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Original Article

Exploring the global transcriptional regulation of autism based on a WGCNA analysis

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Abstract: Autism is a complicated neurodevelopmental disorder, and most studies on autism focus on social contact, communication, and cognitive difficulties related to the condition. Early comprehensive and specific behavioral interventions can enhance social communication, attenuating anxiety and attacks. Therefore, this study, based on a modular comprehensive analysis method, explored the mechanism of neurotransmitter conduction on the pathogenesis of autism. We downloaded autism-related data from the GEO database, performed a differential analysis, a WGCNA analysis, and a GO function, as well as a KEGG pathway enrichment analysis. Finally, non-coding RNAs (ncRNAs) and transcription factors regulating the module were predicted on the basis of hypergeometric testing. In summary, we obtained five co-expression modules, in which the MTA3, PHB2, TNXB, and DCTN2 and RBM23 genes have positive regulatory effects on the dysfunction modules. The enrichment results indicated that the module gene significantly participates in the biological processes involving ion channel activity, the active regulation of cellular amide metabolism, and small molecule catabolism. It also significantly regulates the mRNA monitoring pathways, the calcium signaling pathways, and other signaling pathways such as the neuroactive ligand-receptor interactions and the ECM-receptor interactions. In addition, we found that the ncRNApivot (including CRNDE, miR-103a-3p, miR-106b-5p, etc.) and the TF pivot (including MYC) significantly regulate the dysfunction modules. In general, we decoded a comprehensive network of key gene regulation involving autism. These analyses reveal core dysfunctional modules, the underlying regulatory factors, and the driving genes for autism, helping us to further understand the mechanisms of autism neuronal conduction disorders.

Keywords: Autism, key genes, enrichment, co-expression analysis, regulatory factors

Introduction

Autism is a heterogeneous neurodevelopmental disorder characterized by early onset difficulties, abnormally restrictive repetitive behaviors, and interests in social communication. There is about 1% global incidence, and 70% of autism patients have accompanying complications [1]. It is primarily clinically characterized by cognitive impairment, a lack of social skills, and stereotyped behaviors [2]. Genetic and epidemiological studies have shown that autism is a complicated disease associated with environmental and genetic factors [3]. The environmental factors include genetic mutations or deletions after vaccination, viral infections, and encephalitis. Inflammation results from placental defects, an immature blood-brain barrier, the mother's immune response to infection during pregnancy, preterm birth, encephalitis in postnatal children, or a toxic environment [4].

In autism, a highly heritable disease of universal neurodevelopmental disorders, genetic factors are central to the etiology. Dual specific phosphatase 15 (DUSP15) is considered to be a key regulatory gene for oligodendrocyte differentiation, probably an autism susceptibility gene in the Chinese Han population [5]. Risk alleles and haplotypes near MEGF10 TSS may regulate the transcription activity and leave it susceptible to autism [6]. What's more, it has been shown that PCDHA acts as a potential candidate gene for autism [7]. Currently, previous studies have shown that there are some potential therapeutic targets for autism, such as, miR-486-3p [8], miR-132 [9], MSNP1AS [10], and THRIL [11]. In many cases, early inter-

vention using a variety of techniques can help with autism treatment, but it cannot eliminate the underlying causes [12]. Likewise, currently, drugs cannot completely treat autism but can effectively alleviate its symptoms, such as self-harm, aggression, repetitive and stereotyped behavior, inattention, hyperactivity, and sleep disorders [13]. These studies enhance our comprehension of autism's mechanism and offer ideas for progressive study. However, only a few genes have been clarified in a single study, and autism's mechanism has not been comprehensively explained.

Autism is a complex neurodevelopmental disorder, with the characteristics of impaired social interaction and communication as well as restrictive, repetitive, and stereotypical behavior. There are various controversial and competitive theories on the cause of autism, but the underlying pathogenesis is still unclear. We gathered the genes of iPSC-derived neuronal cells, iPSC-derived neural progenitor cells, and IPSCs in autism patients in the GEO database and analyzed the autism dysfunction modules driven by the transcription factors and the ncRNA regulators based on the differential gene expression profile data for autism to understand the molecular mechanisms of neuronal conduction disorders in autistic patients.

Materials and methods

Data resources

The NCBI Gene Expression Omnibus (GEO) database [15] includes a broad classification of high-throughput experimental data, including single-channel and dual-channel microarray-based assays for mRNA abundance, protein molecules, and genomic DNA. It also contains data relating to non-array-based high-throughput functional genomics and proteomics technologies. First, we gathered a set of related gene expression profiles for autism neuronal cells from GEO, numbered GSE67528 [16]. Then, the ncRNA-mRNA interaction pairs under a score of ≥ 0.5 were screened from the RAID v 2.0 database [17], including 431937 interacting pairs involving 5431 ncRNAs. The RAID v 2.0 database enrolled over 5.27 million RNA-related interactions, containing roughly 4 million RNA-RNA interactions and over 1.2 million RNA-protein interactions, almost referring to 130,000 of the 60 species comprising 130,000 RNA/protein symbols, contributing to our comprehensive observation of the varieties of RNA-related interactions. Among them, we downloaded and used the overall human transcription factor target data in the TRRUST v 2 database [18], which involved 2492 transcription factors and 9396 interaction pairs.

Differentially expressed genes

The R language DESeq2 package [19, 20] was applied to analyze the differential expressions of the gene expression profile data of the research [19, 20]. The R language DEseq2 analysis process consisted of three main steps, normalization, dispersion estimation and differential expression testing. We alleviated the dispersion estimate with respect to each gene to a common estimate of global genes, or a local estimate for genes with a similar expression intensity using a weighted conditional likelihood, detected and corrected low dispersive estimates by simulating the dependence of dispersion on average expression intensity in the whole samples. BBSeq uses the average absolute deviation of the dispersion estimate to simulate the dispersion to the mean to reduce the effect of the outliers. DSS utilized the Bayesian method to estimate the individual genes' dispersion, which can account for the dispersion value heterogeneity of diverse genes. BaySeq and ShrinkBayes estimated the priori of the Bayesian model for all genes, leaving the posterior probability or false discovery rate (FDR) for the differential expressions.

Weighted correlation network analysis (WGCNA)

To explore the drivers of neuronal conduction disorders in autistic patients, we separately targeted ASD-derived iPSC-derived neurons and normal iPSC-derived neurons, ASD-derived iPSC-derived neural progenitors and normal iPSC-derived neural progenitors, and our ASD differential analysis of the IPSCs and normal IPSCs yielded differential gene expression profiles for autism. In the meantime, to explore the synergistic expression of the differential genes in autism, we used WGCNA [21] to analyze the differential expression matrix of autism and search for gene modules that are synergistically expressed. First, the weighting value of the correlation coefficient was applied; in other words, the correlation coefficient was calculat-

ed by taking the gene correlation coefficient to the power of N (the Pearson coefficient) between any two genes. The connections in the network were constrained by the scale-free networks, leaving the algorithm more biologically meaningful. Then, a hierarchical clustering tree was established with correlation coefficients between the genes. Diverse branches of the clustering tree signify diverse gene modules, and varying colors indicate various modules. Based on the size of the gene's regulatory power in each dysfunctional module, the key genes that cause dysfunctional modules were explored and regarded as the key genes in the pathogenesis of neuronal conduction disorders.

Patient and blood samples

The blood samples were identified by experienced pathologists. All the patients signed and provided a written informed consent. All the tissue samples were gathered in conformity to the International Ethical Guidelines for Biomedical Research involving Human Subjects. This research was performed under the approval of the Penglai Traditional Chinese Medicine Hospital and conducted based on the regulations of the Penglai Traditional Chinese Medicine Hospital.

Verification of the key genes using qPCR

Total RNA was extracted from the blood. A reverse transcription kit was used for transcribing the total RNA into cDNA, and a SYBR qPCR detection kit was used to perform the qPCR reaction. The hot-start iTaqTM DNA polymerase was activated using the qPCR program, which starts the initial 3 minute denaturation step at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 45 s. Beta-actin is considered the internal reference gene.

Enrichment analysis

The exploration of the signaling pathways and functions involved in gene expression usually has a favorable effect on studying the molecular mechanisms of disease, and the enrichment analysis of the gene functions and pathways in the dysfunction modules is a practical way to investigate the potential pathogenesis of neuronal conduction disorders in autism. Hence, the R language ClusterProfiler package [22] was used for the Go function, and the KEGG pathway enrichment analysis was performed for the five module genes of autism. ClusterProfiler is a type of bioconductor software package, providing statistical analysis and visualization of functional clustering for gene sets or clusters.

Transcription factors and ncRNAs regulating dysfunctional modules

Non-coding RNA (ncRNA) and the transcription factors (TF) usually act as the core driving force of gene transcriptional and post-transcriptional regulation. Therefore, their effect on the autism dysfunction module were scientifically predicted and tested. Pivot regulators, including ncRNA and TF, are defined as modulators with remarkable regulatory roles in modules during the onset of neuronal conduction in autism. The hypergeometric test required at least two more control connections between each regulator and module, and the significant *p* value of each module's enrichment target was lower than 0.01.

Results

Identification of the autism expression disorder molecules

Many experimental studies have been carried out on the pathogenesis of autism to identify the potential pathogenic genes for the condition. However, the connections of the complicated molecular and overall roles of the genes are unknown. To observe molecular variations in the progression of neuronal conduction disorders in autistic patients, the analysis for differential expression was performed on the basis of microarray data to further identify the differential expressions of genes between the APS-derived iPSC-derived neuronal cells and the normal iPSC-derived neuronal cells, the ASD-derived iPSC-derived neural progenitor cells, and the normal iPSC-derived neural progenitor cells, the ASD-derived IPSCs and the normal IPSCs (DEG) while obtaining 2058, 1460, and 1101 differentially expressed genes, respectively (**Figure 1A-C**). We believe that some genes in these differential genes cause neuronal conduction disorders in autistic patients. In the Venn diagram showing the overlapping differential genes, it was found that 17 of the three sets of differential results overlap.

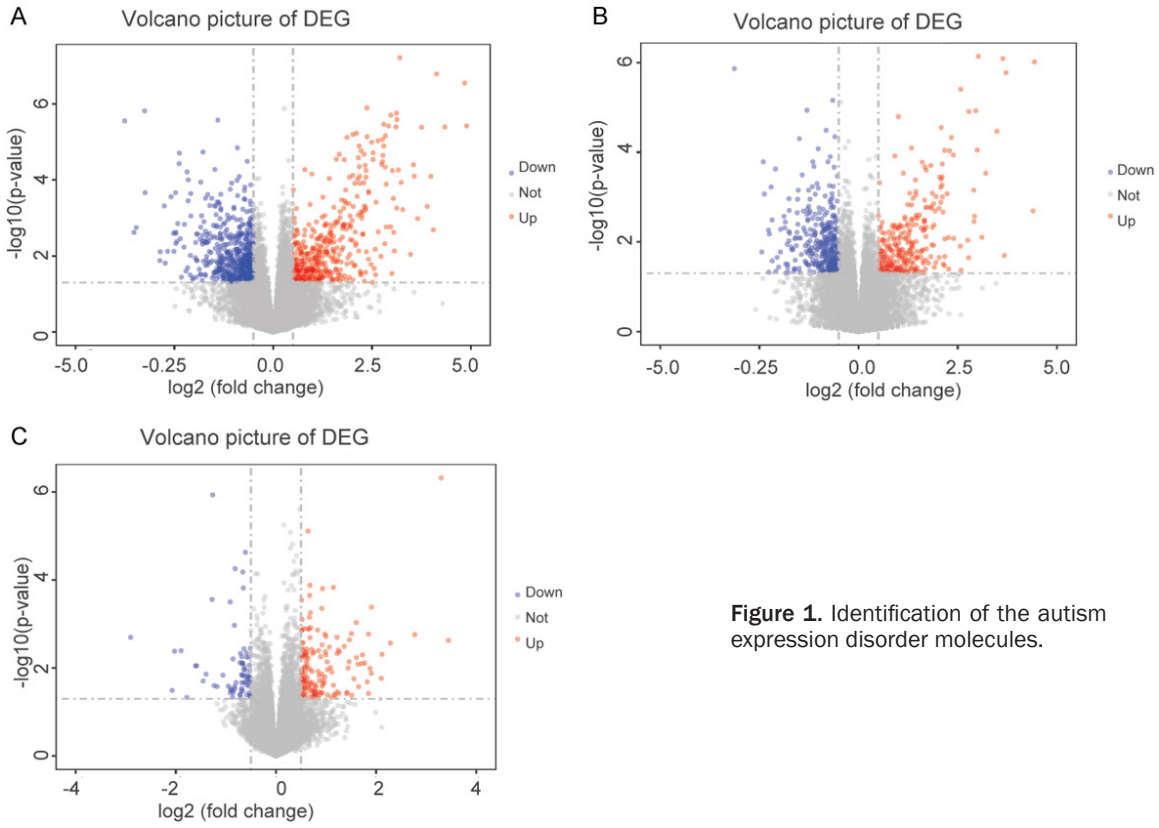

Figure 1. Identification of the autism expression disorder molecules.

Identification of the autism functional disorder modules

The combination of three sets of differential genes resulted in 4192 differential genes that led to neuronal conduction disorders in autistic patients. Based on the 4192 autism differential genes and the interaction genes, the expression profile matrix was established in the samples. Moreover, on the basis of WGCNA, an obvious co-expression of the genes was observed in the samples. Modularity copes with the globally complicated systems and divides them into more detailed and organized subsystems with their own features. As for the elemental gene, the module collects genes with the relationship of synergistic expression, and the genes in same module express consistently. There is also an interactive relationship between each module, with its effect standing for the gross characteristics, constructing a bridge for each gene to operate. It is a great method for converging the expression behavior of the patient samples with autism into modules so as to detect the complex synergistic connections in these genes from the aspect of expressive behavior. Accordingly, by determining the co-expression panel as a module, five functional disorder modules for autism were obtained, including 3395 differential genes (**Figure 2**). The key genes of each module were determined according to the dysfunctional module, and mainly core genes were obtained, including MTA3, PHB2, TNXB, and DCTN2, and RBM23 (**Table 1**). Furthermore, the expression levels of the key genes were verified using qPCR. We found that the expression trends of the key genes was consistent with the previous results.

Functions and pathways involved in the module gene

The functions and pathways are crucial mediators in the physiological response of the disease. To explore the function and pathway in the dysfunctional module gene is conducive to determining the upstream and downstream relations in the same module gene, and is also conducive to constructing a molecular bridge between the modules and the disease in system biology, which deepens the comprehensives with respect to the potential molecular mechanism. GO function and KEGG pathway enrichment analyses were carried out respec-

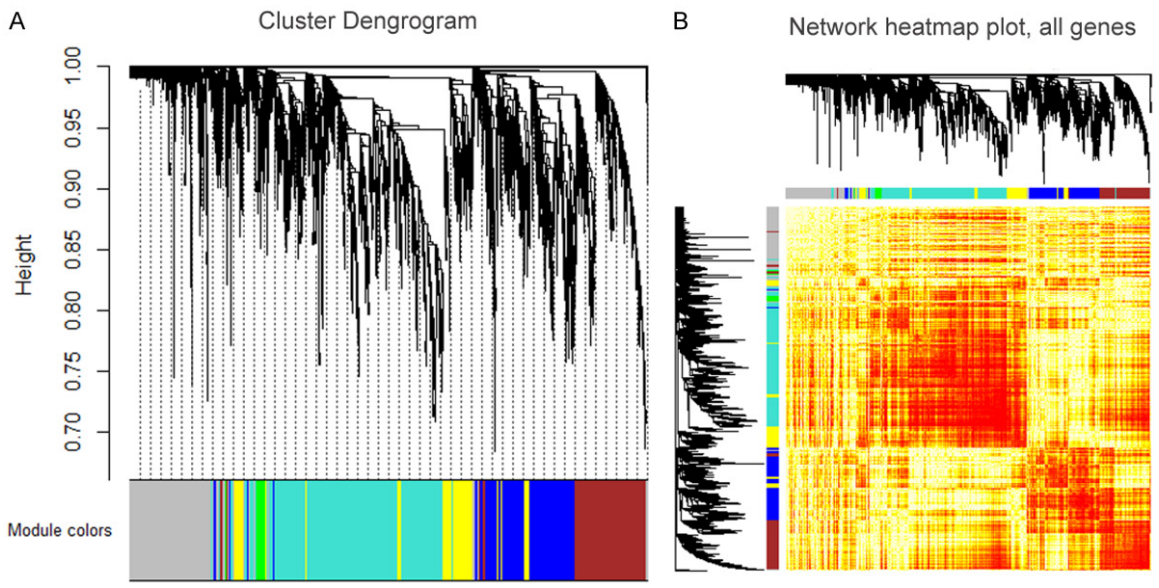

Figure 2. Synergistic expression of differential genes of autism in the patient samples. A. The five co-expression groups via clustering were identified as modules, and five colors represent five co-expression modules. B. Here are heat maps of all the gene expressions in the sample, whose expression behavior is clustered into five co-expression modules.

Table 1. Core genes within the autism-related dysfunction module

color	HubGenes	Module
blue	PHB2	m2
brown	MTA3	m3
green	TNXB	m5
turquoise	DCTN2	m1
yellow	RBM23	m4

tively in five modules and 2997 biological processes, and 553 cells, 768 molecular functions, and 80 KEGG pathways were obtained (**Figure 3**). It was found that the module gene was significantly involved in the biological processes involving ion channel activity, substrate-specific channel activity, the active regulation of cellular amide metabolism, and small molecule catabolism. They also significantly participated in the mRNA monitoring pathways, RNA degradation, calcium signaling pathways, and signaling pathways such as neuroactive ligand-receptor interactions and ECM-receptor interactions.

TF and ncRNA drive the neuronal conduction disorders in autistic patients

In terms of systematic biology and genetics, the gene transcriptional and post-transcriptional regulations have been identified as the pivotal regulators of disease occurrence and development, but the transcription factors and ncRNAs are common regulators for expressions and functions. Though many biologists have valued the single or multiple TF and ncRNA regulations of the pathogenesis of allergic rhinitis, few investigations pay attention to the overall impact on the dysfunctional mechanisms and progression. Therefore, in the present study, a crucial analysis of the co-modules based on the targeted regulatory correlation between TF and ncRNA was carried out to investigate the pivotal transcriptional regulators which can regulate the neuronal transmission disorders in autism. The results revealed that 433 ncRNAs involved 583 ncRNA-module regulatory pairs and 31 transcription factors involved 32 TF-module target pairs. In addition, we statistically analyzed the number of pivot control modules and obtained ncRRA (CRNDE, miR-103a-3p, miR-106b-5p, etc.) and the dysfunctional modules TF (MYC) most regulated. These transcription factors may regulate the progression of neuronal conduction disorders in autistic patients by the signaling pathways involved in gene involvement. Therefore, these underlying regulatory factors were identified as the dysfunctional molecules in the neuronal conduction disorders.

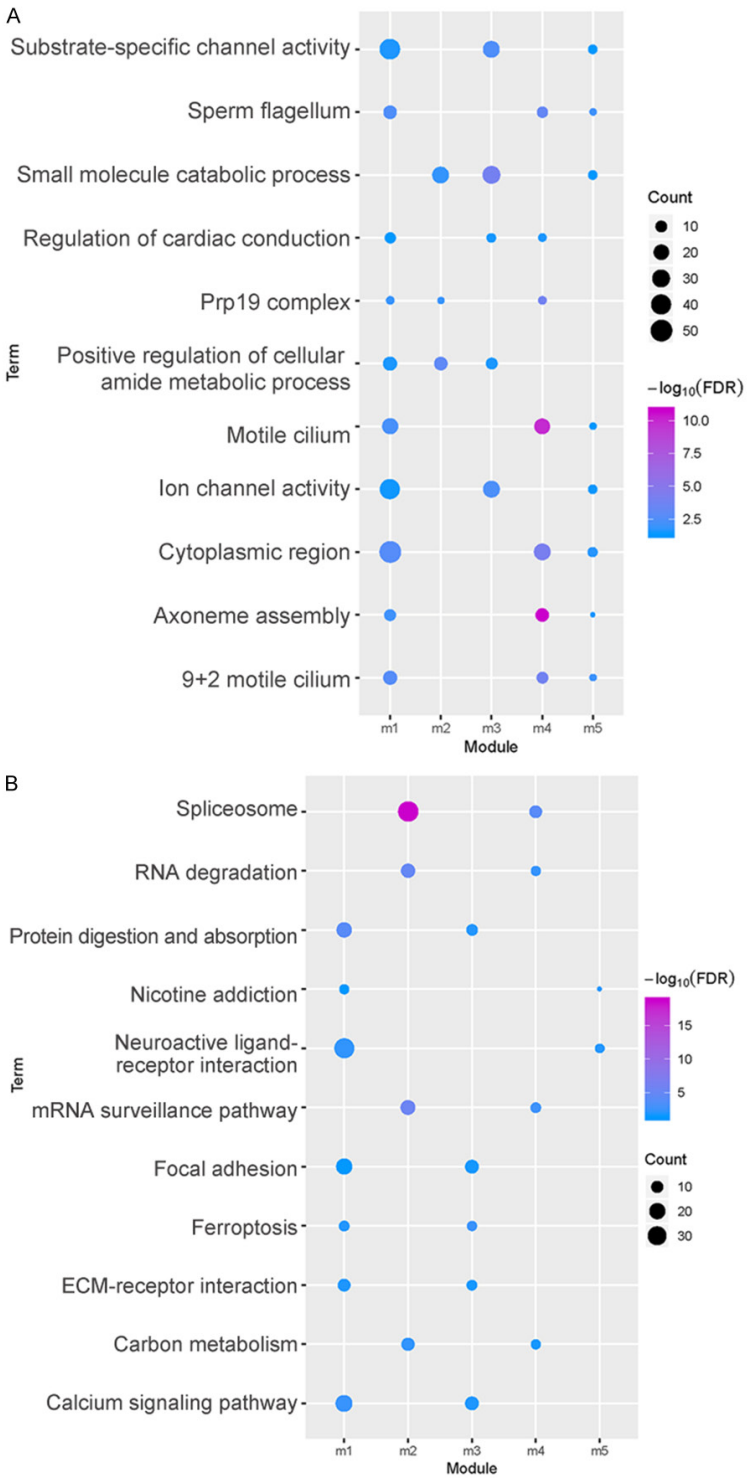

Figure 3. Functional and pathway enrichment analysis excerpts of the module gene. A. A functional enrichment analysis excerpt of module gene GO. From blue to purple, the enrichment increases significantly. The larger the circle, the greater the proportion of the module gene in the GO function entry gene. B. A functional enrichment analysis excerpt of the module gene KEGG. From blue to purple, the enrichment increases significantly. The larger the circle, the greater the proportion of the module gene in the KEGG function entry gene.

Discussion

At the module level, we first noticed that the two modules of the gene are significantly involved in the mRNA monitoring pathways, RNA degradation, the calcium signaling pathways, and other signaling pathways such as the neuroactive ligand-receptor interactions and the ECM-receptor interactions. Also, mRNA monitoring promotes the detection and destruction of mRNAs containing premature stop codons by a process called nonsense-mediated decay, thereby preventing the synthesis of truncated and other abnormal proteins, which may have dominant negative and other harmful effects [23]. The interruption of the RNA degradation process can seriously affect neurons [24].

On the one hand, calcium is one of the most versatile, oldest, and most versatile molecules among the biological signaling molecules, regulating physiological systems from membrane potential and ion transporters to various levels of kinases and transcription factors [25]. On the other hand, Ca^{2+} signaling is involved in synapse formation and dendritic growth in proteins associated with ASD, while several voltage-gated and ligand-gated ion channel mutations that regulate neuronal excitability and Ca^{2+} signaling are also associated [26]. The signal transduction of neuroactive ligand receptors is related to synaptic transmission [27]. In addition, the activation of ECM receptors regulates downstream signaling cascades, controls cytoskeletal dynamics and synaptic activity, and regulates neuro-

nal structure and function, thus affecting animal behavior [28].

We predicted 433 ncRNAs to mediate neuronal conduction disorders in autism through a mediator module. Our statistical analysis demonstrated that CRNDE, miR-103a-3p, miR-106b-5p, etc. have significant effects on three dysfunctional modules. The TLR3-NFκB-cytokine signaling pathway has important effects on LncRNA CRNDE-triggered inflammation [29]. CRNDE promotes the proliferation, invasion, and migration of osteosarcoma cells through the regulation of Notch1 signaling and the epithelial-mesenchymal transition [30]. In addition, miR-103a-3p acts as a tumor suppressor by binding to Linc00152 to regulate the malignant behavior of glioma stem cells [31]. MiR-106b-5p advances the invasiveness and stem cell-like phenotype of renal cell carcinoma by activating Wnt/β-catenin signaling [32]. The results clearly show that CRNDE, miR-103a-3p, and miR-106b-5p, as ncRNAs regulating the most dysregulated ASD dysfunction, may play a potential part in the regulation of neuronal conduction disorders in ASD patients. They may serve as candidates for further molecular experiments. Other ncRNAs that significantly modulate the autism dysfunction module may also take part in the basic process of autism neuronal conduction disorders, as may also be candidates for further molecular validation.

Finally, we identified 31 transcription factors that were differentially expressed to different degrees and that significantly modulated the dysfunctional module of autism. In accordance with the regulatory analysis, MYC remarkably regulated two modules, making difference to allergic rhinitis. Pam, an MYC-associated protein, regulates the growth and proliferation of cells and neuronal function using the TSC/mTOR pathway in mammalian cells [33]. MYC is a transcription factor, which facilitates the expression of many target genes to coordinate apoptosis, proliferation, and metabolism at the cell, tissue, and organism levels [34]. More importantly, the downregulation or inactivation of MYC causes an impaired cell cycle progression [35]. Our study showed that MYC regulated the dysfunctional module at most, suggesting its potential role in neuronal conduction disorders in ASD patients, and it can be a candidate for further molecular experiments. Meanwhile, other transcription factors that significantly regulate the autism dysfunction module may also be involved in the basic process of neuronal conduction disorder in ASD patients, which needs to be confirmed by experiments.

In our study, a systematic modular analysis was conducted. According to the modular analysis, we identified the dysfunction modules and autism-related genes using a weighted correlation network analysis. Moreover, MTA3, PHB2, TNXB, DCTN2, and RBM23 may be the core genes in the underlying dysfunction of autism. CRNDE, miR-103a-3p, and miR-106b-5p may have potential roles in regulating neural conduction disorders in patients with atrial septal defect (ASD).

Disclosure of conflict of interest

None.

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