Research Article:
miR-320 and Inflammation Regulation in Experimental Autoimmune Encephalomyelitis Through Interference With Tumor Growth Factor-β Signaling Pathway

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ABSTRACT

Background: MicroRNAs are small non-coding RNAs that regulate gene expression and involve in many cellular and physiological mechanisms. Recent studies have revealed that dysregulation of microRNAs might contribute to autoimmune disorders such as Multiple Sclerosis (MS). Based on these findings, we examined the potential role of miR-320 isoforms; miR-320-3p and miR-320-5p, in the context of autoimmune neuroinflammation and pathogenesis of EAE, which is an animal model of MS.

Materials and Methods: The expression levels of miR-320-3p and miR-320-5p, and their predicted target genes, TGFBR2 and Smad2, were quantified in the CNS tissue in mice with Experimental Autoimmune Encephalomyelitis (EAE) using RT-PCR method. The expression was also examined in splenocytes macrophages and astrocytes. To examine the interaction of miR-320-3p and miR-320-5p with the 3′-UTR of potential target transcripts, the mimic sequences of both isoforms were transfected into splenocytes and then examined by RT-PCR.

Results: The expression of both isoforms of miR-320 significantly increased in different phases of EAE and activated lymphocytes, whereas the levels of their predicted target genes, Smad2 and TGFBR2 decreased in these cells. Obtained data revealed that miR-320-5p level significantly increased in activated macrophages and astrocytes; however, the miR320-3p level did not show significant changes in these cells after Lipopolysaccharide (LPS) stimulation. The levels of TGFBR2 and Smad2 decreased in transfected splenocytes.

Conclusion: Our findings suggest that upregulation of miR-320 isoforms might be involved in the neuroinflammation and pathogenesis of MS through targeting and suppression of TGFBR2 and Smad2, i.e. protective genes in MS.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) with approximately 22 nucleotides in length. miRNAs are transcribed by RNA polymerase II or III into Primary miRNA (pri-miRNA) and undergo multistep biogenesis, which processes them into precursor and mature miRNAs [1, 2]. In most cases, miRNAs interact with the 3′-UTR of target mRNAs and suppress them, so they act as negative regulators of gene expression [3]. However, studies have reported that miRNAs interact with other regions, including the 5′-UTR, coding sequence, and gene promoters.

Furthermore, miRNAs can activate gene expression in certain conditions [4]. Various studies have shown that miRNAs are critical for normal animal growth as well as numerous biological processes, including cell proliferation, differentiation, and apoptosis by targeting and dysregulating the expression of various protein-coding genes [5]. Also, the aberrant expression of specific miRNAs is the cause of many human diseases, including cancers, infectious diseases, and immune-related disorders [5, 6]. In addition, miRNAs are secreted into extracellular fluids. Recent studies have indicated that the extracellular miRNAs can be used as potential biomarkers for a variety of diseases. They also play a crucial role as signaling molecules to mediate cell-cell communication [7, 8].

Previous studies have shown that various miRNAs play possible roles in major autoimmune disorders, including diabetes, rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis [9]. Studies on PBMC and CNS tissue of multiple sclerosis patients have shown altered expression of various miRNAs [10, 11]. In the present study, we assessed the role of miR-320 in autoimmune neuroinflammation and pathogenesis of Experimental Autoimmune Encephalomyelitis (EAE), an animal model of Mmultiple Sclerosis (MS). Immature miR-320 generates two mature isoforms; miR-320-3p and miR-320-5p. Also, gene ontology analysis of miR-320-3p and -5p predicted targets has revealed that both isoforms have been implicated in the regulation of leukocyte activity and Transforming Growth Factor (TGF)β signaling. In the context of MS, upregulation of miR320 mature isoforms has been reported in miRNA-profiling studies of patients' brain tissue [12, 13].

Nonetheless, the protective or pathogenic role of this miRNA in the disease process is not exactly known. In this study, we first examined the expression levels of miR-320-3p and miR-325p isoforms in the central nervous system tissue of EAE animals at different phases of the disease. Next, the expression levels of miRNA isoforms were assessed in cultures of cells with potential roles in MS/EAE pathogenesis. Overexpression assay in splenocytes was done to observe the interaction of miR-320 isoforms with predicted target genes.

Materials and Methods

Experimental autoimmune encephalomyelitis induction

C57BL/6 wildtype mice (6 weeks old) were obtained from the Pasteur Institute of Iran and maintained in the animal facility of Tehran University of Medical Sciences. EAE was induced in 12 weeks old mice (n=30) by subcutaneous injection of Myelin Oligodendrocyte Glycoprotein (MOG)-35-55 peptide. MOG 35-55 peptide emulsified in Complete Freund's Adjuvant (CFA) was injected subcutaneously on the back (0.1mL of emulsion/site) (EK-2110, Hooke Kit ™ MOG35-55/CFA Emulsion PTX). Pertussis toxin in PBS was injected two intraperitoneal cavity at 200ng/mouse/dose (0.1mL) on the day of immunization and the following day. In the control group, CFA and pertussis toxin were injected subcutaneously and intraperitoneally, respectively.

Following immunization, clinical assessment of EAE was done daily for 30 days after immunization using a 0-15 point scoring system [14]. All experiments were done in accordance with guidelines of the Animal Care Committee of Tehran University of Medical Sciences. The immunized mice were divided into three groups, and their CNS tissues were removed at three time points following disease induction [15]. The first time point was pre-onset (before the appearance of neurological signs, approximately on day 10 post-immunization). The second time point was at the peak of the disease (acute phase), and the third time point was at days 25-30 post-induction (chronic phase).

Cell cultures and treatment

Spleens were separated from the MOG-immunized mice in the pre-onset phase of the disease (7 days after EAE induction); tissues were homogenized, and their splenocytes were isolated using FicollHypaque density gradient centrifugation (Inno-Train). A total of 2×106 cells were cultured in the presence of different concentrations of (MOG 35-55) (MOG 35-55, Hooke labs) in RPMI 1640 medium Gibco supplement. The cells were harvested after 12, 24, and 48h of incubation at 37°C. In similar experiments, splenocytes were

obtained from 6 to 8weekold wild type C57BL/6 mice and were also cultured in RPMI 1640 media and treated with mouse anti-CD3 (0.5µg/mL) and anti-CD28 (0.2µg/mL) (eBioscience) in different time points from 1 to 24h at 37°C [15].

In addition to splenocyte cells, in this study, Bone Marrow-Derived Macrophages (BMDMs) and primary mouse astrocyte cultures were prepared, as previously described [15, 16]. BMDMs were obtained from femur and tibiae bones of C57BL/6 mice. Cells from bone marrow were cultured for 7 days in the presence of 50ng/mL M-CSF (eBioscience) [16]. The differentiated macrophages were treated with LPS (10 and 100ng/mL) for 16 h at 37°C. Astrocyte cells obtained from neonatal mouse brain and then were cultured in DMEM medium supplemented with (20%) FBS. The astrocytes were stimulated with 10 and 100ng/mL LPS (Sigma Aldrich) for 16h at 37°C [17].

RNA isolation, cDNA synthesis, and Real-time PCR

Total RNA was extracted from lumbar spinal cord tissue of EAE mice and cultured cells (stimulated splenocytes, activated macrophages, and astrocytes) using miRNeasy Mini Kit (Qiagen) and stored at −80°C. The RNA concentration was assessed with a Nanodrop. About 1µg of total RNA was used for First-strand cDNA synthesis using miScript II RT Kit (Qiagen) for microRNA assessment and TAKARA kit for mRNA expression analyses, according to the manufacturer's instructions. MicroRNAs (miR-320-3p and miR-320-5p) and their predicted target levels were evaluated by real-time PCR using SYBR Green dye on a Bio-Rad CFX96 system in cells and CNS tissue. MicroRNA expression data were normalized against snord68 and snord72 expression levels (Qiagen) whereas β-actin levels were used to normalize mRNA expression.

miRNA transfection

To investigate the effect of microRNA overexpression on the levels of selected target genes (Smad2, TGFBR2) in cells, mouse splenocyte were transfected with miR-320-3p, and miR-320-5p mimic sequences (50 nM/mL) (Qiagen) with HiPerFect transfection reagent (Qiagen). After 4h, the transfected cells were incubated at 37°C for 48h in the presence of anti-CD3 (0.5µg/mL) and anti-CD28 (0.2µg/mL) (eBioscience). All-Stars negative control siRNA sequence (Qiagen) was used as a control [15].

Statistical analysis

Statistical analyses were performed in SPSS V. 20. The Student's t and Mann-Whitney U-tests were used for two groups comparisons. One-way ANOVA or Kruskal-Wallis tests were performed for comparisons between multiple groups. The obtained data are shown as Mean±SEM.

Results

miR-320 isoforms upregulation in the nervous tissue of the animal model

Expression analyses of inflammationrelated genes showed all three inflammatory cytokines (tumor necrosis factor [TNF]-a, interleukin [IL]-1b and IL-6) as well as CD3e (lymphocyte marker), F4/80 (monocytoid cell marker) and GFAP (astrocyte marker) had been upregulated in CNS of EAE mice in the different phases of disease compared with non-EAE [18] .

To investigate the dysregulation of miR320-3p and miR3205p in the CNS during EAE disease in mice, we analyzed the expression of miRNAs in the MS animal model at different phases of disease. Expression assessment for two miR-320 mature isoforms in the EAE spinal cord tissue showed a significant increase in miR-320-3p isoform at the acute phase and miR-320-5p isoform at all three phases of disease compared with the control mice (Figure 1).

miR-320-3p and miR-320-5p expression increase following immune cell activation

Different cell types, including autoreactive T cells, macrophages, astrocytes, and microglia, are involved in MS/EAE neuroinflammation and pathogenesis. Astrocytes produce numerous inflammatory mediators and thus have a key role in the pathogenesis of MS. To determine the possible influence of these cells on miR-320 upregulation during inflammation, we used different in vitro systems. First, both antigenspecific stimulation and polyclonal activation of T cells were assessed. For antigenspecific stimulation, the splenocytes were isolated from MOGimmunized mice. The cells were restimulated in culture with three concentrations of MOG 35-55 peptide (10, 20, and 40µg/mL) for different time points.

Expression analysis of MOG-stimulated splenocytes by RT-PCR showed a significant increase in miR-320-3p levels at 20 and 40µg/mL MOG after 12h and in 10µg/mL concentration of MOG after 24h. Our findings showed that miR-320-5p significantly increased at 40µg/

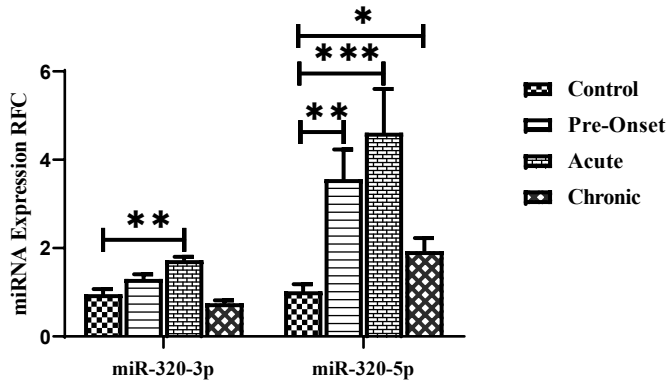

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Figure 1. Expression levels of miR-320-3p and miR-320-5p in spinal cord tissue of EAE at acute and chronic phases of the disease in comparison with the control group

Data presented as Mean±SEM; Number of mice in each group=10; *P<0.05; **P<0.01; ***P<0.001. The Kruskal-Wallis test

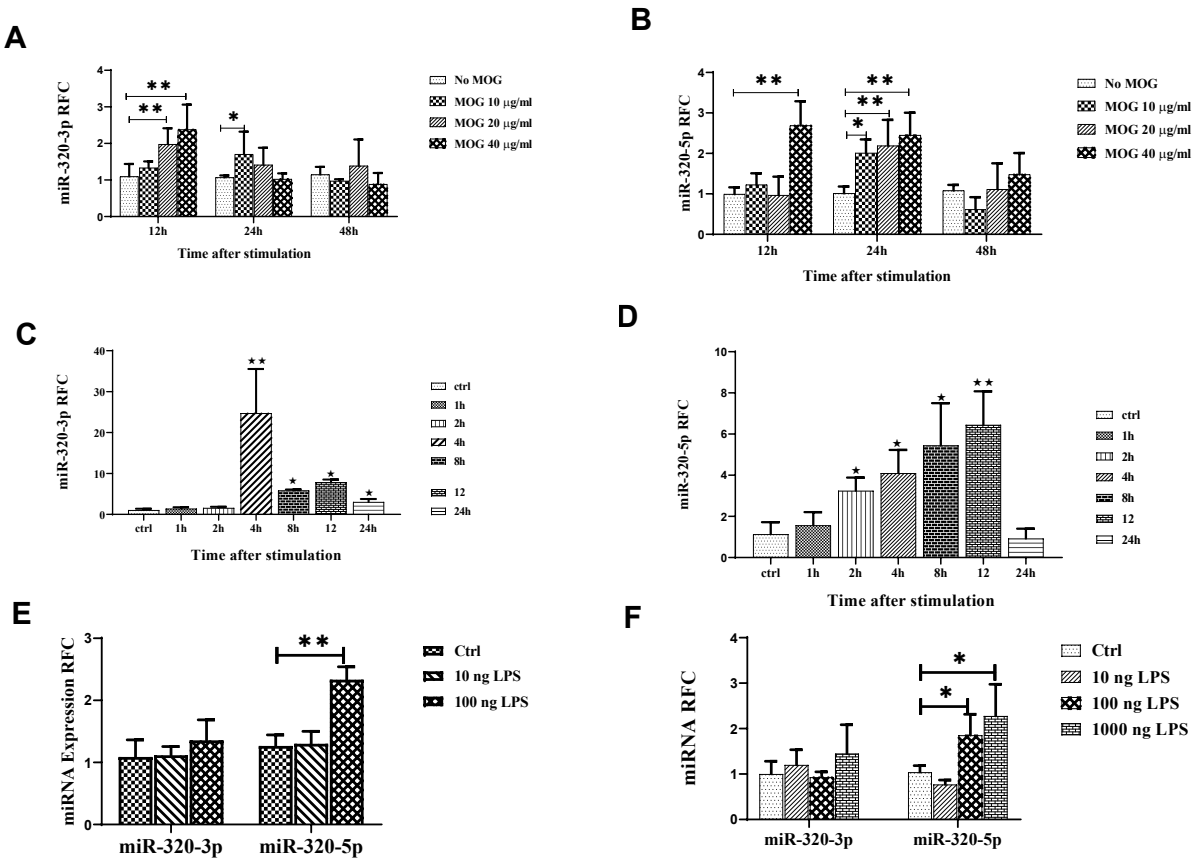

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Figure 2. miR-320-3p and miR-320-5p expression levels in stimulated splenocytes, macrophages, and astrocytes. Expression levels of miR-320-3p and miR-320-5p isoforms were determined by real-time PCR in MOG-treated splenocytes from immunized animals (A, B) and splenocytes activated with anti-CD3 and anti-CD28 for different time points (C, D). miR-320 isoforms expression were analyzed in stimulated bone marrow-derived macrophages (E) and primary astrocytes (F)

Data are presented as Mean±SEM; n=3. The experiment repeated twice; *P<0.05; **P<0.01; The Kruskal-Wallis test

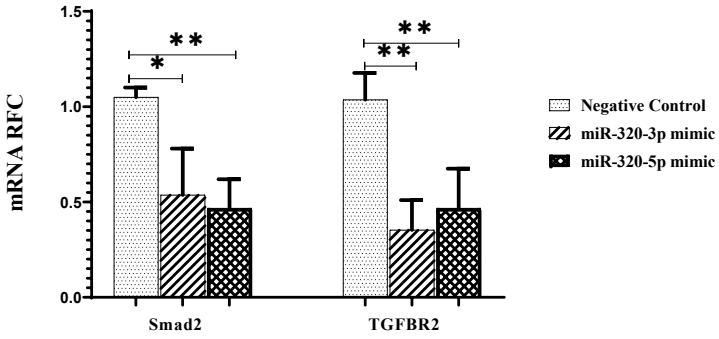

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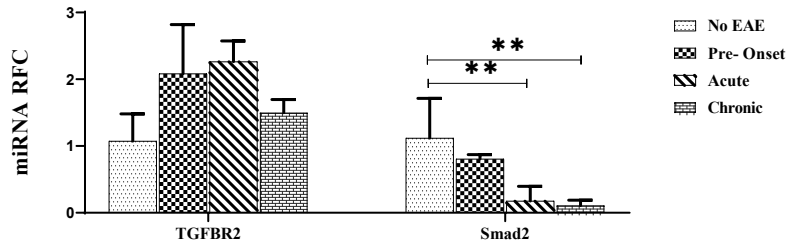
Figure 3. miR-320 isoforms predicted target genes expression levels in miRNA mimic transfected splenocytes. The expression of potential target genes was quantified in stimulated splenocyte cells transfected with miR-320 isoforms mimic. TGFBR2 and Smad2 transcripts were significantly suppressed in miR-320-3p and miR-320-5p transfected cells compared with miRNA negative control overexpressing cells

Data presented as Mean±SEM; **P<0.01; *P<0.05; The Student's t-test

mL concentration of MOG after 12h of treatment as well as in 10, 20 and 40µg/mL concentrations of MOG after 24h (Figure 2. A, B). To determine the expression levels of miR320 isoforms in T cells following polyclonal activation, splenocytes were stimulated with antiCD3/CD28 antibodies and then the levels of both isoforms were measured using quantitative RT-PCR at several time points after activation. The analyses revealed a substantial increase in miR-320-3p levels at 8, 12, and 24h and miR-320-5p levels in 4, 8, and 12h following stimulation (Figure 2. C, D). Overall, these data suggested that activation of immune cells could be associated with upregulation of miR320 isoforms and thus might play a role in the pathogenesis of the disease. miRNA expression was assessed in primary cultures of BMDMs and astrocytes. Mice BMDM cells were stimulated with two different concentrations of LPS (10 and 100ng/mL) for 16h. miR320-5p isoform levels significantly increased after treatment with 100ng/mL LPS. Nonetheless, miR-

A



B

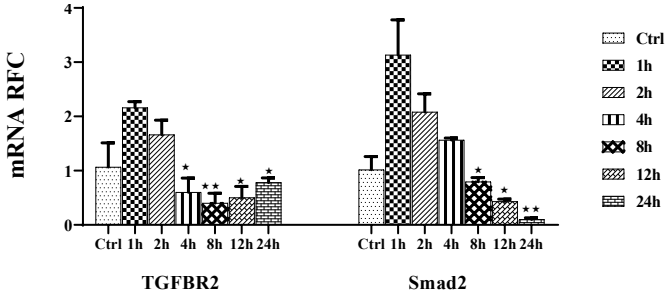


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Figure 4. miR-320-3p and miR-320-5p predicted target genes expression levels in CNS of EAE mice and activated splenocyte cells. TGFBR2 and Smad2 transcripts were determined in the spinal cord of EAE mice by quantitative real-time PCR (A). miR-320 isoforms target genes were also examined in stimulated splenocyte cells compared with unstimulated cells at different time points

320-3p levels did not show any significant changes following LPS stimulation (Figure 2. E).

To examine the miRNA expression levels in astrocyte cells following activation, mouse primary astrocytes were simulated with 100 and 1000ng/mL LPS for overnight. RT-PCR data identified that miR320-5p levels significantly increased in activated astrocytes. However, miR320-3p levels did not show any significant changes after LPS stimulation (Figure 2. F). Overall, these data indicate that the miR-320 isoforms are upregulated in splenocytes, macrophages, and astrocytes after activation.

Regulation of predicted targets expression by miR-320 isoforms

MicroRNAs apply their regulatory effects through interactions with proteincoding transcripts. To examine the potential mRNA transcripts which might be targeted by each miR-320 isoform, we selected some mRNA targets from TargetScan and miRDB databases. Among miR-320-3p and miR3205p predicted targets, we focused on molecules with critical roles in immunoregulation or cytokine signaling. We selected TGFBR-2 and Smad2 as predicted targets for both isoforms of miR-320. TGFBR2 and Smad2 play a key role in the TGF-b signaling pathway and effects of this cytokine on different types of leukocytes, including their effects in promoting Treg differentiation. We first transfected miR-320-3p, and miR-320-5p mimic sequences separately into mouse splenocytes and assessed the predicted target expression levels by real-time RT-PCR. TGFBR2 and Smad2 transcript levels were significantly decreased in miR-320-3p- and miR320-5p-transfected cells compared with cells transfected with a negative control sequence (Figure 3).

Expression of miR-320 isoform target genes and its dysregulation in the central nervous system of experimental autoimmune encephalomyelitis mice and activated splenocytes

Given the increased levels of miR320-3p and miR-320-5p in CNS tissue of EAE mice and the regulation of Smad2 and TGFBR2 expressions by miR320-3p and miR-320-5p, we investigated Smad2 and TGFBR2 levels in lumbar spinal cord tissue of EAE mice. The analyzed data revealed that the expression levels of Smad2 significantly decreased in acute and chronic phases of EAE compared with pre-onset phase and no EAE mice, whereas the expression of Smad2 did not show any changes (Figure 4 A).

We also examined Smad2 and TGFBR2 levels in activated lymphocytes, which showed high expression of miR-320-3p and miR-320-5p in stimulated splenocytes after 4, 8, and 12h. Hence, to investigate the effect of upregulation of miR-320-3p and miR-320-5p on the potential targets expression changes, we analyzed the expression levels of miR-320 isoforms predicted targets, TGFBR2, and Smad2 in stimulated splenocytes. Our findings revealed that the expression levels of both target genes decreased in 4, 8, 12, 24h time points, and there was a negative correlation between Smad2, TGFBR2, and miR-320 isoforms (Figure 4. B).

Discussion

Inflammation is an important and complex biological phenomenon caused by the activation of the immune system in response to pathogens and cell damage. It plays a key role in the clearing of damaged cells and pathogens. Dysregulation of inflammatory responses or excessive inflammation may result in autoimmune disorders such as rheumatoid arthritis and multiple sclerosis [19]. MS is an inflammatory and immune-mediated disease of the brain and spinal cord, which is characterized by an abnormal response of both innate and adaptive immune system [20]. Although the etiology and pathogenesis of MS have not fully identified, migration of autoreactive lymphocytes across the Blood-Brain Barrier (BBB) may be a result of axonal demyelination of neurons [21]. The presence of autoreactive and proinflammatory T cells, including Th1 and Th17 in CNS will result in axonal demyelination, degeneration, and tissue damage [22, 23].

Extensive studies have shown that various dysregulations in the immune system contribute to multiple sclerosis disease, but the impairment of T cell function, especially Th1, Th17, and Treg cells, plays an important role in the pathogenesis of this disease [24]. Th1 and Th17 cells have distinct roles in the pathogenesis of the disease. Treg cells function that limits the immune response to pathogens and prevents the occurrence of autoimmune response and damage to host in normal conditions has been impaired in MS [25, 26]. Autoreactive myelin-specific T-cells are potentially present in healthy persons, but they are under the influence of regulatory factors such as Treg cells and inhibited by these cells.

Thus, the differentiation and function of these cells are valuable in the diagnosis and treatment of this disease. Another regulatory factor is microRNAs that play an important role in the development of multiple autoimmune diseases, such as multiple sclerosis [24]. MicroRNAs are small (usually 19-24 nucleotides), non-coding RNAs

that regulate gene expression at the posttranscriptional level. The association of circulating miRNAs with physiological and pathological processes has been detected in recent studies. miRNAs also have identified as potential biomarkers, therapeutic agents, or drug targets [21]. Several studies showed that several miRNA species were differentially expressed in patients with MS compared with controls and used as potential biomarkers for diagnostic and drug- response.

EAE is a mouse model of multiple sclerosis disease, characterized by CNS autoimmune inflammation resulting from activation and infiltration of autoreactive T cells and leukocytes from the periphery into the CNS. Because the animal model of multiple sclerosis EAE provides extensive knowledge of the pathogenicity of the disease, our assessments were performed in this model. Our findings in this study showed that miR-320 family might be involved in the neuroinflammation. In our study, gene expression assessments showed the levels of inflammatory markers such as proinflammatory cytokines (TNF-a, IL-1, and IL6) and cell markers (CD3e as a lymphocytic marker, F4/80 as a macrophage marker, and GFAP, as an astrocyte marker) significantly increased. The analyzed data indicate that the severe inflammation and presence of immune cells in the spinal cord of the mice at the acute and chronic phases of the disease, create serious response against myelin antigens.

In this study, we assessed the role of miR-320 isoforms (miR-320-3p and miR-30-5p) in autoimmune neuroinflammation. Our gene expression studies revealed that the levels of both miR-320-3p and miR-3205p isoforms significantly increased in acute and chronic phases of EAE. Our findings are consistent with previous miRNA studies in MS CNS tissue. Certainly, two miRNA profiling studies on the brain lesions of MS patients have shown upregulation of miR-320 isoforms [12, 13]. Inflamed tissue has many types of cells that can affect the microRNA content of the tissue. Therefore, due to the difference in the rate of inflammation and cell infiltration between the pre-onset and acute or chronic phases of EAE, the increased expression of miR-320-3p and miR-320-5p at the acute and chronic phases may reflect the content of the microRNAs in the immune cells.

Inflammation in the brain tissue leads to the activation of residual nerve cells, including astrocyte and microglia cells, which intensify inflammation. In addition to the activity of the nerve cells, various types of active immune cells, including T cell and macrophage, enter the inflamed tissue of the brain [20]. Our experiments on activated lymphocytes showed the upregulation of miR-320 isoforms in activated T cells, MOG, and polyclonal stimulated cells. In vitro gene expression studies on monocytoid and astrocyte cells revealed that miR-320-5p isoform significantly increased in activated macrophage and astrocyte cells, whereas the expression level of miR-320-5p isoform did not show any changes.

Studies on blood cells have pointed to the role of miR-320 isoforms in MS pathogenesis. miR-320 was shown to be down-regulated in B cells of MS patients. Further studies showed that miR-320 isoforms could increase the severity of MS by targeting some target genes such as MAPK1, MMP-9, ITGB3, Rac1, NRP-1, and IGF-IR [27].

Our findings suggest that miR-320 isoforms could target transcripts involved in cytokine signaling and Treg cell function. In this study, we showed that TGFBR2 and Smad2 are target genes for miR-320 isoforms using overexpression experiments. TGF-β is a cytokine with pleiotropic functions and has several important roles in development, immunoregulation, survival, growth, and cell differentiation [28]. TGF-β1 plays an important role in the development and function of Treg cells. TGF-β1 contributes to the protection of our body against the auto-reactive T cells by increasing the differentiation of CD4+ T cells into Treg cells. TGF-β also prevents differentiation of CD4+ T cells towards Th1, which leads to a decrease in IFN- γ production and induction of EAE [29]. TGF-β1 has various effects, especially protective one in the context of MS/EAE. These effects could be exerted through the effects of this cytokine on neuronal and lymphocytic cells. TGF-β1 enhances remyelination in MS lesions by increasing the oligodendrocyte differentiation, so plays a protective role in the neuronal cells [30, 31].

In general, TGF-β signaling is initiated by the binding of this cytokine to heteromeric complexes of TGFbRI and TGFbRII receptors on the cell surface. TGF-β signaling is transduced through Smad and non-Smad pathways [32]. Oligomerization of type I and II receptors lead to phosphorylation of cytoplasmic signaling molecules such as Smad2 and Smad3. The Smad2/3 heterodimer then binds to the common-mediator Smad4 and make a complex in the nucleus of cells which involve in the regulation of target gene expression [28, 33, 34].

The findings show that miR-320 isoforms can diminish the TGF-b signaling pathway by targeting TGFBR2 and Smad2 genes. Thus miR-320 isoforms can reduce both neuroprotective and immunomodulatory effects of TGF-

b simultaneously, thereby playing a possible role in the pathogenesis of the MS disease.

The obtained results of this study suggest that miR-320 isoforms increase in the CNS of EAE animals. Overexpression of these microRNAs might be involved in the autoimmune neuroinflammation and pathogenesis of MS/EAE through targeting and suppression of protective genes such as TGFBR2 and Smad2.

Ethical Considerations

Compliance with ethical guidelines

All experiments in this study conformed with guidelines from Research Ethics Committee of Tehran University of Medical Sciences (ethics approval for grant: 90-03-15048)

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Authors' contributions

Designe, optimize, and performed experiments: Farideh Talebi, Samira Ghorbani; Data analysis and writing the manuscript: Farideh Talebi; Supervising and oversaw the research process: Mohammed Vojgani; Conceived the hypotheses and designed and supervised the research: Farshid Noorbakhsh.

Conflicts of interest

The authors declared no conflict of interest.

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