miRNA-485 Regulates Th17 Generation and Pathogenesis in Experimental Autoimmune Encephalomyelitis through Targeting STAT3

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Research Article

Keywords: MicroRNA, Experimental autoimmune encephalomyelitis, Th17, STAT3

Posted Date: October 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2151273/v1

Abstract

Experimental autoimmune encephalomyelitis (EAE) refers to the T-helper (Th) cell-induced autoimmune disease causing demyelination, axonal loss, as well as neurodegeneration of central nervous system (CNS). EAE pathogenesis is highly dependent on T-helper 17 cells (Th17) that generate interleukin-17 (IL-17), and their activity and differentiation are tightly regulated by some cytokines and transcription factors (TFs). In the pathogenic mechanism of various autoimmune disorders, including EAE, certain miRNAs play a role. Our research detected a novel microRNA (miR) that can regulate EAE. According to the results, during EAE, the expression of miR-485 notably lowered while significant increase could be found through STAT3 expression. It was discovered that miR-485 knockdown in vivo upregulated Th17-associated cytokines while aggravating EAE, whereas miR-485 up-regulation down-regulated Th17-associated cytokines while mitigating EAE. The up-regulation of miRNA-485 in vitro inhibited Th17-associated cytokine expression within EAE $CD4^+$ T cells. Furthermore, as revealed by target prediction and dual-luciferase reporter assays, STAT3 was miR-485's direct target, a gene that encodes a protein responsible for Th17 generation. Taken together, miRNAs exert vital functions in Th17 generation and EAE pathogenesis.

Introduction

Multiple sclerosis (MS) represents the chronic spinal cord/brain inflammatory demyelination disorder that mainly affects young adults [1]. Its mean age of onset is 20 – 45 years [2]. Currently, MS is associated with higher incidence and dismal prognostic outcomes [3]. Autoimmunity mediated by $CD4^+$ T cells is extensively recognized as the critical factor related to MS pathogenic mechanism, especially in the early phases of the disease [4, 5]. As an extensively utilized human MS model, experimental autoimmune encephalomyelitis (EAE) represents central nervous system (CNS) inflammation regulated via $CD4^+$ T cells as well as is associated with T helper 1 (Th1)/T helper 17 (Th17) cell infiltration and demyelination [6–8]. Other intriguing findings include that mice exhibiting a reduced number of Th17 cells possess lower EAE susceptibility, and Th17 cells that secrete interleukin-17 (IL-17) could be viewed within brain lesions, as evidenced by autopsy reports of human MS cases [9–11].

Naive $CD4^+$ T cells promoted via antigen undergo activation, proliferation, and differentiation into diverse T helper (Th) cells, including Th1, Th2, Th17, and induced regulatory T cells [12, 13]. Among them, Th17 cells are the effector subpopulation secreting IL-17, IL-21, and IL-22, the vital factors for developing MS and EAE [14, 15]. Th17 cells are differentiated in a complex process under the regulation of various transcription factors (TFs), which include a STAT3 and RORγt [16]. STAT3 triggers RORγt expression and naive $CD4^+$ T cells differentiation in Th17 cells. [17].

MicroRNAs (miRNAs) have been identified as the single-stranded small noncoding RNAs (ncRNAs, 18 – 22 nucleotides long) with a high conservation level that could combine with 3'-untranslated regions (3'-UTRs) in target gene to repress gene expression at the post-transcription level [18, 19]. Abnormal miRNA expression or activity participates in various human disorders, including autoimmunity disorders and

malignant tumors, while changes in miRNA activity might be an effective factor to diagnose some diseases [20, 21]. Numerous researchers have recently revealed the important function of miRNAs in the pathogenic mechanism and progression of MS [22–24]. miRNA expression profiles are identified within lymphocytes, peripheral blood mononuclear cells (PBMCs), whole blood, as well as serum from diverse MS subtypes in comparison to normal subjects [25]. Many researchers have identified miRNAs with up-regulation and down-regulation within diverse samples collected in MS cases or EAE mice, which constitute important effects on the pathogenic mechanism of MS, suggesting that such miRNAs can be used to diagnose and predict prognosis of MS [26]. To take an example, miRNA-326 (miR-326) exhibits significant up-regulation among MS cases and EAE mice and could promote Th17 differentiation. Additionally, miR-326 knockdown in vivo significantly suppresses Th17 cells while evidently reducing the severity of EAE [27]. The deletion of miR-155 in mice generates decreased severity of EAE, which is accompanied by lower CNS inflammation as well as significantly decreased Th17 number [28]. In addition, anti-miR-155 therapy remarkably inhibits the progression of EAE [29]. It is proposed that miR-301a modulates immunity mediated by Th17 cells through the IL-6/23-STAT3 pathway [30]. By targeting STAT3 and RORγt, miR-20b inhibits autoimmune inflammation related to IL-17 within EAE [24]. Collectively, miRNAs are the candidate therapeutic targets for MS.

Roles of miR-485 have been identified in diabetic nephropathy progression and lupus nephritis mice, overexpression of miRNA-485 inhibits the production of proinflammatory cytokines[31, 32]. MiR-485 exhibits down-regulation within $CD4^+$ T cells in MS cases when compared with normal subjects [33]. Nonetheless, the function of miR-485 in Th17 generation, as well as EAE, remains completely unknown.

The present study identified the critical effect of miR-485 in regulating Th17 generation and the pathogenic mechanism of the EAE model. According to our results, miR-485 expression was reduced in CNS during EAE. Overexpression of miR-485 exhibits negative regulation of Th17 generation in-vitro and in-vivo, mitigating EAE through STAT3. In this study, miR-485 was associated with the Th17-induced EAE pathogenic mechanism. Overexpression of miR-485 suppresses the generation of proinflammatory factors (including IL-17 and IFN-γ) in EAE mice. Consequently, it could be deduced that miR-485 could be the candidate diagnostic biomarker and therapeutic target.

Materials And Methods

Experimental Animals

In the current work, each experiment was carried out at the Key Laboratory of Neurology of Hebei Province, Shijiazhuang, Hebei Province, China, which gained approval by Experimental Ethics Committee of the Second Hospital of Hebei Medical University.

In the current work, 8 – 10-week-old C57BL/6 female mice (weight, 18 – 20 g) were provided by Vital River Laboratories (Beijing, China) and raised within the specific pathogen-free (SPF) animal facility under

12:12-h light – dark (LD12:12) cycle conditions. In addition, they were allowed to drink water and eat food freely.

EAE Induction and Assessment

EAE was triggered using a four-point paraspinal subcutaneous injection of an emulsion containing complete Freund's adjuvant (CFA) (0.05 mL, Sigma, St Louis, USA), myelin oligodendrocyte glycoprotein (MOG) 35 – 55 peptide (250 μg, Peptide Biotechnology, Nanjing, China), 0.05 mL of sterilized saline and *Mycobacterium tuberculosis* H37Ra (4 mg/mL, Difco Laboratories, Detroit, USA); subsequently, pertussis toxin (500 ng, List Biological, CA, USA) was given through intraperitoneal injection and again the same amount was injected after 48 h. Following immunization, the body weight of mice was monitored daily. The neurological deficits were evaluated using the international five-point scale. 0 – 5 points indicated the absence of clinical signs, weight loss and tail paralysis, hindlimb weakness, total hindlimb paralysis, hindlimb paralysis accompanied by forelimb paralysis or weakness, and dying or being dead, separately.

Histological Analysis

At 21 days post-immunization (PI), three mice in diverse groups were killed through cervical dislocation, followed by sequential phosphate-buffered saline (PBS) perfusion (20 mL, pH 7.4) as well as 4% (w/v) paraformaldehyde (PFA, 20 mL) fixation. Following the dissection of spinal cords, they were subject to 4% (w/v) PFA fixation overnight, paraffin-embedding, and slicing to the 5-μm transverse sections. For measuring demyelination and infiltration of inflammatory cells separately, luxol fast blue (LFB) or hematoxylin and eosin (H&E) staining was carried out thereafter. Inflammation was evaluated using a 0 – 4 point scale, which indicated none, mild (lymphocyte infiltration surrounding blood vessels and meninges), moderate (1 – 10) severe (11 – 100) and massive inflammation (> 100 lymphocytes within spinal cord in each microscopic field), separately. Demyelination was evaluated using a 0 – 3 point scale, which indicated none, rare, a few, and massive myelin sheath injuries separately.

Cell Isolation

Spleens were collected from mice receiving pre-chilled PBS perfusion ahead of time. Subsequently, the sample was filtered with the 70-μm cell strainer for preparing the single-cell suspension and washed twice in PBS. Thereafter, by adopting the mouse $CD4^+$ T cell Isolation Kit (BD IMag™), magnetic cell sorting was utilized to isolate naive $CD4^+$ T cells from EAE mouse spleens.

Cell Transfection

We cultivated the isolated $CD4^+$ T cells in Roswell Park Memorial Institute medium (RPMI-1640), including 10% fetal bovine serum (FBS). Subsequently, 5 μg/mL plate-bound anti-CD3 along with 2.5 μg/mL anti-CD28 antibodies (eBioscience, San Diego, CA, USA) were supplemented to activate cells for a period of 48 h. The Lipofectamine 2000 reagent was utilized for cell transfection in line with specific instructions. Following counting, cells were inoculated into each plate on day 1 prior to transfection to guarantee optimal cell confluency on the transfection day. For oligonucleotide transfection, miR-485 mimics, inhibitor (GenePharma, Suzhou, China), and negative control (NC) RNA were utilized. The cells

were collected in this study after 48 h. With the aim of evaluating the efficiency of transfection, real-time quantitative reverse transcription PCR (qRT-PCR) was conducted (Table S1).

Vector Construction and Lentivirus Production

Lentiviruses were provided by Suzhou GenePharma Co. Ltd. (China). 293T cells were used to prepare lentivectors. These packaged lentiviruses were referred to as LV-485 and LV-anti485 separately, with empty (untransformed) lentiviral vector LV-Con (control virus) being the endogenous reference. Viral production and target cell infection were accomplished following specific protocols.

qRT-PCR

RNA extraction was carried out with Tri-RNA reagent (ZS-M11008, Supersmart, Tianjin, China), which was subsequently isolated by chloroform, followed by isopropanol precipitation, ethanol washing, and dissolution within RNAase-free water; the absorbance (OD) value was detected at 260/280 nm by adopting UV spectrophotometer, followed by reverse transcription in complementary deoxyribonucleic acid (cDNA) library with SweScript RT II First Strand cDNA Synthesis Kit (G3332, Servicebio, Wuhan, China). Thereafter, interested levels of mRNA expression were analyzed using RT-qPCR with 2×SYBR Green qPCR Master Mix (G3322, Servicebio, Wuhan, China). Interested mRNA expression was standardized based on GAPDH mRNA expression level and then compared with each other by 2-ΔΔCT approach.

Enzyme-linked Immunosorbent Assay (ELISA)

Cytokine contents within mouse tissues were assessed using IL-17A ELISA kits (EK217/2 – 01, Multisciences, Hangzhou, P. R. China) according to specific protocols.

Western blot (WB) assay

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was introduced for sample lysis; thereafter, bicinchoninic acid (BCA) protein assay kit (Beyotime) was adopted for determining protein content. Thereupon, every 20 – 40 μg of the sample was loaded on the gel lanes, and protein separation was attained with 12% SDS-PAGE as well as transferred onto PVDF membrane. Subsequently, 5% defatted milk was added to block membranes for a period of 2 h under ambient temperature. Then, overnight incubation was performed using primary antibody under 4°C. A fluorochrome-conjugated secondary antibody was then added to further incubate membranes, which were developed on a far-infrared laser scanner (LI-COR, Lincoln, USA), while the image of the WB result was examined using ImageJ. Using endogenous control protein, the interested protein expression was adjusted. Table S2 displays antibodies utilized in WB analysis.

Statistical Analysis

Graphpad Prism 9.0.0 and SPSS25.0 were used to perform the statistical analyses. Results were shown by mean ± SD. Two-tailed Student's t-test was utilized to compare two groups for significant differences,

while Mann-Whitney U test was employed, aiming to explore nonparametric data (EAE scores). P-value < 0.05 stood for statistical significance (*p < 0.05, **p < 0.01).

Result

miR-485 Exhibited Down-regulation within EAE Mice

For exploring miR-485 expression, as well as the relation to the severity of EAE disease, analysis of miR-485 and RORγt, STAT3, IL-17, and interferon-γ(IFN-γ) levels, was performed with qRT-PCR at peak acute phase. The expression of miR-485 within lymph nodes (LNs), spleen, and CNS (brain and spinal cord) in EAE and healthy mice were analyzed using qRT-PCR. According to Fig. 1A, miR-485 levels decreased within the spleen and CNS among EAE mice in comparison to healthy counterparts. Brain and spinal cord tissues isolated in EAE mice exhibited significantly increased STAT3, RORγt, IL-17, and IFN-γ mRNA expression in acute phase, whereas remarkably decreased miR-485 levels (Fig. 1B, C).

miR-485 Ameliorated the Clinical Severity of EAE

miRNAs have been widely proposed to regulate gene levels at the post-transcriptional process. Therefore, the role of miR485 in EAE occurrence was analyzed by constructing lentiviral vectors and transfecting them into EAE mice. Through the tail vein, vectors that encoded pre-miR-485 (LV-485), corresponding inhibitor (LV-anti-485), as well as the empty lentiviral vector (LV-Con) carrying about 2 × 107 transforming units of recombinant lentivirus were administered into each mouse. Using qRT-PCR, miR-485 level was verified. Subsequently, qRT-PCR was conducted to estimate miR-485 levels within diverse organs on the seventh-day post injection (dpi). It was found that the expression of miR-485 peaked within the liver. The infected mice revealed around 1.5-fold expression in the brain and spinal cord. Regarding immunity-related organs, LV-miR-485 transfection increased miR-485 expression in the spleen by 1.2 folds (Fig. 2A). On the seventh dpi, MOG peptide 35 – 55 was added for mouse immunization; LV-485-infected mice led to milder EAE, while LV-anti-485-infected counterparts revealed severe EAE (Fig. 2B). As a result of spinal cord section histological analysis, LV-485-transfected mice were found to have mild CNS pathology with decreased demyelination and infiltration levels (Fig. 2C, D). On the basis of the obtained results, it was deduced that miR-485 exerts a vial function in the development of EAE.

miR-485 Modulation of Th1/Th17 Cells within EAE Mice

IFN-γ generated in Th1 cells, together with IL-17 generated in Th17 cells, has important effects on mediating the pathogenic mechanism of EAE. It was analyzed in this study whether miR-485 affected T-helper cell differentiation and promoted proinflammatory cytokine generation. Th1-/Th17-associated cytokines, as well as corresponding transcription factor (TF) levels within brains from lentivirus-transfected EAE mice, were analyzed. As a result, in comparison to the levels within LV-Con-infected mice, RORγt, STAT3, IL-17, and IFN-γ levels significantly decreased within LV-485-infected mice while increased in LV-anti485-infected counterparts (Fig. 3A,B). Likewise, among the three groups, STAT3, RORγt and IL-

17 protein expression was similar to mRNA levels(Fig. 3C,D,E). These datas suggested that miR-485 suppress the production of proinflammatory cytokines in EAE mice. At the same time, we found that p-STAT3 protein was down-regulated in LV-485-infected mice, p-STAT3 protein was up-regulated in LV-anti485-infected mice, and the expression of miR485 was negatively correlated with STAT3 level.

Effect of miR485 on Th17 Generation in EAE

Th17 cells represent a crucial $CD4^+$ T cell subset associated with pathogenic mechanism of EAE, which are related to miR-485 and STAT3 levels. In this study, the association of miR-485 expression with $CD4^+$ T cells in EAE was analyzed. Initially, $CD4^+$ T cells sorted based on splenocytes in EAE mice were activated for a day using immobilized anti-CD3 and soluble anti-CD28. The purity of sorted CD4 + T cells was more than 90%(Fig. 4A). Later, miRNA-NC, miRNA-485, miRNA-NCI, and miRNA-485I were added to transfect cells for a period of 48 h(Fig. 4B). The mRNA levels of Th17-related cytokines (IL-17A, IL-17F), transcription factor RORγt and STAT3 were measured by PCR method. We observed that the mRNA levels of IL-17A, IL-17F, RORγt and STAT3 were down-regulated in miR-485-transfected CD4 + T cells, while up-regulated in miRNA-485I-transfected cells(Fig. 4C-F). At the same time, as revealed by the WB assay, p-STAT3 and RORγt levels within $CD4^+$ T cells decreased following miRNA-485 transfection while increasing after transfection using miRNA-485I. Therefore, miRNA-485 possibly suppressed the growth of Th17 cells within $CD4^+$ T cells during EAE by regulating STAT3 phosphorylation.

STAT3 is miR-485’s Direct Target

For supporting that miR-485 expression showed negative relationship to STAT3 expression in EAE mice, we first adopted TargetScan Mouse (version 6.2, http://www.targetscan.org/mmu_61/) for identifying the possible targets of miR485(Fig. 5A). As a result, the activate site by which miRNA-485 targeted STAT3 was located within 644 − 650 in 3'-UTR. Thereupon, luciferase reporter assay was adopted for determining the role of miR-485 in directly targeting 3'-UTR of STAT3 mRNA in HEK-293T cells. The luciferase reporter vector was inserted with the mutant (mut) or wild-type (wt) target sequence of the STAT3 3’-UTR (mut or wt 3'-UTR) and then transfected into HEK-293T cells to obtain the mut or wt 3’-UTR co-transfected with miR485 mimic vector. Luciferase activity decreased significantly when compared to the miR control. The mut 3'-UTR vector was unaffected by co-transfection with miR485. Furthermore, antimiR485 co-transfected with the wt 3'UTR vector into HEK-293T cells significantly increased luciferase activity(Fig. 5B). STAT3 was the direct target of miR485 in HEK-293T cells.

Discussion

Identifying circulating miRNAs with high sensitivity and specificity is important for monitoring developing human cancer [34–36]. Specific miRNAs are examined to provide potential biomarkers for prognosis and treatment. miRNAs are related to MS occurrence. Besides, targeting certain miRNAs could treat EAE. Some specific miRNAs reveal specific expressions among MS cases. For example, miR-21, miR-142 − 3p,

miR-146a, miR-146b, miR-155, and miR-326 exhibit elevated levels of expression within brain white matter lesions and PBMCs in model mice and MS cases. Furthermore, additional miRNAs, like miR-15a, miR-15b, miR-20b, miR-467b, miR-181c, and miR-328, exhibit down-regulation within MS [26]. Numerous diverse miRNAs are suggested to have important effects on EAE occurrence through diverse Th1/Th17-related pathways by targeting certain proteins. miR-20b could target STAT3 and RORγt and modulate the differentiation of Th17, thus, affecting EAE occurrence [24]. miR-23b, miR-326, and miR-467b have important effects on the pathogenic mechanism of EAE through modulation of Th17 differentiation by targeting Ets-1, eIF4E, TAB2, TAB3, and IKK-a, respectively [27, 37, 38].

miR-485 is identified as the tumor suppressor, which suppresses the growth and migration of cells in small cell lung cancer (SCLC), papillary thyroid cancer (PTC), and esophageal cancer [39–41]. Notably, miR-485 could regulate fibrotic and inflammatory responses [42, 43]. Wu et al. found that miR-485 exhibited significant down-regulation within high glucose (HG)-exposed human mesangial cells (HMCs). Increased expression of miR-485 decreased the growth of HG-mediated HMCs. HMCs with overexpression of miR-485 had reduced levels of proinflammatory factors (TNF-a, IL-1b, IL-6) [32]. As indicated by Tian et al., miR-485 overexpression decreased RhoA, which stimulated the proliferation of renal tubular epithelial cells (RTEC) but suppressed their apoptosis by modulating the RhoA-dependent TGF-β-MAPK pathway among lupus nephritis mice [31]. According to Wang et al., overexpressed miR-485 hinders cell proliferation and stimulates the airway smooth muscle cells (ASMCs) apoptosis via Smurf2-dependent TGF-β/Smads pathway among chronic asthma mice [44]. In this study, the impacts of miR-485 on EAE and T cell differentiation was analyzed. miR-485 was chosen according to miRNA expression profiling within $CD4^+$ T cells in MS cases, and roles for miR-485 in regulating inflammation function were reported [33]. This study identified that miRNA-485, with down-regulation in EAE mouse spleen and CNS, suppressed Th17 differentiation as well as the pathogenic mechanism of EAE, both in vivo and in vitro. Differentiation of $CD4^+$ T cells is regulated via various pathways; typically, JAK2/STAT3 has a critical effect on the mediation of Th17 cell differentiation [45]. STAT3 and RORγt pathways, together with miRNA-485 targets, were investigated; as a result, STAT3 was identified by using creditable algorithms (TargetScan). Luciferase reporter assays revealed that that miRNA-485 co-transfected with STAT3–3'-UTR in HEK-293T cells suppressed luciferase activity, while miRNA-485 up-regulation within HEK-293T cells decreased STAT3. Additionally, miRNA-485 expression decreased within CNS, whereas STAT3 expression increased in EAE, indicating STAT3 as a direct target of miRNA-485.

EAE represents the autoimmune disorder regulated via $CD4^+$ T cells; its pathogenic mechanism is associated with both Th1 cells producing IFN-γand Th17 cells producing IL-17 [8, 46]. RORγt could specifically activate the transcription of Th17 cells and generate excessive IL-17 [47]. Therefore, activating Th17/IL-17 signaling via JAK2/STAT3 pathway might upregulate the factors, such as IL-6, TGF-β, and RORγt [48]. Since miRNAs regulate immune and neuronal activities, miR-485 was adopted to examine their effect on the development of EAE. As a result, among LV-miR-485-infected mice before MOG peptide 35 – 55 immunization, the up-regulation of miR-485 causing the alleviation of the severity of EAE, as well as the CNS impairment/demyelination and inflammatory infiltration (Fig. 2). It suggests the

involvement of miR-485 during EAE pathogenic mechanism and occurrence. Subsequently, this work discovered that the overexpression of miR-485 could reduce RORγt, STAT3, IFN-γ and IL-17 expression within CNS, which further suggests that miR-485 triggers the production of proinflammatory factors and regulates the EAE pathogenic mechanism by acting on Th17 cell production. This result is consistent with the inhibitory effect of miRNA-485 on the production of proinflammatory cytokines in diabetic nephropathy.

In vitro, miRNA-485 up-regulation could lower the levels of IL-17, RORγt, and STAT3 from $CD4^+$ T cells within EAE mouse spleens. Based on WB assay, STAT3 revealed down-regulation in splenic $CD4^+$ T cells with overexpression of miRNA-485. Furthermore, the STAT3 expression within $CD4^+$ T cells increased in miRNA-485I group compared with miRNA-NCI group. It concluded that miR-485 expression revealed negative relation to STAT3 levels within $CD4^+$ T cells of EAE. For determining the function of miRNA-485 in regulating STAT3 expression, STAT3 was the target of miRNA-485 that was predicted by TargetScan. According to luciferase reporter assays, STAT3 was the target of miRNA-485. Moreover, miR-485 regulated the generation of Th17 cells by targeting STAT3.

Collectively, miR-485 has a protective effect on EAE mice. Besides, miR-485 can significantly affect the pathogenic mechanism of EAE by modulating Th17 cell generation in vivo and in vitro through STAT3. Therefore, the above results explores the pathogenic mechanism of MS. In addition, this study could also aid in understanding the function of miRNA as the new and efficient targets to treat MS cases.

Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yumei Xue, Lu Zhang, Ruoyi Guo and Congcong Yuan. The first draft of the manuscript was written by Yumei Xue, Bin Li and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics Approval

This study was approved by the Ethics Committee of The Second Hospital of Hebei Medical University (Shijiazhuang, Hebei, China).

Consent to Participate

This article does not contain any studies with human participants performed by any of the authors.

Consent for Publication

Not applicable.

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Figures

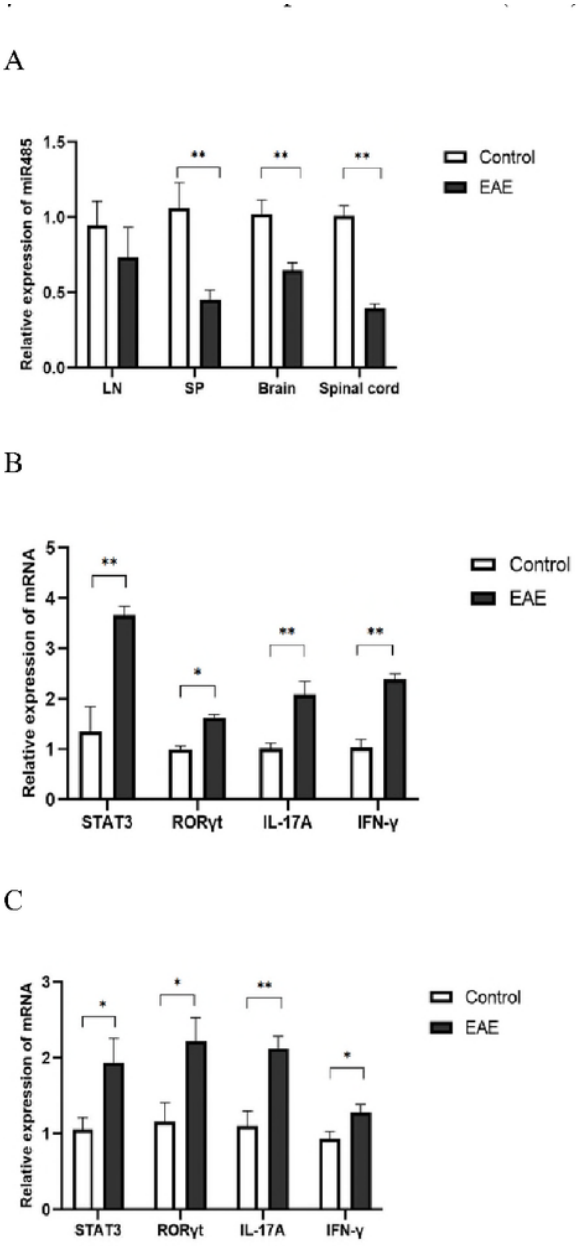

Figure 1

Decreased miRNA-485 expression within EAE mice. A. qRT-PCR assay was conducted to analyze miRNA-485 expression within LNs, spleen, and CNS in EAE and healthy mice (n = 6). B. qRT-PCR assays on STAT3, RORγt, IL-17, and IFN-γlevels within the brain in the context of EAE (n = 6). C. The STAT3, RORγt, IL-17, and IFN-γmRNA levels within spinal cord tissues (n = 6). *p<0.05, **p<0.01 vs. control.

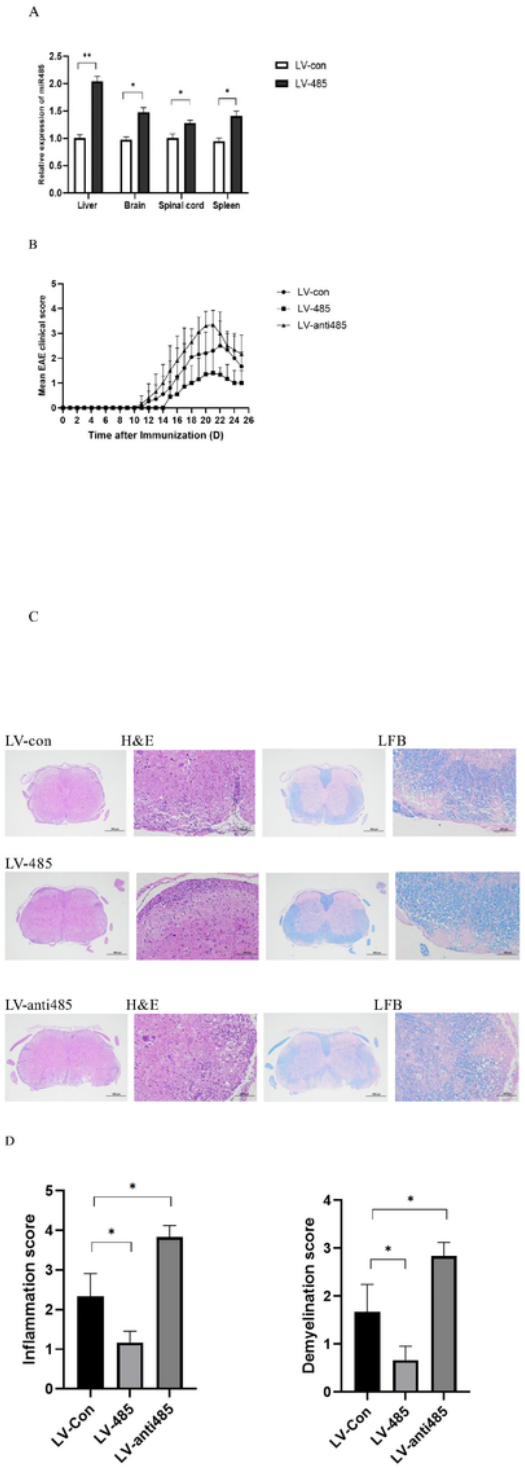

Figure 2

miR-485 regulated the development of EAE. A. qRT-PCR assay on miR-485 levels within liver, brain, spleen and spinal cord of LV-Con or LV-485-infected mice after 7 d (n=3). B. Clinical EAE scores of lentivirus-infected mice (n=10). C. Spinal cord histopathology analyzed in lentivirus-infected mice on 21 dpi (n = 3). On the right (×200), an enlarged diagram of the boxed areas within the left columns (×40) is shown. Scale

bars=100 μm. D. It shows the semi- quantitative demyelination score (based on LFB staining) and inflammation score (based on HE staining). *p<0.05 **p<0.01 vs. control.

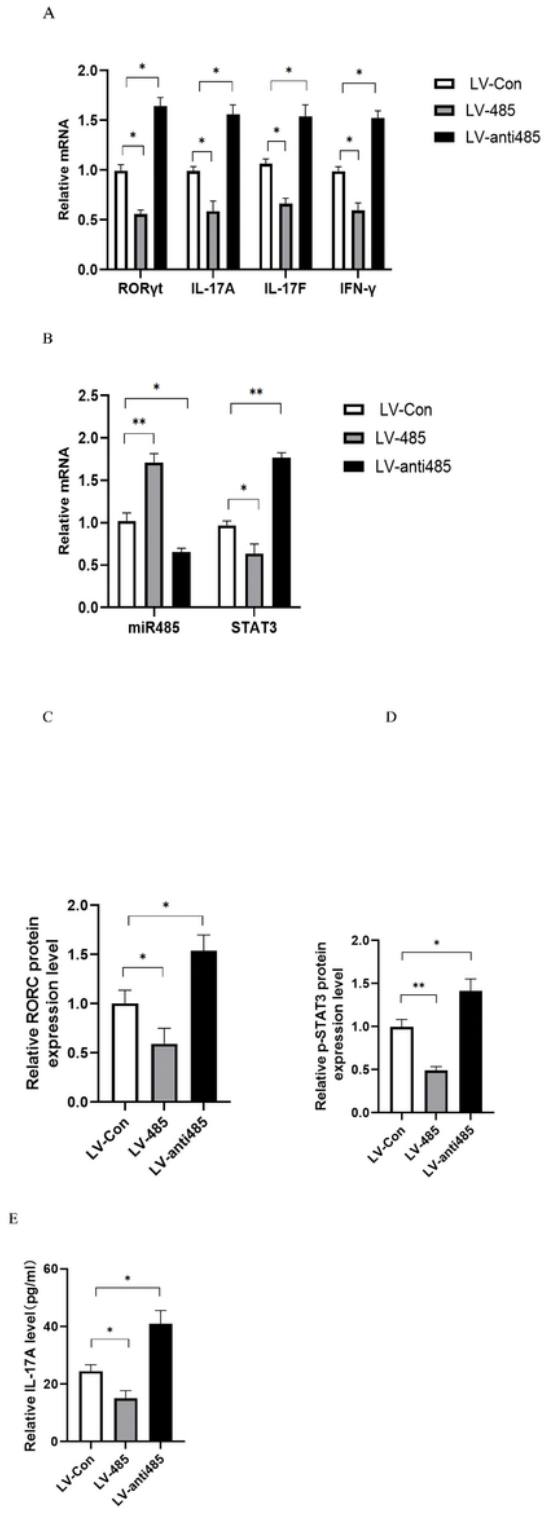

Figure 3

miR-485 modulation of Th1/Th17 cells within EAE mice. A. qPCR assay on RORγt, IFN-γ, and IL17 mRNA levels in lentivirus-infected mice brain tissues (21 days post-immunization; n = 6). B. qPCR analysis

ofSTAT3 and miR485 mRNA levels in brain tissues of lentivirus-infected mice (n = 6; 21 days post-immunization). C-D. Western blotting analysis of p-STAT3 and RORγt protein levels in lentivirus-infected mice brain tissues (21 days post-immunization; n = 3). E. ELISA assay for IL-17A protein expression in lentivirus-infected mouse brain tissues (n = 6). *p<0.05 **p<0.01 vs. control.

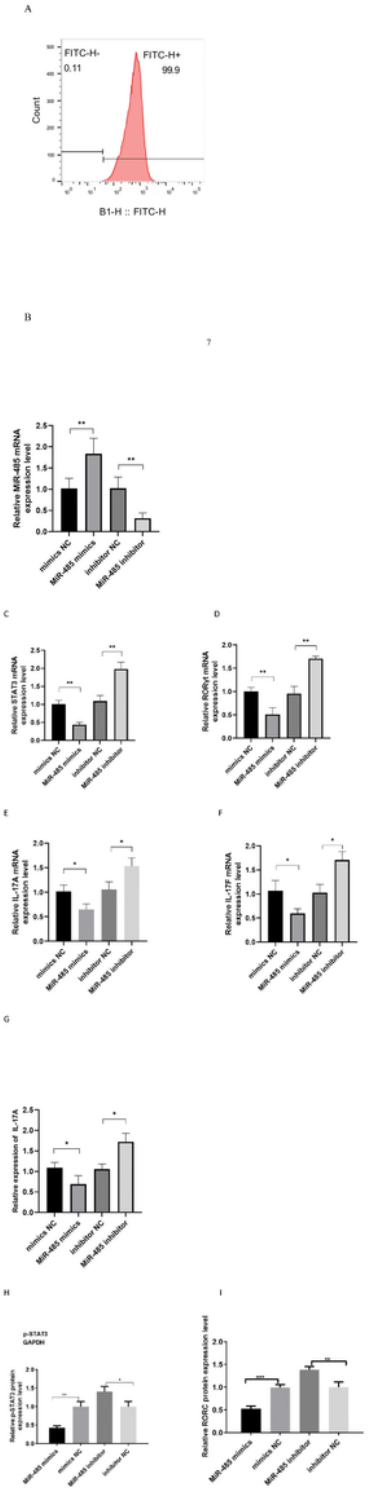

Figure 4

Effect of miR-485 on differentiation of Th17 in EAE. 5 μg/mL plate-bound anti-CD3 along with 2.5 μg/mL anti-CD28 antibodies were introduced to activate $CD4^+$ T cells separated in EAE mouse splenocytes through magnetic cell sorting for a period of 24 h. Thereafter, $CD4^+$ T cells were subjected to 48 h transfection using miRNA-NC/miRNA-485/miRNA-485I. A. Purity of $CD4^+$ T cells sorted. B. miRNA-NC/miRNA-485/miRNA-485I were transfected into activated $CD4^+$ T cells, respectively, for 48 h, and later qRT-PCR was conducted to analyze the miR-485 level (n = 4). C-F. qRT-PCR assay on RORγt, STAT3, IL-17A, IL-17F mRNA levels, within $CD4^+$ T cells subjected to miRNA-NC/miRNA-485/miRNA-485I transfection (n = 4). G. ELISA was conducted to analyze the expression of IL-17A within culture supernatants of transfected cells in Fig. 4B (n = 6). H-I. WB assay was carried out to explore the RORγt and p-STAT3 levels within transfected $CD4^+$ T cells (n = 3). *p<0.05, **p<0.01vs. controls.

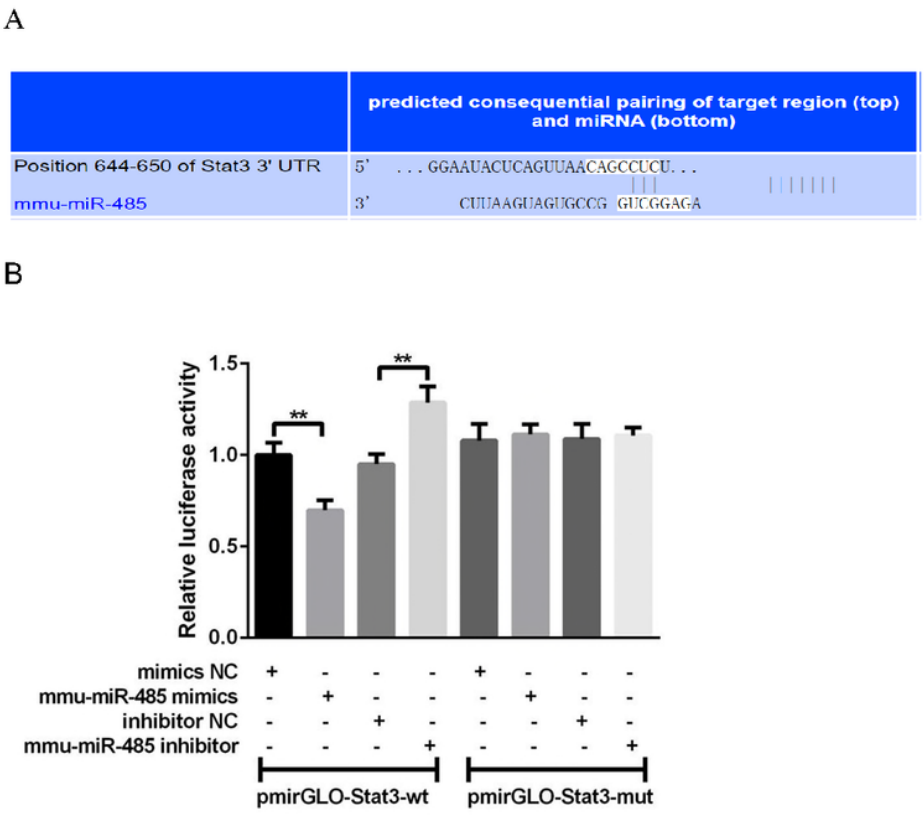

Figure 5

STAT3 is a direct target of miR485 in HEK-293T cells. A. Diagram of the STAT3 3' UTR containing the reporter constructs. B. HEK-293T cell luciferase activity after miR485 mimics or miR485 inhibitor co-transfected with pGLO-STAT3–3'UTR wt or pGLO-STAT3–3' UTR mut for 36 h.

Supplementary Files

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- TableS1TableS2.docx