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Genetic Variation in Intergenic and Exonic miRNA Sequence and Risk of Multiple Sclerosis in the Isfahan Patients

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ABSTRACT

MicroRNAs (miRNAs), have been documented to perform a key role in the pathogenesis of multiple sclerosis (MS), a chronic inflammatory and autoimmune disease. Recent studies have shown that single nucleotide polymorphism in the sequence of the miRNA may change their production and expression which can lead to miRNA dysfunction and pathogenicity. Some studies have reported the relationship between miRNA polymorphism and the increased risk of autoimmune disease. This study was conducted to investigate the association between *mir*155 rs767649, mir196a2 rs11614913 and *mir*23a rs3745453 polymorphism and the risk of multiple sclerosis in the Iranian MS patients in Isfahan.

A population of 80 patients and the same number control were selected. After DNA extraction, genotyping was performed through tetra amplification refractory mutation system-PCR method (T ARMS PCR).

The frequencies of TT, TC and CC genotypes of *mir*23a were 46, 35 and 20% in MS patients and 42, 14 and 24 in healthy subjects respectively. These results showed that individuals carrying the genotypes of rs3745453 TC had a 2.3-fold increased risk of MS (OR=2.3, p=0.048). There was no significant difference between genotypes and allele frequency of *mir*155 and *mir*196a2 in patients and healthy controls (p>0.05).

Our findings specified that CT heterozygosity in *mir*23a gene significantly related with risk of MS. Unlike *mir*155 and *mir*196a2, *mir*23a rs3745453 may have contributed to the etiology of MS in Isfahan patients. However, extensive studies are required to gain more reliable and authentic results.

Keywords: Genetic variation; MiRNA; Multiple sclerosis; T ARMS PCR

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INTRODUCTION

Multiple sclerosis (MS) is a neurological inability of the central nervous system (CNS) characterized with chronic inflammation, myelin and axonal loss, brain atrophy, and progressive neurological dysfunction. It is estimated that 2.5 million people in the world are suffering from MS. Recently the concerned increase in MS patients, has been reported by Iranian neurologist, especially in Isfahan. Despite unclear etiology, genetic susceptibility, environmental factors such as smoking, viral infections especially Epstein-Barr virus (EBV), low exposure to sunlight (vitamin D deficiency) and immunologic factors proposed for susceptibility to this disease.[1-3]

MicroRNAs (miRNAs) are small non-coding RNAs and one of the key elements in regulating gene expression which are involved in various biological processes, such as proliferation, differentiation, and apoptosis[4]. By targeting specific mRNA, they block the translation or induce its degradation. It is estimated that approximately 30 percent of all protein-coding genes are under translational control by miRNAs.[5,6]

A single nucleotide polymorphisms (SNPs) in the sequence of miRNAs may change their production or affinity to target genes that ultimately result in sensitivity to various diseases. Recent study documented abnormal miRNA expression in MS patients compared with controls. Some studies revealed that stable miRNAs in biofluids, such as blood, Cerebrospinal fluid and brain injuries, differentially expressed, therefore, make circulating miRNAs as diagnostic and predictive biomarkers.[7-10]

Even a minor alteration in miRNA coding gene may lead to significant changes in gene expression that confirmed to be correlated with autoimmune inflammatory diseases such as rheumatoid arthritis, type 1 diabetes, and MS.[11-14] *mir*23a is a non-coding RNA intergenic variant that is located at chromosome 19. Based on websites such as the www.microRNA.org and www.pictar.org and Target Scan 6.1, mir23a has a role in immunity and MS pathology. Several studies reported the importance of this miRNA in pathological state of several cancers, cardiac hypertrophy, and multiple sclerosis.[15-22.]Recently *mir155* proposed as an immune-response modulator for both adaptive and innate immune systems.[23] some researchers have specified that miR155 plays a critical role in various physiological and pathological processes such as inflammation, differentiation, cancer, viral infections, and cardiovascular diseases. [24] Moreover, miR155 has been characterized as a component of the primary macrophage response to different types of inflammatory mediators such as IFN-β or Tumor Necrosis Factor-α (TNF-α). [25-29] It has been shown that miR155 is induced by bacterial lipopolysaccharide (LPS) in a human monocytic cell line.[30]

Some study in Egyptian population proposed that mir196a2 rs11614913 polymorphism could be associated with asthma severity.[31] Some of reports showed that polymorphism of mir196a2 rs11614913, as a biomarker associated with some types of cancer such as gallbladder, liver, breast, gastric, prostate, as well as autoimmune disease.[25] Based on the fact that genetic variants can alter the expression and function of miRNAs, we carried out an association study between miRNA polymorphism and MS susceptibility.

In present study, we attempted to specify the frequency of genotypes and alleles regarding *mir* 155 rs767649, *mir*196a2 rs11614913 and *mir-23a* rs3745453 polymorphisms in a subset of Isfahan MS patients and the healthy controls. This study is the first report of the rs3745453, rs767649 and rs11614913 polymorphism, especially in Iranian MS population.

MATERIALS AND METHODS

Sample Collection

Eighty patients with definite MS were selected from Department of Neurology (No. IR.MIAU.REC.1396.801) at Kashani Hospital within 2016-2017.This individuals was recognized according to the revised criteria (Mc Donald, 2010) by neurologists. The same number healthy subjects that were age-matched were selected as control. Normal individuals have been checked for the history of MS and any other inflammatory-demyelinating disease in their families.

DNA Extraction

Peripheral blood with a volume of 2 mL was collected in the tubes containing EDTA from both MS and the healthy groups. DNA extracted with salting out method, Briefly, RBC were lysed using low salt-buffer I (10 mM KCl, 10 mM Tris-HCl (pH: 7.5), 10 mM MgCl2, 2 mM EDTA,). To the pellet added high salt-buffer II (10 mM Tris-HCl, 400 mM NaCl, EDTA

KCl), 10% SDS were added and incubated in 37°C. To remove proteins, 6 M NaCl was added and centrifuged for 5 min at 8000 rpm. For extraction of DNA, 2 volumes of absolute ethanol were added to the supernatant. The extracted DNA was washed with 70% ethanol and dried then sterile water added to vials. DNA was stored at -20°C until experiments.

Quality controls of extracted DNA was detected by spectrophotometer and visualized by electrophoresis on 1% agarose gel

Genotyping

The genotyping of three miRNA are performed using Tetra amplification refractory mutation system-polymerase chain reaction (T ARMS PCR).

For genotyping of samples, DNA was amplified using smar Taq polymerase (Cinnagen, Iran). The PCR reaction tube contains, 5 µL of genomic DNA (100 ng/mL), 2 µL of inner primers (10 pmol), 1.5 µLof outer primers (10 pmol), 0.75 µL of MgCl2 (1.5mM), 0.5 µL of dNTP (20 mM), 1.2 µL of PCR buffer, 0.3 µL of smar Taq polymerase (Cinnagen, Iran) and appropriate amount of ddH2O .

All primers were designed by primer3 software and analysed with oligo7 and BLAST software in NCBI. The features of primers are listed in Table 1.

Amplification of SNPs was carried out in a 20 µL reaction volume and under standard reaction conditions, consisting of initial denaturation for 5 min at 95°C followed by 34cycles of 95°C for 45 s in a Biorad thermocycler (Bio-Rad Laboratories USA), annealing temperatures (58/2 C for rs3745453, 55.8 for rs767649 And 64 for rs11614913) for 1min, 72°C for 40 s, followed by extension Temperature at 72°C for 10 min. PCR products were detected by Ethidium bromide (Sinaclon, Iran) on 2% agarose gel electrophoresis with TBE 1 x buffer (Figure 1).

Statistical Analysis

In this study T-Test was used to compare the distribution of genotypes and alleles frequencies in control and patient groups. Pearson Chi-square method was applied for Hardy Weinberg equilibrium evaluation of genotypes. A *p* lower than 0.05 was considered significant. In order to estimate the relationship between genotype variants and the odds of a disease, odd ratio (OR) with 95% confidence intervals was evaluated.

RESULTS

In this case- control study MS group were consists of 80 patients with an average age of 17-45 years and an equal number as a control group. The ratio of female: male was 60/20 in MS and 61/19 in controls. Most of the MS patients were between 25 and 35 years old. in the present study t tests and Pearson's Chi-square did not show any significant difference in age and gender distribution between patients and healthy groups ($p>0.05$).

Frequency of rs767649 A/T, rs11614913 T/C and rs3745453 T/C Polymorphism in MS Patients and Control Groups

The genotypes frequencies of miRNA polymorphisms in MS and control subjects are shown in Table 2.

Frequency of *mir*23a rs3745453 T/C genotypes in both groups showed that this variant was not perverted from Hardy Weinberg equilibrium ($p>0.05$). The TC genotypes were the most repeated genotypes in the studied groups.

The results revealed that TC genotype of *mir*-23 rs3745453 significantly increased the risk of MS (OR =2.3, $p=0.04$) compared to TT genotype (OR=0/7), while the minor allele frequency (C allele) of rs3745453 was not associated with MS risk (OR=0/5 $p>0.05$). Furthermore, the rs3745453 C allele seems not to be a risk factor for susceptibility to MS (OR=0/5, 95% CI=0.09-0.06). As shown in Table 2, 3 for the *mir* 196a2 rs11614913 polymorphism, CT genotype was significantly more common compared with other genotypes ($p=0.71$, OR=1.1) whereas the TT genotype had a lower frequency in the control and case groups.

The CT genotype was the most common genotype in both case and MS patients groups. For the *mir*155

rs767649, AA genotype frequency was too low to be defined by statistical methods (Table 2). 93% of MS patients and 87% of controls had the T allele while the frequency of A allele was 1% in MS patients and controls. A major difference obtained in the number of alleles T and A between MS and healthy groups, no significant difference was shown between male and female MS patients carrying alleles T or A as well as TT, AT or AA genotypes.

Table 1. T ARMs polymerase chain reaction (T ARMS PCR) primers and their annealing temperature in Isfahan multiple sclerosis patients and controls

Gene	Sequence primer (5´- 3´)	Annealing temperature	Product size
*Mir*23a Outer F	CGAAGCAGAAAGACAGGAGG	58.2	837
*Mir*23a Outer R	CGTTTTGGTTGAGGGACAGT		
*Mir*23a Inner F	AAATGTGCTACATTGACTTAGAGTGC	58.2	C 394
*Mir*23a InnerR	GGCAGGACTTTCCAGAAATAGA		T 490
*Mir*155 Outer F	TGTCTATGACCACTAATTCCCAC	55.8	841
*Mir*155 Outer R	AAATTTGGGTTAAATGATGTCAC		
*Mir*155 InnerF	ATATAACACATTATCAAAAACACCGT	55.8	T 404
*Mir*155 InnerR	ATTAGAGCACTCAGAAAAGCGT		A 485
*Mir*196a2 Outer F	CCTTAGGGAGGTTGTGGGGGC	64	472
*Mir*196a2 Outer R	TTGGAATTGGCTGGACCCTCTTT		
*Mir*196a2 InnerF	GAACTCGGCAACAAGAAACGGC	64	C 310
*Mir*196a2 InnerR	CGAAAACCGACTGATGTAACTCCGA		T 208

Table 2. Distribution of rs3745453, rs11614913 and rs767649 polymorphism genotypes in Isfahan multiple sclerosis patients and controls

Genotype frequencies rs3745453	Patients (n=80)	Controls (n=80) *p*		OR[c] (CI: 95%)[b]
TT	37 (46%)	42 (52%)		0.7(0.1-0.09)
CC	16 (20%)	24 (30%)		0.59 (0.1-0.08)
CT	27 (35%)	14 (17%) 0.04		2.3 (0.1-0.09)
rs11614913 TT	11(13%)	9(11%) NS		1.2(0.082-0.074)
CC	29(36%)	34(42%)		0.8(0.13-0.099)
CT	40(50%)	37(46%)		1.1(0.16-0.10)
rs767649 TT	75(93%)	70(87%)	NS	1.1(0.11-0.04)
AT	5(6%)	10(12%)		0.46(0.06-0.057)
AA	0	0		0

[a]MS; Multiple sclerosis, [b]CI;Confidence of interval and [c]OR; Odds ratio,

Table 3. Distribution of rs3745453, rs11614913 and rs767649 polymorphism alleles in Isfahan multiple sclerosis patients and controls

Allele frequencies rs3745453	Patients %	Controls%	*p*	(CI: 95%)
T	0.63	0.61	(TT+TC)vs.CC	(0.02-0.03)
C	0.37	0.39	0.7	(0.09-0.06)
rs11614913			(CC+CT) vs. TT	(0.082-0.074)
C	0.61	0.63	0.7	(0.13-0.099)
rs767649			(TT+TA) vs. AA	(0.11-0.04)
A	0.1	0.1	0.33	(0.06-0.057)

CI; Confidence interval

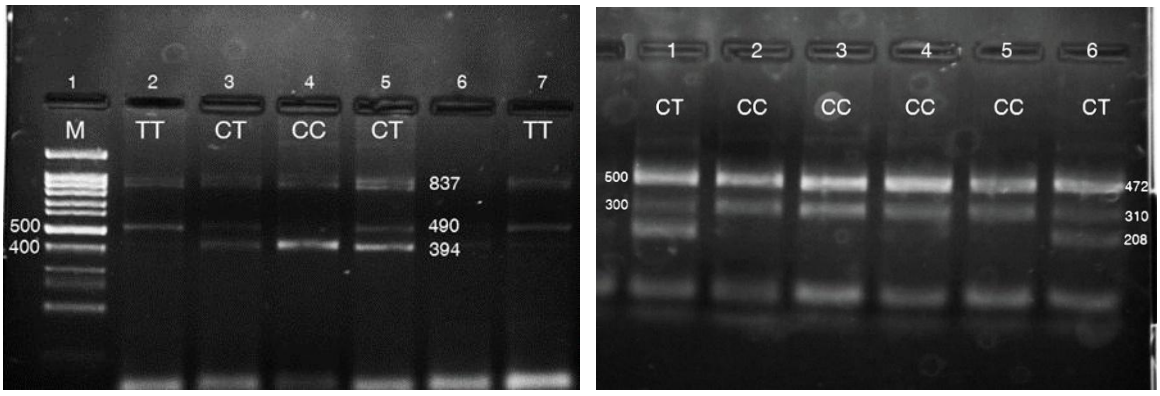

Figure 1. Gel electrophoresis of PCR products of Isfahan MS patients using T ARMS PCR. A. (left, mir23a) Lane 1 represents the 100 bp DNA marker (sinaclon, Iran), lanes 2,7 correspond to the TT homozygous individuals, Lanes 5, 3 correspond to the T/C heterozygous type individuals, lane 4 correspond to the CC homozygous individuals. B. (right, mir196a2) lane1, 6 represents heterozygous type individuals, Lanes 2, 3, 4, 5 correspond to the CC/homozygous genotype

DISCUSSION

In recent years, it has been suggested that miRNAs act as key regulators of gene expression networks. miRNAs have been reported as major cellular modulators that are involved in vital biological processes such as immunity. Like other genes, the genetic variation in the miRNA sequence results in functional changes that are associated with the pathogenesis of autoimmune diseases.[23] In this case control study, we examined the impact of functional *mir*155 rs767649, *mir*196a2 rs11614913 and *mir*23a rs3745453 polymorphism on MS risk in a sample of Isfahan population. We found that *mir*-23a rs3745453 CT variant seems to significantly increase the risk of MS in this population (OR=2.3 $p<0.04$). Compared with Ridolfi et al [15] our results did not support an association between rs3745453 C allele polymorphism and MS pathogenicity ($p=0.16$). The number of samples and variety of studied resources (such as blood, plasma, tissue) could be related to difference of results in countries. In a study on MS in Italy, reported that serum level of mir23 in Ms patients decreases, while the frequency of C alleles (inverse to our study)

increased compared with controls.[15] Therefore, suggested that polymorphism of *mir*-23a rs3745453 have potential to play as a hazard factor for MS. In the context of *mir* 23a, more studies have been conducted on its expression and based on reports of the effect of this miRNA in inflammatory conditions, it is necessary to evaluate the association of its polymorphism with autoimmune diseases in different countries. In another study, *mir*-23a has been implicated in some type of cancers, cardiac hypertrophy, and muscular atrophy.[5,16] Several studies have reported a significant increase in expression of *mir*23a in various types of cancers, such as bladder, malignant cholangiocytes, laryngeal cancer, osteoblast cell line and its reduction in serum of patients with Polycystic ovary syndrome. In a study conducted in China, showed that *mir*-23a in Serum has a potential biomarker for recognition of type 2 diabete.[17-22]

An exonic *mir*-155 with rs767649 (T/A) polymorphism in the upstream region of the pre-*mir*-155 gene is transcribed from the B-cell integration cluster (BIC). It has been suggested that *mir*155 modulate host antiviral immune response by elevating type I IFN signaling, and contribute in diverse biological processes such as immunity. Dysregulation of *mir*-155 has been reported in viral infections and different cancers. Some study reported *mir*155 highly expressed in activated T and B cells as well as macrophage and dendritic cells.[23] Based on the odds ratio calculated, about *mir*155, suggested that rs767649 TT genotype have possible potential to act as a risk factor for MS (OR=1.1, 95% CI=0.11-0.04). However further research is needed to confirmed our results. Unlike our results, in the Chinese population, the TT genotype was reported as a protective factor in Cervical Cancer OR=0.62, 95% CI=0.47–0.82.[24] Present study showed that 93% of patients and 87% of controls had the T allele, and none of the patients and controls had the AA genotype, which was similar to results of Brazilian population genotypes frequency in diabetes mellitus disease. The frequency of TC genotype in our study population was significantly different from that of China (5% versus 51%), Likely due to differences in the geographic areas or different mechanisms of pathogenicity of this locus in different races. Another study in China, reported TT genotype of rs767649 was associated with risk of non-small cell lung cancer (OR=1.28, 95% CI=1.03−1.58).[26]

Recent evidence indicates that *mir*-196a2 rs11614913 has a role in innate immunity, also contributes as a biomarker of cancer.

In *mir*-196a2 rs11614913, our results like another studies of China, USA, Germany, Japan, and Turkey on cancer) [25,29] showed that CT genotype was significantly more common compared with other genotypes (OR=1.1, 95% CI=0.16-0.10), whereas the TT genotype had a lower frequency in the control and case groups. Regarding gastric cancer, miR-196a2 polymirphism represented no significant association with risk of disease in any genetic model test. In Russia, there was a strong correlation between the miR196a2 polymorphism and the severity of MS (OR=3.23, CI: 1.99–5.26).[29] In a study on the Asian population, It was shown that, the *mir*196a2 rs11614913 polymorphism seems to contribute to susceptibility to lung cancer risk, diagnosis, screening and therapy.[25,27] In several recent meta-analysis studies, it was found that there is no significant relationship between gastric cancer and *mir*169a2polymorphism, but in case-control studies in the Egyptian, German and Japanese populations, there was a significant correlation between *mir*196a2 rs11614913 polymorphism and stomach cancer prognosis. [27]

By Comparing other extensive association studies that have been conducted in Asian and European populations with different techniques such as PCR RFLP, Taq man SNP Genotyping and DNA sequencing, on various types of cancer, it confirmed that *mir*196a2 rs11614913 polymorphism significantly increases the risk of breast and the lung cancer. In other words, individuals with CC genotype have a higher risk than CT or TT genotypes for breast, lung and stomach cancer. Consequently, CC homozygotes, which undergo a change in the pattern of the expression of the mir196a2 gene, are more susceptible to cancer.[28]

In conclusion, our findings showed that CT heterozygosity in *mir*23a gene significantly related with risk of multiple sclerosis (OR=2.3, p=0.048). However, extensive studies are required to gain more reliable and authentic results. Present study is the first report of *mir 23a*, *mir155* and *mir 196a2* polymorphism in the Iranian MS patients. We believe that the results of this study could be a prelude to further broader studies on the association of different polymorphisms of miRNA and multiple sclerosis risk.

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