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Research Article

Increased Circulating miR-10a Levels Associated with Multiple Sclerosis

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Abstract

Background: Multiple sclerosis (MS) is an autoimmune disease that causes chronic inflammation of the central nervous system. MicroRNAs (miRNAs) are small non-coding RNAs 19–24 nucleotides long, which are differentially expressed in different tissues. The role of miRNAs in MS remains unclear.

We assessed *miR-10a* transcript levels in MS patients during recurrence and two months after relapse.

Materials and Methods: In this case-control study, we used real-time PCR to examine *miR-10a* expression in the peripheral blood mononuclear cells of 60 patients with relapsing-remitting multiple sclerosis (RRMS), 30 during recurrence and 30 two months after relapse, and 30 healthy subjects who were referred to the MS Clinic of Kashani Hospital, Isfahan Province. In silico analysis was also performed on the validated *miR-10a* targets using miRTarBase.

Results: *miR-10a* expression was higher in RRMS patients during recurrence and two months after relapse ($p < 0.0001$ and $p < 0.0001$, respectively) than in the healthy subjects. Furthermore, in silico molecular signaling enrichment analysis identified 12 mRNAs as validated *miR-10a* targets.

Conclusion: The expression of *miR-10a* was elevated in patients with RRMS compared to healthy subjects, suggesting that *miR-10a* could be a potential biomarker for RRMS diagnosis.

Keywords: Biomarker, miRNA, *miR-10a*, Multiple sclerosis

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1. Introduction

Multiple sclerosis (MS), an inflammatory disease that affects the central nervous system (CNS) of millions of patients worldwide, leads to motile inability by reducing coordination and motor, autonomic, and neurocognitive function.[1] MS is a multifactorial disease, and

a combination of environmental, epigenetic and genetic factors can lead to a continued immune attacks on the CNS.[2] Evidence indicates that the first, second, and third-degree relatives of patients with MS are at increased risk of developing the disease.[3] In developing countries like Iran, and big cities like Isfahan, the frequency of patients with MS has increased, with a current prevalence of 85.8 per 100000 people.[4] MS often presents in women between 20 and 40 years of age; however, the disease affects both males and females and can occur in the young and elderly. This autoimmune disease most frequently presents as a relapsing-remitting disease form (RRMS),[5] with the majority of patients with MS (almost 85 %) exhibiting the RRMS initially. More than half of all patients with MS have progression and reduced mobility between the acute attacks, and develop secondary progressive MS within 10 to 20 years of diagnosis.[6] Although the number of available therapies for MS has increased, little is known about biomarkers that can predict the response of patients to a specific treatment, preventing the development of process-specific therapies. Targeted therapies are becoming increasingly common, and therapeutic biomarkers can be rapidly screened during the early phases of their development.[7] Biomarkers are biological substances that can be objectively evaluated to indicate normal biological processes, pathogenic processes, or pharmacological responses.[8] MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs, which regulate gene expression at the post-transcriptional level by interacting with binding sites in target mRNAs. Recent studies have indicated that miRNAs can also be detected outside cells; thus, these have been termed extracellular miRNAs. Extracellular miRNAs are found in different body fluids, including plasma, serum, saliva, amniotic fluid, sputum, tears, urine, and seminal plasma. The discovery of extracellular miRNAs broadened the scope of miRNA research and prompted many studies. Due to the challenges associated with accessing brain tissue *in vivo*, plasma or serum have been utilized by the majority of studies attempting to identify feasible biomarkers for diverse CNS disorders.[9] Significant developments have been made towards recognizing the genes involved in MS; however, the genetic and phenotypic complexity of MS has considerably hindered progress. MiRNAs have attracted considerable attention since they regulate the expression of up to 30 % of protein-coding genes and may have pivotal roles in the development of numerous complex diseases. The lack of MS studies investigating plasma miRNAs prompted a recent study that identified a circulating miRNA signature for MS,[10] demonstrating that miRNAs are involved in the regulation of the immune system. Other recent studies have proposed that miRNA expression signatures in the blood have the potential to serve as biomarkers for human diseases, indicating that analyzing the miRNA expression of blood cells may be a promising approach for the

blood-based diagnosis of numerous cancers and autoimmune diseases.[11] The *miR-10* gene family has attracted attention due to its position within Hox clusters, which are developmental regulators, and because it is conserved across species.[12] Conflicting evidence has been reported on the role of *miR-10a* in tissue inflammation and autoimmunity, T helper (Th1) and interleukin (IL)-17-producing Th17 cells, glioblastoma multiforme cells, and Treg cells.[13–16] Furthermore, the role of *miR-10a* in patients with RRMS (particularly during recurrence and two months after relapse) remains unknown. Therefore, we explored the role of *miR-10a* in patients with RRMS by investigating the *miR-10a* expression levels in patients with recurrent MS, in patients two months after relapse, and in healthy individuals using qRT-PCR.

2. Materials and Methods

2.1. Patients and controls

A total of 90 blood samples were collected from 60 relapsing-remitting multiple sclerosis (RRMS) patients, 30 of whom were recurring patients and 30 whose MS had relapsed two months earlier, and 30 randomly selected healthy individuals (both male and female) at Kashani Hospital (Isfahan, Iran). The healthy individuals had no history of autoimmune disease, based on medical examinations, whilst the RRMS patients were diagnosed by an expert neurologist using the recommended McDonald diagnostic criteria.[17] Forty-two patients had only received interferon (IFN)-β treatment, whilst all other patients had received no treatment in the two months prior to sampling. Informed consent was obtained from all participants prior to sample collection, then 4 ml of peripheral blood was collected into EDTA-containing tubes and transported to the laboratory on ice.

2.2. Peripheral blood mononuclear cell (PBMC) isolation

PBMCs (monocytes and lymphocytes), which have lower densities than granulocytes (erythrocytes and leukocytes), were isolated from the blood samples by density gradient lymphoprep (Bio Sera, Kansas City, USA) according to the manufacturer's protocol. First, 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. The samples were centrifuged at 800 × *g* for 30 mins, with PBMCs remaining in the intermediate phase and all other cells being deposited. The PBMCs were then transferred from the intermediate phase into a 2 ml RNAase-free microtube and frozen at –70 ˚C.

2.3. miRNA extraction

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

2.4. cDNA synthesis and real-time PCR

cDNA was synthesized using a standard kit (Pars Genome, Tehran, Iran) according to the manufacturer's instructions. Real-time quantitative PCR reactions were carried out in duplicate using an ABI PRISM 7500 instrument (Applied Biosystems, USA). Briefly, 20 ng/µl of cDNA was added to a master mix containing 10 pmol/µl of *miR-10a* primer (Pars Genome) and 5 ml of SYBR premix ExTaq II (TaKaRa, Kusatsu, Shiga Prefecture, Japan), in a total reaction volume of 10 µl. *U6* (Pars Genome) was selected as a housekeeping gene to normalize the data. The PCR reaction conditions were as follows: 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, then a melting curve program (70–95 °C with a temperature transition rate of 1 °C/s and a continuous fluorescence reading). Real-time PCR analysis was performed using the ΔΔCT method, where CT is the cycle threshold.[18] The primer efficiency was verified at five different concentrations (Fig. 1).

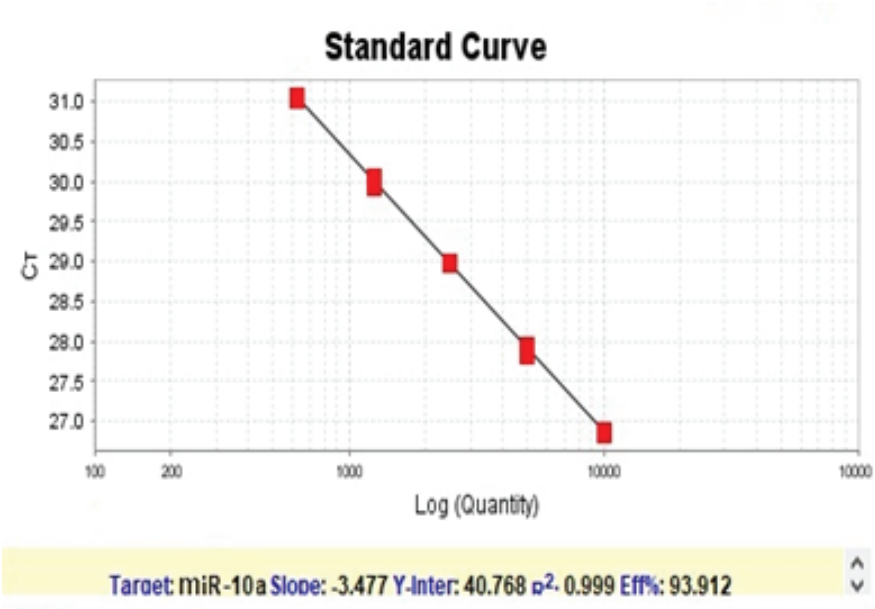

Figure 1: Standard curve of different dilutions of the real-time PCR products of *miR-10a*.

2.5. Statistical analysis

Graph Pad Prism statistical software version 5.01 (Graph Pad, San Diego, CA, USA) was used for statistical analysis. The normality of the data was evaluated using the Kolmogorov–Smirnov test and one-way ANOVA was used to analyze the data from different groups. For all tests, $p \leq 0.05$ was considered statistically significant.

2.6. Molecular enrichment analysis

We used the online in-silico databases miRWalk[19] and miRTarBase[20] to obtain validated *miR-10a* targets in order to perform molecular enrichment analysis on the *miR-10a* targetome and identify the miRNAs associated with MS.

3. Results

3.1. Clinical and biological features of patients

In this study, we investigated 60 RRMS patients, of whom 30 were recurring patients (mean age: 39.20 ± 2.154 years, range: 18-60, 7 male and 23 female) and 30 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). The clinical and biological characteristics of the patients and healthy individuals are shown in Table 1.

Table 1: Clinical and biological characteristics of recurring RRMS patients, those two months after relapse, and healthy individuals.

Characteristics	Control	Recurring patients	Patients two months after relapse
Number of subjects	30	30	30
Sex: Number of males Number of females	10 20	7 23	9 21
Mean age (years)	38.60 ± 1.843	39.20 ± 2.154	33.7 ± 1.522
Range (years)	21-58	18-60	21-51
Mean 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 Non-interferon	- -	18 12	24 6

3.2. Analysis of miR-10a expression

miR-10a expression was evaluated using real-time quantitative PCR for the RRMS patients (recurrent and two months after relapse; n = 60) and healthy subjects (n = 30). Ct values were determined using the $2^{-\Delta\Delta Ct}$ method. *miR-10a* expression was significantly higher in the RRMS patients than in the healthy subjects, by approximately 2.17 and 2.66 for the recurrent patients and those two months after relapse, respectively ($p < 0.0001$ and 0.0001, respectively) (Fig. 2).

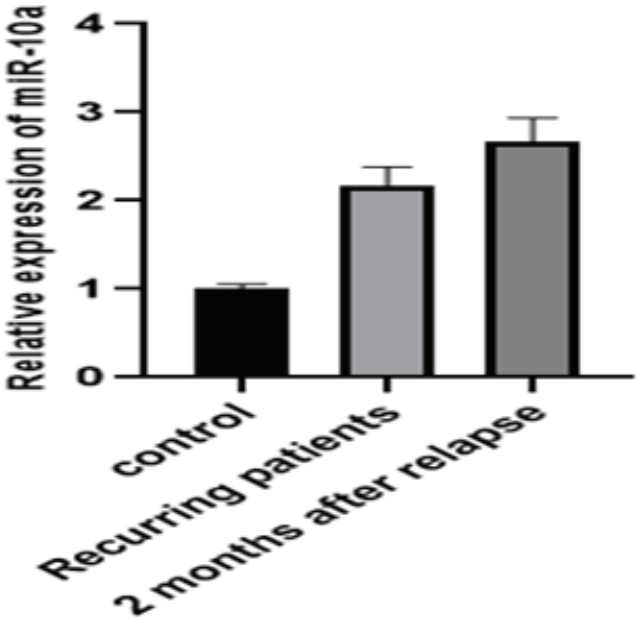

Figure 2: Average relative *miR-10a* expression in recurring RRMS patients and those two months after relapse increased by approximately 2.17 and 2.66, respectively ($p < 0.0001$ and $p < 0.0001$, respectively).

3.3. Molecular enrichment analysis

To determine the role of *miR-10a* in MS, molecular enrichment analysis was conducted. he miRWalk database confirmed *miR-10a* as associated with MS. Using the miRTarBase database, 12 mRNA targets of *miR-10a* were identified, namely *HOXA1, USF2, Hdac4, BTRC, MAP3K7, EPHA4, ACTG1, Klf4, PIK3CG, SERPINE1, Creb1,* and *MMP14.* All validated mRNA targets identified from the miRTarBase were supported by experimental evidence, including RT-qPCR, western blotting, and reporter assays.

4. Discussion

In this study, we showed that *miR-10a* expression was significantly higher in both recurrent RRMS patients and in patients two months after relapse than in healthy individuals. Based on these findings, we hypothesize that *miR-10a* is overexpressed in patients with RRMS compared to healthy individuals; therefore, *miR-10a* could be a potential therapeutic target for inhibiting MS progression. The clinical course of MS is variable; despite the existence of multiple disease-modifying treatments for relapsing forms of MS, patients usually continue to exhibit clinical disease activity and impaired neurological ability months after disease onset. Consequently, biomarkers that predict disease activity and assess therapeutic response are desirable, and there is a need for more effective treatments.[21, 22] miRNA dysregulation has been reported in various immune cells of patients with MS; some studies have demonstrated changes in miRNA expression in the brain tissue and immune cells of patients with MS and associations between MS progression and miRNA expression.[23, 24] Numerous studies have reported a correlation between *miR-10a* and different autoimmune diseases and T cells,[13, 14] revealing that *miR-10a* is expressed at high levels in naturally occurring Treg cells and that Treg cell *miR-10a* expression is inversely correlated with susceptibility to autoimmune disease.[13] As mentioned previously, *miR-10a* probably contributes to other autoimmune diseases via a similar immune pathological mechanism; therefore, we examined the levels of the *miR-10a* transcript in patients with RRMS. Our results suggest *miR-10a* expression is a valuable biomarker in recurring patients and those two months after relapse. Moreover, we observed increased *miR-10a* transcript expression in recurring patients and in those two months after relapse. *miR-10a* stabilizes the Treg gene expression program by repressing non-Treg genes such as Bcl6 and Ncor2; however, the genetic ablation of *miR-10a* does not induce Treg defects or autoimmunity.[25] Additionally, using the miRTarBase database we identified *HOXA1, USF2, Hdac4, BTRC, MAP3K7, EPHA4, ACTG1, Klf4, PIK3CG, SERPINE1, Creb1*, and *MMP14* as validated *miR-10a* targets. Matrix metalloproteinases (MMPs) have been implicated in MS, with MS patients exhibiting higher levels of *MMP2* and *MMP14* than normal individuals.[26] Furthermore, *MMP14* has been validated as a target gene of *miR-10a* in colorectal cancer, since *MMP14* expression reverses *miR-10a*-induced reductions in anoikis resistance activities.[27] It has been proposed that *HDAC* inhibitors could be used to treat MS, with experimental MS models suggesting consistent efficacy.[28] Furthermore, Liang *et al*. reported that the *miR-10a* targets *HDAC4* and *HOXA1* are involved in cell proliferation.[29] It can be assumed that *miR-10a* has multiple target genes which exert various *miR-10a* activities in

different biological processes, indicating numerous directions for future research. Taken together, the results of this study demonstrate the relevance of *miR-10a* in RRMS and suggest that *miR-10a* could be used as a potential biomarker for the early diagnosis and treatment of RRMS patients. Additional *in vitro* and *in vivo* experiments are required to confirm the specific roles of *miR-10a* in MS. In conclusion, we studied the levels of the *miR-10a* transcript in RRMS patients and healthy controls, revealing increased *miR-10a* expression in both groups of RRMS patients (recurring patients and those two months after relapse). *miR-10a* might therefore be able to predict treatment response; however, further cooperative studies are required to address the utility of miRNAs as biomarkers. Identifying the genetic factors involved in MS could provide a better understanding of the pathophysiology and prognosis of MS.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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