Original Article

Up-Regulation of *Tmevpg1* and *Rmrp* LncRNA Levels in Splenocytes and Brain of Mouse with Experimental Autoimmune Encephalomyelitis

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Abstract

Background: Two long noncoding (lnc) RNAs, which have been recognized as *Tmevpg1*/*Ifng-AS1*/*NeST* and *Rmrp* play indispensable roles in the differentiation of T_H1 and T_H17, respectively. The aim of the present scientific study was to analyze the expression levels of the aforementioned lncRNAs in experimental autoimmune encephalomyelitis (EAE) as an animal model for multiple sclerosis (MS).

Materials and Methods: Initially, EAE was induced in C57BL/6 mice via immunization by using MOG peptide. The leukocyte infiltration rate and demyelination of neuronal axons were determined. Secondly, the expression levels of *Tmevpg1*, *Rmrp*, *Tbx21*, and *Rorc* were analyzed in the cultured splenocytes and brain lysates, by using Real-Time PCR assay; eventually, the levels of interferon-gamma and interleukin-17 evaluated by ELISA.

Results: Gene expression analysis revealed that *Rorc* expression in the splenocytes of EAE mice in comparison to the controls was elevated; however, *Tbx21* expression did not show any significant difference. *Tmevpg1* and *Rmrp* levels increased in the splenocytes of EAE mice (4.48 times and 39.70 times, respectively, $p = 0.0001$). Besides, in the brain lysate, the entire genes that have been mentioned were higher than the controls (*Tmevpg1*: 3.35 times $p = 0.02$ and *Rmrp* 11.21 times, $p = 0.0001$).

Conclusion: The marked up-regulation in *Tmevpg1* and *Rmrp* transcripts suggested the essential roles of lncRNAs in the pathogenesis of EAE and multiple sclerosis indeed. Further investigations are necessary to evaluate the values of these lncRNAs as the target for the therapy or molecular marker for disease monitoring.

Keywords: Experimental autoimmune encephalomyelitis (EAE), LncRNA, *Tmevpg1*, *Rmrp*, *Rorc*, *Tbx21*

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Introduction

Multiple Sclerosis (MS) has been distinctly recognized as an autoimmune disease in the central nervous system (CNS), which affects more than 2 million individuals worldwide[1].

Different subsets of lymphocytes including T-helper 1

(T_H1) cells and T_H17 are engaged in crucial aspects of the process of demyelination that occurs in the central nervous systems during the courses of the disease[2]. In this process, T_H1 cells, activate monocytes and macrophages by producing interferon-gamma (IFN-γ); additionally, T_H17 cells play a complementary role in MS by producing the pathogenic cytokines, such as interleukin (IL)-17, and the recruitment of neutrophil cells into an inflammatory tissue[2,3]. The most important molecules in the differentiation of T-helper (T_H) lymphocytes into T_H1 and T_H17 subtypes has identified as *Tbx21* (the murine ortholog of human T-bet gene) and *Rorc* (the murine ortholog of human *RORγt*, gene), respectively[4,5]. The results of the previous studies have revealed that two long noncoding RNAs (lncRNAs) identified as *Tmevpg1* and *Rmrp* to assist *Tbx21* and *Rorc* respectively; in order to control the differentiating of T_H1 and T_H17[6, 7]. LncRNAs are a newly discovered type of regulatory RNAs that exist throughout the genome[8]. Unlike micro-RNAs that prevent the expression of genes, lncRNAs can exert both inhibitory and enhancing effects on gene expression[9].

It has been indicated that *Tmevpg1* (the ortholog of NeST gene in human) is expressed in T_H1 cells and positively correlates with IFN-γ production in mouse and human[6,10]. As well, in T_H17 cells, DEAD-box RNA helicase-5 (DDX5) is an indispensable activator for Rorc-associated transcription responses[11]. The interaction of DDX5 with *Rorc* requires a lncRNA called *Rmrp*[12].

In fact, identifying lncRNAs, which are associated with the pathogenesis of diseases through gene expression regulation, could assist in recognizing the pathogenesis of the diseases and finding new therapeutic targets. Previous studies demonstrated that the expression of NeST was significantly increased in Hashimoto's thyroiditis[13] and Sjogren's syndrome[14]. In despite, the expression of *NeST* in peripheral blood mononuclear cells (PBMCs) of idiopathic thrombocytopenic purpura patients was lower than the controls[15]. Autosomal recessive mutations in *Rmrp* have been detected in cartilage-hair hypoplasia (CHH)[16] a syndrome characterized by short stature, sparse hair, and immunodeficiency. On the other hands, up-regulation of *RMRP* is associated with glioma progression and invasion[17], as well ischemic heart failure was noticed[18]. Recently, Zhang et al, reported that in PBMCs, which were obtained from the patients with MS, 2353 lncRNAs up-regulated and 389 lncRNAs down-regulated; which indicate their essential role in the development of MS development[19]. As of now, there has not been any scientific study reporting the transcription levels of *Nest*/*Tmevpg1* and *RMRP*/*Rmrp* in MS and its animal model, Experimental autoimmune encephalomyelitis (EAE). Regarding the *Tmevpg1* and *Rmrp*'s roles in T cell differentiation, we speculated that the abnormal expression of *Tmevpg1* and *Rmrp* might involve in the pathogenesis of EAE. To evaluate the hypothesis, we explored the expressions of *Tmevpg1*, *Rmrp*, *Tbx21*, and *Rorc* in splenocytes and brains that were obtained from EAE mice.

Methods

Experimental Animals and Induction of Experimental Autoimmune Encephalitis: Female C57BL/6 mice (6–8 weeks) were obtained from the Royan Institute (Iran). Animals were housed in standard polycarbonate cages. They were in a temperature-controlled room (23 ± 1°C). The room had a fixed 12 h light-dark cycle (08:00–20:00) and unlimited access to water and food. The Ethics and Research Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1396.454) approved all procedures. EAE induction was performed base on the protocol which was previously published with some modifications[20]. Briefly, animals were allowed to adapt to the laboratory conditions for at least 1 week before EAE induction. For EAE-induction, 200 µg of myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$; KJ Ross-Petersen AsP, Denmark) was emulsified with an equal volume of complete Freund's adjuvant (CFA; Sigma, USA) consist of 500 µg of heat-killed *Mycobacterium tuberculosis* (100 µL total volume). It was injected subcutaneously in both hind flanks of each mouse. As well, they received by intra-peritoneal injections the pertussis toxin (300 ng in 100 µL PBS; List Biological Lab, USA) when immunization occurred and following 48 h again. The injections were accomplished by using a 1-mL insulin syringe needle. The clinical symptoms every other day were measured

for 27 days after induction of the disease base on the following standards: 0 = no symptoms; 1 = distal limp tail; 2 = complete limp tail; 3 = one hind limb paralyzed; 4 = both hind limbs paralyzed; 5 = Hind limbs and one forelimb paralyzed; 6 = Hind limbs and both forelimbs paralyzed; 7 = moribund/death [21]. Age-matched naïve animals were used as a control. Body weights were recorded every 2 days.

Tissue Collection and Processing for Demyelination and inflammation assay: Twenty-seven days after disease induction, mice were sacrificed; vertebral columns and brains were removed. For the pathological study, all vertebral columns were isolated gently then placed in formalin 10%, after 48 h transferred into 5% Nitric Acid (for 1 week) and again transferred into formalin 10%. Lumbar regions of the spinal cords were used for further tissue processing. The samples were molded by using paraffin, and the molds were cut-off by Microtome (5 µM sections). Hematoxylin and eosin (H&E) staining was applied to evaluate the infiltration of inflammatory cells and for the pathological changes in the spinal cord. Additionally, Luxol Fast Blue (LFB) staining was used to investigate spinal demyelination.

Cell Culture: The animals were sacrificed promptly, by 27 days follow up post immunization. The animals' spleens were taken out; consequently, single-cell suspensions were generated in a petri dish containing incomplete RPMI1640 (Caisson, USA) medium. Splenocytes were then incubated in 24-well micro-titer plates (5×10^5 cells/well), re-stimulated by using PHA (2 µg/mL) or MOG (10 µg/mL). A group of splenocytes without any stimuli was selected as the control. Following 24 hours, cells were gathered for gene expression analysis. For cytokine assay, supernatants were collected after 72 hours. The entire samples were stored at -70°C up to the usage.

Gene Expression Measurements by quantitative reverse transcription–polymerase chain reaction (qRT-PCR): The evaluations of gene expressions were done by using qRT-PCR method. Total RNA was extracted from cultured splenocytes (1×10^6 re-stimulated cells) and homogenized brain (200 µL of lysate) using the QIAamp® RNA Blood Mini Kit (Qiagen, Germany). All procedures were performed according to the kits' protocol. The integrity and purity of RNA were controlled by using denaturing agarose-formaldehyde gel electrophoresis containing MOPS buffer and measuring the absorbance ratio in 260/280 nm. The extracted RNA converted to complementary DNA (cDNA) by using PrimeScript RT kit (Takara, Japan). The Beacon Designer 8 software did the design of the two primers *Tmevpg1* and *Rmrp* and the rest of the primers were chosen from previous reports[22-24]. Beta-Actin was quantified as a control to normalize the differences in total RNA levels. In the cases of *Tmevpg1* and *Rmrp*, the PCR products further were confirmed using Sanger sequencing in both directions on the ABI 3130xl Genetic Analyzer (Applied Biosystems, USA). All primers ordered from Sinaclon (Iran). The primer sequences presents in Table 1. Amplifications were performed in volumes of 20 µL containing 2µL of cDNA, 10µL of RealQ Plus 2x Master Mix Green (Amplicon, Denmark), 1µM of forward and reverse primers, and 7µL PCR grade water. For cDNA amplification in Rotor-Gene 6000 (Corbett, Australia), an initial denaturation at 94°C for 15 min was followed by 40 cycles at 94°C for 15s and 60°C for 1 min. PCR products were checked using melt curve analysis and agarose gel electrophoresis. The relative quantification of gene expression was obtained by comparison with the REST method. All gene expression analyses were done as duplicated.

Cytokine assay: The amounts of IFN-γ and IL-17 in the supernatants of cultured cells were assayed by using ELISA (Mabtech, Sweden) base on the instructions of the manufacture. The tests were performed triplicates.

Statistical tests: The Mann–Whitney U test was performed to analyze the differences in the averages. Gene expression changes were analyzed by REST method[25]. Data were presented in the form of mean±SEM (standard error of means) and $p<0.05$ was considered statistically significant. Statistical analysis was performed using PRISM software version 6.0 (GraphPad Software Inc, San Diego, California, USA).

Results

Characterization of the Experimental Model: Following immunization with MOG, C57BL/6 mice developed acute monophasic disease with 80% incidence, which peaked around days 22-27 after

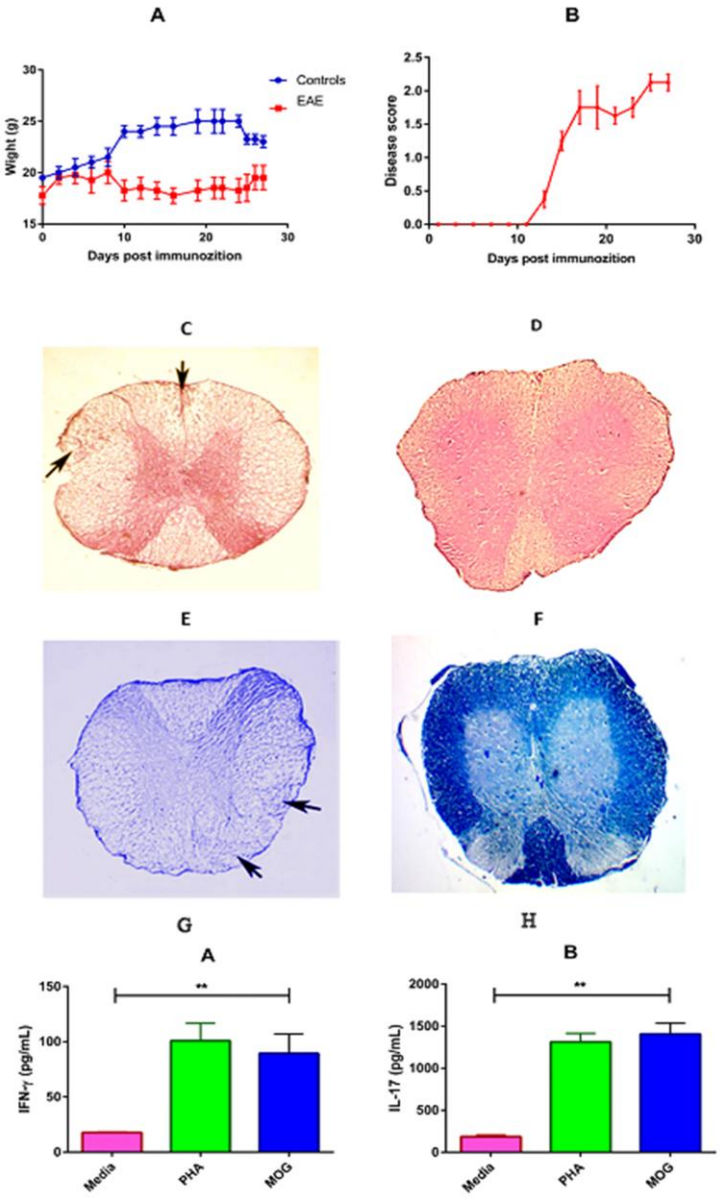

Figure 1. Characterization of the EAE Model. EAE was induced in female C57BL/6 mice following immunization with MOG emulsified in complete Freund's adjuvant. (A) The weight of control (n = 6) and EAE (n = 3) mice were measured every other day. (B) The EAE disease scores were recorded. The infiltrations of immune cells were shown by using H&E staining on spinal cords of EAE mouse (C) in comparison to healthy mouse (D). The arrows point the accumulation of a large number of immune cells in the representative section of the spinal cord of a C57BL/6 mouse with severe EAE. LFB staining (E, F) were used to evaluate the demyelination in spinal cords. The demyelinated area in the spinal cord of an EAE mouse was shown by the arrows (C). The concentration of IFN-γ (G) and IL-17 (H) in MOG-stimulated cells and non-stimulated cells (Media) in EAE group (n = 6) were measured by ELISA. All the tests were performed duplicated. Mann–Whitney U test was performed to analyze the differences in the averages. Values represent as the mean ± SEM (standard error of means). ** $p \leq 0.01$.

immunization. Development of EAE signs was accompanied by body weight lost with maximal loose that coincided with paralysis at the peak of the occurrence of the disease (Figure 1A, 1B). In order to evaluate the presence of inflammatory cells, 27 days after immunization with MOG, brains and spinal cords

Table 1: The sequence of Primers.

Gene	Forward	Reverse
Rmrp	5'-CTGTTTCCTAGGCTACATACGA-3'	5'-CACTCTCTGCCCGTGGTC-3'
Rorc	5'-GACCCACACCTCACAAAT TGA-3'	5'-AGTAGGCCACATTACACTGCT-3'
Tmevpg1	5'-AATTGTCTGTTAGGATGTG-3'	5'-GATTATGCTGTCGTCTTG-3'
Tbx21	5'-CCACAAGCCATTACAGGATGTT-3'	5'-GGAGTCTGGGTGGACATATAAGC-3'
Beta- Actin	5'-AAGCCATGCCAATGTTGTCTCT-3'	5'-CCTAGCACCATGAAGATCAAGATCA-3'

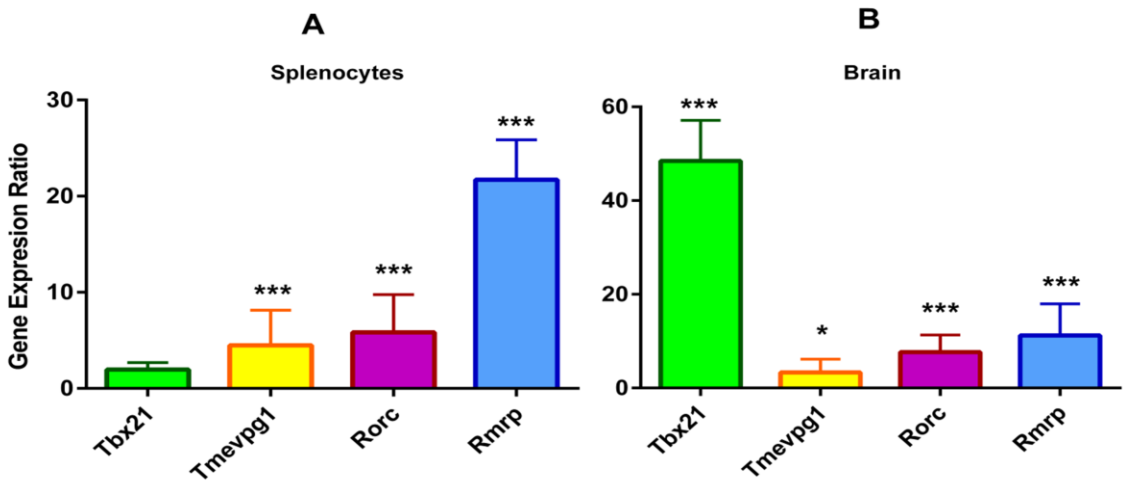

Figure 2. Gene expression analysis of lncRNAs and transcription factors. The expression of *Tmevpg1*, *Tbx21*, *Rmrp*, and *Rorc* in the splenocytes (A) and the lysate of brains (B) were analyzed by using qRT-PCR. The splenocytes of EAE mice (n = 3) were re-stimulated with MOG, and the alterations in the expression of the genes were compared with the controls (n = 3). All the tests were performed triplicated. Values represent as the mean ± SEM (standard error of means). * $p \leq 0.05$; *** $p \leq 0.001$.

were removed and stained by using H&E staining method to observe the infiltration of immune cells in the spinal cord Figure 1C, 1D). Additionally, LFB staining was used to observe the white part of spinal cord demyelination. The results showed that in EAE mice, infiltration of immune cells around spinal cord tissue was increased, and the nerve cells' myelin was lost in some areas (Figure 1E, 1F). The most prominent inflammatory cells accumulation that was detected at the peak of disease was associated with the plaques of demyelination in the spinal cord.

After re-stimulation of splenocytes with MOG, the concentration of IFN-γ and IL-17 cytokines in the supernatant were measured. In EAE; as a result, IFN-γ and IL-17 cytokines increased significantly in MOG-stimulated cells compared to the non-stimulated cells (media) (p = 0.002). Mean concentration of IFN-γ and IL-17 in MOG-stimulated cells were 89.32 ± 17.68 pg/mL and 1406.61 ± 128.94 pg/mL; in addition, in non-stimulating cells were 17.79 ± 1.18 pg/mL and 184.39 ± 51.46 pg/mL, respectively (Figure 1G, 1H).

The expression levels of *Rmrp* and *Tmevpg1* were increased in splenocytes and brains of EAE mice: Apparently, to clarify the involvement of T_H1 and T_H17 related genes in the immunopathology of EAE, we primary assessed the expression of *Tbx21* and *Rorc* mRNA in splenocytes obtained after *in-vitro* re-stimulation, by using qRT-PCR (Figure 2A). In EAE group, in splenocytes that were re-stimulated with MOG peptide, the expression of *Tbx21* was increased 1.196 (SE = 0.720-1.679) fold when compared to the controls, which was not statistically significant (p = 0.579). However, the expression of *Rorc* in splenocytes re-stimulated with MOG was increased 5.841 times (SE = 2.325-13.007), and the observed difference was statistically significant when compared to the mRNA expression splenocytes of the controls,

(p = 0.0001). Accumulating evidence underscores the capability of *Tmevpg1* and *Rmrp* lncRNA in the differentiation of T_H1 and T_H17 subtypes. Therefore, we secondly analyzed the expression pattern of these molecules in the splenocytes of EAE mice after *in-vitro* re-stimulation with MOG (Figure 2A). Contrary to the control group, *Tmevpg1* gene expression in EAE group was significantly higher (4.482 times, SE = 3.523-5.780; p = 0.0001). The *Rmrp* gene expression in EAE group compared to the control group was significantly higher (39.704 times, SE = 31.343-50.344; p = 0.0001).

Subsequently, we evaluated the expression of *Tbx21*, *Rorc*, *Tmevpg1*, and *Rmrp* in the brains of EAE mice (Figure 2B). In order to measure the expression of the genes in the brain, the mice brain lysate of EAE and control group were prepared. *Tbx21* and *Rorc* were significantly up regulated (p = 0.0001, p = 0.0001) in EAE group (in comparison to control group) by a mean factor of 48.48 and 7.67, respectively (SE = 28.64-81.40 and SE = 3.66-14.63, respectively). The expression of *Tmevpg1* in the EAE group showed an increase of 3.35 times (SE = 2.81-5.17; p = 0.02) when compared with the control group. As expected, *Rmrp* was also significantly up regulated in EAE group (in comparison to the control group) by a mean factor of 11.218 (SE = 6.74-16.83; p = 0.0001).

Discussion

The involvement of the *Rmrp* and *Tmevpg1* lncRNA in the differentiation of CD4+ T cell into the T_H17 and T_H1 subtypes was highlighted previously[10, 12]; however, the alterations in *RMRP*/*Rmrp* and *NeST*/*Tmevpg1* RNA expression levels in MS or EAE were not evaluated. The outcomes of the current experiment revealed that the expression of Rmrp and Tmevpg1 lncRNAs in splenocytes of EAE mice were 39.70 and 4.48 times more than the expressions in the controls. As well, the expression of the genes associated to these two lncRNAs, i.e. *Rorc* and *Tbx2*1, were increased 5.84 and 1.20 times, respectively; although the increase in the expression of *Tbx21* gene was not statistically significant; nonetheless, the observed increase was significant from a biological point of view. The results indicated that the production of IFN-γ that was controlled by the *Tbx2*1 transcription factors increased in EAE mice (5 folds in comparison to the non-stimulated cells as the control group). Moreover, the simultaneous increase of the expression of *Tbx21* and *Tmevpg1* may have a synergistic effect on the transcription of *Ifng*. Another explanation for this finding obtained from the experiments, which showed that T_H17 cells secrete IFN-γ besides IL-17. Previous findings revealed that conditional inactivation of DDX5 in T cells rendered *Rorc*+ cells defective for generation of IL-17A as well as IFN-γ[26]. Generally, according to the results, up-regulation of *Tmevpg1* in splenocytes of EAE mice was in accordance with the observed increase in demyelination in neuronal axons, which were mediated by IFN-γ associated responses. The distinct increase that was observed in the level of Tmevpg1 mRNA compared to the *Tbx21* mRNA, proposes *Tmevpg1* as a desirable molecular marker for T_H1 associated diseases. Further studies are needed to specify *Tmevpg1* characteristics as a molecular marker for evaluation of the disease activity in MS.

The more expression of *Tmevpg1* RNA levels in EAE than controls were in concordance with the results of the previous studies in Ulcerative Colitis, Sjogren's syndrome[14], and Hashimoto's thyroid diseases, which implied a significant elevation in the expression of *NeST* transcript[13, 27]. Additionally, in Hashimoto's thyroid patients a positive correlation between the transcript levels of *NeST* with *Tbx21* and *Ifng* transcript levels was reported[13]. Apparently, it could be concluded that *NeST* may contribute to the pathogenesis of Hashimoto's thyroid[13].

Accumulating studies have demonstrated that EAE is a T_H1/T_H17-mediated autoimmune disease, since there are abundant T_H1/T_H17 cells that penetrate the CNS and contribute to the demyelination of neuronal axons[2]. Our results also indicated that the inflammatory cells and T_H17 related genes, *Rorc* expression, were higher in splenocytes and CNS from EAE. Evidently, in EAE mice, the level of IL-17 also increased more than 7.6 times in splenocytes re-stimulated with MOG than non-stimulated cells. Previous studies revealed that *Rorc* is a master regulator of differentiation and function of T_H17[28]. For that reason, therapeutic targeting of the *Rorc* is a goal for finding new drugs for MS therapy[29]. However, targeting the *Rorc* may cause significant side effects. It has been reported that besides T_H17, the development of lymphoid tissue inducer cells [30] and innate

lymphoid cells (ILC3) are entirely dependent on *Rorc*[31]. Similarly, thymocytes require to *Rorc* for their survival[26]. The results of a previous study revealed that the loss of Rmrp had no apparent side effect on immature thymocytes, lymphoid tissue inducer cells and ILC3 development[7]. These findings suggest that targeting *Rmrp* may generate fewer side effects; therefore, propose a new approach to modulate the development of T_H17 via targeting *Rmrp* [26]. Distinctly, the initial step toward finding new drugs is identifying how the transcription level of *Rmrp* is altered in MS. The present experiment is the first study to report the expression of *Rmrp* in EAE mice. Regarding that, the transcript levels of *Rmrp* in comparison to *Rorc* showed a significant increase in EAE mice (39.7 vs. 5.841); maybe targeting *Rmrp* would provide results that are more effective. Considering this, it is suggested to investigate the role of *Rmrp* inhibitors in treating and reducing the symptoms of the inflammatory diseases in another study.

The immunopathologic responses during MS involve the brain in which lesions mark neural damage and acute sites of inflammation, which conduct to the phenotypic symptoms of the disability[2]. The results of the current study showed that in EAE the expression of *Rmrp*, *Tmevpg1*, *Rorc*, and *Tbx21* increased in the brain; it could be due to the infiltration of peripheral immune cells or because of the activation of transcription in CNS-resident cells; such as microglia. It emerges in MS, the initial wave of CNS-infiltrating T cells, particularly T helper 17 (T_H17), enter through the choroid plexus[32]. The primary wave of CNS-infiltrating T cells generates the access of a second T cell wave that migrates tremendously through the blood-brain barrier into the CNS[32]. Vigneau *et al*, showed that in chronic infection by Theiler's virus, which causes a primary demyelination similar to demyelination observed in EAE; *Tmevpg1* gene mainly is expressed in the infiltrating immune cells, but not in the CNS-resident cells; such as, microglia[33]. As represented in Figure 2, the Tbx1/Tmevpg1 ratio in splenocytes is not compatible with this ratio in the brain of EAE mice. Indeed, exact factors that influence on the *Tbx1*/*Tmevpg1* ratio is not studied yet. However, the difference between the ratio of T_H1/T_H17 cells in the splenocytes and brain may explain the observed difference between *Tbx1*/*Tmevpg1* ratio in splenocytes and brains of EAE mice.

Beside the function of lncRNA in gene regulation, the role of lncRNAs in the production of micro-peptides could be notable. Although lncRNAs are recognized to be non-coding RNAs, they contain sequences that can be as an open reading frame (ORF), they can be translated and produce a micro peptide[34]. Therefore, it is possible that *Tmevpg1* and *Rmrp* could be also effective in the production of micro-peptides that participate in the pathophysiology of MS; hence, it is highly proposed to investigate the presence of these hypothetical peptides in future studies.

Conclusion

The current study was a cross-sectional study included a small size population of inbred mice. Therefore, it is suggested that further studies be carried out on an extended statistical population and sampling at different time intervals. Moreover, clinical studies are needed to be employed in order to distinctly define the pattern of expression and roles of these lncRNAs in MS. The results of the current study showed that *Tmevpg1* and *Rmrp* were up regulated in EAE. Further studies are needed to introduce *Tmevpg1* and *Rmrp* as the target molecules for the treatment of MS or molecular markers for assessment of disease progression or responses to treatments indeed.

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