rs28362491 accompanying high IL-6 levels was significant in Hashimoto's thyroiditis patient group [27]. In this study, rs28362491 promoter variation has been analysed in 89 patients with BD and 190 healthy controls. We found that the patients with ins/ins genotype and ins allele of rs28362491 in *NFKB1* gene were in a risk group of having BD disease. Patients with ins/ins genotype and ins allele of rs28362491 have an increased risk of having the disease by 2.5- and 1.8-fold, respectively. These data are compatible with the results of Yalçin *et al.* [35], in which the ins/ins genotype of rs28362491 was determined to be more frequent in patients with ocular involvement in BD in a Turkish population. Interestingly, according to Zou *et al.* [36], there is no association with rs28362491 and certain autoimmune and inflammatory diseases in Caucasian population but in Asians. This may be due to the presence but not the necessity of HLA-B51 allele through Middle East to South Eastern Siberia (along the ancient Silk Road through Asia). This idea demands further investigation.

On the other hand, imbalance of NF-κB and IκB has been involved in various diseases and tumour development. However, it is still unclear how specific polymorphisms of these genes associated with disease development. Recent studies have showed that an SNP polymorphism of the *NFKBIA* gene may have an effect on the development of various inflammatory diseases associated with altered immune response.

The single nucleotide polymorphism in the 3′ UTR region of the *NFKBIA* gene may have a genetic effect on the development of various inflammatory diseases associated with altered immune response. In studies with type 1 diabetes mellitus, AA genotype of rs696 was a risk factor for latent autoimmune diabetes in adults [17]. rs696 variation has also been associated with development of type 2 diabetes and Crohn's diseases, resulting in Th1/Th2 imbalance as the pathogenesis [16, 37]. Recently, Hung *et al.* [38] investigating IκBα -881A, -826T, -550A, -519T and -297C polymorphisms concluded that *NFKBIA* promoter gene polymorphisms (-826 C/T, -826 T/T genotypes) are higher in BD, but only -826 T/T genotype is associated with skin lesions in Taiwanese patients with BD. *NFKBIA* 3′ UTR polymorphism frequency in a Turkish population goes hand in hand with the one in Caucasian [15]. In addition to all these studies, no association studies of *NFKBIA* 3′ UTR A-G polymorphisms (rs696) have been reported with respect to BD development. According to our results, the AA genotype of rs696 in the *NFKBIA* gene was significantly higher in the patients with BD than in the controls. AA genotype had a 2.5 times risk factor for development of BD disease.

Moreover, many studies were present about these polymorphisms in recent years, but no studies have been reported rs28362491 and rs696 polymorphisms' combined effects in BD disease. Combining multiple variants may result in greater predictive power of disease risk. Song *et al.* [39] have found that combined genotype of ins/ins+del/ins and GG was associated with an increased risk of sporadic cancer, and as shown in the previously published research, ins/ins/AG combine genotype has a protective role on another inflammatory disease, Hashimoto thyroiditis [27]. On the contrary, according to the multiple comparisons of combined genotypes of rs28362491 and rs696, ins/ins/AA combined genotype frequency was significantly higher in patients with BD than in control groups in this study. This finding is coherent and well supported by the polymorphism analysis in Table 1. The wild genotypes of both two polymorphisms (ins/ins and AA genotypes), ins/ins/AA combined genotype, and also ins allele frequencies were higher in patients with BD considerably.

In conclusion, our study demonstrates that the both single and combined genotype analysis of the SNPs rs28362491 and rs696 may affect susceptibility to BD and increase risk of developing the disease. However, one of the major limitations of our study is the small sample size which may have influenced the statistical power of our analyses. Therefore, further studies are needed to expand the sample sizes. NF-κB seems to modulate the immune response in BD, but it is questionable how NF-κB in BD leads to an abnormal phenotype in BD; therefore, it still has room for research. Moreover, different population studies are necessary to understand whether these variants play considerable roles in the pathogenesis in BD.

## Conflict of interest

The authors declare no conflict of interest.

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