Contribution of hsa-miR-146a and hsa-miR-223 gene variations in patients with multiple sclerosis reveals association of rs2910164 and rs1044165 with risk of multiple sclerosis susceptibility

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ABSTRACT

MicroRNAs (miRNAs) are a group of non-coding RNAs that play a role in gene regulation. Due to their possible functional importance, genetic variants within miRNA genes have been recognized as candidate biomarkers. Single-nucleotide polymorphisms (SNPs) in miRNA genes can be related to the risk of different autoimmune diseases. Some of these SNPs are rs2910164 in the miR-146a and rs1044165 in the miR-223. The aim of this study was to investigate the relationship between these polymorphisms and the risk of multiple sclerosis (MS) in an Iranian population. In this case–control study, 261 patients with MS and 250 healthy controls that matched by age and geographical region were enrolled. After sampling and genomic DNA extraction, genotyping was determined by PCR–restriction fragment length polymorphism. Allelic and genotypic associations between the SNPs and MS were evaluated by the data analysis conducted by SPSS V.20. The frequencies of rs2910164 and rs1044165 SNPs were significantly different between the patients with MS and healthy controls. C and T alleles in the variants rs2910164 and rs1044165, respectively, are associated with increased risk of MS. Such association was obtained in codominant, dominant, and overdominant models for both variants (OR ~3 and OR ~1.5, respectively). Furthermore, this study determined that the C and T alleles of rs2910164 and rs1044165 are risk factors for MS in the Iranian population.

Significance of this study

What is already known about this subject?

- miR-146a and miR-223 dysregulations are involved in the development of multiple sclerosis (MS).
- Evidence proposes that genetic polymorphisms are associated with the prognosis and progression of many disorders, including neurological disorders.
- Polymorphism studies have different outcomes in different racial groups, and findings can be inconsistent on different ethnicities and regions.

What are the new findings?

- Individuals with miR-146a rs2910164 and miR-223 rs1044165 have a higher risk of developing MS.
- miR-146a rs2910164 has a higher potential pathogenic role than miR-223 rs1044165 on the MS susceptibility.
- For miR-146a rs2910164, patients with CC/GC genotype reach an EDSS score of 5, 3 years earlier than others carrying GG genotype.

How might these results change the focus of research or clinical practice?

- These variants can be potential biomarkers for early detection of MS. They also are likely to be associated with stronger inflammatory responses that might be useful in the therapeutic management of the disease.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that appears with axonal demyelination and interruption between nerves and muscles.[1] MS leads to chronic disability in young adults with stupendous social and individual costs. Concerning clinical manifestation, treatment response, and causes, MS is a heterogeneous disorder that is mainly attributed to the effects of environmental factors in people with a genetic predisposition.[2] Significant advances in the applied genomic platforms have facilitated the discovery of new relationships between various molecular mechanisms and MS pathogenesis.[3] In addition, there is growing evidence that non-coding RNA molecules, including microRNAs (miRNAs), are involved in increased or decreased expression of molecules at the onset of MS or through its progression and exacerbation.[4] miRNAs play an important role in certain types of biological processes by regulating gene expression post-transcriptionally via degrading mRNA or inhibiting translation.[5] miRNAs are involved in important processes such as organogenesis, homeostasis, and cell proliferation,

differentiation and signaling.[6 7] Interestingly, miRNA function can be influenced by their sequence variability.[8] Single-nucleotide polymorphisms (SNPs) in miRNAs sequence may be attributed to their evolution leading to the miRNAs with new or altered functions. These variants can serve as a mechanism to produce an miRNA cluster or can be homologous during evolution.[9] In addition, the SNPs at the seed or stem loop of the miRNAs precursor sequence can significantly affect miRNA production or processing.[10] Minor variation in miRNA production can lead to drastic changes in the expression of target genes and therefore pathological outcomes. To date, different variants of miRNAs have been observed in MS and other autoimmune diseases that can lead to changes in miRNA expression and appropriate binding to the target gene.[11–13] miR-146a is one of the conserved miRNAs that are well known for potently regulating the immune response and inflammation.[14] miR-146a has important variants whose contributions to different diseases such as cancer, diabetes and few autoimmune diseases have been determined. However, there are also inconsistencies in the available findings that may be related to the effects of racial differences. rs2910164 (G>C) variant in miR-146a can affect the expression and maturation of mir-146a.[15] The rs2910164 C allele leads to mispairing inside the stem loop of miR-146a and decreases the expression of the mature form.[16] miR-223 plays a key role in the development and hemostasis of the immune system. The involvement of miR-223 in many inflammatory and autoimmune diseases such as rheumatoid arthritis, Crohn's disease, and MS has been demonstrated.[17 18] The increased expression of miR-223 in the T lymphocytes of patients with rheumatoid arthritis has caused this miRNA to be suggested as a diagnostic biomarker of rheumatoid arthritis.[19] However, findings on MS are inconsistent such that miR-223 expression has been reported to increase in the MS lesions and peripheral blood mononuclear cells (PBMCs). In contrast, this miRNA expression has been demonstrated to decrease in the serum of patients with MS.[20 21] It can be therefore important to determine the exact mechanism of miR-223 function in MS pathogenesis. Rs1044165 is in linked with miR-223 and is located inside the VSIG4 gene, which acts as a potent inhibitor of T-cell activation.[22] The association between this polymorphism and MS disease has been reported in a few numbers of populations. However, given the inconsistency in the available findings, studies with other populations can help determine the role of this polymorphism. Our aim was therefore to investigate the association between genetic variations in miR-146a and miR-223 and predisposition and risk of MS.

MATERIALS AND METHODS

Study population

This study contained 261 patients with MS and 250 healthy controls. The individuals were randomly selected from patients referring to the neurological ward of a hospital. MS diagnosis was confirmed by a specialist using MRI and complementary tests. No drugs were administered before sampling. The control populations were unrelated and matched by age (±5 years) and ethnicity with a negative history of neurological disorders. Written informed consent was obtained from each subject, who completed the questionnaire and provided demographic and clinical data.

SNPs genotyping

The blood sample was collected and DNA extraction was conducted by the phenol–chloroform method and was qualitatively and quantitatively examined by a NanoDrop spectrophotometer. To determine the genotypes of the variants, PCR-RFLP was used. For genotyping rs2910164, the forward primer was altered to create a restriction site in the PCR product. Primers used for extensions were F: 5′-CATG GGTTGTGTCAGTGTCAGAGCT-3′ and R: 5′-TGCC TTCTGTCTCCAGTCTTCCAA-3′ for rs2910164 (miR-146a), and F: 5′-CTTGGTCATCATGCCTACAGAC-3′ and R: 5′-TGGCCTGTGAAATAACAATTTC-3′ for miR-223 (rs1044165). The PCR was directed under the conditions as follows: predenaturation at 95°C for 6 min, 35 cycles including denaturation at 95°C for 40 s, 60°C–63°C annealing temperature (depending on the type of primer) for 30 s and synthesis of the fragments (extension) at 72°C for 30 s, and terminal extension at 72°C for 5 min. To assess the PCR-amplified fragments, electrophoresis in polyacrylamide gel 8% (29:1 acrylamide:bis-acrylamide) at 100 mA for 1.5 hours was performed and bands were visualized by silver nitrate staining. To determine the genotypes, 10 µL of all PCR products was digested at 37°C overnight with 1.5 U of the suitable enzyme. The digested product was separated by 10% polyacrylamide gel electrophoresis. For miR-146a rs2910164, the PCR product (147 bp) was restricted under SacI (Fermentase). The lengths of fragments after digestion were 120 and 27 bp for the GG genotype, 147, 120 and 27 bp for the GC genotype and 147 bp for the CC genotype. For the polymorphism in the miR-223 (rs1044165), the PCR product (164 bp) was restricted by EcoRII, and 104 and 60 bp fragments developed. Digestion is developed if the nucleotide change (C>T) exists in the PCR products.

Statistical analysis

For an allele and genotype frequency evaluation between the healthy people and the patients, Pearson's χ^2 test was used. To estimate the risk factor of disease, ORs and 95% CIs were calculated using statistical software. Statistical analyses were performed using SPSS V.20.0, and the usual p value of ≤ 0.05 was considered as a general significant level. The χ^2 test was used to evaluate deviations from the Hardy-Weinberg equilibrium.

RESULTS

Characteristics of the study population

Clinical and pathological characteristics of patients with MS and the control individuals are presented in table 1. No variances had been found in the distributions of age and gender between the control and patients with MS ($p>0.05$). Most patients were over 45 years and had a 5-year average disease duration. The proportion of patients with MS with relapsing-remitting multiple sclerosis (RRMS) was higher than that of those with other types.

Distribution of SNPs

Table 2 shows the frequency distribution of each variant. No deviation from the Hardy-Weinberg equilibrium was seen in

Table 1 Demographic and clinical characteristics of patients and health controls in this study

Index		MS (261)	Control (250)
Mean±SDE age		49.8±7.9	41.4±5.9
Male (%)		75 (28.73)	108 (43.2)
Female (%)		186 (71.26)	142 (56.8)
Disease duration (years)		5.51±2.01	–
EDSS		4.05±1.8	–
Multiple Sclerosis Severity Score		4.48±2.12	–
Disease subtype			
Male	Relapsing-remitting multiple sclerosis (%)	68 (90.66)	
	Secondary progressive multiple sclerosis (%)	5 (6.67)	–
	Primary progressive multiple sclerosis (%)	2 (2.67)	
Female	Relapsing-remitting multiple sclerosis (%)	164 (88.17)	
	Secondary progressive multiple sclerosis (%)	16 (8.60)	–
	Primary progressive multiple sclerosis (%)	6 (3.22)	

the two groups for miR-146a and miR-223 variants. The overall genotype and allele distributions of the rs2910164 and rs1044165 polymorphisms did differ considerably between patients with MS and healthy controls ($p<0.05$) (table 2) (online supplemental figure S1).

The rs2910164 polymorphism was considerably associated with MS ($p=0.0001$). This finding was applicable to genetic models of association with MS (overdominant, dominant, and co-dominant). In all models, the effect of the C allele was found to increase the risk of MS that odd's ratio displayed in table 2. Concerning the rs1044165 polymorphism, the T allele frequency was greater in the patients with MS, and allele variation in the polymorphism caused an increase in the risk of developing MS (table 2). The C>T change in this polymorphism was directly associated with the risk of MS such that in the overdominant, dominant, and codominant genetic models, the risk of MS increased by 1.5. For each polymorphism, Akaike Information Criterion and Bayesian Information Criterion, which determined the model with minimal expected entropy, were used for selection of the best inheritance model, and results showed that the dominant model is the best fit model in both cases. Regarding the relationship between clinical factors and the two polymorphisms, no association was found between age of onset, disease subtype, and duration.

There was no relationship between the MS type and the studied genotypes. However, the frequency distribution of the GG genotype in miR-146a polymorphism was higher in people with RRMS, and a greater number of people with RRMS had CC genotype of miR-223 (online supplemental table S1). According to gender-based grouping and association with the studied variants, the GC genotype of miR-146a and CT genotype of miR-223 were more frequent in women than in men and healthy controls, but no significant difference in the genotype frequency was observed between men and women (online supplemental table S2).

Association between polymorphisms and EDSS

To investigate the relationship between genotype frequencies and disability level, we divided the patients into two groups: group 1, consisting of patients with EDSS scores of 0–5.5 (mild/moderate inability), and group 2, consisting of patients with EDSS scores of 6–10 (severe inability). The frequencies of the genotypes in patients with EDSS scores of 6–10 did not differ from patients with EDSS scores of 0–5.5 (figure 1). There was no significant association between two variations and disability in patients (data not shown).

Survival analysis

Survival analysis was conducted for the EDSS 5 disease development for different genotypes of miRNAs (figure 2). Accordingly, mean durations to reach EDSS score of 5 in patients with GG and GC+CC genotypes in miR-146a were 8 (HR: 0.625, 95% CI 0.343 to 1.138) and 5 (HR: 1.6, 95% CI 0.878 to 2.91) years, respectively ($p=0.003$). This time reaches 9 years for CT+TT genotypes (HR: 1.159, 95% CI 0.589 to 2.201) and 8 years for CC (HR: 0.889, 95% CI 0.470 to 1.680) in miR-223 ($p=0.14$).

Enrichment analysis

To predict rs2910164 and rs1044165 possible effects on the immune regulatory pathway, we used the Enrichr[23] tool to investigate pathways and biological processes that theoretically can be affected by miR-146a and miR-223 experimentally validated target genes. The results approved their target genes' involvement in various pathways and biological processes that regulate different aspects of immune system responses. Then to identify the central elements of these biological networks, we analyzed protein–protein interactions (PPIs) by string database and Cytoscape software. *cytoHubba* plug-in and MCC method, as the best performing method, was used to rank genes by their network features (figure 3).

DISCUSSION

We studied two polymorphisms in miR-146a and near to the miR-223 sequence in patients with MS to investigate their association with the risk and severity of MS as well as the disability level. We observed that the rs2910164 miR-146a C allele was associated with MS such that the presence of this allele could increase the risk of developing MS by approximately three times. Besides that, the miR-223 rs1044165 variant was found to be associated with the risk of developing MS. miR-146a regulates the functions of acquired immunity, inflammatory responses and antiviral pathways and is likely to play a significant role in astrocyte-mediated inflammation. As a regulator of negative feedback, miR-146a can contribute to the immune response by targeting the TRAF6 and IRAK1 genes as adapter molecules downstream of the Toll-like and cytokine receptors, which are essential for proinflammatory signaling.[24] On the other hand, this miRNA is important for regulatory T-cell function, and decreased expression of miR-146a leads to

Table 2 Association of genetic polymorphisms in miR-146a and miR-223 with MS q1

SNP/genetics model	Control (%) n=250	MS (%) n=261	OR	95% CI	P value	AIC	BIC
rs2910164 (miR-146a)							
Codominant							
GG	190 (76)	136 (52.10)	1.00	–			
GC	53 (21.2)	111 (42.52)	2.926	1.972 to 4.341	0.0001		
CC	7 (2.8)	14 (5.36)	2.794	1.098 to 7.107	0.039	682.1	694.8
Dominant							
GG	190 (76)	136 (52.10)	1.00	–			
GC+CC	60 (24)	125 (47.89)	2.911	1.994 to 4.249	0.0001	680.1	688.6
Recessive							
GG+GC	243 (97.2)	247 (94.63)	1.00	–			
CC	7 (2.8)	14 (5.36)	1.968	0.781 to 4.959	0.14	710	718.5
Overdominant							
GG+CC	197 (78.8)	150 (57.47)	1.00	–			
GC	53 (21.2)	111 (42.52)	2.751	1.862 to 4.063	0.0001	685	693.5
rs1044165 (miR-223)							
Codominant							
CC	191 (76.4)	177 (67.81)	1.00	–			
CT	51 (20.4)	73 (27.96)	1.545	1.023 to 2.332	0.048		
TT	8 (3.2)	11 (4.21)	1.484	0.583 to 3.773	0.48	709.5	722.2
Dominant							
CC	191 (76.4)	177 (67.81)	1.00	–			
CT+TT	59 (23.6)	84 (32.18)	1.536	1.039 to 2.271	0.03	707.5	715.9
Recessive							
CC+CT	242 (96.8)	250 (95.78)	1.00	–			
TT	8 (3.2)	11 (4.21)	1.331	0.526 to 3.366	0.543	711.8	720.3
Overdominant							
CC+TT	199 (79.6)	189 (72.41)	1.00	–			
CT	51 (20.4)	73 (27.96)	1.52	1.001 to 2.269	0.046	708.2	716.6

AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion; MS, multiple sclerosis; SNP, single-nucleotide polymorphism.

deregulation of interferon (IFN)-γ responses and T-cell inhibitory function.[25] The study of Lescher *et al* showed that miR-146a expression increased in the experimental autoimmune encephalomyelitis (EAE) lesions.[26] It also has been shown that the expression of this miRNA markedly increases in PBMCs of patients with RRMS compared with the healthy controls.[27] These changes in expression levels could be due to the important variants of this miRNA. The C allele of rs2910164 polymorphism, located in the passenger strand of miR-146a-5p, leads to decreased production of mature miRNA, which is due to differences in nuclear processing.[16] Any variant that changes the mir-146a level could affect vital pathways modulated by this miRNA. To predict these pathways, experimentally validated targets of mir-146a were obtained from the miRTarBase database.[28] Pathway and gene ontology enrichment analyses showed that the most significant pathways for miR-146a target genes are T-cell receptor regulation of apoptosis and toll-like receptor signaling pathway, and biological processes with the highest score are cytokine-mediated signaling pathway (GO:0019221) and cellular response to type I IFN (GO:0071357). To find miR-146a hub target genes, PPI network was conducted, and among its 20 hub genes, 17 genes were either cytokines or associated with cytokines directly. Therefore, rs2910164 polymorphism that affects

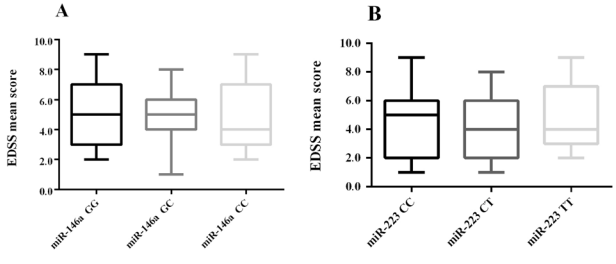

Figure 1 Disability level status measured using EDSS mean score in a patient with multiple sclerosis and various genotypes: (A) miR-146a variant, p=0.28; (B) miR-223 variant p=0.06.

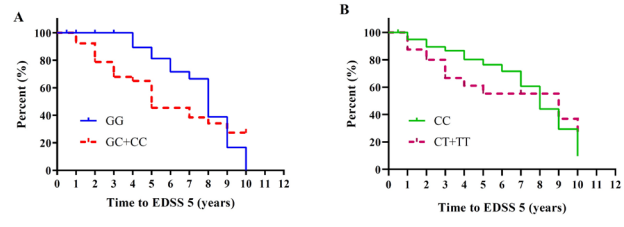

Figure 2 Survival curves displaying the correlation between miR-146a and miR-223 polymorphism and time to EDSS 5 in patients with multiple sclerosis. (A) Genotype GG versus CG+CC in miR-146a (p=0.003, analysis with Wilcoxon test). (B) Genotype CC versus CT+TT (p=0.14, analysis with Wilcoxon test).

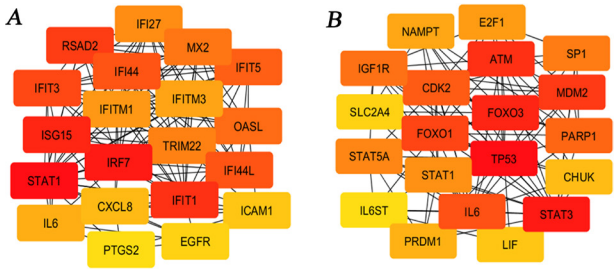

Figure 3 PPI network assessment to find central elements. (A) Hub target genes of miR-146a after PPI analysis; 17 genes are either cytokines or associated with cytokines directly. (B) Top hub target genes of miR-223; the 8 genes of 20 miR-223 hub target genes are either cytokines or associated with cytokines directly. The range of colors is between red and yellow in order of importance. PPI, protein–protein interaction.

the amount of mature miRNA also affects the cytokines profile that is modulated by this miRNA. The association between the presence of the C allele and higher expression of (IFN-γ, tumor necrosis factor alpha and interleukin (IL)-1β) also has been proved experimentally.[29] Regarding the findings of the current study, we can argue that the presence of the C allele leads to an increased risk of developing MS. It is likely due to a decrease in the miR-146a levels and its effects on inflammatory and proinflammatory cytokines However, the findings on miR-146a are inconsistent such that no association between the rs2910164 variant and MS was observed in an Italian population,[30] while the C allele of this SNP was frequently observed in a population of Chinese female patients, with a significant association with the risk of MS.[29] This inconsistency can be due to the difference in evolutionary histories between these two populations. On the other hand, there also is evidence that gender influences the prevalence, pathology, and prognosis of MS. A tentative explanation is the effects of sex hormones, especially estrogen, on MS. Estrogen increases the secretion of proinflammatory cytokines such as IFN-γ and IL-17, probably by influencing the key transcription factors in inflammation or by regulating miRNA expression.[31] Our study also determined that in the people with RRMS, the C allele in miR-146a and T allele in miR-223 frequency was greater compared with other type of MS. Therefore, these variants are likely to be associated with stronger inflammatory responses, or the higher expression of cytokines may be associated with these genotypes in patients with RRMS.

Given the significant role of miR-223 in MS, we also studied an effective variant (rs1044165) linked to miR-223 with the risk of developing MS. A study with an Italian population has reported an association between the rs1044165 variant and MS.[21] On the other hand, inconsistent results regarding miR-223 expression association with MS have been reported by a study with serum samples of the patients with MS, elucidating the expression of miR-223 in these patients has decreased.[30] miR-223 is located on the X chromosome and has different copy numbers in men and women; it is, therefore, challenging to conduct association studies with the current polymorphisms, and it is more logical to study SNP association in gender-stratified populations. In a study with a Russian population, the data on men and women were separately analyzed, with inconsistent findings with the study on the Italian population, which can be attributed to the involvement of different mechanisms in MS pathogenesis in different racial categories. In our population, an association between the rs1044165 variant and the risk of MS was observed, and the presence of the T allele in different genetic models increased this risk by approximately 1.5 times; in women, such association was observed as well. Since rs1044165 is located in VSIG4 gene 3′ untranslated region (UTR) and approximately 3 kb far from the miR-223 location, and there is no study that has investigated rs1044165 effects on miR-223 expression level; it is difficult to explain the mechanism that can lead to this association between rs1044165 variant and MS susceptibility. Because VSIG4 is a potent inhibitor of T-cell activation, one possibility can be a change in miRNA profile that bind to that region in VSIG4 3′UTR considering rs1044165. The miRNAsong tool[32] was used to analyze ±50 nucleotides flanking region of rs1044165, and results showed that the number of potential interactions with the free energy less than −25 kcal/mol, which have a higher chance to be a real miRNA binding site, is more for mRNA containing C allele (85 sites, sum of free energy=−2373.1) compared to T allele (79 sites, sum of free energy=−2206.7). Thus, rs1044165 variant can change miRNA base-regulating mechanisms of the VSIG4 expression and thereby affect the immune responses and MS pathogenesis. Another possibility is the effect of rs1044165 on mir-223 processing. To investigate the possible influence of rs1044165 on RNA secondary structure, we analyzed a fragment of ~3.2 kb from upstream mir-223 promoter to 100 bp after rs1044165 location. It has been proven that non-structured flanking RNA is vital to miRNA processing, and the ssRNA–dsRNA junction is critical to determine the precise cleavage site by Drosha and DGCR8.[33 34]

Interestingly, despite the long distance (2904 bp) between mir-223 and rs1044165 location, the RNAfold web server shows that this variant changes the accessibility of pri-miRNA and might be responsible for an expressional change in this miRNA (figure 4). The mRNA strand with the T allele has extra structured branches that can affect microprocessor complex access to pri-mir-223. Similar results were obtained with longer fragments. If rs1044165 can change, the expression level of mir-223

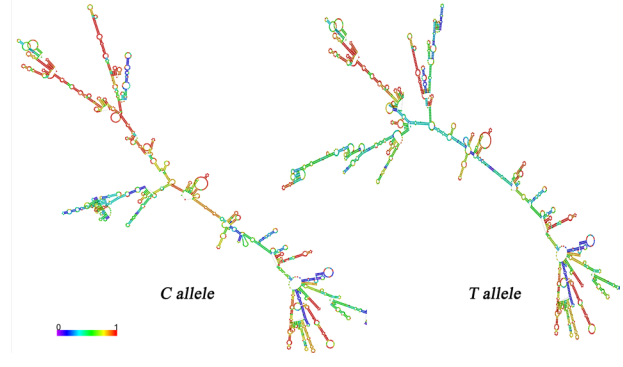

Figure 4 Predicted RNA secondary structure of region included rs1044165. Pre-miR-223 is indicated by a black arrow. The C allele represents more accessibility and less structured branches.

can also affect the immune process modulated by this miRNA. IL-6-mediated signaling pathway (GO: 0070102), IL-27-mediated signaling pathway (GO:0070106), IL-35-mediated signaling pathway (GO:0070757), and T-helper 17 cell lineage commitment (GO:0072540) are the most significant biological processes that have been predicted to miR-223 experimentally validated target genes by Enrichr tool. The 8 genes of 20 mir-223 hub target genes are either cytokines or associated with cytokines directly, and 9 of them are transcription factors. Experimentally, evidence indicates that miR-223 increases in the blood and T cells of patients with MS as well as in the active MS damages.[20] It has also been observed that miR-223 is mainly expressed in myeloid cells and can affect Th17 cell differentiation and therefore can play a part in Th17 cell-associated inflammatory responses.[35] The miR-223 is also involved in immune inflammatory responses by modulation of the nuclear factor kappa B pathway.[36] miR-223 has a modulator role in remyelination, so although its deficiency reduces CNS demyelination, it also has a neuroprotective role, which is required for efficient M2-like activation of microglia and supports remyelination.[37] We also investigated the association of the combination of the two polymorphisms with the risk of developing MS (data not shown). When the two polymorphisms combined, the frequencies of the effective alleles decreased drastically and significant association was not observed.

CONCLUSION

Taken together, it will be helpful to determine the effective genetic factors on disease predisposition, including autoimmune, concerning the significance of racial differences. The current study showed that the presence of two important polymorphisms of miR-146a and miR-223 could increase the risk of developing MS. Although these results were obtained from the patients who were almost 90% RRMS type, because approximately 65% of patients with RRMS will afterward develop SPMS type as the second phase,[38 39] it can be declared that the presence of the C (rs2910164) and T (rs1044165) alleles can contribute to the development and exacerbation of MS. Their coexistence, however, was observed in very few subjects, with no significant association with MS.

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Contributors SS and SOE performed experiments and statistical analysis and cowrote the paper. SR coordinated the study, designed and performed experiments, and supervised the research.

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