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Original Article

Association between *CTLA-4* gene polymorphism and ankylosing spondylitis: a case-control study

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Abstract: Aims: The aim of our study was to evaluate the association between *CTLA-4* polymorphisms (+49A/G, -318C/T and CT60A/G) and ankylosing spondylitis (AS) susceptibility. Methods: A total of 120 AS cases and healthy controls, matched on the age and gender, were enrolled in the study. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) were used to determine the gentypes of +49A/G, -318C/T and CT60A/G polymorphisms. Genotype distribution in control group was assessed by Hardy Weinberg Equilibrium (HWE) test. Odds ratio (OR) with 95% confidence interval (95% CI) were adopted to evaluate the relationship of *CTLA-4* polymorphisms and AS susceptibility. Results: In our study, genotype distribution of the three polymorphisms in control group was consistent with the HWE ($P > 0.05$). The genotype analysis showed that AA genotype of + 49A/G polymorphism could increase the risk for AS (OR=2.357, 95% CI=1.127-4.930). Moreover, the frequency of A allele was also presented as a risk factor for AS. Additionally, AA genotype and A allele of CT60A/G appeared to be related with AS susceptibility (OR=2.610, 95% CI=1.047-6.510; OR=1.751, 95% CI=1.160-2.641). However, the T allele of -318C/T appeared to be a protective factor for AS (OR=0.383, 95% CI=0.228-0.643). Conclusion: In summary, there existed significant association between *CTLA-4* gene polymorphisms and increased or decreased risk for AS.

Keywords: *CTLA-4,* ankylosing spondylitis, +49A/G, -318C/T, CT60A/G

Introduction

Ankylosing spondylitis (AS) is one of the most prevalent prototypical spondyloarthropathies. The disease is more prevalent in Whites population, morbidity of which is 0.1-1%, higher than other population [1-3]. Moreover, it has been demonstrated that the occurrence of AS is highest in northern European countries and lowest in sub-Saharan Africa [4, 5]. The probability to develop AS for individuals who are positive for HLA-B27 is approximately 1-2% and will up to 15-20% when they have a first-degree relative with AS [6, 7]. Although some treatments including medication, surgery and physical therapy have been used to relieve symptoms, there is still no effective therapy for AS [8, 9]. Therefore, it is urgent to find out high-risk population of AS and prevent the disease timely. Recently, it has been reported that specific cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) gene polymorphisms may play crucial role in the pathogenesis of AS.

CTLA-4, also known as cluster of differentiation 152 (CD152), is a negative regulator of T-cell immune response and is expressed primarily in activated regulatory T-cells [10-12]. Stimulating the *CTLA-4* receptor can turn off the attack of T cells, which are a type of lymphocyte (a type of white blood cell). Numerous autoimmune diseases have been reported to be associated with polymorphisms of *CTLA-4* gene. Polymorphisms within *CTLA-4* that was related with the down-regulation of *CTLA-4* could cause autoimmune T cell clonal proliferation and thus result in the occurrence of autoimmune diseases.

Until now, the studies have investigated the relationship of *CTLA-4* +49A/G polymorphism and primary biliary cirrhosis, 318C/T polymorphism and systemic sclerosis, and CT60A/G polymorphism and autoimmune thyroid disease [13-15]. However, there were few studies on the association of above polymorphisms and AS susceptibility.

Table 1. Primers sequences of *CTLA-4* polymorphisms

SNP locus	SNP site	SNP	Sequence
+49A/G	Exon1	rs231775	F: 5'-CCACGGCTTCCTTTCTCGTA-3'
			R: 5'-AGTCTCACTCACCTTTGCAG-3'
-318C/T	Promoter	rs5742909	F: 5'-AAATGAATTGGACTGGATGGT-3'
			R: 5'-TTACGAGAAAGGAGGCCGTG-3'
CT60A/G	3'-flanking region	rs3087243	F: 5'-CACCACTATTTGGGATAT ACC-3'
			R: 5'-AGGTCTTATTTTGAGGAAGGC-3'

The aim of our study was to assess the association of *CTLA-4* + 49A/G polymorphism, 318C/T polymorphism and CT60A/G polymorphism with AS risk.

Subjects and analysis

Subjects

The peripheral blood of AS patients was collected from department of spinal surgery, provincial hospital affiliated to Shandong University, and healthy controls were selected from blood donors. The AS group includes 120 patients with 54 men and 66 women and the average age were 22.4 years and 49.6 years, respectively. One hundred and twenty healthy people, 58 men and 62 women, who had never suffered from any rheumatological disease, were selected as the control group. Their average age was 26.7 and 47.7. Controls were matched with AS cases on the age and gender [16]. The subjects would be excluded if they were met the following items: high blood pressure, diabetes, hyperlipidemia, smoking and obesity. Written informed consent was obtained from all subjects. The experiment was supported by the Hospital's Ethics Committee.

DNA extraction and genotyping analysis

Genome DNA was isolated from peripheral blood samples by the conventional phenol-chloroform extraction method. *CTLA-4* polymorphisms were examined by polymerase ch-|ain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing analyses. Primers were designed by Primer 5.0 software, provided by Shanghai biological engineering technology service co., LTD. Primers sequences were listed in **Table 1**.

20 μL reaction solution contained 10 × PCR buffer 2.5 μL, $MgCl_2$ (25 mmol/L) 1.5 μL, dNTPs (2.5 mmol/L of each) 2.0 μL, each primer (20/μmol/L) 0.4 μL, DNA template 1.5 μL, Taq polymerase (5 U/μL0.2 μL (Nanjing Dulai Biol Technol Co., LTD), and double-distilled water 16.5 μL. Amplifications were performed in 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, 20 s at 95°C, 30 s at 58.7°C, 58.6°C, 58.5°C, 30 s extension at 72°C and a final 5 min extension at 72°C + 49A/G and CT60A/G polymorphism were genotyped by PCR-RFLP. The PCR products were digested by BbvI, and NcoI restriction enzyme (Boehringe, Germany). And the digestion was performed at 37.5°C for BbvI and 65°C for NcoI overnight. All digested products were detected by 20 g/L agarose gel electrophoresis with 80 v for 30 min, then stained with ethidium bromide and analyzed by gel imaging (GeneScan3.1 software, PE Biosystems). And the genotypes of -318C/T were determined by direct sequencing.

Statistical analysis

Odds ratio (OR) and 95% confidence interval (CI), calculated with X^2 test, were employed to analyze the association of genotype or allele and AS susceptibility. For control group, the observed genotype frequencies of the three polymorphisms were assessed with Hardy-Weinberg equilibrium (HWE) by Pearson X^2 test. *P* value less than 0.05 was considered as significant. All the analysis was conducted in SPSS 18.0 software.

Results

The electrophoresis results of enzyme digestion and sequence for three polymorphisms of CTLA-4

For +49A/G, the digested results showed that homozygous AA was corresponded with 328 bp fragment, heterozygous AG with 328 bp, 244 bp and 84 bp fragments and homozygous GG with 244 bp and 84 bp fragments. For CT60A/G, we found that homozygous GG genotype were presented with 216 bp bands, heterozygous AG individuals with 26 bp, 216 bp and 196 bp bands and homozygous AA with 26 bp and 196 bp bands. In addition, -318C/T polymorphism produced three genotypes of CC, CT and TT.

Table 2. Alleles and genotypes distribution of *CTLA-4* polymorphisms in case and control groups

Genotype/Allele	Case (n=120), n (%)	Control (n=120), n (%)	χ^2	*P* value	OR (95% CI)
Genotype					
49A/G					
GG	28 (23.3)	44 (36.7)	-	-	1
AG	62 (51.7)	56 (46.7)	3.344	0.067	1.740 (0.959-3.157)
AA	30 (25.0)	20 (16.6)	5.273	0.022	2.357 (1.127-4.930)
318C/T					
CC	98 (81.7)	70 (58.3)	-	-	1
CT	20 (16.7)	46 (38.3)	14.893	0.000	0.311 (0.169-0.570)
TT	2 (1.6)	4 (3.4)	1.481	0.224	0.357 (0.064-2.004)
CT60A/G					
AA	77 (64.2)	59 (49.2)	4.441	0.035	2.610 (1.047-6.510)
AG	35 (29.2)	45 (37.5)	0.826	0.363	1.556 (0.598-4.050)
GG	8 (6.6)	16 (13.3)	-	-	1
Allele					
49A/G					
G	118 (49.2)	144 (60.0)	-	-	1
A	122 (50.8)	96 (40.0)	5.681	0.017	1.551 (1.080-2.226)
318C/T					
C	216(90.0)	186(77.5)	-	-	1
T	24(10.0)	54(22.5)	13.777	0.000	0.383(0.228-0.643)
CT60A/G					
G	51 (21.3)	77 (32.1)	-	-	1
A	189 (78.7)	163 (67.9)	7.202	0.007	1.751 (1.160-2.641)

Relationship of CTLA-4 polymorphisms (+49A/G, 318C/T and CT60A/G) with risk for AS

As shown in **Table 2**, Genotype distribution of the three polymorphisms in control group were consistent with the HWE (P>0.05). And our study showed that AA genotype of +49A/G polymorphism could increase the risk for AS (OR=2.357, 95% CI=1.127-4.930). Moreover, the frequency of A allele was higher in case group (20.8% vs. 40.0%), which was presented as a risk factor for AS. Additionally, AA genotype of CT60A/G appeared to be related with AS susceptibility (OR=2.610, 95% CI=1.047-6.510). Further analysis suggested that A allele of CT60A/G also showed strong effects on the occurrence of AS (OR=1.751, 95% CI=1.160-2.641). However, the T allele of -318C/T could inhibit the occurrence of AS (OR=0.383, 95% CI=0.228-0.643).

Discussion

Common genetics (human leukocyte antigen [*HLA*] class-I gene, HLA-B27) and common pathology (enthesitis) are related to the spondyloarthropathies. AS is the main disease linked with *HLA* gene (1973) and the direct relationship of AS with *HLA-B27* gene has been determined [17-20], which suggests that genes play crucial role in the pathogenesis of AS.

The effects of *CTLA-4* +49A/G, -318C/T and CT60A/G polymorphisms on other diseases have been extensively reported [21-25]. The study of CHEN et al. indicated that there existed an obvious association between PBC and *CTLA-4* +49A/G polymorphism [13]. For Italian population, a relationship between -318C/T polymorphism with risk of systemic sclerosis has been found in the study of Balbi et al [14]. Moreover, according to Bicek et al's study, CT60A/G polymorphism was related with GD susceptibility, but not with two other types of autoimmune thyroid disease (AITD) (HT and PPT) [15]. In view of the above-mentioned studies, we concluded that *CTLA-4* polymorphisms might serve as risk factor for AS, so the relationship of *CTLA-4* and AS risk was investigated. Our study showed that + 49A/G and

CT60A/G polymorphisms were both genetic-susceptibility factors for AS. While, -318C/T polymorphism was related with decreased risk for AS.

The fact is that the studies about the association of gene polymorphism and disease risk commonly focus on the single SNP. To explore the precise relationship of *CTLA-4* polymorphism, we studied three SNPs located in exon1, promoter and 3'-flanking region of *CTLA-4*, which makes our results much more comprehensive and accuracy. In conclusion, there was significant correlation between *CTLA-4* polymorphisms and AS susceptibility. Further studies with consideration of gene-gene, gene-environment interaction are needed to confirm the results.

Disclosure of conflict of interest

None.

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