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The Effect of Interferon Beta and Natalizumab on miR-20b Expression in Patients with Relapsing-Remitting Multiple Sclerosis is Potentially Mediated by Modulation of the Jak–STAT Signaling Pathway: A Case-control Study

Aysan Jafari Harandi[1,2], Alireza Mirzaee Sedigh[1], Mitra Ataei[1], Sepideh Bayrami[2], Emran Esmaeilzadeh[2], Mohammad Hossein Sanati[1*]

[1]Department of Medical Genetics, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran; [2]Fetal Health Research Center, Hope Generation Foundation, Tehran, Iran

ABSTRACT

Background: The mechanisms of the function of interferon beta (IFN-β) and natalizumab (NTZ) in multiple sclerosis (MS) patients have not yet been fully understood. Over the past decades, many studies have been conducted to evaluate gene expression changes especially regulatory non-coding RNAs such as microRNAs (miRNAs) following therapy in MS patients.

Objective: To assess the changes in the expression of miR-20b in MS patients treated with IFN-β or NTZ.

Methods: Sixty patients with relapsing-remitting MS (RRMS) and 30 healthy controls (HCs) were enrolled. The patients were categorized as untreated (N=20), IFN-β-treated (N=20), and NTZ-treated (N=20). For the expression analysis, real-time PCR was performed on the whole blood. The bioinformatic tools were applied for signaling pathways enrichment analysis of miR-20b targetome.

Results: The relative expression of miR-20b was significantly downregulated in the untreated patients compared with the HCs (-1.726-fold, $p<0.001$), while IFN-β-treated and NTZ-treated patients showed no statistical difference compared with the HCs (0.733-fold, $p=0.99$ for IFN-β and 1.025-fold, $p=0.18$ for NTZ). This indicates the restoration of miR-20b expression to normal level in the treated patients. Additionally, in silico analysis demonstrated that the Jak–STAT signaling pathway is enriched with miR-20b targets ($p<0.0001$).

Conclusion: Our findings suggest that the positive effects of IFN-β and NTZ in the RRMS patients could be potentially mediated by returning miR-20b expression to baseline.

Keywords: Interferon Beta, Natalizumab, miR-20b, Multiple Sclerosis

*Corresponding author:
Mohammad Hossein Sanati, Department of Medical Genetics, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
Email:
mohammadhsanatii@gmail.com

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INTRODUCTION

Multiple sclerosis (MS), the most prevalent immune-based neurodegenerative disease, causes damage to the central nervous system (CNS). The development of this disease is influenced by various factors, including epigenetic changes, genetic predisposition, and environmental stimuli e.g. Epstein-Barr virus exposure and insufficient levels of vitamin D (1). Along with the diverse range of clinical manifestations, the response to disease-modifying therapies (DMTs) varies significantly. The currently available conventional therapies, such as interferon beta (IFN-β), and natalizumab (NTZ) exhibit only partial effectiveness. Moreover, patients often experience unpredictable and suboptimal responses to these treatments (2). Currently, there is a lack of reliable biomarkers for predicting therapy response, leaving neurologists without guidance or tools to determine the optimal therapeutic approach. Clinical examination, magnetic resonance imaging (MRI), and analysis of metabolites are commonly used technics for monitoring the effectiveness of the treatments (3). However, these methods do not capture various pathomechanisms; therefore, identifying biomarkers that accurately reflect treatment responses would enable more optimal management of patients.

MicroRNAs (miRNAs) are a promising group of biomarkers MS research (4). These short non-coding RNAs play a post-transcriptional regulatory role (5). Several works have been performed in MS to explore the miRNA expression in distinct forms of the disease, including relapsing-remitting multiple sclerosis (RRMS). Additionally, researchers have investigated the function of miRNAs in the MS pathogenesis by studying the animal model of the disease, and experimental autoimmune encephalomyelitis (EAE). These investigations have identified promising biomarkers that have the potential to be used for prognosis, diagnosis, and staging (6).

For RRMS patients, IFN-β is currently the established initial therapy. Despite its widespread use, the precise mechanism of IFN-β and its effects on the body remain unclear due to its multiple and diverse impacts (7). Pharmacological interventions can modulate signaling molecular pathways, which can consequently lead to changes in the expression of miRNAs (8). Ongoing research in the field of MS focuses on identifying the dynamics of miRNA expression following drug administration. When it comes to researching miRNA biomarkers for predicting treatment response, the majority of studies have focused on the impact of NTZ (9).

There is a pressing need to continue conducting studies that can shed light on the exact mechanism of IFN-β and NTZ to identify new biomarkers for monitoring therapy. Therefore, our research aimed to assess the effect of IFN-β and NTZ on the miR-20b expression, previously identified as showing significant abnormal expression in RRMS (10, 11). Our study aimed to explore the potential utility of this miRNA in monitoring the effectiveness of therapy with interferon beta in the patients with RRMS as well as elucidating the mechanism of action of IFN-β and NTZ by regulating miR-20b expression. We compared the expression levels of this miRNA in IFN-β-treated and NTZ-treated RRMS patients with the treatment-naïve RRMS patients and the healthy controls (HCs).

MATERIALS AND METHODS

Patients Cohort

Once we received approval from the local Ethics Committee of National Institute of Genetic Engineering and Biotechnology (IR.NIGEB.EC.1398.10.18.C) and obtained signed informed consent, we proceeded to collect blood samples from 20 RRMS patients who had not received any prior treatment, as well as from 40 RRMS patients undergoing treatment with IFN-β-1a (CinnoVex; CinnaGen; N=20) or NTZ

(Tysabri; N=20).Additionally, we collected blood samples from 30 HCs who were matched in terms of age, gender, ethnicity, and with no symptoms of neuropathy or allergic disorders. McDonald criteria was the basis for diagnosis (12).

Total RNA Extraction

To extract total RNA including miRNA, we employed the Hybrid-R™ Blood RNA purification kit (GeneAll, South Korea). The quantity of the extracted RNA was evaluated using a NanoDrop spectrometer (Thermo Scientific, USA) and denaturing gel electrophoresis was used for assessing the RNA's integrity.

cDNA Synthesis and Real-Time PCR

To synthesize cDNA, we employed the Universal cDNA synthesis kit (Exiqon, Denmark), which facilitates the creation of polyadenylated miRNAs and their subsequent transcription into cDNA in a single step. For the real-time PCR analysis, we utilized the ExiLENT SYBR® Green master mix (Exiqon, Denmark) along with miR-20b primers obtained from the same manufacturer. The sequence of forward and reverse primers were, respectively, as follows: 5'-ACACTCCAGCTGG GCAAAGTGCTCATAGT−3' and 5'-TGGTGTCGTGGAGTCG−3'. All reactions were performed in triplicate using the ABI Step One Plus instrument (ABI, USA). To normalize the data, we employed unisp6 RNA spike-in as a reference gene.

Statistical Analysis

The fold change was calculated using the $2^{-\Delta\Delta CT}$ method (ΔCT patients – ΔCT mean HCs). To assess the normality of the data, the Kolmogorov-Smirnov test was conducted using SPSS version 20. Furthermore, the non-parametric Mann-Whitney test was employed to analyze the differences between the groups. The data are presented as the mean±SEM, and a significance level of $p \leq 0.05$ was considered statistically significant.

In Silico Analysis of miR-20b Targets and Related Signaling Pathways

We employed miRTarBase (13) and miRWalk 3.0 (14) databases to gather experimentally verified mRNA targets. Additionally, miRWalk 3.0 was used to obtain predicted target genes, as it incorporates results from 11 different target prediction databases. Subsequently, we utilized the Database for Annotation, Visualization, and Integrated Discovery (DAVID) online database, version 2023q2, to identify enriched signaling pathways involving the validated and predicted target genes (15). The DAVID database provided results from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (16).

RESULTS

Clinicopathologic Features of the Patients

All individuals who received IFN-β and NTZ treatment exhibited a positive response to the therapy, as evidenced by stability in their Expanded Disability Status Scale (EDSS) scores, absence of relapses after starting IFN-β or NTZ consumption, and no significant abnormalities found in their MRI scans. The blood samples were collected from the IFN-β-treated and NTZ-treated subjects immediately following their final injection. These patients were all receiving a weekly intramuscular dose of 30 μg of IFN-β-1a or NTZ for at least one year. Prior to and during the study, the treatment-naïve RRMS patients did not undergo any immunomodulatory or MS-specific therapies. Table 1 contains a summary of the general and clinical data of the patients.

Normalization of Aberrant Expression of miR-20b

According to the Fig. 1, the relative expression of miR-20b was found to be significantly downregulated in the treatment-naïve patients compared with the HCs (-1.726-fold, $p<0.001$), while the IFN-β-treated and

Table 1. Characteristics of patients and HCs

Group	Sex (F/M)	Age (years) Mean±SD	Disease duration (years) Mean±SD	EDSS Mean±SD
HCs	25/5	32.5±7.6	NA	NA
IFN-β-treated	15/5	28.4±5.4	6.4±3.5	2.4±0.7
NTZ-treated	16/4	27.7±6.9	5.7±2.7	2.1±0.9
Treatment naïve	14/6	30.1±8.4	5.9±3.1	3±0.6

F: Female; M: Male; HCs: Healthy controls; NA: Not applicable; EDSS: Expanded Disability Status Scale

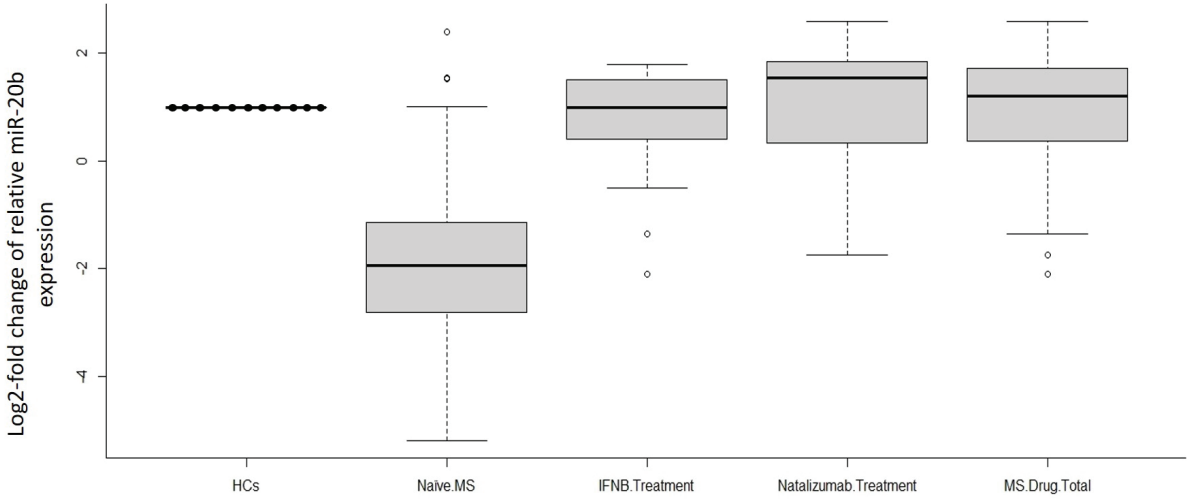

Fig. 1. Normalization of miR-20b expression in IFN-β-treated and NTZ-treated RRMS patients. Analysis of expression level of miR-20b in treatment naïve (n=20), IFN-β-treated (n=20) and NTZ-treated (n=20) RRMS patients and also the healthy controls (HCs; n=30).

the NTZ-treated patients showed no statistical difference compared with the HCs (0.733-fold, p=0.99 for IFN-β and 1.025-fold, p=0.18 for NTZ). Also, the expression of miR-20b was significantly higher in all the treated RRMS patients compared with the treatment-naïve RRMS patients (p<0.001). These findings indicate that the expression of this miRNA is brought back to levels similar to those found in the healthy individuals, suggesting that the mechanism of action of IFN-β and NTZ in the body may involve the regulation of this specific miRNA.

Molecular Signaling Pathway Enrichment Analysis of miR-20b Targetome

The results of bioinformatic analysis can be found in Table 2. Collectively, these findings demonstrate that the targetome of miR-20b is implicated in various signaling pathways, which likely play significant roles in mediating the diverse effects of IFN-β and NTZ. Of particular note, Jak–STAT signaling pathway is enriched with miR-20b targets (p-value of 1.9E-5), propounding that the beneficial effects of IFN-β and NTZ could be mediated by targetome of miR-20b in this pathway.

DISCUSSION

RNA undergoes changes at various disease stages and in response to the treatment. In the near future, it could potentially be utilized as a predictive measure in clinical practice (17). In the context of MS, several studies have been performed to gain understanding of how different medications function in patients and why there is variation in treatment response (18). These studies showed that in the RRMS patients, some genes are associated with a more favorable response to treatment with IFN-β (19).

Table 2. Top 24 most statistically relevant KEGG signaling pathways with miR-20b targetome (DAVID database).

Rank	Kegg pathway	The number of genes in the pathway	Statistical *p*-value
1	Bladder cancer	15	1.2E-7
2	Endocytosis	30	2.8E-6
3	Jak–STAT signaling pathway	35	1.9E-5
4	Adherens junction	15	3.1E-5
5	TGF-beta signaling pathway	18	2.1E-5
6	Cell cycle	21	8.2E-5
7	Long-term potentiation	17	3.4E-4
8	Small cell lung cancer	15	7.3E-3
9	p53 signaling pathway	13	7.1E-3
10	Prostate cancer	15	2.1E-4
11	Melanoma	13	3.3E-6
12	Glioma	12	3.7E-6
13	Lysosome	15	3.7E-2
14	Colorectal cancer	12	1.5E-2
15	MAPK signaling pathway	27	1.6E-2
16	Non-small cell lung cancer	9	1.8E-2
17	Wnt signaling pathway	17	1.6E-2
18	Thyroid cancer	6	1.2E-2
19	Focal adhesion	20	3.6E-2
20	Regulation of actin cytoskeleton	21	4.8E-2
21	PPAR signaling pathway	9	7.4E-1
22	Renal cell carcinoma	9	6.0E-3
23	Apoptosis	10	8.4E-2
24	Ribosome	10	1.3E-4

Another study showed that lower expression in monocytes before initiating treatment can predict a better clinical response (20). Since 2011, studies focusing on miRNA profiling have shifted their attention toward examining changes in miRNA expression in response to specific treatments for MS. Most of these studies have focused on analyzing the changes in miRNA expression patterns following NTZ therapy (21).

In this work, we observed that the expression levels of miR-20b in the RRMS patients who responded positively to IFN-β and NTZ therapy were within a normal range. This suggests that IFN-β and NTZ treatment restores miR-20b expression, previously found to be deregulated (10, 11). In this context, Ingwersen et al. designated a longitudinal study to assess the effect of NTZ on a set of miRNAs in the RRMS patients (22). At bassline, miR-20b downregulated compared with the HCs, but after one year of therapy with NTZ, the miR-20b expression increased to the level of the HCs. Taken together, these findings suggest that this miRNA could serve as potential biomarkers for monitoring the response to IFN-β and NTZ treatment in the RRMS patients. Without a doubt, the effect of IFN-β and NTZ on the kinetics of miRNAs is multifaceted and relies on a complex network of interactions. However, it is believed that indirect effects play a more significant role, whereby changes in miRNA expression levels are primarily a result of alterations in the composition or function of specific cell types (23). Consequently, the elevated expression of miR-20b following treatment initiation may reflect an increase in these cell populations.

The susceptibility of mice to develop EAE even after eliminating key cytokines from the Th1 pathway led researchers to discover the involvement of another group of T-helper cells

called Th17 cells (24). Th17 cells primarily function through the production of Il-17, considered their signature cytokine. Various lines of evidence have emphasized the crucial role of Th17 cells in MS (25). The miRNA-20b specifically targets the Stat3/ROR-ct in Jak–STAT signaling pathway, which plays an important role in the differentiation of $CD4^+$ T cells into Th17 cells and is one of the enriched signaling pathways in our analysis (26). In the RRMS patients, the expression of miR-20b reduced in their blood cells (10, 11). However, when exposed to NTZ or IFN-β, the expression of miR-20b is normalized or even increased (22). We hypothesize that this normalization in miR-20b expression has the potential to reduce the number of Th17 cells. The beneficial effects of NTZ and IFN-β could be mediated through the blood-brain barrier (BBB), which serves as a protective shield for the brain, acting as an immune-privileged organ (27). It achieves this by restricting the free movement of solutes and cells between the blood and the brain parenchyma, both transcellularly and paracellularly. (28). In MS, one of the early signs is the breakdown of the BBB, allowing immune cells from the bloodstream to leak into the CNS (29). miR-20b plays a role in suppressing VEGF-A. This, reduces the activity of CLN-5 and OCLN (30, 31). We believe that the up-regulation of miR-20b by NTZ and IFN-β most probably enhances the functioning of the BBB. Consistent with our bioinformatic analysis, Yang et al. analyzed Gene Expression Omnibus (GEO) data and provided evidence that miRNA-20b plays a pivotal role in the pathophysiology of MS by regulating 34 genes (32).

The dysregulation of miRNAs in diseases can be a result of the disease itself or can directly contribute to the pathobiology of the disease. Therefore, when observing changes in miRNA expression patterns, it is important to validate these findings with functional data to determine whether the changes are indicative of improvement after treatment or if the miRNAs themselves play a role in the therapeutic mechanism (33). Our result emphasizes the active involvement of miR-20b, at least partially, in the therapeutic mechanisms of two DMTs for MS. To address these challenges, it is propounded that the expression experiments should be accompanied by genetic variant analysis of miRNA-encoding genes and miRNA-binding sites of target genes. Longitudinal studies are also deemed more sensible than the case-control studies to achieve a comprehensive understanding (34). The development of pharmacological agents targeting specific miRNAs is gaining momentum to modulate dysfunctional pathways and coordinate complex protein expression programs. (35). Altogether, the results suggest that IFN-β and NTZ may exert their effects by modulating the expression of miR-20b. However, it is important to note that further research with a larger patient cohort undergoing longitudinal examination is necessary for clinical applications.

CONCLUSION

This study demonstrates the restoration of miR-20b expression in the RRMS patients following the treatment by IFN-β and NTZ and also suggests that this restoration positively affects Th17 homeostasis and BBB function. Therefore, future similar studies could shed more light on the association between the treatment and the miRNA expression in MS.

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AUTHORS' CONTRIBUTION

AJH, EE and MHS designed and directed the project. AJH, SB, and EE carried out

the experiment. AMS, MA, and MHS contributed to reviewing the final version of the manuscript. All authors read and approved the final manuscript

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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