Psychiatry Research 282 (2019) 112639

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

An integrative analysis of transcriptome-wide association study and mRNA expression profile identified candidate genes for attention-deficit/hyperactivity disorder

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ARTICLE INFO

Keywords:
Attention-deficit/hyperactivity disorder (ADHD)
Transcriptome-wide association study (TWAS)
Genome-wide association study (GWAS)
Gene ontology (GO)
Pathway

ABSTRACT

Background: Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder, but the genetic mechanism of ADHD remains elusive now.

Methods: Tissue specific transcriptome-wide association study (TWAS) of ADHD was performed by FUSION utilizing a genome-wide association study (GWAS) dataset of ADHD (including 20,183 ADHD cases and 35,191 healthy controls) and gene expression reference from brain and blood. Furthermore, the genes identified by TWAS were compared with the differently expressed genes detected by mRNA expression profiles of ADHD rat model and autism spectrum disorders (ASD) patients. Functional enrichment and annotation analysis of the identified genes were performed by DAVID and FUMAGWAS tool.

Results: For brain tissue, TWAS identified 148 genes with P value < 0.05, such as *TDO2* ($P_{TWAS} = 4.01 \times 10^{-2}$), *CHD1L* ($P_{TWAS} = 9.64 \times 10^{-3}$) and *KIAA0319L* ($P_{TWAS} = 4.05 \times 10^{-4}$). Further 11 common genes were examined in the mRNA expression datasets, such as *ACSM5* ($P_{TWAS} = 3.62 \times 10^{-2}$, $P_{mRNA} = 0.005$), *CCDC24* ($P_{TWAS} = 1.49 \times 10^{-2}$, $P_{mRNA} = 2.35 \times 10^{-3}$) and *MVP* ($P_{TWAS} = 5.55 \times 10^{-3}$, $P_{mRNA} = 5.40 \times 10^{-3}$). Pathway enrichment analysis of the genes identified by TWAS detected 3 pathways for ADHD, including Other glycan degradation (P value = 0.021), Viral myocarditis (P value = 0.034) and Endocytosis (P value = 0.041).

Conclusions: Through integrating GWAS and mRNA expression data, we identified a group of ADHD-associated genes and pathways, providing novel clues for understanding the genetic mechanism of ADHD.

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder characterized by inattentive, hyperactive and impulsive symptoms, which affects 5% of children, 5% adolescents and 2.5% of adults all over the word (Faraone et al., 2015). Genetic factors play a vital role in the etiology of ADHD and its comorbidity with other disorders, the evidence of which are provided by family, twin and adoption studies (Faraone et al., 2005). Family, twin, and adoption studies showed that ADHD has a high heritability of 70–80% in both children and adults, which covered the contribution of both common and rare causal genetic variant of ADHD (Faraone et al., 2005, Larsson et al., 2014). Most research focused on genes which belong to the dopamine neurotransmitter system. Molecular genetic studies support the dopamine hypothesis, with meta-analyses providing evidence for association between ADHD and the dopamine receptor genes (*DAT1*) (Hoogman et al., 2013). Genome-wide association studies (GWAS) have revealed additional candidate genes (e.g., *BAIAP2C, FBXO33D, SPOCK3*) (Ribases et al., 2009-Weber et al., 2014), although these variants have not achieved genome-wide significance (Sanchez-Mora et al., 2015, Neale B et al., 2010). Although there are a wealth of evidence supporting for the role of genetics in ADHD, specific and definitive genetic mechanisms have not been identified.

Genome-wide association study (GWAS) is often used to detect effects of common risk genetic variants on the phenotype. The heritability attributed to the common SNPs can be also estimated using GWAS summary data of ADHD. Using the GWAS summary data, a recent paper reported an ADHD heritability of approximately 10%, which was largely attributed to the common causal genetic variant, but not rare causal genetic variants (Anttila et al., 2018). However, GWAS are also

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https://doi.org/10.1016/j.psychres.2019.112639
Received 18 July 2019; Received in revised form 18 October 2019; Accepted 24 October 2019
Available online 25 October 2019
0165-1781/

likely to ignore the relationship that exists between genetic variants, DNA functional elements (e.g. gene expression/protein levels) and complex traits and diseases. Expression quantitative trait loci (eQTLs) is one of the most effective ways to discover gene regulation networks (Brem and Kruglyak, 2005). The eQTL method measures the variance in gene transcription, followed by mapping the genetic loci affecting gene expression at mRNA level (Jansen and Nap, 2001). Therefore, integrating the GWAS and eQTLs data has the potential to discover novel disease associated genes.

Transcriptome-wide association study (TWAS) has recently been developed to integrate the characteristics of eQTLs studies and GWAS to expound gene-trait relationships (Veturi and Ritchie, 2018). TWAS has successfully identified many genes whose genetically regulated expression is associated to traits and can boost power to detect novel disease genes (Barfield et al., 2017). TWAS is mainly used to train multivariate expression quantitative trait loci (eQTL) models on reference expression panels, use these models to predict unobserved gene expression in large scale GWAS cohorts, and compute association statistics by regressing phenotype directly onto imputed gene expression (Park et al., 2017). For instance, two novel genes have been identified as statistically significantly associated with nonobstructive azoospermia (NOA) susceptibility through TWAS method (Jiang et al., 2017). Moreover, Alexander Gusev et al. have performed TWAS and identified multiple novel genes at risk loci associated with schizophrenia (Gusev et al., 2018).

In this study, we performed an integrative analysis of transcriptome-wide association study and mRNA expression profile to identify genes whose expression is relative to ADHD. First, we performed TWAS analysis using GWAS data of ADHD and gene expression reference from brain, peripheral blood and whole blood. Second, the genes identified by TWAS were further compared with the differently expressed genes identified by the mRNA expression profiles of ADHD rat model and ASD human subjects. Third, gene ontology (GO) and pathway enrichment analysis of the genes identified by TWAS were performed by DAVID.

2. Material and methods

2.1. GWAS summary data of ADHD

The GWAS summary data set of ADHD was driven from the Psychiatric Genomics Consortium (Demontis et al., 2017). Briefly, this GWAS data set comprised of 12 cohorts collected from European, North American and Chinese (20,183 ADHD cases and 35,191 healthy controls). The Illumina PsychChip was used to genotype all the DNA samples. Before the analysis, stringent quality control procedures were conducted on the genotyped markers and individuals. The literature used the Phase 3 reference panel of 1000 Genomes Project to perform genotype imputation. SNP association tests were performed using a logistic regression model for each cohort and using principal components as covariates for correcting population stratification. Finally, the GWAS results of the cohorts were meta-analyzed by an inverse-variance weighted fixed effects model. Detailed information of the subjects, genotyping, imputation and quality control can be found in the published study (Demontis et al., 2017).

2.2. mRNA expression profile of ADHD rats model

The mRNA expression profile of ADHD rats and control rats was downloaded from the published study (Yoshida et al., 2014). Briefly, spontaneously hypertensive rats (*SHR*) were used as rat model of ADHD and Normotensive Wistar-Kyoto rats (*WKY*) were used as control rats in this study. Total RNA was purified from brain tissue with a miRNeasy kit (Qiagen, Hilden, Germany). The 4 × 44 K whole rat genome oligo microarray version 3.0 G2519F was used to perform expression profile, with WKY and SHR at 3 and 6 weeks of age. Total RNA (200 ng) was reverse transcribed into double-stranded cDNA using AffnityScript multiple temperature reverse transcriptase, and amplified. The published literature contained a detailed description of the sample characteristics, experimental design, and statistical analysis of the data set (Yoshida et al., 2014).

2.3. mRNA expression profile of ASD human subjects

To the best of our knowledge, no transcriptome-wide mRNA expression profiling of human ADHD patients have been conducted. ADHD and ASD originate from partly similar familial or genetic factors as supported by family and twin studies (Rommelse N et al., 2010, Ronald et al., 2008). Given the common characteristics of ADHD and ASD (Ronald et al., 2008), the mRNA expression profiles of ASD were also applied in this study (Gupta et al., 2014). ASD expression data comes from post-mortem brains of ASD individuals. Frozen brain samples derived from a total of 57 (40 unique individuals) control and 47 (32 unique individuals) autism samples. The researchers extracted 50 mg of total RNA from post-mortem brain tissue with Trizol reagent (Invitrogen), which was prepared for RNA-Seq libraries. The RNA-Seq reads were mapped to a set of sequences derived from the Genome Reference Consortium Human build 37 (GRCh37) assembly. About 48,260 of the total 62,069 reported Ensembl gene annotations used the Python script 'HTSeq-count' to estimate gene expression levels. The gene count data were normalized by methods implemented in Conditional Quantile Normalization and Exploratory Data Analysis and Normalization for RNA-Seq (EDASeq). Picard command-line tools 'CollectRnaSeqMetrics' and RNA-Seq summary statistics were provided by 'CollectGcBiasMetrics'. Permutation testing was used to estimate the threshold for transcriptome-wide significant differential expression (EDASeq, 400 permutations, $P = = 4.76 \times 10^{7}$). The more detailed information can be found in published studies (Gupta et al., 2014).

2.4. Statistical analysis

FUSION was applied to the GWAS summary data of ADHD for TWAS within brain, whole blood and peripheral blood (Gusev et al., 2016). The gene expression reference weights for brain, whole blood and peripheral blood were obtained from the FUSION website (http://gusevlab.org/projects/fusion/) (Gusev et al., 2016). Briefly, the gene expression weights was first calculated using the prediction models of FUSION. Then, the calculated expression weights were combined with GWAS results to impute association statistics between gene expression levels and target diseases. Bayesian sparse linear mixed model (BSLMM) was used to compute the SNP-expression weights in the 1-Mb cis loci of the gene for a given gene (Zhou et al., 2013). The association testing statistics between predicted gene expression and target diseases was calculated as $Z_{TWAS} = w'Z/(w'Lw)^{1/2}$ (Gusev et al., 2016). Z denotes the scores of ADHD and w denotes the weights. L denotes the SNP-correlation (LD) matrix. In this study, we accounted for linkage disequilibrium (LD) among SNPs and viewed the imputed gene expression data as a linear model of genotypes with weights. A TWAS P value was calculated for each gene within brain, whole blood and peripheral blood, respectively. The genes with P value < 0.05 was considered as significant.

The GO and pathway enrichment analysis of the genes identified by TWAS were performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Bioinformatics Microarray Analysis (https://david.ncifcrf.gov/) (Huang et al., 2009). Homo sapiens was set as the background to decide the degree of enrichment analysis. DAVID tool used a novel biological module-centric algorithm, a set of new fuzzy classification algorithms, to functionally analyze large gene lists (Huang D et al., 2007, Huang D et al., 2007). For the gene list, functional annotation chart analysis was performed to identify the most relevant biological terms (Huang et al., 2007, Dennis et al., 2003), including GO Biological Process, GO Molecular Function, GO Cellular Component, KEGG Pathways. The genes

Table 1
List of top 30 candidate genes identified by TWAS for ADHD.

Gene	Chr	Tissue	P_{TWAS}
NME5	5	brain	1.00×10^{-4}
ABHD5	3	Whole Blood	1.00×10^{-4}
GMPR2	14	Whole Blood	1.00×10^{-4}
ZNF763	19	brain	2.85×10^{-4}
BLOC1S2	10	Peripheral Blood	3.69×10^{-4}
KIAA0319L	1	brain	4.05×10^{-4}
FMNL3	12	Peripheral Blood	8.31×10^{-4}
BLOC1S2	10	Whole Blood	8.59×10^{-4}
CCDC138	2	brain	8.70×10^{-4}
TMBIM6	12	brain	1.63×10^{-3}
SV2A	1	brain	1.79×10^{-3}
TARBP1	1	Whole Blood	1.97×10^{-3}
TATDN3	1	brain	2.97×10^{-3}
ALS2CR8	2	brain	2.98×10^{-3}
ST3GAL3	1	brain	3.27×10^{-3}
ELAVL4	1	brain	3.31×10^{-3}
BLOC1S2	10	brain	3.33×10^{-3}
NBEAL1	2	brain	3.48×10^{-3}
FMNL3	12	brain	3.70×10^{-3}
FGFR3	4	brain	3.76×10^{-3}
ACAD11	3	brain	3.90×10^{-3}
CNPY2	12	brain	3.91×10^{-3}
TMEM206	1	brain	4.47×10^{-3}
BST1	4	Peripheral Blood	4.76×10^{-3}
RASSF4	10	brain	4.88×10^{-3}
SLC6A12	12	Whole Blood	4.95×10^{-3}
RAPGEF5	7	brain	4.99×10^{-3}
MVP	16	Whole Blood	5.55×10^{-3}
RPS20	8	Whole Blood	5.79×10^{-3}
RASGRP4	19	Whole Blood	6.62×10^{-3}

identified by TWAS were further compared with the differently expressed genes identified by mRNA expression profiles. Functional Mapping and Annotation of Genome-wide Association Studies (FUMAGWAS, http://fuma.ctglab.nl/) was used to annotate, prioritize, visualize and interpret the function of the common genes shared by TWAS and mRNA expression profiles.

3. Results

3.1. TWAS

For brain tissue, TWAS identified 148 genes with TWAS *P* value < 0.05, such as *TDO2* ($P_{TWAS}=4.01\times10^{-2}$), *CHD1L* ($P_{TWAS}=9.64\times10^{-3}$), *KIAA0319L* ($P_{TWAS}=4.05\times10^{-4}$) and *CCDC138* ($P_{TWAS}=8.70\times10^{-4}$) (Supplementary Table 1). For peripheral blood and whole blood, TWAS detected 88 genes with TWAS *P* value < 0.05, such as *ABHD5* ($P_{TWAS}=1.00\times10^{-4}$ for whole blood), *GMPR2* ($P_{TWAS}=1.00\times10^{-4}$ for whole blood), *TARBP1* ($P_{TWAS}=1.97\times10^{-3}$ for whole blood) and *BST1* ($P_{TWAS}=4.76\times10^{-3}$ for peripheral blood) (Supplementary Table 1). The top 30 significant genes of ADHD identified by TWAS were showed in table 1.

We further compared the genes identified by TWAS with the differently expressed genes detected by mRNA expression profiles. Eleven common genes were identified, such as *ACSM5* ($P_{TWAS}=3.62\times10^{-2}$, $P_{mRNA}=0.005$ for ADHD rat model), *CCDC24* ($P_{TWAS}=1.49\times10^{-2}$, $P_{mRNA}=2.35\times10^{-3}$ for human ASD patients), *MCM6* ($P_{TWAS}=3.25\times10^{-2}$, $P_{mRNA}=9.84\times10^{-4}$ for human ASD patients) and *MVP* ($P_{TWAS}=5.55\times10^{-3}$, $P_{mRNA}=5.40\times10^{-3}$ for human ASD patients) (Table 2). Fig. 1 showed gene expression heat map of those common genes.

3.2. Gene ontology and pathway enrichment analysis

The results of GO enrichment analysis for genes identified by TWAS were described in table 3. We detected 23 GO terms with DAVID *P*

Table 2
List of common genes identified by TWAS and mRNA expression profile.

Gene	mRNA expression profile		TWAS P value
	Source of profile	P value	
ACSM5	ADHD rat model	0.005	3.62×10^{-2}
ARHGAP27	ASD human	1.62×10^{-3}	4.77×10^{-2}
ASB8	ASD human	1.10×10^{-4}	3.72×10^{-2}
CCDC24	ASD human	7.97×10^{-3}	1.49×10^{-2}
ELAVL4	ASD human	5.39×10^{-4}	3.31×10^{-3}
GMPR2	ASD human	2.04×10^{-5}	1.00×10^{-4}
TMBIM6	ASD human	6.63×10^{-3}	1.63×10^{-3}
MCM6	ASD human	9.84×10^{-4}	3.25×10^{-2}
MVP	ASD human	5.40×10^{-3}	5.55×10^{-3}
POLR3C	ASD human	3.02×10^{-3}	2.80×10^{-2}
TXNL4B	ASD human	5.94×10^{-3}	4.15×10^{-2}

value < 0.05 for ADHD, such as membrane (*P* value $=5.66\times10^{-4}$), cytoplasm (*P* value $=3.54\times10^{-3}$) and MAPK cascade (*P* value $=2.63\times10^{-2}$) (Table 3). Pathway enrichment analysis of the genes identified by TWAS detected 3 pathways, including Other glycan degradation (hsa00511, *P* value = 0.021), Viral myocarditis (hsa05416, *P* value = 0.034) and Endocytosis (hsa04144, *P* value = 0.041).

4. Discussion

Although the cellular and molecular basis of ADHD has been studied over the past few years, the mechanism of ADHD is extremely complex. In this study, we integrated the genetic information of TWAS with mRNA expression profile, which had potential to provide novel insight into the molecular mechanism of ADHD. We found 236 unique genes with transcriptome-wide-significant associations with ADHD. Besides, twenty-three GO terms and three pathways were identified through performing DAVID for these significant genes.

One of the important genes identified by this study is tryptophan 2,3-dioxygenase (*TDO2*), which encodes a heme enzyme that plays an important role in tryptophan metabolism by catalyzing the first and rate-limiting step of the kynurenine pathway. Tryptophan is the serotonin precursor, which controls serotonin level in human body. Abnormalities of serotonin metabolism have been implicated in a wide range of human neuropsychiatric disorders (Comings et al., 1995). Using the IntelliCage, a battery of established tests revealed that deficiencies in TDO differentially affect exploratory behavior and learning performance in mice. Besides, gene deficiency differentially affected the metabolism of kynurenine, serotonin and dopamine in the brain (Too et al., 2016). In addition, a mutation that resulted in increased activity of the *TDO2* could resulted in low serotonin levels by increasing degradation of tryptophan (Comings et al., 1995). Single nucleotide polymorphisms in the *TDO2* gene may be associated with ADHD (Comings et al., 1995), which was consistent with the results of TWAS. However, further research is still needed to explore the underlying mechanism.

Another notable gene was chromodomain helicase DNA binding protein 1 like (*CHD1L*). *CHD1L* (also known as *ALC1*) encodes a DNA helicase protein involved in DNA repair and the protein converts ATP to add poly(ADP-ribose) as it regulates chromatin relaxation following DNA damage (Ahel et al., 2009). In a previous study of 117 unrelated probands with sporadic ADHD, a panel of 26 genes implicated in intellectual disability (ID) and ASD was sequenced to evaluate whether variation in ASD/ID-associated genes were also present in participants with ADHD. It was identified that only one putative deleterious variant in *CHD1L* (Kim et al., 2017).

We also detected 23 ADHD associated GO terms, the main function of which was associated with cellular component and biological process. For instance, MAPK cascade (GO:0000165) lies downstream of many cell surface receptors and cooperate in transmitting various

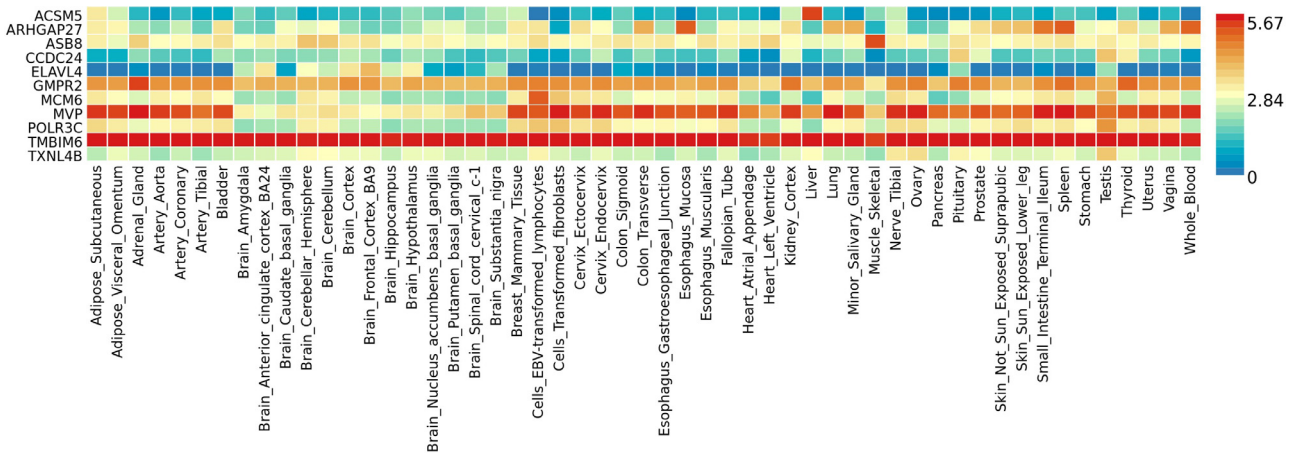

Fig. 1. Gene expression heat map of the detected common genes shared by TWAS and mRNA expression data.

extracellular signals to the nucleus. It has become clear that the RAS-MAPK pathway is essential for normal neurophysiologic functioning within multiple brain circuits (Samuels et al., 2009). The proteins in this pathway are involved in modulating release of inhibitory neurotransmitters in regions of the brain involved in attention regulation and executive functions, including the prefrontal cortex (Shilyansky et al., 2010). The close relationship between intellectual and attentional functioning in Noonan syndrome (NS) could indicate that both capacities may be impacted by neurodevelopmental differences resulting from aberrant RAS-MAPK signaling cascade (Pierpont et al., 2018). That some degree of similarity between the behavioral deficits observed in a portion of children with Noonan syndrome and those with idiopathic ADHD was indicated in the findings (Pierpont et al., 2015).

Several candidate biological pathways were detected for ADHD, such as endocytosis (hsa04144) pathway. Protein kinase C (PKC) sensitive negative endocytic mechanism is implicated to cause dopamine transporter (DAT) function aberrantly in ADHD (Sorkina et al., 2005). The dopamine transporter plays an essential role in the modulation of dopaminergic neurotransmission by mediating the reuptake of dopamine into presynaptic neurons (Daniels and Amara, 1999). Some studies supported that the sustained DAT down-regulation in response to PKC activation was believed mainly to be a result of DAT endocytosis (Sorkina et al., 2005, Melikian and Buckley, 1999). Multiple DAT coding variants have been reported in ADHD, and these DAT mutants exhibit anomalous DA efflux, loss of function, or DAT membrane instability due to endocytic dysfunction (Sakrikar et al., 2012). DAT surface expression is dynamically regulated by endocytic trafficking (Eriksen et al., 2010). As all we know that the low level of Dopamine D2 receptor (DRD2) function in the brain is a risk factors for ADHD, and DRD2 endocytosis is vital for its desensitization (Li et al., 2012).

Although the heritability of ADHD is partly explained by GWAS, they still could miss the genetic variants with expression-trait associations. TWAS used multivariate expression quantitative trait loci (eQTL) models and GWAS summary data to identify the genes associated with the diseases in the expression level (Park et al., 2017). Compared with the previous GWAS of ADHD (Demontis et al., 2017), TWAS is prone to spurious prioritization based on the expression data from ADHD related tissues. Besides, TWAS was more accurate to prioritize candidate causal genes than simple baselines (Gusev et al., 2018). TWAS has been successful in identifying many genes and can boost power to detect novel disease genes (Barfield et al., 2017).

However, there are two limitations that need to be noted in this

Table 3
Gene ontology enrichment analysis results of the genes identified by TWAS.

Category	ID	NAME	P value
GOTERM_CC_DIRECT	GO:0,016,020	membrane	5.66×10^{-4}
GOTERM_CC_DIRECT	GO:0,005,737	cytoplasm	3.54×10^{-3}
GOTERM_BP_DIRECT	GO:0,003,351	epithelial cilium movement	8.69×10^{-3}
GOTERM_BP_DIRECT	GO:0,030,890	positive regulation of B cell proliferation	8.99×10^{-3}
GOTERM_BP_DIRECT	GO:0,021,591	ventricular system development	1.01×10^{-2}
GOTERM_BP_DIRECT	GO:0,016,032	viral process	1.79×10^{-2}
GOTERM_MF_DIRECT	GO:0,050,135	NAD(P)+ nucleosidase activity	2.27×10^{-2}
GOTERM_MF_DIRECT	GO:0,001,147	transcription termination site sequence-specific DNA binding	2.27×10^{-2}
GOTERM_BP_DIRECT	GO:0,000,165	MAPK cascade	2.63×10^{-2}
GOTERM_BP_DIRECT	GO:0,019,915	lipid storage	2.84×10^{-2}
GOTERM_BP_DIRECT	GO:0,003,333	amino acid transmembrane transport	3.07×10^{-2}
GOTERM_CC_DIRECT	GO:0,005,654	nucleoplasm	3.17×10^{-2}
GOTERM_MF_DIRECT	GO:0,003,953	NAD+ nucleosidase activity	3.39×10^{-2}
GOTERM_BP_DIRECT	GO:0,035,556	intracellular signal transduction	3.49×10^{-2}
GOTERM_BP_DIRECT	GO:0,017,144	drug metabolic process	3.54×10^{-2}
GOTERM_MF_DIRECT	GO:0,050,660	flavin adenine dinucleotide binding	3.69×10^{-2}
GOTERM_BP_DIRECT	GO:0,009,058	biosynthetic process	4.29×10^{-2}
GOTERM_MF_DIRECT	GO:0,005,088	Ras guanyl-nucleotide exchange factor activity	4.30×10^{-2}
GOTERM_BP_DIRECT	GO:0,046,629	gamma-delta T cell activation	4.33×10^{-2}
GOTERM_BP_DIRECT	GO:0,045,716	positive regulation of low-density lipoprotein particle receptor biosynthetic process	4.33×10^{-2}
GOTERM_BP_DIRECT	GO:0,010,988	regulation of low-density lipoprotein particle clearance	4.33×10^{-2}
GOTERM_BP_DIRECT	GO:0,005,975	carbohydrate metabolic process	4.42E-02
GOTERM_MF_DIRECT	GO:0,004,553	hydrolase activity, hydrolyzing O-glycosyl compounds	4.59E-02

Abbreviations: Molecular Function (MF); Cellular Component (CC); Biological Processes (BP).

study. First, TWAS has been developed to identify causal genes, the regulated expression of which was associated to target diseases. TWAS may have lower power to detect the causal loci without cis-expression effects on target disease. Second, to the best of our knowledge, few human expression profiling of ADHD was conducted by now. Therefore, the mRNA expression profiles of ADHD rat model and human ASD subject were used in this study. Given these limitations, our results should be interpreted with caution and the further studies are needed to confirm our findings.

In conclusion, this is the first report on ADHD combining TWAS and gene expression profiling datasets. In this work, after a comparison of TWAS and expression profile, 11 common genes were identified to be candidate genes for ADHD. However, in view of some limitations, the results should be carefully explained. Therefore, a further study was needed to verify our results and reveal the potential effect of identified candidate genes in the development of ADHD.

Ethical standards

The manuscript does not contain clinical studies or patient data.

Declaration of Competing Interest

The authors have stated that they have no conflict of interest.

Acknowledgments

This study was funded by the National Natural Scientific Foundation of China(grants number 81472925 and 81673112), the Key projects of international cooperation among governments in Scientific And Technological Innovation (grant number 2016YFE0119100), the Technology Research and Development Program of in Shaanxi Province of China(grant number 2013KJXX-51), and the Fundamental Research Funds for the Central Universities.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.112639.

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