Toll-like-receptor gene polymorphisms in a Tunisian population with Behçet's disease

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Abbreviations:

BD:	Behçet's disease
PCR-RFLP:	polymerase chain reaction – restriction fragment length polymorphism
OR:	odds ratio
CI:	confidence interval

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ABSTRACT

Objective. *The manifestations of BD are considered to have developed as a result of immunological dysfunction, which is suggested to be induced by microbial pathogens. The Toll-like receptor (TLR) genes were known to be associated with a variety of infectious diseases due to their central role in both innate and adaptive immunity. In this report, we investigated the possible association between BD patients and genetic variations within the TLR 2, 4 and 9 genes in a Tunisian population.*

Patients and methods. *135 Tunisian BD patients and 159 healthy blood donors from the same geographical area were genotyped by polymerase chain reaction for the TLR polymorphisms.*

Results. *Among the TLR polymorphisms, only the distribution of TLR9 1486 T/C genotype ($p=0.07$; $\chi^2=3.30$; OR=1.54; 95% CI=0.94–2.51) and allele ($p=0.08$; $\chi^2=2.91$; OR=1.34; 95% CI=0.94–1.92) frequencies was different between BD patients and healthy controls, but did not reach statistical significance. For the TLR9 1237 T/C, the distribution of genotypes and alleles were not significantly different comparing total patients with controls. There were no associations between the studied polymorphisms and the main clinical manifestations of BD.*

The G, T and A allele of the TLR4 1896 A/G, TLR4 11196 C/T and TLR2 12408 G/A polymorphisms were uncommon and absent in the Tunisian population.

Conclusion. *Our results showed that SNPs in the TLR2, 4 and 9 genes were not significantly associated with susceptibility to BD.*

Introduction

Behçet's disease (BD) is a multisystemic inflammatory disorder characterized by oral and genital ulcers and cutaneous, ocular, arthritis, vascular, central nervous system and gastrointestinal involvement (1, 2). The disease is most common along the ancient Silk Road extending from Eastern Asia to the Mediterranean basin. The typical age of onset is in the 30s, and the male to female ratio varies with ethnic origin (2).

BD possibly involves complex interactions of genetic and environmental factors. HLA-B51 is the genetic marker most strongly associated with BD in different populations, with relative risk ranging from 1.38 to 20.70 (3, 4).

The manifestations of BD are considered to have developed as a result of immunological dysfunction, which is suggested to be induced by microbial pathogens in genetically susceptible individuals, includes hyperactivity of neutrophils, overexpression of several proinflammatory and Th1-type cytokines, several phenotypic and functional lymphocyte abnormalities (5, 6).

Herpes simplex virus (HSV) is currently the only virus possibly associated with BD (1, 2). *Streptococcus sanguis* has been suggested as a causative agent, because the bacteria and antibodies against the bacteria are frequently found in the oral flora and serum, respectively, of patients with the disease (7).

Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), are essential components of the innate immune system as recognition of microbial antigens occurs via PRRs that are expressed by innate effector cells. Microbial recognition results in a rapid and efficient immune response against invading microorganisms (8). The TLRs are members of an evolutionary conserved interleukin-1 super-family of transmembrane receptors that recognize pathogen associated molecular patterns (9). Recognition of pathogen-derived ligands such as peptidoglycans, lipopolysaccharide (LPS) and bacterial CpG-DNA by Toll-like receptor 2 (TLR2), TLR4 and TLR9, respectively, has recently been discovered (10, 11).

Due to the role of the innate immune genes, such as TLRs, at the interface between host and environment, genetic variations within these genes could have a major impact on host defence or inflammatory disease pathogenesis. Common TLR polymorphisms are associated with reduced responsiveness to various pathogens and with gram-negative infection, sepsis, and asthma, among other conditions (12).

We speculate that genetic variation in the innate immune genes, like TLR, may play a role in determining susceptibility, not only to infectious diseases but also to chronic inflammatory human diseases such as BD.

For the TLR2 gene, two single nucleotide polymorphisms Arg677Trp and Arg753Gln have been identified. TLR2 recognizes lipoproteins of the cell membrane of Gram-positive bacteria, Gram-negative bacteria, mycobacteria, mycoplasma and other organisms. TLR2 gene mediated a response to bacterial cell wall components (13). Any abnormalities affecting the function of TLR2 could explain the aberrant immune response that causes the symptoms of BD.

Regarding TLR-4, an A to G conversion at position 12874 replaces Asp 299 by a Gly residue and results in a loss of function, whereas a C to T conversion at position 13174 replaces Thr 399 by an Ile residue results in an intermediate phenotype (14). TLR4 forms a complex with CD14 and other cofactors on the surface of immune cells to activate intracellular signalling.

Polymorphisms 1237T/C and 1486T/C within the TLR9 promoter have been recently identified. There imply that genetic variation in *TLR9* could play a role in human diseases potentially associated with altered innate immune responses.

Any abnormalities affecting the function of TLR can play an important role in the development of BD, and could explain the aberrant immune response that causes the symptoms. As patients with BD were rather susceptible to infectious agents, such as streptococci and mycoplasma, both of which are recognized by TLRs, it seemed reasonable to suppose there would be an association between TLR polymorphisms and BD. The current study compared the distribution of the genotype and allele frequencies of TLR2, TLR4 and TLR9 genes among Behçet's patients and healthy controls. The aim was to investigate the possible association between these polymorphisms and susceptibility to Behçet's disease in Tunisian patients.

Patients and methods

We studied 135 native Tunisian patients (93 male and 42 female) with BD, recruited from Rabta Hospital, between September 2005 and May 2007. All patients were diagnosed according to the diagnostic criteria proposed by the international study group for BD (15). The mean (SD) ages of the BD group and controls were 39.5±13 and 41.3±9 years, respectively. The clinical features of the patients are summarized in Table I. A control group of 159 unrelated, healthy Tunisian age- and sex-matched individuals who had no known medical problems on a health-screening questionnaire were enrolled. The study was approved by our National Ethics Committee and was conducted in accordance with the guidelines in the Declaration of Helsinki. Informed consent was obtained from all patients with Behçet's disease and controls participating in this study.

Genotyping

DNA from all study subjects was obtained from peripheral blood and extracted using the salting out procedure as described (16). The samples of the 286 (135 BD patients and 159 healthy controls) subjects were genotyped for the TLR4 (1896 A/G rs 4986790 and 11196 C/T rs4986791), TLR9 (21237 T/C rs5743836 and 21486 T/C rs187084) and finally for the TLR2 (12408 G/A rs5743708) polymorphisms.

A polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method was used to genotype patients and controls for the target polymorphisms. The total volume of the PCR was 25 ml containing 100 ng of genomic DNA, 1xPCR buffer (Fermentas International, Ontario, Canada), 5 mM of a dNTP-Mix Fermentas), 0.5 units of Taq DNA polymerase (Fermentas) and 3 pmol of each primer. The final MgCl2 concentrations are given in Table II. The PCR comprised an initial denaturation step (95°C for 15 min), 35 cycles (94°C for 30 s), primer annealing temperature as given in Table II for 30s, 72°C for 30 s and a final extension step (72°C for 10 min). The volume of the restriction assays was 15 ml containing the appropriate buffer, water

Table I. Clinical characteristics of patients with Behçet's disease and demographic features of healthy control group.

	No. of patients (%) n=135	No. of controls (%) n=159
Age (mean ± SD, years)	39.5 ± 13	41.3 ± 9
Sex (Male)	93 (68.8)	114 (72.6)
Duration of the disease (mean ± SD, years)	8.69 ± 4	_
Familial antecedent	14 (10.3)	_
HLA-B51	31 (22.9)	_
Frequencies of clinical manifestations		
Oral ulceration	135 (100)	_
Genital ulceration	109 (80.7)	_
Skin lesions		
Erythema nodosum	16 (11.8)	_
Folliculitis/acne	65 (48.1)	_
Skin pathergy response	29 (21.5)	_
Ocular inflammtion		
Uveitis	62 (45.9)	_
Retinal vasculitis	21 (15.5)	_
Vascular involvement		
Vascular involvement	28 (20.7)	_
Phlebitis	19 (14.0)	_
Neurologic involvement	28 (20.7)	_
Arthritis	26 (19.2)	_

HLA: human leukocyte antigen.

Table II. Annealing temperature (°C), primers, Taq, DNTP and MgCl2 concentrations used for PCR.

Gene	Polymorphism	Primers (μM)	Taq (U)	Number of cycles	DNTP (mM)	$MgCl_2$ (mM)	Annealing temp (°C)
TLR9	T-1237C	0.8	0.7	38	0.16	2.2	61°C
	T-1486C	0.5	0.7	36	0.16	1.5	60°C
TLR4	Asp299Gly	0.4	0.5	31	0.12	3	61°C
	Thr399Ile	0.4	0.5	31	0.12	2.5	
TLR2	Arg753Gln	0.4	0.7	35	0.2	3	65°C

TLR: Toll-like receptor.

Table III. Primer sequences used in RFLP analyses of TLRs polymorphism.

Genes	Polymorphisms	Primers	
TLR9	1237 T/C	TLR9-Forward	5'-ATGGGAGCAGAGACATAATGGA-3'
		TLR9-reverse	5'-CTGCTTGCAGTTGACTGTGT-3'
	1486T/C	TLR9-Forward	5'-TCCCAGCAGCAACAATTCATTA-3'
		TLR9-reverse	5'-CTGCTTGCAGTTGACTGTGT-3'
TLR4	Asp299Gly	F:5'-GATTAGCATACTTAGACTACTACCTCCATG-3'	
		R: 5 ' -GATCAACTTCTGAAAAAGCATTCCCAC-3'	
	Thr399Ile	F:5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3'	
		R:5'-ACCTGAAGACTGGAGAGTGAGTTAAATGCT- 3'	
TLR2	Arg753Gln	F: 5' -CATTCCCCAGCGCTTCTGCAAGCTCC-3'	
		R: 5'-GGAACCTAGGACTTTATCGCAGCTC-3'	

Table IV. Restriction enzymes and length of the restriction fragments.

Gene polymorphism		Restriction enzyme	Length of the restriction fragments
TLR4	Asp299Gly	Nco I	Wild type (allele A): 188 bp Asp299Gly (allele G): 168 bp + 20 bp
TLR4	Thr399Ile	Hinf I	Wild type (allele C): 124 bp Thr399Ile (allele T): 98 bp + 26 bp
TLR2	Arg753Gln	Msp I	Wild type (allele G): 104 bp + 25 bp Arg753Trp (allele A): 129 bp
TLR9	T-1237C	BstNI	Wild type (allele C) 108pb + 27pb T-1237C (allele T) 60pb+ 48pb + 27pb
TLR9	T-1486C	AflII	Wild type (allele C) 327pb +172pb T-1486C (allele T) 499pb

TLR: Toll-like receptor.

and restriction enzyme (Fermentas) and 10 ml of the PCR product. It was incubated overnight at 37°C and analyzed by electrophoresis on a 4% agarose gel. Additionally all primer sequences are given in Table III and restriction enzymes used are given in Table IV.

Statistical analysis

The χ^2 test was used to compare the distribution of the different mutations between the Behçet's affected individuals and the representative control group. Differences in alleles and genotypes frequencies between patients and healthy controls were compared using the standard Chi squared test (Epistat statistical package). When the expected cell number was less than 5, Fisher's exact test was used. Probability values of 0.05 or less were regarded as statistically significant. The strength of a gene association is indicated by the odds ratio (OR). The odds ratio and the 95% confidence intervals (CI) were calculated whenever applicable.

The subjects used in this study were justified by the Hardy-Weinberg's exact test, and no genetic bias was observed for each SNP (p>0.06 for all analyses).

Results

The distribution of genotypes and alleles frequencies was compared between Tunisian BD patients and healthy controls. G, T and A allele of the TLR4 1896 A/G, TLR4 11196 C/T and TLR2 12408 G/A polymorphisms were uncommon (allele frequencies are 0% for each polymorphism).

The distribution of the TLR9 1237 T/C and 1486 T/C by study group is shown in table V. TLR9 1486 T/C allele (p=0.08; χ^2=2.91; OR=1.34; 95% CI=0.94–1.92) and genotype (p=0.07; χ^2=3.30; OR=1.54; 95% CI=0.94–2.51) were different between BD patients and healthy controls but did not reach statistical significance. The frequency of the C allele was 32% in BD patients and 39% in controls.

For the TLR9 1237 T/C, the distribution of alleles and genotypes were not significantly different comparing total patients with controls. The frequency of the C allele was 10% in BD patients compared to 8.8% in the control group. There were no associations between the studied polymorphisms and the main clinical manifestations of BD (skin lesions, ocular inflammation, vascular involvement, neurologic involvement, arthritis, disease severity).

Also, when analyses were carried out between the TLR polymorphisms and BD patients with HLA-B*51 and those without these characteristics no significant difference was found according to the presence of HLA-B51.

Discussion

The genotype frequencies of the TLR2 and TLR4 polymorphisms were not significantly different between Behçet's patients and controls. The G, T and A allele of the TLR4 1896 A/G, TLR4 11196 C/T and TLR2 12408 G/A polymorphisms were uncommon (allele frequencies are 0% for each polymorphism).

For TLR9 polymorphism, a difference was found in the distribution of 1486T/C allele (p=0.08; χ^2=2.91; OR=1.34; 95% CI=0.94–1.92) and genotype

Table V. Comparison of TLR9 T-1237C and T-1486C polymorphisms between Tunisian BD patients and healthy controls.

SNP	Genotype frequency (%)			χ^2 (*p*-value)	Allele frequency (%)		χ^2 (*p*-value)
1237T/C	T/T	T/C	C/C		T	C	
Patients	111 (82.2)	21 (15.6)	3 (2.2)	0.22	243 (90)	27 (10)	0.25
Controls	134 (84.3)	22 (13.8)	3 (1.9)	0.89	290 (91.1)	28 (8.8)	0.61
1486T/C	T/T	T/C	C/C		T	C	
Patients	66 (48.9)	51 (37.8)	18 (13.3)	3.30	183 (67.8)	87 (32.2)	4.06
Controls	61 (38.4)	72 (45.3)	26 (16.3)	0.07	194 (61%)	124 (39%)	0.08

p=0.07 for genotype frequencies; p-value was obtained when comparing TT genotype to (TC+CC) genotype. *p*=0.08 for allele frequencies.
TLR: Toll-like receptor; BD: Behçet's disease; SNP: single nucleotide polymorphism.

(*p*=0.07; χ^2=3.30; OR=1.54; 95% CI=0.94–2.51) frequencies between BD patients and healthy controls but did not reach statistical significance.

TLR2 12408G/A polymorphism was uncommon in our Tunisian population, and like a previous study (17) we did not find any of its variants in the control group. A recent study found that TLR2 variants were completely absent in Caucasians and Asians (18). Also, our results were in comparison with other autoimmune diseases. In a Turkish study they found that none of the patients from the rheumatoid arthritis (RA) groups had the A allele or A/G genotype and that the TLR2 Arg753Gln polymorphism does not play a role in BD (19). In the Basque population no association was found between TLR2 and TLR4 polymorphisms and Type I diabetes mellitus (20). A very low frequency of the TLR2 Arg753Gln was in systemic lupus erythematous (SLE) patients (21).

For the TLR4, the data of previous studies revealed carriage rates for the Asp299Gly mutation of TLR4 gene ranging from 7% to 12% among healthy Caucasian subjects (22). The rate of TLR4 polymorphism is low, but in our study TLR4 are compromised by the absence of the allele frequency.

There was no suggestion of any association between these polymorphisms and Behçet disease risk. In Tunisian population TLR2 and TLR4 are compromised by the absence of the allele frequency and lack of power. In this study we did not have enough power to detect genetic effects of TLR2 and TLR4 polymorphisms on Behçet's disease.

At present, the impact of the TLR9 1237T/C and 1486T/C polymorphisms on the expression of *TLR9* remains unknown but a dysfunction in bacterial recognition or a lack of an adequate immune response to bacterial can not be excluded. In knockout mice, TLR9 has been shown to be responsible for mediating the maturation of dendritic cells and the production of proinflammatory cytokines including tumor necrosis factor, interleukin-6, and interleukin-12 by macrophages following exposure to unmethylated CpG-rich bacterial DNA (11). To our knowledge, no study has reported an association between TLR9 1237T/C and/or 1486T/C polymorphisms and BD.

Our study showed that there was no relationship between 1237T/C polymorphism and BD. This result was in concordance with those found in Turkish population (19). When studies were done in other autoimmune diseases, the 1237T/C polymorphism was associated with susceptibility to asthma in European Americans, but not in Hispanic or African Americans (23) and recently, has been associated with Crohn's disease (24). There may be different disease associations reflecting different immunopathogenetic mechanisms.

Differences have been reported in the distribution of 1486T/C allele and genotype frequencies between BD patients and healthy controls, but did not reach statistical significance. Ito *et al*. showed that SNPs in the TLR9 gene were not significantly associated with susceptibility to BD in a recent Japanese study (25). This was in concordance with results found in our study but sample size cannot provide enough statistical power to give a significant result and it is necessary to increase the sample size before a conclusion can be reached.

When clinical manifestations were taken account, there were no associations between 1237T/C and 1486T/C polymorphisms and the main clinical manifestations of BD (skin lesions, ocular inflammation, vascular involvement, neurologic involvement, arthritis, disease severity).

In our study, the number of subjects was not large enough to conclude the contribution of this polymorphism on Tunisian BD patients.

Polymorphisms in the TLR9 gene might influence the functional capability of TLR9 to elicit effective defense mechanism against microbial pathogens. It has been shown that the TLR9/MyD88 pathway mediates antiviral cytokine responses by Dentritic cells (DC), and possibly other cell types, which are coordinated to promote effective NK cell function (26).

Despite the same role of important mediators for many autoimmune diseases, conflicting results were shown in Behçet's disease. Touma *et al*. found that CTLA-4 gene variants are not associated with Behçet's disease or its clinical manifestations (27), but CTLA4-A/G polymorphism was found to be associated with Behçet's disease in the Tunisian population (28). Recently, another important receptor in mediating various immune effector functions was found to have clinical implications in Behçet's disease (29).

Our study should be investigated in other ethnic groups using large samples to investigate the mechanism of TLR polymorphisms in BD. This should contribute to better understanding the role of these polymorphisms in BD.

The lack of data regarding TLR9 polymorphisms indicate the need for larger studies to clarify the role of these polymorphisms in infectious and/or autoimmune diseases.

This study has suffered from a lack of power and it is necessary to increase the sample size before a conclusion can be obtained. The present work should be regarded as a hypothesis-testing study with its limitations.

References

1. YURDAKUL S, HAMURYUDAN V, YAZICI H: Behçet syndrome. *Curr Opin Rheumatol* 2004; 16: 38-42.
2. SAKANE T, TAKENO M, SUZUKI N, INABA G: Behçet's disease. *N Engl J Med* 1999; 341: 1284-91.
3. MIZUKI N, OHNO S, TANAKA H *et al*.: Association of HLA-B51 and lack of association of class II alleles with Behçet's disease. *Tissue Antigens* 1992; 40: 22-30.
4. OHNO S, OHGUCHI M, HIROSE S, MATSUDA H, WAKISAKA A, AIZAWA M: Close association of HLA-Bw51 with Behçet's disease. *Arch Ophthalmol* 1982; 100: 1455-8.
5. GÜL A: Behçet's disease: an update on the pathogenesis. *Clin Exp Rheumatol* 2001; 19 (Suppl. 24): S6-12.
6. ZIERHUT M, MIZUKI N, OHNO S *et al*.: Immunology and functional genomics of Behçet's disease. *Cell Mol Life Sci* 2003; 60: 1903-22.
7. LEHNER T: The role of heat shock protein, microbial and autoimmune agents in the aetiology of Behçet's disease. *Int Rev Immunol* 1997; 14: 21-32.
8. WRIGHT SD, RAMOS RA, TOBIAS PS, ULEVITCH RJ, MATHISON JC: CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; 249: 1431-3.
9. TAKEDA K, AKIRA S: Toll receptors and pathogen resistance. *Cell Microbiol* 2003; 5: 143-53.
10. ADEREM A, ULEVITCH RJ: Toll-like receptors in the induction of the innate immune response. *Nature* 2000 17; 406: 782-7.
11. HEMMI H, TAKEUCHI O, KAWAI T *et al*.: A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; 408: 740-5. Erratum in: *Nature* 2001; 409: 646.
12. SCHRODER NW, SCHUMANN RR: Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect Dis* 2005; 5: 156-64.
13. BOCHUD PY, HAWN TR, ADEREM A: Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. *J Immunol* 2003; 170: 3451-4.
14. BEUTLER B, BEUTLER E: Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med* 2002; 347: 1978-80.
15. INTERNATIONAL STUDY GROUP FOR BEHÇET'S DISEASE: Criteria for diagnosis of Behçet's disease. *Lancet* 1990 5; 335: 1078-80.
16. MILLER SA, DYKES DD, POLESKY HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16: 1215.
17. LACHHEB J, DHIFALLAH IB, CHELBI H, HAMZAOUI K, HAMZAOUI A: Toll-like receptors and CD14 genes polymorphisms and susceptibility to asthma in Tunisian children. *Tissue Antigens* 2008; 71: 417-25.
18. MOCKENHAUPT FP, CRAMER JP, HAMANN L *et al*.: Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. *Proc Natl Acad Sci USA* 2006; 103: 177-82.
19. BACANLI A, SALLAKCI N, YAVUZER U, ALPSOY E, YEGIN O: Toll-like receptor 2 Arg753Gln gene polymorphism in Turkish patients with Behçet's disease. *Clin Exp Dermatol* 2006; 31: 699-701.
20. SANTIN I, BILBAO JR, DE NANCLARES GP, CALVO B, CASTANO L: No association of TLR2 and TLR4 polymorphisms with type I diabetes mellitus in the Basque population. *Ann N Y Acad Sci* 2006; 1079: 268-72.
21. SANCHEZ E, OROZCO G, LOPEZ-NEVOT MA, JIMENEZ-ALONSO J, MARTIN J: Polymorphisms of toll-like receptor 2 and 4 genes in rheumatoid arthritis and systemic lupus erythematosus. *Tissue Antigens* 2004; 63: 54-7.
22. KOEDEL U, ANGELE B, RUPPRECHT T *et al*.: Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. *J Immunol* 2003; 170: 438-44.
23. LAZARUS R, KLIMECKI WT, RABY BA *et al*.: Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. *Genomics* 2003; 81: 85-91.
24. TOROK HP, GLAS J, TONENCHI L, BRUENNLER G, FOLWACZNY M, FOLWACZNY C: Crohn's disease is associated with a toll-like receptor-9 polymorphism. *Gastroenterology* 2004; 127: 365-6.
25. ITO A, OTA M, KATSUYAMA Y, INOKO H, OHNO S, MIZUKI N: Lack of association of Toll-like receptor 9 gene polymorphism with Behçet's disease in Japanese patients. *Tissue Antigens* 2007; 70: 423-6.
26. BEUTLER B, BEUTLER E: Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med* 2002; 347:1978-80.
27. TOUMA Z, HAMDAN A, SHAMSEDDEEN W *et al*.: CTLA-4 gene variants are not associated with Behçet's disease or its clinical manifestations. *Clin Exp Rheumatol* 2008; 26 (Suppl. 50): S132.
28. BEN DHIFALLAH I, CHELBI H, BRAHAM A , HAMZAOUI K HOUMAN H: CTLA-4 +49A/G polymorphism is associated with Behçet's disease in a Tunisian population. *Tissue Antigens* 2009; 73: 213-7.
29. AKSU K, KITAPCIOGLU G, KESER G *et al*.: FcgammaRIIa, IIIa and IIIb gene polymorphisms in Behçet's disease: do they have any clinical implications? *Clin Exp Rheumatol* 2008; 26 (Suppl. 50): S77-83.