RESEARCH ARTICLE

HLA-B27 subtypes and tumor necrosis factor α promoter region polymorphism in Iranian patients with ankylosing spondylitis

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ABSTRACT. ***Background.*** **HLA-B27 is an MHC class I molecule that is strongly associated with ankylosing spondylitis (AS). TNF-α, as an important cytokine in inflammatory joint disease, might have a role in the process of AS. This study was performed to determine HLA-B27 subtypes among Iranian patients with AS, and to investigate TNF-α gene polymorphisms in the patient groups.** ***Methods.*** **Ninety seven AS patients (74 HLA-B27-positive and 23 HLA-B27-negative) and 137 healthy normal subjects (2 HLA-B27-positive) were enrolled in this study. HLA-B27 positive patients were screened using the polymerase chain reaction, with sequence specific primers (PCR-SSP), for B*27 subtyping. All patients and the controls were also investigated for determination of TNF-α polymorphisms using the same method.** ***Results.*** **Just two subtypes were detected in our patients, namely B*2705 (63.4%) and B*2702 (36.6%). The study of TNF-α polymorphisms at position -238 showed that the A allele and AA genotype were significantly over-represented in all patient groups in comparison with the control group ($p < 0.001$). At position -308, while the A allele and AA genotype were significantly over-represented in the whole patient group ($p = 0.01$), there was no significant difference between the AS groups and the control group.** ***Conclusion.*** **It could be suggested that a polymorphism within the TNF-α gene at -238 play an important role in AS, although this polymorphism was not related to HLA-B27 subtypes.**

Keywords: ankylosing spondylitis, HLA-B27, MHC class I, polymorphism, TNF-α

The human leukocyte antigen B*27 is known to be a class I antigen of the major histocompatibility complex (MHC) system that is strongly associated with ankylosing spondylitis (AS) [1-3]. AS is the prototype of the seronegative spondyloarthropathies, and is characterized by axial and peripheral arthritis. There is a strong association between the HLA-B27 and AS, which makes this MHC molecule an important genetic marker of the disease [1, 4]. Although the exact pathophysiology of the disease is still unknown, it seems to be a multifactorial disease with environmental and genetic factor involvement [5].

Thirty one HLA-B27 subtypes, with considerable geographic and ethnic differences, have already been reported [1, 5, 6]. A limited number of amino acid residues, occupying defined positions in the peptide-binding groove, differentiates one from another [3]. There are various distributions of the HLA-B27 alleles in different populations [3]. While HLA-B*2701, *2702, *2705, *2708 and *2709 have been reported in Caucasians, HLA-B *2704, *2706, and *2707 have been reported only in Asians [1, 2, 7, 8]. A number of studies have investigated the preferential association of certain subtypes with AS, and found a predisposition to the disease associated with the HAL-B*2705, *2702, *2704, and *2707 subtypes, among the first ten (HAL-B*2701 to *2710) subtypes [1, 2, 7, 8].

Tumor necrosis factor alpha (TNF-α), as a proinflammatory cytokine that plays an important role in the regulation of the immune response, could be implicated in the inflammation of the synovium, and erosion of the cartilage in rheumatological diseases [9, 10]. As erosion of the sacroiliac joints is the prominent feature of AS, TNF might have a role in this disease. The gene encoding TNF-α is considered a risk factor in AS [9, 11, 12]. This gene is located within the MHC class III region, close to the HLA-B locus [13]. Given that certain MHC class II alleles are associated with variations in levels of production of TNF [14], a role for polymorphisms of the TNF

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gene in MHC-associated autoimmune diseases such as AS is suggested. TNF production could be affected by a range of polymorphisms at position -308 and -238, the regulatory region of the TNF gene [15, 16].
This study was performed to determine HLA-B27 subtypes in Iranian patients with AS, and to evaluate the relationship with polymorphisms of the TNF-α promoter region; TNF and the HLA system are located on same chromosome and these genes are inherited as a haplotype.

METHODS AND DONORS

Patients and control

Ninety seven AS patients (89 males and eight females) were selected randomly from the "Iranian AS Association", which includes a mixed population, from all Iranian ethnic groups, during 2005-2006 *(table 1)*. The diagnosis of AS in these patients was made according to the Modified New York Criteria (MNYC) [17]. One hundred and thirty seven Iranians, who had never suffered from any rheumatological diseases, were also randomly selected from blood donors at Iranian blood transfusion organizations for determination of TNF-α polymorphisms.

HLA typing

DNA from subjects' peripheral blood leukocytes was extracted by a modified "salting-out" method [18]. Genetic amplification was performed using the polymerase chain reaction (PCR). Screening of HLA-B27 was accomplished by primers of E2 and E3, which are used to amplify exon 2 and exon 3 of B27 to cover all known B27 subtypes. A primer pair amplifying the third intron of HLA-DRB1 (796bp) was used as an internal control [19].

Allele typing

Allele typing was performed using the PCR-sequence specific primer (PCR-SSP) method with the "Olerup SSP™ HLA-B*27 Kit" (Olerup SSP AB, Sweden). PCR was performed on a Corbett Thermal cycle, model: CG1-96. PCR products were visualized in 2% agarose gel under UV illumination, following ethidium bromide staining, and were documented by photography [18].

Determination of TNF-α

Cytokine typing was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP assay kit; Heidelberg University, Heidelberg, Germany) [24]. Briefly, amplification was carried out using a thermal cycler Techne Flexigene apparatus (Rosche, Cambridge, UK). The polymerase chain reaction products were visualized by 2% agarose gel electrophoresis. After electrophoresis, the gel was placed on an ultraviolet transilluminator, and a picture was taken for interpretation and documentation. Each of the primer mixes contained a control primer pair that amplified either a part of the β-globin gene or a part of the C-reactive protein gene. The β-globin control primers produce an 89-base pair (bp) fragment, while the primer pairs amplifying the CRP gene produced a 440-bp amplicon.

Statistical analysis

Data were analyzed using the chi-square X^2 test. The odds ratio and P value were calculated for each allele in the patient and control groups using SPSS statistical software, version 14 (SPSS Inc, Chicago, IL, USA). A P-value of less than 0.05 was considered significant. For multiple testing corrections, the Bonferroni correction was performed and the resulting p-value of less than 0.008 was considered significant for TNF-α genotypes at position -238.

RESULTS

Of the 97 patients with AS, 74 were HLA-B27 positive (76.3%), while only two of 137 controls were HLA-B27 positive ($p < 0.001$). Two HLA-B27 subtypes were detected in our study, including B*2705, the most frequent subtype (66.2%), and B*2702 (37.8%).
Allele and genotype frequencies for the TNF-α gene in positions -238 and -308 (promoter region) in the control and patient groups, are shown in the *tables 2,3*, respectively. The frequencies of the TNF-α -238 A and G alleles, and also the AG and GG genotypes differ significantly between the patient group and the control group ($p < 0.0001$). The frequencies of the TNF-α -238 A allele and the AA genotype were also significantly over-represented in all patient groups (all HLA-B27 subtypes and HLA-B27 negative) in comparison with the control group *(table 2)*.
The A and G allele frequencies ($p = 0.016$) and also the AG and GG genotypes ($p < 0.01$) of TNF-α-308 differ significantly between the patient and control groups *(table 3)*.
There was no significant difference in the TNF-α genotypes between the two groups of HLA-B*2702 and HLA-B*2705. Although the AG genotype -238 in the patient groups was only detected in patients with HLA-B*2705 (three cases), this was not significantly different to other patient groups ($p = 0.54$). The AA genotype at position -308 was also more common in the

Table 1
Characteristics of of patients with ankylosing spondylitis

Age (years)	Number (total = 97)	Percentage (%)	Male (n = 89)	Female (n = 8)
12-22	47	48.45	43	4
23-32	38	39.18	34	4
33-42	9	9.28	9	0
43-52	3	3.09	3	0

Table 2
Comparisons of allele and genotype frequencies of TNF-α -238 between AS patient groups and controls

Allele/ Genotype	Control (n = 137) N (%)	Patients				Odds ratio (OR) and P-value (P)							
		Total patients (n = 97) N (%)	HLA-B27- (n = 23) N (%)	HLA-B2702 (n = 25) N (%)	HLA-B2705 (n = 49) N (%)	Controls *versus* patients		Controls *versus* HLA-B27-		Controls *versus* HLA-B2702		Controls *vs.* HLA-B2705	
						OR	P	OR	P	OR	P	OR	P
A	215 (78.5)	191 (98.46)	46 (100.0)	50 (100.0)	95 (96.94)	17.47	< 0.0001	13.06	0.002	14.17	0.001	8.69	0.00005
G	59 (21.5)	3 (1.55)	0 (0.00)	0 (0.00)	3 (3.07)	0.06	< 0.0001	0.08	0.002	0.07	0.001	0.128	0.00005
AA	79 (57.7)	94 (96.91)	23 (100.0)	25 (100.0)	46 (93.88)	17.52	< 0.0001*	17.70	0.0006*	19.17	0.0003*	8.67	0.00001*
AG	57 (41.6)	3 (3.10)	0 (0.00)	0 (0.00)	3 (6.13)	0.06	< 0.0001*	0.06	0.0007*	0.05	0.0004*	0.12	0.00002*
GG	1 (0.7)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0.69	0.77	2.85	0.94	2.63	0.98	1.37	0.69

* Significant at 5% level, adjusted for the Bonferroni multiple testing correction.

Table 3
Comparisons of allele and genotype frequencies of TNF-α -308 between AS patient groups and controls

Allele/ Genotype	Control (n = 137) N (%)	Patients				Odds ratio (OR) and P-value (P)							
		Total patients (n = 97) N (%)	HLA-B27- (n = 23) N (%)	HLA-B2702 (n = 25) N (%)	HLA-B2705 (n = 49) N (%)	Controls *versus* patients		Controls *versus* HLA-B27-		Controls *versus* HLA-B2702		Controls *versus* HLA-B2705	
						OR	P	OR	P	OR	P	OR	P
A	235 (85.8)	181 (91.42)	43 (93.47)	48 (96.00)	90 (91.84)	0.43	0.016	0.42	0.23	0.25	0.07	1.87	0.17
G	39 (14.2)	13 (6.57)	3 (6.52)	2 (4.00)	8 (8.17)	2.31	0.016	2.38	0.23	3.98	0.07	0.54	0.17
AA	98 (71.5)	84 (84.85)	20 (86.96)	23 (92.00)	41 (83.68)	2.57	0.01	2.65	0.19	4.58	0.05	2.04	0.14
AG	39 (28.5)	13 (13.14)	3 (3.04)	2 (8.00)	8 (16.33)	0.39	0.01	0.38	0.19	0.22	0.05	0.49	0.14

HLA-B*2702 group, but the difference compared to the HLA-B*2705 group was not significant (92% *versus* 84%, p = 0.48).

DISCUSSION

The association between HLA-B27 and AS has been known about for more than three decades, and is one of the strongest HLA-disease associations [2]. Although HLA-B27 might have a role in antigen presentation, the exact mechanism that allows HLA-B27 to confer susceptibility to AS remains unknown [2, 3].

The frequency of HLA-B27 positive cases in AS patients varies between different countries. In our study, three quarters of the AS patients were HLA-B27 positive, which is similar to the estimated frequency (about 70%) in Iran [20]. It seems that the genetic basis for the pathogenesis is heterogeneous, which could be due to diverse subtypes of the disease in different regions. These epidemiological data might support the hypothesis that genetic factors, in association with environmental factors, could influence the disease [2].

TNF promoter polymorphisms seem to be a predisposing factors for AS. In this context, we determined the distribution of the TNF promoter polymorphisms at positions -238 and -308 in HLA-B27 AS patients and healthy controls. A significant decrease in the frequency of the TNF-238A allele in patients with AS was found, when compared to healthy individuals. Association analysis showed that the TNF-238G allele is in linkage disequilibrium with the HLA-B27 allele. The decreased frequency of the TNF-238A allele in AS patients is therefore likely to be secondary to the HLA-B27 association. In contrast to our results, no difference in the distribution of the TNF-238 allele was reported between AS patients and healthy controls in the Spanish population [21]. Moreover, in these population studies, no evidence for linkage disequilibrium between HLA-B27 and the TNF-238G allele was observed. The discrepancy between the two studies could be explained by the absence of linkage disequilibrium between the TNF-238G allele and HLA-B27 in the Spanish population. This difference could reflect the ethnic differences between the populations studied [9].

The -308 G to A transition seems to be associated with the HLA -A1- B8 -DR3 haplotype, which predispose individuals to autoimmune diseases [22]. In our study, the A allele and the AA genotype of TNF-α (-308) have a significant association with AS, which is in contrast with previous studies [5, 21]. While this difference between HLA-B2702 and HLA-B2705 is not significant, we cannot confirm the hypothesis that the TNF-308A allele and TNF-308A/G genotype will be inherited with just HLA-B2702 as a haplotype. However, overrepresentation of the A allele and the AA genotype could be associated with a higher production of TNF-α [23], confirming the role of elevated TNF-α levels in inflammation [24].

Although there is not enough evidence to support a role for TNF alleles in AS, associations between TNF alleles and infectious and autoimmune diseases have been described [5, 25]. Since polymorphic genes within the MHC could display a high degree of linkage disequilibrium, associations with class I or II alleles could be a consequence of linkage with a nearby, disease-causing TNF gene.

While the role of TNF single nucleotide polymorphisms has previously been reported in some disorders, this study suggests a role for this cytokine polymorphism in AS [26-29]. Although the G to A transition polymorphism at position -238 seems to be associated with a number of autoimmune diseases, independent of HLA-association, the TNF-238 polymorphism has been reported in several studies, to be associated with severity rather than susceptibility in autoimmune diseases. Therefore, such factors could be of significance in determining disease outcome [5, 30]. However, in order to determine whether a relationship between certain TNF alleles and disease outcome exists in AS, further studies are needed to expand the analyses of TNF polymorphisms in a group of AS patients stratified for disease activity.

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REFERENCES

1. Cipriani A, Rivera S, Hassanhi M, *et al.* HLA-B27 subtypes determination in patients with ankylosing spondylitis from Zulia, Venezuela. *Hum Immunol* 2003: 745-9.
2. Oguz F, Ocal L, Diler A, *et al.* HLA- B27 in Turkish patients with spondyloarthropathy and healthy controls. *Disease Markers* 2004; 20: 309-12.
3. Gonzalez S, Garcia-Fernandez S, Martinez-Borra J, Blanco-Gelaz M, Rodrigo L, Sanchez del Rio J. High variability of HLA-B27 alleles in ankylosing spondylitis and related spondyloarthropathies in the population of northern Spain. *Hum Immunol* 2002: 673-6.
4. Varnavidou-Nicolaidou A, Karpasitou K, Georgiou D, Stylianou G, Kokkofitou A, Michalis C. HLA-B27 in the Greek Cypriot population: distribution of subtypes in patients with ankylosing spondylitis and other HLA-B27-related diseases. The possible protective role of B*2707. *Hum Immunol* 2004; 65: 1451-4.
5. Brinkman B, Huizinga T, Kurban S, *et al.* Tumour necrosis factor a gene polymorphisms in rheumatoid arthritis: association with susceptibility to, or severity of, disease? *Br J Rheum* 1997; 36: 516-21.
6. Nicknam MH, Mahmoudi M, Amirzargar AA, *et al.* Determination of HLA-B27 subtypes in Iranian patients with ankylosing spondylitis. *Iran J Allergy Asthma Immunol* 2008; 7: 19-24.
7. Shankarkumar U, Ghosh K, Mohanty D. HLA B27 polymorphism in Western India. *Tissue Antigens* 2002; 60: 98-101.
8. Khan M. Update: the twenty subtypes of HLA-B27. *Curr Opin Rheumatol* 2000; 12: 235-8.
9. Kaijzel E, Brinkman B, van Krugten M, *et al.* Polymorphism within the Tumor Necrosis Factor alpha promoter region in patients with ankylosing spondylitis. *Human Immunol* 1999; 60: 140-4.
10. Sklatvala J. Tumor necrosis factor alpha stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature* 1986; 322: 547-9.
11. Verjans GM, Brinkman BM, Van Doornik CE, Kijlstra A, Verweij CL. Polymorphism of tumour necrosis factor-alpha (TNF-a) at position -308 in relation to ankylosing spondylitis. *Clin Exp Immunol* 1994; 97: 45-7.
12. Verjans GM, Messer G, Weiss EH, van der Linden SM, Kijlstra A. Polymorphism of the tumor necrosis factor region in relation to disease: an overview. *Rheumat Dis Clin North Am* 1992; 18: 177-86.
13. Campbell D, Trowsdale J. Map of the human histocompatibility complex. *Immunol Today* 1993: 14.
14. Jacob C, Fronek Z, Lewis G, Koo M, Hansen J, McDevitt H. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor a: relevance to genetic predisposition to systemic lupus erythematosus. *Proc Natl Acad of Sci USA* 1990; 87: 1233-7.
15. Hamann A, Mantzoros C, Vidal-Puig A, Flier JS. Genetic variability in the TNF-a promoter is not associated with type II diabetes mellitus (NIDDM). *Biochem Biophys Res Com* 1995; 211: 833-9.
16. D'Alfonso S, Momigliano Richiardi P. A polymorphic variation in a putative regulation box of the TNFA promoter region. *Immunogenetics* 1994; 39: 150-4.
17. Braun J, Sieper J. Ankylosing spondylitis. *Lancet* 2007; 369: 1379-90.
18. Nicknam M, Jamshidi A. M GH. Comparison of validity of microlymphocytotoxicity and flowcytometry methods with PCR for HLA-B27 antigen typing. *Med JIR Iran* 2003; 17: 75-9.
19. Voorter C, Swelsen W, van den Berg-Loonen E. B*27 in molecular diagnostics: impact of new alleles and polymorphism outside exons 2 and 3. *Tissue Antigens* 2002; 60: 25-35.
20. Nicknam M, Ganjalikhani Hakemi M, Jamshidi A. Association between HLA-B27 antigen and ankylosing spondylitis in Iranian patients. *Hakim Journal* 2005; 8: 29-34.
21. Fraile A, Nieto A, Beraun Y, Vinasco J, Mataran L, Martin J. Tumor necrosis factor gene polymorphisms in ankylosing spondylitis. *Tissue Antigens* 1998; 51: 386-90.
22. McGuire W, Hill A, Allsopp C, Greenwood B, Kwiatkowski D. Variation in the TNF-a promoter region associated with susceptibility to cerebral malaria. *Nature* 1994; 371: 508-10.
23. Hoffmann SC, Stanley EM, Darrin Cox E, *et al.* Association of cytokine polymorphic inheritance and in vitro cytokine production in anti-CD3/CD28 stimulated peripheral blood lymphocytes. *Transplantation* 2001; 72: 1444-50.
24. Erbek SS, Yurtcu E, Erbek S, Atac FB, Sahin FI, Cakmak O. Proinflammatory cytokine single nucleotide polymorphisms in nasal polyposis. *Arch Otolaryngol Head Neck Surg* 2007; 133: 705-9.
25. Huizinga T, Westendorp R, Bollen E, *et al.* TNF-a polymorphisms, production and susceptibility to multiple sclerosis in different groups of patients. *J Neuroimmunol* 1997; 72: 149-53.
26. Rezaei N, Amirzargar AA, Shakiba Y, Mahmoudi M, Moradi B, Aghamohammadi A. Proinflammatory cytokine gene single nucleotide polymorphisms in common variable immunodeficiency. *Clin Exp Immunol* 2009; 155: 21-7.
27. Mahdaviani SA, Rezaei N, Moradi B, Dorkhosh S, Amirzargar AA, Movahedi M. Proinflammatory cytokine gene polymorphisms among Iranian patients with asthma. *J Clin Immunol* 2009: 28.
28. Amirzargar AA, Bagheri M, Ghavamzadeh A, *et al.* Cytokine gene polymorphism in Iranian patients with chronic myelogenous leukaemia. *Int J Immunogenet* 2005; 32: 167-71.
29. Amirzargar AA, Rezaei N, Jabbari H, *et al.* Cytokine single nucleotide polymorphisms in Iranian patients with pulmonary tuberculosis. *Eur Cytokine Netw* 2006; 17: 84-9.
30. Vinasco J, Beraun Y, Nieto A, *et al.* Polymorphism at the TNF loci in rheumatoid arthritis. *Tissue Antigens* 1997; 49: 74-8.