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ORIGINAL ARTICLE

The fluctuations of expression profiles of critical genes in the miRNA maturation process and pro-and anti-inflammatory cytokines in the pathogenesis and progression of multiple sclerosis

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Abstract

Background Multiple sclerosis (MS) is a central nervous system disease known for immune-mediated demyelination, inflammatory, and neurodegeneration symptoms. Discovering molecular biomarkers to classify RRMS and SPMS patients, monitor the disease activity, and response to particular treatments is one area that has received notable attraction. MicroRNA (miRNA), a single-stranded non-coding RNA molecule, is a significant regulator of gene expression recruited in pathogenic mechanisms in diverse diseases, especially cancer and MS. Also, the relapsing-remitting features of MS exhibit that both inflammatory and anti-inflammatory cytokines are effective in the progression of the disease over time.

Methods and results It was assessed the expression patterns of the genes (*Drosha*, *Pasha (DGCR8)*, and *Dicer*) encoding the critical enzymes in the processing steps of miRNA maturation and major pro-inflammatory and anti-inflammatory cytokines (*IFN-α*, *IFN-β, and IL-6*) in blood cells of 40 MS patients (two groups of 10 men and women in both clinical courses of RR and SPMS patients) in comparison with 20 healthy control group (10 males and 10 females). The highest transcription activity of *Drosha* was observed for RRMS patients (4.2 and 3.6-fold, respectively), and the expression ratio was down regulated in male and female patients with SPMS (3.9- and 3.1-fold, respectively). Considering the studied cytokines, the increase in expression ratio of *IL-6* in SPMS patients and the decrease in transcript abundance of *INF-α*, and *INF-β* cytokines are consistent with the progression of the disease.

Conclusions Our findings showed that the high and low transcriptional levels of the considered genes seem to be effective in the pathogenesis and progression of MS.

Keywords MicoRNA machinery · Multiple sclerosis · Gene expression · Pro-inflammatory and anti-inflammatory cytokines

Abbreviations

ANOVA	Analysis of variance
AGO1	Argonaute-1
BBB	Blood-brain barrier
CNS	Central nervous system
dsRBP	Double-stranded RNA binding protein
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Status Scale score
IL-6	Interleukin 6
IFN	Interferon
MS	Multiple sclerosis
miRNA	MicroRNA
MRI	Magnetic resonance imaging
PBMC	Peripheral blood mononuclear cells
qRT-PCR	Quantitative reverse transcription PCR

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RR	Relapsing-remitting
SP	Secondary progressive
SD	Standard deviation
Th1	T helper 1

Introduction

Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, and neurodegenerative disease of the central nervous system (CNS). This disease is involved in lesions predominantly in the white matter of CNS, which lead to new focal lesions dominantly occurring in the white and gray matter, and neurodegeneration diffusion of the grey and white matter of the brain and spinal cord. This degradation can result in increasing T-helper-type 17 (Th17) lymphocyte migration in the blood-brain barrier (BBB) in which neurological dysfunction attacks the myelin sheaths or the cells that produce and maintain them, leading to nerve conduction impairment [1]. Clinically, the disease symptoms appear most generally in different young adult development stages such as late adolescence, young adulthood, or mid-life, which often cause their disability during most productive years by degradation of axons via autoimmune responses of autoreactive T cells, characterized by both inflammatory and neurodegenerative features (long-term disability) including vision problem, walking or moving a limb problem, vertigo, sensation loss, and lack of coordination [2]. It widely believes that clinical emersion, course, and therapy responses to be heterogeneous and may be influenced by the interaction between genetic, autoimmune, and environmental conditions. These parameters direct the mechanism of the disease, although the detailed etiology is unknown [3].

The patients experience different multiple sclerosis phases that differ in various subtypes of diseases. However, the prediction of the disease course and therapeutic strategy can be more specified by the classification of the disease in relapsing-remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR) subtypes [4]. Most patients (80–85%) are exposed to a common form of the disease like relapsing-remitting, a type of symptomatic attack or exacerbation, that typically lasts for some time and is then eliminated spontaneously, called relapsing-remitting multiple sclerosis (RRMS). This type of MS is the most frequent disease course and distinguished by evident attacks of increasing or new neurologic symptoms. Periods of partial or complete recovery were observed after these attacks or relapses [5]. Approximately, 10–15% and 50% of RRMS individuals will experience transient symptoms followed by a primary and secondary progressive form of multiple sclerosis based on irreversible deficits and neurodegeneration, respectively. Although an immunological attack on the myelin basic protein (MBP) as the main reason causing MS has been considered to be mediated by lack of harmony between T lymphocytes subsets, it is known that B lymphocyte play a crucial role in the pathogenesis of the autoimmune disease [6, 7].

Biomarkers are evaluated as measured parameters and considered as a criterion of pathogenic processes or therapeutic intervention responses. Identifying the dependable diagnostic biomarkers for MS can to improve the diagnose, progress, prediction of MS prognosis, and assessment of treatment responses [8, 9]. Biomarkers can be obtained from peripheral blood mononuclear cells (PBMC), cerebrospinal fluid (CSF) cells, plasma, serum, or circulating exosomes to characterize RRMS, SPMS, and PPMS patients [10]. It was previously shown that dysregulation of miRNA expression is associated with multiple pathological conditions involving the immune system that include the neurological disease, autoimmune disease, and cancer [11]. Several articles have been published recently emphasizing the function of miRNAs in the pathogenesis of MS [4, 12, 13]. Previous studies shown that miRNA has an exigent duty in the myelin sheath formation that surrounds axons (oligodendrocytes). Its function can also be associated with differentiation of Schwann cell precursors (SCPs) to myelinated state. Therefore, the diminution of miRNA in different types of cells terminated in both differentiation arrest and demyelination, emphasizing how to regulate the abundance of miRNA transcript and the intricacy of gene expression regulation that provided by miRNA during the differentiation of myelinated cells [14, 15].

MicroRNAs or miRNAs (small non-coding RNA molecules) include about 20–24 nucleotides that function in the cytoplasm. Approximately half of all human gene expression and protein synthesis are modulated by miRNAs. These single-stranded molecules are encoded by families of highly conserved nuclear genes in various organisms. They are capable of down-regulating protein synthesis of the target mRNAs by binding to 3'UTR or 5'UTR sequences. Imperfect miRNA and RNA sequence match leads to translational inhibition or transcriptional degradation in the target, while perfectly match results in targeted mRNA cleavage [16]. During the last decade, lots of studies have shown that miRNA molecules are not only involved in many physiological and pathological processes and regulating the immune system but also they are critical in the development of MS. It has been reported that miRNAs are notably stable in human blood, that obtained as free nucleic acids from blood circulation or isolated from vesicles, red and white blood cells. miRNA expression patterns in whole blood cells were differed in MS patients, and is believed that different expression patterns would be obtained from active and inactive MS lesions. Therefore, miRNA expression

analysis in serum and blood cells provides the opportunity to serve as biomarkers for blood-based diagnosis of various diseases, including autoimmune diseases [17, 18].

Furthermore, in previous studies, the presence of autoreactive T cells and macrophages, also released mediator cytokines from immune and epithelial cells could determine the active MS lesions. The main characteristic of the active phase of MS is manifested by the interaction of pro-inflammatory and anti-inflammatory cytokines which are functional in the deterioration of the patient's clinical condition [19, 20].

In this study, we tested the mRNA expression patterns of the main components of the biogenesis of miRNA; *Drosha*, *Pasha (DGCR8)*, and *Dicer*, in blood cell samples of patients with relapsing-remitting MS (RRMS) and secondary progressive MS (SPMS) and compared them to the healthy individuals using qRT-PCR analysis. As pro-inflammatory and anti-inflammatory cytokines affect the progression of the disease, we evaluated the expression levels of interleukin 6 (IL-6), interferon (IFN)-α, and β in blood cells of RRMS and SPMS patients. This report demonstrated the major miRNA processing machinery, pro-inflammatory and anti-inflammatory cytokines as beneficial biomarkers for determining a pattern of symptoms of MS. Up- and down-regulation of these critical genes provided distinct tools to distinguish the complicated progression of MS.

Materials and methods

Study subjects and sample collection

As regards the sampling, the patients were selected from Jahrom MS community, located in the southwest of Iran (Jahrom City, Iran). The study was approved by the Human Research Ethics Committees from the Jahrom University of Medical Sciences (registration number IR.Jums. REC.1397.144), and all the participants were requested to fill out the informed consents that focused on lifestyle habits and to donate blood samples by explaining the objective of the study. All patients were examined by a neurologist under primary tests (blood test, brain magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) analyses), secondary test (optic coherence tomography, urodynamic testing, cognitive testing), other tests for differential diagnosis e.g., lumbar puncture for CSF examination, basic CSF biochemistry (glucose, protein, albumin, IgG, and lactate levels), microbiological tests (cell count and other microbial and ELISA tests), cytopathological evaluation (screening for malignant cells), and tests for intrathecal immunoglobulin G (IgG) synthesis). When a clinical course characterized by periods of clinical stability, acute relapses and MRI activity, the patients were classified as RR-MS. Finally, SP-MS was defined in patients presenting an initial RR course followed by a progressive course. In all cases, clinical evaluation, and brain and spine MRI criteria for the MS diagnosis were performed during hospitalization for diagnostic purposes. Also, the patients were diagnosed under the McDonald 2017 criteria for clinically definite MS [21]. Forty patients were considered by two different clinical courses of MS as follows: RRMS; 10 males and 10 females, with the average Expanded Disability Status Scale score (EDSS) value of 5.5 (standard deviation (SD) = 0.8), SPMS; 10 males and 10 females, with a mean EDSS score of 4.5 (SD = 0.65) with a mean disease duration of 3–5 years for two groups of patients (Table 1). The control group including matched age and sex individuals showed no clinical symptoms or laboratory features concerning autoimmune disorders, other infectious diseases, and inflammatory diseases (10 males and 10 females, mean age ± SEM: 36.2 ± 1.2). The clinical characteristics of clinical characteristics of multiple sclerosis patients and healthy controls were assessed (Table 1). None of the two groups of patients had received any immunosuppressive medications in the past three months before participating in this study. Early in the morning, the fresh blood samples were collected in the EDTA tubes by venipuncture and stored at 4 °C for no longer than 24 h.

Table 1 Demographics data and clinical characteristics of multiple sclerosis patients and healthy controls

Description	RRMS patients (N = 16)	SPMS patients (N = 16)	Control (N = 20)
Patient demographics	10, 10	10, 10	10, 10
Jender (male, female)	34.1 ± 1.3	35.0 ± 0.9	36.2 ± 1.2
Age: Mean ± SD (y)			
Disease history	3–5	3–5	
Time since MS diagnosis	5.5 ± 0.8	4.5 ± 0.65	
EDSS score			
Disease activity	10.5	11.0	
Current number of T2 weighted	9.7	16.3	
lesions (mean)	0.4	0.7	
Relapses in total (mean)			
Relapses in the last 12 months (mean)			

RNA isolation from peripheral blood mononuclear cells (PBMC)

Total RNAs were extracted from peripheral blood mononuclear cells (PBMC) for each group of individuals using Trizol reagent manufactured by Sigma Aldrich, USA. The extraction process was carried out by the manufacturer's instructions. PBMC purification was performed based on the density gradient procedure: Whole blood samples were subjected to lysis using Red Blood Cell (RBC) lysis buffer. Then, the samples were incubated for 5 min at 25 °C and

were subsequently centrifuged at 800 g for 5 min to remove the lysis buffer. The supernatant was then aspirated, and the cells were gently resuspended in 1 mL of RBC lysis buffer. Next, the resulting suspension was centrifuged for 10 min at 6000 g and the whole supernatant was discarded. Then, the pellet from each tube was resuspended in 1 mL of sterile phosphate buffer saline (PBS). After washing the pellets, 0.7 mL and 0.15 mL of Trizol reagent and chloroform were subsequently added to the tube and were homogenized using vortex for 15 s, then centrifugated at 8000 g for 15 min. The supernatant was transmitted to a new tube containing 0.6 mL volume of ice-cold isopropanol slowly shaken, and then placed on ice for 20 min. The samples were subjected to centrifugation at a force of 8000 g for 10 min at 4 °C. After discarding the supernatants, the resulting pellets were suspended in a solution of 0.2 mL 75% ethanol and centrifuged at 6000 g for 5 min at 4 °C. Finally, the pellets were left to air-dry for 5–7 min and resuspended in 15 µL of nuclease-free water.

Reverse transcriptase and cDNA synthesis

Firstly, genomic DNA (gDNA) was discarded using *DNase* I enzyme (RNase- Free DNase Set, Fermentase). Then, RNA quality and quantity were assessed by an agarose gel electrophoresis technique and a NanoDrop® ND-1000 spectrophotometer, respectively. Next, the first-strand complementary DNA (cDNA), via RevertAid first strand cDNA synthesis Kit (Fermentas), was produced with 4 µL of total RNA (30 µg) as follows: following manufacturer's instructions, we combined the isolated RNA with a 200 U/µL reverse transcriptase enzyme from Fermentase, Lithonia, as well as 0.5 µg/µL oligo-dT primer. The mixture was then incubated for 60 min at 42 °C, followed by 10 min at 70 °C. Each resulting cDNA sample was divided into two equal portions of 10 µL and preserved at -20 °C for subsequent analysis.

Quantitative RT-PCR

Analysis of expression ratios of genes encoding critical enzymes, Drosha, Pasha, Dicer, IL-6, IFN-α, and β, were performed through quantitative reverse transcription PCR (qRT-PCR). The primer sequences were designed based on the corresponding genes with Primer 3 (http://frodo.wi.mit.edu/primer3/) software (Table 1). Conventional polymerase chain reaction (PCR) was carried out using a Bio-Rad thermocycler. The reaction volume was set as 25 µL per sample and consisted of 1X PCR buffer, 0.2 mM dNTPs, 0.4 µM forward and reverse primers, 1 U/µL Taq DNA polymerase, and 200 ng cDNA. The first step of denaturation was conducted at 94 °C for 5 min. This step was followed by 35 cycles of denaturation at 94 °C for 30 s, with specific annealing temperatures ranging between 60 and 62 °C for 45 s for each primer. The extension process was carried out at 72 °C for 60 s. Finally, the last step was a final extension at 72 °C for 10 min. PCR products were analyzed by ethidium bromide-stained 2% agarose gel electrophoresis in Tris–borate–EDTA buffer. Each of the samples was measured in triplicate by real-time PCR. The qRT-PCR amplification assay was performed by an SYBR® Green master mix Kit™ (Ampliqon, Denmark) and StepOne Real-Time PCR System (Applied Biosystems), in 18 µL reaction mixture (Master Mix 10 µL, diluted cDNA 2 µL, each primer (forward and reverse) 0.6 µL, and PCR-grade water 7.4 µL). The qRT-PCR conditions were 95 °C for 5 min as initial denaturation, then 40 cycles of denaturation at 95 °C for 15 s, 60 °C as annealing temperature for 20 s, and extension at 72 °C for 15 s. The relative expression is defined as the expression ratio of a target gene versus RPL as a reference gene, and the expression levels of genes were determined by REST (Relative Expression Software Tool).

Statistical analysis

Statistical analysis was performed using one-way ANOVA and multiple differences were evaluated using Duncan's multiple range tests to investigate the differences between groups of patients and healthy controls. The statistical significance level was considered to be $p < 0.05$. Also, all obtained data were expressed as the mean ± standard deviation (SD).

Results

Expression changes of Drosha, Pasha, and dicer gene in RRMS and SPMS patients

Due to the requirement of biogenesis of miRNAs in MS pathology, the patients in different courses should inevitably form significant levels of blood miRNAs to describe the timely occurring progressive phase of the disease. This phenomenon requires many alterations in the transcript levels of genes encoding the major miRNA machinery enzymes. To better understand how genes underlying miRNA machinery biogenesis in blood cells that expressed under the two different courses of MS diseases contained relapsing-remitting and secondary progressive, the expression profiles of the three genes (i.e., *Drosha*, *Pasha*, and *Dicer*) at the two courses were determined using qRT-PCR in PBMCs from the forty patients with MS (20 RR-MS and 20 SP-MS patients) and 20 controls as below:

Drosha

Figure 1. To regulate MS progression, the *Drosha* mRNA transcripts were significantly down-regulated in comparison with the healthy control individuals, while the highest increasing of expression levels observed in male RR-MS patients (up to 4.2-fold) (Fig. 1a). The average relative expression levels of *Drosha* in blood cells of patients under relapsing-remitting, however, were considerably higher than the expression under secondary progressive (Table 2). Also, the lowest expression levels (~1) were related to healthy controls. Furthermore, in female patients, it decreased slightly during disease progression (3.6- and 3.1-fold in RRMS and SPMS patients, respectively). The highest considerable variation was observed in the RRMS patients between the male MS patients and the healthy control group.

Pasha

The average transcript abundance of *Pasha* for both RRMS and SPMS, was quickly up-regulated (RRMS patients; 5.373- and 7.681-fold, respectively) contrasted to the control group (Table 2). Regarding female patients, the highest ratio of *Pasha* expression level was obtained (up to 5.7-, and 7.9-fold change in RRMS and SPMS patients, respectively), while for male patients the lower transcript amounts of *Pasha* were obtained in RRMS and SPMS patients (5.1- and 7.1-fold, respectively) (Fig. 1b). In fact, regarding the two types of MS progression courses under study, secondary progressive was considered as the course that exhibited the maximum significant differences with the healthy control.

Dicer

On the subject of male and female patients, it was showed that transcript amounts of *Dicer* gene were negatively

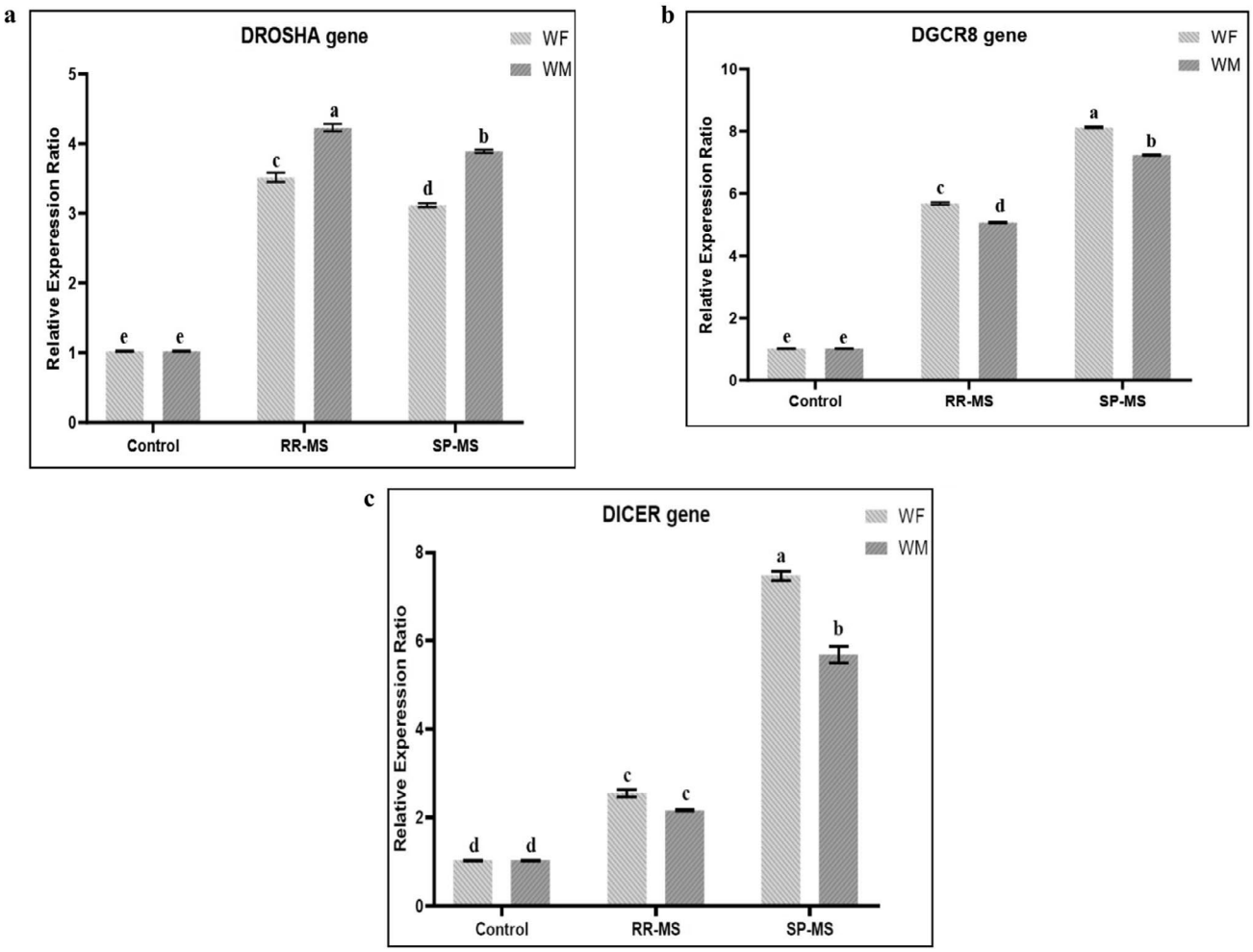

Fig. 1 Relative expression levels of the *Drosha* (a), *Pasha (DGCR8)* (b), and *Dicer* (c) genes in patients with RRMS and SPMS vs. healthy controls. Statistically significant differences at $p \leq 0.05$ (Duncan's multiple range test) represent by different letters (a, b, c) above the bars. WF = female patient, WM = male patient

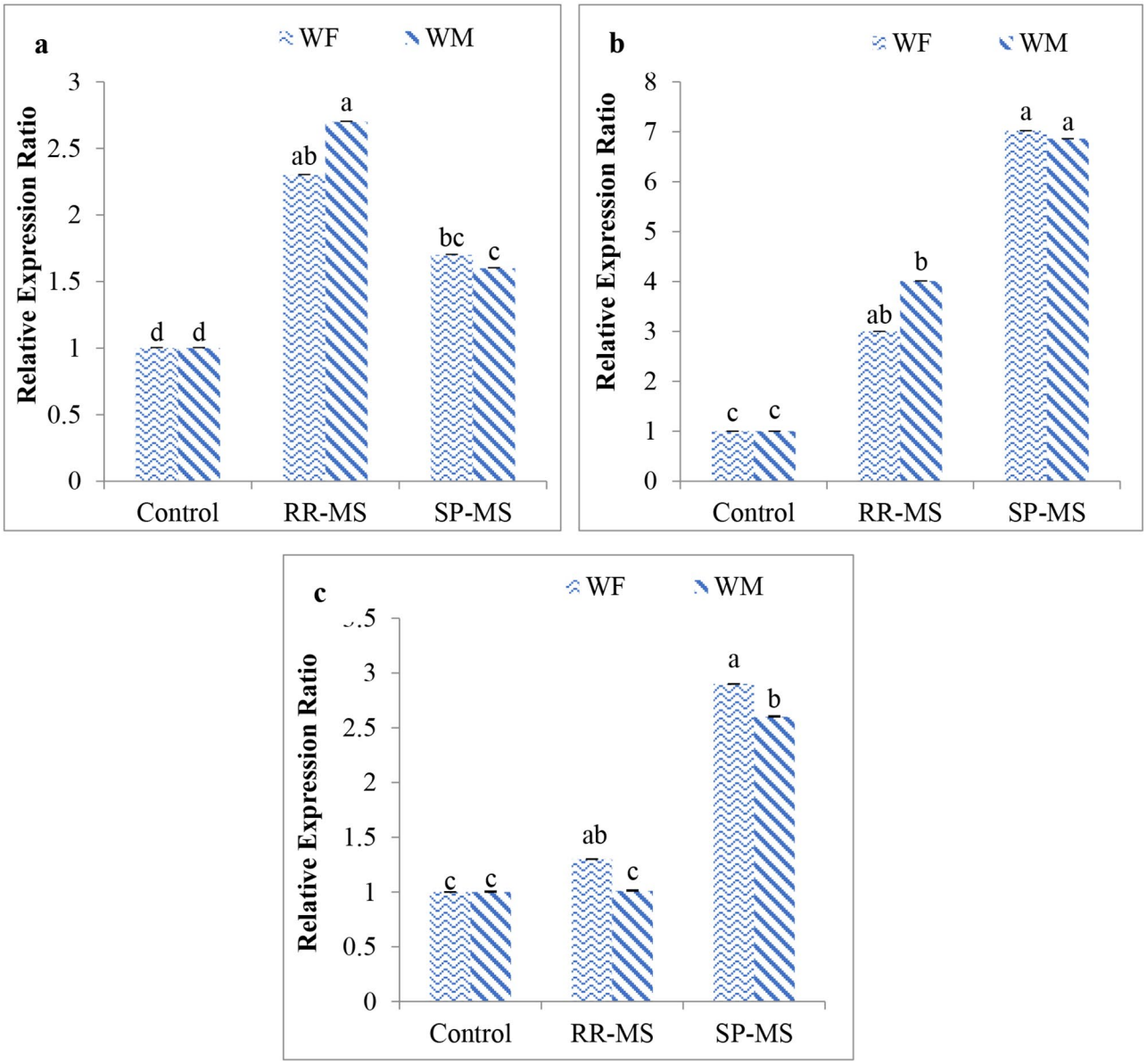

Fig. 2 The transcription activity of *INF-α* (a), *INF-β* (b), and *IL-6* (c) genes in patients with RRMS and SPMS vs. healthy controls. Statistically significant differences at $p \leq 0.05$ (Duncan's multiple range test) represent by different letters above the bars. WF = female patient, WM = male patient

disease progression-dependent. Comparing male and female patients with the healthy control, the lowest transcription activity of *Dicer* was detected for RRMS (2.1- and 2.5-fold change, respectively), and increased sharply for SPMS patients (5.9- and 7.8-fold change, respectively) (Fig. 1c). In addition to any transcription activity of the two other genes in the healthy control group, no detectable transcripts were seen for the *Dicer* gene. The highest *Dicer* transcription activity was detected in SPMS patients, in which females showed the most significant difference with the control group.

MS progression-specific regulation of pro- and anti-inflammatory cytokines genes

The main characteristic of the active period of the MS disease is manifested by the mutual effects of pro-inflammatory and anti-inflammatory cytokines over time with the progress of the disease in the patients. Cytokines are essential for immune response activity and perform an essential function in establishing and maintaining inflammation in the cell. The transcription activity of pro- and anti-inflammatory cytokines genes e.g., *INF-α*, *INF-β*, and *IL-6* by quantitative RT-PCR was performed to investigate the inhibiting

Table 2 The average relative expression levels of *Drosha, Pasha (DGCR8), Dicer*, *INF-α*, *INF-β*, and *IL-6* genes in PBMC cells of patients with relapsing-remitting (RR) and secondary progressive (SP) multiple sclerosis

	Fold change RR-MS vs. HS	Fold change SP-MS vs. HS	p-value
Drosha	3.872	3.502	< 0.0001
Pasha (DGCR8)	5.374	7.681	< 0.0001
Dicer	2.353	6.580	< 0.0001
INF-α	1.738	2.535	< 0.0001
INF-β	3.516	7.01	< 0.0001
IL-6			

HS = healthy subjects, RRMS = relapse-remitting multiple sclerosis, SPMS = Secondary-progressive multiple sclerosis, vs. = versus

and directing function of the cytokines in the progress of MS disease.

INF-α

As it is shown in Fig. 2a, the average transcript abundance of the *INF-α* gene ascertained those male and female patients had the maximum levels of down-regulation at the secondary progressive stage. For both male and female patients, the expression levels of *INF-α* boosted at the start of MS disease (RRMS) (2.7 and 2.3-fold, respectively), and gradually decreased in secondary progressive MS patients (1.6- and 1.7-fold, respectively). As a whole, no significant difference was revealed in the expression ratio of *INF-α* between the male and female RRMS patients, and the highest difference was observed between the male RRMS patients and healthy control group indicating that the relapsing-remitting stage could not be considered as the significant progressive stage.

INF-β

Regarding RRMS patients, either male or female' patients, surprisingly considerable transcript amounts (6.86- and 7.02-fold change) were observed, but for SPMS patients a significant decrease was detected as compared to healthy controls (Fig. 2b): In female patients, the transcript level of *INF-β* was rapidly increased during relapsing-remitting (up to 7.02-fold change) and declined sharply upon the secondary progressive stage (~ 3.0-fold change). The same as male patients, after the progression of the disease, the transcription activity of *INF-β* decreased under the secondary progressive stage in females (4.01-fold change), but higher quantities were detected at the relapsing-remitting stage (6.86-fold). The highest significant difference in *INF-β* transcription activity was detected between RRMS patients and the healthy control group, indicating the importance of its inhibitory function in the progression of MS.

IL-6

Under the progression of MS, an increasing trend occurred for transcript levels of the *IL-6* gene (2.9-fold) for the female patients at the secondary progressive stage. In the following, higher transcription activity was observed for male SPMS patients (2.6-fold), and the minimum expression level was detected for male RRMS patients (1.01-fold change) (Fig. 2c). Overall, the following results can be drawn as follows: "The greater the progression of disease, the higher amounts of the *IL-6* transcript become, and vice versa". Regulation of the *IL-6* gene in MS patients is probably an MS progressive- as well as gender-dependent mechanism.

Discussion

Evaluating the function of microRNAs in the progression of MS in patients is a novel research field. An ameliorated science in examining miRNAs as biomarkers in MS provides a tool to recognize pathological conditions and a new therapeutic objective to identify relapsing and remitting phases of patients with MS [12]. The research by Wang and Liang [10], has shown that the dysregulation of miRNA may be an important key in disrupting immune homeostasis and initiating the induction of main subsets of different immune cell types e.g., Th1, Th17, Treg, and CD^{8+} lymphocytes that contribute the autoimmune pathogenesis process of MS.

Although the existence of miRNA in blood cells has been recognized as a criterion in various human disorders like cancer, heart disease, and brain injury, circulating miRNA in several biological fluids to regulate intercellular gene expression is also detected as a clinical biomarker [22]. Important features of miRNA in the blood e.g., stability against circulating RNase, various methods to storage, freeze-melt, the most known lipid-based formulation, and maximum pH required for packaging make them the best possible candidates to quantify for a new category of non-invasive and sensitive biomarkers [23–25]. Although, additional information is still evolving and the few practical kinds of research performed to date have used diverse biological samples for miRNA expression analysis.

Several studies have documented the investigation of the transcript abundance of various miRNAs in healthy individuals and patients with MS, indicating that the expression profile of miRNA in patients shown significant changes compared to the control group. Although some contradictory observation demonstrated the loss of regulation of the transcript accumulation of miRNA in many patients. This can be somewhat correlated with differences in patient samples or miRNA transcription activity quantification methods, which

are usually performed either as qRT-PCR analysis or microarray [26–30].

Previous results have been reported that miRNAs, miR-25 and miR-106b, are downregulated in Treg cells in patients with MS by changing biological tasks of transforming growth factor β (TGF-β) [31]. In another study using next-generation sequencing (NGS), microarray analysis, and qRT-PCR, the expression pattern analysis of miRNAs was performed in whole blood samples from RRMS patients (n = 25) and healthy control (n = 50). RNA Seq and microarray analysis exhibited that the expression of two miRNAs (miR-20a-5p and miR-7-1-3p) significantly decreased and miR-16-2-3p was upregulated [32]. In addition, previous studies confirmed considerably the lowest expression levels of miR-20a and miR-17 in the progression of MS, indicating a significant role in the activation of immunoregulatory cells and upregulation of several genes in whole blood RNA of patients with MS [33].

So far, more miRNAs have been predicted and their transcription activities have been investigated in different progressive courses of MS disease. However, in two important courses of this disease, RRMS and SPMS, there is little detailed data describing transcript amounts of genes encoding the important miRNA machinery components in MS patients. The critical enzymes in the miRNA biogenesis machinery inside the nucleus e.g., RNase III enzyme (Dicer), and Drosha cleavage and transport *pre-miRNA* into the cytoplasm. Furthermore, a double-stranded RNA binding protein (dsRBP) (Pahsa) with the potential to determine cleavage sites on the pri-miRNA is another important factor in the miRNA synthesis. In addition, the processing of appropriate pri-miRNAs are affected by other factors, including argonaute-1 (AGO1), argonaute-2 (AGO2), as well as double-stranded RNA-binding proteins PACT, TARBP1, and TARBP2 [34, 35]. Because of the importance of these essential elements in the processing of miRNA maturation, regarding the effect of *Drosha*, *Pasha*, and *Dicer* on regulating the pathogenesis of MS, the RNA levels of these genes were evaluated during the RR and SP stages in comparison to healthy controls.

Consequently, for both male and female patients, compared to the healthy control, the highest transcription activity of *Drosha* was observed for RRMS patients (4.2-and 3.6-fold change, respectively), and decreased to its minimum transcript for patients with SPMS (3.9- and 3.1-fold change, respectively). Jafari et al. 2013 displayed that Drosha is believed to be an important element that is correlated with power prognostic factors in the human gastrointestinal cancer cells, describing the role of probable for the various transcriptional levels of Drosha in this tumor and the correlation with pathological features [36].

DGCR8 (*Pasha*) is an Mg^{2+}-dependent endonuclease that is involved in miRNA biogenesis, ribosomal RNA processing, and viral defense [35]. In our study, the means of transcript amounts of *Pasha* up-regulated 5.373-and 7.681-fold in RRMS and SPMS patients versus controls. Regarding male and female RRMS patients, the transcription activity of *Dicer* was 2.1 and 2.5 times higher than the healthy controls, while the transcript abundance of *Dicer* was significantly increased in male and female SPMS patients (5.9- and 7.8-fold, respectively). Changes in miRNA machinery components are progressively identified to play an essential duty in regulating pathogenic genes involved in the developmental stages of MS pathogenesis. These alterations, though dysregulation of miRNAs may occur, are reasoning to clarify unusual miRNA expression levels in various neurological and autoimmune diseases [11]. Consequently, it has been reported that dysregulation of miRNAs might cause disease in two different ways as follows: changes in their transcription activity and the mutations in the binding sites of the corresponding mRNA. Essential stages of miRNA synthesis are crucial in performing the correct function of the immune system, as the removal of miRNA processing mediated by Dicer in Treg cells in mice leads to a lethal autoimmune syndrome [37, 38]. Also, Dicer and Drosha were observed to have low transcription amounts in tumors, which is incoherent to a poor clinical outcome [39, 40]. Also, it has been documented that miRNA levels have important functions in chronic, active, inactive, mature oligodendrocytes, and demyelinated tissue lesions. Moreover, other previous research showed the removal of Dicer and Drosha enzymes in the miRNA synthesis machinery can potentially cause failure of T-cell function and autoimmune diseases [41]. The significance of miRNAs as central regulators in generating new myelin sheaths is discovered by using transgenic models with knockdown of crucial enzymes of miRNAs biogenesis machinery e.g., Dicer. It was shown that Dicer knockdown of oligodendroglia in mice promotes the inhibition of normal development myelination, leading to several damages e.g., oxidative, inflammatory astrocytosis, and microgliosis in the brain. Another study showed Dicer was downregulated in B lymphocyte cells and correlated with transcript abundance of CD80 T lymphocytes, contributing to the dysregulation of the normal MS immune response [42].

Also, the qRT-PCR technique has been utilized to acquire a correlation between the expression levels of important genes involved in the miRNAs biogenesis machinery and consequently changes in the pro-and anti-inflammatory cytokines ratios of *INFα*, *INFβ*, and *IL-6* of RR-MS and SP-MS patients as compared to healthy control. Our results showed a marked increase in amounts of pro-inflammatory (*IL-6*) and lower transcription activity of anti-inflammatory

(*INF-α* and *INF-β*) transcripts in the PBMC of patients with the progression of MS as compared to healthy controls. We found that *INF-α* and *INF-β* cytokines were significantly different between patients with two types of progression (RR, and SP-MS patients) and healthy control. The increase in the expression ratio of *IL-6* in SPMS patients and the decrease in transcription activity of *INF-α*, and *INF-β* cytokines are consistent with disease progression because the expression balance of those seems to be pivotal to the regulation of this process. These findings are in agree with previous findings describing the up-regulation of pro-inflammatory cytokines as exclusively occurring during MS secondary progressive, whereas the increase of anti-inflammatory cytokine levels is believed to occur during relapsing-remitting in patients with RR-MS [43]. Therefore, our results from this research exhibit the affirmation of the hypothesis describing an inconsistency in the transcript amounts of pro-and anti-inflammatory underlying the progression of MS from RRMS to SPMS. Our findings certainly affirm the previous research exhibiting that those pro-inflammatory cytokines are increased while the PBMC of SPMS shows a decline in the level of cytokines with the function of anti-inflammatory [44]. Similarly, it was previously shown that significantly lower expression of TNF-β evaluated in RRMS patients than in SPMS patients, suggesting TNF-β can activate the immune system and some unknown mechanism to regulate amelioration of MS. They found considerable expression levels of numerous pro-inflammatory cytokines including IFN-α, IL-2, IL-6, IL-1β, and TNF-α may promote and exacerbate lesions. IL-6 as a cytokine has several functions by modifying numerous cells' external and internal surfaces of the CNS [45]. Furthermore, it has been shown the significant role of IL-6 on autoreactive effector T cells (Teffs) from MS patients. In patients with active relapsing-remitting MS courses, IL-6 signaling was believed to be important in supporting the resistance to regulate by regulatory T cells (Tregs) effector cells, which may be involved in the disease progression. Another study exhibited the role of IL-6 in both EAE and MS pathogenesis, thereby its function in the lymphoid tissues. Although there are few reports about limited IL-6 actions in CNS of EAE and MS. Lately, some ordinary recovery of EAE symptomatology and histopathology as clinical features have been demonstrated in the mice with Astrocyte-specific deficiency of interleukin-6 (Ast-IL6 KO) [46]. Also, it has been reported that IL-6 was mostly traceable in the CSF samples in patients with MS as compared to samples of healthy control, in which considerable discrepancy appeared between patients' different subtypes of MS e.g., relapsing remitting (RR), secondary progressive (SP) and primary progressive (PP) as compared to healthy individuals. However, studies showed IL-6 was expressed in equal amounts in the CSF samples from all types of MS patients. IL-6 activity in RRMS patients, among other biomarkers, was found to refer to clinical and radiological features of the disease [47].

Moreover, our results based on gender differences showed a considerable discrepancy in the transcript abundance of *INF-α*, *INF-β*, and *IL-6* encoding cytokines between two genders of patients with MS. However, the expression level of INF-β was only notably upregulated in female patients with RR-MS (Fig. 2). Previous studies showed IFN-β is the first utilized strategy in MS treatment, whereas IFN-α is an approved treatment to clinical use for virus infections and cancer. Although the information about its therapeutic mechanism is still unclear, the former document exhibited that IFN-β induces the low expression level of T-cells in the antigen presentation by changing their expression at the protein level. Also, studies indicated that IFN-β is effective in the T cell differentiation from a Th1 cell as a pro-inflammatory response and towards a Th2 response (anti-inflammatory) [48]. Hence, IFN-β could downregulate the expression level of IL-12 and elevate the transcription activity of anti-inflammatory cytokines like IL-27 by macrophages. Most finding on the role of the *IFN* genes in MS pathogenesis has recognized them principally as biomarkers in PBMC of patients with MS that assessed before and after IFN-β therapy. Restoration of immune dysfunction in MS patients was reported by IFN-β treatment to a degree, but not completely [49]. In patients with MS, various strategies with therapeutic targets of memory cells like B-cell depletion therapy, considerably diminish the disease. It seems the foundation of this activity becomes manifest to be associated with the down-regulation of pro-inflammatory cytokines or increased potential of these cells for antigen presentation. Mainly, it has been reported that a significant decrease of cells that form part of the adaptive immune system (memory cell) in PBMC of MS patients by a prerequisite mechanism involved in FAS-R-mediated caspase-3-dependent apoptosis that occurred in IFN-β therapy. However, it was previously shown that immune mechanisms in autoimmune diseases especially in patients with MS reported are resistant to different IFN therapy strategies [50]. Systemic studies of the efficacy of IFN-β therapy have reported low MS progression, a decrease in relapse rate, and the amounts of lesions in CNS. Although its regulatory mechanism involved is not fully understood, previous research results have pointed to the regulatory function of IFN-β in signaling and CNS inflammatory responses [51].

It has been reported that IFN-β can be an effective factor in inducing IFN-α and also has extra straightforward effects on target cells. There are numerous reports on the efficiency of IFN-β, as an approved therapeutic method, that could reduce MS progression by modifying the course of MS. Numerous types of research indicated the specificity

of IFN-α in treatment that increased perception in MS and could be used as a neuroprotective agent and the possibility increased the fertility in women. IFN-β therapy could initiate the IFN signaling pathway and increase signals of subnormal type I IFN in patients with MS. Although, clinical benefits of IFN-β therapy are promoted by the therapeutic effects of other common drugs and vitamins [44].

Numerous types of research have measured the role of different types of MS treatment methods by regulating the function of miRNAs, not only to recognize a biomarker for assessing the therapeutic responses but also to comprehend the mechanism of treatment method at the molecular level. Waschbisch et al. (2011) first reported the transcription activity of five candidate miRNAs in PBMC from patients with RRMS, IFN-β treated patients, and patients who used glatiramer acetate. The results from this study showed no significant changes in the transcript abundance of selective five miRNAs between untreated and IFN-β treated patients [52].

In conclusion, our finding exhibit that the up-regulation of *Drosha*, *Pasha*, and *Dicer* may contribute to the regulation of the pathogenesis process via the control of biogenesis of miRNAs underlying the progression of the MS diseases.

It is necessary to conduct more research on the microRNA biogenesis machinery and the expression levels of predicted miRNAs in the blood and other body fluids of RR and SPMS patients. Also, the correlation of higher levels of the major miRNA's machinery biogenesis, increased proinflammatory cytokines and downregulation of anti-inflammatory cytokines may perform a significant function in determining the severity of MS disease and the pathogenesis of two important MS stages. Future research can contribute to in-depth study of the regulation of specific miRNAs focusing on differentiating patients with RRMS and SPMS from healthy controls, and the correlation between specific miRNAs and transcript amounts of genes regulated by pro- and anti-inflammatory in both RRMS and SPMS patients. It needs to be more investigated as diagnostic biomarkers and new approaches for MS treatment at the transcriptional and post-transcriptional levels in future studies.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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