

Study of The Correlation between miR-106a, miR-125b, and miR-330 on Multiple Sclerosis Patients by Targeting TNFSF4 and SP1 in NF- κ B/TNF- α Pathway: A Case-Control Study

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Received: 21/ October/ 2020, Accepted: 20 /April/2021

Abstract

Objective: Multiple sclerosis (MS) is a complex multifactorial neuro-inflammatory disorder. This complexity arises from the evidence suggesting that MS is developed by interacting with environmental and genetic factors. This study aimed to evaluate the *miR-106a*, *miR-125b*, and *miR330*- expression levels in relapsing-remitting multiple sclerosis (RRMS) patients. The miRNAs' impact on *TNFSF4* and *Sp1* genes through the NF- κ B/TNF- α signaling pathway was analyzed by measuring the expression levels in case and controls.

Materials and Methods: In this in silico-experimental study, we evaluated the association of *miR-106a*, *miR-125b*, and *miR330*- with *TNFSF4* and *Sp1* gene expression levels in 60 RRMS patients and 30 healthy controls by real-time polymerase chain reaction (PCR).

Results: The expression levels of *miR-330*, *miR-106a*, and *miR125-b* in blood samples of RRMS patients were predominantly reduced. The expression of *TNFSF4* in patients demonstrated a significant enhancement, in contrast to the diminishing *Sp1* gene expression level in controls.

Conclusion: Our findings indicated an association between *miR-106a* and *miR-330* and *miR125-b* expression and RRMS in our study population. Our data suggested that the *miR106-a*, *miR125-b*, and *mir330*- expression are correlated with *TNFSF4* and *Sp1* gene expression levels.

Keywords: Biomarker, *microRNA*, Multiple Sclerosis

Cell Journal(yakhteh), Vol 24, No 7, July 2022, Pages: 403-409

Citation: Hadi N, Seifati SM, Nateghi B, Ravaghi P, Khosraviyan F, Namazi F, Fotouhi Firouzabad M, Shaygannejad V, Salehi M. Study of the correlation between miR-106a, miR-125b, and miR-330 on multiple sclerosis patients by targeting TNFSF4 and SP1 in NF- κ B/TNF- α pathway: a case-control study. Cell J. 2022; 24(7): 403-409. doi: 10.22074/cellj.2022.7835.

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Introduction

Multiple sclerosis (MS) disorders are developed due to chronic inflammation of the central nervous system (CNS) and can increase focal lesions in the white matter of the spinal cord and brain (1). Clinical studies have shown that MS affects women 2-fold higher than men, and the progressive phase was between 5 and 35 years after the first onset (2). Relapsing-remitting MS (RRMS) is the typical primary type of MS and accounts for about 85% of MS patients (3). There is no standardized method of how to pursue MS patients to detect disease activity (4). Although MS's exact etiology remains enigmatic, the identification of genetic variations affecting MS disease development has grown in the last few years (5).

Immense evidence has indicated that tumor necrosis factor alpha (TNF- α) plays a pivotal role in MS development (6-8). Consecutive TNF- α expression could induce chronic inflammatory demyelinating disorder,

synaptic instability in the brain, and subsequently sensory and cognitive damage (9). It has been demonstrated that the damaging role of TNF- α in MS might significantly relate to its transmembrane receptors tumor necrosis factor receptor type II (TNF-RII) or receptors, tumor necrosis factor (TNFR-I) (10). TNF receptors are present at the cell surface as monomers, while the monomers' ligand-induced homodimerization activates TNF- α signaling. OX40L is recognized as a ligand for OX40, also known as tumor necrosis factor ligand superfamily member 4 (TNFSF4) and CD252 (Cluster of differentiation 252), and is a type II transmembrane protein that might have a pivotal role in the differentiation and stimulation of T-cells. TNFSF4 is mainly expressed on the surface of T-cell antigen-presenting cell (APC) cells (11). The encoded protein of this gene is primarily expressed on activated CD4⁺ and CD8⁺ T-cells and various B-cells, microglia, vascular endothelial cells, and dendritic cells

(12). The encoded protein is involved in APC interactions and facilitates the adhesion of T-cells to endothelial cells. Interaction between TNFSF4 and CD4+ T-cells by TNFSF4 stimulates nuclear factor-kappa B (NF- κ B) through TNF-R related TRAF1 and TRAF2. Besides, this signaling pathway is related to several immune functional activities, which include the intensification of Th2 responses and the production of Th2 and Th1 cytokines (13).

Sp1 is a transcription factor that binds to many promoters. Moreover, SP1 could involve in many processes such as apoptosis and immune responses. It has been proved that there is a relationship between Sp1 expression and the activity of the inflammatory cytokine TNF (14). Sp1 sites are frequently observed in NF- κ B related genes, which can activate specific promoters in NF- κ B over its binding sites. Sp1 acts through NF- κ B by activating the NF- κ B promoter sites (15).

The association of miRNA with components of the RNA-induced silencing complex (RISC) results in the degradation or translational repression and/or destabilization of their relevant mRNA. It occurs through miRNA binding to the 3'-untranslated region (UTR) of target genes (16). Growing evidence has shown the abnormal miRNA activity in CNS glial cells of patients with MS and peripheral blood immune cells compared with normal individuals, suggesting that miRNA expression could be correlated with MS pathogenesis and immunological features (17).

Honardoost et al. (18) have proposed that miR-106a probably has an inductive function in Th17 differentiation. Sanctuary et al. (19) have revealed that Mir-106a may have a crucial function in inflammatory bowel disease through the TNF- α pathway. Also, the biological role of miR-330-5p has been offered in some cells and cancers (20). According to Mao et al. (21) study on prostate cancer cells, microRNA-330 suppresses cell motility by decreasing the expression of Sp1, which plays the role of a transcription factor through the TNF- α pathway. Furthermore, miR-125b was demonstrated to bind the 3'UTR of TNF- α ; therefore, MicroRNAs could reduce the expression of TNF- α in the innate immune response pathway. In this study, we examined whether the dysregulated expression of miR-106a, miR-125b, and mir-330 can be considered a diagnostic biomarker in RRMS patients with recurrent disease symptoms two months after relapse. This study evaluated the correlation of miRNAs with the TNFSF4 and SP1 mRNA and its pathogenicity by examining the expression levels of miR-106a and miR-125b, and miR-330 in normal and MS samples.

Materials and Methods

Ethical Issue

All experimental procedures were approved by the Ethic Committee of the Isfahan University of Medical Sciences (289271).

miRNA prediction

In this in silico-experimental study, we collected lists of microRNA target genes using miRWalk (22) and a list of gene-disease association export of DisGeNET (23). We chose genes that overlapped between these lists. These genes were studied in several databases for data mining. In this search, we evaluated Reactome (24), DAVID, STRING, and KEGG analysis. Interactions between genes, microRNAs, and pathways are illustrated in the designed network using Cytoscape software (25).

Patients and controls

The Informed consent was taken from all subjects before sample collection. Briefly, we collected 4mL of blood from 90 participants (both male and female), including 60 RRMS patients and 30 healthy subjects. Overall, 30 healthy controls were recruited from Kashani Hospital (Isfahan, Iran), and 60 patients with RRMS were divided into two groups, including 30 who were recurrent and 30 who had recurrences for at least two months. Based on medical examinations, the healthy participants had no family history of autoimmune disease. The RRMS patients were diagnosed by a specific neurologist based on McDonald's criteria (26). 32 of these patients had solely received interferon (IFN)- β treatment two months before sampling, and the rest had not received any treatments. In the next step, we collected.

Preparing peripheral blood human mononuclear cells from blood

Blood samples were isolated through a gradient of density Lymphoprep Peripheral Blood Human Mononuclear Cells (PBMCs, Bio Sera, USA). The cells remained in an intermediate phase after centrifugation, although other cells were deposited. In the next step, we gradually diluted the lymphoprep solution in a falcon tube with physiological saline at a ratio of 1:1, and then the solution was centrifuged at 800 g for 30 minutes (27). Afterward, Peripheral Blood Human Mononuclear Cells were transferred into a 2 ml RNAase-free microtube from the middle phase.

Evaluating the expression of genes and MicroRNAs

RNA extraction from PBMCs was conducted by TRIzol reagent (Invitrogen, USA). The purity of the RNA was evaluated by a NanoDrop spectrometer. Furthermore, cDNA was prepared by a cDNA Synthesis Kit (TaKaRa, Japan). Additionally, the miRNA synthesis of RNA was done using a standard kit (Pars Genome, Tehran, Iran). *U6* was selected as the housekeeping gene for the normalization of the miRNAs data. Also, For the normalization of genes, *GAPDH* was considered a reference gene. The quantitative real-time polymerase chain reaction (qPCR) was accomplished by the Rotor-Gene 6000 (Corbett Life Science, Australia), and the repetition of samples was duplicated. Moreover, the amount of cycle threshold of real-time was determined by $2^{-\Delta\Delta Ct}$ methods. Primers purchased from micro-gene (Korea) are indicated in Table 1.

Table 1: The primer genes list

Genes	Primer sequence (5'→3')	Temperature (°C)
<i>SP1</i>	F: TTG AAAAAG GAG TTG GTG GC	60
	R: TGC TGG TTC TGT AAG TTG GG	
<i>TNFSF4</i>	F: GAAGAAGGTCAGGTCTGTCAAC	58
	R: AATCAGTTCTCCGCCATTAC	
<i>GAPDH</i>	F: TGCCGCCTGGAGAAACC	60
	R: TGAAGTCGCAGGAGACAACC	

Statistical analysis

The GraphPad Prism Ver.9 (San Diego, USA) was applied to analyze the real time polymerase chain reaction (PCR) results. The Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. The one-way ANOVA test was used to analyze expression levels between two case and control groups. $P < 0.05$ was considered statistically significant for the tests.

Results

Clinical and biological features

Sixty RRMS patients participated in this study, of which 30 subjects were selected 2-months after relapse. Furthermore, 30 healthy subjects joined the study as the control group. Patients and controls were sufficiently matched in terms of age and sex. According to the expression analysis results, there was no statistically significant difference in sex and taking interferon and non-interferon drugs between patients and controls ($P=0.619$ and $P=0.388$, respectively). The biological characteristics of two groups of patients (recurrent and recurrences for at least two months) are shown in Table 2.

Table 2: Biological and Clinical characteristics of patients and healthy individuals

Characteristics	Control	Recurring patients	Two months after relapse patients
Number of subjects	30	30	30
Sex			
Males	10	9	9
Females	20	21	21
Drug			
Interferon	-	11	11
Non-interferon	-	19	19
Age (Y)			
Mean age	1.843 ± 38.60	1.522 ± 33.7	1.522 ± 33.7
Range	21-58	21-45	21-45

In-silico analysis

We provided a list including 178 genes that had overlap between microRNAs and genes associated with multiple sclerosis, and we presented these results using a VENN Diagram (Fig.1). Based on the enrichment of these genes, we found that these genes are correlated with NF- κ B and TNF- α signaling pathways as vital pathways in MS. (Fig.S1, See Supplementary Online Information at www.celljournal.org). Also, the enrichment results pointed to the relationship between genes and MS in DisGeNET. We reported an interaction between genes, pathways, and microRNAs targeted genes in the cytoscape network (Fig.2).

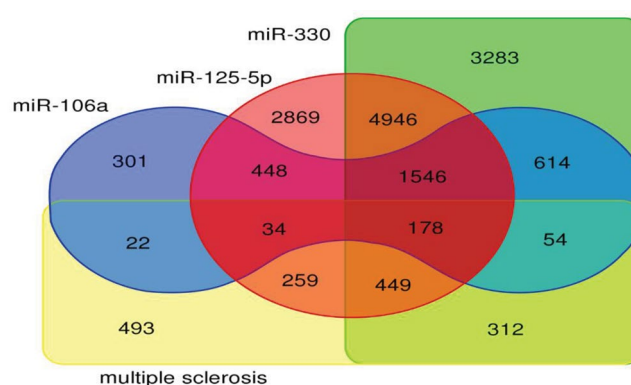


Fig.1: miRNA targets. Venn diagrams were employed to acquire the prediction of miRNAs that target *SP1* and *TNFSF4* genes. The number of 178 indicated the quantity of the selective miRNAs which target the genes.

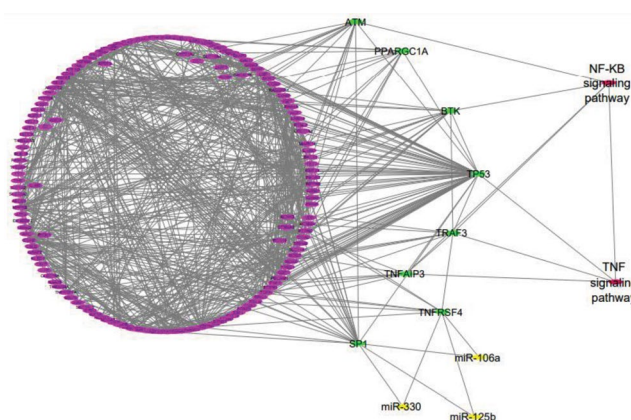


Fig.2: The network mapping. Interaction and correlation of mir and hub genes indicating *miR-330-5p*, *miR-106a*, and *miR-125b* are associated with *SP1*, *TNFSF4* genes in NF- κ B/TNF- α pathway.

miR-330-5p is up-regulated in RRMS patients

miR-330-5p expression was assessed by qRT-PCR in

two groups: The patients, including 30 patients with recurrent presentations and 30 patients two months after relapse (n=60) and healthy subjects (n=30). The results indicated a significant growth (by 3-fold) in the expression of this miRNA in patients (both recurrence and two months after relapse) compared with healthy subjects (P<0.0001 and P<0.0002, respectively, Fig.3A).

Down-regulation of miR-125b and miR-106a expression in RRMS patients

To determine the potential roles of miR-125b and miR-106a in RRMS patients, we investigated both miRNAs' expression levels in patients (two months after relapse and recurrence) versus control individuals. Our results demonstrated that miR-106a expression was significantly down-regulated (by 0.5-fold) in the recurrence and two months after relapse patients compared with the control group (P<0.0001 and P<0.006, respectively). Moreover, data demonstrated that miR-125b was significantly diminished (by 0.7-fold) in both recurrence and two months after relapse patients (P<0.0001 and P<0.0005, respectively) and probably plays an essential role in RRMS patients (Fig.3B, C).

Analysis of the expression level of TNFSF4 and SP1

In this study, two potential targets of *miR-330-5p*, *miR-106a* and *miR-125b* were selected for further analyses. *TNFSF4* and *SP1* were selected as mentioned in the methods. Our data indicated decreased expression of *SP1* (by 0.5- fold) (P<0.0017 and P<0.003, respectively). In contrast, *TNFSF4* expression was increased significantly in recurrence and patients two months after relapse (by 3-fold) (P<0.004 and P<0.02, respectively) compared with healthy (Fig.3D, E).

Analysis of the expression relationship of miR-106a, miR-125b, and miR-330 with selected targets

To assess the hypothesis's validity, we compared the expression level differences between miR-106a and miR-125b in patients and control samples with the expression level differences in *TNFSF4* between patient and control samples. According to data analysis, there was a significant inverse relationship between the reduced expression levels of miR-106a and miR-125b with the increase in *TNFSF4* expression. The Pearson correlations for the association of miR-106 and miR-125b with *TNFSF4* were equal to -0.4970 and -0.6284, respectively, and P values showed significant relationships (P=0.0052 and P=0.0002, respectively, Fig.4).

Moreover, the increase in miR-330 expression was inversely correlated with a decrease in *Sp1* gene expression. The Pearson correlation r was -0.7280, while the P<0.0001 showed the significance of the association (Fig.4).

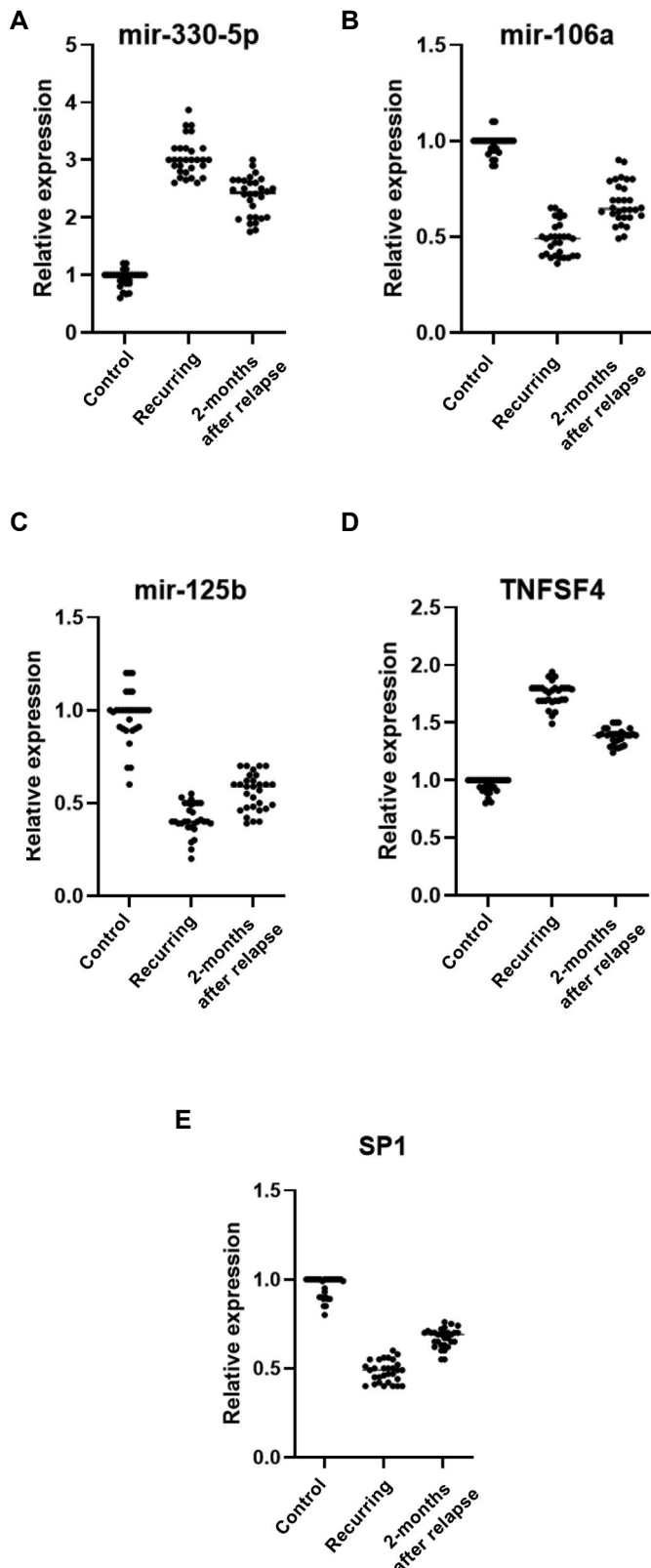


Fig.3: Quantitative real-time polymerase chain reaction (PCR) analysis results. **A.** Relative expression of *miR-330-5p*, **B.** *miR-106a*, and **C.** *miR-125b* in recurring patients and two months after relapse patients and healthy individuals. Relative quantification for the *miR-330-5p* (P<0.0001 and 0.0002, respectively), *miR-106a* (P<0.0001 and P<0.006, respectively) and *miR-125b* (P<0.0001 and P<0.0005, respectively) and **D.** *TNFSF4* (P<0.004 and P<0.02, respectively), and **E.** *SP1* (P<0.0017 and P<0.003, respectively) were significantly different between patients and the control group. All Data were presented as mean ± SD (n=30 per group). Data were calculated using a one-way analysis of variance (ANOVA) and Tukey's post hoc test.

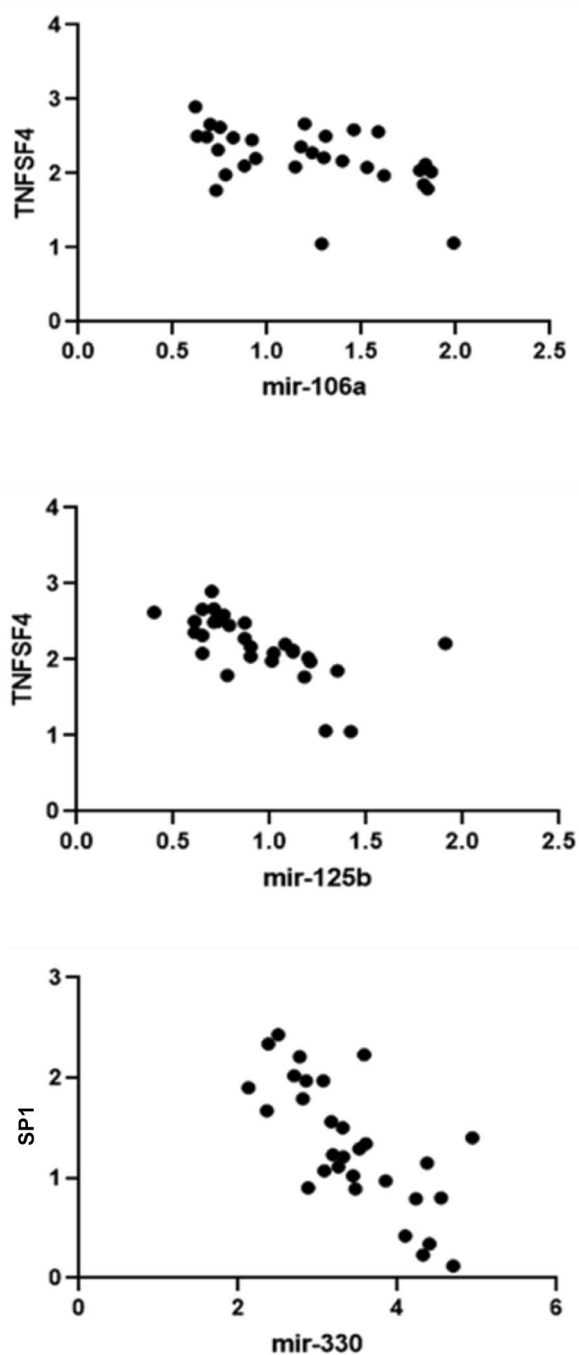


Fig.4: Graphs plotted from the Pearson correlation analysis showed a significant relationship between microRNAs and their targets. This correlation between genes and their targets is inverse. The Pearson correlation for associating miR-106 and *TNFSF4* was equal to -0.497 and $P < 0.0052$ and miR-125b with *TNFSF4* was equal to -0.6284 and $P < 0.0002$. The Pearson correlation for miR-330 and *SP1* was -0.7280 , with a $P < 0.0001$. All data were presented as mean \pm SD ($n=30$ per group).

Discussion

MS's clinical pathophysiology is diverse to the extent that despite the accessibility of therapeutics for the relapsing type of MS, patients have clinical disease activity and neurologic inability within months after disease onset. Due to the need for more effective treatments, biomarkers recruited for predicting inability and therapeutic response assessment are desirable in managing patients (28). In MS,

dysregulation of miRNAs has been indicated in various immune cells. Immense evidence has demonstrated changes in miRNA expression in immune cells and brain tissue of MS patients. Therefore, we explored the correlation between MS progression and the expression of miRNAs (29).

In this study, the correlation of three miRNAs, such as miR-125b, miR-330-5p, and miR-106a expression with MS disease, was investigated. We indicated significantly higher expression of miR-330-5p in both RRMS groups than in healthy subjects. In contrast, a decrease was revealed in the miR-125b and miR-106a expression in RRMS patients. According to the Choi et al. (30) study, the expression of miR-330 increased during brain development and NSC (Neural stem cells) differentiation. In a miRNA microarray analysis, it was indicated that miR-106a was significantly reduced in MS patients (31). It has been proposed that the down-regulated miR-125b expression could serve as a helpful noninvasive biomarker for Alzheimer's disease (32). We investigated these three miRNAs' potential roles via in-silico molecular enrichment analysis, and we found a correlation between miRNA and gene roles in biological processes involved in MS pathology. Notably, we indicated that miR-330-5p, miR-106a, and miR-125b were directly associated with the inflammation pathway. These data are supported our hypothesis that there is a correlation between miR-106a, miR-125b, and miR-330 in MS patients by targeting *TNFSF4* and *SP1* in NF- κ B/TNF- α pathway.

The regulation of immune system responses to nerve cell myelin sheaths is a fundamental matter in MS development. TNF- α /NF- κ B signaling pathway has a crucial role in autoimmune responses regulated by various factors, and Sonar et al. indicated an association between *TNFSF* and autoimmune disease (33, 34).

Another gene examined in this study is a transcription factor called *SP1*, which was predicted to be targeted by miR-330 (21). *SP1* is a transcription factor regulated by the TNF- α /NF- κ B signaling pathway. This transcription factor is activated after binding to the GC-box at NF- κ B binding sites. We found an association between *SP1* and miR-106a, miR-125b, and miR-330 in MS based on the bioinformatic analysis. Moreover, in-silico data have demonstrated that *SP1* could target the NF- κ B/TNF- α pathway. Based on the evidence, NF- κ B decreases through increasing miR-330 expression and subsequently with reduced expression of *SP1*. Contrariwise, it can increase apoptosis in autoimmune diseases such as MS and deteriorate the symptoms. Mao et al. (21) confirmed that miR-330 was downregulated and inversely associated with *SP1* expression. Also, they induced miR-330 expression by transfection of miR-330 mimic, and the outcome was a reduction in expression levels of the *SP1* gene, but the results of *SP1*-knockdown were contrary to previous. Based on their research, miR-330 could target *SP1* directly (14, 15).

Recently, primary MS therapies have been based on the

inhibition of the TNF- α /NF- κ B signaling pathway. Thus, tumor factor α -necrosis factor (α) blockers are a treatment of interest in many inflammatory and autoimmune diseases (35). This therapeutic target can control the demyelination of nerve cells in MS and its recurrence. Therefore, regulatory inhibiting factors such as miR-330 might be acceptable candidates. However, miR-106a and miR-125b are inhibitory candidates for treating MS and other autoimmune conditions.

Conclusion

Our findings illustrated that low miR-106a and miR-125b expression contributes to high TNFSF4 expression, whereas increased miR-330 expression activates the TNF- α signaling pathway by targeting and reducing the *SP1* gene expression. Subsequently, the activated TNF- α signaling pathway may lead to the initiation and progression of autoimmune diseases such as MS.

Acknowledgments

There is no financial support and conflict of interest in this study.

Authors' Contributions

N.H., S.M.S.; Contributed to the conception and design. N.H., F.Kh., F.N., B.N.; Contributed to all experimental work and molecular experiments. F.Kh., P.R.; Contributed to the conception, and design of the database and bioinformatic analysis, statistical analysis, and data interpretation. N.H.; Drafted the manuscript, which was revised by M.S.V.Sh., M.F.F.; Resources and methodology. M.S.; Conceptualization, validation, visualization, and supervision. All authors read and approved the final manuscript.

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