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Differential Expression of Long Noncoding RNAs And Targeted MRNAs in Peripheral Blood Lymphocytes of Neurodevelopmental Disorders

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Abstract

Deregulation of long noncoding RNAs (lncRNAs) is becoming recognized as a major feature of many neurological disorders. In the current study, we aimed to measure the expression of seven lncRNAs and lncRNA-targeted mRNAs in the peripheral lymphocytes of attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD), and intellectual disability (ID) patients via quantitative real-time reverse transcriptase PCR (qRT-PCR). We found that in ADHD, the expression of mRNAs of the *BDNF, SHANK2, HOXB5*, and *HOXA6* genes was down-regulated significantly, but there was no difference in the expression of selected lncRNAs. As for ASD, the expression of the mRNAs of the *HOXA6* and *HOXA13* genes was significantly down-regulated, accompanied by a significant reduction of lncRNA that overlaps with the gene locus of *HOXA13*. A gender-dependent difference in expression of lncRNAs and targeted mRNAs was indicated in ID. In male ID, there was a significantly down-regulated expression of mRNAs of *HOXB5* and *HOXA13*, accompanied by differentially decreased expression of lncRNA-targeted mRNAs of *SYT15, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13*. In female ID, the mRNAs of *HOXB5* and *HOXA6* were significantly down-regulated, with the significantly down-regulated expression of lncRNA-targeted mRNAs of *BDNF, PKNOX2, HOXB5,* and *HOXA6*, and the differentially increased expression of lncRNAs that overlap *SYT15* and *SHANK2*. Our results indicated a differential expression pattern for lncRNAs and targeted mRNAs in the peripheral lymphocytes in different neurological disorders.

Keywords: LncRNA; ADHD; ASD; ID; peripheral blood lymphocytes; qRT-PCR

Abbreviations

Lncrnas: Long Noncoding Rnas; ASD: Autism Spectrum Disorder; ADHD: Attention Deficit/Hyperactivity Disorder; ID: Intellectual Disability; *SYT15*: Synaptotagmin XV; *BDNF*: Brain-Derived Neurotrophic Factor; *PKNOX2*: PBX/Knotted 1 Homeobox 2; *SHANK2*: SH3 and Multiple Ankyrin Repeat Domains 2; *HOXB5*: Homeobox B5; *HOXA6*: Homeobox A6; *HOXA13*: Homeobox A13.

Introduction

Whole-genome and transcriptome sequencing implies that the complexity of an organism may be regulated by noncoding portions of the genome rather than by proteins. Long noncoding RNAs (lncRNAs) refer to RNAs exceeding 200 nucleotides in length (as compared to the ~21–23 nucleotide length of microRNAs (miRNAs)), which do not encode for proteins [1]. It was initially assumed that lncRNAs act merely as primary or precursor transcripts for the production of short ncRNAs (sncRNAs) such as miRNAs or small nucleolar RNAs [2]. Further investigations gradually revealed the complex and special functionality of lncRNAs in various life processes by acting solely or together with proteins. LncRNAs have been shown to be involved in major mechanisms of gene expression regulation, such as targeting transcription factors, initiating chromatin remodeling, directing methylation complexes, and blocking nearby transcription [3]. Multiple studies have emphasized an important role for lncRNAs in epigenetic regulation, development, and disease [4–9]; however, the underlying specific mechanisms for their role in these processes are yet to be clarified.

LncRNAs were also shown to be regulated temporally and spatially during development [10], with the greatest abundance of transcribed lncRNAs found in the central nervous system [11]. LncRNAs are essential to the development, maintenance, and function of the brain. They have been shown to take part in fundamental processes such as synaptogenesis, neurogenesis, and gamma-amino butyric acid (GABA)-ergic interneuron function. Studies analyzing the differential expression of lncRNAs upon differentiating human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) to neurons have identified several lncRNAs as integral components of neurogenesis [12,13]. Additionally, 659 evolutionarily conserved murine lncRNAs have been identified, of which the brain-specific lncRNAs are preferentially (by a 2- to 3-fold increase) located adjacent to brain-expressed protein-coding genes involved in transcriptional regulation or in nervous system development [14]. Synaptogenesis is a pivotal process during neuronal development. Metastasis-associated lung-adenocarcinoma transcript 1 (*MALAT1*) is an lncRNA that was shown to regulate synaptic density and the expression levels of neuroligin 1 (*NLGN1*) and synaptic cell-adhesion molecule (SynCAM1), which are

involved in controlling synapse formation [15]. GABA is one of the most abundant neurotransmitters in the brain and has key roles in development [16]. During fate-specification from neuronal oligodendrocyte bipotent progenitors into GABAergic interneurons, 56 lncRNAs were found to be upregulated [17].

Increasing evidence has indicated the involvement of lncRNAs in neurobehavioral, neurodevelopmental, neurodegenerative, neuroimmunological, and neuro-oncological disorders, highlighting the functional importance of this subclass of brain-enriched RNAs [18–21]. Deregulation of lncRNAs is becoming recognized as a major feature of many types of neurological disorders. Autism spectrum disorders (ASDs) represent various developmental disorders, including autism and pervasive developmental disorder not otherwise specified (PDD-NOS). The common symptoms of ASD include problems of reciprocal social interactions, verbal and non-verbal communication, and rigid and stereotyped behaviors. Many efforts have been invested in the elaboration of the etiology of this disease. Differential expression of lncRNAs has been observed in both postmortem brain tissue and lymphoblastoid cell lines from ASD patients. Ziats and Rennert showed that more than 200 lncRNAs were differentially expressed in a microarray of postmortem prefrontal cortex and cerebellum tissue of ASD patients [22]. Hu et al. identified 20 common lncRNAs that were dysregulated in lymphoblastoid cell lines derived from three subgroups of individuals diagnosed with ASD when compared to controls [23,24]. Intellectual disability (ID) is another cluster of developmental disorder diseases, with the implied involvement of disturbance of synaptogenesis and normal synaptic function through the regulation of gene transcripts by short and long ncRNAs [25]. Attention deficit/hyperactivity disorder (ADHD) is one of the most prevalent psychiatric disorders in childhood and adolescence and has many negative consequences for both the child and the family. The role of lncRNAs in the development of ADHD is suspected but has not yet been proven.

We have previously determined that synaptic vesicle cycling (SVC)–associated, as well as *HOX* gene–associated, lncRNAs were differentially expressed in ASD peripheral blood [26]. In the current study, we aimed to examine the expression of seven lncRNAs and lncRNA-targeted mRNAs in the peripheral lymphocytes of ADHD, ASD, and ID patients via quantitative real-time reverse transcriptase PCR (qRT-PCR) in order to explore lncRNA expression and regulation patterns in different neurodevelopmental disorders. The targeted genes selected for study included *HOXB5*, *HOXA6, HOXA13, SYT15, PKNOX2, SHANK2*, and *BDNF*, which were proven to be involved in neurodevelopment and/or synaptic functions. Our study may provide a new and practical way to investigate the mechanisms underlying ADHD, ASD, and ID, and the differentially expressed lncRNAs that are identified may be used as potential biomarkers for early detection and diagnosis.

Patients, Materials, and Methods

Study subjects

A total of 80 children (40 boys and 40 girls) 4 to 5 years of age who were outpatients and were receiving health care from the department of child healthcare of Hubei maternal and child Health hospital were recruited for this study. Among them, 20 had diagnoses of ASD; 20, of ADHD; and 20, of ID. Each patient met the diagnosis criteria of the diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV). Twenty age-matched, phenotypically and developmentally normal children who received regular health examinations during the same period were selected as the controls. There were no overlapping diagnoses among the different groups. All study subjects were excluded from previously having had epilepsy, brain damage, or any other neurologic or genetic disorder. Informed consent was obtained from the parents of each subject. The Hospital Ethics Committee reviewed and approved the research project.

Peripheral blood lymphocyte preparation

A sum of 3 to 5 ml of heparinized peripheral venous blood was obtained from participants, and the lymphocytes were isolated within 30 minutes by using lymphocyte separation liquid (Tianjin Haoyang Biological Manufacture Company, China). All lymphocyte samples were stored at -70°C until the total RNA was extracted.

RNA isolation and quality control

Total RNA was extracted from lymphocyte samples with a Qiagen Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA quantity was measured with a NanoDrop ND-1000. Agilent Bioanalyzer 2100 was used to assess the RNA integrity for each sample.

qRT-PCR analysis

Five micrograms of total RNA extracted from leukocytes was used for the synthesis of first strand cDNA using the SuperScript III First Strand cDNA Synthesis Kit (Invitrogen, Calsbad, CA). qRT-PCR analysis was performed by using the ABI7900 system (Life Technologies, Grand Island, NY) and SYBR green dye SuperArray PCR master mix (SABiosciences, Frederick, MD). The mRNA of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for quantitative analysis of lncRNAs or mRNAs. The lncRNA or mRNA values were normalized to GAPDH levels. Normalized, relative gene expression was calculated using standard ΔΔCt methods using Applied Biosystem RQ Manager Software (v1.2). Each qRT-PCR reaction was run three separate times, with technical triplicates in each reaction. All data were given in terms of the relative expression of the mean ± S.E. (N=10). The data were subjected to one-way ANOVA followed by an unpaired, two-tailed t-test. Differences were considered significant at $p<0.05$.

Results

General demographic information of study subjects

Among 80 subjects (40 boys and 40 girls) recruited for this study, the average age was 4.8 ± 0.4 years. There was no significant age difference between each group. The gender composition was also the same for each group: 10 males and 10 females (Table 1).

Differential expression of mRNAs of *SYT15, BDNF, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13* in different neurological disorders

qRT-PCR analysis of expression of mRNAs of the *SYT15, BDNF, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13* genes indicated the differential expression pattern in different neurological disorders (Table 2). The expressions of mRNAs of the *HOXB5* and *HOXA6* genes were up-regulated significantly in the ADHD group compared to controls. In the ASD group, a significantly up-regulated expression of mRNAs of the *HOXA6* gene was found compared to in the control group.

Group	Male		Female		P value	Average age
	Number	Age (yrs)	Number	Age (yrs)		(yrs)
ASD	10	4.8 ± 0.8	10	4.9 ± 0.6	>0.05	4.8 ± 0.9
ADHD	10	4.7 ± 0.5	10	4.9 ± 0.9	>0.05	4.8 ± 0.8
ID	10	4.8 ± 0.9	10	4.9 ± 0.8	>0.05	4.9 ± 0.4
Control	10	4.8 ± 0.8	10	4.9 ± 0.7	>0.05	4.9 ± 0.5
ASD: Autism Spectrum Disorder; ADHD: Attention Deficit/Hyperactivity Disorder; ID: Intellectual Disability						

Table 1: The age and gender composition of study subjects.

A different mRNA expression pattern was revealed for ID subjects of different genders. In male ID subjects, there was significantly increased expression of mRNAs of the *HOXA6* gene. The mRNAs of the *HOXB5* gene were significantly down-regulated in female ID subjects compared to in the control group.

Group		*BDNF*	*SYT15*	*SHANK2*	*PKNOX2*	*HOXB5*	*HOXA6*	*HOXA13*
ADHD		0.683	10427.869	0.918	1.172	0.862*	2.395*	2.808
ASD		2.214	0	3.767	3.11	1.115	5.856*	5.202
ID	Male	1.485	0	6.214	5.193	0.894	2.967*	3.476
	Female	0.468	0	0.124	0.459	0.187Δ	1.098	1.199
*Significantly increased expression when compared with the control group ($p<0.05$); ΔSignificantly decreased expression when compared with the control group ($p<0.05$).								

Table 2: Differential expression of mRNAs of selected genes in different groups.

Differential expression of lncRNAs that target mRNAs of *SYT15, BDNF, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13* in different neurological disorders

Also, qRT-PCR analysis of the expression of lncRNAs that target mRNAs of *SYT15, BDNF, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13* found inconsistency in different neurological disorders (Table 3). There was significantly up-regulated expression of lncRNAs that target mRNAs of *BDNF, SYT15, PKNOX2,* and *HOXA6* in both ADHD and ASD. However, the significantly increased expression of lncRNAs targeting mRNAs of *HOXA13* was found in ADHD, and the same for lncRNA targeting mRNAs of *SHANK2* in ASD. The expression of lncRNAs that target mRNAs of *SYT15* and *PKNOX2* was shown to be differentially decreased in male ID as compared to the control group. However, for female ID, no significantly different expression for the lncRNAs targeting mRNAs of *SYT15, BDNF, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13* were found.

Group		lnc-*BDNF*	lnc-*SYT15*	lnc-*SHANK2*	lnc-*PKNOX2*	lnc-*HOXB5*	lnc-*HOXA6*	lnc-*HOXA13*
ADHD		3.714*	5.93*	2.519	4.282*	1.97	6.804*	13.089*
ASD		7.602*	7.375*	5.878*	5.98*	9.386	2.528*	1.488
ID	Male	3.562	3.255*	2.935	3.156*	4.116	1.33	0.597
	Female	0.788	1.408	0.649	0.692	0.888	0.476	0.832
*Significantly increased expression when compared to control group ($p<0.05$).								

Table 3: Differential expression of lncRNAs in different groups.

Discussion

Due to the significantly negative impact of neurodevelopmental and neuropsychiatric disorders such as ASD, ADHD, and ID on individuals and families, a vast amount of effort has been invested in exploration of the pathogenesis and identification of, and intervention for, these diseases. However, until now, there were no generally accepted biomarkers for early screening and/or diagnosis of ASD, ADHD, and ID. Because the examination of samples from brain tissues and related structures is not suitable in clinical settings, we attempted to investigate the possible changes in the peripheral blood lymphocytes related to or involved in the occurrence and development of central nervous system diseases. The participation and functional importance of lncRNAs in neurodevelopmental disorders have been widely identified and proven through human research and animal model

studies. In the present study, we examined differentially expressed lncRNAs and targeted mRNAs of seven development-related genes, including *SYT15, BDNF, PKNOX2, SHANK2, HOXB5, HOXA6*, and *HOXA13* in the peripheral blood lymphocytes of children with ASD, ADHD, and ID. Our results indicated a different expression pattern of selected lncRNAs and targeted mRNAs in different neurological disorders.

Differential expression of lncRNAs and targeted mRNAs of *BDNF* in ADHD, ASD and ID

The *BDNF* gene encodes a member of proteins in the nerve growth factor family. Binding of this protein to its cognate receptor promotes neuronal survival in the adult brain. Expression of this gene is reduced in Alzheimer's, Parkinson's, and Huntington's disease patients. Nowadays, this gene has been demonstrated to possibly be involved in the regulation of the stress response and in the biology of mood disorders [27,28]. It has been hypothesized that *BDNF* is involved in the pathogenesis of ADHD, although the study results are controversial. An increase in plasma *BDNF* levels was found in untreated ADHD patients, and the plasma *BDNF* levels had a significant positive correlation with the severity of inattention symptoms [29]. However, *BDNF* serum levels were significantly lower in adults with ADHD compared to healthy controls ($p<0.0001$) [30], whereas there was no alteration of serum *BDNF* levels in untreated patients with ADHD [31]. In our current study, the significantly up-regulated expression of lncRNAs that target the mRNAs of *BDNF* in the ADHD and ASD groups has been confirmed. No significant difference was indicated for mRNAs of *BDNF* in ADHD, ASD, and ID, and no difference for lncRNAs or targeted mRNAs of *BDNF* in ID. Although the different outcomes among these studies may be partly due to the difference in study subject composition, our finding suggested that an increased expression of lncRNA, which targets the mRNAs of *BDNF* in the peripheral blood lymphocytes of ADHD and ASD, may be in accordance with the possible change in neuron cells in brain correlating to the regulation of secretion of *BDNF*. The finding of no difference in the expression of lncRNAs or targeted mRNAs of *BDNF* in the peripheral blood lymphocytes of ID may imply a different pathogenesis for ID without the involvement of the *BDNF* gene.

Differential expression of lncRNAs and targeted mRNAs of *SYT15* and *SHANK2* in ADHD, ASD, and ID

We found no significant difference in the levels of the mRNAs of *SYT15* and *SHANK2* in peripheral blood lymphocytes in ADHD, ASD, and ID. However, significantly up-regulated expression of *SYT15*-targeted lncRNAs in ADHD, ASD, and male ID was present, accompanied by no significant expression in female ID. Also, significantly up-regulated expression for *SHANK2*-targeted lncRNA was found in ASD.

The *SYT15* gene encodes a member of the Synaptotagmin (Syt) family of membrane trafficking proteins. A study has demonstrated that most synaptotagmins are expressed in the rodent brain in highly distinctive expression patterns, and that individual neurons express variable subsets of different synaptotagmins [32]. Synaptotagmins-1, -2, and -9 are known to have an essential role as calcium sensors for fast synaptic release. Synaptotagmin-7 is a major calcium sensor for the exocytosis of large secretory vesicles in endocrine cells. Unlike related family members, *SYT15*-a is classified as a non-neuronal, Ca_{2+}-independent Syt [33]. Synaptotagmins have been implicated in relation to susceptibility to psychiatric disorders such as ADHD and ASD [34,35]. No significant difference for mRNAs of *SYT15*, with significantly increased expression for *SYT15*-targeted lncRNAs, were found in the peripheral lymphocytes of children with ADHD and ASD in our study, which implied that the function of *SYT15* in these disorders warrants clarification. The expression of *SYT15*-targeting lncRNAs was significantly increased in males, but there was no difference in female ID, with no difference in expression in mRNA levels for both genders, an interesting finding. Whether this finding indicates a distinct mechanism underlying the development of ID between different genders requires further investigation.

The *SHANK2* gene encodes a protein that is a member of the Shank family of synaptic proteins, which may function as molecular scaffolds in the postsynaptic density of excitatory synapses [35]. This gene has been identified in patients with ASD and ID [36,37]. In our study, no difference in the mRNAs of *SHANK2* in ADHD, ASD, and ID was present, with the increased expression of *SHANK2*-targeting lncRNA in ASD, which may reflect a different expression pattern in different cells for *SHANK2*.

Differential expression of lncRNAs and targeted mRNAs of *PKNOX2, HOXB5, HOXA6*, and *HOXA13* in ADHD, ASD, and ID

Our results revealed no significant difference in the expression of mRNAs of *PKNOX2* in ADHD, ASD, and ID, with a significant increase of *PKNOX2*-targeting lncRNAs in ADHD, ASD, and male ID. We also identified a significant, consistently increasing trend for expression of lncRNAs and the targeted mRNAs of *HOXA6* in ADHD and ASD. There was a significantly up-regulated expression of mRNAs of *HOXB5* in ADHD, of *HOXA6* in male ID, and of *HOXA13*-targeting lncRNA in ADHD. However, the significantly down-regulated expression of mRNA of *HOXB5* in female ID was indicated by our results.

PKNOX2 encodes several homeodomain proteins that are sequence-specific transcription factors that share a highly conserved DNA-binding domain and play fundamental roles in cell proliferation and differentiation. *PKNOX2* has been reported to be involved in the pathogenesis of schizophrenia and the formation of substance dependence [38,39]. Because the clinical characteristics of ID and schizophrenia are similar, decreased levels of *PKNOX2*-targeted lncRNA may be involved in the development of ID.

The *HOXA6* and *HOXB5* genes encode the class of transcription factors called homeobox (*HOX*) genes found in clusters named A and B on two separate chromosomes. The expression of these proteins is regulated spatially and temporally during embryonic development. The *HOX* gene family is known to be a classic example of the intimate relationship between embryogenesis and tumorigenesis. Studies have suggested that *HOXB5* acted as a positive modulator, most likely by promoting cells' proliferative response and invasiveness in ER-positive breast cancer [40,41]. Dickson et al. demonstrated that *HOXA6* was directly involved in the fundamental processes of hemopoietic progenitor cell development [42]. Similar to *HOXA6*, *HOXA13* is a homeobox gene that encodes transcription factors regulating embryonic development and cell fate. Dysregulation of *HOXA13* has been implied in the cancer genesis and development of gastric cancer and hepatocellular carcinoma [42,43]. Several studies have linked the disordered proliferation of brain neural cells such as cortical neural progenitor cells and glial cells with the pathogenesis of ASD and ID [44–48]. The differential expression of *HOXB5, HOXA6*, and *HOXA13*, which are involved in the proliferation of cell growth, may have played a specific role in the neurodevelopmental disorder. These *HOX* genes were almost all affected in ADHD, ASD, and ID, in either a separate or a combined manner, with differential expression for

lncRNAs and targeted mRNAs. This finding indicated a fundamental role for *HOX* genes in the neurodevelopmental and neuropsychiatric diseases. The exact function of the lncRNAs and targeting mRNAs of *HOXB5*, *HOXA6*, and *HOXA13* in ADHD, ASD, and ID deserve further exploration.

Conclusion

Through our work, we identified for the first time the differential expression pattern of lncRNAs and targeted mRNAs for several development-related genes (*HOXB5, HOXA6, HOXA13, SYT15, PKNOX2, SHANK2, and BDNF*) in the peripheral lymphocytes of ADHD, ASD, and ID patients. Although the potential expression difference between peripheral blood lymphocytes and cells of brain origin may weaken the strength of our findings, and further study with more samples and more elaborate design was needed to validate these results, our investigation provided a new window for exploring the mechanism underlying neurodevelopmental and neuropsychiatric disorders and the identification of biomarkers that are more practicable in clinic practice.

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Author Contributions

Haiqing Xu coordinated the experiments and participated in sample collection; Zhiwei Zhao, Haiqing Xu, and Lihui Wu drafted the manuscript; Zhiwei Zhao and Hong Wang performed the experimental work; and Qiong Dai and Aiqin Zhou performed the statistical design and analyses. Xiaoyan Wang, Meirong Wu, Xinglian Liu, and Xuan Zhang recruited the patients and obtained samples and informed consents. Nanbert Zhong designed and supervised the studies, finalized the manuscript, and was responsible for all studies in the entire project.

Competing Interests

The authors declare no conflict of interest.

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