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MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with multiple sclerosis and experimental autoimmune encephalomyelitis

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ABSTRACT

Mammalian noncoding microRNAs (miRNAs) are suggested to be involved in immune system function. We found that miR-155 expression was highly correlated with disease severity in patients with multiple sclerosis and mice with experimental autoimmune encephalomyelitis (EAE). Knockdown of miR-155 resulted in low Th1 and Th17 cells and mild EAE, and its overexpression led to more Th1 and Th17 cells and severe EAE. MiR-155 promoted the development of inflammatory Th17/Th1 cell subsets. These findings demonstrate that miR-155 confers susceptibility to EAE by affecting inflammatory T cell responses and can be a new target for therapy of multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating neurodegenerative disease of the central nervous system (CNS). Although the exact pathogenesis of MS remains unclear, $CD4^+$ T cell mediated autoimmunity was suggested as one of the most important aspects of the pathogenesis (Sospedra and Martin, 2005). IFN-γ producing T helper type 1 (Th1) cells were one type of effector helper T cells that mediate the pathogenesis of MS and experimental autoimmune encephalomyelitis (EAE). Recent study suggested that T helper 17 (Th17) cells are critical mediators of chronic and autoimmune inflammation (Bettelli et al., 2006). Subsequent studies demonstrated that inhibition of IL-17 in mice can ameliorate several autoimmune disorders, including experimental autoimmune encephalomyelitis (EAE) (Ivanov et al., 2006). In addition, IL-17-expressing T cells have been found in lesions of brain tissues from patients with multiple sclerosis (Tzartos et al., 2008).

MicroRNAs (miRNAs) are a class of noncoding RNAs that modulate gene expression at the posttranscriptional level and are involved in regulating several aspects of inflammation (Xiao and Rajewsky, 2009; O'Connell et al., 2010a, b). When poorly regulated, miRNAs are critically involved in a range of human diseases (Ma et al., 2007; Thum et al., 2008; Slack and Weidhaas, 2008) and potentially serve as diagnostic, and prognostic markers or therapeutic targets (Lu et al., 2005; Littman and Rudensky, 2010).

Several miRNAs such as miR-155, miR-181a, miR-181b, miR-150, miR-146a, miR-326, miR-124 and miR-92a were identified recently as important regulators for immune cell development and immune responses. MiR-155 is one of the miRNAs that are most highly implicated in autoimmunity, and it was shown miR-155 plays a crucial role in the function of pathogenic immune cells, such as T cells, B cells and dendritic cells (DCs) (Calame, 2007; O'Connell et al., 2010a, b; Zhou et al., 2010). Moreover, aberrant expression of miR-155 has been observed in many human autoimmune conditions (Stanczyk et al., 2008; Wang et al., 2010; Junker et al., 2009) and it was associated with impaired autoimmune development in miR-155 deficient mice.

In the present study, we examined the expression of eight microRNAs which were known to be immunologically relevant. Furthermore,we studied the role of miR-155 in Th1 and Th17 cell differentiation and cytokine production by over-expression and inhibition of miR-155 in EAE.

2. Materials and methods

2.1. Patients and controls

MS patients were admitted in the Second Hospital of Hebei Medical University between January 2010 and December 2012. All MS patients underwent a standard battery of examinations and fulfilled the McDonald's criteria. The MS cohort consisted of 58% females and 42% males. Sera were collected from 31 MS patients, 32 GBS patients (for other neuroimmunological disease control study) and 31 healthy subjects. The healthy control subjects were also residing in Hebei

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region and matched with cases in terms of gender and age (mean age 40.7 years; range 25–56). None of the patients received any immunomodulatory therapy 3 weeks before the blood withdrawal. Moreover, to avoid possible confounder effects due to diurnal variation in immune function, all samples were collected between 6 and 8 in the morning. This study was approved by local Ethical Committees. All recruited subjects signed an informed consent to participate in the study. All the patients and controls were Chinese of Han race.

2.2. Mice

A total of 82 C57BL/6 wild-type mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. Beijing, China. Mice were maintained in a specific pathogen-free condition. All mice were 6–8 wk old at the beginning of experiments. All experiments were in accordance with Guidelines for the Care and Use of Laboratory Animals (Science & Technology Department of Hebei Province, PR China).

2.3. Induction and evaluation of EAE

EAE was induced in C57BL/6 mice by immunization with 250 μg of MOGp35–55. All peptides were dissolved in complete Freund's adjuvant (Sigma, St Louis, MO, USA) containing 4 mg/ml of heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA). At day 0 and 48 h after immunization, C57BL/6 mice were injected with 500 ng of pertussis toxin (Alexis, San Diego, CA, USA) in PBS, intraperitoneally (i.p.). Clinical assessment of EAE was performed after disease induction by the following criteria: 0, no disease; 1, tail paralysis; 2, hindlimb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, forelimb and hindlimb paralysis; 5, death. Mice were randomly divided into five groups: 15 mice in miR-155 control mimic group, 19 mice in miR-155 mimic group, 20 mice in miR-155 control inhibitor group, 20 mice in miR-155 inhibitor group, and 8 mice in no treatment group.

2.4. Histopathology

For histopathological studies, spinal cords were dissected from female mice (n = 4), fixed in 10% formalin in PBS, and embedded in a single paraffin block. The 8 mm thick sections were stained with H&E and luxol fast blue, and stained sections were evaluated for immune cell infiltration and demyelination.

2.5. Analysis of miR-155 expression

For analysis of miR-155 expression, real-time RT-PCR analyses were carried out using TaqMan miRNA assays (Applied Biosystems) and relative expression was calculated using the CT method, and normalized to uniformly expressed U6 (Applied Biosystems) (n = 4).

2.6. MiR-155 mimic and inhibitor treatment

The oligonucleotides of miR-155 mimic and inhibitor (Genepharma, China) were synthesized to overexpress or knockdown miR-155 expressions in mice. The sequence of miR-155 mimic is: sense(5′–3′)UUAA UGCU AAUU GUGA UAGG GGU and antisense(5′–3′)CCCU AUCA CAAU UAGC AUUA AUU, whereas miR-155 inhibitor sequence is: ACCC CUAU CACA AUUA GCAU UAA, which is based on complementarity to sense sequence of the miR-155 mature sequence. 2′OMe was used as chemical modification of the inhibitor to make the modified antisense oligo playing a steady role in the competitive inhibition of the target sense strand. For in vivo miR-155 treatment, 100 μl Entranster™ — in vivo (Engreen Biosystem Co, Ltd., Beijing) was mixed with 50 μg miR-155 mimic or inhibitor or their respective controls according the instruction. And the complexes were administered in vivo to these mice on days 5, 7, 9, 11, 13 and 15 after immunization (n = 10). For EAE reversal, miR-155 inhibitor was administered when a clinical score of 1.5 was observed (n = 5).

2.7. Proliferative responses of T cell and cytokine analysis

Spleens or draining lymph nodes were harvested and pooled from EAE mice, and single-cell suspensions were prepared. Cells were cultured at 5×10^7 cells/well in 24-well U-bottom plates with 10 mg/ml MOG35–55 peptide in complete RPMI 1640 medium (including 10% FCS, 100 units/ml streptomycin, 50 mM beta-mercaptoethanol) (n = 4). For ELISA analyses, supernatants were harvested at 72 h of culture. The concentrations of indicated cytokines were measured by quantitative capture ELISA, according to the guidelines of the manufacturers (BD Biosciences) (n = 4). For the detection of proliferative responses, splenocytes or LN cells were seeded in 96-well plate at 5×10^3 cells per well with 10 mg/ml MOG35–55 peptide and then cultured for 72 h. 10 μl of CCK-8 (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) solution was added to each well of per plate, and incubated the plate at 37 °C for the final 4 h. The absorbance at 450 nm was assayed for proliferation. Experiments were repeated four times, and each series was performed in triplicate.

2.8. Preparation and evaluation of CNS cells

Brains and spinal cords of mice, which were perfused with cold PBS, were dissected and incubated in 2.5 mg/ml collagenase D for 30 min at 37 °C. Single-cell suspensions were prepared. Cells were washed in RPMI 1640 medium, and mononuclear cells were isolated using a discontinuous Percoll gradient (Pharmacia, Piscataway, NJ) (n = 4).

2.9. Statistical analysis

Statistical analysis was performed using the unpaired *t* test. $P < 0.05$ is considered significant. Data are presented as mean ± SEM. ANOVA analysis was used to calculate the differences of various treatments of mice with EAE. All the analyses were done using SPSS 7.0.

3. Results

3.1. Upregulation of miR-155 in patients with MS

The respective clinical characteristics in patients with MS, GBS and healthy individuals were shown in Table 1. Eight extracellular immunologically relevant microRNAs, including miR-155, miR-326, miR-146a, miR-150, miR-181a, miR-181b, miR-124 and miR-92a, were investigated in sera samples from MS and control subjects. Five of those miRNAs were >1 fold up-regulation in MS compared to controls, whereas none of them showed down-regulation. MiR-155 showed the highest increase (fold change = 3.65; $P < 0.001$) (Fig. 1A). Fig. 1B showed that miR-155 expression was significantly higher in sera of patients with MS than those with GBS and healthy individuals. Detailed analysis found a higher expression of miR-155 in MS patients during relapse than those during remission (Fig. 1C).

Table 1
Characteristics of patients with MS and controls.

	MS	Control	GBS
Total	31	31	32
Age	44.4 ± 13.2	40.7 ± 8.4	41.5 ± 12.2
Sex			
Female	18	16	14
Male	13	15	18
Clinical stage			
Relapsing	14		All in the acute phase
Remitting	17		