Up-regulation of circulating miR-93-5p in patients with relapsing-remitting multiple sclerosis

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Received; 12/09/2019 revised; 20/10/2019 accepted; 24/11/2019

Abstract

Introduction: Multiple sclerosis (MS) is an inflammatory demyelinating in which there is no standardized method to detect this disease activity. It has shown abnormal microRNAs (miRNAs) function in peripheral blood immune cells. miRNAs expression is probably responsible for immunological features associated with MS. The purpose of the current study was to investigate the association of miR-93-5p expression with MS disease.
Materials and methods: In this case-control study, a totally of 30 relapsing-remitting MS (RRMS) patients and 30 healthy subjects were enrolled. Following miRNA extraction and cDNA synthesis, miR-93-5p expression was examined in peripheral blood mononuclear cells (PBMCs) using real-time polymerase chain reaction.
Results: The expression of miR-93-5p was significantly increased in RRMS patients compared to healthy subjects (P=0.001). Receiver operating curve (ROC) analysis identified a strong predictive power of miR-93-5p on discriminating MS from healthy individuals, with the area under the curve (AUC) of 0.939 (95% CI; 0.8581-1.000). On the other hand, the samples were analyzed based on the type of drug treatment (interferon and non-interferon), which did not show any considerable differences (P=0.863).
Conclusion: Our findings showed that *miR-93-5p* has highly elevated expression in patients with RRMS compared to healthy subjects. Based on the results *miR-93-5p* may be a prospective biomarker with the potential use for diagnosis of RRMS patients.

Keywords: Multiple sclerosis, MicroRNA, miR-93-5p

Introduction

Multiple sclerosis (MS) is the prototypic disease of the central nervous system (CNS) myelin (1). The disease results in injury to the myelin sheaths, the oligodendrocytes, and, to a lesser extent, the axons and nerve cells themselves. The symptoms of MS modify, depending in part on the location of plaques within the CNS (2). It is guesstimated to affect up to two million people worldwide. Its clinical appearances begin usually in the third and fourth decade of life, with a female: male ratio approaching 3:1(3). The prevalence of MS indicates considerable variability all

over the world (4). Some population-based investigations have shown 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 (5). The risk of acquiring this complex disease is associated with exposure to environmental factors in genetically susceptible people (6). According to the explanations provided, MS grouped into four classes including relapsing-remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR) (7). This autoimmune disease most frequently presents as a relapsing-remitting disease form (RRMS). The progression and severity of symptoms are often unpredictable, highly variable, and can include motor, sensory, and cognitive impairments (8). Identification of biomarkers that could predict the development of MS in high-risk populations would allow for intervention strategies that may prevent evolution to definite disease (9). microRNAs (miRNAs) are attractive as potential biomarkers because their expression pattern is reflective of underlying pathophysiologic processes and they are specific to various disease states. miRNAs are endogenous, noncoding, single-stranded RNAs of 19–25 nucleotides in length (10-11). Due to miRNAs are stable in serum, they are being developed as biomarkers for cancer and other diseases. Suitable non-aggressive biomarkers with high specificity are needed for the diagnosis of various diseases (12-13). Several investigations have indicated that unusual miRNAs function in peripheral blood immune cells as well as CNS glial cells (14). In MS disease, miRNAs dysregulation is suggested in several immune cells. Various studies have demonstrated alternations in miRNAs expression in brain tissue and immune cells from MS patients, and associations between MS progression and miRNAs expression (15-16). Investigation of the deregulated miRNAs in various autoimmune diseases has shown the relation between the deregulation of *miR-93-5p* and MS (17). Thus, to explore whether the deregulated expression of miR-93-5p can be used as a biomarker in RRMS, we have determined the expression of miR-93-5p by quantitative real-time PCR in peripheral blood mononuclear cells (PBMCs) of RRMS patients and healthy individuals.

Materials and methods

The study was approved by the Ethics Committee of the Medical Genetics Research Center of Genome. Informed consent was taken from all participants before sample collection

Patients and controls

Blood samples were collected from MS patients and random samples from healthy individuals (both male and female). Overall, 60 samples including 30 relapsing-remitting MS (RRMS) patients, diagnosed in the Kashani Hospital (Isfahan, Iran) and 30 healthy controls were selected for this study. The healthy subjects had no history of autoimmune diseases based on the medical examinations. MS patients were diagnosed by an expert neurologist based on the recommended McDonald diagnostic criteria (18). Four mL peripheral blood was collected in EDTA-containing tubes and transported to the laboratory on ice.

Peripheral blood mononuclear cells (PBMCs) isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by density gradient lymphoprep (Bio Sera, Kansas City, USA) based on the manufacturer's protocol. Mononuclear cells, monocytes, and lymphocytes have lower densities in comparison with

erythrocytes and leukocytes, therefore, after centrifugation; they remain in an intermediate phase. Briefly, 4 ml of blood was diluted at a ratio of 1:1 with physiological saline and gradually added to the 4 ml lymphoprep solution gradient in a falcon tube. Collected falcons were centrifuged at 800 g for 30 mins and then PBMCs were transferred from the middle phase into a 2 mL RNAase-free microtube and was frozen at −20 °C.

miRNA extraction

miRNA was extracted from PBMCs using miRNA Hybrid-R (Geneall, Seoul, Korea) based on the manufacturer's instructions. The quality of miRNA at a 260/280 nm wavelength ratio was measured by a NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA).

Complementary DNA synthesis and real-time PCR

The synthesis of complementary DNA (cDNA) was done using a standard kit (Pars Genome, Tehran, Iran) based on the manufacturer's leaflet. The real-time quantitative PCR reactions were accomplished in triplicate by using a Rotor-Gene 6000 system (Corbett Life Science, Mortlake, Australia). Briefly, in a total volume of 10 µl, 20 ng µl $^{-1}$ of cDNA was added to a master mix, including 10 pmol µl $^{-1}$ of *miR-93-5p* primer (Pars Genome, Iran) 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 PCR reaction was set at 95 °C for 15 mins 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).

Statistical analysis

Cycle threshold (Ct) values were specified for each sample and comparative CT method ($2^{-\Delta\Delta Ct}$) was used to determine the relative quantification of the *miR-93-5p* transcript (19). Data were analyzed using Graph Pad Prism statistical software, version 5.01 (Graph Pad, San Diego, CA, USA). The normality was evaluated by the Kolmogorov–Smirnov test. Also, the samples were analyzed based on the drug type (interferon and non-interferon). The independent samples T-test were applied to analyze the data between groups. To evaluate the potential of *miR-93-5p* as a diagnostic indicator of MS, the receiver operating characteristic (ROC) curve was created and the area under the curve (AUC) was measured by computing sensitivity and specificity. For all tests, a P-value of 0.05 was considered statistically significant.

Systematic molecular pathway enrichment analysis

To obtain suitable miRNAs for MS, a vast search in the miRWalk 2.0 database was performed. To narrow down the miRNA candidate, high score miRNA was selected. Subsequently, to carry out signaling pathway enrichment analysis was conducted using the DIANA database tool. DIANA-miRPath v3.0 database has been extended to support features such as incorporates KEGG pathways (20). This database supplies the results from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to recognize the signaling pathways with miR-93-5p targetome (21).

Results

Biological features

In this investigation, 30 RRMS patients (mean age: 39.2 years, range: 18-60, 7 male and 23 female) and 30 healthy subjects (mean age: 38.6 years, range: 21-58, 10 male and 20 female) were studied. Patients and controls were sufficiently matched in terms of age and sex. There was no statistically significant difference in interferon and non-interferon drugs (P=0.863). The major biological characteristics of RRMS patients have been briefly shown in Table 1.

Table 1. Biological characteristics of RRMS patients and healthy subjects.

Samples	Age (year)	EDSS	Drug	Disease duration (year)	Number of family history
Patients	39.20 ± 2.154	2.22± 0.12	Interferon: 18 Non-interferon: 12	6.72 ± 0.76	11
Controls	38.60 ± 1.843	-	-	-	-

RRMS: relapsing-remitting multiple sclerosis, EDSS: Expanded Disability Status Scale. Data are presented as mean ±SD.

Analysis of *miR-93-5p* expression

The expression of *miR-93-5p* was measured by a quantitative real-time PCR method in two groups: RRMS patients (n = 30) and healthy subjects (n = 30). The Ct values of real-time PCR were determined by $2^{-\Delta\Delta Ct}$ methods and data analysis using SPSS statistical. The results indicated significant growth in the expression of *miR-93-5p* in RRMS patients compared with healthy subjects. We observed that RRMS was associated with higher levels of *miR-93-5p* expression compared with healthy subjects (P=0.001) (Figure 1). ROC curve analysis determined a strong predictive power of *miR-93-5p* in discriminating MS patients from healthy controls, with the AUC of 0.939 (95% CI; 0.8581-1.000) (Figure 2).

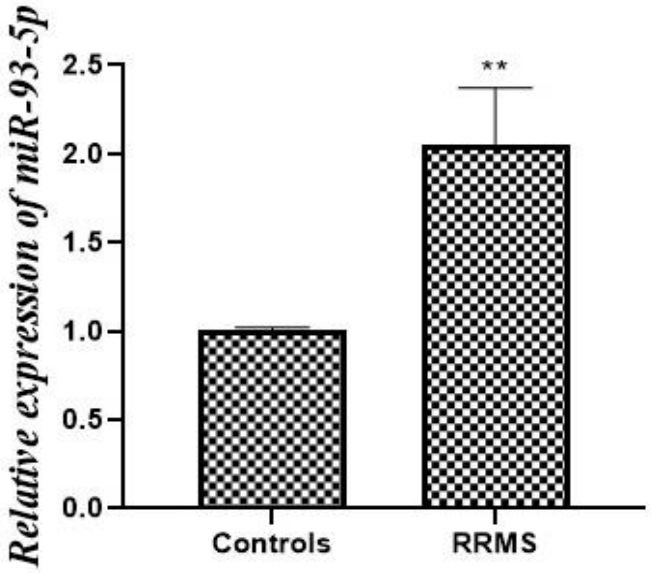

Figure 1. The average of relative expression of *miR-93-5p* in RRMS patients and healthy subjects. Relative expression for the *miR-93-5p* was significantly different between RRMS patients and controls (**P=0.001). Error bars show SE.

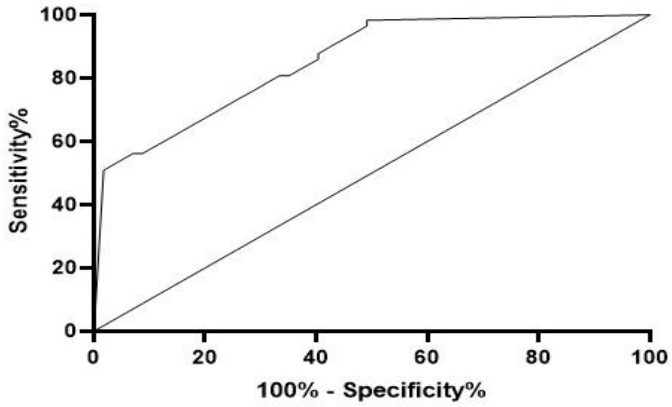

Figure 2. ROC curve of RRMS samples analyzed for relative expression of miR-93-5p in peripheral blood mononuclear cells. This analyses determined the miR-93-5p relative expression to discriminate in RRMS patients from healthy individuals with sensitivity and specificity of 100% and an area under the ROC curve (AUC) of 0.939 (95% CI; 0.8581-1.000).

Molecular signaling pathway enrichment analysis

Attributing gene symbols of chosen miR-93-5p targetome into a helpful annotation tool of DIANA-mirPath v.3 determined statistically significant organization of attribute genes with several KEGG signaling pathways. The set of attribute genes as miR-93-5p targetome was often enriched in 51 KEGG pathways. Six statistically relevant KEGG signaling pathways including Prion diseases, mRNA surveillance pathway, TGF-beta signaling pathway, FoxO signaling pathway, Pathways in cancer, and Chronic myeloid leukemia signaling pathways ranked as top related signaling pathways (Table 2).

Table 2. Top six statistically relevant KEGG signaling pathways with miR-93-5p targetome.

Rank	KEGG pathway	Number of genes (in the pathway)	P value
1	Prion diseases	5	4.28E-19
2	mRNA surveillance pathway	27	1.72E-05
3	TGF-beta signaling pathway	19	1.87E-05
4	FoxO signaling pathway	34	2.56E-05
5	Pathways in cancer	67	4.18E-05
6	Chronic myeloid leukemia	21	8.09E-05

KEGG: Kyoto Encyclopedia of Genes and Genomes, TGF-beta: Transforming growth factor-beta.

Discussion

In this study, *miR-93-5p* was selected from the miRWalk database, as a miRNAs involved in MS disease. Subsequently, the expression of *miR-93-5p* was examined by quantitative real-time PCR in two groups: RRMS patients (n=30), and healthy individuals (n=30). The data indicated an increased expression of *miR-93-5p* in RRMS patients in comparison with healthy individuals. According to our results, we hypothesized that overexpression of miR-93-5p 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. Additionally, in the prion diseases pathway, *CCL5* and *PRNP* genes were observed, both of these genes are *miR-93-5p* targets. Moreover, studies have shown that *CCL5* and *PRNP* genes have a role in MS disease. PRNP,

which is often expressed in CNS, is a vital protein for maintaining myelin. The CCL5 gene also encoding a chemokine protein which is secreted from T cells (22). In the foxo signaling pathway, *STAT3* and *IL10* genes were seen which are targeted for *miR-93-5p*. Genome-wide association investigation in a high-risk isolate for MS demonstrated associated variants in *STAT3* gene (23). It has proposed that *IL-10* suppressor activity is impaired in MS (24). Therefore, based on the bioinformatics findings it can be assumed that *miR-93-5p* has a certain role in MS disease, too. mRNAs–miRNAs interaction analysis on autoimmune-deregulated miRNAs and well-known regulators of Th17 differentiation was applied to explore new targets of miRNAs which might play a certain role in Th17 differentiation. So, some miRNAs were nominated with inhibiting (*miR-20b, miR-93, miR-20a, miR-152, miR-21,* and *miR-106a*) Th17 differentiation via interaction with the negative or positive regulators of this pathway (17). Also, it has shown that *miR-93-5p* regulates the expression of *IL-8* and *VEGF* in neuroblastoma SK-N-AS cells (25). The current study is the first study in the Iranian population about the association of *miR-93-5p* expression with MS, conducted by reverse transcription-quantitative PCR (RT-qPCR). The result of this study revealed an increase expression for *miR-93-5p* in RRMS patients compared to controls. ROC analysis determined the optimal cutoff value for *miR-93-5p* with an area under the ROC curve (AUC) of 0.939 by considering the criteria which were already described in the materials and methods (Figure 2). These results strongly indicate the discriminating potency of *miR-93-5p* expression level as a valuable biomarker of the RRMS patients. However, more studies are needed to confirm this expression cutoff point for clinical use of *miR-93-5p* as a diagnostic biomarker. Understanding the complexity of miRNAs may open up a new view to find individual biomarkers in clinical diagnosis and monitor the efficacy of therapy. In the future, *miR-93-5p* could be used as a molecular goal in designing new RRMS control strategies.

Conclusion

We investigated the transcript levels of *miR-93-5p* in RRMS patients in comparison with healthy subjects. Our results have shown the increased rate of *miR-93-5p* transcripts in RRMS patients. Therefore, *miR-93-5p* could be a potential therapeutic target specifically in the RRMS. It can be concluded that owing to its increasing, *miR-93-5p* may have a prognosis role in RRMS.

Acknowledgments

This study is the result of Mr. Jalil Shafiei's master thesis. We appreciate all patients who participated in this research.

Conflict of interest

The authors declare that they have no conflict of interest.

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