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MicroRNA-29b variants and MxA expression change during interferon beta therapy in patients with relapsing-remitting multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterized by immune-mediated demyelination and axonal injury. Myelin-reactive IFN-γ-producing Th1 cells have been shown to play an important role in the development of MS. MicroRNAs (miRNAs) are a new class of small non-coding RNA molecules about 22 nucleotides long which regulate gene expression post-transcriptionally by binding to 3′ UTR of their mRNA targets, and resulting in degradation or transcriptional repression of the targeted mRNA. Accumulating evidence supports that miRNA dysregulation is linked to the pathogenesis of autoimmune diseases that include MS. miR-29b expression has been shown to be upregulated in memory CD4+T cells from relapsing-remitting MS (RR-MS) patients, which may reflect chronic Th1 inflammation. Interferon beta (IFN-β) benefits patients with MS and reduces symptoms of the RR-MS. MxA is induced by type I interferon and predicts IFN-β response in MS patients. The aim of this study was to evaluate miR-29b variants and MxA expression and serum IFN-γ level in responders and non-responders to IFN-β treatment.
Methods: A total of 70 IFN-β treated RR-MS patients including 35 responders and 35 non-responders were enrolled. We analyzed the expression level of miR-29b variants and MxA using the peripheral blood of MS patients treated with IFN-β for more than one year. Real-time RT-PCR was performed to analyze miR-29b variants and MxA expression one year after initiation of IFN-β therapy. Serum cytokine level was measured by ELISA.
Results: The results indicated that the expression level of miR-29b-3p changed related to IFN-β response. Moreover, miR-29b-5p was downregulated under IFN-β treatment in responders versus non-responders. MxA level was significantly decreased in the responders. There was no change in IFN-γ level following treatment with IFN-β in the MS patients.
Conclusions: Our results might provide fundamentals for the development of new markers of the biological effects of IFN-β therapy.

1. Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterized by immune-mediated demyelination and axonal injury (Trapp and Nave, 2008). The disease is characterized by complex genetic traits and patho-mechanisms that translate into the heterogeneity of clinical manifestations and therapeutic response (Hoffjan and Akkad, 2010). Myelin-reactive IFN-γ-producing Th1 cells have been shown to play an important role in the development of MS (Chitnis, 2007). There is growing interest in the link between regulatory microRNA (miRNA) expression and gene modulation of MS. MicroRNAs are a new class of small non-coding RNA molecules about 22 nucleotides long which regulate gene expression post-transcriptionally by binding to 3′ UTR of their mRNA targets, and resulting in degradation or transcriptional repression of the targeted mRNA (Bartel, 2009). MiRNAs are critically involved in the regulation

of a wide array of biological processes including the immune system function. Accumulating evidence supports that miRNA dysregulation is linked to the pathogenesis of autoimmune diseases that include MS (Thamilarasan et al., 2012).

MiR-29b is a key part of a negative feedback loop that controls the balance of Th1 cells by repressing multiple target genes, including T-bet. The miR-29b expression has been shown to be upregulated in memory CD4 + T cells from RR-MS patients, which may reflect chronic Th1 inflammation. Persistent up-regulation of both miR-29b and IFN-γ in MS is indicative of chronic inflammation (Smith et al., 2012; Steiner et al., 2011).

A number of miRNA species were found to be differentially expressed in patients with MS compared with controls and to have an impact on the development or prevention of the disease. Emerging evidence has demonstrated that various microRNAs are dysregulated in several immune cells of relapsing-remitting multiple sclerosis (RR-MS) patients and can be changed by disease-modifying therapies (DMTs) (Waschbisch et al., 2011; Hecker et al., 2013a,b). Immunomodulatory therapy like recombinant interferon beta (IFN-β) benefits patients with MS and reduces the number of relapses and magnetic resonance imaging (MRI) lesions (Weinstock-Guttman and Jacobs, 2000). The biological activity of IFN is mediated by the induction of intracellular antiviral proteins, including myxovirus resistance proteins (MxA). MxA is transcriptionally induced by type I (alpha and beta) and type III (lambda) IFNs via an autocrine feedback mechanism that initiates up-regulation of MxA (Holzinger et al., 2007). MxA mRNA expression predicts IFN-β response in MS patients (Matas et al., 2014). Individual therapeutic responses to IFN-β vary as some patients with MS do not respond to therapy. However, methods to determine IFN-β responsiveness have not been validated. Our objective was to evaluate an immunologically relevant miRNA to classify IFN-β responder status.

Here, we investigated the miR-29b variants expression changes in peripheral blood mononuclear cells (PBMC) and IFN-γ level in serum of patients with RR-MS in response to IFN-β therapy. Then, we compared the miRNA expression and cytokine level between IFN-β responder and non-responder groups. Also, this study examined the correlation of gene expression of MxA with the response to treatment with IFN-β.

2. Materials and methods

2.1. Patients

We enrolled 70 patients with diagnosis of MS according to McDonald criteria who were characterized as IFN-β responders ($n = 35$) and non-responders ($n = 35$) from Isfahan. All patients were assessed for the Expanded Disability Status Scale (EDSS) by their treating physician. Patients were considered responders when there was no increase in the EDSS score and no relapses during the follow-up period. MS non-responder patients were characterized by failure to respond optimally from initiation of therapy as assessed by clinical measures. All patients were treated with IFN-β for at least one year. The study was approved by the ethics committee of the Isfahan University (no. 296,075) and written informed consent was obtained from all participants.

2.2. Peripheral blood mononuclear cells (PBMCs) isolation

Blood samples were collected one year after starting IFN-β therapy. Immediately after collecting EDTA blood samples, PBMCs were isolated from blood using density gradient centrifugation method by layering the whole blood on Ficoll. Briefly, blood samples were diluted in the proportion of 1:1 with PBS. Diluted blood was gently layered on the top of the Ficoll and then it centrifuged at $800 \times g$ for 30 min. Then, PBMCs were removed from the interface phase and washed twice with PBS. After washing and cell counting, PBMCs were pelleted by centrifugation and then were frozen at $-80\,^{\circ}C$ for further RNA extraction step.

2.3. RNA extraction and cDNA synthesis

Total RNA extraction of Ficoll-isolated PBMCs from each sample was accomplished to measure the levels of miRNAs using a RiboEx Kit (GeneAll). Quality of extracted RNA was determined according to 260/280 absorbance ratio, measured by Nano Drop. cDNA synthesis for miR-29b and MxA was fulfilled using a universal cDNA synthesis kit (Exiqon, Denmark) in poly A tailing manner, according to manufacturer leaflet.

2.4. Real-time RT-PCR

For analysis of miR-29b variants and MxA expression, total RNA was reverse transcribed into cDNA followed by real-time reverse transcription-PCR using RealQ Plus Master Mix Green (Ampliqon, Denmark) in accordance with the manufacturer's instructions on an ABI 7500 system. Fold change of microRNA was expressed relative to the expression levels of RUN48 and SNORD44 as endogenous controls. Expression of MxA gene was also determined by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content. All experiments included at least three replicates per group. Relative quantification was calculated by $2^{-\Delta\Delta CT}$ method.

2.5. Serum cytokine measurement

To evaluate the influence of IFN-β on Th1 cytokine after one-year treatment, we measured the levels of IFN-γ in IFN-β responders and non-responders. Cytokine level was measured in the sera of RR-MS patients using the human IFN-γ ELISA kit (Biolegend, USA) according to the manufacturer's instructions.

2.6. Statistical analysis

All data was analyzed using SPSS software, Version 22. Quantitative expression levels between groups were compared through Student's t-test. Cytokine levels between groups were compared through Mann-Whitney U test. Data are presented as mean ± SD and a $p < 0.05$ was considered significant.

3. Results

3.1. Down-regulation of miR-29b-3p in IFNβ-responder RRMS patients versus non-responders

A set of 70 cases were evaluated to characterize the miR-29b variants and MxA expression level from the peripheral blood of RR-MS patients under IFN-β therapy. We analyzed the expression of miRNA from the PBMC of RR-MS patients treated with IFN-β for more than one year to detect a potential impact of immunomodulatory therapy on deregulated miRNA. The expression pattern of miR-29b-3p was evaluated by quantitative real-time PCR method in three groups including responder RR–MS patients ($n = 35$), non-responder RR-MS patients ($n = 35$) and healthy subjects ($n = 20$). Clinical features of patients are shown in Table 1. Expression data of miRNAs were normalized with a corresponding mean value of endogenous gene small nuclear RNA,

Table 1
Demographics of MS patients and healthy controls.

Demographic data	Healthy controls	Responders	Non-responders
Subjects, *N*	16	35	35
Age (years): mean ± SD	31 ± 6.52	33.72 ± 8.19	35.44 ± 8.06
Gender, women/men	8/7	30/5	29/6
Disease modifying therapy	n/a	IFN-β	IFN-β
EDSS score	n/a	0–5	0–5

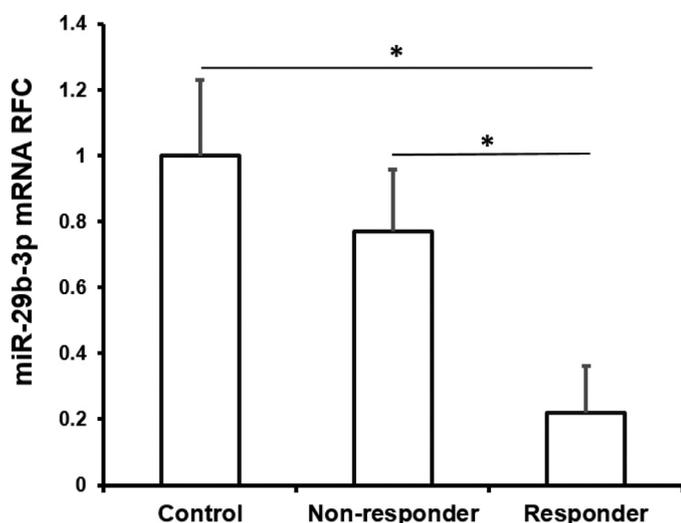EDSS: Expanded Disability Status Scale; n/a: not applicable; SD: standard deviation.

Fig. 1. miR-29b-3p expression levels in RR-MS patients in response to IFN-β treatment.
miR-29b-3p expression levels in IFN-β treated MS patient leukocytes. The expression of miR-29b-3p was studied in the peripheral blood from 35 IFN-β treated responder RR-MS patients and from 35 IFN-β treated non-responder patients, for more than one year IFN-β treatment by microRNA assay-based quantitative RT-PCR following the $2^{-\Delta\Delta CT}$ method. RNU48 was utilized for an endogenous reference to standardize microRNA expression levels. Bars indicate means ± SD, *$p < 0.05$.

RUN48. The miR-29b expression level was significantly decreased in the responder group compared with non-responders (p-value < 0.05, approximately 3.5 fold) and healthy group (p-value < 0.05, approximately 5 fold). MiR-29b-3p expression did not show any significant expression change in the group of IFN-β treated non-responder MS patient (Fig 1).

3.2. Down-regulation of miR-29-5p in responders compared to non-responders

We analyzed relative expression levels of miR-29b-5p in PBMCs derived from IFN-β treated RR-MS patients as compared with healthy controls. Then, we sought to examine miR-29b-5p levels in IFN-β treated responder RR-MS patients versus the non-responder MS patients. No statistically significant differences in miR-29b-5p expression were observed either between responders and healthy subjects or non-responders and healthy group. While miR-29b-5p expression was significantly lower in IFN-β treated responder RR-MS patients compared to the non-responders. Investigating the miR-29b-5p expression level by qRT-PCR exhibited an average of 3 fold down-regulation in responders compared with non-responders (Fig 2).

3.3. Down-regulation of MxA in RR-MS responders to IFN-β therapy compared to non-responders

The expression level of MxA, an IFN induced gene, among RR-MS patients treated with IFN-β showed that MxA level was significantly decreased in the responder group compared with healthy group (p-value < 0.05, approximately 6 fold) or non-responders (p-value < 0.01, approximately 8 fold). However, the expression level of MxA did not differ between the group of non-responders and healthy subjects (p-value = 0.807) (Fig. 3). On the other hand, the patients with good clinical response to IFN-β showed up-regulated expression level of MxA after one year of treatment, while patients with lower expression of MxA had a higher rate of relapses.
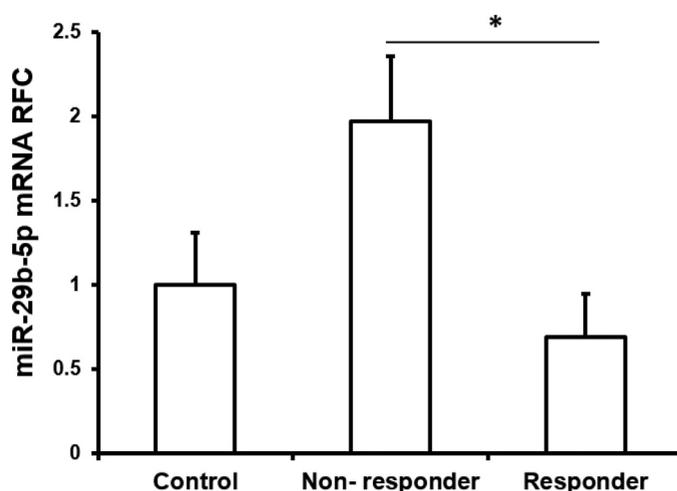

Fig. 2. miR-29b-5p expression levels in RR-MS patients in response to IFN-β treatment.
Quantitative PCR analysis of miR-29b-5p expression level in PBMCs of RR-MS patients in responders ($n = 35$), non-responders of RR-MS patients in responders ($n = 35$), non-responders ($n = 35$) and normal controls ($n = 16$). Results are presented as the ratio of miRNA to the small nuclear RNA RUN48, relative to that in control. Data are presented as means ± SD, *$p < 0.05$.
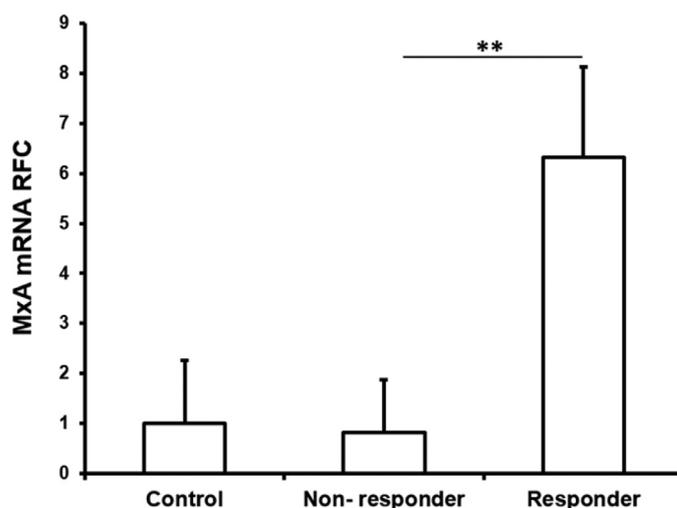

Fig. 3. MxA expression levels in RR-MS patients in response to IFN-β treatment. Investigating the MxA expression levels was done by qRT-PCR analysis. Results are presented as the ratio of MxA to GAPDH, relative to that in control. Values are means ± SD, **$p < 0.01$.

3.4. Serum IFN-γ upon IFN-β treatment

To investigate the long-term effect of IFN-β treatment on IFN-Υ in drug responders and non-responders, we determined serum levels of IFN-γ in MS patients by ELISA. No statistically significant difference was observed in the IFN-γ level during IFN-β exposure. IFN-γ level was not significantly modified following at least one year of IFN-β treatment as depicted in Fig. 4.

4. Discussion

IFN-β has been widely used to treat MS, However, the specific mechanisms behind its therapeutic efficacy are yet to be fully understood. Some of MS patients dose not respond to IFN-β therapy, and there is a lack of markers that can correlate with IFN-β response status in MS. Identifying non-responder patients is essential to define therapy strategies. Consistent data regarding alterations in the expression of
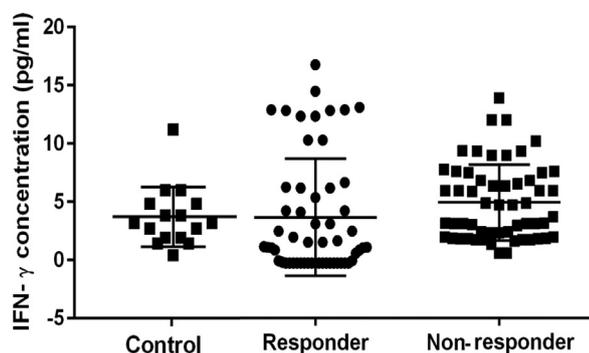
Fig. 4. Levels of serum IFN-γ in RR-MS patients in response to IFN-β treatment. IFN-γ concentration of serum from RR-MS patients under IFN-β treatment and healthy controls was determined using ELISA. Data are shown as means ± SD.

miRNAs in MS are reported and support a pivotal role of miRNAs in the pathogenesis of neurodegenerative diseases including MS (Hébert et al., 2008; Junker et al., 2009). However, little is known about small regulatory RNAs and how they contribute to the mechanism of action of IFN-β treatment in MS patients. The regulation of miRNAs may relate to the immunomodulatory effects of the treatment. Moreover, miRNAs might be markers for characterizing the biological response to IFN-β and treatment monitoring.

In this study, microRNA expression profile was analyzed in PBMCs of MS patients treated with IFN-β to identify the potential impact of treatment on deregulated miRNAs. However, immunomodulatory treatment with IFN-β did not restore the expression of deregulated miRNAs (Waschbisch et al., 2011). It has been reported that some miRNAs such as miR-29b-5p decreased in PBMCs from MS patients in response to IFN-β treatment and might play an important role in the mechanisms of therapy of IFN-β (Hecker et al., 2013a,b). Hecker et al. reported the up-regulation of IFN-β-responsive genes was accompanied by a down-regulation of the mir-29 family one month after the start of therapy (Hecker et al., 2013a,b). Here, we investigated whether the expression of miR-29b variants in responders could be different from non-responders. MiR-29b-3p showed a significant change in the level of expression in IFN-β treated responder RR-MS patients compared to non-responders. Interestingly, miRNAs are stable in blood samples and this proposed that PBMCs miRNA can be used as a potential clinical biomarker (Gandhi, 2015). Therefore, down-regulation of miR-29b-3p in RR-MS responders might be used as a responsiveness discriminating biomarker.

Therapeutic effect of IFN-β treatment has been associated with modulation of the balance between Th1, Th17, Th2 and regulatory T (Treg) cells. We failed to find any evidence of such a correlation. Graber et al. showed a decrease in levels of *ex vivo* cellular production of IFN-γ levels over the course of treatment on any IFN therapy in responders to therapy. Another study investigated whether IFN-β therapy affects serum levels of IL-17, IL-23, IL-10, IL-4, IFN-γ, IL-9 and TGF-β in RR-MS patients 2 months after treatment. They observed a reduction in IL-17 and IL-23 levels, whereas the levels of other cytokines remained unchanged upon treatment (Kurtuncu et al., 2012). Th17 cells can be polarized to Th1-like Th17 subpopulation T helper 17.1 which are highly enriched in CSF of patients with early MS (van Langelaar et al., 2018). Upregulation of miR-29b in CD4 + T cells, which reflect chronic Th1 inflammation, may also associate to Th17.1 cells.

MxA is widely used as a measure of biologic response to IFN-β (Hemmer and Berthele, 2009). A study examining the expression level of MxA among RR-MS patients showed that patients with lower expression of MxA had a higher rate of relapses (van der Voort et al., 2009). Hecker et al. showed downregulation of miR-29 family expression in PBMCs after IFNβ-1b treatment. In addition, their decrease was linked to increased IFN-β-responsive genes (Hecker et al., 2013a,b). Here, this correlation was further reviewed in RR-MS patients under

IFN-β therapy. Our findings provide additional evidence to support the role of MxA, a biomarker for unresponsiveness to IFN-β, with the occurrence of relapses.

To sum up, the results obtained showed the presence of altered regulation of miR-29b-3p in IFN-β treated responder RR-MS patients versus healthy subjects along with different MxA expression after IFN-β therapy. Therefore, miR-29b-3p levels might be used as a diagnostic indicator of responsiveness to IFN-β. However, more studies are needed aiming to confirm this notion.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the Ethics Committee of Isfahan Neurosciences Research Center (Code of Ethics: IR.296075) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

The authors declare that they have no conflict of interests concerning this article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2019.07.034.

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