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LncRNAs associated with multiple sclerosis expressed in the Th1 cell lineage

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Abstract

Multiple sclerosis (MS) is a type of inflammatory and demyelinating disorder of the central nervous system in which immune-mediated inflammatory processes are elicited by secreted cytokines from T helper (Th)-1 and Th17 cells. While some protein-coding genes expressed in T cell types have established involvement in MS disease progression, little is understood about the roles of long noncoding RNAs (lncRNAs) within the disease landscape. LncRNAs, noncoding RNAs longer than 200 nucleotides, likely control gene expression and function of Th1 cells, and offer the potential to act as therapeutic and biomarker candidates for MS. We identified lncRNAs in Th1 cells linked to MS. Expression levels of candidate lncRNAs and genes were evaluated in 50 MS patients and 25 healthy controls using quantitative real-time polymerase chain reaction, and their correlations were assessed. LncRNAs encoded by AC007278.2 and IFNG-AS1-001 showed significantly higher expression in relapsing Phase MS patients whereas IFNG-AS1-003 was elevated in patients in the remitting phase compared with relapsing patients. Collectively, these misregulated lncRNAs may provide valuable tools to understand the relationships between lncRNAs and MS, and possibly other related disorders.

KEYWORDS
biomarker, CNS, lncRNA, multiple sclerosis, T helper 1

Aref Hosseini and Shohreh Teimuri contributed equally to this study.

1 | INTRODUCTION

Multiple sclerosis (MS) is a multifactorial chronic inflammatory disorder which leads to the demyelination of the neuron sheath in the central nervous system (CNS; Ransohoff, Hafler, & Lucchinetti, 2015). The adaptive immune system is hyperactivated in MS, including involvement of T helper 1 (Th1) cells. Myelin-reactive Th1 cells lacking IL-17(+) are highly pathogenic, while Th17 cells lacking interferon (IFN)-γ (+) are not pathogenic, and only Th1 cells infiltrate the noninflamed CNS at primary stages of MS onset, while Th17 cells appear in the CNS at later stages (O'Connor et al., 2008). Although the precise involvement of Th1 cells in autoimmunity is not completely understood, identification of therapeutic approaches that specifically target pathogenic Th1 cells with less effect on other immune cells may be an important approach (Christie & Zhu, 2014; Stromnes, Cerretti, Liggitt, Harris, & Goverman, 2008).

Historically, IFN-γ production was considered as the hallmark of Th1 cells, driving inflammation and autoimmunity (Zhu, Yamane, & Paul, 2009). There is a reduction in serum IFN-γ levels among IFN-β treated MS patients and IFN-β is widely used as a selective drug for MS treatment (Revel, Chebath, Mangelus, Harroch, & Moviglia, 1995). During the differentiation of IFN-γ-secreting Th1 cells, IL-12Rβ2 is upregulated and IL-12 drives the expression of IL-18Rα (Sareneva, Julkunen, & Matikainen, 2000; Szabo, Dighe, Gubler, & Murphy, 1997; Yoshimoto et al., 1998). The *IL18R1* and *IL18RAP* genes encode the heterodimeric IL-18 receptor (the α and β chains, respectively). The cytokine IL-18 is a modulator of innate and adaptive immune responses, and acts by inducing Th1 cell differentiation through its receptor (IL-18R; Gracie, Robertson, & McInnes, 2003; Nakanishi, Yoshimoto, Tsutsui, & Okamura, 2001).

Long noncoding RNAs (lncRNAs) are a special class of transcripts > 200 nucleotides long, with no apparent protein-coding capacity (Cheetham, Gruhl, Mattick, & Dinger, 2013; Ma, Bajic, & Zhang, 2013). About 50% of protein-coding genes that are located less than 50 kb away from a lncRNA gene are coexpressed, and this correlation decreases with increasing distance between them (Spurlock et al., 2015). In addition to being candidate therapeutic targets, lncRNAs are a more practical biomarker than proteins or messenger RNAs (mRNAs) as they are more stable in body fluids, more tissue-specific, and are readily detected by a variety of techniques (Ayers, 2013; Geisler & Coller, 2013; Qi & Du, 2013; Tong & Lo, 2006). LncRNAs participate in a wide range of biological processes whose activities are modulated by lncRNA dose (Lee, 2012; Nagano & Fraser, 2011). Expression of specialized genes are crucial to define cell fate and the unique functions of each cell type, and lineage-specific lncRNAs are likely to be important factors that impact these activities (Teimuri et al., 2018).

In this study, we identify lncRNAs that are expressed specifically in Th1 cells and which are located less than 50 kb from protein-coding genes involved in Th1 cell differentiation. Hence, we identified candidate lncRNAs potentially involved in MS pathogenesis. We validate the expression levels and MS-association of these identified lncRNAs and adjacent protein-coding genes in the PBMC of MS patients.

2 | MATERIALS AND METHODS

2.1 | Data mining

Coding genes of proteins involved in Th1 cell differentiation were retrieved through data mining using the keywords Th1 cell, differentiation, and signaling pathway among published papers up until May 2017 available in PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), Science Direct (https://www.sciencedirect.com/), and Web of Science (https://clarivate.com/products/web-of-science). Next, the Ensembl GRCh37 genome assembly was used to assign chromosomal locations of loci (https://grch37.ensembl.org/index.html). Additionally, whole-genome RNA-seq data containing Th1 lineage-specific expressed lncRNAs were used in this study (Spurlock et al., 2015) as well as chromosomal locations of these lncRNAs which were deduced from Ensembl GRCh37.

2.2 | Proximity analysis

Proximities of selected lncRNAs and the respective genes involved in Th1 cell differentiation were checked through python programming language (version 3.6.2 the Python-RRID:SCR_008394; script is available in Supporting Information File 1). Eventually, to visualize data, we transformed extracted protein-coding genes into STRING database to construct protein–protein interactions (Szklarczyk et al., 2014). Then, STRING database output was transferred to Cytoscape 3.6.0 network visualization software (Shannon et al., 2003; RRID:SCR_003032).

2.3 | Human subject blood sample collections

Patients attended at Alzahra Hospital, Isfahan, Iran were included in the study. MS was diagnosed according to the revised McDonald criteria and patients were also characterized by a neurologist based on the Expanded Disability Status Scale (EDSS), a method for calculating and understanding disability in MS patients. Approximately 10 ml blood samples were obtained from each participant and stored in ethylenediaminetetraacetic acid contained tubes. Patients ($n = 50$) were divided into 25 subjects with relapsing phase who recently were diagnosed, and 25 at the remitting phase who were treated with IFN-β. To minimize the effect of IFN-β, remitting patients were requested to donate blood samples before next dose intake. Relapsing remitting multiple sclerosis (RRMS) patients did not show other CNS pathologic conditions other than MS, as well as infection, tumor(s) and systemic hematologic diseases, and they were not supplemented with any antineoplastic or immunomodulating drug before experiments. Moreover, 25 blood samples were obtained from age/gender-matched healthy individuals with no sign of immune-related diseases and who were taking no medications. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples through density gradient lymphoprep procedure (Stemcell Technologies, Vancouver, Canada). Human subject work was approved by Institutional Review Board of Royan Institute (Project Id. No. 91000573). All protocols and procedures were

TABLE 1 Patients' clinical and demographic data

Characteristics	RRMS patients	Relapse	Remit	Control	*P*-value comparison between patients and controls / relapse, remit, and control samples
Sex: Female/Male	38/12	19/6	19/6	17/8	0.46/0.76
Age (SD)	34 (8.30)	32.48 (7.07)	35.52 (9.26)	32.84 (6.06)	0.49/0.23
Duration of illness (SD)	3.08 (4.41)	0	6.16 (4.47)	–	–
Disease severity (EDSS)	1.47	1.56	1.38	–	–

Note. EDSS: expanded disability status scale; SD: standard deviation.

checked and prepared in accordance with the relevant guidelines and regulations provided to use human specimens. Written informed consents were obtained from all individuals according to the guidance from the Institutional Review Board of Royan Institute.

2.4 | RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from samples in accordance with the TRIzol reagent manufacturer's supplied instruction (Invitrogen, Carlsbad, CA). RNA concentration and purity were assessed using Nanodrop spectrophotometer (Nanodrop 1000; Thermo Fisher Scientific, Waltham, MA). RNase-free DNase (Thermo Fisher Scientific) treatment was used to eliminate possible DNA contamination. Isolated RNA was reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA samples were stored at −80 °C until used.

2.5 | Quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR for mRNAs was performed using SYBR Green Master Mix: SYBR Premix Ex Taq II (TaKaRa, Kusatsu, Shiga Prefecture, Japan) using specific primer pairs in Step One Plus Real-Time PCR thermal cycler (Applied Biosystems, Foster City, CA). RT-qPCR experiments were conducted in triplicate. *UBC* and *YWHAZ* were used as the reference genes with the most stable expression accordance with the analysis of PBMC between controls, relapsing, and remitting patients (Oturai, Sondergaard, Bornsen, Sellebjerg, & Christensen, 2016). Primer sequences are listed in Table 2.

TABLE 2 Primer pairs used in this study

Primer	Sequence 5′–3′	GC (%)	3′Δ*G* (kcal/mol)	Amplicon size (bp)	Ta (°C)
Long noncoding RNAs					
IFNG-AS1-001 F	ACATACTTCCACCAGAGA	44.4	−6.2	143	52
IFNG-AS1-001 R	TTCCACAACACTATCAACT	36.8	−6.3		
IFNG-AS1-002 F	GGAGGAGAAGTCAGTAGC	55.6	−6.6	101	52
IFNG-AS1-002 R	AGCCAAGGGAACACATAC	50.0	−5.4		
IFNG-AS1-003 F	CCAGCACCATAATTCCAG	50.0	−6.9	116	50
IFNG-AS1-003 R	GAAGACATGAGCACTGAC	50.0	−6.6		
AC007278.2 F	ATCATCTGTATGCTGTCTAAC	38.1	−5.4	121	60
AC007278.2 R	AACCATATAATGAGGCTGTC	40.0	−6.6		
AC007278.3 F	TTGAAGAGGAGATTAAGTATTAGG	33.3	−5.7	127	58
AC007278.3 R	TCGATCATCTTCACATTCACATC	39.1	−6.2		
Protein-coding genes					
IFN--γ F	GTTCTCTTGGCTGTTACTG	47.4	−5.8	96	52
IFN--γ R	CATTATCCGCTACATCTGAAT	38.1	−5.7		
IL18R1 F	GAGAAACATTTTGGGTATAAGT-TATG	30.8	−5.0	90	52
IL18R1 R	CTCTATCAGTGAGTGGATTTC	42.9	−5.3		
IL18RAP F	AACACTCTACTCTGGCAAA	42.1	−6.8	119	52
IL18RAP R	ATCCTTTAATTCGCTCTCCT	40	−6.7		
Reference genes					
UBC F	GGATTTGGGTCGCAGTTCTTG	52.4	−6.1	135	58
UBC R	TGCCTTGACATTCTCGATGG	50	−6.5		
YWHAZ F	ACTTTTGGTACATTGTGGCTTC	40.9	−6.9	94	62
YWHAZ R	CCGCCAGGACAAACCAGTA	57.9	−5.8		

Note. F and R represent forward and reverse primers respectively. 3′Δ*G* stands for the Gibbs energy for binding of 3′ part of primer with the respective template. Ta indicates annealing temperature of PCR reactions.

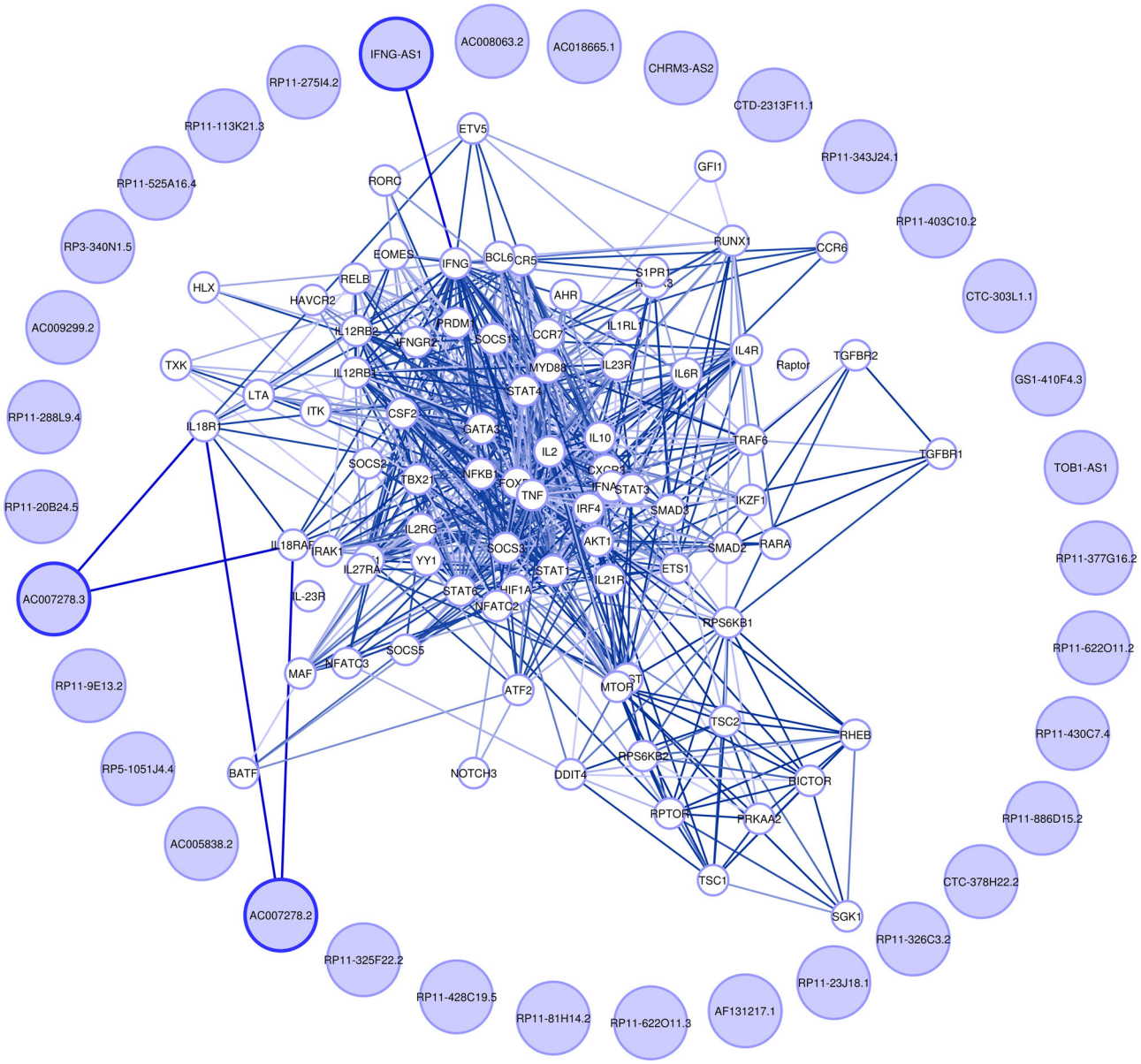

FIGURE 1 Genes involved in Th1 cell differentiation and associated lncRNAs. Th1 cell differentiation genes were retrieved by data mining as elucidated in Section 2. Protein-coding gene associations with lncRNA genes were assigned based on their proximities using a Python algorithm (see supplemental information). Designated lncRNAs (outer ring of circles) within proximity of connected coding genes (inner network of smaller circles) are outlined in blue and connected with solid lines. lncRNA: long noncoding RNAs [Color figure can be viewed at wileyonlinelibrary.com]

2.6 | Statistical analysis

Data normality was checked by Shapiro-Wilk test and were assessed by two-tailed Student's *t*-test and one-way analysis of variance to evaluate the differences followed by pairwise comparisons using Tukey's correction. LncRNA-mRNA coexpression analysis was performed using Pearson correlation coefficient. SPSS 20 software (SPSS, Chicago, IL) and GraphPad Prism (version 6; GraphPad software, California corporation, CA) were used for statistical analyses. All values are represented as mean ± SEM and $p < 0.05$ was considered statistically significant. Co-LncRNA web site was also used to determine an overview of relevant pathways of coexpressed protein-coding genes with selected lncRNAs (Zhao et al., 2015).

3 | RESULTS

3.1 | Identification of Th1-specific lncRNAs and genes

First, we selected 84 Th1 cell differentiation genes, and then categorized them based on their signaling pathway involvements

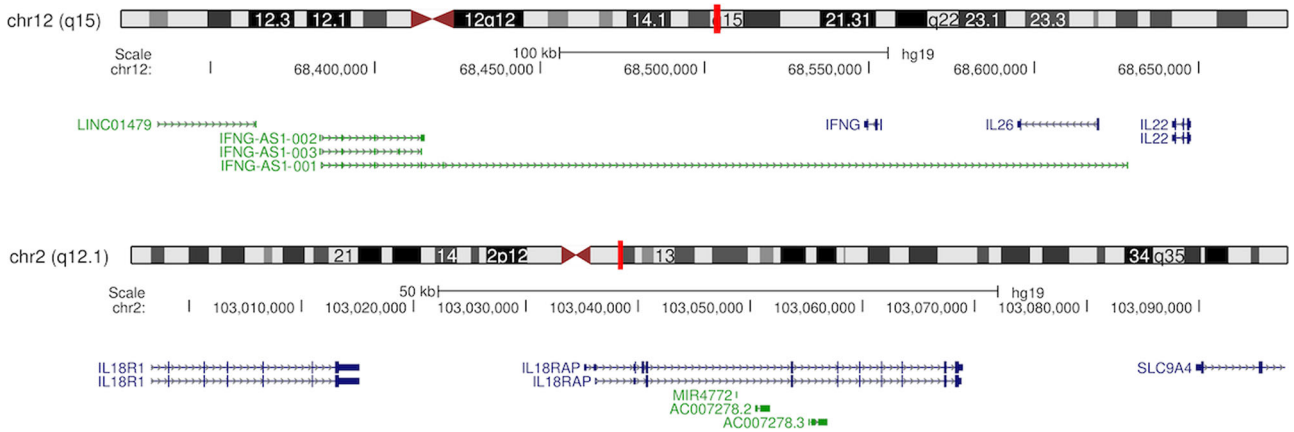

FIGURE 2 Chromosomal organization of genes and lncRNA clusters. Idiograms of chromosome 12 (top) and chromosome 2 (bottom), with the relevant regions marked in red and expanded diagrammatically below each idiogram. Protein-coding genes are drawn in blue and lncRNAs are green; exons are represented as solid boxed lines, and introns are barbed lines that indicate the direction of transcription. *IFNG* is located inside an intron of IFNG-AS1-001, while its distance from IFNG-AS1-002 and IFNG-AS1-003 is more than 100 kbp. On the other hand, AC007278.2 and AC007278.3 are located within the introns of *IL18RAP* and *IL18R1*. Note different sized scale bars for top and bottom diagrams. Chromosomal locations of the genes and lncRNA were obtained from UCSC genome browser at https://genome.ucsc.edu/. IL18R1: interleukin 18 receptor 1; lncRNA: long noncoding RNAs [Color figure can be viewed at wileyonlinelibrary.com]

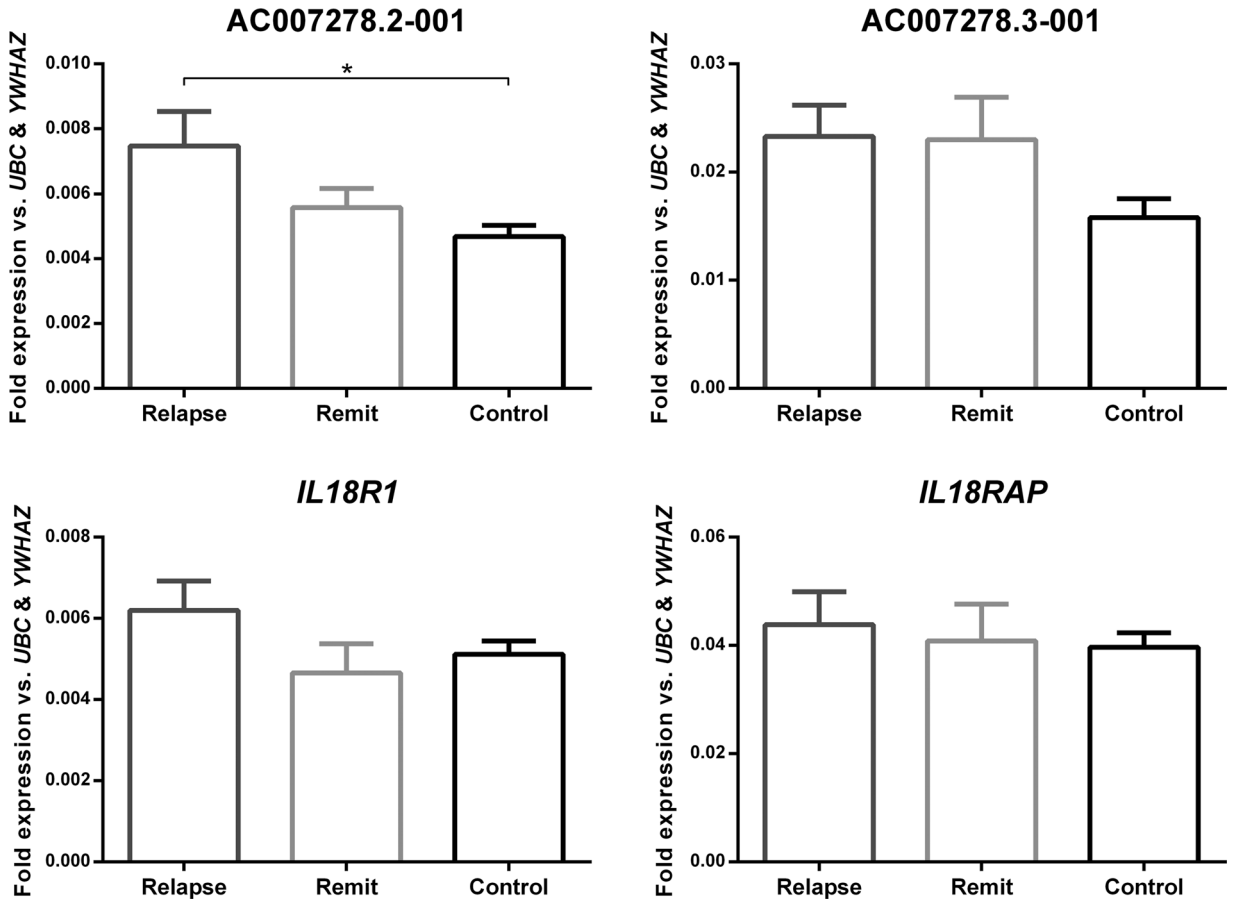

FIGURE 3 Expression level analyses of AC007278.2, AC007278.3, and associated genes. Bar-plots of the expression level of lncRNA genes in MS and control samples were acquired by RT-qPCR and values given as mean normalized expression relative to *UBC* and *YWHAZ*. Stars indicate significant differences (* = $p < 0.05$). See Section 2 for statistical methods used. lncRNA: long noncoding RNAs; MS: multiple sclerosis; RT-qPCR: quantitative real-time polymerase chain reaction

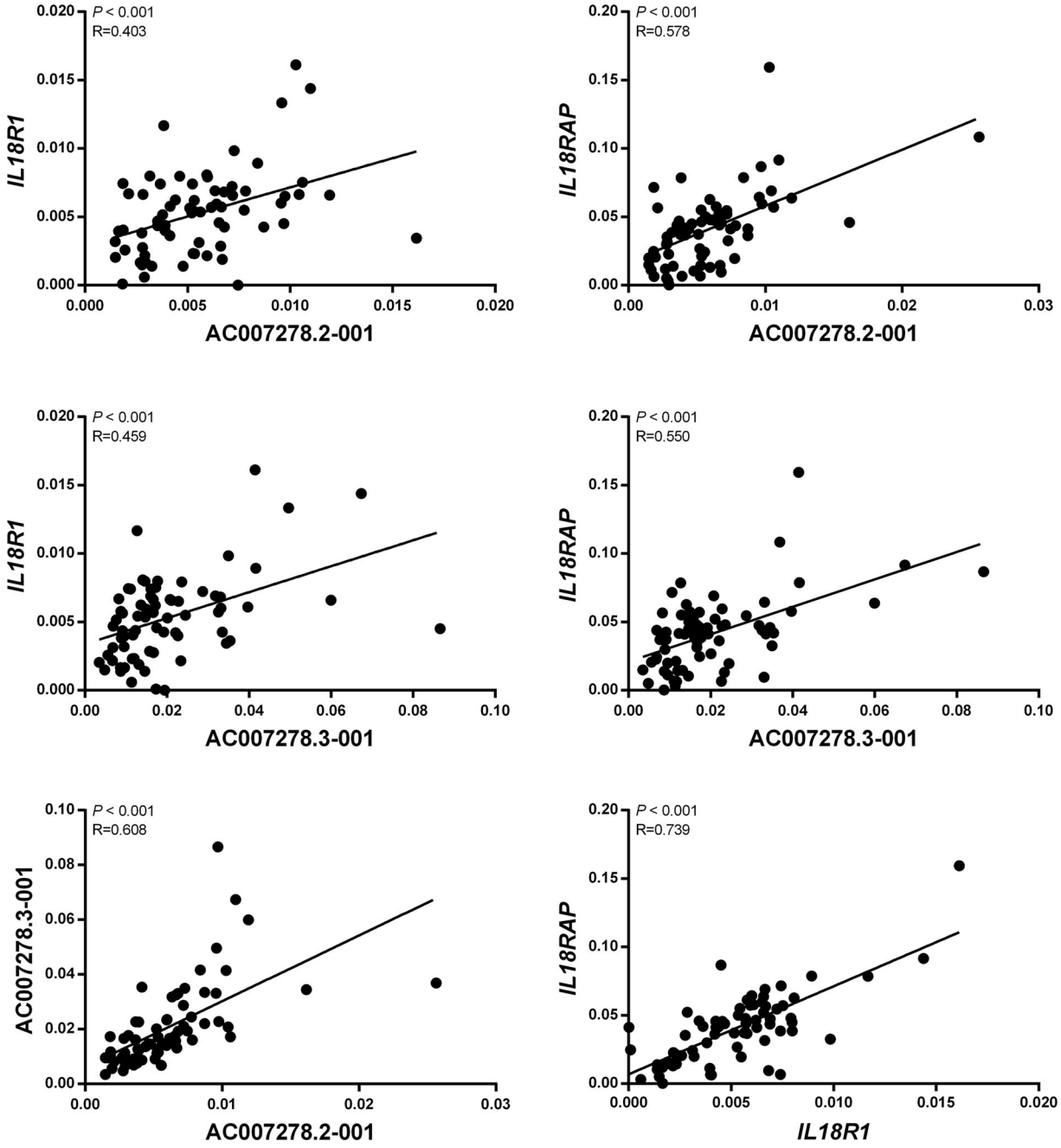

FIGURE 4 Correlation analyses between the expression of lncRNAs and the genes. Correlation between AC007278.2, AC007278.3, and respective protein-coding genes based on Pearson's correlation analysis. *p* value < 0.001 obtained for all tests. Positive correlations were found for all gene comparisons, and *R* values for each correlation is shown. lncRNA: long noncoding RNAs

through a vast data mining experiment (see Section 2; Figure 1). Previously, 34 Th1 lineage-specific lncRNAs were identified by Spurlock et al. (2015), and this study is consistent with those findings and expands the list of Th1-specific genes.

3.2 | LncRNA-protein-coding gene proximity

Chromosomal proximity between specified lncRNAs and protein-coding genes was identified using a program we developed using python programming language. Three Th1 lineage-specific lncRNAs (AC007278.2, AC007278.3, and IFNG-AS1) were identified from 34 candidate lncRNAs that were located proximal to protein-coding genes involved in Th1 cell differentiation (*IL18RAP*, *IL18R1*, and *IFNG*; Figure 1,2).

3.3 | Patient characteristics

The demographics, clinical features, and EDSS scores and main medical therapy of 50 MS patients and 25 healthy controls are

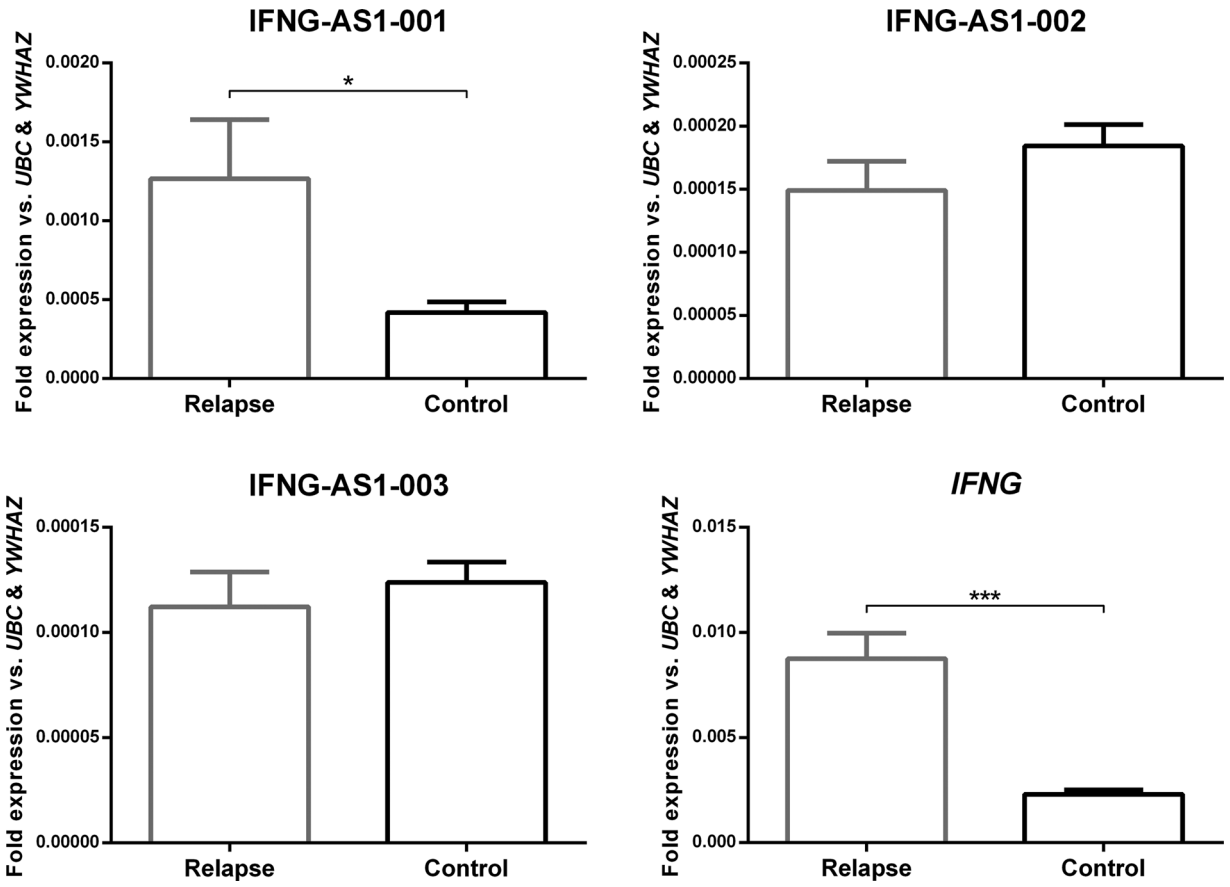

FIGURE 5 Expression of IFNG-AS1 transcripts and respective genes. Bar-plot of the differential expression levels of lncRNA genes in relapsing and control samples. Values are given as mean normalized expression relative to *UBC* and *YWHAZ*. Stars indicate significant differences (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). lncRNA: long noncoding RNAs

summarized in Table 1. Statistical analyses showed no significant differences between each sample group.

3.4 | Expression levels of AC007278.2 and AC007278.3 in relapsing-remitting patients and their correlation with *IL18R1* and *IL18RAP*

To examine lncRNAs levels in PBMC, we performed quantitative real-time PCR detection of AC007278.2 and AC007278.3 in 50 MS patients including 25 in relapsing phase, 25 in remitting phase, and 25 matched healthy controls. Levels of AC007278.2 lncRNA were significantly higher in the PBMC of the relapsing phase group compared with the healthy controls ($p = 0.025$; Figure 3), while there was no significant difference in the expression levels of AC007278.3. Given the potential cis-regulatory roles of these lncRNAs on the protein-coding genes *IL18R1* and *IL18RAP*, *IL18R1* and *IL18RAP* levels were also measured. However, the levels of *IL18R1* and *IL18RAP* were not significantly different between the MS and control groups. Remarkably, significant correlations were observed among expression of both protein-coding genes and their associated lncRNA (Figure 4).

3.5 | Significant changes in the expression level of IFNG-AS1-001 transcript in MS patients and correlation with *IFNG*

IFN-γ, which is produced by Th1 cells and triggers demyelination of CNS lesions in MS patients, is a therapeutic target to inhibit the progress of MS. To eliminate bias in our study, remitting patients who received IFN-β were excluded from statistical analysis as IFN-β can modulate the expression of *IFNG*. Hence, only relapsing phase patients were compared with the controls.

In addition, the levels of expression of three alternative IFNG-AS1 transcripts was measured by RT-qPCR in the MS and control groups. IFNG-AS1-001 levels were higher in the PBMC of the MS relapsing phase compare to the healthy controls ($p = 0.022$). In contrast, IFNG-AS1–003 and IFNG-AS1–002 showed no significant difference between relapsing and control groups (Figure 5). On the other hand, IFNG-AS1.003 appeared to be upregulated in the remitting phase group relative to the relapsing phase group (Figure S1).

Considering the potential cis-regulatory function of IFNG-AS1 on neighboring *IFNG*, we evaluated the expression level of *IFNG*, and found it was upregulated in relapsing phase patients compared with the controls ($p < 0.001$). The qPCR results indicated a

positive correlation between *IFNG* and IFNG-AS1-001 expression (Figure 6).

4 | DISCUSSION

Recent findings have demonstrated important roles for lncRNAs in the regulation of immune functions, elevating the diagnostic and therapeutic potential for this large class of genes (Atianand, Caffrey, & Fitzgerald, 2017; Carpenter et al., 2013; Hu et al., 2013; Ranzani et al., 2015; Sigdel, Cheng, Wang, Duan, & Zhang, 2015; Valadkhan & Plasek, 2018; Wu et al., 2015; Yang, Wu, Zhang, & Ni, 2018). We have identified lncRNAs with potential involvement in MS pathogenesis by implementing a new approach: identification of lncRNA genes with unique expression in the Th1 cell lineage and which are located proximal to protein-coding genes involved in Th1 cell function. Hence, these lncRNAs have high potential for association with MS pathogenesis through their physical association with protein-coding genes in Th1 cells, a key cell lineage with involvement at the primary stages of MS. Furthermore, we found that expression of two lncRNAs correlated significantly with neighboring mRNAs in MS patients compared with controls. One lncRNA correlated with the expression of *IL18R1* and *IL18RAP*, and another correlated with *IFNG* in association with MS disease.

Previous studies linked expression of *IL18R1* and *IL18RAP* as early biomarkers of MS and as promoters of Th1 cell development (Gutcher, Urich, Wolter, Prinz, & Becher, 2006; Yu, Chang, Ahyi, & Kaplan, 2008). We found that AC007278.2 and AC007278.3 lncRNAs were significantly higher in relapsing phase compared with the healthy controls and correlated positively with both *IL18R1* and *IL18RAP*. These correlations could demonstrate the possible regional control of lncRNAs in conjunction with their neighboring protein-coding genes.

Previous studies demonstrated associations between IFNG-AS1 and various disorders including its upregulation in ulcerative colitis (Padua et al., 2016), primary immune thrombocytopenia (Li et al., 2016), and Sjögren's syndrome (Wang et al., 2016). IFN-γ plays a pathological role by triggering demyelination of lesions in the CNS of

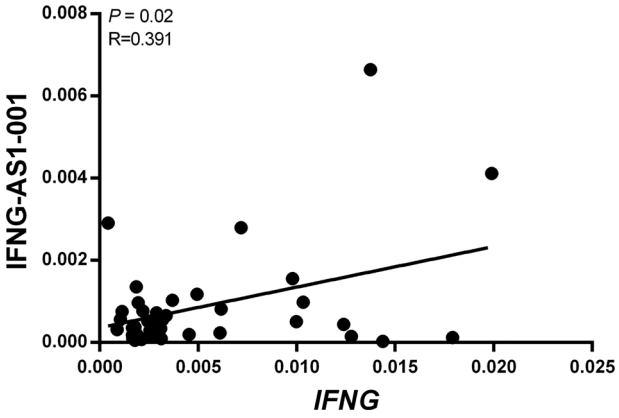

FIGURE 6 Correlation analysis between IFNG-AS1-001 and *IFNG*. Positive correlation between IFNG-AS1-001 and *IFNG* expression based on Pearson's correlation analysis, $p = 0.02$

MS patients (Olsson, 1992), and thus a potential therapy for MS is suppression of IFN-γ. Moreover, a study on IFNG-AS1 demonstrated its regulatory role on the expression of *IFNG* (Peng et al., 2015), making this lncRNA a potential target for MS therapy.

In this study, we evaluated three alternative transcripts of IFNG-AS1. As depicted in Figure 2, the genomic locations of IFNG-AS1–003 and IFNG-AS1–002 were not within near proximity (50 kb) of *IFNG*. Accordingly, expression of these two isoforms of IFNG-AS1 did not correlate with *IFNG*. In contrast, our experimental results indicated that only expression of IFNG-AS1-001, which is within 50 kb of *IFNG*, was correlated with *IFNG*. These results suggest that only the IFNG-AS1-001 isoform may be involved in Th1 cell through regulation of *IFNG*.

Possible roles for AC007278.2, AC007278.3, and IFNG-AS1 in signaling pathways was determined using co-lncRNA (bio-bigdata.hrbmu.edu.cn/Co-LncRNA), and their related protein-coding genes in signaling pathways were determined using Enrichr (amp.pharm.mssm.edu/Enrichr/) databases. These analyses indicated their involvement in multiple signaling pathways (Figures 7 and S2). Several autoimmune and other immune-related disorders are among these pathways.

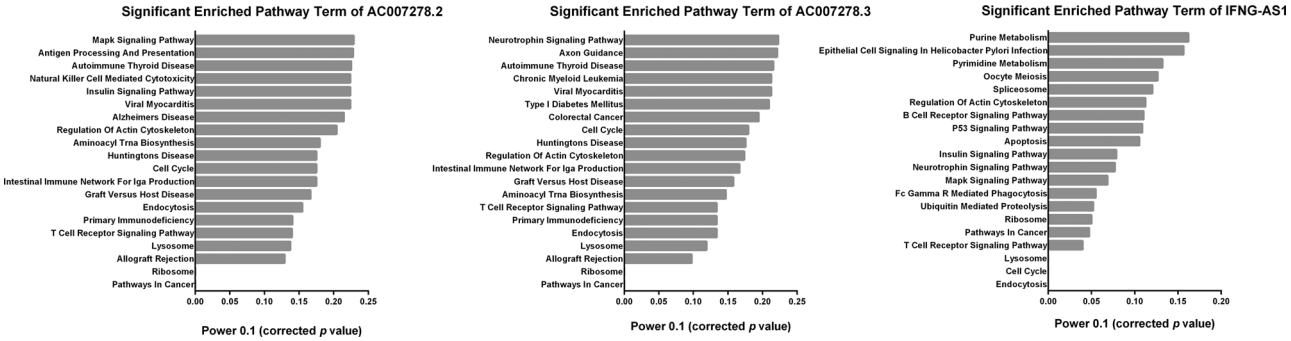

FIGURE 7 Enrichment pathway analysis. Top 20 pathways for AC007278.2, AC007278.3, and IFNG-AS1 lncRNAs coexpressed genes based on KEGG pathways analysis of co-lncRNA. The most significant value (with less *p* value) is at the bottom of the graph. KEGG: Kyoto Encyclopedia of Genes and Genomes; lncRNA: long noncoding RNAs

5 | CONCLUSIONS

Here we identified lncRNAs linked to MS in Th1 cells. The lncRNAs we identified are physically associated with protein-coding genes that themselves are associated with Th1 cell function and MS progression. The lncRNAs identified in this study are potential biomarkers for detection of MS and, because their expression may regulate the associated protein-coding genes, are potential targets for MS therapy. Moreover, the approach used here could be used to identify other lncRNA-protein-coding gene associations in other autoimmune diseases associated with specific cell types.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

A.H.: Designing research studies, conducting experiments, acquiring data, analyzing data, preparing reagents, and writing the manuscript. S.T.: Designing research studies, conducting experiments, acquiring data, analyzing data, preparing reagents, and writing the manuscript. M.E.: Acquiring data, analyzing data, and writing the manuscript. S.M.M.R.: Acquiring data, analyzing data, and writing the manuscript. K.G.: Designing research studies, data interpretation, manuscript writing, and final approval of the manuscript. T.L.M.: Designing research studies, data interpretation, manuscript writing, and final approval of the manuscript.M.E.: Acquiring data, data interpretation, manuscript writing, and final approval of the manuscript. M.H.N.E.: Designing research studies, data interpretation, manuscript writing, and final approval of the manuscript.

DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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