RESEARCH

Evaluation of circulating miRNAs and mRNAs expression patterns in autism spectrum disorder

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Abstract

Background: Autism spectrum disorder is a condition related to brain development that affects a person's perception and socialization, resulting in problems in social interaction and communication. It has no single known cause, yet several different genes appear to be involved in autism. As a genetically complex disease, dysregulation of miRNA expression and miRNA–mRNA interactions might be a feature of autism spectrum disorder. The aim of the current study was to investigate the expression profile of circulating miRNA-128, miRNA-7 and *SHANK* gene family in ASD patients and to assess the possible influence of miRNA-128 and miRNA-7 on *SHANK* genes, which might provide an insight into the pathogenic mechanisms of ASD and introduce noninvasive molecular biomarkers for the disease diagnosis and prognosis. Quantitative real-time PCR technique was employed to determine expression levels of miRNA-128, miRNA-7 and *SHANK* gene family in blood samples of 40 autistic cases along with 30 age- and sex-matched normal volunteer subjects.

Results: Our study revealed a statistical significant upregulation of miRNA-128 expression levels in ASD cases compared to controls (p value < 0.001). A statistical significant difference in *SHANK-3* expression was encountered on comparing cases to controls (p value < 0.001). However, miRNA-7 expression showed no significant difference between the studied groups.

Conclusions: MiRNA-128 and *SHANK-3* gene are emerging players in the field of ASD. They are promising candidates as noninvasive biomarkers in autism. Future studies are needed to emphasize their pivotal role.

Keywords: ASD, miR128, miR7, *SHANK* family

Background

Autism spectrum disorder (ASD) represents a spectrum of multifaceted, pervasive and neurodevelopmental disorders that tend to manifest by 18 to 36 months of age. ASD is distinguished with social, communicative deficits and confined repetitive behavioral patterns. ASD is frequently associated with comorbidities such as epilepsy, intellectual disabilities (IDs), immune dysfunction, hyperactivity and gastrointestinal disorders [1, 2].

The prevalence of ASD is reporting an increased trend all over the world. It is estimated to be 1–2% of the population [3]. Although there are no epidemiological data for ASD in Egypt, an earlier study by Seif Eldin et al. [4] proposed a prevalence rate of 33.6% among Egyptian children with developmental disabilities. In 2017, 800,000 cases with ASD were reported in Egypt by the Social Solidarity Ministry [5]. It is worth noting that the variability in prevalence rates among different countries relies on the availability of screening tools and advanced diagnostic systems [3].

The cause of ASD remains to be elucidated; however, it is postulated that both environmental and genetic factors together play a pivotal role in risk development of ASD [6]. Currently, ASD is considered a primarily

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genetic disorder with 74–84% estimated heritability, the pathogenesis of which can be triggered by both pre- and postnatal environmental factors comprising maternal smoking, infections and pesticide exposure [2]. Moreover, increased risk of ASD can be attributed to prenatal drug exposure such as thalidomide, misoprostol and valproic acid [6].

SH3 and multiple ankyrin repeat domains proteins (*SHANKs*) are scaffolding proteins that are essential to synaptic formation, development and function. Many genetic studies proposed a strong association between the *SHANK* family genes (*SHANK-1*, *SHANK-2* and *SHANK-3*) and ASD as well as other neurodevelopmental and neuropsychiatric disorders [7].

Deletions, duplications and coding mutations in the *SHANK* genes were detected in several patients with syndromic ASD and intellectual disability (ID) with higher severity in mutations in *SHANK-3* and *SHANK-2* genes compared to those in *SHANK-1* [8].

Deletions and point mutations of *SHANK-3* are correlated with Phelan-McDermid syndrome, ASD, intellectual disability, schizophrenia and bipolar disorder. Furthermore, its duplications are related to Asperger syndrome, attention deficit hyperactivity disorder, schizophrenia and bipolar disorder [9].

Point mutations of *SHANK-2* and microdeletions of *SHANK-1* and *SHANK-2* have been implicated in patients with ASD, Alzheimer's disease, mild-to-moderate ID and non-syndromic ID [10, 11].

In view of the multifactorial nature implicated to ASD, it is possible that at least one epigenetic mechanism can be accused in ASD pathogenesis. MiRNAs can be highlighted as potential players in this field. MiRNAs are short noncoding nucleic acids that can influence the expression of entire gene networks by suppressing the transcription of messenger RNA (mRNA) into proteins [12].

MiRNAs are abundantly present in the central nervous system (CNS), producing around 70% of all miRNA species. Furthermore, changes in miRNA expression occur in the different stages of development and across various brain regions [13].

Accumulating evidence reveals that miRNAs are implicated in many biological and physiological processes including neurogenesis, neuroproliferation and synaptic plasticity in the CNS; thus, dysregulation of miRNAs is linked to behavioral and cognitive changes figured out in many neuropsychiatric disorders [6]. Altered peripheral miRNA expressions have been reported in a number of psychiatric disorders. Correlations between changes in neuroendocrine/ neuroimmune responses and different peripheral miRNAs patterns have been documented. Since brain tissue is not easily accessible, blood-based miRNAs, that are inexpensively, noninvasively and easily

obtained, have been presented as clinically applicable biomarkers of psychiatric disorders [14].

In the case of ASD, it has been postulated that differentially upregulated miRNAs with high expression levels mostly suppress genes related to neuronal and synaptic dysfunction, whereas differentially downregulated miRNAs cause abnormal activation of genes involved in inflammatory and compensatory processes [13].

MicroRNA-128 is a brain-enriched miRNA highly expressed in the cortex, cerebellum and maturing cortical neurons. It functions in the context of regulating neurogenesis in the embryonic cortex, as well as proliferation and differentiation of neural progenitor cells (NPCs) by suppressing pericentriolar material 1 (PCM1). Thus, abnormal cortical development that underlies the pathophysiology of ASD can be attributed to aberrant expression of miR-128 [15]. Furthermore, dysregulated miR-128 potentially targets genes which are known genetic causes of ASD including *neurexin 1*, *SHANK-3* and *reelin* genes [16, 17].

Recently, Aamodt identified miR-128 as a potential therapeutic target for ASD. His study postulated that inhibiting miR-128 could normalize microglia reactivity in autistic patients and thus improve clinical symptom severity. As miR-128 is a negative regulator of vocal learning, then reducing its levels in ASD patients could restore plasticity, or their capacity to learn to speak [18].

Another ASD-related miRNA, miR-7, which regulates the expression of the ASD risk gene *SHANK-3*, has a crucial role in proper development of cortical neural progenitors and cortical growth through its interactions with genes in P53 pathway. Thus, its dysregulation can result in abnormal development of hippocampal neurons [9, 19, 20].

In this study, we aimed to investigate the expression profile of circulating miRNA-128, miRNA-7 and *SHANK* gene family in ASD patients in comparison with typically developing children and to explore the posttranscriptional regulation of *SHANK* genes by miRNA-128 and miRNA-7, which might provide an insight into the pathogenic mechanisms of ASD and the possibility of employing the expression profiles of *SHANK* genes and the studied miRNAs as potential diagnostic biomarkers for ASD. A better understanding of ASD pathogenesis will pave the way for the introduction of new therapeutic approaches to improve the clinical symptoms in autistic patients.

Methods
Study design and sample collection
The present case–control study recruited 40 autistic participants; their ages ranged between 2 and 10 years, and 30 age- and sex-matched control subjects. Diagnosis of

autistic disorder was assigned according to routine clinical interview and psychiatric examination using the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM-5) criteria [21] and Autism Diagnostic Interview-Revised (ADI-R) [22]. The Childhood Autism Rating Scale (CARS) was used for the assessment of autism severity [23].

Participants with congenital syndromes, neurological, metabolic disorders or malignancy were excluded from the present study as miRNA dysregulations have been demonstrated in these conditions [24–26]. Furthermore, Kichukova et al. [13], Vaccro et al. [28] and Vasu et al. [29] excluded such cases while studying miRNAs expression in ASD [13, 27, 28].

Full history taking with general emphasis on family history constructing a pedigree, consanguinity status, family history of similar conditions was carried out for all participants.

Five milliliters of peripheral venous blood were withdrawn from each participant under complete aseptic conditions; 3 milliliters was anticoagulated with ethylenediaminetetraacetic acid (EDTA) for immediate processing. Rest of blood was left to clot at room temperature (25 °C) and then centrifuged, and sera were separated and added to QIAZol in uniquely and properly identified sterile tubes for each individual subject and stored at −80 °C.

Procedure

MiRNAs expression assay

Total RNA was isolated using QIAgen miRNeasy Mini isolation kit according to manufacturer's protocol. MiR-128 and miR-7 were reversely transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and specific miRNA reverse transcription (RT) primers according to the manufacturer's instructions in a final volume of 15 μL (incubated for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then maintained at 4 °C) and then stored at −80 °C until analysis.

mRNAs expression assay

Extraction was done from whole blood using QIAamp RNA blood kit (QIAgen, Germany) according to manufacturer's instructions followed by immediate conversion to cDNA using High capacity cDNA Archive kit (Applied Biosystems) in a final volume of 20 μL (incubated for 10 min. at 25 °C, 120 min. at 37 °C, 5 min. at 85 °C and then maintained at 4 °C) and then stored at −80 °C until analysis.

RNA concentration and quality were determined using NanoDrop 2000c spectrophotometer® (Thermo Fisher Scientific Inc. USA). Assessment of A260/A280 ratios

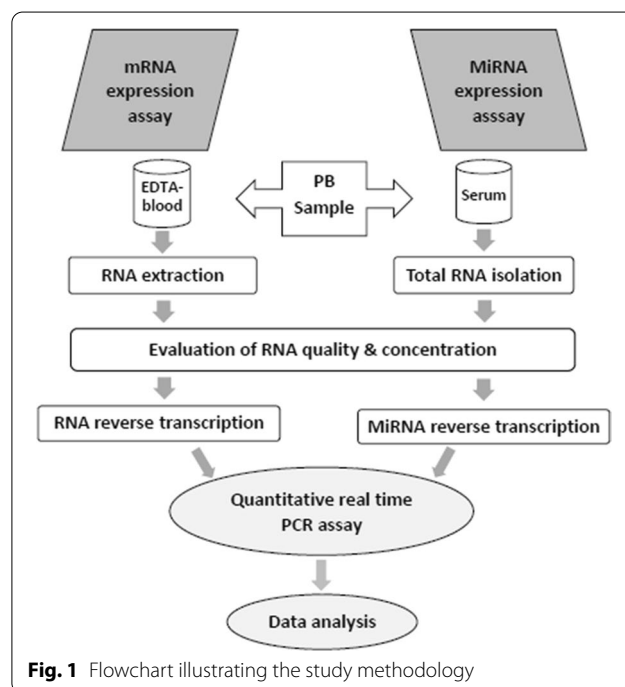revealed that all RNA samples are with sufficient quality for qPCR analysis (1.93–2.10).

For real-time PCR

Two microliters of RT products were mixed with 10 μL of Taqman PCR master mixture (No UNG), 1 μL TaqMan miRNA or mRNA assays and 7 μL nuclease-free water in a final volume of 20 μL.

All reactions were performed on QuantStudio 12 k flex Real-time system (Applied Biosystems) with the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Relative expression of target miRNAs was normalized to miRNA-16, and target mRNAs was normalized to B-actin. The fold changes of candidate miRNAs and mRNA expression were calculated by the equation $2^{-\Delta\Delta Ct}$ (Fig. 1).

Statistical analysis

All data were converted and manipulated by using SPSS software program version 18.0. Data were analyzed; mean and standard deviation were calculated for quantitative data. The quantitative variables were compared, and *t*-test was applied for parametric data. Mann–Whitney was applied for nonparametric data. The difference between groups was considered statistically significant when $p < 0.05$ and considered highly statistically significant when $p < 0.01$.

Fig. 1 Flowchart illustrating the study methodology

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Results

A total of 70 participants aged between 2 and 10 years participated in the current research, comprising 40 autistic patients and 30 age- and sex-matched healthy individuals. The median age of autistic participants was 4 years, while that of controls was 3.6 years, with a statistically insignificant difference between patients and controls ($p = 0.94$, $t = 0.2$). The patient group included 29 (72.5%) males and 11 (27.5%) females, while the control group showed 22 (73.3%) males and 8 (26.7%) females, and the difference was statistically insignificant ($p = 0.93$).

The history and clinical data of the autistic cases are shown in Table 1.

The upregulation of miRNA-128 expression level was statistically highly significant in cases than controls ($p < 0.001$). However, miRNA-7 showed no significant difference between the studied groups ($p > 0.05$) (Table 2; Figs. 2 and 3).

The expression of *Shank-3* was upregulated in cases compared to controls with a statistical significant difference $p < 0.001$. However, both Shank-1 and Shank-2 expressions showed no significant difference in cases compared to that of controls ($p = 0.8$, $p = 0.2$, respectively) (Table 3; Figs. 4, 5, 6).

The difference was insignificant in miR-7, miR-128 and *Shank* genes family expression levels considering gender and consanguinity among the patients group (Tables 4, 5).

Table 1 History and clinical data of the autistic cases

Variable	Cases (*N* = 40)			
	Positive		Negative	
	N	%	*N*	%
Consanguinity	25	62.5	15	37.5
Family history	30	75	10	25
Clinical data				
Obesity	2	5	38	95
Short stature	1	2.5	39	97.5
Epilepsy/EEG	6	15	34	85
Febrile convulsions	2	5	38	95
Dysmorphism	6	15	34	85
GIT problems	4	10	36	90
ADHD	5	12.5	35	87.5
Sleep disorders	3	7.5	37	92.5
Hypotonia	5	12.5	35	87.5
Movement problems	1	2.5	39	97.5
CARS				
Mild-to-moderate autism	24	60		
Severe autism	16	40		

Table 2 MiRNA-128 and miRNA-7 expression among studied groups

Variables	Cases		Controls		*p* value
	Median	IQR	Median	IQR	
miR-7	1.7	1.2–2.3	1.7	0.9–1.8	0.09
miR-128	2.1	1.2–3.2	1.8	0.8–1.9	**< 0.001**

The bold format represents the significant *p* values

Mann–Whitney test

IQR: Interquartile range

p value is considered significant if < 0.05

Considering the family history of neuropsychiatric disorders, a statistically significant difference was detected between it and the expression of both miR-7 and *Shank-2* gene among the patients group ($p = 0.03$) (Table 6).

Table 7 reveals a weak positive correlation between miR-7 and miR-128 ($p = 0.001$).

Discussion

ASD is highly heritable, with genetic contributions representing about 80% of ASD risk, and a higher risk observed autistic children's siblings. Although epidemiological studies link the genetic contribution to autism, less is known about the putative genes involved [29, 30].

The developing brain encompasses many posttranscriptional mechanisms like microRNAs that adjust gene expression with the preservation of the genetic code. Such mechanisms are dysregulated in autistic cases. Altered miRNA expressions are detected in the brain, blood, saliva and olfactory precursor cells of ASD patients [31].

Neural progenitor cells can either divide or become specific types of neurons. If this proper scenario did not take place—for instance, if progenitors did not stop dividing in order to mature—various neurodevelopmental disorders may arise, comprising autism spectrum disorder [15].

MicroRNAs control gene activity and protein expression such as miR-128 that aids the newly formed neurons to travel to the correct region of the cortex, which is crucial for several cognitive processes including thought and language [15].

Our study demonstrated a significantly higher expression of serum miR-128 in autistic patients compared to age- and sex-matched healthy controls ($p < 0.001$), consistent with a study conducted by Zhang et al. [15], who investigated the role of miR-128 in the developing cortex of mice. Their results postulated that miR-128 prohibits cortical NPCs from dividing or development into more specialized cells. Overproduction of miR-128 in
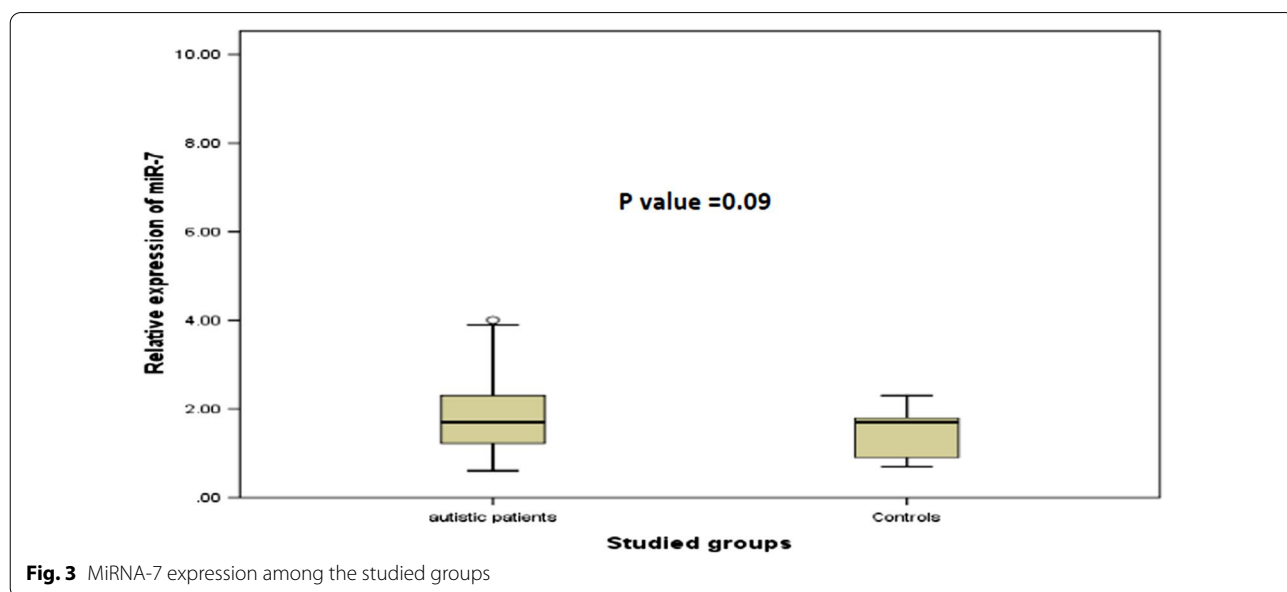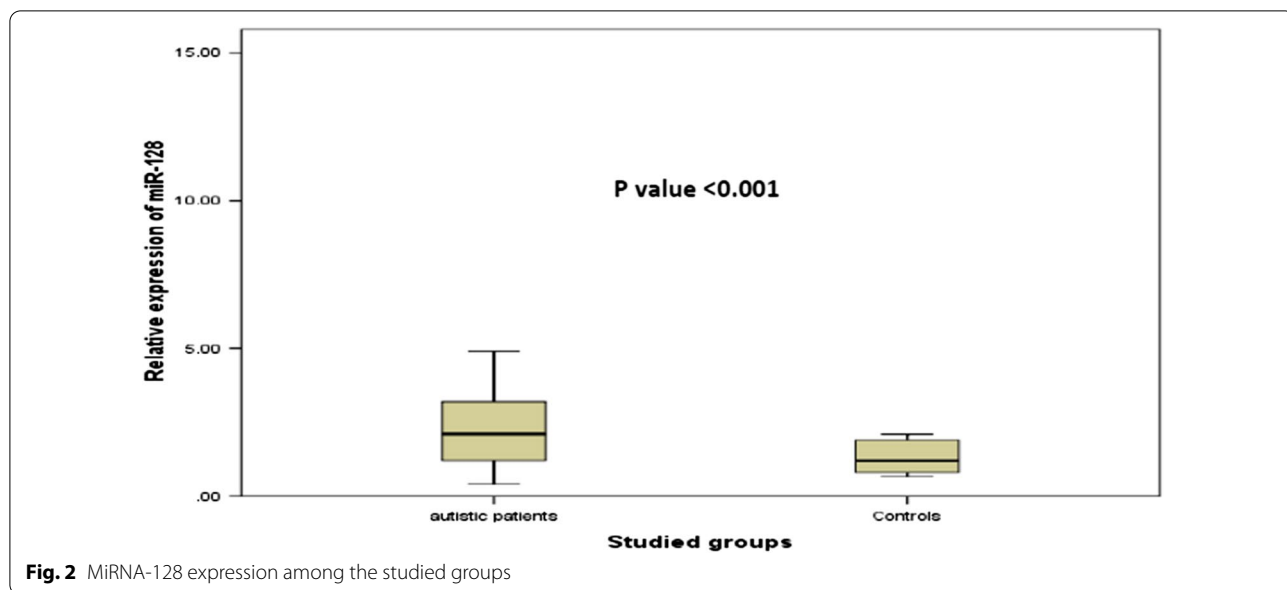
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Fig. 2 MiRNA-128 expression among the studied groups

Fig. 3 MiRNA-7 expression among the studied groups

Table 3 Expression of *SHANK* family genes among studied groups

Variables	Cases		Controls		*p* value
	Median	**IQR**	**Median**	**IQR**	
Shank-1	0.9	0.4–2.1	1.1	0.3–1.7	0.8
Shank-2	1.8	0.8–2.7	1.2	1–1.9	0.2
Shank-3	2.8	1.7–4.5	1.1	0.77–1.2	**< 0.001**

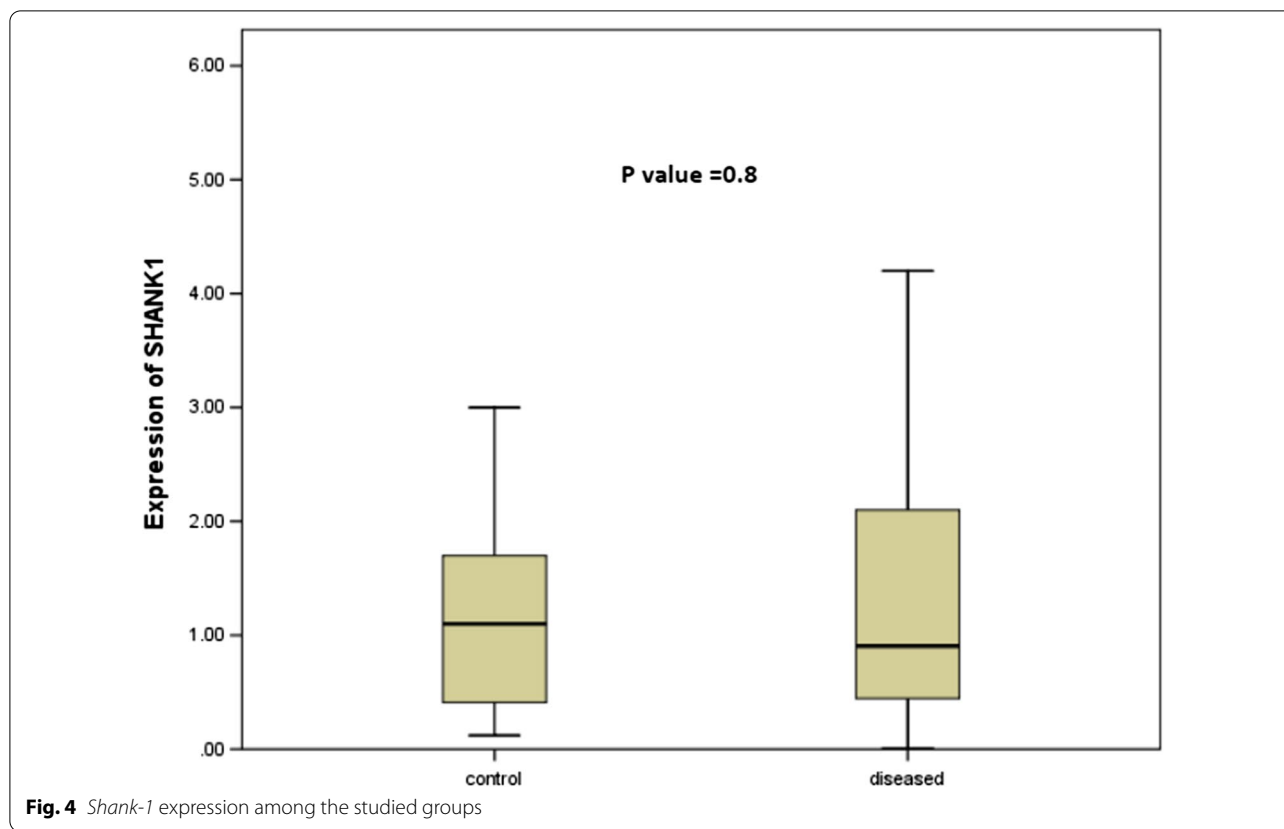The bold format represents the significant *p* values

Mann–Whitney test

IQR: Interquartile range

p value is considered significant if < 0.05

the progenitor cells caused the cells to divide less often and promoted their maturation into neurons. Contrarily, removing miR-128 from the progenitor cells enhanced their division and reduced neurons' formation. Further investigation showed that miR-128 represses the production PCM1 protein that controls the proper division of the cells.

The observed actions of miR-128 are concordant with a Ge et al.'s study [32], who elucidated that PCM1 loss evoked the early exit of NPCs from the cell cycle and encouraged their premature differentiation to neurons. In

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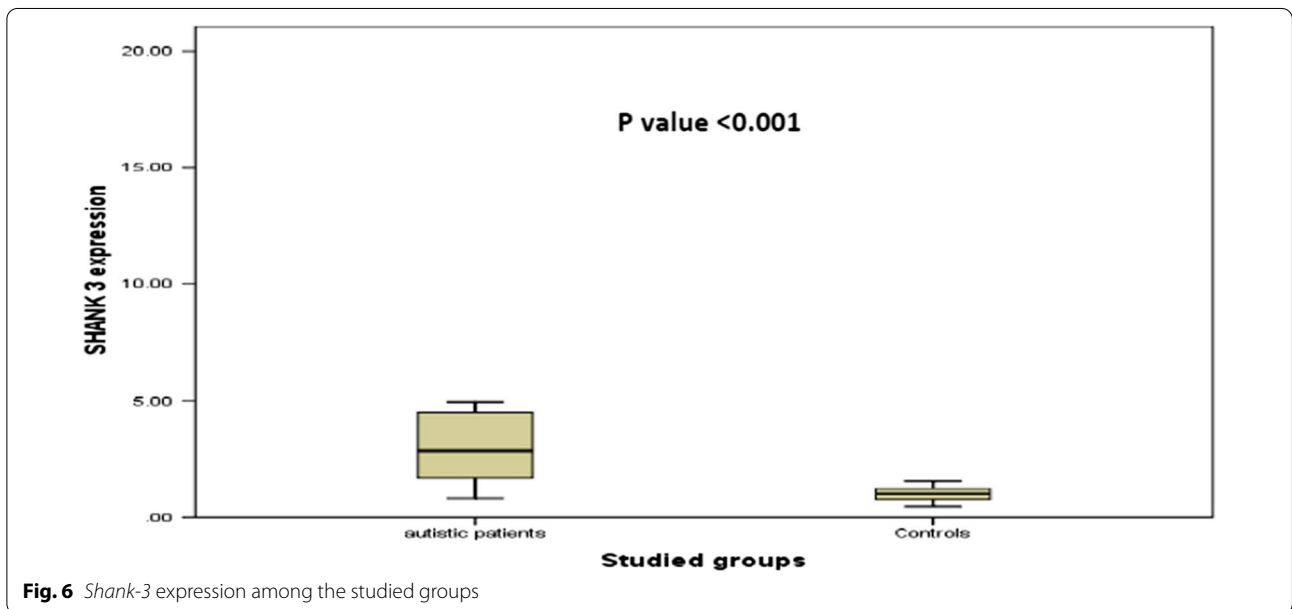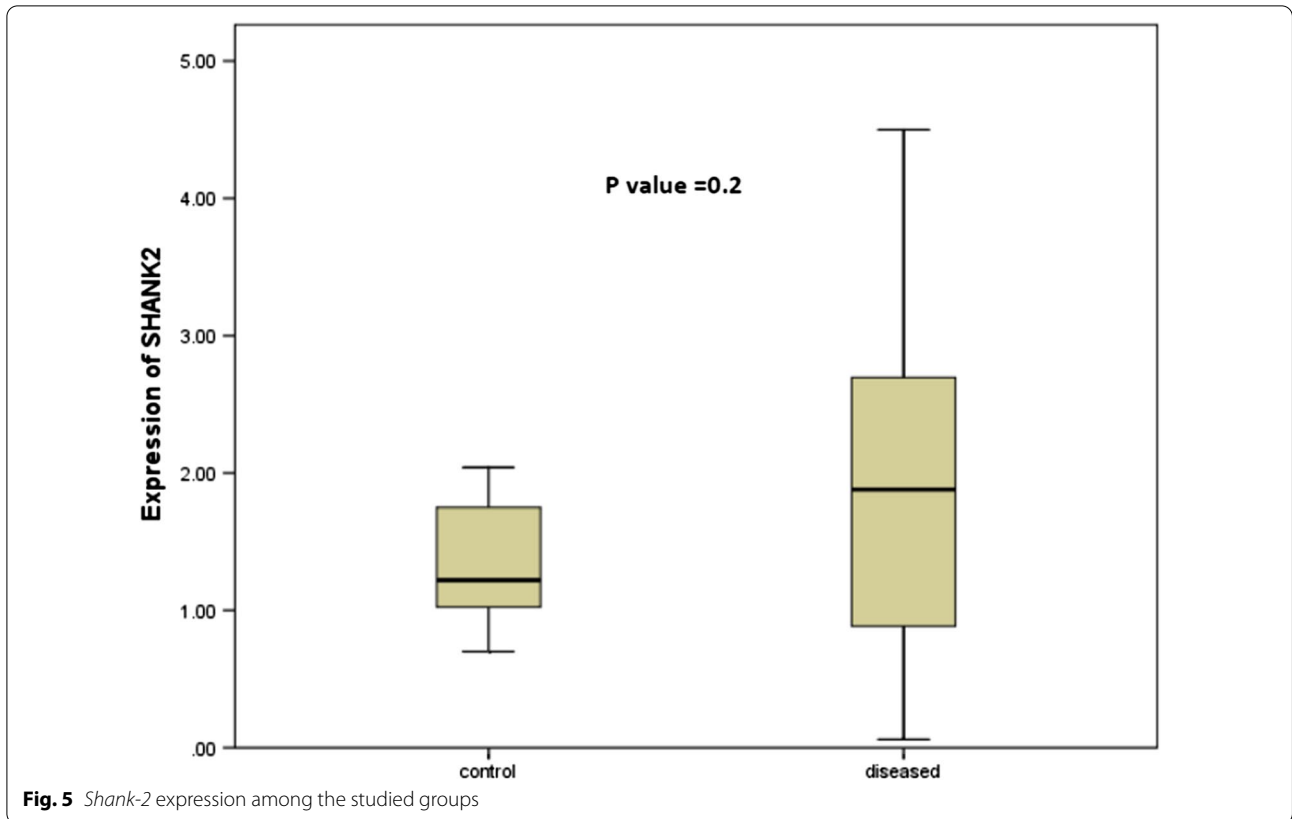**Fig. 4** *Shank-1* expression among the studied groups

their study, knockdown of PCM1 lead to the impairment of interkinetic nuclear migration of NPCs that causes the overproduction of neurons and premature exhaustion of the NPC pool in the developing neocortex.

In our study, miR-7 levels showed no significant difference among the studied patients and controls, which was concordant with a study conveyed by Mundalil Vasu et al. [28], in which miR-7 levels were not differentially expressed in ASD individuals and neither in controls. Contrasting studies were published by Hicks et al. [33] and Sehovic et al. [34], who presented a significant upregulation of miR-7 in saliva of autistic children.

Our study reported a significant association between both miR-7 and *SHANK-2* gene with a family history of neuropsychiatric disorder. This result was revealed also by a study done by Zhang et al. [35] who detected that the circulating level of miR-7 was significantly increased in schizophrenia patients. They reported that overexpression of miR-7 suppressed the expression of *SHANK-3* causing a significantly decline in the levels of *SHANK-3* mRNA and protein. They concluded that miR-7 potentially plays a pivotal role in the pathophysiology of schizophrenia by binding to 3-prime untranslated regions of the mRNA and altering the morphology

and function of neurons. Moreover, a study done by Viorel et al. [36] reported the frequent dysregulation of miR-7 in the brains of patients with neurocognitive disorders and in animal models of Alzheimer, Huntington's and Parkinson's diseases, implying that using agonist or antagonist miRNA oligonucleotides in the correction of miRNA expression might be a promising approach to ameliorate or even to cure such diseases.

Regarding *SHANK-2*, our results were consistent with that offered by Homann et al. [37], who sequenced the genomes of 90 individuals across nine families. Each family comprised at least three available relatives suffering from a psychotic illness and three available unaffected relatives. In one family, seven siblings with schizophrenia spectrum disorders each carried a new private missense variant within the *SHANK-2* gene. This variant lied within the consensus SH3 protein-binding motif by which *SHANK-2* can interact with postsynaptic glutamate receptors. Moreover, previous publications reported the alterations in the *SHANK-2* gene locus in neuropsychiatric patients; they recognized *SHANK-2* variations comprising truncations, missense mutations, deletions and *SHANK-2* promoter regions mutations; all of these findings emphasized the

Fig. 5 *Shank-2* expression among the studied groups

Fig. 6 *Shank-3* expression among the studied groups

causative link of *SHANK-2* variants to ASD, IDs and schizophrenia [38, 39].

Mutations of genes that code synaptic cell adhesion molecules and scaffolding proteins which are important for the formation, stabilization and homeostasis of synapses, like *neuroligins*, *neurexins* and *SHANK* genes have been reported in patients with ASD. *SHANK* family genes code for scaffolding proteins lying in the postsynaptic

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Table 4 Comparison between the expression of miRNA-7, miRNA-128 and *SHANK* family genes regarding gender

Variables	Males		Females		*p* value
	Median	IQR	Median	IQR	
miR-7	1.8	1.3–2.3	1.7	1.1–2.9	0.84
miR-128	2.3	1.2–3.4	1.9	1.3–3	0.91
Shank-1	0.9	0.3–1.9	1.1	0.6–2.3	0.21
Shank-2	1.8	1.2–2.5	1	0.6–3.3	0.79
Shank-3	3.2	1.4–8	2.6	1.9–3.4	0.65

Mann–Whitney test

IQR: Interquartile range

p value is considered significant if < 0.05

Table 5 Comparison between the expression of miRNA-7, miRNA-128 and *SHANK* family genes regarding associated consanguinity

Variables	Positive cases (*N* = 25)		Negative cases (*N* = 15)		*p* value
	Median	IQR	Median	IQR	
miR-7	1.8	1.3–2.4	1.7	1–2.3	0.5
miR-128	2.5	1.5–3.6	1.6	0.7–2.6	0.06
Shank-1	0.8	0.3–2.2	1.1	0.4–2.3	0.7
Shank-2	1.8	0.9–2.5	1.8	0.8–3	0.9
Shank-3	2.8	1.5–6.8	3.7	2.1–4.2	0.6

Mann–Whitney test

IQR: Interquartile range

p value is considered significant if < 0.05

density of glutamatergic synapses. Deletions and mutations of *SHANK-3* are among the major genetic abnormalities in neurodevelopmental disorders, ASD, IDs and schizophrenia [40].

In our study, the *SHANK-3* gene was significantly overexpressed in the patients' group compared to the controls ($p < 0.001$). Our result was consistent with a study conveyed by Moessner et al. [41], who found that overexpression of *SHANK-3* may result in ASD, as proved

Table 6 Comparison between the expression of miRNA-7, miRNA-128 and *SHANK* family genes regarding the family history of neuropsychiatric disorders

Variables	Positive cases (*N* = 25)		Negative cases (*N* = 15)		*p* value
	Median	IQR	Median	IQR	
miR-7	1.9	1.3–4.4	1.4	1.1–1.9	**0.03**
miR-128	2.3	1.4–3	2.1	0.8–3.5	0.6
Shank-1	0.7	0.4–1.6	1.2	0.4–2.2	0.4
Shank-2	2.3	1.3–3.1	1.3	0.6–2.4	**0.03**
Shank-3	2.6	1.9–4.3	3.5	1.8–8.7	0.7

The bold format represents the significant *p* values

Mann–Whitney test

IQR: Interquartile range

p value is considered significant if < 0.05

by a report of Asperger syndrome in an individual with three copies of *SHANK-3* locus, supporting the hypothesis that autism is caused by improper excitatory/inhibitory neuronal ratios in the brain [42].

Moreover, *SHANK-3* overexpression was also observed by Jin et al. [43], who detected an unanticipated increase in *SHANK-3* transcripts in the brain regions that occurred in *SHANK-3* heterozygous knockout mice with partial deletions of specific exons. This increase in *SHANK-3* mRNA was not likely a nonspecific consequence of chromosomal alterations in the *SHANK-3* that mostly occurred in the N-terminal exons in *SHANK-3* mutant mice, which proposes selective compensatory activation of upstream *SHANK-3* promoters in the process. One candidate player for this feedback mechanism is *β-catenin*, which, upon loss of synaptic *SHANK-3*, moves from the synapse to the nucleus to elicit histone deacetylase-2 dependent transcriptional modifications. Whether *β-catenin* directly binds to the upstream *SHANK-3* promoters to stimulate their transcription needs to be confirmed [44].

Table 7 Correlation analysis between the expression of miRNA-7, miRNA-128 and *SHANK* family genes

Variables	miR-7		miR-128		Shank-1		Shank-2		Shank-3	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
miR-7	–	–	0.48	0.02	− 0.1	0.4	− 0.02	0.1	0.1	0.36
miR-128	0.6	**0.001**	–	–	0.07	0.6	0.06	0.8	0.02	0.79
Shank-1	− 0.1	0.4	0.07	0.6	–	–	− 0.1	0.4	− 0.1	0.5
Shank-2	0.1	0.42	0.2	0.08	− 0.1	0.4	–	–	− 0.1	0.3
Shank-3	0.1	0.36	0.02	0.79	− 0.1	0.5	−0.1	0.3	–	–

The bold format represents the significant *p* values

r: correlation coefficient

p value is considered significant if < 0.05

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Conclusions

In conclusion, our results demonstrate that the levels of miRNA-128 and *SHANK-3* mRNA showed a statistical significant upregulation in Autistic patients. We suggest that this may reflect their metabolic imbalance in vivo; hence, this may provide a new insight regarding their molecular clues for recognizing autism disease.

A greater sample size of autistic patients is still needed in order to support these conclusions.

MiRNA-128 and *SHANK-3* are emerging players in the field of ASD; future studies are needed to assess their implementation as diagnostic biomarkers and therapeutic targets in autism.

Abbreviations
ASDs: Autism spectrum disorders; ADI-R: Autism Diagnostic Interview-Revised; CARS: Childhood Autism Rating Scale; ID: Intellectual disabilities; IQR: Interquartile range; MiRNA: MicroRNA; mRNA: Messenger RNA; NRCs: Neural progenitor cells; PCM1: Repressing pericentriolar material 1; SHANK: SH3 and multiple ankyrin repeat domains protein.

Authors' contributions
AH participated in preparing the research design, conducting the laboratory work and preparing the paper for submission and final approval of the version to be published. OM participated in preparing the design of the research, data collection and statistical analysis. NA is responsible for patient's selection and recruiting and diagnosis of the patients. MH participated in performing the laboratory work and revising the manuscript. MM participated in performing the laboratory work and data collection. SN participated in conducting the laboratory work and helped to draft the manuscript. All authors read and approved the final manuscript.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate
Each participant's guardian provided an informed written consent after full acknowledgement about the research. All procedures performed in our study involving human participants were in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the ethical committee of the National Research Centre (Registration number 16373).

Consent for publication
Not applicable.

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

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