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miR-326 and miR-26a, two potential markers for diagnosis of relapse and remission phases in patient with relapsing–remitting multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis is an inflammatory autoimmune disease widely characterized by myelin destruction of CNS. Th-17 cells, have been demonstrated to play a crucial role in pathogenesis of MS. MicroRNAs are a new class of non-coding RNAs that participate in post-transcriptional regulation of gene expression. Previous studies have reported a potential role of various miRNAs in induction of Th-17 differentiation and progress of autoimmune diseases. In recent years, it has been shown that miR-326 and miR-26a involved in progress of Th-17 and MS disease.

Objective: To evaluate expression pattern of miR-326 and miR-26a in peripheral blood lymphocytes of relapsing–remitting MS patients during relapsing and remitting phases compared to healthy control subjects.

Materials and methods: Forty RR-MS patients of Isfahan population were diagnosed as relapsing (n = 20) or remitting phase (n = 20) patients according to clinical manifests and expression level of miR-26a and miR-326 was measured in these groups by quantitative real time PCR method compared to 20 healthy controls. In-silico molecular signaling pathway enrichment analysis was also performed on validated and predicted targets (targetome) of miR-26a by DAVID database to explore possible role of miR-26a in Th17 differentiation.

Results: We observed up-regulation of both miR-326 and miR-26a in relapsing phase of multiple sclerosis patients compared with remitting phase (p value = 0.0001) and healthy controls (p value = 0.0091). ROC curve analysis confirmed valuable and precise potential of miR-326 to discriminate between relapsing and remitting phases of multiple sclerosis with specificity and sensitivity of 100% at a proposed optimum cutoff point. Furthermore, in-silico molecular signaling pathway enrichment analysis detected TGF-β signaling pathway as one of the most statistically relevant pathway with miR-26a targetome.

Conclusion: Our results confirmed potential of miR-326 as a diagnostic biomarker to discriminate between relapsing and remitting phases of multiple sclerosis disease. Similar expression pattern to miR-326 and in-silico molecular enrichment analysis altogether suggest an inducing role of miR-26a in differentiation of pathogenic Th17 cells during pathogenesis of multiple sclerosis by targeting major components of the TGF-β signaling pathway (i.e. SMAD4 and SMAD1) and disarrangement of this signaling pathway.

1. Introduction

Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system (CNS) which is widely characterized by the brain and spinal cord myelin destruction (Bendszus and Storch-Hagenlocher, 2013; Calabresi, 2004). It occurs most commonly in young people with more prevalence in women (Bendszus and Storch-Hagenlocher, 2013). Once it befalls, MS disease causes attacks of neurological dysfunction that leads to disability. There are four courses for MS disease. Relapsing–remitting MS (RR-MS)

Abbreviations: AUC, area under the curve; CNS, central nervous system; DAVID, database for annotation, visualization and integrated discovery; hADSCs, human adipose tissue-derived stem cells; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNAs, microRNAs; MS, multiple sclerosis; PBLs, peripheral blood lymphocytes; PP, primary progressive; PR, progressive-relapsing; ROC, receiver operating characteristic; SP, secondary progressive.

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is characterized by unpredictable acute attacks with worsening of symptoms in relapse period. Relapse usually appears to evolve over several days to week and then its manifestation is spontaneously recovered in remitting period. Approximately, 85% of MS patients represent RR-MS phenotype usually after 10 to 20 years. More than 50% of RR-MS patients ultimately will transmit into secondary progressive (SP-MS) which is characterized by exacerbation of symptoms without any recovery. Other two forms of disease namely, primary progressive (PP-MS) and progressive-relapsing (PR-MS) are more rare phenotypes with prevalence of 15 and 5 percentages in MS patients, respectively (Bendszus and Storch-Hagenlocher, 2013).

Although etiology of MS disease has still unknown, it has been demonstrated that MS disease is an immune-mediated inflammatory response with infiltration of activated monocytes, T and B cells into CNS (Bendszus and Storch-Hagenlocher, 2013; Hauser and Oksenberg, 2006; Lassmann et al., 2001). T helper-17 (Th-17) cells are a subset of $CD4^+$ T helper cells which differentiate from human naïve $CD4^+$ T cell in the presence of IL-6, IL-23 and TGF-β cytokines (Harrington et al., 2005; Lassmann et al., 2001; Park et al., 2005; Veldhoen et al., 2006). The Th-17 cells are regarded as the producing IL-17, IL-21, IL-22 and GM-CSF cytokines that have major role in tissue injuries of various autoimmune diseases such as MS disease (Brucklacher-Waldert et al., 2009; Tzartos et al., 2008), rheumatoid arthritis (Jacobs et al., 2009), systemic lupus erythematous (Yang et al., 2009), and psoriasis (Fujishima et al., 2010). Obvious studies have demonstrated up-regulation of Th-17 cells and IL-17 level in peripheral blood mononuclear cells, cerebrospinal fluid and active lesions of multiple sclerosis patients during relapsing phases compared to remitting phase (Komiyama et al., 2006; Tzartos et al., 2008).

MicroRNAs (miRNAs) are a new class of small non-coding RNAs which regulate gene expression post-transcriptionally by binding to 3′ UTR of their mRNA targets, and resulting in degradation or transcriptional repression of the targeted mRNA (Bartel, 2009). Previous studies have reported involvement of different miRNAs in regulation of Th-17 differentiation from naïve $CD4^+$ T cells in association with pathogenesis of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. Hence, these observations indicate a critical function of miRNAs in regulation of naïve T cell differentiation program into Th-17 subset (Du et al., 2009; Mycko et al., 2012; Niimoto et al., 2010).

In one study Du et al. (2009) introduced miR-326 as a Th17-associated miRNA whose expression elevates in peripheral blood lymphocytes of multiple sclerosis patients during relapsing phase compared with remitting phase and healthy individuals, representing miR-326 correlation with the disease severity. Furthermore their in-vitro studies showed that miR-326 expression level dramatically increases during Th-17 polarizing condition by targeting ETS1, a negative regulator of Th17 differentiation (Du et al., 2009). Finally Du et al. proposed valuable potential of miR-326 as a diagnostic biomarker for multiple sclerosis severity. In a different study Niimoto et al. (2010) reported up-regulation of miR-26a in peripheral blood mononuclear cells (PBMCs) and synovium tissue of 6 patients with rheumatoid arthritis in comparison with healthy subjects. In addition they revealed that the expression level of miR-26a was increased in IL-17 producing T cells during in-vitro polarization of these cells from naïve $CD4^+$ T cells, nominating miR-26a as another possible Th17-associated miRNA.

Discrimination between two periods of relapse and remitting is important especially for analysis of the progress of MS disease and effectiveness of drug therapy evaluation in patients with RR-MS in medical laboratories. Therefore, the aim of the present study was the evaluation of miR-326 and miR-26a levels in PBLs of RR-MS patients in relapse and remitting periods separately.

2. Materials and methods

2.1. Patients and samples

Forty relapsing–remitting MS (RR-MS) patients including 20 patients in relapsing phase and 20 patients in remitting phase were diagnosed based on McDonald criteria in Multiple Sclerosis Clinic of Al-Zahra Hospital (Isfahan) during a course of 8 months. After taking clearly informed consent, 5 ml of their blood was collected in EDTA containing tubes. This was accompanied by collecting 20 healthy blood samples without any infectious or allergic diseases which result in active immune system. Immediately after obtaining blood samples, they were transferred to a laboratory on ice for downstream analysis. All relapsing phase patients were new RR-MS cases with a severe attack, and without any previous consumption of immunomodulatory drugs, whereas inevitably all remitting phase patients were using immunomodulatory β-interferon. However for all remitting patients, drug adjustment was performed in such a way that blood taking was accomplished one week after previous interferon injection and just before the next shot, when the amount of drug and so its effect were in its minimal state. All patients had disease grade (SDSS) of 1. Other routine clinical information and variables of patients comprising sex, age, disease duration, MRI results and symptoms at the onset of MS are represented in Supplementary Table 1.

2.2. PBL isolation from blood

Immediately after collecting blood samples, peripheral blood lymphocytes (PBLs) were isolated by density gradient lymphoprep (STEMCELL Technologies, USA) according to manufacturer protocol. Briefly for each sample, 3 ml of blood was diluted in proportion of 1:1 with physiological saline and was gently poured over the top of 3 ml lymphoprep solution gradient in a falcon tube. Prepared falcons were centrifuged at 800 *g* for 30 min at room temperature. Finally PBLs were removed from interface phase into a 2 ml *RNAase*-free microtube. After washing and cell counting, PBLs were pelleted by centrifugation for 10 min at 250 *g* and then were frozen at −20 °C for further RNA extraction step.

2.3. RNA extraction

Total RNA extraction from PBLs was accomplished using TRizol reagent (Invitrogen, USA) based on manufacturer instruction. Quality of extracted total RNA was determined according to 260/280 absorbance ratio, measured by Nano Drop spectrometer (Thermo Scientific, USA). In order to eliminate any potential contamination with unwanted DNA, total RNA samples undergone RNA-free *DNase* (TaKaRa, Japan) treatment.

2.4. cDNA synthesis and real-time PCR

cDNA synthesis for miR-326 and miR-26a was fulfilled using a "universal cDNA synthesis kit" (Exiqon, Denmark) in poly A tailing manner, according to manufacturer leaflet. Real-time quantitative PCR reactions were carried out as triplicate by using standard protocols with an ABI PRISM 7500 instrument (Applied Biosystems, USA). Concisely, in a total volume of 10 μl, 20 ng/μl of cDNA products was added to a master mix comprising 10 pmol/μl of each miR-326 or miR-26a DNA primers (Exeqon, Denmark) and 2 U of SYBR premix Ex*Taq* II (TaKaRa, Japan). The run method program was set as 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.

2.5. Statistical analysis

Real-time PCR data analysis was performed using the ΔΔCT method in Microsoft office excel 2007 software and final data were normalized by small nuclear RNA, RNU48, expression level as an endogenous