Association of polymorphisms in the programmed cell death 1 (PD-1) and PD-1 ligand genes with ankylosing spondylitis in a Chinese population

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Abstract

Objectives

To investigate the association of the polymorphisms and haplotypes in the PD-1 and PD-1 ligand genes with ankylosing spondylitis (AS) in a Chinese population.

Methods

A total of 196 Chinese patients with ankylosing spondylitis and 180 age- and sex-matched controls of the same ethnic origin were included in the study. The polymorphisms in PD-1 and PD-1 ligand genes were genotyped using polymerase chain reaction-restriction fragment length polymorphism, allele-specific PCR and fluorescence melting curve methods.

Results

The T allele of the PD-1 rs2227982 polymorphism was more frequent in the patient group than in the controls (28.53% vs. 17.22%; p=0.000). The frequency of the CT haplotype (PD-1 rs2227981 C allele and PD-1 rs2227982 T allele) was higher in the AS patient group comparing with the controls (odds ratio (OR)=1.845, 95% confidence interval 1.220-2.789). The C allele of the PD-L1 rs822336 polymorphism was also more frequent in the patients than in the controls (34.69% vs. 27.22%; p=0.007). However, we did not find any evidence of genetic association between PD-L2 rs1009759/rs6476985 polymorphisms and AS (p=0.371 and 0.061, respectively). No significant difference in the distribution of the PD-1 rs2227981 alleles was found in our population when comparing AS patients with control groups (p=0.226).

Conclusions

The results support a major role for PD-1/PD-L1 polymorphisms in disease susceptibility of AS.

Key words

programmed cell death 1, ankylosing spondylitis, polymorphism, genotyping, case-control study

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Introduction

Ankylosing spondylitis (AS) is the second most common form of inflammatory arthritis worldwide with an incidence ranging from 0.5% to 1.0% (1, 2). Genetic factors appear to account for the majority of the susceptibility to AS (3). Family studies indicate that HLA-B27 on chromosome 6 contributes only 16% to 50% of the total genetic risk for the disease (3-5), but on other chromosomes, many interesting areas that may contain additional disease predisposing genes have been identified. (4, 5). To our knowledge, only HLA-B60 has been shown to be attributable so far (6, 7), other predisposing genes therefore need to be identified.

PD-1 is a 55-kDa transmembrane protein with one extracellular IgV-like domain and a 97-amino acid cytoplasmic tail containing one immunotyrosine inhibitory motif (ITIM) and one immunotyrosine switch motif (8). PD-1 is expressed in activated T-cells, B-cells, and myeloid cells. PD-1 conducts negative signals to T-cells upon interaction with its ligands, programmed death-1 ligands (PD-L). PD-1–PD-L interactions lead to cell cycle arrest in G0/G1 but do not increase cell death (9).The PD-1 gene is located on chromosome 2q37. Many studies showed the association between the PD-1 gene polymorphisms and the development of SLE (10) and rheumatoid arthritis (11, 12). However, the results of these reports are controversial.

Both PD-L1 and PD-L2 are located in a 120 kilobasepairs (kb) segment on chromosome 9p24. PD-L1 is 18 kb in size with seven exons, whereas PD-L2 is located 42 kb downstream of PD-L1 and has equally many exons, but stretches up to 60 kb. There is only one genetic study of SLE and the PD-1 ligands that showed lack of association between them (13).

The purpose of the present study is to investigate the associations between the PD-1 and PD-L gene polymorphisms and the susceptibility to AS in a Chinese population.

Patients and methods

Subjects and protocol

A total of 196 AS patients (including 178 males and 18 females) meeting the American College of Rheumatology modified New York Criteria for AS were enrolled in the study. Patients were recruited from the department of rheumatology and immunology, Shandong Provincial Hospital. The diagnosis of AS was established according to the clinical information collected at the same time. The control group was made up of 180 normal individuals who underwent a health examination in the health Center of Shandong Provincial Hospital. They had no previous medical history and no abnormal laboratory results. All the subjects and controls were of Chinese origin and were matched for age and sex. Samples were obtained from subjects after they provided written informed consent. This study was approved by the Ethics Review Committee of Shandong Provincial Hospital.

Genotyping of PD-1 rs2227981

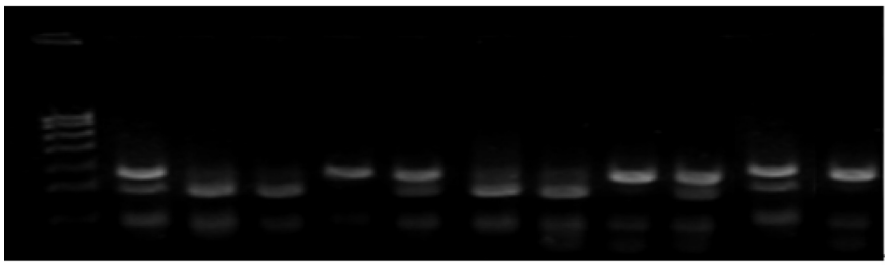
Genomic DNA was extracted from peripheral blood leukocytes using TIANGEN Genomic DNA Purification Kit according to the manufacturer's instructions. PCR amplification was performed with 50 ng of the genomic DNA in a 25 μl reaction volume containing 10 pmol of sense primer in 0.5μl, 10 pmol of antisense primer in 0.5 μl,and a 2×reaction buffer 12.5μl provided by the manufacturer. PCR was performed in a Gene Amp PCR System 9700 as follows: 30 cycles consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C and 1 min of extension at 72°C with an initial denaturation step of 5 min at 94°C and a final extension of 30 min at 72°C. The subsequent restriction-fragment-length polymorphism (RFLP) analysis was performed to determine the PD-rs2227981 SNPs using Alu I (Table I). Figure 1 shows the agarose gel electrophoresis results after RFLP.

Genotyping of PD-1 rs2227982, PD-L1 rs822336, PD-L2 rs6476985 and PD-L2 rs1009759

Genomic DNA was extracted from peripheral blood leukocytes using TIANGEN Genomic DNA Purification Kit according to the manufacturer's instructions. An ABI 7900 instrument

Table I. PCR primers and restriction enzymes (RE).

SNP	PCR primers	RE
PD-1 rs2227981	Forward primer 5-AGACGGAGTATGCCACCATT-3 Reverse primer 5-CACTGTGGGCATTGAGACAT-3	AluI
rs2227982	Allele-specific primer 5-GCGGGCAGGGCGGCCACAGAGAACACAGGCATGG-3 5-GCGGGCCACAGAGAACACAGGCATGA-3 common primer 5-AGCCCCTGAAGGAGGACCCCT-3	
PD-L1 rs822336	Allele-specific primer 5-GCGGGCAGGGCGGCAGGGTATTTGCTCAGCTCGC-3 5-GCGGGCAGGGTATTTGCTCAGCTCGG-3 common primer 5-CCTCACATTACTAATACGCAAATC-3	
PD-L2 rs6476985	Allele-specific primer 5-GCGGGCAGGGCGGCAAAACCTTCCGATGGCTTAG-3 5-GCGGGCAAAACCTTCCGATGGCTTAA-3 common primer 5-CTGGGTTTTACACTGAGTGAAA-3	
rs1009759	Allele-specific primer 5-GCGGGCAGGGCGGCGAATAGGGCACAAATTAACTCGG-3 5-GCGGGCGAATAGGGCACAAATTAACTCGA-3 common primer 5-TCACCTACAGGGAAAT GAAAGGGT-3	



1: Marker Control group: 2: TC, 3: TT, 4: TT, 5: CC, 6: TC
Patient group: 7: TT, 8: TT, 9: CC, 10: TC, 11: TC, 12: CC

Fig. 1. Gel electrophoresis patterns of PD-1 rs2227981.

was used for PCR amplification and fluorescence melting curve analysis. The PCR volume was 5μL containing 50 ng of the genomic DNA,0.02μl each of the three primers, SYBR Green PCR Master Mix 2.5 μl ,and double distilled water 2μl (Table I). PCR was performed in standard 96-well plates, sealed with optical adhesive covers. After amplification, the fluorescence intensity of the PCR product was measured from 60°C to 90°C at a temperature gradient of 0.5°C/min. The ABI 7900 automatically calculates the negative derivative of the change in fluorescence. When graphed, this yields a peak at the Tm of the PCR product.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows version 13.0. Genotypic and allelic frequencies were obtained by direct counting. Statistical analysis to compare distributions was performed by the χ^2 test. Odds ratios (ORs), *p*-value and 95% confidence intervals (CIs) were calculated with respect to the minor allele compared with the major allele. A *p*-value of less than 0.05 was considered significant. Bonferroni correction was used to correct the multiple test. The SHEsis program was used for calculating estimated haplotype frequencies.

Results

The allele frequencies of five SNPs are shown in Tables II and III. The distributions of the genotypes of all SNPs were in Hardy-Weinberg equilibrium and did not differ between the control and patients groups.

PD-1 rs2227981 in AS patients and in controls

One hundred and ninety-six patients and 180 controls were genotyped for PD-1rs2227981 C/T SNPs. The frequencies of C/C, C/T and T/T genotypes were 41.84%, 49.98% and 9.18% among the AS patients; 50.8%, 42.22% and 7.78% among the controls, respectively. The allele frequencies of C and T were 66.33% and 33.67% in the patient group; 71.11% and 28.89% in the controls. There was no significant difference between these two groups for PD-1rs2227981 polymorphism (Table II).

PD-1 rs2227982 in AS patients and in controls

Among the AS patient group, the frequencies of C/C, C/T and T/T genotypes were 9.96%, 36.22% and 54.08% respectively; and in the controls the frequencies were 5%, 24.44% and 70.56% respectively. The allele frequencies of C and T were 28.53% and 72.19% in the patient group; 17.22% and 82.78% in the controls. There was a significant difference between the two groups, with an OR of 1.213 according to the χ^2 test and the 95% CI from 0.833 to 1.767 (Table II).

Then, we evaluated the association between PD-1 genotypes and AS with the effect of the allele T for rs2227981 C>T and rs2227982 C>T assumed to be dominant (CC *vs*. TT and CT) or recessive (TT *vs*. CT and CC). As shown in Table IV, for rs2227982 C>T, the carriers of the T allele (the TT homozygote and the CT heterozygote) showed a significantly higher risk of AS compared with the CC homozygotes under the dominant model (OR=0.492, 95% CI: 0.321-0.753, *p*=0.001).

Haplotype analysis of PD-1rs2227981C/T and PD-1rs2227982T/C

The frequencies of the CT, CC, TT and TC haplotype between the patient

Table II. Genotyping and allele frequency and haplotype of PD-1rs2227981 and PD-1rs2227982 in patients and controls.

SNP	Alleles	Patients (%)	Controls (%)	*p*-value	*p*-value correction	OR (95% CI)
PD-1 rs2227981	CC	82 (41.84)	90 (50)			
	CT	96 (49.98)	76 (42.22)			
	TT	18 (9.18)	14 (7.78)	0.283	0.566	
	C	260 (66.33)	256 (71.11)			
	T	132 (33.67)	104 (28.89)	0.226	0.452	0.825(0.605–1.126)
PD-1rs2227982	CC	106 (54.08)	127 (70.56)			
	CT	71 (36.22)	44 (24.44)			
	TT	19 (9.69)	9 (5)	0.004	0.008	
	C	283 (72.19)	298 (82.78)			
	T	109 (28.53)	62 (17.22)	0.000	0.000	1.213(0.833–1.767)
PD-1 rs2227981/ rs2227982 haplotype	TC	98 (25.1)	82 (23.3)			
	TT	34 (8.6)	19 (5.3)			
	CC	185 (47.1)	211 (59.9)	0.001		0.597(0.446–0.798)
	CT	75 (19.2)	40 (11.4)	0.003		1.845(1.220–2.789)

Table III. Genotyping and allele frequency and haplotype of PD-L1 rs822336, PD-L2 rs6476985 and PD-L2rs1009759 in patients and controls.

SNP	Alleles	Patients (%)	Controls (%)	*p*-value	*p*-value correction	OR (95% CI)
PD-L1 rs822336	CC	19 (9.96)	18 (10)			
	CG	98 (48.98)	62 (34.44)			
	GG	79 (40.31)	100 (55.56)	0.007		
	C	136 (34.69)	98 (27.22)			
	G	256 (65.310	262 (72.78)	0.027	0.054	1.42(1.040–1.940)
PD-L2 rs6476985	CC	48 (24.49)	62 (34.44)			
	CT	95 (48.47)	76 (42.22)			
	TT	53 (27.04)	42 (23.33)	0.106		
	C	191 (48.72)	200 (55.56)			
	T	201 (51.28)	160 (44.44)	0.061		0.76(0.570–1.013)
PD-L2 rs1009759	CC	86 (43.88)	72 (40)			
	CT	81 (41.33)	77 (42.78)			
	TT	29 (14.80)	31 (17.22)	0.695		
	C	253 (64.54)	221 (61.39)			
	T	139 (35.46)	139 (38.61)	0.371		1.145(0.851–1.540)

group and the controls were 19.2% *vs*. 11.4% for CT; 47.1% *vs*. 59.9% for CC, 8.6% *vs*. 5.5% for TT and 25.1% *vs*. 23.3% for TC. The frequency of the CT haplotype was significantly higher in the AS patients than in the controls (p=0.003, OR 1.845; 95% CI 1.220 to 2.789, Table II).

PD-L1 rs822336.PD-L2 rs6476985 and PD-L2 rs1009759 polymorphisms in AS patients and in controls

For PD-L1 rs822336, the frequencies of C/C, C/G and G/G genotypes among the AS patients and the controls were 9.69% *vs*. 10%; 48.98% *vs*. 34.44%; and 40.31% *vs*. 55.56% respectively. Among the 196 AS patients, the allele frequencies of C and G between these two groups were 34.69% *vs*. 27.22% for allele C, and 65.31% *vs*. 72.78% for allele G. A significant difference was found between the two groups (p= 0.027), and OR was 1.42 according to the χ^2 test with the 95% CI from 1.040 to 1.940, (Table III).

For PD-L2 rs6476985 polymorphism between the two groups, the frequencies of C/C, C/T and T/T genotypes were 24.49% *vs*. 34.44%, 48.47% *vs*. 42.22% and 27.04% *vs*. 23.33% respectively (p=0.106). The allele frequencies of C and T were 48.72% *vs*. 55.56% and 51.28% *vs*. 44.44% comparing the patient group with the controls (p=0.061) (Table III).

The frequencies of C/C, C/T and T/T genotypes of PD-L2 rs1009759 between the AS group and the control group were 43.88% *vs*. 40%; 41.33% *vs*. 42.78%; and 14.8% *vs*. 17.22% respectively (p=0.695). And the allele frequencies of C and T between two groups were 64.54% *vs*. 61.39% and 35.47% *vs*. 38.61% respectively (p=0.371) (Table III). No significant differences were detected for PD-L2 polymorphisms.

Then, we evaluated the association between PD-L1 and PD-L2 genotypes and AS with the effect of the allele C for rs822336 G>C assumed to be dominant (CC and CG *vs*. GG) or recessive (CC *vs*. CG and GG). Similarly, for rs6476985 T>C, the effect of the allele C was investigated according to a dominant (CC and CT *vs*. TT) or recessive (CC *vs*. TT and CT) model of inheritance, for rs1009759 C>T, the effect of the allele T was investigated according to a dominant (CT and TT *vs*. CC) or recessive (TT *vs*. CC and CT) model of inheritance.

As shown in Table IV, for rs822336 G>C, the carriers of the C allele (the CC homozygote and the CG heterozygote) showed a significantly higher risk of AS compared with the GG homozygotes under the dominant model (OR=1.851, 95% CI: 1.229-2.789, p=0.003). For rs6476985 T>C, the carriers of the T allele (the TT homozygote and the CT heterozygote) showed a significantly higher risk of AS compared with the TT homozygotes under the recessive model (OR=0.617, 95% CI: 0.394-0.966, p=0.034).

Haplotype analysis of PD-L2 rs6476985C/T and PD-L2 rs1009759

When comparing the AS patients with the controls, the frequencies of the CC, CT, TC and TT haplotype were: 26.6% *vs*. 30.1%; 24.1% *vs*. 23%; 23.4% *vs*. 29.1%; and 25.9% *vs*. 1.8% respectively. No significant difference was detected (Table III).

Discussion

The pathogenesis of AS is still not completely understood so far (14), although there have been some genetic studies in different populations (15-19), we are the first to study the association of polymorphisms in the programmed cell

Table IV. The analysis of inheritance mode for the genotypes of SNPs between the AS patients and controls.

	Mode of inheritance	Genotype	Cases	Controls	*p*-value	OR (95%CI)
PD-1 rs2227981	Recessive	CC+CT	178	166		
		TT	18	14	0.626	0.834 (0.402–1.730)
	Dominant	CC	82	90		
		CT+TT	114	90	0.112	0.719 (0.497–1.081)
PD-1rs2227982	Recessive	CC+CT	177	171		
		TT	19	9	0.083	0.490 (0.216–1.114)
	Dominant	CC	106	127		
		CT+TT	90	53	0.001	0.492 (0.321–0.753)
PD-L1 rs822336	Recessive	CC	19	18		
		GG +CG	177	162	0.921	0.966 (0.490–1.905)
	Dominant	CC +CG	117	80		
		GG	79	100	0.003	1.851 (1.229–2.789)
PD-L2 rs6476985	Recessive	CC	48	62		
		TT+CT	148	118	0.034	0.617 (0.394–0.966)
	Dominant	CC +CT	143	138		
		TT	53	42	0.409	0.821 (0.514–1.311)
PD-L2 rs1009759	Recessive	TT	29	31		
		CC+CT	167	149	0.521	0.835 (0.480–1.450)
	Dominant	CT+TT	110	108		
		CC	86	72	0.447	0.853 (0.566–1.286)

death 1 (PD-1) and PD-1 ligand genes with AS in a Chinese population.

PD-1 gene was isolated in 1992 by subtractive hybridisation technique as a molecule whose expression was enhanced by apoptotic stimuli in two different cell lines (20). The human PD-1 gene is located on chromosome 2q37.3 and composed of 5 exons, the gene product is a 288-amino acid, 55-kDa type I transmembrane protein with a single IgV domain in the extracellular region. The cytoplasmic N terminus region of PD-1 protein contains ITIM, which is essential for the inhibitory function of PD-1 (21-23). Some studies showed that its C terminus tyrosine residues inhibited lymphocyte activation by binding to downstream signal transduction molecules like SHP1 and SHP2 (9, 24). PD-1 has two ligands, PD-L1 and PD-L2. The PD-1 ligand/PD-1 pathway might deliver a negative signal for T cell activation and block the CD28/B7-2 pathway (25, 26).

Many studies focused on the role of the PD-1/PD-L pathway in autoimmune diseases. Studies on animal model show that PD-1 gene knockout mice tend to develop autoimmune diseases such as autoimmune dilated cardiomyopathy, lupous glomerulonephritis, rheumatoid arthritis and type 1 diabetes (27-29). PD-1 gene deficiency might be the cause of graft-*versus*-host diseases. Nishimura *et al*. (28) found that in TCR(2C-TCR) transgenic mice, when PD-1 gene was knocked out, 17 out of 69 2C×PD-$1^{-/-}$ H-$2^{b/d}$ mice died of a graft-*versus*-host-like disease in 10 weeks and others exhibited various degrees of systemic damage while 2C-TCR-PD-$1^{+/+}$ mice were healthy.

Prokunina and colleagues found PD-1.3A correlated with SLE in European and Mexican people (RR=2.6 and 3.5 respectively). They found PD-1.3A could reduce PD-1 transcription, thus impair immune tolerance and induce autoimmune response (10). Some studies suggested that PD-1.3A might be associated with lupus nephritis (30), but others showed controversial results (31, 32). Wang *et al*. who studied the correlation between PD-1 and SLE in Han Chinese population hypothesized that 7872-T/T and 8162-G/G might have a prevention value for SLE (33). For other auto-immune diseases, Nielsen et al found PD-1 associated with type 1 diabetes (34), while Fawwaz's study failed to show this (35). Few studies focused on the association between PD-L1, PD-L2 and auto-immune diseases (12).

In this study, we investigate the association of the PD-1 and PD-1 ligands (PD-L1 and PD-L2) gene polymorphism and the haplotypes with AS in a Chinese population. Our data showed that the frequency of PD-1 rs2227982-T allele was significantly higher in AS patients than in healthy controls. No statistical difference for PD-1 rs2227981C/T was observed between the two groups. Haploid analysis showed PD-1 rs2227981/ rs2227981 CT haploid was significantly higher in AS group than in control group.

In four previous genomewide screening studies (16, 36-38), three of them showed that chromosome 2 was likely to contain predisposing genes for AS. Some researchers have studied the IL-l gene cluster located on 2q13 in AS (17, 39). Our data showed PD-1 that located on chromosome 2 was involved in the immunopathogenesis of AS. PD-1 rs2227982T is located in the exon area, and codes the mutation from Val to Ala, It needs further study to elucidate the effect on protein structural and functional changes.

Some studies showed the binding sites of PD-L1 and PD-L2 for PD-1 were different, PD-L1 and PD-L2 pathway might have different biological influence. By comparing the distribution of PD-L1 rs822336, PD-L2 rs6476985 and rs1009759 between AS patients and healthy controls, we found the frequency of PD-L1 rs822336 C allele was significantly higher in AS patients than in controls. It implies that PD-1/PD-L1 pathway, but not PD-1/PD-L2 pathway, may play an important role in the pathogenesis of AS.

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