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Analysis of Single Nucleotide Polymorphisms in *HLA-DRA*, *IL2RA*, and *HMGB1* Genes in Multiple Sclerosis

Mohsen Asouri[1,2], Hamid Alinejad Rokni[3], Mohammad Ali Sahraian[4], Sadegh Fattahi[2], Nima Motamed[5], Rozita Doosti[4], Hamzeh Rahimi[1], Maryam Lotfi[2], Azam Moslemi[6], Morteza Karimpoor*[1], Fereidoun Mahboudi*[1], Haleh Akhavan-Niaki*[7, 8]

Abstract

Background: Multiple sclerosis (MS) is a common demyelinating neurodegenerative disorder with significant heritability. Previous studies have associated genetic variants in human leukocyte antigen (*HLA*) complex, *IL2RA*, and *HMGB1* genes with the pathophysiology of MS.
Methods: In order to investigate the gene association in the Iranian population, we performed a genotyping study of 36 variants in the mentioned genes using Sanger sequencing in 102 MS patients and 113 healthy controls.
Results: Our results identified significant associations as well as significant allele frequency differences in some of the studied single-nucleotide polymorphisms including rs4935356, rs3177928, and rs7197 from *HLA-DRA* gene, and rs12722489 and rs12722490 variants from *IL2RA* gene ($p< 0.05$). Moreover, the strong linkage disequilibrium of two common haplotypes was estimated from the *HLA-DRA* gene.
Conclusions: This association study may suggest the role of these polymorphisms in the genetic susceptibility of MS in the Iranian population and would facilitate the recognition of causative variants in this disease.

Keywords: *HMGB1*, *HLA-DRA*, *IL2RA*, Multiple sclerosis, Polymorphism.

Introduction

Multiple sclerosis (MS) is the most common autoimmune neurologic disease which affects approximately two million new cases worldwide, especially young adults (1). Three patterns of disease are seen in MS patients: relapsing-remitting (RRMS), in which episodic exacerbations are separated by periods of recovery; secondary progressive (SPMS), people diagnosed with RRMS eventually develop progressive disability; and primary progressive (PPMS), in which disability progresses steadily from disease onset (2). Multiple sclerosis arises when a susceptible individual encounters environmental triggers that stimulate an inflammatory response against self-antigen in the central nervous system (CNS). The immunological studies have shown that dis-

1: Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran.
2: North Research Center, Pasteur Institute of Iran, Amol, Iran.
3: Systems Biology and Health Data Analytics Lab, the Graduate School of Biomedical Engineering, UNSW Sydney, 2052, NSW, AU.
4: Multiple Sclerosis Research Center; Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran.
5: Department of Social Medicine, Zanjan University of Medical Sciences, Zanjan, Iran.
6: Department of Biostatistics, Faculty of Medical Sciences, Arak University of Medical Sciences, Arak, Iran.
7: Zoonoses Research Center, North Research Center, Pasteur Institute of Iran, Amol, Iran.
8: Department of Genetics, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran.
***Corresponding authors: Morteza Karimpoor; Tel: +98 912 280 6133; E-mail: mortezakarimi@pasteur.ac.ir**
& Fereidoun Mahboudi; Tel: +98 912 280 6133; E-mail: mahboudi@pasteur.ac.ir
& Haleh Akhavan-Niaki; Tel: +98 911 125 5920; E-mail: halehakhavan@yahoo.com.
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regulation of cellular and humoral immune responses elicit infiltration of lymphocytes and macrophages into the CNS along with anti-myelin antibodies and complement activation, which leads to episodes of inflammatory demyelination and a progressive neurodegenerative process with axonal transection and neuronal loss as an early event (3). An eruption of focal inflammation is responsible for the episodic RRMS phase, while axonal loss and neurodegeneration cause progressive accumulation of disability (4, 5).

Both environmental and genetic factors are involved in the etiology of MS. Vitamin D deficiency, smoking, Ultraviolet B (UVB)/ sunshine, and some pathogens are also considered as environmental risk factors for the development of MS (6).

Genetic factors are primarily responsible for the increased frequency of the disease in the relatives of affected individuals. Studies on twins and siblings suggest that multiple genes, each exerting different effects, play considerable roles in susceptibility to MS. Therefore, MS is considered as a mutagenic and complex disorder. Candidate-gene studies have validated the human leukocyte antigen (*HLA*) class II (*HLA-DRB1*) as the strongest susceptibility locus for MS. In 2011, the genome-wide association studies (GWAS) and ImmunoChip studies with more than 9,000 MS cases discovered 110 non-*HLA* genetic loci associated with MS (7).

Further studies have shown that genes encoding high mobility group box protein 1 (*HMGB1*) and the IL-2 receptor α chain (*IL-2Rα*) exert critical functions regarding immune responses in the development of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and MS (8). Elevation of peripheral blood mononuclear cells (PBMC), as well as upregulated serum and cerebrospinal fluid (CSF) levels of *HMGB1*, have been implicated in MS patients in several studies (9, 10). Large numbers of macrophages and microglia expressing the endogenous *HMGB1* and its ligands as well as Toll-like receptor 2 (TLR2) and TLR4 are also found in MS patients (11). The cytokine interleukin-2 (IL-2 has a wide range of effects that are essential for the balance between immune response and tolerance. The IL-2 receptor α chain (IL-2Rα), also known as CD25, is a central constituent of the trimeric IL-2 receptor complex that binds to IL-2. It has been shown that single-nucleotide polymorphisms (SNPs) in or near *HLA-DRA*, *IL2RA*, and *HMGB1* genes are associated with the increased risk of immune-mediated diseases including MS. The association of some SNPs in or near the mentioned genes has been studied extensively regarding increased risk of developing this disease. For instance, MS-associated *IL2RA* SNPs rs2104286 and rs11256593 are associated with CD25 expression on $CD4^+$ T cells. Changes in CD25 expression may influence the immune and inflammation signaling cascades, thus affecting $CD4^+$ T cell differentiation and T_{Reg} cells suppressive activity (12).

The purpose of this study was to investigate the frequency of 36 SNPs in three known loci, *HLA-DRA*, *IL2RA*, and *HMGB1* in the Iranian population. A total of 102 MS patients and 112 control subjects were selected and genotyped using polymerase chain reaction (PCR) method and Sanger sequencing.

Materials and methods

Sample collection

A total of 102 MS patients and 112matched controls were selected from the individuals referred to Sina teaching Hospital in Tehran, Iran. The patients were diagnosed based on the McDonald criteria (4), clinical signs and symptoms; all results were confirmed using brain magnetic resonance imaging (MRI). The control group was selected from patients who attended the hospital due to other causes and brain MRI was used to rule out MS. This group was age and sex-matched with the case group. The Ethics Committees of Sina teaching Hospital and Pasteur Institute of Iran approved the study. Written informed consent was taken from all participants.

DNA extraction

Five ml of peripheral blood from patients and healthy controls were collected in K3-EDTA tube and genomic DNA was extracted and purified from whole-blood lymphocytes by Mini QIAamp DNA Mini Kits (Cat. 51104; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA measurement and quality control were performed using spectrophotometry

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observance at 260/280 and 260/230 nm, respectively. The DNA integrity and fragmentation were investigated using 1% agarose gel electrophoresis.

Polymerase chain reaction (PCR)

PCR was performed by using Step One Plus Real-time PCR system (Applied Biosystems, Foster City, USA) to detect the 36 polymorphisms. These SNPs, located in *HLA-DRA*, *IL2RA*, and *HMGB1* genes, were selected by Haploview 4.2 using genotype data from Genome Browser release #27 in the HapMap Project (http://www.hapmap.org). The chromosome location, dbSNP number, and gene annotation of selected polymorphisms are summarized in Table 1. The specific primer sequences for each gene were designed through the Primer3 online software (http://frodo.wi.mit.edu/primer3), and the primer specificity was checked out by Primer-BLAST and SNPCheck V3 tools. The physiochemical properties of primers were further evaluated using Gene Runner software. All primer sequences are shown in Table 2.

Table 1. The genomic properties of studied polymorphism of *HMGB1*, *HLA-DRA*, and *IL2RA* genes that were extracted from UCSC genome browser for investigated genomic regions in this study.

Variant ID	Chr:bp	Allele	MAF	Location	Gene
rs538493533	13:30460237	A/G	0.001	3‘UTR	*HMGB1*
rs577524260	13:30460223	C/T	0.001	3‘UTR	*HMGB1*
rs111892138	13:30460267	T/C	0.022	3‘UTR	*HMGB1*
rs201945336	13:30460287-8	AA/A	-	3‘UTR	*HMGB1*
rs182881863	13:30460196	T/G	0.001	3‘UTR	*HMGB1*
rs149637108	13:30460189-94	CTTCCT/CT	-	3‘UTR	*HMGB1*
rs61338778	13:30460263-5	TTT/TTTT	-	3‘UTR	*HMGB1*
rs55642413	13:30460306-7	GG/G	-	3‘UTR	*HMGB1*
Rs9281809	32444502 & 32444503	-/AACTAACT	0.344	intron	*HLA-DRA*
Rs4935356	6:32444611	T/A/G	0.341 (T)	Intron	*HLA-DRA*
Rs3135390	6:32444618	C/A	0.131 (C)	Intron	*HLA-DRA*
Rs4935354	6:32444621	C/G/T	0.341 (C)	Intron	*HLA-DRA*
Rs3177928	6:32444658	G/A	0.120 (A)	3’ UTR	*HLA-DRA*
Rs7194	6:32444703	G/A	0.341 (G)	3’ UTR	*HLA-DRA*
rs7195	6:32444762	A/G	0.341 (A)	3’ UTR	*HLA-DRA*
Rs1131541	6:32444789	T/A	0.120 (A)	3’ UTR	*HLA-DRA*
Rs7196	6:32444794	A/T	0.221 (A)	3’ UTR	*HLA-DRA*
Rs7197	6:32444803	T/C/G	0.117 (T)	3’ UTR	*HLA-DRA*
Rs1051336	6:32444815	G/A/C	0.120 (A)	3’ UTR	*HLA-DRA*
Rs111471704	6:32444889	T/C	-	3’ UTR	*HLA-DRA*
Rs1157343109	6:32444988	T/C	-	3’ UTR	*HLA-DRA*
Rs1041885	6:32445032	T/A	0.119 (A)	3’ UTR	*HLA-DRA*
Rs12722489	10:6060049	C/T	0.091 (T)	Intron	*IL2RA*
Rs917751277	10:6060048	A/G	-	Intron	*IL2RA*
Rs992067421	10:6060043	C/A	-	Intron	*IL2RA*
Rs959264277	10:6060039	A/G	-	Intron	*IL2RA*
Rs11597542	10:6059981	T/C	0.001 (C)	Intron	*IL2RA*
Rs140860467	10:6059935	T/C	0.007 (C)	Intron	*IL2RA*
Rs17149458	10:6059897	T/A	0.029 (A)	Intron	*IL2RA*
Rs12722490	10:6059828	C/T	0.010 (T)	Intron	*IL2RA*
Rs3118470	10:6059750	T/A/C	0.319 (C)	Intron	*IL2RA*
Rs78556477	10:6059635	G/A	0.059 (A)	Intron	*IL2RA*
Rs41294925	10:6059590	A/G	0.008 (G)	Intron	*IL2RA*
Rs12722491	10:6059467	G/T	0.010 (T)	Intron	*IL2RA*
Rs550805995	10:6059430	G/A	0.001 (A)	Intron	*IL2RA*
Rs12722621	10:6059210	C/A	0.041 (A)	Intron	*IL2RA*

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Table 2. Sequence and amplicon size of primers.

Gene	Forward	Reverse	Length (bp)
IL2RA	ATGCTCTGCCTCTGGAAGACAC	TATCTCAATGGGTTTCCACACTGT	1472
HLA-DRA	TGCCTGCTTTTGCTTCTTTAGTCTC	AGGTGGTTTCAAGAATCAGTCAGAC	1173
HMGB1	AGAATGTATCCCCAAAAGCGTGAG	CACAGCACTGTAACTATCTTGGC	1366

DNA amplification was carried out by PCR with 3 µl of DNA in a 25 µl total reaction mixture containing 12.5 µl master mix (2x) and 0.5 µM of each primer. Thermal cycling conditions were as follows: denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. The amplification was followed by the last extension step at 72 °C for 5 min. The PCR products were monitored after electrophoresis with agarose gel 1.5% using a gel documentation system.

Sanger sequencing

The amplicons after gel purification were sent to the Macrogen Company (Seoul, South Korea) for Sanger sequencing. The results were trimmed and analyzed by BioEdit and Chromas software to ensure the quality and accuracy of the sequencing. Then, the extracted sequences were blasted against the non-redundant (NR) database to validate the annotated regions with sequences of interested genes.

Statistical analysis

The calculated findings were illustrated via reporting odds ratio (OR) and 95% confidence intervals (CI) for each SNP. The differences in allelic and genotypic distribution between the two studied groups were considered as significant if the computed *p* value was less than 0.05. The Hardy-Weinberg equilibrium (HWE) was evaluated through the Chi-square ($\chi 2$) goodness-of-fit test, and allele frequencies and genotype distributions were analyzed using the Chi-square ($\chi 2$) test. Pairwise, linkage disequilibrium (LD) of *HLA-DRA*, *IL2RA*, and *HMGB1* SNPs was operated using Haploview 4.1 software and the obtained data were reported by describing the D′ and r^2 parameters. Moreover, 10.000 permutations were conducted to address significant levels in haplotypes.

Results

In this study, 102 MS patients, (17.6% men and 82.4% women) with a mean age of 35.29±14.66 years and 113 healthy controls (24.8% men and 75.2% women) were compared regarding totally 36 polymorphisms within the *HLA-DRA*, *IL2R*, and *HMGB1* genes, 14, 14 and, 8, respectively, by PCR-sequencing method. The association between various polymorphisms of these three genes and MS disease was evaluated by the chi-square test. Thus, the frequency of related genotypes in each polymorphism was computed in MS patients and control groups, separately. No deviation from HWE was identified in both MS patients and control groups for all SNPs (p> 0.05) (data not shown).

Based on our results, in the polymorphisms of the *HLA-DRA* gene, the rs4935356 (p= 0.001), rs3177928 (p= 0.002), and rs7197 (p= 0.002) SNPs were significantly associated with the risk of MS disease. Moreover, the frequencies of the A allele of rs3135390 (p= 0.026), an allele in rs3177928 (p= 0.027), and C allele in rs7197 (p= 0.001) were significantly altered in our MS patients. The findings of association analysis between *HLA-DRA* SNPs and the risk of MS are exhibited in Table 3.

Results of variants of *IL2RA* revealed that there was a statistically significant association between rs12722489 (p= 0.007) and rs12722490 (p= 0.03) SNPs and the risk of MS; however, other polymorphisms of *IL2RA* gene (rs917751277, rs992067421, rs959264277, rs11597542, rs140860467, rs17149458, rs3118470, rs78556477, rs41294925, rs12722491, rs550805995, and rs12722621) had no significant differences between the two studied groups (p> 0.05). The frequency of T allele in rs12722489 showed significant discrepancies in MS patients compared to healthy controls. Differences in other SNPs of this gene did not

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reach a level of significance (Table 4). Upon analysis of the polymorphisms of *HMGB1* gene, the genotype and allele frequencies of rs146076135, rs201945336, rs111892138, rs200308321, rs149637108, rs538493533, rs577524260, and rs182881863 SNPs were calculated and, finally no significant difference was observed in allele and genotype frequencies in both groups (p> 0.05) (Table 5). Finally, the linkage disequilibrium (LD) patterns of *HLA-DRA*, *IL2RA*, and *HMGB1* SNPs were analyzed. Figure 1 illustrates the strong patterns of LD found in patients of this study. As shown, two haplotype blocks as following were significant predictors of MS disease in the *HLA-DRA* gene: one block of rs7194 and rs7195 SNPs; and another block consisting of rs4935356, rs3135390, rs4935354, rs3177928, rs7194, rs7195, rs1131541, rs7196, and rs7197 SNPs.

Table 3. The frequency of *HLA-DRA* alleles and genotypes in Iranian multiple sclerosis (MS) patients and controls.

Polymorphism	Status		(Frequency of Patients) Percent	(Frequency of Control) Percent	P-value
rs4935356	allele	G	(143) 70.8	(136) 65.4	0.219
		T	(49) 24.25	(53) 25.5	
		A	(10) 4.95	(19) 9.1	
	genotype	A/A	(1) 1.0	(0) 0.0	**<0.001**
		G/A	(6) 5.9	(15) 14.4	
		G/G	(52) 51.5	(50) 48.1	
		G/T	(33) 32.7	(21) 20.2	
		T/A	(2) 2.0	(4) 3.8	
		T/T	(7) 6.9	(14) 13.5	
rs3135390	allele	A	(170)84.15	(191) 91.8	**0.026**
		C	(31)15.35	(17) 8.2	
		T	(1) 0.5	(0) 0.0	
	genotype	A/A	(74)73.3	(89) 85.6	**0.049**
		C/A	(21) 20.8	(13) 12.5	
		C/C	(5) 5.0	(2) 1.9	
		T/A	(1) 1.0	(0) 0.0	
rs4935354	allele	C	(49) 24.26	(55) 26.4	0.611
		T	(153) 75.74	(153) 73.6	
	genotype	C/C	(7) 6.9	(15) 14.4	0.17
		C/T	(35) 34.7	(25) 24.1	
		T/T	(59) 58.4	(64) 61.5	
rs3177928	allele	G	(191) 94.55	(184) 88.5	0.027
		A	(11) 5.45	(24) 11.5	
	genotype	A/A	(1) 1.0	(0) 0.0	**0.002**
		G/A	(9) 8.9	(24) 23.1	
		G/G	(91) 90.1	(80) 76.9	
rs7194	allele	G	(49) 24.26	(51) 24.5	0.951
		A	(153) 75.74	(157) 75.5	
	genotype	A/A	(59) 58.4	(66) 63.5	0.24
		G/A	(35) 34.7	(25) 24.0	
		G/G	(7) 6.9	(13) 12.5	
rs7195	allele	G	(153) 75.74	(159) 76.4	0.868
		A	(49) 24.26	(49) 23.6	
	genotype	A/A	(7) 6.9	(10) 9.6	0.63
		G/A	(35) 34.7	(29) 27.8	
		G/G	(59) 58.4	(65) 62.0	
rs1131541	allele	T	(189) 93.56	(189) 90.9	0.308
		A	(13) 6.44	(19) 9.1	
	genotype	A/A	(0) 0.0	(1) 1.0	**0.49**
		T/A	(13) 12.0	(17) 16.4	
		T/T	(88) 87.1	(86) 82.7	
s7196	allele	T	(165) 81.7	(171) 82.2	0.889

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		A	(37) 18.3	(37) 17.8	
	genotype	A/A	(5) 5.0	(6) 5.8	
		T/A	(27) 26.7	(25) 24.0	0.87
		T/T	(69) 68.3	(73) 70.2	
rs7197	allele	C	(172) 85.1	(198) 95.2	**0.001**
		T	(30) 14.9	(10) 4.8	
	genotype	C/C	(75) 74.3	(95) 91.3	
		C/T	(22) 21.3	(8) 7.6	**0.001**
		T/T	(4) 4.0	(1) 1.0	
rs1051336	allele	G	(189) 93.56	(191) 91.8	0.490
		A	(13) 6.44	(17) 8.2	
	genotype	G/A	(13) 12.9	(17) 16.3	0.57
		G/G	(88) 87.1	(87) 83.7	
rs111471704	allele	T	(201) 99.5	(208) 100	0.493
		A	(1) 0.5	(0) 0.0	
	genotype	T/A	(1) 1.0	(1) 1.0	0.99
		T/T	(99) 99.0	(103) 99.0	
rs1157343109	allele	T	(202) 100	(208) 100	-
	genotype	T/T	(101) 100	(104) 100	
rs1041885	allele	T	(202) 100	(208) 100	-
	genotype	T/T	(101) 100	(104) 100	

Table 4. The frequency of IL2RA allele and genotype in Iranian multiple sclerosis (MS) patients and controls.

Polymorphism	Status		(Frequency of Patients) Percent	(Frequency of Control) Percent	P-value
rs12722489	allele	A	(28) 13.7	(13) 5.8	0.005
		G	(176) 86.3	(211) 94.2	
	genotype	AA	(5) 4.9	(3)2.8	0.007
		G/A	(18) 17.6	(5)4.7	
		G/G	(79) 77.5	(99) 92.5	
rs917751277	allele	T	(204) 100	(224) 100	-
	genotype	TT	(102)100	(107)100	-
rs992067421	allele	G	(204) 100	(224) 100	-
	genotype	GG	(102)100	(107)100	-
rs959264277	allele	T	(204) 100	(224) 100	-
	genotype	TT	(102)100	(107) 100	-
rs11597542	allele	A	(204) 100	(224) 100	-
	genotype	AA	(102)100	(107) 100	-
rs140860467	allele	A	(204) 100	(224) 100	-
	genotype	AA	(102)100	(107) 100	-
rs17149458	allele	A	(204) 100	(224) 100	-
	genotype	AA	(102)100	(107) 100	-
rs12722490	allele	A	(12) 5.9	(7) 3.1	0.167
		G	(192) 94.1	(217) 96.9	
	genotype	AA	(0) 0.0	(1) 0.9	0.03
		GA	(12) 11.8	(5) 4.7	
		GG	(90) 88.2	(101)94.4	
rs3118470	allele	A	(130) 63.7	(150) 67.0	0.482
		G	(74) 36.3	(74) 33.0	
	genotype	AA	(45) 44.1	(52) 48.6	0.83
		GA	(40)39.2	(39)36.4	
		GG	(17)16.7	(16) 15.0	
rs78556477	allele	C	(204) 100	(224) 100	-
	genotype	CC	(102) 100	(107) 100	-
rs41294925	allele	T	(201) 98.5	(224) 100	0.107
		C	(3) 1.5	(0) 0.0	
	genotype	TC	(3) 2.9	(0) 0.0	0.114

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		TT	(99) 97.1	(107) 100	
rs12722491	allele	C	(204) 100	(224) 100	-
	genotype	CC	(102) 100	(107) 100	–
rs550805995	allele	C	(204) 100	(224) 100	-
	genotype	CC	(102) 100	(107) 100	-
rs12722621	allele	G	(204) 100	(224) 100	-
	genotype	GG	(102) 100	(107) 100	-

Table 5. The frequency of *HMGB* allele and genotype in Iranian multiple sclerosis patients and controls.

Polymorphism	Status		(Frequency of Patients) Percent	(Frequency of Control) Percent	P-value
DELINSCrs146076135	allele	C	(183) 97.0	(195) 70.0	
	genotype	C-/-	(0) 0.0	(1) 0.9	0.51
		C/-	(21) 20.6	(27)24.1	
		C/C	(81) 79.4	(84) 75.0	
DELINSTrs201945336	allele	T	(204) 100		-
	genotype	T/T	(102) 100	(112) 100	-
rs111892138	allele	A	(204) 100		-
	genotype	A/A	(102) 100	(112)100	-
DELINSArs200308321	allele	A	(204) 100	(214) 100	
	genotype	A-/A-	(102) 100	(107)100	-
DELINSGAAGrs149637108	allele	GAAG	(204) 100	(222) 91.0	
	genotype	GAAG/-	(0)0.0	(1) 0.9	0.98
		GAAG/GAAG	(102) 100	(111)99.1	
rs538493533	allele	T	(204) 100	(224)100	-
	genotype	TT	(102) 100	(112)100	
rs577524260	allele	G	(204) 100	(224)100	-
	genotype	GG	(102) 100	(112)100	-
rs182881863	allele	A	(204) 100	(224)100	-
	genotype	AA	(102) 100	(112)100	-

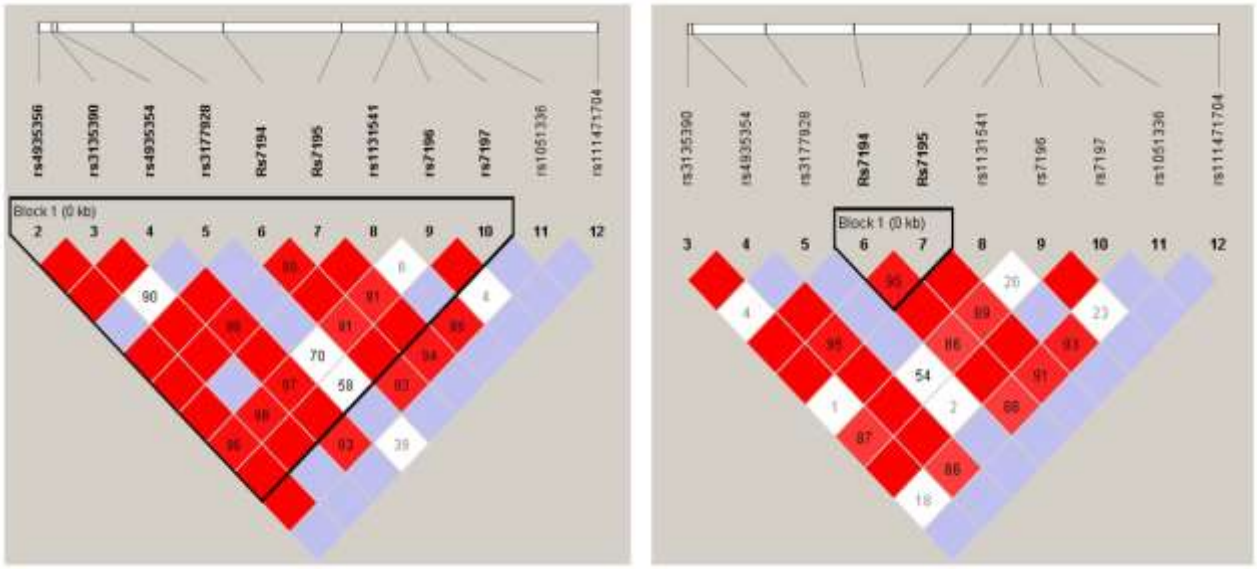

Fig. 1. Linkage disequilibrium (LD) of *HLA-DRA* SNPs using *D´* (left, red color) and r^2 (right, gray color) values for patient (left) and control (right) group. High levels of LD are depicted via increasing scale intensity from 0 to 100, as exhibited by the bars.

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Discussion

In the present study, we investigated the associations between 36 SNPs and the risk of MS in a population in Iran. A significant association ($p< 0.05$) among certain SNPs from *HLA-DRA* and *IL2RA* genes were found. Although a number of non-*HLA* genes have recently been recognized to contribute to susceptibility to MS with a modest effect, the *HLA* region is generally identified as being the strongest risk contributor. Meta-analysis studies suggest that *HLA-DR2*, and specifically the *DRB1*15* allele, are significant risk determinants in Chinese MS patients, nonetheless less strong correlations were revealed in Western MS populations; whereas, *HLA-DR9* alleles appeared to confer the resistance to disease in this population (13).

This study showed significant associations as well as significant allele frequency differences in some of the studied SNPs including rs4935356, rs3177928, and rs7197 from the *HLA-DRA* gene and rs12722489 and rs12722490 variants from the *IL2RA* gene.

The rs4935356 variant from the *HLA-DRA* gene was previously reported to be involved in alcoholism and addiction disorders through influencing the brain, behavior, and immune system (5). Here, by observing significant associations, we found a novel relationship and possibly new disease-related function for rs4935356 at risk of MS condition. In addition, the SNP variant rs3177928, significantly related to increased risk of MS, is reported in novel *HLA-DRA* downstream variants that were independent of *HLA-DRB1* alleles which are associated with non-Löfgren sarcoidosis (NL Sarcoidosis) as a multiorgan inflammatory disorder. Recent non-sarcoidosis studies showed that rs3177928 was associated with lipoprotein metabolism and connected with inflammatory mechanisms (14). Moreover, recently it was discovered that rs7197 SNP is strongly associated with antibody response against viral elements such as Epstein-Barr virus (EBV) capsid antigen. Our results are consistent with this finding and suggest that rs7197 genetic variation might have a role in developing MS disease through immune-mediated and particular microbial genes and susceptibility (15). In addition, the results of LD analysis demonstrated a strong linkage between rs7195 and rs7194, which was confirmed by findings showing almost the same level of non-significance for their genotypes and allele frequencies.

The IL-2/*IL2R* signaling stimulates the proliferation and survival of activated T cells and has a paramount non-redundant role in the production of regulatory T cells (16). Our data showed significant associations of rs12722489 and rs12722490 SNPs in *IL2RA* and the risk of MS. Additionally, allele frequency differences were noted too. The rs12722489 polymorphism is linked with multiple autoimmune conditions such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, and ulcerative colitis. *In silico* analysis suggested significant discrepancies in the affinity of estrogen receptor (ER) binding site between the alternative allelic variants, with a stronger predicted affinity for the risk (G) allele. Electrophoretic mobility shift assessment illustrated that purified human ERα only bound G variant of a 32-bp genomic sequence containing rs12722489 (17). Chromatin immunoprecipitation showed that endogenous ERα in humans interacted with rs12722489 genomic region *in vivo* and DNA pull-down assay confirmed differential allelic binding of amplified 189-bp genomic fragments containing rs12722489 with endogenous human ERα. In a luciferase reporter assay, a kb-long genomic part containing G but not A allele of rs12722489 demonstrated enhancer properties in MT-2 cell line, an HTLV-1 transformed human cell line with a regulatory T cell phenotype (17). Moreover, associations with various autoimmune disorders of polymorphisms in an LD block in which the *IL2*/*IL21* genes map (4q27), and also in genes encoding the *IL2RA* and *IL2RB* subunits located in 10p15 and 22q13, respectively, were identified through GWAS. Polymorphisms in these three genes were studied in 430 MS patients and in 550 ethnically matched controls in Spain. Replication and meta-analysis with results from an independent cohort of 771 MS patients and 759 controls in Spain confirmed the association of polymorphisms in the *IL2RA* gene but did not verify the association for *IL2RB* (18). Regression analyses of the combined cohort in in Spain study revealed the independence of two *IL2RA* association signals: rs2104286 and rs11594656/rs35285258. The

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related role of the *IL2RA* gene on MS susceptibility is well in line with its common effect on autoimmune risk and the suggestive association of IL2/IL21 warrants further investigation (18).

The *HMGB1* belongs to the classification of endogenous damage-associated molecular pattern molecules (DAMPs), also known as alarmins. *HMGB1* is passively released from necrotic cells and it is actively secreted from activated immunocompetent cells, including macrophages (19). Nevertheless, extracellular *HMGB1* has inhibitory effects on phagocytic activity of macrophages (efferocytosis), which is critical to the resolution of inflammation (20). Increasing evidence exists for the role of *HMGB1* in autoimmune disorders. In this context, recent studies have shown associations between *HMGB1* and rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and Sjögren's syndrome (21). Studies on *HMGB1* are scarce in MS. In this examination, we considered the SNPs of *HMGB1*; however, none of the studied polymorphisms was significantly associated with the risk of MS regarding both genotype and allele frequency. Zhen *et al.* examined *HMGB1* levels of peripheral blood mononuclear cells (PBMC), serum, and CSF in MS patients and found a considerably higher *HMGB1* level in serum, PBMC, and CSF compared to healthy individuals and non-inflammatory neurological disorder controls (10). Andersson *et al.* demonstrated that *HMGB1* and its receptors, RAGE (receptor for advanced glycation end-products), are highly expressed in active lesions of MS as well as in its counterpart animal model EAE (experimental autoimmune encephalomyelitis), while being expressed at normal levels in inactive lesions. This suggests a potential interaction of these molecules in the inflammatory process involved in pathogenesis (11). We might suggest that the reason behind not significant differences between the two groups could be the effect of sample size or the fact that simply this genetic variation is not notably altered among Iranian MS patients.

In conclusion, we studied 36 SNPs in *HLA-DRA*, *IL2RA*, and *HMGB1* genes in Iranian MS patients. Our results demonstrated significant associations of these genetic variants and MS disease. Further assessment of these SNPs in larger sample sizes along with other variants of mentioned genes would highly facilitate the recognition of causative variants in MS disease.

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