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ORIGINAL ARTICLE

Expression assay of calcium signaling related lncRNAs in autism

Ashkan Pourtavakoli[1] · Soudeh Ghafouri-Fard[2] · Solat Eslami[3] · Serge Brand[4] · Mohammad Taheri[5,6]

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Abstract

Background Calcium signaling has essential roles in the neurodevelopmental processes and pathophysiology of related disorders for instance autism spectrum disorder (ASD).

Methods and results We compared expression of SLC1A1, SLC25A12, RYR2 and ATP2B2, as well as related long non-coding RNAs, namely LINC01231, lnc-SLC25A12, lnc-MTR-1 and LINC00606 in the peripheral blood of patients with ASD with healthy children. Expression of SLC1A1 was lower in ASD samples compared with control samples (Expression ratio (95% CI) 0.24 (0.08–0.77), adjusted P value = 0.01). Contrary, expression of LINC01231 was higher in cases compared with control samples (Expression ratio (95% CI) 25.52 (4.19–154), adjusted P value = 0.0006) and in male cases compared with healthy males (Expression ratio (95% CI) 28.24 (1.91–418), adjusted P value = 0.0009). RYR2 was significantly over-expressed in ASD children compared with control samples (Expression ratio (95% CI) 4.5 (1.16–17.4), adjusted P value = 0.029). Then, we depicted ROC curves for SLC1A1, LINC01231, RYR2 and lnc-SLC25A12 transcripts showing diagnostic power of 0.68, 0.75, 0.67 and 0.59, respectively.

Conclusion To sum up, the current study displays possible role of calcium related genes and lncRNAs in the development of ASD.

Keywords Autism spectrum disorder · SLC1A1 · SLC25A12 · RYR2 · ATP2B2 · LINC01231 · lnc-SLC25A12 · lnc-MTR-1 · LINC00606 · lncRNA

Introduction

Autism spectrum disorder (ASD) denotes to a range of neurodevelopmental disorders pigeonholed by defects in behavior, communication, interaction, and learning. This disorder is associated with genetic and genomic alterations and epigenetic changes, particularly in the neuroimmunological processes [1]. In spite of a strong genetic component which is reflected in the high concordance in the monozygotic twins versus dizygotic twins, no single gene has a prominent role in ASD [1]. Channelopathies, particularly in calcium channels are regarded as important culprits in the pathogenesis of ASD [2]. Meanwhile, activity of ion channels can be influenced by long non-coding RNAs (lncRNAs) [3]. Based on the prominence of calcium signaling in the pathogenesis of neurodevelopmental disorders, we selected *SLC1A, SLC25A12, RYR2* and *ATP2B2* genes from calcium signaling pathway and found their related lncRNAs through searching in the relevant data bases, namely ncbi.nlm.nih.gov, LNCipedia.org and RNAcentral.org.

✉ Soudeh Ghafouri-Fard
s.ghafourifard@sbmu.ac.ir

✉ Mohammad Taheri
mohammad.taheri@uni-jena.de

1 Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

2 Men's Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

3 Department of Medical Biotechnology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran

4 Center for Affective, Stress and Sleep Disorders, Psychiatric Clinics, University of Basel, Basel, Switzerland

5 Institute of Human Genetics, Jena University Hospital, Jena, Germany

6 Urology and Nephrology Research Centre, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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SLC1A1 is gene encodes a protein which is a member of solute transporters family. This membrane-related molecule is the central transporter that evaporates the activator neurotransmitter glutamate from the extracellular spaces at synaptic regions. This function is essential for appropriate synaptic activation and to avoid neuronal injury associated with disproportionate activation of glutamate receptors. This gene is involved in a variety of neurological disorders, particularly epilepsy [4–6]. Glutamate has an indirect role in calcium signaling, since it can activate quisqualic acid receptors and voltage-gated calcium channels, which together facilitate calcium uptake in lower motor neurons [7]. Moreover, SLC1A1 has been shown enhance mitochondrial sodium/calcium exchange to activate the mitochondrial respiratory chain [8].

SLC25A12 codes for a calcium-binding mitochondrial carrier. This protein is located in the mitochondria and participates in the interchange of aspartate and glutamate through the inner membrane of the mitochondria. *SLC25A12* variants have been shown to be associated with autism [9]. Moreover, *SLC25A12* mutations are known to cause of global cerebral hypomyelination [10].

RYR2 gene encodes a protein which forms channels for transportation of calcium ions within cells. Certain variants in this gene are associated with hypersensitivity to activation by calcium resulting in enhanced tendency to establish calcium waves and delayed afterdepolarizations [11].

ATP2B2 encodes a P-type primary ion transport ATPase described by the development of an aspartyl phosphate intermediate throughout the reaction cycle. This enzyme has an important role in intracellular calcium homeostasis [12].

Literature search and *in silico* analyses have led to identification of LINC01231 [13], lnc-SLC25A12, lnc-MTR-1 and LINC00606 [14] as related lncRNAs with SLC1A1, SLC25A12, RYR2 and ATP2B2, respectively.

We compared expression of SLC1A1, SLC25A12, RYR2, ATP2B2, LINC01231, lnc-SLC25A12, lnc-MTR-1 and LINC00606 in the blood of patients with ASD with healthy children.

Materials and methods

Patients and controls

Totally, 30 ASD cases (11 females and 19 males) and 41 typically developing children (11 females and 30 males) were included in the study. Cases were diagnosed in the associated clinics during 2018–2019, using the Diagnostic and Statistical Manual of Mental Disorders (fifth edition) [15] criteria. Autism Diagnostic Observation Schedule-Generic (ADOS-G) was also used for evaluation of ASD cases [16]. Exclusion criteria were structural brain diseases and systemic disorders. Written informed consent forms were signed by guardians of all children. The study protocol was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1401.199).

Experimental step

RNA was obtained from whole blood using the RNJia Kit (ROJE Technologies, Iran). After this step, 75 ng RNA was used for cDNA synthesis using AddScript kit (AddBio, Korea). SLC1A1, SLC25A12, RYR2, ATP2B2, LINC01231, lnc-SLC25A12, lnc-MTR-1 and LINC00606 levels were quantified in ASD samples and control samples using SYBRGreen Ampliqon master mix (Denmark). B2M was considered as normalizer. Experiments were conducted in in the ABI step one plus PCR machine. Information about primers is presented in Table 1.

Statistical analysis

GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA) was used for this step. Expression levels of SLC1A1, SLC25A12, RYR2, ATP2B2 and their related lncRNAs were compared between ASD cases and healthy controls. Comparative–delta Ct method was used. Distribution of the values was assessed by the Shapiro-wilk test. Unpaired *t* test or Mann–Whitney *U* test was used for determination of differentially expressed genes between two groups. Two-way ANOVA (Type 3 Sum of Squares (SS) ANOVA) and Tukey post hoc tests were used to examine the effect of main factors on gene expression levels in patients and controls subgroups.

Undetermined values were set to a maximum Ct + 1 (41) and included in the expression levels calculations and statistical analysis. Correlations between expression levels were measured with Spearman's rank correlation coefficient since data was not normally distributed.

ROC curves were plotted to evaluate the suitability of expression levels of differentially expressed genes for diagnostic purposes. The optimum threshold was identified using Youden's J parameter. $P < 0.05$ was considered as significant.

Results

General information

Table 2 shows general data of participants in the study.

Expression assays

Substantial differences were identified in the levels of SLC1A1, LINC01231, RYR2 and lnc-MTR-1 between ASD

Table 1 Primers characteristics

Gene	RNA type	locus	F primer	R primer	Length of amplicon	Tm
RyR2	Coding	1q43	ATCCCAACGCAGCAAGGAAA	TGTCTGTAGCACCATCTCAGCC	100	60
lnc-MTR-1	lncRNA	chr1:236907044–236,916,931	AGCCTGATGAACCAGTGTGCT	TCCAGCAATCTGCCTCTTTCCA	156	63
ATP2B2	Coding	3p25.3	GCGAGGGCAACGAAGGATGT	CCGTGACCAGGACCACACAGA	123	62
LINC00606	lncRNA	chr3:10759484–10,764,192	GCTACAAAGGAGCAGCCACGA	TCAGCGGTTGTCACAGCACAT	248	61
SLC1A1	Coding	9p24.2	CGGCGAGGAAAGGATGCGA	AGAGTTGAGAGGTTGCTGTGTTCT	130	63
SLC25A12	Coding	2q31.1	GCGGTCAAGGTGCAGACAACTA	AACGCTCTCCATCAACCTCAGTA	94	63
LINC01231	lncRNA	chr9:3181589–3,200,500	TTCTGGAGGAAAGGGAAGAGATT	GGAGCCCAAGCACAGGTT	137	60
lnc-SLC25A12	lncRNA	chr2:171855927–171,999,859	CAGGTGGGATGGAAGAAGCC	TACTGAGAATGAACTTGGGCAG	80	58

Table 2 General data of ASD patients and controls

Group	Parameter	Value	
Patients	Sex	Males	19
		Females	11
	Age (Years, mean ± SD)	Males	6 ± 1.33
		Females	6 ± 1.73
Controls	Sex	Males	30
		Females	11
	Age (Years, mean ± SD)	Males	6.2 ± 1.88
		Females	5.63 ± 1.28

cases and health controls. In Fig. 1, we used Unpaired *t* test or non-parametric Mann–Whitney *U* test (without considering the gender) to compare the expression of studied genes between patients and healthy controls.

To examine how the two categorical diseases and gender variables, and their interactions, affect the gene expression levels, we used a two-way ANOVA and Tukey post hoc tests (Table 3).

Disease factor had a noteworthy effect on expression levels of SLC1A1, SLC25A12, lnc-SLC25A12 and RYR2. Sex

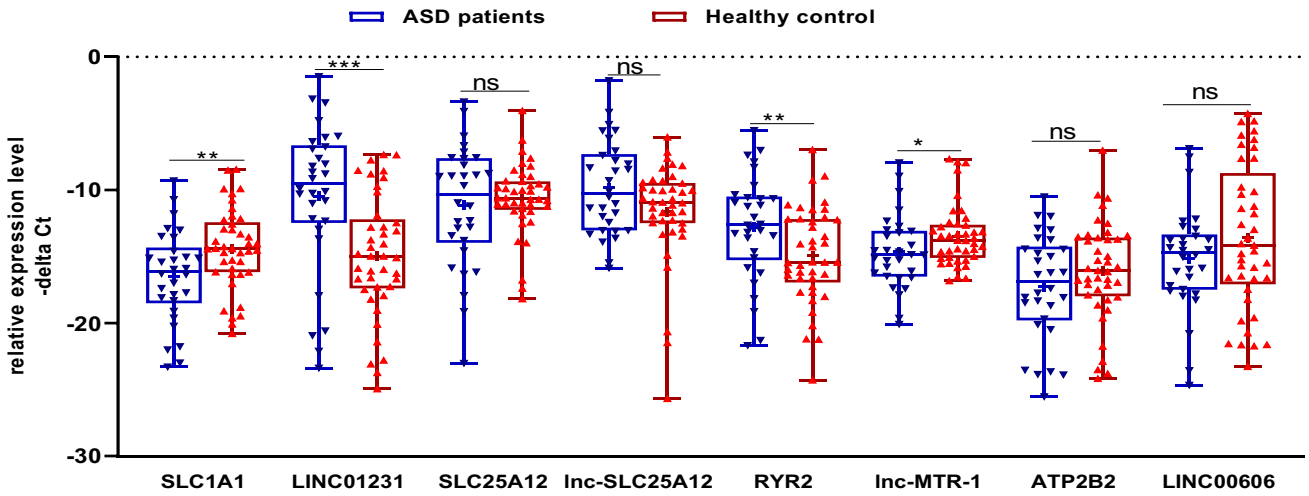

Fig. 1 Expression level of SLC1A1, SLC25A12, RYR2, ATP2B2 and their related lncRNAs, namely LINC01231, lnc-SLC25A12, lnc-MTR-1 and LINC00606 in total ASD patients and controls as described by-delta Ct values. Unpaired *t* test or Mann–Whitney *U* test was used for comparisons (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, *ns* non-significant)

Table 3 Gaphpad prism output from analysis of effect of disease and gender (Tests of Between-Subjects Effects) on expression levels of SLC1A1, SLC25A12, RYR2, ATP2B2 and their related lncRNAs, namely LINC01231, lnc-SLC25A12, lnc-MTR-1 and LINC00606 genes

Source of Variation	Group effect			Gender effect			Interactions		
	SS[1] (TYPE III)	F[2]	P value	SS (TYPE III)	F	P value	SS (TYPE III)	F	P value
SLC1A1	62.08	5.94	**0.017**	19.06	1.82	0.18	2.58	0.24	0.62
LINC01231	326.4	12.9	**0.0006**	71.24	2.81	0.098	0.34	0.013	0.9
lnc-SLC25A12	54.54	4.16	**0.045**	50.5	3.85	0.053	0.73	0.055	0.81
RYR2	70.52	4.95	**0.029**	30.24	2.12	0.14	1.19	0.083	0.77
SLC25A12	6.88	0.51	0.47	0.017	0.001	0.97	3.92	0.29	0.58
lnc-MTR-1	22.44	3.47	0.066	3.48	0.54	0.46	0.81	0.12	0.72
ATP2B2	29.19	1.93	0.16	5.51	0.36	0.54	10.26	0.68	0.41
LINC00606	32.93	1.4	0.23	85.79	3.66	0.059	1.87	0.08	0.77

[1]Sum of Squares

[2]F of Variance

Statistically significant values are shown in bold ($P \leq 0.05$)

factor and interaction of sex and disease factors had no effect on expression of studied genes (Table 3).

SLC1A1 was under-expressed in ASD cases compared with controls (Expression ratio (95% CI) 0.24 (0.08–0.77), adjusted P value = 0.01). Contrary, expression of LINC01231 was higher in cases compared with controls (Expression ratio (95% CI) 25.52 (4.19–154), adjusted P value = 0.0006) and in male cases compared with healthy males (Expression ratio (95% CI) 28.24 (1.91–418), adjusted P value = 0.0009). RYR2 was significantly over-expressed in ASD children compared with controls (Expression ratio (95% CI) 4.5 (1.16–17.4), adjusted P value = 0.029). On the other hand, lnc-MTR-1 had a tendency to be under-expressed in cases compared with controls (Expression ratio (95%

Table 4 Expression of SLC1A1, SLC25A12, RYR2, ATP2B2 and their related lncRNAs in ASD cases compared with healthy controls (Adjusted P values are shown)

Genes		Total patients vs. controls (30 vs. 41)	Male patients vs. male controls (19 vs. 30)	Female patients vs. female controls (11 vs. 11)
SLC1A1	Expression ratio (95% CI)	0.24 (0.08–0.77)	0.18 (0.03–0.97)	0.32 (0.02-4)
	Adjusted P Value	0.017	0.055	0.642
LINC01231	Expression ratio (95% CI)	25.52 (4.19–154)	28.24 (1.91–418)	22.9 (0.45–1152)
	Adjusted P Value	0.0006	0.009	0.160
SLC25A12	Expression ratio (95% CI)	0.97 (0.26–3.6)	0.89 (0.12–6.27)	0.43 (0.02–7.46)
	Adjusted P Value	0.474	0.998	0.869
lnc-SLC25A12	Expression ratio (95% CI)	3.76 (1.03–13.7)	4.38 (0.63–30.3)	3.22 (0.19–53.8)
	Adjusted P Value	0.045	0.194	0.693
RYR2	Expression ratio (95% CI)	4.5 (1.16–17.4)	5.48 (0.73-41)	3.7 (0.19–69.5)
	Adjusted P Value	0.029	0.128	0.644
lnc-MTR-1	Expression ratio (95% CI)	0.42 (0.17–1.06)	0.5 (0.13–1.95)	0.36 (0.05–2.62)
	Adjusted P Value	0.066	0.546	0.537
ATP2B2	Expression ratio (95% CI)	0.37 (0.1–1.5)	0.67 (0.08–5.35)	0.21 (0.01–4.37)
	Adjusted P Value	0.168	0.958	0.537
LINC00606	Expression ratio (95% CI)	0.35 (0.09–1.51)	0.45 (0.03–6.06)	0.27 (0.006-12)
	Adjusted P Value	0.239	0.855	0.809

CI) 0.42 (0.17–1.06), adjusted P value = 0.066) (Table 4). Finally, there was a significant difference for LNC01231 expression between male patients and female controls (P value = 0.0033) (data not shown).

We also distinguished significant pairwise correlation between levels of calcium signaling-related genes and their associated lncRNAs both among ASD cases and healthy children. Table 5 shows these results.

Then, we depicted ROC curves for SLC1A1, LINC01231, RYR2 and lnc-SLC25A12 transcripts showing diagnostic power of 0.68, 0.75, 0.67 and 0.59, respectively (Fig. 2).

The highest sensitivity value was reported for LINC01231 in male cases (sensitivity = 0.79). The best specificity value was demonstrated for Lnc-SLC25A12 among females (specificity = 0.9). Table 6 shows the details of ROC curve analyses in subgroups of ASD patients.

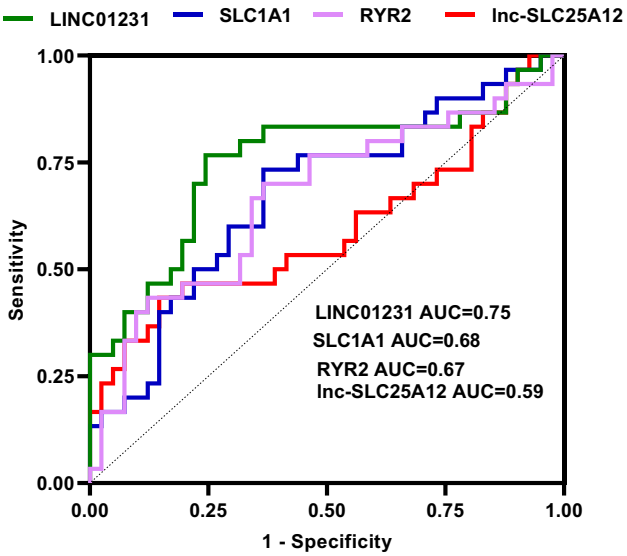

Fig. 2 ROC curves of LINC01231, SLC1A1, RYR2 and lnc-SLC25A12 transcript levels

Discussion

Calcium signaling has essential roles in the neurodevelopmental processes and pathophysiology of related disorders such as ASD [17]. Certain alterations in the calcium signaling might have damaging effects along pathways affecting the function of endoplasmic reticulum mitochondria [17]. Moreover, lncRNAs affect pathophysiology of ASD through different mechanisms [18]. In the current study, we appraised expression of some calcium signaling related mRNAs and their related lncRNAs in the blood of ASD cases versus controls.

In a previous study, the same genes were studied by our team, in patients with refractory epilepsy; and there was a significant correlation among lnc-MTR-1 and ATP2B2, ATP2B2 and lnc-SLC25A12 and lnc-MTR-1 and lnc-SLC25A12 pairs in refractory epileptic patients. The highest correlation was between ATP2B2 and lnc-MTR-1 which is its related long non coding RNA and affects its function in neurons [19]. These two genes play a role in cellular Calcium metabolism by interaction with proteins that involve in signaling, transporting or storage of Calcium ions [20]. Also, in that study we found increased expression of SLC1A1, SLC25A12, lnc-MTR-1 and LINC01231 genes in male patients in compare with healthy male, which demonstrate their up-regulation function in pathogenesis on Refractory Epilepsy; but lnc-SLC25A12 which is a non-coding RNA showed no significant expression different [19].

Expression assays showed under-expression of SLC1A1 and up-regulation of its related lncRNA, LINC01231 in ASD cases compared with controls. On the other hand, RYR2 was remarkably over-expressed in ASD children compared with controls, while lnc-MTR-1 had a tendency to be under-expressed in cases compared with controls.

SLC1A1 has a function in buffering local glutamate concentration at excitatory synapses and modulation of distinctive recruitment of different subtypes of glutamate

Table 5 Spearman's correlations between RNA expression levels among the ASD patients and controls

	LINC01231	SLC25A12	lnc-SLC25A12	RYR2	lnc-MTR-1	ATP2B2	LINC00606
	Patients controls	Patients controls	Patients controls	Patients controls	Patients controls	Patients controls	Patients controls
SLC1A1	−0.09 −0.07	0.34 0.24	0.07 −0.13	0.02 0.13	−0.02 0.13	−0.13 0.31*	0.11 −0.13
LINC01231		0.31 0.38*	0.87** 0.64**	0.82** 0.63**	0.71** 0.38*	0.5* 0.24	0.51* 0.62**
SLC25A12			0.46* 0.56**	0.32 0.53**	0.49* 0.71**	0.19 0.30	0.44* 0.55**
lnc-SLC25A12				0.76** 0.65**	0.74** 0.44*	0.4* 0.16	0.56* 0.51**
RYR2					0.68 0.45*	0.65** 0.37*	0.54* 0.55**
lnc-MTR-1						0.53* 0.29	0.53* 0.54**
ATP2B2							0.27 0.37*

*$p < 0.05$

**$p < 0.001$

Table 6 ROC curve analyses in patients with ASD disease and female and male subgroups

	SLC1A1				LINC01231				RYR2				Lnc-SLC25A12			
	AUC ± SD	Sensitivity	Specificity	P value	AUC ± SD	Sensitivity	Specificity	P value	AUC ± SD	Sensitivity	Specificity	P value	AUC ± SD	Sensitivity	Specificity	P value
Total patients vs. total controls (30 vs. 41)	0.67 ± 0.06	0.73	0.63	0.01	0.75 ± 0.06	0.77	0.76	0.0003	0.67 ± 0.06	0.7	0.63	0.01	0.59 ± 0.07	0.43	0.85	0.18
Female patients vs. Female controls (11 vs. 11)	0.71 ± 0.11	0.73	0.82	0.09	0.70 ± 0.11	0.73	0.73	0.1	0.67 ± 0.12	0.64	0.83	0.17	0.54 ± 0.12	0.27	0.9	0.76
Male patients vs. Male controls (18 vs. 30)	0.67 ± 0.08	0.74	0.57	0.03	0.79 ± 0.07	0.79	0.77	0.0007	0.69 ± 0.07	0.53	0.87	0.02	0.65 ± 0.08	0.53	0.83	0.07

receptors at extrasynaptic sites [21]. The related lncRNA with this gene has a number of variants that are associated with attention deficit hyperactivity disorder, substance abuse, antisocial behavior measurement, educational attainment, mathematical ability and insomnia (https://www.genecards.org/cgi-bin/carddisp.pl?gene=LINC01231).

RYR2 has been among genes whose copy number variations are implicated in ASD as revealed in a population-based investigation in Lebanon [22]. This gene encodes a calcium release channel expressed in the brain. Moreover, RYR2-related modulation of calcium homeostasis is implicated in cognitive functions and neuronal postsynaptic plasticity [23].

Therefore, dysregulated genes in the circulation of ASD patients as revealed in this study have functional roles in the regulation of calcium homeostasis and are possibly contributing to the pathogenesis of ASD.

It is worth mentioning that expression levels of genes in the peripheral blood do not necessarily reflect their levels in the cerebrospinal fluid (CSF). A recent expression study has shown relatively weak correlations between serum protein concentrations and CSF protein concentrations [24]. However, altered expression levels of genes in the peripheral blood of ASD cases might affect some crucial signaling pathways in the blood cells, thus indirectly influence the pathobiology of disorder. In line with this hypothesis, a former exploratory study has suggested that the gene expression profile of peripheral blood specimens of young ASD subjects can be used to detect the biological signatures for ASD [25].

The observed correlations between expression levels of these genes in the ASD cases and controls further support their possible implications in a functional network in the pathophysiology of ASD.

ROC curves for SLC1A1, LINC01231, RYR2 and lncSLC25A12 transcripts showed diagnostic power of 0.68, 0.75, 0.67 and 0.59, respectively. Therefore, LINC01231 is the best transcript among mentioned transcripts for separation of ASD cases from controls. Taken together, the current study shows possible role of calcium related genes and lncRNAs in the development of ASD. However, additional functional studies are required for verification of their functions in the pathoetiology of ASD. Finally, since the sample size is small, it is better to look at the protein level for all protein-coding genes in peripheral blood or repeat RNA quantification in another small set of new cases for proper validation.

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Author contributions AP performed the experiment. SE analyzed the data. SGF wrote the draft and revised it. MT and SB designed and supervised the study. All the authors contribute equally and read the submission.

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Data availability All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Competing interests The authors declare no competing interests.

Ethical approval All procedures performed were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments.

Consent to participate Informed consent forms were obtained from parents of all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1401.199).

Consent for publication Not applicable.

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