Role of Plasma MicroRNAs 145 and 484 in Diagnosis of Multiple Sclerosis, Disease Activity and the Transition from Relapsing Remitting Multiple Sclerosis to Secondary Progressive Multiple Sclerosis

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Abstract

Background: There is a growing need for biomarkers that can help in early diagnosis of multiple sclerosis (MS) and in recognizing patients with MS activity. Moreover, many studies are recently focusing on biomarkers that may help in diagnosis of the transition from relapsing remitting multiple sclerosis (RRMS) to secondary progressive multiple sclerosis (SPMS). Circulating microRNAs (miRNAs) are now considered promising biomarkers.

Objectives: Studying the role of plasma miRNA-145 and miRNA-484 in the diagnosis of MS, disease activity and in diagnosing the transition from RRMS to SPMS.

Patients and Methods: Forty-six subjects of both sexes were included, 31 patients with MS (21 with RRMS, 8 with SPMS and Two patients with primary progressive multiple sclerosis (PPMS)) and 15 healthy controls. Expression analysis of plasma miRNAs; miR-145 and miR-484 were assessed by real-time quantitative polymerase chain reaction (PCR) after miRNA extraction.

Results: MicroRNAs 145 and 484 could significantly discriminate between MS cases and controls, with best cut-off values > 0.6 and > 1.7 respectively. They could also significantly discriminate between active and inactive MS cases, with best cut-off values > 0.8 and > 2 respectively. Plasma miRNA-145 could discriminate between RRMS and SPMS cases, with best cut-off value ≤1.4.

Conclusion: Plasma miRNAs 145 and 484 might be used as promising biomarkers for early diagnosis of MS and in diagnosis of disease activity. Plasma miRNA-145 could be also helpful in diagnosis of the transition from RRMS to SPMS.

Keywords: miRNA-145; miRNA-484; MS Diagnosis; Activity; Transition.

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Introduction

Multiple Sclerosis (MS) is a chronic inflammatory central nervous system autoimmune disease affecting approximately 2.8 million people worldwide. The global prevalence of MS was 35.9 per 100,000 people according to data recorded between September 2019 and March 2020 from 115 countries representing nearly 87% of the world's population **(Walton et al., 2020).** The exact etiology of MS is still unknown; it involves an interaction between epigenetic, genetic and environmental factors **(Olsson et al., 2016).** There is a growing need for biomarkers that can help in early diagnosis of MS and in recognizing patients with MS activity, for early appropriate treatment **(Vistbakka et al., 2022).** Moreover, the diagnosis of conversion from relapsing remitting multiple sclerosis (RRMS) to secondary progressive multiple sclerosis (SPMS) is usually based only on retrospective clinical and radiological evaluation **(Inojosa and Ziemssen, 2021).** Progression of disability - as measured by the Expanded Disability Status Scale (EDSS) - over 1 year is required for the diagnosis of SPMS **(Lublin et al., 2014).** However, EDSS has disadvantages of being mainly dependent on ambulation and lower limb function with high inter-rater variability and low sensitivity **(Ontaneda et al., 2017).** So, studies are currently focusing on imaging and biochemical biomarkers that may help in early diagnosis of the transition from RRMS to SPMS **(Inojosa and Ziemssen, 2021).** Circulating microRNAs (miRNAs) are now considered promising biomarkers in patients with MS due to their high stability and easy detection in serum, plasma, or cerebrospinal fluid (CSF) **(Piket et al., 2019).** In this study we aimed at studying the role of plasma miRNA-145 and miRNA-484 in diagnosis of MS and disease activity as well as in diagnosing the transition from RRMS to SPMS.

Patients and Methods

Forty-six subjects of both sexes were included in this case control-study in the period between June, 2019 to May, 2020. The study was conducted in MS unit of Ain Shams University Hospital. The included subjects were 31 patients with MS (21 with RRMS, 8 with SPMS and Two patients with primary progressive multiple sclerosis (PPMS)) and 15 healthy controls recruited from workers at the hospital. Patients with MS were diagnosed according to Mc Donald criteria, 2017 **(Thompson et al., 2018).** We included patients of both sexes, with age > 18 years old.

Patients selected for this study did not receive treatment with steroids in the last month; or interferon beta, dimethyl fumarate, teriflunomide, glatiramer acetate or fingolimod in the past 3 months; or other disease-modifying therapies (DMTs) in the past 6 months including rituximab, ocrelizumab, natalizumab, methotrexate and cyclophosphamide. We excluded patients younger than 18 years old, patients with any other neurological disorder affecting CNS and patients with malignancies. All patients were subjected to full history taking and meticulous neurological examination. The degree of motor disability was measured by EDSS **(Kurtzke, 1983).** All patients were subjected to brain and cervico-dorsal spine magnetic resonance imaging (MRI) with contrast, and compared with a previous study done within 6-12 months. Defining the clinical course of MS, subtypes and activity followed **Lublin et al. (2014).** Activity was defined clinically (within 1 month) by relapse, acute or sub-acute episode of new or increasing neurological dysfunction followed by full or partial recovery, in absence of fever, infection or evidence of a pseudo-relapse; and/or radiologically (MRI)

by occurrence of new or enlarging T2 hyperintense lesion in the follow up imaging, or presence of contrast enhanced lesion. Progressive disease was defined over 1 year by steadily increasing objectively documented neurological dysfunction/disability without unequivocal recovery.

Three milliliters of venous blood were collected from both patients and controls under complete aseptic conditions in sterile ethylenediaminetetraacetic acid (EDTA) containing tubes. Samples were centrifuged at 1500 rpm for 10 minutes and plasma was separated and stored at -80◦C until subsequent RNA extraction. Expression analysis of miRNAs; miR-145, miR-484 and the housekeeping gene (U6) were assessed by real-time quantitative polymerase chain reaction (PCR) after miRNA extraction using miRNeasy Mini Kit (cat. No. 217184) supplied by #Qiagen (QIAGEN Strasse 1 40724, Hilden, Nordrhein-Westfalen, Germany). Reverse transcription (RT) was then performed on the extracted RNA prepared in the previous step using specific miRNA primers for each miRNA. The reagents used were TaqMan MicroRNA specific RT-primers and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Cat. No. 4366596) supplied by #ThermoFisher (Im Steingrund 4-6 63303, Dreieich, Hessen, Germany). That was then followed by quantitative PCR amplification and finally calculation of the results.

Ethics approval and consent to participate:

The research was conducted after the approval of the Institutional Review Board, Faculty of Medicine, Minia University who confirmed that the study conformed to the principles of "Declaration of Helsinki" (No. 200: 2019; Date: June 17, 2019). All patients signed an informed written consent.

Statistical Analysis

The collected data were revised, coded, tabulated and entered into the SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 20 for Microsoft Windows. Regarding descriptive statistics: mean ± standard deviation (SD) and range were used for numerical parametric data; median and interquartile range (IQR) for numerical non-parametric data; and frequency and percentages of non-numerical data. Mann-Whitney test was used to assess the statistical significance of the difference between two groups of non-parametric numerical data. Receiver operating characteristic curve (ROC curve) was used to assess the diagnostic performance of the miRNAs in diagnosis of MS, disease activity and in diagnosing the transition from RRMS to SPMS, and the area under the curve at the specified cut-off value to calculate sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). P-value < 0.05 = significant.

Results

The studied cases included 25 (80.6 %) females and 6 (19.4%) males; ages ranged from 20 to 50 years old with a Mean ± SD of (33.45 ± 8.17). The studied controls included 5 (33 %) females and 10 (67%) males; age ranged from 18 to 50 years old with mean ± SD of (29.80 ± 9.5). Among the studied cases, there were 21 patients (67.7 %) with RRMS, 8 patients (25.8 %) with SPMS and 2 patients (6.45%) with PPMS. Fourteen patients (45.2 %) were active while 17 (54.8 %) were inactive. The mean age of onset in years was 28.87 (± 8.30 SD). The mean and median duration of illness (in months) were 54.90 (± 49.09 SD) and 48 respectively. The mean total number of relapses was 3.77 (± 3.02 SD). The mean of total EDSS was 3.66 (± 1.84 SD).

With the comparison of microRNAs' relative expression between cases and

controls, it was found that the miRNA-145 and miRNA-484 relative expression was significantly higher in MS patients than in controls (**Table. 1**).

Table 1. microRNAs' relative expression in MS patients and controls:

Variables	MS patients		Controls		Z*	P-value
	Median	IQR	Median	IQR		
miRNA-145	1.00	.30-3.40	.30	.10-2.70	2.09	**0.04**
miRNA-484	2.50	.50-6.20	.40	.10-1.70	2.06	**0.01**

MS: Multiple sclerosis; Bold P-value means significant

It was found that miRNA-145 could significantly discriminate between MS cases and controls, with the best cut-off value > 0.6; sensitivity equals 61.29%, specificity = 73.33 %, PPV = 82.6% and NPV = 47.8%. Moreover, miRNA-484 could significantly discriminate between MS cases and controls, with the best-cut off value > 1.7; sensitivity equals 61.29 %, specificity = 80%, PPV = 86.4 % and NPV = 50% (**Table. 2** and **Fig.1**).

Table 2. Diagnostic performance of different microRNAs relative expression for differentiation between MS patients and controls

Receiver Operating Characteristic (ROC) Curve					
Variables	Area Under the Curve	Standard Error	Asymptotic Significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
miRNA-145	.690	.091	**.036**	.538	.819
miRNA-484	.737	.083	**.005**	.586	.855

MS: Multiple sclerosis; Bold P-value means significant

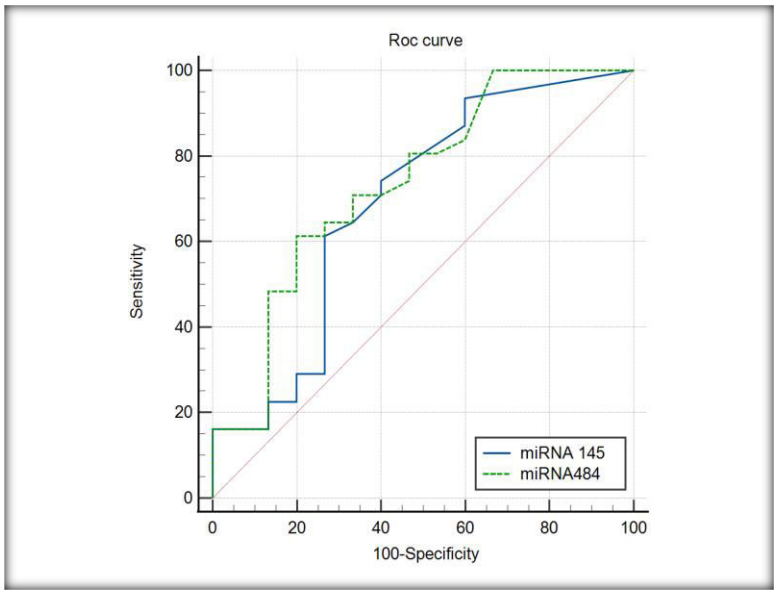

Fig.1. ROC curve for diagnostic performance of both microRNAs' relative expression for differentiation between MS patients and controls

With comparison of microRNAs' relative expression between patients with active MS and patients with inactive MS, it was found that microRNAs' relative expression was higher in active cases in comparison to inactive cases and that was statistically highly significant (**Table.3**).

Table 3.microRNAs' relative expression in active and inactive MS patients

Variables	Active		Inactive		Z*	P-value
	Median	IQR	Median	IQR		
miRNA-145	3.25	1.40-12.90	.50	.30-1.00	2.8	**0.005**
miRNA-484	6.20	4.40-15.50	.60	.20-1.90	4.1	**<0.001**

MS: Multiple sclerosis; Bold P-value means significant

It was found that miRNA-145 could discriminate between active and inactive cases, with the best cut-off value > 0.8; sensitivity equals 92.9%, specificity = 70.6%, PPV = 72.2% and NPV = 92.3%. Similarly, miRNA-484 could discriminate between active and inactive cases, with the best cut-off value > 2; sensitivity equals 100%, specificity = 82.35%, PPV = 82.4% and NPV = 100%. These results were highly significant for both microRNAs (**Table. 4** and **Fig.2**).

Table 4. Diagnostic performance of different microRNAs for differentiation between active and inactive MS patients

Receiver Operating Characteristic (ROC) Curve					
Variables	Area Under the Curve	Standard Error	Asymptotic Significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
miRNA-145	.880	.060	**<0.001**	.713	.969
miRNA-484	.966	.025	**<0.001**	.855	1.000

MS: Multiple sclerosis; Bold P-value means significant

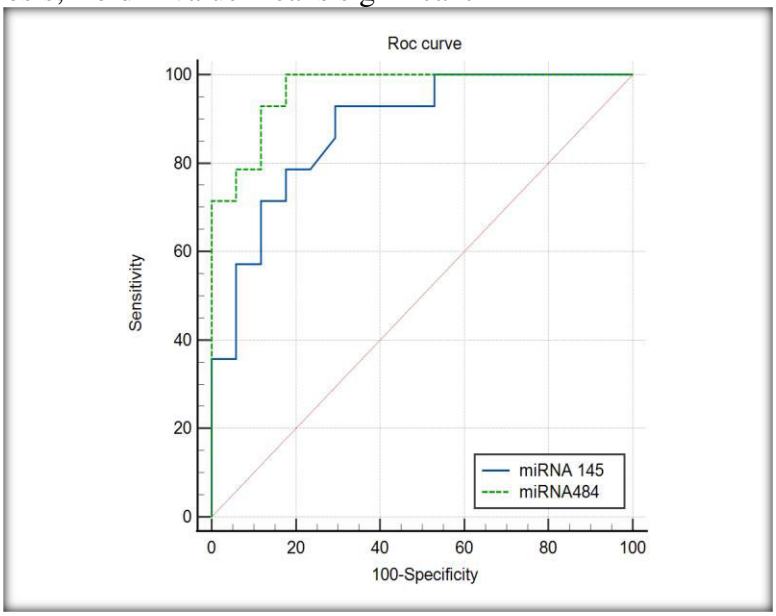

Fig.2. ROC curve for diagnostic performance of both microRNAs' relative expression for differentiation between active and inactive MS patients

With comparison of microRNAs' relative expression between patients with RRMS and patients with SPMS, it was found that microRNAs' relative expression was higher in RRMS cases in comparison to SPMS cases and that was statistically significant with miRNA-145 (**Table.5**).

Table 5. microRNAs' relative expression in RRMS and SPMS patients

Variables	RRMS		SPMS		Z*	P-value
	Median	IQR	Median	IQR		
miRNA-145	1.80	0.50-6.00	.35	.30-.90	2.17	**0.028**
miRNA-484	4.00	.80-7.70	1.35	0.20-2.95	1.54	0.13

RRMS: Relapsing remitting multiple sclerosis; SPMS: Secondary progressive multiple Sclerosis; Bold P-value means significant

It was found that miRNA-145 could discriminate between RRMS and SPMS cases, with the best cut-off value ≤1.4; sensitivity equals 100%, specificity = 52.38 %, PPV = 44.4% and NPV = 100%. These results were statistically highly significant regarding miRNA-145, while miRNA-484 showed non-significant results (**Table.6** and **Fig.3**).

Table 6. Diagnostic performance of different microRNAs for differentiation between RRMS and SPMS patients

Receiver Operating Characteristic (ROC) Curve					
Variables	Area Under the Curve	Standard Error	Asymptotic Significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
miRNA-145	.765	.088	**.003**	.571	.901
miRNA-484	.688	.110	.087	.489	.846

RRMS: Relapsing remitting multiple sclerosis; SPMS: Secondary progressive multiple Sclerosis; Bold P-value means significant

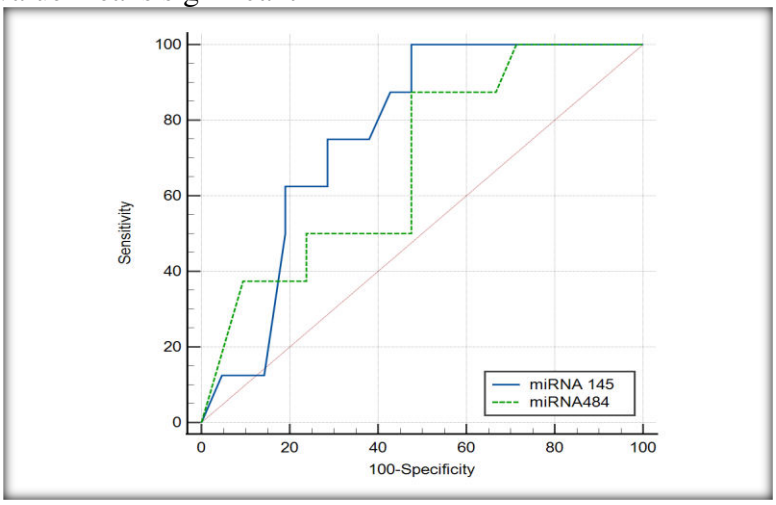

Fig.3. ROC curve for diagnostic performance of both microRNAs' relative expression for differentiation between RRMS and SPMS patients

Discussion

In our study, the two studied microRNAs showed significantly higher relative expression in patients with MS versus controls. That was in agreement with **Gandhi et al. (2013)** who reported that miRNA-145 was significantly upregulated in MS patients versus controls. In the Egyptian study done by **Sharaf-Eldin et al. (2017)** miRNA-145 has been also shown to be significantly upregulated in the serum of MS patients as compared to healthy controls.

Sondergaard et al. (2013) found that miRNA-145 was significantly upregulated in serum and plasma of RRMS patients versus control group. Similarly, **Keller et al. (2009)** found that miRNA-145 was significantly higher in RRMS patients than in controls.

As regard miRNA-484, our results were in line with the international multi-center study for identification of MS-specific serum miRNAs **(Regev et al., 2018).** In the reproducibility phase of this study in which 73 promising miRNAs were analyzed and found that miRNA-484 was upregulated in patients with MS compared to healthy individuals (P = 0.01) with 1.2 fold changes. Also, **Magner et al. (2016)** found that miRNA-484 was significantly upregulated by 1.34 folds in RRMS patients as compared to controls (P = 0.00008)**. Mohamed et al. (2022)** also reported significant upregulation of miRNA-484 in peripheral blood mononuclear cells (PBMCs) of RRMS patients as compared to control group.

The diagnostic performance of miRNA-145 and miRNA-484 relative expression in the diagnosis of MS was assessed in our study and it was found that both microRNAs could discriminate between MS cases and controls, and so might aid in early diagnosis of MS. Best cut-off values were > 0.6 and > 1.7 respectively; sensitivity = 61.29 % for both of them and specificity = 73.33 % and 80% respectively.

That was in agreement with **Keller et al. (2009)** who examined the serum of 39 RRMS patients and found that miRNA-145 differentiated between MS patients and healthy controls with specificity and sensitivity of 89.5% and 90.0% respectively. Similarly, that was in agreement with **Sharaf-Eldin et al. (2017)** who found that miRNA-145 could distinguish MS patients (no=37) from healthy controls (no=20) with 73% sensitivity and 60.9% specificity. **Sondergaard et al. (2013)** also found that miRNA-145 expression in serum was able to predict MS patients with a sensitivity of 85%. Regarding miRNA-484, **Mohamed et al. (2022)** found that its sensitivity and specificity for diagnosis of MS were 88.2% and 86.7% respectively.

Both plasma microRNAs, 145 and 484 examined in our study, showed highly significant upregulation in patients with active MS than in those with inactive type. The diagnostic performance of miRNA-145 and miRNA-484 relative expression in the diagnosis of active MS has been also assessed in our study, and it was found that both microRNAs could significantly discriminate between active and inactive MS, and so might aid in early diagnosis of MS activity. Best cut-off values were > 0.8 and > 2 respectively; sensitivity = 92.9% and100%; specificity = 70.6% and 82.35% respectively.

That was in agreement with **Perdaens et al. (2020)** who found that miRNA-145-5p expression is 2.3 folds upregulated in the CSF of active relapsing cases versus the remitting inactive (P = 0.02). Also, that was supported by a research performed on the spinal cord injury in rat model which reported that miRNA-145-5p inhibition decreased inflammation and oxidative stress, which are prominent features in MS disease activity **(Jiang et al.**,

2021). miRNA-145-5p is one of the two transcripts of miRNA-145 gene locus, together with miRNA-145-3p, and the two transcripts have similar roles and are thought to be co-transcribed **(Ye et al., 2019).**

In addition, that was in agreement with **Kornfeld et al. (2021)** who demonstrated that miRNA-145 gene is a negative regulator of oligodendrocyte differentiation. It controls this process partially by targeting the critical Myelin Regulatory Factor (MYRF) gene in oligodendrocyte progenitor cells, and so prevents their differentiation. This is in favor of the demyelinating process that occurs during disease activity.

Apoptotic protease activating factor 1 (APAF-1) is a potential target gene for miRNA-484 and so, miRNA-484 can play a role in regulation of apoptosis; with upregulation of miRNA-484 there is decrease in expression of APAF-1 and inhibition of apoptosis **(Mohamed et al., 2022).** Apoptosis is considered an anti-autoimmune mechanism that eliminates the immune active lymphocytes from the circulation and CNS, and so prevents the immune-mediated tissue damage. Upregulation of miRNA-484 expression in MS patients promotes the persistence of these pathogenic autoreactive lymphocytes instead of being apoptotic **(Ebrahimiyan et al., 2018).**

In our study, we also found that miRNA-145 might be used as a useful biomarker for diagnosis of the transition from RRMS to SPMS. Best cut-off value was ≤1.4 with sensitivity = 100%, specificity = 52.38 %.

This was in agreement with **Gandhi et al. (2013)** who reported that miRNA-145 was significantly upregulated in RRMS as compared to SPMS patients but they didn´t report sensitivity, specificity or cut-off values.

Limitations of the study

The small sample size in our research was the most important limitation. Further research in much larger number of patients with larger number of different MS subtypes as well as healthy controls is needed for more validated results. We may also need to examine other different MicroRNAs and also to be examined in CSF. Long term follow up for these patients may also add value to future research, emphasizing the impact of different DMTs on MicroRNAs relative expression.

Conclusions

The results of plasma miRNAs 145 and 484 confirm the previous studies, and they could be used as promising biomarkers for early diagnosis of multiple sclerosis and in diagnosis of disease activity. Plasma miRNA-145 could be also helpful in diagnosis of the transition from RRMS to SPMS.

List of abbreviations

APAF-1: Apoptotic protease activating factor 1
CSF: Cerebrospinal fluid
DMTs: Disease-modifying therapies
EDSS: Expanded disability status scale
EDTA: Ethylenediaminetetraacetic acid
IQR: Interquartile range
miRNAs: microRNAs
MRI: magnetic resonance imaging
MS: Multiple sclerosis
MYRF: Myelin regulatory factor
NPV: Negative predictive value
PBMCs: Peripheral blood mononuclear cells
PCR: Polymerase chain reaction
PPMS: Primary progressive multiple sclerosis
PPV: Positive predictive value
ROC: Receiver operating characteristic
RRMS: Relapsing remitting multiple sclerosis
RT: Reverse transcription
SD: Standard deviation

SPMS: Secondary progressive multiple Sclerosis
SPSS: Statistical package for the social science

Consent for publication

- Not applicable.

Availability of data and materials

- The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

- Authors declare no competing interests.

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Authors' contribution

- M.M.I.: revised the results, and wrote the manuscript. N.A.H.: revised the results and statistics. S.I.B.: did the clinical pathology methods. D.A.Z.: revised the clinical data obtained. T.M.D.: recruited patients and controls and collected the needed data. All authors have read and approved the manuscript.

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