Whole-transcriptome analysis of serum neuron-derived exosomes reveals specific signature for autism spectrum disorder in Chinese children

Yannan Qin  
Xian Jiaotong University: Xi’an Jiaotong University

Li Cao  
Xian Jiaotong University: Xi’an Jiaotong University

Haiqing Zhang  
Xi’an Children’s Hospital

Shuang Cai  
Xian Jiaotong University: Xi’an Jiaotong University

Jinyuan Zhang  
Xi’an Jiaotong University

Bo Guo  
Xi’an Jiaotong University

Fei Wu  
Xi’an Jiaotong University

Lingyu Zhao  
Xi’an Jiaotong University

Wen Li  
Xi’an Jiaotong University

Lei Ni  
Xi’an Jiaotong University

Liying Liu  
Xi’an Jiaotong University

Xiaofei Wang  
Xi’an Jiaotong University

Yanni Chen  
Xi’an Children’s Hospital

CHEN HUANG  
Xi’an Jiaotong University  
https://orcid.org/0000-0002-4810-7160

Research
Abstract

Background

Autism spectrum disorder (ASD) is a prevalent developmental disorder that appears in early childhood and manifests repetitive behavioral and social deficits. Reported biomarkers for ASD cannot directly and specifically reflect the abnormality of brain neurons which are preferentially affected and correlated with clinical severity of the disease. Neuron-derived exosomes (NDEs), which carry proteins, lipids and nucleic acids can reflect a variety of brain diseases. However, little research has been reported about NDEs in children with ASD.

Methods

Serum samples were collected from 100 ASD children and 60 age-matched typically developed (TD) children, and were pooled into 5 ASD groups and 3 TD groups (n=20). NDEs from the pooled sera in each group were isolated using L1CAM antibody mediated immunosorbent assay and characterized by nanoparticle tracking analysis, transmission electron microscopy and western blot. Whole-transcriptome of NDEs was detected by IncRNA microarray and RNA-Sequencing. RNAs expressed differently in NDEs from ASD sera as versus those from TD sera were screened, analyzed, and validated.

Results

Serum NDEs were isolated successfully. A total of 1418 mRNAs, 1745 IncRNAs and 11 miRNAs were identified to be differentially expressed, and most of them were down-regulated in ASD. The differentially expressed RNAs were prone to be distributed on chromosome 17. Target gene prediction and bioinformatic analysis revealed that a large portion of RNAs were involved in neuron- and glycan-related networks implicated in ASD. qRT-PCR identified that the levels of EDNRA, SLC17A6, HTR3A, OSTC, TMEM165, PC-5p-139289_26, and hsa-miR-193a-5p changed significantly in ASD, indicating them as potential biomarkers for the disease.

Limitations

The correlation between the expression of candidate biomarkers and disease severity were not examined. Some of our participants were not medication-free. Samples were mainly from a single hospital.

Conclusions

Whole-transcriptome analysis of serum NDEs reveals specific signature for ASD in Chinese children, which may help detect predictive biomarkers and molecular mechanisms of ASD, and provide reference for diagnoses and therapeutic management of the disease.

Background
Autism spectrum disorder (ASD) is a term for a set of early-appearing social communication deficits and repetitive sensory-motor behaviours associated with a strong genetic component as well as other causes [1]. The prevalence of ASD is region-specific, varying from 0.9 case out of 1000 children in India to 1 case within 59 children in the US [2]. In China, the prevalence is now around 1% [3]. Screening for ASD is the first step toward early detection and diagnosis, thereby impacting the likelihood of patients accessing early intervention and, importantly, improving long-term outcomes. Currently, the clinical diagnosis of ASD is still based on the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (9), which is focused on assessing patient behaviors, but it lacks quantizable indicators and cannot accurately diagnose mild or non-typical autism or that in very young patients. There is a need to find useful and reliable biomarkers to assist the diagnosis of autism.

In the past decade, searching for genetic biomarkers has been a hot spot in ASD research. Numerous related genes have been reported, including NRXN1, SHANK3, SHANK2, MECP2, SNC2A, CHD8, DYRK1A, POGL, GRIN2B, KATNAL2, NLGN3, NLGN4, CNTN4, CDH10, CDH9, and SEMA5A [4–6]. Unfortunately, only 10–38% of ASD cases have been reported with known genetic deficits [7, 8]. In recent years, blood/serum biomarkers have drawn much attention due to their accessibility, low cost and rapid detection. In our previous study, we identified four candidate peptides as biomarkers, namely SerpinA5-A, ApoC1-A, FABP1-A and PF4-A [9], and α2-3-linked sialylation of apolipoprotein D (APOD) [10] as potential biomarkers for ASD. A recent study also showed that SLC25A12, LIMK1, and RARS might serve as potential blood protein biomarkers for ASD [11]. However, the biomarkers that have been reported cannot specifically reflect the abnormality of brain neurons which are preferentially affected in autism and the dysregulation of specific genes in neurons correlated with clinical severity [12, 13].

Exosomes are small, single-membrane, secreted extracellular vesicles of ∼30 to ∼200 nm in diameter that have the same topology as the cell and are enriched in selected proteins, lipids, nucleic acids and glycoconjugates. In the central nervous system, almost all types of cells secrete exosomes, which mediate neuron-glial cell communication, promote neuronal repair and growth, and promote the progression of glioblastoma and neurological diseases [14]. Exosomes are very stable in terms of constitution and protect the "biological cargo" they carry from degradation and denaturation in the extracellular environment. Compared with biological fluids, such as cerebrospinal fluid, blood or urine, exosomes can provide more reliable and accurate biomarkers for neurological diseases [14–16]. More importantly, they can cross the blood-brain barrier and have low immunogenicity. Overall, they are a promising source for biomarkers and ideal vehicles for drug delivery, which might be widely used in the diagnosis and treatment of neurological diseases [16]. Currently, it is found that secreted extracellular vesicles increase in the serum of children with ASD and contain IL-1β that stimulates secretion of human microglia cells [17]. Mesenchymal stem cell-derived exosomes can improve autism-like behavior in BTBR mice and may be a cell-free therapeutic tool for ASD [18, 19]. These findings uncover important roles of exosomes, suggesting the necessity of characterizing the detailed molecular status of brain-derived exosomes in ASD.
Recent studies have shown that the surface of exosomes derived from neurons carries neural cell adhesion molecule L1 (L1CAM) that can be utilized to isolate the exosomes from serum/plasma; proteins in neuron-derived exosomes (NDEs) can reflect brain injury, progression from acute mild traumatic brain injury to chronic traumatic brain disease, cognitive dysfunction caused by HIV infection, and neurological abnormalities such as Alzheimer's disease [20, 21]. However, the expression of proteins or RNAs in NDEs of children with ASD is rarely reported.

It is well known that exosomal miRNAs can be used as potential diagnostic and prognostic biomarkers as well as therapeutic tools for a variety of neuropsychiatric diseases, such as dementia, Alzheimer's disease, depression, and schizophrenia [22]. In light of this, the present study was conducted to examine the expression of RNAs in NDEs from children with ASD and to reveal the possible mechanisms of the disease. We collected serum samples from 100 ASD children and 60 age-matched typically developed (TD) children, and pooled the samples into groups (n = 20). NDEs of the pooled sera in each group were isolated using L1CAM antibody mediated immunosorbent assay [20, 21] and were characterized by nanoparticle tracking analysis, transmission electron microscopy and western blot. Whole-transcriptome of the NDEs was analyzed by lncRNA microarray and RNA-Sequencing. RNAs expressed differently in NDEs from ASD sera as versus those from TD sera were screened, analyzed, and validated. In brief, a total of 1418 mRNAs, 1745 lncRNAs and 11 miRNAs were found differentially expressed. Most of these RNAs were down-regulated in ASD and enriched in neuron-related and glycan-related networks associated with ASD. Levels of some potential markers were found significantly changed in ASD.

Methods

Study Approval

Approval for this research was obtained from the Ethics Committee of Xi’an Jiaotong University (Xi’an, China). All parents of the participants signed written informed consent. The experiments were carried out in accordance with the ethical guidelines of the Declaration of Helsinki.

Subjects

The study enrolled 100 children with ASD (between 2.5 and 6 years of age; 90 males) and 60 age-matched TD children (54 males) as control. The ASD children were recruited from Xi’an Children’s Hospital, Xi’an, China. The healthy children were recruited from the same region to minimize the influence of different environments. The ASD children were examined by a developmental behavioral pediatrician and a pediatric neurologist or psychiatrist. All the consultants agreed on the diagnosis of ASD according to DSM-V criteria. Children with tuberous sclerosis complex, Rett syndrome, Prader Willi syndrome, Angelman syndrome, or Fragile X syndrome were excluded. All the participants were screened via a parental interview for current and past physical illnesses. Those who had any type of infection or disease within two weeks before the time of examination were excluded. ASD was evaluated with the autism diagnostic observation schedule (Table 1).
Collection and preparation of serum samples

Venous blood samples were collected by a pediatric nurse. The blood was allowed to clot at room temperature for 30 min, and the clot was then removed by centrifuging at 1,500×g for 10 minutes. The resulting supernatant is immediately transferred to a clean polypropylene tube, and EDTA-free inhibitor cocktail (Halt protease inhibitor, Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) was added at a concentration of 10 μL/mL serum. The obtained serum was aliquoted into small portions and was immediately frozen on dry ice and stored at −80 °C. To tolerate individual variation, 25 μL of each serum sample was collected and every 20 samples were pooled into one subgroup. Altogether, we got 5 ASD subgroups and 3 TD subgroups (n=20). To avoid bias caused by gender difference, proportion of males in each subgroup was the same (90%). The remaining serum in each sample was maintained for further individual validation.

Isolation of serum neuron-derived exosomes (NDEs)

NDEs in the serum samples were isolated as described previously [20,21] with minor modifications. Briefly, 0.5 ml of serum was incubated with 0.15 ml of thromboplastin-D (Fisher Scientific, Inc., Hanover Park, IL) at room temperature for 60 min. Then 0.5 ml of calcium- and magnesium-free Dulbecco's balanced salt solution (DBS−2) with protease inhibitor cocktail (Roche Applied Sciences, Inc., Indianapolis, IN) and phosphatase inhibitor cocktail (Pierce Halt, Thermo Scientific, Inc., Rockford, IL) was added and the mixture was centrifuged at 1,500×g for 20 min. The supernatant was mixed with 252 μl of ExoQuick exosome precipitation solution (EXOQ; System Biosciences, Inc., Mountainview, CA), and incubated for 1 hr at 4°C. The resultant exosome suspension was centrifuged at 1,500×g for 30 min at 4°C and the pellet was re-suspended in 150 μl of DBS−2 with the inhibitor cocktails before immunochemical enrichment of NDEs. Each sample received 100 μL of 3% BSA (1:3.33 dilution of Blocker BSA 10% solution in DBS−2 [Thermo Scientific, Inc.]) and was incubated with 1 μg of mouse anti-human CD171 (L1CAM neural adhesion protein) biotinylated antibody (clone 5G3, eBioscience, San Diego, CA) for 1 hr at 4°C. Following that, 25 μl of streptavidin-agarose resin (Thermo Scientific, Inc.) plus 50 μL of 3% BSA was added and the sample was incubated at 4°C for 30 min. After centrifugation at 200×g for 10 min at 4°C and removal of the supernate, 3% BSA was added again, and centrifugation and supernatant removal were repeated. Each pellet was suspended in 50 μl of 0.05 M glycine-HCl (pH 3.0), incubated at 4°C for 10 min, and re-centrifuged at 4,000×g for 10 min at 4°C. The obtained supernatant was transferred to a new Eppendorf tube containing 5 μL of 1 M Tris-HCl (pH 8.0) and stored at −80°C.

Nanoparticle tracking analysis (NTA)

NDEs suspension at a concentration between 1 x 10⁷/ml and 1 x 10⁹/ml was examined using a Nanosight NS300 (NanoSight Ltd., Amesbury, UK), equipped with a 405 nm laser to determine the size and quantity of particles isolated.

Transmission electron microscopy (TEM)
NDEs solution (20-40 μl) was placed on a copper mesh, post-negatively stained with 2% phosphotungstic acid for 10 min, and then dried for 2 min under incandescent light. The copper mesh was observed and photographed under a transmission electron microscope (H-7650 Hitachi microscope; Hitachi, Tokyo, Japan).

**Western blot analysis**

The NDEs suspension was denatured in 5X sodium dodecyl sulfonate (SDS) buffer and subjected to western blot analysis (10% SDS-polyacrylamide gel electrophoresis; 50 μg protein/lane) using mouse monoclonal antibody CD63 (ab59479), mouse monoclonal antibody CD81 (ab79559) and rabbit polyclonal antibody L1CAM (ab232894; all from Abcam, Shanghai, China). The proteins were visualized on the chemiluminescence detection Syngene GBox (Syngene Europe).

**Extraction of total RNA in NDEs**

Total RNA in NDEs was isolated using the Exosomal RNA isolation kit (Norgen Biotek, 58000) according to the manufacturer’s instructions. Briefly, 200 μl of the transferred supernatant containing purified NDEs was incubated with 300 uL Lysis Buffer A and 37.5 uL Lysis Additive B at room temperature for 10 min, following which 500 uL of 96-100% Ethanol was added to the mixture and mixed well via 10-second vortexing. Then, 500 uL of the mixture was transferred into a Mini Spin column and centrifuged at 3,000×g for 1 min, and the remaining mixture was transferred and centrifuged by repeating the steps. After that, 600 uL Wash Solution A was applied and the column was centrifuged at 3,300×g for 30 seconds twice. The spin column was then moved to a fresh 1.7 mL Elution tube, and 50 uL Elution Solution A was added. Finally, centrifugation was performed at 400×g for 1 min and 5,800×g for 2 min to obtain total RNA.

**Human IncRNA microarray and data analysis**

Total RNA was purified using a RNeasy Mini Kit (Qiagen, Germany) and was checked for a RIN number to inspect RNA integration with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). LC Biotech Human IncRNA Microarray 4×180 K (Agilent Technologies; Santa Clara, CA) was utilized to detect the expression of mRNAs and IncRNAs in NDEs. The microarray slide contains 26,083 mRNA probes and 1,05,135 IncRNA probes, and IncRNA sequencing data are available from Gencode, UCSC, Ensembl, Refseq, LNCipedia, NONCODE, LncRNA Disease, Ernas, NRED and other databases. Amplification of cRNA, fluorescent labeling and hybridization of the microarray were performed by following the protocol of Agilent Technologies. Briefly, equal amount of RNA from each subgroup was reversely transcribed into cDNA, which was then labeled with Cy3 (GE Healthcare; Biosciences, Piscataway, NJ, USA) and hybridized with the microarray slide. After that, the slide was scanned on the Agilent Microarray Scanner G5761A (Agilent Technologies). Data were extracted with Feature Extraction software 12.0.3.1 (Agilent Technologies), and raw data were normalized by Quantile algorithm. Genes with \( p \) value<0.05 and a fold change of at least 2 were selected for further analysis. GO/KEGG pathway enrichment analyses of the
target genes were performed using Fisher's exact test. The function of IncRNAs was predicted by analyzing the functional annotations of mRNAs that were highly co-expressed with IncRNAs.

**Small RNA library construction, sequencing and data processing**

Approximately 1 ug total RNA was used to prepare small RNA library according to the protocol of TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, USA). Single-end sequencing (36 bp) was performed with an Illumina HiSeq2500 at LC-BIO (Hangzhou, China). Briefly, the raw reads were subjected to the Illumina pipeline filter (Solexa 0.3), and the dataset was further processed with an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA), to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with a length of 18~26 nucleotides were mapped to Homo species precursors in miRBase 20.0 by BLAST search to identify known miRNAs and novel 3p- and 5p- derived miRNAs. The hairpin RNA structures containing the sequences were predicted from the flank 80 nt sequences using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). miRNA differential expression based on normalized deep-sequencing counts was analyzed using the Fisher exact test and Student t test, and the significance threshold was set to be 0.01 or 0.05. To predict the genes targeted by most abundant miRNAs, two computational target prediction algorithms (TargetScan 50 and miRanda 3.3a ) were used to identify miRNA binding sites. Finally, the data predicted by both algorithms were combined and the overlaps were calculated. The GO terms and KEGG pathways of these most abundant miRNAs, miRNA targets were also annotated.

**Quantitative real-time PCR**

All primers were designed and synthesized by Takara (TakaraBiotechnology, Dalian, China). To avoid false-positive amplification of contaminating genomic DNA in the mRNA samples, all the primers spanning different exons were designed (Table 2). For mRNA, cDNA was synthesized using a PrimeScript RT reagent kit (Takara Biotechnology Co, Ltd, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed using the IQ5 real-time PCR detection system, and GADPH was taken as a control. Relative quantification of mRNA expression levels was performed using SYBR Premix Ex Taq II on an FTC-3000TM System (Funglyn Biotech Inc., Toronto, Canada). For miRNA cDNA was synthesized by servicebio RT First strand cDNA Synthesis Kit (Servicebio, Wuhan, China), qRT-PCR was carried out using the SYBR Premix Ex Taq™ II (TaKaRa) and U6 was taken as a control. PCR conditions consisted of a 5 min preincubation at 95°C, followed by 40 repeats of 95°C for 10 s and 60°C for 20 s. All samples were run in triplicate and the average values were calculated. The relative levels of mRNAs EDNRA, SLC17A6, HTR3A, OSTC and TMEM165, as well as miRNAs PC-5p-139289_26 and hsa-miR-193a-5p were calculated using the $2^{-\Delta\Delta C_t}$ method.

**Statistics**

All statistical analyses were performed using the software SPSS (version 17) and group statistics are presented as mean ± SD. The t-test for independent variables was used to examine the inter-group
differences, and a significance level of 0.05 was adopted.

Results

Characterization of serum NDEs

Basic characteristics of the participants were shown in Table 1. NDEs in the pooled sera in the ASD group (including 5 subgroups) and the TD group (including 3 subgroups) were isolated using L1CAM antibody mediated immunoadsorption (Fig. 1). Nanoparticle tracking analysis showed a higher NDE concentration in the ASD group ($2.04 \pm 4.35 \times 10^{10}$/ml) than in the TD group ($1.20 \pm 3.28 \times 10^{10}$/ml). The average particle size of NDEs was $61.50 \pm 20.71$ nm in the ASD group and $62.07 \pm 20.75$ nm in the TD group, showing no significant difference (Fig. 2A). Under TEM, both groups of NDEs presented a "saucer" like structure (Fig. 2B). Meanwhile, compared with serum, the obtained NDEs solution was rich in exosomal-specific marker proteins CD63 and CD81 (Fig. 2C). L1CAM expression was higher in NDEs than in total serum exosomes (Fig. 2D).

Differential expression and bioinformatic analysis of mRNAs in serum NDEs of ASD

Based on IncRNA microarray detection and original data normalization, air-wise Pearson’s correlation coefficients of all RNAs among subgroups were shown in Fig. 3A. The coefficients between the biological replicates (subgroups) within each group were obviously higher than those between the two groups (Fig. 3A). mRNAs and IncRNAs with at least twofold differential expression and a P value of less than 0.05 were subjected to further examination. This resulted in 167 up-regulated and 1251 down-regulated mRNAs in ASD serum NDEs (Fig. 3B and Supplemental Table 1). Hierarchical clustering analysis (HCA) of these 1418 differentially expressed mRNAs (DEmRs) showed similar expression profiles among the biological replicates within each group but differential profiles between the two groups (Fig. 3C). Principal component analysis (PCA) of the DEmRs showed that the two groups were separated and the biological replicates in the TD group clustered more closely than those in the ASD group (Fig. 3D). To characterize the distribution of genes for DEmRs on chromosomes and to reveal the susceptible chromosomes, the genes on each chromosome were counted and the ratio of the number of such genes to the total number of genes present on the chromosome (data from Human Genome Resources at NCBI, GRCh37) was calculated. As a result, chromosomes 1 and 2 had the largest number of genes for DEmRs, while chromosomes 21 and Y had the least number of such genes. However, genes with the maximum ratio were on chromosomes 17 and 5, and those with the minimum ratio were on chromosomes 13 and Y (Fig. 3E). GO annotation of DEmRs showed that their products were mainly distributed in cytoplasm, nucleus and plasma membrane; were able to bind with proteins, metal ions and DNA; and were involved mainly in DNA-dependent transcription, small molecule metabolism, transcriptional regulation and other biological processes (Fig. 3F). KEGG analysis revealed that DEmRs participated in mainly three types of processes: (1) the signal transduction processes, such as MAPK signaling pathway, calcium signaling
pathway, PI3K-Akt signaling pathway and cAMP signaling pathway; (2) neuron-related pathways such as
neuroactive reception-ligand interaction, axon guidance and synaptic vesicle circulation; and (3)
glycosylation related pathways such as N-glycan biosynthesis, endoplasmic reticulum protein processing,
sugar binding and glycosaminoglycan degradation (Fig. 3G).

Differential expression and functional prediction of lncRNAs in serum NDEs of ASD

According to the results of lncRNA microarray, 239 lncRNAs were significantly up-regulated and 1506
lncRNAs were significantly down-regulated in ASD serum NDEs (Fig. 4A and Supplemental Table 2). HCA
of these 1745 differentially expressed lncRNAs (DElnRs) showed similar expression profiles among the
biological replicates within each group but differential profiles between the two groups (Fig. 4B). PCA of
the DElnRs separated the subgroups into TD and ASD groups as their natural grouping (Fig. 4C). To
characterize the distribution of DElnRs on chromosomes and to reveal the susceptible chromosomes, the
DElnRs on each chromosome were counted and the ratio of the number of DElnRs to the length of that
chromosome was calculated (data from Human Genome Resources at NCBI, GRCh37; the unit of length
is Mbp). It was found that chromosomes 1 and 2 had the largest number of DElnRs and chromosomes
21 and Y had the least number of DElnRs; whereas, chromosomes 19 and 17 had the maximum ratio and
chromosomes X and Y had the minimum ratio (Fig. 4D). Genes with a distance of less than 100kb from
lncRNA were regarded as the target genes for cis-acting. As a result, 382 DElnRs were predicted to be
positively or negatively correlated with their target genes (R > 0.8) (Supplemental Table 3). Of these genes,
107 were also DEmRs (Fig. 4E and supplemental Table 3). Double-omic analysis
(https://www.omicstudio.cn/tool) of the 107 pairs of DElnR-DEmR revealed that 81.3% pairs were
positively correlated and commonly down-regulated in ASD (Fig. 4F). GO annotation showed that these
107 target genes were mainly cytoskeleton-related proteins (Fig. 4G) with functions such as auxiliary
transport protein activity, protein binding and translation regulation (Fig. 4H), and were involved in protein
metabolism, transport, and cell growth and/or maintenance (Fig. 4I).

Neuron-related network and significant markers in ASD

Notably but not unexpectedly, 104 DEmRs were related to neuron, of which 19 were up-regulated (e.g.,
EDNRA) and 85 (e.g., SLC17A6 AND HTR3A) were down-regulated (Fig. 5A). For lncRNAs, the expression
levels of SNHG1, Inc-C20orf201-1 and Inc-TRPV5-1 were positively correlated with their target genes,
namely CHRM1, OPRL1 and EPHB6 (Fig. 5B). According to GO and KEGG analyses, these neuron-related
RNAs were mainly involved in neuroactive ligand-receptor interaction (e.g., ADORA2A and EDNRA),
pathways of neurodegeneration (e.g., CHRM1 and SNHG1 [lncRNA]), glutamatergic synapse (e.g., GRIA4
and HOMER2), axon guidance (e.g., EPHB6 and Inc-TRPV5-1 [lncRNA]), synaptic vesicle cycle (e.g.,
ATP6V1G1 and SLC17A6), dendrite formation (e.g., TENM2 and IGSF9B), neuron projection development
(e.g., OPRL1 and Inc-C20orf201-1 [lncRNA]), neuron migration (e.g., BARHL1 and DISC1), neuron
apoptotic process (e.g., CLN3 and BCL2L11), calcium signaling pathway (e.g., ADORA2A and CCKAR), as
well as MAPK signaling pathway (e.g., ERBB3 and TNFRSF1A). All of these processes formed a neuron-related signaling network implicated in the molecular mechanisms of ASD (Fig. 5C). The most enriched pathway was neuroactive ligand-receptor interaction, involving 5 up-regulated mRNAs (e.g., EDNRA) and 19 down-regulated mRNAs (e.g., HTR3A) in ASD (Fig. 5D). To validate the differential expression of these RNAs, EDNRA, SLC17A6 and HTR3A were randomly selected and examined by qRT-PCR in individual serum samples. As a result, the level of EDNRA was significantly higher in ASD than in TD \( (p = 0.001) \), while those of SLC17A6 \( (p = 0.03) \) and HTR3A \( (p = 0.007) \) were significantly lower (Fig. 5E). These were consistent with the results of IncRNA microarray.

**Glycan-related network and candidate markers in ASD**

Most eukaryotic proteins are modified by covalent addition of glycan molecules, which modulates the structures and functions of the proteins. Glycans are synthesized in the presence of glycosyltransferases, sulfonyltransferases, glycosidases and other glycan-binding proteins; aberrant expression of these enzymes may result in many complicated pathological conditions, such as inflammation, diabetes, cancer and neurological abnormality [23, 24]. According to IncRNA microarray and bioinformatic analysis, 54 DEmRs were carbohydrate-related genes, of which 5 (e.g., HPSE, and GALM) were significantly up-regulated and others (e.g., OSTC and MAN1B1) were significantly down-regulated in ASD (Fig. 6A). DElnRs including LOC101927919, Inc-KIAA1919-1, Inc-SHCBP1L-1 and RP11-177J6.1 were positively correlated with their predicted target genes NUS1, KIAA1919, NPL and TMEM165, respectively (Fig. 6B). Based on GO and KEGG analysis, these DEmRs and DElnRs were mainly involved in carbohydrate metabolic process (e.g., HPSE and SLC37A4), protein N-linked glycosylation (e.g., MGAT5 and OSTC), carbohydrate binding (e.g., MSIGLEC5 and CLEC1A), glycolysis (e.g., GALM and PGM1), glycosaminoglycan metabolic process (e.g., CHST12 and HPSE), and glycolipid metabolic process (e.g., NEU1 and ST8SIA6) (Fig. 6C). Expressions of OSTC, MAN1B1 and MGAT5 were down-regulated in N-glycan biosynthesis implicated in ASD (Fig. 6D). To validate the differential expression of these RNAs, OSTC and TMEM165 of NDEs were randomly selected and examined by qRT-PCR in individual serum samples. The results showed that the levels of OSTC and TMEM165 were significantly lower in the ASD children \( (p = 0.001 \text{ and } p = 0.02) \) (Fig. 6E).

**Expression of miRNA in serum NDEs**

A total of 4310 mature microRNAs (miRNAs) were examined in the serum NDEs, among which 150 were present in all subgroups (Supplemental Table 4). The miRNA with the highest concentration across all the subgroups was hsa-miR-21-5p_R + 1, accounting for 6.9% in TD and 8.5% in ASD in terms of the normalized read counts (Fig. 7A). Unpaired two-tailed Student’s t test identified 10 miRNA sequences with significant differences \( (p < 0.05) \) between ASD and TD (Fig. 7B). The sequence GATTTCTTCCAGTGCTCTGA was aligned to two pre-miRNAs and was given two names: mmu-mir-6240-p3_1ss8GT and mmu-mir-6240-p5_1ss8GT. Of these 11 miRNA, the one with the biggest variation was PC-3p-38497_124, which was remarkably up-regulated (fold change = 20.32, \( p = 0.029 \)) in ASD relative to TD (Fig. 7B). The miRNA with the most significant change was PC-5p-139289_26, which was absent in
the TD subgroups \((p = 0.0056)\) (Fig. 7B). Two other miRNAs (PC-3p-275123_15 and PC-5p-149427_24) were up-regulated in ASD, and another seven miRNAs (e.g., hsa-miR-193a-5p and mmu-mir-6240-p3_1ss8GT) were relatively down-regulated (Fig. 7B). HCA of these differentially expressed miRNAs (DEmiRs) revealed similar expression profiles among the biological replicates within each group but differential profiles between the ASD and TD groups (Fig. 7C). PCA showed that the biological replicates in the TD group clustered more closely (Fig. 7D).

Targets of these 11 DEmiRs were predicted by both TargetScan 50 and miRanda 3.3a algorithms. According to the results of IncRNA microarray, 25 of the predicted targets were DEmRs and 45 were DElnRs (Fig. 7E and Supplemental Table 5). Notably, 86.2% of the DEmRs and 71.1% of the DElnRs were down-regulated in serum NDEs of ASD. Two miRNAs, namely PC-5p-139289_26 and hsa-miR-193a-5p, had the largest number of predicted targets with high confidence (TargetScan score > 90). For hsa-miR-193a-5p, 3 mRNAs (PIGZ, CEBPG and NETO2) were down-regulated and 2 (PAQR3 and OMD) were up-regulated, while 14 IncRNAs (e.g., CTC-340A15.2 and Inc-CLDN20-3) were down-regulated and 3 (ENST00000532430, CAND1.11 and EPS15L1) were up-regulated in the serum NDEs of ASD (Fig. 7F). For PC-5p-139289_26, 11 mRNAs (e.g., ING3 and BRD7) were down-regulated and 2 (EDNRA and SCUBE3) were up-regulated, while 11 IncRNAs (e.g., RP11-401P9.1 and LOC400043) were down-regulated and 4 (e.g., RSU1P2 and LOC101927027) were up-regulated in ASD (Fig. 7F). GO annotation revealed that most targets were related to signal transduction (41%) and cell communication (36%). The most enriched annotation was glutathione synthesis and recycling (fold enrichment = 263). Other enriched annotations included mannose transferase activity, attachment of GPI anchor to uPAR, gamma-glutamyl cycle, prostanoid ligand receptors and eicosanoid ligand-binding receptors (fold enrichment > 80) (Fig. 7G). To validate the differential expression of these DEmiRs, PC-5p-139289_26 and hsa-miR-193a-5p were randomly selected and examined by qRT-PCR in individual serum samples. The results showed that the expression of PC-5p-139289_26 increased significantly \((p = 0.0008)\) and that of hsa-miR-193a-5p decreased significantly \((p = 0.003)\) in ASD (Fig. 6D). These results were consistent with those of the RNA sequencing.

**Discussion**

At present, the diagnosis of ASD is still based on symptom evaluation, as the underlying pathological mechanism is still unclear. There are no blood-based diagnostic tools or approved drugs for ASD. Research that identifies reliable biological markers of disease status and symptomology in ASD is therefore urgently needed. Neurobiological systems critical to social functioning are arguably the most promising biological sources for ASD biomarkers and therapeutic targets. However, existing methods for brain detection have mostly relied on autopsy or animal models, which are limited because of poor timeliness and species differences. Most cells in the nervous system, including neurons, astrocytes, oligodendrocytes and microglia, secrete exosomes under normal or pathological conditions. Exosomes can reflect the host cell proteins and nucleic acids at the time of secretion and can diffuse across the blood brain barrier into the periphery. Neuron-derived exosomes (NDEs) can be captured by antibodies directed against the cell surface proteins embedded in the vesicle membrane [20, 21]. Although
investigation of NDEs is relatively novel, attractive evidence from other fields suggests that such investigation can afford insight into the pathological mechanisms and processes associated with Alzheimer's Disease and depressive disorder [25, 26]. In the present study, serum NDEs from ASD as well as TD children were collected and characterized scrupulously. By a whole-transcriptome analysis, we screened 1418 mRNAs, 1745 lncRNAs and 11 miRNAs differentially expressed in ASD as against TD. This was validated by examining several candidate RNAs in individual samples. These validated RNAs might be potential biomarkers for ASD diagnosis, which could reflect important molecular events in neurons timely and noninvasively.

Thus far, a putative speech and language region at chromosome 7q31-q33 seems most strongly linked to autism. Cytogenetic abnormalities at the 15q11-q13 locus are fairly frequent in people with autism, and a "chromosome 15 phenotype" is described in individuals with chromosome 15 duplications [27]. Some candidate genes are considered located at chromosomes 7q22-q33 and 15q11-q13 [28], and 21 genes in chromosomal 8p region are identified as most likely to contribute to neuropsychiatric disorders and neurodegenerative disorders [29]. Variant alleles of the serotonin transporter gene (5-HTT) on chromosomes 17q11-q12 are more frequent in individuals with autism than in healthy people [28]. In addition, many mutations on NLGN4X, an X-linked cell adhesion molecule, result in ASD [30]. In the present study, chromosome 17 was the commonly and mostly enriched chromosome for both DEmrS and DElnRs in ASD. A high portion of the DEmrS on chromosome 17 participated in cell communication and signal transduction, which are essential for synapse formation and neurotransmitter release. Abnormal expression of such mRNAs implies the abnormality of these functions in ASD.

Brain-derived exosomes carry and release multiple molecules related to neuronal function and neurotransmission in the brain, which is beneficial for the reciprocal communication between neural cells (e.g., neuron − glia interactions), synaptic plasticity, neuronal development, and neuroimmune communication. In the present study, of the mRNAs differentially expressed in serum NDEs from ASD and TP children, 104 DEmrS (7.3%) were annotated to be related to neuroactive ligand-receptor interaction, pathways of neurodegeneration, glutamatergic synapse, axon guidance, synaptic vesicle cycle, dendrite, neuron projection development, neuron migration and apoptotic process. Most (81.7%) of these neuron-related mRNAs were down-regulated in ASD. As demonstrated in the pathway of neuroactive ligand-receptor interaction (Fig. 3H), 5 receptors (e.g., EDNRA) were up-regulated and 19 (e.g., HTR3A) were down-regulated in ASD. A previous study reported that neuropeptide receptor gene expression was lower in children with autism and the lower neuropeptide receptor gene expression predicted greater social impairments and greater stereotyped behaviors [31]. In the present study, 5-hydroxytryptamine receptor 3A (HTR3A) significantly decreased in the ASD serum NDEs. HTR3A is one of the receptors for 5-hydroxytryptamine (serotonin), a biogenic hormone that also functions as a neurotransmitter and a mitogen. Ample evidence suggests that levels of serotonin and serotonin transporter (SERT) increase significantly in autistic children than in gender and age-matched controls [32, 33]. It thus can be hypothesized that increase of serotonin and SERT may be a kind of cell self-help that compensates for the loss of receptors, but it needs to be experimentally confirmed in the future. Another specific signature is the decreased expression of vesicular glutamate transporter 2 (SLC17A6) in the ASD serum NDEs.
Receptors for glutamate (Glu), GRIK5, GRIK2 and GRIA4 were also down-regulated. Glu acts as an excitatory neurotransmitter at many synapses in the central nervous system. SLC17A6 mediates the uptake of Glu into synaptic vesicles at presynaptic nerve terminals of excitatory neural cells. The postsynaptic actions of Glu are mediated by a variety of receptors expressed on postsynaptic cell membrane. Emerging evidence suggests that imbalance between excitatory (Glu-mediated) and inhibitory (GABA-mediated) neurotransmission may be a common pathophysiological mechanism in ASD [34, 35]. These studies, together with the findings in the present study, suggest that reduction in the expression of Glu transporter and receptors might be the main reason for the abnormalities of Glu-mediated neurotransmission and hence a therapeutic target in ASD.

Glycans and their conjugates (glycoproteins, proteoglycans and glycolipids) are major constituents of the neural cell membrane and extracellular matrix (ECM). Glycans and glycoconjugates participate in nearly every biological process in the developing brain. A potential link between ASD and changes in glycosylation was first observed in patients with congenital glycosylation disorders (CDGs) [36]. Recent advances in genome sequencing have identified many genetic variants that occur in genes encoding glycosylated proteins (proteoglycans or glycoproteins) or enzymes involved in glycosylation (glycosyltransferases and sulfotransferases) [37, 38]. However, it remains unknown whether “glycogene” variants cause changes in glycosylation and whether they contribute to the etiology and pathogenesis of ASDs. In the present study, we analyzed the whole transcriptome of serum NDEs in ASD to screen potential biomarkers and explore the important molecular events in brain neurons of ASD children. Our results showed that a total of 54 DEmRs (3.8%) were glycogenes, and most of them (90.7%) were down-regulated in ASD. The 54 DEmRs mainly participated in carbohydrate metabolic process, protein N-linked glycosylation, carbohydrate binding, glycolysis, glycosaminoglycan metabolic process and glycolipid metabolic process. Thereinto, OSTC, MAN1B1 and MGAT5, translating to key enzymes for N-linked glycosylation, were significantly down-regulated in ASD. In our previous study, we found a significant decrease of STL binding glycans or glycoproteins that contain trimers and tetramers of GlcNAc (core structure of N-glycans) in ASD versus in TD (fold change = 0.54, \( p = 0.0057 \)) [10]. In all, no matter at the gene level, the transcription level, or the level of translation and post-translation modification, abnormalities of glycosylation and carbohydrate metabolism might be an important molecular mechanism of ASD. Moreover, the decrease of receptors and transporters of neurotransmitters may be related with the decrease of glycogenes as most of the receptors and transporters are highly glycosylated. OSTC is a subunit of the oligosaccharyl transferase (OST) complex that catalyzes the initial transfer of a defined glycan (Glc3Man9GlcNAc2 in eukaryotes) from the lipid carrier dolicholpyrophosphate to an asparagine residue within an Asn-X-Ser/Thr consensus motif in nascent polypeptide chains. In the present study, expression of OSTC significantly decreased in ASD serum NDEs, suggesting it as a candidate biomarker for ASD diagnosis.

Recent studies have shown that abnormal expression of miRNAs could be involved in the underlying pathogenesis of ASD. miRNAs are small noncoding mRNAs that regulate gene expression and are often linked to biological processes and implicated in neurodevelopment. A dozen of miRNAs, such as miRNA-125b and miRNA-132, have been observed to regulate the expression of ASD risk genes, act differently on
the morphology of the spine and synaptic plasticity in brain neurons, and participate in ASD etiopathogenesis [39]. However, compared with mRNA and IncRNA, fewer miRNAs were found differentially expressed in ASD serum NDEs in the present study. Among 11 DEmiRs, PC-5p-139289_26 was significantly up-regulated and hsa-miR-193a-5p was significantly down-regulated in ASD, and both of them had the largest number of predicted targets that were differentially expressed in ASD, indicating that these two miRNAs might play important roles in ASD. These targets were mostly involved in glutathione synthesis and recycling and mannosyltransferase activity, which are closely correlated with synthesis of Glu and glycans involved in the neuron- and glycan-related networks in ASD. However, the relationships between miRNAs and their target genes have not yet been verified.

Limitations

This study might have some limitations that merit consideration. Firstly, we did not examine the correlation between the expression of candidate biomarkers and disease severity. This would be addressed in our future research. Secondly, we collected only one blood sample per participant (due to the invasive nature of venipuncture, particularly in children), which limited our ability to assess within-individual consistency of our biological measures. Thirdly, some of our participants were not medication-free. Even though their medications were stable (for at least four weeks) before blood collection, it is possible that our results might be influenced by the medication status. Fourthly, our samples were mainly from a single hospital. Although it is one of the few famous hospitals in the northwest of China that treat ASD children from five neighboring provinces, most of its patients are still from the local regions. Further research involving participants from multiple areas would be a great addition to the present study.

Conclusions

In short, 1418 mRNAs, 1745 IncRNAs and 11 miRNAs were identified differentially expressed in the serum NDEs from the ASD children versus from the TD children. Most of these RNAs were down-regulated and involved in neuron-related and glycan-related networks implicated in ASD. Levels of potential markers, including EDNRA, SLC17A6, HTR3A, OSTC, TMEM165, C12orf49, PC-5p-139289_26, and hsa-miR-193a-5p, changed significantly in the ASD children. Whole-transcriptome analysis of serum NDEs reveals specific signature for ASD in Chinese children, which could serve as predictive biomarkers and provide information for understanding the molecular mechanisms of ASD. Hopefully, our results may provide reference for future diagnostic and therapeutic management of the disease.

Abbreviations

**ASD:** Autism Spectrum Disorder  
**NDEs:** Neuron-derived exosomes  
**TD:** Typically developed
**DSM:** Diagnostic and Statistical Manual of Mental Disorders

**APOD:** Apolipoprotein D

**L1CAM:** Neural cell adhesion molecule L1

**DBS-2:** Dulbecco’s balanced salt solution

**SDS:** Sodium Dodecyl Sulfonate

**qRT-PCR:** Quantitative real-time PCR

**HCA:** Hierarchical clustering analysis

**DEmRs:** Differentially expressed mRNAs

**PCA:** Principal component analysis

**DEInRs:** Differentially expressed IncRNAs

**DEmiRs:** Differentially expressed miRNAs

**HTR3A:** 5-hydroxytryptamine receptor 3A

**SERT:** Serotonin transporter

**SLC17A6:** Vesicular glutamate transporter 2

**Glu:** Glutamate

**ECM:** Extracellular matrix

**CDGs:** Congenital glycosylation disorders

**OST:** Oligosaccharyl transferase

**Declarations**

**Availability of data and materials**

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

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Authors' contributions

Y.Q. carried out analysis of data from lncRNA microarray and RNA sequencing, generated the graphs, and wrote the manuscript; L.C. took charge of preparation of serum samples, isolation and characterization of serum NDEs, and qRT-PCR; H.Z. collected samples from patients and summarize the clinical information of patients; S.C., J.Z. and L.Z. participated in data analysis; B.G. performed extraction of NDEs; F.W., W.L. and X.W. participated in bioinformatics analysis; L.N. and L.L. modified the draft of this paper; Y.C. performed ASD diagnosis and sample collection; and C.H. participated in the design of the project, coordination of the staff and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Xi’an Jiaotong University (Xi’an, China) provided institutional ethical approval for this study. All parents of the participants signed written informed consent.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

References

1. Lord C, Elsabbagh M, Baird G, Veenstra-Vanderweele J. Autism spectrum disorder. Lancet. 2018 Aug;11(10146):508–20. 392(.

2. Hyman SL, Levy SE, Myers SM, COUNCIL ON CHILDREN WITH DISABILITIES, SECTION ON DEVELOPMENTAL AND BEHAVIORAL PEDIATRICS. Identification, Evaluation, and Management of Children With Autism Spectrum Disorder. Pediatrics. 2020 Jan;145(1):e20193447.

3. Sun X, Allison C, Wei L, Matthews FE, Auyeung B, Wu YY, et al. Autism prevalence in China is comparable to Western prevalence. Mol Autism. 2019;10:7.

4. Weiss LA, Arking DE, Gene Discovery Project of Johns Hopkins & the Autism Consortium. Daly MJ, Chakravarti A. A genome-wide linkage and association scan reveals novel loci for autism. Nature. 2009;461:802–8.
5. Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ, et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature. 2012;485:237–41.

6. Krämer J, Beer M, Bode H, Winter B. Two Novel Compound Heterozygous Mutations in the TRAPPC9 Gene Reveal a Connection of Non-syndromic Intellectual Disability and Autism Spectrum Disorder. Front Genet. 2021 Feb;25:11:972.

7. Vorstman JAS, Parr JR, Moreno-De-Luca D, Anney RJL, Nurnberger JI Jr, Hallmayer JF. Autism genetics: opportunities and challenges for clinical translation. Nat Rev Genet. 2017 Jun;18(6):362–76.

8. Lin KZ, Liu H, Roeder K. Covariance-based sample selection for heterogeneous data: Applications to gene expression and autism risk gene detection. J Am Stat Assoc. 2021;116(533):54–67.

9. Yang J, Chen Y, Xiong X, Zhou X, Han L, Ni L, et al. Peptidome analysis reveals novel serum biomarkers for children with autism spectrum disorder in China. Proteomics Clin Appl. 2018;12(5):e1700164.

10. Qin Y, Chen Y, Yang J, Wu F, Zhao L, Yang F, et al. Serum glycopattern and Maackia amurensis lectin-II binding glycoproteins in autism spectrum disorder. Sci Rep. 2017;7:46041.

11. Yao F, Zhang K, Feng C, Gao Y, Shen L, Liu X, Ni J. Protein Biomarkers of Autism Spectrum Disorder Identified by Computational and Experimental Methods. Front Psychiatry. 2021 Feb 25;12:554621.

12. Velmeshev D, Schirmer L, Jung D, Haeussler M, Perez Y, Mayer S, Bhaduri A, Goyal N, Rowitch DH, Kriegstein AR. Single-cell genomics identifies cell type-specific molecular changes in autism. Science. 2019 May 17;364(6441):685–689.

13. Jin X, Simmons SK, Guo A, Shetty AS, Ko M, Nguyen L, Jokhi V, Robinson E, Oyler P, Curry N, Deangeli G, Lodato S, Levin JZ, Regev A, Zhang F, Arlotta P. In vivo Perturb-Seq reveals neuronal and glial abnormalities associated with autism risk genes. Science. 2020 Nov 27;370(6520):eaaz6063.

14. Fayazi N, Sheykhhasan M, Soleimani Asl S, Najafi R. Stem Cell-Derived Exosomes: a New Strategy of Neurodegenerative Disease Treatment. Mol Neurobiol. 2021 Mar 21. doi: 10.1007/s12035-021-02324-x. Epub ahead of print. PMID: 33745116.

15. Wang X, HuangFu C, Zhu X, Liu J, Gong X, Pan Q, Ma X. Exosomes and Exosomal MicroRNAs in Age-Associated Stroke. Curr Vasc Pharmacol. 2021 Feb 8. doi: 10.2174/1570161119666210208202621. Epub ahead of print. PMID: 33563154.

16. Filippone A, Praticò D. Endosome Dysregulation in Down Syndrome: A Potential Contributor to Alzheimer Disease Pathology. Ann Neurol. 2021 Feb 6. doi: 10.1002/ana.26042. Epub ahead of print. PMID: 33547827.

17. Tsilioni I, Theoharides TC. Extracellular vesicles are increased in the serum of children with autism spectrum disorder, contain mitochondrial DNA, and stimulate human microglia to secrete IL-1β. J Neuroinflammation. 2018;15(1):239.

18. Alessio N, Brigida AL, Peluso G, Antonucci N, Galderisi U, Siniscalco D. Stem cell-derived exosomes in autism spectrum disorder. Int J Environ Res Public Health. 2020;17(3):E944.
19. Perets N, Oron O, Herman S, Elliott E, Offen D. Exosomes derived from mesenchymal stem cells improved core symptoms of genetically modified mouse model of autism Shank3B. Mol Autism. 2020 Aug;17(1):65. 11.

20. Goetzl EJ, Peltz CB, Mustapic M, Kapogiannis D, Yaffe K. Neuron-derived plasma exosome proteins after remote traumatic brain injury. J Neurotrauma. 2020;37(2):382–8.

21. Goetzl EJ, Abner EL, Jicha GA, Kapogiannis D, Schwartz JB. Declining levels of functionally specialized synaptic proteins in plasma neuronal exosomes with progression of Alzheimer’s disease. FASEB J. 2018;32(2):888–93.

22. Fries GR, Quevedo J. Exosomal microRNAs as potential biomarkers in neuropsychiatric disorders. Methods Mol Biol. 2018;1733:79–85.

23. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer. 2015 Sep;15(9):540–55.

24. Kronimus Y, Dodel R, Galuska SP, Neumann S. IgG Fc N-glycosylation: Alterations in neurologic diseases and potential therapeutic target? J Autoimmun. 2019 Jan;96:14–23.

25. Neuron-related blood inflammatory markers as an objective evaluation tool for major depressive disorder: An exploratory pilot case-control study. Kuwano N, Kato TA, Mitsuhashi M, Sato-Kasai M, Shimokawa N, Hayakawa K, Ohgidani M, Sagata N, Kubo H, Sakurai T, Kanba S J Affect Disord. 2018 Nov; 240:88–98.

26. Song Z, Xu Y, Deng W, Zhang L, Zhu H, Yu P, Qu Y, Zhao W, Han Y, Qin C. Brain Derived Exosomes Are a Double-Edged Sword in Alzheimer’s Disease. Front Mol Neurosci. 2020 May 29;13:79. doi: 10.3389/fnmol.2020.00079. PMID: 32547364; PMCID: PMC7274346.

27. Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, Inoue K, Tomonaga S, Watanabe Y, Chung YJ, Banerjee R, Iwamoto K, Tanda K, Takao K, Miyakawa T, Bradley A, Takumi T. Abnormal behavior in a chromosome-engineered mouse model for human 15q11-13 duplication seen in autism. Cell. 2009 Jun 26;137(7):1235–46.

28. Muhle R, Trentacoste SV, Rapin I. The genetics of autism. Pediatrics. 2004 May;113(5):e472-86.

29. Tabarés-Seisdedos R, Rubenstein JL. Chromosome 8p as a potential hub for developmental neuropsychiatric disorders: implications for schizophrenia, autism and cancer. Mol Psychiatry. 2009 Jun;14(6):563–89.

30. Nguyen TA, Wu K, Pandey S, Lehr AW, Li Y, Bemben MA, Badger JD 2nd, Lauzon JL, Wang T, Zaghloul KA, Thurm A, Jain M, Lu W, Roche KW. A Cluster of Autism-Associated Variants on X-Linked NLGN4X Functionally Resemble NLGN4Y. Neuron. 2020 Jun 3;106(5):759–768.e7.

31. Oztan O, Jackson LP, Libove RA, Sumiyoshi RD, Phillips JM, Garner JP, Hardan AY, Parker KJ. Biomarker discovery for disease status and symptom severity in children with autism. Psychoneuroendocrinology. 2018 Mar;89:39–45.

32. Abdulamir HA, Abdul-Rasheed OF, Abdulghani EA. Serotonin and serotonin transporter levels in autistic children. Saudi Med J. 2018 May;39(5):487–94.
33. Meyyazhagan A, Balasubramanian B, Easwaran M, Alagamuthu KK, Shanmugam S, Kuchi Bhotla H, Pappusamy M, Arumugam VA, Thangaraj A, Kaul T, Keshavarao S, Cacabelos R. Biomarker study of the biological parameter and neurotransmitter levels in autistics. Mol Cell Biochem. 2020 Nov;474(1-2):277–84.

34. Rojas DC. The role of glutamate and its receptors in autism and the use of glutamate receptor antagonists in treatment. J Neural Transm (Vienna). 2014 Aug;121(8):891–905.

35. Horder J, Petrinovic MM, Mendez MA, Bruns A, Takumi T, Spooren W, Barker GJ, Künnecke B, Murphy DG. Glutamate and GABA in autism spectrum disorder—a translational magnetic resonance spectroscopy study in man and rodent models. Transl Psychiatry. 2018 May;25(1):106.

36. Freeze HH, Eklund EA, Ng BG, Patterson MC. Neurological aspects of human glycosylation disorders. Annu Rev Neurosci. 2015 Jul;8:105–25.

37. Yu TW, Chahrour MH, Coulter ME, Jiralerspong S, Okamura-Ikeda K, Ataman B, Schmitz-Abe K, Harmin DA, Adli M, Malik AN, D’Gama AM, Lim ET, Sanders SJ, Mochida GH, Partlow JN, Sunu CM, Felie JM, Rodriguez J, Nasir RH, Ware J, Joseph RM, Hill RS, Kwan BY, Al-Saffar M, Mukaddes NM, Hashmi A, Balkhy S, Gascon GG, Hisama FM, LeClair E, Poduri A, Oner O, Al-Saad S, Al-Awadi SA, Bastaki L, Ben-Omran T, Teebi AS, Al-Gazali L, Eapen V, Stevens CR, Rappaport L, Gabriel SB, Markianos K, State MW, Greenberg ME, Taniguchi H, Braverman NE, Morrow EM, Walsh CA. Using whole-exome sequencing to identify inherited causes of autism. Neuron. 2013 Jan 23;77(2):259–73.

38. Dwyer CA, Esko JD. Glycan susceptibility factors in autism spectrum disorders. Mol Aspects Med. 2016 Oct;51:104–14.

39. Schepici G, Cavalli E, Bramanti P, Mazzon E. Autism Spectrum Disorder and miRNA: An Overview of Experimental Models. Brain Sci. 2019 Oct 3;9(10):265.

Tables

Table 1 Basic characteristics of the participants
|                              | ASD    | TD     | p-Value |
|------------------------------|--------|--------|---------|
| N                            | 100    | 60     |         |
| Males, (n) %                 | 90 (90%) | 54 (90%) |         |
| Age, years<sup>a</sup>       | 3.5 (2.5-5.5) | 3.5 (2.5-6.0) | 0.980  |

Autism diagnostic observation schedule (ADOS)<sup>b</sup>

|                                   | ASD     | TD     |         |
|-----------------------------------|---------|--------|---------|
| Language and communication        | 5.14±2.78 |        |         |
| Reciprocal social interaction     | 11.36±3.23 |        |         |
| Stereotyped behaviors and restricted interests | 1.59±1.23 |        |         |
| Total score                       | 16.64±4.39 |        |         |

Severity degree

|                      | ASD     | TD     |         |
|----------------------|---------|--------|---------|
| Mild:                 | 17%     |        |         |
| Moderate:             | 64%     |        |         |
| Severe:               | 19%     |        |         |

<sup>a</sup> Median (range). <sup>b</sup> Average±SD

Table 2 All primer sequences for qRT-PCR
| Primer name | Sequence                        |
|-------------|--------------------------------|
| mRNA primers |                               |
| GAPDH-F     | GGAGCGAGATCCCTCCAAAAT          |
| GAPDH-R     | GGCTGTGTCATACCTTCTCATGG       |
| TMEM165-F   | GGCCCAGATGAAGACCTTAGC         |
| TMEM165-R   | ACTGATATGGCAGCGACAAATG        |
| OSTC-F      | CGTGTCCTCTTTTAGT              |
| OSTC-R      | TGCCCATGTTCATCAGTC            |
| SLC17A6-F   | GGGAGACAATCGAGCTGACG          |
| SLC17A6-R   | TGCAGCGGATACCGAAGGA           |
| EDNRA- F    | TCGGGTTCTATTTCTGTATGCC        |
| EDNRA- R    | TGTTTTGCGCATTCTCGACG          |
| C12orf49-F  | GCAGCCCAACGCAACTTC            |
| C12orf49-R  | TGTTCTCATGCTGACGCT            |
| CHRM1-F     | CTCTATACCCAGCTACCTGCTCA       |
| CHRM1-R     | CCGAGTCACGGAGAAGTAGC          |
| HTR3A-F     | GAAGCCAACCAACCTCATC           |
| HTR3A-R     | CCACATCCACGAACTTATTGAT        |
| miRNA primers |                             |
| U6-F        | GCTTCGGCAGACATATACTAAAAT      |
| U6-R        | CGCTTCACGAAATTGCGTGCAT       |
| PC-5p-139289_26-F | ATCCAGTGCGTGTTCGTG  |
| PC-5p-139289_26-R | TGCTGTCAGAGCAGCCAG  |
| hsa-miR-193a-5p-F | ATCCAGTGCGTGTTCGTG          |
| hsa-miR-193a-5p-R | TGCTTGGGTCTTTGCGGCG        |
Figure 1

Schematic flow diagram of the integrated strategy used herein.
Figure 2

Characterization of the serum NDEs. A. Nanoparticle tracking analysis of NDEs in the ASD group and the TD group. B. The image of NDEs under transmission electron microscopy. C. WB analysis of exosomal-specific marker proteins CD63 and CD81 in serum and the obtained NDEs solution. D. WB analysis of the expression of L1CAM in the total serum exosomes and in the NDEs.
Figure 3

Differential expression and bioinformatic analysis of mRNAs in serum NDEs of ASD. A. Pearson's correlation analysis of all RNAs among different subgroups based upon IncRNA microarray. B. The differentially expressed mRNAs (DEmRs) with at least twofold change and a P value of less than 0.05 in ASD. C. Hierarchical clustering analysis (HCA) of the DEmRs in subgroups of TD-1-3 and ASD-1-5. The subgroups were listed in columns, and DEmRs were listed in rows. The color and intensity of each square
indicates expression levels relative to the other data in the row. Red, high; blue, low; white, medium. D. Principal component analysis (PCA) of DEmRs in subgroups of TD-1-3 and ASD-1-5. The color of each circle indicated a subgroup. E. The number and proportion of genes for DEmRs distributed on chromosomes. GO annotation (F) and KEGG analysis (G) of the DEmRs in ASD.

Figure 4
Differential expression and functional prediction of IncRNAs in serum NDEs of ASD. A. The differentially expressed IncRNAs (DElnRs) with at least twofold change and a P value of less than 0.05 in ASD. B. HCA of the DEmRs in subgroups of TD-1-3 and ASD-1-5. The subgroups were listed in columns, and DElnRs were listed in rows. The color and intensity of each square indicates expression levels relative to the other data in the row. Red, high; blue, low; white, medium. C. PCA of DEmRs in subgroups of TD-1-3 and ASD-1-5. The color of each circle indicated a subgroup. D. The number of DElnRs on each chromosome and the ratio of the number of DElnRs to the length of that chromosome. E. Venn diagram of the DEmRs and DElnRs and their targeting relationship (overlapped). F. Double-omic analysis of the 107 pairs of DElnR-DEmR. The color of the dot indicated correlation coefficient. The axis represented log2 fold change (ASD/TD). GO annotation of target genes for DElnRs according to the three grouping classifications of cellular component (G), molecular function (H) and biological process (I).
Figure 5

Neuron-related network and significant markers in ASD. A. The log2 fold changes and p values of 104 DEmRs that were related to neuron in ASD. B. The positive relationship of IncRNAs SNHG1, Inc-C20orf201-1 and Inc-TRPV5-1 with their target genes CHRM1, OPRL1 and EPHB6, respectively. The r value and p value was calculated. The p value was lower than 0.05. C. Neuron-related network associated with ASD according to GO and KEGG analyses. The red square represented neuron related functions; the
yellow circle represented DEmRs; and the blue diamond represented DElnRs. D. The most enriched pathway, neuroactive ligand-receptor interaction, which contained 5 up-regulated mRNAs and 19 down-regulated mRNAs in ASD. Red arrow represented up-regulated and blue arrow represented down-regulated in ASD. E. Individual validation of the differential expression of EDNRA, SLC17A6 and HTR3A by qRT-PCR.
Glycan-related network and candidate markers in ASD. A. The log2 fold changes and p values of 54 DEmRs that were related to glycan metabolism in ASD. B. The positive relationship of IncRNAs LOC101927919, Inc-KIAA1919-1, Inc-SHCBP1L-1 and RP11-177J6.1 with their target genes NUS1, KIAA1919, NPL and TMEM165, respectively. The r value and p value was calculated. The p value was lower than 0.05. C. Glycan-related network associated with ASD according to GO and KEGG analyses. The green square represented carbohydrate associated functions; the orange circle represented DEmRs; and the blue diamond represented DElnRs. D. Expressions of OSTC, MAN1B1 and MGAT5 were down-regulated in N-glycan biosynthesis implicated in ASD. Blue arrow represented down-regulated in ASD. E. Individual validation of the differential expression of OSTC and TMEM165 by qRT-PCR.
Figure 7

Differential expression of miRNA in serum NDEs of ASD. A. Abundance of the miRNAs in TD and ASD. The normalized expression level of each miRNA was represented as the mean of the read counts in biological replicates. B. The normalized expression level of 11 DEmiRs in subgroups of TD and ASD. C. Eleven miRNAs were differentially expressed across subgroups. The 11 DEmiRs (p<0.05) across ASD (ASD_1-5) and TD (TD_1-3) subgroups are clustered in the heatmap using a complete clustering algorithm.
algorithm. D. PCA of DEmiRs in subgroups of TD-1-3 and ASD-1-5. The color of each circle indicated a subgroup. E. The relative network of DEmiRs and their targets, DEmRs and DElnRs, implicated to ASD. Targets of these 11 DEmiRs were predicted by both TargetScan 50 and miRanda 3.3a algorithms. The green triangle represented DEmiRs; the red circle represented DEmRs; and the blue hexagon represented DElnRs. The solid lines represented the targeting relation. F. Two miRNAs, namely PC-5p-139289_26 and hsa-miR-193a-5p, had the largest number of predicted targets with high confidence (TargetScan score>90). G. GO annotation revealed the percentages of genes and the fold enrichments for all cellular activities. H. Individual validation of the differential expression of PC-5p-139289_26 and hsa-miR-193a-5p by qRT-PCR.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Supplentarytables.xlsx