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RESEARCH

Investigation of LncRNA *PVT1* and *MiR-21-5p* Expression as Promising Novel Biomarkers for Autism Spectrum Disorder

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Abstract

The characteristics of ncRNA in children with autism spectrum disorder (ASD) were observed to disclose a theoretical basis for further research on molecular markers for early warning of ASD. Children with ASD and normal control children were recruited to collect peripheral blood RNA samples. The concentration of *PVT1* and *miR-21-5p* was quantitatively analyzed by qRT-PCR. Pearson correlation coefficient method was used to evaluate the link between *PVT1* level and *miR-21-5p* level of the children. Receiver operating characteristic (ROC) curves were applied to reckon the predictive value of *PVT1*, *miR-21-5p*, and their combination in ASD. The interconnection of *PVT1* with *miR-21-5p* was represented by luciferase reporter assay. The targeted genes of *miR-21-5p* were predicted. The enrichment and protein interaction analysis of these genes was carried out to find the important core genes and analyze their value in ASD. In the disease group, the level of *PVT1* was downregulated, while the content of *miR-21-5p* was upregulated. The expression level of serum *miR-21-5p* was negatively correlated with the level of *PVT1*. Luciferase reporter gene assay documented that *PVT1* directly targeted *miR-21-5p*. ROC curve showed that *PVT1*, *miR-21-5p*, and their combination showed clinical value for disease diagnosis. The functional enrichment analysis showed that the targets of *miR-21-5p* participated in ASD by regulating related functions and pathways. Reduced expression of *PVT1* and raised *miR-21-5p* were good diagnostic markers for ASD, which would provide a basis for effective prevention, early diagnosis, and early intervention of ASD.

Keywords *PVT1* · *miR-21-5p* · Early diagnosis, Autism spectrum disorder · Expression

Introduction

Autism spectrum disorder (ASD) is a neurological dysfunction that emerges during childhood and persists throughout the whole life (Lord et al. 2018; Richards et al. 2020). Onset typically begins in infancy, with gradual development noticeable by 18 months of age, and diagnosis attainable by 24 months (Zhang et al. 2020). Studies indicate that ASD is mainly related to genetic factors, maternal pregnancy, perinatal factors, neurobiological factors, neurobiochemical factors, infection and immune factors, and nutritional factors (Alvarez-Arellano et al. 2020). ASD is a complex condition, primarily connected to abnormal nerve development, dysfunction of nerve pathways, abnormal synaptogenesis and nerve connection, and neurotransmitter imbalance (Tran et al. 2019). The primary symptoms of children with ASD are social interaction disorder, communication disorder, and stereotyped repetitive behavior and interest (Kodak and Bergmann 2020; Liu et al. 2022a). The diagnosis of ASD is based on behavioral manifestations and developmental situations (Smith et al. 2019). There are numerous diagnostic scales commonly used in clinics; however, a conclusive diagnosis of ASD often requires the observation and evaluation of professionally trained professional evaluators, or long-term observation and evaluation (Shulman et al. 2020). Early behavior reinforcement intervention can significantly influence development, particularly in behavior, adaptability, and communication aptitude (Landa 2018). Therefore, promoting early detection, verification, and treatment is recommended to improve children's socially acceptable behavior and reduce or eradicate negative behavior.

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Among many possible pathogeneses of ASD, the regulation of long-chain non-coding RNA (lncRNA) has attracted wide attention (Ghafouri-Fard et al. 2022). The expression of *MEG3* was increased in children with ASD, and *MEG3* could distinguish children with ASD from the control group (Taheri et al. 2021). In ASD cases, the concentrations of *DISC2*, *PRKAR2A-AS1*, and *LOC101928237* increased, which could be a possible reference for this disease (Tamizkar et al. 2021). LncRNA *PVT1* leads to possible early development and differentiation of the nervous system. Studies have shown that abnormal expression of *PVT1* could affect the normal development of the central nervous system (Li et al. 2021). The expression of *MIAT* and *PVT1* in exosomes of untreated schizophrenia patients was changed (Guo et al. 2022). *PVT1* of schizophrenic patients is downregulated, leading to a shred of evidence that lncRNA is involved in the pathogenesis of schizophrenia (Safari et al. 2019). It is widely recognized that schizophrenia and ASD are both mental illnesses. Therefore, *PVT1* might potentially be linked to ASD and other neurodevelopmental disorders and could serve as a biomarker for early screening of ASD. The purpose of this observation was to explore the expression characteristics of lncRNA in children with ASD and to unveil a basis for further study on ASD screening biomarkers.

Materials and Methods

Research Objects

This study comprised of 60 patients with ASD treated in a local psychiatric hospital. Their average age was 7.07 ± 2.56 years old, IQ was 68.13 ± 15.86, and they were of Han nationality (Table 1). In this study, ASD was selected based on the diagnostic criteria published by the American Psychiatric Association (Wakefield 2016). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the patients were diagnosed with social communication deficits as well as restricted interests and repetitive behaviors. Patients with other neuropsychiatric diseases such as Wright's syndrome, fragile X syndrome, epilepsy, schizophrenia, obsessive–compulsive disorder, affective disorder, congenital heart disease, trisomy 21 syndrome, and other congenital diseases were excluded from the study. The ADOS score was estimated for each patient in four categories (Table 1). The guardian of each participant was fully informed and provided a written informed consent form before joining the study. The Ethics Committee of Shenzhen Polytechnic University provided the approval for this research.

The peripheral venous blood 2 ml of the study object was collected and stored in the tube with the medical record number and name of each participant clearly marked on the label. These specimens were centrifuged for 10 min using a desktop low-speed centrifuge at 3500 rpm. The serum was then collected in an autoclaved 1.5-ml tube and frozen at − 80 °C.

Clarification of lncRNA and miRNA Expression

Real-time fluorescence quantitative PCR (qPCR) was carried out according to the instructions of TaqMan kit (TaqMan universal mixed kit II, ABI company). *PVT1* and *miR-21-5p* sequences were queried from GenBank. Primer 3 software was used to design primers and quantitative primers were synthesized by Shanghai Jikang Biological Co., Ltd. (China). TRIzol (ThermoFisher, USA) was used to extract RNA. RNA of 1 μg was taken for reverse transcription, and reverse transcriptional reaction was performed as per the instructions of kit (TaqMan RNA and microRNA reverse transcription kits, ABI company, USA). The standard cDNA after reverse transcription was amplified by PCR. The primers used in this detection were as follows: *PVT1* forward, 5′-TGAGAACTGTCCTTACGTGACC-3′ and reverse 5′-AGAGCACCAAGACTGGCTCT-3′; *miR-21-5p* RT primer, 5′-GTCGTATCCAGTGCAGG GTCCGAGGTATTCGCACT GGATACGACTCAACATCAGT-3', forward, 5′-GGCGGT AGCTTATCAGACTGATG-3′, and reverse 5′-GTGCAGGGT CCGAGGTATTC-3′.

Affirmation of Target of PVT1

The Bioinformatics website LncBook 2.0 (https://ngdc.cncb.ac.cn/lncbook/home) was utilized to anticipate the binding sites between *PVT1* and *miR-21-5p*. The luciferase activity assay was then carried out to certify the target relationship. In

Table 1 Basic clinical data of the subjects

Items	Controls group ($n = 58$)	ASD group ($n = 60$)	*Significance* (p)
Age (years)	7.76 ± 2.67	7.07 ± 2.56	0.154
IQ	82.90 ± 22.70	68.13 ± 15.86	< 0.001
ADOS			
Communication	0	7.17 ± 2.02	-
Social interaction	0	6.77 ± 1.81	-
Imagination	0	3.33 ± 1.90	-
Repetitive and restricted behaviors	0	3.67 ± 1.78	-

ASD autism spectrum disorder, *IQ* intelligence quotient, *ADOS* autism diagnostic observation schedule. All the data are presented as mean ± standard deviation

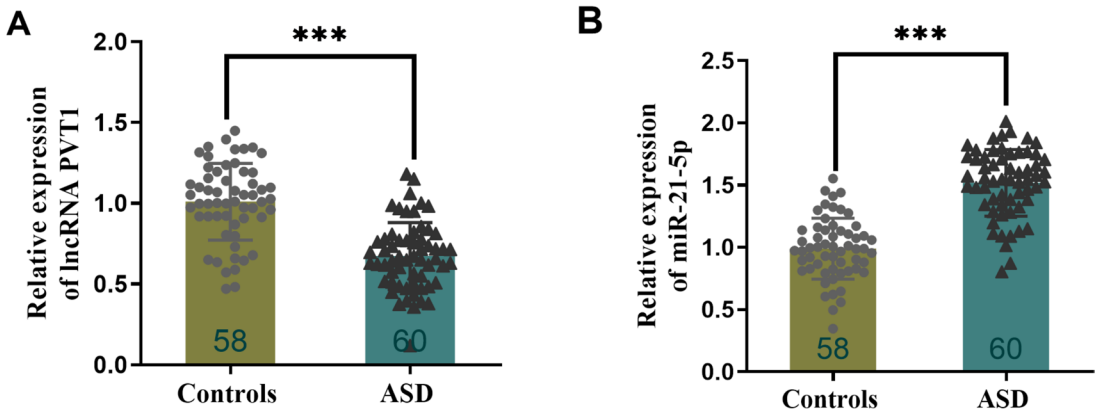

Fig. 1 We unveiled the levels of *PVT1* and miR-210 in ASD patients. **A** The relative quantification of *PVT1* between the two groups. **B** The assessments of *miR-21-5p* content in the two groups. *** $P < 0.001$

this assay, we constructed recombinant plasmids pmirGLO-*PVT1*-wide type (WT) and pmirGLO-*PVT1*-mutant (MUT). *PVT1*-WT and its mutant derivative without *miR-21-5p* putative site were sub-cloned into the luciferase gene coding region located downstream of HEK 293T. The cells were cultured in 24-well plates and co-transfected 48 h after transfection, using Lipofectamine 2000 reagent. The fluorescence values in each group were gained using a luciferase system (Promega, USA).

GO Functional Enrichment and KEGG Signaling Pathway Analysis of Target Genes

The targeted genes of *miR-21-5p* were predicted on EVmiRNA, miRDB, TargetScan, and miRtarbase. The intersection of predictive genes was performed using a Venn diagram.

The DAVID data library (https://david.ncifcrf.gov/) was used for Gene Ontology (GO) analysis of targeted genes, including biological process (BP), cellular component (CC), and molecular function (MF). The Kyoto Encyclopedia of Genes and Genomes (KEGG) signal path enrichment results were also obtained in this library. The enrichment conditions were $P < 0.05$ as the threshold.

Construction of Protein–protein Interaction (PPI) networks

The corresponding PPI network for targeted genes was constructed using the online database STRING (https://string-db.org/). The interaction score > 0.500 was selected and the disconnected nodes in this network were hidden.

Statistical Analysis

All statistical analyses were reckoned with SPSS 20.0 software. The *t*-test analysis, one-way, and two-way analyses of variance (ANOVA) were utilized for comparing discrepancies. Pearson analysis was the method for correlation certification. The clinical values of the single lncRNA, miRNA, and combination were evidenced by the receiver operating characteristic (ROC) curve. $P > 0.05$ was on behalf of statistical significance.

Results

PVT1 and miR-21-5p Expression

Content of non-coding RNAs was reflected by the qPCR, and the results were displayed in Fig. 1. The relative expression levels of *PVT1* in the ASD group were diminished compared with controls ($P < 0.001$, Fig. 1A). *MiR-21-5p* was overexpressed in patients with ASD ($P < 0.001$, Fig. 1B). Collectively, ASD development might lead to the abnormal expression of *PVT1* and *miR-21-5p*. To validate the link between *miR-21-5p* and

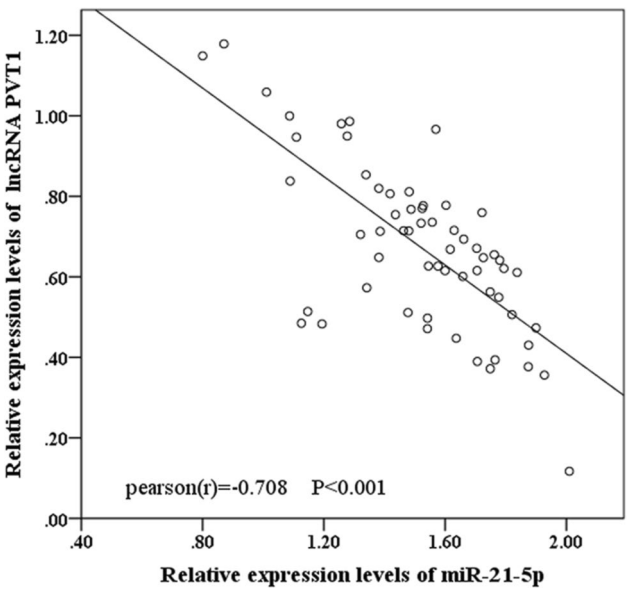

Fig. 2 *PVT1* correlated with *miR-21-5p* in ASD patients

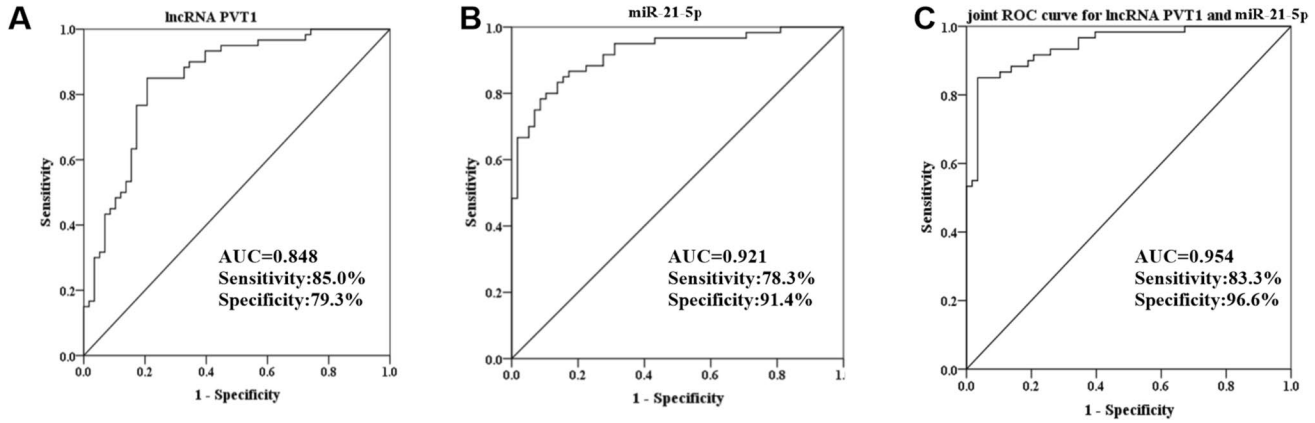

Fig. 3 ROC findings for diagnosing ASD patients. Diagnostic value analysis of **A** *PVT1*, **B** *miR-21-5p*, and **C** their combination for disease

PVT1, the Pearson analysis was employed to determine their correlations in patients. As depicted in Fig. 2, the elevated content of *miR-21-5p* was inversely proportional to the quantification of *PVT1* (r = -0.708, P < 0.001).

Predictive Value of PVT1 and miR-21-5p in Patients with ASD

The predictive significance of *PVT1*, *miR-21-5p*, and their combination in patients with ASD was presented in Fig. 3. ROC curve analysis showed that the lower area under the curve (*AUC*) for *PVT1* was 0.848 (95% *CI* = 0.78–0.92) with a sensitivity and specificity of 85.0% and 79.3%, respectively (Fig. 3A). The expression of *miR-21-5p* also showed a diagnostic ability in ASD patients (*AUC* = 0.921, sensitivity = 78.3%, specificity = 91.4%, Fig. 3B). When *PVT1* was combined with *miR-21-5p*, the *AUC* increased to 0.954 with a 95% *CI* of 0.91–0.98, suggesting that *miR-21-5p* and *PVT1* combination had high accuracy in predicting ASD patients (Fig. 3C). The results above indicated that

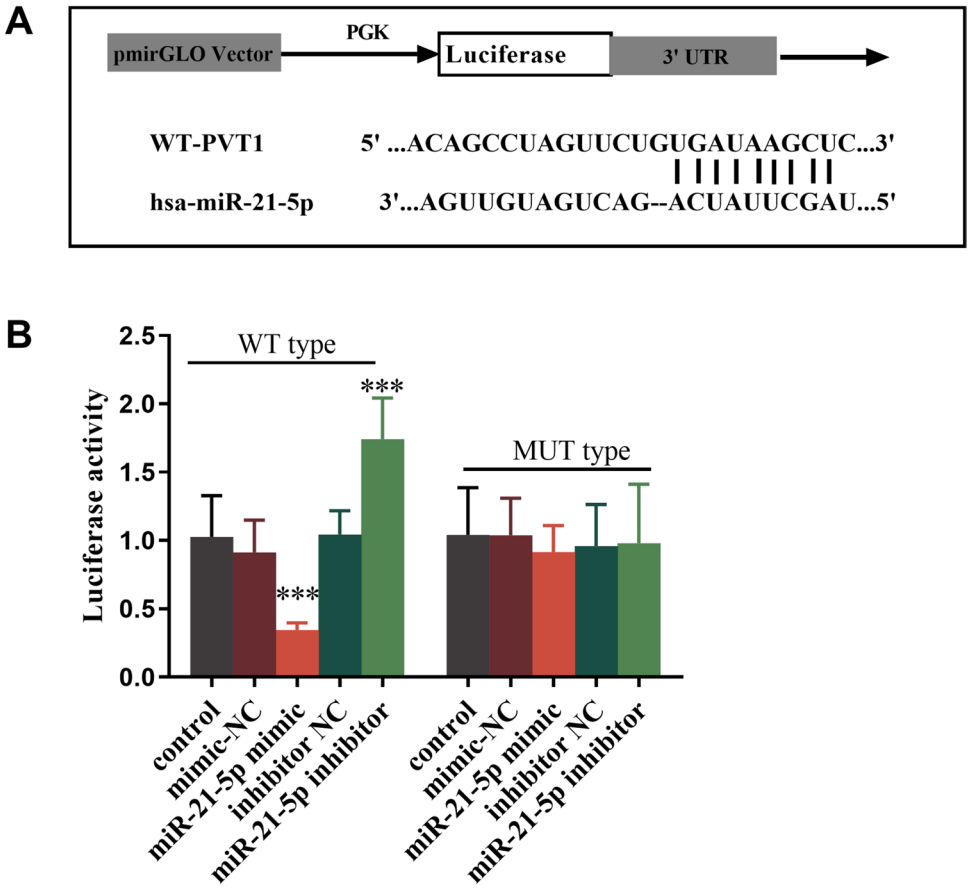

Fig. 4 Validation of the targeting link between *PVT1* and *miR-21-5p*. **A** Binding sites of *miR-21-5p* and *PVT1*. **B** Transfection of *miR-21-5p* mimic significantly inhibited luciferase activity of wild-type *PVT1* 3'-UTR, while *miR-21-5p* inhibitor did the opposite. *** P < 0.001

both *PVT1* and *miR-21-5p* alone could distinguish between ASD children and healthy individuals. Additionally, an improvement in diagnostic value was observed when the two genes were combined.

The Targeted Connection Between miR-21-5p and PVT1

The putative regions between *PVT1* and *miR-21-5p* were depicted in Fig. 4A. The results from double luciferase reporter gene detection showed that the high expression of *miR-21-5p* significantly inhibited the fluorescence intensity of pmirGLO-*PVT1*-WT plasmid, while *miR-21-5p* inhibitors promoted the luciferase activity ($P < 0.001$, Fig. 4B). However, there was no effect on fluorescence intensity in the pmirGLO–*PVT1*-MUT group ($P > 0.05$, Fig. 4B).

GO Enrichment Analysis and KEGG Pathway Analysis of Targets

Through bioinformatics analysis, the EVmiRNA, TargetScan, miRDB, and miRtarbase identified 1667, 384, 469, and 649 target genes, respectively (Fig. 5). A total of 95 shared genes were screened by the Venn method (Fig. 5).

The results of the GO enrichment analysis characterized that the targeted genes were enriched in the asymmetric synapse, post-synaptic specialization, neuron to neuron synapse, and neurotrophin receptor binding (Fig. 6A). The

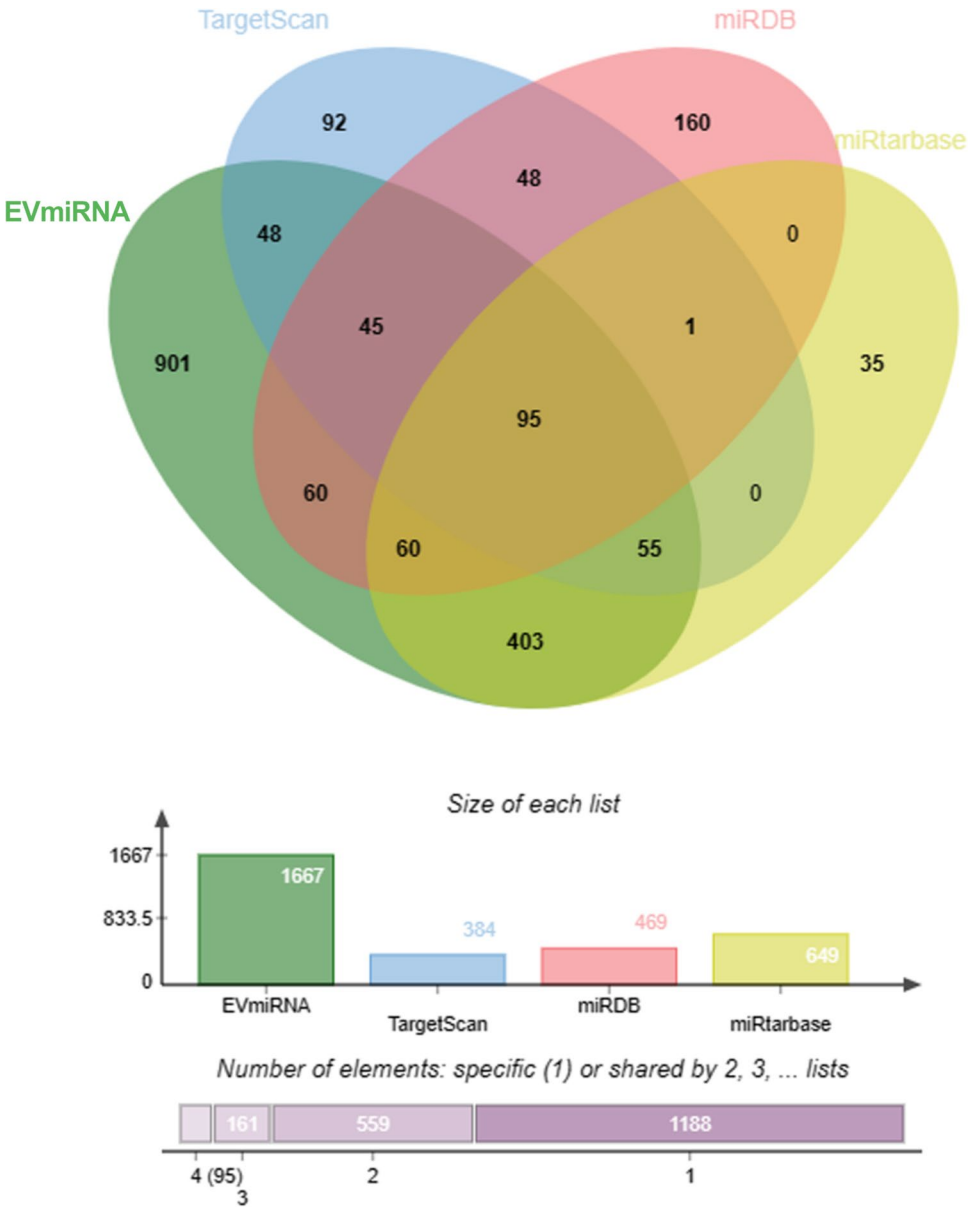

Fig. 5 The downstream genes of *miR-21-5p*

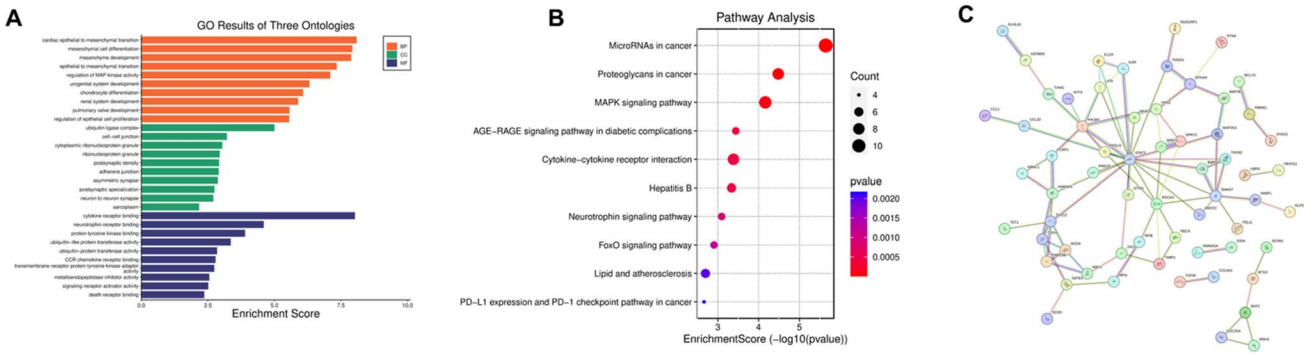

Fig. 6 The bioinformatic results. **A** The GO findings of three ontologies. **B** The top ten pathways. **C** The PPI network

Table 2 The top 10 nodes of autism spectrum disorder-related PPI network

Node	Description	Degree
STAT3	Signal transducer and activator of transcription 3	19
PDCD4	Programmed cell death protein 4	8
SMAD7	Mothers against decapentaplegic homolog 7	8
PIK3R1	Phosphoinositide-3-kinase regulatory subunit alpha/beta/delta	7
SUZ12	SUZ12 polycomb repressive complex 2 subunit	6
CBX4	E3 SUMO-protein ligase CBX4	5
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	5
AGO4	Eukaryotic translation initiation factor 2c	4
EPHA4	Ephrin type-A receptor 4	4
FRS2	Fibroblast growth factor receptor substrate 2	4

KEGG analysis recognized that pathways mainly included the *MAPK* signaling pathway, neurotrophin signaling pathway, and FoxO signaling pathway (Fig. 6B).

The PPI of target genes was constructed by the STRING database, which consisted of 140 nodes and 62 edges (Fig. 6C). The top ten nodes of this PPI network were shown in Table 2; *STAT3*, *PDCD4*, and *SMAD7* were identified as the main genes related to ASD.

Discussion

ASD is a type of neurodevelopmental disorder (Hu et al. 2020). According to the estimation of the World Health Organization, the global prevalence rate of autism spectrum disorder is 6.25‰ (Ilijoski et al. 2022). The prevalence of ASD is around four times higher in males than in females and has been increasing over time (India State-Level Disease Burden Initiative Mental Disorders Collaborators 2020). There is currently no specific medication or treatment available for the core symptoms, often leading to complications with other diseases. As a result, the prognosis for patients is generally bleak and may require lifelong care (Iles 2021). It seriously destroys the quality of patients' lives. Accordingly, identifying the underlying cause of the disease is of utmost importance.

LncRNAs could potentially act as biological markers for early detection of ASD. Several foreign experiments have been undertaken to identify biological markers linked to ASD, including complement system and synaptogenesis (Aspra et al. 2022; Mansur et al. 2021). As of yet, no definitive biological markers have been found for early clinical screening and diagnosis. Publications documented that lncRNAs were involved in ASD and other neurodevelopmental disorders. The study by Ziats et al. showed abnormal changes in LncRNAs in the brain tissues of ASD patients (Ziats and Rennert 2013). LncRNA *IFNG-as1* expression declined in children with ASD, which might be a contributing indicator to chronic inflammation of this disease (Fallah et al. 2020). *PVT1* played an important regulatory role in complex diseases where environmental and genetic factors interact (Lv et al. 2019; Tang et al. 2022b). *PVT1* might be a target for the treatment of peripheral neuropathy induced by diabetes and crush-injured sciatic nerves, promoting that *PVT1* could correlate to crush-injured sciatic nerves (Chen et al. 2018; Pan et al. 2023). In the current research, the expression of *PVT1* was decreased in ASD patients, documenting that ASD appearance might lead to inhibited *PVT1* expression. Schizophrenia is a chronic mental disorder. Safari et al. established that *PVT1* was down-regulated in Schizophrenic patients, lending a piece of evidence that *PVT1* played an inhibitory role in mental disorder diseases (Safari et al. 2019). *MiR-21-5p* is an attractive target in various conditions due to its critical role in many biological functions and diseases, including nerve injury, ionizing radiation, and malignancies (Liu et al. 2022b; Mahmoudi et al. 2022; Singh et al. 2021). The genome of *miR-21-5p* is located on chromosome 17 (17q.23.1) in the intron region of *TMEM49* gene (Tang et al. 2022a). The outcome of this research indicated enhanced expression of *miR-21-5p* in ASD patients, declaring its association with ASD occurrence. Additionally, the

negative relationship between *miR-21-5p* and *PVT1* was observed in ASD patients, further suggesting their close interaction in ASD.

It is acknowledged that lncRNA and miRNA have the potential as biomarkers of disease diagnosis and prognosis. Cui et al. confirmed that *PVT1* had potential diagnostic value in early lung cancer, and the overexpression of *PVT1* in cancer tissues indicated a poor prognosis (Cui et al. 2016). In a publication on clinical engagement in sepsis, *PVT1* revealed the possibility of acting as a predictive marker in sepsis patients (Chen et al. 2022a). A study has confirmed that in patients with schizophrenia, *miR-21-5p* was linked with the development of schizophrenia and could function as a biomarker for schizophrenia patients (Liu et al. 2017). The results of ROC curve analysis in this study showed that *PVT1* and *miR-21-5p* displayed comparable diagnostic values in children with ASD. Additionally, the combined ROC curve of *PVT1* and *miR-21-5p* demonstrated superior diagnostic capability. Therefore, it was conceivable that the diagnostic value of the combination of the *PVT1* and *miR-21-5p* was more satisfactory to that of single diagnostic approach. Although the diagnostic value of *PVT1* has been substantiated through experimental methods, and the combination of *PVT1* and *miR-21-5p* has been preliminarily confirmed for ASD, the specific mechanism of *PVT1* and *miR-21-5p* in ASD and how they regulate the disease remain elusive. Future studies should be performed to verify the results of this study.

LncRNAs usually serve as the "miRNA sponge" to inhibit the expression of miRNAs, thereby exerting a fundamental regulatory effect. In diabetic foot ulcer and hypoxia/reoxygenation injury, *PVT1* sponged *miR-21-5p*, suggesting the targeted relationship between them (Chen et al. 2022b; Wu et al. 2021). In the sequence test of ASD brain specimens, the expression of *miR-21-5p* was overexpressed, and it might exert a biological function in the brain of ASD (Mor et al. 2015). Thus, miR-21-5p is selected as the target of PVT1. Gill et al. found that increasing the expression level of *miR-21-5p* was verified in ASD using sequencing profiles, involving the proliferation and death of neuronal cells (Gill et al. 2022). The targeted interconnection between *miR-21-5p* and *PVT1* was identified by the luciferase reporter test, which showed *miR-21-5p* was a ceRNA of *PVT1*. In this article, we predicted the targets of *miR-21-5p* and found 95 shared targets. The bioinformatic analysis was used to support the function of these targets. Through the GO enrichment method, the targets were mainly enriched in asymmetric synapse, post-synaptic specialization, neuron to neuron synapse, and neurotrophin receptor binding. The synapse was composed of asymmetric synapse, post-synaptic specialization, neuron to neuron synapse, which is the foundation of information exchange and schizophrenia pathophysiology (Zhang et al. 2023). The neurotrophin receptor binding was essential in nerve growth (Conroy and Coulson 2022). Briefly, the targeted genes might participate in ASD by regulating the above-mentioned biological elements or functions. Through KEGG analysis, the targets participated in the *MAPK* signaling pathway and neurotrophin signaling pathway. The *MAPK* signaling pathway was the top enrichment pathway, which is correlated to ASD by modulating neuronal physiology (Vithayathil et al. 2018). The neurotrophin signaling pathway is a cell signaling mechanism that plays a crucial role in the development, plasticity, and repair of the nervous system (Liang et al. 2019). *STAT3*, *PDCD4*, and *SMAD7* were the main genes in the PPI network. Among these genes, *STAT3* takes part in ASD by regulating relative pathways, *PDCD4* regulates the central nervous system, and *SMAD7* may influence the glucose metabolism in neurons (Di Paolo et al. 2020; Li et al. 2022; Yuan et al. 2020).

To sum up, this study confirmed the declined expression of *PVT1* in the serum of children with ASD, while the expression of *miR-21-5p* was enhanced, and the level of *miR-21-5p* was raised with the decrease of *PVT1* level. ROC curve proved the clinical value of *PVT1* and *miR-21-5p* as biomarkers in the diagnosis of diseases. In addition, *PVT1*/*miR-21-5p* axis might regulate neuropathologic function, thus relating to ASD.

Author Contribution All the authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Mingjun Jiang and Guanwen Chen. The first draft of the manuscript was written by Mingjun Jiang, and all the authors commented on the previous versions of the manuscript. All the authors read and approved the final manuscript.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. The Ethics Committee of Shenzhen Polytechnic University provided the approval for this research.

Consent to Participate The guardian of the study knew and signed the informed consent form before joining the study.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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