PDCD1 Single Nucleotide Polymorphisms in Iranian Patients With Juvenile Idiopathic Arthritis

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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

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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 compared with 202 sex and ethnically matched healthy 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**

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

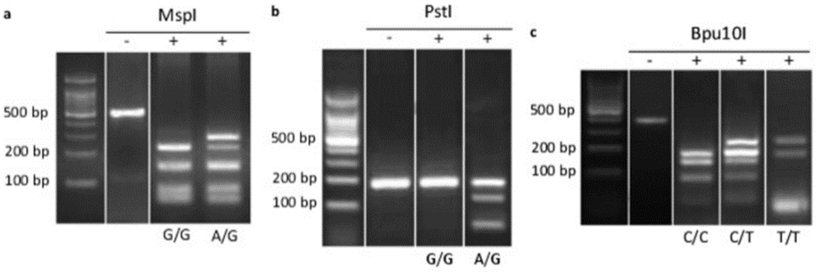

Figure 1. Gel electrophoresis patterns of PD-1.1, PD-1.3, and PD-1.9. (a) The amplified fragments of PD-1.1 were digested with MspI, the PCR product was 552 bp, with a durable digestion point of 282 bp. If the product was G, the 282 bp fragment was digested to 227 and 54 bp; if not, it was characterized 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 allel 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.

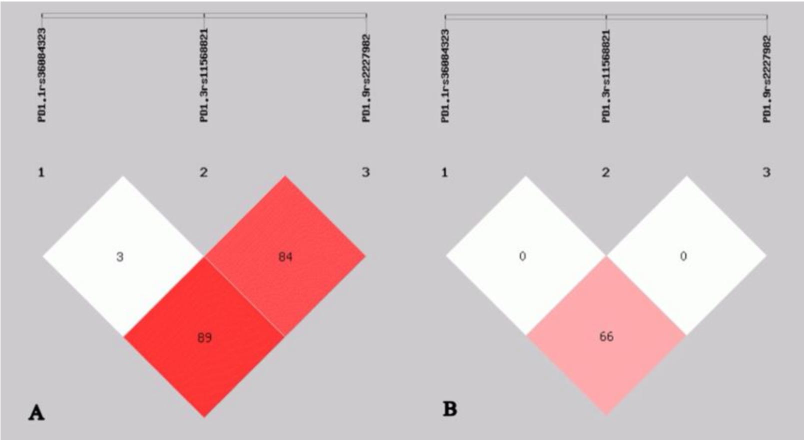

Figure 2. Pairwise linkage disequilibrium (LD) among three SNPs in PDCD1 gene. (A) LD was calculated by the D′ statistic using the data from all participants. A *D′* value of 100 attests a complete LD between 2 markers, and a *D′* value of 0 attests a complete linkage equilibrium; (B) r^2 for the three 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 2. Allele and genotype distribution of *PDCD1* gene in Iranian patients with JIA and healthy controls

dbSNP	Alleles/ Genotypes	JIA (n=50) N (%)	Control (n=202) N (%)	*P*	Adj. P^a	OR (95% CI)
PD1.1 **rs36084323**	G	96 (96)	398 (98.5)	0.106	0.159	0.361 (0.1-1.3)
	A	4 (4)	6 (1.5)	0.106	0.159	2.763 (0.764-9.987)
	GG	46 (92)	196 (97)	0.102	0.153	0.352 (0.095-1.298)
	AG	4 (8)	6 (3)	-	-	2.84 (0.77-10.47)
	AA	0 (0)	0 (0)	-	-	-
HWE			0.83			
PD1.3 **rs11568821**	G	87 (87)	348 (86.1)	0.822	0.822	1.076 (0.563-2.057)
	A	13 (13)	56 (13.9)	0.822	0.822	0.928 (0.486-1.77)
	GG	37 (74)	146 (72)	0.806	0.806	1.091 (0.54-2.205)
	AG	13 (26)	56 (28)	-	-	0.916 (0.453-1.85)
	AA	0 (0)	0 (0)	-	-	-
HWE			0.022			
PD1.9 **rs2227982**	C	95 (95)	397 (98.3)	0.055	0.159	0.335 (0.104-1.078)
	T	5 (5)	7 (1.7)	0.055	0.159	3.028 (0.919-9.97)
	CC	46 (92)	195 (96.5)	0.091	0.153	0.412 (0.116-1.47)
	CT	3 (6)	7 (3.5)	-	-	1.778 (0.443-7.135)
	TT	1 (2)	0 (0)	-	-	
HWE			0.802			

JIA, Juvenile idiopathic arthritis; ^{a}Adjusted *P* for multiple testing using Benjamini-Hochberg method; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium test.

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.

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

Block 1 Haplotypes				Frequencies			
Row	**rs36084323**	**rs11568821**	**rs2227982**	**Hap.freq (JIA)**	**Hap.freq (Control)**	**95% CI***	***P***
1	A	G	T	0.03	0.012	0.615-11.431	0.174540
2	G	A	C	0.12	0.136	0.458-1.745	0.743117
3	G	G	C	0.82	0. 847	0.514-1.760	0.873777

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

§ If a CI does not contain 0, frequencies of haplotypes in case and control groups are significantly different at α=0.05.

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.

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