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 (RRm) 1.78; 95% CI 1.13, 2.81] and the GA genotype (RRm 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 (RRm 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.

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...
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 blockade 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 the promoter region (−536) was identified as G to A (rs36084323) [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 and 330 matched controls 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’-GTC 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 TTG TCC AT-3’ and 5’-AGT GGC TCA 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 combined relative risks (RRm) 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 (RRm = 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 (RRm = 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 (RRm = 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
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-L1 CT genotype, so we combined subjects with the PD-L1 AC and AA genotypes and those with PD-L1 CC genotype as reference. Compared with this reference group, PD-L1 CC carriers with the PD-1 GG and GA 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 [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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AS patients, n (%) (n = 330)</th>
<th>Normal controls, n (%) (n = 330)</th>
<th>RRm (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-1 G-536A genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>81 (24.5)</td>
<td>64 (19.4)</td>
<td>1.78 (1.13, 2.81)</td>
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<tr>
<td>GA</td>
<td>163 (49.4)</td>
<td>143 (43.3)</td>
<td>1.59 (1.09, 2.31)</td>
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<tr>
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<td>86 (26.1)</td>
<td>123 (37.3)</td>
<td>1.00 (reference)</td>
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</tr>
<tr>
<td>G</td>
<td>325 (94.2)</td>
<td>271 (81.1)</td>
<td>1.37 (1.09, 1.73)</td>
<td>0.007</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td>1.00 (reference)</td>
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</tr>
<tr>
<td>PD-L1 A8923C genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8 (2.4)</td>
<td>3 (0.9)</td>
<td>3.00 (0.77, 11.74)</td>
<td>0.113</td>
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<tr>
<td>AC</td>
<td>58 (17.6)</td>
<td>61 (18.5)</td>
<td>0.95 (0.62, 1.46)</td>
<td>0.828</td>
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<tr>
<td>AA</td>
<td>264 (80.0)</td>
<td>266 (80.6)</td>
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<tr>
<td>C</td>
<td>74 (11.2)</td>
<td>67 (20.2)</td>
<td>1.15 (0.80, 1.67)</td>
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<tr>
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<td>593 (89.8)</td>
<td>1.00 (reference)</td>
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<tr>
<td>PD-L2 C47103T genotype</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CT</td>
<td>0 (0.0)</td>
<td>86 (26.1)</td>
<td>0.01 (0.001, 0.06)</td>
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<tr>
<td>CC</td>
<td>330 (100)</td>
<td>244 (73.9)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0 (0.0)</td>
<td>86 (26.1)</td>
<td>0.01 (0.001, 0.07)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>660 (100)</td>
<td>574 (87.0)</td>
<td>1.00 (reference)</td>
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<tr>
<td>PD-1/PD-L1/PD-L2 genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG + GA /CC /CC</td>
<td>6 (1.8)</td>
<td>2 (0.6)</td>
<td>6.63 (1.28, 34.46)</td>
<td>0.025</td>
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<tr>
<td>AA /CC /CC</td>
<td>2 (0.6)</td>
<td>1 (0.3)</td>
<td>4.33 (0.36, 52.64)</td>
<td>0.250</td>
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<tr>
<td>GG + GA /AC + AA /CC</td>
<td>238 (72.1)</td>
<td>149 (45.1)</td>
<td>3.05 (2.16, 4.32)</td>
<td>&lt;0.001</td>
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<tr>
<td>AA /AC + AA /CC</td>
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<td>92 (27.9)</td>
<td>1.00 (reference)</td>
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<td>GG + GA /CC /CT</td>
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<td>0 (0)</td>
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</tr>
<tr>
<td>GG + GA /AC + AA /CT</td>
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<td>56 (17.0)</td>
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<tr>
<td>AA /CC /CT</td>
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<td>0 (0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA /AC + AA /CT</td>
<td>0 (0)</td>
<td>30 (9.1)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Combined with PD-1 AA/PD-L1 AC + AA/PD-L2 CC, and all PD-L2 CT as reference.*
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 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 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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**Disclosure statement:** The authors have declared no conflicts of interest.

**References**

10. Latchman Y, Wood CR, Chernova T et al. PD-L2 is a second ligand for PD-1 and its ligands in vivo is complex and modulated by other transcription or post-transcription factors, such as cytokines.
16. Latchman Y, Wood CR, Chernova T et al. PD-L2 is a second ligand for PD-1 and its ligands in vivo is complex and modulated by other transcription or post-transcription factors, such as cytokines.


