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Immune-related miRNA expression patterns in peripheral blood mononuclear cells differ in multiple sclerosis relapse and remission

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ABSTRACT

MiRNAs were shown to participate in development of autoimmune inflammatory process in multiple sclerosis (MS). To investigate miRNAs involvement in relapse-remission MS course, we analyzed expression of immune-related miRNAs in PBMC of treatment-naïve relapsing and remitting MS patients and healthy controls. The upregulation of miR-126-3p, miR-146b-5p, miR-155, miR-196a-5p, miR-21-5p, miR-223-3p, miR-326 and miR-379-5p in remission compared to relapse was observed; when apply gender stratification, miR-223-3p and miR-379-5p were upregulated only in men. Therefore, miRNAs play essential role in maintaining stable MS course and this process has certain gender-specific differences.

1. Introduction

Multiple sclerosis (MS) is a severe autoimmune disease of central nervous system (CNS), which is characterized by a complex of immune-related pathological processes, leading to demyelination and axonal loss and therefore giving rise to loss of neurological functions and disability. The most common MS course is relapsing-remitting, which is defined by alternations of acute inflammatory attacks of neurological impairments (called “relapse”) lasting at least 24 h and of partial or complete recovery (called “remission”) (Lublin and Reingold, 1996). The mechanisms underlying the occurrence of relapses remain to be fully elucidated but the role of immune system, where immune cells being activated infiltrate the central nervous system and, thereby, provoke neuroinflammatory processes, have been demonstrated to be triggering for MS relapses (reviewed in Mills et al., 2017).

The functioning of immune system and regulation of the autoimmune inflammatory processes was shown to be mediated by miRNAs – 19-24 nucleotide-long non-coding RNAs (reviewed in Baulina et al., 2016; Dai and Ahmed, 2011). MiRNAs regulate gene expression on post-transcriptional level through the complementary binding of the miRNA seed region (6–8 nucleotide sequence) mainly with the 3′-untranslated region of the target mRNAs. This binding leads to the target mRNA degradation in case of complete complementarity or to the inhibition of the mRNA translation in case of incomplete complementarity (Bartel, 2004; Carthew and Sontheimer, 2009). Generally, miRNAs act as inhibitors of the target mRNA expression, and due to degeneracy (redundancy) and pleiotropy of their action, miRNAs regulate the expression of > 60% protein-coding genes (Friedman et al., 2009).

In the past years, the identification of MS-related miRNA differentially expression patterns led miRNA to be considered as the new potential prognostic biomarkers for disease development and progression (Gandhi, 2015). Up to date, MS-specific miRNA were found in whole blood (Keller et al., 2015; Keller et al., 2014) and its different components, namely peripheral blood mononuclear cells (PBMC) (Martinelli-Boneschi et al., 2012; Otaegui et al., 2009; Sondergaard et al., 2013), PBMC subpopulations (Ahmadian-Elmi et al., 2016; Fenoglio et al., 2011; Sanders et al., 2016; Sievers et al., 2012; Wu et al., 2017), plasma (Gandhi et al., 2013; Siegel et al., 2012; Sondergaard et al., 2013) and serum (Regev et al., 2016), as well as in brain lesion tissues (Junker et al., 2009) and cerebrospinal fluid (Haghikia et al., 2012). Besides though these studies elucidate the molecular insights in MS pathogenesis, many of them did not take into account diverse parameters of the disease such as MS course, gender prevalence and therapeutics. In addition, these studies are characterized by different design of experiments (*i.e.* usage of different starting material, miRNA expression analysis platforms, algorithms of raw data analysis and normalization) that in general raises the need for results validation and for interpretation of functional and pathological implication of miRNA in disease course.

Abbreviations: EAE, experimental autoimmune encephalomyelitis; FC, fold change; PBMC, peripheral blood mononuclear cells; RRMS, relapse-remitting multiple sclerosis; Th, T helper

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Here, we focused on the search of immune-related miRNAs in PBMCs as an informative and easily accessible biological material in order to find out miRNAs involved in MS relapse-remission dynamics. To reduce variability, we included only those RRMS patients, who were free from any disease modifying treatments and from any other inflammatory disorders. Due to men and women differ in susceptibility to RRMS (Koch-Henriksen and Sorensen, 2010), and the influence of sex on miRNA expression patterns was observed in MS patients (Munoz-Culla et al., 2016), we applied gender-based approach to find the possible gender differences in miRNA expression patterns in relapse and remission, assuming that the different molecular pathological mechanisms may underlie such effects in different sex.

2. Material and methods

2.1. Patients and controls

Forty-four unrelated patients with relapsing remitting multiple sclerosis (RRMS) diagnosed according to the McDonald Criteria (Polman et al., 2011) as well as twenty four age and sex pair matched healthy controls (HC) were enrolled in the study. Among the RRMS patients, 20 were undergoing clinical relapse (samples were collected in 24–36 h after relapse manifestation, not associated with fever or infection, and before the first corticosteroid administration) whereas the other 24 were in a clinically stable phase for at least 6 months (remission). All patients were treatment-naïve, *i.e.* did not receive any disease modifying treatments for multiple sclerosis. 24 healthy controls were volunteers who were excluded from acute or chronic infections. All RRMS patients and healthy individuals lived in the Moscow region and self-reported as Russians. 4 relapsing MS patients, 4 remitting MS patients and 4 healthy controls (all men) were enrolled in the screening miRNA PCR array analysis. The rest individuals were involved in RT-qPCR analysis. Demographic and clinical characteristics of all the subjects enrolled in the study are shown in Table A.1. The local ethics committee approved the study, and written informed consent had been obtained from each patient.

2.2. Preparation of blood samples

Blood samples were collected in EDTA containing tubes. For miRNAs extraction, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient method (Sigma-Aldrich, St. Louis, MO, USA) within 3 h of sampling.

2.3. RNA isolation

Total RNAs including small RNAs were extracted using miRNeasy Mini Kit (Qiagen, Hildren, Germany) according to the manufacturer's instructions. RNA quality and quantity were assessed using the NanoDropTM spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. MiRNA PCR array analysis

MiRNA profiling was performed with Human Immunopathology miRNA PCR array (SABiosciences, Frederick, MD, USA), which focuses on analysis of immune-related miRNAs. This array contains 84 miRNA specific primer assays (Table A.2) and is considered sufficient for rapid profiling of miRNAs that involved in regulation of normal and pathological immune response. Briefly, RNA including small RNAs was reverse transcribed using miScript II RT Kit (Qiagen, Hildren, Germany); a SYBR Green–based qPCR was then performed in the Applied Biosystems StepOnePlus Real-Time PCR System instrument, as follows: 15 min at 95 °C and 40 cycles of 15 s at 94 °C, 30 s at 55 °C, and 30 s at 70 °C. Due to exploratory nature of miRNA PCR array analysis, no adjustments for multiple testing were made; miRNA PCR Array Data Analysis software version 3.5 was used for data analysis (http://pcrdataanalysis.sabiosciences.com/mirna/arrayanalysis.php) as recommended by manufacture. Expression values were normalized to six endogenous controls: SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-6P (RNU6B). The miRNAs relative expression was calculated with the Delta-Delta Ct method, where ΔΔCt for each miRNA = [(average of Ct minus average of Ct of six endogenous controls in the MS group) minus (average of Ct minus average of Ct of six endogenous controls in the healthy control group)] (Livak and Schmittgen, 2001) Statistical significance was determined using Student's *t*-test. MiRNA considered to be deregulated between two groups if the FC ≥ 2 or ≤ 0.5 and $p < 0.05$. Individual differences in each group were expressed as mean ± SD; the coefficient of variability in groups ranged from 0.97 to 7.99% (Pearson, 1896). The median power of the array analysis was calculated according to Patnaik et al. (2017).

2.5. RT-qPCR analysis

TaqMan assays were employed for the quantitative analysis of the ten miRNA expression in the extended groups. Briefly, the input RNAs were reverse transcribed using the TaqMan miRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA were then subjected to qPCR in triplicate using the TaqMan miRNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. MiRNA differential expression in RT-qPCR TaqMan assays was calculated using the Delta-Delta Ct method as described in “MiRNA PCR array analysis” section except that RNU6B was used as endogenous control as it showed the smallest variance among samples in preliminary array analysis. The miRNA expression among different patient groups was compared by means of Mann–Whitney *U* test. *p*-Values lower than 0.05 were considered significant. Individual differences in each group were expressed as mean ± SD; the coefficient of variability in groups ranged from 1.52 to 8.96% (Pearson, 1896). The Benjamini-Hochberg correction for multiple comparisons was used (Benjamini and Hochberg, 1995).

2.6. MiRNA target analysis

For miRNA target analysis we used a web application miRNet, that integrates data from eleven different miRNA databases and allows creating miRNA-target interaction networks based on experimental data at different confidence levels (Fan et al., 2016). This tool was used to visualize target genes of dysregulated miRNAs with 1.0 and 2.0 degree cutoffs for target nodes.

3. Results

In order to find out immune-related miRNAs involved in MS relapse-remission dynamics, we performed preliminary analysis of miRNA expression in PBMCs on restricted number of remitting and relapsing MS patients and healthy controls using Human Immunopathology miRNA PCR array. To exclude possible bias in miRNA expression levels in blood due to hormonal changes during the menstrual cycle or use of oral contraceptives, the screening analysis was carried out in men only. Results of comparison of miRNA expression in PBMC between MS remission *vs* healthy controls, MS relapse *vs* healthy controls and MS remission *vs* MS relapse are presented in Table 1. MS patients in remission when compared to healthy controls were characterized by significant upregulation of 18 miRNAs: miR-126-3p, miR-128-3p, miR-143-3p, miR-145-5p, miR-148a-3p, miR-16-5p, miR-17-5p, miR-182-5p, miR-185-5p, miR-191-5p, miR-203a-3p, miR-223-3p, miR-23b-3p, miR-26a-5p, miR-27b-3p, miR-451a, miR-98-5p, and miR-99b-5p, with Fold Change (FC) = 2.0–6.2 and $p = 0.009$–0.043. While comparing MS patients in relapse to healthy controls we identified three differentially expressed miRNAs: miR-135b-5p, miR-379-5p and miR-451a, which were significantly upregulated as well (FC = 2.3–5.6;