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The Association of miRNA-146a Gene Variation and Multiple Sclerosis in The Iranian Population

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Abstract

Background: Multiple sclerosis (MS) is a complex human autoimmune-type inflammatory disease of the central nervous system (CNS). MicroRNA-146a (miR-146a) belongs to an endogenous and non-coding RNA family with 18-22 nucleotides long, which modulates the innate and adaptive immune response.

Methods: Our study aimed to investigate a possible association between rs2910164 and rs2431697 polymorphisms of the miR-146a gene and multiple sclerosis in the Iranian population.

A total of 60 MS cases and 100 controls were recruited. Single nucleotide polymorphism (SNP) rs2431697 was genotyped by utilizing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and SNP rs2910164 was genotyped by using Tetra-primer ARMS-PCR. Statistical Analysis conducted by the chi-squared test utilizing SPSS version 21.0 Software. The Hardy-Weinberg equilibrium assumption was evaluated.

Results: The results of the present study suggest the miR-146a gene rs2431697 polymorphism is not associated with multiple sclerosis. However, there is a significant relationship between polymorphism rs2910164 of the miR-146a gene and multiple sclerosis in the population studied ($P = 0.012$).

Conclusion: Our data provide evidence that the miR-146a gene may be involved in creating the susceptibility to MS in the Iranian population.

Keywords: Multiple sclerosis, miR-146a, Tetra-primer ARMS-PCR., PCR-RFLP

Conflicts of Interest: *None declared*
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Introduction

Autoimmune disease is an abnormal response of the immune system in which the immune system attacks the host tissue. This condition results in tissue destruction and chronic inflammation. One example is multiple sclerosis, a CNS disease that affects approximately two million people worldwide and is characterized by acute astrogliosis, demyelination, and axonal damage. A mall group of parents was diagnosed with primary progressive phase (PPMS) compared to relapsing-remitting MS (RRMS) which affects 80 percent of MS patients and is characterized by irregular relapses followed by periods of remission with no new signs

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↑What is "already known" in this topic:
MicroRNA-146a belongs to an endogenous and none coding RNAs family which modulates the innate and adaptive immune response and involved in the negative regulation of Toll-like receptors (TLRs) signaling. Furthermore, have been demonstrated miR-146a has significant role in MS pathophysiology.

→What this article adds:
Investigating the relationship between two types of miRNA-146a gene polymorphisms (rs2910164, rs2431697) and the risk of MS in the Iranian population and the practical use of these polymorphisms for early diagnosis and prevention of this disease, which has a high prevalence in the young population.

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of disease activity. The precise mechanism of MS is still unclear. The disease is most likely caused by a complex interaction between multiple genes and environmental factors, resulting in inflammation-mediated central nervous system degeneration (1-3). A number of genomic studies have confirmed the importance of the immune system in the development of multiple sclerosis, including genetic association studies that have dramatically expanded the list of MS susceptibility genes beyond the nearly 40-year-old HLA association (4).

MicroRNAs (miRNAs), which are small single-stranded regulatory RNAs capable of interfering with intracellular messenger RNAs (mRNAs) containing either complete or partial complementarity, are beneficial for the development of new therapies against cancer polymorphism and viral mutation. Multiple microRNAs have been reported to induce RNA interference (RNAi), a post-transcriptional gene silencing mechanism. In addition, recent evidence suggests that they participate in the transcriptional regulation of genome activities. They were initially identified in Caenorhabditis elegans as native RNA fragments that modulate a wide variety of genetic regulatory pathways during embryonic development; they are now recognized as small gene silencers transcribed from the noncoding regions of a genome (5).

MiR-146a is a miRNA that is expected to negatively regulate the innate immune, inflammatory response, and antiviral pathway. MicroRNA-146a (miR-146a) belongs to an endogenous and non-coding RNA family with 18-22 nucleotides long which modulates the innate and adaptive immune response. miRNA-146a is located in the LOC285628 gene on chromosome 5 and plays crucial roles in regulating IRAK1 and TRAF6 in the NF-kB signaling pathway, which suppresses the synthesis of pro-inflammatory cytokines in myeloid and T cells. Moreover, miR-146a is involved in the negative regulation of Toll-like receptors (TLRs) signaling and airway remodeling-associated proteins (MMP-13 and collagen II). Disruptive miR-146a can lead to many autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome. Furthermore, some studies revealed that miR-146a was unregulated in active MS lesions, which demonstrated the significant role of miR-146a in MS pathophysiology (6-11).

Polymorphism in miRNA expression and maturation may be associated with disease susceptibility. It has been shown that three SNP (rs2910164, rs57095329, rs2431697) may have affected the functions of the miRNAs (12). For example, Luo et al. showed that the rs57095329 A>G polymorphism in miRNA-146a promotor correlated with reducing the affinity of the Ets-1 transcription factor and consequently, increased the risk of systemic lupus erythematosus in the Chinese population (13). Similarly, rs2910164 G>C and rs2431697 reduce the expression levels of miRNA-146a (12, 14). Different studies investigated the association between miRNA-146a polymorphism and autoimmune diseases but the results were not conclusive and inconsistent (12, 15-17). Turning to MS susceptibility, limited studies investigated the association between these three SNPs with MS and a discrepancy can be seen in reported data. For instance, a study in the Caucasian population reported no association between miR-146a rs2910164 and MS (18). However, Li et al. reported a significant association between rs2910164 variants and increased risk of MS in the females of the Chinese population (11).

In this study, we investigated the association between two variants of miRNA-146a gene polymorphism (rs2910164, rs2431697) and with the risk of MS in the Iranian population. We first examined the miRNA-146a gene rs2431697 SNP with RFLP-PCR, and subsequently, SNP rs2910164 was genotyped by utilizing the Tetra-primer ARMS-PCR. Finally, the genotype frequencies were calculated, and the statistical analysis was performed by the chi-squared test.

Methods

Patients and controls

The current study included a total of 160 individuals consisting of 100 control subjects and 60 patients Diagnosed with MS by a well-trained neurologist. All the patients were selected from a range of 20 to 60 years old from the MS Society of Tehran and sampled at the Endocrinology and Metabolism Research Institute, Tehran, Iran. In addition, patients with other autoimmune diseases or genetic disorders were excluded from our study. All subjects provided written informed consent for participation in this study.

Genotyping

A minimum of 4-5 ml peripheral blood samples were taken from subjects and poured into the tube containing EDTA as an anticoagulant agent. The samples were held at -70 degrees Celsius (-70 °C). Genetic DNA was isolated after proteinase K digestion utilizing a DNA extraction kit (MBST, Iran) according to the manufacturer's instructions. After the extraction, isolated DNA was purified and qualified with spectrometry technique using nanodrop (Thermo Fisher Scientific, USA). An SNP within the miR-146a (rs2431697) was targeted and genotyped employing the Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The primers used for PCR-RFLP were designed utilizing Primer3, Gene runner, Primer blast, and UCSC software, including a forward primer (5'-AGAGGGGGTGAAAGAAGGAA-3') and reverse primer (5'-TTCTCAGTGCCAATGTGAGG-3'). The conditions of PCR are as follows: the initiation step of PCR was performed at 95 °C for 1-5 minutes. The denaturation, annealing, and extension were performed at 95, 52, and 72 °C, respectively and lasted for 50 seconds each. The final extension was conducted at 72 °C for 10 minutes. Eventually, the restriction enzyme digestion was accomplished utilizing Taq1 (Thermo Scientific) with a concentration of 10 U/ul. This enzyme cut the 260bp sequence in rs2431697 into 199bp and 60bp fragments with T nucleotide. However, in the presence of a C nucleotide, the enzyme doesn't cut the 260bp sequence. The digestion was carried out in the hot plate at 36 °C for 16 hours and the digestion products were visualized with 3% agarose gel electrophoresis.

Moreover, another SNP (rs2910164) in miR-146a was in-

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Table 1. Primer design information for rs2910164 and rs2431697

Name	Primer name	Primer sequence (5'-3')	Tm	Product size
rs2910164	Forward inner (G allele)	CATGGGTTGTGTCAGTGTCAGACGACGTG	61.1	170
	Revers inner (C allele)	GATATCCCAGCTGAAGAACTGAATTTGAG	58.7	236
	Forward outer	CCAGATGTTTATAACTCATGAGTGCCAGG	60.1	351
	Reverse outer	ATACCTTCAGAGCCTGAGACTCTGCCTT	61.4	351
rs2431697	Forward	AGAGGGGGTGAAAGAAGGAA	51.8	
	Reverse	TTCTCAGTGCCAATGTGAGG	51.8	260

vestigated utilizing the Tetra primer amplification refractory mutation system (Tetra-primer ARMS-PCR). In this technique, four primers were designed, including two Inner primers and two Outer primers. The information about Primers and restriction enzymes is depicted in Table 1. The PCR conditions gene were similar to PCR-RFLP for the rs2431697 SNP except for the initiation denaturation phase which was conducted at 95 °C for 15 seconds and the annealing phase Conducted at 62 °C for 50 seconds in Tetra-primer ARMS-PCR. Finally, the PCR products are visualized by the agarose gel electrophoresis.

Sanger Sequencing

4 samples were randomly sequenced for each polymorphism to confirm the accuracy of PCR results.

Statistical Analysis

In this study, the statistical analysis was conducted by the chi-squared test utilizing SPSS version 21.0 Software. The Hardy-Weinberg equilibrium assumption was evaluated and the logistic regression procedure was utilized to achieve the adjusted odds ratio (OR) for genetic polymorphism. Besides, the P-value of less than 0.05 was considered statistically significant.

Results

This study included 160 subjects, with 100 healthy persons as the control group and 60 with MS diagnosis in Iran. The DNA obtained from the subjects was characterized by utilizing a spectrophotometer and agarose gel electrophoresis which showed an appropriate DNA extraction method.

In the presence of T nucleotide in RFLP-PCR, the Taq1 enzyme digested the 260bp sequence to 60bp and 199bp sequences. However, the 260bp sequence remains uncut in the presence of C nucleotide. Consequently, The Taq1 restriction enzyme produces one band in gel electrophoresis for homozygote genotypes and two bands for heterozygotes. As shown in Figure 1, the heterozygous TC showed two bands in 260bp and 199bp. Moreover, the homozygous CC and TT have one band in 260bp and 199bp, respectively (Figure 1). The results of the sequence were consistent with the results of PCR-RFLP and Tetra-primer ARMS-PCR, which are shown in Figures 2, 3, 4, 5 and 6. Indeed, the frequency of the rs2431697 in the miR-146a gene was analyzed and the related information for alleles and genotype frequencies for patients and controls is presented in Table 2. The C and T allele frequencies of this polymorphism were calculated at 57% and 43% for controls, and 64.2% and 35.8% for patients, respectively. Consequently, there was no significant association in miR-146a gene rs2431697 polymorphism was seen between patients and control groups. Turning to rs2910164, we observed GG genotypes in 170bp and the GC genotypes showed three bands in 351, 236, and 170bp in gel electrophoresis. The genotype frequencies for rs2910164 polymorphism were calculated, and the result is depicted in Table 3.

The GG, GC, and CC genotype frequencies were calculated at 77.0%, 20.0%, and 3.0% for the control group and 60.0%, 40.0%, and 0.0% for patients respectively (Table 2), and logistic regression analysis revealed the significant relation between the rs2910164 and MS and the C allele considered a risk factor in GC.

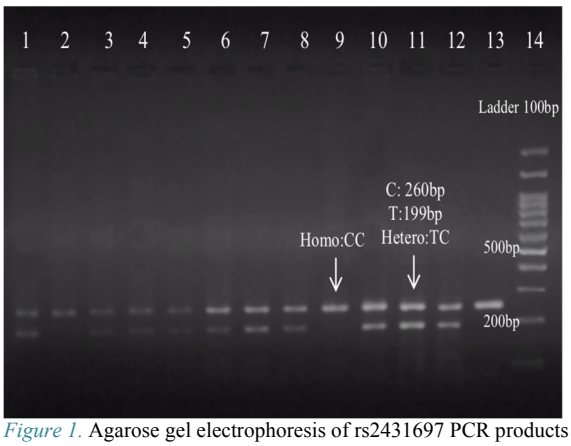

Figure 1. Agarose gel electrophoresis of rs2431697 PCR products

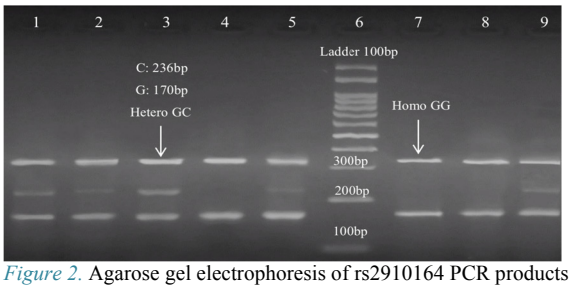

Figure 2. Agarose gel electrophoresis of rs2910164 PCR products

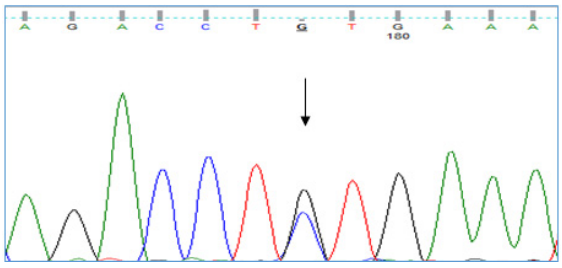

Figure 3. rs2910164, miR-146a gene (GC genotype)

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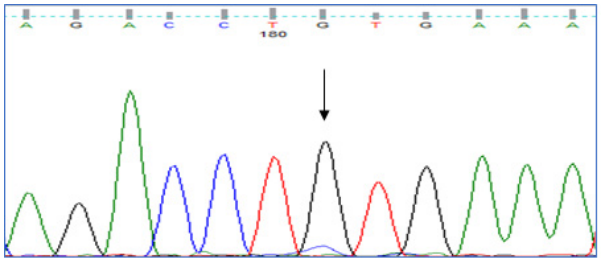

Figure 4. rs2910164, miR-146a gene (GG genotype)

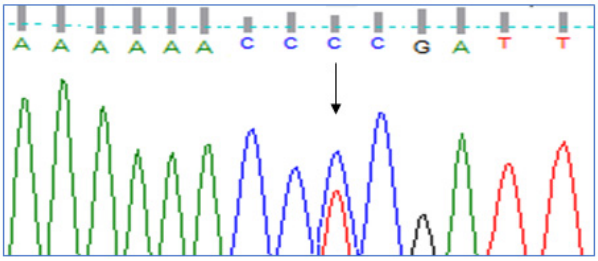

Figure 5. rs2431697, miR-146a gene (CT genotype)

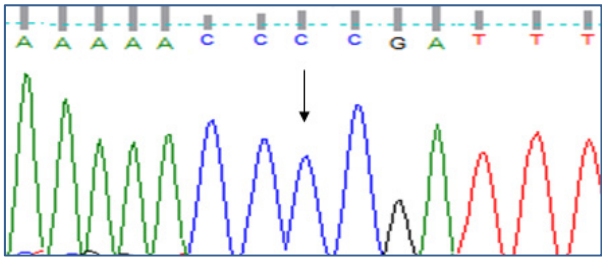

Figure 6. rs2431697, miR-146a gene (CC genotype)

Discussion

We intended to investigate the association between two SNPs of miRNA-146a gene (rs2431697, rs2910164) and MS. Several reported results indicated the importance of miRNAs in biological processes such as cell proliferation, apoptosis, stem cell maintenance, and embryonic development (19, 20). As mentioned before, miRNA-146a can modulate the innate and adaptive immune response. Increased expression of miRNA-146a affects the downstream signaling pathways of TLRs in the adaptive immune response by downregulation of the TRAF6/IRAK1 and consequently increases the production of tumor necrosis factor-a (TNFa). Moreover, the IRAK1 molecule can induce innate immune tolerance in neonates. Therefore, it can be concluded that miRNA-146a may play an important role in the pathogenesis of autoimmune diseases (21-23). For example, a mismatch of a G: U pair to a C: U in the stem structure of miRNA-146a precursor, which is the result of rs2910164 G>C polymorphism, can affect the expression of this miRNA leading to diverse functional alterations (14, 24). However, the contradictory results can be seen in other case-control and meta-analysis studies. For instance, Chen et al. did a comprehensive study among 2197 patients with autoimmune diseases and 3042 controls to analyze the correlation between miR-146a rs2910164 polymorphism and autoimmune diseases. The results showed that both allele and genotype frequencies have no significant association with autoimmune disease susceptibility in rs2910164 polymorphism (15).

However, Li et al. did a comprehensive study and demonstrated the significant association of the rs2910164 G>C polymorphism with autoimmune diseases (17). Furthermore, another meta-analysis confirmed this association by Yang and co-workers. They also showed that rs3746444 polymorphism was correlated with autoimmune diseases (16). Recently, a study by Park and his team reported a positive association between rs2910164, rs57095329, and rs2431697 and autoimmune diseases. Moreover, they found that the rs2910164 C allele reduces the risk of psoriasis, asthma, uveitis, and Behcet's disease but it increases the risk of MS (12).

Turning to MS, Li et al. revealed that the rs2910164 polymorphism contributes to the MS in female patients by affecting the mature miRNA-146a and the release of pro-inflammatory cytokines in MS patients. They investigated the effect of this polymorphism on the level of TNF-α, IFN-γ and IL-1β expression. Patients with GC and CC genotypes showed an elevated expression of TNF-α and IFN-γ in RRMS (11). Interestingly, in the present study, none of the rs2431697 genotypes were associated with an increased risk of MS, but rs2910164 genotypes were associated with

Table 2. Genotype frequency, allelic frequency, and significant level of the rs2431697 polymorphism in patient and control groups

rs2431697	Control (n=100)	Patients (n=60)	Sum (n=160)	*P*-value
	n(%)	n(%)	n(%)	
C	114 (57.0%)	77 (64.2%)	191 (59.7%)	
T	86 (43.0%)	43 (35.8%)	129 (40.3%)	0.206
CC	19 (19.0%)	17 (28.3%)	36 (22.5%)	
TC	76 (76.0%)	43 (71.7%)	119 (74.4%)	0.100
TT	5 (5.0%)	0 (0.0%)	5 (3.3%)	

Table 3. Genotype frequency, allelic frequency, and significant level of the rs2910164 polymorphism in patient and control groups

rs2910164	Control (n=100)	Patients (n=60)	Sum (n=160)	*P*-value
	N (%)	N (%)	N (%)	
C	26 (13.0%)	24 (20.0%)	28 (15.6%)	0.095
G	174 (87.0%)	96 (80.0%)	270 (84.4%)	
CC	3 (3.0%)	0 (0.0%)	3 (1.9%)	
GC	20 (20.0%)	24 (40.0%)	44 (27.5%)	
GG	77 (77.0%)	36 (60.0%)	113 (70.6%)	0.012

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an increased risk of MS. Of course, it should be noted that the results are different in other populations, which is certainly due to differences in geographical location, environmental conditions, lifestyle and race. It has been found that environmental factors influence genetic factors and since environmental factors are the drivers of genetic factors, therefore, examining the food pattern, geographical location and quality of life can be influenced by these polymorphisms. And their relationship with the occurrence or tendency of the disease to be effective. Since there is no definitive report on the significance or non-significance of these polymorphisms with MS disease, it is not possible to confidently comment on the pattern followed by the Iranian population. gave a decisive Therefore, more and more detailed studies are needed in a wider dimension to make a definitive opinion about the relationship between the two polymorphisms rs2431697 and rs2910164 of the miR-146a gene with MS disease.

Conclusion

In conclusion, the investigation of the association between the two SNPs (rs2431697, rs2910164) of the miRNA-146a gene and multiple sclerosis (MS) reveals contradictory results in different studies and populations. While some studies suggest a significant association between the rs2910164 G>C polymorphism and autoimmune diseases, including MS, others report no significant association.

Furthermore, in this study, it is observed that the rs2910164 genotypes are associated with an increased risk of MS, whereas none of the rs2431697 genotypes show an association with increased MS risk.

Given the influence of environmental factors on genetic factors and the variability among populations, more detailed studies are necessary to establish a definitive opinion on the relationship between these miR-146a gene polymorphisms and MS.

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Ethical Consideration

All procedures performed in this study were in line with the ethical standards of the EMRI institution at which this study was conducted. Informed consent was obtained from all subjects involved in the study.

Conflict of Interests

The authors declare that they have no competing interests.

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