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Original Article

Circulating *miR-193b-3p* and *miR-376a-3p* Involved in Iranian Patients with Multiple Sclerosis

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Multiple sclerosis (MS) is an inflammatory disease that affects the central nervous system. MicroRNAs (miRNAs) are small non-coding RNAs that are usually 18-24 nucleotides long, which may have a pivotal role in the expansion of many complex diseases. The objective of this study was to evaluate the transcript levels of *miR-193b-3p* and *miR-376a-3p* in relapsing-remitting multiple sclerosis (RRMS) patients. In this case-control study, *miR-193b-3p* and *miR-376a-3p* expression in 60 RRMS patients, of which 30 were recurring patients and 30 were two months after relapse patients, and 30 healthy subjects were examined in peripheral blood mononuclear cells using real-time PCR reaction. Our results showed that the expression of *miR-193b-3p* was significantly reduced in recurring patients and two months after relapse patients in comparison with healthy subjects ($P < 0.004$ and $P < 0.0001$, respectively). In contrast, *miR-376a-3p* showed an increased expression in recurring patients and two months after relapse patients ($P < 0.0001$). Based on the findings, it can be assumed that *miR-193b-3p* and *miR-376a-3p* may be prospective biomarkers with the potential use for diagnosis of RRMS patients.

Keywords: Multiple sclerosis, biomarker, miRNA, *miR-193b-3p*, *miR-376a-3p*

Multiple sclerosis (MS) is an inflammatory autoimmune disease specified by myelin demolition of the central nervous system (CNS) (1). MS attacks the myelinated axons in the CNS and annihilates the myelin and axons (2). Some population-based studies have demonstrated a pointy increase in the prevalence of MS in Iran. Isfahan is currently globally known for its high prevalence of MS during the last decade (3). Disease clinical signs begin usually in the third and fourth decade of life, and the sex ratio is approaching 3:1 in female and male, respectively (4). Neurologists suggested that MS patients may be grouped into four classes including relapsing-remitting MS (RRMS) which is the commonest class, composing about 85% of MS patients, secondary progressive MS (SPMS), primary progressive MS (PPMS) representing about 10% of MS patients, and progressive-relapsing MS (PRMS) which is an uncommon form (2, 5). Some individuals with RRMS have a benign disease period with minimal disease impairment. However, many patients finally convert to SPMS (6). Although the exact etiology of MS remains still enigmatic, during the last few years the identification of genetic variants affecting the development of MS disease has grown (7). Biomarkers reflecting the prediction of inability and assessment of therapeutic response are desirable in the management of patients. Biomarkers are biological materials that are objectively evaluated as an indicator of normal biological processes or pathogenic events (8-9). MicroRNAs (miRNAs) are

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a family of endogenous, noncoding RNA molecules with approximately 22 nucleotides length which regulate gene expression post-transcriptionally by binding to the 3′-untranslated region (UTR) of their mRNA targets. Currently, it is clear that miRNAs can potentially regulate every aspect of cellular activity, including differentiation, metabolism, proliferation, apoptosis, tumorogenesis (10-12). Several studies have demonstrated that abnormal miRNAs function in peripheral blood immune cells as well as CNS glial cells (13). In MS disease, miRNAs dysregulation is proposed in several immune cells. Various studies have indicated changes in miRNAs expression in brain tissue and immune cells from MS patients, and associations between MS progression and miRNAs expression (14,15). The main challenge in MS is to develop biomarkers that could help in diagnosing MS disease (16). Therefore, we focused on two miRNAs as potential candidates for MS biomarkers and the expression of *miR-193b-3p* and *miR-376a-3p* was determined by quantitative real-time PCR in peripheral blood mononuclear cells (PBMCs) of RRMS patients and healthy individuals. In order to explore whether the deregulated expression of *miR-193b-3p* and *miR-376a-3p* could be used as a biomarker in RRMS.

Materials and methods

Patients and samples

Blood samples were collected from RRMS patients and random samples from healthy subjects (both male and female). Overall, 90 samples including 60 patients with RRMS, of whom 30 were recurring patients, 30 were two months after relapse patients, and 30 healthy subjects who were referred to MS Clinic of Kashani Hospital, Isfahan Province, were selected for this study. The healthy subjects had no history of autoimmune disease based on the physician examination. Recurring patients and two months after relapse patients were diagnosed by an expert neurologist based on the recommended McDonald diagnostic criteria (17). Forty-two patients had only received β-interferon (IFN-β) for treatment, and all other remaining patients had not received any treatment for at least two months prior sampling. Four ml blood was collected in EDTA-containing tubes and immediately transported on ice to the laboratory. Written informed consent was obtained from all participating individuals before sample collection, and all procedures were performed according to the institutional ethical guidelines.

Peripheral blood mononuclear cells (PBMCs) isolation

At the first step, the peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using density gradient lymphoprep (Bio Sera, Kansas City, USA) based on the manufacturer's protocol. Briefly, 4 ml of blood was diluted at a ratio of 1:1 with physiological saline and gradually added to 4 ml lymphoprep solution gradient in a falcon tube. The tubes were centrifuged at 800 ×*g* for 30 min, and then PBMCs were transferred from the middle phase into 2-ml RNAase-free microtubes and frozen at −70 °C until next step.

MiRNA extraction

MiRNA was extracted from PBMCs using miRNA Hybrid-R (Geneall, Seoul, Korea) based on the manufacturer's instructions. Quality of the extracted miRNA was determined according to the 260/280 absorbance ratio, measured by NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA).

Complementary DNA synthesis and real-time polymerase chain reaction

Complementary DNA (cDNA) synthesis for *miR-193b-3p* and *miR-376a-3p* was carried out using a universal cDNA synthesis kit (Exiqon, Denmark) using poly-A tailing, according to manufacturer's instructions. Real-time quantitative PCR reactions were carried out in triplicate by using standard protocols with an ABI PRISM 7500 instrument (Applied Biosystems, USA). Briefly, in a total volume of 10 µl, 20 ng/µl of cDNAs were

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added to a master mix comprising 10 pmol/µl of each *miR-193b-3p* or *miR-376a-3p* primers (Exeqon, Denmark) and 5 ml of SYBR premix ExTaq II (TaKaRa, Kusatsu, Shiga Prefecture, Japan) and *U6* was selected as a housekeeping gene for normalization of data.. The program for the run was set as follows: 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR reaction was followed by a melting curve program (70–95 °C with a temperature transition rate of 1 °C s^{-1} and a continuous fluorescence reading). Real-time PCR data analysis was performed using the ΔΔCT method, where CT is the cycle threshold (18).

Statistical analysis

For the statistical study, Graph Pad Prism statistical software, version 6.05 (Graph Pad, San Diego, CA, USA) was used. The normality was evaluated by the Kolmogorov–Smirnov test. P≤ 0.05were considered as statistically significant.

Results

Clinical characteristics

In this study, 60 RRMS patients of whom 30 were recurring patients (mean age: 39.20 ± 2.154 years, range: 18-60, 7 male and 23 female), 30 patients that were two months after relapse (mean age: 33.7 ± 1.522, range: 21-51, 9 male and 21 female), and 30 healthy subjects (mean age: 38.60 ± 1.843, range: 21-58, 10 male and 20 female) were investigated. Clinical characteristics of patients (recurring patients and two months after relapse patients) are shown in Table 1.

Down-regulation of *miR-193b-3p*

To explore the potential roles of *miR-193b-3p* in patients with RRMS, we investigated the expression level of *miR-193b-3p* in recurring patients and two months after relapse patients versus healthy individuals by qRT-PCR. Our results showed that the expression level of *miR-193b-3p* was significantly down-regulated in both recurring patients and two months after relapse patients compared to that in healthy individuals (P < 0.004 and P < 0.0001, respectively) (Figure 1). These results indicate that *miR-193b-3p* was significantly decreased in the RRMS patients and probably plays an important role in MS disease.

Up-regulation of *miR-376a-3p*

The expression of *miR-376a-3p* was evaluated by qRT-PCR method in two groups: RRMS patients including 30 recurring patients, 30 two months after relapse patients (n=60), and healthy individuals (n=30). The results demonstrated significant growth in the expression of *miR-376a-3p* in both RRMS patients (recurring patients and two months after relapse patients) compared with healthy individuals (P < 0.0001). We observed that RRMS was associated with higher levels of *miR-376a-3p* expression compared with healthy individuals (Figure 2).

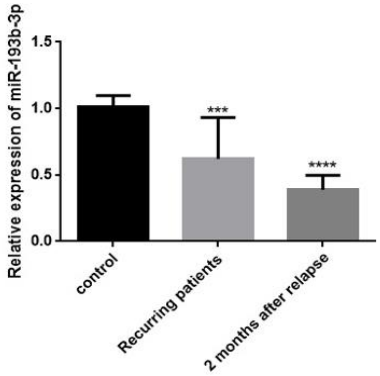

Figure 1. Relative expression of *miR-193b-3p* in recurring patients and two months after relapse patients and healthy individuals. Relative quantification for *miR-193b-3p* was significantly different between patients and control groups (***P < 0.004; ****P < 0.0001).

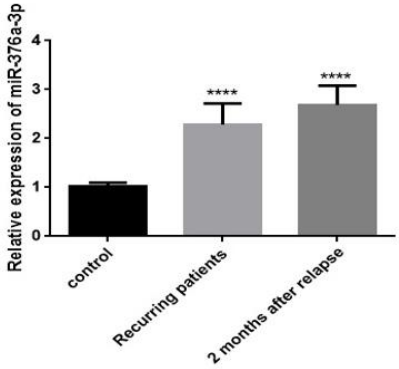

Figure 2. Relative expression of *miR-376a-3p* in recurring patients and two months after relapse patients and healthy individuals. Relative quantification for the *miR-376a-3p* was significantly different between patients and control groups (****P < 0.0001).

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Table 1. Clinical characteristics of recurring patients and two months after relapse patients

Characteristics	Control	Recurring patients	Two months after relapse patients
Number of subjects	30	30	30
Mean of disease duration (years)	-	6.72 ±0.76	5.81±0.77
Range (years)	-	0.5-16	0.5-20
Family history	-	11	8
Drug			
Interferon	-	18	24
Non-interferon	-	12	6

Discussion

Circulating miRNAs might have important role in MS development and prognosis (19, 20). In this study, *miR-193b-3p* and *miR-376a-3p* were selected from miRWalk database, as two miRNAs involved in MS disease. Subsequently, the expression of *miR-193b-3p* and *miR-376a-3p* was determined by quantitative real-time PCR in two groups: RRMS patients including 30 recurring patients and 30 two months after relapse patients (n=60), and healthy individuals (n=30). The data revealed a reduced expression of *miR-193b-3p* in both recurring patients and two months after relapse patients in comparison with healthy individuals. According to our results, down-regulation of *miR-193b-3p* may have tumor-suppressing role in RRMS. Additionally, we observed that the expression of *miR-376a-3p* increased significantly in recurring patients and two months after relapse patients in comparison with healthy individuals. Based on the results, we hypothesized that overexpression of *miR-376a-3p* in the RRMS group in comparison with the control group could be studied as a potential therapeutic target to inhibit RRMS progression in the future. It has proposed that changes in the expression of *miR-193b-3p* and *miR-376a-3p* have been associated with various autoimmune diseases. Iwamoto et al. have determined the expression of *miR-193b* in patients with systemic sclerosis (SSc) and healthy controls using TaqMan-based real-time PCR. They find that *miR-193b* targets urokinase-type plasminogen activator and is downregulated in SSc and probably *miR-193b* downregulation contributes to proliferative vasculopathy in SSc (21). On the other hand, TaqMan array analysis indicated that *miR-376a* was significantly increased in CD^{4+} T cells from PBMCs of RRMS patients (22). Moreover, 24 dysregulated miRNAs were reported in the epidermis of psoriatic skin and 37 other miRNAs were found to be dysregulated in the dermal inflammatory infiltrates of the corresponding patients. Among those, *miR-193b* was downregulated in Th17 cells (23). A previous microarray screening identified *miR-376a-3p* as a differentially expressed (up-regulated) miRNA in MS disease (24). The identification of genetic factors involved in MS could be useful for better understanding of the prognosis and treatment of this disease and probably other autoimmune diseases sharing a common underlying mechanism with MS (25, 26). Taken together, the results of this study indicated the relevance of *miR-193b-3p* and *miR-376a-3p* to RRMS. This means that in the future, *miR-193b3p* and *miR-376a-3p* may be considered as potential biomarkers for the early diagnosis of RRMS patients. Understanding the complexity of miRNAs may open up a new way to find biomarkers for clinical diagnosis of some serious diseases and to monitor the efficacy of various therapies. However, additional *in vitro* and *in vivo* experiments are required to address the biomarker usefulness of

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these miRNAs.

Conflict of interest

The authors declared no conflict of interest.

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