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ORIGINAL ARTICLE

Variants in Intron 4 of PD-1 Gene are Associated with the Susceptibility to SLE in an Iranian Population

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ABSTRACT

Background: *Programmed cell death protein 1* (*PD-1*) is a negative co-stimulatory molecule with immunomodulatory properties. Recently, *PD-1* gene defects have attracted attention in the pathogenesis of SLE. **Objective:** Here, we assessed the association of *PD-1* gene polymorphisms in intron 4 and haplotypes with the susceptibility to SLE. **Methods:** Seventy-six SLE patients and 159 healthy controls were included. We screened the polymorphisms by amplifying the intron 4 of the *PD-1* gene with the specific primers followed by sequencing. **Results:** Two distinct SNPs were identified (rs6705653 and rs41386439) within the intron 4 of the *PD-1* gene. The AA genotype of +7499 (G/A) SNP was associated with the higher risk of SLE [OR=3.31, 95% CI (1.25–8.76), p-value=0.045], while A allele was identified as a risk allele [OR=1.75, 95% CI (1.10–2.76), p-value=0.015]. However, no significant association was observed between the allele and the genotype frequencies of +7209 (C/T) polymorphic region of the *PD-1* gene and susceptibility to SLE. Haplotype analysis results showed the significantly higher presence of H2 haplotype (AC; +7499/+7209) [OR=1.70, 95% CI (1.24–2.33), p-value=0.0012] in SLE patients. **Conclusion:** To the best of our knowledge, this is the first report of the significant association of *PD-1* +7499 (G/A) SNP with the SLE susceptibility and the first detection of both polymorphic loci in a population from Iran. However, more investigations are necessary to confirm these findings.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex immunogenetic and chronic disease, defined by the destruction of self-tolerance, imbalanced immune response against self-antigens, augmentation of autoantibodies, and disturbed clearance of immune complexes and apoptotic cells (1), which may eventually lead to irrecoverable end organ damage (2-4). Different environmental and infectious agents such as continuous exposure to sunlight (UV-irradiation) and EBV virus may trigger the onset of SLE among predisposed susceptible individuals(5,6). Although SLE is widespread in most countries and ethnicities, it is more prevalent in Asians, Afro-Americans, Afro-Caribbean and Hispanic Americans than in the Caucasians (7). The incidence of SLE is also higher in female patients, especially during child-bearing ages(8,9). SLE is a common disease in Iran with the estimated prevalence of 40 patients in 100,000 population (10). *Programmed cell death protein 1* (PDCD1 or PD-1) is an inhibitory or co-stimulatory molecule, abundantly found on the surface of activated T lymphocytes, B lymphocytes, natural killer (NK) cells and mononuclear cells (11). PD-1 is also an immunoregulatory molecule regulating lymphocyte activation and cytokine production through interaction with its specific ligands, PD-L1 and PD-L2 (12). It belongs to the CD28 superfamily and plays a crucial role in regulating the immune balance needed for self-tolerance (13). *PD-1* gene is located on chromosome 2q37.3, contains six distinct introns, and its transcription produces three alternatively spliced mRNAs (14). *PD-1* gene polymorphisms and its deregulated expressions have been attributed to the development and propagation of various autoimmune disorders such as SLE (15-21). One of the most important polymorphic regions among *PD-1* intronic loci is intron 4, which contains particular transcription sites for several regulatory elements such as NFκB, RUNX1 and E-BOX (22). Possible polymorphisms in intron 4 may affect the specific binding sites of these factors and alter the transcription process or even the expression of *PD-1*. However, there is still lack of evidence expressing the role of *PD-1* gene polymorphisms among SLE patients, particularly in intron 4 (17). In the present study, we aimed to investigate the association between *PD-1* gene polymorphisms in intron 4 and susceptibility to SLE in an Iranian population in the southeast of the Caspian Sea, Iran.

MATERIALS AND METHODS

Patients, Controls and Sample Collection. Seventy-six (76) patients complying with at least 4 items of the revised American College of Rheumatology (ACR) principles for SLE diagnosis (23) and 159 healthy subjects were recruited in this study. The successfully sequenced and reliable genotyping results were used for further analyses, while samples without genotyping results in the region of interest were not included. All patients were referred to Sayyad Shirazi Educational Hospital, Rheumatology Department, Golestan University of Medical Sciences. Table 1 lists the demographic data, clinical characteristics and laboratory parameters of SLE patients and healthy subjects. All patients or controls who were diagnosed with or claimed to suffer from an active inflammatory condition, pregnant participants or individuals with the family history of all other autoimmune diseases were not included. All participants were notified about the objectives and possible outcomes of the study, and a signed informed consent was obtained according to the Declaration of Helsinki guidelines (24). The Ethics Committee of Golestan University of Medical Sciences (GoUMS) also approved the methods of the current research dealing with human specimens.

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Table 1. Clinical characteristics and laboratory findings of patients with systemic lupus erythematosus and healthy subjects.

Characteristics		SLE patients (N=76)	Healthy subjects (N=159)
Age		38.10±13.42	42.94±15.47
Gender	Female	71 (93.4%)	91 (57.2%)
	Male	5 (6.6%)	68 (42.8%)
Anti-dsDNA titer		123.37±17.42	-
Leukopenia		29 (38.2%)	-
ESR		27.75±17.04	-
C3		1.27±0.45	-
C4		0.31±0.18	-
CH50		48.95±13.56	-
MPO		1.24±0.25	-
ANA		2.64±2.19	-
ANCA		2.72±2.46	-
CRP		14 (18.4%)	-
Lupus Nephritis		21 (27.6%)	-
SLEDAI		17.17±13.15	-

*Values are the number (%) of participants and/or means ± SD (standard deviation). Anti-dsDNA: anti–double-stranded DNA; Leukopenia: White blood cell counts lower than 3000 per µL; ESR: erythrocyte sedimentation rate; C3: complement component 3; C4: complement component 4; CH50: Total complement activity; MPO: myeloperoxidase (MPO); ANCA: anti-neutrophil cytoplasmic antibody; CRP: C-reactive protein; ANA: Antinuclear antibodies; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Genomic DNA Extraction and Screening *PD-1* SNPs within Intron 4. A total volume of 5 ml whole blood was taken from all participants. Genomic DNA was extracted from peripheral blood leukocytes using the NucleoSpin® Blood mini spin DNA preparation kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocols. Extracted DNA was stored at -70ºC until polymerase chain reaction (PCR) amplifications were conducted. The locus was amplified, and sequencing was the method of choice to screen the polymorphisms within the intron 4 of the PD-1 gene. The sequences of intron 4 specific primers obtained from Nielsen *et al.* (25) were as follows: forward: 5'-ACAATAGGAGCCAGGCGCA-3'; reverse: 5'- GGGTCCTCCTTCTTTGAGG -3'. PCR amplifications were performed in a final volume of 25 µL reaction mixture (Genetbio, Daejeon, Republic of Korea) containing the

following reagents: 100 ng genomic DNA, 10 pmol of each primer, 1 unit of Taq DNA polymerase, 10X PCR buffer, 2 mM $MgCl_2$ and 0.2 mM dNTP mixture. The amplification of the desired site was carried out in an Eppendorf Thermal Cycler (Hauppauge, New York, USA) with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 30 sec at 95°C, 40 sec at 61°C,72°C for 40 sec with a final extension step at 72°C for 5 min. The amplified PCR products were visualized under UV radiation as a single band with a length of 695 base pairs after electrophoresis on a 2% agarose gel containing the SYBR safe DNA stain (Sinaclon Co., Iran). The PCR products were then transferred to the Topaz Gene Research Company and subjected to sequencing by a 3730XL automatic DNA sequencer (ABI, USA). Finally, the CodonCode Aligner 6.0.2 software (CodonCode Corporation, USA) was used to assemble the sequencing results of SLE patients and healthy subjects with the reference sequence and to identify polymorphic sites.

Statistical Analysis. Pearson's goodness of fit test was used to analyze the Hardy-Weinberg equilibrium (HWE) for each polymorphic region in both groups of SLE patients and healthy subjects. The SNPstats software (http://bioinfo.iconcologia. net/SNPStats) (26) and the Arlequin software package 3.5.2.2 (http://cmpg.unibe.ch/ software/arlequin35/) (27) were used to reconstruct haplotypes upon adjusting sex and age covariates to each other. Statistical analysis was performed using the SPSS 22.0 (SPSS, Chicago, USA) software. To evaluate case-control study associations, the odds ratio (OR) and 95% confidence interval (CI) were determined. The genotype and haplotype frequencies in each group were examined by the *Chi* square goodness of fit test ($\chi 2$). p-values less than 0.05 were considered significant.

RESULTS

Analysis of the sequencing results of SLE patients compared to 69 recognized polymorphic loci within the intron 4 of the *PD-1* gene revealed two distinct SNPs (rs6705653 and rs41386439).

The Association of Distinguished Alleles and Genotypes with the Susceptibility to SLE.
Four different inheritance models were applied to investigate the association of identified *PD-1* gene polymorphisms with SLE and to evaluate the distribution of related alleles and genotypes. We demonstrated that the GG genotype of *PD-1* +7499 (G/A) was more commonly observed in healthy controls. By setting the GG genotype as the reference prior to adjustment by covariates (gender and age), the association of AA genotype with SLE was delineated [OR=3.03, 95% CI (1.39–6.61), p-value=0.02]. The evaluation of allele frequencies also conceded a remarkable association of A allele with SLE [OR=1.68, 95% CI (1.15–2.46), p-value=0.0072]. After adjustment by covariates, AA genotypes were associated with the elevated risk of SLE under the co-dominant model [OR=3.31, 95% CI (1.25–8.76), p-value=0.045]. Likewise, the A allele was significantly associated with SLE susceptibility after sex- and age-adjustment [OR=1.75, 95% CI (1.10–2.76), p-value=0.015]. Table 2 illustrates the frequencies of genotypes of *PD-1* +7499 (G/A) under other inheritance models before and after adjustment by covariates.

Table 2. The frequencies of alleles and genotypes of rs6705653 (+7499, G/A) within the intron 4 of the PD-1 gene among SLE patients and healthy subjects under different inheritance models.

Genotypes and alleles		Healthy subjects (n=149)	SLE patients (n=76)	OR (95% *CI*)	*P*-value	OR (95% *CI*)	*P*-value
		Number (%)	*Number (%)*	*Not adjusted*		*Adjusted by covariates**	
	G	202 (68%)	82 (54%)	Reference			
	A	96 (32%)	70 (46%)	**1.68 (1.15-2.46)**	*0.0072*	**1.75 (1.10-2.76)**	*0.015*
	Co-dominant model						
	GG	71 (47.6%)	26 (34.2%)	Reference			
	AG	60 (40.3%)	30 (39.5%)	1.37 (0.73-2.56)	*0.02*	1.49 (0.72-3.08)	*0.045*
rs6705653 (+7499, G/A)	**AA**	18 (12.1%)	20 (26.3%)	**3.03 (1.39-6.61)**		**3.31 (1.25-8.76)**	
	Dominant model						
	GG	71 (47.6%)	26 (34.2%)	Reference			
	AG+AA	78 (52.4%)	50 (65.8%)	1.75 (0.99-3.10)	*0.053*	1.89 (0.97-3.68)	*0.059*
	Recessive model						
	GG+AG	131 (87.9%)	56 (73.7%)	Reference			
	AA	18 (12.1%)	20 (26.3%)	**2.60 (1.28-5.28)**	*0.0085*	**2.74 (1.11-6.77)**	*0.025*
	Over-dominant model						
	GG+AA	89 (59.7%)	46 (60.5%)	Reference			
	AG	60 (40.3%)	30 (39.5%)	0.97 (0.55-1.70)	*0.91*	1.07 (0.55-2.09)	*0.84*
X^2 HWE* (*P*-value)		4.12 (0.35)	3.21*(0.071)*				

*The level of significance: 0.05. Significant associations are also shown in bold. To standardize risk assessment, sex and age adjustment was conducted. Fisher's exact test was also performed for *Hardy-Weinberg equilibrium*. The unreliable sequencing results are not included and regarded as missing data.

Regarding the *PD-1* +7209 (C/T) polymorphic region, the CC genotype with the highest prevalence in healthy subjects was set as reference. However, no significant association was observed between TT [OR=1.29, 95% CI (0.36–4.61)] or CT [OR=1.39, 95% CI (0.72–2.6.7)] genotypes and the susceptibility to SLE under the co-dominant model before sex- and age-adjustment (p-value=0.6). Analysis of genotypes by adjusted covariates did not

significantly alter the odds ratios. Under the co-dominant model, both TT [OR=1.28, 95% CI (0.23–7.06)] and CT [OR=1.39, 95% CI (0.63–3.06)] genotypes were not associated with the susceptibility to SLE (p-value=0.7). Moreover, no significant association was observed between *PD-1* +7209 (C/T) genotypes and SLE under other inheritance models (Table 3).

Table 3. The frequencies of alleles and genotypes of rs41386439 (+7209, C/T) within the intron 4 of the PD-1 gene among SLE patients and healthy subjects under different inheritance models.

Genotypes and alleles		Healthy subjects (n=158)	SLE patients (n=76)	OR (95% CI)	P-value	OR (95% CI)	P-value
		Number (%)	Number (%)	Not adjusted		Adjusted by covariates*	
rs41386439 (+7209, C/T)	C	271 (86%)	125 (82%)	Reference			
	T	45 (14%)	27 (18%)	1.25 (0.77-2.02)	0.37	1.27 (0.69-2.33)	0.45
	Co-dominant model						
	CC	120 (76%)	53 (69.7%)	Reference			
	CT	31 (19.6%)	19 (25%)	1.39 (0.72-2.67)	0.6	1.39 (0.63-3.06)	0.7
	TT	7 (4.4%)	4 (5.3%)	1.29 (0.36-4.61)		1.28 (0.23-7.06)	
	Dominant model						
	CC	120 (76%)	53 (69.7%)	Reference			
	CT+TT	38 (24.1%)	23 (30.3%)	1.37 (0.74-2.52)	0.31	1.37 (0.65-2.89)	0.4
	Recessive model						
	CC+CT	151 (95.6%)	72 (94.7%)	Reference			
	TT	7 (4.4%)	4 (5.3%)	1.20 (0.34-4.23)	0.78	1.19 (0.22-6.47)	0.84
	Over-dominant model						
	CC+TT	127 (80.4%)	57 (75%)	Reference			
	CT	31 (19.6%)	19 (25%)	1.37 (0.71-2.62)	0.35	1.37 (0.63-3.01)	0.43
X^2 HWE* (P-value)		1.58 (0.20)	0.35 (0.56)				

The level of significance: 0.05. Significant associations are also shown in bold. To standardize risk assessment, sex and age adjustment was conducted. Fisher's exact test was also performed for *Hardy-Weinberg equilibrium*. The unreliable sequencing results are not included and regarded as missing data.

The *PD-1* Haplotypes within Intron 4 and Association with SLE.
The EM algorithm was adopted to build up haplotypes between two identified polymorphic regions of the *PD-1* gene, which disclosed a higher prevalence of *H2* (AC; +7499/+7209) [OR=1.70, 95% CI (1.24–2.33), p-value=0.0012] haplotype among patients, thereby demonstrating an elevated risk of SLE in *H2* careers (Table 4). The relative LD between two *PD-1* SNPs was formed according to the D′ values (D′ values limited to zero show the absence of LD, while those approaching 1 represent complete LD). The SNP stats analysis revealed a nearly complete LD among the tested *PD-1* SNPs [D′ value = 0.9411, r=0.5249 (p-value<0.0001)].

Table 4. Analysis of haplotype frequencies of PD-1 SNPs within the intron 4 and their association with SLE susceptibility; Adjusted by covariates (Sex and Age).

Haplotype	+7499 (G/A)	+7209 (C/T)	SLE patients (2n=152) N (%)	Healthy Subjects (2n=298) N (%)	OR (95% CI)	P-value
H1	G	C	82 (53.95 %)	198 (66.44 %)	Reference	
H2	**A**	**C**	**44 (28.29 %)**	**58 (19.46 %)**	**1.70 (1.24 – 2.33)**	**0.0012**
H3	A	T	26 (17.76)	40 (13.43 %)	1.54 (1.08 – 2.21)	0.19
H4	G	T	rare	2 (0.67 %)	NA	NA
Global haplotype association *p*-value: *0.039*; NA: not applicable for rare haplotypes						

Lack of Association between Disease Activity, *PD-1* Genotypes and Haplotypes.
We also evaluated the association of *PD-1* genotypes and haplotypes with SLEDAI scores. However, no significant difference of SLEDAI score was demonstrated between any of the genotypes and constructed haplotypes in both polymorphic loci.

DISCUSSION

SLE as a complex autoimmune and a chronic inflammatory disorder encompasses diverse clinical and laboratory manifestations (1,2,22). Environmental and infectious agents among individuals with genetic background may trigger the onset of the disease. Genetic predisposition may also play a role in the deterioration and propagation of disease (3). PD-1 is an immunoregulatory surface molecule involved in the modulation of self-tolerance (15,19). Intron 4 of the PD-1 gene is comprised of several transcription binding sites for regulatory elements (17). The polymorphisms in these loci may result in gene expression alterations. The presence of polymorphisms within the intron 4 of the *PD-1* gene could also be associated with susceptibility to various disorders such as SLE (19), rheumatoid arthritis (RA) (16), ankylosing spondylitis (AS) (18), type I diabetes (28), multiple sclerosis (MS) (29) and viral infections, including HCV (30), HIV (31) and HBV (32). In the present

study, we determined *PD-1* gene polymorphisms within intron 4 among SLE patients and healthy subjects by PCR-sequencing and recognized two distinct polymorphic loci (rs6705653 and rs41386439). Regarding the allelic frequencies and genotype distributions of *PD-1* +7499 (G/A), the AA genotype was significantly higher among SLE patients. In accordance with previous studies (25), the homozygous AA and heterozygous GA genotypes were more prevalent among SLE patients, and the GG genotype was higher among healthy subjects (Table 2). Thus, the presence of A allele, and consequently AA genotype within this locus could be attributed to the elevated risk of SLE. Moreover, the presence of G allele and GG genotype within +7499 locus of the *PD-1* gene could have protective effects against SLE. According to the lack of experimental reports on the association of +7499 locus with SLE (25), to the best of our knowledge (33,34), this is the first study reporting the significant association of this locus with SLE as well as the first study investigating *PD-1* +7499 (G/A) SNP in an Iranian population. However, further studies should be conducted to highlight the functional importance of SNPs in this site. Analysis of the allelic frequencies of *PD-1* +7209 (C/T) polymorphisms among SLE patients compared to healthy subjects showed no significant difference. Previous studies revealed that *PD-1* +7209 (C/T) polymorphisms may weaken the binding potency of NF-κB and RUNX1 transcription factors, alter the expression of PD-1 among SLE patients and deteriorate the disease status (35). Several exclusive studies in Poland (35) and Taiwan (19) reported the association of *PD-1* +7209 (C/T) polymorphisms with the risk of SLE, which were inconsistent with ours but not significant. These findings denote that T allele could be introduced as a possible SLE risk allele and C allele could be protective. According to the literature, this is the first study investigating the association of rs41386439 polymorphisms with SLE in an Iranian population. We also analyzed the haplotypes of identified polymorphisms within the intron 4 region of the *PD-1* gene. Our findings revealed that the AC (+7499/+7209) haplotype was significantly associated with the increased risk of SLE. In accordance with previous studies (25,28), the co-occurrence of A and C alleles in patients could be introduced as risk alleles. Thus, individuals with CC/GG haplotype might be less likely to develop SLE, as described previously (28). The relative LD between two tested *PD-1* SNPs was almost complete. Although previous studies on Iranian subjects did not investigate the association of these SNPs with SLE, further investigations should be conducted to confirm or reject our findings. The investigation of the distribution of alleles, genotypes and haplotypes based on the disease severity resulted in no significant findings, all genotypes and haplotypes were distributed equally regarding SLEDAI scores. Therefore, these polymorphic loci are probably not suitable to estimate the disease activity in SLE patients. In conclusion, analysis of the sequencing results of the intron 4 within the *PD-1* gene revealed a significant association between occurrence of A allele and AA genotypes of *PD-1* +7499 (G/A) SNP with a higher risk of developing SLE, while G allele was significantly higher in healthy subjects. Moreover, the association of the AC (+7499/+7209) haplotype with SLE was explicit. However, no significant relation was noticed between PD-1 +7209 (C/T) SNP and SLE. Likewise, the genotypes and haplotypes were not related to the SLEDAI scores. Although our findings indicate compelling differences in the allelic frequencies and genotype distribution of SLE patients compared to healthy subjects in an Iranian population, further investigations are needed due to the limited sample size of our research to confirm current results and determine the effects of these genetic changes on increasing the chance of developing SLE.

ACKNOWLEDGEMENTS

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