Research Article

The *HLA rs9267649* and *CYP24A1 rs2248359* Variants are Associated with Multiple Sclerosis: A Study on Iranian Population

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Background: Studies have shown that MS results from synergism between genetic and environmental factors. As a genetic factor, the *rs9267649* variant through the regulatory effect on the *HLA-DRB1* expression is involved in the MS development. In addition, vitamin D deficiency through involvement of *rs2248359* variant of *CYP24A1* has shown to play important role in the risk of MS.

Objectives: The aim of this study was to investigate both the *HLA rs9267649* and *CYP24A1 rs2248359* variants with risk of multiple sclerosis (MS) in Iranian population.

Materials and Methods: The *rs9267649* and *rs2248359* variants were genotyped in 82 Iranian Relapsing-Remitting Multiple Sclerosis (RRMS) patients and 100 matched healthy controls, using the PCR-RFLP method. The genotype and allele frequencies were calculated and statistically analyzed.

Results: A significant difference was found in the allele distribution for the both *rs9267649* and *rs2248359* variants, such that the A allele of *rs9267649* and the C allele of *rs2248359* were found to be more frequent in MS patients than in the healthy controls (p-value: 0.009, OR: 2.264, 95% CI: 1.211-4.231 and p-value: 0.028 OR: 1.594, 95% CI: 1.052-2.415), respectively.

Conclusions: The present research results provide further evidence on the association of the two variants: *rs9267649* of the *HLA* and *rs2248359* of the *CYP24A1* gene with MS etiology and an increased risk of MS in Iranian RRMS patients. However, further large-scale investigations in various ethnicities and in the functional genomics level are demanded to confirm our findings.

Keywords: CYP24A1, HLA, Multiple Sclerosis, *rs9267649, rs2248359*

1. Background

It is estimated that more than 2.5 million people are affected by MS worldwide. Similar to many other type of diseases, the prevalence of MS is influenced by the differences in the ethnic origin, age and size of the population, as well as environmental and geographical variations. Regarding Iran, recent reports have proposed a trend toward high-prevalence for the MS (1,2).

With an unknown detailed molecular etiology, MS is suggested as a multifactorial disorder; a chronic autoimmune disease, and the leading cause of neurological disability in young adults characterized by inflammation, neurodegeneration, and demyelination of the central nervous system (CNS) (3,4). Among the genetic risk factors, the human leukocyte antigen (*HLA*) super locus on chromosome 6p21 composed of

Class I, II, and III has widely been recognized as the most influential factor in the MS etiology. Belonging to the class II genes, the *HLA-DRB1*15:01* allele is widely recognized as the most significant factor with a key role in the immune system function through encoding and involvement in the antigenic peptide presentation to the T cells. It has been proposed that *HLA-DRB1*15:01* allele alters the beta-chain amino acid repertoire of the *HLA* class II, as a result, structural alteration of the antigenic peptide-binding groove results in a change in the T cell response and consequently the autoimmune responses in the central nervous system (CNS) (5,6)Other factors could play a profound role in the MS etiology among which the degree of gene methylation in case of *HLA-DRB1*. In an unmethylated state, *HLA-DRB1* shows a higher level of expression in monocytes in *DRB1*15:01* carriers. In agreement with this notion recent studies have shown that *rs9267649* variant is associated with an increased DNA methylation of the *HLA-DRB1*; as a result a reduced expression of *HLA-DRB1* which modulates the *DRB1*15:01* effects (7).

Among the environmental risk factors, low blood vitamin D levels in the majority of MS patients have shown to be associated with the pathogenesis of MS, presumably through the influence of vitamin D on immune cells by shifting cytokines ratio towards an anti-inflammatory state (8,9). The most active form of vitamin D, the 1,25-Dihydroxyvitamin D3 (Calcitriol), reduces myelin loss and promotes remyelination in the CNS. Calcitriol level is regulated by 24-hydroxylase, an enzyme coded by cytochrome P450 family 24 subfamily A member1 (*CYP24A1*) gene. This enzyme is responsible for hydroxylation of C24, thus, conversion of calcitriol to an inactivated form (i.e. calcitroic acid) (10,11).

2. Objectives

In the present study, we have probed the association between these two variants: *rs9267649* and *rs2248359* with MS in Iranian patients. The first variant is located on the *HLA* region of the *HLA* class III loci on the short arm of human chromosome 6 and the second in the promoter region of *CYP24A1* gene on the long arm of human chromosome 20. Applying PCR-RFLP, herein we report the association of these two variants and the related alleles with the etiology of RRMS.

3. Materials and Methods

3.1. Type of MS and Sample Size

In the present investigation, a total number of 182 subjects were included in the study consisting of 82 RRMS; the most common type of MS. The disease was diagnosed by the neurologist according to the McDonald criteria. The control group was consisted of 100 healthy subjects without previous history of autoimmune disease. The two groups were also matched for age, gender, as well as ethnicity.

All participants were informed about the purpose of the study and the written consent was obtained. The ethics of the study was approved by the Ethical Committee at the National Institute of Genetic Engineering and Biotechnology, Iran.

3.2. Sample Collection and Genomic DNA Extraction

A total volume of 3 mL of whole blood was collected by venipuncture in a sterile tube containing EDTA (1.5 mg.mL^{-1}). Genomic DNA was isolated from 200 µL of the whole blood using Gene All DNA Blood Mini Kit (Exgene Clinic SV, Korea), and concentration of the extracted DNA was determined applying spectrophotometer (NanoDrop 2000 Thermo-Fisher Scientific, USA). The quality of the extracted DNA samples was assessed through running on a 1% agarose gel and stored at –20 °C until use.

3.3. Genotyping

Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) was used for single nucleotide polymorphism identification in the *HLA* region and *CYP24A1* gene, respectively. The primers used for genotyping were either designed applying OLIGO7 (i.e. *rs9267649* primer set) and tested with the UCSC In-Silico PCR program available online (https://genome.ucsc.edu/cgi-bin/hgPcr) or obtained through referring to the publications (for *rs2248359*; Skalli, *et al*) (12).

Polymerase Chain Reaction PCR was performed in a total volume of 25µL under standard reaction condition using thermal cycler (TechneFlexigene, UK). The primer sequences and details of PCR conditions are presented in **Table1**.

The PCR-generated amplicons were analyzed by running on a 1.5% agarose gel electrophoresis. Subsequently, PCR products of the both *rs9267649* and

rs2248359 were digested overnight at 37 °C applying RsaI and SacII restriction enzymes (Fermentas) for analysis of the both variants, respectively.

Applying RsaI restriction enzyme on the PCR product of the *HLA* results in the generation of two blunt-ended products. This enzyme recognizes the G allele and digests double-stranded DNA at the 5′-GTAC-3' restriction site. The products with the approximate sizes of 82 bp and 318 bp are identifiable in the subsequent an agarose electrophoresis which represents the GG (ancestral) genotype. A single band of 400 bp represents the AA genotype (variant), while, production of all the three bands represents the GA (heterozygous) genotype, respectively. The SacII restriction enzyme recognizes 5′-CCGCGG-3' restriction site of the *CYP24A1* gene. Following enzymatic digestion, those *CYP24A1* variants which are carrier of the first C nucleotide give rise to two sticky end restriction products (98 bp and 228 bp). On the other hand, in T allele carriers, this recognition site is lost and a single band will be generated when exposed to the restriction enzyme with an approximate size of 326 bp in the subsequent electrophoresis. All of the digested products were separated on a 2.5% agarose gel. The results obtained through restriction fragment length polymorphism analysis are summarized in **Table 1**. Further verification on the obtained genotypes was obtained through application of direct Sanger sequencing (ABI 370Xl Analyzer, Applied Biosystems) on the PCR products. Subsequently, chromatograms were checked using Chromas2.6.6 (Technelysium Pty Ltd**,** Australia)

(https://chromas.software.informer.com/2.6/) and aligned against multiple sequences through application of ClustalW algorithm of the MEGA7.0.26 software (https://www.megasoftware.net/) (13).

3.4. Statistical Analysis

The association of the two variants; *rs9267649* and *rs2248359* with the risk of MS was evaluated by comparing genotypic and allelic frequencies between the cases and controls. The difference in the genotypic and allelic distribution was determined by using Chi-Square statistical method of analysis. Furthermore, the risk estimates were calculated using logistic regression and odds ratios (ORs) with 95% confidence intervals (CIs) for the dominant, co-dominant, recessive, and allelic models. Statistical analysis was conducted using SPSS software (version 24, SPSS Inc., Chicago, IL, USA). Data with p-values ≤ 0.05 were considered statistically significant.

4. Results

4.1. Demographic Characteristics

Table 2 represents a summary of the demographic and clinical features of the participants in the present study. Running Shapiro-Wilk normality test indicated an abnormal distribution of the age data between the case and control groups (p-value of 0.000). Checking for the significance of the age difference between the two groups, Mann-Whitney test was used; result of which

Table 1. Primer's sequences, PCR condition and the expected product length before and after digestion

SNP	Primer sequence (5'-3')	Product length (bp)	PCR condition	Restriction enzyme (Restriction site)	Length of fragments following RFLP
rs9267649	**F:** ACCTGACCCTTCCCTGTGTAT **R:** GGGAGGATGGCTTGATCTCTTTG	400	35 cycles of 95 °C for 30 sec, 63 °C for 20 sec and 72 °C for 30 sec	RsaI (G/A)	318 bp and 82 bp
rs2248359	**F:** AGTTAGGAAATGCGCCTTGAG **R:** GGATCAGGTTGAAAGGATTCG	326	35 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec	SacII (C/T)	228 bp and 98 bp

Table 2. Demographic and clinical characteristics of the study participants in the present study.

Characteristics	Healthy Controls (n=100)	MS Patients (n=82)
Gender		
Female	82	66
Male	18	16
(Female/Male ratio)	4.55	4.12
Age (years)(mean±SD)	31.4±6/03	33.01±7.03
Disease diagnosis (years)(mean±SD)	n/a	3.7±2.1
EDSS*(mean± SD)	n/a	2.54±1.43
MS-type (n)	n/a	RRMS(82)

*Expanded disability status scale.

showed that there isn't a significant difference in the mean age between the two groups (p-value of 0.117). As well, we could not find a significant difference between the sexes in the two groups using Chi-Square test (p-value of 0.795).

4.2. Target Polymorphisms and Association with MS
The frequencies of the *rs9267649* and *rs2248359* variants were examined for Hardy-Weinberg equilibrium (HWE) through application of the Chi-Square respectively; all of which were found to be consistent with HWE (p-value>0.05).

The number and sizes of the PCR-RFLP fragments for the two polymorphisms: the *rs9267649* of the *HLA* system and *rs2248359* of the *CYP24A1* were determined by digestion of the PCR products with the respective restriction enzymes; RsaI (for *rs9267649*) and SacII (in case of *rs2248359*) and subsequent running on a 2.5% agarose gel.

As **Figure 1** represents the presence of all the three respective fragments (i.e., 400, 318, and 82 bp for *rs9267649* variant and 326, 228, and 98 bp regarding *rs2248359* variant) could be observed on the studied subjects in the electrophoresis.

Additionally, applying direct Sanger sequencing confirmed PCR-RFLP pattern and the obtained electropherograms have indicated the respective genotypes for the *rs9267649* and *rs2248359* variants, as well (**Fig. 2**).

The allelic distribution and genotypic frequencies of the *rs9267649* and *rs2248359* variants are summarized

in **Table 3**. The estimated frequencies for all genotypes of the *rs9267649* variant (i.e., GG, GA, and AA) in cases were statistically different from those of controls (68.3% vs 83.0% (GG), 26.8% vs 16.0% (GA) and 4.9% vs 1.0% (AA), with a p-value of 0.043). Accordingly, a significant difference was found between the genotypes of r*s2248359* variant (CC, CT, TT) in the subjects when compared to those of controls (28.0% vs 24.0% (CC), 56.1% vs 41.0% (CT), 15.9% vs 35.0% (TT), and p-value of 0.013) (**Fig. 3**).

Furthermore, a significant difference in the genotype frequency was detected in the dominant model of the *rs9267649* variant, dominant, and co-dominant models of the *rs2248359* variant, respectively (**Table 3**). We also were able to find a significant difference in the allelic frequencies for the *rs9267649* and *rs2248359* variants between cases and controls. The A allele of the *rs9267649* variant and the C allele of the *rs2248359* variant were associated with the risk of MS (p-value of 0.009, OR: 2.264, 95% CI: 1.211-4.231, and p-value of 0.028, OR: 1.594, 95% CI: 1.052-2.415, respectively).

5. Discussion
Studies conducted through application of Genome-Wide Association Studies (GWAS) have led us to the identification of a number of variants such as SNPs involved in susceptibility to the MS. Thus far, around 200 loci have been found to play a role in the susceptibility to MS development. While *HLA* region was proposed as the main susceptibility locus for MS;
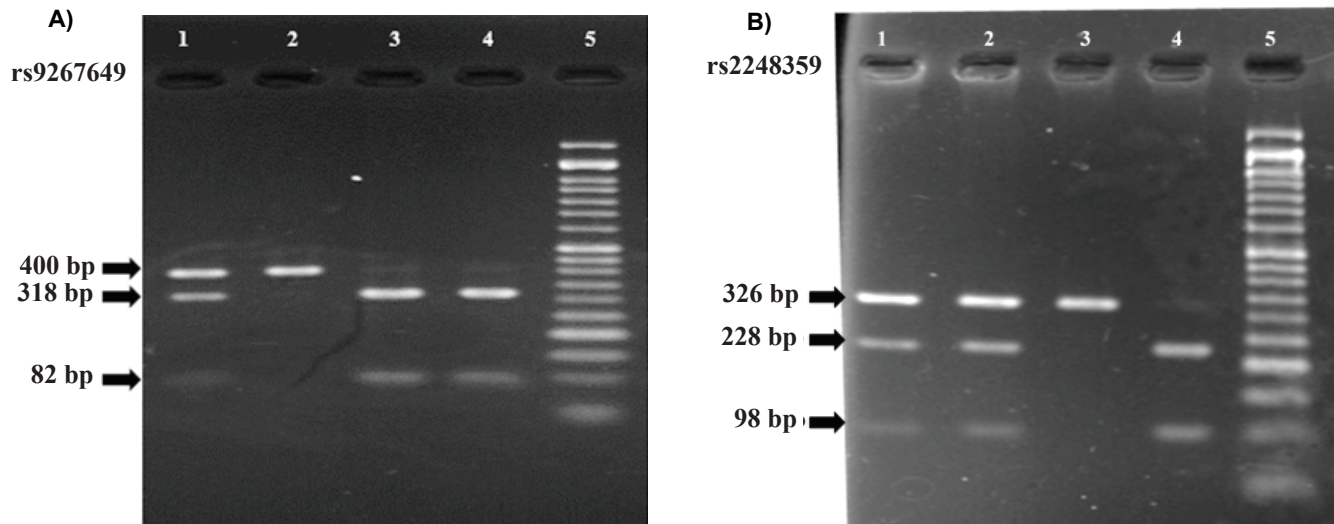
rs9267649 , Figure 1A	RsaI	Fragment size (bp)
1	2	400, 318, 82
2	0	400
3,4	1	318, 82

0: Not Cleaved, 1 Cleaved, 2: Both or Heterozygote

rs2248359, Figure 1B	SacII	Fragment size (bp)
1,2	2	326, 228, 98
3	0	326
4	1	228, 98

0: Not Cleaved, 1 Cleaved, 2: Both or Heterozygote

Figure 1. The electrophoretic pattern of PCR-RFLP; **A)** rs9267649 variant; 1: heterozygous product (3 bands; 82 bp, 318 bp and 400 bp), 2: undigested PCR product (1 band; 400 bp), 3,4: homozygous products (2 bands; 82 bp and 318 bp), 5: DNA ladder 50 bp (17 bands; 50-1500 bp) from left to right, respectively. **B)** rs2248359 variant; 1,2: heterozygous products (3 bands; 98 bp, 228 bp and 326 bp), 3: undigested PCR product (1 band; 326 bp), 4: homozygous product (2 bands; 98 bp and 228 bp), 5: DNA ladder 50 bp (17 bands; 50-1500 bp) from left to right, respectively.

as shown by GWAS, nevertheless several other genes within non-*HLA* region have also been proposed to contribute in the disease development (14,15). Among the two variants analyzed in the present study, a strong association was reported formerly by Kular *et al* between *rs9267649* variant and MS. According to their study, *rs9267649* is associated with an increased *HLA-DRB1* DMR methylation, resulting in a reduced expression of the gene, and a modulated risk effect of *DRB1*15:01* (7).

Formerly, *rs9267649* variant of the HLA system has been subject of study in the other parts of the world. For instance, Kular *et al*., have carried out a meta-analysis to investigate the association between *rs9267649* and MS through three cohort studies carried out in Sweden, Germany, and Iceland on a total number of 14,259 cases and a larger population of the control (i.e. 171,347) on European ethnicities (7). Nevertheless, the association of this variant with MS has not been well studied in the other parts of the world such as Asian ethnicities and Persians (Iranian) in particular. Our study shows a positive association for the A allele of *rs9267649* with the risk of MS (18.3% vs. 9.0%, with a p-value of 0.009).
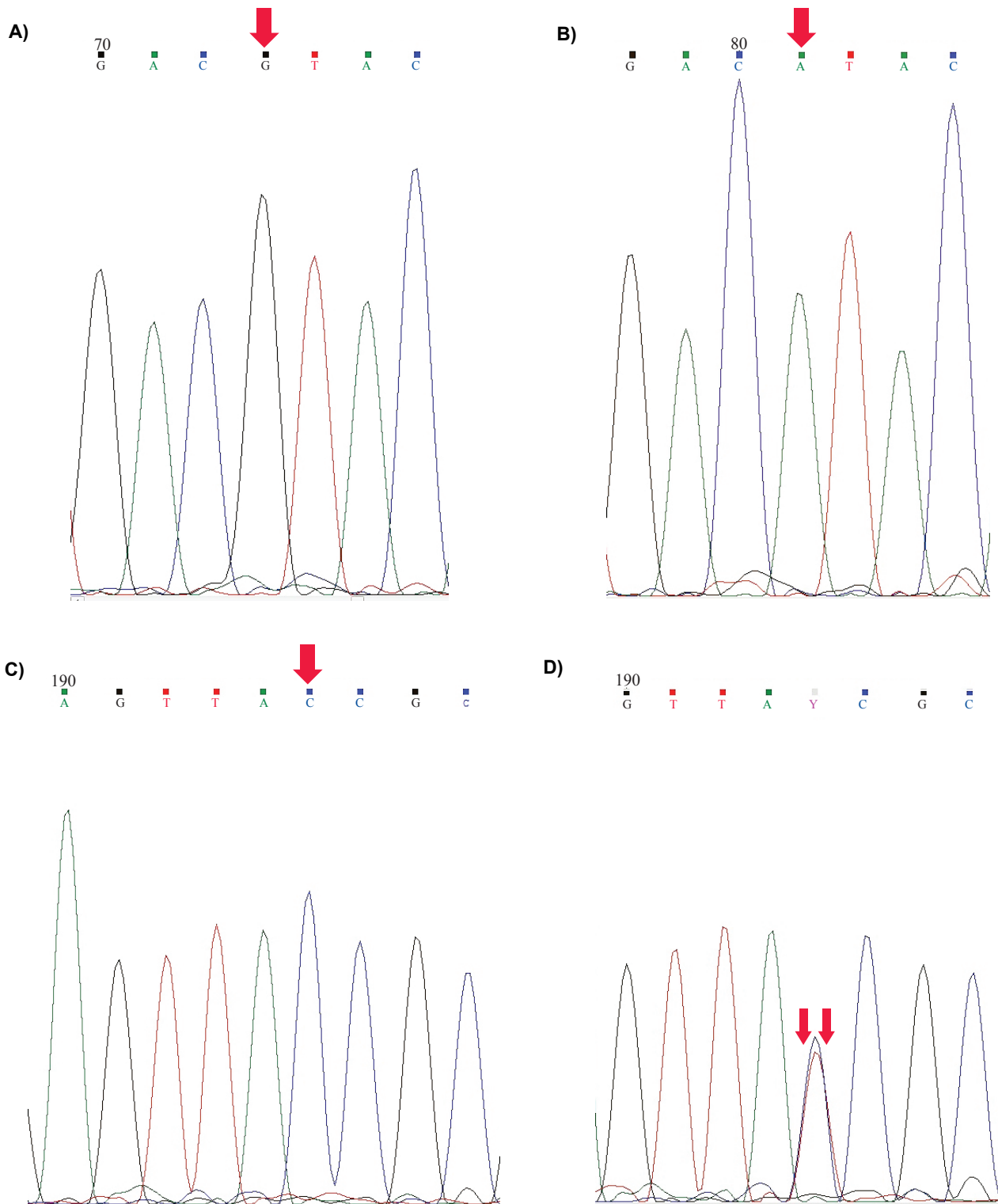
Figure 2. Direct Sanger sequencing; PCR was carried out and product was subjected to the Direct Sanger sequencing for both variants: rs9267649 and rs2248359. Panels: **A)** and **B)** indicate the genotypes of GG and AA homozygotes of the rs9267649 variant. Panel **C)**, and **D)** indicate the genotypes of CC homozygote and the CT heterozygote of the rs2248359, respectively.

In all panels; Black graph: G nucleotide, Green graph: A nucleotide, Blue graph: C nucleotide, Red graph: T nucleotide

Table 3. Distribution of the allele and genotype frequencies of the rs9267649 and rs2248359 studied in the RRMS cases and controls.

| SNP	Genotype/Allele	Case (n=82) (%)	Control (n=100) (%)	X²	P value	OR (95%CI)
rs9267649	GG	56 (68.3%)	83 (83%)	6.273	0.043 df=2	
	GA	22 (26.8%)	16 (16.0%)			
	AA	4 (4.9%)	1 (1.0%)			
Recessive Genetic Model	AA	4 (4.9%)	1 (1.0%)	2.536	0.111 df=1	5.077 (0.556-46.340)
	GA+GG	78 (95.1%)	99 (99.0%)			
Dominant Genetic Model	AA+GA	26 (31.7%)	17 (17.0%)	5.401	0.020 df=1	2.267 (1.127-4.560)
	GG	56 (68.3%)	83 (83.0%)			
Codominant Genetic Model	GA	60 (41.7%)	84 (58.3%)	3.198	0.074 df=1	0.519 (0.252-1.072)
	GG+AA	22 (57.9%)	16 (42.1%)			
Allelic Model	A	30 (18.3%)	18 (9.0%)	6.797	0.009 df =1	2.264 (1.211-4.231)
	G	134 (81.7%)	182 (91.0%)			
rs2248359	CC	23 (28.0%)	24 (24.0%)	8.697	0.013 df =2	
	CT	46 (56.1%)	41 (41.0%)			
	TT	13 (15.9%)	35 (35.0%)			
Recessive Genetic Model	CC	23 (28.0%)	24 (24.0%)	0.386	0.535 df =1	1.234 (0.635-2.401)
	CT+TT	59 (72.0%)	76 (76.0%)			
Dominant Genetic Model	CC+CT	69 (84.1)	65 (65%)	8.506	0.004 df =1	2.858 (1.390-5.878)
	TT	13 (15.9%)	35 (35 %)			
Codominant Genetic Model	CT	46 (56.1%)	41 (41.0%)	4.116	0.042 df =1	1.839 (1.018-3.320)
	CC+TT	36 (43.9%)	59 (59.0%)			
Allelic Model	C	92 (56.1%)	89 (44.5%)	4.848	0.028 df =1	1.594 (1.052-2.415)
	T	72 (43.9%)	111 (55.5%)			

A)

B)
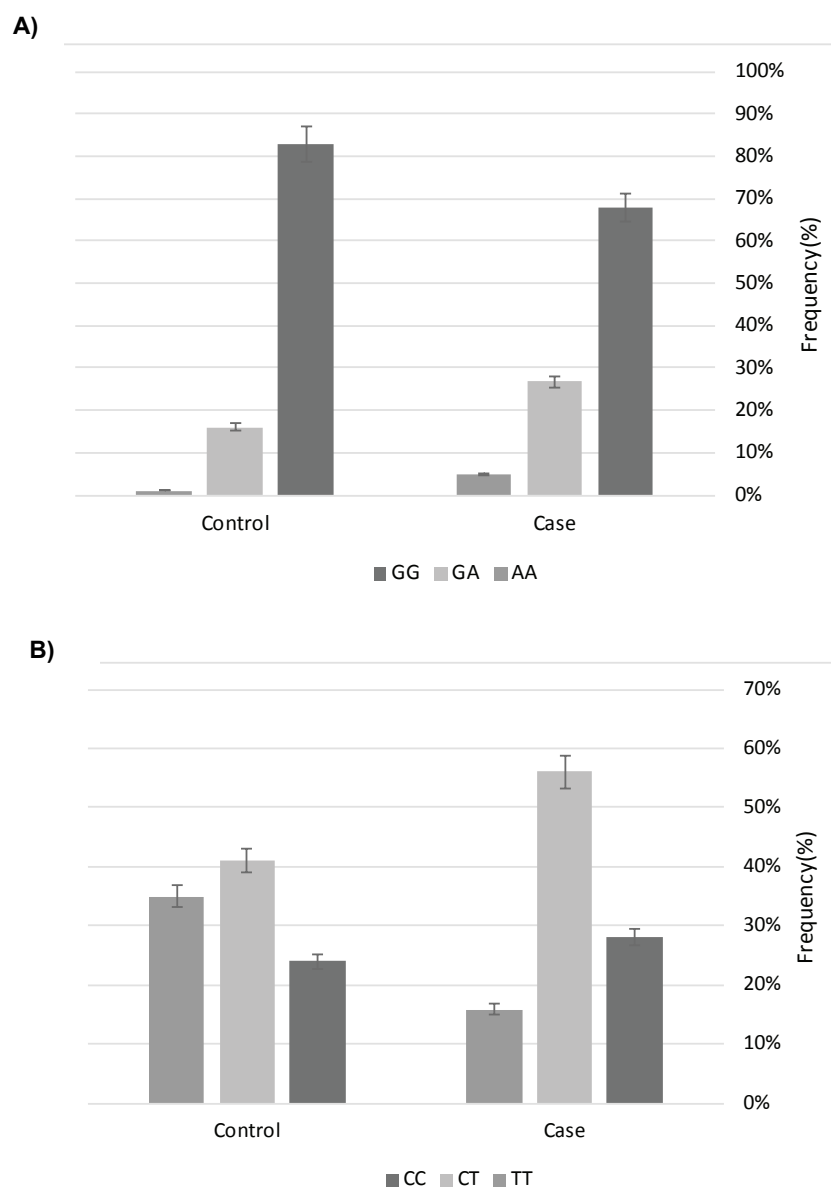
Figure 3. Genotypes' frequency distribution in RRMS cases and healthy controls counterparts. **A)** The estimated frequencies for all genotypes of the rs9267649 variant (GG, GA, AA) were statistically different from those in the controls (estimated p value: 0.043). And **B)** The estimated frequencies for all genotypes of the rs2248359 variant (CC, CT, TT) were statistically different from those in the controls (estimated p value: 0.013).

As well, this finding indicates a protective effect of the G allele of *rs9267649* on the development of MS, if not at all, but at least among Iranians.

Located on the promoter region of the *CYP24A1*, the *rs2248359* variant which is highlighted by the involvement in the vitamin D metabolism (16) is among the non-*HLA* loci predicted by the GWAS to have a strong linkage potential with the MS (15).

Vitamin D modulates immune response; a function supported by many studies that serum vitamin D deficiency and polymorphisms in the genes involved in vitamin D metabolism could lead to autoimmune diseases (17). While GWAS has been conducted in a large population of the European ancestry, however, due to the diversity of the polymorphisms worldwide, in addition to the socio-behavioral factors such as vitamin D deficiency, insufficient exposure to sunlight, melanin composition of the skin, etc., additional studies

on the other populations and ethnicities, and among which, Iranians is mandatory. Our investigation shows an increased C allele frequency in the *rs2248359* variant in MS patients. The C allele carriers (hetero or homozygous carriers) are more likely to develop MS than non-carriers (56.1% vs 44.5%, p-value 0.028) through changes in the *CYP24A1* promoter efficiency; resulting in the transcriptional output and mRNA levels. Studies by other investigators suggest that the C allele of rs2248359 variant leads to an enhanced *CYP24A1* expression in the human brain. As a result, an increased 24-hydroxylase expression and an exacerbated degradation of the $1,25(OH)_2D3$ (Calcitriol) through hydroxylation confers susceptibility to MS (10,16). While we carried out our study on a small population size, however, our results are in conformity with the GWAS study conducted by IMSGC/WTCCC2 on a large number of European subjects (9,772 MS cases and 17,376 controls) (15) as well as the study of Beechman *et al.*, (14,498 MS cases vs. 24,091 controls) (14), in addition to the study carried out on African Americans subjects (803 MS cases vs. 1516 controls) (18). In contrast, other studies carried out on Germans (19), Chinese (20) Danish (21), Moroccan (12) and Atlai ethnicities (22) indicate a lack of a significant association between *rs2248359* and MS risk. Also, a recent study among European population did not show a significant association between *rs2248359* and MS AAO (age at onset) (23). Such disparity could be attributed to the ethnic, socioeconomics differences, as well as geographical and environmental influences, respectively.

According to the previous study among the Iranian population to analyze *CYP24A1* gene expression status in 50 RRMS patients, the expression level of *CYP24A1* compared with a normal individual has shown a decrease, however, no reached statistical significance. Besides, that study did not observe a gene expression-phenotype correlation for *CYP24A1* which may be due to limited statistical power as a result of the small sample size. So, further studies are required to identify the contribution of genes involved in MS, particularly *CYP24A1* (24),

The involvement of the *rs2248359* variant is not only limited to the MS but also studies on several other inflammatory diseases have documented a significant correlation between this variant and those other diseases, among which, Asthma (25), Atopic dermatitis (26), Type 2 diabetes and Hepatitis C (28).

The relationship of the gene-environment with respect to Vitamin D was shown in other studies, among which, the interaction between vitamin D and the *HLA DRB1*1501* allele, as vitamin D response element (VDRE), has been identified in the promoter region of the *HLA DRB1*1501* gene (29).

Therefore, it could be concluded that SNPs related to vitamin D metabolism can affect MS susceptibility through gene-environment and gene-gene interactions.

6. Conclusion

In the present study, we have attempted to investigate both the *HLA* and non-*HLA* region variations in parallel. In conclusion, our data suggest that the *HLA rs9267649 A/G* genotype and the non-*HLA rs2248359 C/T* genotype are associated with the increased susceptibility to MS. However, large-scale investigations with more sample sizes among Iranian and various ethnicities are required to confirm our results. Also, further studies at the functional genomics level will help us to clarify the underlying correlation between DNA polymorphisms and susceptibility to MS.

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