

MicroRNAs (20a, 146a, 155, and 145) expressions in a sample of Iraqi patients with multiple sclerosis

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Abstract:

Background: Multiple sclerosis is a devastating central nervous system autoimmune disorder that is characterized by a series of inflammations, demyelinations, and neurodegenerations that affect the brain and spinal cord. The epigenetic studies specially micro Ribonucleic acid expression represent an important field of researches that probably uncover the obscurities behind the multiple sclerosis pathogenesis.

Objectives: to study the expression of micro Ribonucleic acids (20a, 146a, 155, and 145) in multiple sclerosis patients by the use of real time polymerase chain reaction.

Patients and Methods: A case-control study was performed using real time polymerase chain reaction technique to measure the relative expression of micro Ribonucleic acids (20a, 146a, 155, and 145) in peripheral blood leukocytes of 25 newly diagnosed untreated multiple sclerosis patients and comparing them with that of 25 clinically apparent healthy controls .

Results: Studying of micro Ribonucleic acids expression in multiple sclerosis patients revealed a significant down-regulation in micro Ribonucleic acid-20a while up- regulation of micro Ribonucleic acid-155 expression in multiple sclerosis patients in comparison to controls. Micro Ribonucleic acids -146a and 145 were not associated with significant changes in its expression in multiple sclerosis patients in comparison to controls.

Conclusion: multiple sclerosis is associated with significant changes in micro Ribonucleic acids expression including micro Ribonucleic acid-20a, and micro Ribonucleic acid-155 but not micro Ribonucleic acid 146a and-145 that can be measured by real time polymerase chain reaction technique.

Key words: MicroRNAs, Real time PCR, Gene expression, Multiple sclerosis.

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Introduction:

Multiple sclerosis is a devastating central nervous system autoimmune disorder that is characterized by a series of inflammation, demyelination, and neuro-degeneration which affect the brain and spinal cord (1-3). Today, many researches in multiple sclerosis disease have been directed toward the epigenetic studies which are related to the inheritable and potentially reversible DNA and chromatin changes that regulate the expression of genes without DNA sequence alteration (4). Anyhow, this work was conducted to study part of the epigenetic role in multiple sclerosis disease that are related to the microRNAs which recently have been proposed to be one of the important contributors in MS pathogenesis (5,6). MicroRNAs are small noncoding conserved RNAs present in many species, from viruses to humans (7). They are 18–24 nucleotides in length and formed from a precursor by an enzymatic procedure (8). The immune system cells,

like any other mammalian cell type, depend on miRNAs to regulate their development, proliferation, migration, and differentiation. Usually, these activities are harmonized by both ubiquitous expression and cell type-specific miRNA species (9,10). They have been determined as regulatory molecules just about 20 years ago (11). They regulate the gene expression at the posttranscriptional level by degradation or repression of their target mRNA (12). The emerging roles of miRNAs in modulating immune responses make it possible that dysregulation in their expression may participate in the pathogenesis of autoimmune diseases (13). Furthermore, the study of miRNAs expression profile have demonstrated that their dysregulation in different cellular compartments of immune system can affect pathogenesis of MS (14). This work included the study of some microRNAs which were microRNA-20a, microRNA-146a, microRNA-155, and microRNA-145.

Method

A case control study was performed using two steps reverse transcription polymerase chain reaction technique to study the

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gene expression of some miRNAs including miR-20a, miR-146a, miR-155, and miR-145 in patients with MS disease. The study included 25 patients who were newly diagnosed as having MS disease and before receiving any steroid treatment or immunomodulatory therapy who attend Multiple Sclerosis Clinic, or are admitted to the Neuromedical ward in Baghdad Teaching Hospital, and Special Nursing Home in Baghdad Medical City as well as from the Neuromedical Consultancy Unit in Al-Yarmouk Teaching Hospital in Baghdad city. Multiple sclerosis diagnosis was achieved according to the 2010 McDonald criteria (15) by the neurologist senior depending on clinical history, physical examination, brain and spinal cord MRI, Visual evoked potential if required, and laboratory tests as necessary to exclude other demyelinating diseases and other possible MS differential diagnosis. The patients were asked for their permission to give blood samples and signed an informed consent for their agreement to participate in the research project. Data information for each patient were collected including clinical history, Physical signs, Laboratory tests, and MRI findings. Regarding controls, they included 25 apparently healthy medical staff with no history of having any autoimmune disease or receiving any steroid or immunomodulatory therapy.

All the studied blood samples of the patients and controls were collected, tested, and analyzed during the period from 1st of April 2015 to 31st of March 2016. The samples included 2 mls of blood which were collected from the participant under aseptic technique, and were evacuated into K3-EDTA blood tube (Human, Germany) to be followed with their suitable processing for measuring their relative genes expression. All samples were collected in time period between 9am–12am for both the MS patients and healthy controls in order to avoid measuring the effects of diurnal variation on immune function.

A molecular experiment was conducted to find the genes expression of the peripheral white blood cells for both the patients and controls. This included the gene expression of four types of miRNAs (20a, 146a, 155, and 145). The expression of all the above genes in each individual was normalized to the expression of one of his/ her reference genes which was RNU6-2 gene that was also obtained from their peripheral white blood cells. After finding the gene expression of each target and for each participant and normalizing it with their own reference genes, they were all compared to the normalized target gene expression of a single calibrator (that was considered to be equal to 1) in order to find the final fold change in gene expression for each target. In details, this experiment included the following steps:

Leukocytes separation by the use of erythrocyte lysis Buffer (Qiagen, Germany) according to the manufacturer protocol

(16).

Purification of Total RNA, Including miRNAs which was performed by the use of miRNeasy Mini Kit (Qiagen, Germany). This Kit combines phenol/guanidine based lysis of samples and silicamembrane-based purification of total RNA. These steps were performed according to the manufacturer's protocol (16).

Assessment of the extracted RNA purity and concentration that were automatically measured by micro-volume UV spectrophotometer. The purity was measured for each sample depending on the absorbance at 260nm (A_{260}) and 280nm (A_{280}) ratio (A_{260}/A_{280}). The sample which had RNA purity of 1.9 - 2.1 was accepted and included in the study (17).

Two-step- RT-PCR reaction: Total RNA containing miRNA was the starting material in RT-PCR reaction which was performed in two steps.

Step 1- RNA Reverse Transcription: Measuring the expression of target genes by qPCR requires the conversion of RNA into cDNA through the process of reverse transcription that was done by the use of miScript II RT Kit (Qiagen, Germany). All RNA species were converted into cDNA, where polyadenylation of mature miRNAs was done by poly(A) polymerase, and by the use of oligo-dT primers they were reverse transcribed into cDNA. The oligo-dT primers carry a universal tag sequence on the 5' end to allow amplification of mature miRNA in the step of real-time PCR. Polyadenylation and reverse transcription were performed in parallel in the same tube. All the steps were conducted according to manufacturer instructions (17).

Step 2- Real-time PCR: Following the conversion of RNA into cDNA, real time PCR reaction was conducted on the cDNA template by the usage of SYBR Green reporter to detect the amplified target. For detection of mature miRNA, cDNA prepared in a reverse transcription reaction serves as a template for real-time PCR analysis using an miRNA-specific miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit which contains the miScript Universal Primer (act as reverse primer) and QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany). The reaction setup was prepared according to the manufacturer's recommendations (17). At the end of qPCR reaction, analysis of data was performed. Mx3000P software associated with the Stratagene Agilent real time PCR machine was used to find C_T values and to demonstrate the amplification plots as well as the dissociation curves for each reaction. Once data capture completed, expression analysis was performed by the relative quantification.

Statistical analysis: The results were analyzed by the use of Statistical-Package for the Social Sciences (version 23.0 software), and the Microsoft-Office Excel software, 2007. Independent T- test, Mann-Whitney U test, were used to

calculate the significance of the differences. P value less than 0.05 was considered to be a statistically significant.

Results:

From a total of 25 patients with MS, 23 (92%) were females and 2 (8%) were males with a female to male ratio (F:M) of 11.5:1. The ages of these patients ranged between 18 - 55 years. the mean age was 33.32 years with a standard deviation of ± 9.45 years. The controls group was selected to be sex and age matched with the patients group, where they also consisted of 23 (92%) females and 2 (8%) males with a female to male ratio (F:M) of 11.5:1. The mean age of the controls group was 33.84 years with a standard deviation of ± 9.05 years, and

range of 19-54 years. Regarding the type of multiple sclerosis, there was only two type of patients. Relapsing remitting MS patients comprised 23 (92%) individual, and the remaining 2 (8%) patients were of primary progressive MS type. In this study, the mean of the Log fold change values for miR-20a gene expression in cases with multiple sclerosis (-0.924) was lower than that of the controls (-0.337). The observed difference was statistically significant where the p-value was less than 0.05. This indicates that the miR-20a gene expression in the patients were significantly down-regulated in comparison to the control. (Figure 1). Effect size for the means difference in miR-20a gene expression between patients and controls was also tested, where Cohen's d was (-0.54).

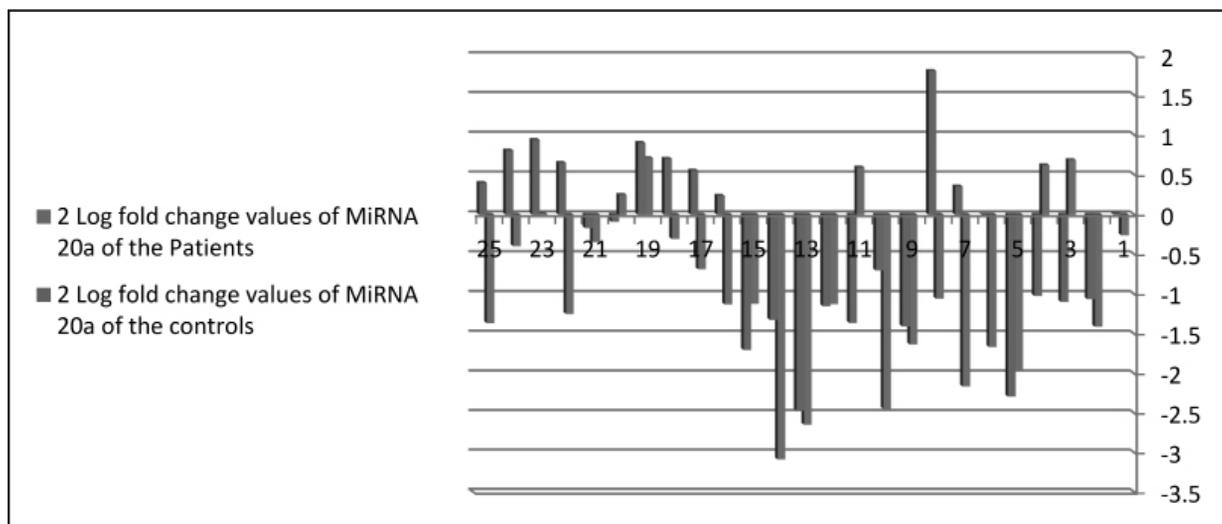


Figure 1: MiRNA 20a gene expressions in patients and controls

This figure shows miR-20a gene expressions in patients and controls, where the Bars which are above the zero line refer to the genes up-regulation, while those below it represent genes down-regulation. The blue bars are for patients, while the red bars are for controls. For miR-146a, the mean of the Log fold change values of the gene expression showed no statistically significant difference between patients (-0.789) and controls (-0.751), where the p-value was more than 0.05. (figure 2).

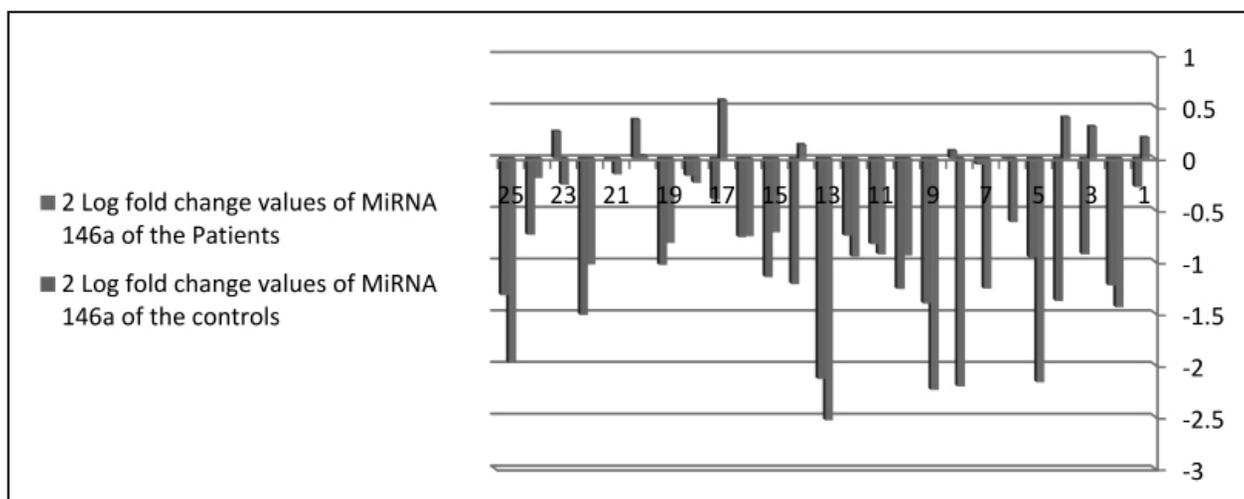


Figure 2: MiRNA146a gene expressions in patients and controls

This figure shows miR-146a gene expressions in patients and controls, where the Bars which are above the zero line refer to the genes up-regulation, while those below it represent genes down-regulation. The blue bars are for patients, while the red bars are for controls.

The Log fold change values for miR-155 gene expression in MS patients (-0.272) was higher than that of controls (-1.029). The observed difference was statistically significant where the p-value was less than 0.05. This indicates that the miR-155 gene expressions in the patients were significantly up-regulated in comparison to the controls. (figure 3). Effect size for the means difference in miR-155 gene expression between patients and controls was also tested, where Cohen's d was (0.67).

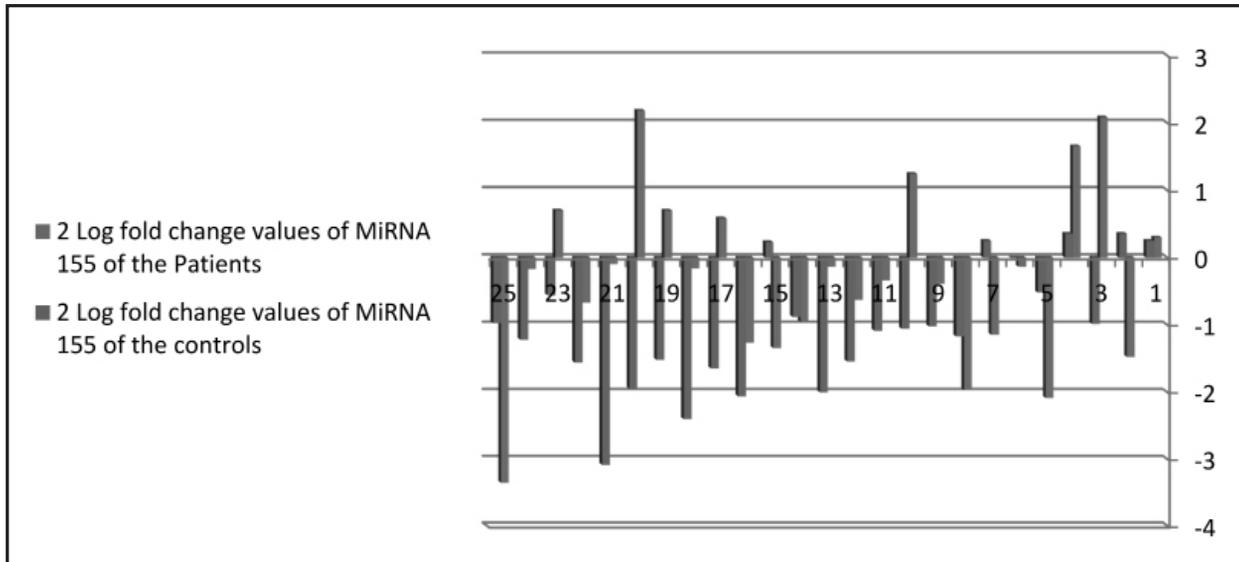


Figure 3: MiR-155 gene expressions in patients and controls

This figure shows miR-155 gene expressions in patients and controls, where the Bars which are above the zero line refer to the genes up-regulation, while those below it represent genes down-regulation. The blue bars are for patients, while the red bars are for controls.

For miR-145, the mean of the Log fold change values of gene expression showed no statistically significant difference between patients (-0.477) and controls (-0.724), where the p-value was more than 0.05. (figure 4).

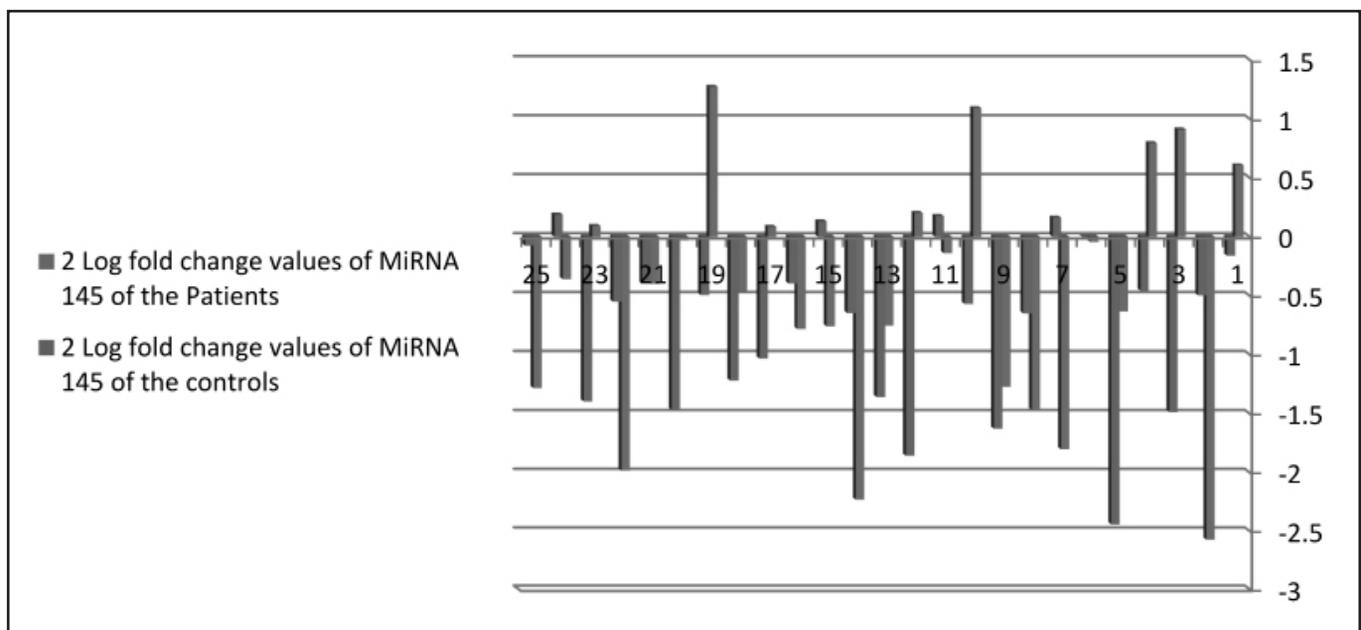


Figure 4: MiR-145 gene expressions in patients and controls

This figure shows miR-145 gene expressions in patients and controls, where the Bars which are above the zero line refer to the genes up-regulation, while those below it represent genes down-regulation. The blue bars are for patients, while the red bars are for controls.

The means rank of the Log fold change values of miRNAs (20a, 146a, 155, and 145) genes expression for the female patients were higher than that of male patients but they failed to reach the statistical significance, where their P-values were more than 0.05. In addition, there was no statistically significant differences in the means rank of the Log fold change values of miRNAs (20a, 146a, 155, and 145) genes expression between the RRMS and PPMS where their P-values were more than 0.05.

Discussion

The female to male ratio (F:M) was 11.5:1. This higher percent of MS in female in comparison to male was consistent with the reports of other studies (18,19) who found also a higher prevalence of the disease in females. But, female to male ratio (F:M) which is 11.5:1 was somewhat higher than the reported ratio by other studies which reported range of F:M ratios of (2.3–3.5:1) (18,19,20,21). Such differences in the ratio could be due to the selected criteria for the included patients in this study, where only the newly diagnosed cases with no previous steroid treatment were included, thus it does not reflect the exact F:M ratio for the disease. However, Many factors may play a role in the difference in sex prevalence including the genetic differences, the effect of gonadal hormones, differences in modern lifestyle and environmental exposures in men and women (22,23).

MicroRNA-20a gene expression was significantly down-regulated in MS patients in comparison to controls. This was consistent with other studies which also reported down-regulation of this miRNA in MS patients in contrast to controls (24,25). Where, Cox et al. (24) investigated the miR-20a gene expression in peripheral blood samples of 59 MS patients before receiving treatment, and of 37 controls. The results were obtained by the use of miRNA microarray analysis which later on were confirmed by the use of RT-PCR. However, miR-20a was significantly down-regulated in MS patients in comparison to controls. Regarding Keller et al. (25), they examined miR-20a gene expression in the whole blood sample of 25 treatment-naïve RRMS patients and 50 healthy controls by the use of next generation sequencing, microarray analysis, and qRT-PCR. They also reported a significant down-regulation of miR-20a gene expression in MS patients.

MiR-20a was suggested to be a regulator of genes that are involved in T-cell activation which were up-regulated in the blood of MS patients (24). Moreover, another study documented that overexpression of miR-20a decreases the production IL-2,

IL-6 and IL8, which are considered as essential regulators of inflammatory responses (26). Thus, miR-20a down-regulation may have a role in MS pathogenesis which supports the idea that miR-20a or its analogue are supposed to be a useful target to provide a new therapeutic approach (24). However, This significant difference between patients and controls in miR-20a gene expression with a medium effect size (Cohen's $d = -0.54$) make it possibly a biological marker for the diagnosis of MS with diagnostic accuracy/AUC of (0.65), sensitivity of (60%), and specificity of (56%) at cut off point of (≤ -0.54) fold change.

Regarding miR-146a, there was no statistically significant difference in its expression between MS patients and controls. On the contrary, another study revealed an increase in miR-146a expression in MS patients in comparison to controls (27). This discrepancy in findings could be attributed to the relatively short half-life of miR-146a which is about 1.5–2 h in the CNS and tissues cells of human (28). The time of sample collection was different from patient to patient (may reach up to two months) since the last attack. Such delay in sample collection could be responsible for the decrease in the expression of the up-regulated miR-146a.

For miR-155, there was a statistically significant difference in its expression between MS patients and controls, where it was up-regulated in patients in comparison to controls. This finding was in accordance with other studies which also documented up-regulation of miR-155 gene in MS patients (29,30,31). MiR-155 may exert its action through promoting myelin phagocytosis mediated by macrophages through down-regulation of brain resident cells CD47 by targeting its 3'-UTR (32). Furthermore, miR-155 induces IFN- γ -producing Th1 and Th17 development which are responsible for the MS inflammatory degeneration (33). This significant up-regulation in miR-155 in MS patients in comparison to controls with medium effect size (Cohen's $d = 0.67$) make it an appropriate biological marker for diagnosis of MS with diagnostic accuracy AUC of (0.68), sensitivity (72%), and specificity (64%) at cut off point (≥ -0.96) fold change.

Regarding miR-145, in this work there was no statistically significant difference in its expression between MS patients and controls, while other studies reported a dramatic increase in miR-145 expression in MS patients (34,35). A reasonable explanation for such discrepancy is not found. According to the finding in this work, miR-145 is not considered a suitable biological marker for MS diagnosis.

It is notable that the majority of autoimmune diseases predominate in females. Such autoimmune disease sex bias is complex and possibly involves sex hormones, sex chromosomes, and epigenetic regulation of genes, mainly by miRNAs (36,37). However, very few studies are available to discuss the role of miRNAs in sex biased diseases (38).

In this work, there were no statistically significant differences in miRNAs (20a, 146a, 155, and 145) gene expression in MS patients according to sex. Similar studies with such classification to be compared with, were not found except one study which also documented an absence of a significant difference in miR-146a expression according to sex in MS patients (14). This indicates that these genes are unlikely to be responsible for the sex bias in the MS disease.

This study included two types of MS, the RRMS and PPMS. MiRNAs (20a, 146a, 155, and 145) did not show any significant differences in their expressions between these two types. Another study with such finding was not available except one study which also documented an absence of a significant difference in miR-146a expression in different MS types (14). These findings indicate that these genes are probably not associated with the bias toward one of these two types, RRMS or PPMS.

Authors' Contributions:

Zainab A. Aljawadi: Study conception, design, samples processing, data analysis and interpretation, writing the research.

Aida R. Al-Derzi : First supervisor, critical revision

Ban A. Abdul-Majeed : Second supervisor, critical revision

Akram M. Almahdawi: Consultant neurologist provided the clinical cases of multiple sclerosis patients

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