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Association between circulating MicroRNAs (hsa-miR-92a-1* and hsa-miR-454) and multiple sclerosis phenotypes and activity status

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Abstract

Efficient diagnosis of multiple sclerosis (MS) disease along with early prediction of its progression will ultimately lead to better management, control of complications and improvement of therapeutic outcomes and patient's well-being. Blood based biomarkers like circulating microRNAs represent a non- invasive, fast, and easily measured markers with a promising potential. This work intended to assess the relative expression of circulating hsa-miR-454 and hsa-miR-92a-1* as a diagnostic and prognostic tool among Egyptian MS patients in terms of correlation to disease type and severity. hsa-miR-454 and hsa-miR-92a-1* relative expression was measured in the plasma of 31 MS patients, relapsing remitting MS (RRMS, n=21) and progressive MS (PMS, n=10) and 20 age and sex matched normal controls by using reverse transcription followed by real time PCR. Disease severity assessment was done in the form of patient expanded disability status scale (EDSS) evaluation. Relative expression of hsa-miR-454 and hsa-miR-92a-1* did not show a statistically significant difference between MS cases and controls. However, hsa-miR-454 was significantly higher among RRMS patients in comparison to PMS patients (P = 0.04). Additionally, both markers showed a statistically significant upregulation among patients in disease exacerbation in comparison to patients in remission (P = <0.01) and both showed a negative correlation with EDSS. In conclusion, microRNAs may represent potential valuable non-invasive biomarkers for assessment of MS type (RRMS vs PMS), as well as for prediction of disease activity and severity in MS patients.

Keywords: Multiple sclerosis, miR-92a-1*, miR-454, EDSS

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Introduction

Multiple sclerosis (MS), an inflammatory demyelinating and neurodegenerative disease, is considered the most common disabling non-traumatic disease to affect young adults.[1] Worldwide, the estimated number of MS patients increased to 2.8 million people in 2020 with global prevalence about 35.9 per 100,000

persons.[2] The estimated number of MS patients in Egypt was about 25,000 subjects and this is the highest number of MS patients in the Middle East region. [3]

In most of MS cases, the disease follows a relapsing-remitting course that includes short-term episodes of neurological deficits which resolve almost completely. A minority of the patients experience progressive neurological deterioration.[4]

MicroRNA (miRNA) is a class of small noncoding RNAs (nearly 19–22 nucleotides in length) capable of regulating gene expression. It was estimated that miRNAs target 33% of human genes approximately. [5] They have been considered as key regulators in the homeostasis of many biological systems and implicated as modulators of many pathobiological processes.[6] Moreover, they have been linked to cancers, autoimmune diseases, inflammation, viral infections, heart diseases, nervous system diseases, obesity, and inherited diseases.[7]

One of the earliest studies that used microarray in the analysis of miRNA expression in MS patients showed that some miRNAs were significantly up-regulated, whereas others were significantly down-regulated in peripheral blood of all MS patients subtypes including primary progressive, secondary progressive and relapsing-remitting disease in comparison to control subjects.[8] In another study, analysis using miRNA PCR array in plasma samples indicated that plasma levels of 29 miRNAs increased in the patients with relapsing remitting MS (RRMS).[9]

Circulating miRNAs are very stable in plasma and serum, this is the reason why many studies extensively assessed their potential as an ideal immune biomarker.[10] Studying the dysregulation of miRNAs and their functions in the CNS will be of great benefit to our understanding of MS pathogenesis and translation of that knowledge to clinical applications. For example, astrocytes contained miRNAs that were strongly upregulated in active MS lesions and were predicted to target CD47 so that serving to release macrophages from inhibitory control and subsequently promoting phagocytosis of myelin.[11]

Collection of peripheral blood is less invasive and easier to be obtained than collection of CSF. So, presence of blood-derived biomarkers that can aid diagnosis in non-invasive way, detect disease activity and distinguish the different MS phenotypes may be advantageous and of great value in the field of personalized MS treatment and prediction of response to treatment.

Being highly stable and non-invasive biomarkers, the present study aimed to evaluate circulating miRNAs; hsa-miR-92a-1* and hsa-miR-454 as diagnostic tools among Egyptian MS patients and to correlate their relative expression with the disease activity and severity in terms of expanded disability status scale (EDSS).

Subjects and Methods

Subjects

This case control study included 31 MS patients. Of these, 21 were relapsing remitting MS patients (RRMS) and 10 progressive MS patients (PMS) fulfilling the McDonald's criteria for diagnosis and classification of MS (2017).[12] They were recruited from the MS unit, Neurology Department, Ain Shams University Hospitals. There were 6 male patients and 25 female patients, their age ranged from 18 to 50 years with mean and SD 31.13 ± 8.58 years.

Assessment of disease activity was done according to clinical picture and radiological findings. MS patients were divided according to disease activity into two groups: 14 patients in the active group (exacerbation) and 17 patients in the inactive group (remission). Patients receiving disease modifying treatment or steroids within 1 month prior to sampling and patients having other demyelinating diseases were excluded. Twenty age and sex matched apparently normal subjects were included too as a control group.

Laboratory work was conducted in the Clinical Pathology Department, Ain Shams University Hospital from August 2019 to March 2020. Prior to initiation, the study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Ain

Shams University (FMASU MD 152/2019) and all participants provided written informed consent.

Sample collection & miRNA expression analysis

Three milliliters of venous blood were collected from each study participant under complete aseptic condition in a sterile EDTA vacutainer tube. Samples were transported to the laboratory in ice box to avoid any possible RNA degradation. Samples were centrifuged at 380 xg for 10 minutes and plasma were separated and stored at -80◦C until subsequent RNA extraction. Hemolyzed samples were discarded, and repeated freezing and thawing was avoided. The isolation of extracellular miRNA from plasma was carried out using miRNeasy Mini Kit reagents (cat. No. 217184, QIAGEN Strasse 1 40724 Hilden, Germany). Reverse transcription (RT) of the miRNA extract to cDNA was done using a TaqMan® MicroRNA Reverse Transcription Kit (cat. No. 4366596, ThermoFisher Scientific, Frankfurter Strasse 129 b, 64293 Darmstadt, Germany) according to the manufacturer's instructions. The standard MicroRNA TaqMan® assays (ThermoFisher Scientific, Germany) used for miRNA amplification and quantification were the following: hsa-miR-454 (cat no. 4427975, test ID: 001996), hsa-miR-92a-1* (cat no. 4427975, test ID: 002137) and the housekeeping gene U6 (cat no. 4427975, test ID: 001973). Twenty microliters reaction mix required for the quantitative PCR according to the manufacturer's instructions containing 5 µl cDNA of the product from the RT reaction, 10 µl of TaqMan Universal PCR Master Mix, 1 µl of TaqMan microRNA assay (20×), and 4 µl nuclease-free water. Thermal cycling profile was performed at 95 °C for 10 min and then, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The reaction was performed using PCR detection system 5 Plex Rotor Gene Real-Time PCR Analyzer (Qiagen, Germany). Finally, the relative quantitation of hsa-miR-92a-1* and hsa-miR-454 in each sample was calculated using delta-delta Ct ($2^{-\Delta\Delta CT}$) method.

Statistical analysis

The statistical analysis was carried out using Statistical Package for Social Science (SPSS 20). Data are expressed as mean and SD for quantitative parametric measures, median and interquartile range for quantitative non-parametric measures and percentage for qualitative measures. The normality of data distribution was tested using the Shapiro-Wilk W test. Chi-Square test was used to examine the relationship between two qualitative variables. Mann Whitney test was used to assess the statistical significance of the difference between two groups of non- parametric numerical data. Spearman's correlation was used to assess the direction and significance of association between two groups of nonparametric variables. A p value of <0.05 was considered statistically significant. Receiver operating characteristic curve (ROC curve) was used to study the diagnostic performance of miRNAs.

Results

Descriptive characteristics of the study participants

Thirty-one MS patients and 20 age and sex matched controls were included in the study. The main demographic and clinical characteristics are shown in Table 1.

Table 1. Demographic and clinical data of patients and controls.

	MS patients (n=31)	Controls (n=20)
	Mean ± SD	Mean ± SD
Age	31.13 ± 8.58	34 ± 12.31
Gender (M: F)	1:4	1:3
	Mean ± SD	
Age of disease onset in years	28.39 ± 8.89	NA
EDSS	3.69 ± 1.92	NA

Table 1. Continued.

	MS patients (n=31) Mean ± SD		Controls (n=20) Mean ± SD
9 HP test Rt hand (sec.)	32.74 ± 16.53		NA
9 HP test Lt hand (sec.)	33.95 ± 16.52		NA
	Median (25^{th} – 75^{th} percentile)		
Duration of disease in months	48 (21 - 72)		NA
No. of relapses	8.7 (7.15 – 14.15)		NA
25 fw test 1^{st} time (sec.)	8.7 (7.3 - 13.3)		NA
25 fw test 2^{nd} time (sec.)	8.8 (7.8– 14.4)		NA
MS Type N (%)	RRMS	PMS	NA
	21 (67.7%)	10 (32.3%)	
MS Activity N (%)	Relapse	Remission	NA
	14 (45.2%)	17 (54.8%)	

EDSS: Expanded disability status scale, 9 HP test: Nine-hole peg test, 25 fw test: Timed 25-foot walk test, NA: not applicable.

Comparative Analysis of the relative expression of hsa-miR-454 and hsa-miR-92a-1 in different study groups*

There was no statistically significant difference between the relative expression of both microRNAs hsa-miR-454 and hsa-miR-92a-1* in the patient and control groups. However, the relative expression of both miRNAs was upregulated among RRMS patients in comparison to PMS patients but only hsa-miR-454 showed a statistically significant difference (*P* = 0.04) (Table 2).

Table 2. Comparison of miRNAs relative expression between RRMS and PMS patients.

	Relative Expression of miRNAs		*P* value	FC (RRMS/PMS)
	RRMS (n= 21)	PMS (n= 10)		
	Median (25^{th} – 75^{th} percentile)	Median (25^{th} – 75^{th} percentile)		
hsa-miR-454	1.2 (0.3- 6)	0.3 (0.1-1.1)	0.04	4
hsa-miR-92a-1*	1.6 (0.6- 5.4)	0.6 (0.2-1.3)	NS	2.7

RRMS: relapsing remitting MS, PMS: progressive MS, FC: fold change. *P* value>0.05 is not significant (NS).

Comparative analysis of hsa-miR-454 and hsa-miR-92a-1* relative expression, as an activity marker of MS, revealed that both miRNAs were strongly upregulated in patients who were in exacerbation when compared to patients in remission with a statistically significant difference (p = < 0.01 for both miRNAs) (Table 3).

Table 3. Comparison of miRNA relative expression between exacerbation and remission patients' groups.

	Relative Expression of miRNAs		P value	FC (Active/ Not active)
	Active (exacerbation) (n= 14) Median (25^{th} – 75^{th} percentile)	Not active (remission) (n= 17) Median (25^{th} – 75^{th} percentile)		
hsa-miR-454	2.6 (1.2-9.1)	0.2 (0.1-0.8)	< 0.01	13
hsa-miR-92a-1*	5 (1.6-9.1)	0.5 (0.2-0.8)	< 0.01	10

FC: fold change. *P* value<0.05 is significant

Diagnostic performance of circulating plasma miRNAs in discriminating different MS patient groups

Diagnostic performance analysis using ROC curve with multiple cutoff levels analysis for discrimination between patients in disease relapse and patients in remission was carried out. It showed that both hsa-miR-454 and hsa-miR-92a-1* relative expression can discriminate between patients who are in disease activity (relapse) and those who are not with an optimum cutoff ≥ 1 for hsa-miR-454 and ≥ 1.15 for hsa-miR-92a-1*. Diagnostic sensitivity and specificity were 85.7% and 82.4% while PPV and NPV were 80% and 87.5%, respectively for both miRNAs (Figure 1). The area under the curve (AUC) was 0.88 and 0.92 with 95% confidence interval (CI) of (0.76 – 1) and (0.83-1) for hsa-miR-454 and hsa-miR-92a-1*, respectively.

For discrimination between RRMS and PMS, ROC curve analysis showed that hsa-miR-454 relative expression can discriminate between RRMS and PMS with an optimum cutoff value > 0.60. Diagnostic sensitivity and specificity were 66.7% and 70% while PPV and NPV were 82.4% and 50%, respectively. The AUC was 0.73 with 95% CI of (0.56 – 0.9) (Figure 2).

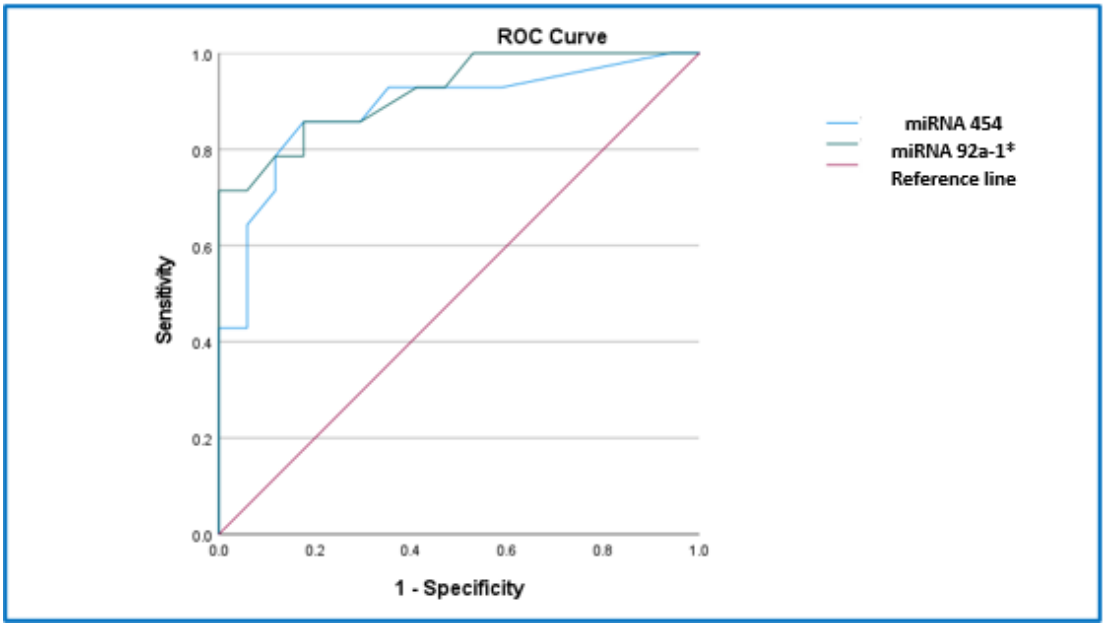

Figure 1. ROC curve illustrating diagnostic performance of miRNA 454 and miRNA 92a-1* in differentiation between relapsed and remittent MS patients.

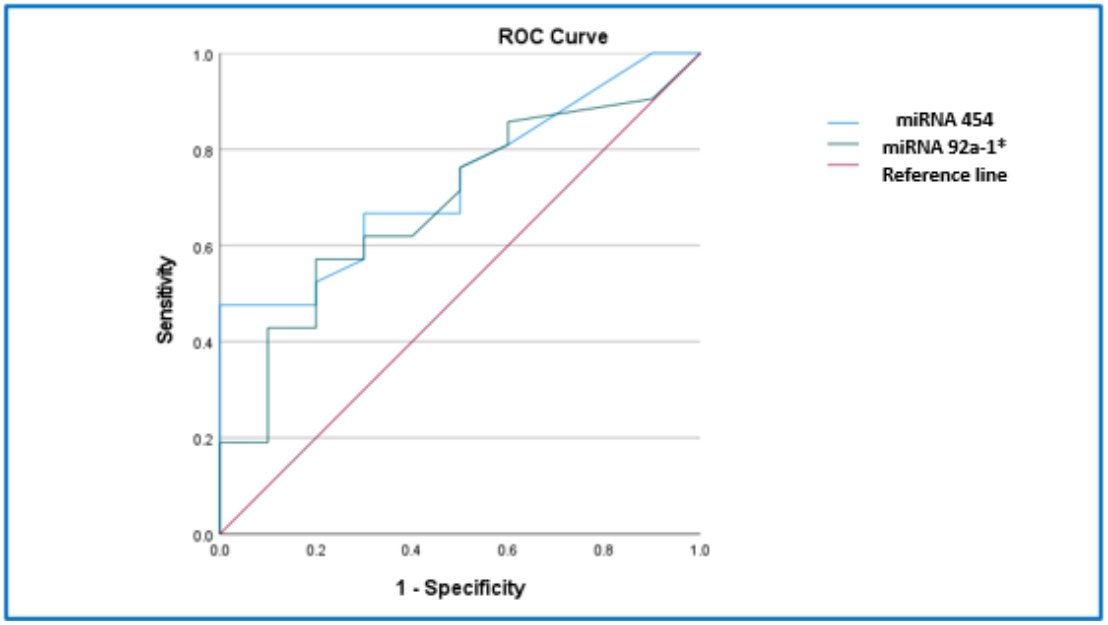

Figure 2. ROC curve illustrating diagnostic performance of miRNA 454 and miRNA 92a-1* in differentiation between RRMS and PMS.

Correlation study of hsa-miR-454 and hsa-miR-92a-1 relative expression and EDSS*

Using Spearman's correlation analysis among all MS patients, the relative expression of hsa-miR-454 and hsa-miR-92a-1* showed significant negative correlation with EDSS as a measure of disease severity (r -0.48 and -0.43, *P*= 0.01 and 0.02, respectively) (Figure 3 & 4). Additionally, in the group of active disease patients, relative expression of both hsa-miR-454 and hsa-miR-92a-1* showed a significant negative correlation with the EDSS (r -0.69 and -0.54, *P*= 0.01 and 0.05, respectively).

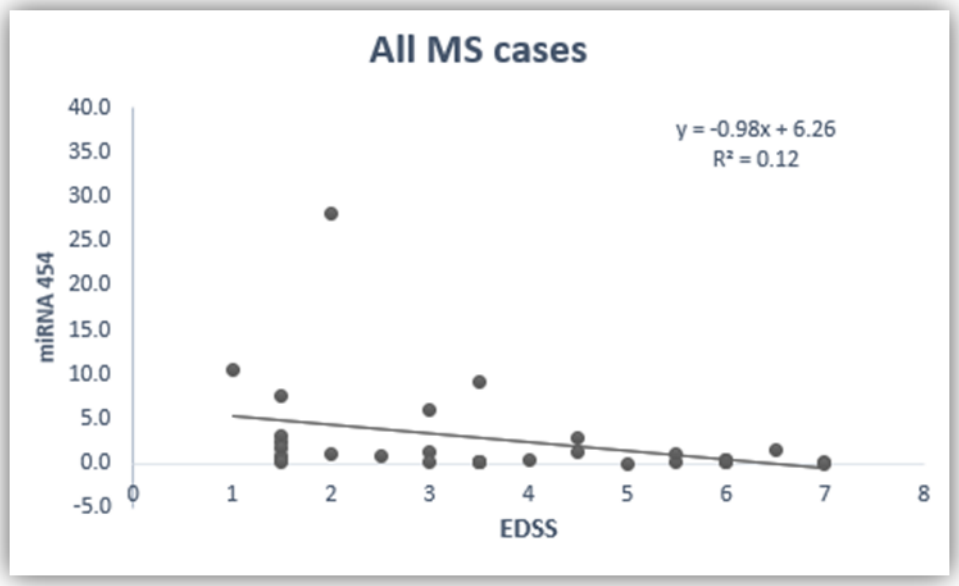

Figure 3. Correlation between miR-454 and EDSS in all patient group.

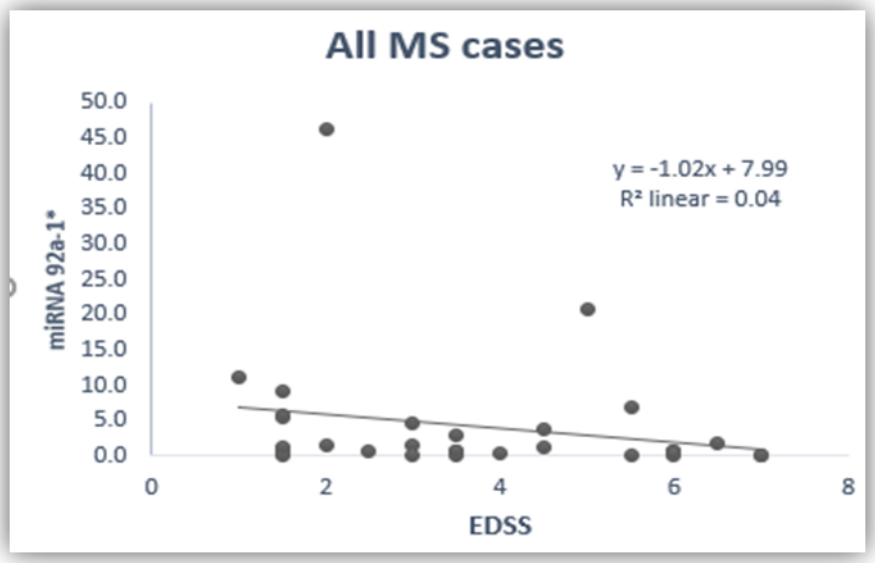

Figure 4. Correlation between miR-92a-1* and EDSS in all patient group.

Discussion

Multiple Sclerosis remains a significant cause of morbidity with a high incidence rate. The discovery of non-invasive biomarkers for MS diagnosis and disease activity status assessment with adequate sensitivity and specificity is a major challenge to reduce the related morbidity, improve response to treatment and quality of life.

In the present study, we tried to evaluate circulating miRNAs; hsa-miR-92a-1* and hsa-miR-454 as diagnostic tools and activity markers in MS patients. The relative expression of both miRNAs did not differentiate neither RRMS patients nor PMS patients from study controls. These finding are different from those reported by Gandhi et al., 2013 who found that hsa-miR-454 was upregulated in RRMS in comparison to controls.[9] This difference in the results may be attributed to the smaller sample size. However, our comparative analysis of RRMS and PMS patients revealed that both hsa-miR-454 and hsa-miR-92a-1* were upregulated among RRMS patients in comparison to PMS patients but only hsa-miR-454 showed a significant difference. These results are in accordance with Gandhi et al., 2013 findings which revealed that the relative expression of both miRNAs showed a significant upregulation among RRMS patients in comparison to secondary progressive MS (SPMS) patients.[9]

The cause of higher relative expression of some miRNAs in RRMS rather than PMS patients is not yet clear. However, miRNAs that differentiated RRMS from progressive forms were found to target crucial immune functions and the neuronal homeostasis maintenance.[13] For example, miR-27a, the strongest miRNA that is upregulated among RRMS patients and differentiated it from progressive forms, showed a strong link with both the neurotrophin signaling pathway and the T cell receptor (TCR) signaling pathway.[14] In addition, miR-27a targets many proteins involved in intracellular signaling networks that regulate the nuclear factor kB (NF-κB) and mitogen activated protein kinases activity. [14] Thus, miR-27a inhibits dendritic cell mediated differentiation of Th1 and Th17 cells in vitro and vivo and promotes accumulation of Tr1 and Treg cells in vivo.[14] These immune functions and pathways were noticed to be affected by hsa-miR-454 and hsa-miR-92a expression upon studying other diseases.[15] It was found that miR-454 downregulates mRNA of CYLD which is a de-

ubiquitination enzyme that regulates the NF-κB signaling pathways.[15] Also, it was hypnotized that decreased level of miR-92a expression leads to severe reduction of naive TCR expression [16] in addition to its role in regulating the neurotrophin protein tropomyosin receptor kinase A whose expression was found to be reduced under the effect of miRNA-92a mimics in experimental studies performed on neuroblastoma cells.[17] These observations may give an explanation regarding different miRNAs relative expression (miR-454 and miR-92a-1*) among RRMS patients in comparison to PMS patients as they are related to the same intracellular pathways that were found to be influenced by miR-27a.

In the context of disease activity, our results showed a statistically significant upregulation of both circulating miRNAs hsa-miR-454 and hsa-miR-92a-1* among MS patients who were in exacerbation (relapse) when compared to patients in remission and ROC curve blotting for diagnostic performance analysis showed relatively high sensitivity and specificity. Our results came in agreement with these of Pesch et al., 2018 who found that miR-92a expression was upregulated in CSF samples of relapsed MS patients. [18] Our findings together with these of Pesch et al., 2018 throw lights indicating that miR-92a and miR-454 miRNAs may be potential novel biomarkers that can differentiate between relapsed/active and stable patients. Note that our results are based on using minor invasive sample collection methods.

Contradictory results were reported by Kacperska et al., 2015 who found that miR-92a expression did not change significantly with the disease activity. However, their results showed a trend related to the number of disease relapses demonstrating that the lower miR-92a expression, the more frequent flare-ups to occur. [19] This difference from our results might be attributed to using a different assay primers/probes showing a slightly different sequence which binds to a different site in real time PCR analysis but detects a similar product or attributed to the racial difference.

In the present study, EDSS was used as a measure to assess patient's disability and disease severity. Our results revealed that both circulating hsa-miR-454 and hsa-miR-92a-1* showed a strong negative correlation to EDSS of MS patients. Similar results were shown by Kacperska et al., 2015 while studying the relative expression of miR-92a, their results showed a negative correlation with EDSS in MS patients which reached statistical significance among relapsed patients.[19]

Diagnostic performance analysis in the present study provided certain cutoff levels of circulating miRNA relative expression to discriminate between RRMS and PMS in addition to discrimination between patients who are in disease exacerbation and those who are not. This would be of a great help in the prediction of upcoming flare ups along the disease course. Literature search of previous studies did not show definite discrimination cutoff values and the suggested cutoffs should be tested by further research including larger sample size.

In conclusion, circulating hsa-miR-454 and hsa-miR-92a-1* profiles in MS may possibly serve as non-invasive biomarkers of disease activity and phenotype discrimination. They may qualify to discriminate between subjects with MS active disease (exacerbation patients) from these with inactive disease (patients in remission).

Author Contributions

HAA; designed and approved the whole research protocol. SIB and NTF; contributed to the protocol design, revised laboratory work, and approved the final paper version to be published. RM; monitored data collection process and the laboratory work, interpreted the data, and critically revised the paper. DZ; supervised sample collection according to inclusion criteria, revised clinical data, diagnosis, and patient classification. AAM; collected the samples and patient's clinical data, carried out the laboratory work and analyzed it, carried out statistical analysis and drafted the paper. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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Ethical approval

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Ain Shams University (FMASU MD 152/2019).

Informed consent

All participants provided written informed consent.

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