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MiR-1-3p facilitates Th17 differentiation associating with multiple sclerosis *via* targeting ETS1

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Abstract. – OBJECTIVE: T helper 17 (Th17) cells are involved in the pathogenesis of multiple sclerosis (MS). The present study aimed to explore the role of miR-1-3p in Th17 cell differentiation associated with MS.

PATIENTS AND METHODS: Expression of miR-1-3p in periphery blood mononuclear cells (PBMC), cerebrospinal fluid (CSF), CD4+ T cells, CD8+ T cells, non-T cells and differentiated CD4+ T cells derived from healthy donors and MS patients during remitted and relapsed stages was detected. Level of ETS1 in PBMC in MS-relapse patients was examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Correlations among neurofilament light (NF-L), C-reactive protein (CRP), estrogen receptor 1 (ESR), interleukin 17A (IL-17A) in serum, NF-L, C-X-C motif chemokine ligand 13 (CXCL13), chitinase 3 like 1 (CHI3L1), RAR related orphan receptor C (RORC) in CSF, and ETS proto-oncogene 1 (ETS1), RORC in PBMC and miR-1-3p expression were analyzed. The target gene of miR-1-3p was analyzed by performing Dual-Luciferase reporter assay, and the IL-17A+ CD4+ (Th17) cells were detected by flow cytometer. Gene expressions of IL-17A, RORC and Th17 pathogenic were determined by qRT-PCR.

RESULTS: Upregulated miR-1-3p was observed in MS-relapse patients and Th17 cells, and expression of miR-1-3p was positively correlated with MS severity. MiR-1-3p overexpression in naïve CD4+ T cells promoted the differentiation of Th17 cells by upregulating the level of inflammation-associated markers. The expression of ETS1, which was predicted to be the target gene of miR-1-3p, was reduced in PBMC from MS-relapse patients, while the upregulation of ETS1 inhibited the expression of pathogenic genes of Th17.

CONCLUSIONS: The study demonstrated the positive role of miR-1-3p in Th17 differentiation associated with MS *via* targeting ETS1.

Key Words:
MiR-1-3p, ETS1, Multiple sclerosis, Th17 differentiation.

Introduction

Multiple sclerosis (MS), which is a chronic disease caused by multiple factors, is characterized by a series of clinical symptoms including sensory loss, vision problems, myasthenia and coordination and language problems[1]. MS development initially undergoes actuate inflammatory injury, and then, evolves into destroy of blood-brain barrier, and neurodegenerative disorder within the central nervous system (CNS) is regarded as main causes to MS[2,3]. Though the exact pathogenesis of MS remains currently unclear, generally, it is a combination of environmental and genetic factors accompanied by a serious of deregulations of immune responses that lead to the initiation and progression of MS[4]. $CD4^+$ helper T (Th) cells are l known for their immune protection against the pathogenic disturbance from outside world[5]. At the initial stage, naïve $CD4^+$ T cells, which is a type of Th cells, can be differentiated when stimulated by a specific external environment, and then, transferred into several types of Th cell subsets, which play a specific role in different parts of immune systems[6]. According to polarization state, Th cells can be mainly divided into Th1, Th2, Th17 and $Foxp3^+$ regulatory T (Treg) cells and the recently discovered follicular helper T cells (Tfh), Th9 and Th22 cells[7]. Th17 cells belongs to Th cell family, and mainly secrete pro-inflammatory cytokines IL-17A, IL-17F and could express RAR-related orphan receptor gamma (RORC), which is RORγt in mice[8]. Th17 cells are mainly involved in autoimmune diseases, including in systemic lupus erythematosus, psoriatic, allergy and asthma, MS. In bronchoalveolar lavage fluid of patients, the increase of Th17 cytokines, such as IL-17A, IL-17F and IL-22 were found to be largely associated

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with moderate and severe asthma phenotypes[9]. It was reported that umbilical cord-derived mesenchymal stem cells could alleviate systemic lupus erythematosus patients by regulating the balance between Treg and Th17[10]. Li et al[11] showed that the proportion of Th17 cells in peripheral blood and the expression levels of IL-17 and IL-23 in serum were elevated in MS patients, indicating that Th17 cells and their secretions may be involved in the initiation of MS.

MiRNAs are a category of non-coding single-stranded RNA molecules with the length of approximately 22 nucleotides. MiRNAs involve in the post-transcriptional gene expression regulation in plants and animals. MiR-1-3p expression was downregulated in many types of cancers[12,13], and it was found to suppress the invasion of prostate cancer[14]. Furthermore, increased miR-1-3p was correlated with reverse remodeling after human myocardial infarction[15]. The function of miR-1-3p includes the inhibition of viral replication *via* targeting ATP6V1A to suppress the progression of viral myocarditis[16]. However, the effect of miR-1-3p on the initiation and development in MS was rarely reported. Hence, the present study aimed to explore the role of miR-1-3p in the differentiation of Th17 cells and the association between miR-1-3p and MS.

Patients and Methods

Patients

All patients and healthy donors (33 healthy controlled samples, 36 MS patients during remitting stage and 42 during relapsing stage) of peripheral blood, cerebrospinal fluid (CFS) and serum were recruited from the Second Affiliated Hospital of Harbin Medical University. The patients and healthy donors were required to fast overnight before sample collecting. One part of periphery blood samples was stored in tubes supplemented with ethylenediaminetetraacetic acid (EDTA) anticoagulant for obtaining peripheral blood mononuclear cell (PBMC), while another part was let stand for 1 h, and then, was centrifuged at 2500 x g for 15 min to collect the upper supernatant. CSF samples were obtained from lumbar puncture during diagnosis, and all the samples were maintained in a refrigerator under 4°C. All the patients were informed with the present study and signed the consent form, the Institutional Review Board for Clinical Research of the Second Affiliated Hospital of Harbin Medical University approved the study.

T Cell Isolation

PBMCs in peripheral blood derived from healthy donors were isolated by Ficoll density gradient centrifugation. For the separation of CD^+ 4 T cell and $CD8^+$ T cell, PBMC suspensions were prepared with the supplement of CD4 T cell MicroBeads (Miltenyi Biotechnology, Bergisch-Gladbach, Germany) (10 µL per 10^7 cells), cultured for 10 min at 4°C, and then, purified in MACS column (Miltenyi Biotec, Bergisch-Gladbach, Germany). Then, the CD8 T cell MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) was added into flow-through device to isolate $CD8^+$ T cells. The isolation purity of $CD4^+$ T cell was determined by flow cytometry. Naïve $CD4^+$ cell isolation kit II (130-124-420, CD163 MicroBead Kit, Human, Miltenyi Biotec, Bergisch-Gladbach, Germany) was used to isolate naïve $CD4^+$ T cells. The PBS buffer containing 0.5% (v/v) BSA and 2 mM EDTA was prepared to suspend PBMCs, which was then added with 10 µL naïve $CD4^+$ cell MicroBead Cocktail II and incubated for 5 min at 4°C. Subsequently, the suspension with 30 µL buffer and 20 µLnaïve $CD4^+$ T cell MicroBead Cocktail II was cultured for 10 min at 4°C. MACS column was used to purify the cells under the magnetic field, and the unlabeled cells in the flow-through device were collected as well.

CD4+ Cells Differentiation

The obtained naïve $CD4^+$ cells from PBMCs were collected and differentiated into 4 different types of cells, namely, Th1, Th2, Th17 and Treg cells. A 24-well culture dish was prepared with 250 µL of anti-CD3 antibodies (2 µg/mL in PBS, clone 2C11, eBioscience, San Diego, CA, USA), 4 x 10^5 naïve $CD4^+$ T cells were dissolved into the differentiation culture medium containing Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) with 10% FBS, nonessential amino acids (NEAA, Invitrogen, Carlsbad, CA, USA), antibiotics, and 55 µM β-mercaptoethanol (Invitrogen, Carlsbad, CA, USA) and added into each well coated with anti-CD3 antibodies. The incubator was plated in a 95% humidifier at 37°C for 96 h. The supplements in each differentiating medium were presented in Table I.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokine Determination

Serum NF-L and IL-17A levels and CSF, CXCL13 and CHI3L1 levels from CSF were detected by ELISA using Multi-Analyte ELISA array Kit (Qiagen, Hilden, Germany).

Table I. Supplements in different CD4+ T cells culture medium.

Cell line	Supplements
Th1	1 μg/mL anti-CD28 (clone PV1.17, eBioscience, San Diego, CA, USA) and 10 μg/mL anti-IL-4 antibodies (clone 11B11, eBioscience, San Diego, CA, USA), 2 ng/mL IL-2 (Pepro-tech, London, UK) and 5 ng/mL IL-12 (Peprotech, London, UK)
Th2	1 μg/mL anti-CD28 and 10 μg/mL anti-IFN-γ antibodies (clone R4-6A2, eBioscience, San Diego, CA, USA), 2 ng.mL IL-2, and 20 ng/mL IL-4 (Peprotech, London, UK)
Th17	1 μg/mL anti-CD28, 10 μg/mL anti-IFN-γ, 10 μg/mL anti-IL-4 antibodies, 10 ng/mL human IL-6 (R&D, McKinley Place, MN, USA), 2 ng/mL TGF-β1 (R&D, R&D, McKinley Place, MN, USA)
Treg	1 μg/mL anti-CD28, 2 ng/mL IL-2, and 2 ng/mL TGF-β1

Westergren Method

ESR was detected by Westergren method using ESR analyzer. 0.8 mL EDTA- anticoagulated blood was added into the plastic vial filled with 0.2 mL 3.8% sodium citrate diluent and fully mixed. After 60 min, the level to which cells fall in one hour is assessed, and the level of ESR was read as the unit of mm/h.

Turbidimetric Inhibition Immunoassay

The CRP value of collected serum was determined by Turbidimetric inhibition immunoassay using C-reactive protein assay kit (E023-1-1, Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocol. 7600 automatic biochemical analyzer (Hitachi, Tokyo, Japan) was used to access the CRP. The experiment was conducted under 37°C.

Cell Transfection

MiR-1-3p mimic (miR10000416-1-5) and its negative control were obtained from Rib-Bio, Science Park (Guangzhou, China). The synthesized pCMV6-XL4-ETS1 vector (SC125359) and its equal quantity of negative control were purchased from OriGene (Rockville, MD, USA). The naïve $CD4^+$ T cells were seeded into a culture plate before cell transfection. The cell transfection was conducted using Lipofectamine™ 2000 transfection reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The concentration of miR-1-3p mimics used for transfection was 100 nM, and 4 μ gpCMV6-XL4-EST1 plasmid and its control empty plasmid were used.

After the naïve $CD4^+$ T cells had been transfected with miR-1-3p mimic, mimic control and control, partial cells were separated and divided into two groups. One group of cells was stimulated by 10 μg/mL anti-CD3 with 1 μg/mL anti-CD28 and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS), nonessential amino acids, antibiotics and 55 μM β-mercaptoethanol for 5 days. Another group of cells were incubated in Th17 culture medium for 5 days.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The miR-1-3p expression level was compared in PBMC and CSF from normal, remitted and MS-relapse patients, then among $CD4^+$, $CD8^+$ and non-T cells. $CD4^+$ T cells were then differentiated into Th1, Th2, Th17 and Treg cells. In the PBMC and CSF from MS-relapse patients, the expression levels of RORC and ETS1 were detected. Moreover, the miR-1-3p, RORC, IL-17A, CXCL3, CSF2, IL-23R, AHR, IL-10 and MAF expressions were measured in naïve $CD4^+$ T cells transfected with miR-1-3p mimic or synthesized pCMV6-XL4-ETS1 vector, or the combination of the two. Total RNAs in cells were collected using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), while the total RNAs in CSF were extracted using miRNeasy 96 kit (Qiagen, Hilden, Germany). After the total RNA extraction, the quantitative PCR process of miR-1-3p was conducted using All-in-One miRNA qRT-PCT Detection Kit (GeneCopoeia, Rockville, MD, USA). For quantitative analyses of RORC, IL-17A, CXCL3, CSF2, IL-23R, AHR, IL-10, MAF and ETS1, the whole process was conducted using 5All-InlOne RT MasterMix Kit (Applied Biological Materials Inc., Dalian, China) according to the manufacturer's protocol. U6 served as internal reference of miR-1-3p, and GAPDH was for the rest of genes. All the primers sequences were shown as Table II.

Flow Cytometry

The isolation of $CD4^+$ T cells was performed using Naïve $CD4^+$ T cell isolation kit II, while the isolation of human (130-094-131, Miltenyi Bio-

Table II. Primer sequences used for qRT-PCR.

Primer		Sequences
miR-1-3p	forward	5′ ACACTCCAGGTGGGTGGAATGT 3′
	reverse	5′ CTCAACTGGTGTCGTGGAG 3′
RORC		
	forward	5′ TTTTCCGAGGATGAGATTGC 3′
	reverse	5′ CTTTCCACATGCTGGCTACA 3′
IL-17A	forward	5′ CCTCAAGTTCCACTT 3′
	reverse	5′ CACCAGCATCTTCTCCAC 3′
CXCL3	forward	5′ CTGCGCTGCCAGTGCTT 3′
	reverse	5′ ACCTTACATTCACACTTTGGATGTTC 3′
CSF2	forward	5′ TGGAAGCATGTAGAGGCCATCA 3′
	reverse	5′ GCGCCCTTGAGTTTGGTGAAAT 3′
IL-23R	forward	5′ GCCAAGCAGCAATTAAGAAC 3′
	reverse	5′ GACACAGGT TACTTCATCAGG 3′
AHR	forward	5′ TCCTTGGCTCTGAACTCAAGCTGT 3′
	reverse	5′ GCTGTGGACAATTGAAAGGCACGA 3′
IL-10	forward	5′ GGCACCAGAACTCTCCTCTG 3′
	reverse	5′ TGGGTTGAACGTCCGATATT 3′
MAF	forward	5′ AACATATTCCATGGCCAGGG 3′
	reverse	5′ GGATGGCTTCAGAACTGGCA 3′
ETS1	forward	5′ CATATCAGGTTAATGGAGCC 3′
	reverse	5′ GTAGTCGAAGCTGTCATAGG 3′
U6	forward	5′ GCTTCGGCAGCACATATACTAAAAT 3′
	reverse	5′ GCTTCGGCAGCACATATACTAAAAT 3′
GADPH		
	forward	5′ CGGAGTCAACGGATTTGGTCGTAT 3′
	reverse	5′ AGCCTTCTCCATGGTGGTGAAGAC 3′

technology, Bergisch Gladbach, Germany) was conducted using flow cytometry. For detection of IL-17A $^{+}$ CD4^{+} T cells, the cells were isolated and then fixed by 4% paraformaldehyde in PBS for 20 min at room temperature, and filtered through 0.2% Triton X-100 for 15 min. Subsequently, the samples were suspended in PBS containing 5 mg/mL BSA, and then stained by APC anti-human IL-17A (17-7179-42, eBioscience, San Diego, CA, USA) and anti-human CD4 (14-0049-82, eBioscience, San Diego, CA, USA). The identification was carried out by FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA), and the analysis was performed using BD CellQuest Pro software (version 0.3, Franklin Lakes, NJ, USA).

Dual-Luciferase Reporter Assay

The downstream target gene of miR-1-3p was predicted by TargetScan and verified by Dual-Luciferase assay. The WT and mutant with ETS1 3'UTR region were amplified and cloned into pMIR-REPORT miRNA vector (Thermo Fisher Scientific, Waltham, MA, USA) as ETS1-WT reporter plasmid and ETS1-MUT reporter plasmid. Then, naïve CD4^{+} T cells were first treated by miR-1-3p or control miR and transfected with reporter plasmid. *Renilla* plasmid served as a reference. After 48 h, the activities of firefly and *Renilla* Luciferases were measured using Luciferase reporter assay system (Promega, Madison, WI, USA).

Western Blot

The ETS1 expression in naïve CD^{+}4 T cells transfected with pCMV6-XL4-ETS1 plasmid or miR-1-3p mimic was determined using Western blot. Naïve CD^{+}4 T cells were lysed by Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, Shanghai, China), and the total protein level was detected by bovine serum albumin (BCA) Assay Kit (Pierce Chemical Co., Rockford, IL, USA). Briefly, the proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA, USA), which was blocked by 5% skimmed milk for an hour at room temperature. Then, the membrane was incubated with primary antibodies for ETS1 (ab26096, 1:1000, Abcam, Cambridge, MA, UK) at 4°C overnight and subsequently cultured with secondary goat anti-rabbit (ab150077, 1:1000, Ab-

cam, Cambridge, MA, UK) for 2 h at room temperature. The bands were analyzed using Western blotting reagent (Santa Cruz Biotechnology, CA, USA) according to the instructions.

Statistical Analysis

The data were shown as a format of means ± standard deviation (SD) and analyzed in SPSS (SPSS Corp., Armonk, NY, USA). The comparison between two groups was performed by student *t*-test, while multiple comparisons were performed by one-way analysis of variance (ANOVA). The experiments in the present study were independently performed in triplicate. $p<0.05$ was considered to be statistically different.

Results

The Expression Level of MiR-1-3p in Patients with MS

The expressions of miR-1-3p in periphery blood mononuclear cells (PBMC) and cerebrospinal fluid (CSF) from 33 healthy donors, 36 MS patients at remitting stage, and 44 at relapsing stage were determined. High miR-1-3p expression level was observed in both PBMC and CSF from MS patients at relapsing stage as compared with healthy patients and those at remitting stage (Figure 1A and 1B). Subsequently MS-related cytokine levels were examined to analyze their correlations with miR-1-3p. In the serum from MS patients at relapsing stage, the level of NF-L, ESR and CRP were respectively detected by ELISA, Westergren and Turbidimetric inhibition immunoassay. The results showed that the three cytokine levels were positively correlated with miR-1-3p expression level (Figure 1C, 1D and 1E). The levels of NF-L, CXCL13 and CHI3L1 in CSF samples were also examined, and similar positive correlation with miR-1-3p level was also observed in CSF ($p<0.001$, Figure 1F, 1G and 1H).

Elevated MiR-1-3p Expression in Th17 Cells

The $CD4^+$, $CD8^+$ and non-T cells were isolated from PBMCs derived from healthy individuals and their expressions of miR-1-3p were determined. The highest miR-1-3p expression level was detected in $CD4^+$ T cells, while significant lower miR-1-3p expressions were observed in $CD8^+$ and non-T cells (Figure 2A). Then, $CD4^+$ T cells were collected, induced and differentiated into Th1, Th2, Th17 and Treg cells *in vitro*, and the miR-1-3p expression was examined as well. In Th17 cells, the expression of miR-1-3p was the highest among those four groups, while in Treg cells, miR-1-3p presented the lowest expression level (Figure 2B). Moreover, the IL-17A and RORC levels were measured in serum and CSF from MS patients at relapsing stage, and the results showed that both IL-17A and RORC levels were positively correlated with miR-1-3p expression (Figure 2C-2F).

MiR-1-3p May Participate in the Differentiation of Th17 Cells

The miR-1-3p mimic was transfected into naïve $CD4^+$ T cells and the transfection efficiency was detected, and we observed that the expression of miR-1-3p was significantly increased with the transfection of miR-1-3p mimic (Figure 3A). Subsequently, the mRNA expressions of IL-17A and RORC were examined, and the data revealed that mRNA expressions of IL-17A and RORC were greatly upregulated by the transfection of miR-1-3p as compared with the control and mimic control groups (Figure 3B). After separated by flow cytometry, production of $CD4^+$ T cells was more than 90% (Figure 3C), and the proportions of $IL\text{-}17A^+$ $CD4^+$ T cells (Th17 cells) were also greatly increased in the cells transfected with miR-1-3p mimic (Figure 3D). For comparison, the naïve $CD4^+$ T cells were transfected with miR-1-3p as described above and then incubated in differentiating culture medium of Th17 for 5 days to further detect the index. The mRNA expressions of IL-17A and RORC were significantly increased in the group of miR-1-3p mimic as compared with the control and mimic control groups (Figure 3E). Furthermore, some pathogenic (CXCL3, CSF2, IL-23R) and nonpathogenic (AHR, IL-10, MAF) genes related to Th17 cells were examined, and the results demonstrated that the mRNA expressions of CXCL3, CSF2 and IL-23R were significantly high in the cell group transfected with miR-1-3p mimic, while the mRNA expressions of AHR, IL-10 and MAF were inhibited in mimic group (Figure 3F). Flow cytometry analysis also indicated that the percentage of $IL\text{-}17A^+$ $CD4^+$ T cells were significantly increased by miR-1-3p mimic (Figure 3G).

MiR-1-3p Could Affect the Differentiating Process of Th17 in MS Patients by Targeting ETS1

The downstream gene of miR-1-3p was predicted by TargetScan (Figure 4A) and further tested by Dual-Luciferase reporter assay. The group of

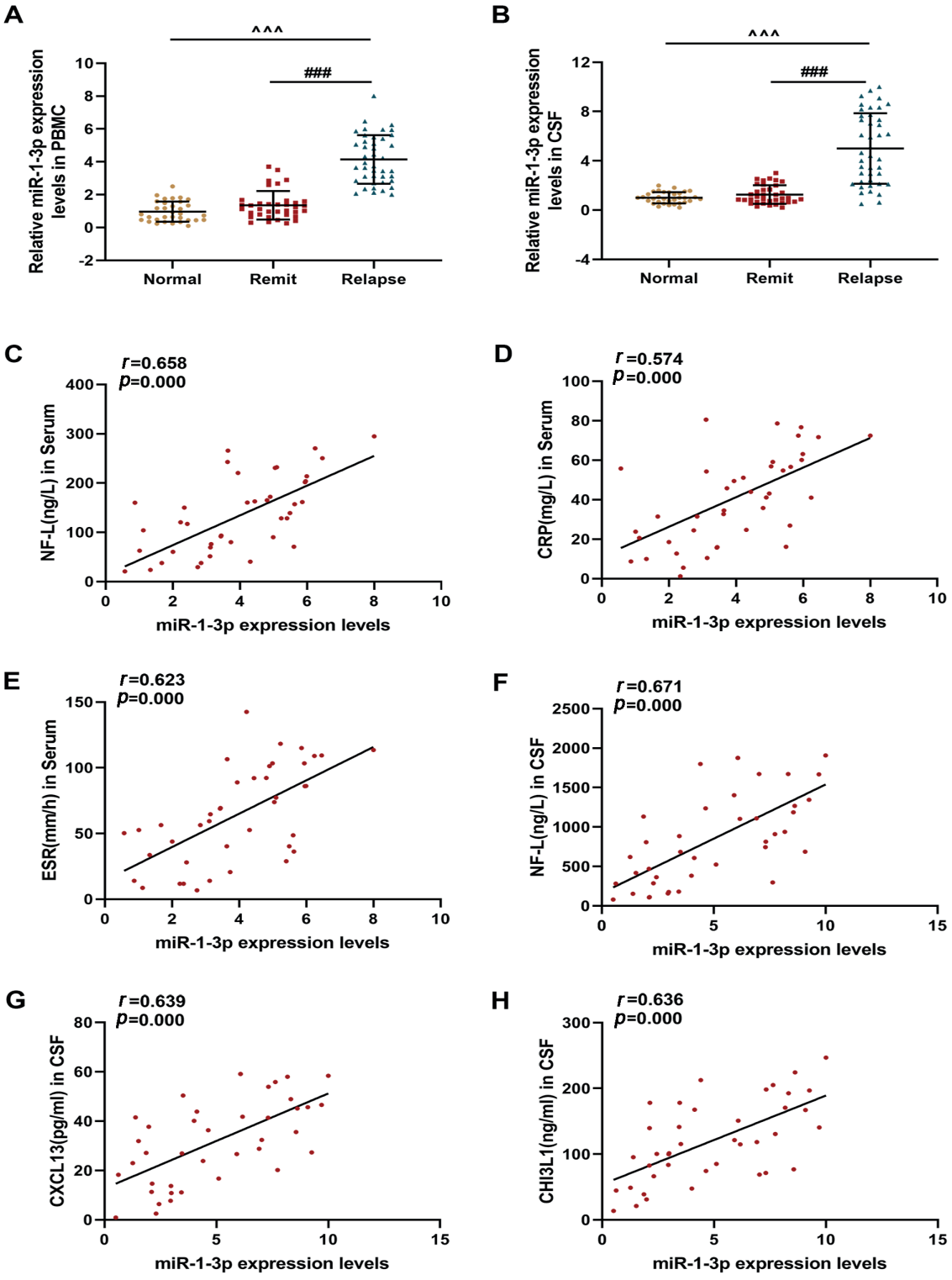

Figure 1. The expression level of miR-1-3p in patients with MS. **A-B,** The miR-1-3p expression in periphery blood mononuclear cell (PMBC) and cerebrospinal fluid (CSF) from MS patients at relapsing stage (n=42), MS patients at remitting stage (n=36) and healthy donors (n=33) detected by quantitative real-time quantitative polymerase chain reaction (qRT-PCR). **C-E,** NF-L, CRP and ESR expression levels in serum from MS patients at relapsing stage examined by enzyme-linked immunosorbent assay (ELISA), Westergren and Turbidimetric inhibition immunoassay, and their correlation with miR-1-3p. **F-H,** NF-L, CXCL3 and CHI3L1 expression levels in CSF from MS patients at relapsing stage assayed by ELISA and their correlation analysis with miR-1-3p. $^{\wedge\wedge\wedge}p<0.001$, *vs.* Normal; $^{\#\#\#}p<0.001$, *vs.* Remit.

ETS1-WT showed a significantly low Luciferase activity when the cells were transfected with miR-1-3p mimic, indicating that miR-1-3p negatively regulated ETS1 (Figure 4B). Moreover, the ETS1 expression was measured in PBMC from healthy individuals, MS patients at remitting and relapsing stages, and the lowest ETS1 expression level was observed in MS patients at relapsing stage as compared with healthy individuals and MS patients at remitting stage, moreover, the negative correlation between miR-1-3p expression and ETS1 was observed (Figure 4C and 4D). In or-

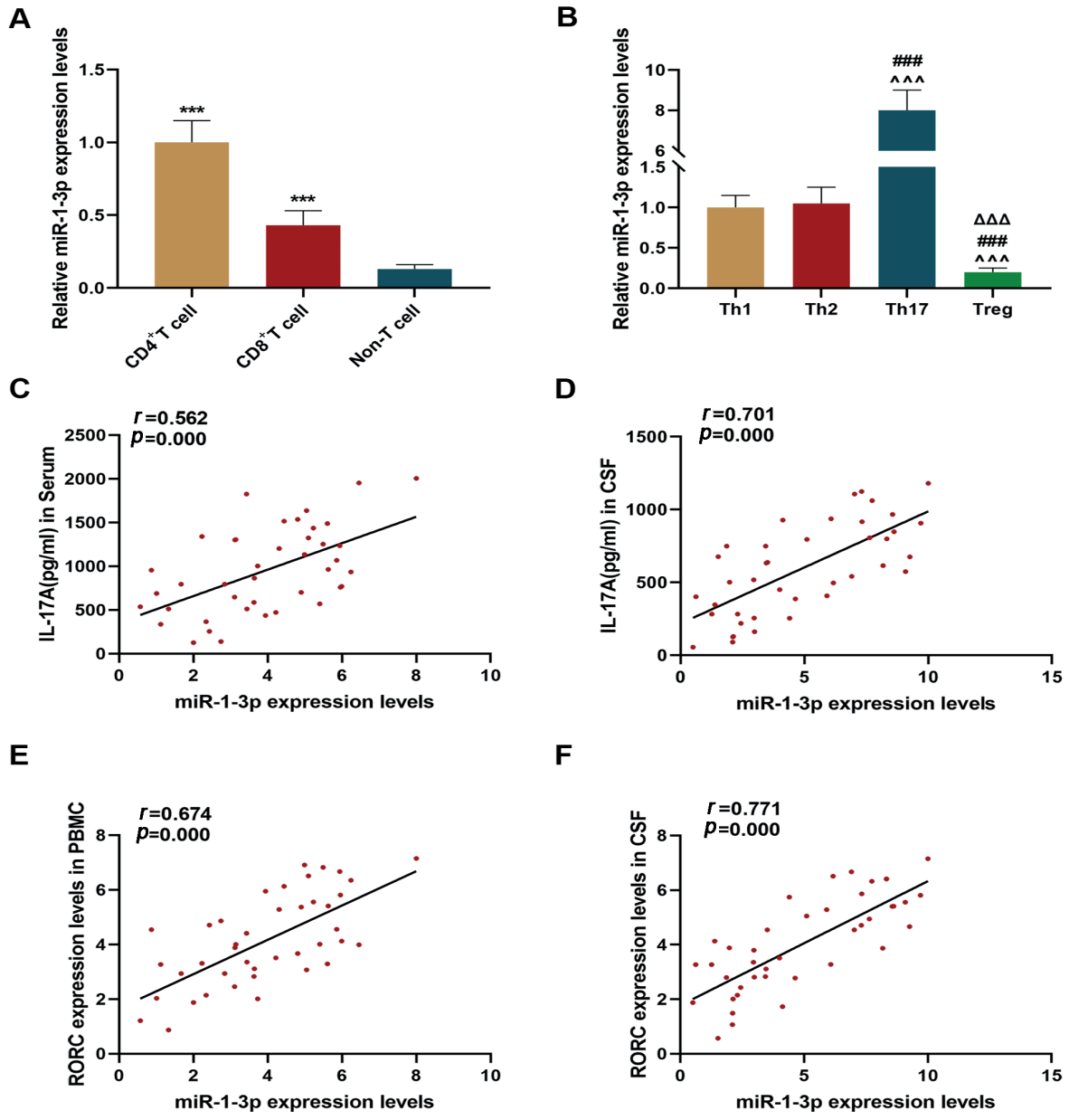

Figure 2. Elevated miR-1-3p expression in Th17 cells. **A,** Analysis of miR-1-3p expression level in different subsets of immune cells including $CD4^+$, $CD8^+$ and non-T cells deriving from periphery blood in healthy donors, the determination of miR-1-3p was carried out by qRT-PCR. **B,** Analysis of miR-1-3p expression level in differentiated naïve $CD4^+$ T cells, including Th1, Th2, Th17 and Treg cells, was detected by qRT-PCR. **C-D,** Positive correlation between IL-17A in serum and CSF derived from MS patients at relapsing stage and miR-1-3p, IL-17A was revealed by ELISA. **E-F,** Positive correlation between RORC in PBMC and CSF derived from MS patients at relapsing stage and miR-1-3p, RORC was determined by qRT-PCR. $^{***}p<0.001$, *vs.* Non-T cell.

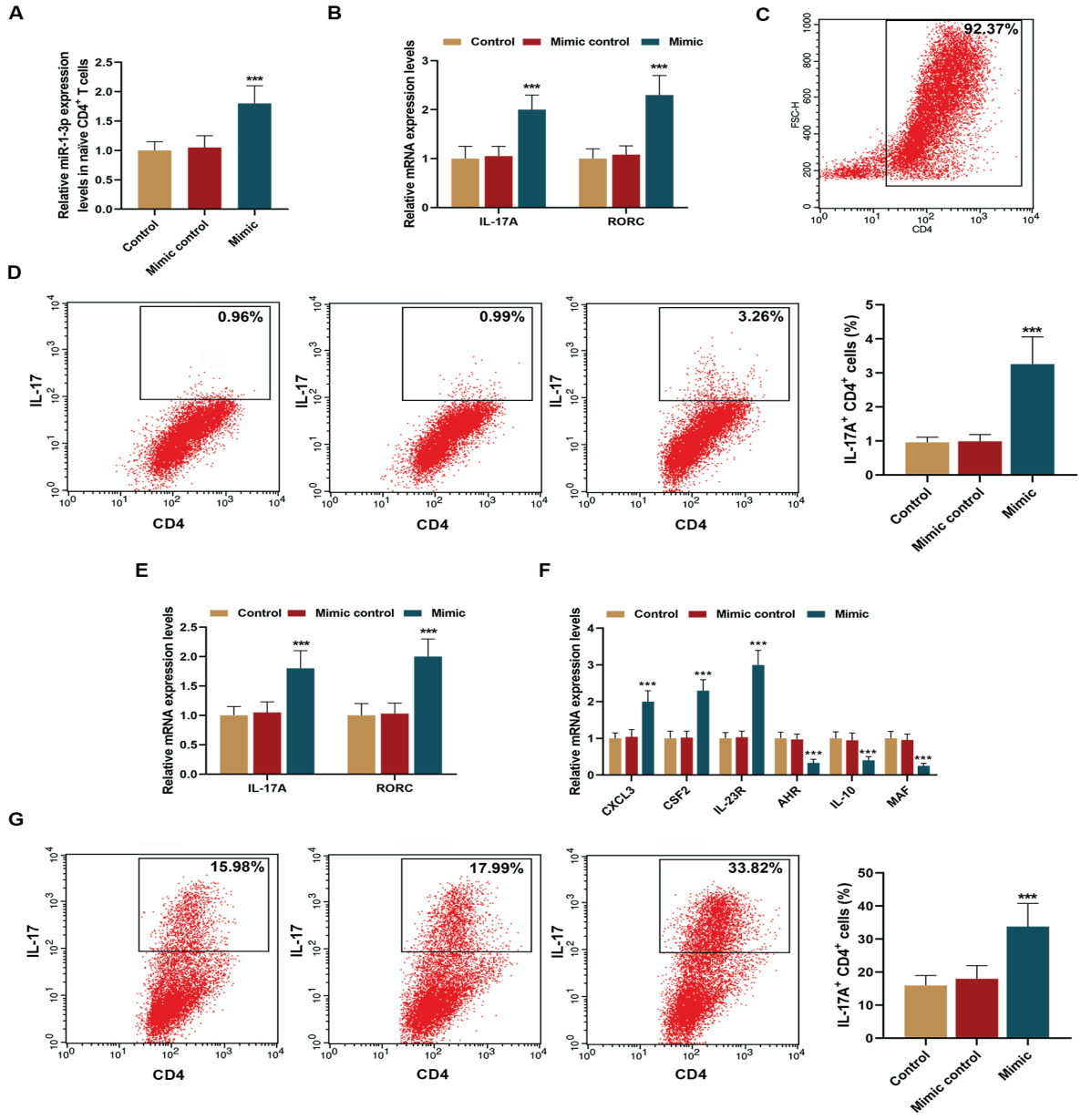

Figure 3. MiR-1-3p may participate in the differentiating process of Th17 cells. **A,** transfection efficiency of miR-1-3p in naïve CD4+ T cells was determined by qRT-PCR, and the groups were divided into control, mimic control and mimic. **B,** The expression of IL-17A and RORC in naïve CD4+ T cells transfected as described above stimulated with anti-CD3 and anti-CD28 for 5 days was identified by qRT-PCR. **C,** Identification of CD4+ T cells using flow cytometry. **D,** Intracellular expression of IL-17A was analyzed by flow cytometry and the percentage of IL-17A+ CD4+ T cells was shown in bar chart. **E,** Naïve CD4+ T cells transfected as described above were cultured in Th17-skewing condition for 5 days, then the IL-17A and RORC expressions determined were by qRT-PCR. **F,** The Th17-related pathogenic (CXCL3, CSF2, IL-23R) genes and the nonpathogenic (AHR, IL-10, MAF) genes expression assayed by qRT-PCR. **G,** The expression of IL-17A and CD4 was analyzed by flow cytometry and the percentage of IL-17A+CD4+ T cells was shown in bar chart. $^{***}p<0.001$, *vs.* Mimic control.

der to explore the role of ETS1 in the differentiation of Th17 cells derived from MS patients, the overexpression of ETS1 plasmid was transfected into naïve CD4+ T cells with or without miR-1-3p mimic of healthy individuals, and the expression of ETS1 was found greatly upregulated after the transfection (Figure 4E and 4F). 5 days after incubation in Th17 culture medium, the expression levels of IL-17A and RORC were determined, and we observed that the upregulation of ETS1 sig-

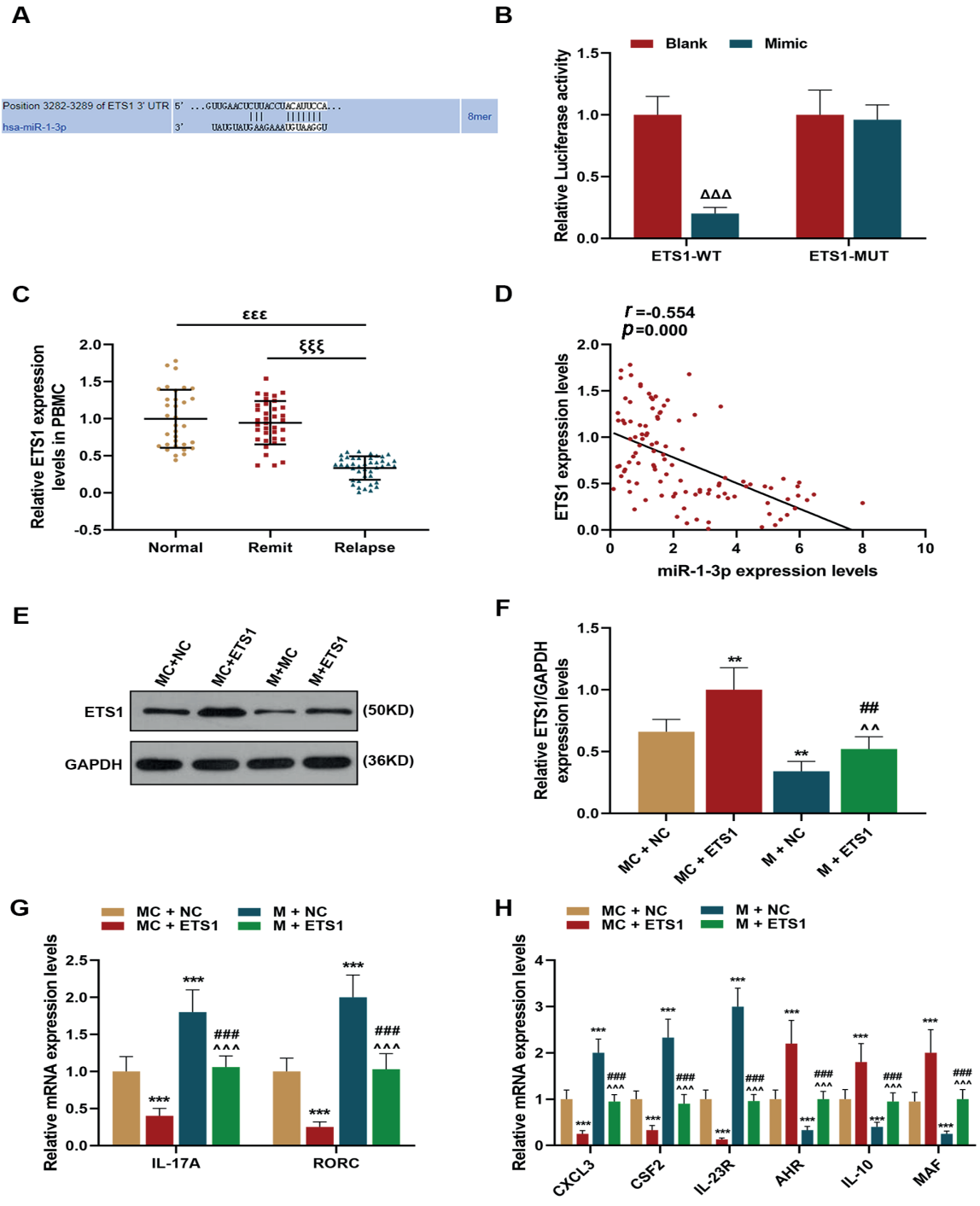

Figure 4. MiR-1-3p targets ETS1. **A,** Prediction of miR-1-3p target gene by TargetScan. **B,** Verification of miR-1-3p targeting ETS1 by dual-luciferase reporter assay. $^{\Delta\Delta\Delta}P<0.001$, *vs.* Blank. **C,** The ETS1 expression in periphery blood mononuclear cell (PMBC) from MS patients at relapsing stage (n=42), MS patients during remitting stage (n=36) and healthy donors (n=33) by qRT-PCR. **D,** Correlation of ETS1 expression and miR-1-3p in healthy individuals, MS patients at remitting and relapsing stages, ETS1 expression level was determined by qRT-PCR. $^{\varepsilon\varepsilon\varepsilon}p<0.001$, *vs.* Normal; $^{\xi\xi\xi}p<0.001$, *vs.* Remit. **E-F,** Transfection efficiency of ETS1 in transfected naïve CD4$^+$ T cells detected by Western blot, and the groups were divided into MC+NC, MC+ETS1, mimic+NC, mimic+ETS1. **G,** Naïve CD4+ T cells in the groups above were cultured in Th17-skewing condition for 5 days, then the IL-17A and RORC expressions were indicated by qRT-PCR. **H,** The Th17-related pathogenic (CXCL3, CSF2, IL-23R) and the nonpathogenic (AHR, IL-10, MAF) genes expressions were observed by qRT-PCR. $^{*}p<0.01$. $^{***}p<0.001$, *vs.* MC+NC. $^{\wedge\wedge}p<0.01$, $^{\wedge\wedge\wedge}p<0.001$, *vs.* MC+ETS1; $^{\#}p<0.01$, $^{\#\#\#}p<0.001$, *vs.* M+NC. MC: mimic control; NC: negative control for overexpression; EST1: EST1 overexpression.

nificantly suppressed IL-17A and RORC expressions, which could be partially reversed by miR-1-3p mimic (Figure 4G). Upregulation of ETS1 suppressed the expression levels of pathogenic gene (CXCL3, CSF2, IL-23R), but promoted the expression levels of nonpathogenic genes (AHR, IL-10, MAF), which were found alleviated by miR-1-3p mimic (Figure 4H).

Discussion

Th17 cells are newly discovered T helper cells that can secret IL-17. During differentiation of Th17 cells, transcriptional growth factor-β (TGF-β) and inflammatory cytokines IL-6, IL-21, IL-1β and IL-23 play important regulatory roles[17]. Nevertheless, the secretion of IL-17 and IL-22 was observed to strongly interfere with the close junction of blood-brain barrier, meanwhile, an elevated level of cytolytic enzyme granzyme B (Granzyme B) has been identified. As a result, T cells and other immunocytes will transfer into CNS, thus disturbing the balance inside CNS[18]. Researches have been conducted to determine the role of Th17 in the pathogenesis of MS. MS is a disease closely related to CNS, while the cytokines produced by Th17 play a negative role in blood-brain barrier and CNS. Waisman et al[19] demonstrated that MS associated the most closely with Th17 cells and the presence of IL-17, moreover, the level of IL-17 was positively correlated with severity and progression of the disease.

Though the association between Th17 and pathogenesis of MS has been widely studied, some studies explored the underlying mechanism and exact role of Th17 in the initiation and progression of MS. MiRNAs, which have been investigated for their regulatory functions in the development of many tumors, have also drawn research attention for their modulation abilities in immune responses[20-22]. Differential expressions of miRNAs could regulate the differentiation of Th17 and autoimmunity progression. MiR-146a contribute to the development of autoimmune diseases, as it can regulate auto-reactive Th17 cell differentiation and organ-specific autoimmunity by suppressing autocrine IL-6 and IL-21[23]. By the establishment of EAE model in mice, miR-201a was found to promote the progression of Th17 subset by sponging IL-6/23-ATAT3 pathway. Meanwhile, the expression level regulated by the specific antagomir was able to control the severity of EAE[24]. However, miR-1-3p expression level was increased in PBMC and CSF from MS patient at relapsing stage in the present study, suggesting the possible relationship between miR-1-3p and MS. Then, positive correlations among NF-L, CRP, ESR, CXCL13, CHI3L1, IL-17A and RORC and the expression level of miR-1-3p were confirmed. Neurofilament light chain (NF-L) is MS molecular marker, and is widely used to indicate degree and severity of MS[25], moreover, serum C-reaction protein (CRP) and erythrocyte sedimentation rate (ESR) expression levels are found to be upregulated in MS patients[26,27]. CXCL13 and CHI3L1 have been reported related to the pathogenesis of MS and could negatively affect CNS[28,29]. IL-17A and RORC are cytokines produced by Th17 cells and contribute to the degradation of CNS, which is related to the pathogenesis of MS as previously explained. Nevertheless, the expression of miR-1-3p in Th17 cells was also significantly high, and upregulated miR-1-3p was shown to increase the pathogenicity of Th17 cells by increasing expressions of inflammation-related genes CXCL3, CSF2 and IL-23R.

Moreover, our study further explored the target gene of miR-1-3p affecting the pathogenesis of MS by regulating ETS1 in the differentiation of Th17 cells. ETS1 transcription factor belongs to ETS gene family and is known as the regulator in many immune cell functions. In Behcet's disease, ETS1 is seen as the target gene of miR-155 in regulating Th17 immune response[22]. In severe atopic dermatitis, suppressed expression level of ETS1 was observed, and further experiments revealed that ETS1 acted as protector in the development of atopic dermatitis disease by suppressing pathogenic T cell responses[30]. As shown by Moisan et al[31], Th cells could be differentiated more efficiently with downregulated ETS1, however, the ETS1-deficient mice presented an aberrantly high expression level of IL-17 transcripts and an elevated mucus secretion by airway epithelial cells in IL-17-related behaviors, thus, ETS1 acted as a suppressor in the differentiation process of Th17 cells. Here, we found that miR-1-3p expression was negatively correlated with ETS1 expression in the MS patients at relapsing stage, and that the upregulation of ETS1 could facilitate the promotion of Th17 cell differentiation and inflammatory molecular caused by overexpression of miR-1-3p.

Conclusions

Our study demonstrated that miR-1-3p functioned together with increased expression of

pathogenic Th17 subset in MS patients at relapsing stage. MiR-1-3p might promote the differentiation process of Th17 cells and the levels of inflammatory cytokines *via* targeting ETS1.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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