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Investigation of Toll-like Receptor (TLR)-7 Gene Polymorphisms in Patients with Behçet Uveitis

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ABSTRACT This candidate gene study was conducted to analyze the association between the toll-like receptor 7 *(TLR7)* gene polymorphisms and the pathogenesis of Behçet uveitis in Turkish patients. This study included 198 participants (89 patients and 109 controls). DNA was isolated from peripheral blood. The *TLR7* gene rs179009 and rs179008 polymorphisms were determined by polymerase chain reaction and Sanger DNA sequencing method (ABI 3730). As a results of this study, no statistically significant difference was detected between the patients and controls ($p>0.05$). This study's findings suggest that *TLR7* gene rs179009 and rs179008 polymorphisms are not associated with Behçet uveitis in Turkish population. To the researchers' knowledge, this is the first study in Turkey to examine *TLR7* gene polymorphisms in patients with Behçet uveitis. Further studies with larger study groups are needed to confirm these results.

INTRODUCTION

Behçet disease (BD) is a chronic multisystem disorder and presented with recurrent attacks. Behçet uveitis is one of the most devastating involvements of the disease and may cause a decrease in visual acuity and, consequently, significant morbidity. Although the etiopathogenesis of the disease has not yet been fully clarified, it is believed that environmental factors may trigger an enhanced immune response resulting in systemic vasculitis in immunogenetically susceptible individuals (Gul 2005, 2015; Keino and Okada 2007; Tugal-Tutkun 2010). Human leukocyte antigen (HLA)-B51 seems to have the strongest association with the disease (de Menthon et al. 2009; Gul and Ohno 2012; Kirino and Nakajima 2019; Mizuki et al. 2020). It also has been found to be associated with ocular involvement of BD in many studies (Elfishawi et al. 2019; Horie et al. 2017; Louthrenoo et al. 2020). Genomewide association studies (GWAS), which are a valuable method to clarify the genetic aspects of the pathogenesis of diseases, have confirmed the role of HLA-B51 and revealed other associated genes, including ERAP-1, IL23R, IL10, CCR1, STAT4, KLRC4, GIMAP2/GIMAP4, UBAC2, TLR4, MEFV, and NOD2 (Fei et al. 2009; Hou et al. 2012; Kirino et al. 2013; Lee et al. 2013; N Mizuki et al. 2010; Remmers et al. 2010). Despite its strong association with the MHC region, which may indicate the adaptive immune component, BD has also common features with autoinflammatory diseases, including episodic inflammatory attacks, overexpression of proinflammatory cytokines, low prevalence of autoantibodies and effectiveness of colchicine (Gul 2005, 2015). It is believed that the innate immune system, which is the first line of the host's defense, has an important role in initiating autoinflammatory diseases (Gul 2005; McGonagle and McDermott 2006). Toll-like receptors (TLRs) are members of the family of pattern recognition receptors (PRRs) (Chang et al. 2006; Takeda and Akira 2005). They are believed to be responsible for establishing a connection between infection and inflammatory diseases. These receptors mediate the production of cytokines and the attraction of inflammatory cells to the site of inflammation. At least 10 TLRs have been identified in humans. Genetic variables in genes that code for receptors in the innate immune system affect the susceptibility to immune-mediated diseases (Kawai and Akira 2011).

Toll-like receptor 7 (TLR7) is specialized in the recognition of single-stranded RNA. It also recognizes imidazoquinoline antiviral drugs (Kawai

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and Akira 2011). Moreover, the involvement of TLR7 in the recognition of DNA viruses was demonstrated (Varani et al. 2007; Zucchini et al. 2008). To date, a limited number of genetic studies on the relationship between TLR7 polymorphisms and BD has been reported. Although an association between single nucleotide polymorphisms (SNPs) of *TLR7* and BD was not found in an earlier study conducted in Japan (Sada et al. 2011), copy number variations (CNVs) of *TLR7* were found to be associated with ocular involvement of BD in a different study in China (Fang et al. 2015). Therefore, the researchers decided that more investigations would be important for clarifying the association between *TLR7* gene polymorphism and BD in different polymorphic regions.

Objectives

Disease-associated polymorphisms of genes may be ethnic specific and may be associated with clinical presentation of the disease, such as ocular, neurological, or intestinal involvement. Therefore, the present study was conducted to investigate the relationship between two SNPs (rs179009 and rs179008) of *TLR7* and ocular involvement of BD in the Turkish population.

METHODOLOGY

Patients

This study involved 198 individuals (89 patients with Behçet uveitis and 109 controls) recruited from the Department of Ophthalmology, Faculty of Medicine, Ondokuz Mayis University (Samsun, Turkey). There were 65 (73%) males and 24 (27%) females in the patient group. All the patients had recurrent uveitis and met the classification criteria of the International Study Group for Behçet disease ("Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease" 1990). They were included in the study regardless of their activity status. Patients who had any different diseases were excluded from the study. All the individuals in the control group were healthy except for refractive errors and were selected by excluding the diagnosis of BD. The patient and control groups were matched in terms of age and ethnic background. The study protocol was approved by the ethics committee of the Faculty of Medicine, Ondokuz Mayis University (OMU KAEK 2015/184). Written informed consent was obtained from all participants. The tenets of the Declaration of Helsinki were followed in all procedures.

The data collection sheet included information such as age, gender, age at presentation, extraocular clinical manifestations, type of uveitis, laterality, and ocular complications.

A complete clinical evaluation, including best-corrected visual acuity on a Snellen scale, slit-lamp biomicroscopy, applanation tonometry, and indirect ophthalmoscopy, was performed for all patients.

Due to financial restriction, we could only randomly choose two SNP regions (rs179009 and rs179008) in the *TLR7* gene.

Genotype Determination

DNA was extracted from 2 ml venous blood according to the kit procedure (Sigma, USA) and stored at -20 °C. *TLR7* rs179009 and rs179008 genotypes were determined by polymerase chain reaction (PCR). A 388 base pair fragment including the polymorphic site was amplified. Primer: sense oligo was f 5'-GGAGTTTGGAAAT TAGGATTATGTT-3' and antisense oligo was r 5'-ACTTTGGCAGTG AATCTATGGC-3. Final volume of 50 µl, containing 2 mM MgCl2, 0.2mM dNTP (Geneun), 0.2 mM F primer, 0.5 mM, 0.2 mM R primer of each 2.5 U Taq DNA polymerase (Fermentas). 35 cycles with denaturation at 95 °C for one minute, annealing at 57 °C for 1 minute 45 seconds, hybridization at 56°C for one minute and extension at 72 °C for one minute applied using a thermal cycler (Techne, USA). Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) and run on the ABI 3730 DNA analyzer (Applied Biosystems). All the sequence analyses were done using the Sequencher 4.8 (Gene Codes, Ann Arbor, MI) by Macrogene. BLAST analysis was done by the MultAlin program (http://multalin.toulouse.inra.fr/multalin/). The genotype distribution, allele frequencies, and composite genotype based on the clinical findings were analyzed.

Statistical Analysis

SPSS 15.0 (Chiccago, USA) program and OpenEpi computer programs were used for sta-

tistical analysis. Chi-square test was applied for allele frequency and genotype frequency analysis. When the p value was less than 0.05 it was considered statistically significant. Minitab program was used for power analysis and defining sample size.

RESULTS

The patient's characteristics and variables are shown in Table 1. There were 65 (73 %) males and 24 (27 %) females in the patient group and 67 (61 %) males and 42 (39 %) females in the control group. The mean age was 35.01 ± 12.35 years in the patient group and 33.75 ± 14.48 years in the control group. The mean duration of Behçet uveitis was 8.1 ± 7.5 years. Of the 89 patients, 33 (37%) had unilateral involvement, while 56 (63%) had bilateral involvement. Eight (9%) of them had anterior uveitis, while 81 (91%) had posterior uveitis. Genotype and allele frequencies of *TLR7* rs179009 T>C, *TLR7* rs179008 A>T for the patient and control groups are presented in Table 2. There was no statistically significant difference in genotype distribution for *TLR7* rs179009 gene T/C genotypes between the patient and control groups (p = 0.73). There was also no statistically significant difference between the two groups for *TLR7* rs179008 gene A/Tgenotypes (p = 0.39).

In the composite genotype analysis, no statistical significant difference was detected (p = 0.09; Table 3). In addition, no association between genotype frequencies and the clinical status of the patients was detected (Table 4).

Table 1: Baseline characteristics of the patients with Behçet uveitis

Gender n (%)	Male	75 (73)
	Female	24 (27)
Mean duration	Behçet Disease	9.8 ± 8.0
of(Year)	Behçet Uveitis	8.1 ± 7.5
Laterality n (%)	Unilateral	33 (37)
	Bilateral	56 (63)
Type of uveitis	Anterior	8 (9)
n (%)	Posterior/pan-uveitis	81 (91)
Visual acuity in	<20/200	29 (33)
bad eye n (%)	20/200 – 20/40	12 (13)
	>20/40	48 (54)
Treatment n (%)	No treatment	18 (20)
	Conventional	51 (57)
	Biologics	20 (23)

DISCUSSION

Although the pathogenesis of BD remains unclear, it is thought that microbial agents or other environmental factors may have a trigger effect at the initiation of the disease in genetically susceptible individuals. Genetic studies may be important to determine the associations between genetic alterations and disease involvement, severity, and response to therapy (Deng et al. 2018).

Toll-like receptors are an important family that are triggered by microbial pathogens and initiate

Table 2: The results of genotype and allele frequencies of *TLR-7* rs179009 T>C and *TLR-7* rs179008 A>T polymorphisms for the patients and controls

SNP	*Genotype/ allele*	*Patients (n=89) (%)*	*Controls (n=109) (%)*	χ^2	*P Value*	*OR (%05 Cl)*
*TLR-7*rs179009	T T	75(84.0)	92 (84.0)	0.621*	0.73	
	TC	2(2.00)	1 (1.00)			
	CC	12(14.0)	16 (15.0)			
	CC+TC : TT	14:75	17:92	0.006	0.97	1.01 (0.459-2.198)
	TC+TT : CC	77:12	93:16	0.057	0.81	0.90 (0.390-2.043)
	C	26	33	0.021	0.88	0.959 (0.545-1.66)
	T	152	185			
*TLR-7*rs179008	T T	12 (13.40)	13 (12.0)	2.983	0.39	
	AT	2 (2.30)	7 (6.4)			
	AA	75 (84.30)	89 (81.6)			
	AT+TT : AA	14:75	2:89	0.236	0.62	0.831 (0.385-1.762)
	AA+AT : AA	12:77	13:96	0.107	0.74	1.150 (0.486-1.702)
	T	26	33	0.020	0.88	0.959 (0.545-1.676)
	A	152	185			

χ^2= Chi-square, **P<0.05, OR= odd ratio, Cl= confidence limit, *Fisher exact test

Table 3: Composite genotype analysis

SNP-SNP	*SNP genotypes*	*Patients n (%)*	*Controls n (%)*	χ^2	*P-value*
rs 179009 (T→C)	AATT	72 (45.9)	85 (54.1)	0.355	0.09
and rs 179008	AACC	3 (42.9)	4 (57.1)		
(A→T)	ATTC	2 (66.7)	1 (33.3)		
	TTTT	3 (100.0)	0 (0.0)		
	TTGG	9 (40.9)	13 (59.1)		
	ATTT	0 (0.0)	6 (100.0)		
Total		89	109		

SNP= Single nucleotide polymorphism, A= Adenine, T= Thymine, C= Cytosine, χ^2= Chi-square, $^{**}P<0.05$

the immune response. Genetic variables in genes that code for receptors in the innate immune system affect the susceptibility to immune-mediated diseases. The association between the TLR genotypes and the pathogenesis of disease has been investigated in patients with BD. In a study, eight SNPs in *TLR2*, *TLR4*, *TLR 8* and *TLR9* were investigated, and only *TLR2* was found to be associated with ocular BD (Fang et al. 2013). In other studies, SNPs in *TLR4* were shown to be associated with BD (Horie et al. 2009; Kirino et al. 2013; Meguro et al. 2008). It was also shown that there is an association between homozygous genotypes and homozygous diplotype configuration of the TLR9 SNPs and susceptibility to BD in the Japanese population (Sakamoto et al. 2012).

In their research, Fang et al. (2015) analyzed copy number variations (CNVs) of TLRs in BD, VKH syndrome, AAU, and healthy controls. They found increased copy number in only *TLR7* in both male (more than one copy) and female (more than two copies) patients with BD. Their results also showed that mRNA expression and, consequently, protein expression of TLR7 were higher in patients who have high copy number of *TLR7* (Fang et al. 2015).

In this study, the researchers investigated the association between the polymorphism of two SNPs (rs179009 and rs179008) of *TLR7* and the susceptibility to ocular involvement of BD.

The researchers did not find any association between these SNPs of *TLR7* and ocular involvement. Furthermore, they analyzed the association between these genotypes and extraocular manifestation of BD, including oral ulcer, genital ulcer, skin involvement, pathergy results, joint involvement, neurological involvement, and thromboembolia. The researchers also analyzed the association between these genotypes and some features of Behçet uveitis, including visual loss, anatomical involvement, and laterality. The researchers did not find any statistically significant association between any of them. Sada et al. (2011) investigated the frequency of eight SNPs in the *TLR7* gene in 200 Japanese patients with BD and 102 control subjects. Similar to the current research, they concluded that *TLR7* gene polymorphism was not associated with susceptibility to BD (Sada et al. 2011).

Although higher expression of TLRs has been shown in previous studies (Fang et al. 2015; Liu et al. 2013), the association between TLR genotypes and susceptiblity to BD still remains inconsistent. This may suggest that changes in TLR expression and function may be more associated with the pathogenesis than gene polymorphisms. Previous GWAS studies also failed to identify common gene variants in TLR genes in BD (Fei et al. 2009; Hou et al. 2012; Lee et al. 2013; N Mizuki et al. 2010; Remmers et al. 2010). Only Kirino et al. (Kirino et al. 2013) identified rare and low frequency variants in *TLR4* genes by performing deep resequencing of targeted genes. We thought that TLRs may have an important role in the pathogenesis of BD; however, there are other additional genetic factors that may affect the development of the disease.

CONCLUSION

In the present study, the researchers did not find any relationship between two SNPs at the *TLR7* gene loci and Behçet uveitis in a Turkish population. No association between these genotypes and the other clinical features of BD, including oral ulcer, genital ulcer, skin involvement, pathergy results, joint involvement, neurological involvement, and thromboembolism, was detected as well. To the researchers' knowledge, this is

Table 4: Genotype frequencies according to the clinical findings

	rs179008 / n (%)			χ^2	*P value*	*rs179009 / n (%)*			χ^2	*P value*
	A/A	*A/T*	*T/T*			*T/T*	*C/T*	*C/C*		
Pathergy										
+	22 (29.3)	1 (50.0)	4 (33.3)	0.179	0.88	23 (30.7)	1 (50.0)	3 (25.0)	2.974	0.56
-	31 (41.3)	1 (50.0)	4 (33.3)			28 (37.3)	1 (50.0)	7 (58.3)		
N/A	22(29.3)	0 (0.0)	4 (33.3)			24 (32.0)	0 (0.0)	2 (16.7)		
Genital Ulcer										
+	50 (66.7)	2 (100.0)	10 (83.3)	2.251	0.32	51 (68.0)	2(100.0)	9 (75.0)	1.131	0.57
-	25 (33.3)	0 (0)	2 (16.7)			24 (32.0)	0 (0)	3 (25)		
Skin Involvement										
+	52 (69.3)	1 (50.0)	7 (58.3)	0.852	0.65	53 (70.7)	1 (50.0)	6 (50.0)	2.294	0.32
-	23 (30.7)	1 (50.0)	5 (41.7)			22 (29.3)	1 (50.0)	6 (50.0)		
Arthritis										
+	36 (48.0)	1 (50.0)	6 (50.0)	0.019	0.99	37 (49.3)	1 (50.0)	5 (41.7)	0.246	0.884
-	39 (52.0)	1 (50.0)	6 (50.0)			38 (50.7)	1 (50.0)	7 (58.3)		
Thromboembolism										
+	8 (10.7)	0 (0.0)	0 (0.0)	1.641	0.44	8 (10.7)	0 (0.0)	0 (0.0)	1.641	0.44
-	67 (89.3)	2 (100.0)	12(100.0)			67 (89.3)	2(100.0)	12 (100.0)		
Neurological Involvement										
+	4 (5.3)	0 (0.0)	1 (8.3)	0.297	0.86	4 (5.3)	0 (0.0)	1 (8.3)	0.297	0.86
-	71 (94.7)	2 (100.0)	11 (91.7)			71 (94.7)	2(100.0)	11 (91.7)		
Vision In Bad Eye										
<20/200	26 (34.7)	2 (100.0)	3 (25.0)	4.859	0.30	26 (34.7)	2(100.0)	3 (25.0)	6.810	0.15
20/200-20/40	17 (22.7)	0 (0.0)	2 (16.7)			18 (24.0)	0 (0.0)	1 (8.3)		
>20/40	32 (42.7)	0 (0.0)	7 (58.3)			31 (41.3)	0 (0.0)	8 (66.7)		
Anatomic Localization										
Anterior	8 (10.7)	0 (0.0)	0 (0.0)	1.641	0.44	8 (10.7)	0 (0.0)	0 (0.0)	1.641	0.44
Posterior	67 (89.3)	2 (100.0)	12(100.0)			67 (89.3)	2(100.0)	12(100.0)		
Laterality										
Bilateral	48 (64.0)	2 (100.0)	6 (50.0)	2.075	0.35	49 (65.3)	2(100.0)	5 (41.7)	3.689	0.16
Unilateral	27 (36.0)	0 (0.0)	6 (50.0)			26 (34.7)	0 (0.0)	7 (58.3)		

A= Adenine, T= Thymine, C= Cytosine, χ^2= Chi-square, $^{**}P<0.05$

the first study in Turkey to examine *TLR7* gene rs179009 and rs179008 polymorphisms in patients with Behçet uveitis. Therefore, the results of this study would contribute to the literature. However, further studies with more polymorphic regions and a larger study group may be helpful to confirm the results of this study. Besides, additional studies are needed to identify other genetic factors that are effective in establishing the interaction between TLRs and BD.

RECOMMENDATIONS

Understanding the pathogenesis of the disease may provide better preventive and treatment strategies and eventually lead to its remission. Genetic studies provide insight into the underlying pathogenic mechanisms of the disease. Due to the multifactorial and complex nature of the disease, selecting the right candidates is a challenge but is also crucial. Based on the results of GWASs, it would be an appropriate approach to conduct studies involving large, well-defined patient groups.

LIMITATIONS

One of the most important limitations of the current study is the small sample size. Due to the methodology and financial restriction, the researchers studied a limited number of cases that were enough for power analysis. Likewise, limited polymorphic regions were studied because of financial restriction (rs179009 (T>C) and rs179008 (A>T)).

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ABBREVIATIONS

BD: Behçet disease
CCR: C-C chemokine receptor
CIs: Confidence intervals
CNV: Copy number variations
ERAP: Endoplasmic reticulum aminopeptidase
GIMAP: GTPase of the immunity-associated protein
GWAS: Genomwide association studies
HLA: Human leukocyte antigen
IL: Interleukin
KLR: Killer cell lectin-like receptors
MEFV: Mediterranean fever
MHC: Major histocompatibility complex
NOD: Nucleotide-binding oligomerization domain-containing protein
OR: Odds ratio
PAMP: Pathogen-associated molecular patterns
PRR: Pattern recognition receptor
PCR: Polymerasse chain reaction
SD: Standard deviation
SNP: Single nucleotide polymorphisms
STAT: Signal transducer and activator of transcription
TLR: Toll-like receptor
UBAC: Ubiquitin-associated domain-containing protein

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