Original Article

Evaluation of the expressed miR-129 and miR-549a in patients with multiple sclerosis

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Abstract

Background: The expression of microRNAs (miRNAs) as circulating biomarkers has been underlined in multiple sclerosis (MS) in the last decade. Due to the presence of a possible relationship between expressed miRNAs and heterogeneous appearances of the pathological processes in MS, the present study attempts to evaluate the expression of miR-129 and miR-549a in patients with MS in comparison with healthy control (HC) group. **Materials and Methods:** Peripheral blood mononuclear cells were separated from fifty patients with MS (subtypes including relapsing–remitting MS and secondary progressive MS) in the Kashani Hospital, Isfahan, Iran, and fifty people as HC group. After RNA extraction and complementary DNA synthesis, the expression of miR-129 and miR-549a was evaluated in patients with MS in comparison with the HC group using a quantitative real-time polymerase chain reaction assay. The data were analyzed using the Kolmogorov–Smirnov and Mann–Whitney tests. Spearman's correlation coefficient was used to examine the relationship between miR-129 and miR-549a with age. **Results:** The results showed that the expression of miR-129 and miR-549a was not significant in patients with MS in comparison with the HC group. Furthermore, the relationship between such miRNAs and age and gender was not significant. **Conclusion:** We suggest the expression of miR-129 and miR-549a as circulating miRNAs in peripheral blood mononuclear cells could not be considered a biomarker for diagnosis and Para clinical.

Keywords: *MicroRNAs, miR-129, human, miR-549a, multiple sclerosis*

Introduction

MicroRNAs (miRNAs) are considered as the major factors involving in the regulation of gene expression at the level of posttranscription through mRNA inhibition and degradation. The other critical functions of miRNAs in the regulation of the immune system have been underlined in the last decade. The important roles of miRNAs in antibody switching, the expression of chemokines and cytokines, proliferation of monocytes and neutrophils, and also development and differentiation of the T- and B-cells elucidate the relationships of miRNAs and autoimmune diseases such as multiple sclerosis (MS).[1-3]

MS is defined as an inflammatory neurodegenerative disease of the central nervous system with diverse clinical appearances and different responses to treatment.[4,5] Although the exact etiology of MS is not clear till now, genetic predisposition, occupational exposure, virus infections, lack of vitamin D, and toxins might be related to the occurrence of this disease.[6,7] Four disease courses including primary progressive MS (PPMS), secondary progressive MS (SPMS), progressive-relapsing MS, and relapsing–remitting MS (RRMS) have been described for MS in recent years.[8]

According to the aforementioned relationship between miRNAs and autoimmune diseases such as MS, performing more investigations regarding miRNAs and MS might open a new venue for discovering diagnostic and prognostic biomarkers in MS. The high stability of the miRNAs in biological samples including cerebrospinal fluid (CSF) and serum proposes such markers as potential circulating biomarkers.[1,9] Recently, miR-129-3p and miR-549a-3p were selected as an appropriate and involved miRNA in MS disease.[10,11] Moreover, using a bioinformatics approach to analyze differential expression profiles of mRNA and miRNA, MS patients showed

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significant differences in mRNA and miRNA expression when compared with normal controls.

Previous studies have identified and reported different miRNAs in MS.[1,4,12-17] Due to the presence of a possible relationship between expressed miRNAs and heterogeneous appearances of the pathological processes in MS, further investigations can unlock the ambiguous questions concerning pathological implications and mediatory duties of miRNAs in the etiopathological mechanisms of MS. For example, one study miR-17-5p has been reported as a deregulated miRNA in MS,[18] and another report has indicated that miR-326 and miR-26a could be a candidate biomarker for MS.[19-21]

Th17 cells perform an essential function in the development of a number of autoimmune disorders (such as MS, inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus [SLE], and psoriasis) and allergy and asthma.[22-25] Interleukin-17 (IL-17) also acts as a powerful inducer of neutrophil aggregation into the inflammatory tissues.[26] A study in 2016 has reported that osteopointin inhibition of miR-129-3p enhances IL-17 expression and monocyte migration in rheumatoid arthritis. This is done by interfering with the signaling pathway of the AKt/PI3K/Syk.[27] In 2015, Odenthal *et al*. state that, in patient with adenocarcinoma, miR-549 will sever as a prognostic and predictive marker in gastric cancer, and this miRNA will be regulated by adjuvant injection into these patients.[28] Recent work published by LOffreda *et al*. in 2020 miR-129-5p is a novel therapeutic target for amyotrophic lateral sclerosis.[29] A study in 2012 mentioned that some miRNA, including miR-549, were regulated translating and mRNA stability of IL-17 cytokines and receptor through 3′ untranslated region-dependent mechanisms.[30] MiR-549 also identified as potential biomarkers for therapy responses in psoriasis with effect on pathological T cells and dendritic cells in keratinocytes.[31] MiR-129 can also suppress the expression and activity of tumor necrosis factor, IL-10, and IL-6 which are associated with a collagenase-induced intracerebral hemorrhage (ICH) rat model.[32] In another study, decreased expression levels of miR-549a in the peripheral blood mononuclear cells and extracellular vesicles in myalgic encephalomyelitis/chronic fatigue syndrome could be cited as an important risk factor in MS, SLE, or other diseases presenting overlapping symptoms.[33] The investigations have shown that miR129-5p inhibits the growth and metastases of neuroendocrine tumors.[34] Another study in 2017 elucidated the function of miR-129-5p in a signaling pathway in the revascularization in collagenase-induced ICH rat models.[32] Furthermore, the inhibitory role of miR129-5p has been suggested in the development of encephalomyelitis-related epilepsy.[35] Regarding miR-549, such miRNA, has been introduced as a diagnostic and prognostic biomarker in patients with gastric cancer.[28,36] Despite the aforementioned data about the miR129-5p and miR-549, information concerning to the circulating miR-129 and miR-549a in patients with MS are scarce so far. In addition, the identification of novel miRNAs might facilitate the diagnosis, treatment, and treatment following MS in near future. To bridge this scientific gap, this study aimed to evaluate and compare the expression level of miR-129-3p and miR-549a in peripheral blood mononuclear cells (PBMCs) of MS patients (RRMS and SPMS subtypes) with healthy individuals.

Materials and Methods

Ethics statement

This study was approved following guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation of the Shahid Sadoughi University of Medical Sciences. Informed consent was obtained from all participants in this study.

Patients and sample collection

Fifty individuals who were patient with MS (mean age = 37.7 years; 42 females/8 males), which was identified by lumber puncture and MRI methods, along with fifty people as the healthy control (HC) group (mean age: 37.8 years; 42 females/8 males) were selected from the MS clinic of Kashani Hospital, Isfahan, which was confirmed by a neurologist. Participants in the HC group had no background of any autoimmune diseases, acute, chronic, allergic diseases during the treatment period, and the duration of the study in themselves and first-degree relatives and taking any anti-inflammatory drugs in the last 2 months. The case group is new patients with MS. MS patients in this study were contained two subtypes including RRMS (n = 45) and SPMS (n = 5). The diagnosis of MS was confirmed using the McDonald′ criteria and the New York International Standard.[37] Patients were classified based on the modified Rio score (MRS).[38] The MRS is a simplified version of Rio score, excluding the expanded disability status scale (EDSS) progression and modified items of the relapse rates and MRI activity. The EDSS of all patients was 0 to 6 (mean: 2.69 ± 1.61). Patients and controls were age and sex matched in this study. The demographic information of participants in this study is shown in Table 1.

Table 1: Demographic characteristics of the participants in the study

Demographics	Patients (n=50)	Controls (n=50)
Age (years), mean±SD	37.7±9.1	37.8±9.2
Average, *n* (%)	<40:29%	<40:29%
	≥40:21%	≥40:21%
Gender (Female/male)	42/8	42/8
Subtype (RRMS/SPMS)	45/5	-
EDSS (0-6), mean±SD	2.69±1.61	-

RRMS: Relapsing-remitting multiple sclerosis, SPMS: Secondary-progressive multiple sclerosis, EDSS: Expanded disability status scale. SD: Standard deviation

Venous blood samples (3 mL for each person) were taken in evacuated tubes (Becton Dickinson, USA). Ficoll-Hypaque density gradient centrifugation method was used for the isolation of PBMCs. Then, PBMCs were stored at −80°C for performing further experiments.

microRNAs extraction

PBMCs were washed two times in ice-cold phosphate-buffered saline; then, total RNA was extracted from PBMCs using RNA Hybrid R™ Kit (Gene All, Korea) according to the manufacturer's procedure. Synthetic miRNA was used to check the accuracy of RNA recovery in samples. NanoDrop spectrophotometer (Thermo Scientific, USA) was applied to indicate RNA integrity, purity, and concentration (OD 260/280 nm).

Complementary DNA reverse transcription (complementary DNA synthesis)

Seven μL of RNA was used for retro-transcription reactions (in a final volume of 20 μl). The experiments were performed in triplicate using PrimeScript RT Reagent Kit (Takara, Japan) and miR-specific stem-loop primers. A synthetic control sample was utilized for checking the efficiency of complementary DNA (cDNA) synthesis and the absence of quantitative real-time polymerase chain reaction (qRT-PCR) inhibitors.

Quantitative real-time polymerase chain reaction

qRT-PCR-detection of miRNAs was done on two miRNAs including miR-129 and miR-549a. qRT-PCR was conducted on the diluted cDNA template with assay-specific primers and probes, using RealQ Plus 2x Master Mix Green (Takara, Japan). All qRT-PCR reactions were performed in duplicate, using a StepOnePlus™ RT-PCR System (Applied Biosystems, USA).

An expression index was measured by the $2^{-\Delta CT}$ method for relative quantification. The level of miRNA expression was normalized to the small nucleolar RNA (U6 snRNA); as an internal normalization control.

Statistical analysis

SPSS software (version 20, SPSS Inc, Chicago, IL) was used for data analysis, and data are presented as the mean ± standard deviation. Kolmogorov–Smirnov test was applied for the evaluation of the normal distribution of data. The key difference between parametric (ANOVA) and nonparametric (Mann–Whitney U) test is that the parametric test relies on statistical distributions in data, whereas nonparametric do not depend on any distribution. Nonparametric does not make any assumptions and measures the central tendency with the median value. Due to the fact that the data distribution was skewed and abnormal, the nonparametric Mann–Whitney U-test was conducted to compare the groups (patients [RRMS and SPMS] and HC) in this study. Median (Interquartile range: 25%–75%) was expressed as scale data and $P < 0.05$ was determined as a statistically significant level. Due to the large dispersion and nonparametric of data, we had to use a box plot instead of a bar or column chart to show the best evaluation between the groups and both miRNAs.

Results

In this study MiRanda (http://www.microrna.org/microrna/home.do) and TargetScan (http://www.targetscan.org/), bioinformatic instruments were applied to indicate miRNA potential target mRNAs.

Demographic information

As aforementioned, this is a case–control report that fifty patients (42 female, 8 males; mean age: 37.7 ± 9.1) with MS (45 RRMS and 5 SPMS) and fifty individuals as the HC group (mean age: 37.8 ± 9.2) were age and sex matched in this study. The EDSS of all patients was 0 and 6. As shown in Table 1, the results of the Chi-square test indicated that the frequently distribution regarding gender and sex was non-significant.

The expression of miR-129 and miR-549a in peripheral blood mononuclear cells, in multiple sclerosis patients and healthy control group

The analysis of qRT-PCR results using a non-parametric Mann–Whitney U-test showed that the expression of miR-129 and miR-549a was not significant in the MS patients group (RRMS and SPMS subtypes) in comparison with HC group [P Value $_{\text{miR-129}} = 0.107$, P Value $_{\text{miR-549a}} = 0.499$, used $2^{-\Delta CT}$ method, Figure 1].

The expression of miR-129 and miR-549a in peripheral blood mononuclear cells, in multiple sclerosis patients and healthy control group according to the age

The age average of the participants in this study was considered <40 and ≤40 [Table 1]. The analysis of qRT-PCR results using a nonparametric Mann–Whitney U-test showed that there was no significant relationship between the expression of miR-129 and miR-549a and the age of the participants in MS patients group (RRMS and SPMS subtypes) and individuals in the HC group [P Value miR-129 in MS patients = 0.723, P Value miR-549a in MS patients = 0.575, P Value miR-129 in HC group = 0.783, P Value miR-549a in HC group = 0.35, used $2^{-\Delta CT}$ method, Figure 2].

The expression of miR-129 and miR-549a in peripheral blood mononuclear cells, in multiple sclerosis patients, and healthy control group according to the gender

Generally, 84 women and 16 men participated in this study [Table 1]. The analysis of qRT-PCR results using a nonparametric Mann–Whitney U-test showed that there was no significant relationship between the expression of miR-129 and miR-549a and the gender of the participants in MS group (RRMS and SPMS subtypes)

and HC group [*P* Value $_{\text{miR-129}}$ in MS patients = 0.785, *P* Value $_{\text{miR-549a}}$ in MS patients = 0.649, *P* Value $_{\text{miR-129}}$ in HC group = 0.612, *P* Value $_{\text{miR-549a}}$ in HC group = 0.668, used $2^{-\Delta CT}$ method, Figure 3].

Discussion

Circulating miRNAs as potential biomarkers have been investigated in MS in recent years.[39-41] For instance, the improper levels of miRNA have been indicated in T and B lymphocytes and peripheral blood mononuclear cells.[42] MRI evidence has been shown the diverse miRNA profiles in active and chronic lesions in MS.[42] In addition, differentially expression of circulating miRNAs in blood and CSF has been reported in different subtypes of MS.[43] It has been shown that miR-223, miR-23a, and miR-15b downregulate in the serum of MS patients with PPMS and RRMS subtypes.[44] In another study in 2018, the association of miR-128-3p and miR-24-3p with disease activity and disability accumulation have been reveled in MS, respectively.[1]

The results of our study indicated the expression of miR-129 and miR-549a was not significant in MS patients group in comparison with the HC group. In the first impression, it is concluded that miR-129 and miR-549a could not suggest as circulating miRNAs in MS. Due to the rare information concerning the expression of miR-129 and miR-549a in MS patients, further studies are needed to confirm our results in future. The effect of treatment was not checked on the expression of miR-129 and miR-549a in RRMS and SPMS subtypes of MS in this study. More investigations in this regard might propose such miRNAs as therapeutic targets or markers for following treatment of MS in future.

In previous studies, age and gender have been highlighted as two important involving factors in MS;[45-47] therefore, our study focused on the possible relationships between the expression of miR-129 and miR-549a and these factors. Although our study showed a statistically nonsignificant correlation between age, gender, and the expression of miR-129 and miR-549a in MS and HC groups, because of the small sample size. Conducting similar studies with the larger statistical population will clarify the exact relationship of age and gender in the expression of miR-129 and miR-549a in MS patients.

These results may be explained by the fact that the sites and contexts as well as stage of diseases are highly important. It seems that results from studies have been investigated miR-129 and miR-549a expression in the site of inflammation and in patients with active phase of

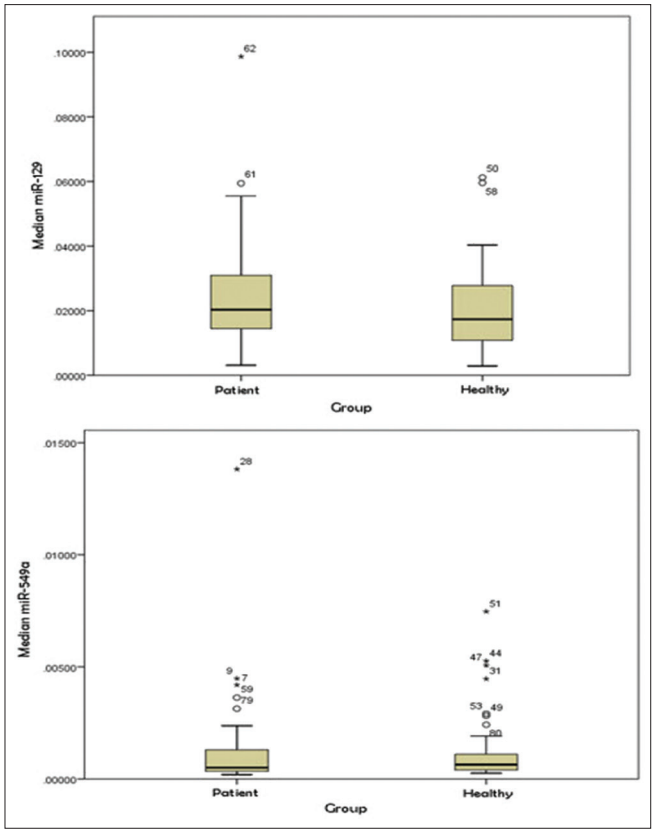

Figure 1: The expression of miR-129 and miR-549a in PBMCs in MS patients in comparison with HC group; (*P* Value miR-129 = 0.107, *P* Value miR-549a = 0.499, used $2^{-\Delta CT}$ method)

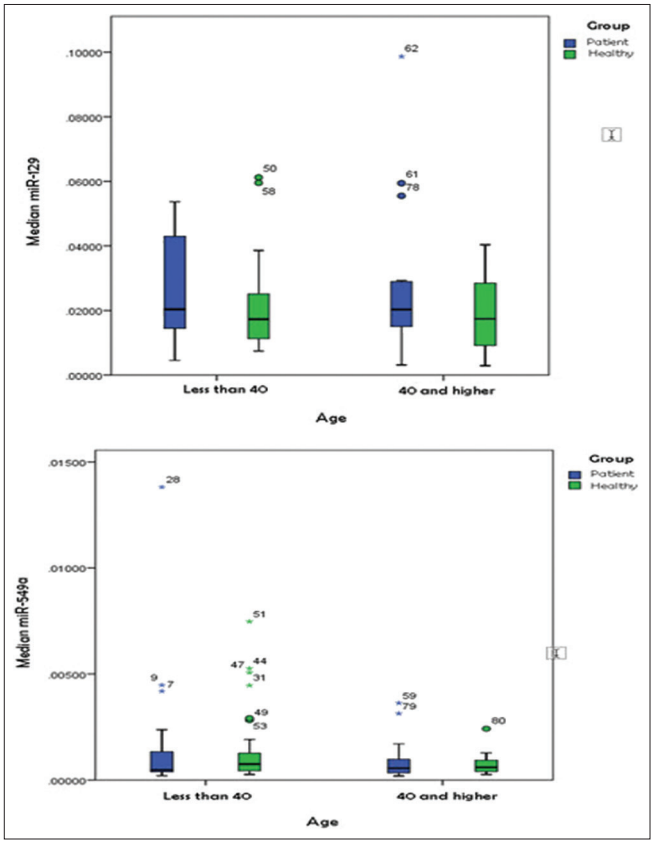

Figure 2: The expression of miR-129 and miR-549a in PBMCs in MS patients and HC group according to the age (*P* Value miR-129 in MS patients = 0.723, *P* Value miR-549a in MS patients = 0.575) (*P* Value miR-129 in HC group = 0.783, *P* Value miR-549a in HC group = 0.35, used $2^{-\Delta CT}$ method)

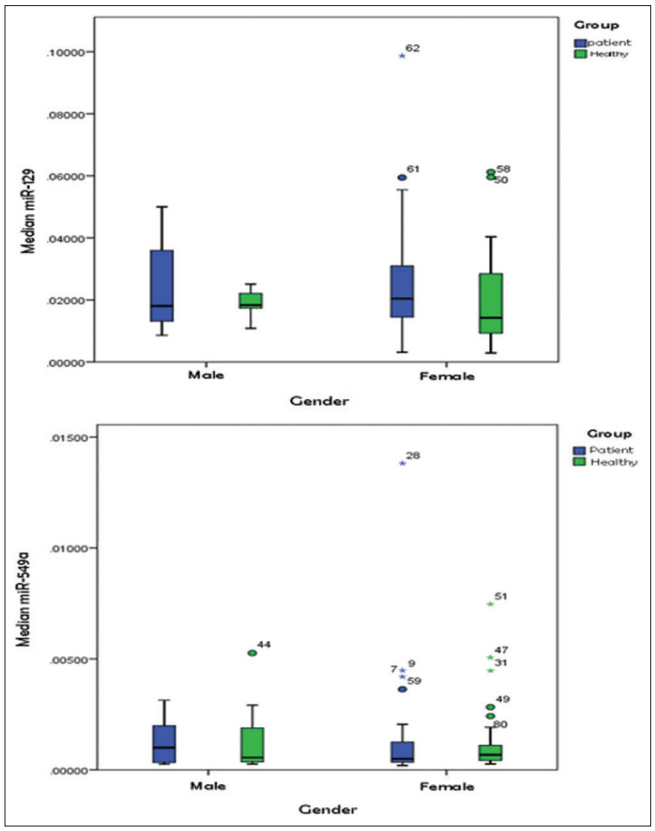

Figure 3: The expression of miR-129 and miR-549a in PBMCs, in MS patients and HC group according to the gender (*P* Value miR-129 in MS patients = 0.785, *P* Value miR-549a in MS patients = 0.649) (*P* Value miR-129 in HC group = 0.612, *P* Value miR-549a in HC group = 0.668, used $2^{-\Delta CT}$ method)

autoimmune disease; miR-129 and miR-549a expressed at the highest levels rather than studies have been investigated miR-129 and miR-549a expression in noninflammatory sites such as peripheral blood and in patients with inactive phase of disease. On the other hand, the difference in the level of expression at T-cells in different autoimmune diseases by T-cells is also evidence of the complexity of the immune system's function against each disease.[29,48,49]

The literature lacks strong evidence regarding miR-129 and miR-549a expression, we recommend the future studies evaluating miR-129 and miR-549a expression in CSF and its level in different courses of MS disease.

Conclusion

Recently, miRNAs have been suggested as important circulating biomarkers in the diagnosis, treatment, and following treatment of diseases such as MS.[50-53] Although different factors including gender, age, and different subtypes of MS might be involved in the expression miRNAs level in MS patients, expression of miR-129 and miR-549a was not significant in our study regarding age and gender. However, the validation of the expression of the miR-129 and miR-549a using further experiments is warranted in MS patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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