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Original Article

Association of *HLA* rs3135388 and rs9271366 Single-Nucleotide Polymorphisms With Multiple Sclerosis in the Chaharmahal and Bakhtiari Province, Iran

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Abstract

Background and aims: Genetics and environment are synergistic in multiple sclerosis (MS). Human leukocyte antigen (*HLA*) class II has a strong genetic association with MS. The aim of this study was to determine the association of *HLA* rs3135388 and rs9271366 single-nucleotide polymorphisms (SNPs) with MS.

Methods: The rs3135388, rs9271366, and rs422951 SNPs were genotyped in 173 Iranian relapsing-remitting MS (RRMS) patients and 200 matched healthy controls, using the polymerase chain reaction-restriction fragment length polymorphism method. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using statistical software to estimate the risk factor for disease. The allele and genotype frequencies of SNPs were determined based on data analysis using SPSS, version 20.

Results: Our results identified a strong association in the allele distribution for both rs3135388 and rs9271366 SNP, such that the A allele of rs3135388 (OR = 1.765; 95% CI: 1.071–2.909) and the G allele of rs9271366 were found to be more frequent in MS patients than in healthy controls (OR = 1.861; 95% CI: 1.025–3.3378). The mutated G allele for rs422951 SNP was relatively frequent (OR = 1.128; 95% CI: 0.745–1.707).

Conclusion: Our findings revealed the critical role of the rs3135388 and rs9271366 SNPs in MS disease progression. Genotyping MS patients could facilitate personalized medical management.

Keywords: Multiple sclerosis, Single-nucleotide polymorphisms, *HLA*, rs3135388, rs9271366

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Introduction

Multiple sclerosis (MS) is an extremely serious chronic inflammatory and degenerative disorder of the central nervous system (CNS).[1-4] The estimated number of people worldwide living with MS has increased to 2.8 million in 2020, which is a 30% increase compared to 2013,[5] and in Iran, the prevalence of the disease was estimated to be 29.3/100 000.[6] As the progression of MS is highly varied and uncertain, most patients initially experience reversible neurological deficits followed by progressive neurological deterioration over time.[7] MS has negative effects on the quality of life and deleterious effects on patients' psychological, social, and physical health aspects.[8,9]. There are four types of MS, including relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS).[10] It is supposed that immune dysregulation results in an autoimmune attack on the myelin of the CNS. Afterward, axonal injury and demyelination play key roles in disability progression.[11] Nevertheless, the etiology of MS remains unknown, but it seems to involve a combination of genetic susceptibleness and a non-genetic trigger that leads to repeated immune attacks on the CNS.[7] The current theories indicate an association between genetic factors such as human leukocyte antigen (*HLA*) class II in MS pathogenesis.[3,12-15]

The major histocompatibility complex (*MHC*) that is described in human *HLA* is a set of genes that play an important role in the rejection of transplants and the activation of specific cellular responses. In various populations, linkage studies have demonstrated that the *HLA* gene on chromosome 6p21.3 is the only part that is consistently associated with MS.[16-18] This gene system in MS is divided into three main sub-regions, including class I, class II, and class III regions. Many other genes, in addition to the *HLA* genes, are encoded in this region, which has many immunological functions and strong genetic associations with autoimmune diseases.[19] Some alleles of the *HLA DRB1* gene (*HLA* class II) have been related to increasing the risk of MS, while others may be considered protective factors for the disease.[20] Genetic studies suggest that the interaction between genetic predisposition and the immune system plays a significant role in MS pathogenesis.[18,21] An analysis of 47 429 MS patients and 68 374 control subjects identified 26 395

significant SNPs.[22] Accordingly, in the present report, we explored genetic associations between *HLA-DRB*1501* (rs3135388 and rs9271366) SNPs and *MHC* Class III SNP in NOTCH4 (rs422951) of *HLA* loci and MS in Chaharmahal and Bakhtiari province.

Materials and Methods

Study Population: Patients and Healthy Controls

This cross-sectional study was performed at the Cellular and Molecular Research Center of Shahrekord University of Medical Sciences from 2012 to 2015. A descriptive-analytical study was conducted, including 173 patients with MS and 200 healthy controls between the ages of 18 and 65 (Mean: 42.9 ± 5.8) from the same geographical area. The inclusion criteria for MS were defined according to the standard criteria for MS and the recommended diagnostic criteria for MS according to McDonald's criteria. Based on these criteria, if an individual has suffered from at least two clinical attacks and there is clear evidence of damage in at least two separate areas of the brain, they can be definitively diagnosed with MS. This is because they meet the requirements for dissemination in both space and time.[23,24] During the clinical evaluation of the patients, neurologists selected a healthy individual without a history of autoimmune or neurodegenerative disease as a control. Written informed consent was obtained from all participants, and blood samples were collected after completing a questionnaire for obtaining clinical data comprising demographics, age, gender, and employment.

DNA Extraction and SNP Genotype Analysis

Three SNPs were determined (rs335388, rs9271366, and rs422951). After searching the literature and the *National Center for Biotechnology Information* database for variations in the *HLA* locus. Blood samples (2 mL) were collected in 5% ethylenediaminetetraacetic acid tubes and stored at -20 ˚C until performing genotyping. Genomic DNA was extracted from the whole blood samples of patients and controls by a phenol-chloroform protocol, and the quality of the DNA was assessed by utilizing the NanoDrop Lite Spectrophotometer. Polymerase chain reaction (PCR)-restriction fragment length polymorphism was utilized for genotyping these SNPs. Table 1 presents the suitable primers used to amplify the matching target DNA sequence by PCR and the names of the enzymes.

PCR containing 2.5 µL PCR buffer (10X), 1.5 µL MgCl2 (50 mM), 0.5 µL of dNTP mix, 0.3 µL each of the 3 primers (50 pmol), 100 ng of genomic DNA (1 µL), and 0.5 U of Taq DNA polymerase 5 U/µL (0.1 µL) was performed in a final reaction volume of 25 µL. The annunciation temperature, product size, and restricted fragment of each variant are listed in Table 1. PCR products underwent electrophoresis in polyacrylamide gel (PAGE) 8% at 50 mA for 1.5 hours, followed by silver staining. For SNP genotyping, SNPs in the *HLA* locus (rs335388, rs9271366, and rs422951) were digested using the appropriate restriction enzyme (Fermentas). After digestion, the PCR product for each SNP was separately run in 8% polyacrylamide gel electrophoresis at 35–40 mA for 3 hours. DNA bands were visualized following the silver staining of the gel.

Statistical Analysis

Statistical analysis was performed using SPSS (version 20.0, SPSS Inc, Chicago, IL) for the Windows operating system [25]. The Chi-square test and Fisher's exact test were used for the evaluation of the genotypic and allelic frequencies between MS patients and healthy control individuals. "Utility Programs for Analysis of Genetic Linkage" were utilized to assess the concordance genotype distribution of variation and Hardy-Weinberg equilibrium.[26] A one-way ANOVA test was employed for the evaluation of SNP polymorphism levels within MS and genotypic subgroups, and a t-test was also used to compare healthy and sick people. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using statistical software to estimate the risk factor for disease. The usual *P* value of ≤ 0.05 was considered a generally significant level.

Results

The characteristics of 173 MS patients and 200 healthy controls enrolled in the study are summarized in Table 2.

We set out by studying all SNPs individually for association with MS and applying a trend test. Table 3 provides the analysis of the genotype frequency of rs3135388, rs9271366, and rs422951 SNPs in an *HLA* gene in MS cases and healthy controls about *P* values and OR.

The genotype frequencies for all genotypes did not deviate from the Hardy–Weinberg equilibrium in healthy controls and MS patients. In the rs3135388 SNP, a significant difference was found between MS patients and controls ($P = 0.039$). In other words, the results showed a significant association between MS and the rs3135388 SNP. In comparison, the G allele was distinguished as a

Table 1. Primer Sequences for Genotyping Three SNPs in *HLA* Loci (rs335388, rs9271366, rs422951, and RFLP Fragment) Restriction Fragment Length Polymorphism

SNP ID	Primer Sequence	Annealing Temperature	Restriction Enzyme	Product Size (bp)	Restricted Fragment (bp)
rs3135388	F: GGTCCTGGGGAATATATGTG R: AGAGATCTCCCAACAAACC	54	*MmeI*	197	38, 159
rs9271366	F: TTGATCAATTTCATTTAGTTCA R: TAGATAAGTCAACTTATCACG	50	MnlI	*301*	35, 266
rs422951	F: CCCTACTCATAGGGCTCC R: CCACCTCCTGATACCCTC	61	*HaeIII*	349	91, 258

Note. SNP: Single-nucleotide polymorphisms; RFLP: Restriction fragment length polymorphism.

Table 2. Demographic Features of MS Patients and Healthy Controls

Index	MS (173)	Control (200)
Age (Mean ± SD)	45.8 ± 5.5	42.9 ± 5.8
Male	32	44
Female	141	156
RRMS female/male	114/27	-
SPMS female/male	24/5	-
PPMS female/male	3/NS	-

Note. MS: Multiple sclerosis; SD: Standard deviation; RRMS: Relapsing-remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis; PPMS: Primary progressive multiple sclerosis; NS: Not seen.

significant factor in MS pathogenesis, and a significant association was observed between this allele and MS (OR = 1.765; 95% CI: 1.071–2.909). Accordingly, the AA genotype represented a significant association between the genotype and MS ($P = 0.021$). The genotype distribution of rs9271366 SNP also was different between MS patients and healthy controls ($P = 0.010$). In this SNP, the G allele is reported as a risk variant in MS progression; the G allele, in comparison to the A allele, is distinguished as a significant factor for MS pathogenesis, and a significant association was found between this allele and MS (OR = 1.861; 95% CI: 1.025–3.3378). Accordingly, in the AG genotype, a significant association was detected between genotype and MS ($P = 0.005$). However, for the rs422951 SNP of the *HLA*, genotype distribution was not significantly different between MS patients and healthy controls ($P = 0.20$). Based on the analysis of the *HLA* genotypes and MS subtypes, no association was observed between MS subtypes and this SNP ($P > 0.05$, Table 4).

Discussion

The purpose of this study was to determine whether three SNPs associated with MS clinical course and disability progression were related to participants with a first neurological presentation of symptoms suggestive of CNS demyelination. The results of this study mainly demonstrated the association of rs3135388 and rs9271366 polymorphisms in the *HLA* gene with MS, while there was no association between MS and the other polymorphism (rs422951). For the rs3135388 variant, AA genotypes significantly increased the risk of MS, and for the rs9271366 variant, the AG genotype significantly increased the risk of MS. SNP rs422951 polymorphism showed no relationship with MS in this group. These findings indicate the importance of SNs rs3135388 and rs9271366 SNs polymorphisms in the population of western Iran.

In the United Kingdom, the United States, Australia, New Zealand, Germany, Switzerland, and the Netherlands, genome-wide association study signals reveal a linkage disequilibrium block containing SNPs (rs3135388, rs3129889, rs9271366, and rs3104373) associated with MS in Caucasian populations.[27-31] In line with this study, another study reported that there is an association between MS and five genetic variants (*INAVA* rs7522462, *EOMES* rs11129295, *C6orf10* rs3129934, *CD86* rs9282641, and *GPR65* rs2119704). The rs3129934 polymorphism in the major histocompatibility region showed the strongest association (OR = 2.16, CI: 1.85–2.74, $P = 2.53 \times 10^{-13}$).[32]

For rs3135388 SNP, the AA genotypes significantly enhanced the risk of MS, and for rs9271366 SNP, the AG genotypes considerably enhanced the risk of MS. In agreement with the literature, we represent some of these considerations in the following section.

Based on rs3135388 investigations, Zivkovic et al demonstrated a high frequency of the tag SNP for *HLA DRB1*1501*, rs3135388, in MS patients. The rs3135388 SNP is practicable as a marker for the *DRB1*1501* allele; this marker serves to detect this polymorphism in MS patients' clinical tests.[33,34] Alcina et al discovered that *DRB1*1501*, an allele of rs3135388 is associated with high expression of *DRB1*, *DRB5*, and *DQB1* genes in a Caucasian population. It has been found that the MS-risk AA genotype carriers of rs3135388 were associated with higher expression of *DQB1*, *DRB5*, and *DRB1*, than the non-risk GG genotype.[35] Benešová et al investigated the association of *HLA-DRB1*1501* tagging rs3135388 SNP with MS susceptibility, disability, and gender differences. Their findings indicated a significant difference in genotype distribution and allele frequency between MS patients and healthy controls. These findings also revealed that AA homozygotes and GA heterozygotes were more frequent in MS patients.[36] In concurrence with the rs9271366 investigations, Field et al reported that rs9271366 was strongly associated with SNP, a proxy for the *HLA-DRB1*15:01* allele.[11,37] According to rs422951 investigations, Huang et al demonstrated that the G allele of NOTCH 4 rs422951 was negatively linked with MS. However, this allele was also significantly associated with MS as an independent resistant allele for this disease in Japanese. Conversely, in this study, the G allele was not associated with any clinical factors.[33,38] The haplotype formed by rs422951 and *HLA-DRB1*15* influences MS pathogenesis and development, according to Huang and colleagues' findings.[38] Genetic factors contribute to MS susceptibility, but clinical manifestations and course are also influenced by variants. To provide appropriate counseling, open new avenues for drug development, and help individuals select the most appropriate treatment for their condition, it is critical to understand the genetic determinants of this period of the disease's clinical course. Anti-inflammatory, immunomodulatory, and immunosuppressive drugs, which act through cytokine signaling, need to be evaluated for their role in predicting responses to variants. To be certain that our results hold up, they must be replicated in other longitudinal cohorts.

Conclusion

The results showed that MS disease is associated with the A allele of the rs3135388 polymorphism. The frequency of this allele in the control group and MS patients was different and statistically significant, and there was also a statistical relationship between the G allele of the

Table 3. The Analysis of Genotype Frequencies of Three SNPs in *HLA* in Multiple Sclerosis Cases and Healthy Controls

Polymorphism	Genotype	Control (200)		MS (173)		OR	95% CI	*P* Value
		No.	%	No.	%			
rs3135388	*GG*	*153*	*76.5*	113	65.31	1		^{Trend}0.039
	GA	27	13.5	29	16.77	1.454	(0.816-2.592)	0.200
	AA	20	10	31	17.91	2.099	(1.138-3.872)	0.021
	[GA] + [AA] vs [GG]					1.713	(1.090-2.693)	0.019
	[A] vs [G]					1.765	(1.071-2.909)	0.025
rs9271366	*AA*	159	79.5	114	65.90	1		^{Trend}0.010
	AG	*40*	*20*	56	32.37	1.953	(1.218-3.129)	0.005
	GG	1	0.5	3	1.5	4.184	(0.431-40.74)	0.200
	[AG] + [GG] vs [AA]					2.007	(1.260-3.197)	0.003
	[G] vs [A]					1.861	(1.025-3.337)	0.039
rs422951	*AA*	76	38	67	38.72	1		^{Trend}0.20
	AG	94	47	69	39.89	0.833	(0.530-1.301)	0.4
	GG	30	15	37	21.39	1.399	(0.781-2.506)	0.25
	[AG] + [GG] vs [AA]					0.970	(0.638-1.474)	0.88
	[G] vs [A]					1.128	(0.745-1.707)	0.57

Note. MS: Multiple sclerosis; SNP: Single-nucleotide polymorphism; MS: Multiple sclerosis; OR: Odd ratio; CI: Confidence interval; N: Number, vs: Versus.

Table 4. Association Between *HLA* Genotypes and MS Subtypes

Polymorphism	rs3135388				rs9271366				rs422951			
	GG	GA	AA	*P* Value	*AA*	AG	*GG*	*P* Value	*AA*	AG	*GG*	*P* Value
RRMS	*74*	*17*	*21*	0.75	78	42	3	0.21	46	46	51	0.78
SPMS	28	9	7	0.22	25	9	0	0.57	13	13	11	0.33
PPMS	11	3	3	0.14	11	5	0	0.76	8	*7*	*7*	0.30

Note. RRMS: Relapsing-remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis; PPMS: Primary progressive multiple sclerosis.

rs9271366 polymorphism and the MS disease. There was no significant difference between the genotype distribution of rs422951 polymorphism between MS patients and healthy controls, and it can be stated that the AG genotype is a risk factor for the development of MS disease. However, no significant relationship was found between these polymorphisms and subtypes of MS disease, and these polymorphisms cannot be used as biomarkers to diagnose subtypes of the disease.

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Competing Interests

The authors declare no conflict of interests.

Ethical Approval

The Research Ethics Committee of Shahrekord University of Medical Sciences approved the study (ID: 92-9-7).

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