Differential expression of microRNA-7188-5p and miR-7235 regulates Multiple Sclerosis in an experimental mouse model- an Biomarker approach

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Differential expression of microRNA-7188-5p and miR-7235 regulates Multiple Sclerosis in an experimental mouse model- an Biomarker approach

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ABSTRACTS:

The short non-coding microRNAs (miRNAs), have emerged as reliable modulators of various pathological conditions including autoimmune diseases in mammals. The current study, aims to identify new potential differential expressed miRNAs and their downstream mRNA targets of the autoimmune disease, Multiple sclerosis (MS). First, we used a computational tool to identify a new set of miRNA(s) that are probably implicated in MS. Preliminary, computational screening reveals that miR-659-3p, miR-659-5p, miR-684, miR-3607-3p, miR-3607-5p, miR-3682-3p, miR-3682-5p miR-4647, miR-7188-3p, miR-7188-5p and miR-7235 are specifically elevated in the secondary lymphoid cells of EAE mice. In addition, expression of the downstream target genes of these miRNAs such as FXBO33, SGMS-1, ZDHHC-9, GABRA-3, NRXN-2 were reciprocal to miRNA expression in lymphoid cells. These confirmed by applying the mimic and silencing miRNA models, these data suggesting new inflammatory target genes of these promising miRNA biomarkers. The *in vivo* adoptive transfer model revealed that the suppression of miRNA-7188-5p and miR-7235 changed the pattern of astrocytes and CNS pathophysiology. The current study identified set of miRNAs and their mRNA targets as reciprocal regulator in MS disease. The absence of miRNA-7188-5p and miR-7235 enhanced the disease alleviation. These optimized results highlight new set of miRNA's based biomarkers with therapeutic potential in experimental MS.

Keywords

Multiple sclerosis; experimental autoimmune encephalomyelitis; Micro-RNA; Therapeutic biomarkers.

INTRODUCTION:

Multiple sclerosis (MS) is a neurological autoimmune disease characterized by infiltration of T lymphocytes and macrophages into the central nervous system (CNS), that leading to multifocal areas of demyelination in the CNS with loss of oligodendrocytes and results in microglial mediated pathological conditions [1,2]. Clinically, MS patients showed a variable pattern of relapsing remittance and intermittent inflammatory exacerbations. Biomarkers for diagnosing MS are proteins or specific antibodies including chemokines, glycoproteins, IgG and IgM antibodies, and cell surface markers of inflammation [3]. However, these biomarkers are not well-correlated with the disease course of MS [4]), indicating an urgent need of developing and validating biomarkers correlate MS using different detection methods [5].

Although the pathological events of MS are well-established, the epigenetic involvement in the pathogenesis of MS is still not completely understood [6-8]. A class of non-coding single-stranded RNA called miRNA which is 19–24 nucleotides, it regulates post transcriptional modulation in genome of the host . MiRNA could interact to 3′ untranslated region (UTR), or rarely 5′ UTRs, of mRNA transcripts, which made perfect and imperfect complementary binding, leads to redundant translational inhibition of mRNA targets [9-12]. In addition, the computational approach in the identification of novel and disease-related miRNA has been considered as a reliable and cost-effective tool [13-15], that reflect the progression of the disease in clinical samples [16]. Strategies such as qPCR, next-generation sequencing (NGS) and the microarray analysis has been utilized to analyze the miRNA expression pattern in various body fluids, such as blood, serum, plasma, cerebrospinal fluid (CSF) and urine in MS patients [17]. The levels of miRNA are significantly altered in clinical fluids with MS progression and in responses to treatment [18-21]. These researches implicate miRNAs are promising biomarkers in the MS autoimmune diseases and studying the role of miRNAs in MS has attracted attention in recent years [12]

Similar to clinical samples, dysregulated miRNA expression is noticed in experimental autoimmune encephalomyelitis (EAE) animal model, a well-characterized experimental model for the human MS disease, that exhibited CNS inflammation and pathological features of MS including ascending paralysis and motor neuron damage and death [22,23. Typically, the EAE model is induced through active immunization with myelin-derived proteins or peptides (e.g., myelin oligodendrocyte glycoprotein (MOG) in an adjuvant to sensitize the T cells in the peripheral lymphoid tissue [24]. Moreover, *in*

vivo injection of the lipopolysaccharides (LPS) induces EAE in T cell receptor (TCR) transgenic mice and relapse of encephalomyelitis in normal mice [25]. Recent reports also identified miR-155 as a key regulator of these inflammatory responses, in mice resulted in a decrease in Th1 and Th17 cellular differentiation in the CNS as well as peripheral lymphoid organs. However, animal models for MS can produce chronic inflammatory events, the in vivo approach for studying miRNAs role in neuronal degeneration and regeneration is still lacking. In this study, we aim to fill this knowledge gap. Here, we applied bioinformatics approach to recognize a new set of miRNAs that correlate with MS progression and quantified the expression levels of identified miRNAs and their targets in EAE/MS disease mice model.

MATERIALS AND METHODS:

Collection of reference miRNA and EST sequences:

Expressed Sequence Tags (EST) related to MS was used for the identification of miRNAs (Figure 1) as reported earlier [14]. The above EST sequences (15,042 ESTs as of December 2018) were extracted from NCBI using the search term "Multiple sclerosis". The published pre-miRNA (38589 as of December 2018) and mature miRNA (48885 as of December 2018) were retrieved from the miRBase (http://www.mirbase.org/). After eliminating redundant and poor quality sequences, local nucleotide database was created for MS specific EST sequences. The above nucleotide database was searched for their homolog among the miRNAs dataset.

Identification of miRNAs and their precursor sequences:

The mature miRNAs were used as reference and its homolog were searched against the created local MS specific nucleotide sequence database at e-value threshold <0.01 using BLAST 2.2.22+ program with all other parameters as default [26]. The FASTA formats of all the candidate sequences were saved. Reference precursor and mature miRNA sequence was aligned against the corresponding singleton ESTs using ClustalW [27] multiple sequence alignment tool. Selected EST sequences with not more than three mismatches were validated for their non-protein encoding phenomenon using BLAST against protein database at NCBI using BLASTx with default parameter [28]. EST sequences were aligned to reference pre-miRNA sequences, the aligned region were extracted and considered as candidate pre-miRNA sequence.

Validation of precursor candidate miRNAs and identification of target:

The candidate pre-miRNAs extracted were validated for secondary structure using Mfold v 3.2 (http://www.mfold.rna.albary.edu/). While selecting a candidate miRNA precursor from the EST resource, the following criteria were used according to Zhang et al. [29]. (a) RNA sequence must fold into an appropriate stem-loop hairpin secondary structure, (b) mature miRNA sequence site in one arm of the hairpin structure, (c) miRNAs should have less than seven mismatches with the opposite miRNA sequence in the other arm, (d) predicted secondary structures had higher negative energy MFEs (≤−18 kcal/mol) and 40–70 % A + U contents. The TargetScan algorithm [30, 31] was used to identify potential targets for miRNA regulation in multiple sclerosis.

Mice and ethics statement

The female 6-8-week-old C57BL/6 mice for all experiments were maintained under specific pathogen-free conditions of animal facility of College of science, King Faisal University, Saudi Arabia. All in vivo and in vitro experiments were performed in accordance with protocols approved by the Research Ethics Committee (KFU-REC/2017-3-1) of King Faisal University, Saudi Arabia. The humane endpoints included 25% body weight loss, paresis or forelimbs paralysis for 24 hr.

EAE models

EAE was induced following a modified method described previously [32]. Briefly, MOG35-55(125lg/mL; Pep-tides International, KY, USA) emulsified in complete Freund's adjuvant (CFA; Sigma-Aldrich) containing H37RA (Mycobacterium tuberculosis; Difco Laboratories, NJ, USA) was injected at the base of the tail. On days 0 and 2, the mice received intraperitoneal injections of pertussis toxin (300 ng; Sigma-Aldrich). The scoring system was adopted as described elsewhere [32] and assessed by expert who is blinded of experimental details; 0,normal; 1, limp tail; 2, hind limb paresis; 3, forelimb weakness; 4,paralysis; and 5, moribund. For adoptive transfer, CD4+T cells isolated from EAE mice 10 days post immunization were cultured for72 h with MOG35-55(25 μg/mL) and IL-23 (25 ng/mL). The encephalitogenic cells ($1x10^7$cell/mouse) were injected intravenously into EAE mice.

Isolation of mononuclear cells from CNS

Mice were perfused intracardially with PBS before the dissection of the CNS, which subsequently minced and homogenized. Mononuclear cells were segregated from cell mixture using 37–70% (vol/vol) Percoll gradients [33]

Cell isolation and differentiation

The naïve (CD4+CD62L+) T cells was isolated usinga MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The conditions for the differentiation of Th17 and Treg cells were adopted from report with modifications [34]. Briefly, naïve CD4+T cells were cultured in the presence of Mouse T-Activator CD3/CD28 Dynabeads (Invitrogen, CA, USA). To generate Th17 cells, the culture media was supplemented with IL-6 (25 ng/mL; R&D Systems, MN, USA), TGF-β1 (3 ng/mL; R&D Systems), and anti-IFN-γ and anti-IL-4 antibodies (10 μg/mL; BioLegend, CA, USA). To generate Treg cells (TH2), the cell culture was supplemented with TGF-β1 (4 ng/mL; R&D Systems) and IL-2 (25 U/mL; R&D Systems).

Macrophage and astrocyte cell cultures and treatment

Macrophages and astrocytes cultures were prepared, as previously described [35]. Briefly, brain cortex was removed from euthanized C57BL/6 mice under sterile conditions. Cells were extracted using cell strainer (80 μm) cells were cultured for 5 days in the presence of M-CSF 50 ng/ (eBioscience) [36]. Differentiated macrophages were treated with lipopolysaccharide (LPS) (10 and 100 ng/mL) for 12 hr at 37 °C before RNA extraction. For astrocyte cultures, cells were removed as above and placed in Dulbecco's Modified Eagle Medium (DMEM/F12) medium under sterile conditions. Brain tissues were dissected, and astrocyte cells were cultured in (DMEM/F12) medium supplemented with 20% FBS. Astrocytes were stimulated with 10 and 100 ng/mL LPS (Sigma Aldrich, Taufkirchen, Germany) for 12 hr at 37 °C [36].

Quantitative real-time PCR

cDNA was synthesized using a TaqMan reverse transcription kit and amplified by using a ViiA7 system. Kits, probes, and reagents for miRNA expression and gene expression assays and target gene primers (Table TS.1) for coding genes and microRNA primers were obtained from Applied Biosystems (CA, USA). The relative expression of mRNAs and miRNAs were calculated by the ΔΔCt method [37].

Transfection and luciferase activity

CD4+ cells were transfected with oligonucleotides using Primary Cell Nucleofector kits and a 4D-Nucleofector system (Lonza, Basel, Switzerland). An antisense miRNA specific for miR-7188 and miR-7235 (150 nmol/L), a scrambled siRNA (75 nmol/L), an antisense (as)-of specific targeted miRNA (250 μmol/L) and a scrambled control (250 μmol/L) were obtained from (Ambion, Austin, TX, USA). qPCR and immunoblotting were used to confirm transfection efficiencies. Reporter plasmid and luciferase activities in the presence of siRNA were assessed following a modified method described elsewhere[28]. A miRNA sequence of differentially expressed mouse miRNA mature sequences was amplified using the following oligonucleotide primers (Figure S1). The scrambled or specific miRNA promoter-encoding expression vector (100 ng) was co-transfected with Si-NS into HEK293K cell-conditions electroporation. Luciferase activity quantified using the Dual-Luciferase Reporter System (Promega) following the manufacturer's instructions.

Protein quantification

Cell lysates from transfected and un-transfected CD4 cells were prepared using the RIPA Lysis Buffer System. Target proteins in the lysates were detected by rabbit polyclonal antibodies specific for NRXN-2 (dilution; 1:1000), GABRA-3 (dilution; 1:500), ZDHCC-9 (dilution; 1:1500), MAPK-6 (dilution; 1:500), FXBO-33 (dilution; 1:1000), mouse monoclonal antibodies specific for β-actin (dilution; 1:1,000) and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000). The Lysis Buffer System and antibodies were purchased from Santa Cruz Biotechnology. Band intensity was quantified by ImageJ software (version 1.48; https://imagej.nih.gov/ij/download.html). To quantify the serum and supernatant cytokine levels, ELISA kits for IL-17a, IL-6, TNF-α, TGFβ (Invitrogen), and IL-10 (GenWay, CA, USA) were used following the manufacturer's instructions.

Immunohistochemistry

To detect T cell infiltration and demyelination in EAE spinal cords, immunohistochemical staining for CD3 T cell marker was performed on lumbar spinal cord sections. Briefly, formalin-fixed paraffin-embedded spinal cord sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the sections in 0.01 M trisodium citrate buffer (pH =

6). Sections were next blocked in 10% normal goat serum containing 0.1% triton X-100 and then incubated overnight at 4°C with antibodies against CD3 (1:100; Santa Cruz Biotechnology Inc. CA, USA) and myelin basic protein (1:500; Sternberger Monoclonal) followed by washing. Sections were then incubated with HRP-conjugated secondary antibodies (1:500, Abcam, MA, USA) followed by color development using DAB substrate solution.

Intracellular staining and flow cytometry

To detect intracellular expression of interleukin (IL)-17A, in transfected CD4+ T cells, cells were surface-stained with anti-CD4 and anti-CD3 antibodies and then fixed with 1 mL/tube BioLegend's Fixation Buffer, at room temperature in the dark for 20 min. The cells were stained with flurochrome-conjugated anti- IL-17A, antibody (Biolegend, CA, USA). Stained cells were assayed with (FlowSight system, Darmstadt, Germany) and results were analyzed with Idea Flow software.

Isolated CD4+ T cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 800 ng/mL ionomycin (Sigma-Aldrich, Taufkirchen, Germany) for 5 hr, with Protein Transport Inhibitor (Invitrogen) added for the final 2 hr. An Intracellular Staining kit (Life Technologies, CA, USA) and phycoerythrin (PE)-conjugated anti-IL-17 antibodies (eBioscience, CA, USA) were used following the manufacturer's instructions. The analysis was performed using (FlowSight system, Darmstadt, Germany).

Analysis of miRNA in MS patients.

Blood and CSF were obtained from 45 consecutive patients referred for diagnostic lumbar puncture. The Institutional Review Board approved the study and informed consent was obtained from all subjects. Diagnosis of MS was based on 2010 revisions to the McDonald diagnostic criteria [38]. Totally, 22 subjects (10 male and 12 female) had evidence of inflammatory CNS demyelination, manifested either as MS, or as a clinically isolated syndrome suggestive of MS in combination with radiology evidences. Selection of patients based on the non-treated with immunomodulatory drugs and exposed acute exacerbation during examination. Non-MS Patients were grouped into diagnostic categories of adult or pediatric inflammatory. Patients who did not fulfill these criteria were classified as non-immuno neuro diseases.

Statistical analysis

Data were pooled from three independent experiments performed in triplicate using three mice per experiment unless otherwise indicated. The mean values were tested for statistical significance by one-way ANOVA. The values of cytokines and PCR expression fold are analyzed using student's t tests parametric and non-parametric mean comparisons between the two groups. Data are shown as mean + SEM. Significance will be considered $P \leq 0.05$.

Results:

Computational identification of miRNAs and their targets

A total of 15094 ESTs from multiple sclerosis samples were retrieved from NCBI and then used for the prediction of miRNAs associated with MS. After careful evaluation of fewer than four mismatches among the mature, premature miRNAs and the respective matched EST sequences and secondary structure analysis, 11 miRNA candidates were proposed to be differentially expressed under multiple sclerosis conditions (Figure 1A-B). Details of the predicted miRNAs are shown in Table TS.1. Among these 11 miRNAs hsa_miR-684, hsa_miR-7188-3P and hsa_miR-7188-5P are reported here for the 1st time in humans, based on their homology with transcripts in *Macaca mulatta* and *Mus musculus* (Figure 1B and Table TS. 1).

Validation of identified miRNA in EAE mice.

The miRNAs that qualified the validation of the comparison between MS and non-MS were analyzed based on their expression profiles. Next, we estimated the expression pattern of the candidate miRNAs from demyelinated tissues which were validated in the splenocytes of control healthy and EAE mice (Figure 2A). The validation of differential miRNA in disease symptom patients and volunteered CSF CD4 cells using quantitative real time PCR analysis (Figure 2B). The results revealed that, the parallel overexpression of miR-684, miR-4647, miR-7188-3p, miR-7188-5p and miR-7235 were noted in clinical patients sample compared to control volunteer subjects. The EAE clinical score and disease incidence of EAE mice were assessed for 22 days post immunization. The EAE clinical score increased initially on 12th day of immunization, profoundly peaked on the 18th day, and started to decline after 20th day of MOG immunization (FigureS1 A-B). The histochemical and microscopy analyses showed damaged glial cells and astrocytes in addition to other pathological lateral modifications in EAE mice. (Figure S1 C-D). The

histology score of myelin degradation and the infiltration of secondary immunological cells were significantly increased in EAE-diseased mice. The demyelination score of EAE-diseased mice as denoted by laxol fast blue score at 22 days of MOG immunization increased significantly relative to the control mice (Figure S1 E-F). The expression of miR-659-3p, miR-659-5p, miR-684, miR-3682-3p miR-3682-5p, miR-4647, miR-7188-3p, miR-7188-5p and miR-7235, were significantly increased in EAE mice compared to control mice. Of these miRNAs, miR-684, miR-4647, miR-7188-3p, miR-7188-5p, and miR-7235 showed >10-fold increase relative to that in the non-EAE mice. Similarly, the serum cytokine levels of IL-6, IL-1β and TNF-alpha in naïve and EAE mice after 24 days of MOG immunization were significantly increased compared to those in control naïve mice (Figure 3A). Therefore, in this study, these miRNAs were selected for further examination.

Select miRNA expression is upregulated in activated splenocytes, macrophages, and astrocytes.

The involvement of other immune cells in the inflammatory response induced in EAE mice and their contribution to the activation of subsequent pathological processes were further analyzed.. RT-PCR data showed that miR-7188-3p, miR-7188-5p and miR-7235 levels were significantly increased in MOG-treated splenocytes. Typically, miR-7188-3p and miR7188-5p levels were increased significantly at 16 hr of MOG treatment. However, the expression levels of miR-684 and miR-4647 not show significant variation relative to those of the other tested miRNAs (Figure 3B). In studies evaluated the expression of miRNAs in naïve CD4 cells activated by anti-CD3 and anti-CD28 antibodies, a considerable increase in the expression of miR-684, miR-4647, miR-7188-3p, miR-7188-5p and miR-7235 at 16 hr and greater time points (Figure 3C). Since the MOG peptide partially activated the splenocytes, the expression of miRNAs was considerably increased at anti-CD3/CD28 stimulation compared to after MOG stimulation.

Furthermore, the expression of these miRNAs was analyzed in microglial-based macrophages and astrocytes extracted from the brain and the spinal cord. The stimulation of these cells using LPS improved the features of monocyte/glial cell inflamed activation in multiple sclerosis conditions. Although the expression of the identified miRNAs was upregulated by LPS stimulation, we did not observe any dose-dependent variation in the expression levels (Figure 3D-E). However, macrophages delivers the downregulated

expression of miR-7188-3p at LPS (50 ng/mL) concentration. Additionally, LPS-stimulated primary astrocytes showed differential expression of miR-4647 and miR-7235 but no significant expression differences were observed for other selected miRNAs (Figure 3E). Interestingly, the miRNAs were found to be differentially expressed in different *in vitro* conditions and in different cell types, which indicates that astrocytes are not greatly involved in post transcriptional processes, whereas astrocytes could regulate other autoimmune cytokine mediators. The results of ELISA estimation of cytokines showed increased secretion of TNF-α, IL-1β and IL-6 in these conditions. In a parallel study using T lymphocytes, the expression levels of some cytokines such as IL-17 and IL-10, were increased, whereas the expression level of TGF-β was decreased in EAE mice (Figure 3F).

Expression of miRNA targeted transcripts regulated by mimics and siRNA.

To investigate the potential mRNA transcripts that might be targeted by miR-684, miR-4647, miR-7188-3p, miR-7188-5p, and miR-7235, we mined a list of predicted mRNA targets from TargetScan tool and Miranda databases. Considering the large number of potential targets (Table TS.1), we selected miRNA-targeted genes with known roles in cytokine signaling, myelin regulation and signal receptors in multiple sclerosis conditions (Figure S1). We selected SGMS-1, ZDHHC-9 and NRXN-2, FBXO-33, GABRA-3 and MAPK-6 as the target of miR-684, miR-4647, and miR-7188-3p, miR-7188-5p, and miR-7235, respectively. The mature miRNA sequences and the mRNA binding sites in the 3' untranslated regions (3' UTRs) of these genes are conserved between humans and mice and the binding sites are the same (Fig-S1). The immunohistochemical analysis of demyelination in the spinal cord showed reduced accumulation of different target proteins in EAE mice (Figure 4A-B). The mRNA expression levels of selected transcripts in stimulated splenocytes at different time points (8 hr, 16 hr and 24 hr, Figure 4C) showed that the expression of these targets was downregulated significantly after 8 hr of stimulation. The expression of the tested miRNAs varied significantly in stimulated splenocytes; with the exception of GABRA-3, the expression of other targets was downregulated by 30%. These results are similar to the observations in the western blot analysis of these targets at 16 hr and 24 hr time points (Figure 4D-E).

Furthermore, we overexpressed or silenced the expression of miR-684, miR-4647, miR-7188-3p, miR-7188-5p and miR-7235 via mimic and siRNA sequence transfection in splenocytes and astrocytes and analyzed the expression of the respective target mRNAs using RT-PCR. In both cell types, the overexpression of miRNAs via mimic-miRs produced the downregulation of the expression of the target mRNA transcripts (Figure 5A-F). However, the silencing of the miRNAs via si-miRs upregulated the expression of all target mRNAs in both cell types (Figure 5A-F). SGMS mRNA expression levels showed significant inverse regulation by miR-684 mimic and siRNA target. The significant expression of target genes was observed in siRNA transfected cells compared to mimic transfected cells. Interestingly, astrocytes showed more expression of SGMS-1 compared to splenocytes (Figure 5A). FBXO-33 expression levels showed reciprocal regulation by miRNA sequences. The significant down regulation was observed in siRNA transfected cells compared to mimic transfected cells of miR-4647 sequences. Whereas, the mimic affects a similar expression in both cells, but siRNA showed significant increased expression in astrocytes compared to splenocytes (Figure 5B). GABRA-3 is an signaling receptor in neuronal cells, which also act as regulator of CNS and astrocytes. GABRA-3 was target of miR-7188-5p. The mimic and siRNA of miR-7188-5p was potential regulator of GABRA-3 protein. The mimic masks the GABRA-3 transcripts and down-regulates its level in astrocytes compared to splenocytes. Despite, siRNA of miR-7188-5p was up-regulate the GABRA-3 transcripts in both splenocytes and astrocytes (Figure 5C). miR-7188-3p targets the MAPK-6 transcripts which act on cellular regulations such as, differentiation, development and polarization of lymphoid cells. The mimic of miR-7188-3p was not significantly altered the MAPK-6 in splenocytes and astrocytes. Whereas, siRNA of miR-7188-3p regulate significant increased expression of MAPK-6 in astrocytes compared to splenocytes (Figure 5D). The transcript of ZDHHC-9 and NRXN-2 expression were parallel on both cells. Particularly, ZDHHC-9 target increased in siRNA based transfected cells of splenocytes and astrocytes. Whereas, NRXN-2 was not significant increased expression in splenocytes but this transcript expressed significant in astrocytes (Figure 5 E-F). These findings correlated with those of the western blot and luciferase activity assays in transfected cells.

Reduction of mRNA transcripts by the direct interaction of miRNA

To validate the identified miRNAs, PGL3 vectors encoding 3'UTR region of candidate mRNA transcripts including MAPK-6, GABRA-3, ZDHHC-9, NRXN-2, FBXO-33 and

SGMS-1 were cotransfected into HEK293T cells in the presence or absence of the corresponding miRNA mimics. The reciprocal expression of Renilla and firefly luciferase caused significant degradation of the luciferase signal in the mimic and negative control transfection systems (Figure 4G-K). Transcript suppression was observed for all transfected miRNA. However, the miR-684, miR-4647, miR-7188-3p, miR-7188-5p, and miR-7235 mimic-transfected cells showed relatively less significant differences than the miR-7188-3p- and miR-7235-transfected cells (Figure 5G-K). Taken together, these findings suggest that MAPK-6, ZDHHC-9 and NRXN-2 are directly targeted by the identified miR-7188-5p and miR-7235 miRNAs.

miR-7188-5p and miR7235 attenuation alleviates EAE by myelin-dependent CD4 cell polarization.

The reciprocal correlation between Th-17 and Treg cells is a hallmark in EAE pathogenesis. Adoptive transferred of $CD4^+$ T cells from MOG immunized mice attenuated miR-7188-5p and miR-7235 and scrambled miRNAs, which restimulated with naïve control mice (Fig-6A). The death incidence was calculated by humane endpoints and the disease recovery was calculated and noted for disease and Si-miR- 7188-5p and miR-7235 respectively (Fig-6A).The effect of Si-miR-7188-5p and miR7235-transfected CD4+ T cells transferred from EAE mice on Th17 polarization was also investigated, shown in (Fig- 6B). Th17 cell differentiation was significantly reduced in EAE mice that received plain $CD4^+$ T cells compared to those that received-antisense miR7188-5p- and miR-7235-treated $CD4^+$ T cells. Furthermore, the histological and immunohistochemical changes were examined; the microscopy-based examinations revealed that, the infiltration of astrocytes and macrophages was comparatively suppressed in si-miRNA transfected mice (Figure 6C-E, I). The protein histology revealed that the accumulation of ZDHHC-9, NRXN-2 and GABRA-3 protein in lumbar spinal cord and cerebellar tissues increased significantly in Si-miRNA transferred mice (Figure 6F-H,J,K). The regained protein may reduce EAE pathology; this result is correlated with the results of the newly resigned ELISA (Figure 6L). The miRNA and mRNA targets were confirmed in patients CSF leukocytes were confirmed by quantitative realtime PCR analysis (Figure6M,N).

Discussion

In the present study, we suggest a promising set of miRNAs as potential biomarkers of MS using the EAE mouse model. None of these identified miRNAs using the

computational approach has been reported previously to be involved in MS disease progression. In addition, we suggest new inflammatory target genes of these promising biomarkers and report hsa_miR-684, hsa_miR-7188-3P and hsa_miR-7188-5P for the first time in humans as novel inflammation-related miRNAs.

The current EAE model displayed the pathological characteristics of MS disease, including glial cell and astrocyte damage associated with a marked increase in myelin degeneration and infiltration of secondary immunological cells [2]. In synchronization with the EAE pathological features, we scored significant increase in serum levels of IL-6, IL-1β, and TNF-alpha in EAE mice after 24 days of MOG immunization, which is consistent with previous studies[39,40]. The expression of nine out of eleven identified miRNAs increased significantly in EAE mice. Of these miRNAs, miR-684, miR- 4647, miR-7188-3p, miR-7188-5p and miR-7235 showed greater than 10-fold increase in the expression level compared to that in the non EAE mice. The qPCR data also showed that miR-7188-3p, miR-7188-5p and miR-7235 levels are significantly increased in MOG-restimulated splenocytes *in vitro* after 16 hr of MOG treatment. Interestingly, among these differentially expressed miRNAs, no miRNA other than miR-4647 has been reported to be expressed in inflammatory autoimmune disease [41,42]. Actually, the miRNAs profile in splenocytes was shown to be dysregulated in mice model of human MS. Also, miRNAs upregulation in the spinal cord tissues and splenocytes from EAE mice (such as miR-92a) were recently reported to be correlated with the progression of MS [43]. Therefore, in addition to analyzing the expression in splenocytes, we analyzed the role of these identified miRNAs in other cell types of EAE mice. In general, our *in vitro* study showed that there is a significant increase in the expression of the five miRNAs in LPS-stimulated macrophages and astrocytes compared to control cells. Consistently, the proinflammatory cytokines (TNF-α, IL-1β and IL-6) released by LPS-stimulated macrophages from EAE mice are significantly elevated relative to those released by macrophages from non-EAE mice. In addition, IL-17 and IL-10 expressions is upregulated and TGF-β expression but decreased in EAE mice. Actually, both clinical and experimental data indicated that proinflammatory cytokines, including TNF-α, IFN-γ and IL-6, produced by macrophages and other immune cell types establish an inflammatory microenvironment that facilitates damage induction at the myelin sheath and the surrounding cells [39, 40]. Related to that, Barin and colleques [44] reported the participation of macrophages in IL-17-mediated inflammation. An imbalance between the proinflammatory properties and immunosuppressive activities of TGF-β was found to

rise and then fall during inflammatory responses [45] may explain the decrease in the TGF-β level in the present study.

Target prediction using TargetScan tools revealed that most of the identified targets are involved in the process of neural biogenesis, signaling and inflammation. The mimic of miR-7188-5p masked the neuronal cell signaling receptor GABRA-3 and downregulated its expression in astrocytes. However, the siRNA targeting miR-7188-5p upregulated the GABRA-3 expression in both splenocytes and astrocytes. Here, we identified GABRA-3, which was previously reported to be a risk factor for MS in patients who died of their disease [46], as a target for miR-7188-5p.

MAPK-6 that involved on various cellular processes, such as differentiation, development and the polarization of lymphoid cells, was selected as an important target of miR-7188-3P. The mimic of miR-7188-3p did not significantly alter MAPK-6 expression in either splenocytes or astrocytes. However, siRNA targeting miR-7188-3p significantly increased the expression of MAPK-6 in astrocytes compared to splenocytes. The expression of mitogen-activated protein kinase 6 has been reported to be downregulated under MS conditions[47]. Among other targets of the miR-7188-5P, neurocalcin delta (NCALD), was found earlier to be downregulated in Alzheimer's disease [48]. The ST3 beta-galactoside alpha-2,3-sialyltransferase 2 (ST3GAL2) was also noticed as a target of miR-7188-5P. Silencing the expression of St3gal2 enhanced the demyelination in mice [49]. In addition, the miR-7188-3P target, myosin, plays a major role in neurodevelopment and regulation of neurological disorders [50]. Therefore, miR-7188-5P seems to target set of genes involved in regulating of MS progression.

Moreover, our results indicate that the adoptive transfer of miRNA-7188-5p-and miR-7235-silenced CD4+ T cells from control mice to EAE mice alleviates EAE disease and changes the pattern of astrocytes and CNS pathophysiology. Among the targets of miR-7235 is the palmitoyltransferase DHHC-type containing 9 (ZDHHC9), which is involved in regulation of neurological disorder [51]. Both the mRNA and protein levels of the adhesion molecule neurexins are decreased during MS demyelination [52]. Therefore, suggesting NRXN-2, as a target of miR-7235 in the current model, may further potentiate MS pathogenesis. The miR-684 was among miRNAs that are upregulated by at least 50% in paclitaxel chemotherapy of mice breast cancer. Further, treatment with glial growth factor reduced inflammation in brain and spinal cord of

Theiler's Murine Encephalomyelitis Virus model of MS and down regulated the expression of neurexophilin targeting miR-684[42].

In the current model, in both astrocytes and splenocytes, SGMS-1 mRNA expression levels significantly dysregulated by the miR-684 mimic and SiRNA. SGMS-11 plays an important role in the regulation of neurodegenerative diseases and the proliferation rate of Neuro-2a cells [53]. Phosphatidylinositol-5-phosphate 4-kinase (PIP4K2A) is also targeted by miR-684 and regulates neurodegenerative disorders such as schizophrenia. However, among the targets of miR-4647, the F-box protein FBXO33 plays a key role in progressive neurodegenerative disorders [54], and its expression is downregulated in relapsing-remitting MS (RRMS) compared with primary progressive MS (PPMS) [55]. Brain-derived neurotrophic factor (BDNF) involved in immune cell-mediated prevention of axonal and neuronal damage after various pathological insults of multiple sclerosis lesions, it was also reported to a target of miR-659-3P. The upregulation of miR-659-3P expression under EAE conditions targets the mRNA encoding the above target genes, leading to subsequent pathogenesis of MS.

Here, the study identifies new set of miRNAs as biomarkers with therapeutic potential in experimental MS and the mRNA targets of these miRNAs. The miRNA mimic, siRNA and adoptive transfer model results highlights miRNA-7188-5p and miR-7235 as potential therapeutic biomarkers for MS disease.

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Competing interests

The authors declare that they have no competing interests.

Authors' contribution

HI participated in research design, performed the experiments and wrote the manuscript. AA assisted with research design and experimental troubleshoots. HH participated equally with HI for research design and experimental troubleshoots. KT participated in research design, computational analysis and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Figures

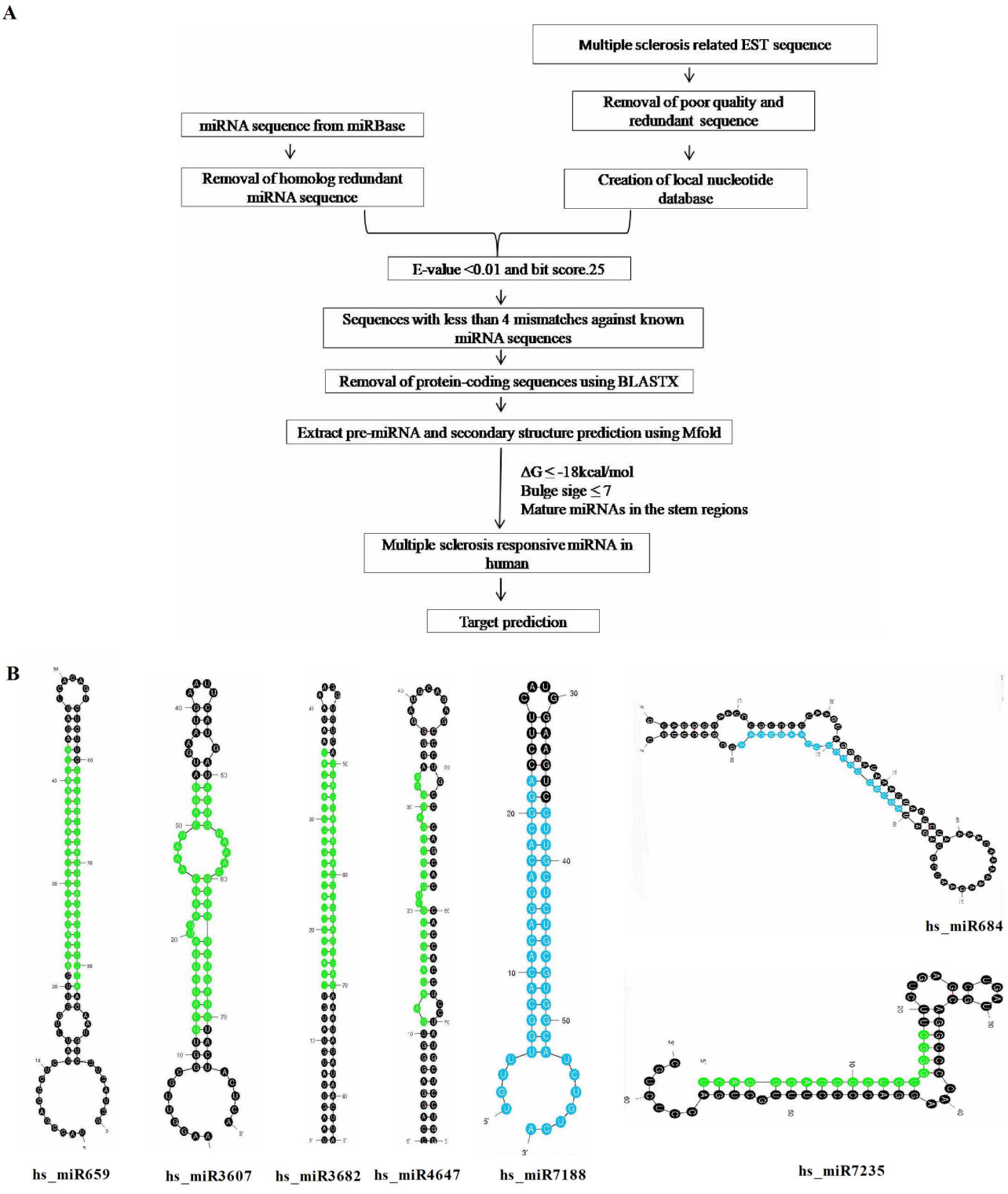

Figure 1

Identification of differential miRNA using insilico approach. A. Schematic illustration of steps involved in computational identification of multiple sclerosis responsive miRNAs. B. Secondary structure of identified

multiple sclerosis responsive miRNAs (hsa-miR-659, hsa-miR-684, hsa-miR-3607, hsa-miR-3682, hsa-miR-4647, hsa-miR-7188 and hsa-miR-7235)

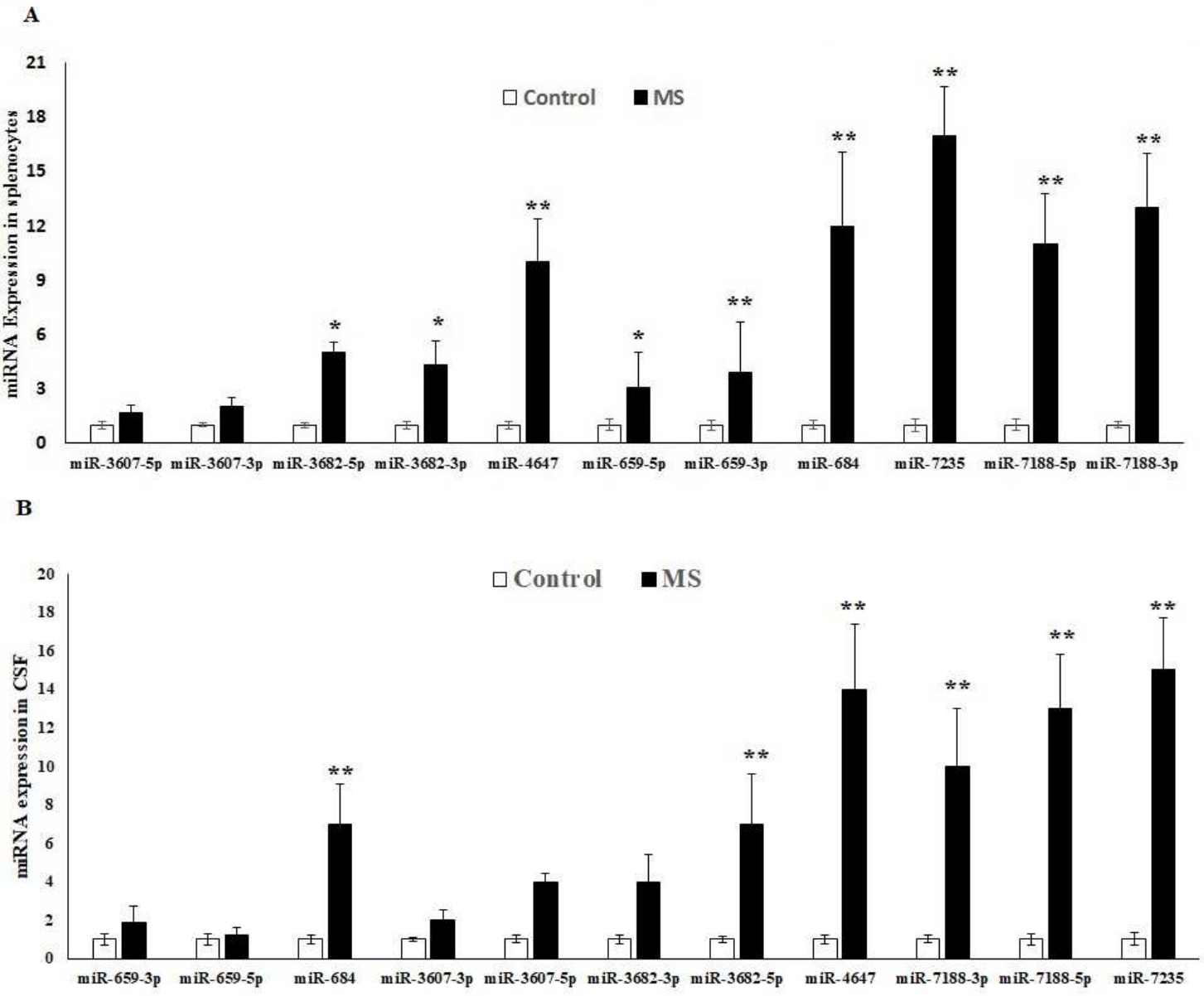

Figure 2

EAE induction and validation of different computationally identified miRNAs.

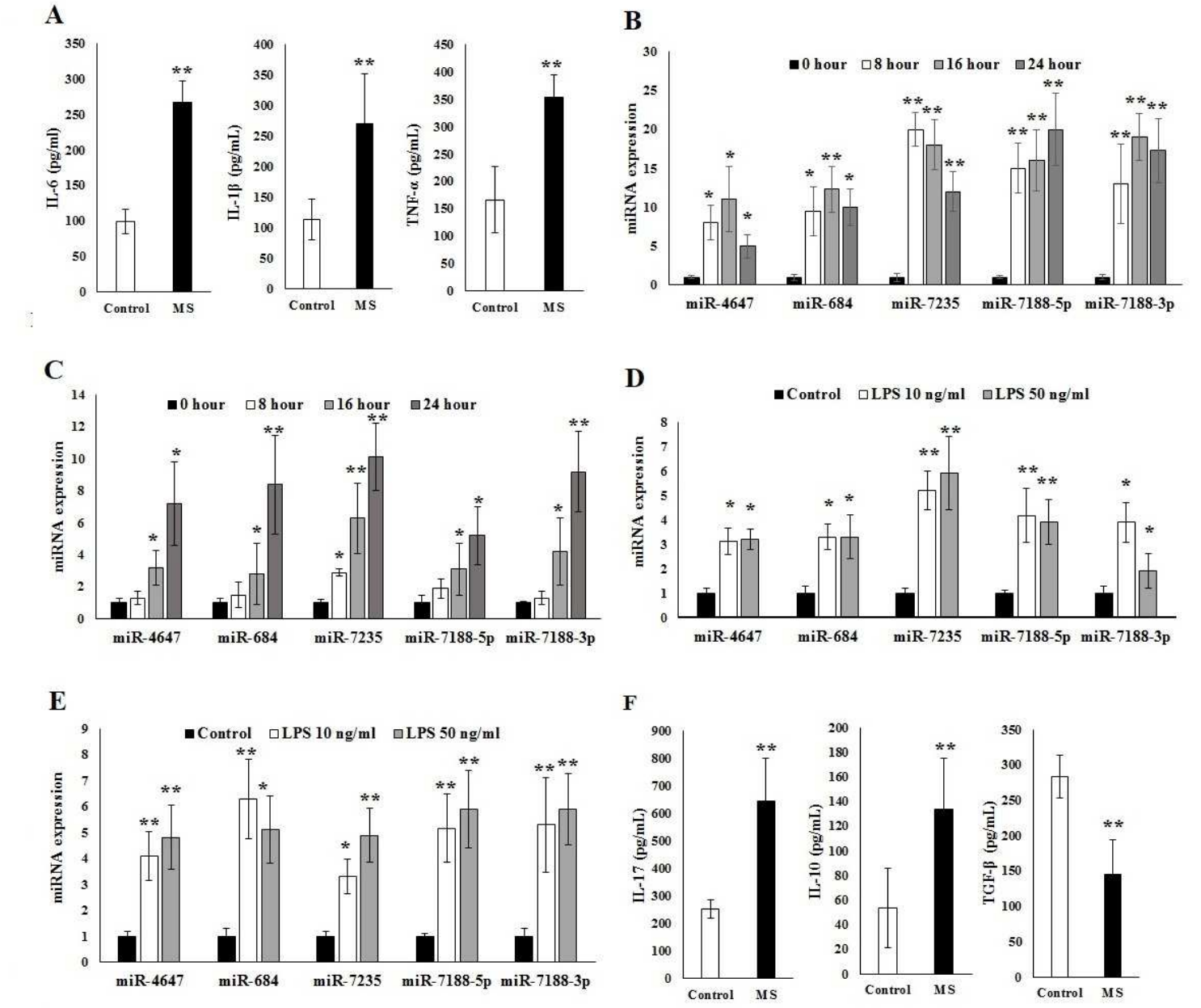

Figure 3

miRNA profile in lymphoid cells and glial derived macrophages and astrocytes. The miRNA expression was evaluated in different cellular types using quantitative real time PCR and identified highly expressed miRNAs in multiple sclerosis based cell types. (A) Serum cytokine levels of IL-6, IL-1Beta and TNF-α were quantified in 24 days after MOG immunization. (B) The splenocytes were isolated from EAE mice after 10 days of MOG immunization and stimulated with MOG 10 ng/ml for 4 hrs. The expression levels of miR-684, miR-4647, miR-7188-3p, miR-7188-5p and miR-7235 were analyzed in stimulated cells at three different time intervals (8 hr, 16 hr, and 24 hr). (C) The selected miRNAs were analyzed in anti-CD3 and anti-CD28 stimulated CD4 cells. Collect the stimulated cells after 0 hr to 24 hr and evaluated the miRNA expression. These different time points revealed that significant miRNA expression takes place after 16 hr of stimulation. (D) The glial macrophages were used for evaluating the miRNA expressions. The isolated

macrophages were LPS stimulated two concentrations for 12 hr (10 ng/ml and 50 ng/ml). (E) The astrocytes were used for evaluating the selected miRNA expressions. The isolated astrocytes were LPS stimulated in two concentrations (10 ng/ml and 50 ng/ml) for 12 hr. (F) Cytokine levels of IL-17, TGF-Beta and IL-10 in culture supernatant of CD4+ cells restimulated with MOG for 72 hr. Data were pooled from three independent experiments and shown as mean ± SD. p< 0.05. Data shown as mean ±SEM, n= 8. Experiment was repeated twice. p<0.05 using T test.

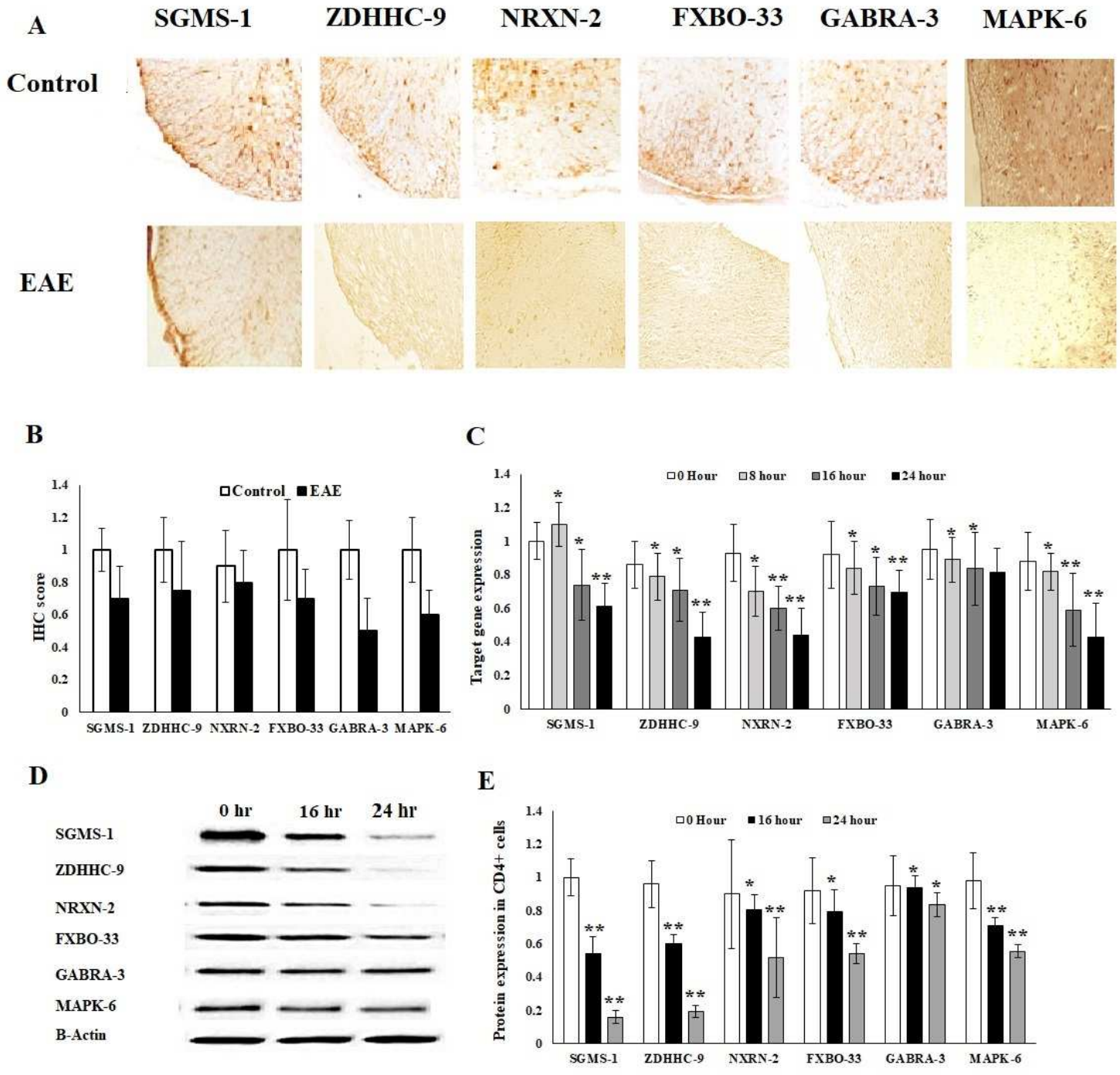

Figure 4

Regulation of targeted genes and protein in tissues and splenocytes. The immunohistochemistry of targeted protein were analyzed in immunoslides. (A-B) These pathological slides revealed the expression level of miRNA targeted protein in tissues at EAE mice after MOG immunization. (C) miRNA predicted target gene expression in activated splenocytes at different time points. Target genes such as FBXO-33, SGMS-1, ZDHHC-9, NRXN-2, GABRA-3 and MAPK-6 were analyzed their expression in 8 hr, 16 hr and 24 hr time points in stimulated splenocytes. The target genes were initially upregulated followed by significant reduction in its expression above 8 hr of activation. (D,E) The protein blot analysis of target gene in activated splenocytes. Data are shown as mean ± SD, n=4. Experiment was repeated twice. $p < 0.05$, student's t test.

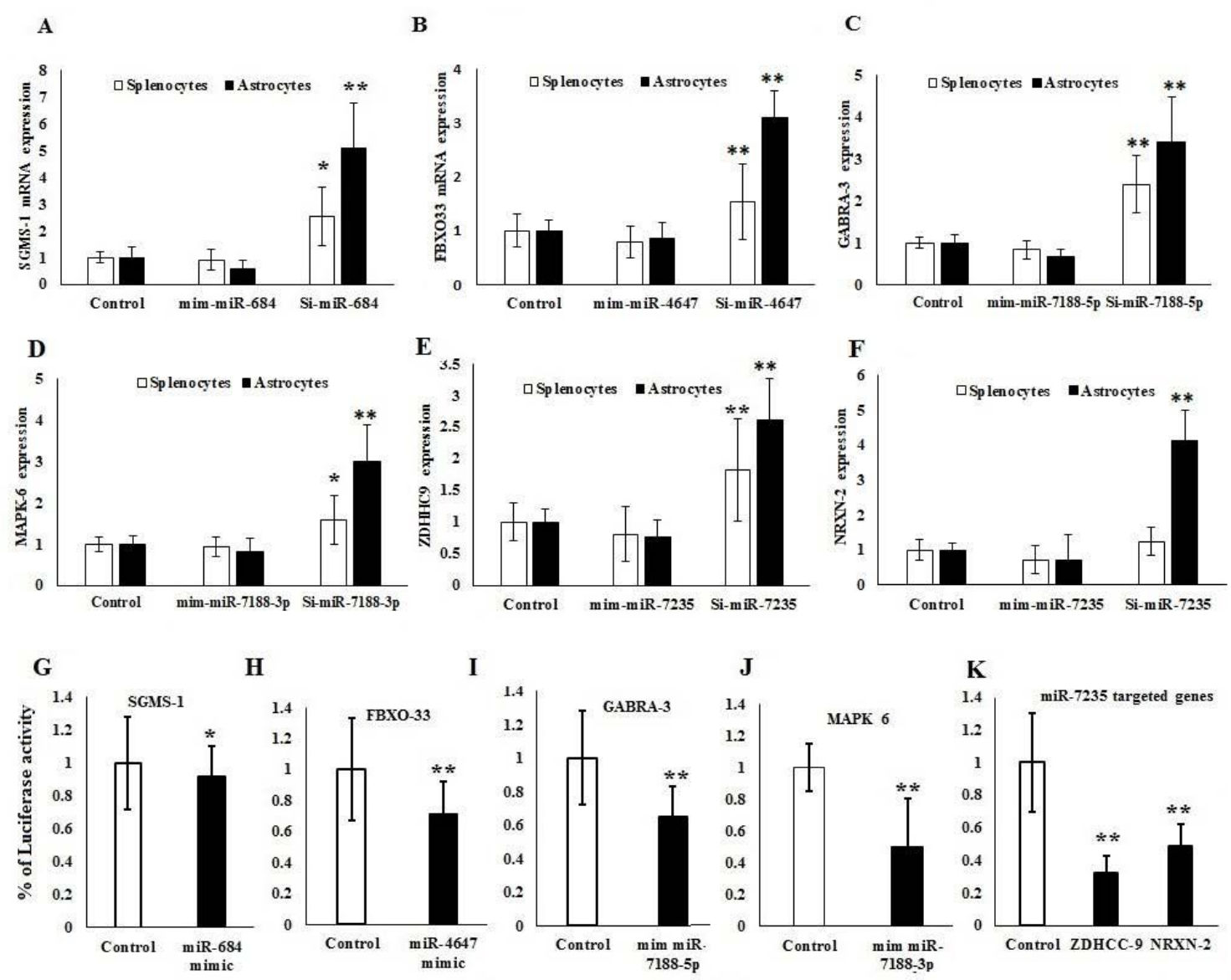

Figure 5

Effect of overexpression and silencing of miRNA sequences in splenocytes and astrocytes. The mRNA expression of target genes was examined in cells transfected with miRNA sequences by quantitative real

time PCR. (A) SGMS-1 gene was significantly suppressed by mimic miR-684 sequences and reciprocal to silencing miR-684 sequences. (B) FBXO-33 gene targeted by mimic miR-4647 sequences and reciprocal to significant upregulation at silencing miR-4647 sequences. (C) GABRA-3 gene expression was downregulated in mimic as well; it was upregulated with silencing miR-7188-5p approach. (D) MAPK-6 transcripts significantly suppressed in CD4 cells of overexpressing miRNA-7188-3p, compared with control. The target mRNA transcripts were upregulated at silencing miR-7188-3p. (E, F) ZDHCC-9 gene was not altered by mimic miR-7235 sequences whereas; target gene NRXN-2 was significantly downregulated by mimic and reciprocal to significant upregulation at silencing miR-7235 sequences. (G) The transfected miRNA mimic sequences into HEK293K cell lines and measured the target expression levels by luciferase assay system. Co-transfection of HEK293K cells containing luciferase 3' UTR construct from together with miR mimics showed significant suppression of luciferase activity in comparison with cells transfected with a control miRNA sequence. mimic of miR-684 target SGMS-1 untranslated region. (H) mimic of miR-4647 target FBXO-33 untranslated region. (I) mimic of miRNA-7188-5p target GABRA-3 untranslated region. (J) Mimic of miR-7188-3p target MAPK-6 untranslated region. (J, K) mimic of miR-7235 targets ZDHCC-9 and NRXN-2 untranslated region. Data are shown as mean ± SD, n=4. Experiment was repeated twice. $p < 0.05$, student's t test.

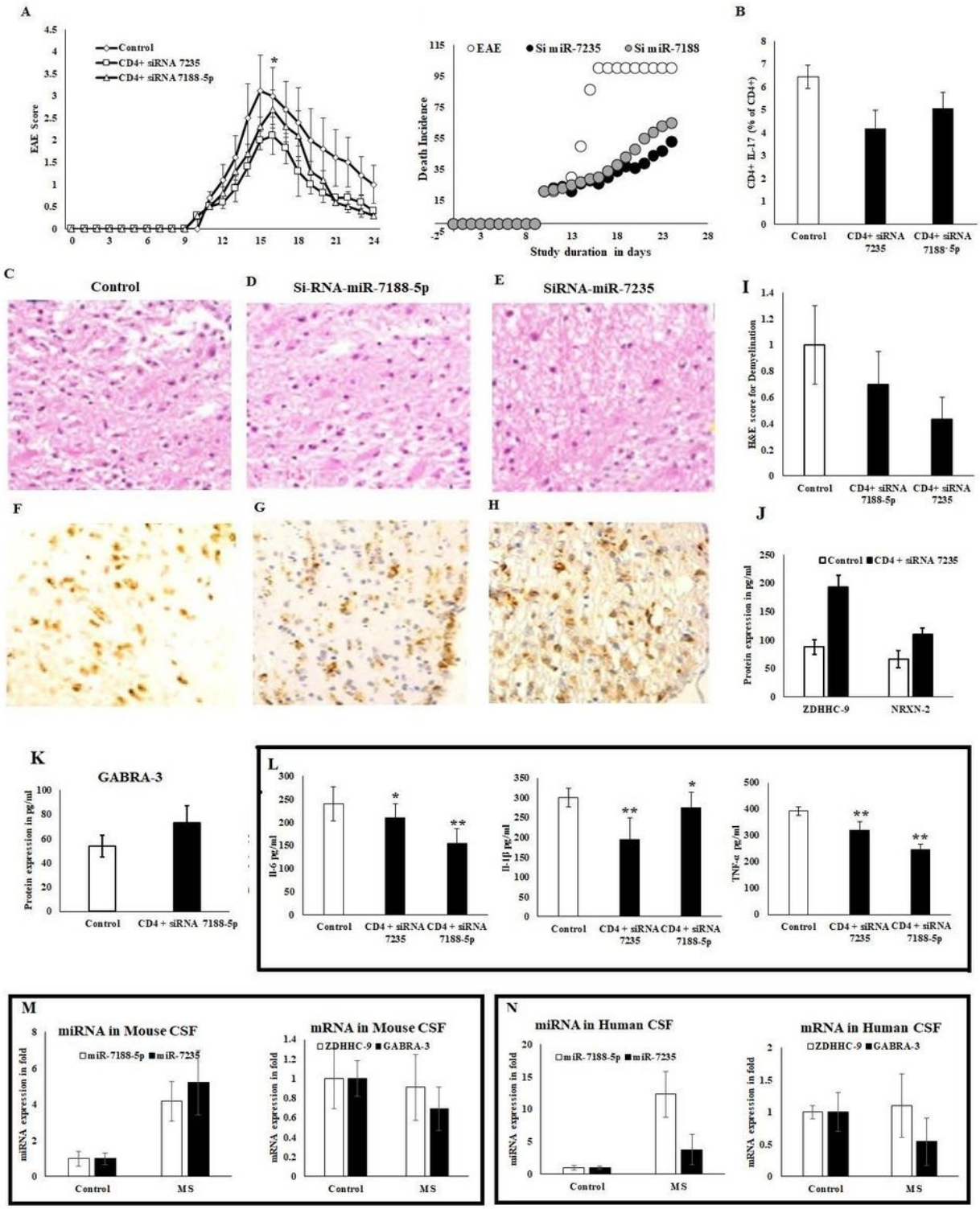

Figure 6

Silencing of miRNA attenuates MOG induced EAE model by LNA (locked nucleic acid) approach. (A) Clinical scores of EAE induced by adoptive transfer of silencing miRNA sequences transfected CD4 cells from naïve mice. Antisense (as)- miR-7188-5p and miR-7235 treated EAE mice were evaluated for clinical sign and macroscopic studies. (B) CD4 population in LNA transfected EAE mice. Frequency of CD4 Il-17 T cells in total CD4 T cells isolated from spinal fluid and tissues after 18 days of MOG immunization and

LNA transfection. (C, D and E) Histopathology of antisense miRNA transfected EAE mice were recorded. (F, G and H) Immuno histochemistry of miRNA targeted gene specific protein using primary anti-ZDHHC-9 and anti-GABRA-3 protein. The expression of target gene was upregulated in silencing miRNA specific sequence EAE mice. (I, J, K) IL-6, IL-1β and TNF-α cytokines were quantified in serum of adoptive transferred mice with miRNA antisense transfected EAE mice. (L,M) Estimation of protein target of miR-7188-5p and miR-7235 using conventional standardized ELISA method. (N) Evaluation of death incidence of anti-miRNA and scrambled transfected EAE. Data are shown as mean ± SD, n=4. Experiment was repeated twice. $p < 0.05$, student's t test.

Supplementary Files

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