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Association of rs12487066, rs12044852, rs10735781, rs3135388, rs6897932, rs1321172, rs10492972, and rs9657904 Polymorphisms with Multiple Sclerosis in Iranian Population

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ABSTRACT

Objectives: Multiple sclerosis (MS) is a chronic disease of the central nervous system. The pathogenesis of MS is best described by a multifactorial model incorporating interactions between genetic and environmental factors with the role of genetic factors increasingly taken into account. The main goal of this study was to investigate the associations of rs12487066, rs12044852, rs10735781, rs3135388, rs6897932, rs1321172, rs10492972, and rs9657904 polymorphisms with MS in the Iranian population. ***Methods***: A total of 83 patients with MS (82.0% female and 18.0% male; mean age = 35.2±8.6 years) and 100 physically and mentally healthy subjects (81.0% female and 19.0% male; mean age = 40.4±6.4 years) were selected using convenient sampling. A 5 mL blood sample was taken from each case and control patient. We used the tetra-primer ARMS-PCR method to genotype the desired polymorphisms. The associations between polymorphisms and the disease were studied based on codominant, dominant, recessive, and overdominant models. ***Results:*** The rs10735781 polymorphism was codominantly (p = 0.029), overdominantly (p = 0.008), and dominantly (p = 0.009) associated with the disease. The rs6897932 was also found to be codominantly (p = 0.012), dominantly (p = 0.019), and recessively (p = 0.011) associated with the disease. ***Conclusions:*** We found an association between the rs10735781 and rs6897932 polymorphisms on the *EVI5* and *IL7RA* genes, respectively, with increased MS in the Iranian population. Therefore, single nucleotide polymorphisms in the *EVI5* and *IL7RA* genes can be considered a prognostic marker of MS.

Multiple sclerosis (MS) is a chronic and progressive disease of the central nervous system, affecting about two million individuals worldwide.[1] It is an autoimmune-mediated demyelinating disorder of the central nervous system that has four clinical forms: secondary progressive (SPMS), relapsing-remitting (RRMS), primary progressive (PPMS), and progressive relapsing (PRMS).[2] It usually affects people between the ages of 20 and 50 years old. It is diagnosed more often in women than men and is estimated to be the most common cause of neurological disability in young adults.[1] Common early signs of MS include vision problems, pain spasm, tingling, numbness, weakness, fatigue, and cognitive impairments. With the progression of the disease, it can disrupt the daily activities of the affected individuals.[3]

For many years, MS was considered an autoimmune disorder, mainly of interest to immunologists. Researchers believed that the placement of viral antigens in the structure of the myelin sheath could cause the immune system to attack and eliminate the neuron.[4] However, in the

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mid-1990s, it was recognized that a neurodegenerative process was responsible for MS development that was unresponsive to immunosuppression. The neuropathology of MS emphasized the axonal changes followed by demyelination, which leads to neural cell death and disease progression.[5] Autoreactive pathogenic helper T cells play a prominent role in the pathogenesis of MS.[6]

The pathogenesis of MS is best described by a multifactorial model incorporating interactions between genetic and environmental factors, including nutrition, climate, and infection.[7,8] The role of genetic factors is increasingly taken into account, as the environmental effects are also largely influenced by the genetic characteristics of individuals.

The primary efforts to identify susceptibility genes were performed through linkage analysis in families with a high prevalence of the disease. Although the studies failed to find a prominent genetic association, the human leukocyte antigen region was identified to have a very strong association with MS.[9] The first genome-wide association study (GWAS) in MS was carried out by the International Multiple Sclerosis Genetics Consortium (IMSGC), and several GWASs and candidate gene association studies provided evidence for the association of more than 200 susceptibility variants with MS.[1]

For example, a 2014 study demonstrated a significant association between the ApaI polymorphism in the vitamin D receptor gene and MS risk in the homozygous and codominant models.[10] Another study in the same year showed a link between polymorphism in the interleukin (IL-2) receptor alpha gene and MS.[11] In 2015, another study investigated the association between 3061AG polymorphism located in the very-late antigen 4 gene and the risk of MS,[12] and more recently the authors of one study suggested that the aryl hydrocarbon receptor nuclear translocator-like rs3789327 CC genotype is associated with a higher risk for MS.[13]

The main goal of our study was to investigate the association of rs12487066 *(CBLB)*, rs12044852 *(CD58)*, rs10735781 *(EVI5)*, rs3135388 (*HLA)*, rs6897932 *(IL7R)*, rs1321172 *(PDE4B)*, rs10492972 *(KIF1B)*, and rs9657904 *(CBLB)* polymorphisms with MS in the Iranian population. The results of this study can, in addition to, confirm or reject the results of previous studies, consider the importance of the examined polymorphisms as biomarkers for better diagnosis of MS.

METHODS

The case group consisted of 83 patients (82.0% females and 18.0% males) with MS referred to Loghman Hospital in Tehran, Iran. Moreover, in this study, all procedures were performed on human participants.

Two experienced neurologists confirmed MS in all cases. All patients were also evaluated with McDonald criteria. The McDonald criteria maintained a scheme for diagnosing MS-based solely on clinical grounds and try to prove the existence of demyelinating lesions.[14] According to their reports, as well as clinical evaluations by relevant specialists, patients did not experience any other neurological or mental disorder. Besides the case group, 100 physically and mentally healthy subjects (81.0% female and 19.0% male) were selected as a control group. None of the cases and controls had a drug addiction or alcohol abuse. The mean age of the case group was 35.2±8.6 years, and the mean age of the control group was 40.4±6.4 years. All subjects participated in the study with informed consent. The ethical committee of the Taban Genetics Center, Tehran, Iran, approved this study.

A 5 mL blood sample was taken from each case and control patient and transferred to the lab for genetic analysis in an EDTA tube. DNA isolation was performed using GENET BIOkit (Global Gene Network, Korea) based on the company protocol. The quantity and quality of DNA were evaluated by the Denovix NanoDrop device (Model Ds-11, USA) and gel electrophoresis, respectively. Amplification-refractory mutation system in conjunction with polymerase chain reaction (tetra-primer ARMS-PCR) was used to genotype the desired polymorphisms, rs12487066, rs12044852, rs10735781, rs3135388, rs6897932, rs1321172, rs10492972, and rs9657904. Using four specifically designed primers, tetra-primer ARMS-PCR allows the amplification of sample DNA only when it contains the target variant.

The tetra-primer ARMS-PCR was carried out in a final volume of 25 µL comprising 1 µL of the DNA sample, 2.5 µL of each outer primer, 2.5 µL of the forward inner primer, 2.5 µL of the reverse inner primer, 0.5 µL dNTPs, 1 µL $MgCl_2$, 2.5 µL Buffer and 0.2 µL of TaDNA polymerase (Sinaclon, Iran). Amplicons were analyzed by 1% agarose gel electrophoresis in TAE 0.5 Xbuffer (Sinaclon, Iran), containing SYBR safe stain. Also, 10% of the

Table 1: All of the primers used in this study.

SNPs	Genes	Sequence
rs12487066	*CBLB*	FO: TTTTCTACTATTGGGTACCCAGAGC RO: CTTTTGTGGACTTCTTCCTCCC FI: CAAGAAAAACTTAACGACTAAAAGTGCTT RI: TTTGCTACAGCACCTTGTCAGTTAG
rs12044852	*CD58*	FO: CAAGGAAGTCATGCTGGAACTGACAT RO: CATGGACTTCATTGCTACAGCATTGA FI: GGATACACACGTGATTCCTAACGGC RI: CCCTTGCCCTCCTCATTCCAT
rs10735781	*EVI5*	FO: AAATGCTAACAGAATTCATCAC RO: CTTGTCTTTTTTCACTGTTGTT FI: CAGACAAAAGTATAAAACTTACTGC RI: GGATCATCCTTTTTGTAACAC
rs3135388	*HLA*	FO: GTCTAACAGAATGGGTAAGGCCAGTCTT RO: GGTCCTGGGGAATATATGTGATCCTTT FI: CAGTAGAGATCTCCCAACAAACCCAC RI: GTCCTCATCAGGAAAACCTAAAGTGTGA
rs6897932	*IL7RA*	FO: CCCTCCATAAAGCTGTCAAATATGTC RO: CAATAAATGGGGCTTAAGCTCTGACT FI: AGGGGAGATGGATCCTATCTTACTCAT RI: GAGAAAAAACTCAAAATGCTGAGGG
rs1321172	*PDE4B*	FO: ATCCTATTGAGCGGGGCTCTCAAATTT RO: GTGCAAGAGAATGCAAAAAGAAGTGAA FI: GATTCTCTGCTCCACTAAGGAGTAACTGC RI: CTCACTCTCTTGCTCATTGCAAATCTC
rs9657904	*CBLB*	FO: ACTAACTTGTACCACTGCATCTTCCTC RO: CCAAAATGTATGATAGGACCTTCAGTTG FI: TTTCAAGTAGCTAAGGCTGAACTAATCCT RI: TTGTTCTTTTTTTTTTTTTTTATGAGTGGG
rs10492972	*KIF1B*	FO: ATTTCAAGTGACCTCACATTGGCTA RO: TTAAAACTGTGAAAACCACTAACCTTTCA FI: TTCCTGTGGTTTTCGCTACAATTATC RI: CAGGATAGAAAAACCTGACCCGT

SNPs: single nucleotide polymorphisms; FO: forward outer primer; RO: reverse outer primer; FI: forward inner primer; RI: reverse inner primer.

samples were sequenced to ensure the amplification of the target regions. All of the primer sets used in this study were designed using the Primer 1 online tool (primer1.soton.ac.uk/primer1.html) and are shown in Table 1.

The initial denaturation cycle of PCR was carried out at 95 °C for 5 minutes for all samples. The amplification of all samples was performed through 30 cycles including denaturation at 95 °C for 30 seconds, annealing [Table 2], and extension at 72 °C for 30 seconds, following by a final extension step at 72 °C for 10 minutes.

The validity of the design of the primer sets was checked by NCBI BLAST before synthesis (Sinaclon, Iran).

We used central and dispersion indices to describe the results statistically. We used the Shapiro-Wilks test to test for normality. To compare the mean age

Table 2: PCR temperature protocol.

Genes	Annealing
HLA (rs3135388)	61°- 30 s
IL7RA (rs6897932)	59°- 30 s
KIF1B (rs10492972)	60°- 30 s
EVI5 (rs10735781)	59°- 30 s
CD58 (rs12044852)	62°- 30 s
CBLB (rs12487066)	58°- 30 s
PDE4B (rs1321172)	61°- 30 s
CBLB (rs9657904)	57 °- 30 s

PCR: polymerase chain reaction.

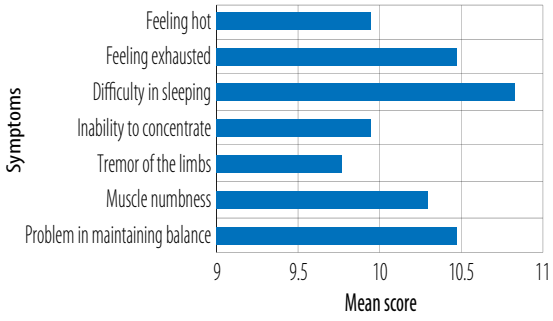

Figure 1: The mean frequency of symptoms associated with multiple sclerosis in patients.

Table 3: The allele frequency of all examined polymorphisms.

SNPs	Allele frequency	Case group n (%)	Control group n (%)	Case/control difference (χ^2, *p*-value)
rs10735781	C	73 (88.0)	95 (95.0)	2.99, 0.080
	G	10 (12.0)	5 (5.0)	
rs6897932	C	43 (51.8)	65 (65.0)	3.26, 0.070
	T	40 (48.2)	35 (35.0)	
rs12044852	C	72 (86.7)	88 (88.0)	0.06, 0.790
	A	11 (13.3)	12 (12.0)	
rs1321172	G	56 (67.5)	68 (68.0)	0.005, 0.930
	C	27 (32.5)	32 (32.0)	
rs12487066	C	29 (34.9)	45 (45.0)	1.90, 0.160
	T	54 (65.1)	55 (55.0)	
rs3135388	C	60 (72.3)	78 (78.0)	0.79, 0.370
	T	23 (27.7)	22 (22.0)	
rs9657904	T	71 (85.5)	86 (86.0)	0.007, 0.920
	C	12 (14.5)	14 (14.0)	

SNPs: single nucleotide polymorphisms.

between the two groups, the independent *t*-test or 'Wilcoxon's nonparametric test was used, based on how data were distributed. The associations between polymorphisms and the disease were studied based on the codominant, dominant, recessive, and overdominant models. All statistical analyses were performed using the IBM SPSS Statistics 25 software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

RESULTS

We found no significant difference between the case and the control group in terms of the male/female ratio (χ = 0.03, p = 0.870*)*. The assessment of the symptoms of the disease indicated that the most common symptoms included sleep disorders, difficulty in maintaining balance, and feeling exhausted [Figure 1].

Independent *t*-test showed that the mean age of the two groups were significantly different (t = -4.54, $p < 0.001$).

Statistical analysis showed that the allele frequencies were not significantly different between the two groups [Table 3].

Genetic analysis demonstrated that rs10735781 polymorphism was associated with MS codominantly (p = 0.029), overdominantly (p = 0.008), and dominantly (p = 0.009), and considering the significance levels the overdominant association seemed to be the preferred model [Table 4]. The rs6897932 polymorphism was found to be codominantly (p = 0.012), dominantly (p = 0.019), and recessively

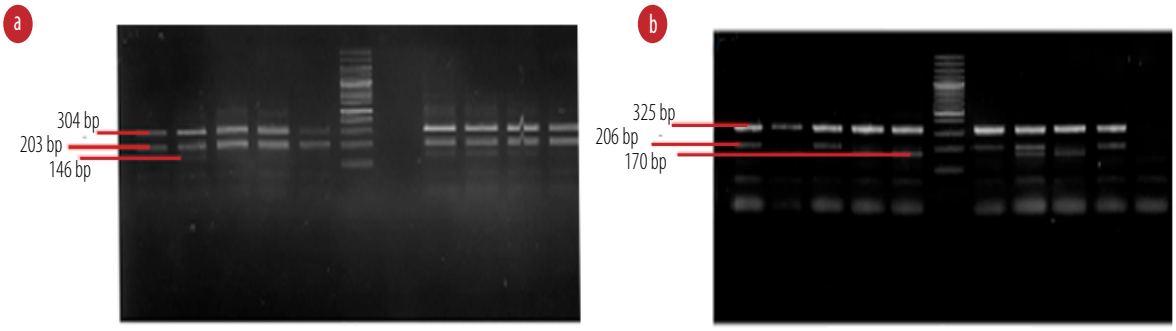

Figure 2: The amplified sequences of **(a)** rs10735781 and **(b)** rs6897932 through the tetra-primer amplification-refractory mutation system in conjunction with polymerase chain reaction method.

Table 4: Genetic analysis results based on the multiple sclerosis model.

Association with response status (n = 183)					
Model	**Genotype**	**Case group n (%)**	**Control group n (%)**	**OR (95% CI)**	***p*-value**
rs10735781					
Codominant	C/C	64 (77.1)	91 (91.0)	1.00	0.029
	C/G	18 (21.7)	8 (8.0)	0.31 (0.13–0.76)	
	G/G	1 (1.2)	1 (1.0)	0.70 (0.04–11.45)	
Dominant	C/C	64 (77.1)	91 (91.0)	1.00	0.009
	C/G-G/G	19 (22.9)	9 (9.0)	0.33 (0.14–0.78)	
Recessive	C/C-C/G	82 (98.8)	99 (99.0)	1.00	0.890
	G/G	1 (1.2)	1 (1.0)	0.83 (0.05–13.45)	
Overdominant	C/C-G/G	65 (78.3)	92 (92.0)	1.00	0.008
	C/G	18 (21.7)	8 (8.0)	0.31 (0.13–0.77)	
Log-additive	-	-	-	0.40 (0.18–0.87)	0.016
rs6897932					
Codominant	C/C	22 (26.5)	43 (43.0)	1.00	0.012
	C/T	40 (48.2)	46 (46.0)	0.59 (0.30–1.15)	
	T/T	21 (25.3)	11 (11.0)	0.27 (0.11–0.65)	
Dominant	C/C	22 (26.5)	43 (43.0)	1.00	0.019
	C/T-T/T	61 (73.5)	57 (57.0)	0.48 (0.26–0.90)	
Recessive	C/C-C/T	62 (74.7)	89 (89.0)	1.00	0.011
	T/T	21 (25.3)	11 (11.0)	0.36 (0.16–0.81)	
Overdominant	C/C-T/T	43 (51.8)	54 (54.0)	1.00	0.770
	C/T	40 (48.2)	46 (46.0)	0.92 (0.51–1.64)	
Log-additive	-	-	-	0.53 (0.34–0.81)	0.003
rs12044852					
Codominant	C/C	63 (75.9)	75 (75.0)	1.00	0.980
	C/A	19 (22.9)	24 (24.0)	1.06 (0.53–2.11)	
	A/A	1 (1.2)	1 (1.0)	0.84 (0.05–13.70)	
Dominant	C/C	63 (75.9)	75 (75.0)	1.00	0.890
	C/A-A/A	20 (24.1)	25 (25.0)	1.05 (0.53–2.07)	
Recessive	C/C-C/A	82 (98.8)	99 (99.0)	1.00	0.890
	A/A	1 (1.2)	1 (1.0)	0.83 (0.05–13.45)	
Overdominant	C/C-A/A	64 (77.1)	76 (76.0)	1.00	0.860
	C/A	19 (22.9)	24 (24.0)	1.06 (0.53–2.12)	
Log-additive	-	-	-	1.03 (0.55–1.94)	0.920
rs1321172					
Codominant	G/G	39 (47.0)	47 (47.0)	1.00	1.000
	C/G	36 (43.4)	43 (43.0)	0.99 (0.54–1.83)	
	C/C	8 (9.6)	10 (10.0)	1.04 (0.37–2.88)	
Dominant	G/G	39 (47.0)	47 (47.0)	1.00	1.000
	C/G-C/C	44 (53.0)	53 (53.0)	1.00 (0.56–1.79)	
Recessive	G/G-C/G	75 (90.4)	90 (90.0)	1.00	0.930
	C/C	8 (9.6)	10 (10.0)	1.04 (0.39–2.77)	
Overdominant	G/G-C/C	47 (56.6)	57 (57.0)	1.00	0.960
	C/G	36 (43.4)	43 (43.0)	0.98 (0.55–1.77)	
Log-additive	-	-	-	1.01 (0.65–1.57)	0.970
rs12487066					
Codominant	T/T	35 (42.2)	30 (30.0)	1.00	0.150
	C/T	38 (45.8)	50 (50.0)	1.54 (0.81–2.93)	
	C/C	10 (12.1)	20 (20.0)	2.33 (0.95–5.75)	

Table 4: Genetic analysis results based on the multiple sclerosis model.

-continued

Model	Genotype	Association with response status (n = 183) Case group n (%)	Control group n (%)	OR (95% CI)	*p*-value
Dominant	T/T	35 (42.2)	30 (30.0)	1.00	0.087
	C/T-C/C	48 (57.8)	70 (70.0)	1.70 (0.92–3.13)	
Recessive	T/T-C/T	73 (88)	80 (80.0)	1.00	0.140
	C/C	10 (12.1)	20 (20.0)	1.82 (0.80–4.15)	
Overdominant	T/T-C/C	45 (54.2)	50 (50.0)	1.00	0.570
	C/T	38 (45.8)	50 (50.0)	1.18 (0.66–2.12)	
Log-additive	-	-	-	1.53 (0.99–2.35)	0.050
rs3135388					
Codominant	C/C	43 (51.8)	60 (60.0)	1.00	0.330
	C/T	33 (39.8)	36 (36.0)	0.78 (0.42–1.44)	
	T/T	7 (8.4)	4 (4.0)	0.41 (0.11–1.49)	
Dominant	C/C	43 (51.8)	60 (60.0)	1.00	0.270
	C/T-T/T	40 (48.2)	40 (40.0)	0.72 (0.40–1.29)	
Recessive	C/C-C/T	76 (91.6)	96 (96.0)	1.00	0.210
	T/T	7 (8.4)	4 (4.0)	0.45 (0.13–1.60)	
Overdominant	C/C-T/T	50 (60.2)	64 (64.0)	1.00	0.600
	C/T	33 (39.8)	36 (36.0)	0.85 (0.47–1.55)	
Log-additive	-	-	-	0.71 (0.44–1.15)	0.160
rs9657904					
Codominant	T/T	60 (72.3)	72 (72.0)	1.00	0.970
	C/T	21 (25.3)	25 (25.0)	0.99 (0.51–1.95)	
	C/C	2 (2.4)	3 (3.0)	1.25 (0.20–7.73)	
Dominant	T/T	60 (72.3)	72 (72.0)	1.00	0.970
	C/T-C/C	23 (27.7)	28 (28.0)	1.01 (0.53–1.94)	
Recessive	T/T-C/T	81 (97.6)	97 (97.0)	1.00	0.810
	C/C	2 (2.4)	3 (3.0)	1.25 (0.20–7.68)	
Overdominant	T/T-C/C	62 (74.7)	75 (75.0)	1.00	0.960
	C/T	21 (25.3)	25 (25.0)	0.98 (0.50–1.92)	
Log-additive	-	-	-	1.03 (0.59–1.82)	0.910

OR: odds ratio; CI: confidence interval.

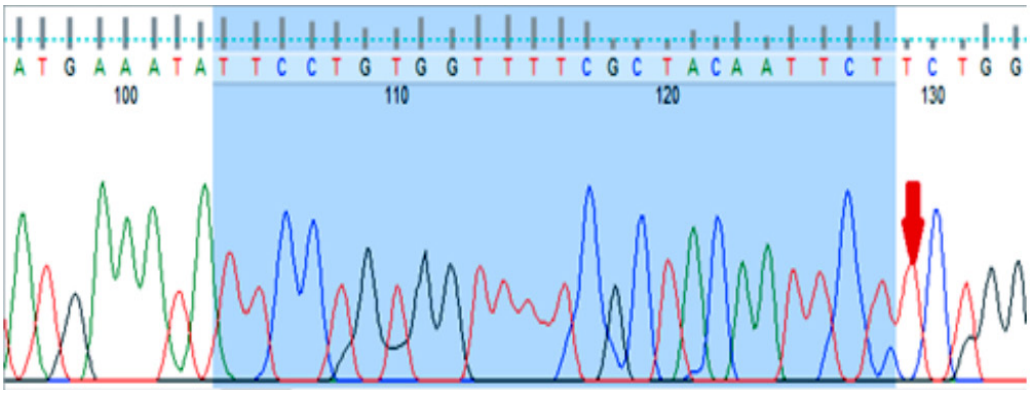

Figure 3: Image of gene sequencing *KIF1B* gene (rs10492972). This image related to homozygous normal genotype (TT).

(p = 0.011) associated with the disease, and considering the significance levels, the recessive association was the preferred model. The rest of the polymorphisms did not show any significant association with the disease [Table 4]. The amplified sequences of the two polymorphisms are shown in Figure 2. The rs10492972 polymorphism genotype was similar in all subjects (TT) and, therefore, it was not analyzed. The amplified sequence of rs10492972 is shown in Figure 3.

DISCUSSION

We investigated the association between rs12487066, rs12044852, rs10735781, rs3135388, rs6897932, rs1321172, rs10492972, and rs9657904 polymorphisms and MS in an Iranian population. Our results demonstrated that the rs10735781 polymorphism is overdominantly associated with the disease. Additionally, rs6897932 polymorphism is codominantly (p < 0.050) and recessively (p < 0.050) associated with the disease. No significant association was found between other polymorphisms and the disease.

The minor allele frequency of rs10735781 polymorphism in the general population is reported to be about 0.35,[15] while in our study, it was 0.12 in the case group and 0.05 in the control group. Although achieving a precise conclusion requires studying larger populations, it could be inferred that the minor allele is associated with certain health conditions. This polymorphism is located at the *EVI5* gene, which regulates cell cycle progression and cytokines by stabilizing the F-Box gene product.[15]

A 2008 study demonstrated a significant association between *EVI5* and MS.[16] The authors of the study further validated the risk effect of *EVI5* for the general MS population in an independent set of 1318 MS patients. Although a more recent study found no significant association of *EVI5* with MS,[15] our study showed a significant association between this gene and the disease. Perhaps the main reason for the mentioned contradictions is the difference in the size and ethnicity of the study samples.

It is still ambiguous how the rs10735781 polymorphism plays a role in the pathology of the disease. It remains to be investigated how and to what extent it could influence T cell lymphomagenesis and function. Functional immunological studies stratified according to *EVI5* genotype will clarify the role of this gene in MS pathology.

The minor allele frequency of rs6897932 in the general population is reported to be 0.17,[17] while in our study it was 0.40 in the case group and 0.35 in the control group. Both of which have a great deal of difference with the general population.[17]

One of the most important hypotheses about the incidence of MS is the hypothesis of autoimmunity, which suggests that MS may be an autoimmune disease. Furthermore, most current MS therapies are based on drugs that attenuate the immune response.[18] Therefore, it is not unlikely that genetic abnormalities in immune system cells are involved in MS pathology.

Some studies have confirmed that variation within the major histocompatibility complex (MHC) exerts a great risk in MS incidence.[19] Furthermore, using the ImmunoChip genotyping assay, a study by the IMSGC found 103 discrete loci outside of the MHC region.[20]

There is a significant association between genetic variation in the *IL7R* gene and patients with progressive MS.[21] More recently, an epistatic interaction that controls the *IL7R* splicing and increases MS risk was identified.[22] Additionally, three *IL7RA* loci (rs3194051, rs987107, and rs11567686) are thought to be significantly associated with increasing MS risk.[23] Our findings are in line with previous studies.[23]

Although the association of rs6897932 polymorphism with MS has not been investigated, its association with other autoimmune disorders, such as systemic lupus erythematosus,[24] and asthma has been established.[25] It is believed that rs6897932 polymorphism regulates monocyte surface *IL7R* induction by lipopolysaccharides and tumor necrosis factor stimulation.[26]

On the other hand, the associations of rs12487066,[27] rs3135388,[28] rs6897932,[29] rs1321172,[30] rs9657904, and rs10492972[31] polymorphisms with MS have been found in previous studies, but we did not find such associations. The reason for this difference can be related to other risk factors. For example, that ethnicity,[32] geographical coordinates,[33] and family history[34] can affect the risk of MS. Also, differences in the sample size and the male/female ratio can also lead to differences in results.

CONCLUSION

MS is a multifactorial disease, thus determining the genetic polymorphisms of genes might be an essential strategy as a marker of genetic predisposition to MS. This study is illustrated the association between the two polymorphisms, rs10735781 and rs6897932 on *EVI5* and *IL7RA* genes, respectively, with increased MS in the Iranian population. *EVI5* is mainly involved in cell cycle regulation and cytokinesis, while *IL7RA* predominantly acts as an immune response mediator, and their role in MS has been somewhat identified in previous studies. Thus, these results suggest that evaluation of *EVI5* and *IL7RA* genetic polymorphisms could be considered a prognostic mechanism to identify individuals at higher risk of developing MS. However, structural and functional genetic studies in the future on larger populations can better capture the importance of these two genes in the onset of the disease.

Disclosure

The authors declared no conflicts of interest. No funding was received for this study.

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