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Concise report

Effects of genetic polymorphisms of programmed cell death 1 and its ligands on the development of ankylosing spondylitis

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Abstract

Objectives. There is a known association of imbalanced peripheral tolerance and autoimmune diseases. The binding of programmed cell death 1 (PD-1) with its ligands 1 and 2 (PD-L1 and PD-L2) inhibits T-cell proliferation through a negative signal via recruitment of src homology 2-domain-containing tyrosine phosphatase 2. Therefore we evaluated the effect of the *PD-1*, *PD-L1* and *PD-L2* genotypes on the occurrence of AS in a population of Taiwanese patients.

Methods. Genetic polymorphisms of *PD-1* G-536A, *PD-L1* A8923C and *PD-L2* C47103T were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for 330 AS patients and 330 healthy controls who were matched by age and gender.

Results. Subjects with the *PD-1* GG genotype [matched relative risk (RR_m) 1.78; 95% CI 1.13, 2.81] and the GA genotype (RR_m 1.59; 95% CI 1.09, 2.31) had significantly greater risk for AS than those with the AA genotype. Subjects with the *PD-L2* CT genotype had lower risk for AS than those with the CC genotype (RR_m 0.01; 95% CI 0.001, 0.06). Interestingly, the combined genotypes of *PD-1* G-536A, *PD-L1* A8923C and *PD-L2* C47103T also appear to be associated with AS development.

Conclusions. Our results suggest that *PD-1* G-536A, *PD-L1* A8923C and *PD-L2* C47103T polymorphisms are associated with the presence of AS.

Key words: Ankylosing spondylitis, Programmed cell death 1, Programmed cell death 1 ligands, Polymorphism.

BASIC SCIENCE

Introduction

AS is an autoimmune disease with a significant genetic basis [1]. Research has established an association between the *HLA-B27* gene and AS [2], but the *HLA-B27* may only account for 16% of the genetic variation of AS [3]. An imbalance of peripheral tolerance is associated with numerous autoimmune diseases [4]. Circulating $CD4^+$ and $CD8^+$ T cells are more highly expressed in AS patients than in healthy subjects [5, 6], so an imbalance of peripheral tolerance may also be associated with AS.

Importantly, the negative signal of activated T cells has a crucial role in the balance of peripheral tolerance [4]. The interaction of the T-cell receptor with the MHC on the surface of antigen-presenting cells (APCs) regulates the activation of T cells [7]. T-cell activation also requires a co-stimulatory signal involving CD28 and CD40 with B7-1 (CD80) and B7-2 (CD86) on the APC [8]. Programmed cell death 1 (PD-1), a membrane protein with an immunoreceptor tyrosine-based inhibitory motif, is induced in lymphocytes and monocytes following activation [7]. Binding of PD-1 with its ligands 1 and 2 (PD-L1 and PD-L2) inhibits T-cell proliferation and CD28-mediated co-stimulation through a negative signal by recruitment

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of src homology 2-domain-containing tyrosine phosphatase 2 [9–11]. A previous study indicated that C57BL/6-PD-1(−/−) mice spontaneously developed lupus-like proliferative arthritis and glomerulonephritis (GN), suggesting that PD-1 is involved in the maintenance of peripheral tolerance [12]. Other *in vivo* studies reported that blockage of PD-L1 and PD-L2 led to diabetes [13] and experimental autoimmune encephalomyelitis [14]. Therefore, blocking the interaction of PD-1 and its ligands may be associated with AS.

The human *PD-1* gene is located on chromosome 2q37 [15]. A single nucleotide polymorphism (SNP) in the promoter region (−536) was identified as G to A (rs36084323) [16], and this SNP, located in the transcription start site, is associated with the function of *PD-1*. The *PD-1* G-536A polymorphism is also associated with the development of RA [17] and type 1 diabetes mellitus [18]. The *PD-L1* and *PD-L2* genes are both located on chromosome 9p24 [18]. The *PD-L1* A8923C (rs1970000) polymorphism is near or within the transcriptional factor binding sites [19], and may influence the binding of transcriptional factors. In addition, the C47103T (rs7854303) genotype in exon 5 of the *PD-L2* gene changes the amino acid of the transmembrane domain from serine to phenylalanine [20]. However, the associations of the *PD-1*, *PD-L1* and *PD-L2* genes with AS development are unclear. In this case–control study, we assess the association of the *PD-1*, *PD-L1* and *PD-L2* genotypes with AS development.

Materials and methods

Patients and controls

A total of 330 AS patients were recruited from the Arthritis clinic of Chung Shan Medical University Hospital (Taichung, Taiwan). Qualified rheumatologists confirmed diagnoses based on the modified New York criteria [21]. All patients were at least 18 years old and gave informed consent. Physicians recorded their clinical histories, including age at initial symptoms, family history of AS and extraspinal manifestations. Age at time of initial symptoms was defined as the time when the first symptom, whether it was an axial symptom, peripheral arthritis, uveitis or enthesitis, developed. Delayed diagnosis was defined as the interval between the onset of the first symptom and the correct diagnosis of AS. A 1 : 1 ratio of case to control subjects was used. The controls, who were matched for age (±5 years) and gender with the AS patients, were randomly selected from sequentially admitted patients and had no rheumatic or autoimmune symptoms. The study conformed to the Declaration of Helsinki. The study was approved by the institutional review board of Chung Shan Medical University Hospital.

Genetic polymorphisms

The *PD-1* G-536A [16], *PD-L1* A8923C [19] and *PD-L2* C47103T [20] genotypes were identified in all subjects based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primers used for amplification of the *PD-1* G-536A genotype were 5′-GAT CTG GAA CTG TGG CCA TG-3′ and 5′-GGC TGC CCA CAG CCT CT-3′. Primers used for amplification of the *PD-L1* A8923C genotype were 5′-AAT GGC TTG TTG TCC AGA GAT G-3′ and 5′-GTA CCA CAT GGA GTG GCT GC-3′. Primers used for amplification of the *PD-L2* C47103T genotype were 5′-GCT TCA CAT TTT CAT CCC AT-3′ and 5′-AGT GGC TCA TGC TGC AGA C-3′. Direct sequencing of 10% of all subjects was used to validate the PCR-RFLP results.

Statistical analysis

Hardy–Weinberg equilibrium was performed to test *PD-1* G-536A, *PD-L1* A8923C and *PD-L2* C47103T genotypes for goodness of fit. Chi-squared-test was used to compare the prevalence of *PD-1*, *PD-L1* and *PD-L2* genotypes and alleles in the case and control groups. The conditional logistic regression model was used to calculate the matched relative risks (RR_m) and 95% CIs in AS development for *PD-1*, *PD-L1* and *PD-L2* genotypes and the combined effects of these genotypes. All *P*-values were calculated using two-tailed statistical tests. SAS 9.1 (SAS Institute, Cary, NC, USA) for Windows was used for all statistical analysis.

Results

A total of 330 AS patients and 330 matched controls were recruited. The mean (S.E.) age of patients and controls was 43.8 (0.6) and 44.5 (0.6) years, respectively, and 69.4% subjects were male in both groups. Among AS patients, age on initial symptom was 31.8 (0.7) years, disease duration was 12.0 (0.6) years and delayed diagnosis was 64.7 (5.1) months.

In our controls, *PD-1* G-536A ($P = 0.06$) and *PD-L1* A8923C ($P = 0.81$) polymorphisms were in Hardy–Weinberg equilibrium. The *PD-L2* C47103T polymorphism was not identified in our AS patients. Compared with subjects with the *PD-1* AA genotype, those with *PD-1* GG and GA genotypes had a 1.78-fold (95% CI 1.13, 2.81) and a 1.59-fold (95% CI 1.09, 2.31) increased risk for AS, respectively (Table 1). Similarly, those with the G allele of the *PD-1* G-536A genotype had a significantly greater risk of AS than those with the A allele (RR_m 1.37; 95% CI 1.09, 1.73). Those with the *PD-L1* CC genotype also had a higher risk for AS than those with the AA genotype (RR_m 3.00; 95% CI 0.77, 11.74). In addition, all AS patients had the *PD-L2* C47103T C allele; the *PD-L2* TT genotype was not present in the controls. Interestingly, compared with subjects with the *PD-L2* CC genotype, *PD-L2* CT carriers had a significantly decreased risk of AS (RR_m 0.01; 95% CI 0.001, 0.06).

Subsequently we evaluated the combined effects of *PD-1*, *PD-L1* and *PD-L2* genotypes on AS. Our results indicate that the subjects with the *PD-1* GG and GA genotypes had an increased risk of AS. Further, we combined subjects with the *PD-1* GG and GA genotypes and compared them with subjects who had the *PD-1* AA genotype. In addition, subjects with the *PD-L1* A8923C C allele have poorer transcriptional activity than those with the *PD-L1*

Table 1 Frequency of *PD-1*, *PD-L1* and *PD-L2* polymorphisms in AS patients and normal controls

Characteristic	AS patients, *n* (%) (*n* = 330)	Normal controls, *n* (%) (*n* = 330)	RR_m (95% CI)	*P*-value
PD-1 G-536A genotype				
GG	81 (24.5)	64 (19.4)	1.78 (1.13, 2.81)	0.013
GA	163 (49.4)	143 (43.3)	1.59 (1.09, 2.31)	0.016
AA	86 (26.1)	123 (37.3)	1.00 (reference)	
G	325 (49.2)	271 (41.1)	1.37 (1.09, 1.73)	0.007
A	335 (50.8)	389 (58.9)	1.00 (reference)	
PD-L1 A8923C *g*enotype				
CC	8 (2.4)	3 (0.9)	3.00 (0.77, 11.74)	0.113
AC	58 (17.6)	61 (18.5)	0.95 (0.62, 1.46)	0.828
AA	264 (80.0)	266 (80.6)	1.00 (reference)	
C	74 (11.2)	67 (10.2)	1.15 (0.80, 1.67)	0.455
A	586 (88.8)	593 (89.8)	1.00 (reference)	
PD-L2 C47103T *g*enotype				
TT	0 (0.0)	0 (0.0)	–	–
CT	0 (0.0)	86 (26.1)	0.01 (0.001, 0.06)	<0.001
CC	330 (100)	244 (73.9)	1.00 (reference)	
T	0 (0.0)	86 (13.0)	0.01 (0.001, 0.07)	<0.001
C	660 (100)	574 (87.0)	1.00 (reference)	
PD-1/*PD-L1*/*PD-L2* genotypes				
GG + GA /CC /CC	6 (1.8)	2 (0.6)	6.63 (1.28, 34.46)	0.025
AA /CC /CC	2 (0.6)	1 (0.3)	4.33 (0.36, 52.64)	0.250
GG + GA /AC + AA /CC	238 (72.1)	149 (45.1)	3.05 (2.16, 4.32)	<0.001
AA /AC + AA /CC	84 (25.5)	92 (27.9)	1.00 (reference)[a]	
GG + GA /CC /CT	0 (0)	0 (0)		
GG + GA /AC + AA /CT	0 (0)	56 (17.0)		
AA /CC /CT	0 (0)	0 (0)		
AA /AC + AA /CT	0 (0)	30 (9.1)		

[a]Combined with *PD-1* AA/*PD-L1* AC + AA/*PD-L2* CC, and all *PD-L2* CT as reference.

A allele [19]. We combined subjects with the *PD-L1* AC and AA genotypes and compared them with subjects who had the *PD-L1* CC genotype. There were no AS patients with the *PD-L2* CT genotype, so we combined subjects with the *PD-1* AA, *PD-L1* AC and AA and *PD-L2* CC genotypes and those with *PD-L2* CT genotype as reference. Compared with this reference group, *PD-L2* CC carriers with the *PD-1* GG and GA and *PD-L1* CC genotypes, the *PD-1* AA and *PD-L1* CC genotypes and the *PD-1* GG and GA and *PD-L1* AC and AA genotypes had a 6.63-fold (95% CI 1.28, 34.46), 4.33-fold (95% CI 0.36, 52.64) and 3.05-fold (95% CI 2.16-4.32) increased risk for AS, respectively (Table 1).

Discussion

Research has proposed that the interaction of PD-1 with its ligands and CD28-mediated co-stimulation play critical roles in peripheral tolerance [9–11]. The possible relationships of several *PD-1* polymorphisms and AS development were also investigated [22, 23]. There is a G to A polymorphism at position −536 in the transcription start site of the *PD-1* gene [16]. Kong *et al*. [17] reported that Hong Kong Chinese with the *PD-1* −536 AA genotype were less likely to have RA than those with the *PD-1* GG genotype. Wang *et al*. [16] reported that the *PD-1* G-536A polymorphism was not associated with SLE in Taiwanese individuals, but their result might be restricted to the small sample size. In the current study we observed that subjects with the *PD-1* −536 GG and GA genotypes had a significantly elevated risk for AS compared with those with the AA genotype. The *PD-L1* A8923C polymorphism is near or within the transcriptional factor binding sites, and may influence the binding of transcriptional factors [19]. Hayashi *et al*. [19] observed that Japanese individuals with the *PD-L1* A8923C C allele had an increased risk for Graves' disease compared with those with the A allele. Our results also indicate that *PD-L1* C allele carriers had a greater risk for AS than those with the A allele, although this effect was not statistically significant. The C47103T genotype in exon 5 of the *PD-L2* gene has an altered amino acid (serine to phenylalanine) in the transmembrane domain [20]. However, the association of the *PD-L2* C47103T genotype and autoimmune diseases seems to be variable. Wang *et al*. [20] reported that individuals with the *PD-L2* C47103T T allele had an increased risk for SLE than those with the C allele, but that individuals with the *PD-L2* C allele were more likely to have RA than those with the T allele [24]. The reasons for these inconsistent results may be due to differences in the frequency of the

PD-L2 C47103T genotype in healthy controls, differences in pathogenesis and/or the use of small sample sizes. In the present study, all our AS patients had the *PD-L2* C47103T C allele, as confirmed by direct sequencing. Further, we observed that individuals with the *PD-L2* CT genotype were protected from AS than those with the CC genotype. *PD-L2* T allele carriers appear unlikely to develop AS.

We evaluated the combined effect of *PD-1*, *PD-L1* and *PD-L2* polymorphisms on AS. In our controls, there were no individuals with the *PD-L2* T allele. We combined subjects with the *PD-1* AA, *PD-L1* AC and AA and *PD-L2* CC genotypes, and those with the *PD-L2* CT genotype as reference. Subjects who simultaneously carried the *PD-1* GG and GA, *PD-L1* CC and *PD-L2* CC genotypes had the highest risk for AS. Subjects with *PD-1* AA, *PD-L1* CC and *PD-L2* CC genotypes also had a greater risk for AS, but there were only three such subjects in our study population. In addition, individuals with *PD-1* GG and GA, *PD-L1* AC and AA and *PD-L2* CC genotypes also had a significantly increased risk for AS. Our results suggest that subjects with the *PD-L2* C47103T CT genotype may be significantly protected against AS, and that those who simultaneously carry the susceptible genotypes (*PD-1* G-536A, *PD-L1* A8923C and *PD-L2* C47103T CC) have an increased risk for AS.

In our controls, the frequency of the *PD-1* A allele was 58.9%, comparable with that previously reported for controls in Chinese and Japanese populations (49–55%) [16, 17, 25]. The frequency of the *PD-1* G-536A and *PD-L1* A8923C genotypes in our controls also conformed to Hardy–Weinberg equilibrium. Our PCR-RFLP results for the *PD-L2* C47103T genotype were confirmed by direct sequencing, reinforcing our genotyping techniques.

In our study, patients and controls were matched by age and gender to reduce possible selection bias. We also tried to minimize possible bias by selecting control subjects of the same ethnicity as our AS subjects. It is also possible that the polymorphisms of *PD-1* and its ligands may only be susceptibility factors for AS in certain ethnic populations. Additional studies including more subjects from different populations may shed light on this question. Further, the regulation of expression of PD-1 and its ligands *in vivo* is complex and modulated by other transcription or post-transcription factors, such as cytokines.

In summary, our results indicate that the *PD-1* G-536A GG and GA genotypes were associated with increased risk for AS development. The *PD-L2* C47103T CT genotype had a protective effect for AS. The combined genotypes of *PD-1* G-536A, *PD-L1* A8923C and *PD-L2* C47103T also appear to be associated with AS.

Rheumatology key messages

- *PD-1* G-536A genotypes may be associated with the development of AS.
- *PD-L2* C47103T genotypes had a protective effect for AS.

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