**PDCD1** Single Nucleotide Polymorphisms in Iranian Patients With Juvenile Arthritis

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Received: 09 Sep. 2016; Accepted: 15 Apr. 2017

**Abstract**—Juvenile idiopathic arthritis (JIA) is a clinically heterogeneous cluster of complex diseases, in which both the genetic and environmental factors seem to play a role in the development of the disease. The current study aims to assess the association of programmed cell death 1 (PDCD1, also called PD-1) gene variants with JIA vulnerability in Iranian population. In this case-control association study, we investigated a group of 50 Iranian patients with JIA in comparison with 202 healthy controls and evaluated the frequency of alleles, genotypes, and haplotypes of PDCD1 single-nucleotide polymorphisms (SNPs), comprising PD-1.1 G/A, PD-1.3 G/A and PD-1.9 C/T, using PCR-RFLP method. Both the allelic and genotype frequencies of PD-1.1, PD-1.3 and PD-1.9 were similar in two groups of patients and controls. Moreover, no significant difference was observed between the two groups of patients and controls for GGC (PD-1.1 G, PD-1.3 G, PD-1.9 C), GAC (PD-1.1 G, PD-1.3 A, PD-1.9 C), and AGT (PD-1.1 A, PD-1.3 G, PD-1.9 T) haplotypes. Our results did not show any association between PDCD1 SNPs and the development of JIA in Iranian population.

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**Keywords:** PD-1; Single nucleotide polymorphism; Juvenile idiopathic arthritis; Children; Autoimmunity

**Introduction**

Juvenile idiopathic arthritis (JIA), the most prevalent cause of childhood disability, is a clinically heterogeneous cluster of complex diseases, with an onset before the age of sixteen years (1). Although the exact etiology of JIA remains unclear, it is believed that the involvement of both genetic and environmental factors is associated with the development of this disease.

The programmed death 1 (PD-1) molecule, encoded by the programmed cell death 1 (*PDCD1*) gene, which is located on 2q37.3, is a member of the immunoglobulin receptor superfamily that encodes a 55-kd type 1 transmembrane inhibitory immunoreceptor, which is localized on the surface of activated B cells, activated monocytes, CD8+ T cells, CD4+ T cells, and natural killer T cells, and contains a tyrosine-based inhibitory motif in its cytoplasmic tail (2-4). Binding of PDCD1 to its corresponding ligands, namely, PDL-1 (B7-H1 or CD274), expressed on macrophages, B cells, T cells, some tumor cells and dendritic cells (DC), and PDL-2 (B7-DC or CD273), located on cultured bone marrow-derived mast cells, macrophages, and DCs, results in the inhibition of both cytokine secretion by previously activated lymphocytes as well as B and T cell proliferation, which itself regulates the maintenance of peripheral tolerance (4,5).

Multiple association studies have been recently
performed to examine the probable associations of different single nucleotide polymorphisms (SNPs) within PDCD1 gene, known to influence the level of PD-1 production, with the susceptibility to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) thus far (6-8).

Although the evidence mentioned above indicates that PDCD1 is a strong candidate gene for vulnerability to autoimmune disorder, including RA, the association of PDCD1 with JIA remains dubious, presumably due to the paucity of data regarding the influence of PDCD1 gene variants on JIA susceptibility. We, therefore, assessed whether PDCD1 is correlated with JIA in the Iranian pediatric population. Three SNPs were investigated, comprising PD-1.9 (C/T, rs2227982, position +7625 of exon 5), PD-1.3 (G/A, rs11568821, position +7146 of intron 4), and PD-1.1 (G/A, rs36084323, position -538 from transcription start site). To the best of our knowledge, the current study is the first one evaluating the probable role of PDCD1 SNPs in JIA proneness in a group of Iranian patients.

Materials and Methods

Participants
Fifty Iranian pediatric patients, diagnosed as having JIA based on the ILAR classification criteria for JIA (9), were recruited. The patients had been referred to the Rheumatology Clinic of the Children’s Medical Center Hospital, the Pediatrics Center of Excellence in Iran, and the Pediatrics Center of Excellence in Iran, and compared with 202 sex and ethnically matched healthy unrelated controls that were randomly selected from unrelated controls that were randomly selected from adults with no family history or clinical manifestation of any rheumatologic and autoimmune disorders.

The ethical committee of Tehran University of Medical Sciences ratified the study. Written informed consents were obtained from all entrants to this study prior to the enrollment.

Sampling and Genotyping
For all participants, peripheral blood was collected into tubes with EDTA as an anticoagulant. The genomic DNA was obtained from the whole blood by standard phenol-chloroform method (10,11). The extracted DNA samples were kept at -20° C until investigation. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was applied for genotyping of PD-1.9 (C/T, rs2227982), PD-1.3 (G/A, rs11568821), and PD-1.1 (G/A, rs36084323) SNPs.

PD1.1. PCR-RFLP on PD1.1 (+538 G/A Promoter) was carried out with 100 ng of the genomic DNA in 25-μL reaction volume containing 0.5 μL of 10 pmol/μL forward primer, 0.5 μL of 10 pmol/μL reverse primer, 0.2 μL of 10 mmol/L dNTPs, 1 U of Taq DNA polymerase, 2.5 μL of 10×PCR buffer, PCR was performed under the following conditions: initial denaturation at 95° C for 3 min followed by 35 cycles of denaturation at 95° C for 40 s, annealing at 60° C for 50 s, and extension at 72° C for 40 s. A final extension at 72° C for 5 min was performed. The 552-bp PCR product was digested with 5 U of MspI (fermentase Inc) at 37° C overnight and was resolved on a 2% agarose gel with Gel Green DNA Staining (Table 1, Figure 1).

PD1.3. The PCR–RFLP on PD1.3 (+7146 G/A intron 4) was carried out with 100 ng of the genomic DNA in a 25 μL reaction volume containing 0.6 μL of 10 pmol/μL forward primer, 0.6 μL of 10 pmol/μL reverse primer, 0.2 μL of 10 mmol/L dNTPs, 1 U of Taq DNA polymerase, 2 μL of 10×PCR buffer. Thermal cycling conditions were 95° C for 10 min followed by 35 cycles of 95° C for 15 s, 60° C for 30 s, and 72° C for 15 s. A final extension at 72° C for 5 min was performed. The 180-bp PCR product was digested with 10 U of PstI (fermentase Inc) at 37° C overnight and was resolved on a 3% agarose gel with Gel Green DNA Staining (Table 1, Figure 1).

PD1.9. The PCR condition of PD1.9 was as same as PD1.1. The 408-bp PCR product was digested with 5 U of Bpu10I (fermentase Inc) at 37° C overnight and was resolved on a 2% agarose gel with Gel Green DNA Staining (Table 1, Figure 1).

The sizes of specifically digested segments were discovered as follows. In the case of PD1.1: 227 bp for G allele and 282 bp for A allele; PD1.3 180 bp product digested to 130 and 50 bp. If the product was digested, the allele was determined as A; if not, it was determined as G; and in the case of PD1.9 the product size 408 bp, with a permanent digestion site to 234 and 174 bp. If the product was C, the 234 bp fragment was digested to 145 and 89 bp; if not, it was determined as T. Fragments with lengths of 145 bp for C allele and 234 bp for T allele are yielded.

Primers were created according to the sequence of the human PDCD1 gene (GeneBank Accession Number: NM_005018). Details of primers’ sequences and restriction enzymes are demonstrated in Table 1.
Table 1. *PDCD1* SNPs, method, enzymes and primers, used in this study

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Reference SNP</th>
<th>SNP position</th>
<th>Location</th>
<th>Method</th>
<th>Restriction Enzyme</th>
<th>PCR Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD 1.1</td>
<td>rs36084323</td>
<td>-538 G/A</td>
<td>Promoter</td>
<td>PCR-RFLP</td>
<td>MspI</td>
<td>F: TTC TAG CCT CC/ GAC GGA ATT CT; R: CTC AAC CCC ACT CCC ATT CT</td>
</tr>
<tr>
<td>PD 1.3</td>
<td>rs11568821</td>
<td>7146 G/A</td>
<td>Intron 4</td>
<td>PCR-RFLP</td>
<td>PstI</td>
<td>F: CCC CAG GCA GCA ACC TCA AT; R: GAC CGC AGG CAG GCA CAT AT</td>
</tr>
<tr>
<td>PD 1.9</td>
<td>rs2227982</td>
<td>7625 C/T</td>
<td>Exon 5</td>
<td>PCR-RFLP</td>
<td>Bpu10I</td>
<td>F: GGA CAG CTC AGG GTA AGC AG; R: AGG GTC TGC AGA ACA CTG GT</td>
</tr>
</tbody>
</table>

Figure 1. Gel electrophoresis patterns of PD-1.1, PD-1.3, and PD-1.9. (a) The amplified fragments of PD-1.1 were digested with MspI, the PCR product was 552 bp, with a durable digestion point of 282 bp. If the product was G, the 282 bp fragment was digested to 227 and 54 bp; if not, it was characterized as A. (b) The amplified fragments of PD-1.3 were digested with PstI, the PCR product size was 180 bp. If the product was digested, the allele was distinguished as A; if not, it was distinguished as G. (c) The amplified fragments of PD-1.9 were digested with Bpu10I, the PCR product was 408 bp with a permanent digestion site for 234 bp. If the allele was C, the 234 bp fragment was digested to 145 and 89 bp, if not, it was T.

Statistical Analysis

The allele and genotype frequencies were evaluated by direct gene counting and compared with the controls using the chi-square test. The odds ratio (OR) and 95% confidence interval (CI) were examined for each allele and genotype. SHEsis online software was used to analyze haplotype, genotype, Hardy-Weinberg equilibrium (HWE), as well as linkage disequilibrium (12). The \( D' \) and \( r^2 \) values for the three before-mentioned *PDCD1* SNPs, were evaluated, and the haplotype blocks were assessed by SHEsis online software (Figure 2). The genotype distributions of PD-1.1 (G/A, rs36084323), PD-1.3 (G/A, rs11568821), and PD-1.9 (C/T, rs2227982), were tested for deviation from the Hardy-Weinberg equilibrium in control group using the \( \chi^2 \) test in R-Genetics Package (http://cran.um.ac.ir/web/packages/genetics/genetics.pdf). \( P \) of less than 0.001 was considered statistically significant for HWE (13). Therefore, all of the *PDCD1* alleles evaluated in this study comply with Hardy-Weinberg equilibrium. To adjust for multiple testing the Benjamini-Hochberg method (14) to control the false discovery rate (FDR) was used. \( P \) of less than 0.05 was considered to be statistically significant.

Figure 2. Pairwise linkage disequilibrium (LD) among three SNPs in *PDCD1* gene. (A) LD was calculated by the \( D' \) statistic using the data from all participants. A \( D' \) value of 100 attests a complete LD between 2 markers, and a \( D' \) value of 0 attests a complete linkage equilibrium; (B) \( r^2 \) for the three SNPs evaluated in the *PDCD1* gene.
Results

The allelic and genotype frequencies in Iranian patients with JIA and controls are depicted in Table 2. The distribution of these genotypes did not show any significant deviation from the Hardy-Weinberg equilibrium in healthy controls.

<table>
<thead>
<tr>
<th>dbSNP</th>
<th>Alleles/ Genotypes</th>
<th>JIA (n=50) N (%)</th>
<th>Control (n=202) N (%)</th>
<th>P</th>
<th>Adj. P*</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>96 (96)</td>
<td>398 (98.5)</td>
<td>0.106</td>
<td>0.361</td>
<td>(0.1-1.3)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4 (4)</td>
<td>6 (1.5)</td>
<td>0.106</td>
<td>2.763</td>
<td>(0.764-9.987)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>46 (92)</td>
<td>196 (97)</td>
<td>0.102</td>
<td>0.352</td>
<td>(0.095-1.298)</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>4 (8)</td>
<td>6 (3)</td>
<td>-</td>
<td>-</td>
<td>2.84</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

HWE

G 87 (87) 348 (86.1) 0.822 0.822 1.076 (0.56-2.057) 0.928
A 13 (13) 56 (13.9) 0.822 0.822 0.928 (0.486-1.77) 1.091 (0.54-2.205)

AG 13 (26) 56 (28) - - 0.916 (0.453-1.85)
AA 0 (0) 0 (0) - - -

HWE

C 95 (95) 397 (98.3) 0.055 0.159 0.335 (0.104-1.078) 3.028 (0.919-9.97)
T 5 (5) 7 (1.7) 0.055 0.159 0.412 (0.116-1.47) 1.778 (0.443-7.135)

HWE

Both the allelic and genotype frequencies of PD-1.1, PD-1.3, and PD-1.9, were similar in two groups of patients with JIA and healthy individuals.

Additionally, no significant difference was observed between the two groups of patients with JIA and healthy controls for GGC (PD-1.1 G, PD-1.3 G, PD-1.9 C), GAC (PD-1.1 G, PD-1.3 A, PD-1.9 C), and AGT (PD-1.1 A, PD-1.3 G, PD-1.9 T) haplotypes (Table 3).

Table 3. Overall haplotype associations of the single nucleotide polymorphisms according to Haploview

<table>
<thead>
<tr>
<th>Block 1 Haplotypes</th>
<th>Frequencies</th>
<th>95% CI*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 G A T</td>
<td>0.03</td>
<td>0.012</td>
<td>0.615-11.431</td>
</tr>
<tr>
<td>2 G A C</td>
<td>0.12</td>
<td>0.136</td>
<td>0.458-1.745</td>
</tr>
<tr>
<td>3 G G C</td>
<td>0.82</td>
<td>0.847</td>
<td>0.514-1.760</td>
</tr>
</tbody>
</table>

JIA, Juvenile idiopathic arthritis; *95% confidence interval for the difference between Hap.freq case-control.

Both the allelic and genotype frequencies of PD-1.1, PD-1.3, and PD-1.9, were similar in two groups of patients with JIA and healthy individuals.

Additionally, no significant difference was observed between the two groups of patients with JIA and healthy controls for GGC (PD-1.1 G, PD-1.3 G, PD-1.9 C), GAC (PD-1.1 G, PD-1.3 A, PD-1.9 C), and AGT (PD-1.1 A, PD-1.3 G, PD-1.9 T) haplotypes (Table 3).

Figure 2 depicts the LD structure of the evaluated SNPs (rs36084323/rs11568821/rs2227982). Evaluation of LD between each pair of loci suggested that rs36084323 was in relatively strong LD with rs2227982 ($D^* = 0.89$). However, none of the other two pairs of loci were observed to be in significant LD.
Discussion

The contribution of various genetic factors towards the development of autoimmune diseases, including SLE and RA, has been a topic of intensive research recently. Nevertheless, a multitude of investigations has focused on disease susceptibility in adult populations, while there is a paucity of data regarding the effects of genetic variations on autoimmune diseases’ proneness in pediatric patients (15-25). In the present study, we have mainly focused on the associations between PD-1.1, PD-1.3 and PD-1.9 SNPs and the development of JIA in a group of Iranian patients.

Considering the broad pattern of expression of PDL-1, one of the PD-1 ligands, in activated endothelial and epithelial cells, it has been previously propounded that PD-1 molecule could act as a major regulator of lymphocyte activation at the level of synovial tissue (26). Moreover, the negative costimulatory PD-1/PDL-1 pathway has been suggested as the modulator of peripheral T cell responses in both murine and human RA (27). Additionally, CD4+PD-1+ T cells have been identified to amass as anergic cells in the synovial fluid of patients with RA (28). Collectively, this evidence implicates the importance of PD-1 in the pathogenesis of RA.

More than 30 SNPs have currently been distinguished in the human PDCD1 gene, several of which have been associated with susceptibility to autoimmunity in different ethnicities (29). In this research, we examined three potentially functional polymorphisms, namely, PD-1.1 -538 G/A (rs36084323, suggested to influence the transcription and activation of PDCD1 gene following the occurrence of mutations in promoter (26)), PD-1.3+7146 G/A (rs11568821, identified as an enhancer-like region within intron 4 with several binding sites for different transcription factors (30)), and PD-1.9+7625 C/T (rs2227982, SNP in exon 5, which is known to cause a switch in the synthesized amino acid from valine to alanine) and evaluated the correlation between these three gene polymorphisms and the risk of JIA in Iranian pediatric patients. We found no associations between the aforementioned PDCD1 SNPs with JIA vulnerability in Iranian pediatric patients. Furthermore, we observed no correlation between GGC (PD-1.1 G, PD-1.3 G, PD-1.9 C), GAC (PD-1.1 G, PD-1.3 A, PD-1.9 C), and AGT haplotypes (PD-1.1 A, PD-1.3 G, PD-1.9 T) and individuals’ susceptibility to JIA. Our results are in line with the findings of a study conducted by Iwamoto et al., (31), which revealed no associations between PD-1.1 and PD-1.3 SNPs and RA susceptibility in the Japanese population. Contrarily, Kong et al., observed that the AA genotype of PD-1.1 SNP was correlated with a diminished risk for developing RA in Chinese population (32). However, they reported PD-1.3 SNP to be nonpolymorphic in the Chinese population (32). In addition, Prokunina et al., detected an association between allele A of PD-1.3 SNP and RA negative for both rheumatoid factor and the shared epitope in Swedish population (33). In another investigation performed by Liu et al., (26), the GG genotype of PDCD1 rs36084323 SNP was found to be associated with an elevated risk for developing RA in a group of Han Chinese population, although they observed no correlation between PDCD1 rs11568821 and rs2227982 SNPs with RA susceptibility (26). The other research conducted by Tahoori et al., revealed PD1.1 A allele to be associated with an increased risk of RA disease in Iranian population (34). However, they reported no significant differences in PD-1.3 and PD-1.9 SNPs between RA cases and controls (34).

In conclusion, the present study suggests that PD-1.1, PD-1.3, and PD-1.9 SNPs could not affect JIA susceptibility in Iranian population. However, validation by a study with larger sample size from diverse ethnicities is required so as to verify these results.

Acknowledgement

This study was supported by a grant from Deputy of Research, Tehran University of Medical Sciences (grant number: 92-01-30-22087).

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PDCD1 SNPs in JIA

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