Association of programmed cell death-1 (*PDCD-1*) gene polymorphisms with rheumatoid arthritis in Iranian patients

M.T. Tahoori[1], A.A. Pourfathollah[1], M. Akhlaghi[2], S. Daneshmandi[1], M.H. Nicknam[3], N. Soleimanifar[3]

[1]Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran; [2]Rheumatology Research Centre, and [3]Department of Immunology, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

Introduction

Programmed cell death 1 (PDCD-1, also named PD-1, CD279, and SLE_{B2}), a negative T cell regulator to maintain peripheral tolerance, induces negative signals to T cells during interaction with its ligands and is therefore a candidate gene in the development of autoimmune diseases such as rheumatoid arthritis (RA). Herein, we investigate the association of PDCD-1 polymorphisms with the risk of RA among Iranian patients and healthy controls.

Methods

Genomic DNA was extracted from the whole blood samples using DNA Purification kit (DNG-plus). Using the PCR- RFLP method, 3 PDCD-1 SNPs, including PD1.1G/A, PD1.3G/A, and PD1.9C/T were genotyped in 120 RA patients as well as 188 healthy controls. The genotype and allele frequencies of these SNPs were analysed by statistical tests for the significant association between RA patients and controls. Haplotype constructions of these SNPs were performed. Clinical diagnosis of the RA patients was confirmed by the Rheumatology Research Centere of Tehran University of Medical Sciences.

Results

Our study revealed that PD1.1 A allele at position -538 in the promoter region of PDCD-1 gene is associated with an increased risk of RA disease compared to controls (2.9% vs. 0.7%, OR= 3.735, 95% CI= 0.956–14.588, p=0.046). There were no significant differences in other alleles and genotypes of PDCD-1 SNPs between RA cases and controls.

Conclusion

Our results indicate that among the polymorphisms which we evaluated only the PD1.1A allele in the promoter region of PDCD-1 gene is significantly associated with RA susceptibility in Iranian patients.

Key words

programmed cell death-1, rheumatoid arthritis, RFLP, single-nucleotide polymorphisms, PD1.1A allele, DNA purification, promoter, restriction enzyme, haplotype

Mohammad Taher Tahoori, MSc
Ali Akbar Pourfathollah, PhD
Maassoumeh Akhlaghi, MD
Saeed Daneshmandi, MSc
Mohammad Hosein Nicknam, PhD
Narjes Soleimanifar, MSc

Please address correspondence and reprint requests to:
Dr A.A. Pourfathollah,
Department of Immunology,
Faculty of Medical Sciences,
Tarbiat Modares University,
Jalal Ale Ahmad Highway,
P.O. Box 14115-111,
Tehran, Iran.
E-mail: pourfa@modares.ac.ir

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Introduction

Rheumatoid arthritis is a common chronic inflammatory autoimmune disease characterised by destruction of the joint cartilage and bone associated with inflammation of the synovium and pathological infiltration of lymphocytes in target organs. Both cell-mediated and humoral immune responses may contribute to the development of synovitis. T cells are the dominant type of cells that infiltrate the synovial membrane in RA. Although the pathogenesis of RA remains unclear, it seems likely that dysregulated lymphocyte activation causes this autoimmune disease through breakdown of tolerance (1).

Programmed cell death 1 (PDCD-1) molecule is a negative regulator of T cells (2). It belongs to co-stimulatory receptor family, and is a member of an Ig superfamily. This molecule encodes a 55kDa type I transmembrane protein (3-7). PDCD-1 is expressed as monomeric on the cell surface, because it lacks the membrane-proximal cysteine residue which is required for homodimerisation of other members of the CD28 family (7).

PDCD-1 cytoplasmic domain has an immunoreceptor tyrosine-based inhibitory motif (ITIM) which plays a crucial role in down-regulation and inhibitory function of T and B cells for maintenance of peripheral tolerance (8). As an immunoinhibitory receptor, PDCD-1 is expressed on T cells, B cells, and myeloid cells upon activation and binding to its ligands from B7 family and it also has an inhibitory role in proliferation and cytokine production of these cells (6, 9). PDCD-1 deficient mice (C57BL/6) show lupus-like glomerulonephritis and progressive arthritis, suggesting that it is a susceptibility gene for negative regulation of immune system in development of autoimmunity (10). Thus based on this finding, single nucleotide polymorphism (SNP) in this gene can be associated with autoimmune diseases such as RA, SLE, T1D, and MS in human. To date, more than 30 SNPs in the PDCD-1gene have been identified and evaluated associated with different autoimmune diseases (11-16). In this study, 3 SNPs were selected, and analysed in the case control association study in Iranian RA patients compared to controls. These 3 SNPs include: PD1.1G/A (position -538 from the translation start) in the promoter (12); PD1.3G/A (position +7146) in the fourth intron, as a part of an enhancer that allele A of this SNP can inhibit the binding of RUNX1 transcription factor to this enhancer and result in decreased expression of PDCD-1 gene (11); and PD1.9C/T (position +7625, alanine-to-valine) in exon 5 (16).

Patients and controls

Study subjects

All 120 RA patients (16 men and 104 women, age range 17 to 76 years) were acquired from the Rheumatology Research Centre at Shariati Hospital (Tehran, Iran), and diagnosis was established by a group of rheumatologists according to the criteria of the American College of Rheumatology (17). For all 120 RA patients, RF and ESR laboratory tests were carried out (68.3% were RF positive and mean for ESR was 29.5±16.6 SD).

Participants in the healthy control group consisted of 188 individuals (65 men and 123 women, age range 17 to 63 years) who had no background of autoimmune diseases and were matched with the study group. Approval of this study was obtained from the medical ethics committee at Tehran University of Medical Sciences. The study was explained to all the people who were recruited and they gave their written informed consent.

Materials and methods

Genomic DNA extraction

EDTA-treated whole blood samples were taken from patients and control donors. Initially, whole blood samples were washed three times with lysing buffer for RBCs lysis in whole blood, and then genomic DNA was extracted from the leukocyte sediments acquired in the previous step using a DNG-plus DNA Purification kit (Cinnagen, Iran) according to the manufacturer's procedure. Genomic DNA used as a PCR template was stored at minimum concentration of 30 ng/μL and -20˚C with Tris-EDTA buffer.

Table I. PDCD1 SNPs, genotyping methods and primer.

SNP ID	SNP position	Location	Method	Restriction enzyme	PCR primers
PD1.1	-538G/A	Promoter	PCR-RFLP	Msp I	Forward TTCTAGCCTCGCTTCGGTTA; reverse CTCAACCCCACTCCCATTCT
PD1.3	7146G/A	Intron 4	PCR-RFLP	Pst I	forward CCCCAGGCAGCAACCTCAAT; reverse GACCGCAGGCAGGCACATAT
PD1.9	7625C/T	Exon 5	PCR-RFLP	Bpu10 I	Forward GGACAGCTCAGGGTAAGCAG; reverse AGGGTCTGCAGAACACTGGT

SNP: single-nucleotide polymorphism; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

Genotyping

Three SNPs, including PD-1.1, PD1.3, and PD1.9 investigated in this study were defined by restriction fragment length polymorphism (RFLP) technique using their corresponding restriction enzymes (Fermentase). Different fragment sizes were detected in different SNPs after digestion of PCR products with restriction enzymes and running gel electrophoresis. Primers were designed based on the sequence of the human PDCD1 gene, as shown in Table I.

PCR was set up for amplification of fragments involving desired polymorphism. PCR products were then digested with specific restriction enzymes for each SNP. PCR was achieved by following conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 40s, annealing at 60°C for 50 s (for PD1.3 60.5°C), and an extension at 72°C for 40 s and then a final extension phase at 72°C for 3 min for all SNPs in this study. PCR mixture contained 30 ng of genomic DNA, 1X PCR buffer, 0.2 mM of each dNTP, 6 pmoles of each primer (MWG, Germany), 1 mM $MgCl_2$ (for PD1.3 1.5mM), 0.5 units of *Taq* DNA polymerase (Cinnagen, Iran) and distilled water was then added up to 25 µL at final volume. The PCR product sizes were 552 bp, 180 bp, and 408bp, for PD1.1, PD1.3, and PD1.9, which were digested with MspI, PstI and Bpu10I restriction enzyme (Fermentas), respectively. This restriction enzyme digestion was performed at 37°C overnight. The genotypes of individuals were identified by length of digested fragments subsequent to 2.5% agarose gel electrophoresis stained by ethidium bromide. The sizes of specifically digested fragments were achieved as follows. In the case of PD1.1: 227 bp for G allele and 282 bp for A allele; PD1.3 lacks a PstI site for G allele, so no digestion occurs and the initial 180bp PCR products remain intact, whereas in the case of A allele the PCR product is digested to 123 bp and 57 bp segments; and in PD1.9, segments with lengths of 145 bp for C allele and 234 bp for T allele are yielded.

Statistical analysis

The allele, genotype and haplotype frequencies of case and control groups were compared using chi-square statistical test with a 2×2 contingency table and one degree of freedom. *P*-values less than 0.05 were considered statistically significant. Two-sided *p*-values or Fisher's exact test, odds ratio (OR) and 95% confidence interval (CI) were calculated using SPSS statistical software (version 15.0). We also did statistical analysis using chi-square and *t*-test for association of RA with RF and ESR, respectively.

Results

Allele frequencies

We found that the frequency of PD1.1A allele at position -538 located in the promoter region of the PDCD-1 gene in the patients with RA was significantly higher than that in the controls (2.9% in the patients *vs*. 0.7% in the controls, p=0.046, OR=3.7, CI=0.9–14.5). Interestingly, we observed that the frequency of the PD1.1G allele was 99% and is considered a common allele in Iranians, while the PD1.1A allele is common in Chinese and Mexican-Indian populations (11-12). There was no significant difference between the RA case and controls for other alleles of SNPs in this study, including PD1.3A or G and PD1.9C or T, as shown in Table II.

Allele and genotype frequencies of PDCD-1 SNPs in RA patients compared to controls, as analysed by chi-square test.

Genotype frequency

We compared genotype distribution of PD1.1, PD1.3, and PD1.9 SNPs between Iranian RA patients and healthy controls in this study. Although we observed higher frequencies of PD1.1G/A (4.1% *vs*. 1.5%) and also PD1.9C/T (4.1% *vs*. 2.6%) in RA patients *versus* controls, it was not statistically significant. Also, for other genotypes no significant difference was found in the genotype distribution among them. Frequency of each genotype of these three SNPs was compared between Iranian RA patients (n=120) and healthy controls (n=180). The results obtained did not show any significant association for each genotype of SNPs (Table II). Moreover, as shown in Table II, no minor homozygote genotypes such as PD1.1A/A in controls and PD1.9T/T in both RA patients and controls were observed in our study.

Haplotype frequency

Haplotype of PDCD1 was characterised by combining PD1.1G/A, PD1.3G/A and PD1.9C/T single-nucleotide polymorphisms (SNPs) in this study (Table III). Haplotype frequencies were estimated and association analysis performed in patients compared to control groups. GGC haplotype containing PD1.1G, PD1.3G and PD1.9C was common haplotype in both patient and control groups. The statistical analysis of different haplotype does not show any significant association between two groups as shown in Table III.

Table II. Allele and genotype frequencies of PDCD1 SNPs in RA patients and controls.

SNP	Genotype	Allele	RA patients (n=120)	Controls (n= 88)	OR (95% CI)	*p*-value[a]
PD1.1	PD1.1AA		1 (0. 83)	0 (0)	2.58 (2.241-2.969)	0.38
	PD1.1GA		5 (4.16)	3 (1.59)	2.681 (0.629-11.432)	0.15
	PD1.1GG		114 (95)	185 (98.4)	0.308 (0.076-1.256)	0.08
		PD1.1A	7 (2.91)	3 (0.79)	3.735* (0.956-14.588)	0.046*
		PD1.1G	233 (97.08)	373 (99.20)		
PD1.3	PD1.3 AA		1 (0.83)	1 (0.531)	1.571 (0.097-25.363)	0.062
	PD1.3 GA		26 (21.66)	36 (19.148)	1.168 (0.663-2.057)	0.69
	PD1.3 GG		93 (77.5)	151 (80.319)	0.844 (0.482-1.477)	0.65
		PD1.3 A	28 (11.66)	38 (10.10)	1.175 (0.700-1.971)	0.63
		PD1.3 G	212 (88.33)	338 (89.89)		
PD1.9	PD1.9 C/C		115 (95.833)	183 (97.340)	0.628 (0.178-2.218)	0.33
	PD1.9 C/T		5 (4.166)	5 (2.659)	1.591 (0451-5.618)	0.33
	PD1.9 T/T		0 (0)	0 (0)		
		PD1.9 T	5 (2.08)	5 (1.32)	1.579 (0.452-5.512)	0.34
	PD1.9 C		235 (97.91)	371 (98.67)		

SNP: single-nucleotide polymorphism; OR: odds ratio; 95% CI=95% confidence interval.
[a] *p*-values for each allele and genotype frequencies are calculated by chi-square test with 2×2 contingency Table. *PD1.1 A allele significantly associated with RA compared to controls (*p*-value<0.05).

Table III. PDCD1 haplotype structure and frequencies.

PD1.1/PD1.3/PD1.9 (Haplotype)	RA patients	Control groups	*p*-value	Odds ratio (CI)
1 GGC	118	187	0.24	0.74 (0.45–1.21)
2 GAC	27	37	0.85	1.09 (0.61–1.94)
3 AGC	6	3	0.10	3.03 (0.66–15.51)
4 AGT	5	3	0.17	2.51 (0.51–13.42)
5 GGT	4	5	0.52	1.18 (0.26–5.17)
6 GAT	0	1	0.59	0.0 (0–25.65)
Total	160	236		

Frequency of different haplotype analysed in the case control by chi-square test. *P*-value, odds ratio and confidence interval for each one calculated. CI = Confidence Interval.

Discussion

Lymphocyte activation is precisely regulated by positive and negative signals using various immunoregulatory receptors (18). A fault in the negative signals from immunoinhibitory receptors may lower the threshold of autoreactive lymphocyte activation, break down the peripheral tolerance, and initiate the development of autoimmune diseases. This finding has been made on the basis of the expression of the autoimmune phenotype or lymphocyte hyperactivity in genetically manipulated mice with a deficiency in an immunoinhibitory receptor (Fcγ RIIB, CD22, CTLA-4, or PD-1) (19-22). Thus, negative signals conducted by immunoinhibitory receptors appear to play an important role in the maintenance of peripheral tolerance in mice. For humans, these immunoinhibitory receptors FcγRIIB, CD22, and CTLA-4 have been used as candidate genes in SNP case-control association studies to test their possible association with the human autoimmune diseases SLE and RA. Considering the fact that mutant allele (A) of SNPs is located in promoter region of PDCD-1 gene, it can probably influence the expression of this gene; so the present research was designed to study such association. Our findings suggest that PD1.1A allele can be considered as a risk factor for RA because of its significantly increased frequencies in RA patients in comparison with control. Conversely, Kong *et al*. reported reduced frequencies of PD1.1A allele in Chinese RA patients and considered the PD-1.1A allele to be a protective allele (12). We found that the PD1.1G allele (99%) is a common allele in Iranian individuals in our study. Consistently, frequencies of this allele are also common in Europeans and Africans (Prokunina-Olsson *et al*., unpublished observations) while PD-1.1A is a common allele in Chinese and Mexican-Indian population (11-12). Based on the results of this study, PD1.1 SNPs at promoter of PDCD-1 gene (wild allele to mutant allele or G to A) may result in dysfunction of this co-inhibitory pathway and cause autoimmune diseases such as RA. The accession number for the complete sequence of PDCD-1 from GeneBank is AF363458.

It should be mentioned that, the PD1.1 A allele was associated with susceptibility to RA in our study, which is consistent with the results of Kong *et al*. in Hong Kong on Chinese RA patients (12). Among the seven SNPs investigated by Prokunina *et al*., there was strong association between PD1.3 polymorphism (7146G/A as a regulatory polymorphism) and SLE susceptibility in Europeans and Mexicans (11). In the case of PD1.3G/A SNP (position 7146), A allele can inhibit the binding of RUNX1 transcription factor to the enhancer in the fourth intron and lead to a reduction in expression of PDCD-1 gene. Based upon the results of this study, PD1.3 SNPs at enhancer of PDCD-1 gene have no association with RA susceptibility in Iranian patients; on the contrary, it has been previously reported by Prokunina *et al*. to be associated with SLE and RA in their country (11, 23). Considering the fact that mutant allele (A) of SNPs is located in promoter, it can probably influence the expression of PDCD-1 gene, so the present research was designed to study such association. Further studies are also required to elucidate whether other SNPs of this gene are associated with RA.

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