



miR-455-5p downregulation promotes inflammation pathways in the relapse phase of relapsing-remitting multiple sclerosis disease

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Abstract

MicroRNA-455-5p (miR-455-5p) seems to have an anti-inflammatory role in the immune system since its expression is induced by IL-10 cytokine. Multiple sclerosis (MS) is a chronic demyelinating neurodegenerative disease of the central nervous system that is caused by an autoimmune inflammatory attack against the myelin insulation of neurons. The expression level of miR-455-5p and its role in MS pathogenesis has yet to be elucidated. We found that miR-455-5p expression was highly correlated with disease severity in MS patients. miR-455-5p expression inversely correlates with its inflammatory-predicted targets (MyD88 and REL) in relapse- and remitting-phase patients. Luciferase assays confirm that MyD88 and REL are direct targets of miR-455-5p. This study represents the first report of the miR-455-5p acts as an anti-inflammatory role in MS, at least partially through targeting MyD88 and REL. This study may provide important information for the use of miR-455-5p as a novel strategy to improve the severity of disease and control inflammation and attack in MS patients.

Keywords Multiple sclerosis · Inflammation · miR-455-5p · MyD88 · REL

Introduction

Multiple sclerosis (MS) is one of the most common autoimmune disorders in young adults manifested by chronic inflammatory demyelination of the CNS. The disease is clinically heterogeneous, with about 80% of patients developing the relapsing-remitting MS (RR-MS) subtype (Du et al. 2009).

Although the cause of MS is still unknown, several epidemiological and gene expression studies have been elucidated that immune system is the greatest genetic contributor to disease susceptibility. IFN- γ -producing Th1 cells and IL-17-producing Th17 cells are main pro-inflammatory mediators of cellular immunity that are responsible for autoimmune reactions in CNS leading to axonal degeneration, demyelination, and

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ultimately irreversible tissue damage of patients with MS. In fact, CNS autoimmunity is controlled by the balance between inflammatory cytokines IL-17 and IFN- γ , and the opposing regulatory cytokines IL-10 and the type I IFNs which produced due to toll-like receptors (TLR) ligation (Guo 2016).

Today, recombinant interferon beta 1b and 1a (rIFN-b-1b, rIFN-b-1a) are used to slow down disease progression both in relapsing-remitting (RR) and secondary progressive (SP) MS (Marziniak and Meuth 2014). One of the mechanisms by which IFN-b-1b may exert its beneficial effects in MS is through an impact on cytokine production and secretion such as IL-10 (Ersoy et al. 2005). IL-10 expresses from Th0, Th1 and Th2 cells, B cells, and macrophages and has been considered to have a potential therapeutic role in experimental autoimmune encephalomyelitis (EAE) mouse model and prevents the development of the disease (Guo 2016).

MicroRNAs (miRNAs) have begun to emerge as an important component in the development and progression of MS (Wu and Chen 2016). miRNAs are small non-coding RNAs that regulate their mRNA targets at the post-transcriptional level through binding to complementary RNA sequences in the 3'-untranslated region (3'UTR) of the targets (Kouhkan et al. 2016; Kouhkan et al. 2013; Obeidi et al. 2016; Tasharofi et al. 2017; Zomorrod et al. 2018). The expression of miRNAs in MS was first indicated in the cells derived either from MS patients' blood samples or from active lesions. Several miRNAs such as miR-155 (Zhang et al. 2014), miR-181a (Li et al. 2007), miR-326, miR-150, miR-146a (O'Connell et al. 2010), miR-181b (Pekarsky et al. 2006), miR-124, and miR-92a (Zhang et al. 2014) were identified as important regulators for several aspects of immune cell development and immune responses. miR-455-5p is one of the miRNAs that are most highly induced in response to IL-10 secretion. Its stimulation by IL-10 suggests that miR-455-5p may have an unknown anti-inflammatory function in the immune system (Cardwell and Weaver 2014).

In the present study, we examined the expression level of miR-455-5p in PBMCs of RR-MS patients in relapse and remitting phases. Furthermore, for the first time, in order to clarify the anti-inflammatory role of miR-455-5p, its predicted targets involved in the inflammation pathway were selected and investigated.

Material and methods

Patients and controls

Blood samples were collected from 42 RR-MS patients including 22 patients in relapsing and 20 patients in remitting phases in the MS Clinic of Tehran Sina Hospital in the period 2016–2018. All MS patients fulfilled the McDonald's criteria and had Expanded Disability Status Scale (EDSS) of < 5.5

(Kurtzke 1983; Polman et al. 2011). The MS cohort consisted of 60% females and 40% males. The healthy control samples were also taken from the Tehran Sina Hospital. Patients having newly diagnosed or not received any immunomodulatory therapy 3 weeks before the blood withdrawal. Remitting-phase patients were with no relapses at least in the last 3 months. Relapsing-phase patients were with a severe attack, demonstrating any of the following symptoms: problems with balance and coordination, trouble with vision, issues with bladder, numb or tingling feelings (pins and needles), problems with memory and trouble concentrating that lasting more than 24 h after the period of at least 30 days of improvement, or stability.

Healthy subjects matched with cases in terms of gender and age (mean age 40.7 years; range 25–56). All patient and control subjects signed an informed consent to participate in the study.

Database prediction of miRNA targets

miR-455-5p targets were predicted using the TargetScan algorithm (www.targetscan.org, version 5.1). Then, biological processes and pathways of the given target list, including at least 500 predicted targets, were analyzed using PANTHER (www.pantherdb.org, version 7). In the “ontology” analysis of PANTHER, “pathway” category was investigated and targets in inflammation mediated by chemokine and cytokine signaling pathway and toll receptor signaling pathway that acquired the highest score in the TargetScan were selected to continue the study including MyD88 and REL.

PBMC isolation from blood and RNA extraction

Immediately after sampling, fresh blood was diluted with 2–4 \times the volume of the sterile phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll (Ficoll-Paque PREMIUM GE Healthcare) density-gradient centrifugation according to the manufacturer's protocol. Finally, obtained PBMCs were washed twice in sterile PBS.

Total RNA was purified from all obtained PBMCs using TRIzol (Invitrogen) according to the manufacturer's protocol.

Gene expression analysis

Total RNA reverse transcribed to cDNA using random hexamers and M-MuLV Reverse Transcriptase (Promega) for evaluation of the MyD88 and REL.

For miRNAs evaluation, total RNA first polyadenylated by poly (A) polymerase. Then, reverse transcription was performed using poly (A)-tailed total RNA, general RT primer,

and reverse transcriptase according to the manufacturer's protocol (BONmiR).

Real-time PCR was performed on ABI 157 PRISM 7500 real-time PCR System (Applied Biosystems) with 1 μ l of cDNA product, 1 \times Quantitect SYBR Green PCR Master Mix (Takara), and 0.5 μ l of each forward and reverse primer. Then, the reactions were incubated at 95 $^{\circ}$ C for 3 min, followed by 40 cycles of 95 $^{\circ}$ C for 30 s and 60 $^{\circ}$ C for 30 s. All of the experiments were run in triplicate. The relative expression level of mRNA and miRNA were analyzed and normalized to endogenous expression of β -actin and SNORD47 RNA, respectively, as an internal control using the $2^{-\Delta\Delta CT}$ method. QRT-PCR primer sequences are listed as follows: miR-455-5p F: GTG CCT TTG GAC TAC ATC G, SNORD47 F: ATC ACT GTA AAA CCG TTC CA, MyD88 F: GAA TGT GAC TTC CAG ACC AA, MyD88 R: GAC AGT GAT GAA CCT CAG GAT, REL F: GAA TAA AGG CAG GAA TCA ATC, REL R: TTG GAG CAC GGT TGT CA, beta Actin F: CTT CCT TCC TGG GCA TG, and beta Actin R: GTC TTT GCG GAT GTC CAC.

In target investigations, the mean of miR-455-5p and SNORD47 Cts was determined in healthy samples and compared with obtained Cts of each patient. Based on that, MS patients were divided into two groups: a group that miR-455-5p gene expression level was lower than healthy samples and the other that miR-455-5p gene expression level was higher than or equal to healthy samples. Subsequently, relative gene expression of MyD88 and REL was determined using the comparison of obtained Cts in MS patients with mean Cts in normal samples.

Plasmids, viral vectors construction, and luciferase assay

To determine whether miR-455-5p directly inhibits inflammatory factors MyD88 and REL, luciferase assays were performed. The human miR-455-5p locus on chromosome 9 with its \sim 500-base pair flanking sequences was PCR amplified from genomic DNA and inserted into the pCDH-turbo GFP expression vector. On the other hand, a genomic fragment spanning the 3'UTR lengths of MyD88 and REL carrying the putative miR-455-5p binding sites were cloned into pSICHECK2 vectors. For the construction of Mut-miR-455-5p, two mutations were created in the seed sequence of miR-455-5p and mutated form was sub-cloned into the pCDH-turbo GFP vector.

Human embryonic kidney 293 (HEK293) cells were seeded in 96-well plates and transiently cotransfected with each of the MyD88-3'UTR or REL-3'UTR luciferase vectors in combination with pCDH-miR-455-5p plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The pSICHECK2 empty vector and the

mutated miR-455-5p (Mut-miR-455-5p) were used as the blank and the negative control, respectively.

Luciferase activity was assayed 48 h after transfection using the dual-luciferase reporter assay system (Promega,) and the Renilla luciferase signal normalized to the firefly luciferase signal activity for control of transfection efficiency. All tests were run in triplicate. Three transfection assays were performed to obtain statistically significant data.

Statistical analysis

QRT-PCR data analysis was carried out by the $\Delta\Delta CT$ method using REST 2009 software (Qiagen). Statistical analysis was implemented by GraphPad Prism 6.02 (GraphPad Software Inc., LaJolla, USA). All experiments were performed at least three times, presented as mean \pm SD and analyzed by Student's *t* test. ANOVA analysis was used to calculate the differences of various comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

miR-455-5p downregulated in RR-MS patients in relapse phase

miR-455-5p expression was investigated in PBMC samples from RR-MS and control subjects. We found that miR-455-5p expression was significantly lower in relapsing-phase patients than in those of age-matched controls or patients in remitting phase (Fig. 1a, b). The expression level of miR-455-5p was downregulated up to 70% ($p < 0.05$) in relapsing-phase patients compared with the healthy samples. However, the expression level of miR-455-5p showed no substantial changes between healthy subjects and remitting patient samples which proposed a specific association of miR-455-5p expression with the relapsing phase of MS.

miR-455-5p had an inverse relationship with the expression level of MyD88 and REL in patient samples

In order to determine the role of miR-455-5p in the pathology of MS, analysis of conserved mRNA targets of miR-455-5p (\sim 256 targets) was carried out using the TargetScan program and then gene classification was performed by PANTHER. Several different goals were identified for miR-455-5p in MS-related pathways but the current project studies were more focused on inflammation. So, MyD88 and REL were selected to continue the study.

The mRNA expression levels of MyD88 and REL were investigated in the relapsing and remitting patients. Patients with relapsing MS had significantly higher MyD88 and REL

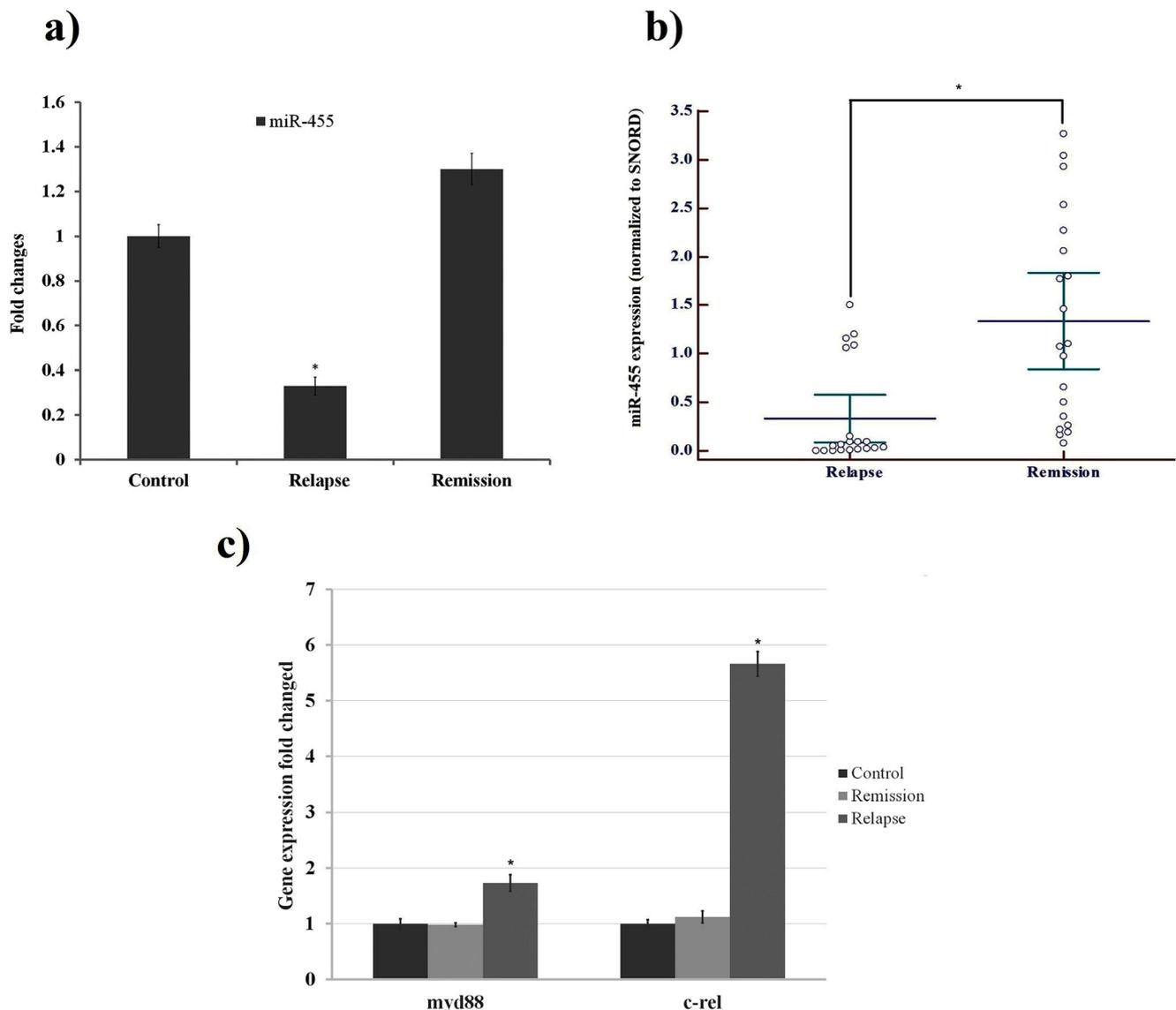


Fig. 1 The expression levels of miR-455-5p and its predicted target genes in samples. **a** Data from QRT-PCR analysis of miR-455-5p expression in PBLs from normal controls (ctrl; $n = 20$) and MS patients in relapse ($n = 20$) and remitting phase ($n = 20$). miR-455-5p expression was significantly downregulated in relapse-phase patients. **b** Distribution of MS patients in relapse and remitting phase according to miR-455-5p expression. Data are normalized to those of controls and are presented relative to expression of the SNORD47 using $2^{-\Delta\Delta C_t}$ method. Data are

representative of three independent experiments. (* $p < 0.05$, versus control; using Student's t test). Horizontal lines represent the mean. Circles represent the expression levels for individual patients. **c** Expression level of miR-455-5p-predicted genes, REL and MyD88, were significantly downregulated in relapse patients compared to the control samples and remitting-phase patients. β -Actin was used as an internal control. Columns and bars show mean of three different experiments and SD respectively; (* $p < 0.05$)

expression (~1.7- and 5.6-fold, respectively, $p < 0.05$) but those from patients with remitting MS did not. (Fig. 1c).

Next, to investigate whether MyD88 and REL expression level is affected by miR-455-5p, MS patients in relapsing and remitting phases were separated into two groups based on the mean expression level of miR-455-5p in normal samples. The first group had lower expression than the reference (≤ 1) and the other group showed higher or equal expression levels compared with the reference (≥ 1 ; $p < 0.05$). MyD88 and

REL showed increased median expression levels in the relapsing patients expressing lower levels of miR-455-5p compared to those expressing higher levels of miR-455-5p (~2.5- and 9.8-fold, compared to 0.85- and twofold respectively, $p < 0.05$, Fig. 2a–d), Whereas the median expression level of MyD88 and REL in remitting patients expressing lower levels of miR-455-5p is high against those expressing higher levels of miR-455-5p (~1.54- and 1.81-fold, compared to 0.47- and 0.53-fold respectively, $p < 0.05$, Fig. 2e–h).

Obtained results indicated that miR-455-5p expression level could affect the expression of predicted target genes, and therefore it can be inferred that miR-455-5p can target MyD88 and REL.

miR-455-5p can directly target inflammatory targets

Given the main focus of the present study on inflammatory pathways, we performed luciferase assay for inflammatory targets, MyD88 and REL, to investigate the role of miR-455-5p in this regard. According to TargetScan, there are two predicted interaction site for miR-455-5p in the MyD88 3'UTR, whereas three sites are predicted to interact with miR-455-5p in the REL 3'UTR, respectively (Fig. 3a). We cloned two psiCHECK-2 plasmids driven by the cytomegalovirus (CMV) basal promoter harboring the 224 nt and 210 nt from REL and MyD88 3'UTRs, respectively, at the 3' position of the luciferase reporter gene.

In a parallel experiment, the conserved seed sequence of miR-455-5p was specifically mutated (Fig. 3b). A marked reduction in the luciferase/Renilla ratio was seen for REL and MyD88 constructs transfected with wild type of miR-455-5p but not with the control or empty vector (Fig. 3c). Furthermore, the observed luciferase/Renilla reduction was abrogated when we cotransfected Mut-miR-455-5p with two mutations at the seed sequence of miR-455-5p.

Discussion

In all forms of MS, inflammation always exists when active demyelination and neurodegeneration take place (Lassmann 2018; Leibowitz and Yan 2016). Immune-triggered inflammation due to releasing of inflammatory factors including reactive oxygen or nitric oxide species (ROS and RNS), excitotoxins as glutamate, and cytotoxic cytokines including IFN- γ , TNF- α , IL-17, IL-21, IL-22, and IL-26 is critically driving further damage and degeneration of CNS elements in MS. So, modulators of inflammation are considered to have valuable potential for clinical applications in the diagnosis or treatment of this category of complicated immune disorders (Chitnis and Weiner 2017; Matute et al. 2001).

IL-10 produced by innate immune cells as well as T and B cells has been considered to have a potential therapeutic role in MS because it promotes the development of an anti-inflammatory cytokine pattern by inhibiting the IFN- γ production of T cells and natural killer (NK) cells, particularly via the suppression of IL-12 synthesis in accessory cells (Guo 2016). IL-10 suppresses the synthesis of many Th1-cell-related cytokines (IFN- γ , TNF- α , TNF- β , IL-1, IL-2, IL-6), and T cell proliferation and downregulates MHC class II expression on monocytes, and inhibits cytotoxic functions of

activated macrophages by inhibiting nitric oxide production (Cunha et al. 1992; de Waal Malefyt et al. 1991).

On the other hand, recent studies demonstrate that IL-10 secretion also could constrain Th17 differentiation and function and could suppress Th17-mediated inflammation through amplifying a negative regulatory loop of IL-10 production and IL-10R signaling (Guo 2016).

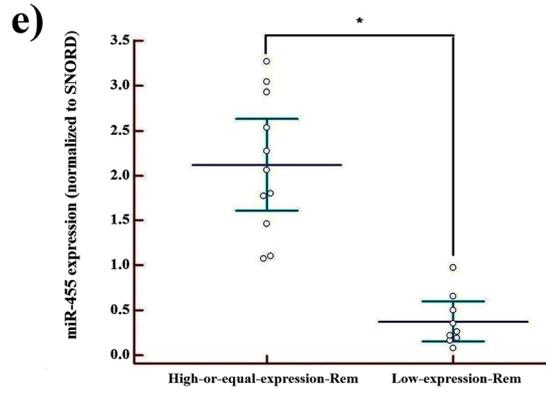
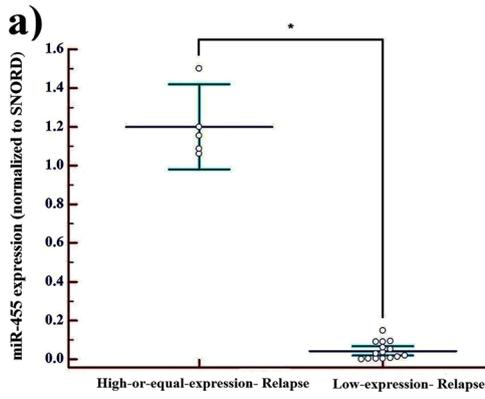
A subsequent report indicated that miR-455-5p super induced as result of TLR stimulation through the IL-10-mediated anti-inflammatory response in mouse macrophages. However, it remains to be determined whether miR-455-5p plays a functional role in the IL-10-mediated anti-inflammatory response in the human system (Cardwell and Weaver 2014).

Thus, in this study, we sought to address the hypothesis that whether miR-455-5p could involve in MS pathogenesis through the act in an anti-inflammatory manner to selectively suppress the expression of certain pro-inflammatory genes.

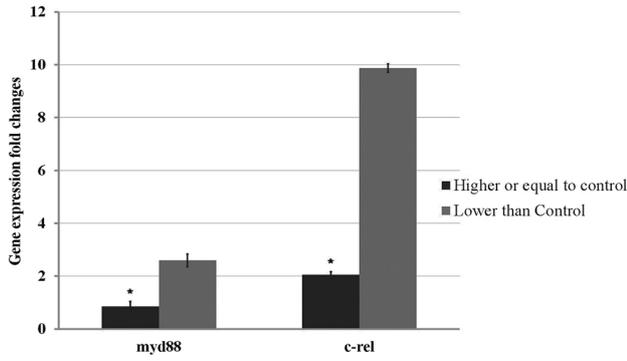
We have shown that miR-455-5p expression sharply down-regulated in peripheral blood of patients in relapsing phase of MS. Then, to obtain the correlation between miR-455-5p and inflammation, we tried to identify putative targets of this miRNA. By recruiting PANTHER algorithm, we found MyD88 and REL genes that have the important role in inflammation path. Next, patients were categorized based to the miR-455-5p expression level. Our results indicated that high expression level of miR-455-5p is correlated with lower expression levels of MyD88 and REL genes in relapse-phase patients, thereby demonstrating its probable anti-inflammatory effects in MS patients. We further confirmed that predicted genes are direct targets of miR-455-5p by luciferase assay.

MyD88, as an miR-455-5p-predicted target, is expressed by dendritic cells, macrophages, and B lymphocytes and plays a key role in the pathogenesis of EAE because MyD88 $-/-$ mice were completely EAE resistant (Miranda-Hernandez et al. 2011). MyD88 necessary for TLR-mediated activation of the transcription factor nuclear factor- κ B (NF- κ B) that promotes the expression of pro-inflammatory cytokines and chemokines, such as IL-1 β , IL-6, IL-8, IL-12, IL-23, and TNF- α , as well as the subsequent priming of Th17 cells and their maintenance (Fig. 4a). Our results suggested that downregulation of miR-455-5p in relapse MS patients leads to overexpression of MyD88 and subsequently increase in the inflammation and severity of the disease through promoting the differentiation and maintenance of the Th17 cells and production of inflammatory cytokines.

Another predicted target is REL. REL is a canonical NF- κ B member expressed primarily in lymphoid tissues by lymphoid and myeloid cells and plays different roles in immunity and inflammation. REL activity may enhance recruitment of inflammatory cells and production of pro-inflammatory mediators like IL-1 β , IL-6, IL-8, IL-12, and



b) Gene expression in relapse group according to miR-455 level



f) Gene expression in remission group according to miR-455 level

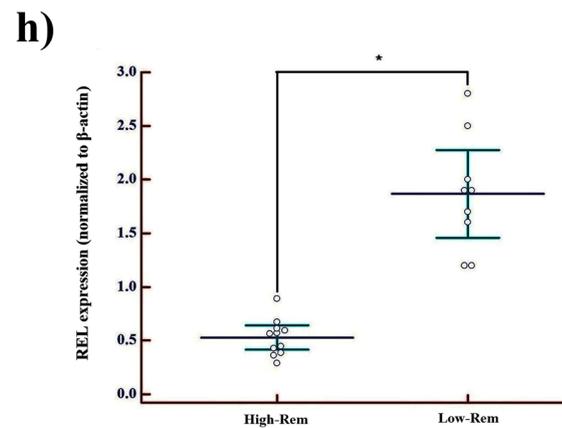
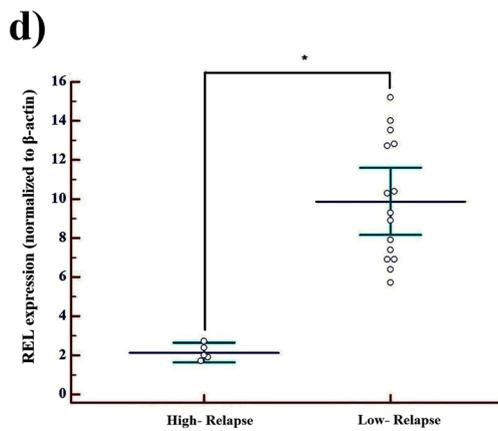
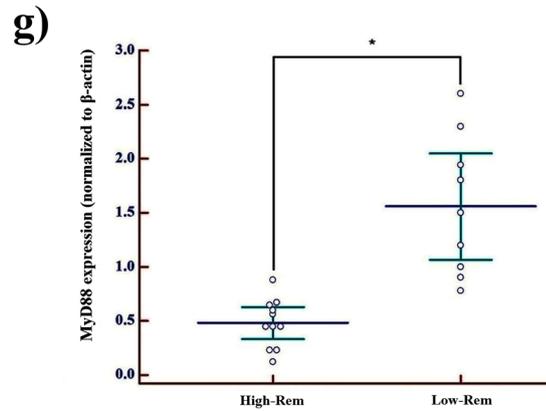
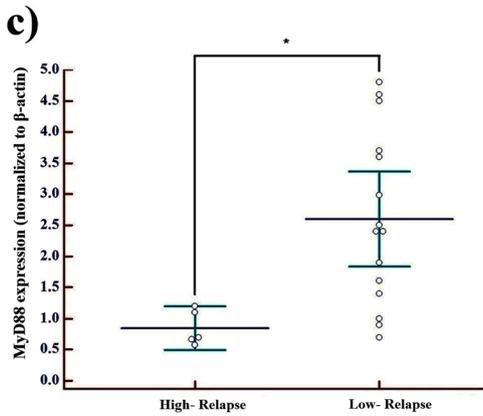
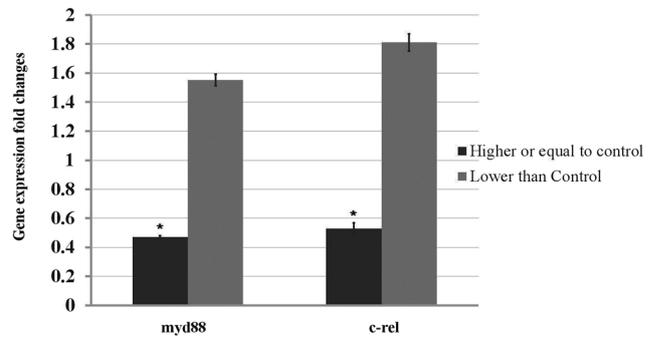
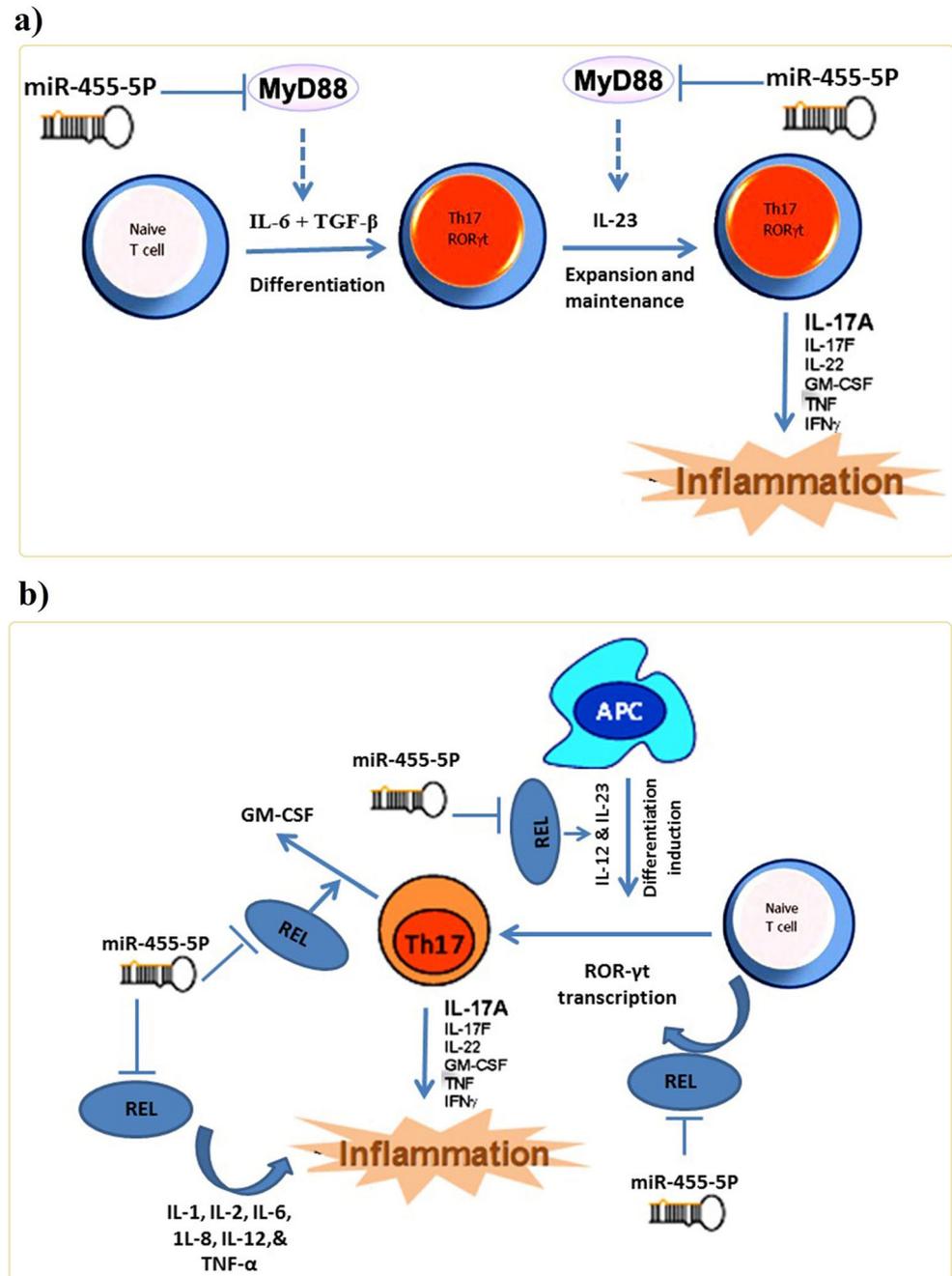


Fig. 4 Model of anti-inflammatory role of miR-455-5p through MyD88 and REL targets. **a** miR-455-5p could inhibit MyD88 that has direct role in the production of IL-6 and IL-23 in myeloid cells, which in turn drives Th17 cell differentiation and activation. In fact, Th17 cells are not primed in the absence of MyD88. **b** Another way through which miR-455-5p exerts its influence is via REL. REL not only has a vital role in differentiation of TH17 cells through activation of ROR γ t, but also involve in differentiation induction of these cells through production of IL-12 and IL-23 from APC cells. REL also directly leads to inflammation through production of IL-1, IL-2, IL-6, IL-8, IL-12, and TNF- α



patients via restriction of Th17 differentiation and prevention of inflammatory cytokines production (Fig. 4b).

Collectively, we elucidated that miR-455-5p is downregulated in relapsing MS patients compared with remitting-phase patients. The exact underlying mechanism that is responsible for the anti-inflammatory role of miR-455-5p has yet to be identified in MS patients, but there is evidence that the direct targeting of MyD88 and REL could be the reason for this phenomenon. Overall, our findings introduce miR-455-5p as one of the anti-inflammatory microRNAs enhances the

differentiation of Th17, so could be useful therapeutic approaches in getting a better control of inflammation and disease severity in MS patients.

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