
Toll-like receptor 4 (TLR4) gene polymorphisms in Italian patients with Behçet's disease

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ABSTRACT

Objective. To investigate potential associations between toll-like receptor 4 (TLR4) gene polymorphisms and susceptibility to, clinical features, and severity of Behçet's disease (BD).

Methods. A total of 189 Italian patients who satisfied the International Study Group criteria for BD and 210 healthy age- and sex-matched blood donors were genotyped for two coding single nucleotide polymorphisms of TLR4 (Asp299Gly and Thr399Ile) by molecular methods. The patients were subgrouped according to the presence or absence of clinical manifestations. Severity score was calculated.

Results. The distribution of allele and genotype frequencies did not differ significantly between the BD patients and the healthy controls. No significant associations were found when BD patients with and those without clinical manifestations were compared. No association between TLR4 polymorphisms and severity score was observed.

Conclusion. Our data suggest that the TLR4 gene polymorphisms are not associated with susceptibility to, clinical expression of, and severity of BD in Italian patients.

Introduction

Behçet's disease (BD) is a disease in which multiple genetic factors, in combination with environmental risk factors such as infectious agents, are probably of importance in determining disease susceptibility and clinical expression (1, 2). Although BD is strongly associated with HLA-B51 and MICA-A6 alleles (3), other genetic factors may also confer a risk for the development of BD in Italian population (4-6).

Vasculitis is the pathological lesion underlying most clinical manifestations of BD (1, 2). In one immunopatho-

logical study, Kobayashi *et al.* showed that the predominant lesion in BD is a neutrophilic vasculitis involving the vasa vasorum (7). These authors also found that neutrophils and endothelial cells of the vasa vasorum are activated. Furthermore, several investigations have shown that various functions of neutrophils in peripheral blood, such as chemotaxis, phagocytosis and generation of reactive oxygen species (ROS) are enhanced in BD (8-13) and that patients with BD have increased myeloperoxidase activity (14-15). Therefore, abnormal activation of the innate immune system may play a significant role in the pathogenesis of BD. However, the immunopathological mechanisms remain to be fully elucidated. Persistent activation by exogenous agents such as heat shock proteins (HSPs) and lipopolysaccharides (LPS) may activate the innate immune system through Toll-like receptors (TLRs), provoking inflammation in BD. Recently, two co-segregating single nucleotide polymorphisms (SNPs) within the gene encoding TLR4, Asp299Gly and Thr399Ile, have been characterized (16) and studied in different inflammatory and infectious conditions (17-25). These polymorphisms have been shown to impair the efficacy of LPS-induced signaling and its capacity to elicit inflammation (16). The aim of this study was to examine the relationship between Asp299Gly and Thr399Ile polymorphisms and the susceptibility to, clinical expression of, and severity of BD in a cohort of Italian patients.

Materials and methods

Study population

Case patients were 189 consecutive patients with BD who were followed up in 9 different Italian referral centers over

a 7-year period (1999-2005). All patients fulfilled the criteria developed by the International Study Group for BD (ISG) (26). The control group consisted of 210 gender- and age-matched healthy subjects who were unrelated volunteer blood donors. The median age of the controls was 36 years (range 19-44). 50% of controls were males. All study subjects were Caucasians who had been residing in Italy for at least one generation. No ethnic differences were present between patients and controls. The study was approved by the Ethics Committees of the participating centers and written informed consent was obtained from patients and controls before inclusion in the study.

The diagnosis of deep vein thrombosis (DVT) and subcutaneous thrombophlebitis was based on clinical data and confirmed by ultrasonography or contrast venography. Severity score was calculated according to the method proposed by Krause *et al.* (27)

HLA class I typing. Serological HLA class I typing was performed by a standard microlymphocytotoxicity technique, using peripheral blood lymphocytes. Out of the 189 Italian patients with BD, 152 were typed for HLA-B51 allele. The control group consisted of 228 Italian healthy blood donors who were different from those genotyped for Asp299Gly and Thr399Ile polymorphisms.

DNA extraction and genotyping. DNA was obtained from whole blood using phenol/chloroform method, according to standard procedures (28). PCR was performed to amplify two small regions of TLR4 gene (29), a fragment of 248 bp containing rs4986790 Ex4+636A>G Asp299Gly polymorphism (forward primer 5'GATTAGCATACTAGACTACTACCTCCATG3' and reverse primer 5'GATCAACTTCTGAAAAAGCATTCCCAC3') and a fragment of 405 bp containing rs4986791 Ex4+936C>T Ile399Thr polymorphism (forward primer 5'GGTTGCTGTTCTCAAAGTGATTTGGGAGAA3' and reverse primer 5'CCTGAAGACTGGAGAGTGAGTTAAATGCT3'). Both forward primers have an altered nucleotide (underlined bases) that is useful to create a restriction site for the endonucleases Nco I and Hinf I re-

Table I. Demographic and clinical features of 189 Italian patients with Behçet's disease.

	Number	Percentage
Female/male	90/99	47.6/52.4
Mean age at disease onset \pm SD (yrs)	30 \pm 12	
Mean disease duration (yrs)	11 \pm 8	
Oral ulcers	189	100
Cutaneous lesions	154	81.5
Papulopustular lesions	101	53.4
Erythema nodosum	77	40.7
Genital ulcers	117	61.9
Epididymitis	13	6.9
Eyes lesions	106	56.1
Anterior uveitis	59	31.2
Posterior uveitis and retinal vasculitis	84	44.4
Arthritis	79	41.8
Central nervous system involvement	30	15.9
Venous thrombosis*	48	25.4
Deep venous thrombosis	33	17.5
Positive pathergy test**	42/96	43.7
HLA-B51***	99/151	65.6
Severity score [§]	6.7 \pm 2.3	

*Subcutaneous thrombophlebitis + deep vein thrombosis; **Pathergy test was performed on 96 patients; ***HLA-B51 was performed on 151 patients; [§]Severity score was calculated according to the method proposed by Krause *et al.* (27).

spectively. PCR amplifications was performed in 25 μ l reaction medium containing 100 μ M of each dNTP, 20 pmol each primer, 1 units Taq polymerase. Amplification profile for two amplicons was as follows:

- initial denaturation 95°C for 2 min 35 cycles of: 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec final extension at 72°C for 3 min for 248 bp amplicon
- initial denaturation 95°C for 2 min 35 cycles of: 94°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec final extension at 72°C for 3 min for 405 bp amplicon

Digestion was performed on 10 μ l PCR products of two amplicons using Nco I and Hinf I restriction endonucleases. These enzymes can reveal the alleles because Nco I cuts the amplicon only if A allele is replaced by G and Hinf I cuts the 405 bp amplicon if C nucleotide is replaced by T. Different genotypes were shown by electrophoresis analysis of digested PCR products in 2.5% agarose gel stained with ethidium bromide (0.5 μ g/ml).

Statistical analysis

Statistical analysis was done using SPSS statistical package (SPSS Inc.,

Chicago, IL, USA). The frequencies of the alleles and genotypes in patients and controls were compared by chi-squared test. Odds ratios (ORs) were calculated with 95% confidence intervals (95% CIs). We performed a power calculation for an unmatched case-control study and estimated relative risk using Power and Sample Size Calculation version 2.1.31 software.

Results

Table I shows the clinical and demographic characteristics of the 189 patients with BD.

The allele and genotype frequencies of the TLR4 Asp299Gly polymorphism in BD patients and in the control group are shown in Table II. Of the 189 BD patients, 16 were heterozygous for the Asp299Gly TLR4 allele, while out of the 210 controls, 17 were heterozygous. No homozygous subjects were found in either group. In these 16 BD patients and in these 17 controls, co-segregation of the Thr399Ile polymorphism was observed, while no BD patients or controls had an isolated Asp299Gly polymorphism. The distribution of the TLR4 Asp299Gly did not differ significantly between BD patients and controls.

Table II. Frequencies of alleles, genotypes and carriage rates of Toll-like receptor (TLR) 4 polymorphism Asp299Gly in patients with Behçet's disease and controls.

	Behçet's disease (n=189)	Controls (n=210)	p-value	OR (95% CI)
Alleles				
G	16/378 (4.2)	17/420 (4.0)	NS	1.1 (0.5-2.1)
A	362/378 (95.8)	403/420 (96.0)		
Genotype				
G/G	0 /189 (0.0)	0/210 (0.0)	NS	
G/A	16/189 (8.5)	17/210 (8.1)		
A/A	173/189 (91.5)	193/210 (91.9)		
Carriage rate				
G/A+G/G	16/189 (8.5)	17/210 (8.1)	NS	1.1 (0.5-2.1)
A/A	173/189 (91.5)	193/210 (91.9)		

Values are the number/total number examined (%). OR: odds ratio.
95% CI: 95% confidence interval.

Allele G frequency (4.2% vs. 4.0%) and carriers of the G allele (G/A+ G/G) (8.5% vs. 8.1%) were similar in BD patients and controls.

Given the sample size (189 patients with Behçet disease and 210 controls) and the allele frequencies of the polymorphism examined, we can conclude with 80% certainty that there is a genetic relative risk of 2.9 at rs4986790, Ex4+636A>G, (Asp299Gly) TLR-4 polymorphism.

We also investigated possible TLR4 Asp299Gly polymorphism associations with BD, stratifying by HLA-B51. No significant associations were observed in HLA-B51 positive and HLA-B51 negative patients, respectively (data not shown).

The associations between the TLR4 Asp299Gly polymorphism and the clinical manifestations of BD defined in Table I were evaluated in the 189 BD patients, comparing patients with and without different manifestations. The following variables were studied: oral ulcers, genital ulcers, cutaneous lesions, papulopustular lesions, erythema nodosum, eye lesions, anterior uveitis, posterior uveitis and retinal vasculitis, arthritis, central nervous system involvement, venous thrombosis, deep venous thrombosis and positive pathergy test. No significant associations were found (data not shown).

There were no significant differences in male/female frequency (58.8/41.2 vs. 51.7/48.3), age at disease onset (27.3±13.1 vs. 30.1±12.3) and disease severity score (6.7±1.9 vs. 6.6±2.3) be-

tween the carriers of the G allele (GG or GA) and AA homozygosity.

Discussion

It has been shown that endogenous and exogenous molecules as HSP and LPS are able to trigger an inflammatory response via TLR2 and TLR4 (30). Both HSPs and LPS have been proposed as possible BD candidate antigens (1). HSPs are upregulated in BD lesions, while serum HSPs levels are significantly higher in BD patients than in controls (31, 32). Similarly, LPS-stimulated production of tumor-necrosis-factor- α , interleukin (IL)-1, IL-6, and IL-8 is significantly elevated in BD patients (12). Monocytes of patients with active BD are activated (33) and express higher concentrations of TLR2 and TLR4 relative to controls (34). Intestinal lesions of BD have also been demonstrated to express both TLR-2 and TLR-4 mRNA, as well as HSP60 mRNA and protein (35). On the same line, Kirino *et al.* (36) demonstrated that levels of TLR4 mRNA, but not of TLR2 mRNA, were constitutively increased in peripheral blood mononuclear cells from patients with BD, regardless of disease activity. Therefore, TLR4 may play an important role in the pathogenesis of BD.

In 2002, Arbour *et al.* described two cosegregating polymorphisms of the human TLR4 gene, Asp299Gly and Thr399Ile (16). These two SNPs are constituted by an A/G transition causing an aspartic acid/glycine substitution at amino acid location Asp299Gly

(rs4986790), and a C/T transition causing a threonine/isoleucine switch at amino acid location Thr399Ile (rs4986791). Arbour *et al.* also pioneered the studies on the functional consequence of these TLR4 polymorphisms (16). They reported that individuals with either the Asp299Gly and/or Thr399Ile polymorphisms had a blunted response to inhaled LPS. Furthermore, transfected cells with TLR4 polymorphisms have a decreased NF- κ B activity compared with wild-type TLR4, leading to reduced cytokine production.

Since their identification, these TLR4 polymorphisms have been studied in some rheumatic conditions with conflicting results. There was no evidence for a role of these mutations in susceptibility to ankylosing spondylitis in 4 studies, while a modest association only was observed in a Canadian study (18, 19, 37-39). Discordant data have also been reported regarding the association of these polymorphisms with susceptibility to RA and response to treatment (21, 22, 40). In a recent study we demonstrated that these polymorphisms were not associated with Italian patients with giant cell arteritis (41).

In this study, we evaluated Asp299Gly and Thr399Ile TLR4 polymorphisms in an ethnically homogeneous and large group of Italian patients with BD. Our results indicate that these polymorphisms are not associated with BD in Italian patients, suggesting that these two TLR4 polymorphisms do not play an important role in the pathogenesis of BD. Both polymorphisms occur with an allelic frequency of approximately 3% to 6% in the Caucasian populations (42). In our control population we found an allelic frequency (6.8%) similar to that reported in a previous Italian study (7.2%) (43). Furthermore, consistent with the very rare occurrence of homozygous mutations, we did not find homozygous in our patients and controls.

A second aim of this study was to determine whether these TLR4 polymorphisms might be associated with the clinical expression of BD in our cohort of Italian patients. However, when patients with and without different mani-

festations were compared, no associations were found. Further, the studied TLR4 polymorphisms were not associated with gender, age at disease onset, and disease severity as assessed by the severity score proposed by Krause *et al.* (27).

However, our study is probably insufficiently powered to detect significant associations for subgroup analyses.

Recently, Meguro *et al.* reported that a polymorphism in the 3' untranslated region (UTR) of the TLR4 (rs7037117) was significantly associated with the risk of developing BD in Japanese patients (44). These authors also showed a significant association between some of the studied TLR4 polymorphisms and incomplete-type BD, age of disease onset, and several manifestations including oral and genital ulcers, skin lesions, and ocular disease.

TLR2 gene polymorphisms have been also recently investigated in BD patients. Similar negative results have been reported in two different populations. Bacanlı *et al.* demonstrated that the TLR2 polymorphism, Arg753Gln, was not associated with Turkish BD (45). Tomyama *et al.* confirmed the lack of associations between common polymorphisms of the TLR2 gene and BD in Japanese patients (46).

TLR9 gene polymorphisms were also not significantly associated with susceptibility to BD in a Japanese population (47).

One of the most important causes of failure to replicate findings in genetic association studies of BD is the inadequacy of sample sizes. Multicentre collaborations to recruit an adequate number of patients are thus required.

Conclusion

We did not find any association between Asp299Gly and Thr399Ile TLR4 polymorphisms and susceptibility to, clinical expression of, and severity of BD. These results do not support the hypothesis that a genetically determined regulation of TLR4 signaling may predispose to the development and clinical expression of this vasculitis in Italian patients. Further, larger studies are required to confirm our findings in other populations.

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