



Assessment of expression profile of microRNAs in multiple sclerosis patients treated with fingolimod

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Abstract

Fingolimod is an immunotherapeutic drug approved in certain countries as first-line therapy for relapsing–remitting multiple sclerosis (RRMS). The drug has been shown to alter the expression of several coding and non-coding genes. In the current study, we assessed the expression of miR-506-3p, miR-217, miR-381-3p, miR-1827, miR-449a and miR-655-3p in peripheral blood of patients with RRMS undergoing treatment with fingolimod compared with healthy controls. We also compared the expression of these miRNAs between fingolimod responders and non-responders to determine their relevance with regard to response to fingolimod. Expression of miR-381-3p was significantly higher in responders than in controls (RE difference = 3.903, $P = 0.005$), while expression of miR-655-3p was significantly lower in both responders and non-responders compared with controls (RE difference = -1.03 , $P = 0.014$; RE difference = -1.41 , $P < 0.0001$, respectively). No difference was found in the expression of other miRNAs between study subgroups. In addition, there was no significant difference in the expression of any miRNA between responders and non-responders. Although there were significant pairwise correlations between expression levels of all of the assessed miRNAs in controls, MS patients exhibited differences in correlation patterns. Expression of miR-381-3p was correlated with age in responders. However, expression of other miRNAs did not correlate with age in any study subgroup. The current study indicates a possible role for miR-655-3p and miR-381-3p in the pathogenesis of MS or possible effects of fingolimod on the expression of these miRNAs. Future studies are needed to verify these results in larger patient populations.

Keywords Fingolimod · multiple sclerosis · miRNA · expression

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Introduction

Fingolimod is an immunotherapeutic drug which targets the sphingolipid-regulated signaling system (Brinkmann et al. 2002), and exerts effects on immune cells by binding with the sphingosine 1-P1 receptor. This binding leads to internalization and degradation of the sphingosine 1-P1 receptor and subsequent lymphocyte sequestration in peripheral lymphatic tissues. Thus, autoaggressive T cells cannot invade the central nervous system (CNS) (Chiba et al. 1998). As the first orally administered medicine for treatment of relapsing–remitting multiple sclerosis (RRMS), its efficacy in decreasing the rate of relapse has been proven in multiple clinical trials (Ayzenberg et al. 2016). Fingolimod is approved as a first-line treatment in the USA and certain other countries, and as a second-line treatment in other countries for patients who do not respond to first-line therapeutic approaches (Ayzenberg et al. 2016). This immunotherapeutic drug has been shown

Table 1 Demographic information for MS patients and controls

Variables	MS patients	Controls
Female/male [no. (%)]	58 (74%)/20 (26%)	44 (55%)/35 (45%)
Age (mean ± SD, years)	36.28 ± 8.8	34.41 ± 10.08
Age range (years)	20-58	24-63
Age at onset (mean ± SD, years)	31.12 ± 8.18	–
Disease duration (mean ± SD, years)	5.2 ± 4.5	–
Expanded Disability Status Scale (mean ± SD)	3.25 ± 1.15	–

to modulate the expression of several coding and non-coding genes. Fiess et al. investigated transcriptomes of CD4⁺ T cells and found differential expression of 890 genes 3 months after initiation of fingolimod therapy. Notably, numerous microRNA (miRNA) precursors, including those for miR-216b, miR-142 and miR-548c, have been among the altered genes (Friess et al. 2017). Fenoglio et al. reported a slight decrease in serum levels of miR-15b, miR-23a and miR-223 after fingolimod treatment (Fenoglio et al. 2013). Marked changes in serum levels of these microRNAs (miRNAs) were found after 6 months of continuing fingolimod treatment (Fenoglio et al. 2016).

In the current study, we assessed the expression levels of miR-506-3p, miR-217, miR-381-3p, miR-1827, miR-449a and miR-655-3p in peripheral blood of RRMS patients undergoing treatment with fingolimod compared with healthy controls. Although other miRNAs have been shown to affect patient response to fingolimod (Nuzziello et al. 2020), we selected these miRNAs based on their roles in immune response in order to gain greater insight into the relationship between these miRNAs and the response to fingolimod. We also compared the expression of these miRNAs between fingolimod responders and non-responders to determine their relevance with regard to response to fingolimod. These miRNAs have

Fig. 1 Box plot with median and interquartile values showing relative expression of miRNAs in MS patients (responders and non-responders) and controls. Relative expression of each miRNA was measured using the Ln (Efficiency^ΔCt) formula. Outliers are shown by the plus (+) sign

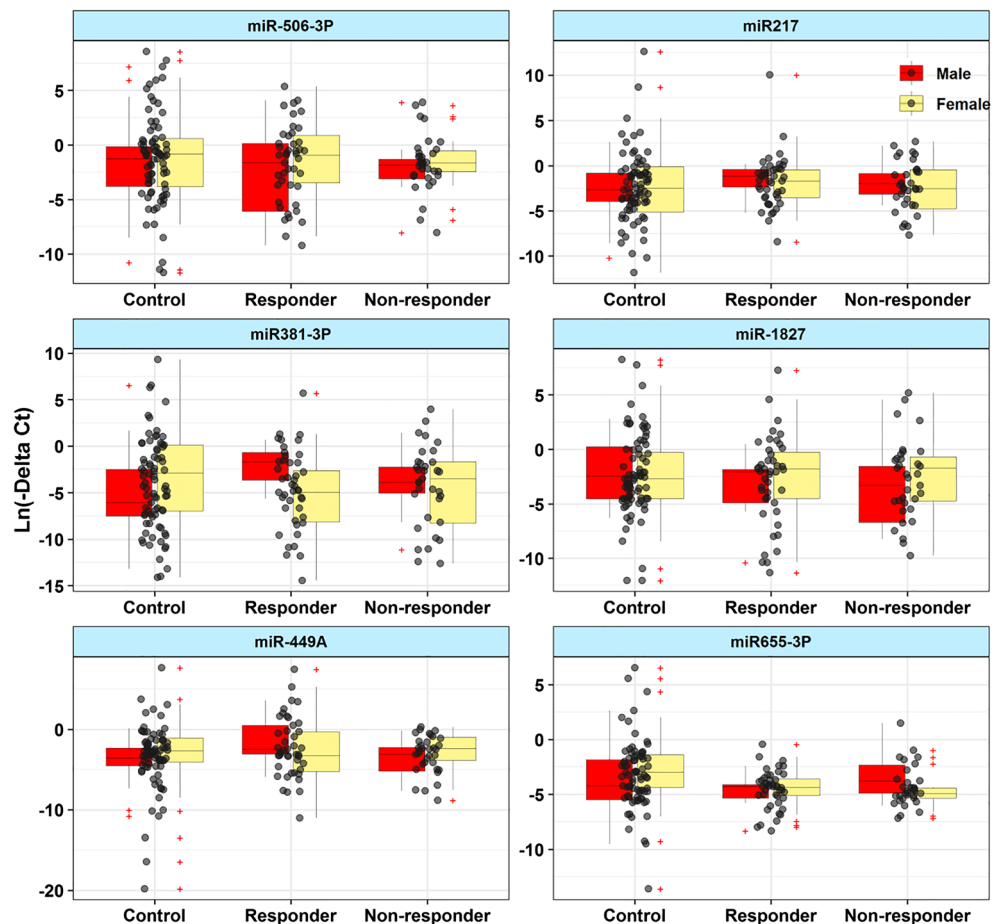


Table 2 Results of Bayesian multilevel model for comparison of relative expression (RE) of miRNAs between MS patients and controls (^areference group: controls)

Gene	Group	RE difference ^a	SE	P value	95% Credible interval for RE difference
miR-506-3p	Responder	-0.249	0.579	0.991	[-1.43, 0.89]
	Non-responder	-0.816	0.568	0.084	[-1.93, 0.3]
miR-217	Responder	0.858	0.62	0.266	[-0.4, 2.03]
	Non-responder	0.233	0.627	0.874	[-0.99, 1.47]
miR-381-3p	Responder	3.903	1.333	0.005	[1.13, 6.45]
	Non-responder	1.984	1.336	0.715	[-0.63, 4.66]
miR-1827	Responder	0.546	0.633	0.4	[-0.78, 1.71]
	Non-responder	-0.006	0.847	0.958	[-1.69, 1.59]
miR-449a	Responder	0.546	0.633	0.866	[-0.78, 1.71]
	Non-responder	-0.006	0.847	0.575	[-1.69, 1.59]
miR-655-3p	Responder	-1.03	0.41	0.014	[-1.85, -0.21]
	Non-responder	-1.41	0.3	<0.0001	[-2, -0.82]

a putative role in the regulation of immune responses, pathobiology of MS or effects of fingolimod. For instance, miRNA-506 can directly bind with its 3'-untranslated region (3'-UTR) of STAT3, thus inhibiting its expression (Su et al. 2019). STAT3 is involved in the process of fingolimod-mediated protection against ischemic white matter damage (Qin et al. 2017). miR-217 and miR-1827 are among the miRNAs found to modulate innate and adaptive immune responses through different mechanisms including modulation of STAT3 expression (Felix et al. 2019). miRNA-381 is a known negative regulator of toll-like receptor 4 (TLR4) signaling (Xu et al. 2015) and suppressor of antigen presentation by dendritic cells (Wen et al. 2016). miR-449a has a role in activation of CD4⁺ T cells during

induction of alloimmune reactions through modulation of metabolic status (Huang et al. 2018). Finally, miR-655-3p is possibly involved in the suppression of immune responses by cyclooxygenase 2 (COX-2) (Majumder et al. 2018).

Materials and Methods

Enrolled individuals

The current investigation was conducted in 78 patients with MS and 79 healthy subjects. MS patients were assessed in Farshchian Hospital, Hamadan, Iran. Poser's criteria

Table 3 Results of Bayesian multilevel model for comparison of relative expression (RE) of miRNAs between responders and non-responders (^anon-responder-responder)

Gene	Group	RE difference ^a	SE	P value	95% Credible interval for RE difference
miR-506-3p	Total	-0.551	0.649	0.435	[-1.79, 0.78]
	Male	-0.304	1.697	0.912	[-3.32, 3.38]
	Female	-0.32	0.764	0.419	[-1.74, 1.27]
miR-217	Total	-0.631	0.676	0.371	[-1.94, 0.7]
	Male	-0.313	0.999	0.561	[-2.25, 1.66]
	Female	-0.503	0.928	0.321	[-2.31, 1.29]
miR-381-3p	Total	0.747	0.983	0.518	[-1.17, 2.66]
	Male	-1.89	1.4	0.198	[-4.66, 0.86]
	Female	1.384	1.112	0.375	[-0.81, 3.54]
miR-1827	Total	-0.529	0.891	0.574	[-2.29, 1.16]
	Male	-0.129	1.536	0.583	[-3.17, 2.93]
	Female	0.014	0.985	0.942	[-2.03, 1.85]
miR-449a	Total	-0.529	0.891	0.809	[-2.29, 1.16]
	Male	-2.274	1.22	0.619	[-4.55, 0.2]
	Female	0.396	0.842	0.173	[-1.31, 2]
miR-655-3p	Total	-0.529	0.891	0.518	[-2.29, 1.16]
	Male	0.638	0.88	0.49	[-0.95, 2.45]
	Female	0.396	0.842	0.158	[-1.31, 2]

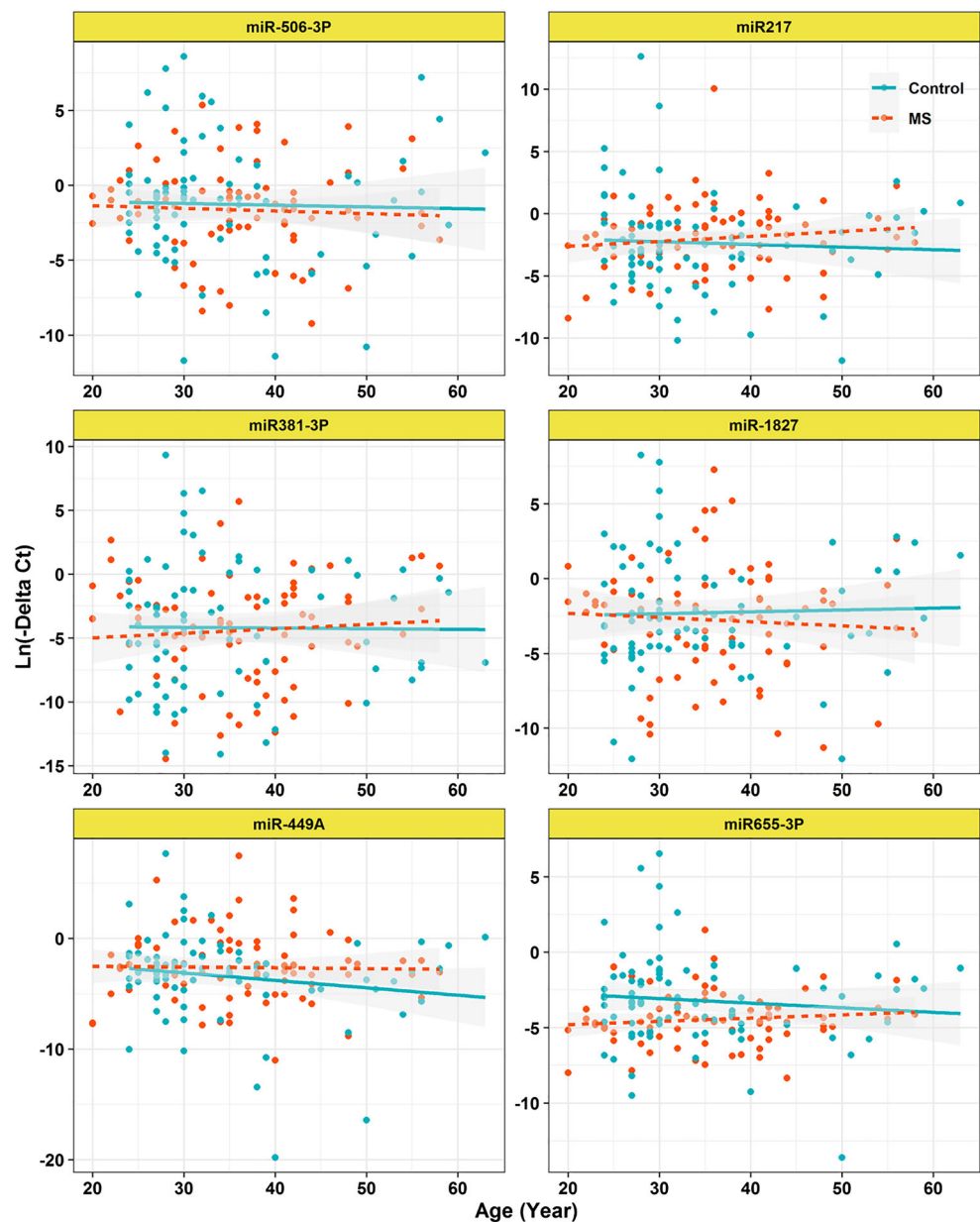
(Fangerau et al. 2004) were used for assessment and diagnosis. All MS patients were being treated with 0.5 mg fingolimod administered orally once daily for at least 6 months. Subsequently, they were recognized as responders if there was no increase in the Expanded Disability Status Scale (EDSS) score, no relapse during the follow-up period (Eftekharian et al. 2019) and no evidence of disease activity based on the MRI data. Occurrence of at least one relapse during this period or a one-point increase in the EDSS score (lasting for 6 months) were regarded as indication of non-responsiveness. In total, 49 patients met the criteria for responsiveness, while 29 patients were regarded as non-responders. Controls were selected from age-matched healthy volunteers who had no history of inflammatory or chronic diseases or

malignancy. The study protocol was approved by the local ethics committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1397.610). Written informed consent forms were obtained from all study participants.

Expression analysis

Three milliliters of peripheral blood were obtained from all enrolled individuals. These samples were used for RNA extraction using the Hybrid-R™ Blood RNA Kit (GeneAll, Seoul, South Korea). First-strand cDNA was synthesized from RNA samples using the TaqMan® MicroRNA Reverse Transcription Kit (ABI, Forest City, CA, USA). Expression of miRNAs was measured using the TaqMan® MicroRNA

Fig. 2 Correlation between expression of miRNAs and age of study participants



Assay (ABI, Forest City, CA, USA). The *RNU6B* gene was used as normalizer. Amplification was carried out in duplicate using the Corbett Rotor-Gene 6000 real-time PCR system.

Statistical analysis

The packages "quantreg" and "brms" in R 3.4.2 software were used for statistical assays. The relative expression (RE) of each miRNA in each sample was calculated using the $\text{Ln}(\text{Efficiency}^{-\Delta\text{CT}})$ formulation. Mean values of miRNA expression were compared between study subgroups using a Bayesian multilevel model using the probabilistic programming language Stan. Two-sided 95% credible intervals (95% CrI) were used to determine the significance of differences. The Spearman rank correlation coefficient was measured to evaluate correlations between miRNA expression and patient age.

Results

Demographic information for MS patients and controls

Demographic information for MS patients and controls is summarized in Table 1.

Relative expression of miRNAs in MS patients and controls

Figure 1 shows the relative expression of miRNAs in MS patients (responders and non-responders) and controls.

The expression of miR-381-3p was significantly higher in responders than in controls (RE difference = 3.903, $P = 0.005$), while expression of miR-655-3p was significantly lower in both responders and non-responders compared with controls (RE difference = -1.03, $P = 0.014$; RE difference = -1.41, $P < 0.0001$, respectively). The expression of other miRNAs did not differ between study subgroups. Table 2 shows the results of Bayesian multilevel modeling for comparison of the RE of miRNAs between MS patients and controls.

There was no significant difference in the expression of any miRNA between total responders and total non-responders. Moreover, no difference was found in the expression of any miRNA when expression of miRNAs in responder cases were compared with sex-matched non-responders (Table 3).

There was no correlation between the expression of any of the miRNAs and the age of study participants (Fig. 2).

Significant pairwise correlations were found between expression levels of all assessed miRNAs in controls, with the

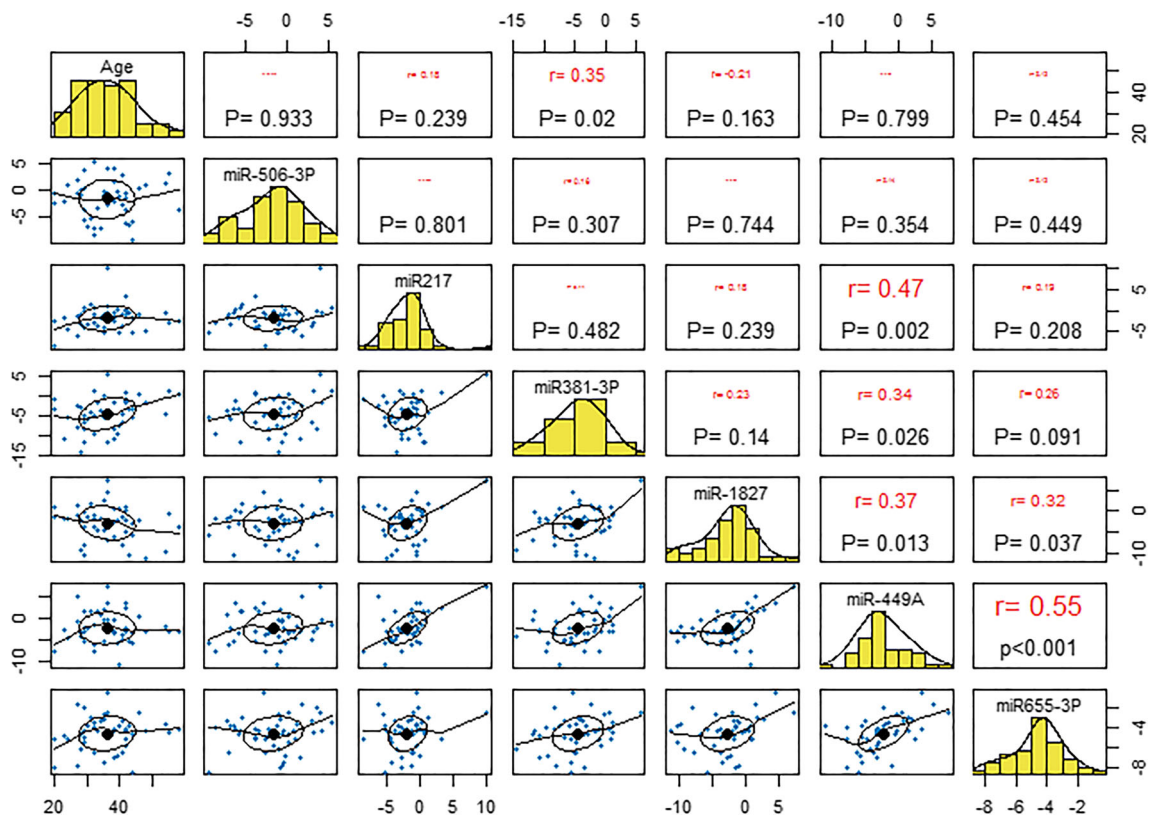


Fig. 3 Scatter matrix depicting correlation between expression of miRNAs in responders. The diagonal presents the distribution of expression values for each miRNA. The left diagonal has a scatterplot

of the two variables above it and to its right; the right diagonal shows the correlation coefficient for the same variables, with the font size increasing for higher correlation

most robust correlation being detected between miR-1827 and miR-655-3p.

However, MS patients exhibited differing correlation patterns. Expression of miR-381-3p was correlated with the age of responders, while expression of miR-449a was correlated with the expression of miR-1827, miR-381-3p, miR-217 and miR-655-3p in responders. Expression of miR-655-3p was also correlated with the expression of miR-1827 in these patients (Figure 3).

No significant correlation was detected between expression of any miRNA and age in non-responders. There were significant correlations between the following miRNA pairs: miR-506-3p/miR-381-3p, miR-381-3p/miR-217, miR-506-3p/miR-449a, miR-217/miR-449-3a, miR-217/miR-655-3p and miR-1827/miR-655-3p (Fig. 4).

Discussion

In the present case–control study, we compared the expression of six immune-response-associated miRNAs between fingolimod-responder MS patients, fingolimod-non-responder MS patients and healthy subjects. We found higher levels of miR-381-3p expression in MS patients who responded to fingolimod compared with healthy controls.

However, no difference in expression of this miRNA was found between non-responders and healthy controls. miR-381-3p binds with the 3'-UTR of the *CD1c* gene, thus suppressing its translation (Wen et al. 2016). CD1c is a transmembrane glycoprotein that participates in the presentation of lipid and glycolipid antigens of self or microbial sources to T cells (Posch and Hurley 2011). A previous study indicated that fingolimod was associated with a reduction in both the proliferation of CD1c⁺ dendritic cells and the release of proinflammatory cytokines from these cells (Luessi et al. 2015). Thus, the higher expression of miR-381-3p in fingolimod responders than in healthy controls might be associated with lower levels of CD1c in these patients compared with controls. Consequently, it is possible that the effect of fingolimod on CD1c expression is mediated through its effects on the expression of miR-381-3p. Meanwhile, miRNA-381 has been shown to be involved in the downregulation of TLR4 signaling (Xu et al. 2015). Our results are in accordance with the results of Andersson et al., who reported upregulation of TLR4 and its endogenous ligand in the CSF mononuclear cells of MS patients compared with healthy subjects (Andersson et al. 2008). However, Haegert et al. showed an unexpected correlation between increased TLR4 expression and a positive response of MS patients to fingolimod (Haegert et al. 2018). Thus, the correlation between miR-

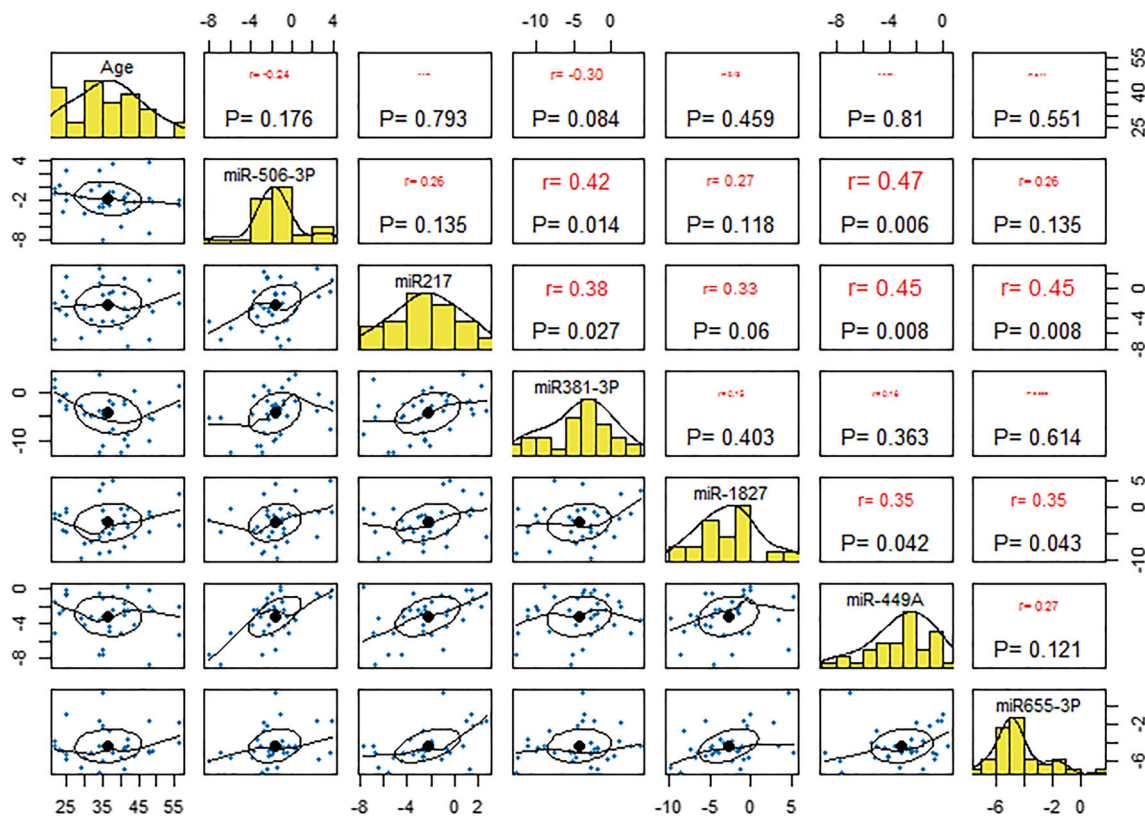


Fig. 4 Scatter matrix depicting correlation between expression of miRNAs in non-responders. The diagonal shows the distribution of expression values for each miRNA. The left diagonal has a scatterplot

of the two variables above it and to its right; the right diagonal shows the correlation coefficient for the same variables, with the font size increasing for higher correlation

381-3p, TLR4 and response to fingolimod should be assessed in future studies.

We also detected lower levels of miR-655-3p in both responders and non-responders compared with controls. Downregulation of this miRNA has been associated with malignant phenotype in hepatocellular carcinoma and gastric cancer (Zhao et al. 2017; Wu et al. 2016; Alsaadoni et al. 2019). Moreover, this miRNA may be involved in the suppression of immune responses by COX-2 (Majumder et al. 2018). COX is involved in different pathogenic events in the evolution of MS, and COX-2 inhibitors have been effective in reversing these processes in animal models (Palumbo 2017). The downregulation of miR-655-3p observed in MS patients undergoing treatment with fingolimod suggests an effect of the drug on the attenuation of COX-2-mediated neuro-inflammatory processes. However, future functional studies are needed to verify this speculation.

Although there were significant pairwise correlations between expression levels of all assessed miRNAs in controls, MS patients exhibited differing correlation patterns. These differences might be due to the presence of disease-associated mechanisms, or alternatively may reflect the distinct effects of fingolimod in responders and non-responders.

Expression of miR-381-3p was correlated with age in fingolimod responders but not in the other study subgroups. The practical significance of this finding should be assessed in future studies. Expression of other miRNAs was not correlated with age in any study subgroup.

Taken together, the results of the current study indicate possible roles for miR-655-3p and miR-381-3p in the pathogenesis of MS or possible effects of fingolimod on the expression of these miRNAs. Future studies are needed to verify these results in larger patient populations including drug-naïve patients.

Our study has some limitations. First, there were no in vivo or in vitro data to support the meaningful impact of these alterations in the context of MS. Moreover, we did not assess expression of miRNAs in peripheral blood mononuclear cells or individual immune cell subsets. Whole blood miRNA measurements are not functionally meaningful for elucidation of key cellular and immune events that are responsible for driving disease pathology or responsiveness to therapy in MS. Finally, we did not assess expression of other miRNAs that have been reported to influence response to fingolimod.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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