

Original data



Abnormal level of VDR-associated lncRNAs in patients with multiple sclerosis

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ARTICLE INFO

Keywords:

Multiple sclerosis
 lncRNA
 SNHG6
 SNHG16
 LINC00346

ABSTRACT

Long non-coding RNAs (lncRNAs) influence pathoetiology of multiple sclerosis (MS). We identified expression levels of three vitamin D receptor-associated lncRNAs, namely *SNHG6*, *SNHG16* and *LINC00346* in the blood of MS patients compared with healthy subjects. *SNHG6* level was significantly lower in MS cases compared with controls (expression ratio (ER) (95 % CI)= 0.39 (0.22–0.69), $P = 0.0015$) and in female patients compared with female controls (ER (95 % CI)= 0.28 (0.13–0.59), $P = 0.0001$). *SNHG16* was also under-expressed in total MS patients compared with controls (ER (95 % CI)= 0.24 (0.1–0.57), $P = 0.0001$). *LINC00346* level was lower in entire patients versus controls (ER (95 % CI)= 0.03 (0.009–0.09), $P < 0.0001$). Such down-regulation was also detected in patients of both sexes compared with corresponding controls ($P = 0.0008$ and <0.0001 for males and females, respectively). There was no difference in expressions of *SNHG6*, *SNHG16* and *LINC00346* between male and female patients. There was no remarkable correlation between expressions of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs and age, disease duration, age at onset or EDSS. *LINC00346* had the AUC values of 0.84, 0.82 and 0.94 in differentiation of total MS patients from total controls, female patients from healthy females and male patients from healthy males, respectively. *SNHG6* could separate female patients from female controls with AUC of 0.79. Finally, female patients from female controls could be separated by *SNHG16* levels with AUC of 0.73. Taken together, these lncRNAs might be proposed as putative peripheral markers for MS.

Introduction

As a chronic inflammatory disorder, multiple sclerosis (MS) has been shown to be associated with dysregulation of vitamin D receptor (VDR) signaling. Numerous genetic studies have indicated that different common MS-related polymorphisms reside within or in the neighborhood of genes participating in the vitamin D metabolism (Lu et al., 2018). Notably, novel high throughput studies as well as functional assays have confirmed that the associations between vitamin D and this disorder can

be explained by the widespread and distinctive genomic binding of the VDR (Lu et al., 2018). In fact, VDR cistrome has significant impact on the epigenomic modeling of the enhancer landscapes influencing the maturation and differentiation of immune cells (Lu et al., 2018). A recent retrospective case-control investigation has revealed association between the *FokI* polymorphism within *VDR* gene and risk of MS development (Cancela Díez et al., 2021). Moreover, a former meta-analysis has revealed associations between *TaqI* polymorphism of *VDR* and risk of MS (Imani et al., 2019).

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<https://doi.org/10.1016/j.bionps.2023.100082>

Received 13 July 2023; Received in revised form 6 November 2023; Accepted 30 November 2023

Available online 1 December 2023

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Recent developments in identification of disease-associated long non-coding RNAs (lncRNAs) have facilitated recognition of several regulatory transcripts which partake in the pathogenesis of MS (Hosseini et al., 2019, Ghafouri-Fard and Taheri, 2020). Meanwhile, a number of lncRNAs have been detected through in silico analyses that are functionally related with VDR signaling. Notably, expressions of these lncRNAs have been found to be dysregulated in diverse disorders related with abnormalities in VDR (Taheri et al., 2021, Ghafouri-Fard et al., 2021b).

We chose three VDR-related lncRNAs, namely *SNHG6*, *SNHG16* and *LINC00346* to measure their relative expressions in the peripheral blood of MS patients. *SNHG16* has been found to modulate expression of IL-10 through sequestering let-7c-5p in a competing endogenous RNA manner (Wang et al., 2020). *LINC00346* has been found to induce JAK1/STAT3 signaling (Li and Wen, 2020), thus it is possibly contributing to the pathogenesis of immune-related disorders. Finally, *SNHG6* has a regulatory role on expression of IL-6 and TNF- α (Shan et al., 2020). Therefore, we hypothesized that mentioned lncRNAs can influence pathoetiology of MS through VDR-related or VDR-independent pathways.

Patients and methods

Study participants

This research was conducted on blood specimens of 50 MS cases (12 males and 38 females) and 50 healthy unrelated controls with the same sex ratio. Controls had no history or sign of neurologic or autoimmune disorders. The revised McDonald criteria (Polman et al., 2011) was used for confirmation of MS diagnosis. Patients were treated with IFN- β (CinnoVex, Cinagene Company, Iran) and had sufficient levels of vitamin D. Informed consent forms were signed by all individuals. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences.

Expression studies

Four mL of whole peripheral blood was acquired via venipuncture from MS patients and healthy subjects. Hybrid-RTM blood RNA extraction kit (GeneAll Biotechnology Co Ltd., South Koera) was used for retrieval of RNA. After verification of appropriate quality and concentration of RNA, cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Expression of *SNHG6*, *SNHG16* and *LINC00346* were enumerated in the rotor gene 6000 Corbett Real-Time PCR machine by using SYBR® Premix Ex Taq™ (TaKaRa, Japan). *B2M* gene was the normalizer gene. The sequences of primers were similar to our previous study (Mazdeh et al., 2019).

Statistical methods

SPSS v.18.0 (Chicago, IL) was used for statistical assessment. Graphs were depicted using GraphPad Prism v9.0 (GraphPad Software, La Jolla California, USA). Expressions of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs in each sample were calculated using the Efficiency adjusted Ct method. The normal/gaussian distribution of values was assessed by the Shapiro-wilk test. Mann-Whitney U test was used to detect differentially expressed genes between the MS cases and healthy controls group. A two-way ANOVA was used to analyze the effects of main factors (disease and gender) and their interaction on expression level of lncRNAs. Tukey post hoc test was used for multiple comparisons between subgroups. Correlations between expression levels of lncRNAs in each group were measured with Spearman's rank correlation coefficient and Bonferroni correction for multiple comparisons since they were not normally distributed. The partial correlation between expression levels and age of study participants, age at onset, disease duration, Expanded Disability Status Scale (EDSS) and Age Related Multiple Sclerosis Severity Score

(ARMSS) was described by r and P values (Controlled for sex). The receiver operating characteristic (ROC) curves were illustrated to judge the diagnostic power of expression levels of *SNHG6*, *SNHG16* and *LINC00346*. Youden's J parameter was measured to find the optimum threshold. P value < 0.05 was considered as significant.

Results

Demographic and clinical data

General data of MS patients and controls is demonstrated in Table 1. Mann-Whitney U test was used to compare differences in age parameter between MS patients and healthy controls. There was no significant difference between two groups (p value=0.2).

Expression analyses

Expression of *SNHG6* was significantly lower in MS patients versus controls (expression ratio (ER) (95 % CI)= 0.39 (0.22–0.69), adjusted P value= 0.0015) and in female patients compared with female controls (ER (95 % CI)= 0.28 (0.13–0.59), adjusted P value= 0.0001). *SNHG16* level was also lower in total MS patients compared with entire controls (ER (95 % CI)= 0.24 (0.1–0.57), adjusted P value= 0.0001). Expression of *LINC00346* was lower in total patients compared with controls (ER (95 % CI)= 0.03 (0.009–0.09), adjusted P value< 0.0001). Such down-regulation was also detected in patients of both sexes compared with corresponding controls (adjusted P values =0.0008 and <0.0001 for males and females, respectively). There was no significant difference in expressions of *SNHG6*, *SNHG16* and *LINC00346* between male and female patients (Table 2).

Fig. 1 shows the expression of genes in total MS patients versus total controls. The results showed substantial difference in expression of *SNHG6*, *SNHG16* and *LINC00346* between two groups.

Fig. 2 illustrates expressions of *SNHG6*, *SNHG16* and *LINC00346* genes in MS patient subgroups versus control subgroups. As illustrated in this figure, there was no significant deference in gene expressions between males and females among either patients or controls.

There was no significant correlation between expressions of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs and age, disease duration, age at onset, EDSS and ARMSS (Table 3).

There were significant correlations between expressions of *SNHG6* and *SNHG16* as well as expressions of *SNHG16* and *LINC00346* in MS patients (P values<0.0001 and =0.0007, respectively). Such correlations were not detected among healthy controls (Table 4).

Figs. 3 and 4 show correlation between expressions of *SNHG6*, *SNHG16* and *LINC00346* genes in MS patients and normal controls, respectively.

LINC00346 had the AUC values of 0.84, 0.82 and 0.94 in differentiation of total MS patients from total controls, female patients from

Table 1
General data of study participants.

Subgroups	Parameter	Value	
Patients	Sex (number)	Male	12
		Female	38
	Age (Years, mean \pm SD)	Male	37.5 \pm 10.8
		Female	40.13 \pm 9.52
	Duration (Years, mean \pm SD)	Male	4.5 \pm 3.03
		Female	7.26 \pm 6.18
	age of onset (Years, mean \pm SD)	Male	32.83 \pm 11.23
EDSS	Female	32.86 \pm 9.32	
	Male	3.41 \pm 1.57	
Controls	Sex (number)	Female	2.85 \pm 1.26
		Male	12
	Age (Years, mean \pm SD (range))	Female	38
		Male	44.08 \pm 15.91
		Female	44.1 \pm 15.38

Table 2

The results of expression study of *SNHG6*, *SNHG16* and *LINC00346* in blood of MS patients compared with controls (Asterisks show significant P values).

lncRNAs	Parameters and values	Total patients vs. Controls (50 vs. 50)	Male patients vs. Male Controls (12 vs. 12)	Female patients vs. Female Controls (38 vs.38)	Female patients vs. Male patients (38 vs.12)
<i>SNHG6</i>	Expression ratio (95 % CI)	0.39 (0.22–0.69)	0.54 (0.14–1.98)	0.28 (0.13–0.59)	0.6 (0.2–1.73)
	Adjusted P value	0.0015*	0.61	0.0001*	0.5908
<i>SNHG16</i>	Expression ratio (95 % CI)	0.24 (0.1–0.57)	0.13 (0.018–0.96)	0.25 (0.14–1.32)	0.83 (0.17–4.16)
	Adjusted P value	0.0015*	0.0449*	0.2129	0.9917
<i>LINC00346</i>	Expression ratio (95 % CI)	0.03 (0.009–0.09)	0.02 (0.001–0.26)	0.04 (0.01–0.18)	0.76 (0.09–6)
	Adjusted P value	< 0.0001*	0.0008*	< 0.0001*	0.98

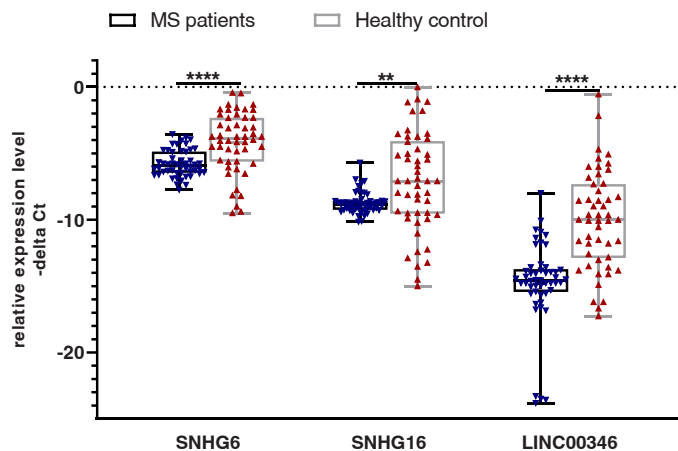


Fig. 1. Expression level of *SNHG6*, *SNHG16* and *LINC00346* genes in MS patients (total) and healthy controls (total) as described by $-\Delta Ct$ values. $-\Delta Ct$ Data were plotted as box and whisker plots. Mann-Whitney U test was used to find differentially expressed genes between two groups. (** P value < 0.01 and **** P value < 0.0001).

healthy females and male patients from healthy males, respectively. *SNHG6* could separate female patients from female controls with AUC of 0.79. Finally, *SNHG16* could separate female patients from female controls with AUC of 0.73 (Table 5).

Figs. 5–7 show ROC curves of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs transcript levels in total MS cases.

4. Discussion

lncRNAs contribute to the development of MS through various convergent or unrelated routes. Since recently, contribution of lncRNAs

in MS risk has been attributed to their roles in the regulation of immune response, particularly expansion and maturation of immune cells (Ghaderian et al., 2020). Recent studies have indicated participation of these transcripts in more diverse cellular functions such as regulation of signaling pathways (Ghafouri-Fard et al., 2021a, Ghafouri-Fard et al., 2021c). In the present case-control study, we compared expression levels of VDR-associated lncRNAs *SNHG6*, *SNHG16* and *LINC00346* between MS patients and healthy subjects. Our results revealed significant decreased levels of all lncRNAs in patients compared with controls. In addition to its participation in the regulation of VDR signaling, *SNHG16* can induce TGF- β /SMAD5 signals through miR-16–5p/SMA-D5-regulatory axis, thus stimulating expression of CD73 expression in $\gamma\delta$ 1 T cells (Ni et al., 2020). A former assay in experimental autoimmune encephalomyelitis has shown essential role of CD73 in effective entrance of lymphocytes into the central nervous system (Mills et al., 2008). On the other hand, TGF- β plays an important role in the development and functions of CD4 regulatory T cells, a group of T cells which are malfunctioning in MS (Lee et al., 2017). Similarly, *SNHG6* can induce TGF- β /Smad signaling pathway (Wang et al., 2019). Thus, participation of *SNHG16* and *SNHG6* in MS pathogenesis relies on its regulatory roles on different pathways.

There was no significant difference in the expressions of *SNHG6*, *SNHG16* and *LINC00346* between male and female patients, indicating similar roles of these lncRNAs in the pathogenesis of MS in difference gender-based groups. There was no significant correlation between expressions of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs and age, disease duration, age at onset or EDSS. This finding might be attributed to small simple size of the study.

The observed correlations between expressions of lncRNAs among MS but not among healthy subjects might reflect the impact of disease condition in changing the functional relationship between these lncRNA. Alternatively, this observation might be due to influence of IFN- β treatment.

We also evaluated diagnostic power of *SNHG6*, *SNHG16* and

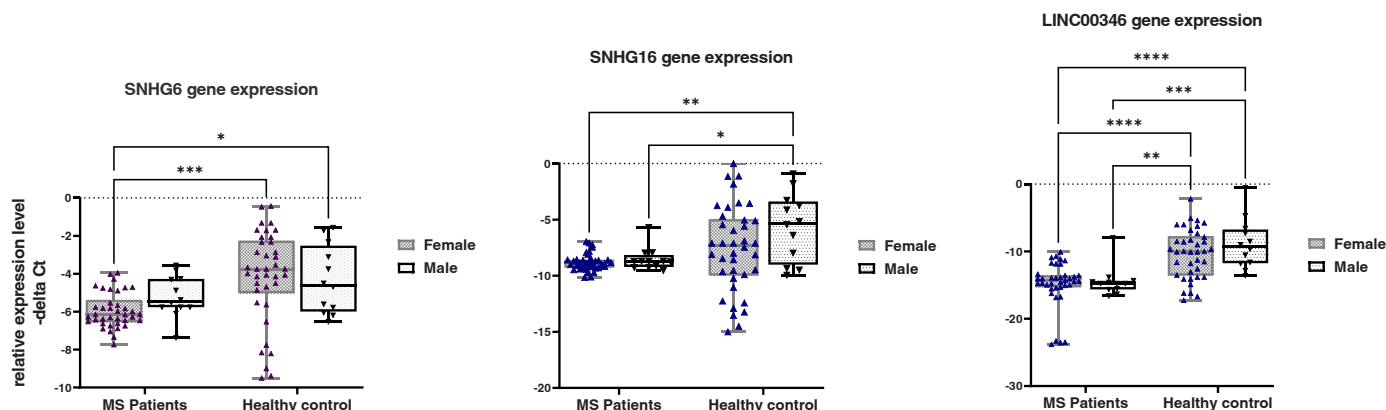


Fig. 2. Expressions of *SNHG6*, *SNHG16* and *LINC00346* genes in MS patient subgroups (male and female) vs. control subgroups (male and female) (* P value < 0.05, ** P value < 0.01, *** P value < 0.001 and **** P value < 0.0001).

Table 3

The results of partial correlation between expression of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs and age, disease duration, age at onset, EDSS and ARMSS (Controlled for sex).

Parameters	Age		<i>SNHG6</i> expression		<i>SNHG16</i> expression		<i>LINC00346</i> expression		age of onset		Disease duration		EDSS	
	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value
Age	1	0	0.039	0.79	-0.006	0.96	-0.026	0.85	0.75	< 0.0001*	0.13	0.34	0.09	0.52
Disease duration	0.14	0.34	-0.14	0.34	-0.09	0.53	0.12	0.4	-0.34	0.01*	1	0	-0.19	0.19
age of onset	0.75	< 0.0001*	0.073	0.61	-0.022	0.87	-0.14	0.31	1	0	-0.34	0.01*	0.097	0.5
EDSS	0.09	0.52	0.18	0.19	0.11	0.44	0.16	0.26	0.097	0.5	-0.19	0.18	1	0
ARMSS	0.59	< 0.0001*	-0.07	0.62	-0.08	0.6	0.07	0.62	0.33	0.01*	0.04	0.78	0.24	0.09

Disease duration was classified into 3 ranges (1–3, 4–10 and more than 10 years). Age of onset was classified into 3 ranges (<30, 31–40 and more than 40 years). EDSS was classified into 2 ranges (1–2, and greater than 2). ARMSS was calculated as follows: (Average rank of EDSS score at a specified age (± 2years)/ Number of patients in the age group+ 1) × 10.

Table 4

Correlation between expression of *SNHG6*, *SNHG16* and *LINC00346* in study groups (R values are shown; after correction for multiple comparisons using Bonferroni correction, P values less than 0.0125 were regarded as significant).

lncRNA	Group	<i>SNHG6</i>		<i>SNHG16</i>	
		r	P value	r	P value
<i>SNHG16</i>	Controls	0.01	0.8		
	Patients	0.74	<0.0001*		
<i>LINC00346</i>	Controls	0.001	0.9	0.3	0.03
	Patients	0.1	0.48	0.46	0.0007*

LINC00346 in MS patients and sex-based subgroups. *LINC00346* had the AUC values of 0.84, 0.82 and 0.94 in differentiation of total MS patients from total controls, female patients from healthy females and male patients from healthy males, respectively. *SNHG6* could separate female patients from female controls with AUC of 0.79. Finally, *SNHG16* could separate female patients from female controls with AUC of 0.73.

Although we demonstrated possible application of these lncRNAs in diagnostic purposes in the current study, this data should be validated over time across different cohorts of patients. After these validation steps, these lncRNAs might be proposed as putative peripheral markers for MS and potential contributors in the pathophysiology of MS. The

results of current study need to be confirmed by functional studies.

This study was among the first studies showing possible participation of VDR-associated lncRNAs in the pathogenesis of MS using a homogeneous population of MS patients being under a certain treatment. However, our study has some limitations. First, as vitamin D has large seasonal variations, it is necessary to evaluate expression of these lncRNA over time to find whether their expression is also influenced by season. Moreover, the *in-silico* method used for selection of these lncRNAs is based on the correlation in expression of these lncRNAs and VDR. Thus, there is no explicit data about the regulatory impact of vitamin D on expression of these lncRNAs. Moreover, the small number of included male subjects limits the statistical power in sex-based analyses. Finally, the possible impact of IFN-β therapy on expression of the studied genes should be assessed in upcoming assays comparing expression of these genes between drug-naïve MS patients and those under treatment with this drug. Other limitations of the current study include lack of expression assays in the initial course of disease, exacerbation phase, remission phase and relapsing phase for each patient, lack of access to drug-naïve patients and lack of assessment of VDR levels and polymorphisms.

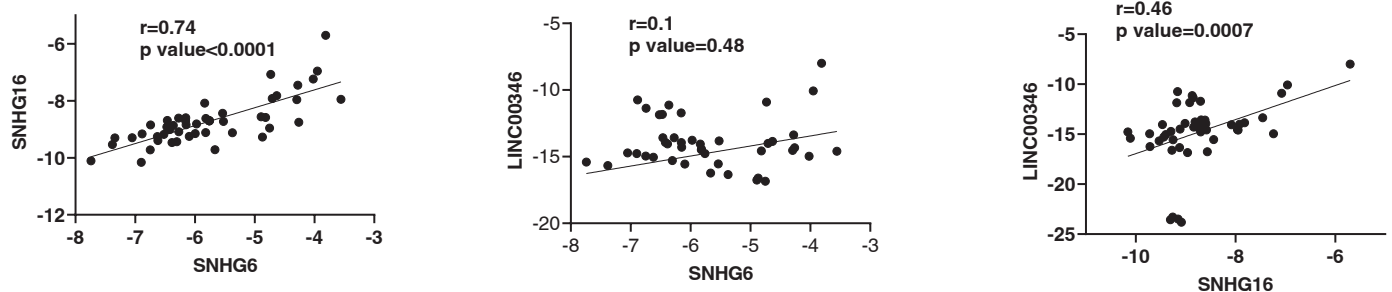


Fig. 3. Correlation between expressions of *SNHG6*, *SNHG16* and *LINC00346* genes in MS patients.

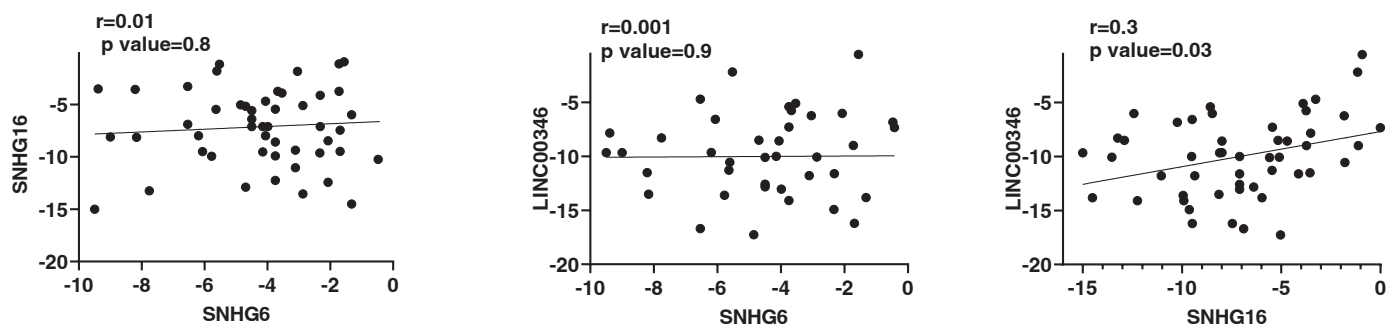


Fig. 4. Correlation between expressions of *SNHG6*, *SNHG16* and *LINC00346* genes in normal controls.

Table 5
ROC curve analyses in different groups of MS patients (AUC: area under curve, SD: standard deviation).

	<i>SNHG6</i>				<i>SNHG16</i>				<i>LINC00346</i>			
	AUC ±SD	Sensitivity	Specificity	P Value	AUC ±SD	Sensitivity	Specificity	P Value	AUC ±SD	Sensitivity	Specificity	P Value
Total patients vs. total controls (50 versus 50)	0.76 ± 0.05	0.84	0.7	<0.0001	0.66 ± 0.06	0.94	0.54	0.0058	0.84 ± 0.04	0.8	0.8	<0.0001
Female patients vs. Female controls (38 versus 38)	0.79 ± 0.06	0.92	0.71	<0.0001	0.63 ± 0.07	0.94	0.5	0.04	0.82 ± 0.04	0.97	0.57	<0.0001
Male patients vs. Male controls (12 versus 12)	0.59 ± 0.12	0.91	0.41	0.41	0.73 ± 0.11	0.91	0.66	0.04	0.94 ± 0.05	0.91	1	0.0002

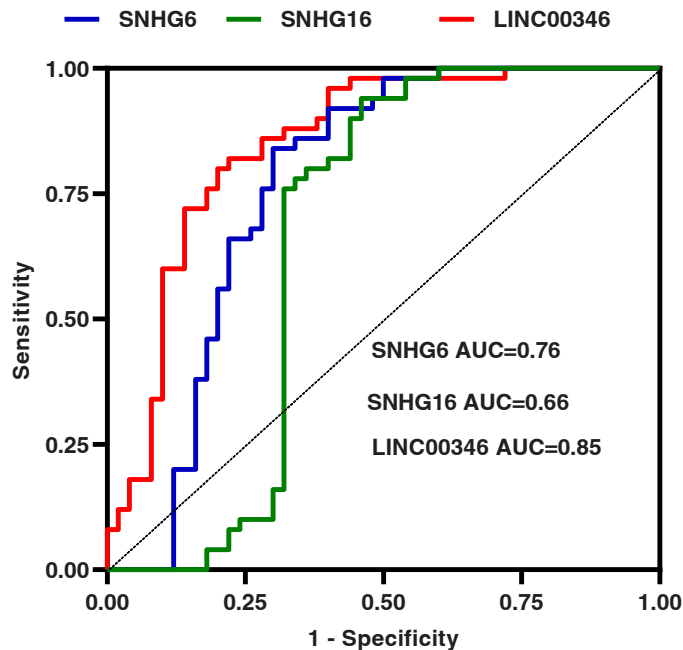


Fig. 5. ROC curves of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs transcript levels in total MS cases.

Ethics approval and consent to participant

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations.

Funding

Not applicable.

CRediT authorship contribution statement

SGF wrote the manuscript and revised it. MT and SF supervised and designed the study. SJ, BMH and HHJ performed the experiment. SE and SH analyzed the data. MD was the clinical consultant and assessed patients for inclusion in the study. All authors read and approved the submitted version.

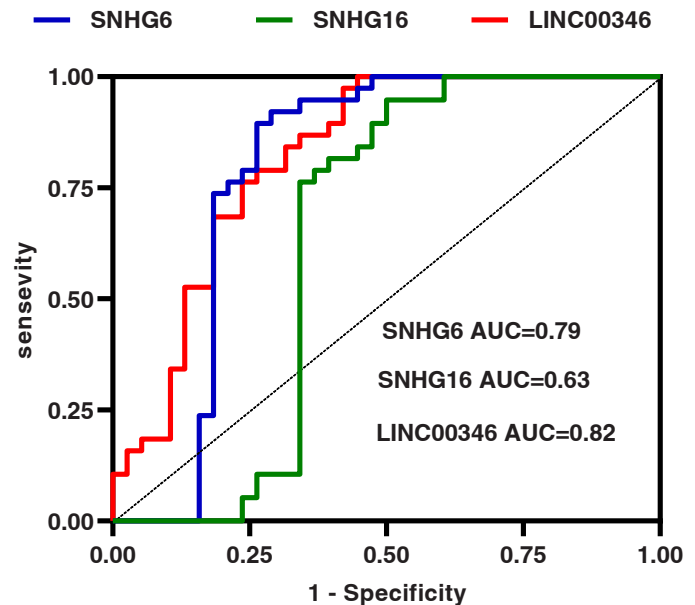


Fig. 6. ROC curves of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs transcript levels in female MS cases.

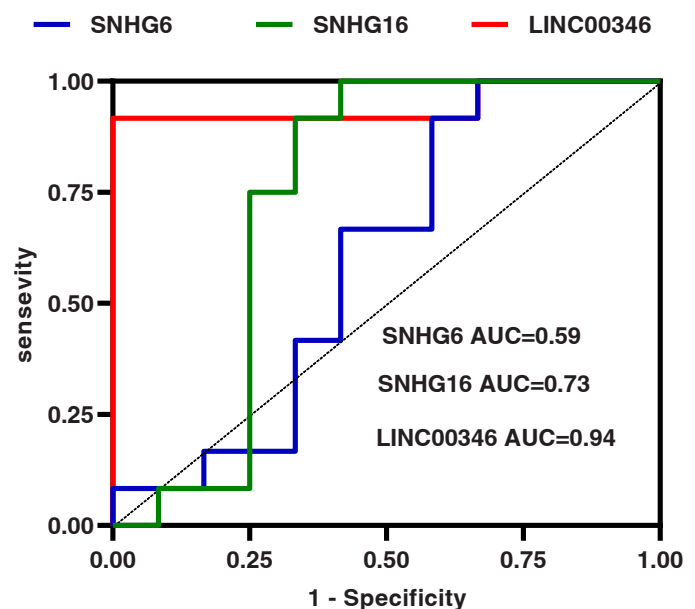


Fig. 7. ROC curves of *SNHG6*, *SNHG16* and *LINC00346* lncRNAs transcript levels in male MS cases.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

Acknowledgement

This study was financially supported by Grant from Medical School of Shahid Beheshti University of Medical Sciences.

Consent of publication

Not applicable.

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