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Expression and diagnostic values of MIAT, H19, and NRON long non-coding RNAs in multiple sclerosis patients

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Abstract

Background: Multiple sclerosis (MS) is a chronic inflammatory disease. Various long non-coding RNAs (lncRNAs) appear to have an important role in the pathophysiology of MS. This study aimed at evaluating the expression levels of lncRNAs, *MIAT*, *H19,* and *NRON* in peripheral blood of MS cases to a healthy control group. We collected blood samples of 95 MS cases (76 relapsing–remitting (RR) and 19 secondary progressive (SP) MS) and 95 controls. We used quantitative real-time PCR for the evaluation of gene expression. The correlation between expression with clinical parameters was analyzed by a multiple linear regression model. Receiver operating characteristic (ROC) curve analysis was carried out to detect the diagnostic potential of lncRNAs levels according to the area under the curve (AUC).

Results: *MIAT, H19*, and *NRON* were significantly increased in the RRMS and SPMS subgroups compared to the controls. We found that the *H19* and *MIAT* expression significantly were higher in SPMS compared with RRMS. Patients with RRMS had a greater level of the average *NRON* expression is compared with SPMS patients. The expression level of *H19* significantly was higher in females relative to male patients. Based on the area under curve (AUC) values, *NRON* had the best performance in the differentiation of MS patients from controls (AUC = 0.95, $P < 0.0001$). A combination of *MIAT*, *H19*, and *NRON* expression levels could be useful in differentiating MS patients with 93.6% sensitivity, 98.9% specificity, and diagnostic power of 0.96 ($P < 0.0001$).

Conclusions: The levels of *MIAT*, *H19*, and *NRON* in peripheral blood could be important biomarkers for MS diagnosis.

Keywords: LncRNA, Multiple sclerosis, Biomarkers, Real-time PCR, Gene expression

Background

Multiple sclerosis (MS) is a chronic complex autoimmune disease that causes the demyelination of neurons in the central nervous system (CNS) [1]. MS is especially common in young female adults [2]. A broad range of symptoms may appear in MS patients, such as pain, visual sensory disturbance, motor impairments, cognitive deficits, and fatigue. The different factors such as autoimmunity, genetic predisposition, and environmental influenced the pathophysiology of MS diseases. Evidence indicates that the reaction between abnormal T cells and CNS autoantigens can cause inflammation, demyelination, and neurodegeneration [3]. In addition, B cells act as an important source of plasma cells that generate antibodies while also regulating T cell production and autoimmune processes. B cells may affect the pro-inflammatory process of other immune cells [4].

Many genomic studies have demonstrated the presence of a large portion of DNA that doesn't code for proteins, but is transcribed to the different types of RNAs. The

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lncRNAs are a type of non-coding RNAs known to possess the ability to regulate cell proliferation through different mechanisms [5]. The dysregulation of lncRNAs is considered in the cause of many neurological disorders [6–8]. Research works have indicated that the expression of lncRNAs is significantly dysregulated in MS patients [9–13].

Immune regulation was affected by the function of different lncRNAs. A previous study revealed an important connection between the lncRNA *MIAT* (Myocardial infarction-associated transcript) and inflammatory response [14]. *MIAT* regulates the differentiation of neural stem cells into oligodendrocytes in the CNS [15]. The *NRON* (Non-Coding Repressor Of NFAT) lncRNA is an endogenous ribonucleic acid that is detected in human T cells and can regulate IL-2 expression and NFAT Ca^{2+}-activated transcription factor in activated T cells [16].

The lncRNA *H19* is a lncRNA with a critical role in immune response regulation. It is also an imprinted gene that is expressed only from the maternal allele [17, 18].

Although the role of *MIAT*, *H19*, and *NRON* in cellular pathways and different cancers has been assessed, only one study has been conducted to evaluate the expression level of *NRON* lncRNA in 10 MS cases. Because there is evidence showing that lncRNAs are stable in human serum, circulating serum RNA levels could serve as a biomarker in non-invasive diagnostic applications [19, 20].

Research on the role of lncRNAs in MS pathogenesis is just beginning. The significance of the present study becomes more apparent due to high incidence and prevalence of MS in Iran [21]. Therefore, we aimed to assess the effectiveness of using known lncRNAs as potential biomarkers for the diagnosis of MS.

Methods

Study subjects

The present case and control study was carried out to assess the expression levels of three lncRNAs (*MIAT*, *H19*, and *NRON*) in 95 MS cases, who were compared to 95 controls from August 2018 to August 2020 at Namazi Hospital in Shiraz. Among all patients, 76 individuals were relapsing–remitting (RR) and 19 were secondary progressive (SP). Controls comprised a representative sample of Shiraz's population; sex- and age-matched controls were randomly selected from neighbor controls at the nearest case's place matched.

All participants in the present study were clinically diagnosed by an expert neurologist according to McDonald's criteria [22]. All cases included in this study showed EDSS progression with no evidence of a relapse in the last 24 months. In the present study, we considered the following characteristics as exclusion criteria: recent infection, smoking history, malignancy, alcohol abuse, and the presence of any other inflammatory or autoimmune diseases. The examined subjects were aged 20–56 years.

Ethics approval for this research was obtained by the local ethics committee of the Kazeroon Branch, Islamic Azad University, Iran (IR.IAU.KAU.REC.1398.034). The subjects completed a written informed consent and voluntarily agreed to participate in the present study as a part of a large prospective project.

Collection of blood samples

After obtaining an informed consent form, the peripheral venous blood samples were collected from MS cases and control individuals in ethylene diamine tetra acetic acid-coated (EDTA) tubes as an anticoagulant.

RNA extraction and cDNA synthesis

A total RNA extraction kit (Favorgen, Taiwan) was used to isolate Total RNA from peripheral blood according to the manufacturer's instructions. cDNA synthesis Kit (Yektatajhiz, Iran) was employed for cDNA synthesis from RNA samples. For cDNA synthesis, RNA samples with theA260/A230 and A260/A280 ratios greater than 1.7 were selected.

Quantitative RT-PCR measurement of the lncRNA levels

Primer Express and Gene Runner software v.3.0 were applied to design and analyze the oligonucleotide primers. By searching the BLAST website, the primers were confirmed to avoid non-specific PCR product formation. Specific primers' evaluations of *MIAT, H19*, *NRON*, and the *TBP* gene (used as a reference gene) are shown in Table 1. Gene expression was normalized relative to the housekeeping genes GAPDH and TBP. TBP was selected as the preferential reference gene given its low variance in Ct between the different samples (data not shown).

Table 1 The nucleotide sequence of primers

Gene name	Primer sequences	Primer length (bp)	Product length (bp)
MIAT	TCCCATTCCCGGAAGCTAGA	20	274
	GAGGCATGAAATCACCCCCA	20	
H19	GCAGACAGTACAGCATCCA	19	91
	CTCCTGAGAGCTCATTCACTC	21	
NRON	CGGCAGCTCGCCCTTAAATA	20	184
	GAACCCCCAAACCTTCCGAT	20	
TBP	CCCGAAACGCCGAATATAATC	21	134
	TCTGGACTGTTCTTCACTCTTG	22	

The relative abundance of the lncRNAs' levels was calculated by real-time RT-PCR using Real Q Plus 2 × Master Mix Green Low ROX™ (Ampliqon, Denmark). We used the thermal-cycling settings of 10 min at 95 °C (one repeat) accompanied by 40 cycles for 15 s at 95 °C and 1 min at 60 °C. Every complete amplification phase was accompanied by a melting phase (15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C). Cycle threshold (Ct) values were used to express the differences in relative levels. ΔCt indicates the difference between the Ct values of *TBP* and *lncRNAs*. The relative *lncRNAs* expression levels for each subject were determined using $2^{-\Delta Ct}$ [23].

Statistical analysis

The *IncRNAs* relative expression levels were reported as mean ± SE. Student's *t* test was employed to compare the mean expression of each *lncRNA* gene between case and control groups. The Beta coefficient of lncRNAs expression levels was estimated in univariate and multiple linear regression analyses. Using a multiple linear regression model, we adjusted for the most important clinical factors. Receiver operating characteristic (ROC) curve analysis was carried out to detect lncRNAs levels as a diagnostic biomarker for MS according to the area under the curve (AUC). We used the multiple logistic regressions to combine the age and sex variables in ROC analysis. The analyses were performed using the GraphPad Prism 5.0 and the SPSS software (version 19.0). The *P* value of < 0.05 was regarded as statistically significant.

Results

Clinical and demographic characteristics

Clinical and demographic characteristics of MS cases and healthy controls are summarized in Table 2. No significant difference was demonstrated in sex and age ratio ($P > 0.05$).

LncRNAs (MIAT, H19 and NRON) expression levels

Figure 1 demonstrates that *MIAT*, *H19*, and *NRON* levels were significantly higher in MS cases than in healthy controls ($P < 0.0001$), the median $\log_2$ fold changes were as follows, respectively 1.10, 2.79, and 1.30. The significantly high expression of *MIAT*, *H19*, and *NRON* can distinguish MS cases from healthy cases (Fig. 1A). Then, lncRNAs expression levels were compared in RRMS and SPMS subtypes with healthy controls separately. *MIAT, H19*, and *NRON* expression levels were significantly increased in the RRMS and SPMS subgroups when compared to the controls separately ($P < 0.001$). One-way ANOVA analysis showed that the *MIAT* and *H19* expression in SPMS significantly was higher than RRMS, while *NRON* level in RRMS was higher relative to SPMS (Fig. 1B).

Table 2 Demographic and clinical characteristic of healthy controls and patients

Variables	MS patients ($n = 95$)	Healthy subjects ($n = 95$)	*P* value
Female	80 (84.2%)	80 (84.2%)	1.00[a]
Male	15 (15.8%)	15 (15.8%)	
Age, y	36.81 ± 8.37	36.81 ± 8.35	1.00[b]
Age of onset, y	27.71 ± 7.96	–	
Duration, y	7.82 ± 6.00	–	
EDSS	2.36 ± 2.19	–	
MS type			
RR	76 (80%)	–	
SP	19 (20%)	–	

Data were shown as mean ± standard deviation (SD) or as *n* (%)

Abbreviations: EDSS, expanded disability status scale; RR, relapsing remitting; SP, secondary progressive

[a] Chi-square Test

[b] Independent two-sample T test

Association between clinical variables and LncRNAs expression

Multiple linear regression analysis was done to detect the association between RRMS and SPMS patients and lncRNAs expression levels after adjusting for several clinical confounders (Table 3). The lncRNAs expression levels were significantly associated with RRMS and SPMS.

Patients with SPMS had a greater level of the average MIAT expression compared with RRMS patients [Beta = 1.48, 95% CI (0.62–2.35) $P = 0.001$]. Compared with RRMS patients, patients with SPMS have a higher levels of H19 expression [Beta = 1.92, 95% CI (0.54–3.29) $P = 0.007$]. Also, H19 expression in female patients was significantly higher than male patients [Beta = 2.76 95% CI (1.25–4.28) $P = 0.001$]. Patients with RRMS had a greater level of the average NRON expression compared with SPMS patients [Beta = 1.49, 95% CI (0.48–2.51) $P = 0.004$].

Predictive power of MIAT, H19, and NRON in MS diagnosis

ROC curve analyses were done to assess the diagnostic values of *MIAT*, *H19*, *NRON*, and whole lncRNAs expression levels and their usefulness in predicting MS. The results of these analyses are presented in Fig. 2 and Table 4. Three lncRNAs were shown to be effective in differentiating MS cases from controls. *MIAT* had an AUC of 0.861 (95% CI: 0.804–0.918;

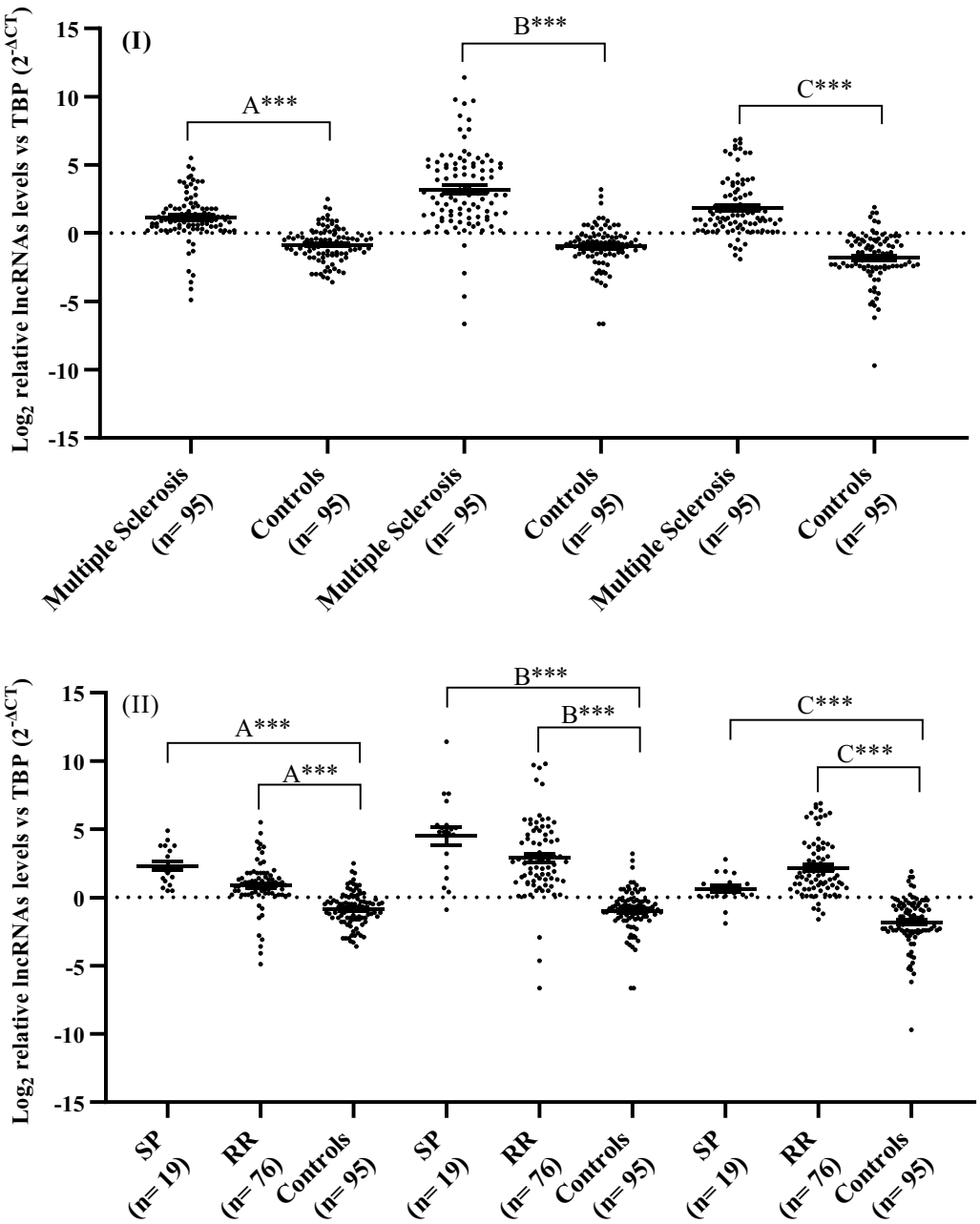

Fig. 1 The LncRNAs of MIAT, H19 and NRON versus the TBP gene levels in the MS cases and control, (I) the levels of MIAT (**A**), H19 (**B**), and NRON (**C**) in controls were significantly lower compared to MS patients, (II) the relative expression of MIAT (**A**), H19 (**B**), and NRON (**C**) (with $\log_2$ fold change) in MS subgroups in comparison with normal healthy controls. Data were shown as mean ± SE. The data were analyzed using Student's t test or one-way ANOVA. $2^{-\Delta Ct}$ values for each individual were used to create each figure. ***$P < 0.001$ significant differences vs. control subjects. Abbreviations: RR, relapsing–remitting; SP, secondary progressive

sensitivity = 89.47%, specificity = 80.00%), *H19* had an AUC of 0.941 (95% CI: 0.903–0.979; sensitivity = 91.58%, specificity = 91.58%), *NRON* had an AUC of 0.951 (95% CI: 0.922–0.981; sensitivity = 93.68%, specificity = 91.58%), and whole lncRNAs expression had an AUC of 0.966 (95% CI: 0.935–0.997; sensitivity = 93.68%, specificity = 98.95%). These findings

Table 3 Association between MIAT, H19, and NRON levels with clinical parameters in MS patients: using univariate and multiple linear regression models

Characteristics	Univariate			Multiplevariate *		
	Beta	95% CI	*P* Value	Beta	95% CI	*P* Value
MIAT relative expression (log_2)						
Female sex	0.21	−0.78, 1.19	0.681	0.38	−0.58, 1.33	0.436
> 30 age-old	0.16	−0.744, 1.06	0.731	0.28	−0.61, 1.17	0.530
Type MS (SP vs. RR)	1.45	0.60, 2.29	0.001	1.48	0.62, 2.35	0.001
Severity MS (≥ 0.5 vs. < 0.5)	−0.28	−1.02, 0.46	0.462	−0.16	−0.89, 0.57	0.664
H19 relative expression (log_2)						
Female sex	2.41	0.86, 3.96	0.003	2.76	1.25, 4.28	0.001
> 30 age-old	0.79	−0.69, 2.26	0.291	1.34	−0.07, 2.75	0.063
Type MS (SP vs. RR)	1.62	0.17, 3.07	0.028	1.92	0.54, 3.29	0.007
Severity MS (≥ 0.5 vs. < 0.5)	−0.11	−1.33, 1.12	0.846	0.14	−1.01, 1.29	0.809
NRON relative expression (log_2)						
Female sex	0.63	−0.51, 1.77	0.276	0.57	−0.55, 1.69	0.314
> 30 age-old	0.39	−0.66, 1.43	0.463	0.28	−0.76, 1.32	0.594
Type MS (RR vs. SP)	1.53	0.53, 2.53	0.003	1.49	0.48, 2.51	0.004
Severity MS (≥ 0.5 vs. < 0.5)	−0.20	−1.06, 0.66	0.640	−0.25	−1.10, 0.59	0.556

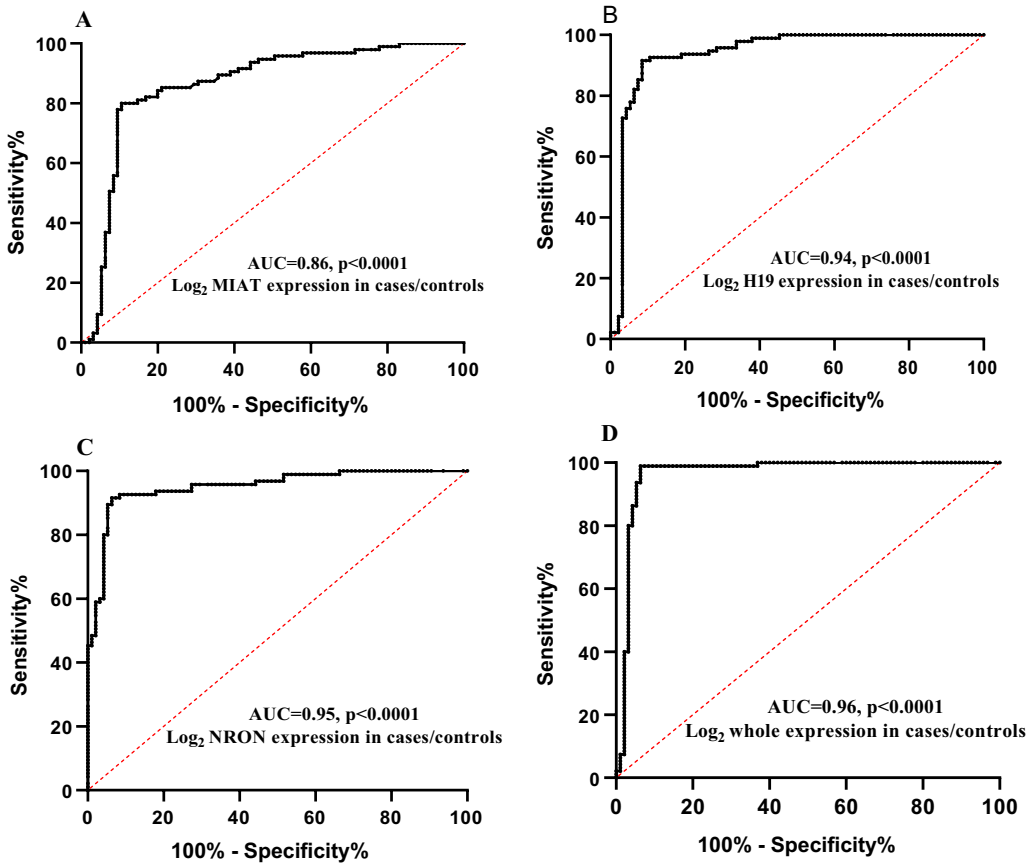

Fig. 2 ROC curves. **A** ROC curve analysis of *MIAT* for diagnosis MS patients from the controls, **B** ROC curve analysis of *H19* for diagnosis MS patients from the controls, **C** ROC curve analysis of *NRON* for diagnosis MS patients from the controls, **D** ROC curve analysis of *whole expression* for diagnosis MS patients from the controls

Table 4 Prediction of diagnostic value by selected biomarkers (log_2 fold change)

Parameter	AUC ± SE	95% CI	*P*	Se (%)	Sp (%)
Diagnostic value					
Expression MIAT	0.861 ± 0.029	0.804–0.918	< 0.0001	89.47	80.00
Expression H19	0.941 ± 0.019	0.903–0.979	< 0.0001	91.58	91.58
Expression NRON	0.951 ± 0.015	0.922–0.981	< 0.0001	93.68	91.58
Combination of all genes	0.966 ± 0.016	0.935–0.997	< 0.0001	93.68	98.95

Abbreviations: AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity

The diagnostic analyses were conducted after combining with age and sex, using multiple logistic regression. $P < 0.05$ was considered statistically significant

demonstrated that *MIAT*, *H19*, *NRON,* and whole lncRNAs expression can be considered important biomarkers for MS diagnosis.

Discussion

The present case–control study was performed on southwest Iranian individuals. We reported an upregulation of *MIAT, H19*, and *NRON* lncRNAs in the peripheral blood of MS patients in comparison with healthy subjects, the median log_2 fold changes were as follows, respectively, 1.10, 2.79, and 1.30. The previous studies reported the dysregulation of various lncRNAs in MS cases [9–11]. For example, Shaker et al. found a significant increase in the expression levels of *lnc-DC* and *MALAT1* in MS patients [10]. Dastmalchi et al. showed that *NEAT1*, *PANDA*, and *TUG1* lncRNAs were over-expressed in MS cases when compared with controls [11]. One previous study [13] showed that three lncRNAs (*NEAT1*, *TUG1*, and *RN7SKRNA*) were up-regulated in RR patients. The downregulation and upregulation of six lncRNAs in MS cases were investigated in another study [24]. We found that the expression levels of *MIAT* and *H19* in SP patients were significantly higher than in RR patients. However, *NRON* expression levels in RR patients showed significantly higher expression relative to SP patients.

NRON is a lncRNA repressor that acts as a specific regulator of NFAT nuclear trafficking and interacts with the importin-beta superfamily [25]. NFATs regulate the transcriptional induction of genes that encode for immune activators/modulators such as GM-CSF, IL- 2, IL-3, IL-4, IL-5, IL-13, L-8, IFNγ,CD5, CD25, CD28, and CD40 [26]. In T cells, NFAT proteins govern gene expression, thereby regulating their activation, differentiation, and development in addition to the maintenance and induction of T cell tolerance [27]. The expression level of the *NRON* lncRNA was significantly higher in MS patients than in controls. This outcome is contrary to that of Fenoglio et al., who found that *NRON* was downregulated in peripheral blood of 10 MS cases in comparison with controls. They showed that *NRON* correlates with MS duration [12]. In Fenoglio study, the downregulation of *NRON* in 10 cases (5 RRMS and 5 PPMS) was evaluated, while in our study, lncRNAs expression was evaluated in 95 MS patients (76 RRMS and 19 SPMS). We also reported the significant upregulation of *NRON* in two subtypes of MS relative to control. Additionally, our results showed no significant correlation between the expression levels of *NRON* with disease duration, EDSS score, and age among MS patients. The mean disease duration in our study was 7.82 ± 6.00 years it seems that the difference in duration of the MS disease in the two studies has led to this controversy. *NRON* expression is likely to be high in the early years of the MS disease and then gradually decreases.

The *H19* lncRNA has an important role in the regulation of immune response [17]. *H19* is an imprinted gene that is expressed from its maternal allele [18]. The deletion of *H19* genes facilitates cell cycle progression in hematopoietic stem cells through the induction of maternal IGF2 [28]. *H19* might contribute to the pathogenesis of MS based on its role in the IGF system and the importance of hematopoietic stem cells in the regulation of immune response [29, 30]. One previous investigation demonstrated the participation of *H19* in rheumatoid arthritis (RA) pathology according to its overexpression in RA cases and its hypersensitivity to cytokine regulation/starvation in these cases [31]. The upregulation of *H19* has been reported in ischemic stroke patients with a potential for the early diagnosis of IS [32]. Zeis et al. demonstrated that over-expression of the IGF1 and IGF2 genes was found in inactive demyelinated lesions [33]. We demonstrated that the expression level of *H19* was significantly up-regulated in the blood of MS cases in comparison with controls. We also observed that *H19* expression levels correlated with sex so that the higher expression in females was detected compared with male patients. The female-specific elevated expression of H19 was reported in a previous study [34]. Adriaenssens et al. reported that 17-beta-estradiol stimulated the endogenous H19 gene in MCF-7 cells [35]. On the other hand, the estrogen–ERα–H19 signaling axis may also be important in the development of breast cancer [36]. This gene

acts as an estrogen receptor modulator [37]. As many articles have suggested the importance of sex hormones in determining the onset and outcome of multiple sclerosisin in females [38, 39]. These findings have raised the challenging question of whether estrogen may be present in the pathophysiology of MS disease by overexpression in *H19* lncRNA? Further studies with a larger sample size are needed to confirm the strong correlation between sex and *H19* expression level in MS patients. Our results also showed that the *H19* expression in SPMS patients was 5.56 times higher than that in RRMS patients. SPMS patients are usually older than RRMS with higher disability and hospitalization rates relative to RR [40]. But in our study, patients with SPMS ($n=19$) had not have higher EDSS scores and age compared with RRMS (76). Thus, we can suppose that a larger sample size for SPMS patients is needed to clarify the significant difference between age and EDSS with RRMS. This result indirectly suggests that *H19* expression in MS disease may have a positive correlation with the disability score of patients but larger sample size is needed to reach this correlation.

MIAT is a well-characterized lncRNA that affects cellular functions such as apoptosis, invasion, and proliferation in many human diseases. The regulatory mechanism of *MIAT* is very complex [41]. In patients with coronary artery disease (CAD) compared with controls, the serum level of *MIAT* was significantly increased and positively correlated with serum IL-6 and TNF-α [42]. Previous research works have shown that the *MIAT* level can be used as a biomarker for the diagnosis and prognosis of different diseases such as CAD [42, 43], various cancers [44, 45], and ischemic stroke [46]. Zhu et al. demonstrated that the expression levels of *MIAT* were significantly up-regulated in the leukocytes of ischemic stroke (IS) patients in comparison with controls [46]. We demonstrated that the expression level of *MIAT* was significantly higher in MS cases than in controls. Also, in the present study, we showed that *MIAT* expression was 5.93 times higher in SP cases than in RR cases. We could not detect the positive correlation between *MIAT* expression and EDSS score but significantly high expression of *MIAT* in SPMS relative to RRMS indirectly may show the positive association between *MIAT* and MS severity.

A valuable method for the early screening of MS is developing a high-sensitive non-invasive blood biomarker. Evidence shows that circulating lncRNAs could be considered MS biomarkers [10, 11, 47]. Elevated *MIAT*, *H19*, and *NRON* expression levels have been reported as characteristics of various disorders (48–53). Therefore, they could also be used as a non-specific biomarker for MS. Among the three lncRNAs investigated in the present study, *NRON* showed the best efficiency as a biomarker-based on its sensitivity, specificity, and AUC values.

In the present study, all patients were chosen consecutively from hospitals during the same period. The participants represented a variety of ethnic groups, though selection bias could not be avoided. More accurate findings could be observed with larger sample sizes to verify our results. Also, in the current investigation, we evaluated the expression levels of lncRNAs without performing a full transcriptome analysis. Furthermore, more investigations are needed to detect the exact molecular mechanisms by which *MIAT*, *H19*, and *NRON* participate in MS pathology. Although the current study indicates that circulating *MIAT*, *H19*, and *NRON* can be used as diagnostic biomarkers for MS, further research is required to find reliable blood biomarkers for clinical use.

Conclusions

This research showed the upregulation of three specific lncRNAs (*MIAT*, *H19*, and *NRON*) in the peripheral blood of 95 MS cases. Additional research with larger sample size is needed to confirm the present findings and clarify the molecular mechanisms of these lncRNAs in the pathogenesis of MS. Their dysregulation profiles indicate that they could be considered potential biomarkers for predicting the course of MS or patients' responses to treatment.

Abbreviations

MS: Multiple sclerosis; lncRNAs: Long non-coding RNAs; RR: Relapsing–remitting; SP: Secondary progressive; AUC: Area under the curve; CNS: Central nervous system; EDSS: Expanded disability status scale; RA: Rheumatoid arthritis; PR: Progressive-relapsing; PP: Primary-progressive; Ct: Cycle threshold; OR: Odds ratio; ROC: Receiver operating characteristic; IS: Ischemic stroke.

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Authors' contributions

MA contributed to study concept and design, acquisition of data. MJ contributed to study concept and design, acquisition of data, analysis and interpretation of data, study supervision, drafting/revising the manuscript for content. MB contributed to study concept and design, acquisition of data drafting/revising the manuscript for content. AS contributed to acquisition of data drafting. MD contributed to acquisition of data drafting. RT contributed to analysis and interpretation of data. AB contributed to study concept and design, acquisition of data, study supervision, drafting/ revising the manuscript for content. All authors read and approved the final manuscript.

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Availability of data and materials

Data and materials will be available if needed.

Declarations

Ethics approval and consent to participate
Ethics approval for this research was obtained by the local ethics committee of the Kazeroon Branch, Islamic Azad University, Iran (IR.IAU.KAU.REC.1398.034). The subjects completed a written informed consent and voluntarily agreed to participate in the present study as a part of a large prospective project.

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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