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CLINICAL STUDY

Expression analysis of vitamin D receptor-associated long noncoding RNAs in patients with relapsing-remitting multiple sclerosis

Mahdis RAHIMI NAIINI[1], Kolsoum SAEIDI[1], Arezoo AZARIAN[2], Kiana BAHRAMZADEH[2], Mahdieh NAZARI-ROBATI[3]

Department of Clinical Biochemistry, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran.
mnazari@kmu.ac.ir

ABSTRACT
BACKGROUND: Vitamin D is a neuroactive steroid that carries out its biological functions through the vitamin D receptor (VDR). The *VDR* gene interacts with certain long noncoding RNAs (lncRNAs). The present study is aimed at evaluating the expression levels of the *VDR* gene as well as those of HOTAIR, H19, MALAT1, and P21 lncRNAs in patients with relapsing-remitting multiple sclerosis (RRMS).
METHODS: This research was conducted on 38 RRMS patients and 38 healthy individuals. The expression levels of VDR and selected lncRNAs in peripheral blood as well as those of vitamin D in the plasma were measured.
RESULTS: The results revealed a significant increase in the expression of lncRNA H19 in the RRMS group compared to the control group. The analysis of the receiver operating characteristic (ROC) curve for H19 gene expression demonstrated a diagnostic value of 0.699 (95% CI: 0.575–0.823). Positive correlations were detected between VDR and lncRNA HOTAIR (r = 0.446, p = 0.008), H19 (r = 0.351, p = 0.042), MALAT1 (r = 0.464, p = 0.006), and P21 (r = 0.512, p = 0.002) in MS patients.
CONCLUSION: The findings of this study suggest that lncRNA H19 could serve as a potential biomarker for MS diagnosis *(Tab. 4, Fig. 1, Ref. 34)*. *Text in PDF www.elis.sk*
KEY WORDS: multiple sclerosis, VDR, lncRNA, HOTAIR, H19, MALAT1, P21.

List of abbreviations:
AUC – area under the curve, β_2M – beta-2-microglobulin, CNS – central nervous system, EDTA – ethylenediaminetetraacetic acid, LncRNAs – long noncoding RNAs, MMP9 – matrix metalloproteinase 9, MS – multiple sclerosis, NO – number, PBMCs – peripheral blood mononuclear cells, PCR – polymerase chain reaction, ROC – receiver operating characteristic, RRMS – relapsing-remitting multiple sclerosis, RXR – retinoid X receptor, SEM – standard error of the mean, SNPs – single-nucleotide polymorphisms, SPMS – secondary progressive MS, VDR – vitamin D receptor

[1]Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran, [2]Department of Medical Genetics, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran, and [3]Department of Clinical Biochemistry, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran

Address for correspondence: Mahdieh NAZARI-ROBATI, Department of Clinical Biochemistry, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran, 7616914115 Kerman, Iran.
Phone: +98 34 33257660, Fax: +98 34 33257448

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Introduction

Multiple sclerosis (MS) is a chronic progressive disease of the central nervous system (CNS) and is characterized by demyelination and inflammatory immune responses. This condition affects a large number of adults around the world, and similar to other autoimmune disorders, its cause remains poorly understood. However, it has been established that both genetic and environmental factors play a role in the development of this disease (1, 2).

A growing body of evidence supports the role of vitamin D in susceptibility to MS, e.g., it has been shown that areas with less UV exposure have a higher prevalence of MS. Moreover, extensive prospective studies examining vitamin D status before the onset of MS have indicated that individuals with low serum vitamin D levels are at a greater risk of developing this disease. Furthermore, clinical studies have reported that vitamin D supplementation in MS patients reduces the risk of disease recurrence. Additionally, low vitamin D levels at the onset of MS are associated with worse cognitive function and neuronal integrity. However, the precise mechanism of action of vitamin D in modulating the onset and progression of MS has not been fully elucidated (3).

Vitamin D plays a crucial role in regulating immune system function since it is a potent immunomodulator that controls both innate and acquired immune responses. Studies have shown that

vitamin D acts through the vitamin D receptor (VDR), which is an intracellular receptor expressed in almost all types of cells in the body, including immune cells. VDR is a ligand-activated transcription factor that mediates the genomic effects of 1, 25-dihydroxyvitamin D. The *VDR* gene is located on chromosome 12 (12q13.11) and comprises nine exons with different allelic variants, some of which impact its function. According to the literature, certain polymorphisms in the *VDR* gene have been linked to an elevated risk of several autoimmune diseases, including MS (2, 4). More recently, it has been found that the expression of the *VDR* gene is influenced by a number of long noncoding RNAs (lncRNAs) (5).

LncRNAs are transcripts with more than 200 nucleotides in length that lack the potential to encode proteins. These molecules are primarily transcribed by RNA polymerase II from intergenic and intronic regions of the genome (6). Studies have indicated that lncRNAs, which represent a novel class of gene modulators, play a role in the regulation of a wide range of physiological immune processes, including the activation of innate immune responses, as well as the development, differentiation, and activation of T cells (7). Although the research in the field of lncRNAs and their role in nervous system diseases is still in its early stages, changes in lncRNAs expression have been reported in CNS diseases, particularly in those associated with inflammatory responses (8–11). For example, a study conducted in 2019 on an experimental autoimmune encephalomyelitis (EAE) animal model showed a decrease in MALAT1 expression in the nervous system of EAE-affected mice. The study reported increased neuroinflammation through an increase in macrophage differentiation toward the Th1/Th17 phenotype (12). On the other hand, studies conducted on serum samples of patients with secondary progressive MS (SPMS) and leukocytes of patients with relapsing-remitting MS (RRMS) reported an increase in MALAT1 expression in these patients (13, 14). Furthermore, the investigation of several single-nucleotide polymorphisms (SNPs) within the *HOTAIR* gene in MS patients revealed a significant association between the risk of the disease and one of SNPs (15). In addition, a study on peripheral blood mononuclear cells (PBMCs) of patients with RRMS showed an elevated expression of HOTAIR and its positive correlation with matrix metalloproteinase 9 (MMP9) (16).

Based on the results of previous studies, the present research investigated the expression levels of the *VDR* gene and several lncRNAs associated with VDR, including H19, HOTAIR, P21, and MALAT1, as well as examined the relationship between VDR and these lncRNAs in RRMS patients. In addition, the plasma vitamin D level was measured to assess its potential relationship with VDR expression levels.

Materials and methods

Study participants

This case-control study was approved by local ethics committees (IR.KMU.REC.1402.030) and conducted according to the Helsinki Declaration. All participants signed an informed consent form. The research was performed on 38 patients with RRMS and 38 healthy individuals who were matched in terms of age and gender. RRMS diagnosis was made by a neurologist based on the revised McDonald's criteria (17). All patients were in state of remission and had not undergone any type of immunomodulatory treatment for a minimum of three months. Healthy controls did not meet the diagnostic criteria for MS and reported no history of autoimmune or neurological diseases.

Sample collection and RNA extraction

Briefly, 5 mL of blood was collected in an EDTA-containing tube from each participant in this study. Then PBMCs were isolated using Ficoll density gradient centrifugation. After washing the PBMCs twice with phosphate-buffered saline solution, total RNA was extracted using Trizol (GeneAll, South Korea) (18). Subsequently, cDNA was synthesized from the extracted total RNAs using the cDNA synthesis kit (Parstous, Iran).

Quantitative real-time polymerase chain reaction (PCR)

The expression levels of the *VDR* gene and lncRNAs H19, MALAT1, P21, and HOTAIR were examined by the real-time PCR technique using specific primers and SYBR Green (SmoBio, Taiwan). The beta-2-microglobulin (*β2M*) gene was utilized as an internal control. The primer sequences used in this study are presented in Table 1. All reactions were carried out in duplicate using the Mic PCR system (BMS, Australia). The temperature profile of PCR reactions was as follows: 95 °C for 15 min, followed by 40 cycles including 95 °C for 40 s, 62 °C for 25 s, and 72 °C for 15 s (18, 19).

Measurement of vitamin D

Plasma samples were collected after subjecting the whole blood to centrifugation at 2,500 ×g for 10 min (4). Then, 25-hydroxyvitamin D concentration was measured using a commercial ELISA kit (Monobind Inc., USA) according to the company's protocol.

Statistical analysis

Data were analyzed using SPSS version 26. The Kolmogorov-Smirnov statistical test was used to assess the normal distribution of data. Student's t-test was employed to compare variables between the two groups. Correlations between the expression levels of genes were determined using Pearson's correlation analysis. An analysis of the receiver operating characteristic (ROC) curve was conducted to evaluate the diagnostic value of differentially expressed genes.

Tab. 1. Primer sequences used for real-time PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (kb)
VDR	TTGCCATACTGCTGGACGC	GGCTCCCTCCACCATCATT	102
MALAT-1	GCTCTGTGGTGTGGGATTGA	GTGGCAAAATGGCGGACTTT	179
HOTAIR	GCACCGCTTTTCTAACTGGC	CAGGGTCCCACTGCATAATCA	141
P-21	AGGACCAGAATAACCCGAGC	CTGGGGTCCAGGATGCATAG	109
H19	CACGGCTTTCTCAGGCCTAT	TACAGCGTCACCAAGTCCAC	238
β2M	CTCCGTGGCCTTAGCTGTG	TTTGGAGTACGCTGGATAGCCT	69

Tab. 2. Demographic characteristics and vitamin D levels of healthy controls and MS patients.

Variable	Control	MS
NO.	38	38
Female	28 (73.7%)	28 (73.7%)
Male	10 (26.3%)	10 (26.3%)
Age (year)	38.24±1.38	38.55±1.34
Age of onset (year)	–	29.60±1.06
Vitamin D (ng/ml)	44.19±1.21	47.86±1.40

Values are expressed as mean±SEM or as n (%), : MS – multiple sclerosis

The diagnostic power was obtained by assessing the area under the curve (AUC). The optimal cut-off point was estimated using Youden's index. The relative expression method using the $2^{-\Delta\Delta Ct}$ formula was employed to evaluate changes in gene expression. For this purpose, the beta-2-microglobulin (*β2M*) reference gene was used to normalize gene expression. p-values less than 0.05 were considered statistically significant. GraphPad Prism software was used to generate graphs.

Result

General characteristics of study participants

Demographic characteristics of MS patients and healthy controls are summarized in Table 2. A total of 38 MS patients and 38 healthy subjects were studied. Each group contained 28 females and 10 males. In light of evidence demonstrating the association of vitamin D deficiency with MS susceptibility and disease progression, we measured vitamin D levels of MS patients and healthy controls. The results revealed no remarkable difference in the levels of vitamin D between healthy subjects and MS patients ($p > 0.05$).

VDR and selected lncRNAs expression levels

Figure 1 demonstrates the relative gene expression levels of *VDR* and lncRNAs HOTAIR, H19, MALAT1 and P21 between MS and control groups. Our data showed that H19 was significantly upregulated in MS patients compared with healthy cases ($p = 0.001$). Meanwhile, no statistically significant difference was found regarding other lncRNAs between these two groups ($p > 0.05$). ROC curve of lncRNA H19 expression level was plotted between sensitivity % on y-axis and (100 – specificity %) on x-axis. Every point on the ROC curve reflects a chosen cut-off value (Fig. 1F). ROC curve analysis of lncRNA H19 expression level indicated an AUC of 0.699 (95% CI = 0.575–0.823, $p = 0.0049$). The sensitivity and specificity of lncRNA H19 were calculated using a cut-off value of 2.465 for lncRNA H19 expression level (Tab. 3).

Correlations between VDR and selected lncRNAs expression levels

According to our results, there were no significant correlations between the relative gene expression levels of *VDR* and lncRNAs HOTAIR, H19, MALAT1 and P21 in control group ($p > 0.05$). However, we found a significant moderately positive correlation between the expression levels of *VDR* and lncRNAs HOTAIR,

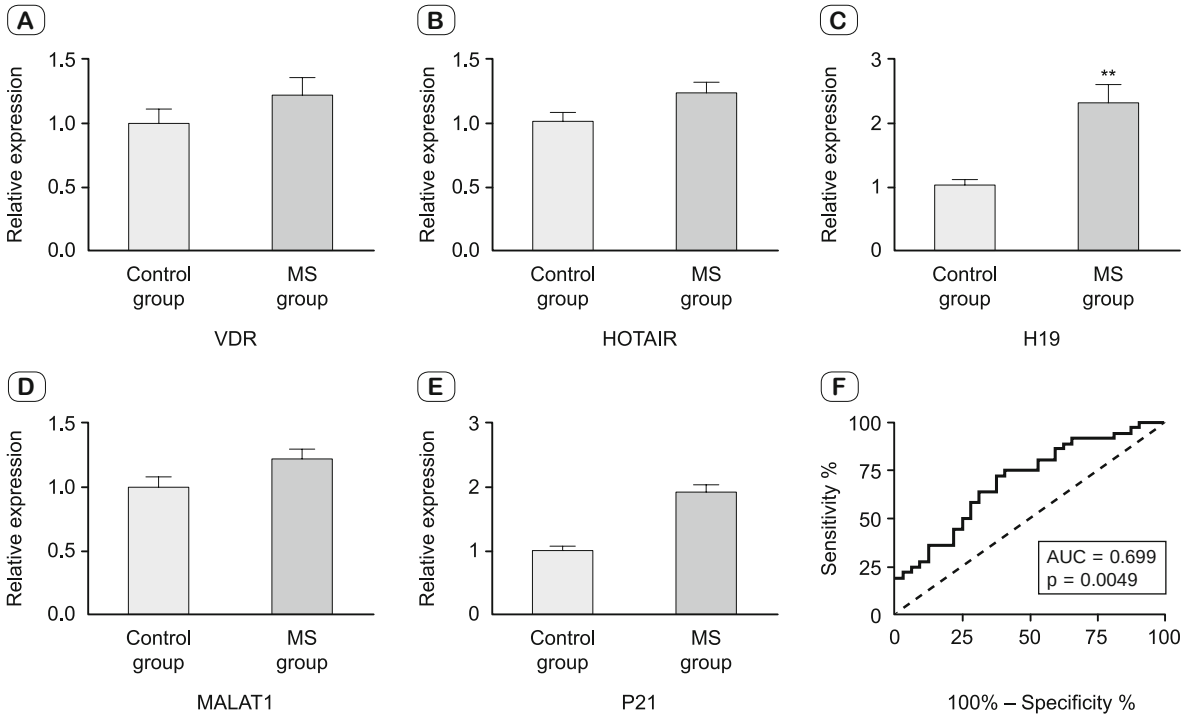

Fig. 1. The relative gene expression levels of *VDR* (A), HOTAIR (B), H19 (C), MALAT1 (D) and p21(E) in MS patients in comparison with healthy controls as well as ROC curve for relative gene expression level of lncRNA H19 for MS diagnosis (F). All data presented as the normalized gene expression to an endogenous reference gene (beta-2-microglobulin). ** $p < 0.01$ compared with healthy control. AUC: area under the curve.

Tab. 3. ROC curve analysis for the relative expression level of lncRNA H19 for MS diagnosis.

	Area Under the Curve (AUC)	p	95% Confidence Interval (CI)		Cut-off point	Sensitivity (%)	Specificity (%)
			Lower Bound	Upper Bound			
H19	0.699	0.0049	0.575	0.823	2.465	72.22	62.50

Tab. 4. Correlations between the relative gene expression levels of *VDR* and four selected lncRNAs.

	Control (n=38)		MS (n=38)	
	r value	p value	r value	p value
HOTAIR	0.185	0.296	0.446	0.008 *
H19	0.132	0.485	0.351	0.042 *
MALAT1	0.213	0.227	0.464	0.006 *
P21	0.142	0.424	0.512	0.002 *

The asterisk (*) indicates statistically significant ($p < 0.05$). MS: Multiple sclerosis

H19, MALAT1 and P21 in MS patients ($p = 0.008$, $p = 0.042$, $p = 0.006$ and $p = 0.002$, respectively) (Tab. 4).

Discussion

Multiple sclerosis is a progressive chronic inflammatory CNS disease with an unknown cause, which leads to demyelination and axonal damage through the pathological activation of the immune system as well as leukocyte infiltration into the CNS. It has been found that a variety of genetic and environmental factors contribute to the onset and progression of this disease. However, the etiology and pathogenesis of MS are still not fully understood (20, 21). Vitamin D is a neuroactive steroid that plays a role in neuronal development. The biological activity of vitamin D is mediated by the receptor VDR. Research suggests that low serum vitamin D levels and VDR polymorphisms contribute to the pathogenesis of several neurological disorders, including MS. Although low levels of VDR expression and vitamin D have been reported in patients with MS, the underlying mechanism of these observations is not elucidated (4, 22, 23). In contrast to these findings, our investigation revealed no significant difference in plasma levels of vitamin D and mRNA levels of VDR between the control and patient groups. Given that vitamin D sufficiency is defined as 25-hydroxyvitamin D levels ≥ 30 ng/mL, both of the studied groups had sufficient vitamin D levels. Although all participants stated that they did not take vitamin D supplements for at least three weeks prior to sampling, other factors essential for maintaining adequate vitamin D levels including diet and sunlight exposure, were not assessed in this study. In agreement with our results, the study by Sultan et al indicated that the difference in serum levels of vitamin D between MS patients and healthy individuals was not statistically significant (24). Similarly, Pistono et al reported no significant difference in VDR expression between MS and control groups (4). Additionally, some studies have shown the presence of various polymorphisms in the *VDR* gene promoter, as well as modifications such as DNA methylation and splicing site abnormalities in the *VDR* gene, which can cause different responses to vitamin D (2). Moreover, even though the concentration of the active form of vitamin D is critical in the expression of VDR, its high concentrations can affect the outcome by increasing the binding of VDR to retinoid X receptor (RXR) and activating the genes involved in the breakdown of 1,25-dihydroxyvitamin D (25). Furthermore, the regulation of VDR is complicated and its expression at the transcriptional level is regulated by other molecules, such as calcium, hormones (e.g., parathyroid hormone), and retinoic acid, which were not evaluated in this study (26).

In the current investigation, the expression pattern of four VDR-related lncRNAs was examined in the peripheral blood of MS patients and healthy subjects. LncRNAs are one class of noncoding RNAs that have been suggested as crucial regulators of CNS function and can be used in the diagnosis and treatment of CNS disorders. The significance of lncRNAs in various autoimmune, neurodegenerative, and oncological conditions has been described. The directive role of lncRNAs in the immune response has been demonstrated by extensive alterations in their expression throughout the innate immune response in addition to the differentiation of immune cells (27). Different lncRNAs have been shown to be dysregulated in MS (14, 28, 29). Notably, we observed higher levels of lncRNA H19 expression in MS patients compared to healthy individuals. LncRNA H19 is an imprinted gene that is only expressed from the maternal allele, and while the role of imprinted genes in development and growth is well established, the molecular mechanism of their impact on the function of the immune system and inflammatory diseases, such as MS, is largely unknown (27). However, some studies suggest that lncRNA H19 is implicated in CNS diseases through the JAK/STAT pathway and the activation and proliferation of astrocytes and microglia (30). Consistent with our findings, Amiri et al found that the expression of lncRNA H19 is significantly higher in SPMS and RRMS subgroups compared to healthy individuals (31). However, in the study by Meber et al, it was reported that the serum levels of lncRNA H19 in MS patients were lower than in healthy subjects (27). After observing the significant difference in lncRNA H19 expression between the studied patients and the control group, we used a ROC curve to determine the diagnostic power of lncRNA H19. This gene could differentiate MS patients from control subjects with an AUC value of 0.699. Our results indicated that lncRNA H19 could serve as a putative biomarker in the panel of biomarkers for MS diagnosis. In this regard, other studies have shown that lncRNA H19 plays a crucial role in various CNS disorders, such as Parkinson's and Alzheimer's diseases, and may be a potential diagnostic and prognostic indicator for CNS diseases (32).

Our findings revealed no significant difference in the expression of MALAT1, HOTAIR, and P21 lncRNAs between the studied groups. These results do not necessarily indicate the lack of involvement of HOTAIR, MALAT1, and P21 in the pathogenesis of MS and may be due to the small size of the analyzed sample. In contrast to our results, Pahlevan et al measured the serum level of

lncRNA HOTAIR and vitamin D in patients with RRMS and came to the conclusion that this lncRNA can contribute to the pathogenesis of MS through mechanisms affecting inflammation (7). In one of the first investigations on the expression of lncRNAs in MS, Fenglio et al evaluated the expression of 90 lncRNAs using qPCR arrays in the PBMCs of patients with RRMS. In their study, it was reported that the expression of MALAT1 shows a significant reduction in the patients compared to the healthy individuals (33). However, in subsequent studies on patients with SPMS and RRMS, an increase in MALAT1 levels was reported in MS patients compared to the control group (13, 14). Dastmalchi et al also examined the expression of lncRNA P21 in the peripheral blood of RRMS patients, and the results of their study revealed that the expression of lncRNA P21 was elevated in the patients (29). The discrepancy in the aforementioned findings raised a question challenging the link between the expression of VDR and its related lncRNAs. Our results showed no significant association between the expression levels of HOTAIR, H19, MALAT1, and P21 lncRNAs and VDR in the control group. On the other hand, a significant positive correlation was observed between the expression of VDR and HOTAIR, H19, MALAT1, and P21 lncRNAs in the studied MS patients. This significant correlation suggests that the aforementioned lncRNAs may be involved in the pathogenesis of MS, particularly in VDR-mediated immune processes. Interactions between vitamin D signaling and the studied lncRNAs have been reported in other diseases, so it makes sense that many of their functions should be correlational (34).

Conclusion

Taken together, our study demonstrated that lncRNA H19 levels were increased in MS patients. The ROC curve analysis suggested the potential diagnostic application of lncRNA H19 in the detection of MS patients. In addition, our findings detected the interactions between VDR and the selected lncRNAs, which may contribute to the pathogenesis of MS. However, additional research is required to clarify the molecular mechanisms underlying these interactions.

Learning points

- Multiple sclerosis (MS) is a chronic inflammatory disease.
- Long noncoding RNAs (lncRNAs) appear to have an important role in the pathophysiology of MS.
- LncRNAs HOTAIR, H19, MALAT1, and P21 may be involved in the pathogenesis of MS through targeting vitamin D receptor-mediated immune processes.
- LncRNA H19 could serve as a putative biomarker in the panel of biomarkers for MS diagnosis.

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