Gene 659 (2018) 109–117

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Analysis of the expression of mir-34a, mir-199a, mir-30c and mir-19a in peripheral blood CD4 + T lymphocytes of relapsing-remitting multiple sclerosis patients

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ARTICLE INFO

Keywords:
mir-19a
miR-30c
miR-34a
miR-199
Multiple sclerosis
Th17

ABSTRACT

Background: Multiple sclerosis is an immune-mediated inflammatory disease of central nervous system. MicroRNAs play important roles in autoimmune diseases such as MS.
Objectives: The aim was to evaluate the expression pattern of miR-34a, miR-199a, miR-30c and miR-19a in peripheral blood derived CD4+ T lymphocytes of both relapsing and remitting phases of MS.
Methods: Blood samples from 40 RRMS patients (20 in relapsing and 20 in remitting phase) and 20 healthy volunteers were taken. CD4+ T cells were isolated. The expression level of miR-34a, miR-199a, miR-30c and miR-19a, and the percentage of Th17 and Treg cells were measured. Expression of master transcription factors of Th17 and Treg cells and several targets of these miRNAs were also evaluated.
Results: Data indicated an increased expression of miR-34a, miR-30c and miR-19a in relapsing phase and decreased expression of miR-199a in remitting phase. ROC curve data add other prestigious information of miR-34a, miR-199a, miR-30c and miR-19a by defining relapsing and remitting phase and also healthy cases with high specificity and sensitivity at a proposed optimum cut-off point.
Conclusion: Collectively, we showed a correlation between the four miRNAs with different phases of MS and their possible involvement in differentiation pathways of Th17 cells, as the most important players in MS.

1. Introduction

Multiple sclerosis (MS), also known as disseminated sclerosis or encephalomyelitis disseminate, is an inflammatory autoimmune disease which severely affects people globally. MS disease onset normally occurs in young adults, and like other autoimmune diseases, it is more common in women rather than men (Milo and Kahana, 2010; Etemadifar et al., 2014). As a result of this disease, the insulating covers of nerve cells in the brain and spinal cord are damaged.

Epidemiologic studies of MS in Isfahan province of Iran, indicates that there is a harsh growth in the frequency of affected patients with an overall prevalence of 85.8 in 10^5 (Etemadifar et al., 2014). MS is

Abbreviations: CD, cluster of differentiation; Foxp3, forkhead box P3; IBD, inflammatory bowel disease; IL, interleukin; MiR, micro RNA; MS, multiple sclerosis; RA, rheumatoid arthritis; RORγt, retinoid-related orphan receptor gamma t; SMAD, mothers against decapentaplegic homolog; SOCS3, suppressor of cytokine signalling 3; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; Th, T-helper cell; Treg, regulatory T cell; UTR, un-translated region

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https://doi.org/10.1016/j.gene.2018.03.035
Received 28 October 2017; Received in revised form 13 January 2018; Accepted 14 March 2018
Available online 15 March 2018
0378-1119/

considered as a multi-factorial disease, and it is still unclear that how the disease initiates and becomes chronic. The underlying mechanism is supposed to be either destruction by the immune system or failure of the myelin-producing cells. Possible causes for this include genetics and environmental factors such as infections (Milo and Kahana, 2010). It has been proven that T-cell-mediated inflammation is critical in the pathogenesis of MS (Lassmann, 2006; Bendszus and Storch-Hagenlocher, 2013). It is now believed that Th17 cells essentially exert their effects via secreting IL-21, IL-22, IL-17 and GM-CSF, which are necessary for autoimmune neuro-inflammation. In the presence of TGF-β, IL-1β and IL-23, and following the activation of specific transcription factors of RORγt/STAT3, naïve $CD4^+$ T cells differentiate into Th17 subset (Kebir et al., 2007; Zhu and Paul, 2010; Hakemi et al., 2011). Similar to the other autoimmune diseases, MS is neatly related to deficiencies in immune regulation through suppression of regulatory T cells (Treg) (Kebir et al., 2007) as their differentiation occurs through activation of STAT5/Foxp3 (Bartel, 2009).

MicroRNAs (miRNAs) are a set of small non-coding RNAs that bind to 3′ UTR region of their target mRNAs and can either repress translation or induce mRNAs degradation (Bartel, 2009). Plenty of studies have shown that posttranscriptional regulation by miRNAs is an important procedure in differentiation pathways and in a lot of other biological processes (Baltimore et al., 2008; Liu et al., 2010). For example, miRNAs possess vital roles in haematopoiesis and functions of immune cells including T cells (Baltimore et al., 2008; Zibert et al., 2010). Mir-34 precursor family set grounds for production of three major mature miRNAs in mammals. These family members are located on chromosome 1 and 11 (Hermeking, 2010). The precursor miRNA stem-loop is processed in the cytoplasm of the cell, and the predominant miR-34 mature sequence is excised from the 5′ arm of the hairpin (Mráz et al., 2009). Meanwhile, miR-199 is related to miR-214 and both miRNAs are transcribed together in the form of a common family (Loebel et al., 2005). The miR-199 family is located on chromosomes 19 and 1 (Chen et al., 2008). MiR-199a/214 cluster genes are encoded by the DNM30s (Dynamin 3 opposite strand). MiR-30c in humans and vertebrate cells plays an important role in intracellular functions (Lagos-Quintana et al., 2001). The miR-30c locus is found on chromosome 6 and 1 (Weber, 2005). Pre-construction products of miR-30 have a regulatory effect on a number of mRNAs. The cluster of miR-17-92, codes 6 mature miRNAs that one of them is miRNA-19 family (Ambros, 2001). MiR-19a family has been identified in vertebrates and consists of 89 different sequences, and is located on chromosome 13 in human. This is involved in the regulation of gene expression (Houbaviy et al., 2003).

Various studies on miR-34a, miR-199a, miR-30c and miR-19a show that these miRNAs are involved in different autoimmune diseases and cancers. Studies on inflammatory bowel disease (IBD), psoriasis, rheumatoid arthritis (RA) and other immune-related diseases have revealed deregulation of miR-34a, miR-199a, miR-30c and miR-19a in patients versus healthy groups (Chen et al., 2007; Iborra et al., 2013; Zibert et al., 2010). For example, miR-30c was reduced in samples taken from superficial skin lesions of patients with Psoriasis (Sonkoly et al., 2007) and had impaired regulation in biopsies of patients with IBD (Fasseu et al., 2010) and in the plasma samples of RA patients (Murata et al., 2013). Biopsy samples from patients with the impaired regulation of miR-19a also indicate that this set of genes get involved in development of autoimmune and lymphoproliferative disorders in rats, and lupus-like autoimmunity (Rigby and Vinuesa, 2008; Xiao et al., 2008). In one study, regulation of miR-19a was impaired in biopsy specimens from patients with IBD (Iborra et al., 2013). Hence, these microRNAs may have similar immunopathogenic roles in MS.

The most important players in immunopathogenesis of many autoimmune diseases like MS are Th17 cells. The miR-34a, miR-199a, miR-30c and miR-19a might have a role in differentiation of Th17 cells. Thus, their deregulation could be consistent with the increase or decrease the number of Th17 cells during relapsing and remitting phases of MS.

The aim of the current study was to evaluate miR-34a, miR-199a, miR-30c and miR-19a expression levels in $CD4^+$ T cells of relapsing versus remitting phase of MS patients. Through this, we tried to pinpoint the miRNAs potential role in differentiation or suppression of Treg or Th17 cells via assessing their correlation in different phases of MS. Moreover, possible targets of miR-34a, miR-199a, miR-30c and miR-19a that could be considered to be involved in differentiation of T helper cells were established *in-silico*, and their accuracy was studied in vitro.

2. Materials and methods

2.1. Patients and control samples

Informed consent was obtained for experimentation with human subjects before sampling. This study was approved by the Institutional Medical Ethics Committee of Royan Institute, Tehran, Iran. All clinical information of patients including age, sex, disease duration and MRI results are summarized in Supplementary Table 1. Ten mL blood samples (with EDTA) were collected from 20 patients with relapsing and 20 with remitting MS had been referred to Hasht-behesht Policlinic in Isfahan. Sampling was done when the patients were newly diagnosed (as relapsing phase) and one week after their first injection, just before they receive their second dose of medication, (as remitting phase). Moreover, 20 control blood samples were obtained from healthy volunteers. Patients were diagnosed based on Mc-Donald criteria in MS by a neurologist.

2.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient Lymphoprep (STEMCELL Technologies, USA) according to the manufacturer's instructions. $CD4^+$ T cells sub-population were isolated from PBMCs using human $CD4^+$ T cells isolation kit II (Germany, Miltenyi Biotec) according to the manufacturer's instructions.

2.3. Flow cytometry analysis of T cells

Cells were analyzed for FOXP3, RORγt and CD4 expression using flow cytometry method. Isolated cells were stained with Anti-Human/Mouse RORγ (t)-PE, Anti-Human Foxp3-PE, Anti-Human CD3 FITC and Anti-Human CD4-Alexa Fluor 488 (eBioscience, USA) antibodies and were analyzed with Becton Dickinson flow cytometer (FACSCalibur, USA) using Cell Quest Pro software. Then, the data were analyzed versus Mouse IgG1 K isotype Control-PE, Rat IgG2a K isotype Control-PE, Mouse IgG2b K isotype Control-Alexa Fluor 488 (eBioscience) and unstained cells.

2.4. Gene expression evaluation with real-time PCR

Total RNA (including miRNAs) was isolated using TRizol reagent (Invitrogen, USA) based on the manufacturer's instruction. The cDNA synthesis for miR-34a, miR-199a, miR-30c and miR-19a was performed with a “universal cDNA synthesis kit” (Exiqon, Denmark) using a poly-A tailing method, as stated by manufacturer. Quantitative real-time PCR (Q-PCR) reactions were performed using standard protocols in an ABI PRISM 7500 instrument (Applied Biosystems, USA). Briefly, in a total volume of 10 μL, 20 ng/μL of cDNA products were added to a master mix comprising 10 pmol/μL of the miRNAs pre-designed primers (Exeqon, Denmark) and 2 U of SYBR premix ExTaq II (TaKaRa, Japan). The run method program was set at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s,60 °C for 20 s and 72 °C for 30 s. RNU48, small nucleolar RNA, was quantified as a control to normalize the results. Synthesis of cDNA for *RORC2*, *TGF-β*, *IL-17A* and *FOXP3* on total RNA

was carried out using Revert Aid First Strand cDNA synthesis Kit (Fermentas, Lithuania) using random hexamer primers. Q-PCR was performed using specific pre-designed primer pairs in ABI PRISM 7500 instrument (Applied Biosystems, USA). The run method program was set at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 30 s. Expression levels of these genes were normalized against 18srRNA as the reference gene. All reactions were carried out in triplicate. Data were evaluated and reported based on relative quantification method (Ma et al., 2014).

2.5. Poly acrylamide gel & T/A cloning

To specify the miR-34a, miR-199a, miR-30c and miR-19a primers, Q-PCR products were run on 12% polyacrylamide gel to see solo band with approximate size of 80 bp (data not shown). Samples were diluted in sample buffer (Tris-HCl 0.5 mM, pH: 6.8, 20%glycerol, 0.025% blue bromophenol) and were loaded on poly acrylamide gel. Product bands were observed as the sole bands of Q-PCR. To further confirm this result, T/A cloning was performed. Briefly, Q-PCR products were cloned into pTZ57R/T (Fermentas, Lithuania) and transformed into competent *E. coli* cells. After selection of the appropriate recombinant colonies, plasmid preparation was performed and one clone of each miRNA was selected for sequencing.

2.6. Molecular signalling pathway enrichment analysis

In order to accomplish molecular enrichment analysis on miR-34a, miR-199a, miR-30c and miR-19a targetome and to find the most related signalling pathways that might be involved, we used online *in-silico* databases like miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) (Dweep et al., 2011) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.phpA) (Hsu et al., 2010) to attain predicted and validated targets of miR-34a, miR-199a, miR-30c and miR-19a. We also used DIANA miRPath v.2.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index) (Vlachos et al., 2012) to visualize pathways of validated targets of miR-34a, miR-199a, miR-30c and miR-19a as a heat map. Then, using UniGene database (http://www.ncbi.nlm.nih.gov/unigene/) and subsequent EST profiling, we studied their expression in lymph nodes and thymus since it will be indispensable if they are involved in differentiation of T helper cells. Finally, miR-34a, miR-199a, miR-30c and miR-19a targetome expressed in lymph nodes and thymus were assigned to DAVID bioinformatics database (https://david.ncifcrf.gov/) (Delgoffe et al., 2011), to categorize the most applicable pathways and molecular networks. The signalling pathways analyzed were:

1. JAK/STAT signalling pathway and the effects of miR-34a, miR-199a and miR-30c on it (DAVID; Fig. 1).
2. MTOR signalling pathway and the effects of miR-34a and miR-19a on it (DAVID, Fig. 2).
3. TGF-β signalling pathway and the effects of miR-34a, miR-30c and miR-19a on it (DAVID, Fig. 3).

2.7. Statistical analysis

All statistical tests were performed using SPSS 20 (Chicago, IL, USA). Comparison between groups was carried out using one way ANOVA test. Data are presented as mean ± SEM and considered significant at $p < 0.05$. In order to measure and achieve the potential of miR-34a, miR-199a, miR-30c and miR-19a as diagnosis accurate factors of diseases to distinguish MS relapsing and remitting phases and healthy persons, receiver operating characteristic (ROC) curves were performed and the area under the curve (AUC) was gauged by calculating sensitivity and specificity for each possible cut-off point of the miR-34a, miR-199a, miR-30c and miR-19a expression level. The p value 0.05 was considered as statistical significance for all tests.

3. Results

3.1. The number of Th17 cells increased significantly in relapsing phase, while Treg cells increased in remission phase of MS

At the first stage, we evaluated the number of Th17 and Treg cells in different phases of MS. Results have revealed that the percentage of $RORC2^+$ $CD4^+$ T cells was significantly increased in relapsing group as compared to the remitting and the healthy group as controls ($p = 0.0002$, $p = 0.0003$, respectively), while the remitting phase patients displayed a significant increase in the percentage of $Foxp3^+$ $CD4^+$ T cells compared to the patients in the relapsing ($p = 0.003$) and healthy controls ($p = 0.001$) (Fig. 4).

3.2. Expression of master transcription factors involved in Th17/Treg differentiation along with IL-17A gene expression in CD4+ T cells

To confirm the results of flow cytometry, we analyzed whether the expression level of master markers of Th17 and Treg cells have changed during the course of the disease from relapsing to remitting. Expression level of IL-17A was significantly increased in relapsing phase compared to remitting phase ($p = 0.023$). Analysis of RORC expression demonstrated a significant rise in relapsing group compared to both remitting and control groups ($p = 0.0007$ and $p = 0.002$, respectively). TGF-β expression level showed a significant increase in relapsing versus control group ($p = 0.008$). However, it was also up regulated in remitting group versus control group ($p = 0.029$). Finally, the expression level of

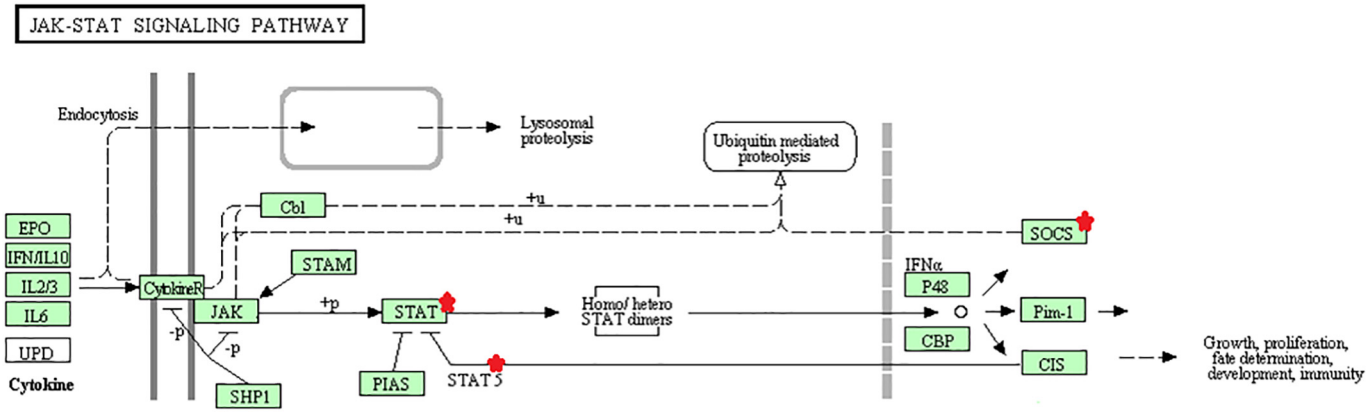

Fig. 1. The miR-34a, miR-199a, miR-30c and miR-19a targetome has joint several important pathways including TGF-β, JAK/STAT and mTORC. Their partial diagram is demonstrated which selected from KEGG pathway. JAK/STAT pathway is a joint one for miR-34a, miR-199a and miR-30c. The targets of these mRNAs are SOCS and STAT5.

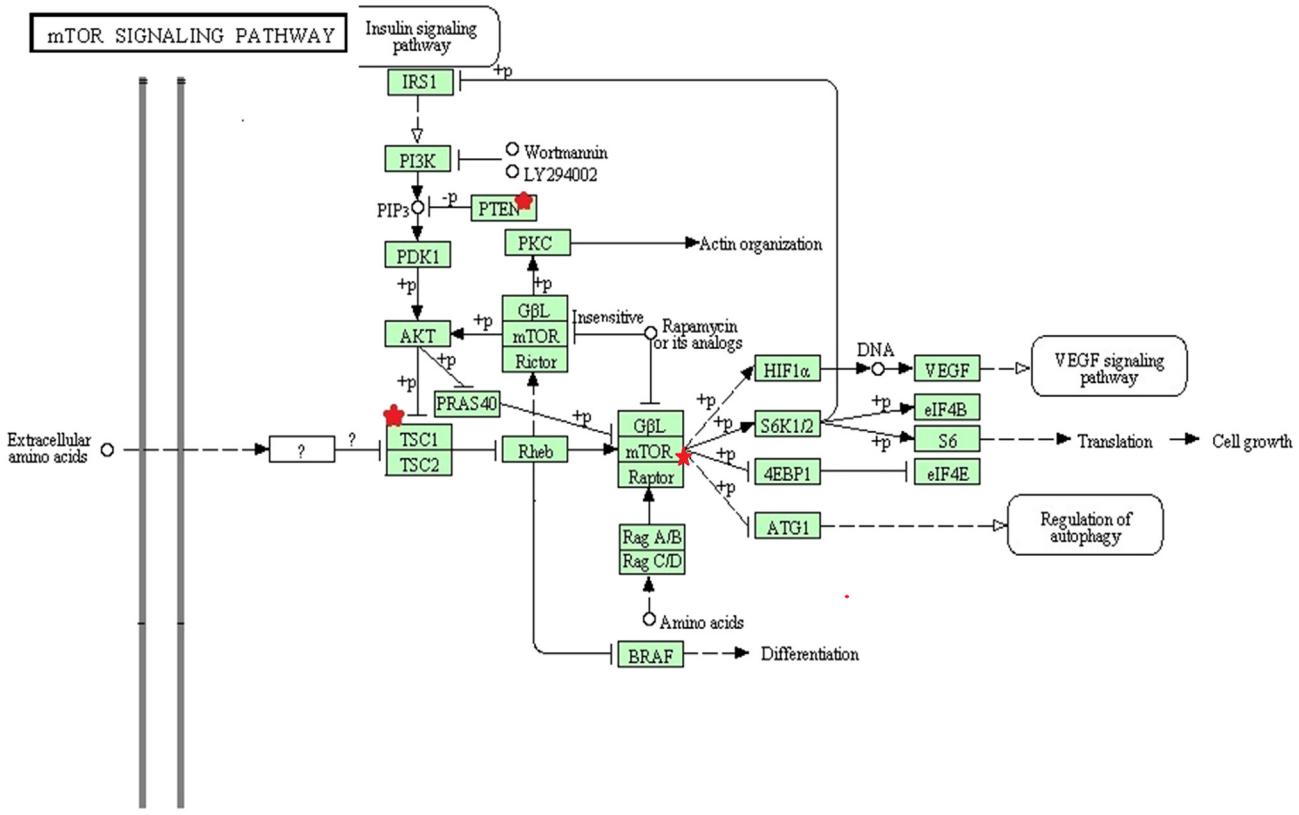

Fig. 2. The mTORC pathway is introduced as a joint pathway between miR-34a and miR-19a. The targets of these mRNA are Tsc1, mTOR and PTEN, as indicated with red stars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

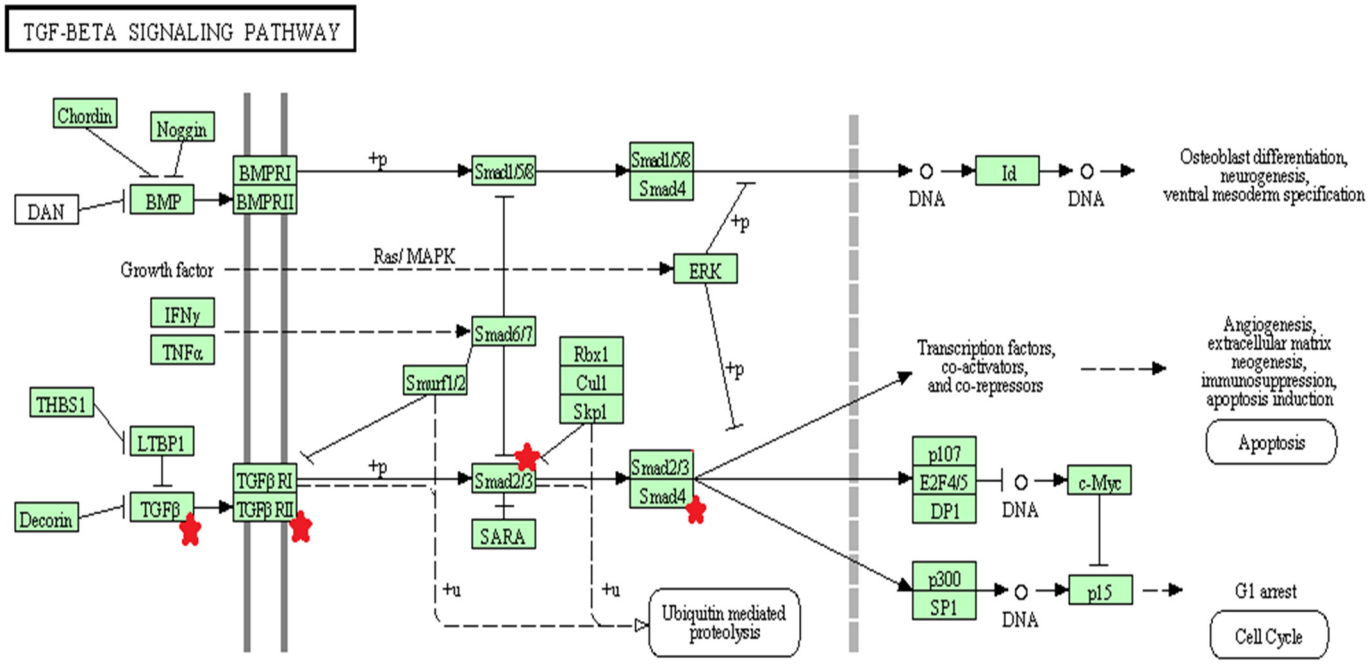

Fig. 3. TGF-β pathway is selected as another pathway for each miR34a, miR-30 and miR-19a. The targets of these mRNAs are TGF-βR1, SMAD2, 3 and 4, as indicated with red stars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Foxp3 was significantly decreased in relapsing compared to control group ($p = 0.046$).

3.3. Up-regulation of miR-34a, miR-30c, miR-19a and down-regulation of miR-199a in relapsing phase of MS

The expression level of miR-34a was significantly higher in relapsing group compared to control and remitting groups ($p = 0.043$ and $p = 0.031$, respectively). Nevertheless, no meaningful difference was

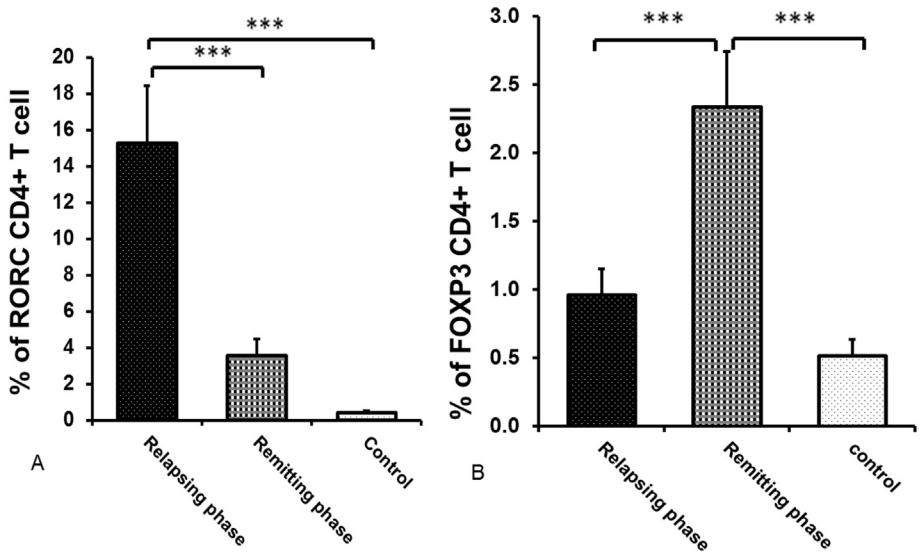

Fig. 4. Flow cytometric analysis of FoxP3$^+$ CD4$^+$ T cells and RORC2+ CD4+ T cells. CD4$^+$ T cells were isolated with human CD4+ T cell isolation kit II and stained with respective antibodies and evaluated in relapsing and remitting phases of MS patients and in healthy controls. (a: Relapsing patients (n = 20), b: remitting patients (n = 20), c: healthy volunteers (n = 20).) (A) Percentage of RORγt +CD4+ T cells shows meaningful increase in relapsing group (B) while percentage of FoxP3$^+$ CD4$^+$ T cells were elevated in remitting group (*p < 0.05, **p < 0.01 and ***p < 0.005).

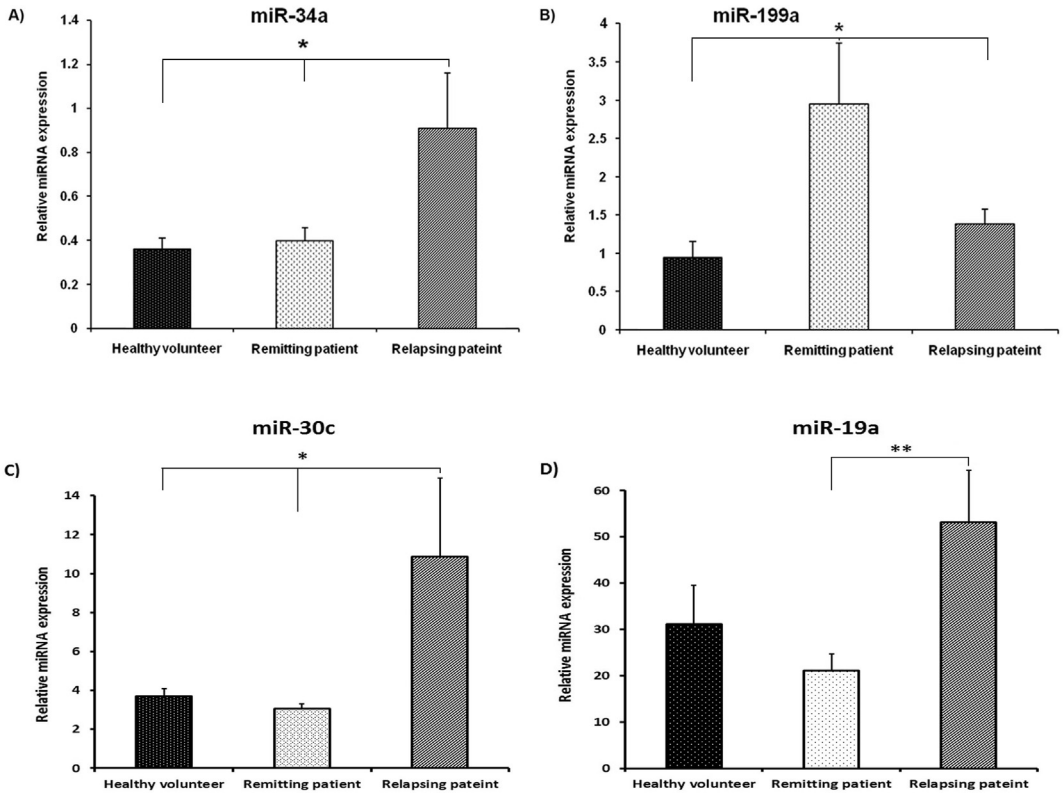

Fig. 5. Up-regulation of miR-34a, miR-30c, miR-19a and down regulation of miR-199a in relapsing phase of MS patients. (A) Expression level of miR-34a in CD4$^+$ T cells of MS patients in relapsing phase (n = 20), remitting phase (n = 20) and healthy controls (n = 20). (B) Expression level of miR-199a in CD4$^+$ T cells of MS patients in the same groups. (C) Expression level of miR-30c in CD4$^+$ T cells of MS patients in relapsing phase. (D) Expression level of miR-19a in CD4$^+$ T cells of MS patients is as the same. Results are normalized versus RNU48 as reference gene (*p < 0.05, **p < 0.01 and ***p < 0.005).

observed between control and remitting groups (p = 0.874) (Fig. 5A).

Expression pattern of miR-199a was opposite to miR-34a expression. MiR-199a expression level elevated significantly in remitting group versus control and relapsing group (p = 0.043 and p = 0.044, respectively). There was also no significant difference between control and relapsing group (p = 0.644) (Fig. 5B).

The expression level of miR-30c was more in relapsing group compared to remitting group and healthy controls (p = 0.014 and p = 0.049, respectively). Nevertheless, there were no meaningful differences between remitting patients and control individuals (p = 0.860) (Fig. 5C).

Transcript level of miR-19a was significantly elevated in relapsing group versus remitting group (p = 0.008) but there was no meaningful difference between healthy volunteers and both remitting and relapsing patients (p = 0.550 and p = 0.186, respectively) (Fig. 5D).

3.4. The potentials of miR-34a, miR-199a, miR-30c and miR-19a as discriminating biomarkers

We compared expression level of miR-34a, miR-199a, miR-30c and

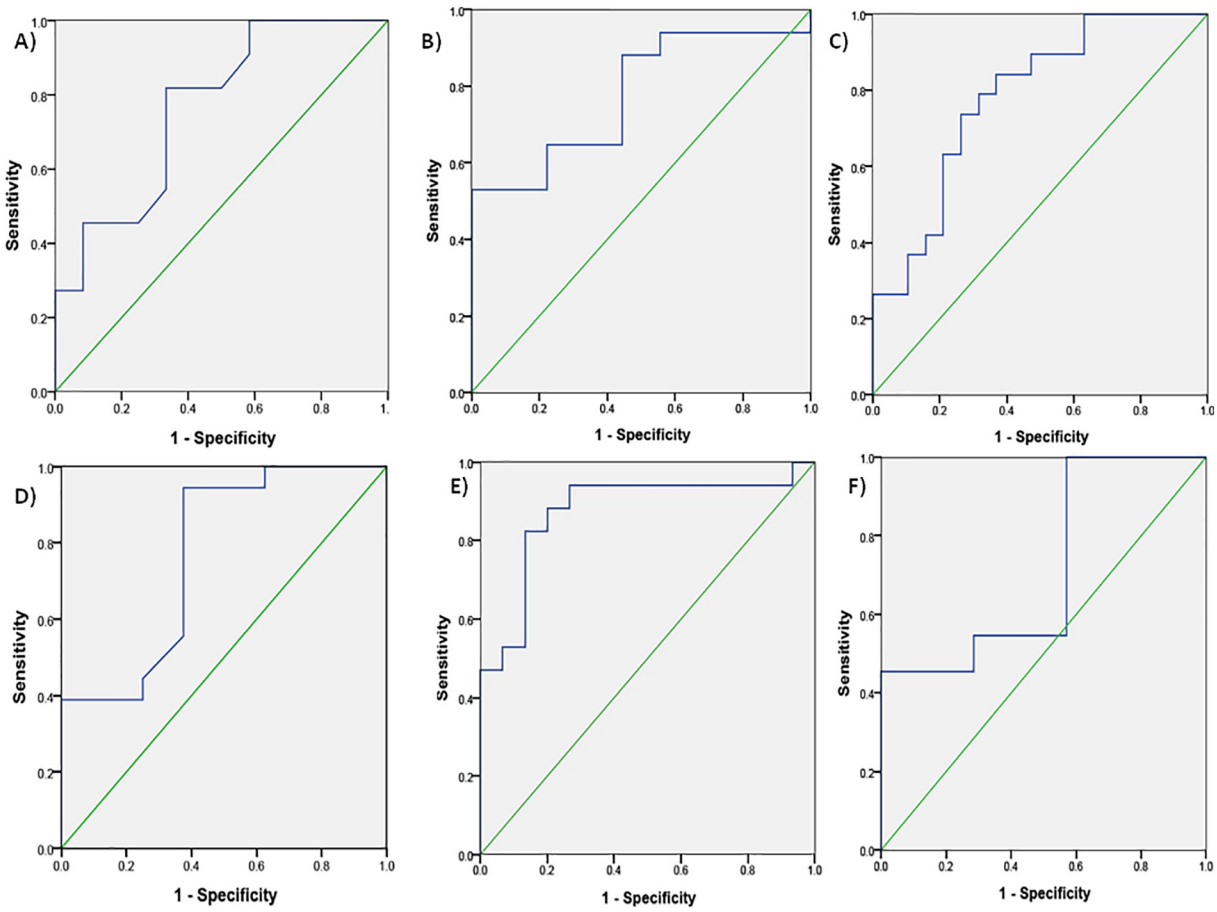

Fig. 6. ROC curve of RR-MS sample sets (relapsing, remitting patients and healthy volunteers) analyzed for relative expression level of miR-34a, miR-199a, miR-30c and miR-19a in CD4+ T cells. These analyses indicated the optimal relative expression level cut off value of 0.13 for miR-34a (A), 1.65 for miR-30c (B) and 6.11 for miR-19a relative (C) to identify remitting and relapsing RR-MS patients. The sensitivities and specificities were 82% and 67% for miR-34a (A), 82% and 87% for miR-30c (B), and 84% and 63% for miR-19a (C). The optimal relative expression level cut-off values were 0.19 for miR-199a (D) and 3.12 for miR-30c relative expression levels (E) to distinguish the patients from healthy persons with the sensitivity and specificity of 94% and 63% (D), and 53% and 100% (E), respectively. At the cut off value of 0.53 (F), miR-34a can distinguish between patients and healthy group with sensitivity and specificity of 46% and 100%, respectively. Areas under the ROC curve of 0.765, 0.875, 0.781, 0.771, 0.778 and 0.714 were respectively belonging to miR-34a, miR-30c, miR-19a, miR-199a, miR-30c and miR-34a (again), regarding to the criteria explained in the materials and methods.

Table 1
ROC analysis of miR-34a, miR-199a, miR-30c and miR-19a for distinguishing between relapsing and remitting phases of MS as well as healthy controls.

miRNA	Cut off value	Sensitivity	Specificity	Area under the curve	Distinguish between
miR-34a	0.13	82%	67%	0.765	Remit and relapse
miR-34a	0.53	46%	100%	0.714	MS and healthy
miR-199a	0.19	94%	63%	0.771	MS and healthy
miR-30c	1.65	82%	87%	0.875	Remit and relapse
miR-30c	3.12	53%	100%	0.778	MS and healthy
miR-19a	6.11	84%	63%0	0.781	Remit and relapse

miR-19a in CD4+ T cells from the three groups by ROC analysis to recognize the potentials of miR-34a, miR-199a, miR-30c and miR-19a expression level as diagnostic biomarkers for distinguishing among relapsing, remitting and healthy conditions. ROC data characterized the optimal relative expression level cut-off value of 0.13 for miR-34a, 1.65 for miR-30c and 6.11 for miR-19a to identify the remitting and relapsing phases in RR-MS patients (Fig. 6A, B and C, respectively). The optimal relative expression level cut-off values were 0.19 for miR-199a, 3.12 for miR-30c and 0.53 for miR-34a to distinguish the MS patients from healthy persons (Fig. 6D, E and F, respectively). This analysing also demonstrated sensitivity, specificity and area under the ROC curve of all of the studied miRNAs (Table 1).

3.5. Molecular signalling pathway enrichment analysis of miR-34a, miR-199a, miR-30c and miR-19a targetome proposes possible role of miR-34a, miR-199a, miR-30c and miR-19a in differentiation of Th17 cells

To recognize the potential role of miR-34a, miR-199a, miR-30c and miR-19a in Th17 differentiation, molecular signalling pathway enrichment analysis was conducted as described in Materials and methods section. Using miRWalk and miRTarBase databases, for miR-199a, 14,153 predicted and 40 validated mRNA targets were found. Moreover, for miR-34a, 13,547 predicted and 525 validated mRNAs targets were specified. In addition, 8192 predicted and 242 validated mRNAs for miR-30c and 3897 predicted and 41 validated mRNAs for miR-19a were specified (data not shown). All predicted targets in MiRWalk were confirmed through at least five predictor databases. Additionally, all validated mRNA targets collected from miRTarBase had been supported by experimental evidences. Heat map view of validated targets designed by DIANA miRPath v.2.0 showed that the pathways involved in differentiation of Th17 cells, which miR-34a, miR-199a, miR-30c and miR-19aa might influence them, are mTOR signalling pathway and TGF-β signalling pathway. These mRNAs affect SOCS, STAT5 expression, as the negative regulators of Th17 pathway (Xiao et al., 2008; Edwards et al., 2011).

Lastly, validated and predicted genes with defined expression in lymph node and thymus were chosen for extra molecular enrichment analysis (not shown in this paper). A combined view of statistically meaningful pathways associated with above genes in KEGG (http://www.genome.jp/kegg/pathway.html), BIOCARTA (http://www.biocarta.com/), and PANTHER (http://www.pantherdb.org/pathway/) pathways were acknowledged by inputting official gene symbols of miR-34a, miR-199a, miR-30c and miR-19a targetome into the functional annotation tool of DAVID. MiR-199a targetome was commonly enriched in cancer and JAK/STAT signalling pathways. On the other hand, miR-34a targetome was recognized in mTOR, TGF-β pathways and JAK/STAT signalling pathways demonstrating the combined intended schematic KEGG signalling pathways (Supplementary Tables 2 and 3). JAK/STAT signalling pathways, TGF-β pathways, adherent junction, chronic myeloid leukemia, ALK in cardiac myocytes, some cancers and etc. were enriched for miR-30c and miR-19a targetome. The mTORc and JAK/STAT pathways were also selected as other probable pathways for miR-19a and miR-30c. All miR-30c and miR-19a common targets are indicated with red stars in Fig. 2. TGF-β was selected as meant pathway for miR-30c and miR-19a (Fig. 3). In mTOR pathway, miR-34a and miR-19a affect TSC1 and mTOR. These genes are negative regulators of Th17 differentiation. In TGF-β pathway, miR-34a, miR-19a and miR-30c inhibit SMAD2, 3 and 4 genes expression. These genes are negative regulators of Th17 pathway and cause of differentiation to Treg pathway.

4. Discussion

Th17 cells are a subset of CD4+ T cells involving in the defence against extra cellular bacterial and fungal infections. However, they are well-known for their roles in pathogenesis of autoimmune diseases like MS (Tesmer et al., 2008). Therefore, factors that are involved in Th17 cells differentiation are considered as potential therapeutic targets for T cell mediated autoimmune diseases.

Our flow cytometric data showed increased frequency of Th17 cells in relapsing phase while Treg cells were elevated in remission phase. To confirm the results, the expression level of *IL-17A*, *RORC*, *TGF-β* and *FOXP3* in different phases of MS were assessed. Our experiments showed up-regulation of *RORC*, and *IL-17A* in relapsing phase of MS patients. Thus, elevated frequency of Th17 cells in relapsing phase was confirmed. The level of *FOXP3* expression as a master transcription factor of Treg cells has been decreased in relapsing phase compared to healthy controls, whereas there was not a large difference between remitting group and healthy volunteers. This means that normal Treg is necessary to end inflammation in relapsing phase. *TGF-β* was increased in both relapsing and remitting phases of MS which may be correlated with its dual role in differentiation of both Th17 and Treg cells. At high concentrations, *TGF-β* promotes *FOXP3* expression which leads to Treg cell generation, while low concentrations of *TGF-β* stimulates Th17 cell differentiation via RORγt and STAT3 expression (Hakemi et al., 2011; Peters et al., 2011).

In the present study, we evaluated the expression of miR-34a in two phases of MS (remitting and relapsing) compared with healthy individuals. The results showed that the expression of miR-34a was significantly increased in relapsing phase, in comparison with remitting and control group.

Previously, it has been reported that miR-34a is up-regulated in active lesion of MS patients (Junker et al., 2009). This result, along with ours, confirms the possible involvement of miR-34a in suppression of Treg formation and deviation towards Th17 development. The miR-34a influences some target genes such as STAT3, Foxp3 and TGF-β that their involvement in differentiation to Th17 cells was predicted in the current study using miRTarBase database. Elevation of *STAT3* expression in MS patients has previously been reported (Chung et al., 1997; Chen et al., 2007). SOCS3 can inhibit STAT3 that is necessary for differentiation of Th17 cells (Chen et al., 2006). Up-regulation of miR-34a could result in decrease of SOCS3 which leads to increase of STAT3 and elevation of Th17 cells in MS patients. Foxp3, involved in Treg signalling pathway, is another predicted target of miR-34a. Therefore, the more miR-34a expresses the less Treg will generate; as we observed in the present study. Some studies have reported reduced levels of TGF-β in $CD4^+$T cells of MS patients (Acosta-Rodriguez et al., 2007; Lee et al., 2009). Surprisingly, we observed elevated transcript levels of TGF-β1 both in relapsing and remitting phases. However, as TGF-β1 is important for differentiation of both Th17 and Treg cells in a dose dependent manner (Hakemi et al., 2011), these contradictory results could be addressed.

On the other hand, one group has reported that the expression of miR-34a in CD4+ T cells is decreased in patients with RR-MS (Lindberg et al., 2010). As they didn't specify the phase of illness at the time of sampling, this discrepancy might be due to the fact that the evaluation was presumably performed in remitting phase rather than relapsing.

Among the other predicted targets of miR-34a, RUNX3 and RORC are involved in Th17 differentiation pathway (Klunker et al., 2009a; Hakemi et al., 2011). Hence, decreasing of Th17 specific markers could be assumed when miR-34a is elevated in relapsing phase. Nevertheless, this was not happened in the current study. As these targets have not yet been experimentally validated for miR-34a, this conclusion is not necessarily correct.

Also we measured the expression of miR-199a in this study. We showed that the expression of miR-199a was not only significantly increased in remitting phase compare to relapsing phase and controls, but also highly reduced in relapsing phase. Simultaneously, the frequency of Treg cells was also highly elevated in the remitting group. In one study, the expression level of miR-199a found to be dramatically decreased in the PBMCs from patient with MS compared with control group (Ma et al., 2014). Another study has reported that this miRNA is up-regulated in active brain white matter lesions of MS patients (Munoz-Culla et al., 2013). Elevation of miR-199a expression has been shown the in some autoimmune diseases such as atopic eczema (AE) and systematic lupus erythematosus (SLE) (Dai et al., 2007). In EAE (the experimental model of MS), high level of miR-199a was observed (Bergman et al., 2013). These results are contradictory and could not be compared with ours as the phase of illness at the time of sampling is not specified. However, its increase in SLE and AE in which Th17 cells are not the main players, could be consistent with our results in remitting phase.

In one study, the researchers have found that miR-199a by suppressing HIF1α and SIRT1, may play a role in Treg cell differentiation (Rane et al., 2009; Rane et al., 2010). This could be correlated with our findings. Thus, one can conclude that miR-199a can inhibit some genes involving in Th17 differentiation while activate some other genes that are crucial in Treg development.

We showed that the expression of RORγt, a predicted target for miR-199a, was significantly higher in relapsing phase versus remitting phase and healthy controls. RORγt is lineage specific transcription factor for Th17 differentiation. Thus, up-regulation of miR-199a must be correlated with less frequent Th17 cells and lower expression of RORγt in remitting phase, as we showed in the current study.

Also, our results showed a significant increase in expression of miR-30c in patients CD4+ T cells with relapsing phase. A relatively little reduction was observed in expression of miR-30c in remitting patients in compared with healthy controls. In one study by Jarnas M. et al., reduction of miR-30c expression in peripheral blood T cells in RRMS patients was shown (Jernås et al., 2013). Rejerkerk A. et al. in another study also showed that the expression of miR-30c is reduced in lesional capillaries in patients with MS (Reijerkerk et al., 2013). These seem to be contrary with our findings, however, as they have not defined the phase of the illness in the time of sampling, their results might represents the situation exists in remitting phase. As we also find miR-30c reduction in remitting phase, these results may be consistent with ours in this aspect. Moreover, the samples have been used in those studies

were different from ours (PBMC, brain tissue versus CD4+ T cells).

We also showed a significant elevation of miR-19a expression in MS patients with relapsing phase. Similar to miR-30c, a little decrease in the level of miR-19a in remitting phase of MS patients in compared with healthy controls was observed. Menaka C.et al. introduced miR-19a as a miRNA that plays a critical role in CNS function and diseases of the nervous system damage (Thounaojam et al., 2013). This is consistent with our findings. De Santis et al. and Raija L P et al. separately have shown that miR-19a expression had no differences in CD4+ T cell between RRMS patients and healthy volunteers (De Santis et al., 2010; Lindberg et al., 2010). This is not correlated with our results; however, as they didn't specify the phase of the disease at the time of sampling, this could not be necessarily controversial. In addition, Paraboschi et al. showed increased level of miR-19a in PBMC of patients with MS in remission phase (Paraboschi et al., 2011) which is contrary with our findings. However, this may be related to different studied samples (PBMC vs. CD4+ T cells).

According to our bioinformatics surveys, miR-30c and miR-19a target several important negative regulators of Th17 differentiation pathway such as SMAD2, SMAD4, TGFβR2, SOCS3, FOXO3 and TSC1. ETS1, BCL6 and STAT1 are specific targets for miR-30c and STAT5B, RUNX3, TGF-β1 and PTEN are targets for miR-19a. We nominate mTORC and JAK/STAT pathways which are affected by miR-19a and miR-30c, respectively. TSC1and PTEN are negative regulators for Th17 differentiation by mTORC pathway (Sauer et al., 2008; Park et al., 2013). ETS1 is an negative regulator for IL-2 and STAT5 signalling pathway (Moisan et al., 2007). STAT1 and STAT5B are also predicted as Th17 inhibitors. STAT5B is a important protein in Treg differentiation (Ivanov et al., 2007; Laurence et al., 2007). One of the recent studies estimated the BCL6 as a target protein of miR-30c which cause a negative impression of Th17 differentiation (Honardoost et al., 2015). It was also shown that BCL6 protein can repress RORγt expression in CD4 T cells and inhibit Th17 differentiation (Yu et al., 2009). SOCS proteins are the strongest negative regulators in STAT pathways (Krebs and Hilton, 2001). For example, specific deletion of SOCS3 increases STAT3 phosphorylation and IL-17 production (Alexander and Hilton, 2004). FOXO proteins are important factors in differentiation of Treg cells from naïve T cells through binding to FOXP3 promotor (Zhu and Paul, 2010). RUNX3 play as one of the important proteins which induce Treg cells generation (Klunker et al., 2009b). SMAD2, SMAD4 and TGFβR2 are also some downstream targets in TGF-β pathway (Breitkopf et al., 2006). Our investigations suggest that TGF-β pathway is probably affected by both miR-30c and miR-19a. As mentioned before, we witnessed increasing of TGF-β in both relapsing and remitting phases. Nonetheless, due to the increased expression of miR-19a and miR-30c in relapsing phase, it seems that the expression level of TGF-β downstream signalling molecules like SMAD2, SMAD4, TGFβR2 might be decreased in relapsing phase, leading to reduction of Treg cells frequency similar to our previous report (Naghavian et al., 2015). According to our data which confirm Th17 up-regulation in relapsing and Treg up-regulation in remitting phase, it could be suggested that increased expression of miR-30c and miR-19a in relapsing phase might suppresses all these Th17 negative regulators and hence, promotes differentiation towards Th17 cells.

We found that increasing of Th17 cells and decreasing of Treg cells frequency in relapsing phase is correlated with reduction of FOXP3 elevation of RORC, IL-17A and IL-23R. This is also correlated with elevation of miR-30c and miR-19a in the same phase. Based on these findings and the fact that these miRNAs target a number of genes affecting Th17/Treg ratio, we can conclude that these targets (as FOXO3, SOCS3, TSC1) could be suppressed by miR-30c and miR-19a in relapsing phase leading to elevation of Th17 cells. However, this should be proved with more experiments.

Finally, ROC analysis demonstrated a high and reliable ability for miR-34a, miR-30c, miR-19a and miR-199a as potential biomarkers with high sensitivity and specificity for distinguishing between relapsing and remitting phases of RR-MS as well as between illness and healthy condition in this disease. However, it is obvious that more studies in larger scales are needed to confirm this expression for clinical use of miR-34a, miR-199a, miR-30c and miR-199a as diagnosis biomarkers for relapsing period from remitting in RR-MS patients.

5. Conclusion

In the present study, we showed up-regulation of miR-34a, miR-30c, miR-19a and down-regulation of miR-199a in relapsing phase of MS which was correlated with more frequent Th17 cells in the same phase of the illness and severity of the disease. In other word, these miRNAs are differentially involved in Th17 and Treg cells development and hence, influence the disease manifestations. Therefore, miR-34a, miR-199a, miR-30c and miR-19a could now be used as biomarkers for monitoring the relapsing and remitting phases of MS in molecular level and maybe as potential therapeutic targets at near future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.03.035.

Acknowledgements

We wish to thank all the patients and healthy volunteers who took part in this study. Without their help, doing this work was impossible. This work was funded by the Vice Chancellor of Research (grant # 292240) and Cellular & Molecular Immunology Research Center (grant #292282), Isfahan University of Medical Sciences, Isfahan, Iran.

Conflict of interest

There is no conflict of interest to disclose and all authors support submission to this journal.

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