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Association of *CTLA4* Gene Polymorphism in Iranian Patients with Ankylosing Spondylitis

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Abstract
Introduction Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a costimulatory molecule expressed by activated T cells. This study was performed to investigate the allele and genotype frequencies of *CTLA4* gene polymorphisms in Iranian patients with ankylosing spondylitis (AS).
Methods One hundred and fifty-seven patients with AS and 103 controls were included in this study. Polymorphisms of *CTLA4* gene at positions +49 (in exon 1), −318, and −1,147 (in the promoter region) were studied on the genomic DNA using PCR restriction fragment-length polymorphism method.
Results The frequencies of the T allele at position −1147 in the patients with AS was significantly increased in comparison with the control group (11% vs. 5%, P=0.004); whereas the frequencies of C allele at the same position were significantly decreased in the patient group (89% vs. 95%, P=0.004). Comparison of genotype frequencies at this position showed that the frequency of CT genotype in comparison with other genotypes was overrepresented in the patient group (20% vs. 8%, P=0.012), while the CC genotype in comparison with other genotypes was decreased (79% vs. 91%, P=0.012). There was no significant difference on frequencies of genotypes at the positions −318 and +49.
Conclusion This study could suggest an association between specific allele in the promoter region of CTLA4 gene and AS disease.

Keywords Ankylosing spondylitis · CTLA4 · gene polymorphism

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Introduction

Ankylosing spondylitis (AS) is a chronic autoimmune disease, characterized by inflammatory axial arthritis. AS is the prototype of a group called seronegative spondyloarthropathies, which seems to have common clinical characteristics and genetic susceptibility [1–4]. Although exact pathogenesis of AS is unclear, it seems to be a multifactorial disease, in which environmental and genetic factors could be involved [5].

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a costimulatory molecule which is expressed by activated T cells and interacts with the B7 molecule on the surface of antigen-presenting cells to induce down-regulation of T cell activation. CTLA-4, which is encoded by *CTLA4* gene (OMIM*123890) located on chromosome 2p33, is a structural homologue of T cell costimulatory protein CD28, but plays a negative regulatory role in T cell response [6–8].

Association of some genetic factors like HLA-B27 with AS are well described and several polymorphisms in genes that susceptible individuals to autoimmune diseases

are described [1–3]. Theoretically, polymorphism within *CTLA4* that lead to reduction of CTLA-4 expression may cause autoimmune T cell clonal proliferation and therefore contribute to the pathogenesis of autoimmune diseases [9]. Association of *CTLA4* gene polymorphisms with several autoimmune diseases such as Graves' disease, Hashimoto's thyroidism, Addison's disease, type 1 diabetes, celiac disease, rheumatoid arthritis, and multiple sclerosis have been studied [10–12]. This study was performed to investigate a possible association between the polymorphisms of the *CTLA4* gene with AS.

Patients and Methods

Subjects

One hundred and fifty-seven patients with AS (135 male and 22 female), with age range of 18–79 years, were randomly selected from the "Iranian AS Association". The diagnosis of AS was made based on the Modified New York Criteria (MNYC) [13]. One hundred and three healthy subjects, who had never suffered from any rheumatological disease, were also selected as the control group from the "Iranian Blood Transfusion Organization". All enrolled patients and controls in this study were unrelated. The study was approved by the local ethics committee of Tehran University of Medical Sciences. Informed consent was obtained from all participants before sampling.

DNA Analysis

Blood was collected in EDTA tubes; DNA extraction was performed from blood leukocytes by a modified salting-out method. Amplification of the position +49 in the exon 1 and −318 in the promoter region of the *CTLA4* gene was performed using PCR with the following primers: forward (5′-TCTTTTCCGCCTATTTTCAGTT-3′) and reverse (5′-CCCTGGAATACAGAGCCAGC-3′).

For amplification of position −1147 in the promoter region, the following primers were used: forward (5′-GCT GAGGTGTGGACCATGG-3′) and reverse (5′-TCAGGT GTTCTTAAAAGCCTTAAC-3′; Table 1).

The reaction was carried out in 20 μl of solution, containing a standard PCR buffer, 200 ng DNA, 0.2 μM of each primer, 0.5 U Taq polymerase, 1.5 mM of $MgCl_2$ and 200 μM of dNTP mix. Amplifications were performed in 35 cycles of 20 s at 95°C, 30 s at 60°C and 1 min at 72°C and a final 10-min extension at 72°C.

Using the restriction fragment-length polymorphism (RFLP) technique [14, 15], phenotyping of three polymorphic sites, two in the 900-bp fragment (−318C/T and 49A/G) and one in the 481-bp fragment (−1147C/T) of the *CTLA4* gene was performed. FOK I (BseG I), Bbv I (BSeX I), and Mse I (Fermentase, Lithuania) enzymes were used for digestion of the amplified products. Restriction enzyme digestion was performed at 37°C (1 h) for BseGI and at 65°C (3 h) for BSeX I and at 65°C (2 h) for Mse I in water bath (Table 2). All digested products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

Statistical Analysis

Data analysis was performed using the Epi Info statistical software (version 6.2, World Health Organization, Geneva, Switzerland). Allele frequencies were estimated by direct gene counting. Data were analyzed using the X^2 test. The odds ratio and *P* value were calculated for each allele and genotype in the patient and control groups. Significance of any deviation from Hardy–Weinberg Equilibrium was tested in the control group for all three SNPs that did not show significant difference. *P* value of less than 0.05 was considered significant. In order to adjust multiple comparisons, Bonferroni correction method was utilized in computing of confidence intervals and rejection of tests in 0.05 level.

Results

Allele Frequencies

The frequency of the T allele at position −1147 of the *CTLA4* gene in the patients with AS was significantly higher than the controls (11% in the patients vs. 5% in the controls, $P=0.004$). However, the frequency of C allele in the patient group was significantly lower than the controls (89% vs. 95%, respectively, $P=0.004$). There were no significant differences on alleles' frequencies at other positions (Table 3).

Table 1 Primers and Amplicon Sizes Used in this Study

Name	Sequence	Amplicon size (bp)	T_m (°C)
CTLPF	5′ TCT TTT CCg CCT ATT TTC AgT T 3′	900	60°
CTLPR	5′ CCC T gg AAT ACA gAg CCA Gc 3′		64°
CTLA4PR1F	5′ gCT gAg gTg Tgg ACA Atg g 3′	481	60°
CTLA4PR1R	5′ TCA ggT gTT CTT AAA AgC CTT AAC 3′		60°

Table 2 Conditions for PCR Product Digestion with Restriction Enzymes

Amplicon	SNP	Enzyme	Enzyme concentration, U	Temperature and duration of digestion	Products of digestion visible on gel (bp)
481 bp	−1147 C/T	FokI (BseGI)	0.5	37°C, 1 h	C, 481 T, 446
900 bp	−318 C/T	Tru9I (Mse I)	2	65°C, 2 h	C, 626 T, 531
	+49 A/G	BbvI (BSeXI)	1	65°C, 3 h	A, 700 G, 500

Genotype Frequencies

Comparison of genotype frequencies at position −1147 of the CTLA4 gene showed that the frequency of CT genotype in comparison with other genotypes was overrepresented in the patient group (20% in the patients vs. 8% in the controls, P=0.012), while the CC genotype in comparison with other genotypes at the same position was decreased in the patient group (79% in the patients vs. 91% in the controls, P=0.012). However, these differences were not statistically significant when adjusted for Bonferroni multiple comparison correction.

There was no significant difference on frequencies of genotypes at the positions −318 and +49. A lower frequency of AA genotype at the position +49 was detected in the patient group (50% in the patients vs. 64% in the controls, P=0.039), which was not statistically significant after adjusting for Bonferroni multiple comparison correction (Table 3).

Discussion

Ankylosing spondylitis is an inflammatory autoimmune disease and its association with HLA system is very well described worldwide [1–4]. It is estimated that up to 95% of patients with AS can be HLA-B27-positive; however, only 2% of HLA-B27-positive individuals in the general populations could be affected by AS, which could suggest the role of other genetic–environmental factors in the pathogenesis of the disease [1, 3, 4, 16].

As a defect in the B7-CD28/CTLA-4 pathway could change T cell response and subsequently lead to auto immune diseases, single-nucleotide polymorphisms within

Table 3 Comparison of Alleles and Genotypes Frequencies of CTLA4 Gene Polymorphisms in the Patients with AS and Healthy Controls

Position	Allele	Genotype	Patients no. (%)	Controls No. (%)	P value	Odds ratio (95%CI)
−1147			N=150	N=103		
	C		226(88.7)	196(95.1)	0.004	0.34(0.15<OR<0.74)
	T		34(11.3)	10(4.9)	0.004	2.95(1.36<OR<6.56)
		CC	118(78.7)	94(91.2)	0.012	0.35(0.15<OR<0.82)
		CT	30(20)	8(7.8)	0.012	2.97(1.23<OR<7.40)
		TT	2(1.3)	1(0.9)	0.64	1.38(0.10<OR<38.92)
−318			N=153	N=103		
	C		285(93.1)	187(90.8)	0.41	1.38(0.69<OR<2.76)
	T		21(6.9)	19(9.2)	0.41	0.73(0.36<0R<1.45)
		CC	134(87.6)	87(84.4)	0.59	1.30(0.60<OR<2.81)
		CT	17(11.1)	13(12.6)	0.86	0.87(0.38<OR<2.00)
		TT	2(1.3)	3(2.9)	0.39	2.44(0.05<OR<3.31)
+49			N=157	N=103		
	A		217(69)	159(77.2)	0.055	0.66(0.43<OR<1.01)
	G		97(31)	47(22.8)	0.055	1.51(0.99<OR<2.31)
		AA	79(50.3)	66(64.1)	0.039	0.57(0.33<OR<0.98)
		AG	59(37.6)	27(26.2)	0.076	1.69(0.95<OR<3.03)
		GG	19(12.1)	10(9.7)	0.69	1.28(0.54<OR<3.11)

CTLA4 gene were investigated in this study. Although an association of certain *CTLA4* gene polymorphisms with several autoimmune diseases were previously described [10–12], the only study on 54 Korean patients with AS failed to show any significant association between the polymorphisms of the *CTLA4* (exon 1 +49 and promoter −318) and SA [17]. Our study was in agreement with that study, while no association was found between the polymorphisms of these regions of *CTLA4* gene and AS; the CC genotype was the most common genotype at promoter −318 in both groups of patients and controls, which is in agreement with a previous study [17]. At the exon 1 position +49 of the *CTLA4* gene, A allele is the wild-type and therefore AA genotype is the most common genotype in the Iranian normal population. However, the frequency of this genotype is less common in the Korean study [17]. G allele and GG genotype which could be associated with reduced surface expression and reduced soluble CTLA-4 were less common types in the patient group as well as normal population. In another study, which was recently performed in the USA in a group of patients with anterior uveitis, a condition strongly linked to HLA-B27, no association with polymorphisms of *CTLA4* −318 and +49 SNP was found in those patients with AS and anterior uveitis [18].

Although ethnic and geographical variations can change frequency of specific polymorphisms in different regions, the position +49 region of *CTLA4* gene could be studied in other regions with interest, while the polymorphisms of this exon region is linked to reduced expression of CTLA-4 on the T cell surface that could lead to reduced soluble CTLA-4 production and subsequently impaired inhibitory function [19].

To our best knowledge, this is the first time that the position −1147 of the *CTLA4* gene is investigated in the patients with AS. Although higher frequency of the CT genotype and lower frequency of CC genotype in the patient group was detected in our study, further studies with more patients are needed to evaluate possible contribution of such polymorphisms to the pathogenesis of AS. Moreover, as there is not enough evidence on functional importance of −1147 promoter region of CTLA4, further functional investigations are recommended.

This study could suggest that regardless of the strong association of AS with HLA-B27, other genetic factors could be considered in this autoimmune disease. CTLA4 gene polymorphisms could have role in the pathogenesis of disease, but further studies are required in different regions of world to elucidate different aspects of this hypothesis.

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