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Analysis of *MicroRNA-18a* Expression in Multiple Sclerosis Patients

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Abstract

Background: In multiple sclerosis (MS), the immune system acts against myelin lesions of the central nervous system, destroying neuronal fibers resulting in signal transmission disturbances in the nervous system. MicroRNAs play important roles in the post-transcriptional regulation of gene expression and in the regulation of disease activity and its response to treatment. The goal of this study was to determine the role of *miR-18a-5p* by comparing its expression in MS patients and healthy subjects.
Methods: RNA was isolated from blood samples of 32 MS patients and 32 healthy individuals, and miR-18a-5p expression was determined by real-time polymerase chain reaction (real-time PCR).
Results: *miR-18a-5p* expression was significantly less in MS patients than in healthy subjects.
Conclusions: The reduction of *miR-18a-5p* expression may be via pathway signaling. Altered signaling plays an important role in MS pathogenesis and the *miR-18a-5p* expression profile in blood cells can be described as a prognostic biomarker and identifier of high-risk individuals in MS.

Keywords: MicroRNA (miRNA), MiRNA-18a-5p, Multiple Sclerosis (MS).

Introduction

Multiple sclerosis (MS), one of the most common neurodegenerative diseases, is characterized by demyelinating lesions in the brain, spinal cord, and optic nerve (1). This disease leads to impaired transmission of neurologic signals by removal of the myelin sheath (2), and is associated with neurologic attacks that lead to disability and impaired neurologic function (3).

Multiple sclerosis symptoms usually begin to appear in people between the ages of 20 and 40 years (4). It is uncommon in children (5), and more common in women than in men (6). Multiple sclerosis results from a combination of environmental and genetic factors. Genetic evidence suggests several essential factors in MS pathogenesis. Genome-wide association studies (GWAS) have provided data on genes associated with MS risk (7). Multiple sclerosis can present as one of four clinical phenotypes. These phenotypes are important not only to predict the course of the disease, but also to decide on the appropriate therapeutic approach:

1- Relapsing-remitting multiple sclerosis (RR-MS)
2. Primary-progressive multiple sclerosis (PP-MS)
3-Secondary-progressive multiple sclerosis (SP-MS)
4-Progressive-relapsing multiple sclerosis (PR-MS) (8).

MicroRNAs (miRNAs) are a small group of non-coding, single-stranded RNAs composed of 18 to 24 nucleotides (9). Gene regulation is affected by post-genomic regulation mechanisms, one of which is miRNA-mediated regulation (7).

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miRNAs regulate gene expression at the post-transcriptional and protein synthesis levels and are involved in basic biological processes.

Understanding the regulatory role of miRNAs not only helps to clarify the expression mode and gene function, but they can also be used as biomarkers for disease diagnosis and treatment (10). Antisense oligonucleotides (anti-miRNAs) can also be used to block certain miRNAs to inhibit expression of target genes (RNA silencing), which is one of the introduced therapeutic methods (11). Therefore, the detection of miRNAs as biomarkers and the use of anti-miRNAs can be a step toward the treatment of some diseases.

Dysregulation of miRNA expression is associated with several pathological processes including viral infections, cancer, and immune system disorders. Understanding the role of miRNAs in these processes may contribute to our understanding of diseases associated with the immune system such as MS (12). miRNAs play an important role in MS because of their overexpression in immune cells, which are mediators of the disease, as well as in central nervous system cells (13). All miRNAs with the same seed sequence probably overlap in the target genes and are classified in families that can have multiple members. The miR-17-92 cluster, which contains six members including *miR-18a, miR-17, miR-19a/b, miR-92a,* and *miR20a,* has been well studied (14).

Genetic research on MS has identified a risk locus in the 13q31 region. No functional elements have been defined in this region, but it is adjacent to the miR-17-92 cluster (7). Stimulation of the miR-17- 92 cluster has been shown to increase active B and CD4+ T cell levels, and slightly increase the number of CD8+ T cells, which in turn causes autoimmune disorders (15). T helper 17 (Th17) cells are involved in the pathogenesis of several autoimmune and inflammatory diseases, including MS and psoriasis. Also, in mouse models, the precise in vitro and in vivo functions of miR-18a in active CD4+ T cells were analyzed, and a negative effect of this miRNA on Th17 cell differentiation was shown. This function is different from those of other miR-17-92 members (16).

In miRWalk 2.0 and miRTarBase bioinformatics prediction databases, SMAD 2/3/4, RORA, BCL2, TP53, and FAS, are among the *miR-18a-5p* targets, which have been reported in various studies as genes involved in the development of MS (17, 18). It should also be noted that one of the most important functional mechanisms of miRNAs is their attachment to the 3′UTRs of complementary mRNAs and regulation of gene expression and contribution in diseases by this mechanism (19).

Given the regulatory role of *mir-18a-5p* and its importance in MS, we therefore used the real-time polymerase chain reaction (real-time PCR) to compare *mir-18a-5p* expression in RR-MS patients and healthy controls.

Materials and methods

Study population

In collaboration with the Pajoohesh private laboratory, Shohadaye Tajrish and Imam Hossein Hospitals in Tehran, informed consent was obtained from all the study participants according to the Ethical Guidelines of the National Institute of Genetics and Biotechnology. Multiple sclerosis was confirmed in patients by complementary and diagnostic tests, and patients with RR-MS were identified by a neurologist. Healthy subjects had no family histories of MS or autoimmune diseases. Blood samples were collected from 32 healthy individuals and 32 patients. The characteristics of these two groups are presented in Table 1. Three ml of venous blood were transferred to Falcon® tubes containing ethylenediaminetetraacetic acid (EDTA) and shaken gently to prevent clotting.

RNA extraction total and cDNA synthesis

Total RNA was extracted from 0.5 ml of peripheral blood according to the RNX-PLUS kit protocol (Sinoclon, Iran). The extracted RNA was quantified using Nanodrop (Thermo Scientific™) and its concentration and 260/280 nm absorption ratio were determined. For qualitative measurements, extracted RNA was electrophoresed on 2% agarose gels and the band of interest was observed.

miRNA fragments generally contain 18-24 nucleotides. For the next steps of the procedure, including PCR, these sequences were lengthened to minimize unwanted errors in analysis by adding poly-A tails to the 3’UTRs using poly (A) polymerase (TAKARA, Japan).

cDNA was synthesized using reverse transcriptase according to the M-Mulv Reverse Transcriptase Kit protocol (Sinoclon, Iran) for 60 minutes at 42 ˚C. The samples were then incubated for 10 minutes at 70 ˚C to inactivate the enzyme.

Real-Time PCR:

Real-time PCR was performed in an Applied Biosystem StepOne System and Master Mix containing LightCycler® FastStart DNA Master plus SYBR Green I (Roche). The thermal-time program was set up in three steps (Table 2).

Primers were synthesized by the Biomed Company for the *5srRNA* and *miR-18a-5p* genes (Table 3). The melting temperature (Tm) for *5s rRNA* and *miR-18a-5p* were 58.9 and 59.9 °C, respectively. Primer specificity was confirmed by the melting curve peak and 2% agarose gel electrophoresis. The melting curves were analyzed to characterize the various reaction products and ensure the specificity of the amplification (Figs. 1 and 2). *5s rRNA* was used as an internal control to normalize the *miR-18-a-5p* reaction in healthy and patient samples.

Table 1. Age range, the percentage of women and men of all samples from healthy subjects and patients, and the number of individuals in each group.

Group	Number	Males (%)	Females (%)	Age range	Average Age (SD) year
Healthy	32	28%	72%	18-50	**33/7 (9/2)**
Patient	32	39%	61%	19-46	**32/4 (7/8)**

Table 2. Three-step Real-time PCR protocol.

step	duration	temperature	cycles
Initial denaturation	10 min	95	45
Denaturation	30 S	95	45
Annealing	30 S	59/9	45
Elongation	30 S	72	45

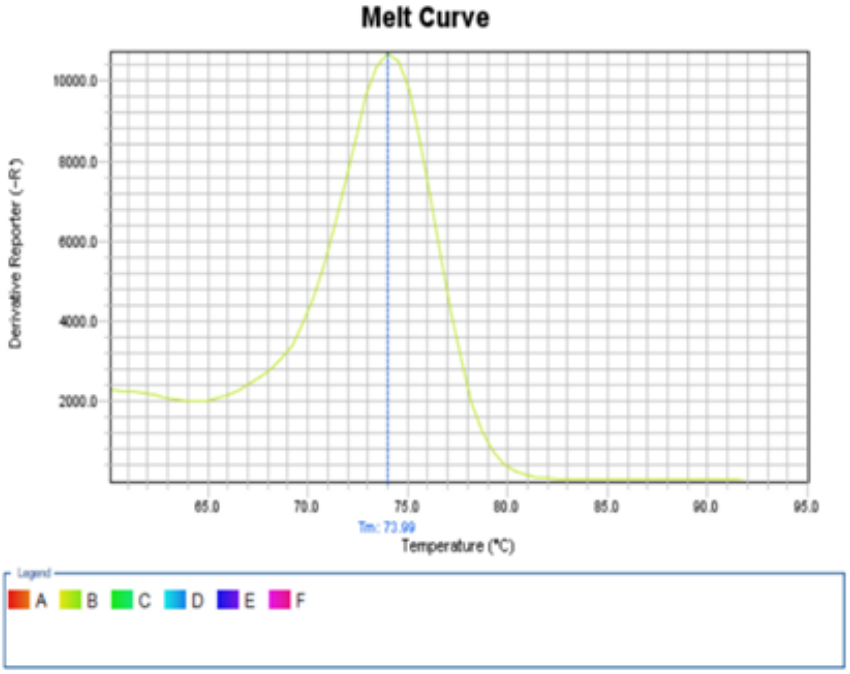

Fig. 1. The melting curve for *miR-18a-5p* is shown. By analyzing the specific and exclusive peak of *miR-18a-5p*, we confirmed the specificity of real-time PCR amplification. The melting temperature (Tm) of *miR-18a-5p* was73.99 ºC.

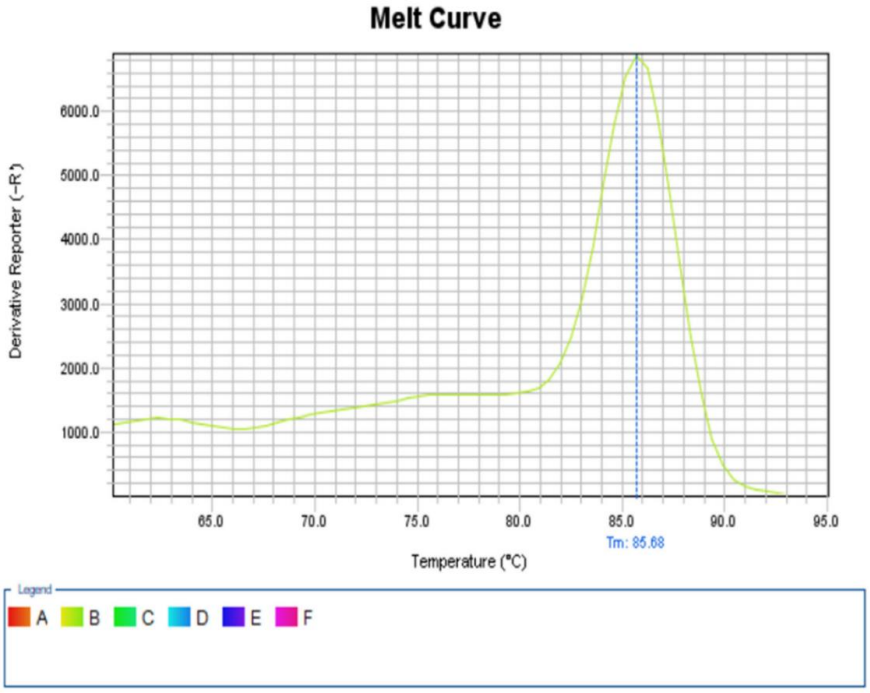

Fig. 2. The melting curve of 5srRNA is shown. The melting temperature (Tm) was 85.68 °C.

Statistical analysis

To compare *miR-18a-5p* expression in the patient and healthy subject groups, the fold change was calculated using Livak law or $2^{-\Delta\Delta Ct}$. Rest software was also used to analyze the data. P values < 0.05 were considered significant.

Results

In this study, *miR-18a-5p* expression was significantly less in patient than in healthy control samples (Fig. 3), while 5s *rRNA* expression was not significantly different between the two groups. The fold change was calculated using the formula $2^{-\Delta\Delta Ct}$ (Fig. 4).

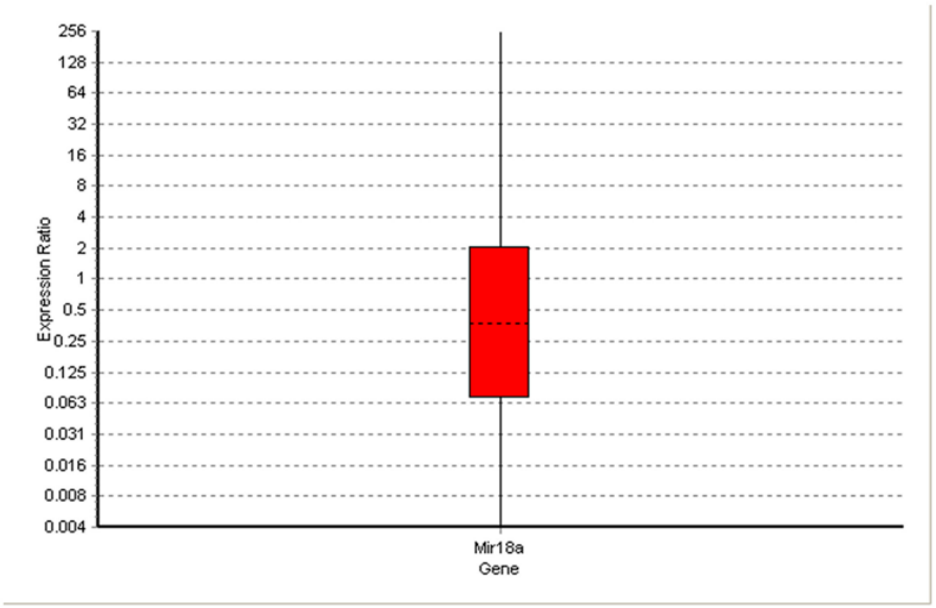

Fig. 3. The box represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. ($p < 0.05$ considered as significant). miR18a is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0.396 (S.E. range is 0.032 - 5.352). miR18a sample group is different to control group. P (H1) =0.048.

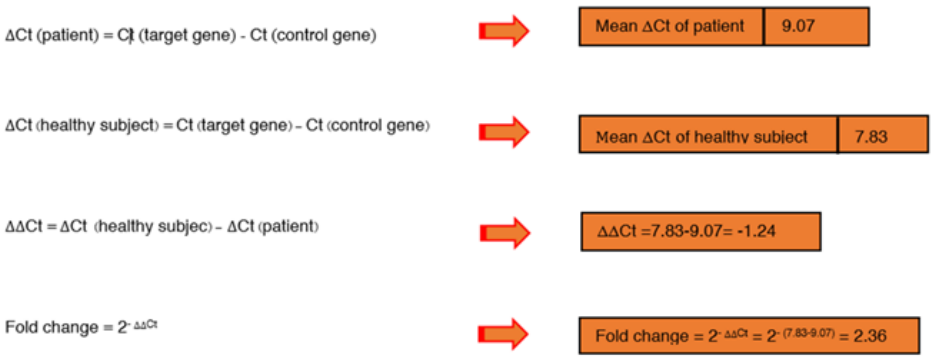

Fig. 4. calculating fold change using Lewack's law.

The mean ΔCt was used in this formula. The mean ΔCts of patients and healthy controls are shown in Fig. 5. The expression reduction rate using the formula $2^{-\Delta\Delta Ct}$ was 2.36; thus, *miR-18a-5p* expression was 2.36 times less in patients than in healthy subjects (Fig. 6).

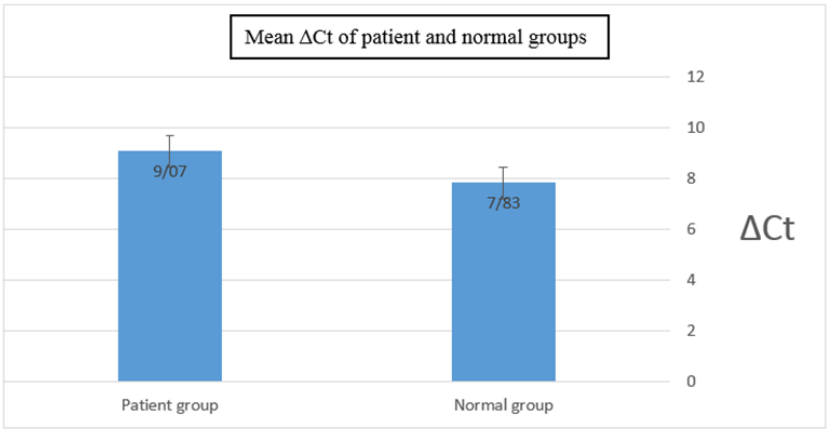

Fig. 5. The columns show the mean ΔCts in the patient and normal groups. According to the inverse relationship between Ct and expression levels, *miR-18a-5p* expression was less in patients than in the normal group.

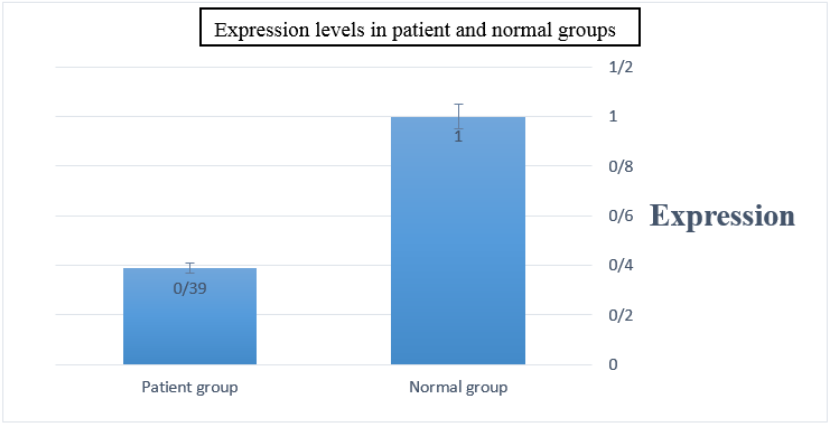

Fig. 6. 18a-5p miR expression in patient and normal groups. The columns represent *miR-18a-5p* expression in the two groups.

In this study, miRTarBase, miRWalk, TargetScan, and Tarbase databases were analyzed for genes involved in various signaling pathways that interact with *miR-18a-5p*, including those in the p53, MAPK, and apoptosis signaling pathways (Table 4). These databases predict the potential targets of miRNAs. Some of these target genes are involved in multiple signaling pathways, and due to the regulatory role of *miR-18a-5p*, contribute to MS pathogenesis.

Table 3. Primer sequences and their product lengths

Gene name	Primers sequence	Product length
5srRNA	F: ′3 - CGGCCATACCACCCTGAAC′ - 5	19
	R: ′3 - CCTACAGCACCCGGTATTC′ - 5	19
miR-18a-5p	F: ′3 - GGTGCATCTAGTGCAGATAG′ - 5	20
	R: ′3- GCGAGCACAGAATTAATACG′ - 5	20

Table 4. *miR-18a-5P* target genes in various signaling pathways

Rank	KEEG Pathway	The number of genes in the pathway	Data base	Genes
1	**p53 signaling pathway (hsa04115) (20)**	13	Tarbas	CCNG1, ZMAT3, CCNB1, CDK4, CCND2, ATM, CCND1, SHISA5, CDKN1A, RRM2, PTEN, PPM1D, MDM2
		1	TargetScan	ATM
2	**MAPK signaling pathway (hsa04010) (20)**	23	Tarbase	BRAF, MAPK8IP2, STK4, RASA2, ELK4, PAK2, CACNB4, MAPK14, TAOK2, GNG12, MAP4K4, MAP3K1, TAOK1, JUN, MAPK8, HSPA8 DUSP3, STMN1, MAP3K2, NFATC3, DUSP1,
		3	TargetScan	ELK4, MAP3K1, FGF1
3	**Melanoma (hsa05218) (20)**	8	Tarbas	BRAF, E2F1, CDK4, CCND1, RB1, CDKN1A, PTEN, MDM2
4	**Hippo signaling pathway (hsa04390) (20)**	3	TargetScan	FGF1, PARD6B, INADL
		23	Tarbas	ACTB, SMAD2, CCND2, SMAD3, MOB1B, WWTR1, CCND1, CTNNB1, CTNNA1, AXIN2, Smad4, SMAD7, MOB1A, PPP2R1B, PARD6B, INADL TGFBR2, BMP4, AJUBA, CTGF, PPP1CB, BIRC2,
5	**Apoptosis pathway (hsa04210) (17, 18)**	13	Mirwalk	P53, PTEN, FAS, RORA, TGFBR2, SMAD4, TGFA, CDK19, IFNAR2, SMAD3, MCM5, LRRC41, BCL2
		13	Tarbase	P53, PTEN, FAS, RORA, TGFBR2, SMAD4, MYC, BCL2L11, CDK19, TNFAR2, BCL2, SMAD3, MCM5

Discussion

To date, scientists have identified several MS biomarkers, including some miRNAs (21). Various studies have shown that some of these miRNAs can be used as diagnostic biomarkers for MS in the early stages or before the onset of clinical symptoms. In addition, unique expression patterns for specific miRNAs have been identified during recovery and recurrent disease stages (22).

Results of previous studies indicate that correcting aberrant miRNA expression can be a therapeutic strategy for MS patients. We found reduced *miR-18a-5P* expression in our MS patients compared to healthy controls. Several studies have examined miRNA expression in MS patients and shown that the expression dysregulation in these patients may be associated with the pathogenic mechanisms and pathophysiology of MS or with disease activity (3). The *miR-18a-5P* expression reduction observed in MS patients indicates that this miRNA may affect immune mechanisms.

Montoya et al. (2017) discovered a specific and essential role for *miR-18a* in limiting Th17 cell differentiation. They showed that *miR-18a* had the greatest effect of all the miR-17-92 cluster miRNAs in upregulating activated T cells. In this

study, a unique role for *miR-18a* as a highly-induced inhibitor of Th17 differentiation was reported (16).

Ingwersen et al. (2015) showed that *miR-18a*, *miR-20b*, *miR-29a*, and *miR-103* were upregulated and mainly expressed in CD4+ T cells in MS patients. The linear manner of analysis used by these researchers showed that expression of these miRNAs is negatively related with disease severity, and *miR-18a* is associated with MS pathogenesis (23). Huang et al. (2016) reported that increased *miR-18a* expression can be used as a biomarker to predict MS patient responses to treatment (24). Junker et al. (2009) found that *miR-18a* expression was decreased in brain lesions of MS patients (25). Gandhi et al. (2013) showed that members of the miR-17-92 cluster, including *miR-18a*, are involved in CD4+ T cell replication and activation in MS patients (12).

The miR-17-92 cluster plays a key role in the development of T helper 1 (Th1) cells, which have been proposed as the most important cells in the pathogenesis of MS. These cells secrete gamma interferon, which has been shown to exacerbate inflammatory symptoms in MS patients (26, 27). One reason for the effect of miRNAs in MS could be their high effect of expression level in immune cells, which are mediators of the disease, as well as central nervous system cells. most nucleated cells are (lymphocytes, monocytes, and granulocytes) in blood components of the immune system. Given the role in of miRNAs in T cell development and differentiation, it has been suggested that T cell activation against myelin and the resulting myelin degradation could explain the decreased expression of *miR-18a-5p* in patients and be considered as a factor contributing to MS pathogenesis.

The findings of this research and the previous studies indicate the regulatory role of *miR-18a-5P* in the immune system. Based on the roles of the immune system in MS pathogenesis and *miR-18a-5P* regulation on immune function, this decreased *miR-18a-5p* expression may increase the risk and be considered as a prognostic factor for MS. It is also suggested as a prenatal biomarker in individuals predisposed to MS.

Currently, it seems the best way to overcome immune system diseases is to identify their causes and mechanisms, and address those, rather than treating symptoms with multiple drugs. One of these mechanisms is the reduced expression of suppressor miRNAs, which eventually increases the expression of genes involved in immune mechanisms. Therefore, reduced expression of miRNAs, such as *miR-18a* in blood cells of MS patients, represents the possible function of these miRNAs as suppressors that affect the expression of immune system-associated genes. Thus, the effect of *miR-18a* and other miRNAs on MS patient immune systems can be found in other mechanisms, including the pathways listed in Table 4.

The RORA gene, one of the *miR-18a-5P* target genes, encodes a regulator of the apoptosis pathway (28) and an important chemical receptor for Th17 cell migration to the brain and mucosa. These cells are involved in the pathogenesis of autoimmune and inflammatory MS. Also, SMAD 2/3/4 genes in the TGF-b signaling pathway contribute to Th17 cell differentiation (16).

Studies have also shown that increased FAS and FASL levels are associated with progression to permanent disability in MS patients by increasing apoptosis in T cells activated against myelin. In addition, a reduction in serum FASL has been reported during disease recurrence, while the FAS level increases dramatically (29, 30).

Finally, it is suggested that according to *miR-18a* target genes, *miR-18a* antisense oligonucleotide (anti-miRNA) could be used to prevent aberrant expression of the target genes involved in various signaling pathways associated with MS. Decreased *miR-18a-5p* expression can be a prognostic marker for MS. In addition, evaluation of the expression profile of this miRNA may be useful to identify high-risk individuals. Using this prognostic data, it should be possible to fine-tune treatment in high-risk individuals, increasing treatment efficacy and potentially preventing recurrence in MS patients.

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