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Report

Up-regulation of miR-485-3p in Iranian patients with relapsing-remitting multiple sclerosis by targeting HLADRB1

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ABSTRACT

Background: Multiple sclerosis (MS) is an autoimmune disease characterized by chronic inflammation in the central nervous system (CNS). MicroRNAs (miRNAs) are tiny molecules that act as regulators within cells, influencing various processes linked to diseases like MS. Understanding the specific role of miRNAs in MS is crucial for developing new treatment strategies. This study focused on an Iranian population with relapsing-remitting MS. The researchers aimed to examine the levels of a particular miRNA, miR-485-3p, and its target gene, HLADRB1, over a minimum two-month period. By investigating these molecules, the study sought to shed light on the potential involvement of miR-485-3p in the pathogenesis of MS. **Methods**: This study investigated the relationship between miR-485-3p and relapsing-remitting multiple sclerosis (RRMS) using a case-control design. The researchers analyzed the expression levels of both miR-485-3p and its potential target gene, HLADRB1, in peripheral blood mononuclear cells (PBMCs) collected from participants. The study included 90 individuals: 30 diagnosed with RRMS who were experiencing a relapse, 30 diagnosed with RRMS who had been in a relapse state for at least two months, and a control group of 30 healthy subjects. **Results**: The expression of miR-485-3p was different in the two groups studied ($P < 0.0002$ and $P < 0.001$, respectively). RRMS patients in relapse and those at least two months post-relapse showed increased expression compared to the normal group. Additionally, we found increased expression of HLADRB1 in RRMS patients compared to healthy control subjects ($P < 0.0001$ and $P < 0.0003$, respectively). **Conclusion**: According to the study's findings, miR-485-3p, at least in the Iranian population studied, is likely to be an important biomarker for the early diagnosis of RRMS. However, HLADRB1 might be a crucial target for the development of this illness. Nevertheless, more research is required to provide a definitive answer.

Key words: miRNA, miR-485-3p, HLADRB1, Multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease characterized by chronic inflammation in the central nervous system (CNS), the control center of our body. The destruction of axons, damage to myelin, progressive exacerbation, relapses, and recovery are some of the main characteristics of this disease [1–3]. It is estimated that up to two million people worldwide are affected by this disease. The disease usually occurs in people in their thirties and forties, and women are diagnosed about three times as often as men [4–6]. Studies show that MS occurrence is increasing in developing countries; in Isfahan, one of the largest cities in Iran, the prevalence is 85.8 per 100,000 people [7]. MS can affect people of all ages and genders, but it affects more women aged 20 - 40. This autoimmune disease is usually relapsing, with relapses alternating with remissions. Relapsing-remitting MS (RRMS) is the most common form, affecting around 85% of MS patients. It's characterized by phases of worsening symptoms (relapses) followed by recovery phases (remissions). Secondary progressive MS (SPMS) develops from RRMS, in which the relapses become less frequent, but the symptoms continue to worsen over time. Primary progressive MS (PPMS) is rarer than RRMS, with gradual worsening of symptoms from the beginning, without pronounced relapses or remissions. Progressive-relapsing MS (PRMS) is the least common form, characterized by a steady progression of symptoms with occasional flare-ups of new or worsening symptoms [8–10].

The most prevalent type of MS is RRMS, characterized by flare-ups and remissions in symptoms [11]. The course of the disease is often unpredictable, and the severity of symptoms can vary greatly. These symptoms can affect the ability to move, feel, and think. The lack of a standardized detection method complicates the diagnosis of active MS. Recently, promising progress has been made in identifying the genetic factors associated with the development of MS [12–15]. A class of short, non-coding RNAs with an aver-

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age length of 22 nucleotides is referred to as miRNAs. miRNAs are essential for various biological functions, such as differentiation, proliferation, and programmed cell death [16–20].

MS may be associated with abnormal miRNAs that malfunction in immune cells of the blood and glial cells of the CNS. These abnormal miRNAs are likely responsible for the immune system abnormalities observed in MS patients. Studies have gradually shown that changes in the patterns of miRNA expression in the immune cells and brain tissue of MS patients are linked to the progression of the disease [21–23]. The exact role and underlying mechanisms of miRNAs in MS are still unclear. In this study, a database called miRWalk was used to identify miR-485-3p as a potentially important miRNA in MS. This suggests that miR-485-3p may play a crucial role in the development of MS. Research into the role of miRNAs in MS paves the way for the development of new miRNA-based therapies. This study, therefore, investigated whether changes in the levels of miR-485-3p and HLA-DRB1 could serve as diagnostic markers for patients with RRMS.

METHODS

Study Population

In this case-control study, blood samples from two groups were compared. A total of 60 RRMS patients were included; half (30) suffered relapses, while the other half (30) had persistent relapses for at least two months. Healthy controls: 30 healthy individuals were recruited from the MS research center in Isfahan. These individuals matched the patient group in terms of age and gender and had no history of autoimmune diseases, which was confirmed by a medical examination. All MS diagnoses were confirmed by a neurology expert using the established McDonald diagnostic criteria [17]. To ensure medications wouldn't confound the results, the researchers only included patients with recurrent disease who hadn't undergone any treatment for at least two months before the study began. Within this group, they specifically selected patients who had been treated only with beta-interferon (IFN-β) for at least two months prior to disease recurrence. All subjects provided their informed consent, and the study was ethically approved by the Islamic Azad University's Ashkezar Branch Ethics Committee. For the analysis of miR-485-3p expression, the researchers collected 4 ml of peripheral blood in EDTA tubes. These blood samples were stored on ice during transportation to the laboratory for analysis by qRT-PCR.

PBMCs Isolation

This study isolated peripheral blood mononuclear cells (PBMCs) from blood samples using a density gradient centrifugation technique, following the manufacturer's instructions for Lymphoprep. Here's a breakdown of the process: (a) Blood Dilution and Layering: Four milliliters of blood were first diluted 1:1 with physiological saline. This diluted blood was then carefully layered on top of a 4 ml volume of Lymphoprep solution in a Falcon tube. (b) Centrifugation and PBMC Collection: The layered Falcon tubes were centrifuged at 800 g for 30 minutes. This centrifugation process separates the blood components based on their density. PBMCs, located in the middle layer, were then carefully transferred to a separate, RNase-free microtube with a capacity of 2 ml. (c) Storage: Finally, the collected PBMCs were frozen at -20°C for later analysis.

RNA Isolation, cDNA Synthesis, and RT-qPCR

Firstly, RNA was isolated from samples using the RNA hybrid R kit. The quality and quantity of the extracted RNA were assessed using gel electrophoresis and NanoDrop. To synthesize cDNA for further analysis, two approaches were used: miR-485-3p: A standard cDNA synthesis kit was employed following the manufacturer's instructions. HLADRB1: cDNA synthesis was performed directly on the total RNA using a separate cDNA synthesis kit according to its specific protocol. The Rotor-Gene 6000 system was used for RT-qPCR; the assay was performed following the instructions. U6 and GAPDH were used as housekeeping genes for analyzing the expression change of HLADRB1 and miR-485-3p. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

The GraphPad Prism software (GraphPad, USA, version 5.01) was used for the statistical analysis. The Kolmogorov–Smirnov test, a nonparametric test, was performed to determine normality, and ANOVA was used to analyze data between groups. For all tests, $p \leq 0.05$ was set as the significance level.

RESULTS

Characteristics of Study Population

A total of 90 participants were enrolled in the present study, including 60 RRMS patients. Among them, 30 had a recurrence (mean age: 32.28 $\pm$ 2.58 years, 11 men and 19 women) and 30 patients who had experienced a recurrence for at least two months (mean

Table 1: Characteristics of recurring patients and two months after relapse patients and healthy individuals

Characteristics	Control (N = 30)	Recurring patients (N = 30)	Two months after relapse patients (N = 30)
Sex			
Number of males	13	11	10
Number of females	17	19	20
Mean age	33.72 ± 6.12	32.28 ± 2.58	34.54 ± 1.25
Mean of disease duration (years)	-	7.63 ± 0.72	6.25 ± 1.82
Family history	-	9	12
Drug: Interferon	-	22	16

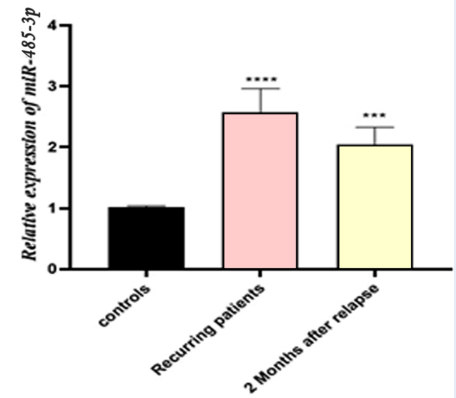

Figure 1: **The average of relative expression of miR-485-3p in recurring patients and two months after relapse patients had an increase** (P < 0.0002 and P < 0.001, respectively).

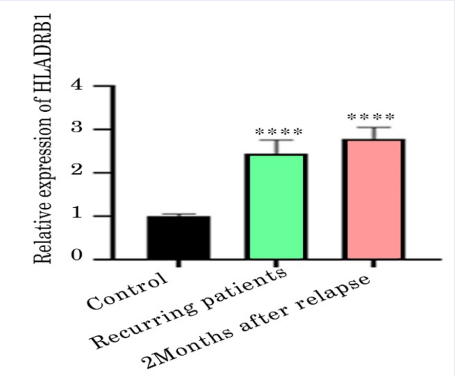

Figure 2: **The average of relative expression of HLADRB1 in recurring patients and two months after relapse patients had an increase** (P < 0.0001 and P < 0.0003, respectively).

age: 34.54 ± 1.25 years, 10 men and 20 women), age- and sex-matched healthy individuals, and 30 healthy subjects (mean age: 33.72 ± 6.12 years, 13 men and 17 women). There was no significant correlation between men and women (p-value = 0.28). The biological characteristics of the patients (with recurrences and recurrences lasting for at least two months) and the healthy subjects are listed in **Table 1**.

Analysis of miR-485-3p Expression

Our research has shown that the relative quantification (RQ) was different across the groups. In our data analysis, there was a significant increase in the expression of miR-485-3p in RRMS patients in comparison to control subjects (P < 0.0002 and P < 0.001, respectively) (**Figure 1**). Thus, our results suggest that miR-485-3p could be valuable as a novel biomarker for RRMS patients.

Analysis of the Expression Level of miR-485-3p's Target

The HLADRB1 gene, which is involved in MS and for miR-485-3p according to the miRWalk 2.0 database, was investigated in patients with relapsing and recurrent disease for at least two months compared to control subjects. Our results showed an increased expression of HLADRB1 in patients with recurrent and at least two-month relapses compared to healthy individuals (P = 0.0001 and P = 0.0003, respectively) (**Figure 2**).

DISCUSSION

In this study, miR-485-3p was nominated as a miRNA intricately involved in RRMS disease. Subsequently, the expression of miR-485-3p was analyzed by real-time quantitative PCR in two groups: RRMS patients

(recurrent and relapsed for at least two months) (n = 60) and healthy individuals (n = 30). Data analysis revealed an increased expression of miR-485-3p in patients with recurrent disease and relapses of at least two months compared to controls. Considering that the most ideal method to investigate the role of miR-485-3p in RRMS is to collect samples from nervous system tissue, we used PBMC samples due to the difficulty of sample collection and the utilization of the biomarker potential of miR-485-3p. We also found that the expression of HLA-DRB1 was significantly increased in RRMS patients compared to controls.

We postulated that, in light of our observations, the overexpression of miR-485-3p in the RRMS group relative to the control group may one day be studied as a possible target for therapy. The course of a patient's MS might vary; months after the disease first manifests, they typically continue to experience neurological impairment and clinical disease activity. For patient care, biomarkers that indicate the likelihood of a therapeutic response are ideal[24–26]. Several studies have shown the correlations between the progression of MS and the aberrant expression of miRNAs[27–29]. The results of the differential expression study in RRMS samples revealed the overexpression of eight out of nine highly dysregulated miRNAs, including miR-485-3p[30]. TaqMan array analysis showed that miR-485-3p was significantly increased in $CD4^+$ T cells from peripheral blood samples of RRMS patients[31]. Ten miRNAs, including miR-485-3p, were identified in miRNA profiling studies in $CD4^+$ T cells from RRMS patients using TLDA[32]. The outcomes of earlier research support our findings and corroborate the conclusions of our investigation.

There is sufficient evidence for the role of HLA II in MS. HLA-DRB1 expression on the cell surface has specifically increased, according to flow cytometric analysis[33,34]. One of the most crucial requirements for creating a miRNA-based biomarker for MS, or any other disease, is the capacity to quantify miRNAs from a range of samples with adequate sensitivity, precision, and repeatability. Gaining insight into the intricacy of miRNAs may provide new opportunities for identifying personalized biomarkers for clinical diagnosis and tracking treatment effectiveness. At the very least, for the Iranian population, miR-485-3p may prove to be a useful biomarker and molecular target in the future when devising novel approaches to manage RRMS. Since miR-485-3p likely regulates multiple genes, it might play a diverse range of functions in biological processes related to MS. This opens exciting avenues for future research to explore these additional roles. This study highlights the potential significance of miR-485-3p and HLA-DRB1 in MS. They could potentially serve as biomarkers for early diagnosis of RRMS or as targets for developing new treatment strategies. However, further studies are crucial to definitively establish their exact role in MS.

CONCLUSIONS

The results indicate that the deregulation of miR-485-3p is likely associated with RRMS and poor prognosis and may have functional significance through its target. This miRNA and its target could potentially serve as valuable biomarkers for the early diagnosis of RRMS and the prediction of treatment response. Identifying genetic factors involved in MS could contribute to a better understanding of the pathophysiology, prognosis, and treatment of this disease.

ABBREVIATIONS

MS - Multiple Sclerosis, **CNS** - Central Nervous System, **miRNA** - MicroRNA, **RRMS** - Relapsing-Remitting Multiple Sclerosis, **PBMCs** - Peripheral Blood Mononuclear Cells, **SPMS** - Secondary Progressive Multiple Sclerosis, **PPMS** - Primary Progressive Multiple Sclerosis, **PRMS** - Progressive-Relapsing Multiple Sclerosis, **IFN-β** - Interferon beta, **EDTA** - Ethylenediaminetetraacetic acid, **qRT-PCR** - Quantitative Real-Time Polymerase Chain Reaction, **cDNA** - Complementary DNA, **RQ** - Relative Quantification, **HLA** - Human Leukocyte Antigen

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AUTHOR'S CONTRIBUTIONS

All authors equally contributed to this work, read and approved the final manuscript.

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None.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All subjects provided their informed consent, and the study was approved ethically by the Islamic Azad University's Ashkezar Branch Ethics Committee.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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