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Associations of IL-6 polymorphisms with Behçet's Disease in Denizli, province of Turkey

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Abstract

Behçet's Disease is a chronic, multisystemic disease and the etiopathogenesis of the disease develop as a result of the interaction of environmental and genetic factors. IL-6 is a proinflammatory cytokine with functions on immune response regulation and chronic inflammation demonstrated by increased serum levels and gene expression studies in Behçet's disease. We genotyped IL-6; rs1800797, rs1800796 and rs1800795 polymorphisms in 78 patients with Behçet's disease and 130 healthy controls using PCR-RFLP based approach. No statistically significant difference in allele or genotype frequencies of IL-6, rs1800797 and rs1800796 was found in between groups. We determined that in the BD patients with genital ulcers, arthritis and without vascular involvement was found to have significantly different IL-6, SNP frequencies compared with healthy controls. In addition, the IL-6 rs1800795 GC genotype and C allele may contribute to the development of the disease in female BD. The haplotype distributions of all loci of IL-6 were examined and GGG was found as the highest frequency haplotype in both groups. In conclusion, the results of our study showed that IL-6 rs1800795 may associate with the susceptibility to the BD. Studies should be conducted with more SNPs in large and different populations need to evaluate the contribution of responsible alleles for the disease and its clinical features.

Keywords: Behçet's disease, Interleukin-6, haplotype, SNPs

Introduction

Behçet's disease (BD) is a chronic multisystemic disease defined with recurrent oral ulcers, genital ulcers and uveitis, is also characterized by inflammatory lesions started with infiltration of lymphocytes and neutrophils into the releated systems such as eyes, skin or brain [1]. To explain the pathogenesis of disease, cytokines, chemokines and their receptor studies have been made at various serum or gene levels [2,3]. Many cytokines play an important role in BD, especially in inflammatory attacks of active phase [4].

Interleukin-6 (IL-6) with well-defined polymorphisms in chronic inflammation and autoimmune diseases is an important proinflammatory mediator [5]. IL-6 stimulates a variety of innate and adaptive immune responses and can be released in response to immune attacks or tissue damages [6]. Cytokines such as IL-1, IL-6, IL-8 or TNF-α IL-6 which are effective in the development and activation of BD, cause an increase in tissue damage by stimulating the migration of neutrophils to the site of inflammation [7]. It has been reported that the serum levels of IL-6 or its receptors are increased in active BD patients [3,7,8]. High levels of these cytokines might be responsible from pre-activated neutrophils in BD patients [9]. IL-6 functions on cell differentiation, immune response regulation and chronic inflammation demonstrated by increased serum levels and gene expression studies in Behçet's disease [7,10].

Human IL-6 gene has been located on chromosome 7p21, including a 28- amino-acid signal peptide [6]. There are many identified single-nucleotide polymorphisms (SNPs) in the IL-6 gene, but only three promoter region SNPs, rs1800797 (−597, G/A) rs1800796 (−572,G/C) and rs1800795 (−174, G/C), have been extensively studied in several immune-related disorders, such as romatoid arthritis, multiple sclerosis, diabetes, allergic diseases, chronic periodonditis, systemic sclerosis or cancer [11-17]. Also a number of recent studies, particularly in different populations and in a Turkish population, have focused on IL-6 gene polymorphisms in BD [18,19] and suggesting the view that IL-6 gene may be a promising candidate gene involved in BD susceptibility [20].

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Considering the location of Turkey, Denizli province and the importance of cytokines/chemokines in BD has led us to study BD and polymorphisms of cytokines. We previously investigated the associations of SNPs and haplotypes of CXCL8 (IL-8), CXCR1, CXCR2 and CXCL5, which play important roles in neutrophil migration and activation in BD [21,22]. As far as we know, this study was the first study on genetic associations of IL-6, rs1800795, rs1800796, and rs1800797 polymorphisms and haplotypes together and their possible contributing effects on the development of BD in Denizli province of Turkey.

Materials and Methods

DNA samples of patients and healthy individuals from the anonymous DNA bank established within the framework of TUBITAK-SBAG-118S662, TUBITAK-113S163 and TUBITAK-SBAG-2388 projects were used in our study. DNA samples of 78 BD patients (48 male and 40 female) diagnosed according to the International Study Group for the classification [23] were included in our study. The clinical features of patients with BD were shown in Table 1. Vascular, neurological and gastrointestinal involvements were excluded from the study due to observed in less than ten patients. 130 healthy controls (HCs) (66 male and 67 female) were selected from the individuals who have no allergy or autoimmune disease.

Table 1. Clinical features of patients with BD (n=78)

CLINICAL FEATURES	BD (%)
Male (M)	40 (51)
Female (F)	38 (49)
Oral ulcer (OU)	78 (100)
Genital lesions (GL)	60 (77)
Erythema nodosum (EN)	32 (41)
Papulopustular lesions (PL)	49 (63)
Positive Pathergy test (PPT)	33 (42)
Ocular involvement (OI)	38(49)
Arthritis (A)	49 (63)
Vascular involvement (VI)	7 (0,9)
Neurological involvement (NI)	8 (10)
Gastrointestinal involvement (GI)	2 (0.03)

Genotyping

To investigate the association of the genetic polymorphisms and BD, genotypes of IL-6 (OMIM: 147620) [rs1800797 (G>A), rs1800796 (C>G), rs1800795 (G>C)] were investigated by polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP). All PCR reaction conditions, PCR primers, restriction enzymes and products were designed as in previous published reports [14,24,25] (Table 2).

Table 2. SNPs and PCR-RFLP conditions

SNP	Primers	PCR	RFLP	Ref
-597rs1800797 (G/A)	F: 5'-GGAGTCACACACTCCACCT -3' R: 5'-CTGATTGGA AACCTTATTAAG -3'	525 bp	GG:525bp AA: 468+57bp	[14]
-572rs1800796 (G/C)	F: 5′-CTCCTCTAAgTgggCTgAAg -3′ R: 5′-CAAgCCTgggATTATgAAgA -3′	212 bp	GG:139+73bp CC:212 bp	[24]
-174rs1800795 (G/C)	F: 5'-TgACTTCAgCTTTACTCTTTg -3' R: 5'-CTgATTggAAACCTTATTAAg -3'	198 bp	GG=30+168 bp GC=30+49+168+119bp CC=49+119bp	[25]

Statistical analysis

All statistical analyzes were performed according to our previous studies (21,22).

Results

The genotype and allele frequencies of *IL-6* polymorphisms detected in BD patients and HCs are shown in Table 3. According to pairwise linkage disequilibrium (LD) analysis of IL-6 strong pairs of BD and HCs were shown in Figure 1. The genotype frequencies of the three SNPs in the *IL-6* gene were found to be compatible with HWE in both the BD patients and HCs groups (data not shown). According to OR calculations, it was found that the distribution of genotype and allele frequencies of rs1800797 (G/A) and rs1800796 (G/C) polymorphic regions did not differ significantly between the BD and HCs. The results indicate that there is a statistically significant difference in the frequencies of the rs1800795 (G/C) CG and GG genotypes, C (mutant) and G (wild) alleles between the BD and HCs, but not in the frequency distributions of the rs1800795, CC genotype. However, the statistical significance of rs1800795 genotypes is lost when Bonferroni correction is applied (Table 3).

Clinical findings of BD with statistically significance of *IL-6* SNPs were summarized in Table 4. As a result of statistical calculations, the fact that *IL-6* rs1800795 GC genotype and C allele frequency distributions were statistically significant between female BD and female HCs groups indicates that this SNP contributed to the development of the disease in the female group. Interestingly, the distribution ratio of the rs1800797 GG genotype was significantly different in patients with ocular lesions compared with BD patients without ocular lesions, and the distribution ratio of rs1800796 GG, CG genotypes and C allele in BD patients with arthritis and BD patients without arthritis were found to be significantly different. However, with Bonferroni correction, significant differences in the groups of female BD and female HCs, with and without ocular lesions, and with and without arthritis disappear (Table 4).

IL-6 haplotype analyzes using the Arlequin ver 3.5 program indicated that the content and frequency distribution of the five different haplotypes were similar in both BD and HCs (Table 5). The haplotype distributions of all loci of *IL-6* were examined and GGG (60.3±3.9% and 64.6±2.9%) was found as the highest frequency haplotype in both groups, respectively. To be able to understand the importance of the haplotype data, we analyzed the global test of differentiation among samples in between BD with/without clinical features, gender or BD patients and HCs by Arlequin program. The results of the global differentiation test showed that the possible

IL-6 haplotypes were not statistically different in the distribution of haplotype frequencies when the BD and the HCs were compared. Global test of differentiation results indicated that in BD patients with genital ulcers, arthritis and without vascular involvement have significantly different frequencies compared with HCs (p=0.03852±0.0045, p=0.04517±0.0056 and p=0.03670±0.0028, respectively). In addition, global tests differentiation showed that probable *IL-6* haplotype frequencies may be significantly different in BD patients without ocular lesions and arthritis compared with female HCs and in patients with arthritis and without vasculitis compared with male HCs. According to global differentiation test results BD patients with arthritis show important significantly different frequencies compared with BD patients without arthritis (p=0.02676±0.0013).

Table 3. Genotype and allele frequencies of the IL-6 gene polymorphisms of BD and HCs

	BD n=78 (%)	HCs n= 130 (%)	OR* (95% Cl)	P	Pc
rs1800797 (G/A), -597					
AA	4 (5)	9 (7)	0.73 (0.22-2.44)	0.6060	ns
AG	36 (46)	49 (38)	1.42 (0.80- 2.50)	0.2302	ns
GG	38 (49)	72 (55)	0.76 (0.44-1.34)	0.3515	ns
A	44 (28)	67 (26)	1.13 (0.72-1.77)	0.5867	ns
G	112 (72)	193 (74)	0.88 (0.57-1.38)	0.5867	ns
rs1800796 (G/C), -572					
CC	1 (1)	1 (1)	1.68 (0.10-27.17)	0.7166	ns
GC	15 (19)	19 (14)	1.39 (0.66-2.93)	0.3848	ns
GG	62 (80)	110 (85)	0.70 (0.34-1.46)	0.3453	ns
C	17 (11)	21 (8)	1.39 (0.71-2.73)	0.3354	ns
G	139 (89)	239 (92)	0.72 (0.37-1.41)	0.3354	ns
rs1800795 (G/C), -174					
CC	1 (1)	1 (1)	1.68 (0.10-27.17)	0.7166	ns
GC	27 (35)	26 (20)	2.12 (1.12-3.99)	**0.0204**	ns
GG	50 (64)	103 (79)	0.47 (0.25-0.88)	**0.0177**	ns
C	29 (19)	28 (11)	1.89 (1.08-3.32)	**0.0263**	ns
G	127 (81)	232 (89)	0.53 (0.30-0.93)	**0.0263**	ns

OR: Odds ratio, 95% Cl: 95 % confidence interval, Pa: Statistically significant difference between patients with BD and control subjects (P < 0.05), Pc: Bonferroni correction. NS: not statistically significant

Table 4. Alleles and genotypes in the IL-6 gene that were found to be statistically different between those with and without BD clinical findings

		SNP	Alelle/Genotype	OR (95%CI)	P	Pc
1	**BD-F**	**rs1800795 (G/C)**	GC	2.86 (1.14-7.22)	0.0257	ns
2	**BD-F**	**rs1800795 (G/C)**	GG	0,35 (0.14-0.88)	0.0257	ns
3	**BD-F**	**rs1800795 (G/C)**	C	2.44 (1.05-5.70)	0.0388	ns
4	**BD-F**	**rs1800795 (G/C)**	G	0.41 (0.18-0.96)	0.0388	ns
5	**OI**	**rs1800797 (G/A)**	GG	2.56 (1.03-6.37)	0.0439	ns
6	**A**	**rs1800796 (G/C)**	GC	11.20 (1.39- 90.44)	0.0234	ns
7	**A**	**rs1800796 (G/C)**	GG	0.08 (0.01-0.65)	0.0181	ns
8	**A**	**rs1800796 (G/C)**	C	11.12 (1.43-86.26)	0.0212	ns
9	**A**	**rs1800796 (G/C)**	G	0.09 (0.01-0.70)	0.0212	ns

BD-F: female Behçet's disease patients, OI: Ocular involvement, A: Arthritis, OR: Odds ratio, 95% Cl: 95% confidence interval, P: Statistically significant difference (P < 0.05). Pc: Bonferroni correction, ns: not significant.

Table 5. Haplotypes and frequencies of IL-6 in patients with BD and HCs

	Haplotype	BD%(±s.d)	HCs%(±s.d)	OR (95%CI)	P	Pc
1	GGG	0.603 (0.039)	0.646 (0.030)	0.8077 (0.4552-1.4333)	0.4655	ns
2	AGC	0.173 (0.030)	0.092 (0.018)	2.0710 (0.8755-4.8989)	0.0975	ns
3	AGG	0.109 (0.025)	0.165 (0.023)	0.6034 (0.2670-1.3636)	0.2246	ns
4	GCG	0.109 (0.025)	0.081 (0.017)	1.2669 (0.4856-3.3049)	0.6287	ns
5	GGC	0.006 (0.006)	0.015 (0.008)	0.4949 (0.0442-5.5478)	0.5684	ns

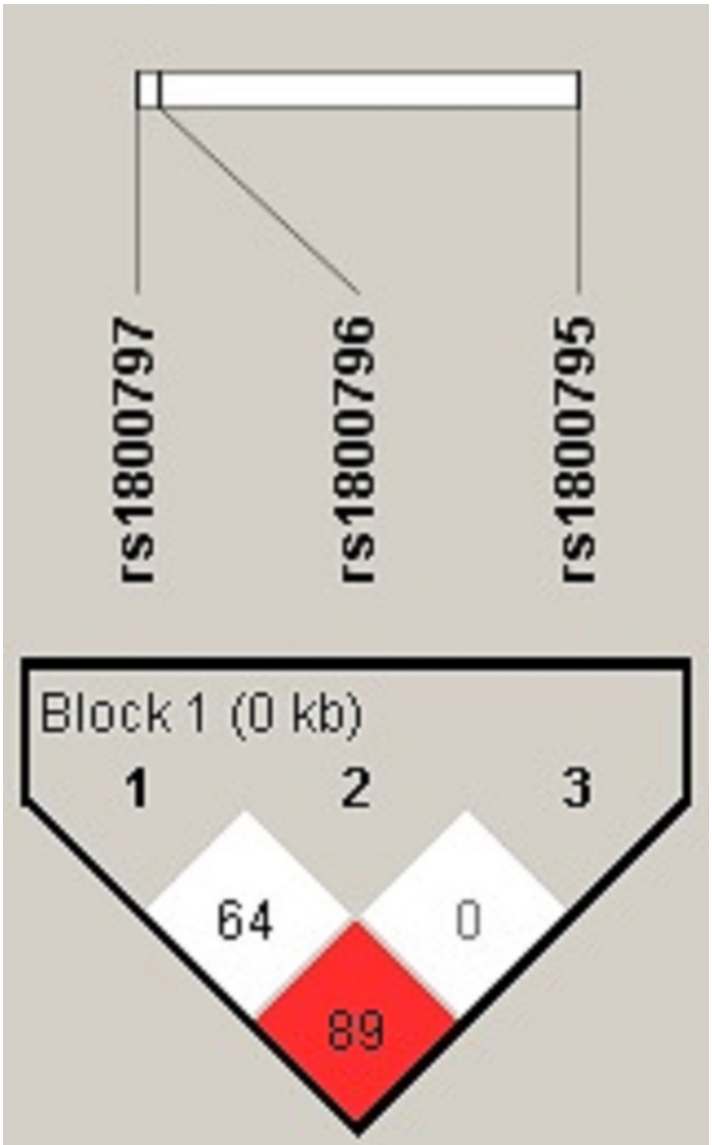

Figure 1. Plot of pairwise linkage disequilibrium (LD) analysis of IL-6 standart D' was shown in the plot. Empty squares indicate a high degree of LD between pairs of markers. Numbers indicate the D' value expressed as a percentile. Red squares indicate pairs in strong LD with LOD scores for LD ≥2; white squares, D' <1.0 and LOD ≤2

Discussion

Behçet's disease is defined as a systemic vasculitic disease as well as different tissue and organ involvements confirm that it is also an inflammatory disease affected by different environmental and genetic factors [26,27]. Determining the relationships between diseases and genetic polymorphisms of cytokines (IL-1, IL-6, IL-8, IL-17, IFN-γ, TNF-α etc.) and their receptors which have important roles in the immune system contributes to the pathogenesis of BD [28].

In the framework of immunopathogenesis of BD, the relationships between clinical findings and serum levels or gene polymorphisms of various cytokines were investigated [21,29]. Some studies reveal that the levels of *IL-6*, is elevated in BD patients in active period [7]. Another study suggested that there were no association between BD and serum levels of *IL-6* [30]. When investigating the gene expression f *IL-6* in BD patients Yamakawa et al. suggested that gene expression is not specific for BD [2]. Besides Jiang et al showed that increased secretion in BD, Maghrebi presented increased expressions of interleukins such as *IL-6* or IL-10 may help to understand the pathophysiology of neuro-inflammatory disorders like multiple sclerosis (MS) and BD [10,31]. All these inconsistent results have led to researchers to study the associations and possible susceptible polymorphisms of *IL-6* in BD patients.

Although the genotype and allele frequencies of the rs1800797 and rs1800796 genes were insignificant in the BD, we determined that only *IL-6*, rs1800795, CG, GG genotypes, C and G alleles were found statistically significant between BD and HCs. However, the statistical significance of rs1800795 genotypes is lost when Bonferroni correction is applied and these results are consistent with other studies in BD patients in different populations. In these studies examining the relationships between *IL-6* rs1800795 and BD and its clinical findings, no correlation was found between SNP and the disease [18,19,32]. Results of a meta-analysis of BD and cytokine gene polymorphisms indicate that the s1800795 C allele may be a protective factor for BD [33]. At the same time, studies on different loci and diseases suggest that *IL-6* polymorphic regions may be markers or novel therapeutic targets for the diseases [34,35]. In addition, when our results were evaluated in terms of gender, although the number of men and women is balanced, similar results were obtained in female patients in the rs1800795 loci. Other haplotype studies with *IL-6* polymorphic regions have shown that functional haplotype and polymorphisms may be protective for the related diseases such as Behçet's disease, osteoarthritis, diabetes or cancer [17,20,35,36].

In our previous studies, a difference was found in terms of haplotypes of IL-8 SNP loci in BD with arthritis, genital ulcer, ocuar lesions, PPT, papulopustular lesions and erythema nodosum [21]. In addition CXCR1 haplotypes were also found different in BD patients with arthritis and erythema nodosum and a statistical difference was observed in receptor CXCR2 haplotypes in patients with genital and ocular lesions [22]. We also determined that there were significant differences in the IL-17A and IL17A/

IL17F haplotype frequency distributions between the HCs and BD patients with clinical findings such as PPT, papulopustular lesions, erythema nodosum, ocular involvement, genital ulcers and arthritis (not published).

When the haplotypes were examined in our study, although there was no statistically significant difference between BD and HCs, the fact that haplotypes with different frequencies were obtained in both groups (Table 5), indicates the diversity of polymorphisms in *IL-6* genes. This suggests that polymorphisms and haplotypes in cytokines have a wide variety and importance in BD patients.

In this study we determined that in the BD patients with genital ulcers, arthritis and without vascular involvement was found to have significantly different *IL-6*, SNP frequencies compared with HCs. Also *IL-6* rs1800795 GC genotype and C allele may contribute to the development of the disease in female BD. Another important result of global differentiation test is the significant difference of BD patients with arthritis. It is noteworthy that arthritis is a common clinical feature in BD patients outcome in all our studies with haplotype analysis of cytokines and receptors [21,22]. These results show the importance of SNPs and haplotypes in following an important clinical finding such as arthritis. This type of research emphasizes the importance and evalution of genetic factors and haplotype studies in disease development.

Conclusion

In conclusion, the results of our study showed that polymorphism of *IL-6*, rs1800795 (G/C) and haplotypes of promoter region rs1800797, rs1800796 and rs1800795 SNPs together may associate with the susceptibility to the BD and clinical features of the disease. Further studies on cytokines or their specific receptors SNPs should be examined to be able to understand the possible disease-cytokine relationships. Investigating Behçet's disease and *IL-6* haplotype relationships with more SNPs in large and different populations need to evaluate the contribution of responsible alleles for the disease and its clinical features. This study may contribute to the new approaches in the BD research.

Conflict of interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

DNA samples of patients and healthy individuals from the anonymous DNA bank established within the framework of TUBITAK-SBAG-118S662, TUBITAK-113S163 and TUBITAK-SBAG-2388 projects were used in our study.

Ethical approval

Ethical Approval No: 2013/02/27, 2013.28 and No: 2018/04/21, 20.02.18, Ethics Committee of Pamukkale University, Denizli/TURKEY.

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