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GENETICS AND EPIGENETICS OF PSYCHIATRIC DISEASES - VOLUME II

Topic Editors:
Cunyou Zhao, Southern Medical University, China
Zhexing Wen, Emory University, United States
Weihua Yue, Peking University Sixth Hospital, China

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EDITED AND REVIEWED BY
Michael E. Symonds,
University of Nottingham,
United Kingdom

*CORRESPONDENCE
Cunyou Zhao,
zhaocunyou@gmail.com
Weihua Yue,
dryue@bjmu.edu.cn
Zhexing Wen,
zhexing.wen@emory.edu

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Editorial: Genetics and epigenetics of psychiatric diseases—volume II

Qiyang Li[1,2], Yuanyuan Gai[2], Weihua Yue[3]*, Zhexing Wen[4]* and Cunyou Zhao[1,2]*

[1]Department of Rehabilitation, Zhujiang Hospital of Southern Medical University, Guangzhou, Guangdong, China, [2]Department of Medical Genetics, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, China, [3]Peking University Sixth Hospital & Institute of Mental Health, Beijing, China, [4]Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA, United States

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Editorial on the Research Topic
Genetics and epigenetics of psychiatric diseases—volume II

Towards psychiatric disorders: Advance in understanding the role of mediating associations between genetic risk burden, environmental or epigenetic risk exposure and phenotype in psychiatric pathogenesis.

In this Research Topic, we have hosted 2 in-depth reviews and 10 original research articles introducing the potential mechanisms of how genetics, epigenetics and environmental factors interact and contribute to the etiology of psychiatric disorders.

Psychiatric disorders, including schizophrenia, bipolar disorder, major depressive disorder, and autism, are complex polygenic mental disorders which arise from their multifactorial nature: genetic, and epigenetic or environmental factors. Over the past decades, genome-wide association studies (GWASs) have reported a number of risk loci that are robustly associated with these disorders. These risk loci have not only provided pivotal insights into the genetic and biological bases of psychiatric disorders but also facilitated the translation of GWAS findings into potential therapeutics. Despite the great success of GWASs, GWAS loci are often hard to be interpreted: most common variants have a weak effect size on traits and exhibit combinatorial patterns of inheritance, thus probably conveying risks for the diseases through molecular networks and interactions. Furthermore, since the clinical presentations and severity of various subtypes of psychiatric disorders vary, genetic differences in various subtypes of psychiatric disorders remain unclear due to inadequate sample sizes or substandard clinical classifications. In addition, how environmental factors affect gene expression through epigenetic modifications and lead to psychiatric disorders remain unclear. Therefore, pinpointing the potential regulatory networks between genetic risk burden,

environmental or epigenetic risk exposure and phenotype remains a challenge in psychiatric pathogenesis.

At the level of genomics, to find out the genetic differences in various subtypes of bipolar disorder, Huang et al. explored the potential causal associations between two bipolar disorder subtypes and lithium responses by comparing the difference in genetic structures among four different psychiatric traits with cross-phenotype analysis. This study illustrated genetic convergence and divergence between bipolar disorder I and II and provided new biological insights into psychiatric disorders. Given the important roles of *BDNF* and *CREB* in the development of schizophrenia, Ping et al. used a case-control design and discovered that rs11030101, rs2030324, rs6265, rs6740584 and rs2551640 are associated with schizophrenia. By using GWAS in Chinese students, Zhang et al. found out that rs80263879 and rs72478903 of *EPHX2* are candidate genetic loci for static spatial working memory. To identify hub genes in modules of major depressive disorder, Yang et al. used Weighted Gene Co-Expression Network Analysis and found six hub genes (*ADM*, *CITED2*, *IER5*, *NFKBIZ*, *SERTAD1*, *TNF*) with similar co-expression and dysregulation patterns in major depressive disorder. In addition, as *ULK4* is a rare susceptibility gene for psychiatric disorders, Luo et al. discussed the roles of ULK4 in neurodevelopmental and neuropsychiatric disorders which are helpful for the development of ULK4-based therapeutic strategy.

It the term of epigenetics, Zhang et al. conducted brain epitranscriptomic analysis in septic patients and identified that A-to-I RNA editing led to dysregulated gene expression, which eventually contributed to brain dysfunction. Given that RNA editing is understudied in the psychiatric field, this study thus provides valuable information to disease pathogenesis and open up a novel research direction. Moreover, Wang et al. identified 10 miRNAs which are dysregulated in Chinese autistic patients. As DNA methylation is an important epigenetic modification, Zhao et al. discovered that hypomethylation of *SSTR4* may contribute to the development of alcohol dependence by using BeadChip array and pyrosequencing. In addition, Jahangir et al. discussed the potentially etiological roles of long interspersed nuclear elements 1 in the development of schizophrenia.

Lipidomics has recently been developed as a powerful tool to investigate the natural characteristics of psychiatric disorders. Tao et al. suggested that peripheral blood lipidomic profile alterations could facilitate the identification of homogeneous transdiagnostic subtypes across psychiatric disorders which might help identifying patients with differential biological characterizations. Li et al. observed that *ERα* rs2234693 and rs9340799 polymorphisms are associated with susceptibility to major depressive disorder in women. The widely used atypical antipsychotics often associate with high prevalence of metabolic disorders in patients with schizophrenia. Yang et al. illustrated that mice with *Tcf7l2* deletion were more vulnerable to suffer metabolic abnormalities which may be mediated by GLP-1R after olanzapine administration.

Currently, the studies on the regulatory role of mediating associations between genetic risk burden, environmental or epigenetic risk exposure and phenotype in psychiatric disorders are still far from enough. Although it is premature to translate these newly found molecule biomarkers and pathogenesis into potential therapeutics, they do provide new insights into the etiology and treatment of psychiatric disorders that can ultimately guide clinical decision and therapeutics.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Altered Expression of Brain-specific Autism-Associated miRNAs in the Han Chinese Population

Ziqi Wang[1], Tianlan Lu[1], Xianjing Li[1], Miaomiao Jiang[1], Meixiang Jia[1], Jing Liu[1], Dai Zhang[1,2,3], Jun Li[1] and Lifang Wang[1]**

[1]National Clinical Research Center for Mental Disorders (Peking University Sixth Hospital), NHC Key Laboratory of Mental Health (Peking University), Peking University Sixth Hospital, Peking University Institute of Mental Health, Beijing, China, [2]Chinese Institute for Brain Research, Beijing, China, [3]Guangdong Key Laboratory of Mental Health and Cognitive Science, Institute for Brain Research and Rehabilitation (IBRR), South China Normal University, Guangzhou, China

Autism is a complex neurodevelopmental disorder. However, its etiology is still unknown. MicroRNAs (miRNAs) are key post-transcriptional regulators. They play an important role in neurodevelopment and brain functions and may be involved in the pathogenesis of autism. Previous studies indicated altered expression of miRNAs in patients with autism. However, the findings were not consistent, and further explorations were needed. This study aimed to investigate whether miRNAs were dysregulated in autism. We examined the expression of 30 brain-specific autism-associated miRNAs in 110 patients with autism and 113 controls in the Han Chinese population using quantitative reverse transcription–polymerase chain reaction. The results demonstrated that 10 miRNAs (hsa-miR-191-5p, hsa-miR-151a-3p, hsa-miR-139-5p, hsa-miR-181a-5p, hsa-miR-432-5p, hsa-miR-181b-5p, hsa-miR-195-5p, hsa-miR-328-3p, hsa-miR-106a-5p, and hsa-miR-484) were significantly differentially expressed (false discovery rate <0.05). All of them were up-regulated in patients with autism compared with controls. The targets of these miRNAs were enriched for genes and pathways related to neurodevelopment, brain functions and autism. These findings suggested the participation of these 10 miRNAs in the pathogenesis of autism in the Han Chinese population.

Keywords: autism, miRNAs, miRNA expression profiling, qRT-PCR, plasma

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Cunyou Zhao,
Southern Medical University, China

Reviewed by:
Ming Li,
Kunming Institute of Zoology (CAS), China
Su-Xia Li,
Peking University, China

****Correspondence:***
Jing Liu
ljyuch@163.com
Lifang Wang
lifangwang@bjmu.edu.cn

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INTRODUCTION

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders. The core symptoms of ASD include significant deficits in social communication and interaction, repetitive behaviors, and restricted interests that commonly appear within the first 3 years of life and last throughout life. Autism affects about 1–2% of the world population with a higher incidence in boys than in girls (Maenner et al., 2020). In China, the prevalence of autism was estimated as 0.7% among 6- to 12-year-old children (Zhou et al., 2020). Studies have shown heritability of 80–90% in ASD; hundreds of genes and loci were indicated to be associated with the disorder (Geschwind, 2011). Recently, noncoding RNAs (ncRNAs), particularly microRNAs (miRNAs), were implicated in the central nervous system (CNS) functions and likely influenced the development of autism (Fabian and Sonenberg, 2012; Treiber et al., 2019). However, the contributions of these ncRNAs in autism are not completely understood yet.

MiRNAs comprise a class of evolutionarily conserved ncRNAs consisting of 19–25 nucleotides. They play an important role in post-transcriptional gene silencing and participate in a multitude of biological processes through base pairing to the 3′ untranslated regions of target messenger RNAs (mRNAs) to degrade the mRNAs or inhibit the translation (Fabian and Sonenberg, 2012; Treiber et al., 2019). A single miRNA could bind to multiple mRNAs. Together, these miRNAs could regulate around two thirds of human mRNAs, and 70% of them were expressed in the CNS (Tonacci et al., 2019). MiRNAs might be essential regulators in brain functions including neuronal plasticity and neuronal development (Rajasethupathy et al., 2009; Follert et al., 2014). Previous studies suggested the association between a number of miRNAs and various neuropsychological diseases including autism (Kocerha et al., 2015; Van Den Berg et al., 2020). Significant changes were detected in the expression of miRNAs in patients with autism using a variety of biomaterials such as postmortem brain, peripheral blood, and saliva. These dysregulated miRNAs might affect the expression of genes related to autism and neurodevelopment (Sarachana et al., 2010; Mor et al., 2015; Wu et al., 2016).

However, most of the current studies about the expression changes of miRNAs in autism focused on European and American individuals. Only two studies explored the miRNA expression in Chinese individuals, both using peripheral blood samples with a relatively small sample size. However, the findings of these studies were not consistent. To explore whether these potentially autism-associated miRNAs were dysregulated in autism, we selected brain-specific miRNAs with at least two consistent reports and analyzed their expression profiles in patients with autism and healthy controls in the Han Chinese population.

MATERIALS AND METHODS

Participants

This study included 110 patients with autism (93 male and 17 female) and 113 typically developing unrelated age- and sex-matched controls (95 male and 18 female). All participants were of Han Chinese ancestry and recruited at the Peking University Sixth Hospital (Beijing, China). The median age of diagnosis for children with autism was 4.39 (range 3.07–5.97) years.

Only children with typical autism were recruited to decrease heterogeneity. They should meet the following criteria under the independent assessment by two senior child psychiatrists: 1) fulfilling the Diagnostic and Statistical Manual of Mental Disorders, fourth edition criteria for autism; 2) Autism Behavior Checklist score ≥53; and 3) Childhood Autism Rating Scale score ≥35 (Krug et al., 1980; Schopler et al., 1980). Healthy controls were evaluated by two psychiatrists through unstructured interviews to confirm that they were not affected by autism. Any participant with Asperger syndrome, pervasive developmental disorder not otherwise specified, fragile X syndrome, tuberous sclerosis, a previously identified chromosomal abnormality, other neurological conditions, familial/inherited diseases (such as congenital deaf-mutism, hemophilia, and familial adenomatous polyposis), or severe mental disorders (such as schizophrenia, schizoaffective disorder and bipolar disorder) was excluded from the present study.

RNA Extraction and Quantitative Reverse Transcription–Polymerase Chain Reactions

The peripheral blood samples were collected from all participants and then centrifuged at 3,500 rpm at 4°C for 10 min to separate plasma from blood cells. Total RNA was extracted from 200-μL plasma samples using the Qiagen miRNeasy Serum/Plasma Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's protocols. The *Caenorhabditis elegans* miR-39 (cel-miR-39) mimic from miRNeasy Serum/Plasma Spike-in Control (Qiagen) was added to the lysed samples for internal normalization. The total RNA samples were then reverse transcribed with TransGen TransScript miRNA First-Strand cDNA Synthesis SuperMIX (TransGen, Beijing, China). Each cDNA was further diluted with RNase-free water and stored at −20°C until use. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed with TransGen PerfectStar Green qPCR SuperMix (TransGen, Beijing, China) on a LightCycler 96 Instrument (Roche, Switzerland). The qRT-PCR was performed in triplicate with a preincubation of 94°C for 30 s, followed by 45 cycles of 94°C for 5 s and 60°C for 30 s. Data were normalized where appropriate with the exogenous control cel-miR-39. All primers are listed in **Supplementary Table S1**. The relative quantitation for miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

Autism-Associated and Brain-specific miRNA Selection

PubMed, Google Scholar, and Web of Science databases were searched for case–control studies exploring the differentially expressed miRNAs between patients with autism and healthy controls using the search terms of "microRNA/miRNA" AND "autism/autism spectrum disorder" until 1 November 2021. The significant threshold values of p and fold change (FC) were set according to the original studies.

Human miRNA expression data were obtained from the miRmine database (http://guanlab.ccmb.med.umich.edu/mirmine). It comprised miRNA expression data from 304 high-quality experiments, including 16 different types of human tissues and biofluids: bladder, blood, brain, breast, hair follicle, liver, lung, nasopharynx, pancreas, placenta, plasma, saliva, semen, serum, sperm and testis (Panwar et al., 2017). Brain-specific miRNAs were defined as miRNAs whose expression in the brain ranked in the top five among all

tissues and were higher than that of 90% of all miRNAs in the brain (Teng et al., 2020).

Bioinformatics Analysis

The targets of each miRNA were predicted with MiRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/). The genes were selected for subsequent analyses if the miRNA-mRNA interactions were experimentally validated or predicted using both TargetScan and miRDB.

Gene Ontology (GO) and KEGG pathway enrichment analyses for the target genes were performed using the R package clusterProfiler (Yu et al., 2012). ASD-related genes were obtained from the Human Gene Module of Simons Foundation Autism Research Initiative database (ASD_SFARI, https://gene.sfari.org/database/human-gene/), which comprised 1023 candidate ASD risk genes (2 September 2021, release). The genes affected by likely gene-disrupting (including nonsense, splice site, and frame-shift) and missense rare *de novo* variants (DNVs) detected in ASD were also included (ASD_DNVs_LGD, 353 genes; ASD_DNVs_missense, 1771 genes) (Iossifov et al., 2014). Further, 401 genes involved in the intellectual disability (ID_all) were acquired from the study by Parikshak, N. N. et al., 2013 (Parikshak et al., 2013). The genes in ASD_SFARI but not in ID_all (ASD_only), genes in both ASD_SFARI and ID_all (ASD&ID overlap), and genes in ID_all but not in ASD_SFARI (ID_only) were analyzed for gene sets enrichment. ASD-associated mRNA coexpression modules were indicated by Parikshak, N. N. et al., 2013, including 2 down-regulated modules ASD_CoexDown_M2 (1042 genes) and ASD_CoexDown_M3 (996 genes) and 3 up-regulated modules ASD_CoexDown_M13 (870 genes), ASD_CoexDown_M16 (492 genes) and ASD_CoexDown_M17 (1042 genes). The markers for different types of neural cells (neurons, 1484 genes; astrocytes, 1960 genes; oligodendrocytes, 1614 genes; microglias, 364 genes) were obtained from the study by Werling et al., 2016 (Werling et al., 2016). The fisher's exact test was performed to evaluate whether a gene set was enriched over background (~20,000 protein-coding genes in the whole genome). *p*-values were adjusted for multiple comparisons using Benjamini–Hochberg correction to assess the false discovery rate (FDR).

Statistical Analyses

Data were analyzed using SPSS Statistics 24 and R 4.0.5. software. The homogeneity for age and sex between patients with autism and controls was assessed with the Student's *t*-test and the chi-squared test following the examination of the normality of distribution using the Kolmogorov–Smirnov test. The differences in relative expression for each miRNA between patients with autism and controls were examined using nonparametric Mann–Whitney U test (two-tailed). FDR was used for multiple comparison corrections. The threshold of significance accepted for all statistical analyses was the *p*-value or FDR less than 0.05.

RESULTS

Based on previous studies, 311 miRNAs (DE-miRNAs) were indicated differential expression between patients with ASD and controls (**Supplementary Table S2**). 74 DE-miRNAs were described in multiple studies, of which 49 showed consistent up-regulation or down-regulation. Besides, 106 DE-miRNAs were detected with qRT-PCR. Together, 119 miRNAs were selected as autism-associated miRNAs (**Figure 1**). Using transcription data from the miRmine database, we defined 177 miRNAs as brain-specific miRNAs (**Supplementary Table S3**). We selected 30 miRNAs that were both autism-associated and brain-specific for further validation (**Figure 1** and **Supplementary Table S4**).

10 DE-miRNAs exhibited significantly differential expression between patients with autism and controls (FDR <0.05). All of them were up-regulated. The expression of five miRNAs in autism increased to greater than twofold of that in the controls, including hsa-miR-191-5p (FC = 2.40, FDR = 2.63E-05), hsa-miR-151a-3p (FC = 2.45, FDR = 4.67E-05), hsa-miR-139-5p (FC = 2.03, FDR = 2.06E-03), hsa-miR-432-5p (FC = 2.22, FDR = 4.60E-03), and hsa-miR-106a-5p (FC = 2.73, FDR = 0.03; **Table 1** and **Figure 2**). Considering the difference in prevalence and clinical manifestations between male and female patients with autism, we further compared the expression of these miRNAs between patients with autism and controls using samples only from males. Six of the 10 DE-miRNAs were significantly dysregulated, including hsa-miR-191-5p, hsa-miR-151a-3p, hsa-miR-139-5p, hsa-miR-181a-5p, hsa-miR-432-5p, and hsa-miR-195-5p. All of them were increasingly expressed in patients with autism (**Supplementary Table S5** and **Supplementary Figure S1**).

The targets prediction revealed miRNA–mRNA interactions between the 10 significant DE-miRNAs and 1732 genes. The GO analysis indicated that these target genes were significantly related to neurogenesis, neuron projection, and synapse functions (FDR <0.05). For KEGG pathway analysis, the target genes over-represented multiple brain-related KEGG pathways including MAPK signaling pathway (FDR = 2.87E-04), PI3K–Akt signaling pathway (FDR = 4.46E-03), axon guidance (FDR = 0.01), and Wnt signaling pathway (FDR = 0.02, **Figure 3A**). The 10 miRNAs also targeted 176 candidate ASD risk genes from the SFARI database [FDR = 3.97E-19, OR (95% CI) = 2.28 (1.93–2.69)] and genes harboring rare DNVs detected in ASD, including representative ASD-related genes such as *MECP2* (hsa-miR-106a-5p, hsa-miR-181a-5p, hsa-miR-195-5p, and hsa-miR-328-3p), *FMR1* (hsa-miR-139-5p, hsa-miR-181a-5p, and hsa-miR-181b-5p), *DDX3X* (hsa-miR-139-5p and hsa-miR-181b-5p), *PTEN* (hsa-miR-181a-5p and hsa-miR-181b-5p), and *RELN* (hsa-miR-195-5p) (**Figure 3B** and **Supplementary Table S6**). Besides, the target genes were significantly enriched for ASD-associated mRNA coexpression down-regulated modules ASD_CoexDown_M2 and ASD_CoexDown_M3 and up-regulated module

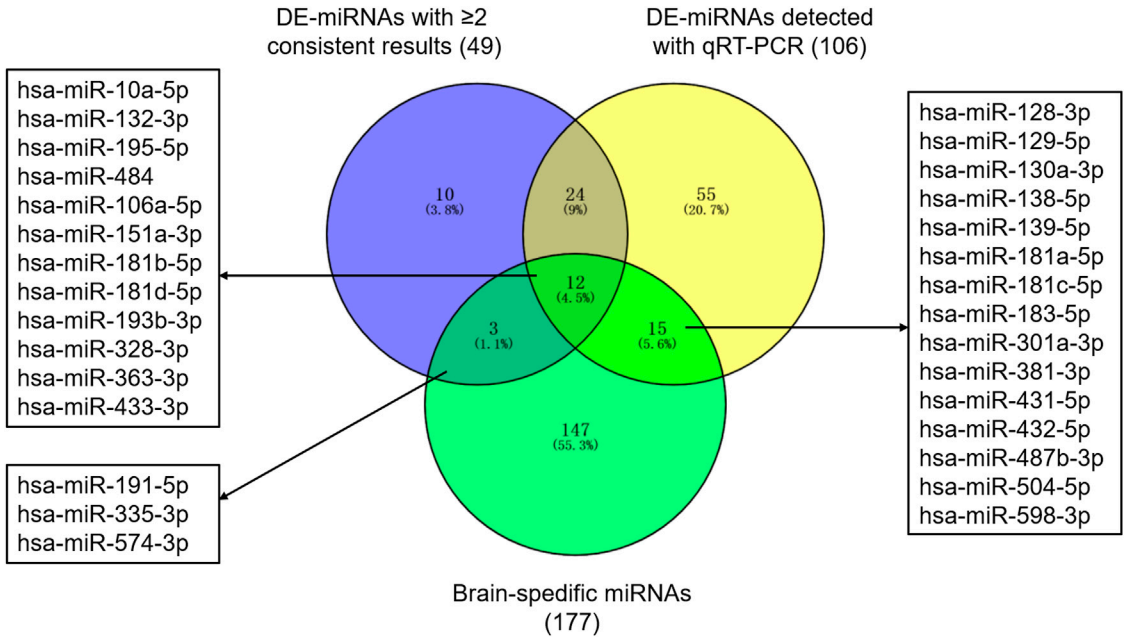

FIGURE 1 | Autism-associated and brain-specific miRNAs. Abbreviations: DE-miRNAs, differentially expressed miRNAs; qRT-PCR, quantitative reverse–transcription polymerase chain reactions.

TABLE 1 | Expression of 30 brain-specific autism-associated miRNAs in patients with autism and controls.

miRNA	FC	p^a	FDR[b]	Regulation	miRNA	FC	p^a	FDR[b]	Regulation
hsa-miR-191-5p	2.40	8.76E-07	**2.63E-05**	up	hsa-miR-335-3p	1.37	0.11	0.20	up
hsa-miR-151a-3p	2.45	3.11E-06	**4.67E-05**	up	hsa-miR-431-5p	1.56	0.13	0.21	up
hsa-miR-139-5p	2.03	2.06E-04	**2.06E-03**	up	hsa-miR-128-3p	2.55	0.14	0.21	up
hsa-miR-181a-5p	1.85	4.30E-04	**3.23E-03**	up	hsa-miR-130a-3p	1.34	0.14	0.21	up
hsa-miR-432-5p	2.22	7.67E-04	**4.60E-03**	up	hsa-miR-181d-5p	1.39	0.14	0.21	up
hsa-miR-181b-5p	1.48	3.47E-03	**0.02**	up	hsa-miR-183-5p	1.61	0.16	0.22	up
hsa-miR-195-5p	1.45	8.18E-03	**0.03**	up	hsa-miR-129-5p	1.26	0.17	0.24	up
hsa-miR-328-3p	1.51	9.37E-03	**0.03**	up	hsa-miR-598-3p	1.54	0.23	0.30	up
hsa-miR-106a-5p	2.73	9.93E-03	**0.03**	up	hsa-miR-574-3p	2.03	0.28	0.33	up
hsa-miR-484	1.28	1.09E-02	**0.03**	up	hsa-miR-138-5p	0.73	0.28	0.33	down
hsa-miR-363-3p	1.37	2.71E-02	0.07	up	hsa-miR-181c-5p	1.48	0.33	0.38	up
hsa-miR-193b-3p	0.40	2.80E-02	0.07	down	hsa-miR-381-3p	0.44	0.40	0.44	down
hsa-miR-504-5p	0.63	3.15E-02	0.07	down	hsa-miR-10a-5p	1.06	0.49	0.52	up
hsa-miR-132-3p	1.47	3.62E-02	0.08	up	hsa-miR-487b-3p	0.98	0.76	0.78	down
hsa-miR-433-3p	1.56	4.58E-02	0.09	up	hsa-miR-301a-3p	1.06	0.88	0.88	up

Abbreviations: FC, fold change; FDR, false discovery rate.
[a]*Mann–Whitney U test (two-tailed).*
[b]*Bold values indicate FDR <0.05.*

ASD_CoexUp_M16, as well as markers for neurons, oligodendrocytes, and astrocytes (FDR <0.05, **Figures 3C,D**).

DISCUSSION

This study investigated the altered expression of 30 brain-specific ASD-associated miRNAs in a Han Chinese cohort. The results revealed that 10 DE-miRNAs (hsa-miR-191-5p, hsa-miR-151a-3p, hsa-miR-139-5p, hsa-miR-181a-5p, hsa-miR-432-5p, hsa-miR-181b-5p, hsa-miR-195-5p, hsa-miR-328-3p, hsa-miR-106a-5p, and hsa-miR-484) were significantly differentially expressed in plasma of patients with autism than in controls (FDR <0.05). All the 10 DE-miRNAs were up-regulated in autism and might be involved in neurodevelopment and multiple brain-related functions

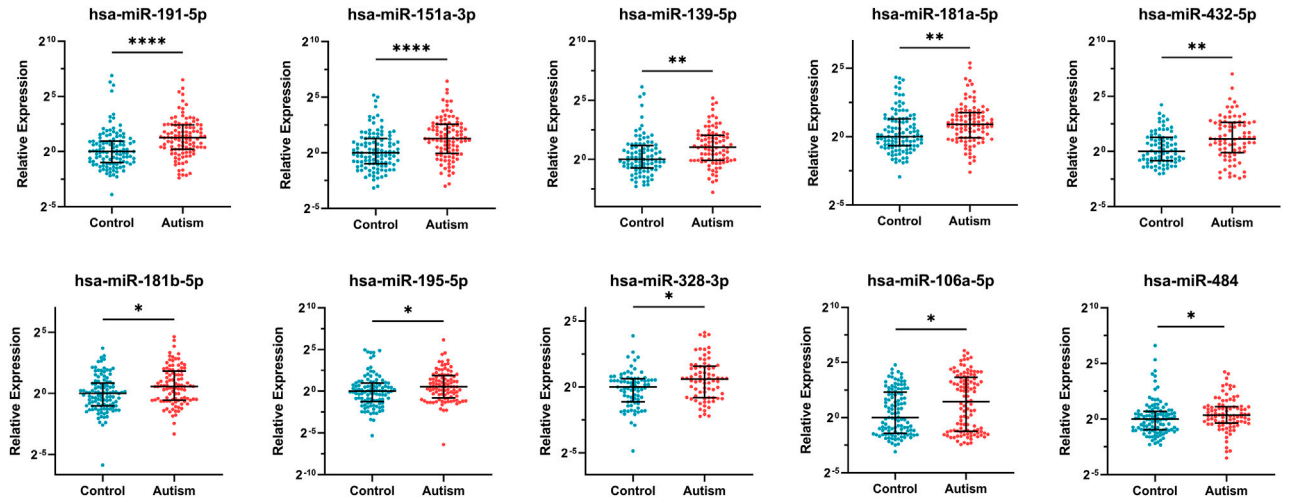

FIGURE 2 | Expression of 10 significantly differentially expressed miRNAs in patients with autism and healthy controls. Mann–Whitney U test was used for statistical analysis and false discovery rate (FDR) was used for multiple testing correction. Data are presented as the median and interquartile range (lines) with each dot representing an individual. * FDR <0.05, ** FDR <0.01, **** FDR <0.0001.

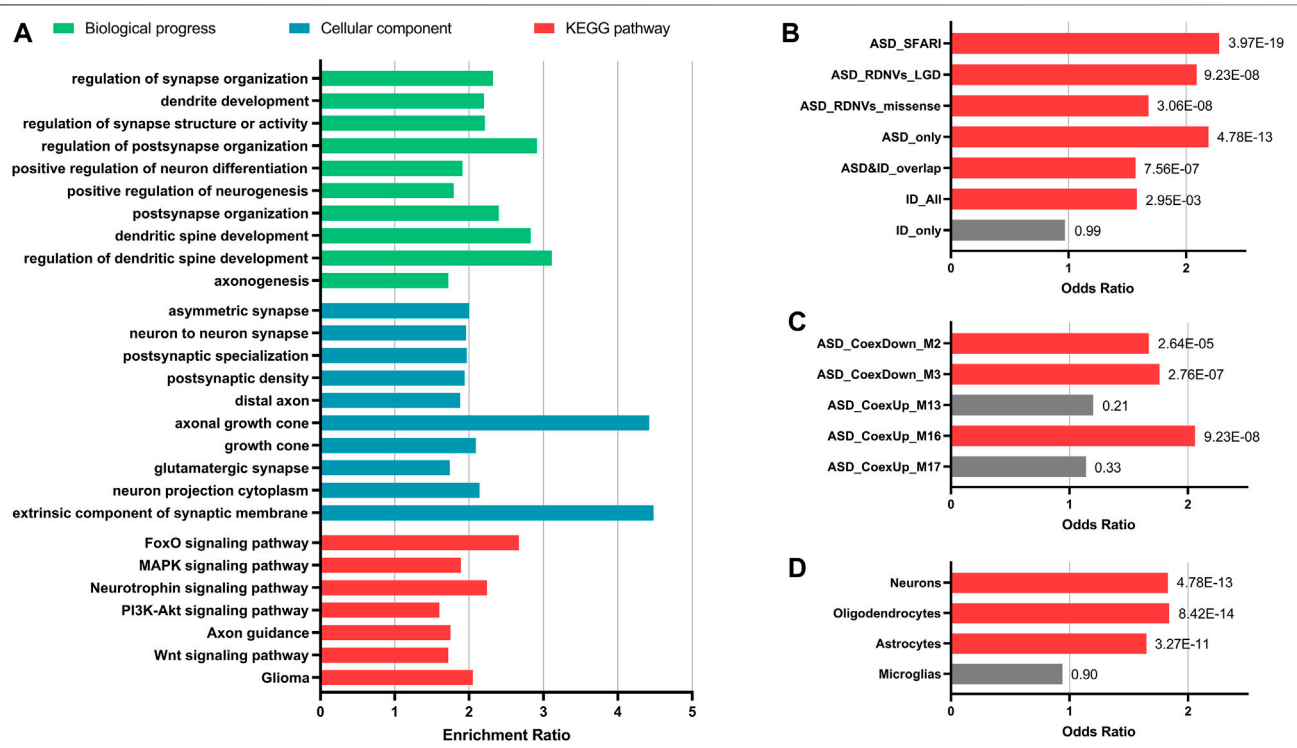

FIGURE 3 | Enrichment analyses for the target genes of 10 significantly differentially expressed miRNAs. **(A)** Enrichment for GO and KEGG pathways that were involved in brain functions. **(B–D)** Enrichment for ASD-related gene lists **(B)**, ASD-associated gene coexpression modules **(C)**, and markers for different types of neural cells **(D)**. Fisher's exact test (two-tailed) with false discovery rate (FDR) correction was applied for enrichment. FDR value of each gene set was indicated. Gene sets with FDR <0.05 are showed with red bars and the others are showed with grey bars. Abbreviations: ASD, autism spectrum disorder; ID, intelligent disability; DNVs, *de novo* variants; LGD, likely gene-disrupting.

and pathways. Besides, the targets of these miRNAs were significantly enriched in ASD-related genes.

Previous studies proved that circulating miRNAs ubiquitously existed in different body fluids, including the peripheral blood plasma (Weber et al., 2010). Plasma or brain miRNAs could physically cross the blood–brain barrier (BBB), and the BBB endothelium also released miRNAs into the circulation (Langford and Masliah, 2001; Witwer et al., 2011). Circulating miRNAs might reflect the pathogenesis of the brain. In the present study, 10 miRNAs were found to be significantly differentially expressed (FDR <0.05), suggesting the altered expression levels of circulating miRNAs in autism. Five of them (hsa-miR-191-5p, hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-195-5p, and hsa-miR-328-3p) were detected consistent up-regulation in the peripheral blood of patients with autism in the previous studies. Besides, the up-regulation of hsa-miR-484 was described in two studies using postmortem brain samples (**Supplementary Table S4**). These findings suggested the potential of circulating miRNAs to be biomarkers for the diagnosis of autism.

We further explored the role of 10 DE-miRNAs targets in brain-related functions and autism. The results showed that these DE-miRNAs might regulate genes and pathways involved in neurodevelopment and neuronal and synaptic functions. The DE-miRNAs targets were significantly enriched for ASD-related genes but not for ID-only genes, indicating a stronger association for ASD and a relatively weaker association for ID. As for ASD-associated gene coexpression modules, the targets of DE-miRNAs were enriched for two down-regulated modules (ASD_CoexDown_M2 and ASD_CoexDown_M3), which were most highly expressed in early human fetal development. Besides, these brain-specific DE-miRNAs might bind to the markers for neurons, oligodendrocytes, and astrocytes, but were not associated with microglia, suggesting that these up-regulated miRNAs were more involved in neuronal functions.

Previous studies demonstrated the regulatory roles of these DE-miRNAs in neurodevelopment and brain functions. For example, hsa-miR-139-5p might act as a negative regulator for neural stem cell proliferation and neuronal differentiation, and modulate cortical neuronal migration by targeting *Lis1* (Huang et al., 2014; Wei et al., 2020). Hsa-miR-484 played an essential role in neurocognition and regulated mitochondrial functions crucial for maintaining synaptic function (Allach El Khattabi et al., 2020; Wingo et al., 2020). Hsa-miR-151a-3p was implicated in SSRI responsiveness and possibly in the clinical response to antidepressant drugs *via* down-regulating CHL1 expression (Oved et al., 2017). Hsa-miR-195-5p was increasingly expressed and targeted BDNF in rats with the rapid onset of heavy alcohol use (Ehinger et al., 2021). The altered expression of these DE-miRNAs was also found in individuals with other comorbid psychiatric disorders sharing genetic overlap with autism, including major depressive disorder, attention-deficit/hyperactivity disorder, and Alzheimer's disease (Mendes-Silva et al., 2016; Sánchez-Mora et al., 2019; Zadehbagheri et al., 2019). These findings, along with those of the present study, supported the relationship between miRNAs and autism, suggesting that up-regulated miRNAs might inhibit the expression of down-regulated genes related to neuronal and synaptic functions (Gupta et al., 2014; Wu et al., 2016). The circulating miRNAs might have the potential to become biomarkers for the diagnosis of autism and provide clues for understanding the pathogenesis of the disorder. Further research is needed to investigate the expression profile and regulatory roles of miRNAs in the CNS and neuropsychiatric disorders.

Some miRNAs did not exhibit consistent changes across different studies. The possible influencing factors might include the type of biomaterials, expression measurement, population/ethnicity, heterogeneity of ASD, and limitation of sample sizes. Besides, most postmortem brain samples used in previous studies were from adults. Although the results were more likely to reflect the pathogenesis in the CNS, the variability in the miRNA expression profile could not be ignored (Mariani et al., 2015; Prieto-Fernández et al., 2020).

This study had several limitations. First, the sample size was relatively small and the miRNAs examined were limited. Second, only patients with typical autism were included in the present study; expression changes of miRNAs in patients with mild symptoms were not investigated. Third, the targets of DE-miRNAs were predicted based on the online database. Further experiments should be performed to identify the actual targets for these miRNAs.

In summary, this study suggested significantly altered expression of 10 miRNAs (hsa-miR-191-5p, hsa-miR-151a-3p, hsa-miR-139-5p, hsa-miR-181a-5p, hsa-miR-432-5p, hsa-miR-181b-5p, hsa-miR-195-5p, hsa-miR-328-3p, hsa-miR-106a-5p, and hsa-miR-484) in patients with autism. These miRNAs might be involved in neurodevelopment and brain functions. Further studies are required to explore the dysregulation of miRNAs in autism and its underlying mechanisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Peking University Sixth Hospital (Beijing, China). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JiL, DZ, JuL, and LW conceived and designed the study. MeJ, JiL, and LW enrolled and evaluated all the participants. TL

and MiJ collected peripheral blood samples. ZW and LW conducted the miRNAs selection. ZW examined the expression profile of the miRNAs and processed data for analyses. ZW and XL performed bioinformatics and statistical analyses. ZW wrote the original manuscript. XL, JiL, DZ, JuL, and LW revised the article and contributed to the final version of the manuscript. All authors have reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.865881/full#supplementary-material

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A Polymorphism in the *BDNF* Gene (rs11030101) is Associated With Negative Symptoms in Chinese Han Patients With Schizophrenia

Junjiao Ping[1,2†], *Jie Zhang*[1,2,3†], *Jing Wan*[4], *Caiying Huang*[4], *Jiali Luo*[1,2], *Baoguo Du*[5*] and *Tingyun Jiang*[1*]

[1]Department of Psychiatry, The Third People's Hospital of Zhongshan City, Zhongshan, China, [2]Joint Laboratory of Psychiatric Genetic Research, The Third People's Hospital of Zhongshan, Zhongshan, China, [3]Department of Psychiatry, Gannan Medical University, Ganzhou, China, [4]Department of Early Intervention, Third People's Hospital of Zhongshan City, Zhongshan, China, [5]Department of Clinical Psychology, The Third People's Hospital of Zhongshan City, Zhongshan, China

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Weihua Yue,
Peking University Sixth Hospital, China

Reviewed by:
Xingguang Luo,
Yale University, United States
Jian-Huan Chen,
Jiangnan University, China

***Correspondence:**
Baoguo Du
dubaoguo99@sina.com
Tingyun Jiang
jzshantou@126.com

[†]These authors have contributed equally to this work

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Objective: This study aimed to investigate the association between brain-derived neurotrophic factor (*BDNF*) and cyclic adenosine monophosphate response element binding protein (*CREB*) gene polymorphisms and schizophrenia.

Methods: This study used a case-control design, and diagnoses were made based on the Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition criteria. One hundred and thirty-four patients with schizophrenia were recruited from the Third People's Hospital of Zhongshan City from January 2018 to April 2020. Sixty-four healthy controls were recruited from the same region. Genotypes at the *BDNF* gene single nucleotide polymorphisms rs11030101, rs2030324, and rs6265 and the *CREB* gene single nucleotide polymorphisms rs6740584 and rs2551640 were determined using a MassARRAY mass spectrometer. Linkage disequilibrium and haplotype analyses were performed, and genotype and allele frequencies were compared between groups. The positive and negative symptom scale (PANSS) was used to evaluate the association between the *BDNF* and *CREB* gene polymorphisms and schizophrenic symptoms.

Results: There was no significant difference in genotype or allele frequencies for rs11030101, rs2030324, rs6265, rs6740584, or rs2551640 between schizophrenic patients and controls ($p > 0.05$). In addition, there were no significant differences in rs11030101, rs2030324, rs6265, rs6740584, or rs2551640 genotype frequencies between the two groups in the dominant, recessive, or over-dominant models ($p > 0.05$). Three loci in the BDNF gene and two loci in the CREB gene were in a state of strong linkage disequilibrium. The frequency of haplotype AAC (rs11030101/rs2030324/rs626), composed of three loci in the BDNF gene, was significantly increased in schizophrenic patients compared with control subjects. There were significant differences in the subscores of PANSSS for negative symptoms, in patients with different rs11030101 genotypes of the *BDNF* gene ($p < 0.05$). There was also

significant differences in the PANSS scores for the general symptom G12 (judgment and lack of insight) in patients with different rs6265 genotypes of the *BDNF* gene ($p < 0.05$).

Conclusion: The *BDNF* gene rs11030101/rs2030324/rs6265 AAC haplotype was potentially associated with an increased risk of schizophrenia. In addition, genotypes at the rs11030101 and rs6265 loci may affect the negative symptoms and general symptoms of schizophrenic patients, respectively.

Keywords: schizophrenia, brain derived growth factor, negative symptoms, polymorphism (genetics), CREB (cAMP response element binding protein)

INTRODUCTION

Schizophrenia is a serious mental illness. The course of the disease is often prolonged, the global prevalence rate is approximately 1%, and approximately half of patients with schizophrenia eventually develop a mental disability, which imparts a heavy burden on society, the family, and thepatient. The clinical symptoms of schizophrenia are complex. Patients generally have either i) no disturbance of intelligence or consciousness, ii) mainly positive symptoms, iii) negative symptoms, iv) cognitive impairment of the three core symptoms and emotional symptoms, or iv) other symptoms as the main symptom types (Duan et al., 2010; Müller, 2014; McMeekin et al., 2016; Lewis, 2018). A large number of clinical and basic research studies have shown that genetic factors play an important role in the pathogenesis of schizophrenia (Giegling et al., 2017). In recent years, association and linkage analysis studies have also confirmed that schizophrenia is a complex polygenic disease (Karl and Arnold, 2014). Schizophrenia is thought to be a brain disease characterized by neurodevelopmental disorders, leading to minor pathological changes in the brain. Meanwhile, the interaction of genetic, biological, and environmental factors plays an important role in the pathogenesis of schizophrenia (Flint and Munafò, 2014).

The product of the brain-derived neurotrophic factor (*BDNF*) gene is widely distributed in the central nervous system. It is a protein that promotes neuronal differentiation, growth, and development, and it plays an important role in maintaining normal brain function (Wang et al., 2015). *BDNF* is located on human chromosome 11p13 and contains 11 exons, with a total length of 70 kb. The rs11030101, rs2030324, and rs6265 loci in *BDNF* play an important role in the occurrence and development of schizophrenia. rs11030101 is located in an intron of the *BDNF* gene and plays an important role in the regulation of gene expression. rs2030324, also known as C270T, is located in the promoter region of the *BDNF* gene, and it affects BDNF expression (Ma et al., 2012). rs6265, also known as G196A (Val66Met), is a single nucleotide substitution at position 196 of the *BDNF* gene, resulting in the conversion of valine (Val) to methionine (Met) at codon 66. This variant reduces the secretion of BDNF by inhibiting its entry into secretory granules, thus affecting BDNF function (Ribasés et al., 2005). At present, there is no consistent conclusion on whether the rs11030101, rs2030324, and rs6265 loci of the *BDNF* gene are susceptibility genes for schizophrenia (Nieto et al., 2013; Zhang et al., 2016a).

BDNF is the most abundant neurotrophic factor in the human body. It activates intracellular signaling; induces the phosphorylation of cyclic adenosine monophosphate response element binding protein (CREB); activates related pathways; and, finally, activates CREB after binding to the related kinase tropomyosin receptor kinase B (Vyssotski et al., 2002). The *CREB* gene, located on chromosome 2q34, is a member of a family of active transcription factors. It is considered to be a potential regulator of the overall survival program of neurons, and it plays a key role in the central nervous system. Some studies have shown that CREB is dysfunctional in patients with schizophrenia. Ren et al. (2014) studied the expression and function of CREB protein and mRNA in the prefrontal cortex and cingulate gyrus. They found that the expression and activity of CREB protein and mRNA in the cingulate gyrus were lower in schizophrenic patients than in control subjects, suggesting that CREB abnormalities in the cingulate gyrus may be related to schizophrenia. It has been speculated that *CREB* gene polymorphisms may be involved in abnormal CREB function and may be related to schizophrenia (Ren et al., 2014; Forero et al., 2016).

Thus, the purpose of this study was to explore the associations between the *BDNF* gene polymorphisms rs11030101, rs2030324, and rs6265 and the *CREB* gene polymorphisms rs6740584 and rs2551640 and schizophrenia.

SUBJECTS AND METHODS

Subjects

Patients with schizophrenia admitted to the Third People's Hospital of Zhongshan City from 2018 to 2020 were recruited. They were included in the study if they: i) met the diagnostic criteria for schizophrenia, as recommended in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition and were diagnosed jointly by two attending physicians who had received consistent training; ii) were aged 16–60 years; and iii) were of Han ethnicity. Patients with major somatic diseases (e.g., diabetes, hypertension, or cancer), nervous system diseases, or other mental disorders were excluded.

The control group was recruited from workers, nurses, volunteers, and patients undergoing health check-ups at the Third People's Hospital of Zhongshan City. Subjects were included in the control group if they: i) had no history of mental illness after psychiatric screening; ii) had no family

history of mental illness; iii) passed a physical examination; iv) were of Han ethnicity; and v) were aged 18–60 years. Individuals who were adopted or from single-parent families with an unknown family history or had major somatic diseases, such as diabetes, hypertension, or cancer, were excluded from participating in the control group.

This study was approved by the Ethics Committee of Zhongshan Third People's Hospital, and either patients or their guardians provided informed consent.

Methods

General Data and Clinical Information Collection

General demographic data, including age, sex, and educational level, were collected from all subjects. The clinical information of patients with schizophrenia, such as the age of onset, was also collected.

Psychotic Symptom Assessment

A medical history was obtained from patients with schizophrenia, and a psychiatric examination was performed on the same day. The positive and negative symptom scale (PANSS) was used to evaluate the psychotic symptoms of the patients. This scale includes a positive symptom score, negative symptom score, and general psychopathological symptom score. Patients were evaluated independently by two attending physicians who had received consistent training. The consistency between evaluators was high ($r > 0.80$).

Genomic DNA Extraction

Peripheral venous blood was collected into an ethylenediaminetetraacetic acid-containing vacuum anticoagulant tube and centrifuged at 3,300 rpm at room temperature. The leukocytes in the middle layer were collected and stored in a cryopreservation tube at −80°C for subsequent use. A TIANamp Genomic DNA Kit (centrifugal column type; DP304; Tiangen, Beijing, China) was used to extract genomic DNA, and the optical density was determined using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Genomic DNA extraction results were evaluated by gel electrophoresis.

Primer Synthesis

The MassARRAY single nucleotide polymorphism (SNP) genotyping platform (Agena Bioscience, San Diego, CA, United States) was used to genotype the *BDNF* and *CREB* gene SNPs. The Agena primer design tool (http://agenacx.com/) was used to design primers for the rs11030101, rs2030324, rs6265, rs6740584, and rs2551640 loci. The polymerase chain reaction (PCR) primers were diluted to 100 μM, and the PCR primer mixture was prepared according to the 1EXT 200 protocol. The extension primer mixture was prepared using 1:25 dilutions of the primers. After the configuration of the extension primer mixture was finalized, 2 μl of each primer were diluted 1:25 for mass spectrometric analysis. The ratio of the extension primers of individual loci was adjusted according to the test results.

PCR Amplification

A 1.5-ml Eppendorf tube was used to prepare a PCR master mix with a total volume of 4.1 μl, consisting of 1.850 μl of water, 0.625 μl of PCR buffer with 15 mM $MgCl_2$, 0.325 μl of $MgCl_2$, 0.100 μl of deoxy-ribonucleoside triphosphate (dNTP) mix, 0.1000 μl of primer mix, and 0.200 μl of HotStarTaq (Qiagen, Hilden, Germany). An 8- or 12-channel pipette was used to add 4 μl of master mix into each well of a 384-well plate, after which 1 μl of genomic DNA (20 ng/μl) was added. The samples were then mixed and centrifuged at 1,000 rpm for 1 min. The 384-well plates were carefully covered with a sealing film to prevent evaporation during the PCR procedure. The PCR amplification procedure was as follows: 94°C for 5 min; 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min; 72°C for 3 min; and hold at 4°C.

Alkaline Phosphatase Treatment

The PCR products were treated with shrimp alkaline phosphatase (SAP) to remove the free dNTPs in the system. An alkaline phosphatase reaction mix (1.53 μl of water, 0.17 μl of SAP buffer, and 0.3 μl of SAP) was prepared in a 1.5-ml Eppendorf tube, and 2 μl of the reaction mix were added to each well of a 384-well PCR plate containing 5 μl of PCR product. The SAP reaction was performed in a PCR machine using the following steps: 37°C for 20 min, 85°C for 5 min, and hold at 4°C.

Single-Base Extension Reaction

After alkaline phosphatase treatment, a single-base extension reaction was performed, as previously described by our research team (Ping et al., 2021) and Shah et al. (Shah et al., 2020)

Resin Purification and Chip Sampling

After PCR, Na^+, Mg^{2+}, K^+, and other salt ions were removed using a cation exchange resin to avoid too many peaks in the analysis spectrum produced by the mass spectrometer, which would affect the analysis. Using a MassARRAY nanodispenser, a microvolume of sample was loaded onto the SpectroCHIP to prepare the co-crystalline film of the chip matrix and the sample.

Mass Spectrometric Detection and Data Analysis

The prepared chip was loaded onto the MassARRAY Analyzer four system for sample detection. Typer 4.0 software was used to obtain the original data and construct a cluster diagram for bioinformatics analysis.

Statistical Analysis

Data were input into Microsoft Excel and sorted and were then analyzed using SPSS 20.0 statistical software (IBM, Armonk, NY, United States). Data that were normally distributed are expressed as the mean ± standard deviation and were analyzed by analysis of variance. Data that did not conform to a normal distribution are expressed as the median (upper quartile, lower quartile) and were analyzed using nonparametric statistical methods. Categorical data were analyzed by the chi-square test. Hardy–Weinberg equilibrium was assessed by the chi-square test using SPSS 20.0, and the genotype and allele frequencies were compared between the schizophrenia and the control groups. Pairwise SNP linkage disequilibrium analysis and association analyses of SNPs and haplotypes were performed using the online software SHEsis (http://analysis.bio-x.cn/SHEsisMain.htm/). Odds ratios (OR) and 95% confidence intervals (95% Cls) were obtained.

Linkage disequilibrium between paired SNPs was analyzed, and the degree of linkage disequilibrium between each pair of SNPs was

expressed as D′. The value of D′ ranges from 0 to 1, with a higher value indicating a higher degree of linkage disequilibrium between the two loci. Polymorphic loci were grouped according to their D′ values, and SNPs with a high degree of linkage disequilibrium were combined into groups for haplotype analysis using the "Pair-loci D'/r2 value" option in SHEsis.

The "Haplotype analysis" option in SHEsis was used to analyze haplotypes. The haplotype frequency was estimated using the expectation-maximization method, and the threshold value was set at 0.03. Taking the allelic genomic combination with the highest frequency as a reference, the ORs and 95% Cls of other allelic combinations were calculated. The overall p value was used to determine whether the haplotype was associated with schizophrenia, using $p < 0.05$ to indicate a significant difference. The results showed that the allelic combination with the highest frequency was the haplotype associated with schizophrenia. Power analysis was performed using G*Power 3.1 software.

RESULTS

Comparison of General Demographic Data Between the two Groups

In the schizophrenia group, there were 92 males and 42 females, aged from 16 to 58 years. The duration of education, age of onset, total PANSS score, positive symptom score, negative symptom score, and general psychotic symptom score were 9.17 ± 2.33 years, 23.78 ± 7.60 years, 90.83 ± 29.85, 13.45 ± 5.98, 17.66 ± 8.55, and 28.80 ± 8.71, respectively. There were 64 individuals in the control group, including 38 males and 26 females, aged from 22 to 59 years, with an average age of 45.6 ± 5.47 years. The duration of education in the control group was 10.62 ± 4.58 years. There was no significant difference in the male:female ratio between the two groups ($p > 0.05$), but there were significant differences in age and the duration of education between the two groups ($p < 0.05$).

Results of Genomic DNA Extraction

The OD values of the extracted genomic DNA showed DNA concentrations greater than 20 ng/μl, with OD 260 nm/280 nm ratios between 1.6 and 2.2 and OD 260 nm/230 nm ratios greater than 0.6230. There was no absorption peak and the DNA bands were intact, without serious degradation, which met the requirements for subsequent SNP analysis. The gel electrophoresis results of the DNA samples are shown in **Supplementary Figure S1**.

Test of Hardy-Weinberg Equilibrium

Genotype data for the rs11030101, rs2030324, and rs6265 polymorphisms in the *BDNF* gene and the rs6740584 and rs2551640 polymorphisms in the *CREB* gene for 134 patients with schizophrenia were tested for Hardy–Weinberg equilibrium. There were no significant differences between the observed genotype frequencies at these five loci and their expected population frequencies, as shown in **Tables 1**, **2** ($p > 0.05$).

The distribution of genotype frequencies at each locus showed that the genotype at the *BDNF* locus, rs11030101, was predominantly AA in both the schizophrenia and control groups; the rs2030327 genotype was predominantly AG; and the rs6265 genotype was predominantly CT. The *CREB* genotype at the rs6740584 locus was predominantly CT (43.3%) in the schizophrenia group and CC (48.4%) in the control group, whereas the genotype at the rs2551640 locus was predominantly AG in both the schizophrenia and control groups.

Results of Genotype Analysis

As shown in **Tables 3**, **4**, there were no significant differences in genotype frequencies at the *BDNF* gene loci rs11030101, rs2030324, or rs6265 between the two groups ($p > 0.05$). Genotype frequencies at the *CREB* gene loci rs6740584 and rs2551640 also showed no significant differences between the two groups ($p > 0.05$). Moreover, there were no significant differences in these genotype frequencies according to gender ($p > 0.05$).

Results of Allelic Analysis

As shown in **Table 5**, there were no significant differences in rs11030101, rs2030324, rs6265, rs6740584, or rs2551640 allele frequencies between the two groups ($p > 0.05$), and there were no significant differences in allele frequencies according to Gender.

Distribution Analysis of the Genetic Model

As shown in **Table 6**, there were no significant differences between the patient and control groups in the dominant, recessive, or over-dominant models of the three *BDNF* gene loci, rs11030101, rs2030324, and rs6265, or the two *CREB* gene loci, rs6740584 and rs2551640 ($p > 0.05$).

Linkage Disequilibrium Analysis

Linkage disequilibrium (i.e., allelic association) between two loci is generally indicated by a D′ value >0.8. Because of the large distance between the three polymorphic loci of the *BDNF* gene and the two polymorphic loci of the *CREB* gene, the estimated r^2 values were relatively low. Therefore, the D′ value was used to determine the degree of linkage disequilibrium between loci. Strong linkage disequilibrium was observed between rs11030101, rs2030324, and rs6265, as shown in **Table 7**. The D′ value for rs6740584 and rs2551640 was 0.953, suggesting strong linkage disequilibrium between these two polymorphic loci of the *CREB* gene.

Analysis of the Association Between *BDNF* and *CREB* Haplotypes and Schizophrenia

We analyzed the distribution of *BDNF* (rs11030101, rs2030324, and rs6265) and *CREB* gene (rs6740584 and rs2551640) haplotypes in the schizophrenia and control groups. When constructing the haplotypes for analysis, only those with a frequency of at least 3% were selected to explore their association with schizophrenia. There was a significant difference in the *BDNF* gene rs11030101/rs2030324/rs6265 AAC haplotype frequency between the schizophrenia and control groups ($p < 0.05$, **Table 8**).

TABLE 1 | Hardy–Weinberg equilibrium test for *BDNF* genotypes.

Locus	Group (*n*)	Genotype	Frequency (%)	X^2	*p*
rs11030101	schizophrenia group (134)	AA	79 (58.9)	0.42	0.81
		AT	46 (34.3)	—	—
		TT	9 (6.7)	—	—
	control group (64)	AA	40 (62.5)	0.24	0.88
		AT	22 (34.4)	—	—
		TT	2 (3.1)	—	—
rs2030324	schizophrenia group (134)	AA	44 (32.8)	0.08	0.96
		AG	67 (50.0)	—	—
		GG	23 (17.2)	—	—
	control group (64)	AA	21 (32.8)	1.27	0.53
		AG	35 (54.7)	—	—
		GG	8 (12.5)	—	—
rs6265	schizophrenia group (134)	CC	37 (27.6)	0.93	0.63
		CT	72 (53.7)	—	—
		TT	25 (18.7)	—	—
	control group (64)	CC	12 (18.8)	0.33	0.85
		CT	34 (53.1)	—	—
		TT	18 (28.1)	—	—

TABLE 2 | Hardy–Weinberg equilibrium test for *CREB* genotypes.

Locus	Group (*n*)	Genotype	Frequency (%)	X^2	*p*
rs6740584	schizophrenia group (134)	CC	56 (41.8)	0.42	0.81
		CT	58 (43.3)	—	—
		TT	20 (14.9)	—	—
	control group (64)	CC	31 (48.4)	0.15	0.93
		CT	28 (43.8)	—	—
		TT	5 (7.8)	—	—
rs2551640	schizophrenia group (134)	AA	52 (38.8)	0.00	1.00
		AG	63 (47.0)	—	—
		GG	19 (14.2)	—	—
	control group (64)	AA	29 (45.3)	0.53	0.77
		AG	30 (46.9)	—	—
		GG	5 (7.8)	—	—

Analysis of the Association Between Genotypes and Clinical Symptoms of Schizophrenia

The associations between *BDNF* (rs11030101, rs2030324, and rs6265) and *CREB* genotypes (rs6740584 and rs2551640) and clinical psychiatric symptoms in patients with schizophrenia are shown in **Table 9**. There were significant differences in the PANSS negative symptom scores, N2 (emotional withdrawal), N3 (communication disorder), N6 (lack of spontaneity and fluency of conversation), and N7 (stereotyped thinking) and total negative symptom scores in patients with different *BDNF* gene rs11030101 genotypes. Moreover, there were significant differences in the PANSS general symptom score G12 (judgment and lack of insight) in patients with different *BDNF* gene rs6265 genotypes ($p < 0.05$).

Evaluation of Statistical Power

The G*Power program was used to perform the power calculation. The size of the sample used in this study had a power of 92.372% to detect a significant association ($\alpha < 0.05$) with a given effect size index value of 0.5.

DISCUSSION

The findings of the current study can be represented by the following two aspects. First, we found a significant increase in the rs11030101/rs2030324/rs6265 AAC haplotype frequency in schizophrenic subjects compared with controls. Second, genotypes at the rs11030101 and rs6265 loci of the *BDNF* gene were associated with either negative or clinical pathological symptoms, which suggested that *BDNF* gene polymorphisms may be associated with negative symptoms in schizophrenic subjects in southern China.

The neurodevelopmental hypothesis is currently the main etiological explanation of schizophrenia. Under this hypothesis, schizophrenia is thought to be a consequence of disorders in the development and maturation of neurons and neural pathways in the embryonic brain. Meanwhile, the symptoms of schizophrenia are induced by an abnormal environment of the outside world (Zhao

TABLE 3 | Comparison of *BDNF* rs11030101, rs2030324, and rs6265 genotype frequencies between the two groups.

Group	Gender	Cases(*n*)	rs11030101			rs2030324			rs6265		
			AA	At	TT	AA	AG	Gg	CC	CT	TT
schizophrenia group	Male	92	55	29	8	29	43	20	25	51	16
control group	Male	38	25	12	1	13	19	6	8	19	11
schizophrenia group	female	42	24	17	1	15	24	3	12	21	9
control group	female	26	15	10	1	8	16	2	4	15	7
p	male	—	0.452	0.742	0.321	—	—	—	—	—	—
p	female	—	0.935	0.916	0.456	—	—	—	—	—	—
p	—	—	0.578	0.675	0.205	—	—	—	—	—	—

TABLE 4 | Comparison of *CREB* rs6740584 and rs2551640 genotype frequencies between the two groups.

Group	Gender	Cases(*n*)	rs6740584			rs2551640		
			CC	CT	TT	AA	AG	Gg
schizophrenia group	Male	92	37	44	11	34	48	10
control group	male	38	19	18	1	19	18	1
schizophrenia group	female	42	19	14	9	18	15	9
control group	female	26	12	10	4	10	12	4
p	male	—	0.209	0.183	—	—	—	—
p	female	—	0.807	0.663	—	—	—	—
p	—	—	0.337	0.217	—	—	—	—

TABLE 5 | Comparison of rs11030101, rs2030324, rs6265, rs6740584, and rs2551640 allele frequencies between the two groups.

Group	Gender	Cases(*n*)	rs11030101		rs2030324		rs6265		rs6740584		rs2551640	
			A	T	A	G	C	T	C	T	A	G
schizophrenia group	male	92	139	45	101	83	101	83	118	66	116	68
control group	male	38	62	14	45	31	35	41	56	20	56	20
schizophrenia group	female	42	65	19	54	30	45	39	52	32	41	33
control group	female	26	40	12	32	20	23	29	34	18	32	20
p	male	—	0.331	0.523	0.194	0.136	0.099	—	—	—	—	—
p	female	—	0.951	0.747	0.290	0.683	0.492	—	—	—	—	—
p	—	—	0.428	0.661	0.088	0.178	0.129	—	—	—	—	—

and Shi, 2015). In this study, 134 schizophrenic patients and 64 healthy controls were recruited from the Han population in Guangdong Province, China. The allele and genotype frequencies of three SNPs (rs11030101, rs2030324, and rs6265) in the *BDNF* gene and two SNPs in the *CREB* gene (rs6740584 and rs2551640) were compared between the schizophrenia and control groups. During the past 2 decades, many small- and large-scale studies have explored the association between *BDNF* gene polymorphisms (rs11030101, rs2030324, and rs6265) and schizophrenia, mainly with negative results. Moreover, *BDNF* is not included in the list of index schizophrenia loci from the Psychiatric Genomics Consortium's genome-wide association study of tens of thousands of cases and controls (Pardiñas et al., 2018). In the current study, the distribution of *BDNF* SNP genotypes (rs11030101, rs2030324, and rs6265) was not different between healthy controls and patients with schizophrenia, which is in accordance with the results of previous studies (Zhang et al., 2016b).

A haplotype refers to a random set of multiple alleles at closely linked loci on a given chromosome. The effectiveness of genetic analysis of a single genetic marker is limited. Haplotype analysis of multiple loci makes effective use of genetic information at each locus and increases the testing power. Specific haplotypes containing disease susceptibility genes or resistance genes can be identified using this analysis method, and multiple susceptibility or resistance loci can be identified by analyzing the composition and frequency of haplotypes composed of different SNPs on the same chromosome. Our haplotype analysis showed that the *BDNF* gene rs11030101/rs2030324/rs6265 AAC haplotype was more common in the schizophrenia group than in the control group, suggesting that this haplotype may be related to an increased susceptibility to schizophrenia.

PANSS is one of the most commonly used scales to evaluate the clinical symptoms of patients with schizophrenia. It includes 33 items assessing the positive, negative, and general symptoms of schizophrenia (Kay et al., 1987; He and Zhang, 1997). We found differences in negative scale scores in schizophrenic patients with different *BDNF* genotypes at the rs11030101 locus. Moreover, different genotypes had an effect on the PANSS negative symptom scores, with the AA genotype showing the greatest effect

TABLE 6 | Genetic model distribution between two groups (n).

Locus	Model	Genotype	Schizophrenia group (n)	Control group (n)	X^2	*p*	Or (95% CI)
rs11030101	dominant	AA + AT	125	62	1.065	0.302	0.448 (0.094,2.137)
		TT	9	2	—	—	—
	recessive	AA	79	40	0.227	0.634	0.862 (0.467,1.589)
		AT + TT	55	24	—	—	—
	over-dominant	AA + TT	88	42	0.00	0.995	1.002 (0.535,1.876)
		AT	46	22	—	—	—
rs2030324	dominant	AA + AG	111	56	0.714	0.398	0.689 (0.290,1.639)
		GG	23	8	—	—	—
	recessive	AA	44	21	0.00	0.997	1.001 (0.531,1.888)
		AG + GG	90	43	—	—	—
	over-dominant	AA + GG	67	29	0.381	0.537	1.207 (0.664,2.193)
		AG	67	35	—	—	—
rs6265	dominant	TT + CT	97	52	1.827	0.177	0.605 (0.291,1.259)
		CC	37	12	—	—	—
	recessive	TT	25	18	2.284	0.131	0.586 (0.292,1.177)
		CC + CT	109	46	—	—	—
	over-dominant	CC + TT	62	30	0.006	0.936	0.976 (0.537,1.773)
		CT	72	34	—	—	—
rs6740584	Dominant	CC + CT	114	59	1.986	0.159	0.483 (0.173,1.352)
		TT	20	5	—	—	—
	Recessive	CC	56	31	0.777	0.378	0.764 (0.420,1.390)
		CT + TT	78	33	—	—	—
	over-dominant	CC + TT	76	36	0.004	0.951	1.019 (0.559,1.858)
		CT	58	28	—	—	—
rs2551640	Dominant	AA + AG	115	59	1.648	0.199	0.513 (0.182,1.442)
		GG	19	5	—	—	—
	Recessive	AA	52	29	0.759	0.384	0.765 (0.419,1.398)
		AG + GG	82	35	—	—	—
	over-dominant	AA + GG	71	34	0.000	0.985	0.994 (0.548,1.806)
		AG	63	30	—	—	—

TABLE 7 | Results of linkage disequilibrium analysis.

Locus	rs2030324	rs6265
rs11030101	0.975	1.000
rs2030324	—	0.943

and the TT genotype showing the smallest effect. Clinical negative symptoms were more prominent in schizophrenic patients with the AA genotype at rs11030101. Genetic theory states that if multiple genes are associated with a disease, one of them is associated with a dominant phenotype of the disease. Previous studies (Li et al., 2013; Zhai et al., 2013) have shown that there is a significant association between *BDNF* gene polymorphisms and clinical negative symptoms of schizophrenia. The same results were obtained in this study, suggesting that the rs11030101 locus of the *BDNF* gene plays an important role in the occurrence of clinical negative symptoms of schizophrenia, which provides some clues for clinical diagnosis.

CREB is involved in the intersection of several intracellular signal transduction pathways. In fact, the cyclic adenosine monophosphate, mitogen-activated protein kinase, calcium-dependent protein kinase, and glycogen synthase kinase three pathways form four upstream pathways of CREB. These four pathways eventually regulate the expression of *BDNF* and other downstream genes, thus affecting neuronal plasticity and neurotransmitter synthesis (Lu et al., 2008). As a nuclear regulatory factor in eukaryotes, CREB plays an important role in neuronal regeneration, synaptic plasticity, learning, and memory. CREB is thought to be involved in the differentiation, survival, and migration of early hippocampal

TABLE 8 | Analysis of haplotype distribution between the schizophrenia and control groups.

Gene	Haplotype	Cases, *n* (frequency)	Controls, *n* (frequency)	*p*	Or (95%CI)
BDNF	AAC	35 (0.132)	8 (0.063)	0.037[a]	2.288 (1.032,5.072)
	AAT	118 (0.442)	69 (0.538)	0.084	0.688 (0.450,1.053)
	AGC	49 (0.181)	26 (0.203)	0.645	0.883 (0.519,1.503)
	TGC	61 (0.227)	24 (0.187)	0.347	1.288 (0.759,2.185)
CREB	CA	163 (0.608)	88 (0.688)	0.215	0.749 (0.474,1.184)
	TG	94 (0.350)	38 (0.297)	0.215	1.335 (0.845,2.109)

[a]$p < 0.05$.

TABLE 9 | Relationships between different *BDNF* and *CREB* genotypes and PANSS scores in the schizophrenia group $p < 0.05$.

PANSS	rs11030101		rs2030324		rs6265		rs6740584		rs2551640	
Items	X^2	p	X^2	p	Z	p	X^2	p	X^2	p
P1	0.782	0.676	5.192	0.075	2.909	0.233	1.724	0.422	2.021	0.364
P2	4.175	0.124	0.904	0.636	1.655	0.437	0.435	0.804	0.019	0.991
P3	0.977	0.613	2.561	0.278	2.142	0.343	1.695	0.428	2.997	0.223
P4	3.944	0.139	1.332	0.514	1.343	0.511	1.027	0.598	1.747	0.417
P5	0.963	0.618	0.154	0.926	0.585	0.747	1.323	0.516	2.265	0.322
P6	1.894	0.388	4.259	0.119	5.380	0.068	2.048	0.359	2.921	0.232
P7	5.423	0.066	1.990	0.370	3.742	0.154	0.269	0.874	0.927	0.629
p (total)	0.084	0.959	2.941	0.230	2.443	0.295	1.468	0.480	1.314	0.518
N1	3.275	0.194	0.330	0.848	0.208	0.901	0.541	0.763	3.272	0.195
N2	6.774	0.034*	0.404	0.817	1.089	0.580	0.110	0.946	2.024	0.363
N3	8.390	0.015*	2.118	0.347	1.818	0.403	0.114	0.944	0.788	0.674
N4	5.874	0.053	1.106	0.575	0.412	0.814	1.097	0.578	6.989	0.030
N5	5.620	0.060	0.259	0.878	0.057	0.972	0.344	0.842	0.345	0.841
N6	9.773	0.008*	1.582	0.453	0.018	0.991	0.653	0.722	0.184	0.912
N7	6.329	0.042*	0.217	0.897	2.554	0.279	0.732	0.693	3.600	0.165
N (total)	8.257	0.016*	0.470	0.791	0.065	0.968	0.106	0.948	2.076	0.354
G1	4.553	0.103	1.883	0.390	1.325	0.516	1.607	0.448	0.634	0.728
G2	3.943	0.139	1.183	0.554	1.088	0.580	0.617	0.735	0.502	0.778
G3	3.244	0.198	0.175	0.916	3.151	0.207	2.388	0.303	1.709	0.426
G4	5.016	0.081	0.827	0.661	0.490	0.783	0.018	0.991	1.693	0.429
G5	1.505	0.471	0.670	0.715	0.294	0.863	1.154	0.561	1.283	0.527
G6	1.813	0.404	0.055	0.973	0.054	0.974	0.053	0.974	1.206	0.547
G7	0.914	0.633	0.583	0.747	0.211	0.900	1.033	0.597	2.740	0.254
G8	1.586	0.452	1.766	0.414	0.218	0.897	2.225	0.329	0.090	0.956
G9	0.544	0.762	2.322	0.313	1.716	0.424	0.844	0.656	1.576	0.455
G10	4.822	0.090	0.402	0.818	1.580	0.454	4.321	0.115	3.201	0.202
G11	1.632	0.442	0.575	0.750	0.255	0.880	0.588	0.745	1.789	0.409
G12	2.442	0.295	4.556	0.102	9.338	0.009*	2.371	0.306	1.327	0.504
G13	1.620	0.445	1.917	0.383	0.166	0.921	1.463	0.481	1.541	0.463
G14	0.032	0.984	0.370	0.831	1.450	0.484	0.223	0.894	2.811	0.245
G15	3.929	0.140	2.222	0.329	0.334	0.846	2.028	0.363	4.494	0.106
G16	4.462	0.107	1.023	0.600	0.730	0.694	0.344	0.842	0.902	0.637
G (total)	5.164	0.076	1.155	0.561	1.084	0.582	0.180	0.914	1.417	0.492
PANSS (total)	5.173	0.075	0.153	0.926	0.316	0.854	0.205	0.902	0.558	0.757

progenitor cells (Kandel, 2012). In 1999, Kawanishi et al. (1999) found that schizophrenic patients with the C allele at the *CREB* gene T933TC polymorphism had general clinical manifestations and certain unique symptoms. We found no significant differences in rs6740584 or rs2551640 genotype or allele frequencies between schizophrenic patients and healthy control subjects. Moreover, no positive associations were found in genetic model, haplotype, and symptom analyses, which was consistent with the results reported by Forero et al. (2016) and Bai Lijuan et al. (Bai et al., 2016). To some extent, this study showed that *CREB* gene polymorphisms are not related to susceptibility to schizophrenia. In addition, these findings indicate that other factors may be involved in the pathogenesis of schizophrenia. Generally speaking, as an important susceptibility gene for fever in schizophrenic patients, polymorphisms of the *BDNF* gene may be related to the susceptibility to schizophrenia and the severity of symptoms.

The statistical power of this study was evaluated using G*Power 3.1 software. The total sample size used for the allele, genotype, and haplotype frequency analyses gave a statistical power of 92.372% for $\alpha < 0.05$. Thus, although the sample size was small, the total sample had good statistical validity and the results were statistically significant. Similarly, Kumar et al. (2020) obtained significant results with a small sample size. They found that the serum BDNF concentration was significantly lower in schizophrenic patients than healthy individuals, and that the rs6265 polymorphism was not associated with schizophrenia in a case-control study of 50 schizophrenic patients and 50 healthy individuals.

A major limitation of this study was the small sample size, which translates to an underpowered study in terms of *BDNF* (rs11030101, rs2030324, and rs6265) and *CREB* (rs6740584 and rs2551640) genotype distributions. This may be considered a serious methodological limitation that prevents definitive conclusions about the role of the *BDNF* gene in schizophrenia in the Chinese Han population. In the future, more large-scale studies in different populations are needed to verify the reliability of the association and contribute to a deeper understanding of the etiology and mechanism of schizophrenia. This study provides the basis for revealing the genetic mechanisms of susceptibility to schizophrenia and the severity of the associated symptoms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directly to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee of the Third People's Hospital of Zhognshan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BD and TJ contributed to the conception and design of the study, and provided the approval for publication of the content. JP and JZ organized the database and performed the statistical analysis, and were responsible for manuscript writing and modification. JL was responsible for the testing of experimental samples. JW and CH were responsible for clinical data collection and clinical evaluation. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Co-Expression Network Modeling Identifies Specific Inflammation and Neurological Disease-Related Genes mRNA Modules in Mood Disorder

Chunxia Yang[1], Kun Zhang[2], Aixia Zhang[1], Ning Sun[1,3], Zhifen Liu[1] and Kerang Zhang[1]**

[1]Department of Psychiatry, First Hospital of Shanxi Medical University, Taiyuan, China, [2]Shanxi Medical University, Taiyuan, China, [3]Nuring College of Shanxi Medical University, Taiyuan, China

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Edited by:
Weihua Yue,
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Tianjin Anding Hospital, China
Yi Li,
Shanxi University of Finance and Economics, China

***Correspondence:**
Zhifen Liu
liuzhifen5518@163.com
Kerang Zhang
atomsxmu@vip.163.com

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Objectives: Mood disorders are a kind of serious mental illness, although their molecular factors involved in the pathophysiology remain unknown. One approach to examine the molecular basis of mood disorders is co-expression network analysis (WGCNA), which is expected to further divide the set of differentially expressed genes into subgroups (i.e., modules) in a more (biologically) meaningful way, fascinating the downstream enrichment analysis. The aim of our study was to identify hub genes in modules in mood disorders by using WGCNA.

Methods: Microarray data for expression values of 4,311,721 mRNA in peripheral blood mononuclear cells drawn from 21 MDD, 8 BD, and 24 HC individuals were obtained from GEO (GSE39653); data for genes with expression in the bottom third for 80% or more of the samples were removed. Then, the top 70% most variable genes/probs were selected for WGCNA: 27,884 probes representing 21,840 genes; correlation between module genes and mood disorder (MDD+BD vs. HC) was evaluated.

Results: About 52% of 27,765 genes were found to form 50 co-expression modules with sizes 42–3070. Among the 50 modules, the eigengenes of two modules were significantly correlated with mood disorder ($p < 0.05$). The saddlebrown module was found in one of the meta-modules in the network of the 50 eigengenes along with mood disorder, 6 (IER5, NFKBIZ, CITED2, TNF, SERTAD1, ADM) out of 12 differentially expressed genes identified in Savitz et al. were found in the saddlebrown module.

Conclusions: We found a significant overlap for 6 hub genes (ADM, CITED2, IER5, NFKBIZ, SERTAD1, TNF) with similar co-expression and dysregulation patterns associated with mood disorder. Overall, our findings support other reports on molecular-level immune dysfunction in mood disorder and provide novel insights into the pathophysiology of mood disorder.

Keywords: mood disorder, co-expression network analysis (WGCNA), hub genes, inflammation, neurological

INTRODUCTION

Mood disorders including major depressive disorder (MDD) and bipolar disorder (BD) are a kind of serious mental illness and are the third leading cause of the global disease burden (Collins et al., 2011; Murray et al., 2012; Jabbi et al., 2020). The molecular factors involved in the pathophysiology of MDD remain challenging (Gagne et al., 2020). Despite their diagnostic distinction, multiple approaches have shown considerable sharing of risk factors across the mood disorders (Coleman et al., 2020). Various hypotheses regarding the pathogenesis of mood disorders, such as the hypothesis of disturbed neuroplasticity (Christoffel et al., 2011) and the inflammatory (Leonard and Maes, 2012; Zeng et al., 2019), have been proposed. Many studies suggested that neural immune activation may be a primary pathway influencing the observed changes in key neuroendocrine and neurotrophic systems (Miller et al., 2013). Substantial evidence supports the changes in mRNA expression in proinflammatory genes and the elevated levels of peripheral inflammatory markers in mood disorder patients (Kohler et al., 2018; Wiedlocha et al., 2018). However, there is no definitive evidence to support the belief of shared inflammation and neurological abnormalities of molecular biology in mood disorders (Savitz et al., 2013).

Mood disorders share several genetic associations, and can be combined effectively to increase variant discovery (Coleman et al., 2020). Several genome-wide association studies (GWAS) in MDD and BD have indicated that the genetic heterogeneity architecture of mood disorder is complex, with many polymorphisms of small effect contributing to the clinical phenotype (Okbay et al., 2016; Ciobanu et al., 2018; Wray et al., 2018). A recent meta-analysis was conducted using results from the Psychiatric Genomics Consortium (PGC) genome-wide association studies for MDD and BD using data including those from 23andMe and UK Biobank to identify numerous shared and disorder-specific associations between mood disorders. In addition, clinical heterogeneity has been recognized as a major limiting factor for robust characterization of gene expression alterations in MDD. For example, the first RNA sequencing study of 463 lifetime MDD cases, consisting of a mixture of individuals with current and remitted MDD, found no differentially expressed genes between cases and controls (Mostafavi et al., 2014). For BD, neuroimaging-guided RNA-sequencing in two studies showed gene-expression changes associated with disease morbidity and related suicide mortality in an independent postmortem cohort (Jabbi et al., 2020).

To elucidate the relationship between inflammation and neuroimaging abnormalities, Savitz et al. conducted a whole genome expression analysis of peripheral blood mononuclear cells and identified 12 differentially expressed genes including TNF and others that related to neurological disorders and/or apoptosis between patients with a mood disorder and healthy controls. There was mounting evidence that was associated with functional and chemical abnormalities within and beyond the neural reward circuitry and was linked to elevated peripheral levels of inflammatory biomarkers in depression (Ely et al., 2021). An Ingenuity Pathway Analysis on these differentially expressed genes yielded two gene networks centered around TNF and related to cell circle and kinase anomalies, respectively. The authors also found that the expression levels of some of these differentially expressed genes were significantly correlated with morphometric abnormalities of the left sgACC, hippocampus, and caudate. However, there are some limitations in a traditional pathway analysis using IPA, for example. One of the limitations is that the gene networks and regulatory indicated in these networks are modeled based only on currently available knowledge. To fully utilize the gene expression information captured by the microarray data, in this study, we conducted a co-expression network analysis for the microarray data generated in and downloaded from GSE using the WGCNA approach, which was a systems biology approach developed for creating gene network models to explore and identify key functional modules and hub genes.

As far as molecular biology is concerned, genes do not act in isolation. In mood disorder, genes interaction within each other with complex networks might be disrupted. At the same time, gene expression data do not function in isolation but rather are highly multidimensional with complex non-linear biological processes. Molecular interactions are not captured by traditional statistical methods (Ciobanu et al., 2020). Weighted gene co-expression network analysis (WGCNA) is a hypothesis-free systems biology approach that identifies "modules" of co-regulated, and therefore functionally related, genes in a given phenotype (Langfelder and Horvath, 2008), extending classic bivariate approaches (Ciobanu et al., 2018). WGCNA: A systems biology approach developed for analysis of transcriptomic data, providing more information than a set of differentially expressed genes. Used sophisticated algorithms and information on correlation patterns among genes, WGCNA is expected to further divide the set of differentially expressed genes into subgroups (i.e., modules) in a more biologically meaningful way, fascinating the downstream enrichment analysis (Wang et al., 2017).

In this study, we aimed to investigate the relationship between global gene co-expression profiles and mood disorder subgroups. Microarray data for expression values of 4,311,721 mRNA in peripheral blood mononuclear cells drawn from 21 MDD, 8 BD, and 24 HC individuals were obtained from GEO (GSE39653). We applied WGCNA and explored the correlation of co-expressed modules *1*) construct a gene-gene similarity network; *2*) divide the network into modules (group genes with similar expressions); *3*) correlate traits to gene modules; and *4*) identify hub genes in modules. We then sought molecular-level immune dysfunction in mood disorder and provide novel insights into the pathophysiology of mood disorder.

METHODS

mRNA Microarray Data Acquisition

Microarray data of GSE39653 was downloaded from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.org/geo/) database, which includes expression levels of 4,311,721 mRNA in peripheral blood

mononuclear cells drawn from 21 MDD, 8 BD, and 24 HC individuals. Details on the recruitment of subjects, sampling and processing of the blood samples, and microarray experiment were given in. Briefly, the mood disorder patients met DSM-IV criteria for recurrent primary MDD in a current major depressive episode or BD in a current major depressive episode with a moderate-to-high Hamilton Depression Rating Scale score and did not receive any psychotropic medications for at least 3 weeks. The healthy control individuals had no personal or family history of psychiatric illness. Quantile normalization and log-transformation were performed for the expression data.

Construction of Weighted Gene Co-Expression Network

First, the microarray data were preprocessed as follows. Data for genes with expression in the bottom third for 80% or more of the samples were removed (Ballouz et al., 2015). The top 70% most variable genes/probs were selected for the construction of the co-expressed network. Using the preprocessed and transformed data, a co-expressed network was constructed using the WGCNA R package (Zhang and Horvath, 2005). Briefly, a correlation matrix for all pair-wise correlations of transcripts was calculated and then transformed into a weighted adjacency matrix with a soft threshold power set to beta = 5 to achieve approximate scale-free topology (model fit $R^2 > 0.88$ while the mean connectivity was kept as large as possible). The connection strengths were then used to calculate the topological overlap (TO), which is a pair-wise measure of two genes' similarity with other genes in the network. Genes were then hierarchically clustered using 1-TO as the distance measure and modules of genes were identified using a dynamic tree-cutting algorithm using the following parameters: minimum modulesize = 30, deepSplit = 4, mergeCutSize = 0.15, and maximumBlockSize = 5000.

Quantification of Module–Trait Associations

The first principal component of each module defined the module eigengene (ME). Genes weakly corrected with the ME (Pearson correlation coefficient<0.3) were removed from the module. For each gene, Pearson correlation coefficient was calculated with the eigengenes of all modules and defined as the model membership (kME). If a gene had the highest correlation and with correlation $p < 0.05$ with the eigengene of a module other than the module it was assigned to initially in the hierarchical clustering, it would be reassigned to this module. Associations between mood disorder (MDD or BD) and MEs were determined by Pearson correlations. Finally, MEs along with the traits were clustered based on their correlation, and the meta-modules were identified to represent groups of correlated modules and/or the traits, which was the affection status of mood disorder in this case.

Identification of Hub Genes, Functional Annotation, and Gene Ontology Analysis

For modules, significantly associated with mood disorder, the top hub genes were identified as those with the highest intramodular connectivity K_IM, representing the highly connected genes within a module. Functional annotation was performed for those hub genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). Gene Ontology (GO) analysis was then performed using the function hyperGTest in the R package Gostats.

Functional Annotation and Enrichment Analysis

We drew a histogram by mapping the GO (Ashburner et al., 2000; Tweedie et al., 2009) function of genes in modules of interest to the corresponding secondary features. The Pearson Chi-Square test was applied to indicate significant relationships between the two input datasets if all the expected counts were greater than. The top five annotation clusters for each analysis were focused on as these clusters are more likely to contain biologically meaningful annotations as these clusters have the highest enrichment score. Then we implemented GO enrichment analysis based on a hypergeometric test. The p-value <0.05 was used as the enrichment cut-off criterion.

RESULTS

Data Preprocessing

After removing genes with expression in the bottom third for 80% or more of the samples, the top 70% most variable probes (27,884, representing 21,840 genes) for 29 patients with mood disorder (21 MDD and 8 BD) and 24 HC were selected for WGCNA.

WGCNA Analysis

Weighted gene co-expression networks construction and gene modules identification. Using the preprocessed data for all 53 participants, a weighted co-expression network was constructed using the WGCNA package. The hierarchical clustering procedure and the dynamic tree-cutting algorithm resulted in 50 modules (**Figure 1** and **Supplementary Table S1**), each of which is assigned a unique color label and visualized in the color band underneath the cluster tree in **Figure 1**. These modules ranged in size from 42 genes in the thistle2 module to 3070 in the turquoise module. Among all 27,765 probes, 13,240 (47.7%) were found to belong to none of the 50 proper modules and were put in an improper module (gray).

Co-Expression Modules Correlated With Mood Disorder

To identify modules related to mood disorder, we correlated each of the 50 module eigengenes with the mood disorder status. As shown in **Table 1**, the saddlebrown module and the lightcyan module were significantly associated with mood disorder. The saddlebrown module, which was positively associated with mood disorder, included 10 out of the 26 genes that were found in Coleman et al. (2020) to be differentially expressed between patients with mood disorder and heathy controls. Among these 10 genes, the following 6 are protein coding genes:

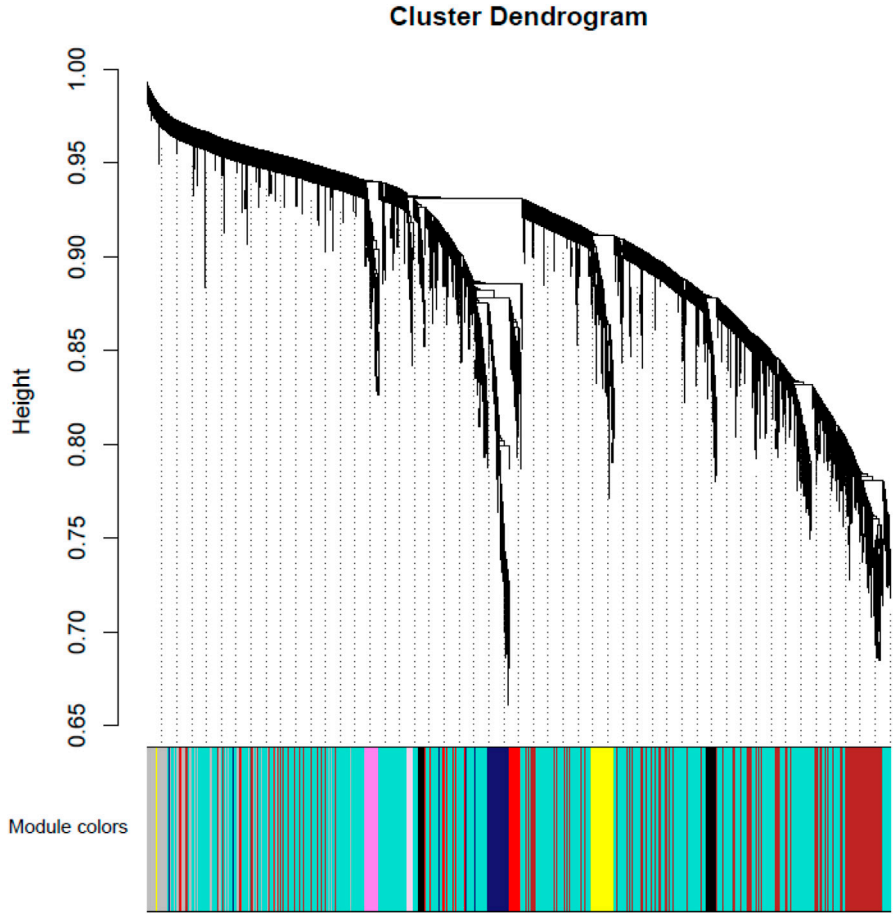

FIGURE 1 | Module-MD vs. Control relationship. Each row corresponds to a module eigengene. Each cell contains the corresponding correlation in the first line and the *p*-value in the second line. The table is color-coded by correlation according to the color legend. Among them, red represents a positive correlation and blue represents a negative correlation.

ADM, CITED2, IER5, NFKBIZ, SERTAD1, and TNF, which were mostly related to neurological features or psychiatric illness. The lightcyan module was negatively associated with mood disorder, and did not include any of the 26 differentially expressed genes identified in Coleman et al. (2020). This implies that the lightcyan module might represent some pathway which may not be identified using differential expression analysis of individual genes. It should be noted that 6 coding genes in the saddlebrown module that were differentially expressed are all over-expressed in the mood disorder cases. The saddlebrown module was also found in the same meta-module in the network of the 50 eigengenes along with mood disorder, as shown in **Figure 2**. In the same meta-module, there were two more eigengenes representing the darkmagenta module and the darkolivegreen module.

Identifying Hub Genes From Candidate Modules

Hub genes for the saddlebrown and lightcyan1 modules were extracted and ranked based on the intramodular connectivity values (Wang et al., 2017). Thus, we identified the hub genes from the saddlebrown module (**Table 1** for the saddlebrown module). Among the 12 genes identified in Savitz et al. (2013) as differentially expressed between controls and cases (mood disorder), 6 appeared in the saddlebrown module (**Supplementary Table S2**).

Functional Annotation of Mood Disorder Correlated Modules and GO Analysis

Hub genes for the saddlebrown and lightcyan1 modules were extracted and ranked based on the intramodular connectivity values. The top hub genes were annotated using the GeneCard website. Most of the hub genes in the saddlebrown module were related to the similar function of RNA processing, in which mRNA 5′-splice site recognited, mRNA cis spliced *via* spliceosome, and branching involved in labyrinthine layer morphogenesis. Moreover, most of the hub genes in the lightcyan1 module were related to the similar function of regulating steroid hormone secretion. GO analysis for the hub genes of these two modules suggested the genes were enriched in

TABLE 1 | Two candidate modules speculated the critical role for the pathophysiology of MD.

Module	Spearman_CC (*p*-value)	Module	Spearman_CC (*p*-value)
MEmediumpurple3	−0.012 (0.9)	MElightcyan	0.017 (0.9)
MEskyblue	0.045 (0.8)	MEviolet	0.15 (0.3)
MEpaleturquoise	−0.2 (0.1)	MEgrey60	−0.059 (0.7)
MEmagenta	−0.077 (0.6)	MEorange	−0.21 (0.1)
MEsteelblue	−0.21 (0.1)	MEfloralwhite	−0.027 (0.8)
MElightgreen	0.052 (0.7)	MEplum1	0.097 (0.5)
MEroyalblue	−0.042 (0.8)	MEbrown	−0.15 (0.3)
MEcyan	−0.13 (0.3)	MEpurple	−0.15 (0.3)
MEdarkgrey	−0.087 (0.5)	MEsalmon	−0.14 (0.3)
MEdarkred	0.037 (0.8)	MEivory	−0.11 (0.4)
MEorangered4	−0.082 (0.6)	MElightsteelblue1	−0.042 (0.8)
MEdarkorange2	−0.059 (0.7)	MElightcyan1	−0.33 (0.02)
MEdarkturquoise	−0.03 (0.8)	MEthistle2	−0.28 (0.05)
MEgreenyellow	0.079 (0.6)	MEdarkgreen	0.047 (0.7)
MEbisque4	−0.045 (0.8)	MEdarkslateblue	0.17 (0.2)
MEturquoise	0.025 (0.9)	MElightyellow	−0.17 (0.2)
MEred	−0.02 (0.9)	MEdarkorange	0.079 (0.6)
MEwhite	−0.12 (0.4)	MEplum2	0.28 (0.04)
MEblack	0.12 (0.4)	MEbrown4	0.23 (0.1)
MEsienna3	0.089 (0.5)	MEmidnightblue	0.037 (0.8)
MEdarkmagenta	0.18 (0.2)	MEpink	0.11 (0.4)
MEdarkolivegreen	0.18 (0.2)	MEskyblue3	0.079 (0.6)
MEsaddlebrown	0.42 (0.002)	MEblue	0.14 (0.3)
MEtan	0.16 (0.2)	MEgreen	0.2 (0.1)
MEyellow	0.17 (0.2)	MEyellowgreen	0.22 (0.1)
		MEgrey	−0.15 (0.3)

the regulation of corticosteroid hormone secretion. Interestingly, 10 of the 19 GO terms pathway in these two modules were associated with the steroid hormone secretion pathway, including negative regulation of steroid hormone secretion (GO:2000832, $p = 2.29E{-}05$) and regulation of corticosteroid hormone secretion (GO:2000846 $p = 6.31E{-}04$) (**Table 2**).

DISCUSSION

In this study, we utilized the WGCNA to explore the gene co-expression modules networks for expression values of 4,311,721 mRNA in peripheral blood mononuclear cells drawn from MDD, 8 BD, and 24 HC individuals were obtained from GEO (GSE39653) (Savitz et al., 2013). We identified 50 co-expression modules in which the number of eigengenes ranged in size from 42 to 13,240 genes. Two co-expression modules (saddlebrown and lightcyan1) showed striking correlation with the phenotypic trait between MD and healthy controls. Among the 12 genes identified in Savitz et al. as differentially expressed between controls and cases (mood disorder), 6 (IER5, NFKBIZ, CITED2, TNF, SERTAD1, ADM) appeared in module saddlebrown. Based on the GO pathway analysis, biological function of the saddlebrown module and lightcyan1 module were found to be focused on inflammation and neurological response and RNA processing.

The algorithm of WGCNA software could construct a gene co-expression network to provide the expanded explanation of gene expression information. As it has some advantages over traditional approaches to differential expression analysis, the software has been conducted for the gene expression pattern in the mental illness (Geng et al., 2020). WGCNA analysis has been widely used in transcriptional analysis of major depression, schizophrenia, autism, and Alzheimer's disease (Miller et al., 2008; Voineagu et al., 2011; Ciobanu et al., 2020). Recently, Belzeaux et al. collected a discovery queue and two duplicate queues with similar designs by using WGCNA analysis, 9 of the 59 modules were associated with clinical improvement (Belzeaux et al., 2016). Another study also reported that WGCNA analysis explored candidate modules and central genes associated with subsyndromic depressive symptoms (SSD). Gene expression studies of SSD observed different patterns between cases and controls, which may provide new insights into the molecular mechanisms of SSD (Geng et al., 2020). To the best of our knowledge, this is the first study that used WGCNA to explore candidate modules and hub genes associated with MD.

In the current study, the 6 hub genes (IER5, NFKBIZ, CITED2, TNF, SERTAD1, ADM) appeared in module saddlebrown were among the 12 differentially expressed genes identified in Savitz et al. (2013) This indicates that a significant proportion of differentially expressed genes related to mood disorder may be tightly co-regulated, functionally related, or in the same pathway. IER5, as an immediate early genes/transcription factor, was likely to affect basic cellular functions such as RNA and protein synthesis, neural plasticity, neurotransmission, and metabolism (Cirelli and Tononi, 2000). IER5 gene encodes an activator of HSF1 which was to control hippocampal PSA-NCAM levels through the transcriptional regulation of

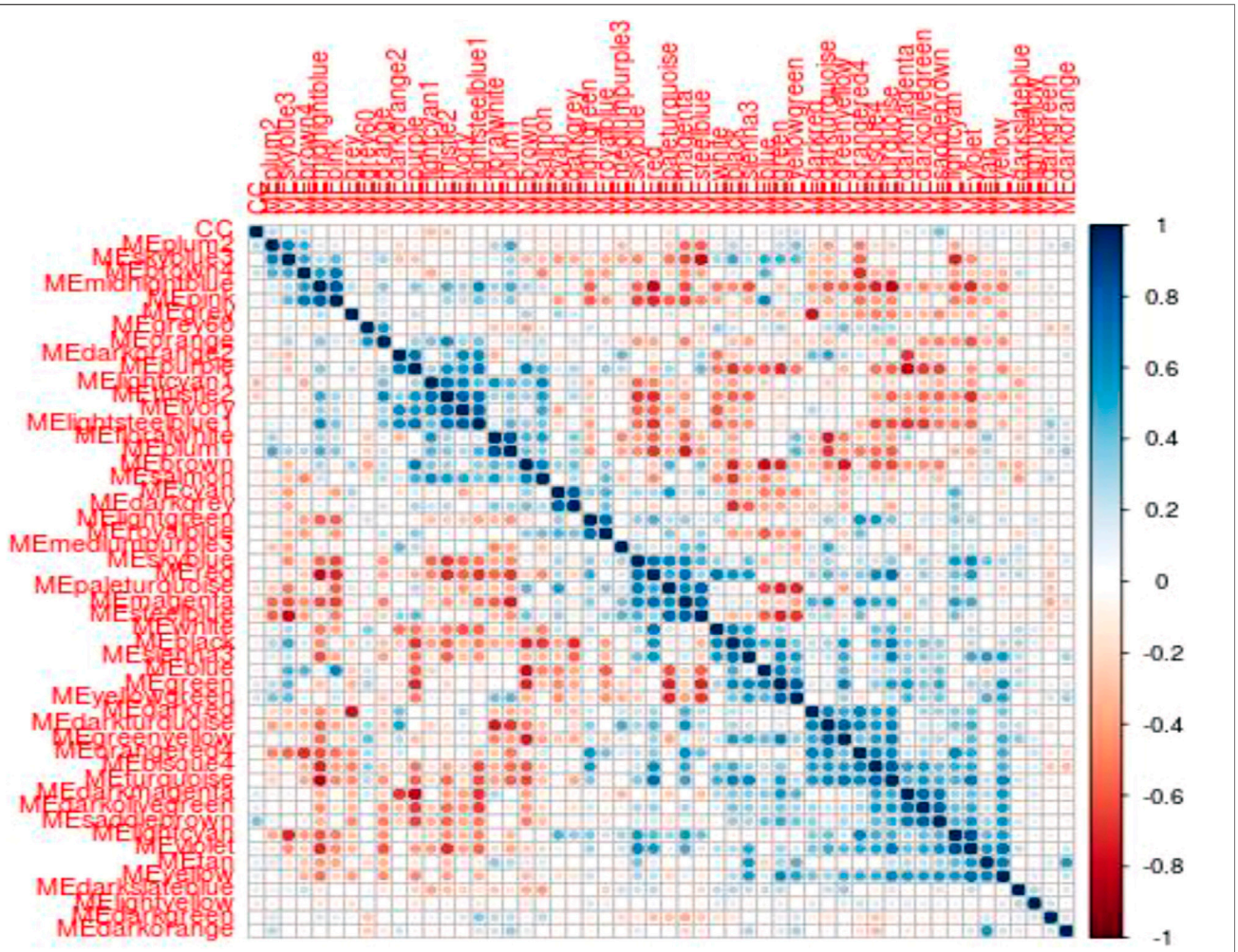

FIGURE 2 | Eigengene heatmap: The scatterplots of MM vs. GS in saddlebrown module exhibited very significant positive correlations.

TABLE 2 | Functional annotation of modules and network analysis.

Module	GOBPID	*p* value	OddsRatio	ExpCount	Count	Size	Term
lightcyan1	GO:2000832	2.29E−05	0	0.01	2	2	Negative regulation of steroid hormone secretion
lightcyan1	GO:2000847	2.29E−05	0	0.01	2	2	Negative regulation of corticosteroid hormone secretion
lightcyan1	GO:2000850	2.29E−05	0	0.01	2	2	Negative regulation of glucocorticoid secretion
lightcyan1	GO:0035933	2.27E−04	142.69	0.024	2	5	Glucocorticoid secretion
lightcyan1	GO:2000849	2.27E−04	142.69	0.024	2	5	Regulation of glucocorticoid secretion
lightcyan1	GO:0035929	6.31E−04	71.33	0.039	2	8	Steroid hormone secretion
lightcyan1	GO:0035930	6.31E−04	71.33	0.039	2	8	Corticosteroid hormone secretion
lightcyan1	GO:2000831	6.31E−04	71.33	0.039	2	8	Regulation of steroid hormone secretion
lightcyan1	GO:2000846	6.31E−04	71.33	0.039	2	8	Regulation of corticosteroid hormone secretion
saddlebrown	GO:0006396	1.11E−05	5.19	3.215	13	678	RNA processing
saddlebrown	GO:0000395	6.59E−05	437.17	0.014	2	3	mRNA 5′-splice site recognition
saddlebrown	GO:0000185	6.06E−04	72.83	0.038	2	8	Activation of MAPKKK activity
saddlebrown	GO:0046886	6.06E−04	72.83	0.038	2	8	Positive regulation of hormone biosynthetic process
saddlebrown	GO:2000271	6.06E−04	72.83	0.038	2	8	Positive regulation of fibroblast apoptotic process
saddlebrown	GO:0060670	7.77E−04	62.42	0.043	2	9	Branching involved in labyrinthine layer morphogenesis
saddlebrown	GO:0008584	8.83E−04	10.4	0.432	4	91	Male gonad development
saddlebrown	GO:0046546	8.83E−04	10.4	0.432	4	91	Development of primary male sexual characteristics
saddlebrown	GO:0045292	9.68E−04	54.61	0.047	2	10	mRNA cis splicing via spliceosome
saddlebrown	GO:0060712	9.68E−04	54.61	0.047	2	10	Spongiotrophoblast layer development

polysialyltransferases, a process that might be involved in neuronal and behavioral development in mice (Yamano et al., 2020). Transcription of NFKBIZ mediates the transcriptional response to TNF and IL-17A. In fibroblasts, CUX1 and NFKBIZ mediate the synergistic inflammatory response to TNF and IL-17A in stromal fibroblasts (Slowikowski et al., 2020). Moreover, Harrison et al., Inagaki et al., and Savitz et al. showed the correlations between hemodynamic response of the amygdala to sad faces and genes such as CFD and NFKBIZ which are involved in the inflammatory response (Harrison et al., 2009; Inagaki et al., 2012; Savitz et al., 2013). Su et al. reported that the NF-κB was activated in the hippocampi of wild-type (WT) mice after CUMS exposure by regulating the expression of cytokines. Previous studies demonstrated that depression-like behaviors caused by stress were dependent on HMGB1/TLR4/NF-κB and TNF-α/TNFR1/NF-κB signalling pathways in CUMS-exposed mice (Su et al., 2017; Liu et al., 2019; Lu et al., 2019). Arctigenin exerts antidepressant-like effects by attenuating microglial activation and neuroinflammation through the HMGB1/TLR4/NF-κB and TNF-α/TNFR1/NF-κB signalling pathways (Xu et al., 2020). CITED2 represses innate immune cell pathogenic response by modulating broad inflammatory gene programming in macrophages and protecting the host from pathogenic inflammation (Pong Ng et al., 2020). SERTAD1, which appeared to be essential for neuron death in trophic support deprivation *in vitro* and *in vivo* and in models of DNA damage, was associated with Alzheimer's disease (Biswas et al., 2010). It may therefore be a suitable target for neuropsychiatric diseases, such as MD. Adrenomedullin (ADM) has been confirmed as a vasorelaxant that is part of the first-line protective (i.e., anti-inflammatory) response to toxic or aversive stimuli such as lipopolysaccharide (LPS) (Wong et al., 2005). Genome-wide association study (GWAS) implicated a single nucleotide polymorphism (SNP) in the vicinity of the ADM gene in a sample of subjects with type II BD. In addition, a functional SNP in the ADM gene was associated with response to paroxetine, an SSRI antidepressant (Glubb et al., 2010). Recently, a whole transcriptome RNA-sequencing study revealed 30 genes (included *ADM*) differentially expressed in MDD compared to controls (Mahajan et al., 2018). Together, these data implicate neuro-inflammation in a large number of genes and functional pathways in MD and playing a crucial role in MD. A growing number of studies suggest behavioral and genetic function of the central nervous system, as well as their involvement affected in many neurologic and psychiatric conditions, such as neurodegenerative diseases and mood disorders (Jeremic et al., 2021). This mounting evidence on the involvement of inflammatory/immune systems and their relationships with neurotransmitters seems to represent intriguing avenues for the development of real innovative therapeutic strategies of mood disorders (Mucci et al., 2020). Neuro-inflammation is potentially important in the pathophysiology of MD. Thus, the current study has confirmed the 6 hub genes (IER5, NFKBIZ, CITED2, TNF, SERTAD1, ADM) of neuro-inflammation in MD.

Interestingly, we also found that the GO about the MD was associated with the saddlebrown and lightcyan1 module. Ten of the 19 GO terms pathway in two modules were associated with the steroid hormone secretion pathway, which included negative regulation of steroid hormone secretion (GO:2000832, p = 2.29E−05) and regulation of corticosteroid hormone secretion (GO:2000846 p = 6.31E−04).

Growing evidence implicates involvement of endogenous glucocorticoids in adverse health effects beyond neurological/neurobehavioral outcomes (neurodegenerative disease, cognitive decline, perceived stress, depression, and suicide) (Thomson et al., 2016). These data provided insight into potential biological mechanisms underlying health impacts and susceptibility in neuropsychiatric diseases, such as MD.

Comparing the results of the WGCNA here with what Savitz et al.'s results were, we think they found that the network of saddlebrown module is clustered based on the similar function of RNA processing. In our study, the RNA processing of mRNA 5′-splice site was recognized, mRNA cis spliced via spliceosome, and branching involved in the labyrinthine layer morphogenesis. Recently, regulating gene expression through splicing, as a novel mechanism, has been described and could contribute to depression by changing gene expression (Le Francois et al., 2018). Alternative splicing is a prevalent modification, especially in human neuronal genes (Kang et al., 2011), resulting in a greater diversity of RNA transcripts (Darnell, 2013; Raj and Blencowe, 2015).

CONCLUSION

In this study, we applied WGCNA to transcriptomic data from 21 MDD, 8 BD, and 24 HC individuals that were obtained from GEO (GSE39653). We found a significant overlap for 6 hub genes (ADM, CITED2, IER5, NFKBIZ, SERTAD1, TNF) with similar co-expression and dysregulation patterns associated with mood disorder. Interestingly, we also found that the GO about the MD was associated with the saddlebrown and lightcyan1 modules. These pathways in two modules were associated with the steroid hormone secretion pathway and function of RNA processing, which have been described could contribute to depression. Our findings support other reports on molecular-level immune dysfunction in mood disorder and provide novel insights into the pathophysiology of mood disorder.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.ncbi.nlm.org/geo/.

ETHICS STATEMENT

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Zhang and NS designed and supervised this study. NS and CY were responsible for data analysis and manuscript drafting. ZL revised the manuscript. AZ and KZ participated in sample collection and carried out the experimental procedures. All authors reviewed and approved the final manuscript.

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ULK4 in Neurodevelopmental and Neuropsychiatric Disorders

Shilin Luo[1,2], *Nanxi Zheng*[3]* and *Bing Lang*[3]*

[1]Department of Pharmacy, The Second Xiangya Hospital, Central South University, Changsha, China, [2]Hunan Provincial Engineering Research Center of Translational Medicine and Innovative Drug, Changsha, China, [3]Department of Psychiatry, National Clinical Research Center for Mental Disorders, The Second Xiangya Hospital of Central South University, Changsha, China

The gene *Unc51-like kinase 4 (ULK4)* belongs to the *Unc-51-like* serine/threonine kinase family and is assumed to encode a pseudokinase with unclear function. Recently, emerging evidence has suggested that ULK4 may be etiologically involved in a spectrum of neuropsychiatric disorders including schizophrenia, but the underlying mechanism remains unaddressed. Here, we summarize the key findings of the structure and function of the ULK4 protein to provide comprehensive insights to better understand ULK4-related neurodevelopmental and neuropsychiatric disorders and to aid in the development of a ULK4-based therapeutic strategy.

Keywords: ULK4, neurodevelopmental disorder, neuropsychiatric disorder, pseudokinase, schizophrenia

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Konstantinos Zarbalis,
University of California, Davis, United States
Xueqin Song,
First Affiliated Hospital of Zhengzhou University, China

***Correspondence:**
Nanxi Zheng
208211058@csu.edu.cn
Bing Lang
bing.lang@csu.edu.cn

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INTRODUCTION

Neuropsychiatric disorders are a wealth of debilitating brain diseases with overlapping etiologies, including genetic variants and environmental stress. The concordance rate is high and the heritability is substantial, although the influence of *de novo* mutations cannot be ignored especially in autism spectrum disorders (ASDs) (Alonso-Gonzalez et al., 2018). During the past decades, genome-wide association studies (GWASs) have reported numerous genetic alleles with single nucleotide polymorphisms (SNPs) (Uffelmann et al., 2021). In addition, recent progress in whole genome interrogation has also demonstrated massive genetic variants that are not covered by GWAS(Rao et al., 2021). The advances in research methodologies have expanded our understanding of the genetic architecture of psychiatric patients but also revealed further complexity. Hence, it is compelling to identify the predisposing risk alleles and to fully elucidate the associated mechanisms underpinning neuropsychiatric disorders. Unfortunately, thus far, only limited success has been achieved. Intriguingly, recent studies have revealed overwhelming evidence in neurodevelopmental elements in neuropsychiatric disorders (Cristino et al., 2014; Cardoso et al., 2019; Al-Naama et al., 2020). Various genetic alterations that occur during the embryonic stages can lead to pathological brain development and may precipitate the onset of psychosis in adolescence. These developmental insults are believed to disturb the neuronal connectivity and cellular architecture within the brain. The most common neurodevelopmental and neuropsychiatric disorders include depression, schizophrenia, autism spectrum disorders (ASD), bipolar disorder, attention deficit hyperactivity disorder, and X-linked intellectual disability, among others. The prevalence of these disorders is growing rapidly, which has caused a tremendous socioeconomic burden, primarily due to their high incidence in children and adolescents (Androutsos, 2012; Robertson et al., 2015; Hansen et al., 2018; Ghandour et al., 2019; Post and Grunze, 2021). During the past several decades, strenuous research has been performed in these fields. Unfortunately, the etiology and underlying mechanisms remain poorly understood.

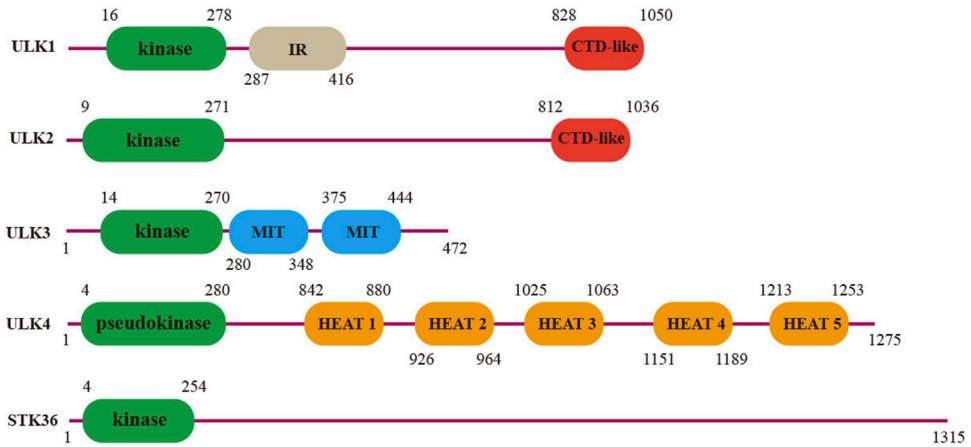

FIGURE 1 | Domain architecture of the human ULK family. Protein interaction domains are annotated as interaction domain (IR), C-terminal domain (CTD) (ULK1 and ULK2), microtubule interacting, and trafficking molecule (MIT) (ULK3), and HEAT domains (ULK4).

In 2014, we first reported that *Unc-51-like kinase 4* (*ULK4*) is crucial for neuritogenesis and neuronal motility and, when defective, may predispose people to neuropsychiatric disorders including schizophrenia (Lang et al., 2014). Since then, accumulating evidence has strongly suggested that ULK4 participates in corticogenesis, cilia maintenance, myelination, and white matter integrity, although the precise downstream signaling pathways and interacting substrates remain elusive. Recently, we have provided evidence that ULK4 deletion can cause decreased intermediate neural progenitors and increased apoptosis, which strongly disrupt normal cortical development (Hu et al., 2021). In addition, ULK4 can form an interactome by physically binding with PP2A and PP1α, the two most abundant phosphatases, and is responsible for over 90% of total Ser/Thr dephosphorylation in eukaryotes. This interactome closely regulates the expression of p-Akt and p-GSK-3α/β, and mice with ULK4-targeted deletion in the excitatory neurons of the forebrain present a spectrum of core features of schizophrenia. These data collectively suggest that *ULK4* is a rare susceptibility gene for psychiatric disorders, especially schizophrenia. In this review, we will summarize the current knowledge of the roles of ULK4 in neurodevelopmental and neuropsychiatric disorders.

MAIN TEXT

Unc-51-like Serine/Threonine Kinase (ULK) Family

In 1998, a novel mouse ortholog of the *Caenorhabditis elegans* serine/threonine kinase uncoordinated-51 (UNC-51) was first cloned (Yan et al., 1998), and thereafter, five related genes in total were found and grouped into the UNC-51-like serine/threonine kinase (ULK) family: ULK1, ULK2, ULK3, ULK4, and serine/threonine kinase 36 (STK36). The kinase domains of ULKs are conserved and located at the N-terminus, and the C-terminal region contains protein interaction motifs important for substrate recruitment (**Figure 1**). In mammals, ULK1 and ULK2 are evolutionarily conserved serine/threonine kinase orthologs of the yeast autophagy-related (ATG) family member ATG1, and play a necessary but somewhat redundant function in proper autophagy initiation (Wang et al., 2018). The high-resolution structure analysis shows that ULK1 and ULK2 share a high degree of conservative domain architecture, including an N-terminal catalytic kinase, extensive middle linker, and C-terminal domain essential for interaction with their binding partners (Lazarus et al., 2015; Chaikuad et al., 2019). During autophagy, the canonical early regulatory complex consists of ULK1/ULK2, ATG13, RB1-inducible coiled-coil protein 1 (RB1CC1, also known as FIP200), and ATG101, which translate upstream nutrient and energy signals (e.g., mTOR and AMPK) into the downstream autophagy pathway (Ganley et al., 2009; Jung et al., 2009; Wong et al., 2013; Lin and Hurley, 2016). Disrupting ULK1 expression in mice leads to defective autophagy-mediated clearance of mitochondria, and mice lacking both ULK1 and ULK2 die shortly after birth due to a defect in glycogen metabolism, which is similar to what occurs with other autophagy-defective mice (Kundu et al., 2008; Cheong et al., 2014). Apart from these processes, ULK1/ULK2 also regulates TrkA receptor trafficking and signaling, which instructs filopodia extension and neurite branching during sensory axon outgrowth (Zhou et al., 2007). Knockdown of ULK2 reduced asymmetric neuropil elaboration and affected habenular development in the brain (Taylor et al., 2011). Recently, *Kang et al.* revealed an association between ULK2 polymorphisms and schizophrenia in the Korean population (Kang et al., 2022).

The other three homologs, ULK3, ULK4, and STK36, contain kinase domains homologous to ULK1/2 but do not have a conserved C-terminal sequence, and they participate in many physiological processes to maintain tissue homeostasis. ULK3 has been reported to be involved in the autophagy induction during senescence (Young et al., 2009). It also has a dual function in the Sonic hedgehog signal transduction pathway, which controls a variety of developmental processes and is implicated in tissue homeostasis and neurogenesis in adults (Fuccillo et al., 2006;

Maloverjan et al., 2010). STK36 is essential for the central pair apparatus and cilia orientation of motile cilia in mice. The cilia of STK36$^{-/-}$ mice are stiff and exhibit significantly reduced stroke amplitude or even immotile movement, which eventually promotes the occurrence of hydrocephalus (Merchant et al., 2005; Nozawa et al., 2013). According to the database in the Swiss Institute of Bioinformatics (SIB), ULK4 is widely expressed in different systems, especially in the secretion system, immune system, and nervous system, but its precise function remains largely unclear. Since we first reported that ULK4 may be a rare susceptibility gene for schizophrenia in 2014, research on this gene has been springing up in the neuropsychiatric field.

ULK4 Protein Structure

ULK4 is a large protein (142 kDa) encoded by the gene *Unc51-Like Kinase 4*, which is located on human chromosome 3p22.1 (Went et al., 2019). Unlike the homolog family member ULK1-3, the ULK4 protein contains a pseudokinase domain at the N-terminus and is thus predicted to be catalytically inactive. There are five HEAT repeats at the C-terminus of ULK4 (842–880, 926–964, 1,025–1,063, 1,151–1,189, and 1,213–1,253) (**Figure 1**), which are commonly found in large proteins, such as mTOR, and are presumably involved in protein scaffolding or interaction (Andrade et al., 2001; Perry and Kleckner, 2003). The crystallized high-resolution structure of ULK4, including its small-molecule inhibitor and ULK4-ATP-rS, has been recently interpreted by two independent research groups (Khamrui et al., 2020; Preuss et al., 2020). Notably, ULK4 can bind to ATP in an unusual Mg^{2+}-independent manner, and the affinity is higher than that of any known pseudokinase (Khamrui et al., 2020). Because some pseudokinases are capable of binding to ATP and allosterically regulating the catalytic functions of kinases using compensatory motifs, even though ULK4 has no apparent phosphotransferase activity (Zeqiraj and van Aalten, 2010), it is assumed that like many others, ULK4 may work as the sensor of ATP and undergo conformational changes upon the binding which subsequently promotes its roles as a scaffold for substrate recruitment. Indeed, *Preuss et al.* predicted many ULK4 interacting partners including active kinases and phosphatases, which require further functional validation (Preuss et al., 2020).

Similar to the working mechanism of STRAD/LKB1, the pseudokinase domain of ULK4 specifically interacts with STK36. This strongly indicates that ULK4 can regulate active kinases directly, despite it being deemed catalytically inactive (Zeqiraj et al., 2009). The unique C-terminal HEAT repeats may enable ULK4 to bind to proper substrates or interacting proteins using a similar recruitment mechanism as ULK1/2. This hypothesis was further substantiated by Preuss and his colleagues, who have revealed that these repeated regions interacted uniquely with calmodulin-regulated spectrin-associated protein 1 (CAMSAP1), oral-facial-digital syndrome 1 (OFD1), and poly(A)-specific ribonuclease subunit 2 (PAN2) (Preuss et al., 2020). However, thus far, there has not been any report that there is an interaction partner of the ULK4 HEAT repeats at the C-terminal of STK36. Domain mapping of ULK4 provides a structural framework for its roles in diseases.

ULK4 and *Unc-51*

The *unc-51* gene was initially described in the nematode *C. elegans* by Brenner in 1974 and showed extensive expression during embryonic brain development when neurons were actively extending their axons, particularly in the head region of late embryos (Brenner, 1974). Surprisingly, worms with the *unc-51* mutation were mostly paralyzed, egg-laying defective, and dumpy (McIntire et al., 1992; Ogura et al., 1994). These data strongly suggested that the unc-51 protein is essential for axon maintenance and elongation. In the brains of *Drosophila* individuals, unc-51-mediated membrane vesicle transport is pivotal in the targeted localization of guidance molecules and organelles that regulate the elongation and compartmentalization of developing neurons as well as motor-cargo assembly (Mochizuki et al., 2011). Similarly, the unc-51 protein was reported to localize in the vesicular structures of growth cones of cerebellar granule cells and spinal sensory neurons in mice, which controls axon formation in granule cells through the endocytic membrane trafficking pathway (Tomoda et al., 1999; Tomoda et al., 2004). As a homologous serine/threonine kinase of unc-51 in humans, ULK4 was initially reported to be associated with blood pressure and hypertension (Levy et al., 2009; Ehret and Caulfield, 2013; Konigorski et al., 2014). Meanwhile, it may be involved in cell cycle control, as its polymorphisms (rs1052501 and rs2272007) were associated with multiple myelomas (Broderick et al., 2011; Greenberg et al., 2013). Inspired by the physiological functions of unc-51, we reanalyzed the common and rare variants of ULK4 in the databases of the International Schizophrenia Consortium (ISC) and among the bipolar Icelandic cases genotyped by deCODE Genetics, and we discovered that it may serve as a rare susceptibility gene for human mental disorders, especially schizophrenia (Lang et al., 2014). Our subsequent functional study further revealed that ULK4 is involved in the remodeling of cytoskeletal components, such as acetylation of α-tubulin, and in this way regulates neurite branching and elongation as well as cell motility.

ULK4 and Neurogenesis

Both *in vivo* and *in vitro* studies have suggested that ULK4 may play a key role in neurogenesis and corticogenesis during developmental stages. In *Xenopus* embryos, ULK4 mRNA is mostly expressed in the ventricular (VZ) and subventricular zones (SVZ) zones and distributed throughout the brain after the closure of the neural tube. Constant expression of ULK4 has also been found in neural stem cells in adult *Xenopus* (Domínguez et al., 2015). Similarly, Ulk4 transcripts are widely found in the VZ, SVZ, and cortical plate in the E15.5 cortex in mice, and ULK4 protein is widely expressed in all cortical layers after postnatal Day 7. Knockdown of ULK4 at E15.5 significantly inhibited cell proliferation and corticogenesis in mice (Lang et al., 2016). Meanwhile, the size of the neural stem cell pool in the forebrain that is important for adult neurogenesis was remarkably reduced in ULK4 null knockout mice at birth (Liu et al., 2016a). Although normal cortical lamination was preserved, the knockout mice showed a thinner cortex due to defective cell proliferation. As abnormal neurogenesis is often associated with neurodevelopmental or neuropsychiatric diseases (Kang et al.,

2016; Guarnieri et al., 2018), it is therefore believed that ULK4 may contribute to the development of these diseases. *Liu et al.* further identified that ULK4 expression was dependent on the cell cycle, with a peak expression in the G2/M phases, and it decreased during both embryonic and adult neurogenesis in ULK4 mutant mice, probably because of a dysregulated Wnt signaling pathway (Liu et al., 2017).

ULK4 and Neurite Arborization

It has been well documented that Unc-51 regulates the dendritic development in the brains of individuals of the genus *Drosophila* through kinesin-mediated membrane transport (Mochizuki et al., 2011). In *C. elegans*, Unc-51 mutation often leads to abnormal axonal elongation and structures (Ogura et al., 1994). Consistently, appropriate neurite arborization is important in establishing synaptic connectivity and neuronal plasticity, which is critical for preventing the onset of schizophrenia (Mochizuki et al., 2011; Mizutani et al., 2019). Therefore, it is assumed that the ULK family plays an important role in the establishment of the appropriate neural network and, when defective, may promote the development of neurological diseases. In line with this hypothesis, our data suggest that the proper expression of ULK4 is critical for neurite branching and brain development. Knockdown of ULK4 in SH-SY5Y cells led to less expression of acetylated α-tubulin, which may underlie the reduced dendrite length and/or branching and compromised neuronal migration (Lang et al., 2014). Defective neuritogenesis may involve multiple signaling pathways including protein kinase C (PKC), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinases (JNK) (Lang et al., 2014). Similarly, our *in utero* electroporation study *in utero* also demonstrated that knockdown of ULK4 caused perturbed neurite arborization in the pyramidal neurons of the cortex (Lang et al., 2016).

ULK4 and the Integrity of White Matter

Children's performance in cognition, intelligence, processing speed, and problem solving is closely associated with the thickness of the white matter, such as the corpus callosum and defective myelination is a hallmark related to neurodevelopmental and neuropsychiatric disorders (Liu et al., 2018b). We previously showed that ULK4 null knockout mice displayed impaired genesis of the corpus callosum (Lang et al., 2014). *Liu et al.* further reported a 50% decrease in myelination in ULK4$^{-/-}$ mice together with a general reduction in myelin components (Liu et al., 2018b). Myelin is produced by oligodendrocytes and controls impulse conduction speed along the axon, which is important to cognitive performance. Children with a less myelinated white matter in their brains often display developmental delay problems. Meanwhile, ULK4 mutant mice also present thin axons and extensive neuroinflammation, which also promote the occurrence of hypomyelination. In addition, ULK4 deficiency significantly attenuated the enrichment of oligodendrocyte transcription factors, the newly formed oligodendrocytes, and myelinating oligodendrocytes (Liu et al., 2018b). These data collectively indicate that ULK4 may be a crucial factor for the integrity of white matter and myelin.

ULK4 and Ciliopathy

The cilium is an antenna-like structure that protrudes from the surface of almost all mammalian cells. It participates in multiple signaling transduction pathways and when defective, can result in a series of inherited disorders called "ciliopathies". The most common features of ciliopathy include cystic liver and/or kidney, blindness, neural tube defects, brain anomalies, mental disability, skeletal abnormalities, obesity, and infertility, among others (Oud et al., 2017). Genomic and bioinformatics research has revealed that some primary cilia genes are linked to psychiatric disorders, such as the genes *CC2D2A* and *Disc1*, which are involved in ciliogenesis (Shen et al., 2008; Marley and von Zastrow, 2010; Veleri et al., 2014), and their defects can lead to psychiatric disorders, including Joubert syndrome (Bachmann-Gagescu et al., 2012), mental retardation (Noor et al., 2008; Shi et al., 2012), Meckel syndrome (Tallila et al., 2008), and Bardet Biedl syndrome (BBS) (Haq et al., 2019). In addition, several signaling pathways and crucial factors highly associated with schizophrenia, such as Wnt signaling, the fibroblast growth factor signaling system, neuronal migration, and the dopamine hypothesis, are dependent on the complete functionality of the cilium, although the specific mechanism is not yet well understood (Marley and von Zastrow, 2010; Muraki and Tanigaki, 2015; Narla et al., 2017; Hoseth et al., 2018). In the mouse brain, ULK4 is strongly expressed in the choroid plexus and ependymal cells lining the ventricles (Lang et al., 2014). Both ULK4 null knockout and hypomorphic mice present disturbed motile cilia development and disorganized ciliary beating which impair CSF flow and eventually lead to congenital hydrocephalus (Vogel et al., 2012; Liu et al., 2016b). These data strongly indicate the potential connection between ULK4 haploinsufficiency and ciliopathy. Acetylated α-tubulin is an important cytoskeletal component of cilia that is instrumental for cilium assembly. Our study, however, revealed that knockdown of ULK4 in human neuroblastoma cells (SH-SY5Y) and the mouse brain led to reduced expression of acetylated α-tubulin (Lang et al., 2014; Lang et al., 2016). In addition, whole-genome RNA sequencing also revealed massive disruption of genes closely related to ciliogenesis including Foxj1, Pcm1, Tubb4a, Dnah9, Rsph4a, Gsn, Kif5a, Lgals3, Lgals3bp, and Dnal1 in ULK4 mice carrying hypomorphic alleles. Interestingly, it has been reported that Foxj1 may target downstream substrates including Spag6, Rsph9, Rsph4a, Dnah9, Dnal1, Ttll6, and Tekt2 which consequently impairs ciliary development and results in hydrocephalus (Liu et al., 2016b). A recent study also reported that patients with a microdeletion of the *ULK4* gene and a microduplication of the *BRWD3* gene manifested core features of ciliopathy such as psychomotor delay, epilepsy, autistic features, hearing loss, obesity, minor facial dysmorphisms, peculiar ear malformations, and skeletal abnormalities (such as dorsal kyphosis and/or valgus knees and flat feet) (Tassano et al., 2018). Thus, it is highly likely that ULK4 contributes to ciliopathies. The results demonstrate that ULK4 is crucial for ciliogenesis and ciliopathies.

TABLE 1 | Summary of ULK4 variants and relevant manifestation in human patients.

SO Term	Ref Allele	Alt Allele	SNP Number	Related Disease	Ref
intron	C	T	rs17210774	bipolar disorder	Lang et al. (2014)
intron	T	C	rs1722850	depressive disorder	Lang et al. (2014)
5 UTR	A	G	rs7651623	risk of discontinuing use of antipsychotic medications in the patients with schizophrenia	Ou et al. (2019)
intron	C	T	rs2030431	risk of discontinuing use of antipsychotic medications in the patients with schizophrenia	Ou et al. (2019)
missense (A542P/A542T)	C	G/T	rs1052501	ASD/multiple myeloma	(Broderick et al., 2011; Greenberg et al., 2013)
missense (K39R/K39T)	T	G/C	rs2272007	ASD/multiple myeloma	Ou et al. (2019)
intron	T	A/C	rs1717027	diastolic blood pressure	Franceschini et al. (2013)
missense (I224F/I224V)	T	A/C	rs1716975	ASD	Ou et al. (2019)
intron	T	G	rs4973978	ASD	
intron	T	C	rs9824775	ASD	
intron	T	C	rs6599175	ASD	
intron	G	A	rs6783612	ASD	
intron	C	T	rs9852303	ASD	
intron	A	G	rs4973893	ASD	
intron	T	C	rs1716670	ASD	

The Progress of Current Research on ULK4 in Mental Disorders

Although previous GWAS studies have suggested that ULK4 is a risk locus for multiple myeloma and interindividual diastolic blood pressure variation, emerging evidence also supports the idea that *ULK4* genetic variants may cosegregate people with multiple neuropsychiatric disorders (Levy et al., 2009; Broderick et al., 2011). In our previous research using the cohort data from the International Schizophrenia Consortium, we identified four schizophrenia patients with *ULK4* intragenic fragment deletions spanning from exon 21 to exon 34 among 3,391 schizophrenia patients (Lang et al., 2014). Another study implicated that SNPs rs7651623 and rs2030431 of *ULK4* are associated with the risk of discontinuing the use of antipsychotics in patients with schizophrenia (Ou et al., 2019). In the Decode database, ULK4 deletion was also enriched in patients with schizophrenia (2/708), bipolar disorder (2/1,136), and autism (1/507) (Lang et al., 2014). In addition, association signals were observed at SNPs rs1052501, rs1716975, and rs2272007, which are located in exons 2, 7, and 17 of *ULK4*, respectively, for allelic transmission disequilibrium from parents to their children with ASD (Ou et al., 2019). Similarly, SNP rs17210774 of *ULK4* is significantly associated with bipolar disorder in Caucasians and another SNP rs1722850, which is close to but downstream of *ULK4,* is related to major depressive disorders (Lang et al., 2014) (**Table 1**). A recent study of the brain-body genetic resource exchange (BBGRE) cohort also reported an incidence in a population of 1.2‰, showing ULK4 copy number variation and exhibiting pleiotropic neurodevelopmental problems including learning difficulties and language delay (Liu et al., 2016a). In addition, a recent clinical study revealed 2 cases with ULK4 intragenic microdeletion (together with partial microduplication of BRWD3) that showed autistic features (Tassano et al., 2018).Consistently, in the follow-up functional analysis, we have revealed that knockdown of *ULK4* altered the activity of Wnt, PKC, MAPK, ERK1/2, and JNK signaling pathways commonly found in human mental disorders, especially schizophrenia (**Figure 2**). In addition, both *ULK4* knockout and hypomorphic mice presented congenital hydrocephalus featuring dilated ventricles and CSF accumulation. Interestingly, a proportion of schizophrenia patients also display increased global or regional CSF(Vogel et al., 2012; Lang et al., 2014). Moreover, *Liu et al.* revealed that ULK4 heterozygous mice displayed anxiety-like behavior with reduced GABAergic neurons in the basolateral amygdala and hippocampus (Liu et al., 2018a), and ULK4$^{-/-}$ mice showed a significant hypomyelination phenotype (Liu et al., 2018b). All these studies strongly suggest that ULK4 may be a rare risk factor for neuropsychiatric disorders including schizophrenia but more evidence is warranted in the future.

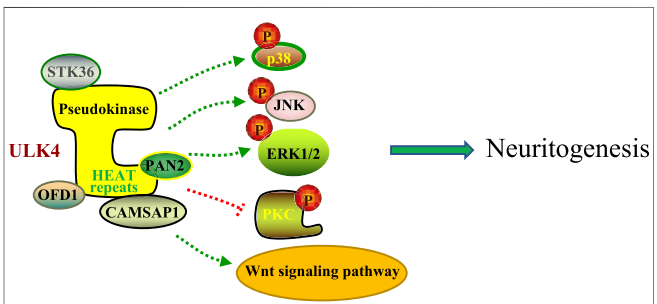

FIGURE 2 | A schematic representation of altered activities of multiple signaling pathways including p38 MAPK, JNK, ERK1/2, PKC, and Wnt signaling pathways by ULK4. These alterations contribute to deficient neuritogenesis, a common feature frequently represented by human mental disorders.

CONCLUSION AND PERSPECTIVES

Although ULK4 is a member of the Unc-51-like kinase family, unlike its ortholog members ULK1-3 and STK36, it is predicted to be catalytically inactive and to function as a pseudokinase. Initially, ULK4 was found to be associated with blood pressure and hypertension but further research has indicated its important functions during neurodevelopment. Knockdown of ULK4 *in vitro* also altered the activities of multiple signaling pathways, including Wnt, PKC, p38 MAPK, ERK1/2, and JNK, and mice with ULK4 deletion showed anxiety-like behaviors, perturbed neurogenesis, and decreased myelination. As mentioned above, ULK4 may be a rare risk factor for a range of psychiatric disorders, including schizophrenia, ASD, bipolar disorder, and depression, whose genetic variants were found in relevant patients and are crucial for ciliogenesis and ciliopathies. Further studies are warranted to fully understand the important function of ULK4, especially in neurodevelopment, and the specific underlying mechanisms for psychiatric disorders. With the successful resolution of the protein structure of ULK4 and further elucidation of its function, a series of small molecules targeting ULK4 may be developed to alleviate relevant neurodevelopmental and neuropsychiatric disorders in the future.

AUTHOR CONTRIBUTIONS

This work was primarily written by SL, NZ, and BL. Figure was produced by SL. All authors read and approved the final manuscript.

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BRIEF RESEARCH REPORT
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Brain Epitranscriptomic Analysis Revealed Altered A-to-I RNA Editing in Septic Patients

Jing-Qian Zhang[1,2,3†], *Jia-Qi Pan*[1,2,3†], *Zhi-Yuan Wei*[1,2,3], *Chun-Yan Ren*[1,2,3], *Fu-Xia Ru*, *Shou-Yue Xia*[1,2,3], *Yu-Shan He*[1,2,3], *Kaisheng Lin*[4*] and *Jian-Huan Chen*[1,2,3*]

[1]*Laboratory of Genomic and Precision Medicine, Wuxi School of Medicine, Jiangnan University, Wuxi, China,* [2]*Joint Primate Research Center for Chronic Diseases, Wuxi School of Medicine, Jiangnan University and Institute of Zoology, Guangdong Academy of Sciences, Jiangnan University, Wuxi, China,* [3]*Jiangnan University Brain Institute, Wuxi, China,* [4]*Jieyang People's Hospital, Jieyang, China*

Recent studies suggest that RNA editing is associated with impaired brain function and neurological and psychiatric disorders. However, the role of A-to-I RNA editing during sepsis-associated encephalopathy (SAE) remains unclear. In this study, we analyzed adenosine-to-inosine (A-to-I) RNA editing in postmortem brain tissues from septic patients and controls. A total of 3024 high-confidence A-to-I RNA editing sites were identified. In sepsis, there were fewer A-to-I RNA editing genes and editing sites than in controls. Among all A-to-I RNA editing sites, 42 genes showed significantly differential RNA editing, with 23 downregulated and 19 upregulated in sepsis compared to controls. Notably, more than 50% of these genes were highly expressed in the brain and potentially related to neurological diseases. Notably, cis-regulatory analysis showed that the level of RNA editing in six differentially edited genes was significantly correlated with the gene expression, including HAUS augmin-like complex subunit 2 (*HAUS2*), protein phosphatase 3 catalytic subunit beta (*PPP3CB*), hook microtubule tethering protein 3 (*HOOK3*), CUB and Sushi multiple domains 1 (*CSMD1*), methyltransferase-like 7A (*METTL7A*), and kinesin light chain 2 (*KLC2*). Furthermore, enrichment analysis showed that fewer gene functions and KEGG pathways were enriched by edited genes in sepsis compared to controls. These results revealed alteration of A-to-I RNA editing in the human brain associated with sepsis, thus providing an important basis for understanding its role in neuropathology in SAE.

Keywords: RNA editing, sepsis-associated encephalopathy, human brain, cis-regulatory analysis, epitranscriptome

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Emory University, United States

Reviewed by:
Qiyang Li,
Southern Medical University, China
Jianmin Yuan,
Nanjing Medical University, China

***Correspondence:**
Kaisheng Lin
kason2000@126.com
Jian-Huan Chen
cjh_bio@hotmail.com

[†]*These authors have contributed equally to this work*

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INTRODUCTION

Sepsis is a life-threatening systemic infectious disease caused by bacteria, viruses, or other factors, with high mortality worldwide (Singer et al., 2016; Rello et al., 2017; Salomao et al., 2019). Septic patients experience damage to multiple organs and systems, including sepsis-associated brain dysfunction. Sepsis-associated brain dysfunction (SABD) is also known as sepsis-associated encephalopathy (SAE). It has been found that up to 70% of patients affected with sepsis could develop SAE, which is the most common organ dysfunction in sepsis (Czempik et al., 2020). Its clinical manifestation is diverse, ranging from mild delirium to coma (Gofton and Young, 2012).

Adenosine-to-inosine (A-to-I) RNA editing is an epigenetic process of adenosine (A) to inosine (I) conversion mediated by the adenosine deaminase acting on RNA (*ADAR*s) family (Christofi and Zaravinos, 2019; Wang et al., 2020). It is recognized as guanosine (G) in reverse transcription and translation (Nishikura, 2016). A-to-I RNA editing has an important regulatory role in inflammatory diseases and neurological diseases (Gélinas et al., 2011; Chung et al., 2018). The potential role of *ADAR* has been reported in sepsis. *ADAR* is highly expressed in the small intestine of septic mice, which inhibits inflammation and plays a protective role against sepsis (Shangxun et al., 2020), providing a new potential therapeutic target for sepsis (Chen et al., 2017). Nevertheless, the role of *ADAR*-mediated A-to-I RNA editing played in sepsis remains unelucidated, especially in SAE.

Herein the current epitranscriptomic study analyzed A-to-I RNA editing from postmortem brain (the parietal cortex) tissues from septic patients and controls at the transcriptomic level and explored editing sites associated with sepsis and their cis-regulatory effects on the gene expression, providing new insight into the molecular mechanism involving A-to-I RNA editing in SAE.

METHODS

RNA-Seq Data

RNA sequencing raw data were obtained from NCBI's Gene Expression Omnibus (GEO) database. The dataset contained brain tissues (parietal cortex gray matter) from 12 patients who died from sepsis and 12 controls who died from noninfectious diseases (GSE135838) (Bustamante et al., 2020). Sepsis patients and controls were balanced for age, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) score, dementia diagnosis, and length of hospital stay. Detailed information can be found in the original report.

RNA-Seq Data Alignment

The obtained sequencing data were processed as previously described (Tao et al., 2021). In brief, quality control analysis was performed using FASTQC. Alignment of reads to the reference human genome sequence (UCSC hg38) was performed using RNA STAR (version 2.7.0e) (Dobin et al., 2013), with multiple-mapped reads and deduplication removed using SAMtools (version 1.9) (Li et al., 2009), and base quality score recalibrated using GATK (version 4.1.3) (Walker et al., 2018).

Identification and Annotation of RNA Editing Sites

RNA single-nucleotide variation (SNV) was identified using VarScan (version 2.4.3) software (Koboldt et al., 2012) using a standard pipeline described previously (Tao et al., 2021). Annotation of SNVs was performed using the Ensembl Variant Effect Predictor (VEP) (McLaren et al., 2016). Furthermore, only A-to-G SNVs with editing levels ≥ 1% observed in at least two samples or annotated as known editing variants in the REDIportal V2.0 database (Mansi et al., 2021) were retained as high-confidence variants.

Quantification and Differential Analysis of Gene Expression

Alignment files generated by RNA STAR were analyzed using FeatureCounts to obtain counts of RNA expression (Liao et al., 2014), and normalized gene expression levels (transcript per million, TPM) were calculated.

Enrichment Analysis of Gene Ontology and Pathways

Enrichment analysis of differentially edited genes were performed using DAVID online prediction tools (https://david.ncifcrf.gov/tools.jsp) and Enrichr (https://maayanlab.cloud/Enrichr/) with false discovery rate (FDR) < 0.05 as the significance cutoff (Kuleshov et al., 2016).

Statistical Analysis

The intergroup levels of RNA editing or gene expression were compared using the Kruskal–Wallis (KW) non-parametric test. Frequency data were analyzed using the Fisher's exact test. Cis-regulatory effects on RNA editing on the expression of edited genes were analyzed using the Spearman correlation to calculate the correlation coefficients *(r)* and *p*-values. Principal component analysis (PCA) was performed and visualized using R (version 3.6.3).

RESULTS

A-to-I RNA Editing in Human Brain Tissues

From transcriptomic data of the brain tissues from septic patients and controls, 3024 high-confidence A-to-I RNA editing sites in 1,192 genes were found (**Figure 1A**). These editing sites covered a variety of functional categories, including 2021 intronic variants, 467 3′-untranslated region variants (3′-UTR), 218 non-coding transcript intronic variants, 138 missense variants, 106 non-coding transcript exonic variants, 42 synonymous variants, 31 5′-untranslated region (5′-UTR) variants, and 1 stop-loss variant (**Figure 1B**). SIFT predicted 55 out of the 138 missense variants to have a potential impact on protein functions (**Figure 1C**). The expression levels of RNA editing enzymes *ADAR* and *ADARB1*, as well as the numbers of editing genes and editing sites in the brain tissues of septic patients, were lower than those in controls (**Supplementary Figures S1A,B**, **Figures 1D,E**). Of all these RNA editing sites, 118 were detected exclusively detected in septic patients and 236 in controls, and 2,670 were common in both groups (**Figure 1F**, **Supplementary Tables S1, S2**).

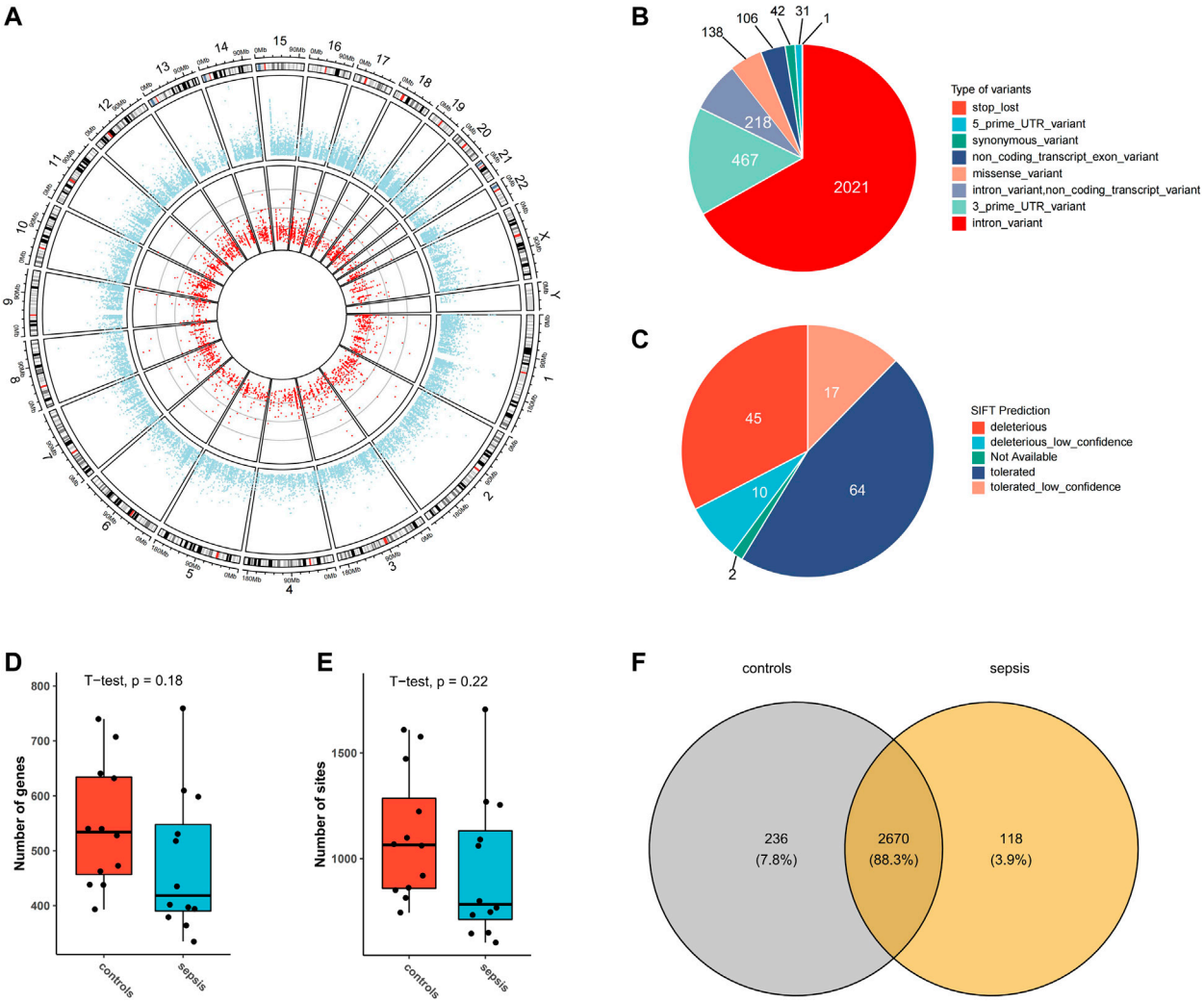

FIGURE 1 | A-to-I RNA editing sites identified from human brain transcriptome in the current study. **(A)** Circos plot of transcription gene expression (outer circle) and A-to-I RNA editing sites (inner circle) in the human brain. **(B)** Functional categories of A-to-I RNA editing detected. **(C)** SIFT prediction of missense variants Boxplot of the number of editing genes **(D)** and sites **(E,F)** Venn plot of A-to-I RNA editing sites in sepsis and controls.

Sequence Preference for Specific Editing Sites in Sepsis

The A-to-I RNA editing sites unique to the sepsis were then analyzed for sequence preference of 6 bp upstream and downstream of the editing sites. The results showed that, in most of the variant categories, G was suppressed 1 bp upstream of the editing sites. In addition, all editing sites preferred G 1 bp downstream the editing sites (**Supplementary Figure S2**).

Differential A-to-I RNA Editing Between Sepsis and Controls

In order to analyze differential A-to-I RNA editing in sepsis, the RNA editing levels of the sites among different groups were compared by the KW test, and a total of 43 differentially edited sites in 42 genes were found**,** with 23 genes downregulated and 19 genes upregulated in sepsis compared to controls (**Figure 2A**; **Supplementary Tables S3, S4**). Forty of these differentially edited sites were known sites. Among the 43 differentially edited sites, 23 were significantly downregulated and 20 upregulated in sepsis compared to those in controls. PCA using these differentially edited sites revealed separation of clustering between sepsis and control samples, with the contribution of PC1 and PC2 to be 33.01% and 19.53%, respectively (**Figure 2B**). Functional enrichment analysis of the differentially edited genes by DAVID revealed that 29 genes were related to protein binding and 16 were related to the cytosol (**Supplementary Table S5**). The results also showed that protein phosphatase 3 catalytic subunit beta (*PPP3CB*), kinesin light chain 2 (*KLC2*), proteasome 20S subunit beta 2 (*PSMB2*), and Matrin 3

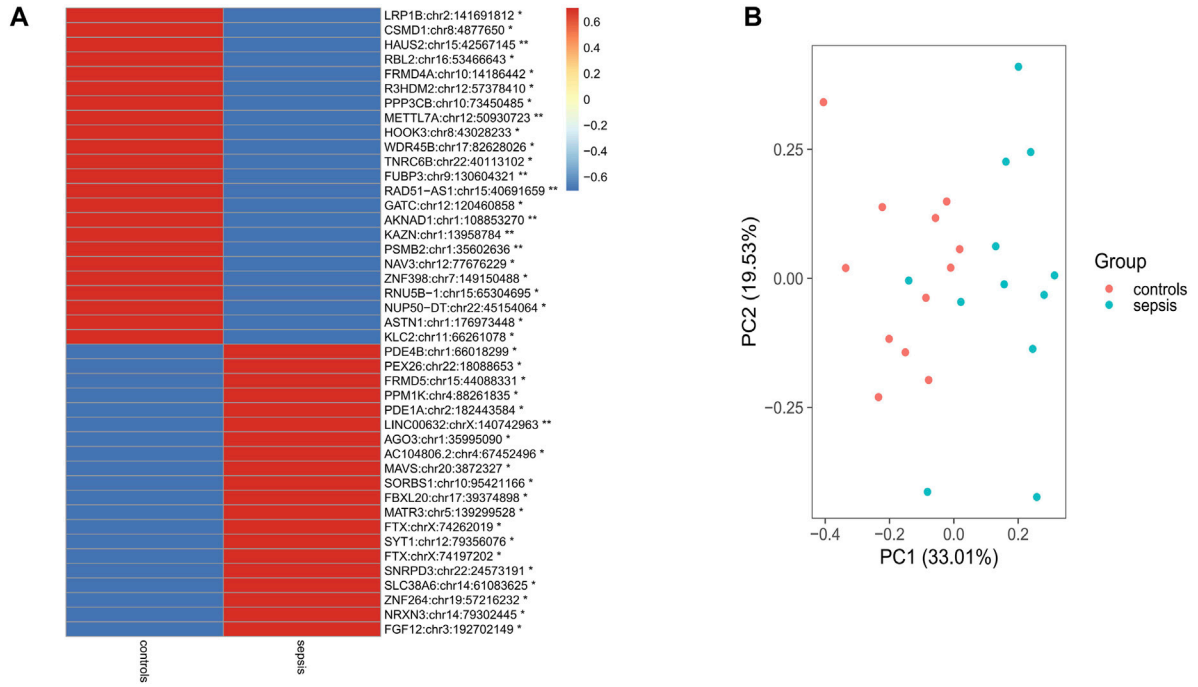

FIGURE 2 | Differential RNA editing sites in the brain between sepsis and controls. **(A)** 43 sites that show statistically different editing levels. *: $p < 0.05$; **: $p < 0.01$; p-values are calculated using the Student's-test. **(B)** Principal component analysis of the 43 differential editing sites between sepsis and controls.

(*MATR3*) were associated with amyotrophic lateral sclerosis, and *PPP3CB*, *KLC2*, and *PSMB2* were associated with prion disease, Alzheimer's disease, and pathways of neurodegeneration (**Supplementary Table S6**), pointing to the association of sepsis with neurological damage and the important role of A-to-I RNA editing in it.

Cis-Regulatory Effects of Differential Editing on Expression

Correlation analysis between sites' editing levels and corresponding gene expression levels was performed to investigate whether they would influence gene expression through RNA editing. A p-value cutoff of 0.05 was used to identify sites with higher correlation. Of the 43 differential editing sites previously found, six sites showed a correlation with the gene expression level ($p < 0.05$). Among them, the editing levels of *HAUS2*:chr15:42567145 ($r = 0.61$), *PPP3CB*:chr10:73450485 ($r = 0.59$), *HOOK3*:chr8:43028233 ($r = 0.49$), and *CSMD1*:chr8:4877650 ($r = 0.41$) were positively correlated with the gene expression level (**Figures 3A–D**). In contrast, *METTL7A*:chr12:50930723 ($r = -0.64$) and *KLC2*:chr11:66261078 ($r = -0.41$) were negatively correlated with the gene expression level (**Figures 3E,F**). In addition, these six sites all had a significantly lower editing level in sepsis than in controls: *HAUS2*:chr15:42567145 ($p = 0.0081$), *PPP3CB*:chr10:73450485 ($p = 0.045$), *HOOK3*:chr8:43028233 ($p = 0.023$), *CSMD1*:chr8:4877650 ($p = 0.043$), *METTL7A*:chr12:50930723 ($p = 0.0049$), and *KLC2*:chr11:66261078 ($p = 0.047$) (**Supplementary Figures S3A–F**).

Functional Enrichment in A-to-I RNA Editing in Sepsis

In order to understand the biological function of A-to-I RNA editing in the human brain affected with sepsis, enrichment analysis was performed using all sites in each group. Among the top enriched GO terms, biological processes including retrograde axonal transport, regulation of microtubule depolymerization, and axon development, cellular components including trans-Golgi network, and AMPA glutamate receptor complex, and molecular functions including actin binding, sodium channel regulator activity, and sodium channel activity were unique to sepsis (**Figures 4A–C**). In contrast, biological processes including membrane organization, Wnt signaling pathway (calcium modulating), neuron cell–cell adhesion, cell junction assembly, protein autophosphorylation, and regulation of presynapse organization and assembly, and cellular component cortical cytoskeleton, and molecular functions including glutamate receptor binding were enriched in controls. KEGG pathway analysis revealed that numerous pathways were enriched in controls but not in sepsis, including GnRH signaling pathway, gastric acid secretion, cholinergic synapse, ErbB signaling pathway, thyroid hormone synthesis, growth hormone synthesis, secretion and action, calcium signaling pathway, GABAergic synapse, axon

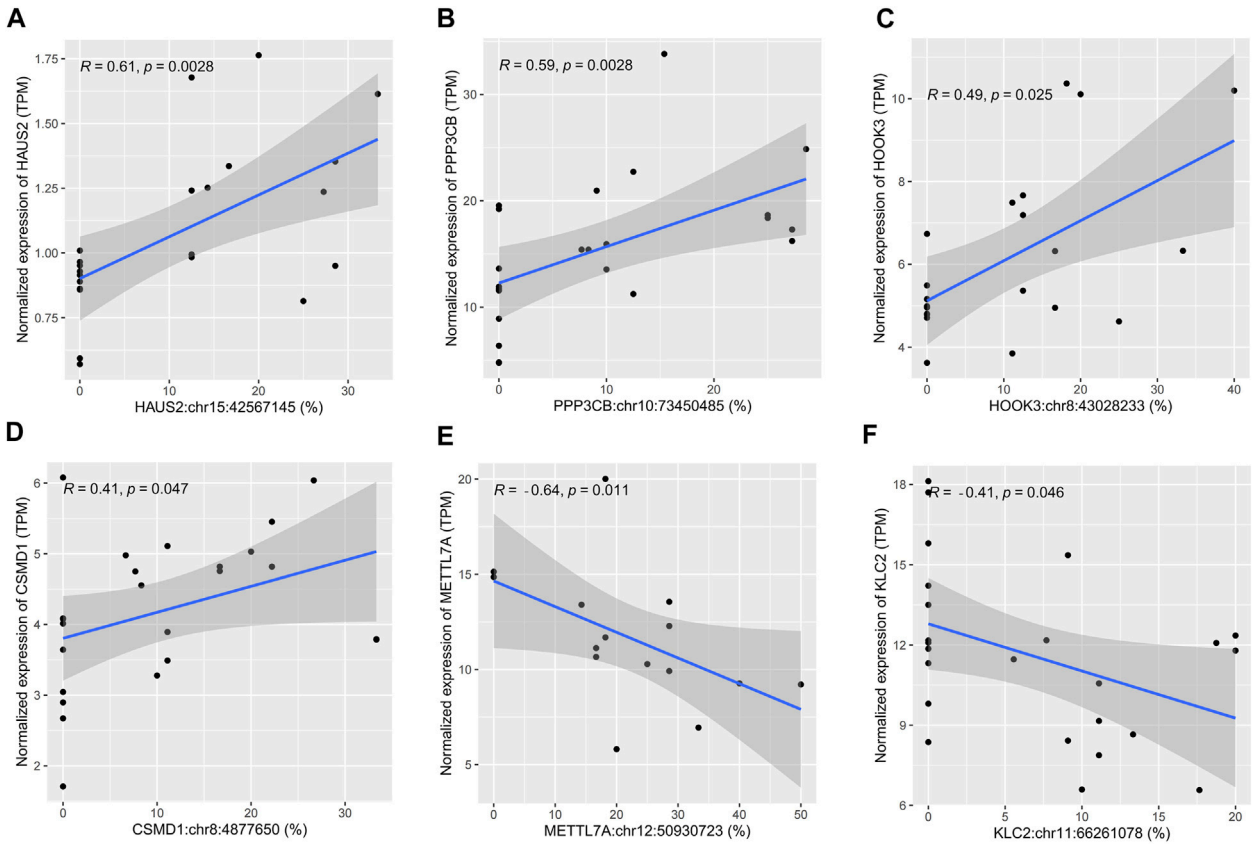

FIGURE 3 | Scatter plots showing cis-regulatory effects of A-to-I RNA editing on gene expression. **(A–F)** The correlation between the editing level and gene expression level in human brain samples.

guidance, and serotonergic synapse (**Figure 4D**). Overall, a reduction of enriched gene functions and pathways in sepsis compared to controls is consistent with decreased editing enzyme expressions, and fewer editing genes and sites in sepsis.

DISCUSSION

Recent studies suggest that RNA editing is involved in brain dysfunction and neurological diseases. Our current study systematically investigated A-to-I RNA editing in human brain tissues and revealed its changes associated with sepsis on a transcriptome-wide scale.

It has been reported that A-to-I RNA editing is widespread in the nervous system. It is associated with the normal development of the nervous system and a variety of neurological diseases (Behm and Öhman, 2016). A-to-I RNA editing has a regulatory role in a variety of neurological diseases, such as amyotrophic lateral sclerosis, developmental epileptic encephalopathy, depression, and schizophrenia (Yang et al., 2021). In the current study, we explored the distribution of A-to-I RNA editing in sepsis-associated brain dysfunction in clinical samples. Previous studies have shown that *ADAR* is highly expressed in macrophages and has a protective effect on sepsis (Shangxun et al., 2020). Our results showed that both the levels of *ADAR* expression and A-to-I RNA editing in the brain decreased in sepsis, which could be in line with a protective role of *ADAR* and A-to-I RNA editing against sepsis.

More than 50% of the differentially edited genes in sepsis were highly expressed in the central nervous system, indicating their potential functional importance. Although no role of these RNA editing sites has been reported, the edited genes have been associated with neurological diseases. *KLC2* may exert its function through factors involved in microtubule motor activity and kinesin binding and is associated with a variety of neurological diseases such as hereditary spastic diseases, optic atrophy, and SPOAN syndrome (Hedera, 1993; Melo et al., 2015). *PPP3CB* encodes a calcium-dependent protein phosphatase that acts intracellularly on Ca (2+)-mediated signal transduction (Chen et al., 2019; Zhang et al., 2019), and its expression is significantly correlated with human brain aging (Hu et al., 2018) and glioblastoma multiforme patients' overall survival (Lou et al., 2019). Its dysregulation has been reported in schizophrenia (Genis-Mendoza et al., 2013; He et al., 2021).

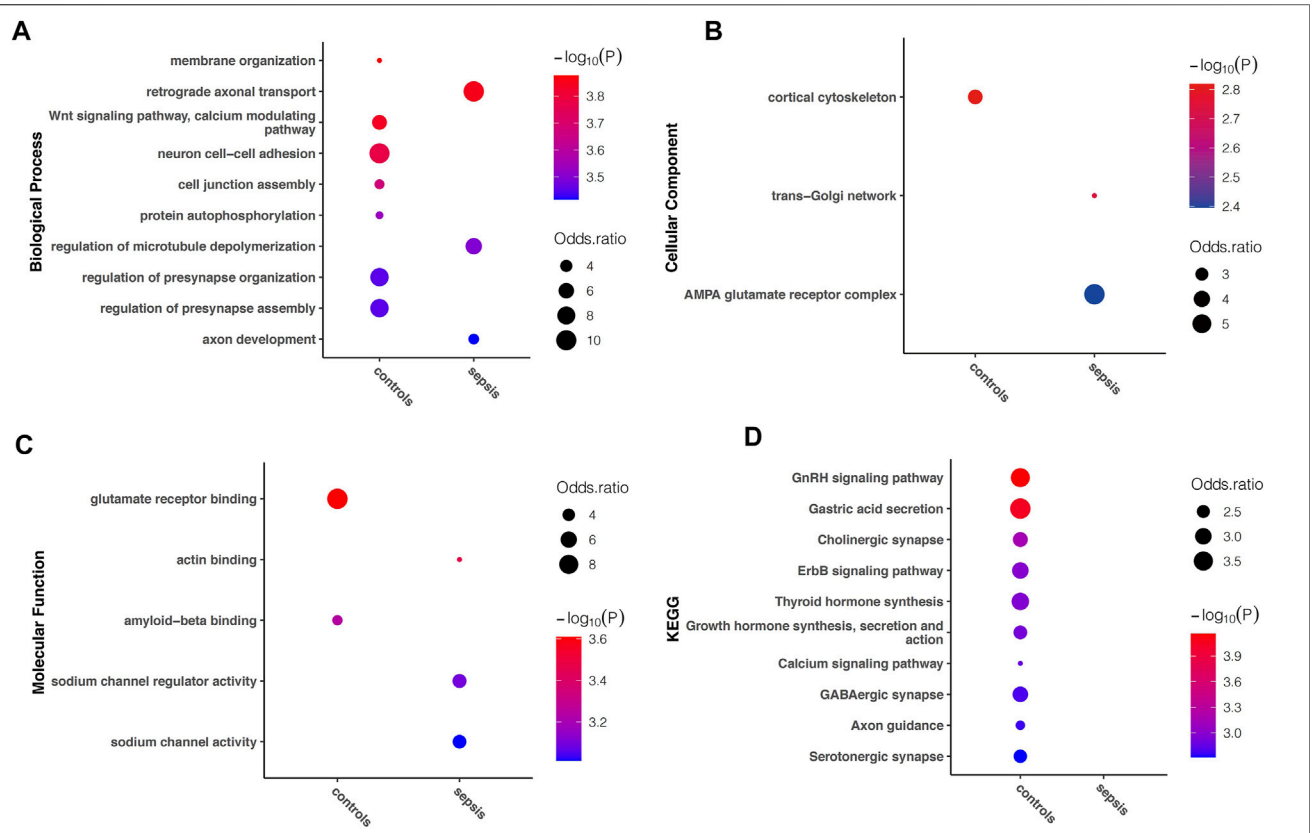

FIGURE 4 | Difference in Gene Ontology and KEGG pathway enriched in sepsis and controls, respectively. The top (showing up to 10) terms with FDR < 0.05 are shown for **(A)** biological processes **(B)** molecular functions, and **(C)** cellular components, as well as **(D)** KEGG pathways uniquely enriched in either sepsis or controls.

Methyltransferase-like 7A (*METTL7A*) encodes a methyltransferase mainly involved in DNA methylation and the innate immune system (Lee et al., 2021). Its role in the hippocampus and neuropathic pain has been implicated (Gong et al., 2021). HAUS augmin-like complex subunit 2 (*HAUS2*) interacts with the γ-tubulin ring complex and is involved in spindle assembly (Lawo et al., 2009), and one of its paralogs are associated with glioblastoma (Ding et al., 2017). The hook microtubule tethering protein 3 (*HOOK3*) gene is involved in protein binding and microtubule binding (Kendrick et al., 2019; Wortzel et al., 2021). Its role has been implicated in neurological diseases such as Alzheimer's disease (Herrmann et al., 2015). Expression of CUB and Sushi multiple domains 1 (*CSMD1*) is correlated with the development and treatment of schizophrenia (Liu et al., 2019). In addition, small nuclear ribonucleoprotein D3 polypeptide (*SNRPD3*) and *PSMB2* are also related to neurological diseases (Martinez and Peplow, 2017; Christodoulou et al., 2020). Notably, mutations of these differentially edited genes have been reported in neurological diseases. For example, familial ALS and distal myopathy were associated with mutations in *MATR3* (Senderek et al., 2009; Johnson et al., 2014). In addition, it has been shown that sepsis could cause long-term cognitive impairment and functional limitation in patients. *CSMD1*, *PPP3CB*, *METTL7A*, and *KLC2* have been reported to be associated with cognitive impairment or cognitive performance (Melo et al., 2015; Stepanov et al., 2017; Gong et al., 2021; Yu et al., 2021). Meanwhile, sepsis can also cause post-traumatic stress disorder and depression. *KLC2* (Du et al., 2010), *PPP3CB* (He et al., 2021), and *CSMD1* (Xu et al., 2014) were associated with mood disorders such as major depressive disorder or bipolar disorder.

Cis-regulation analysis showed that the editing level of six sites were highly correlated with the gene expression. It has been suggested that, in cancer, RNA editing can regulate mRNA abundance and thus modulate immune pathways (Chan et al., 2020). RNA editing in the 3′-UTR might affect mRNA degradation by regulating the RNA secondary structure stability or miRNA accessibility of the edited genes (Brümmer et al., 2017). One of the possible mechanisms is that the editing of *HAUS2*, *HOOK3*, and *METTL7A* mRNA may regulate their gene expression by influencing the binding of regulatory RNAs or proteins to these genes. For example, the expression of *METTL7A* as a tumor suppressor gene can be inhibited by ADAR-mediated RNA editing in the 3′-UTR (Qi et al., 2017). These results thus warranted further studies.

Gene functions and pathways of edited genes showed that the enrichment was weaker in sepsis than in controls, implicating that the sepsis-associated brain dysfunction may be related to the loss of these functions in RNA editing. Among the functions unique to sepsis, the regulation of microtubule depolymerization was noteworthy. Several studies have shown that microtubules are important in the nervous system, and their dysregulation is highly associated with neurological dysfunction (Baas and Ahmad, 2013; Diwaker and Wilson, 2019). A-to-I RNA editing could be closely related to such a biological process.

In conclusion, this study systematically investigated A-to-I RNA editing in the human brain tissues and revealed dynamic alterations in A-to-I RNA editing associated with sepsis. Our results provide a basis for further understanding how RNA editing is involved in SAE.

ADDITIONAL INFORMATION

URLs: Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/); Ensembl Variant Effect Predictor (VEP) (https://www.ensembl.org/vep); REDIportal V2.0 database (http://srv00.recas.ba.infn.it/atlas/index.html); Enrichr (https://maayanlab.cloud/Enrichr/).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

J-QZ and J-QP performed the bioinformatic analysis and drafted the manuscript. Z-YW improved the data analysis pipeline. C-YR, F-XR, S-YX and Y-SH participated in the data interpretation and discussion. KL and J-HC conceived the project and planned the study. All authors contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.887001/full#supplementary-material

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Promoter Specific Methylation of *SSTR4* is Associated With Alcohol Dependence in Han Chinese Males

Rongrong Zhao[1], *Huihui Shi*[1], *Jiajun Yin*[2], *Zhen Sun*[3] *and Yahui Xu*[3]*

[1]*The First Affiliated Hospital and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, China,* [2]*Brain Science Basic Laboratory, The Affiliated Wuxi Mental Health Center with Nanjing Medical University, Wuxi, China,* [3]*The Second Affiliated Hospital of Xinxiang Medical University, Xinxiang, China*

Alcohol dependence (AD), a disease can be affected by environmental factors with epigenetic modification like DNA methylation changes, is one of the most serious and complex public health problems in China and worldwide. Previous findings from our laboratory using the Illumina Infinium Human Methylation450 BeadChip suggested that methylation at the promoter of *SSTR4* was one of the major form of DNA modification in alcohol-dependent populations. To investigate whether DNA methylation levels of the *SSTR4* promoter influence alcohol-dependent behaviors, genomic DNA was extracted from the peripheral blood sample of 63 subjects with AD and 65 healthy controls, and pyrosequencing was used to verify the results of BeadChip array. Linear regression was used to analyze the correlation between the methylation levels of *SSTR4* promoter and the scores of alcohol dependence scales. Gene expression of *SSTR4* in brain tissue was obtained from the Genotype-Tissue Expression (GTEx) project and Human Brain Transcriptome database (HBT). We found the methylation levels of *SSTR4* in AD group were significantly lower than healthy controls (two-tailed *t*-test, $t = 14.723$, $p < 0.001$). In addition, only weak to moderate correlations between the methylation levels of the *SSTR4* promoter region and scale scores of Alcohol Use Disorders Identification Test (AUDIT), Life Events Scale (LES) and Wheatley Stress Profile (WSS) based on linear regression analyses (AUDIT: $R^2 = 0.35$, $p < 0.001$; LES: $R^2 = 0.27$, $p < 0.001$; WSS: $R^2 = 0.49$, $p < 0.001$). The hypomethylated status of *SSTR4* may involve in the development of AD and increase the risk of AD persistence in Han Chinese males.

Keywords: alcohol dependence, gene expression, hypomethylation, SSTR4, Han Chinese

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Edited by:
Cunyou Zhao,
Southern Medical University, China

Reviewed by:
XiongJian Luo,
Kunming Institute of Zoology (CAS), China
Min Zhao,
Shanghai Mental Health Center, China

***Correspondence:**
Yahui Xu
18737354427@163.com

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INTRODUCTION

Alcohol dependence (AD) is a common chronic disorder which imposes a substantial burden on global health. According to World Health Organization (WHO) reports, there were approximately 3.3 million alcohol-related deaths worldwide in 2014, including 320,000 young individuals aged 15 to 29 (Organización Mundial de la Salud, 2014). It is estimated that more than 1.8 million persons were dependent on alcohol, and 1.6 million persons had a lifetime history of alcohol abuse in Germany (Batra et al., 2016). Family, twin and adoption studies have indicated genetic basis for AD susceptibility (Reilly et al., 2017), with the variation in heritability from a range of 40%–70% (Enoch and Goldman, 2001; Agrawal and Lynskey, 2008; Lynskey et al., 2010; Zhang et al., 2012). In addition, environmental factors may play

important roles in AD development through epigenetic regulation of gene expression without DNA sequence alterations (McCutcheon et al., 2012).

Epigenetic regulatory mechanisms could induce stable changes in gene expression with a range of phenotypic outcomes via DNA methylation, histone acetylation, chromatin remodeling, and noncoding RNA regulation (Kouzarides, 2007; Krishnan et al., 2014). Cytosine methylation at CpG dinucleotides-rich regions (CpG islands) is the common epigenetic modification found in DNA where the methylation plays a pivotal role in mediating gene transcription regulation by affecting transcription factor binding. Numerous studies have indicated although most genomic CpGs were stably methylated, CpG islands near or within the promoter regions maintained commonly low methylation levels to allow the transcriptional activation of related gene dynamically, and its dysregulated methylation contributed to disease progression in cases of environmental challenges (Egger et al., 2004; Moore et al., 2013). It was thought that disturbances of epigenetics also participate in pathophysiological processes of AD (Basavarajappa and Subbanna, 2016). Other studies have also found hypomethylation of several genes such as *GDAP1* correlated with increased alcohol consumption (Brückmann et al., 2016), and elevated N-methyl-D-aspartate 2b receptor subunit gene (Biermann et al., 2009) and proopiomelanocortin gene (Muschler et al., 2010) promoters methylation was detectable in DNA from peripheral blood of patients with AD. These alterations in DNA methylation might impact the transcriptional profile and the susceptibility to AD (Wilson et al., 2019). For example, in animal models of AD, up-regulation of *Gdnf* expression due to altered methylation of core promoter or negative regulatory element has been observed in Nucleus Accumbens, which are key brain regions associated with reward and addictive behaviors (Maier et al., 2020). Moreover, specific genetic variants at methylation quantitative trait loci might also influence AD susceptibility via altering DNA methylation status (Zhang et al., 2014). However, evidence from laboratory-based data may not be conclusive, and epidemiological studies are required to better understand the biological mechanisms of alcohol addiction, which could aid in the clinical treatment or prevention of AD.

Somatostatin receptor 4 (SSTR4) is a brain-specific G-protein-coupled receptor as known substrate of somatotropin-release inhibitory factor implicated in the pathophysiological processes of anxiety and depression-like behavior (Günther et al., 2018). Previous studies have shown *SSTR4* is expressed in areas involved in learning and memory processes, and the activation of hippocampal *SSTR4* leads to a switch from hippocampus-based memory to dorsal striatum-based behavioral responses (Gastambide et al., 2009). In addition, experimental data suggest *SSTR4* might represent important therapeutic targets for the treatment of Alzheimer's disease and seizures, yet the direct evidence for the role of *SSTR4* in alcoholism is still lacking.

Our previous genome-wide study based on methylation detection utilizing the Illumina Infinium Human Methylation450 (Illumina Inc., San Diego, California) on DNA extracted from peripheral blood (PB) of 10 AD subjects and 10 paired siblings without AD revealed 1,581 differentially methylated CpG positions (including 865 hypomethylation islands and 716 hypermethylation islands), which were associated with 827 well-annotated reference genes (Zhao et al., 2013). Our data suggested novel potential epigenetic targets relevant to AD. DNA pyrosequencing technology has also been to examine the 2 top-ranked hypo or hypermethylation AD-related genes from Illumina microarrays determined by DAVID. Linear regression analysis showed good correlation between DNA microarrays and pyrosequencing results. In alcohol-dependent subjects, the most prominent hypomethylated CG dinucleotide sites were located in the promoter of *SSTR4*. The objective of current research was to validate the demethylated status of *SSTR4* in Han Chinese alcohol-dependent males.

MATERIALS AND METHODS

Subjects

The current research utilized clinical and methylation microarrays (Illumina Infinium Human Methylation450) data extracted from our previous analyses and newly recruited subjects. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Xinxiang Medical University (2015 Ethics number 27), and written or oral informed consent was obtained from each participant. Blood samples of validation cohort included 128 male participants (63 AD and 65 healthy controls) recruited from community or medical clinic settings of northern Henan Province. A consistent diagnosis of AD was made by at least two psychiatrists according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (American Psychiatric Association, 1994). The Alcohol Use Disorders Identification Test (AUDIT, score range 0–40) was utilized to measure quantity-frequency of alcohol consumption, and the score of AUDIT greater than or equal to 8 suggested and problematic drinking and AD tendency (Babor et al., 2001). The Life Events Scale (LES) (Trivedi et al., 2010) and Wheatley Stress Profile (WSS) (Wheatley, 1990) were used to assess negative life events and possible stress factors associated with AD. Controls were screened to exclude those with alcohol or drug abuse or dependence. We also ruled out subjects with other substance misuse, comorbidity in major psychiatric disorders, serious medical complications, severe neurological or somatic illnesses.

DNA Extraction and Amplification

The QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was utilized to extract and purify genomic DNA from PB. Forty microliters of DNA solution were treated with the CT conversion reagent included in the EpiTect Plus LyseAll Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of bisulfite-treated DNA was determined using a Thermo Nanodrop 2000 spectrophotometer, and the DNA volume was determined to be at least 3 μl. Because the nucleotide composition of DNA is changed and the DNA fragments are smaller after bisulfite conversion, the results of subsequent experiments are not ideal. Therefore, the whole genome was amplified after bisulfite transformation, and the sequence after transformation was maintained. One hundred nanograms of bisulfite-treated DNA were amplified using an EpiTect Whole Bisulfitome Kit (Qiagen,

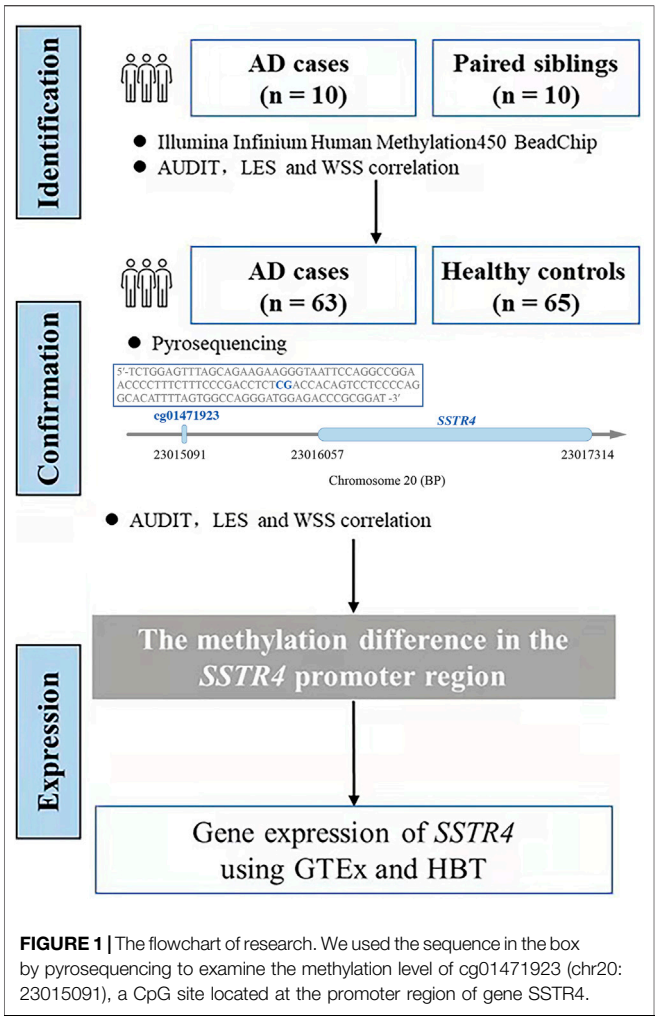

FIGURE 1 | The flowchart of research. We used the sequence in the box by pyrosequencing to examine the methylation level of cg01471923 (chr20: 23015091), a CpG site located at the promoter region of gene SSTR4.

Hilden, Germany) under the following conditions: 8 h at 28°C and 5 min at 95°C.

Pyrosequencing

Primers were designed using Pyrosequencing Assay Design Software (Biotage AB, Uppsala, Sweden): *SSTR4*1f (PCR forward, biotin-labeled), 5′-TTTTTGGAGTTTAGTAGAAGA AGGGTAAT-3'; *SSTR4*1r (PCR reverse), 5′-CACCCTATA ACCTAATTCAATCATTATC-3'; S*STR4*1s (PCR sequencing), 5′-ATCCCTAACCACTAAAATA-3'. PCR was performed using 10 ng of bisulfite-treated DNA using a PyroMark PCR Kit (Qiagen, Hilden, Germany) under the following conditions: the Initial PCR activation step was 15 min at 95°C, followed by 50 cycles of 30 s at 94°C (denaturation), 30 s at 56°C (annealing), 30 s at 72°C (extension), and a final extension of 10 min at 72°C.

Pyrosequencing was performed using PyroMark Gold Q96 Reagents (Qiagen, Hilden, Germany). We applied the Biotage PyroMark MD System (Biotage) to conduct pyrosequencing reactions via sequential nucleotide additions in the predetermined orders based on the instructions of manufacturer. RAW sequencing data were quantitatively analyzed by using Pyro Q-CpG 1.0.9 software (Biotage). The methylation levels of CpG regions were assessed by the percentage of methylated cytosines (M) over the total methylated and unmethylated cytosines (M + U) in the genome.

Statistical Methods

The DNA methylation microarray from Illumina were utilized in reference to our previous findings. DNA methylation levels between 10 AD subjects and 10 paired siblings were compared using the two-tailed paired Student's t-test based on the unequal variance assumption. The methylation value of the *SSTR4* promoter region in validation cohort (63 subjects with AD and 65 healthy controls) was analyzed using a two-tailed unpaired *t*-test with unequal variance. Linear regression analysis was used to examine the associations between the methylation levels of the *SSTR4* promoter region and scale scores of AUDIT, LES and WSS. Gene expression of *SSTR4* was confirmed by the Genotype-Tissue Expression database (GTEx, www.gtexportal.org) and Human Brain Transcriptome database (HBT, www.hbatlas.org) (Kang et al., 2011; Ramasamy et al., 2014; GTEx Consortium, 2015). Two-tailed p value less than 0.05 were considered statistically significant. An overview of subject recruitment and promoter methylation levels analysis was presented as a flow chart in **Figure 1**.

RESULTS

The Correlation Between the Methylation Levels of the *SSTR4* Promoter Region in Blood and the AUDIT, LES, WSS Scores

Considering potential gender effect on genome-wide DNA methylation, only male subjects were recruited in this study. Overall, the mean age of subjects were similar between AD group (mean age 39.1 ± 7.3) and healthy control (mean age 39.6 ± 8.1), with no significant difference (p = 0.722). The scale scores of AUDIT, LES and WSS were higher for AD subjects than for the control group (25.4 ± 7.4 vs. 8.4 ± 3.7, 22.2 ± 5.6 vs. 10.2 ± 3.5, 25.2 ± 5.1 vs. 9.2 ± 2.9, respectively, p-value < 0.001). Analysis results were summarized in **Table 1**. Linear regression analysis revealed the methylation levels of *SSTR4* were only weak to moderate correlations with the scores of AUDIT, LES and WSS as shown in **Figure 2** (AUDIT: R^2 = 0.35, p < 0.001; LES: R^2 = 0.27, p < 0.001; WSS: R^2 = 0.49, p < 0.001).

The Methylation Difference in the *SSTR4* Promoter Region in 10 Paired Siblings Using Microarray Compared to Case-Controls Eith Pyrosequencing

The results of previous DNA methylation microarrays showed that the level of methylation of the *SSTR4* promoter region between cases and paired siblings was statistically significant (t = 2.348, p = 0.043, **Figure 3A**). Likewise, the level of methylation of the *SSTR4* promoter region confirmed by pyrosequencing in cases and controls was statistically significant (t = 14.723, p < 0.001), and hypomethylation of the *SSTR4* promoter region was observed in AD cases (**Figure 3B**).

TABLE 1 | Basic characteristic of the study population.

Feature characteristic	AD cases (*n* = 63)	Healthy controls (*n* = 65)	*t*	*p*
Age (mean ± S.D)	39.1 ± 7.3	39.6 ± 8.1	0.357	0.722
AUDIT (mean ± S.D)	25.4 ± 7.4	8.4 ± 3.7	16.301	<0.001
LES (mean ± S.D)	22.2 ± 5.6	10.2 ± 3.5	14.441	<0.001
WSS (mean ± S.D)	25.2 ± 5.1	9.2 ± 2.9	21.779	<0.001

S.D, standard deviation.

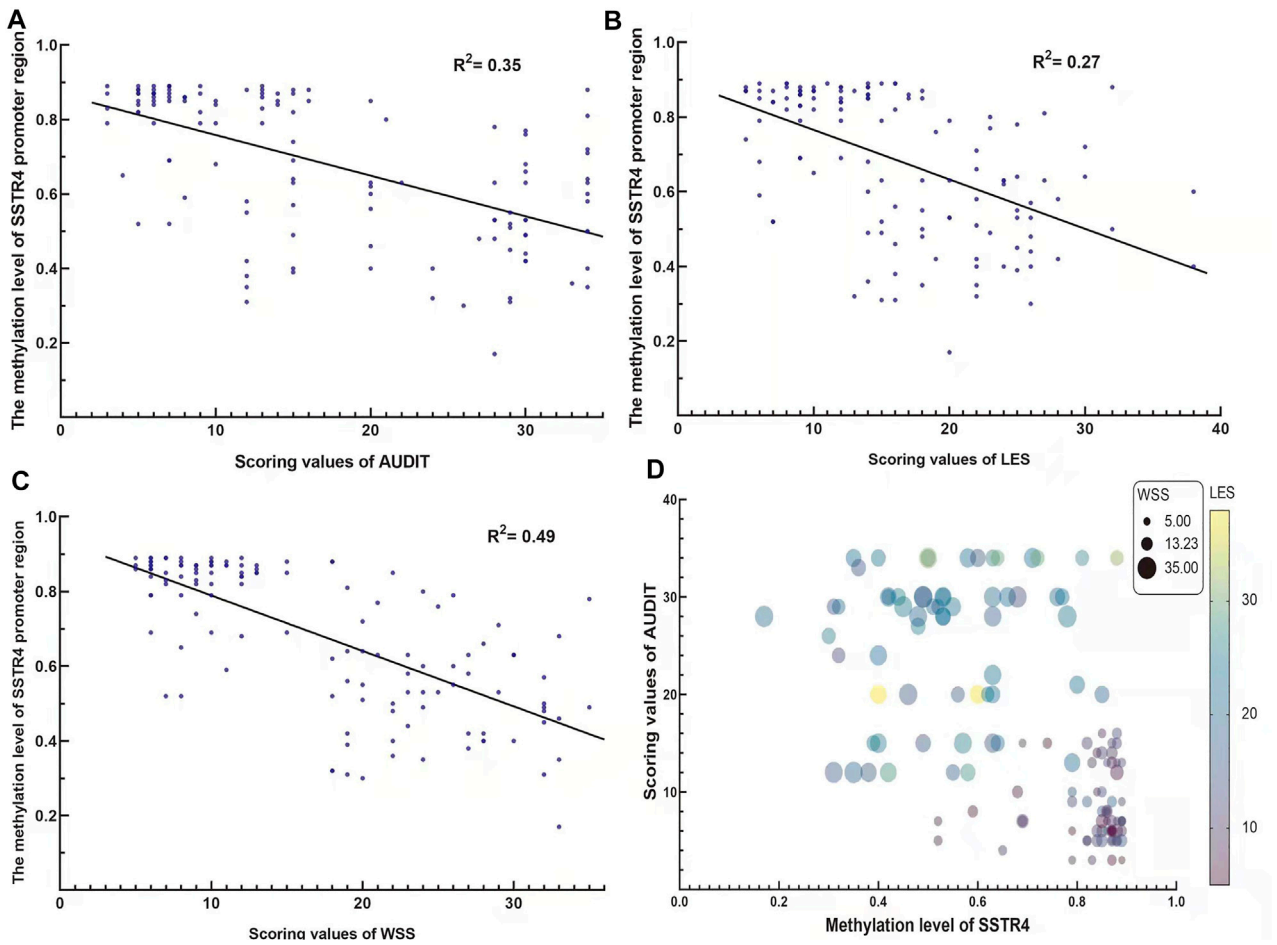

FIGURE 2 | The correlation between the methylation value of the *SSTR4* promoter region and AUDIT, LES and WSS scores. **(A)** The methylation value of the *SSTR4* promoter region and the AUDIT score were negatively correlated. **(B)** The methylation value of the *SSTR4* promoter region and the LES score were negatively correlated. **(C)** The methylation value of the *SSTR4* promoter region and the WSS score were negatively correlated. **(D)** Bubble plot for the visualization of association of SSTR4, AUDIT4, LES and WSS.

Gene Expression of *SSTR4* Using GTEx and HBT

Expression of *SSTR4* in various tissues revealed relatively strong expression in brain tissue. Although *SSTR4* is highly expressed in the cerebellar hemisphere and cerebellum, it is moderately expressed in the nucleus accumbens (NAC), prefrontal cortex (PFC), amygdala (AMY) and hippocampus (HPC) (**Figure 4A**). These regions are related to the reward pathway of addiction in the brain. Temporal expression analyses showed that the expression level of *SSTR4* was relatively stable across lifespan (**Figure 4B**).

DISCUSSION

These findings revealed that compared to controls, AD patients experienced more negative life events (LEs) and higher stress

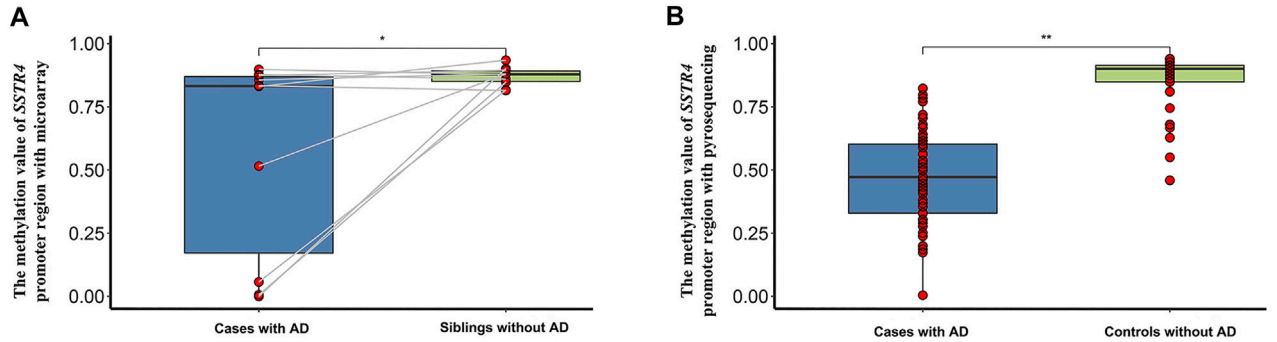

FIGURE 3 | The methylation difference of the SSTR4 promoter region in 10 paired siblings with microarray and case-controls with pyrosequencing. **(A)** Box plot of the methylation value of the SSTR4 promoter region with microarray and 10 paired siblings. **(B)** Box plot of the methylation value of the SSTR4 promoter region with pyrosequencing and case-controls. $^{*}p < 0.05$,$^{**}p < 0.001$.

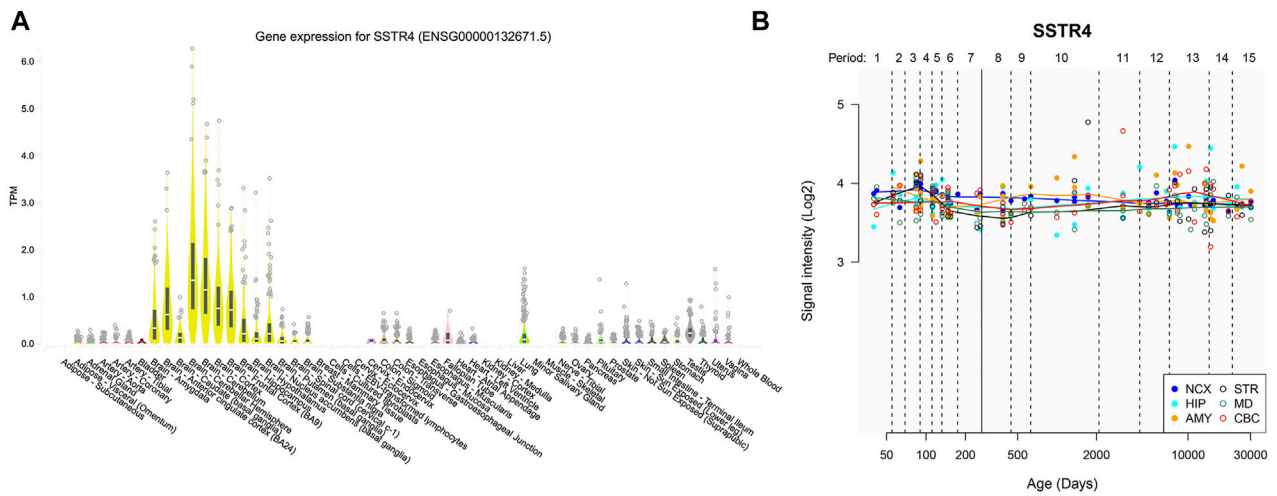

FIGURE 4 | Gene expression of *SSTR4* by GTEx and HBT. **(A)** Spatial expression pattern of the *SSTR4* gene in human brain regions from GTEx. TPM = transcripts per kilobase million. Expression threshold: >0.1 TPM and ≥6 reads in 20% or more of samples. Box plots are shown as median and 25th and 75th percentiles; points are displayed as outliers if they are above or below 1.5 times the interquartile ranges. Data Source: GTEx Analysis Release V8 (dbGaP Accession phs000424. v8. p2). **(B)** Dynamic expression pattern of the *SSTR4* gene in 6 human brain regions across lifespan from HBT. NCX, neocortex; CBC, cerebellar cortex; MD, mediodorsal nucleus of the thalamus; STR, striatum; AMY, amygdal; HIP, hippocampus. Period 1, Embryonic development; Period 2, Early fetal development; Period 3, Early fetal development; Period 4, Early mid-fetal development; Period 5, Early mid-fetal development; Period 6, Late mid-fetal development; Period 7, Late fetal development; Period 8, Neonatal and early infancy; Period 9, Late infancy; Period 10, Early childhood; Period 11, Middle and late childhood; Period 12, Adolescence; Period 13, Young adulthood; Period 14, Middle adulthood; Period 15, Late adulthood.

levels, which indicated that environmental factors play a role in the formation and maintenance of AD. This result is also consistent with our previous clinical research of 10 AD cases and 10 paired siblings without AD as controls (Zhao et al., 2013). The study of Linda Azucena Rodríguez Puente et al. (2019) showed that stressful events occur that have the potential to trigger the consumption of substances, such as alcohol. Stressful events are greater in those who consume alcohol than in those who do not consume alcohol. Likewise, the study of Marketa Krenek showed that although alcohol use severity did not predict changes in recent LEs, the emergence of LEs is associated with subsequent increases in drinking severity. This article also provided partial support for the hypothesis that distal LEs influence changes in both LEs and heavy alcohol use over time (Krenek et al., 2017). Ethan H indicated that although LEs may not necessarily contribute to the maintenance of long-term alcohol abuse among heavy drinkers with high addiction severity, daily stressful events predicted increases in daily drinking the whole time for all heavy drinking, and stress may influence the emergence of early drinking behaviors (Mereish et al., 2018). These studies' findings were consistent with our research.

In addition, the follow-up results of our study revealed that the lower the methylation value of *SSTR4* was, the higher the AUDIT, LES and WSS values were. According to this result, stressful events (higher values of LES and WSS) may

contribute to alcohol use disorder and AD (higher value of AUDIT), and then influence the methylation of *SSTR4* (hypomethylation). In contrast, hypomethylation of *SSTR4* may induce addictive behavior. It can be inferred that stressful events that lead to the hypomethylation of *SSTR4* mediate alcohol abuse. A study by Scheich et al. revealed that activation of *SSTR4* in the central nervous system plays a role in modulation of behavioral responses to acute stress and neuroendocrine changes induced by mild chronic stress in mice, suggesting involvement of *SSTR4* in anxiety and depression-like behavior (Scheich et al., 2016; Scheich et al., 2017), consistent with our research. Through these studies, we can better understand how LEs and higher stress act as high risk factors for AD. This result offers treatment options for reducing the negative effect on LEs and higher stress to reduce the germination and maintenance of AD.

AD and drugs of abuse have a moderate to high heritability component (Goldman et al., 2005). In addition to the variation of basic sequences, epigenetic modification of gene sequences may also be associated with substance dependence (Zhang et al., 2012). The present study suggested that there was significantly lower DNA methylation of the *SSTR4* promoter region in AD cases than in healthy controls. Sample sizes of AD cases and controls were increased to perform theoretical verification, which was used to confirm the results based on the research of 10 AD cases and 10 paired siblings without AD as controls.

Somatostatin (SST), also known as somatotropin-release inhibitory factor, is a cyclopeptide that plays an important role in inhibiting hormone secretion and neuronal excitability (Günther et al., 2018). Somatostatin receptor 4 (SSTR4) belongs to the SSTR family of G protein-coupled transmembrane receptors (GPCRs) comprised of five members (SSTR1–5), which trigger various transmembrane signaling pathways (Reisine and Bell, 1995; Csaba and Dournaud, 2001; Zou et al., 2019). *SSTR4* is expressed in areas involved in learning and memory processes (Günther et al., 2018). Gastambide et al. (2009) found that hippocampal *SSTR4* is functionally involved in a switch from hippocampus-based memory to dorsal striatum-based behavioral responses. Through a biological database, we found that *SSTR4* is highly expressed in brain tissue, and moderately expressed in the NAC, PFC, AMY and HPC. Psychostimulants are involved in the major brain regions including the ventral tegmental area (VTA), NAC, PFC, AMY, and HPC (Peña et al., 2014). Furthermore, a study by Moneta D indicated that SSTR4 enhanced (α-amino-3-hydroxy-5-methyl-4-isoxazoleioni-c axid, AMPA)-receptor-mediated excitatory signaling (Moneta et al., 2002) and that AMPA receptors were related to addiction (Godino et al., 2015). These results suggest that *SSTR4* may be related to reward and addiction. Temporal expression analyses showed that the expression level of *SSTR4* was relatively stable over time. However, our study showed hypomethylation of *SSTR4* in AD cases, which indicated a potential high expression of *SSTR4*. According to this, expression of *SSTR4* might be an upstream regulator of alcohol abuse, which can be inferred from previous findings, and suggests that alcohol abuse may ultimately affect *SSTR4* expression.

At present, there are few reports about the methylation of *SSTR4* related to AD. Dominika Berent interviewed 176 AD cases and 127 healthy controls to assess genotyping for the *SSTR4* rs2567608 polymorphism. The result revealed that AD cases and the controls did not differ significantly according to the *SSTR4* rs2567608 genotype and allele frequencies (Berent et al., 2017a). This study involved the relationship between the *SSTR4* genotype and AD, but did not examine the methylation of *SSTR4*. Another study interviewing the same participants revealed that the *SSTR4* promoter region was methylated in 21.6% of patients with AD and only 2.3% of controls (Berent et al., 2017b), suggesting a difference in methylation levels of *SSTR4* between AD cases and controls. This result is consistent with our present research in some respects.

The present study has several limitations. First, the sample size was relatively small, so further research will enlarge the sample size to verify the methylation levels of *SSTR4* by pyrosequencing. Second, we did not examine *SSTR4* expression levels in blood samples because they were unavailable for RNA extraction, and further research will analyze the correlation between DNA methylation and *SSTR4* expression in blood samples. Third, it still remains unclear whether epigenomic changes in peripheral cells could fully reflect the true DNA methylation status of brain. Nevertheless, tissue biopsies in every alcohol dependent subject are neither ethical nor practical, and previous studies have showed methylation of CpG positions occurring in PB might track part of the changes in central nervous system (Hillemacher et al., 2009). And last, AD in males were relatively easy to recruit, so subjects in this study were only males. But in view of the potential effect of gender on methylation, this may be a limitation of this study. In the further research, we may be recruit AD in females.

In summary, the promoter region of *SSTR4* differs between AD cases and controls. This study provides novel insights that heavy drinking likely results in alteration of epigenetic modification, which might in turn promote AD development. The hypothesis would integrate the understanding of methylation mechanism in the process of gene-environment interactions in alcohol-dependent patients. In addition, the *SSTR4* gene may represent a new biomarker for AD, which offers new ideas for the treatment of AD. Given these findings, additional effective therapeutic options may be developed in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Xinxiang Medical University

(2015 Ethics number 27), and written or oral informed consent was obtained from each participant.

AUTHOR CONTRIBUTIONS

RZ and JY were responsible for the study concept and design. RZ and HS contributed to the acquisition of clinical experiment data and drafted the manuscript. JY and ZS performed the data analysis and interpreted the findings. YX provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.915513/full#supplementary-material

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REVIEW
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L1 Retrotransposons: A Potential Endogenous Regulator for Schizophrenia

Muhammad Jahangir, Li Li, Jian-Song Zhou, Bing Lang and Xiao-Ping Wang**

Department of Psychiatry, National Clinical Research Center for Mental Disorders, The Second Xiangya Hospital of Central South University, Changsha, China

The long interspersed nuclear elements 1 (LINE-1/L1s) are the only active autonomous retrotransposons found in humans which can integrate anywhere in the human genome. They can expand the genome and thus bring good or bad effects to the host cells which really depends on their integration site and associated polymorphism. LINE-1 retrotransposition has been found participating in various neurological disorders such as autism spectrum disorder, Alzheimer's disease, major depression disorder, post-traumatic stress disorder and schizophrenia. Despite the recent progress, the roles and pathological mechanism of LINE-1 retrotransposition in schizophrenia and its heritable risks, particularly, contribution to "missing heritability" are yet to be determined. Therefore, this review focuses on the potentially etiological roles of L1s in the development of schizophrenia, possible therapeutic choices and unaddressed questions in order to shed lights on the future research.

Keywords: retrotransposons, LINE-1, schizophrenia, somatic mutation, chromatin remodelling

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Weihua Yue,
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Wei Yan,
University of Michigan, United States

****Correspondence:***
Bing Lang
bing.lang@csu.edu.cn
Xiao-Ping Wang
xiaop6@csu.edu.cn

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INTRODUCTION

Retrotransposons are a group of "jumping genes" which constitute ~17% of the human genome. In fact, nearly half of the human genome (~45%) is derived from insertions of transposable elements (Lander et al., 2001). The mechanism and further details of retrotransposons have already been extensively reviewed elsewhere (Terry and Devine, 2020). Retrotransposons are divided into LTR or non-LTR retrotransposons with the presence or absence of long terminal repeat sequences (LTR), respectively. LTR retrotransposons are also known as endogenous retroviruses (ERVs) due to similar mechanism and structure to simple retroviruses (Garcia-Perez et al., 2016). Non-LTR RTEs (Retrotransposable elements), are further classified into autonomous RTEs LINEs (Long interspersed nuclear elements) and Non-autonomous RTEs (Alu) (Lander et al., 2001). Non-autonomous non-LTR retrotransposons are collectively known as SINEs (short interspersed elements) and there are also SVA SINEs in humans, in addition to Alu (Ostertag et al., 2003; Wang et al., 2005). All these actively promote genetic diversity, mutations as well as human diseases (Lander et al., 2001). Although there are three major LINE families (L1, L2, and L3), only the L1 family can transpose in the human genome (Moran et al., 1996). L1 performs reverse transcription at the genomic target site, in a process known as target-primed reverse transcription (Luan et al., 1993). LINE-1 (L1) mediated insertions are regarded to be a kind of endogenous mutations capable of causing deletions/insertions and copy number variation which are all well-established risk factors for neurological disorders including Alzheimer's disease, autism and schizophrenia (Baillie et al., 2011; Guffanti et al., 2014). Increased level of L1 copy number has also been reported in the blood of animal

models, major depressive disorder (MDD) patients (Liu et al., 2016) and post-traumatic stress disorder (PTSD) subjects due to hypomethylation (Rusiecki et al., 2012). Discussing all aspects of transposable elements and their consequences on genome health for each disorder is beyond the scope of this article, therefore, this review is however mainly focused on L1 and its involvement in schizophrenia development.

L1 Retrotransposition in Brain Cells

Retrotransposable elements (RTEs) are only known to be de-repressed in the brain during human life. Accumulation of approximately 13.7 novel somatic L1 insertions have been noticed in human hippocampus (Upton et al., 2015). Although this number remains arguably controversial (Sanchez-Luque et al., 2019), the consensus conclusion is that mature neuronal cells support somatic L1 retrotransposition which has been evidenced in non-dividing neurons *via* engineered L1 retrotransposition (MacIa et al., 2017). Furthermore, L1 mis-regulation in brain tissues has been found associated to neurological diseases, and the putative reasons may be: 1) Increased RTE expression/activity due to mutations of RTEs-regulating genes, 2) genetic and environmental components and 3) time-dependent accumulation of L1 copy number, neuronal degeneration and phenotypes associated with aging (Terry and Devine, 2020).

L1s' mobilization occurs more frequently during differentiation of neurons than non-neuronal cell types (Coufal et al., 2009). However, L1s can also be mobilized in postmitotic neurons (MacIa et al., 2017). Likewise, the rat hippocampus also presents L1 retrotransposition activity during adult neurogenesis, indicating a strong retrotransposition activity in neural progenitor cells even at adult stage (Muotri et al., 2009). L1 copy numbers are also significantly higher in various areas of healthy adult human brains (especially the hippocampus) when compared to the liver and heart of the same person (Coufal et al., 2009; Upton et al., 2015). Terry et al. have proposed that in the context of findings by Muotri and Coufal et al., somatic L1 retrotransposition seems to occur at all phases of neuronal life, including mature or developing neurons, differentiating neural stem cells and neuronal progenitor cells (Coufal et al., 2009; Muotri et al., 2009; Terry and Devine, 2020). In light of these aforementioned studies, it is assumed that probably the L1 frequency is associated with cellular active engagement into neuronal circuits. Cells with more activity may have higher retrotransposition rate. However, many questions still remain unaddressed. For example, is L1 associated with increased pyramidal cell activity or reduced activity of inhibitory neurons? Is retrotransposition active at specific time window only or throughout the life span of a cell? If it is active throughout the life, then which stage of life is associated with the harmful effects? Studies using proper animal models would help to answer these questions.

Most L1s integrate into non-exonic regions and won't cause any recognized phenotypes. So far, no hotspots for L1 insertion have been discovered in the genome. The question of whether L1 insertion is random or guided by environmental factors, hormone influence, or inherited genetic print remains unanswered.

L1 Retrotransposition, a Putative Risk Factor of Schizophrenia

The insertion of L1 has long been proposed to predispose people with the risk of schizophrenia (Doyle et al., 2017). The significant increase in copy number of L1 has been confirmed in the postmortem prefrontal cortex of schizophrenia patients (Bundo et al., 2014). In addition, L1 copy number in neurons was markedly increased in contrast to non-neuronal cells in schizophrenia patients (Bundo et al., 2014). Moreover, genomic analyses of brain tissues from animal models which utilized poly I:C and chronic epidermal growth factor to produce schizophrenia-like phenotypes also revealed an increase of L1 copy number, implying the impact of prenatal and postnatal stress (Bundo et al., 2014). Antipsychotics have no influence on L1 copy numbers. Moreover, consistent increase of L1 copy number has been observed in iPS cell-derived neurons of schizophrenia patients with 22q11 deletion (Bundo et al., 2014). This suggests that a well-defined substantial genetic risk factor indeed contributes to the concentration of L1 in the brain (Bundo et al., 2014). Moreover, Whole Genome-Sequencing (WGS) data have suggested that L1s preferentially insert into genes related to synaptic functions (Bundo et al., 2014; Doyle et al., 2017). Baillie et al. also determined that L1s are mostly enriched in genes responsible for the neuronal synapse, axogenesis, postsynaptic density and presynaptic membrane (Baillie et al., 2011; Bundo et al., 2014; Doyle et al., 2017), which indicates the L1 retrotransposition specifically affects activity at neuropil. However, the exact reason for possible integration of L1s into synaptic genes is unknown.

It is worth noting that the retrotransposition itself can cause many by-products which may have detrimental consequences. For example, because of its endonuclease activity, the ORF2p product of L1 might generate mutations and instability. The expression of faulty protein, RNA or DNA in the cytosol may also trigger immune response, inflammation and neuron degeneration (Terry and Devine, 2020). Dysregulated expression of retrotransposable elements (RTEs) can be extremely harmful for a number of reasons. First, high levels of RTE proteins, RNAs, or extrachromosomal cDNA copies can cause cellular toxicity and activate inflammatory response pathways. Second, such expression allows for functional RTE replication, which could result in insertional mutagenesis, activation of the DNA damage response, or even programmed cell death (Dubnau, 2018). Immune activation models simulating both viral infection and inflammation have been used to investigate possible links between perinatal environmental risk factors for schizophrenia and L1 activity. In both the mouse and macaque models, an increased L1 copy number in the brain was observed in response to these two perturbations, indicating that the L1 content in the brain is likely influenced by early environmental factors (Bundo et al., 2014). Although L1 mobilisation can occur during neurogenesis as well as later stages of neuronal development which eventually leads to

individual somatic mosaicism (Lupski, 2013; McConnell et al., 2013; Poduri et al., 2013), it remains unaddressed why the schizophrenia symptoms appear later in life. Is there any difference between L1 retrotransposition pattern and frequency at early and adult developmental stages? Is there any difference in L1s frequency between inhibitory and excitatory neurons? Moreover, genes responsible for synaptogenesis have been reported dysfunctional in schizophrenia (Gandal et al., 2018). Do L1s also integrate into the open reading frame of synaptogenesis genes? Do L1s induce the disruption of synapse-forming genes alone or in combination with other stimuli?. Nevertheless, it is uncertain how the L1s target precisely at those genes. Are there specific sequences in genes for synaptogenesis, which are sensitive towards L1s? Or a parallel protein is transcribed along with L1s which is specific for some nucleotide sequences and exclusively recruits L1s towards genes for synaptogenesis? Though L1 is emerging as a possible cause of schizophrenia, it may be equally possible that L1 retrotransposition may be the pathological consequence of schizophrenia. These questions remain largely unclear and shall be warranted for further study. Moreover, Bundo et al., 2014 have studied L1 retrotransposition in multifaceted clinical settings; Postmortem brain tissue-iPSCs-animal model. However, further clinical studies are essential to determine the unexplored aspects of L1 and schizophrenia molecular biology from real biological environment (particularly postmortem studies) to provide additional evidence.

L1, Interferons and Schizophrenia

Activation of endonuclease-dependent L1 retrotransposon can increase the expression of endogenous IFN-β and IFN-stimulated genes which in-turn suppress L1 propagation (Yu et al., 2015). *In vitro* study also suggested that endogenous IFN signaling limits the propagation of L1 retrotransposon. Collectively it is suggested that IFN may play a protective role against L1 retrotransposon activation and propagation. The activation of L1 possibly activates the expression of low levels of IFN, which in turn antagonize the subsequent L1 retrotransposition. This hypothesis is supported by a correlation between L1 and IFN-β mRNA expression and the capacity of exogenous L1 to induce the expression of IFN-β and downstream substrates in vitro (Yu et al., 2015). It is not clearly understood whether INF-β can be harnessed as a therapeutic option for schizophrenia. Excessive accumulation of L1 DNA in the cytosol of neurons [due to three-prime repair exonuclease I (TREX1) deficiency] can precipitate type 1 interferon (IFN-1) inflammatory response and subsequent apoptosis (Thomas et al., 2017). The response of IFN-1 could be ameliorated by inhibitors of the L1 reverse transcriptase, implicating that L1 reverse transcriptase is an appropriate target for the treatment (De Cecco et al., 2019). But questions still remain unanswered. For example, which and when L1 should be inhibited? Cognitive symptoms often precedes psychosis (Mintz and Kopelowicz, 2007), therefore it is intriguing whether administration of L1 reverse transcriptase inhibitor in cognitively impaired mice would provide more mechanistic insights for schizophrenia. Nevertheless, negative correlation between IFN-γ and cognition in patients with schizophrenia has been reported recently (Wilson et al., 2018).

Whilst L1s exploit the cellular machinery to achieve replication, the host cells also have developed a number of defense mechanisms to counteract L1 toxicity. Innate cellular immunity and inhibitory elements for L1 retrotransposition include IFNs, RNA mediated regulation, post transcriptional silencing *via* DICER and siRNA, L1 RNPs binding partners, Poly A binding proteins (PABPs), PCNA and other regulatory elements have been extensively elaborated somewhere else (Pizarro and Cristofari, 2016). TAR DNA binding protein 43 (TDP-43) is a protein which binds with the RNA transcript of L1. Mutated TDP-43 presents reduced binding with L1 RNA which in turn results in elevated L1 transcripts (**Figure 1**) (Li et al., 2012). Nevertheless, the exact inhibitory mechanism and associated elements contributing to the process are not fully understood. Of note, despite of the cell's precise mechanisms for regulating transposable elements (TEs) activity, certain TEs are still able to escape repression and produce new integration in germ cells during early embryonic development and in somatic tissues later in life (Baillie et al., 2011; Kazazian, 2011; Lee et al., 2012).

The interaction of L1-IFNs-schizophrenia pathology is still vague and not explored enough. For example, how does L1 trigger INF response and how INFs fail to respond to L1 and drive schizophrenia remain entirely unclear. Do the truncated L1 transcripts or their translated protein accumulation causes inflammatory response/toxicity and ultimately serves as a cause of schizophrenia development? How does the L1 escape the regulation of IFN? Does the escape occur in a cell/time specific manner? Do the IFNs only work against truncated L1 or also the full length L1s? Further research is warranted to address these questions.

L1s and Somatic Mutations

The L1 elements are non-LTR transposons which can transpose in neural progenitor cells during brain development and might contribute to intra-individual difference in brain function (Coufal et al., 2009; Evrony et al., 2012; Erwin et al., 2016). Single cell sequencing and genome wide analysis confirmed few L1 somatic insertions in normal human caudate and cortical neurons, which however argues that L1 retrotransposition is the main source of neuronal diversity in the human cerebral cortex and caudate nucleus (Evrony et al., 2012). Human specific L1 (L1Hs) elements integrate favorably into genes linked with neuronal functions and diseases (Jacob-Hirsch et al., 2018). For example, the DLG2 gene, which is frequently mutated in schizophrenia, had 141 somatic insertions of L1 in the brain samples (Jacob-Hirsch et al., 2018) and thus was considered as a target for somatic L1-associated variants (Erwin et al., 2016). In brain tissues, the number of retrotranspositions is higher than non-brain samples, and even higher in brains suffering from tuberous sclerosis complex, Rett syndrome, ataxia-telangiectasia or non-syndromic autism (Jacob-Hirsch et al., 2018). Most of somatic brain retrotransposons incorporate into pre-existing repetitive elements, favorably A/T-rich L1 sequences, and form nested insertions. Those pre-existing retrotransposons may serve as "lightning rods" for new insertions, which allows deliberately-regulated gene expression in order to safeguard detrimental outcome. Therefore, the

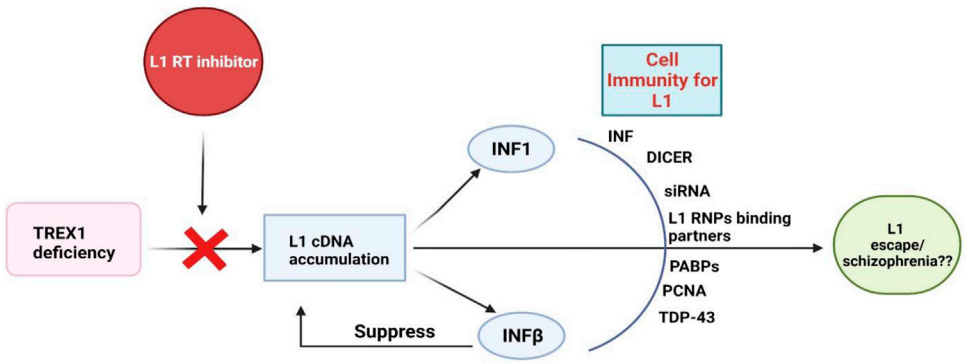

FIGURE 1 | Cellular immune responses to L1s. **TREX1:** Three Prime Repair Exonuclease 1, **INFs**: Interferons, **PABPs:** poly-A binding proteins, **PCNA:** Proliferating cell nuclear antigen, **TDP-43:** TAR DNA binding protein-43.

dysregulated retrotransposition may compromise this safety mechanism and increase the likelihood of detrimental mutagenesis in neurodevelopmental diseases (Jacob-Hirsch et al., 2018). However, the mechanism for selective target site insertion of L1 is not known and remains open for debate, for example, which L1s prefer nested insertion and which one goes for harmful mutagenesis predisposing to the development of schizophrenia? Moreover, the utilization of single cell sequencing approach will not only assist to study the role of L1 retrotransposition, but the different behaviors and functions of various cell types in context of their microenvironment which may have impact on L1 retrotransposition and schizophrenia.

The postzygotic somatic mutations (PZMs), which include epimutations (changes in histone modifications and promoter methylation that affect gene expression but not the DNA sequence), usually result in somatic mosaicism. Compared to other tissues, the PZMs are comparatively common in mammalian brains especially in schizophrenia patients (Singh et al., 2020). Moreover, It has been found that harmful somatic mutations found in schizophrenia brains were enriched in schizophrenia-related pathways including dopaminergic and glutamatergic pathways or long-term potentiation process (Kim et al., 2021). The brain somatic mutations, particularly in GRIN2B (one of the subunit of NMDA receptor), disrupt the localization of GluN2B to dendrites and impair proper synapse formation (Kim et al., 2021).

L1 Hypomethylation and Schizophrenia

The promoter region of DNA regulates gene transcription and its methylation shields the binding of transcription factors, ultimately silencing the gene expression. Hypomethylation exposes promoter to transcription factors and allows the subsequent transcription or protein expression. In mammalian genomes, L1 is the active autonomous retrotransposon, and hypomethylation of L1 is associated with higher retrotransposon activity. Analysis of peripheral blood samples revealed significant hypomethylation of L1 in schizophrenia patients (Misiak et al., 2015; Liu et al., 2016) both in first episode schizophrenia and chronic schizophrenia (Murata et al., 2020). However, it is intriguing whether the L1 methylation in peripheral blood is truly indicative of L1 methylation in the brain. Hypomethylation of L1 has also been noticed in other mental disorders like MDD and PTSD (Saurez et al., 2018). It is not yet clear whether the pattern of L1 retrotransposition in schizophrenia is similar with or different from other psychiatric disorders. However, DNA methylation is reported variable to adapt to neuronal activity alteration (Guo et al., 2011) and it could likely mediate or contribute to the integration of environmental stimuli into diseased cell features, resulting in neuronal dysfunction (Linde and Zimmer-Bensch, 2020).

Paradoxically, hypermethylation of L1 in brain tissue of schizophrenia patients has also been reported (Fachim et al., 2018), which indicates a globally elevated DNA methylation in schizophrenia. DNA-methyltransferases (DNMTs) help to establish the DNA methylation pattern since embryonic stages and up-regulated DNMTs have been detected in the brains with schizophrenia (Grayson et al., 2006; Zhubi et al., 2009). However, each variant of DNMTs may contribute in different capacity to the onset of schizophrenia. Recently, it was reported that DNMT3B rs2424932 was strongly associated with gender and DNMT3B rs1569686 associated early age onset of schizophrenia while DNMT3L rs2070565 associated with family history and early onset of schizophrenia. Altered activity of DNMTs indeed suggests that the genetic nature of methyltransferases should be taken into account when dealing with epigenetic events mediated by methylation in schizophrenia, (Saradalekshmi et al., 2014). In human neural progenitor cells (hNPCs), deletion of DNMT1 results in hominoid-specific L1's transcriptional activation and chromatin remodeling. The activated L1s act as alternate promoter for several neuronal protein-coding genes affecting neuronal functions, suggesting a hominoid-specific L1-based transcriptional network influenced by DNA methylation that influences neuronal protein-coding genes (Jönsson et al., 2019).

DNA methylation is generally thought to hamper the binding of transcription factors through the action of methyl-CpG-binding domain proteins, thus considered a repressive epigenetic feature (Curradi et al., 2002). The L1 promoters are C–phosphate–G (CpG) rich regions and are highly methylated and silenced under normal conditions (Steinhoff and Schulz,

2004). Because non-LTR retrotransposons encompass one-third of all CpG sites in humans (Cordaux and Batzer, 2009), silencing L1 expression *via* CpG DNA methylation and histones modification is a key repressive mechanism preventing mutagenic events from accumulation (Bourc'his and Bestor, 2004; Castro-Diaz et al., 2014; Jacobs et al., 2014). A robust molecular tool, dCas9-MQ1^{Q147L} system, has been recently developed to introduce *in vivo* site-specific DNA methylation editing with high specificity and activity (Lei et al., 2017). Although dCas9-MQ1^{Q147L} has not been tested in schizophrenia pathology, it has opened a window towards *in vivo* methylome editing and personalized medicine to alleviate the disease phenotype. Further improvement of the technique and skills are required to refine the editing efficiency.

Methyl CpG binding protein (MeCP2) is a protein that plays a role in global DNA methylation as well as neurodevelopmental disorders. MeCP2 appears to be crucial for normal functioning of nerve cells and serve as an inhibitory factor for L1 retrotransposition (Muotri et al., 2010). L1 retrotransposition can be manipulated in a tissue-specific fashion, and disease-related genetic alterations can affect neuronal L1 retrotransposition frequency (Muotri et al., 2010). DNA methylation may suppress L1 production in neural stem cells by attracting MeCP2, as evidenced by a group of CpG sites within the L1 promoter that showed a tendency to de-methylate during neuronal differentiation (Muotri et al., 2010).

L1, Chromatin Remodeling, and Schizophrenia

The structural alteration of histone proteins within the nucleosome mediates transitions between euchromatin and heterochromatin, which are associated with active and inactive transcription respectively (Grayson and Guidotti, 2013). DNA methylation can target retrotransposons and result in a repressive chromatin conformation that can access and silence the coding sequences in their proximity (Singer et al., 2010). Insertions of retrotransposons into open chromatin (Euchromatin) is assisted, whereas, insertions into condensed chromatin (heterochromatin) is unlikely. Another possibility is that L1 inserts randomly in accessible chromatin, impacting many genes and producing a wide range of transcriptional alterations (Singer et al., 2010). It is speculated that *de novo* L1 insertions selectively target CpG-poor promoters of NPC-specific genes because L1 endonuclease identifies an A+T-rich sequence motif. In fact, insertions into A+T-rich introns of housekeeping genes are equally possible (Singer et al., 2010). A recent report has proposed that pre-existing L1s within A/T rich sequences in the genome may serve as lightning rods for retrotransposons and support nested insertion to avoid harmful mutations (Jacob-Hirsch et al., 2018).

Chronic drug treatment may activate certain intergenic repetitive sequences, eventually leading to abnormally expressed retrotransposable elements. It has been demonstrated that repeated cocaine administration decreases histone H3 Lys9 trimethylation (H3K9me3) binding and activates several specific retrotransposons: L1, SINEs, and LTRs) but had dramatically increased L1 expression in NAc of the brain (Maze et al., 2011). Cocaine may potentially have nonspecific or random effects on H3K9me3 enrichment across the genome, with inconsistent or little effect on neuronal function (Maze et al., 2011). It is speculated that each environmental stimulus may have variable effects on brain regions. However, how does the cocaine-chromatin interaction precipitate the onset of schizophrenia is unclear, although histone modifications, DNA methylation and chromatin structure in schizophrenia has been detailed (Abdolmaleky and Thiagalingam, 2014; Duan, 2019).

It is reported that SIRT6 is a potent suppressor of L1 retrotransposon activity (Van Meter et al., 2014). SIRT6 binds to the 5′-UTR of L1 loci, where its mono-ADP ribosylates the nuclear corepressor protein KAP1 and assists its interaction with the heterochromatin factor HP1α. HP1α then contributes to the packaging of L1 elements into transcriptionally suppressed heterochromatin (**Figure 2**). Depletion of SIRT6 from L1 loci in response to DNA damage allows previously silenced retroelements re-activated (Van Meter et al., 2014). But this mechanism has not been investigated in the context of schizophrenia biology.

In neurodevelopmental disorder cohorts, mutations in genes which encode chromatin remodelers are overrepresented (Mossink et al., 2021). Chromatin remodelers impacts the growth, migration, and circuit integration of cortical cells including GABAergic/glutamatergic neurons and glia (Mossink et al., 2021). A study has showed that GAD67 mRNA levels are lower in schizophrenia patients, and the amount of methylation at the associated promoter in these patients is significantly lower in the repressive chromatin fraction (Huang and Akbarian, 2007). However, the effects of chromatin remodeling in individual brain cell type and its association with L1 retrotransposition or schizophrenia is still a loop which needs further investigation. Chromodomain helicase DNA binding protein 2 (CHD2) gene is involved in neurogenesis, chromatin remodeling, and gene expression, and detrimental mutation in CHD2 has been found relevant to the onset of schizophrenia in children (Poisson et al., 2020). However, the precise roles of CHD2 in L1 retrotransposition is yet to be established, which needs further investigation to delineate the mechanism for the treatment of schizophrenia.

A study in cancer cells has identified 142 genes capable of activating or repressing L1 retrotransposition (Liu et al., 2018). These genes, which are widely associated to human diseases, regulate the life cycle of L1 at the transcriptional or post-transcriptional level, depending on the endogenous L1 nucleotide sequence, highlighting the intricacy of L1 regulation. HUSH and MORC2 preferentially bind evolutionarily young, full-length L1s in in euchromatic setting which is transcriptionally permissive, and trigger the deposition of histone H3 Lys9 trimethylation (H3K9me3), which silences transcription (**Figure 2**) (Liu et al., 2018). Of note, these silencing events usually occur inside introns of transcriptionally active genes, resulting in HUSH, MORC2, and L1-dependent downregulation of host gene expression. This is an excellent example illustrating how epigenetic silencing of transposable elements helps to rewire host gene expression programs (Liu et al., 2018). HUSH and MORC2 work together to preferentially

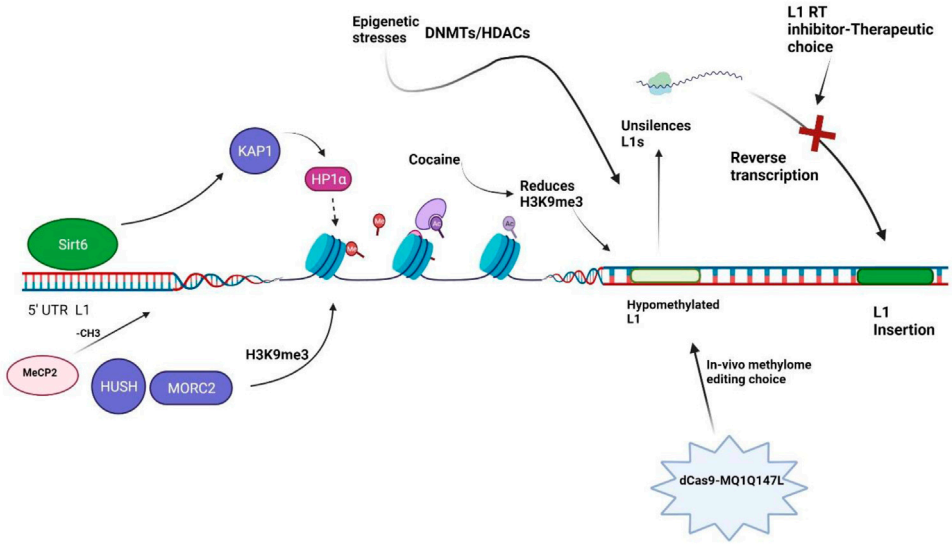

FIGURE 2 | Illustration of chromatin remodelers. **Sirt6:** Sirtuin 6, **KAP1:** Krüppel-associated box 1/KRAB-associated protein 1, **HP1α:** Heterochromatin Protein 1α, **H3K9me3:** methylation of lysine 9 of histone H3, **HUSH:** human silencing hub-complex, **MORC2:** Microrchidia CW-type Zinc Finger 2, **DNMTs:** DNA methyltransferases, **HDACs:** Histone Deacetylases, **MeCP2**: Methyl-CpG-binding protein 2, **L1 RT**: L1 reverse transcriptase.

target young, full-length L1s in transcriptionally permissive euchromatic areas (Liu et al., 2018). These L1s are actually the greatest threat to genomic stability and integrity, as a subclass of them remains active and mobile (Liu et al., 2018). The HUSH- and MORC2-dependent L1 silencing mechanism needs to be explored in the schizophrenia patient-derived cells. It is intriguing whether the HUSH-MORC2 molecular apparatus can be harnessed to rescue the L1 insertion in genes for better treatment of schizophrenia.

Missing Heritability, Schizophrenia and L1s

As opposed to more common disorders such as anxiety or depression (Sullivan et al., 2013; Meier et al., 2019), schizophrenia has much higher heritability estimates of the genetic components (Ripke et al., 2014) Previous GWAS study has estimated approximately 23% of the variance in liability in schizophrenia (Blanco-Gómez et al., 2016; Maroilley and Tarailo-Graovac, 2019). This is in marked difference to results from family studies, which show that heritability for schizophrenia accounts for 60–80% of disease risk, and even beyond 80% in twin studies (Sullivan et al., 2003; Avramopoulos, 2018). However, the current data from genetic analysis does not directly corroborate the heritability estimates, resulting in the so-called "heritability gap" in psychiatry (van Calker and Serchov, 2021). Furthermore, the majority of genetic alterations identified by GWAS studies falls into non-coding regions of DNA (intergenic regions and introns) (Welter et al., 2014), which makes it difficult to validate their potential pathogenic roles in psychiatric illnesses.

It has been proposed that transposable elements contribute in genome expansion and modification not just through transposition but also through the generation of tandem repeats (Ahmed and Liang, 2012) and tandem repeats contribute in schizophrenia pathology (Grube et al., 2011). The KCNN3 is a schizophrenia potential risk gene that encodes a small conductance calcium-activated potassium channel (SK3) that regulates neuronal firing patterns. It has been reported that the short tandem repeats (STR) affects the SK3 potassium channel function and the cognition of schizophrenia patients (Xiao et al., 2021). Longer CAG repeats can reduce the SK3 channel activity in transfected HEK293 cells, which was consistent with the dysfunctional higher cognitive abilities caused by SK3 overexpression in animals (Grube et al., 2011; Martin et al., 2017). However, it should be noted that tandem repeats are typically multiallelic (Nithianantharajah and Hannan, 2007) which makes them difficult to genotype using SNP-based GWAS array platforms. This may contribute greatly to the "missing heredity" of psychiatric disorders (Xiao et al., 2021). In line with this, Kuhn et al. have suggested that SNP array-based GWAS studies would have overlooked possible phenotypic impacts of L1s (Kuhn et al., 2014), and that L1s may play a role in the "missing heritability" (Manolio et al., 2009). Though, the transposable elements contribute in generating tandem repeats but L1s specific contribution in generation of tandem repeats and eventually to "missing heritability" is the unexplored aspect, and further study will assist to find out the unseen heritable risks and their mechanism of development consequencing into schizophrenia.

Animal Studies

Many studies using mouse models have revealed that joint exposure to peripubertal stress and prenatal immune challenge induces synergistically pathological effects on neurochemistry and adult behavior. The offspring of poly-I:C mouse model had worsened schizophrenia-like phenotypes, if subjected to environmental stress in puberty, signifying that early environmental stress can lower the threshold for the onset of schizophrenia (Giovanoli et al., 2013). That is to say, the environmental stress can increase the frequency of L1

insertions and increase the susceptibility to schizophrenia, probably *via* altered expression of synaptic or other schizophrenia-related genes in neurons (Bundo et al., 2014). Several environmental factors, such as alcohol and cocaine consumption (Maze et al., 2011; Ponomarev et al., 2012), stress (Ponomarev et al., 2010; Hunter et al., 2012) and exercise (Muotri et al., 2009) have been confirmed to alter L1 expression in the adult brain. Early life experience such as maternal care overrides the activity of L1 in mice and alters DNA methylation (Bedrosian et al., 2018), implicating early life bereavement or stress could increase the occurrence of L1 retrotransposition. Further investigation is needed to determine whether the L1 activity in early life depends on the severity of environmental stimuli or duration of exposure. A recent study revealed that L1 retrotransposition is up-regulated in the adult hippocampus after novel exploration (Bachiller et al., 2017). This shows that activities of L1 retrotransposition may underpin hippocampal activation-based memory formation in the adult brain (Bachiller et al., 2017). Consistently, Coufal and colleagues also discovered an enrichment of L1 ORF2 copy number in the adult mouse hippocampus compared to other brain areas. Furthermore, the activity of engineered human L1 retrotransposition can be detected in neural progenitor cells derived from human embryonic stem cells or isolated from human fetal brain, demonstrating that L1 components might be activated as early as the formation of the central nervous system (Coufal et al., 2009). More than one third of non-reference L1s are found within the open reading frames of protein-coding genes implicated to schizophrenia (Guffanti et al., 2016) which strongly suggests the close association between L1 retrotransposition and schizophrenia (Saurez et al., 2018).

It has been evidenced that L1 retrotransposition is required for physiological neuronal activity during memory formation in the hippocampus. L1 can affect memory formation in a time-dependent manner, specifically the Long-term memory (LTM) (Bachiller et al., 2017) which is largely based on the functional strengthening of existing synapses as well as the formation of new synapses (Radwanska et al., 2011). Intriguingly, L1 is specifically enriched in genes responsible for synaptic function in schizophrenia (Baillie et al., 2011; Bundo et al., 2014; Doyle et al., 2017). How L1 contributes to memory formation during novel exploration and the onset of schizophrenia is not clear. Probably two classes of L1s exist: "good" and "Bad" L1s, based on temporal activation, integration site or L1 guiding elements. There is also a scope for developing poly I:C animal model to check time dependent L1s profile which could be intervened *via* siRNA capable of crossing Blood-brain barrier. It is also needed to explore whether the pattern of L1 retrotransposition is heritable.

Furthermore, only ~ 100 retrotransposition-competent (RC) different classes of L1s are found in individual human (Brouha et al., 2003; Beck et al., 2010) whereas, approximately 3000 RC L1s are found in mice (DeBerardinis et al., 1998; Naas et al., 1998; Goodier et al., 2001; Sookdeo et al., 2013). The different promoter sequences regulating L1 transcription in human and mouse, and linked differences in their regulation, may likewise consequence in divergent spatiotemporal patterns of L1 expression (Faulkner and Billon, 2018). Therefore, the critical evaluation of using mice as a model to study L1 in human neurological disorders is required. Different number of retrotransposition competent L1s between human and mouse makes it more difficult and challenging to develop a model which can reliably mimic human molecular biology for L1 and its role in schizophrenia development.

Although the detailed methodology to study the L1 retrotransposition rate in neurons have been reviewed (Faulkner and Billon, 2018), but limited literature is available to dissect roles of L1 in schizophrenia and related mechanism using animal models. Manipulating neuronal subtypes with engineered L1s *in-vitro*/*in-vivo* may uphold the promise for cell-type specific investigation, although it's a time consuming, labor-demanding and technique-challenging task.

CONCLUSION

Accumulating evidence suggests that L1s prefer to re-insert in genes responsible for synapse formation in schizophrenia patients. It is warranted to explore further how L1 retrotransposition affects the synapse health chemically or physically? Does it cause hinderance in production, intracellular packaging and synaptic transmission of neurotransmitters? Moreover, currently available antipsychotics have no effects on L1 retrotransposition (Bundo et al., 2014), therefore, L1 reverse transcriptase inhibitor could be harnessed as a therapeutic choice in iPSCs derived from patients with schizophrenia and cognitively impaired mice. But the paradigm is yet to be established for proper administration of the reverse transcriptase inhibitors. Moreover, L1s preferably integrate into A/T rich region whereas nickase Cas9 can convert A into G in the target site. Therefore, nickase Cas9 would likely be a helpful tool to exploit for *in-vivo* genome correction. However, the challenge of identifying off-target editing and control still remains and further technological improvement is required. The CRISPR-based genome editing has been tested for neurological diseases like autism spectrum disorder, Alzheimer's, epilepsy, Parkinson's and Huntington's diseases (Lubroth et al., 2021) which possibly heads towards clinical trials. It has not been tested for schizophrenia and is still in infancy.

Schizophrenia symptoms usually appear in adolescence and dysfunctional GABAergic signaling is the common phenotype consistently found in schizophrenia pathophysiology. As the functional maturation of GABAergic interneurons can prolongs up to post-adolescence period in primate prefrontal cortex, it is highly likely that L1s may also preferentially expressed in GABAergic interneurons. Brain has wide diversity of neuronal cells in terms of function and location, and L1' behavior in specific cell-types needs to be explored to identify cell specific effect. Moreover, it also remains unaddressed whether the sex hormones help to trigger chromatin remodeling, L1 activation and then schizophrenia.

Genome sequencing of families with schizophrenia would possibly provide a clue about the heritable pattern of L1.

Analysis of post-mortem brain of patients with schizophrenia and mouse models for schizophrenia exposed to various environmental insults at different times and exposure durations, will probably help to strengthen the loop in understanding. Maternal care in early life can modulate L1 activity, but how does its lacking translate into L1s escape and molecular pathology of schizophrenia is uncertain. There is a need to develop classification system in order to determine whether schizophrenia can be caused by L1 retrotransposition or other mechanisms? Categorization of L1s into "good or bad" and development of improved molecular reporter system to trace L1s in the genome are imperative. Thus, evidences regarding L1 retrotransposons being the cause of schizophrenia are insufficient. Further efforts are required for the development of techniques and skills to investigate the mechanism and epigenetic regulation of L1s leading to schizophrenia.

AUTHOR CONTRIBUTIONS

MJ and LL: Writing original draft, J-SZ: review and edition: BL and X-PW: Conceptualized the study.

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Olanzapine Promotes the Occurrence of Metabolic Disorders in Conditional TCF7L2-Knockout Mice

Ye Yang[1†], Manjun Shen[2†], Li Li[1], Yujun Long[1], Lu Wang[1], Bing Lang[1] and Renrong Wu[1]**

[1]National Clinical Research Center for Mental Disorders, Department of Psychiatry, The Second Xiangya Hospital of Central South University, Changsha, China, [2]Shenzhen Nanshan Center for Chronic Disease Control, Department of Psychiatry, Shenzhen, China*

Objectives: Schizophrenia (SCZ) patients display higher incidence of metabolic syndrome (MetS) and comorbidity of type II diabetes. Both atypical antipsychotics and genetic variants are believed to predispose the patients with the risk, but their interplay remains largely unknown. TCF7L2 is one of the most common genes strongly associated with glucose homeostasis which also participates in the pathogenesis of schizophrenia. In this study, we aimed to explore the regulatory roles of TCF7L2 in atypical antipsychotics-induced MetS.

Methods: Mice with pancreatic β-cell–specific Tcf7l2 deletion (CKO) were generated. The CKO mice and control littermates were subjected to olanzapine (4 mg/kg/day) or saline gavage for 6 weeks. Metabolic indices, β cell mass, and the expressing levels of TCF7L2 and GLP-1R in the pancreatic tissue were closely monitored.

Results: Tcf7l2 CKO mice displayed a spectrum of core features of MetS, which included remarkably increased rate of weight gain, higher fasting insulin, higher values of blood lipids (cholesterol, triglyceride, and low-density lipoprotein), impaired glucose tolerance, and hypertrophy of adipocytes. Notably, these effects could be further exacerbated by olanzapine. In addition, Tcf7l2 CKO mice with the olanzapine group showed significantly decreased expressions of GLP-1R protein and a trend of reduced pancreatic β-cell mass. RT-qPCR revealed that the CKO mice presented a significantly less transcription of Sp5, an important element of the Wnt signaling pathway.

Conclusion: Our study illustrates that mice with pancreatic β-cell–targeted Tcf7l2 deletion were more vulnerable to suffer metabolic abnormalities after olanzapine administration. This impairment may be mediated by the reduced expression of GLP-1R.

Keywords: TCF7L2, CKO, olanzapine, metabolic disorders, GLP-1R

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Weihua Yue,
Peking University Sixth Hospital, China

Reviewed by:
Ghislaine Guillemain,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Lei Zhang,
Tongji University, China
Wei Yan,
University of Michigan, United States

****Correspondence:***
Bing Lang
bing.lang@csu.edu.cn
Renrong Wu
wurenrong@csu.edu.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

Schizophrenia (SCZ) is a chronically disabling brain disorder which affects around 1% of the global population (Stilo and Murray, 2010). Despite the strenuous research in the past several decades, SCZ still remains largely elusive. Both genetic and environmental factors are implicated, and the hereditary is high (60%–80%) (Khavari and Cairns, 2020); however, the precise genetic landscape and environmental interaction mechanism remain incredibly understudied. As a

result, SCZ patients suffer a considerably short life expectancy compared with the general population (Correll et al., 2017). Amounting epidemiological evidence has suggested a high occurrence of metabolic syndrome (MetS) which strongly contributes to the shorter life span presented by SCZ patients (Jayatilleke et al., 2017). MetS features glucose intolerance, insulin resistance, dyslipidemia, type 2 diabetes mellitus (T2DM), and obesity which predispose to an excess cardiovascular mortality. In a recent review, individuals with schizophrenia concomitant diabetes ranged from 1.26% to 50% across studies, with median 13% (Ward and Druss, 2015), highlighting a high comorbidity of diabetes and SCZ. Intriguingly, SCZ patients with untreated diabetes showed poorer overall cognitive performance (Takayanagi et al., 2012), which could be effectively ameliorated by anti-diabetic drugs such as metformin (Wu et al., 2008). Meanwhile, patients diagnosed with diabetes are more likely to exhibit cognition impairment and higher risk to develop major mental illnesses such as SCZ or bipolar disorder. As such, a bi-directional promotion model has been proposed, and existence of common pathophysiological mediators in both diabetes and schizophrenia has been hypothesized (Takayanagi et al., 2020).

It is considerably arguable to identify the common mediators to the comorbidity of MetS and SCZ. The widely used atypical antipsychotics (APPs) often associate with high prevalence of metabolic disorders (35.3%) in patients with schizophrenia, indicating a critical role of APPs in the comorbidity of MetS in SCZ patients (Jeon and Kim, 2017). Olanzapine is one of the most widely used APPs in clinics with established effects of weight gain and severe MetS, which has been well demonstrated by meta-analyses (Huhn et al., 2019), randomized clinical trials (RCTs) (McEvoy et al., 2007), and animal studies (Coccurello et al., 2006). Largely due to these unpleasant adverse effects, the withdrawal rate of olanzapine is 2–8 times higher than that of other antipsychotics (Lieberman et al., 2005).

Apart from the APPs, the polygenic nature of schizophrenia also predisposes patients with the higher risk of the onset of MetS. Glucose intolerance is one of the core features of MetS which, however, is frequently reported in drug-naïve psychotic patients including SCZ (Fernandez-Egea et al., 2009; Garcia-Rizo et al., 2016; Greenhalgh et al., 2017). This strongly indicates that the higher incidence of MetS in SCZ patients also originates from intrinsic vulnerability to metabolic disturbance. However, up to date, there is almost no literature report to describe the interaction between genetic variants and APP in SCZ patients or the underlying mechanism.

TCF7L2 is a Wnt signaling-associated transcription factor which is strongly expressed in several tissues, especially the pancreas. It can stimulate the pancreatic β-cells' proliferation (Jin, 2016) *via* Wnt signaling (Yi et al., 2005) and stands as one of the best-established candidate genes for type 2 diabetes (Lyssenko et al., 2007). Decreased TCF7L2 protein closely correlates with downregulated GIP- and GLP-1 receptors, two key determinants for β-cells' proliferation and survival, as well as glucose-stimulated insulin secretion (Shu et al., 2009a). Intriguingly, recent genetic data have demonstrated TCF7L2 polymorphisms as high-risk alleles for human SCZ across world population (Hansen et al., 2011; Alkelai et al., 2012; Liu et al., 2017). In mice, manipulation of the Tcf7l2 expression through gain/loss-of-function approaches leads to anxiety-like behavior and dosage-dependent contextual fear learning impairment (Savic et al., 2011). Deletion of Tcf7l2 across the pancreatic tissue (da Silva Xavier et al., 2012) or specifically in β-cells disrupts the healthy glucose tolerance and insulin secretion (Mitchell et al., 2015). Thus, TCF7L2 has been hypothesized as an important risk gene co-shared by type 2 diabetes, MetS, and psychiatric disorders, especially SCZ (Postolache et al., 2019).

While the aforementioned evidence suggests that TCF7L2 mutation may impair proper pancreatic function and cognition, there is scarce literature deciphering the regulatory roles of TCF7L2 in APP-induced MetS. Previously, we have shown that olanzapine challenge associates an increased expression of Tcf7l2 in non-pancreatic tissues and concomitant dyslipidemia (Li et al., 2018), highlighting an active interaction between olanzapine and Tcf7l2. In the present study, we further addressed this question using pancreatic β-cell–specific Tcf7l2-knockout mice. Our results support a central role of TCF7L2 in β cells for olanzapine-induced metabolic abnormalities and when disrupted, promote the progress of MetS.

MATERIALS AND METHODS

Animals

The $Tcf7l2^{tm1a(EUCOMM)Wtsi}$-knockout-first mice were purchased from the European Mouse Mutant Archive (https://www.infrafrontier.eu/procedures/animal-welfare-and-ethics/emma-repository). The knock-out-first construct contained the sequence of FRT-En2SA-IRES-LacZ-PolyA-loxP-hbactP-neo-PolyA-FRT-loxP and was used to target sequences of intron 5. A further loxP was introduced prior to exon 6, which enables exon 5 flanked by two loxp insertions and the potential to create conditional Tcf7l2-knockout mice if required. The detailed vector diagram is shown in **Figure 1A**. The $Tcf7l2^{tm1a(EUCOMM)Wtsi}$-knockout-first mice were crossed with Flp transgenic mice in order to remove the sequence between the two FRT sites and to create $Tcf7l2^{fl/fl}$ mice. To specifically delete Tcf7l2 in β cells of the pancreas, $Tcf7l2^{fl/fl}$ mice were crossed with Ins2-Cre mice to generate $Tcf7l2^{fl/fl}$: In2-cre (referred as Tcf7l2 CKO) mice. Both the Ins2-Cre and Flp mice were obtained from the Shanghai Model Organisms Center. All the mice were maintained on a C57BL/6 background with free access to regular chow and water. An average of three to five animals was housed per cage under a 12 h light-dark cycle. All experiments were performed in accordance with the Guidelines and Regulation of Laboratory Animals Used for Biomedical Studies of Xiangya Second Hospital, China. Animal care practices and all experiments were reviewed and approved by the Animal Committee of Xiangya Second Hospital, Central South University, Changsha, China.

Tcf7l2 CKO mice and control littermates were obtained from mating of $Tcf7l2^{fl/fl}$: In2-cre mice to achieve $Tcf7l2^{fl/fl}$ and In2-cre

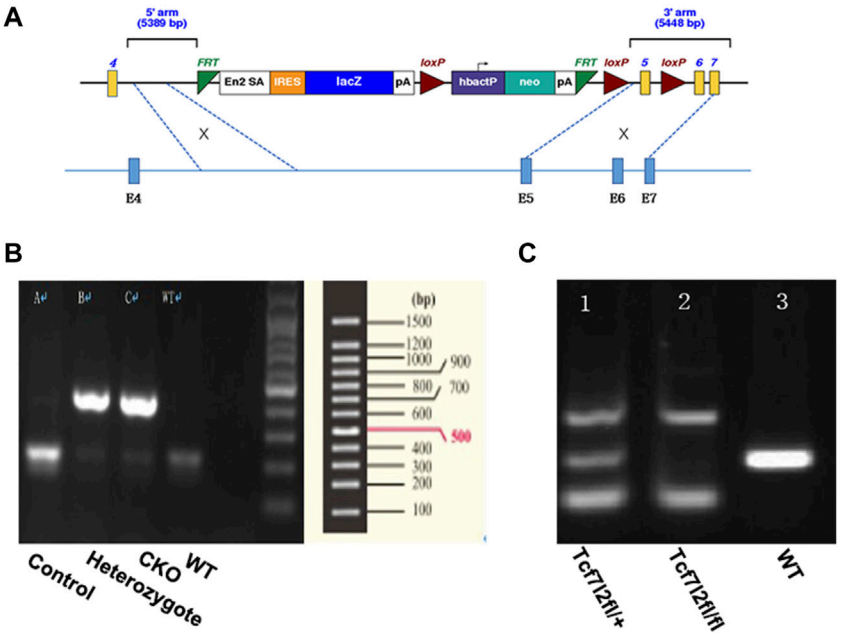

FIGURE 1 | Generation of mice with pancreatic β-cell–targeted Tcf7l2 deletion. **(A)** Diagram of the construct used to generate Tcf7l2$^{fl/fl}$ mice. It should be noted that exon 5 was flanked by two loxp sites and was supposed to be deleted at the presence of cre-recombinase. **(B)** PCR results showed mice with cre-recombinase (400 bp, lanes 2 and 3) or littermate control (endogenous band at 200 bp, lane 1). The sample at lane 4 was from a C57 mouse as a blank control. **(C)** PCR results showed genotypes of Tcf7l2$^{fl/+}$ (lane 1, with the PCR products at 139 bp, 281 bp, and 500 bp) and Tcf7l2$^{fl/fl}$ mice (lane 2, with the PCR products at 139 bp and 500 bp). Lane 3 displayed the PCR product (281 bp, endogenous band) from a C57 mouse as a blank control.

TABLE 1 | Sequence of paired primers and the expected PCR product size.

	Name	Sequence	Expected PCR product size
Pair 1	Tcf7l2_47987_F	GGAGAGAGACGGGGTTTGTG	Mutant: 139; WT: 139
	CAS_R1_Term	TCGTGGTATCGTTATGCGCC	
Pair 2	Tcf7l2_47987_F	GGAGAGAGACGGGGTTTGTG	Mutant: 139, 500; WT: 139
	Tcf7l2_47987_R	CCCACCTTTGAATGGGAGAC	
Pair 3	Cre_F	TCGATGCAACGAGTGATGAG	Mutant: 400
	Cre_R	TCCATGAGTGAACGAACCTG	
Pair 4	Control_F	CAAATGTTGCTTGTCTGGTG	WT: 200
	Control_R	GTCAGTCGAGTGCACAGTTT	

(referred as CKO) and Tcf712$^{fl/fl}$ as controls. Mice were born at the expected Mendelian ratios with no apparent abnormalities. Each mouse was genotyped using genomic DNA with pairs of primers listed in **Table 1** Two bands (139 bp and 500 bp) can be detected for mice with successful exon 5 deletion, while a 281 bp band will be amplified for WT allele. CKO mice were further confirmed by a 400-bp PCR product for cre-recombinase, whereas a 200-bp PCR product for WT control.

Drug Administration

Due to the high risk to develop metabolic syndrome in women, we used female animals in our experiments. Female mice (2-month-old) were used in order to exclude sex differences (Li et al., 2016; Oxenkrug and Summergrad, 2020) and were randomly divided into four groups (eight per group): 1) control mice with saline (0.9%, oral gavage) for 6 weeks; 2) control mice with olanzapine (4 mg/kg/d, oral gavage) for 6 weeks; 3) Tcf7l2 CKO mice with saline (0.9%, oral gavage) for 6 weeks; and 4) Tcf7l2 CKO mice with olanzapine (4 mg/kg/d/, oral gavage) for 6 weeks. Olanzapine (Hansoh Pharmaceutical Co. Ltd., Jiangsu, China) was dissolved in saline (0.9%) at a concentration of 0.8 mg/ml, and rigorous shaking was always performed prior to oral gavage. All drugs were administered at a fixed time (9:00 a.m.–11:00 a.m.) every day.

Body Weight Measurement and Oral Glucose Tolerance Test

Body weight was monitored at the same time (11:00 a.m.) once each week which was used to adjust drug dosage. The rate of

weight gain was calculated as follows: (body weight at week 6F02D body weight at week 0)/ body weight at week 0. For OGTT, mice were deprived of food for 16 h (at the end of week 6) and administered with 2 g/kg glucose (50%) in saline through oral gavage. Blood was sampled from the tail at 0, 30, 60, and 120 min after glucose administration. Blood glucose levels were analyzed by using a glucometer (Bayer, Germany).

Measurement of Plasma Hormones and Lipid Levels

Blood samples from the mice that were fasted for 16 h were collected by eyeball extirpating under deep anesthesia (pentobarbital sodium, Sigma). Plasma was collected after centrifugation (3,000 rpm, 10 min, 4°C) in heparin-coated microvette tubes containing EDTA. Plasma concentrations of triglycerides, total cholesterol, HDL, and LDL were determined by using an automatic biochemical analyzer (Rayto, China). Plasma insulin, proinsulin, and glucagon levels were assessed by ELISA (Mercodia).

Western Blot Analysis

For immunoblotting, mice were sacrificed and the pancreatic proteins were extracted and sonicated in RIPA buffer containing complete protease and phosphatase inhibitors (Roche) on ice. Then, the supernatant was collected after centrifuging at 12,000 rpm for 5 min at 4°C, and the protein concentration was determined by using the BCA protein assay kit. Equal amounts of protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then blocked in 5% non-fat milk for 1.5 h, followed by incubation with primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit anti-Tcf7l2 (1:2000 dilution; Novus), rabbit anti-Glp-1R (1:1000 dilution; Novus), and rabbit anti-GAPDH (1:2000 dilution; Abcam). After that, the membranes were incubated with HRP-conjugated secondary antibodies (1: 2000 dilution; Sigma) and developed using an enhanced chemiluminescence kit (Millipore). The density of the bands was analyzed by ImageJ.

HE Staining and Quantifying the Adipocyte Area

Abdominal adipose tissues were removed and were fixed in 4% paraformaldehyde solution (Solarbio), embedded in paraffin wax, and sectioned at the thickness of 5 μm with a cryostat (Leica). After deparaffinization, adipose sections were stained with hematoxylin solution for 5 min and then rinsed in distilled water. Then, the sections were stained with eosin solution for 5 min. Stained adipose sections were examined and photographed with a microscope equipped with an imaging system (Nikon Eclipse E100, Japan). The size of adipocytes was quantified by ImageJ 1.51s with the following protocol: 1) selecting a fixed area size (1,060 × 900 μm) within each adipocyte image; 2) randomly selecting 30 adipocytes in the area and measuring the absolute pixel area of each cell; 3) quantifying the amount of adipocytes in each desired adipocyte size group; and 4) calculating the percentage of adipocyte size group and then making comparison.

Immunofluorescence Staining and Calculations of Beta Cell Mass

Isolated pancreases were fixed in 4% paraformaldehyde solution for 24 h and embedded in paraffin wax. The sections (from head to tail) were cut at 5 μm thickness and deparaffinized and rehydrated followed by rinsing with 0.01% PBS (pH 7.4). The slides were incubated overnight at 4°C with rabbit anti-Tcf7l2 (1: 100 dilution; Novus) and mouse anti-insulin antibody (1:300 dilution; Servicebio), or rabbit anti-GLP-1R (1:200 dilution; Novus) and mouse anti-glucagon (1:150 dilution; R&D). After a full rinse, the slides were then incubated with Cy3-conjugated goat anti-rabbit (1:300 dilution; Wuhan Google Biotechnology Co., Ltd.) or FITC-conjugated goat anti-mouse (1:400 dilution; Wuhan Google Biotechnology Co., Ltd.) secondary antibodies, followed by nuclear counterstaining with DAPI (1:1000 dilution; Servicebio). Images were captured with an inverted fluorescence microscope (Nikon ECLIPSE TI-SR, Japan). The fluorescence intensity of each slide was quantified by ImageJ 1.51s. The whole pancreas section was scanned using Caseviewer 2.0 (3D HISTECH, Pannoramic 250/MIDI, and Hungary), and the area sizes of the pancreas and beta cells were measured for all sections. A complete pancreatic section (from head to tail) of the pancreas from each animal, including representative sections of the head, body, and tail of the pancreas, was analyzed, and all islet cells in this section per mouse were counted in each group. The beta cell mass was estimated, as previously described: pancreas mass × (insulin positive area/the size of pancreas).

Quantitative Real-Time PCR Analysis

Total RNA was isolated from the pancreas and extracted using TRIzol (Sigma), as described previously. cDNA was synthesized from total RNA (2 mg) using the M-MLV Reverse Transcriptase Kit (TAKARA, Dalian, China). Subsequently, qRT-PCR was performed using a SYBR Premix EX Taq™ Kit (TAKARA, Dalian, China) by an ABI 7500 Real-Time PCR system (7900 HT system, ABI, United Kingdom) according to the manufacturer's instructions. The synthesized primer sequences are listed in **Table 1**. The following thermocycling conditions were used for qPCR: an initial step of 93°C for 120 s, followed by 40 cycles of denaturation at 93°C for 60 s, and annealing and extension at 60°C for 60 s. Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene U6. RT-qPCR was performed in triplicate.

Statistical Analysis

All statistical analyses were performed by SPSS software (version 19.0), and the values were represented as mean ± SEM. The body weight and the glucose levels of OGTT at different time points were analyzed by repeated measures ANOVA. The body weight rate, lipid and hormone levels, and adipocyte area were analyzed using two-way ANOVA. The statistical significance was set at a value of $p < 0.05$.

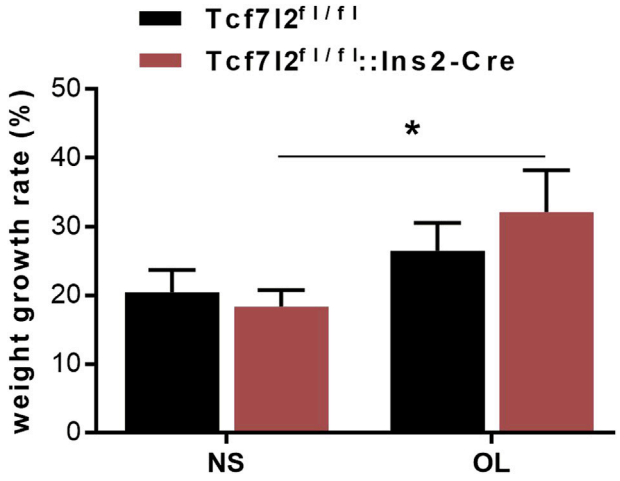

FIGURE 2 | Comparison of the body weight growth rate between different treatment groups during the 6-week intragastric olanzapine administration. Tcf7l2$^{fl/fl}$: Ins2-Cre + OL represented Tcf7l2 CKO mice challenged with olanzapine (red). Tcf7l2$^{fl/fl}$: Ins2-Cre + NS were Tcf7l2 CKO mice administered with saline (blue). Tcf7l2$^{fl/fl}$ + OL and Tcf7l2$^{fl/fl}$ + NS were the control mice subjected to olanzapine (black solid line) or saline (black dotted line) treatment. All of the results were expressed as the mean ± SEM, *$p < 0.05$, Ol, olanzapine. NS, saline. $n = 8$ in each group.

RESULTS

Olanzapine Administration Accelerated the Speed of Weight Gain in Tcf7l2 CKO Mice

Previous reports have demonstrated a well-established MetS-facilitating role of olanzapine in rodents (Huhn et al., 2019). To investigate the possible interplay between Tcf7l2 and olanzapine, Tcf7l2 CKO mice (Tcf7l2fl/fl:In2-cre) and littermate controls (Tcf7l2$^{fl/fl}$) were orally gavaged with olanzapine (4 mg/kg/day) or saline for 6 weeks. The body weight was monitored every day, and the dosage of olanzapine was adjusted every week to adapt to the body weight changes as appropriate. Although the CKO mice in the olanzapine group exhibited the highest body weight gain, no difference in body weight between individual groups of mice was detected at any time during the 6 weeks. However, a robust effect of olanzapine treatment [F (1, 28) = 5.579 and $p = 0.025$] can be observed in the weight gain rate by two-way ANOVA analysis. Compared to saline treatment, Tcf7l2 CKO mice presented larger increment of weight gain and the trend of increased body weight when challenged with olanzapine [F (1, 28) = 5.409 and $p = 0.027$] (**Figure 2**).

Effects of Olanzapine Treatment on Oral Glucose Tolerance Test in Tcf7l2 CKO and Control Mice

As Tcf7l2 is critically associated with blood glucose regulation, we then carried out the OGTT test to compare the performance of glucose tolerance between Tcf7l2 CKO and control mice after olanzapine treatment for 6 weeks. Although the time to the peak glucose all occurred at 30 min after gavage in the four groups, the CKO mice with olanzapine administration showed significantly impaired glucose tolerance as revealed by the highest peak value of plasma glucose ($p < 0.001$, red curve, **Figure 3A**). Compared with Tcf7l2$^{fl/fl}$ mice, Tcf7l2 CKO mice displayed significantly higher glucose levels despite the treatment with olanzapine (red line) or saline (blue line) at 30 min ($p = 0.034$ and $p < 0.001$, respectively). In addition, the Tcf7l2 CKO mice also exhibited higher plasma glucose values at 60 and 120 min after oral olanzapine challenge ($p < 0.05$). When the blood glucose concentration was normalized against the fasting blood glucose concentration at various time points, group differences at different time points were also detected (**Figure 3B**). Tcf7l2 CKO mice displayed significantly higher glucose concentration than Tcf7l2$^{fl/fl}$ mice at 30 min, regardless of olanzapine administration (all $p < 0.001$). Similarly, the Tcf7l2 CKO mice administered with olanzapine also showed higher concentration at 60 and 120 min than Tcf7l2$^{fl/fl}$ mice (all $p < 0.05$); the concentration in the Tcf7l2 CKO mouse saline group was also higher than that of the control olanzapine group at 60 min ($p = 0.028$). For the AUC values, two-way ANOVA analysis revealed a main effect of olanzapine treatment [F (1, 28) = 31.623 and $p < 0.001$], a main effect of Tcf7l2 gene knockout [F (1, 28) = 19.043, $p < 0.001$] and an interaction effect [F (1, 28) = 4.227 and $p = 0.049$]. Tcf7l2 CKO mice in the olanzapine group exhibited higher AUC values than control mice in the olanzapine group [F (1, 28) = 20.607 and $p < 0.001$] and the Tcf7l2 KO mouse saline group [F (1, 28) = 29.487 and $p < 0.001$]. The AUC values were also significantly higher in the Tcf7l2$^{fl/fl}$ mouse olanzapine group than the Tcf7l2$^{fl/fl}$ mouse saline group [F (1, 28) = 6.363 and $p = 0.0181$] (**Figure 3C**).

Olanzapine Treatment Altered the Expression Profile of Plasma Hormones and Lipids in Tcf7l2 CKO and Control Mice

As clinical administration of olanzapine has long been proposed to cause insulin resistance and dyslipidemia, we then examined the main effects of olanzapine treatment to insulin levels. As expected, the plasma insulin level was markedly lower in Tcf7l2 CKO mice with olanzapine [F (1, 20) = 7.545 and $p = 0.012$] than that in Tcf7l2 CKO mice administered with saline [F (1, 20) = 8.043 and $p = 0.01$] and Tcf7l2$^{fl/fl}$ mice administered with olanzapine [F (1, 20) = 5.271, $p = 0.033$] (**Figure 4A**). However, no significant difference was observed in plasma proinsulin and glucagon between different groups (**Figures 4B,C**). We then measured the levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein (LDL-C), and triglyceride in serum from different groups to rule out the possibility of dyslipidemia. For the plasma TC levels, main effects of olanzapine treatment [F (1, 26) = 5.579; $p = 0.015$] and interaction effect [F (1, 26) = 4.754; $p = 0.038$] were revealed by two-way ANOVA analysis. No main effect of Tcf7l2 gene knockout [F (1, 26) = 1.507; $p = 0.231$] was confirmed. Compared to Tcf7l2 CKO mice with olanzapine, both Tcf7l2 CKO mice with saline [F (1, 26) = 11.496; $p = 0.002$] and Tcf7l2$^{fl/fl}$ mice with olanzapine [F (1, 26) = 6.222; $p = 0.019$] showed lower TC levels (**Figure 4D**). Similarly, two-way ANOVA also revealed a main effect of olanzapine treatment [F (1, 26) = 6.367, $p = 0.018$] but no main effect of Tcf7l2 gene

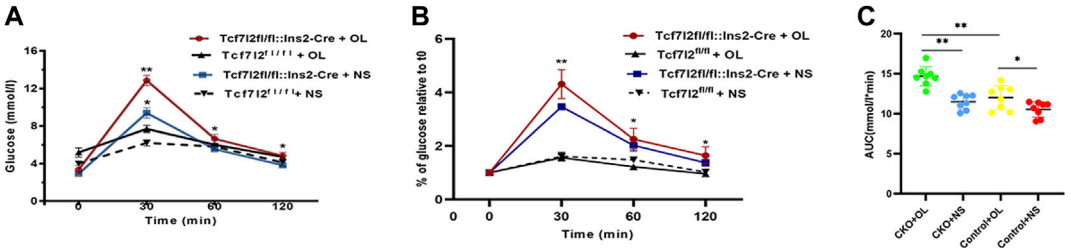

FIGURE 3 | Tcf7l2 CKO mice displayed impaired oral glucose tolerance after intragastric administration of olanzapine for 6 weeks. **(A)** Oral glucose tolerance test (OGTT) on overnight fasted mice from different groups. The blood glucose level was measured at 0, 30, 60, and 120 min after glucose administration (2 g/kg body weight). $Tcf7l2^{fl/fl}$: Ins2-Cre + OL is the Tcf7l2 CKO mouse olanzapine group (red), $Tcf7l2^{fl/fl}$:Ins2-Cre + NS is the Tcf7l2 CKO mouse saline group (blue), $Tcf7l2^{fl/fl}$ - + OL is control mice treated with olanzapine (black solid line), and $Tcf7l2^{fl/fl}$ + NS is control mice treated with saline (black dotted line). *$p < 0.05$ and **$p < 0.001$. **(B)** Comparison of the percentage of blood glucose relative to fasting blood glucose at various time points among different groups. $Tcf7l2^{fl/fl}$: Ins2-Cre + OL is the Tcf7l2 CKO mouse olanzapine group (red), $Tcf7l2^{fl/fl}$: Ins2-Cre + NS is the Tcf7l2 CKO mouse saline group (blue), $Tcf7l2^{fl/fl}$ - + OL is control mice treated with olanzapine (black solid line), and $Tcf7l2^{fl/fl}$ + NS is control mice treated with saline (black dotted line). *$p < 0.05$ and **$p < 0.001$. **(C)** Area size under the blood glucose curve of four groups of mice after 6 weeks of treatment. **$p < 0.001$, OI vs. NS group in Tcf7l2 KO mice, and Tcf7l2 KO mice vs. control mice in olanzapine group. *$p < 0.05$, OI vs. NS group in control mice. $n = 8$ for each group. OL, olanzapine; NS, normal saline.

deletion [F (1, 26) = 0.359; $p = 0.554$] or interaction between Tcf7l2 deletion and olanzapine treatment in the plasma TG levels [F (1, 26) = 0.607; $p = 0.443$]. The TG levels were significantly increased in Tcf7l2 CKO mice treated with olanzapine [F (1, 26) = 5.454; $p = 0.028$] compared with Tcf7l2 CKO mice treated with saline (**Figure 4E**). For the plasma LDL levels, two-way ANOVA revealed a main effect of Tcf7l2 gene deletion [F (1, 26) = 15.590; $p = 0.001$] but no main effect of olanzapine treatment [F (1, 26) = 4.125, $p = 0.053$] and no interaction [F (1, 26) = 2.295, $p = 0.142$]. Moreover, the LDL levels were higher in the Tcf7l2 CKO mice with olanzapine group than the Tcf7l2fl/fl mice with olanzapine group [F (1, 26) = 15.989; $p < 0.001$) and the Tcf7l2 CKO mice with saline group [F (1, 26) = 6.286; $p = 0.019$] (**Figure 4F**). In line with the previous literature, no significant differences in plasma HDL levels were detected between different groups (**Figure 4G**).

Effects of Olanzapine Treatment to Beta Cell Mass in Tcf7l2 CKO and Control Mice

We further investigated whether Tcf7l2 CKO mice had altered pancreatic β-cell mass, especially after administration of olanzapine. The mass of β cells was evaluated, as previously reported (Mitchell et al., 2015), and the results are shown in (**Figure 5**). Although the Tcf7l2 CKO mice with olanzapine group had the least amount of pancreatic β-cell mass among four groups, no significant difference was revealed ($p > 0.05$, two-way ANOVA). This result indicates a propensity of reduced β-cell mass in Tcf7l2 CKO mice when treated with olanzapine, and this may prime the mice with the vulnerability of dysregulated β-cell function.

Comparison of the Size and Number of Adipocytes Between Tcf7l2 CKO and $Tcf7l2^{fl/fl}$ Mice After Olanzapine Treatment

Previous studies have reported that olanzapine may contribute to the hypertrophy of adipocytes in mice. In our study, we also observed higher growth rate of weight gain in Tcf7l2 CKO after olanzapine challenge, but it is unclear whether Tcf7l2 is involved in olanzapine-induced adipocyte hypertrophy. We then performed HE staining with white fat tissue and compared the size and number of adipocytes to determine fat accumulation between different groups (**Figure 6A**). In the saline group, the size of the adipocytes in $Tcf7l2^{fl/fl}$ mice was roughly between 1 and 11×10^3 μm^2, and more than 40% of the cells had the size between 3 and 5×10^3 μm^2. As opposed, populations of the adipocytes in the Tcf7l2 CKO mice were larger than 11×10^3 μm^2, and less than 30% of the cells had the size between 3 and 5×10^3 μm^2 ($p < 0.05$). In the olanzapine group, most of the adipocytes of the Tcf7l2 CKO mice exhibited a cell size larger than 13×10^3 μm^2, whilst the majority of the adipocytes of $Tcf7l2^{fl/fl}$ mice had the size between 5 and 13×10^3 μm^2. The detailed distribution pattern of the adipocyte area size is shown in (**Figure 6B**). For the cell size of $17–20 \times 10^3$ μm^2, variance analysis showed a main effect of Tcf7l2 gene deletion [F (1, 12) = 6.328; $p = 0.027$]. In addition, Tcf7l2 CKO mice had more adipocytes than control mice after olanzapine administration [F (1, 12) = 6.307; $p = 0.027$]. Specifically, Tcf7l2 CKO mice with olanzapine had more adipocytes with cell size over 20×10^3 μm^2 [F (1, 12) = 6.618; $p = 0.024$]. For the cells with body size between 15 and 17×10^3 μm^2, variance analysis supported a main effect of olanzapine treatment [F (1, 12) = 6.658; $p = 0.024$], and the number of adipocytes in Tcf7l2 KO mice with olanzapine is significantly larger than that of Tcf7l2 CKO mice with saline [F (1, 12) = 6.945; $p = 0.022$]. However, for the area size of $5–15 \times 10^3$ μm^2, no difference in the proportion of adipose cells among the four groups was observed ($p > 0.05$). However, for the area of $3–5 \times 10^3$ μm^2, variance analysis showed a main effect of olanzapine treatment [F (1, 12) = 21.051; $p = 0.001$], and both the Tcf7l2 CKO mice and control mice in the olanzapine group displayed fewer numbers of adipocytes than those treated with saline [F (1, 12) = 5.031; $p = 0.045$ and F (1, 12) = 18.026; $p = 0.001$, respectively]. However, for the area size of $1–3 \times 10^3$, no difference in the proportion of adipose cells among the four groups was observed ($p > 0.05$).

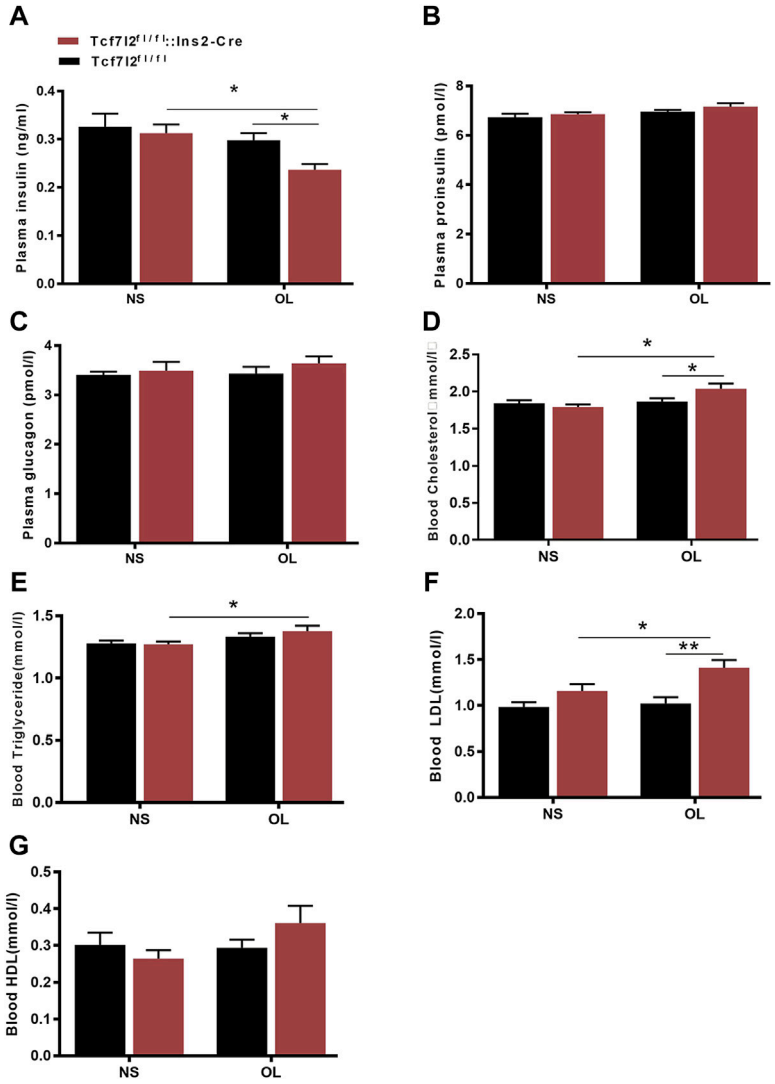

FIGURE 4 | Comparison of the plasma hormones and blood lipid between different treatment groups in Tcf7l2 CKO and control mice. Tcf7l2 CKO (Tcf7l2fl/fl: Ins2-Cre, red) and control ($Tcf7l2^{fl/fl}$, black) mice were subjected to olanzapine (4 mg/kg/day) or saline for 6 weeks. Effects of different treatment groups on insulin **(A)**, proinsulin **(B)**, glucagon **(C)**, total cholesterol **(D)**, triglyceride **(E)**, LDL-C **(F)**,, and HDL-C **(G)** at the end of 6-week treatment. All of the results are expressed as the mean ± SEM. *$p < 0.05$ and **$p < 0.001$.

Olanzapine Treatment Decreased the Pancreatic GLP-1R Expression in Tcf7l2 KO Mice

Previous studies have demonstrated a strong association between impaired β-cell function and reduced Tcf7l2 and GLP-1R expression in the pancreas (Shu et al., 2009b). To test this possibility in our model, we performed immunofluorescence staining to confirm the reduced expression of Tcf7l2 and to determine the expressing profile of GLP-1R in the pancreas before and after olanzapine challenge. Compared to the control group (**Figures 7A–C**), reduced Tcf7l2-positive staining (**Figures 7H,I,K,L**) in β cells (co-stained with anti-insulin, **Figures 7G,J**) of Tcf7l2 CKO mouse pancreas was observed, regardless of the presence of olanzapine in the current study. Intriguingly, a reduced expression of Tcf7l2 was also observed in $Tcf7l2^{fl/fl}$ mice subject to olanzapine treatment (**Figures 7D–F**), suggesting a strong inhibitory role of olanzapine to the expression of Tcf7l2 protein. Similarly, as opposed to the $Tcf7l2^{fl/fl}$ mice with saline (**Figures 8A–C**) or olanzapine treatment (**Figures 8D–F**), an overtly decreased expression of GLP-1R was also detected in Tcf7l2 CKO mice with (**Figures 8G–I**) or without (**Figures 8J–L**) olanzapine challenge. Intriguingly, the expression of glucagon seemed unaltered (**Figures 8B,E,H,K**). We then run Western blotting to quantify the expression levels of Tcf7l2 and GLP-1R protein in the pancreatic tissue of the four groups (**Figure 9**). As expected, Tcf7l2 CKO mice displayed significantly less expression of Tcf7l2 protein which was further reduced after olanzapine treatment [$F(1, 12) = 22.428$; $p < 0.001$, **Figure 9C**]. Two-way ANOVA

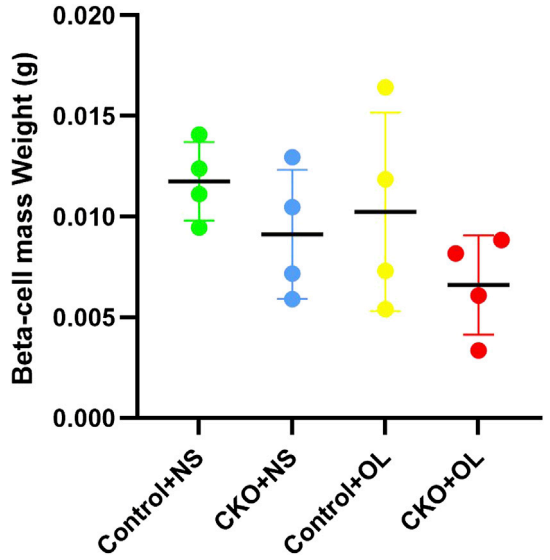

FIGURE 5 | Comparison of the beta cell mass between different treatment groups in Tcf7l2 CKO and control mice. Tcf7l2 CKO (Tcf7l2fl/fl: Ins2-Cre) and control (Tcf7l2$^{fl/fl}$) mice were subjected to olanzapine (4 mg/kg/day) or saline for 6 weeks. All of the results are expressed as the mean ± SEM. OL, olanzapine; NS, normal saline.

revealed the main effects of Tcf7l2 gene deletion [F (1, 12) = 19.236; $p = 0.001$] and olanzapine treatment [F (1, 12) = 28.061; $p < 0.001$], with no interaction with the expression of Tcf7l2 levels [F (1, 12) = 8.019, $p = 0.015$, **Figure 9A**]. Similarly, the Tcf7l2 CKO mice treated with olanzapine showed a markedly downregulated GLP-1R when compared with Tcf7l2$^{fl/fl}$ mice treated with olanzapine [F (1, 12) = 33.041; $p < 0.001$] or Tcf7l2 CKO mice treated with saline [F (1, 12) = 26.048; $p < 0.001$] (**Figure 9B**). We further quantified the expressions of Sp5 and Axin2, two important components of the Wnt signaling pathway at the transcriptional level. Compared with the Tcf7l2 CKO mice with saline group, only Tcf7l2 CKO mice treated with olanzapine displayed significantly less expression of Sp5 mRNA (F = 4.845; $p = 0.048$, **Figure 8D**), highlighting a disturbed Wnt signaling pathway in the pancreas of the CKO mice.

DISCUSSION

Increasing evidence strongly suggests that a high percentage of SCZ patients display MetS and are subjected to increased risk of T2D (Postolache et al., 2019), which in turn considerably exacerbates their cognitive function. Specific psychotropic drugs like olanzapine have shown profound effects to increase MetS dysregulation, but the pleiotropy in genetic vulnerability, especially those loci leading to activated immunometabolic or endocrine pathways, also plays a pivotal role in the development of T2D and SCZ. In this study, we have determined the multifaceted roles of Tcf7l2, one of the strongest genetic determinants for T2D in humans (Hansen et al., 2011) and in the development of MetS upon olanzapine challenge in mice. We showed that mice with pancreatic β-cell–specific Tcf7l2 deletion were more vulnerable to suffer MetS after long-term administration of olanzapine. We further revealed that conditional deletion of Tcf7l2 may lead to the reduced expression of GLP-1R and a tendency of less islet β-cell mass in mouse pancreas. Our results suggest that Tcf7l2 may regulate islet cell function *via* GLP-1R in the pancreas and when defective, may enhance the deleterious effects of olanzapine and promote the occurrence of metabolic abnormalities.

How TCF7L2 orchestrates the body weight management on its own or in combination with olanzapine remains arguably inconsistent. Both Tcf7l2 dominant-negative mice and mice with pancreatic Tcl7l2 deletion displayed normal body weight (da Silva Xavier et al., 2012; Takamoto et al., 2014). Clinical research also found that TCF7L2 risk SNP allele rs7903146 is not associated with obesity, and T2D patients with TCF7L2 gene variants exhibit no heterogeneity in body weight and BMI compared to healthy controls (Ferreira et al., 2018). However, Zhang et al. (2016)have reported a close association between

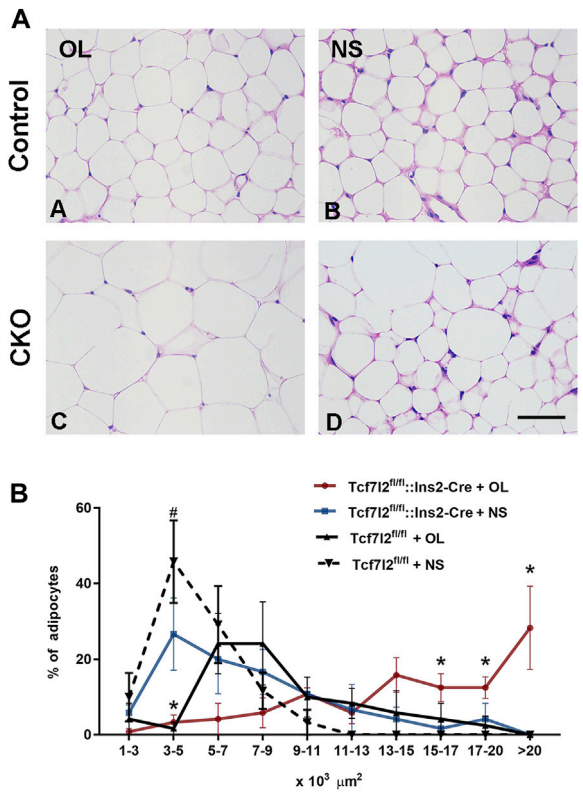

FIGURE 6 | Comparison of the size and number of adipocytes between different treatment groups in Tcf7l2 CKO and control mice. **(A)** Representative images of hematoxylin and eosin (H&E)-stained abdominal adipose tissues harvested from different groups of mice after 6 weeks of treatment. A) Tcf7l2$^{fl/fl}$ mouse olanzapine group. B) Tcf7l2$^{fl/fl}$ mouse saline group. C) Tcf7l2 CKO mouse olanzapine group. D) Tcf7l2 CKO mouse normal saline group. **(B)** Percentage of different area sizes of adipocytes in different groups of mice after 6 weeks of treatment. *$p < 0.05$, OI vs. NS group in Tcf7l2 CKO mice and Tcf7l2 CKO mice vs. Tcf7l2$^{fl/fl}$ mice in olanzapine group. $^{\#}p < 0.05$, OI vs. NS group in control mice. $n = 4$. OI, olanzapine; NS, normal saline. Scale bar = 50.

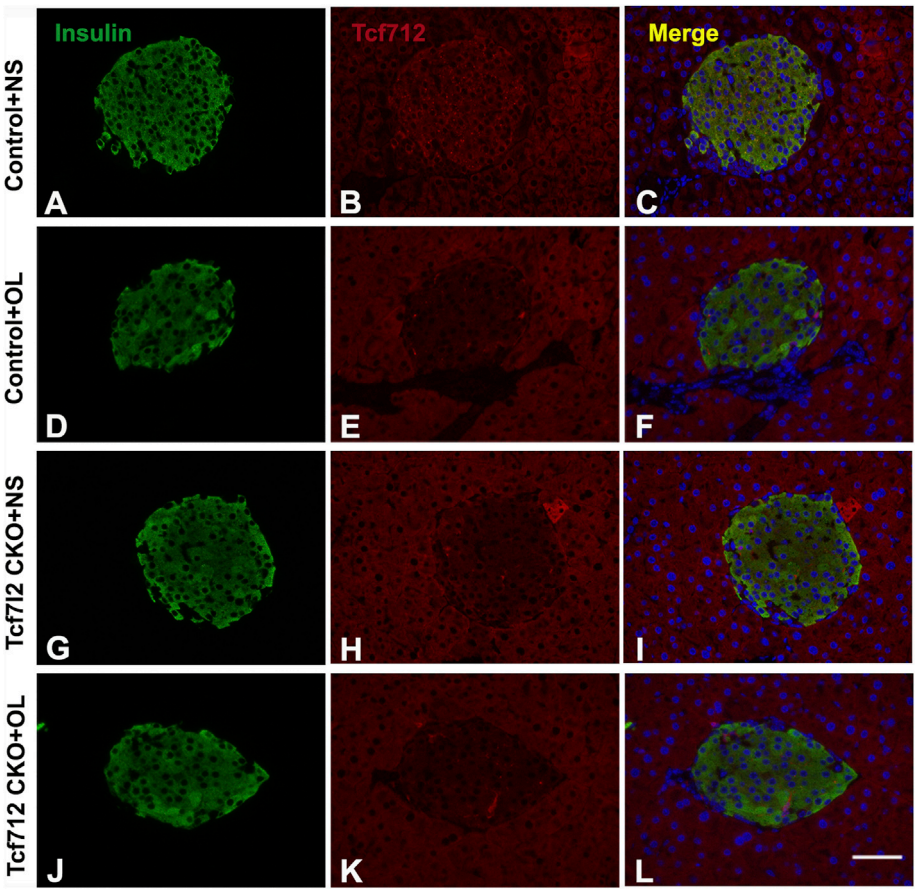

FIGURE 7 | Representative immunofluorescence images of TCF7L2 in the pancreas islets of Tcf7l2 CKO and Tcf7l2$^{fl/fl}$ mice before and after the olanzapine challenge. Immunostaining results of insulin (green) in control mice treated with saline **(A)**, control mice treated with olanzapine **(D)**, Tcf7l2 CKO mice treated with saline **(G)**, and Tcf7l2 CKO mice treated with olanzapine **(J)**. Immunostaining results of Tcf7l2 (red) in control mice treated with saline **(B)**, control mice treated with olanzapine **(E)**, Tcf7l2 CKO mice treated with saline **(H)**, and Tcf7l2 CKO mice treated with olanzapine **(K)**. Merge of insulin (green) and Tcf7l2 (red) in control mice treated with saline **(C)**, control mice treated with olanzapine **(F)**, Tcf7l2 CKO mice treated with saline **(I)**, and Tcf7l2 CKO mice treated with olanzapine **(L)**. Scale bar = 50.

several TCF7L2 polymorphisms and obesity in first-episode diabetes among Chinese population. In the present study, we found that Tcf7l2 CKO mice present higher body weight growth rate when exposed to olanzapine. This is consistent with recent clinical observation that healthy Chinese people with TCF7L2 rs7093146 have larger increment of weight gain when treated with olanzapine (10 mg/d) (Li et al., 2017). These results strongly indicate that TCF7L2 is highly likely to be involved in the body weight management regulated by olanzapine.

In line with previous studies (Minet-Ringuet et al., 2007; Hou et al., 2018), our results also confirmed that chronic administration of olanzapine in mice can lead to significant adipocyte hypertrophy. This effect is even more prominent after Tcf7l2 deletion. In our case, Tcf7l2 CKO mice have more adipocytes with an area size over 17×10^3 μm^2 after continuous exposure to olanzapine for 6 weeks. Consistently, Yang et al. 2007 have shown that olanzapine induces triacylglyceride accumulation and promotes SREBP-1-related adipogenesis in differentiating 3T3-L1 preadipocytes. In addition, Minet-Ringuet et al. (2007) have revealed that adipocytes derived from olanzapine-treated rats displayed reduced lipolytic activity and increased fatty acid synthase activity, thus contributing to the excess of weight gain (Minet-Ringuet et al., 2007; Yang et al., 2007). Our study further revealed that Tcf7l2 CKO mice display significantly hypertrophic adipocytes in the presence of olanzapine, indicating a synergic effect of genetic deficit and stress. In fact, Tcf7l2 can inhibit adipogenic differentiation of 3T3-L1 and prevent adipogenesis, hence playing a role against olanzapine-induced fat deposition (Tian et al., 2017). Deletion of Tcf7l2 in mature adipocytes in mice leads to whole-body glucose intolerance and hepatic insulin resistance, which is concomitant with increased subcutaneous adipose tissue mass and adipocyte hypertrophy (Kaminska et al., 2012; Chen et al., 2018). Importantly, the TCF7L2 mRNA expression is downregulated in humans with impaired glucose tolerance and adipocyte insulin resistance. All these data strongly support that TCF7L2 may work closely with olanzapine to regulate adipocyte development and metabolism.

Consistent with previous reports (Ikegami et al., 2013; Li et al., 2018), the present study further confirmed overtly dysregulated

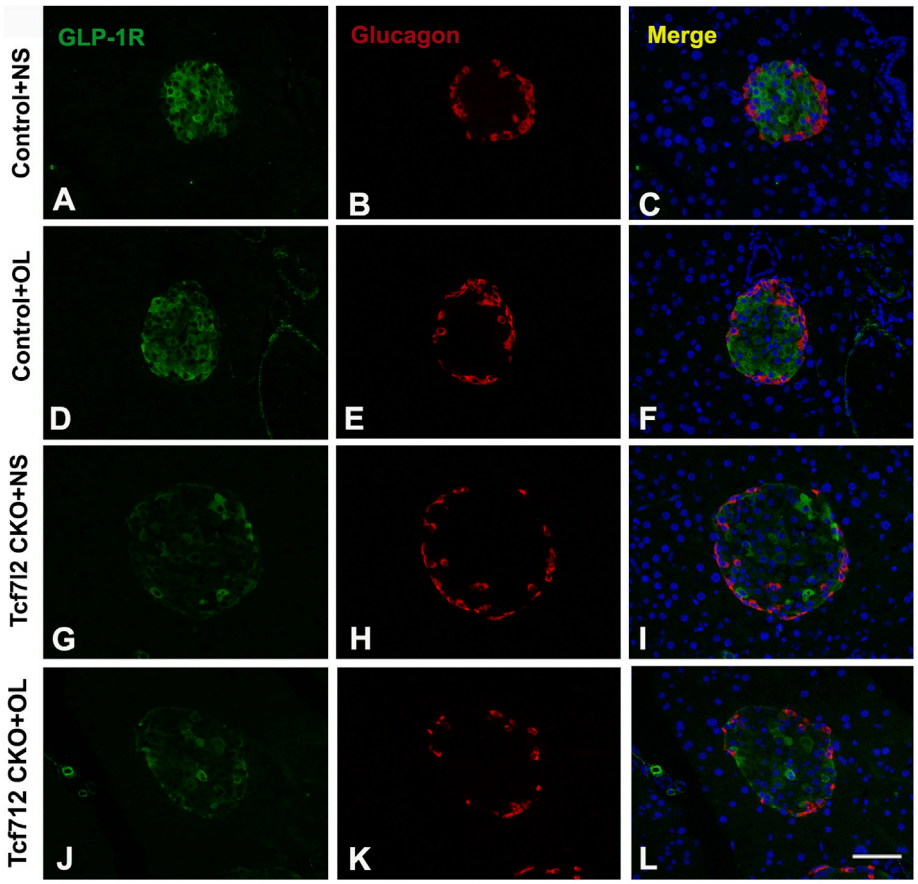

FIGURE 8 | Representative immunofluorescence images of GLP-1R in pancreas islets of Tcf7l2 CKO and Tcf7l2$^{fl/fl}$ mice before and after olanzapine challenge. Immunostaining results of GLP-1R (green) in control mice treated with saline **(A)**, control mice treated with olanzapine **(D)**, Tcf7l2 CKO mice treated with saline **(G)**, and Tcf7l2 CKO mice treated with olanzapine **(J)**. Immunostaining results of glucagon (red) in control mice treated with saline **(B)**, control mice treated with olanzapine **(E)**, Tcf7l2 CKO mice treated with saline **(H)**, and Tcf7l2 CKO mice treated with olanzapine **(K)**. Merge of GLP-1R (green) and glucagon (red) in control mice treated with saline **(C)**, control mice treated with olanzapine **(F)**, Tcf7l2 CKO mice treated with saline **(I)**, and Tcf7l2 CKO mice treated with olanzapine **(L)**. Scale bar = 50.

glucose homeostasis and impaired glucose tolerance in mice challenged with olanzapine. Similarly, Tcf7l2-knockout mice also showed increased blood glucose levels and impaired glucose tolerance. In addition, mice with selective deletion of Tcf7l2 in β cells displayed impaired oral and intraperitoneal glucose tolerance at postnatal 8 and 16 weeks, respectively (Mitchell et al., 2015). In agreement with these data, we have revealed that olanzapine treatment for 6 weeks significantly exacerbates periphery glucose intolerance in Tcf7l2 CKO mice. Although a peripheral insulin resistance could not be fully ruled out, a previous study has revealed impaired glucose tolerance rather than insulin resistance when Tcf7l2 was specifically deleted in the pancreatic β cells which indicated that the impairment of glucose tolerance mainly arose from decreased secretion of insulin (Mitchell et al., 2015). These studies strongly suggest that Tcf7l2 may work synergically with olanzapine to regulate glucose homeostasis, and deletion of Tcf7l2 further promotes glucose intolerance induced by olanzapine. Our data indicate that this dysregulation may be at least partially mediated by dysfunctional GLP-1 signaling in Tcf7l2 CKO mice.

Previous reports have shown that acute administration of olanzapine has no effects on plasma insulin and glucagon levels in mice (Savoy et al., 2010; Ikegami et al., 2013). Interestingly, deletion of the Tcf7l2 gene in mouse pancreatic tissue did not disturb the fasting blood insulin, proinsulin, and glucagon concentrations (Mitchell et al., 2015). As opposed, we have demonstrated that olanzapine exposure is associated with abnormal blood insulin secretion in mice with pancreatic-specific Tcf7l2 gene deletion but not in wild-type mice. These data strongly suggest that dysfunctional Tcf7l2 may prime the mice with the vulnerability to glucose metabolism, and olanzapine may precipitate the abnormal secretion of insulin after Tcf7l2 deletion. Notably, CKO mice presented lower baseline blood glucose, indicating a disruption of insulin sensitivity may not be fully ruled out. Additionally, a previous study has also shown that islets isolated from 20-week-old Tcf7l2 gene-deficient mice displayed impaired glucose-stimulated insulin secretion (da Silva Xavier et al., 2012). Whether olanzapine administration further exacerbates insulin secretion in Tcf7l2 CKO mice requires further research in the future. Dyslipidemia is a core symptom

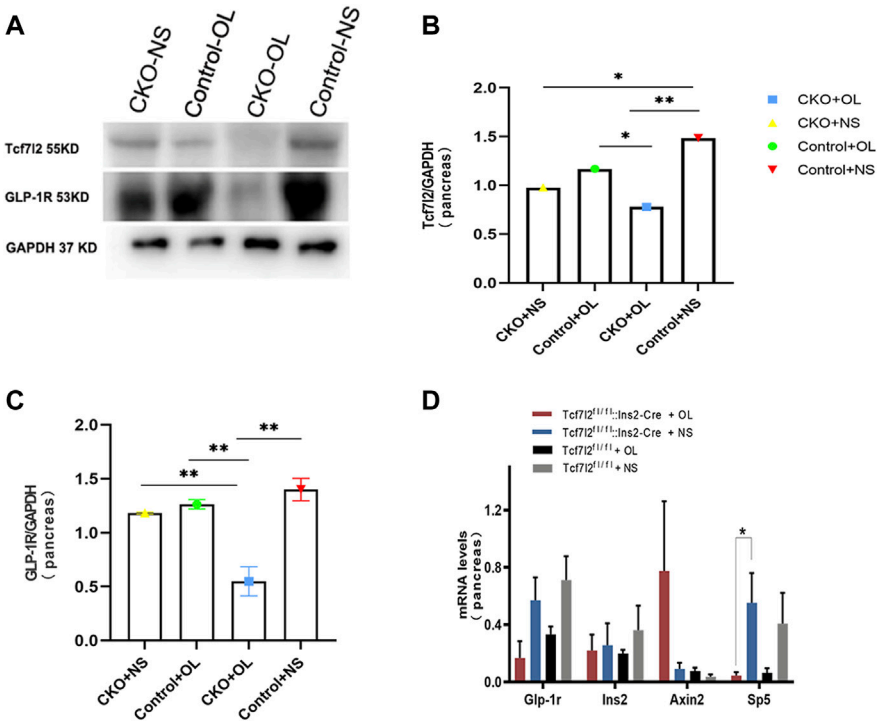

FIGURE 9 | Expressions of TCF7L2 and GLP-1R in the pancreas islets of Tcf7l2 CKO and Tcf7l2$^{fl/fl}$mice before and after the olanzapine challenge. **(A)** Protein expressions of Tcf7l2 and GLP-1R in the pancreatic tissue of Tcf7l2 CKO (Tcf7l2fl/fl: Ins2-Cre) and control (Tcf7l2fl/fl) mice. **(B)** Quantified protein expression of Tcf7l2 in the pancreatic tissue of Tcf7l2 CKO (Tcf7l2fl/fl: Ins2-Cre) and control (Tcf7l2fl/fl) mice. *$p < 0.05$ and **$p < 0.001$. OL, olanzapine; NS, normal saline. **(C)** Quantified protein expression of GLP-1R in the pancreatic tissue of Tcf7l2 CKO (Tcf7l2fl/fl: Ins2-Cre) and control (Tcf7l2fl/fl) mice. **$p < 0.001$. OL, olanzapine; NS, normal saline. **(D)** RT-qPCR analysis revealed a markedly reduction of sp5 mRNA in the pancreatic tissue of the Tcf7l2 CKO mouse olanzapine group (red) compared with the Tcf7l2 CKO mouse normal saline group (blue), $n = 4$.

of metabolic disorders. Notably, individuals with at-risk alleles of Tcf7l2 displayed abnormal concentrations of TC, LCL, HDL, or very low-density lipoprotein (VDLL) in periphery blood (Gallardo-Blanco et al., 2017), indicating a strong association of Tcf7l2 and dyslipidemia. In line with these findings, we have also uncovered that deletion of the Tcf7l2 gene could aggravate olanzapine-induced dyslipidemia in mice. These results support an important involvement of Tcf7l2 in the lipid metabolism.

It has been proposed that Tcf7l2 may modulate the pancreatic secretion *via* regulating the development of β-cells. Takamoto et al. (2014) have created several lines of Tcf7l2 dominant-negative transgenic mice and revealed significant reduced beta cell area and whole-pancreas insulin content in both the adult and newborn mice. On the contrary, utilizing tamoxifen-inducible pancreatic β-cell–specific Cre-recombinase mouse strain, Boj et al. (2012) found no differences in pancreatic islets and beta cell mass when tamoxifen was given after weaning. Interestingly, da Silva Xavier et al. (2012) produced pancreas-specific Tcf7l2-knockout mice which only displayed decreased oral glucose tolerance from 20 weeks with no altered β-cell mass. As opposed, they have observed 30% β-cell mass reduction in mice with β-cell–specific Tcf7l2 deletion (Mitchell et al., 2015). In our study, we have revealed that the CKO mice presented lower insulin level together with a trend of reduced β-cell mass. This feature was further exacerbated after the treatment of olanzapine. We assume that Tcf7l2 may predispose β cells to the risk of olanzapine-induced MetS, and a normal expression of Tcf7l2 can alleviate the negative effects of olanzapine and help the maintenance of proper pancreatic function and glucose homeostasis. To further reveal how Tcf7l2 affects the mass of β cells at different developmental stages, a longitudinal study utilizing the tamoxifen-inducible pancreatic β-cell–specific Cre-ER mouse strain shall be warranted in the future.

The glucagon-like peptide-1 (GLP-1) is a multifaceted hormone with broad pharmacological potential. In pancreas, it can enhance β-cell proliferation, inhibit apoptosis, and promote insulin transcription and biosynthesis (Lee et al., 2018). GLP-1 receptor agonists are successfully in clinical use for the treatment of type 2 diabetes. Intriguingly, we have detected a synchronized downregulation of Tcf7l2 and GIP-R in the CKO mice after olanzapine administration, indicating a functional synchronization between Tcf7l2 and GIP-1. We also determined the expressions of Axin and Sp5, two important components of the Wnt signal pathway. Consistent with the previous report (Boj et al., 2012), we did not detect any change in the Axin expression among different groups. However, a significantly reduced mRNA expressing level was revealed in the Tcf7l2 KO mice challenged with olanzapine, highlighting a close involvement of the Wnt signaling pathway.

This study has several limitations. For example, only a single dose of olanzapine was used, and thus, dosage-dependent effects

could not be fully ruled out. Meanwhile, we did not monitor the concomitant insulin concentration during the OGTT because of the limited blood withdrawn from the vein of live mice. Another issue is that *ex vivo* insulin secretion experiments were not performed. Therefore, it is difficult to fully characterize the insulin secretion in absence of Tcf7l2 and in response to olanzapine treatment. In addition, although we have detected an overt reduction of GIP-R in the pancreas of the CKO mice, a detailed cellular and molecular mechanism still needs to be established.

In summary, our study illustrates that pancreatic Tcf7l2-knockout mice were more vulnerable to suffer metabolic abnormalities after olanzapine administration. This impairment may be mediated by the reduced expression of GLP-1R. Tcf7l2 can potentially protect pancreatic β-cell, maintain glucose homeostasis, and thus alleviate MetS caused by olanzapine. Our study provides a theoretical basis for proper application of olanzapine at clinics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Committee of Xiangya Second Hosptial, Central South University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YY contributed to analysis and drafted the manuscript, and MS collected data. LL and YL contributed to experiments. RW designed and critically revised the manuscript; RW contributed to conception and design and contributed to interpretation; BL contributed to analysis and interpretation; RW and BL contributed to conception and design. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Patterns of Convergence and Divergence Between Bipolar Disorder Type I and Type II: Evidence From Integrative Genomic Analyses

Yunqi Huang[1,2,3†], Yunjia Liu[1,2,3†], Yulu Wu[1,2,3†], Yiguo Tang[1,2,3], Mengting Zhang[1,2,3], Siyi Liu[1,2,3], Liling Xiao[1,2,3], Shiwan Tao[1,2,3], Min Xie[1,2,3], Minhan Dai[1,2,3], Mingli Li[1,2,3], Hongsheng Gui[4,5] and Qiang Wang[1,2,3]**

[1]Mental Health Center and Psychiatric Laboratory, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University, Chengdu, China, [2]West China Brain Research Center, West China Hospital of Sichuan University, Chengdu, China, [3]Sichuan Clinical Medical Research Center for Mental Disorders, Chengdu, China, [4]Center for Health Policy & Health Services Research, Henry Ford Health System, Detroit, MI, United States, [5]Behavioral Health Services, Henry Ford Health System, Detroit, MI, United States

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Edited by:
Cunyou Zhao,
Southern Medical University, China

Reviewed by:
Xiao Xiao,
Kunming Institute of Zoology (CAS), China
Jian-Huan Chen,
Jiangnan University, China

***Correspondence:**
Hongsheng Gui
hgui1@hfhs.org
Qiang Wang
wangqiang130@scu.edu.cn

†These authors have contributed equally to this work and share first authorship.

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Aim: Genome-wide association studies (GWAS) analyses have revealed genetic evidence of bipolar disorder (BD), but little is known about the genetic structure of BD subtypes. We aimed to investigate the genetic overlap and distinction of bipolar type I (BD I) & type II (BD II) by conducting integrative post-GWAS analyses.

Methods: We utilized single nucleotide polymorphism (SNP)–level approaches to uncover correlated and distinct genetic loci. Transcriptome-wide association analyses (TWAS) were then approached to pinpoint functional genes expressed in specific brain tissues and blood. Next, we performed cross-phenotype analysis, including exploring the potential causal associations between two BD subtypes and lithium responses and comparing the difference in genetic structures among four different psychiatric traits.

Results: SNP-level evidence revealed three genomic loci, *SLC25A17, ZNF184*, and *RPL10AP3*, shared by BD I and II, and one locus (*MAD1L1*) and significant gene sets involved in calcium channel activity, neural and synapsed signals that distinguished two subtypes. TWAS data implicated different genes affecting BD I and II through expression in specific brain regions (nucleus accumbens for BD I). Cross-phenotype analyses indicated that BD I and II share continuous genetic structures with schizophrenia and major depressive disorder, which help fill the gaps left by the dichotomy of mental disorders.

Abbreviations: BD, Bipolar disorder; BDI, Bipolar disorder type I; BDII, Bipolar disorder type II; condFDR, Conditional false discovery rate; conjFDR, Conjunctional false discovery rate; eQTL, Expression quantitative trait locus; FUMA, Functional mapping and annotation of GWAS; GSMR, Generalized summary-data–based Mendelian randomization; GWAS, Genome-wide association study; HEIDI, Heterogeneity in dependent instruments; IVs, Instrumental variants; IVW, Inverse variance weighted; LD, Linkage disequilibrium; MAF, Minor allele frequency; MAGMA, Multi-marker analysis of genomic annotation; MDD, Major depressive disorder; MR-PRESSO, MR pleiotropy residual sum and outlier; NHGRI-EBI, National Human Genome Research Institute–European Bioinformatics Institute; OLS, Ordinary least squares; PGC, Psychiatric Genomics Consortium; pleioFDR, pleiotropy-informed conditional false discovery rate; RDoC, Research Domain Criteria; SCZ, Schizophrenia; SMR, Summary-data–based Mendelian randomization; SNP, Single nucleotide polymorphism; TWAS, Transcriptome-wide association analyses.

Conclusion: These combined evidences illustrate genetic convergence and divergence between BD I and II and provide an underlying biological and trans-diagnostic insight into major psychiatric disorders.

Keywords: bipolar disorder, genome-wide association studies, transcriptome-wide association analysis, Mendelian randomization, bipolar type I, bipolar type II

INTRODUCTION

Bipolar disorder (BD) is one of the most severe psychiatric disorders, characterized by mood state fluctuation. As one of the top causes of disability worldwide, BD affects more than 40 million people worldwide with a lifespan prevalence of 1%~4% (Merikangas et al., 2011; Huang et al., 2019), early-onset in adolescents, and elevated risk of suicide (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2018).

Population and molecular studies have proved evidence of the complex etiology of BD. Twin and family studies have estimated that the heritability of BD is over 70% (Edvardsen et al., 2008; Bienvenu et al., 2011). GWAS have brought a deeper insight into BD (Stahl et al., 2019; Li et al., 2021) compared with previous population genetics studies (Bertelsen et al., 1977; Kieseppa et al., 2004). The largest-scale GWAS of BD was recently processed by the Psychiatric Genomic Consortium Bipolar Disorder Working Group (PGC BD) (Mullins et al., 2021), and 64 genome-wide significant loci were identified. However, it failed to display increasing single nucleotide polymorphism (SNP)–level heritability (h^2_{SNP}) of BD (Psychiatric 2011; Mullins et al., 2021).

BD can be categorized into several major subtypes: BD type I (BD I) and type II (BD II), cyclothymia, and other specified bipolar and related disorders, according to the Diagnostic and Statistical Manual Disorders, Fifth Edition (DSM-5). BD I requires manic episodes at least once despite depression states, and BD II is defined as more than one depressive and hypomanic state. The worldwide lifetime prevalence of BD I (0.4%–1.2%) (Bebbington and Ramana 1995; Merikangas et al., 2011; Huang et al., 2019) differs from BD II (0.1%–2.5%) (Bebbington and Ramana 1995; Merikangas et al., 2011; Huang et al., 2019). In addition, clinical presentations and severity vary in the two subtypes (Judd et al., 2008; Merikangas et al., 2011); however, their genetic differences remained unclear due to insufficient sample size or substandard clinical classification (Charney et al., 2017; Bipolar Disorder and Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2018).

Under the dichotomic diagnostic system, BD is hard to be distinguished from schizophrenia (SCZ) and major depressive disorder (MDD). BD with psychotic symptoms or manic BD performs and behaves similarly to SCZ; and oppositely, depressive BD is always misdiagnosed as MDD, resulting in limited therapeutic effects. Additionally, second-generation antipsychotics play an important role in the treatment of BD, SCZ, and MDD, indicating potential shared biological mechanisms among these phenotypes. Linkage disequilibrium (LD) score analysis indicated that BD I is much more genetically correlated with SCZ, whereas the genetic correlation of BD II with MDD is higher (Mullins et al., 2021). These provide a new perspective on the genetic correlation between BD I, BD II, SCZ, and MDD. Moreover, molecular genetic studies have uncovered overlapped risk factors between the genomic architecture of psychiatric disorders (Psychiatric 2011; Charney et al., 2017; Stahl et al., 2019; Mullins et al., 2021); however, current diagnostic systems failed to elucidate it clearly.

To better understand BD etiology and taxonomy, our study aimed to provide more evidence through post-GWAS analyses. Integrative Omics approaches were adopted to navigate functional genes expressed in the influenced brain regions. Additionally, we explored cross-phenotype genetic structure in adult psychiatric disorders. We are trying to enrich the Research Domain Criteria (RDoC) and re-evaluate and provide new evidence for the cross-disease diagnosis of mental disorders.

METHODS AND MATERIALS

Study Design and GWAS Data Resources

The genome-wide association (GWA) meta-analysis summary data of BD I and II were from the PGC BD, containing BD I (25,060 cases and 449,978 controls) and BD II (6,781 cases and 364,075 controls), respectively. All participants were of European descent, and the diagnosis was explicitly based on international consensus criteria (DSM-IV, ICD-9, or ICD-10). Details on participant and cohort information and quality control can be accessed at Consortium (2020). Nominal significant instrumental variants (IVs) of response to lithium salt in bipolar disorder (Hou et al., 2016b; Song et al., 2016; International Consortium on Lithium et al., 2018) were downloaded from National Human Genome Research Institute–European Bioinformatics Institute (NHGRI-EBI) GWAS Catalog (Buniello et al., 2019) (https://www.ebi.ac.uk/). The latest and biggest GWA meta-analysis summary statistics for SCZ (53,386 cases and 77,258 controls) (Trubetskoy et al., 2022) and MDD except samples in the 23andMe dataset (59,851 cases and 113,154 controls) (Wray et al., 2018) were from PGC website. Non-rsID SNPs were converted using ANNOVA (Wang et al., 2010), and those without rsID were removed. Beta was computed by log (OR). The overall post-GWAS analysis pipeline is shown in **Figure 1**.

Single-Marker Analysis

PleioFDR Analysis

For genetic overlap, we used pleiotropy-informed conditional false discovery rate (pleioFDR) methods (Andreassen et al., 2013), including conditional FDR (condFDR), an extension of the standard FDR, and conjunctional FDR (conjFDR) analysis, defined in turn as the maximum of the two condFDR values. The pleioFDR provided a conservative estimate of the FDR associated

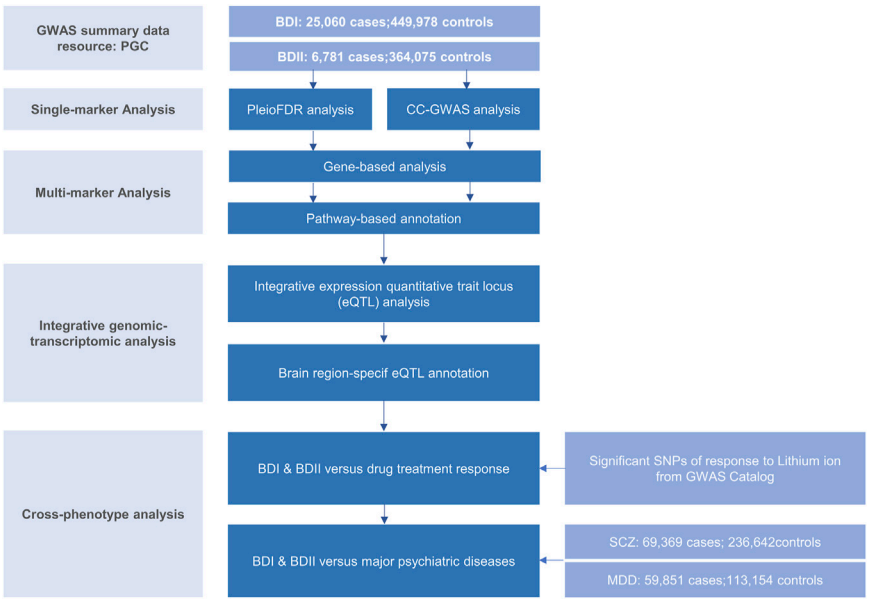

FIGURE 1 | Workflow of key methodological steps in this study. PGC: psychiatric genomics consortium, BD I: bipolar disorder type I, BD II: bipolar disorder type II, SCZ: schizophrenia, MDD: major depressive disorder.

with both phenotypes and was applied to identify specific shared loci. Based on an empirical Bayesian statistical framework, this statistical framework increased statistical power in detecting SNPs that did not reach genome-wide significance. Independent significant SNPs were defined with condFDR <0.01.

CC-GWAS Analysis

For genetic uniqueness, we applied the CC-GWAS method (Peyrot and Price, 2021). CC-GWAS perceives differences in minor allele frequencies (MAF) across two traits by analyzing case-control GWAS summary statistics for each other. It weighted the effect size using two methods, ordinary least squares (OLS) weights and exact weights. To avoid suspect null–null SNPs, SNPs with $p_{OLS} < 5 \times 10^{-8}$ were excluded. Then, those that failed to pass the required level of significance of CC-GWAS ($p_{EXACT} > 1 \times 10^{-4}$) were excluded to effectively control the type I error rate caused by suspect stress test SNPs.

Multi-Marker Analysis

Gene-Based Analysis

Independent genomic loci were mapped by shared and trait-specific SNPs from GWAS summary data of BD I (PGC), BD II (PGC), and CC-GWAS (this study) using ANNOVAR employed in functional mapping and annotation of GWAS [FUMA (Watanabe et al., 2017)] online platform (https://fuma.ctglab.nl/). Significant SNPs were first selected by LD $r^2 > 0.6$ within a 10 kb window. Second, we narrowed lead SNPs with LD $r^2 > 0.1$ with the same window. Genomic risk loci were identified by merging lead SNPs if they were closer than 250 kb, thus, containing multiple lead SNPs. The European samples retrieved from the 1,000 Genomes Project phase 3 (1000G EUR) (Genomes Project et al., 2015) were used to calculate LD. To further define independent genomic loci diverged in BD I and II, we utilized MAGMA v1.6 implemented in FUMA (Watanabe et al., 2017). Gene locations and boundaries were from the NCBI Build GRCh37 assembly.

Pathway-Based Annotation

Functional annotation was performed to uncover the likely biological mechanisms linking and distinguishing BD I and II. Enrichment for the genes mapped to all (candidate, genes nearest to lead and lead) SNPs in the identified shared loci was evaluated by the Molecular Signatures Database (MsigdB) *via* a hypergeometric test implemented in FUMA (Watanabe et al., 2017). Genes without unique Entrez ID or pathways containing less than two genes were removed. The results were adjusted by Benjamini–Hochberg false discovery rate (BH FDR) of 0.05.

Integrative Genomic-Transcriptomic Analysis

Integrative Expression Quantitative Trait Locus Analysis

To detect important but non-genome-wide significant sites, we first used summary-data–based Mendelian randomization (SMR) (Zhu et al., 2016) to estimate loci with strong evidence of causal effects of blood [eQTLGen Consortium (Vosa et al., 2021), 31,684 whole blood samples] and a large-scaled meta-data for brain resources [GTEx Consortium et al., 2017; Qi et al., 2018), CMC(Fromer et al., 2016) and ROSMAP (Ng et al., 2017), 1194 estimated effective sample] *via* gene expression in BD I and BD II risk. SMR analysis was limited to significant cis eQTL ($p_{eQTL} < 5 \times 10^{-8}$), with MAF >0.20, and passing heterogeneity in dependent instruments outlier (HEIDI-outlier) test ($p \geq 0.01$) due

to its conservativeness (Zhu et al., 2016; Zhu et al., 2018). Significant loci were filtered after multiple testing and within 1 MB distance from each probe.

Brain Region-specific eQTL Annotation

Likewise, we conducted brain-specific analyses using e-MAGMA (Gerring et al., 2021) and FUSION(Gusev et al., 2016), transcriptome-wide association studies (TWAS) to map genes based on precomputed tissue-specific eQTL statistics leveraging 13 brain tissues of GTEx v8 (Consortium 2020) and test whether SNPs influencing gene expression are associated with BD I and II. The 1000G EUR were used as a reference dataset to account for LD between SNPs. FDR correction was also applied to control the multiple tests performed on the numbers of genes in each process.

Cross-Phenotype Analysis

BD I and II Versus Lithium Treatment Responses

We then investigate bi-directional causal relationships between BD I and II and lithium salt response using the "two-sample MR" (Version 0.5.3) and "Mendelian randomization" (Version 0.5.1) R packages. We selected independent SNPs with p-value $< 5 \times 10^{-6}$ and harmonized exposure and outcome data by removing SNPs with large MAF differences or different reference alleles. For two-sample MR analysis, we used inverse variance weighted (IVW), weighted median, and MR-Egger as primary methods. MR-Egger intercept test and MR pleiotropy residual sum and outlier (MR-PRESSO) test (Verbanck et al., 2018) were used to evaluate potential horizontal pleiotropy.

BD I and II Versus Major Psychiatric Diseases

To explore genetic causal associations between BD I, BD II, SCZ, and MDD, we conducted a bi-directional MR analysis between each pair of the heritable variables using generalized summary-data–based Mendelian randomization (GSMR) (Yang et al., 2011). The estimated effect size and its standard error from multiple instrumental variants were associated with the exposure trait at a genome-wide significant level ($p < 5 \times 10^{-8}$). Attribute to insufficient instruments included in analyses, a p-value threshold of 5×10^{-5} was used. In GSMR, genetic instruments with pleiotropic effects are detected and eliminated by the HEIDI-outlier procedure, the same with SMR. We used default options in GSMR with HEIDI testing for the detection of instrumental outliers (LD $r^2 < 0.05$, and at least 10 SNPs were required).

Finally, MiXeR (Frei et al., 2019; Holland et al., 2020) was applied as a polygenic overlap analysis. Univariate models estimated polygenicity (estimated number of variants) and discoverability (the average magnitude of additive genetic associations across variants) of each phenotype. Bivariate Gaussian mixture models were also applied to estimate the number of variants influencing each trait that explained 90% of h^2_{snp} and their overlap with each other. MiXeR calculated a Dice coefficient, a ratio of shared variants to the total number of variants, to evaluate the polygenic overlap. In line with the Akaike information criterion (AIC), MiXeR evaluated model fitting based on the current power of input summary statistics.

RESULTS

Genetic Overlaps Between Bipolar Type I and II

For signals shared by BD I and BD II, the conjFDR analysis identified 74 significant SNPs ($p < 0.01$) that are mapped to three genomic loci (**Table 1**; **Supplementary Table S1**; **Figure 2A**): *ZNF184* (zinc finger protein 184), mapped by *rs67240003* (ALT:T, REF:G, MAF:0.044, $p_{FDR} = 5.26 \times 10^{-3}$) and *RPL10AP3* (ribosomal protein L10a pseudogene 3), mapped by rs6990255 (ALT:T,REF:C, MAF:0.042, $p_{FDR} = 7.54 \times 10^{-3}$). The third one is consistent with the newest BD GWAS: *SLC25A17* (solute carrier family 25 member 17), mapped by rs5758064 (ALT:C, REF:T, MAF:0.49, $p_{FDR} = 7.47 \times 10^{-3}$). The shared genomic loci, candidate independent SNPs, allelic association, and novelty for BD are summarized in **Supplementary Table S1**. The stratified conditional quantile–quantile (Q–Q) plots showed SNP enrichment for BD I condition on association with BD II and vice versa (**Supplementary Figure S1**), suggesting the existence of polygenic overlap.

Genetic Distinction of Bipolar Type I and II

CC-GWAS analysis was applied to the publicly available summary statistics for BD I and BD II. The only one CC-GWAS BD I versus BD II SNP was *rs12154473* (ALT:G, REF:A, MAF: 0.56), mapping *MAD1L1* (mitotic arrest deficient 1 like 1, $p_{OLS} = 2.83 \times 10^{-8}$; $p_{EXACT} = 6.07 \times 10^{-5}$; **Table 2**; **Supplementary Tables S2 and S3**; **Supplementary Figure S2**). The Manhattan plot of CC-GWAS results is shown in **Figure 2B**.

In the gene-based analysis, CC-GWAS displayed nine significant genes between BD I and II after multiple testing ($p < 0.05/18{,}626 = 2.68 \times 10^{-6}$). A total of 129 genes were significant for BD I ($p < 0.05/18{,}847 = 2.65 \times 10^{-6}$). *CACNA1C* (gene calcium voltage-gated channel subunit alpha1 C, $p = 2.80 \times 10^{-11}$), *MAD1L1* ($p = 7.56 \times 10^{-11}$), and *TMEM258* (transmembrane protein 258, $p = 9.48 \times 10^{-11}$), were the top three genes of BD I. The only significant gene of BD II ($p < 0.05/18{,}830 = 2.66 \times 10^{-6}$) was slit guidance ligand 3 (*SLIT3*, $p = 7.92 \times 10^{-9}$) (**Supplementary Tables S4 and S12**).

In the pathway analysis, 11, 6 and 1 pathways were significantly enriched by the genes through MAGMA analysis for BD I, BD II, and CC-GWAS summary statistics ($p_{Bonferroni} < 0.05$), respectively. As for BD I, the "neuron part", "somatodendritic compartment", "high voltage-gated calcium channel activity", and "voltage-gated calcium channel activity involved in cardiac muscle cell action potential" were gene ontology pathways verified by CC-GWAS and BD I. As for BD II, the only significant pathway was the "Hirsch cellular transformation signature up" ($p = 9.84 \times 10^{-5}$) (**Supplementary Table S5**).

TABLE 1 | Conjunction FDR; pleiotropic loci in BD type I (BD I) and BD type II (BD II) (BD I & BD II) at conjFDR <0.01.

Locus	CHR	SNP	Position	Neighbor gene	A1	A2	ConjFDR BD I&BD II	Zscore_BD I	Zscore_BD II
1	6p22.1	rs67240003	27443202	ZNF184	T	G	0.00526327	−4.74249	−4.3186
2	8p12	rs6990255	34126948	RPL10AP3	T	C	0.0075384	4.350196	4.21441
[a]3	22q13.2	rs5758064	41153879	SLC25A17	C	T	0.00747462	−4.74249	−4.21714

[a]*Same locus identified in previous BD genome-wide association studies.*

Independent complex or single gene loci ($r^2 < 0.2$) with SNP(s) with a conjunctional FDR (conjFDR) < 0.05 in BD I and BD II. All SNPs with a conjFDR value of 0.05 (bidirectional association) and association with BD I & BD II are listed and sorted in each LD block. We defined the most significant SNP in each LD block based on the minimum conjFDR. Chromosome (CHR), minor allele (A1), and major allele (A2), Z-scores for each pleiotropic locus are provided. All data were first corrected for genomic inflation. Locus name is based on exonic lead SNPs. Remaining locus name is based on the nearest gene and does not refer to any inferred biological function. Details are in ***Supplementary Table S1****.*

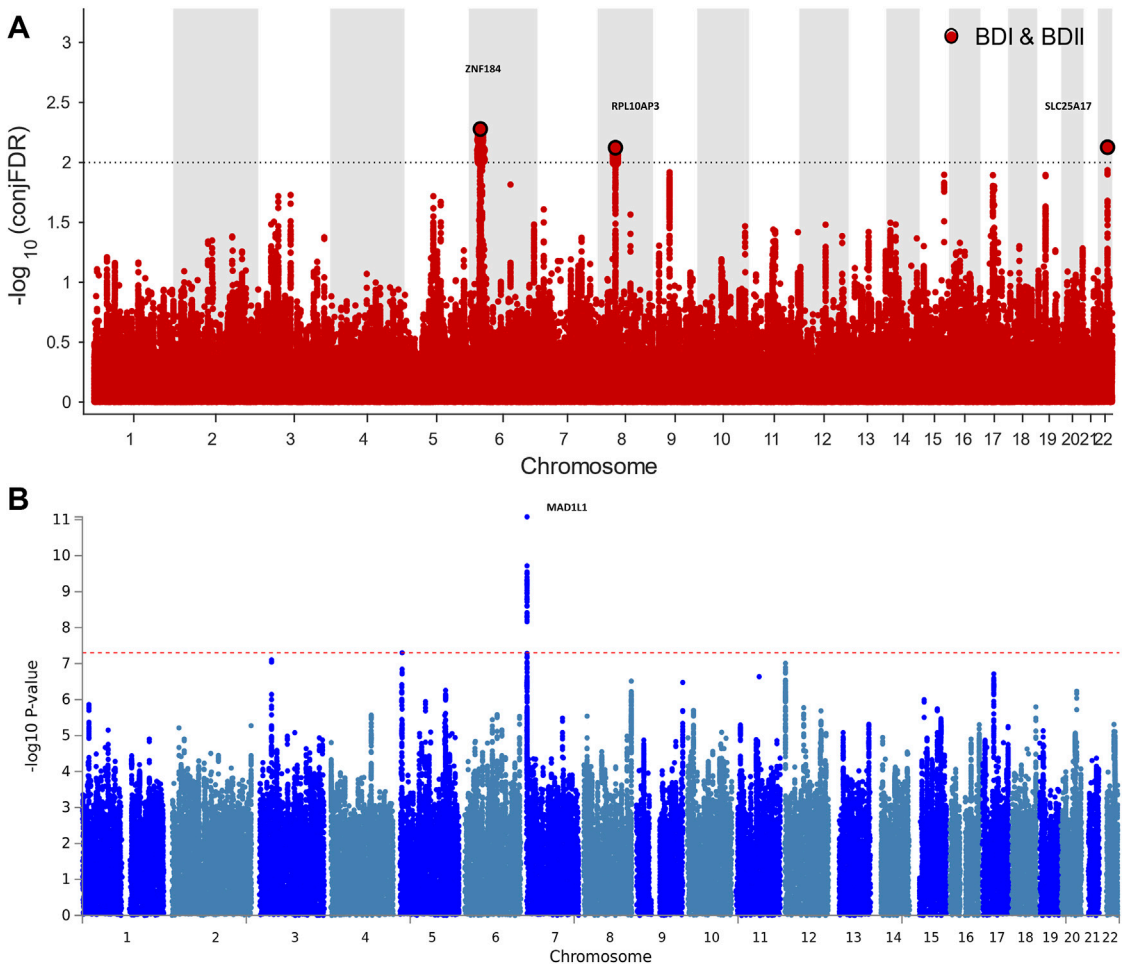

FIGURE 2 | Manhattan plots showing the association statistics for single marker analysis of BD I and II genetic overlap (**Figure 2A**) and distinctness (**Figure 2B**). The *y*-axis shows the GWAS −log10 *p*-values per SNP across chromosomes 1-22. **(A)** SNPs with conditional *p*-value < 1×10^{-2} are shown with large black points. **(B)** SNPs identified by case–case genome-wide association analysis (CC-GWAS) with $p < 5 \times 10^{-8}$. The figures show the localization of significant loci. Details about the loci are provided in **Tables 1** and **2**.

TWAS Analyses in Blood and Brain Regions

In the SMR process, 11 in brain ($p_{SMR} < 6.61 \times 10^{-6}$) and 49 in blood ($p_{SMR} < 3.19 \times 10^{-6}$) putative BD I-associated genes were identified after multiple testing corrections and a heterogeneity test. The top loci were *NMB* (neuromedin B) and *FADS1* (fatty acid desaturase 1) for BD I in blood and brain, respectively (**Supplementary Figure S3**). We did not observe significant results for BD II after multiple testing. The related genomic

TABLE 2 | Distinguished loci between BD I and BD II by CC-GWAS results at OLS <5 × 10^{-8} and EXACT <1 × 10^{-4}.

Disorders	SNP	CHR	Position	Locus	A1B1(OLS)			A1B1 (Exact)		
					Beta	Se	*p*	Beta	Se	*p*
BD I & BD II	rs12154473	7	1982181	MAD1L1	0.0117	0.00211	2.83E-08	0.0435	0.0108	6.07E-05

For the CC-GWAS–specific locus, the lead CC-GWAS SNP and its chromosome, physical position, locus name, respective case–control effect sizes and p-values and the CC-GWAS OLS and exact case–case effect size, standard error(se), and p-value. Details are in ***Supplementary Table S3****.*

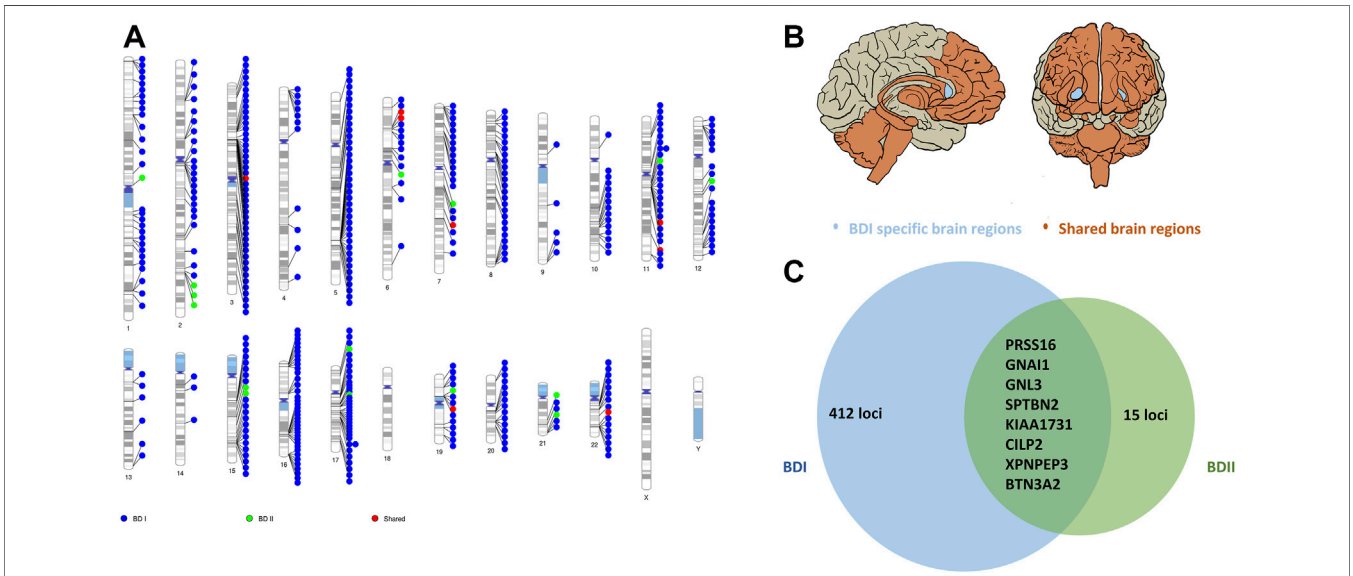

FIGURE 3 | Shared and trait-specific eQTLs of BD I and BD II. **(A)** Genomic regions that are specific to BD I (blue points), specific to BD II (green points), and shared (red points). **(B)** Associated 13 brain regions with BD I and BD II: cortex, frontal cortex, anterior cingulate cortex, caudate, putamen, hypothalamus, amygdala, hippocampus, substantia nigra, cerebellum, cerebellar hemisphere, spinal cord cervical c-1 (orange area), and BD I specific brain region: nucleus accumbens (light blue area). **(C)** Prioritized genes for shared and trait-specific regions. Genes presented reached significance (after FDR correction) in the TWAS test and were identified by two methods (FUSION and e-MAGMA). The numbers represent trait-specific genes identified by at least one method. Details are in **Supplementary Tables S8–S12**.

loci, candidate SNPs, and allelic association for BD I are summarized in **Supplementary Tables S6 and S7**.

In the brain region-specific TWAS analysis, e-MAGMA identified 148 loci (p_{FDR} <0.05) of BD I. *FADS1* (p_{FDR} = 1.87 × 10^{-7}), *PLEC* (plectin, p_{FDR} = 3.10 × 10^{-7}), and *ITIH4* (inter-alpha-trypsin inhibitor heavy chain 4, p_{FDR} = 3.10 × 10^{-7}) were the top ones. These genes encompassed three brain regions, including the hypothalamus, amygdala, and cerebellum (**Supplementary Table S8**). Similarly, FUSION indicated 336 genes (p_{FDR} <0.05, 9 hits of previous GWAS of BD achieved nominal significance) of BD I (**Supplementary Table S9**) among all the 13 brain regions.

As for BD II, two genes showed significant association with BD II by e-MAGMA after correction for multiple testing: *GNAI1* (G protein subunit alpha I 1 on chromosome 7, p_{FDR} = 2.64 × 10^{-7}) and *PRSS16* (serine protease 16 on chromosome 6, p_{FDR} = 1.40 × 10^{-5}). Both were over-expressed in the cerebellum (**Supplementary Table S10**). A total of 21 genes were significant by FUSION after multiple tests among brain regions without nucleus accumbens (NAc) (**Supplementary Table S11**). The top hit was AC005932.1 (p_{FDR} = 2.07 × 10^{-4}), a novel nominal significant gene for BD II (**Figure 3**; **Supplementary Figures S4 and S5**; **Supplementary Table S12**).

Correlations Between BD I and II and Response to Lithium

We selected 16 SNPs from the GWAS catalog mapped to "response to lithium ion". After selection and harmonization, ten SNPs were included in the final bi-directional Mendelian randomization between BD I and response to lithium ion. The estimate effect was positive and statistically significant in weighted median [beta = 1.89; standard error (SE) = 0.426; p = 9.57 × 10^{-6}] but not significant in IVW (beta = 2.22; SE = 1.66; p = 0.180). Nine SNPs were included in the analysis between BD II and response to lithium ion, showing negative results in IVW (beta = 3.27; SE = 2.28; p = 0.153) or weighted median (beta = 3.29; SE = 2.21; p = 0.136), even estimates were higher compared to BD I. Based on different hypotheses of whether there is a dose-response relationship between the shared genetic instruments of exposure and outcome with an intercept versus whether omitting each genetic variant from analysis differs from

the original model, MR-Egger and MRR-PRESSO displayed similar results for the heterogeneity test. The relationship between BD I and response to lithium failed to pass MR-Egger intercept analysis (MR-Egger intercept = 0.39; $p = 0.01$) or MR-PRESSO (p_{Global} test <0.001), indicating the possible existence of horizontal pleiotropy (**Supplementary Table S13**; **Supplementary Figure S6**).

Genetic Overlaps Between BD I and II and Other Traits

GSMR analyses provided evidence that genetically SCZ provided a 0.50-fold and 0.32-fold causality increase in BD I and BD II, respectively. Inversely, MDD increased causality with BD I and BD II by 0.23-fold and 1.11-fold, respectively. In the other direction, BD I provided 0.32-fold causal effect on SCZ (beta = 0.32, SE = 0.019, $p = 3.70 \times 10^{-62}$), comparing with less effect on MDD (beta = 0.043, SE = 0.018, $p = 1.36 \times 10^{-2}$). Since the number of SNPs ought to be over 10, p-value threshold was set to be 5×10^{-5} when BD II was computed into clumping as exposure trait. The causal relationship between BD II and SCZ (beta = 0.073, SE = 0.008, $p = 1.12 \times 10^{-21}$) were very close to BD II and MDD (beta = 0.050, SE = 0.008, $p = 7.71 \times\times 10^{-11}$). BD I (beta = 0.505, SE = 0.044, $p = 5.18 \times\times 10^{-31}$) and BD II (beta = 0.173, SE = 0.012, $p = 2.78 \times\times 10^{-50}$) were presented with causality with each other (**Supplementary Table S14**).

MiXeR estimated that approximately 7.88 k (SE = 0.26 k) variants influence BD I, which was comparable to the case of SCZ (9.82 k; SE = 0.22 k), lower than that for major depression (21.6 k, SE = 2.64 k) and 19.82 k (SE = 21.12 k) variants influenced BD II. The deficiency of sample size may explain the odd statistics in BD II. MiXeR also revealed a higher polygenicity in BD II and MDD than in BD I and SCZ. In BD I and BD II, 7.47 k (SE = 0.29 k) and 7.47 k (SE = 1.73 k) variants were associated with SCZ; 5.44 k (SE = 0.59 k) and 13.23 k (SE = 5.91 k) variants were associated with MDD, respectively. Consistent with LD score regression, MiXeR showed that BD I enjoyed higher genetic overlap with SCZ (rg = 0.70) than with MDD (rg = 0.39), and oppositely, BD II possessed higher genetic overlap with MDD (rg = 0.68) than with SCZ (rg = 0.61) (**Supplementary Figure S7**; **Supplementary Table S15**).

DISCUSSION

The present study is the first comprehensive post-GWAS analysis of BD I and II using the largest BD dataset (Mullins et al., 2021). Different from the original study that aimed to identify novel genes and drug targets using overall BD as the primary phenotype (Mullins et al., 2021), our integrative genomic analyses directly answered the following question: what are the shared and distinct genetic components of BD subtypes? In this study, we corroborated and expanded evidence from previous clinical and genetic studies that there did exist a partially shared genetic basis between BD I and II and provided further insights into their genetic divergence. When compared to other earlier studies (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Charney et al., 2017; Huckins et al., 2017; Mullins et al., 2021) on the same research question (**Supplementary Table S16**), our study is innovative from different aspects: 1) largest sample size for BD I and II; 2) more systematic statistical genetics analyses within BD itself and across major psychiatric disorders; and 3) new biological explanation to the distinction of BD subtypes.

For genetic convergence, all of these loci identified shared by BD I and II were previously reported to be associated with bipolar disorder, depression, ADHD, autism spectrum disorder, or schizophrenia (Cross-Disorder Group of the Psychiatric Genomics 2013; Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium, 2017; Ferguson et al., 2018; Wu et al., 2020), underpinning their contribution to mental disorder risk. *ZNF184* has been reported to be likely associated with subcortical volume (van der Meer et al., 2020), indicating a potential biological function in BD neurodevelopment (Valli et al., 2019). Research for links between mental and physical disorders is also proposed (Van Veldhoven 2010; Liu et al., 2017; Wain et al., 2017; Ferguson et al., 2018).

For genetic divergence, notably, *MAD1L1*, which was reported to be genome-wide significant in the two previous BD GWAS (Hou et al., 2016a; Ikeda et al., 2018) in Asian samples, nominally distinguished BD I and II in this study. This gene contributes to cell cycle control through the regulation of mitosis and has been shown to have a pleiotropic effect on psychosis and BD (Ruderfer et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Charney et al., 2017). Moreover, *MRM1* (mitochondrial rRNA methyltransferase 1), *ZNHIT3* (zinc finger HIT-type containing 3), *DHRS11* (dehydrogenase/reductase 11), and *GGNBP2* (gametogenetin binding protein 2) were first reported as significant in the gene-based test. *SLIT3* was identified to be BD II-specific by gene-based analysis. *SLIT3* was reported to increase schizophrenia susceptibility (Shi et al., 2004). Population difference (EUR vs. Han) and phenotype heterogeneity across countries may explain this interesting observation. *SLIT3* has also been shown to play a critical role in the formation and maintenance of the nervous system (Vargesson et al., 2001), indicating a generally shared genetic association among psychiatric disorders. However, it was not discovered to be associated with BD I, which indicated *SLIT3* could be a marker to distinguish BD II.

Enriched gene-sets of BD I were involved in neuronal and postsynaptic compartments as well as calcium channel activity, triggering presynaptic signaling, which reconfirmed cross-phenotype correlation across BD, SCZ, ASD, and cognitive deficiency (Pescosolido et al., 2013; Cupertino et al., 2016; Forstner et al., 2017; Bipolar, Schizophrenia Working Group of the Psychiatric Genomics Consortium. Electronic address et al., 2018). These pathway processes indicate that BD I primarily represents BD biological features and point to deeper research into common biological pathogenesis among mental disorders. In comparison, the BD II-specific pathway effects are generally linked with cancer and inflammatory and metabolic diseases (Hirsch et al., 2010), suggesting that larger

cohorts are required to provide a mechanic prompt for further research on BD II.

Interestingly, from the integrative Omics analysis, we found that *FADS1*, one of the three top eQTL-associated loci shared by both brain and blood, is presented with opposite directions of effect on gene expression in the two different tissues. This observation also suggests that *FADS1* possibly plays a role in the tissue-specific gene regulation of BD I. The possible reason was that *FADS1* is strongly associated with blood cell and lipid and glucose metabolite (Sabatti et al., 2009; Tintle et al., 2015; Tabassum et al., 2019; Chen et al., 2020),and is, thus, highly expressed in blood. Brain region-specific eQTL analysis yielded 15 genes specific for BD II. These eQTLs provide promising candidate genes for subsequent functional experiments, especially *NOS2* (Nitric oxide synthase 2) and *CASP8* (Caspase-8), participate in drug metabolism (Whirl-Carrillo et al., 2012), despite no correlation to psychosis was yet found. While several of these genes are implicated by genome-wide significant loci, many of them are not the closest gene to the index SNP, highlighting the value of probing underlying molecular mechanisms to prioritize the most likely causal genes in each corresponding locus and moving from genes to functional mechanism. However, most genes are not overlapped among gene-based and TWAS analyses due to different hypotheses on how SNPs affect gene expression (**Supplementary Table S12**).

In addition, BD I and BD II significantly differ in biosignatures as revealed by gene expression differentiation in functional brain regions and drug response in this study. Gene expression differentiation in NAc might represent an endophenotype of BD I addressing dysfunction of brain circuits. By regulating dopamine release and the midbrain dopamine system, NAc contributes to the onset of SCZ (Eastwood et al., 2005; Kozlovsky et al., 2006), especially for delusion and hallucination. It is also a contributor to the pathophysiology of BD, as shown in a postmortem brain analysis (Kunii et al., 2019). Even though we did not find direct causal relationships between BD I, BD II, and lithium response, there indeed exists a linkage with lithium response following the guidelines (Yatham et al., 2018): lithium was first-line to BD I, but not to BD II. Lithium is more effective for patients sharing etiological homogeneity; based on longitudinal stability and familial clustering, lithium response has been suggested to define a distinct genetically based BD nosology (International Consortium on Lithium et al., 2018). Therefore, biological indicators such as treatment response, clinical prognosis, and progression of BD I and BD II should be included in genetic analysis to enable improved precise clinical decision-making. It is also the RDoC standard that a combination of neuroscience research will be helpful for future genetic research, even altering clinical management (Insel et al., 2010).

Another interesting finding of this study is that, despite bidirectional causal associations from GSMR and mixed directional overlap from MiXeR, whether the causal relationships driven by other covariates (Yang et al., 2011) is unclear, and meanwhile, MiXeR analysis prompted a high clinical heterogeneity for BD I-BD II pair, when compared with BD I-SCZ or BD II-MDD pair. One explanation could be that BD I and II may help fill the gap across mental disorders by revealing transdiagnostic biotypes (Cross-Disorder Group of the Psychiatric Genomics Consortium. Electronic address and Cross-Disorder Group of the Psychiatric Genomics 2019). Insights into such continuous genetic structure, rather than completely independent disease entities, may greatly contribute to clinical decision-making in prophylaxis or management of the disorder. The findings of this study will also trigger larger studies on BD II and other biotypes, such as psychosis bipolar disorder and cyclothymia, because current BD GWAS mainly reflected the genetic characteristics of BD I, the majority of overall BD cases. Although a large sample size of GWAS is always important in nowadays genomic studies, the statistical power will be greatly reduced when there is nonnegligible clinical heterogeneity caused by the classification system within disease phenotype (Mitchell et al., 2021). Therefore, as RDoC emphasized, large-scale transdiagnostic investigations are urgently needed to untangle whether impairment or symptoms can be regarded as subtype-specific, and so do multi-omics analysis (Cuthbert 2020).

One of the potential limitations of our study is that the population imbalance of BD I and BD II GWAS may be susceptible to reduced power; however, it did not lead to inflated type I error in our post-GWAS analysis. Another limitation is that we failed to achieve individual genotypes, leading to the incompleteness of some important analyses, such as polygenic risk score (PRS) calculation. Finally, our study only obtained GWAS summary statistics of BD I and BD II, lacking data from other BD subtypes. Once other characteristics of clinical subtypes (psychotic symptoms) are available in PGC or other groups, further refined genetic architecture for BD should be explored by a more systematic comparison in future.

In summary, genetic evidence deepens our understanding of the biological etiology of BD and prioritizes a set of candidate genes distinguishing BD I and II for functional follow-up experiments and indicates a spectrum connecting psychiatric disorders, which enable better ways to optimize nosology and precise treatments in psychiatry.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study were derived from the following resources available in the public domain: https://www.med.unc.edu/pgc/download-results/.

AUTHOR CONTRIBUTIONS

HG and QW have full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. YH, YL, and YW are joint first authors, and HG and QW are corresponding authors. HG and QW designed this

work. YH, YL, YW, YT, SL, LX, and MZ acquired and analyzed GWAS summary data and interpreted the results of the data analyses. YH, YL, and YW drafted this manuscript. ST, MX, MD, ML, HG, and QW substantively revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.956265/full#supplementary-material

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Association Between Polymorphisms in Estrogen Receptor Genes and Depression in Women: A Meta-Analysis

Cuifen Li[1†], Manli Xie[2†], Weiwei Wang[5], Yanyan Liu[1], Dan Liao[1], Jingwen Yin[4] and Hao Huang[3*]*

[1]Department of Gynecology, Songshan Lake Central Hospital of Dongguan City, Dongguan, China, [2]Department of Obstetrics and Gynecology, Qingxi Hospital of Dongguan City, Dongguan, China, [3]Department of Obstetrics and Gynecology, Affiliated Hospital of Guangdong Medical University, Zhanjiang, China, [4]Department of Psychiatry, Affiliated Hospital of Guangdong Medical University, Zhanjiang, China, [5]Affiliated Hospital of Guangdong Medical University, Zhanjiang, China

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***Correspondence:**
Jingwen Yin
amysays@live.com
Hao Huang
hao_huang@gdmu.edu.cn

[†]*These authors have contributed equally to this work*

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Objective: It is suggested that estrogen receptors (ERs) might be associated with the disproportionate vulnerability of women to depressive episodes. Several variants in ER-alpha (ERα) and ER-beta (ERβ) have been linked to depression, but the results were not consistent. Hence, we conducted a meta-analysis to evaluate the association between ERα/ERβ and depression in a cohort of women.

Methods: A comprehensive literature search was performed in public databases. The genetic association between polymorphisms in Erα/ERβ and depression risk in a cohort of women was evaluated by odds ratios (ORs) and 95% confidence intervals (CIs). Cochran's Q test and the I^2 index were used to evaluate heterogeneity.

Results: In total, 10 studies and 4 SNPs (rs2234693, rs9340799, rs4986938, rs1256049) were included in our meta-analysis. rs2234693 genotype was significantly associated with the risk of depression in women by dominant model (CC + CT vs TT, OR = 1.30, 95% CI: 1.09–1.55, $p = 0.0031$), recessive model (CC vs CT + TT, OR = 1.64, 95% CI: 1.00–2.67, $p = 0.0478$), additive model (CC vs TT, OR = 1.93, 95% CI: 1.12–3.35, $p = 0.0189$) and allelic model (C vs T, OR = 1.24, 95% CI: 1.10–1.39, $p = 0.0003$). For rs9340799, the frequencies of risk genotypes according to the dominant (GG + GA vs AA, OR = 1.47, 95% CI = 1.10–1.98, $p = 0.0096$, $I^2 = 0\%$, $p = 0.43$) and allelic (G vs A, OR = 1.33, 95% CI: 1.04–1.69, $p = 0.0236$, $I^2 = 0\%$, $p = 0.39$) models were significantly lower in women with depression than in controls within the Asian subgroup. For rs1256049, risk genotypes were significantly more frequent in depressed subjects than in controls under the dominant model (AA+ GA vs GG, OR = 1.62, 95% CI: 1.19–2.21, $p = 0.0024$) and the allelic model (A

Abbreviations: DSM-IV, Diagnostic and Statistical Manual of Mental Disorders-4th edition; ICD, international classification of diseases; BDI, beck depression inventory; HAMD, hamilton rating scale for depression; SDS, Self-rating Depression Scale; CCMD-3, chinese classification and diagnostic criteria of mental disorders third edition; SCID, Structured Clinical Interview for DSM, disorders; EPDS, edinburgh postnatal depression scale; MADRS, Montgomery-Asberg Depression Rating Scale; MINI, international neuropsychiatric interview; HWE, Hardy–Weinberg equilibrium; CES-D, Centre for Epidemiology Studies–Depression Scale; PCR, polymerase chain reaction; RFLP: Restriction Fragment Length Polymorphism. KASPar, KBioscience's competitive allele-specific PCR, amplification of target sequences and endpoint fluorescence genotyping.

vs G, OR = 1.35, 95% CI: 1.07–1.72, $p = 0.012$) after sensitivity analysis by omitting one study which induce the heterogeneity.

Conclusions: The current meta-analysis is the first and most comprehensive investigation of the association between ERs and depression in women, and the findings support the concept that ERs participate in the etiology of sex heterogeneity in depression.

Keywords: ERα, Erβ, polymorphism, women, depression

1 INTRODUCTION

Depression is characterized by a persistent depressed mood and/or loss of pleasure in activities (Greenberg et al., 2012). There is a notable sex difference in the epidemiology of depressive episodes, with a higher prevalence in women than in men (Parker and Brotchie, 2010) from early life through the mid-50s, especially among women in the menopausal and postpartum periods (Blazer, 2003; Pearlstein, 2015; Sassarini, 2016). This observation indicates that the factors involved in the production and regulation of steroid hormones might be involved in the sex heterogeneity of depression.

There is a great deal of evidence that the sex heterogeneity of depression is largely due to estrogens and estrogen receptors (ERs) (Schuit et al., 2005; Sundermann et al., 2010; Nalvarte et al., 2021). Withdrawal of estrogens plays a key role in the onset of depression in animal models (Stoffel and Craft, 2004). Clinical evidence also indicates that women in the postpartum and menopausal periods show increased vulnerability to depression due to their drastically reduced estrogen levels (Sassarini, 2016). Hormone replacement therapy has been shown to have the benefit of preventing depression (Grigoriadis and Kennedy, 2002; Dwyer et al., 2020). Moreover, estrogen is involved in the regulation of multiple neural molecular processes and neurotransmitters that have been strongly implicated in affective disorders (Hwang et al., 2020); for example, processes and systems associated with estrogen include neurodevelopment, neurodegeneration, synaptic plasticity, neuroinflammation, dopamine signaling, the serotonin (5-HT) system and the hypothalamic–pituitary–adrenal (HPA) axis (Ostlund et al., 2003; Varshney et al., 2017; Baghel and Srivastava, 2020; Hwang et al., 2020; Brand et al., 2021; Varshney et al., 2021). Estrogen may play a key regulatory role in "windows of vulnerability" to depression in women (Schiller et al., 2015).

The cellular effects of estrogens are mainly due to the activation of two estrogen receptors, known as ER alpha (ERα) and ERβ (Heldring et al., 2007). These two receptors belong to class 1 of the superfamily of nuclear hormone receptors (Sundermann et al., 2010). Both ERα and ERβ are expressed throughout the brain (Enmark et al., 1997), especially in brain regions associated with core deficiencies in depression, such as cognitive function and emotion (González et al., 2007). ERα is predominantly expressed in the hypothalamus and amygdala, which indicates that this receptor may be involved in the regulation of autonomic function and emotional regulation. ERβ is predominantly expressed in the hippocampal formation and entorhinal cortex and thalamus, indicating that this receptor may modulate cognitive function, memory and motor functions (Osterlund and Hurd, 2001). Thus, there is evidence that ERs might underlie the neuropathology of psychiatric disorders such as depression.

To date, several variants in these two receptors have been associated with depression, especially in women; these variants include rs2234693, rs9340799, rs4986938 and TA repeat in ERα as well as rs1256049 and rs2077647, rs1271572 in Erβ (Tsai et al., 2003; Kim et al., 2010; Ryan et al., 2012; Pinsonneault et al., 2013; Zhang et al., 2014; Kang et al., 2015; Różycka et al., 2016; He et al., 2017; Zhang et al., 2017; Tan et al., 2018). However, such results have not been consistently reported in population-based studies. The generalizability of these results is limited by the small sample size, which may lead to insufficient statistical power to detect a true effect. It is necessary to combine sample sizes through a meta-analysis in order to more precisely identify the genetic association of ERα/ERβ variants with depression in women.

2 MATERIALS AND METHODS

The present study was performed and prepared in accordance with the guidelines proposed by the Cochrane Collaboration in the Cochrane Handbook for Systematic Reviews of Interventions (http://www.cochranehandbook.org) and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Liberati et al., 2009).

2.1 Search Strategy and Inclusion/Exclusion Criteria

A literature search was performed in PubMed, Science Direct, Wiley Online Library, the Cochrane Library, Google Scholar, and the Chinese National Knowledge Infrastructure (CNKI) from inception to 2 March 2022. No limitations were imposed on publication year or language. The search terms used to identify studies were a combination of terms identifying the genes ('ERα', 'ER1', 'ESRα', 'ESR1', 'estrogen receptor 1' and 'estrogen receptor alpha' or 'ERβ', 'ER2', 'ESRβ', 'ESR2', 'estrogen receptor 2' and 'estrogen receptor beta') and terms identifying the phenotypes of interest ('MDD', 'depression'). Studies that met the following criteria were included in the analysis: 1) the study was performed on adult women (≥18 years old) with a diagnosis of depression; 2) the study reported data from at least one independent sample; and 3) the study provided sufficient data to calculate the odds

TABLE 1 | Assessment of the risk of bias in the pooled studies.

Study	Year	Clearly stated objective and hypothesis	Clear eligibility criteria for participants	Information bias in genotyping	Selection bias	Information bias in assessment of environmental factors	Information bias in assessment of depression	Restrictions on the ethnicity of participants	Replicable statistical methods	Assessment of HWE	Sufficient descriptive data	Stated genotype frequencies
Tan et al	2018	Yes	Yes	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes
He et al	2017	Yes	No	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes
Zhang et al	2017	Yes	Yes	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes
Różycka et al	2016	Yes	No	QC	No	NA	Without diagnostic criteria	Yes	Yes	Yes	Yes	Yes
Kang et al	2015	Yes	Yes	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes
Zhang et al	2014	Yes	Yes	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes
Pinsonneault et al	2013	Yes	Yes	QC	No	NA	No	No	Yes	Yes	Yes	Yes
Ryan et al	2012	Yes	Yes	QC	Women aged 70–77	NA	No	Yes	Yes	Yes	Yes	Yes
Kim et al	2010	Yes	Yes	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes
Tsai et al	2003	Yes	Yes	QC	No	NA	No	Yes	Yes	Yes	Yes	Yes

QC, details given of quality control procedures; NA, not available; HWE, Hardy-Weinberg equilibrium.

ratios (ORs) and their 95% confidence intervals (CIs). Studies were excluded if 1) they were performed on youth under 18 years old or on men, or 2) there was no control group. The reference lists of all primary studies were also reviewed. For multiple publications covering the same sample, the one with the most complete and recent report was included.

2.2 Data Extraction and Quality Assessment

Two reviewers independently extracted the data and study characteristics, including the following: first author name and publication year, study design, participant information (population, ethnicity, sample size, mean age, sex), diagnostic criteria, assessment, genotyping methods, genotype and allelic distribution.

2.3 Risk-Of-Bias Assessment

A quality assessment was performed according to the guidelines laid out in the HuGE Review Handbook (Little et al., 2008). We applied a checklist designed to assess the quality and risk of bias in human genomic epidemiological studies, as described in a previous study (Elwood et al., 2019). The checklist included the following items: a clearly stated objective and hypothesis, clear eligibility criteria for participants, information bias in genotyping, selection bias, information bias in assessment of environmental factors, information bias in assessment of depression, restrictions on the ethnicity of participants, replicable statistical methods, assessment of Hardy-Weinberg equilibrium (HWE), sufficient descriptive data, and stated genotype frequencies. The assessment was conducted by two reviewers, and discrepancies were resolved by consensus. The results of the assessment are shown in **Table 1**.

2.4 Statistical Analysis

HWE was tested using Pearson's chi-squared test. Four genetic models were used in the meta-analysis: the dominant model, the recessive model, the additive model and the allelic model. ORs with 95% CI were used to assess the different distributions of ER gene polymorphisms in the depression and control groups within this cohort of women. Cochran's Q test and I^2 index based on a chi-square distribution were applied to estimate heterogeneity. I^2 ranges from 0 to 100%. Low, moderate, high and extreme heterogeneity correspond to0-25%, 25–50%, 50–75% and75-100%, respectively (Liberati et al., 2009). If the result of the Q test was $p > 0.05$ and $I^2 < 50\%$, the Mantel–Haenszel method was used to estimate the pooled odds ratio, assuming a fixed effects model. Otherwise, ORs were pooled according to a random-effects model (DerSimonian and Laird). Sensitivity analysis for the overall effect was performed by omitting one study at a time. Potential publication bias was evaluated by Begg's funnel plots, Egger's (Egger et al., 1997) and Peters' (Peters et al., 2006) regression tests. The two-tailed p values were used in all analyses, and $p < 0.05$ was regarded as statistically significant. The statistical analyses were performed using the *meta* package in R version 3.6.2 (R Core Team, Vienna, Austria) and R Studio version 1.2.1 (Certified B Corporations, Boston, United States).

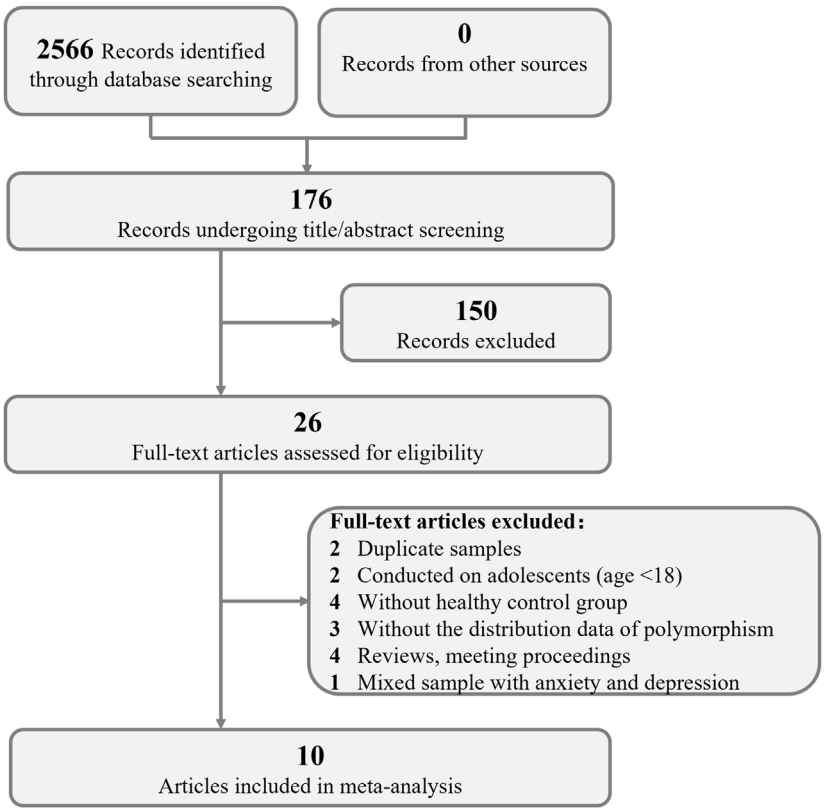

FIGURE 1 | PRISMA flow chart of the inclusion and exclusion of studies. PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

3 RESULTS

In total, 2566 articles were obtained from the initial search. The flow diagram of the process of selection is illustrated in **Figure 1**. A total of 176 studies were screened based on their titles, abstracts and contexts; 26 studies passed this screening. These 26 studies were evaluated by reading the full-text articles; at this stage, 15 studies were excluded because they were duplicate records, meeting proceedings, reviews or because they had insufficient data, no control groups or were conducted on adolescents. One study was excluded because it used a mixed sample of patients with menopausal anxiety and patients with depression (Zhou et al., 2012). Ultimately, data from 10 studies were included in our meta-analysis of the roles of ERα and ERβ in susceptibility to depression in women (Tsai et al., 2003; Kim et al., 2010; Ryan et al., 2012; Pinsonneault et al., 2013; Zhang et al., 2014; Kang et al., 2015; Różycka et al., 2016; He et al., 2017; Zhang et al., 2017; Tan et al., 2018). Three of these studies were conducted in Caucasians, and 7 were conducted in Asians. Data on 7 different SNPs were collected, four of which were reported in 3 independent samples and thus included in our meta-analysis (rs2234693, rs9340799, rs4986938, rs1256049). The data on rs2234693 in Kim's (Kim et al., 2010) study and rs2077647 in Zhang's (Zhang et al., 2014) study were excluded from the meta-analysis because the genotype distribution in the control group showed deviation in the HWE test. rs2077647 and rs1271572 in ERβ and TA repeat in ERα were not included in the meta-analysis due to the insufficient number of studies. The main characteristics of these studies are presented in **Table 2**.

For rs2234693 in ERα, we collected 4 studies reporting the genotype and allelic distributions in 752 female depression patients and 4247 controls. A significant pooled OR was found in the dominant model (CC + CT vs TT, OR = 1.30, 95% CI: 1.09–1.55, p = 0.0031 under a fixed-effects model), the recessive model (CC vs CT + TT, OR = 1.64, 95% CI: 1.00–2.67, p = 0.0478 under a random-effects model), the additive model (CC vs TT, OR = 1.93, 95% CI: 1.12–3.35, p = 0.0189 under a random-effects model) and the allelic model (C vs T, OR = 1.24, 95% CI: 1.10–1.39, p = 0.0003 under a fixed-effects model) (**Figure 2A**). The heterogeneity analysis showed high heterogeneity in the recessive model (I^2 = 60%, p = 0.06) and the additive model (I^2 = 61%, p = 0.05). The sensitivity analysis was performed, and the data showed that no individual study qualitatively altered the pooled ORs in the four models (**Figure 2B**). This indicated the stability of the result. The shape of the funnel plots showed no publication bias (**Supplementary Figure S4**, **Supplementary Table S1**).

For the meta-analysis of rs9340799 in ERα, we collected 6 studies with 960 female depression patients and 4619 controls

TABLE 2 | Characteristics of studies investigating polymorphisms of ERα and ERβ in women with depression.

Study	Year	Country	Ethnicity	Genotyped SNPs	Diagnostic criteria and assessment	Disease	Methods	HWE(*p*)
Tan et al	2018	Singapore	Asian	rs2234693, rs9340799, rs4986938, rs2077647, TA repeat	DSM-IV, EPDS	Perinatal depression	PCR-RFLP	>0.05
He et al	2017	China	Asian	rs2234693, rs9340799, rs4986938, rs1256049	SDS, HAMD, CCMD-3	Perinatal depression	PCR-PFLP	>0.05
Zhang et al	2017	China	Asian	rs4986938, rs1256049	DSM-IV, HAMD	Depression	PCR	>0.05
Różycka et al	2016	Poland	Caucasian	rs9340799	HAMD	Menopausal Depression	PCR-RFLP	>0.05
Kang et al	2015	China	Asian	rs4986938, rs1256049, rs1271572	BDI, DSM-IV	Perimenopausal depression	SNaPshot	>0.05
Zhang et al	2014	China	Asian	rs1256049, rs2077647	BDI, DSM-IV	Postpartum depression	PCR	<0.05 for rs2077647
Pinsonneault et al	2013	Canada	Caucasian (91%), with 2% Asian, 2% Hispanic and 4% other	rs2077647, TA repeat	DSM-IV, EPDS, MADRS	Postpartum depression	SNapShot,PCR-PFLP	>0.05
Ryan et al	2012	Frence	Caucasian	rs2234693, rs9340799	DSM-IV, CES-D	Menopausal depression	KASPar	>0.05
Kim et al	2010	Korean	Asian	rs2234693, rs9340799, rs4986938, rs1256049	DSM-IV, BDI	Post-menopausal depression	PCR-RFLP	<0.05 for rs2234693
Tsai et al	2003	China	Asian	rs2234693, rs9340799	DSM-IV, HAMD	Depression	PCR	>0.05

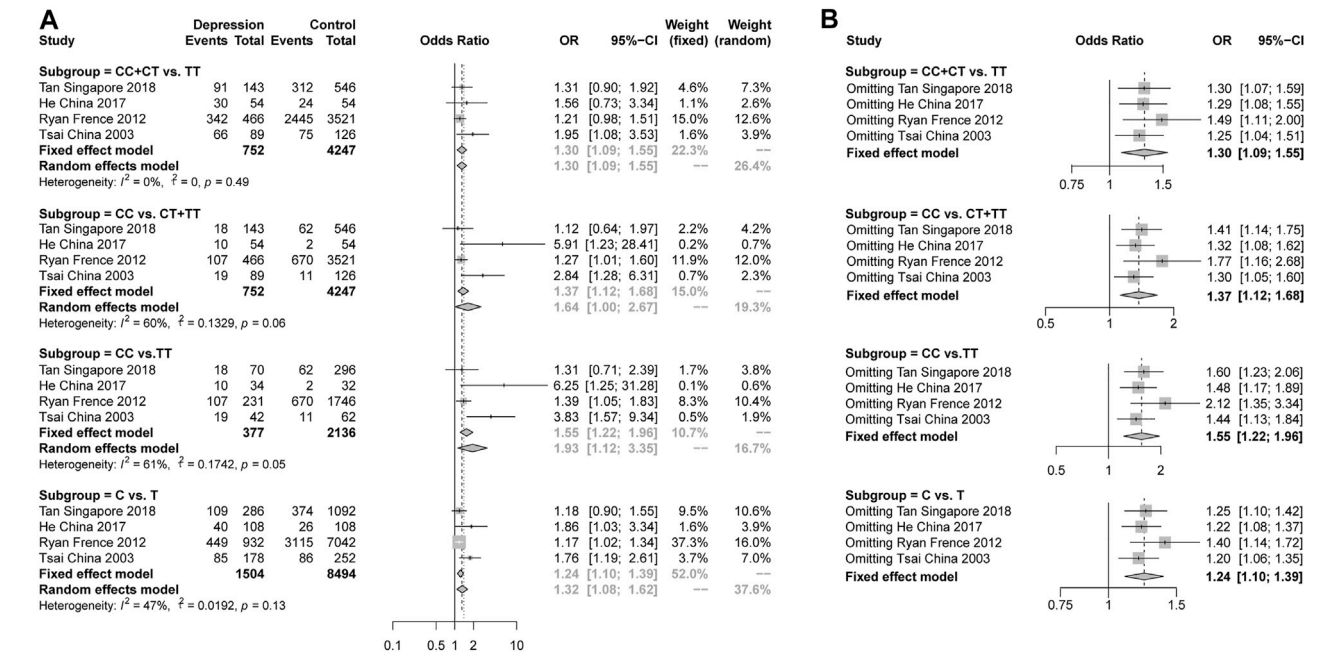

FIGURE 2 | (A), Forest plot of studies comparing the risk of the rs2234693 polymorphism of ERα between women with depression and controls; **(B)**, Meta-analysis with a fixed-effects model with individual studies omitted.

to calculate the pooled OR. In the results, a slightly significant difference was found in the recessive model (GG vs GA + AA) under a fixed-effects model (OR = 1.26, 95% CI: 1.01–1.58, p = 0.0405) with low heterogeneity (I^2 = 0%, p = 0.55) (**Figure 3**). There was no significant difference and high heterogeneity shown in the dominant model, the additive model and the allelic model (**Figure 3**). Due to the high heterogeneity, the pooled sample was then evaluated using stratification analysis of ethnicity. In the Asian subgroup, there were 4 studies with 327 female depression patients and 784 controls pooled in the

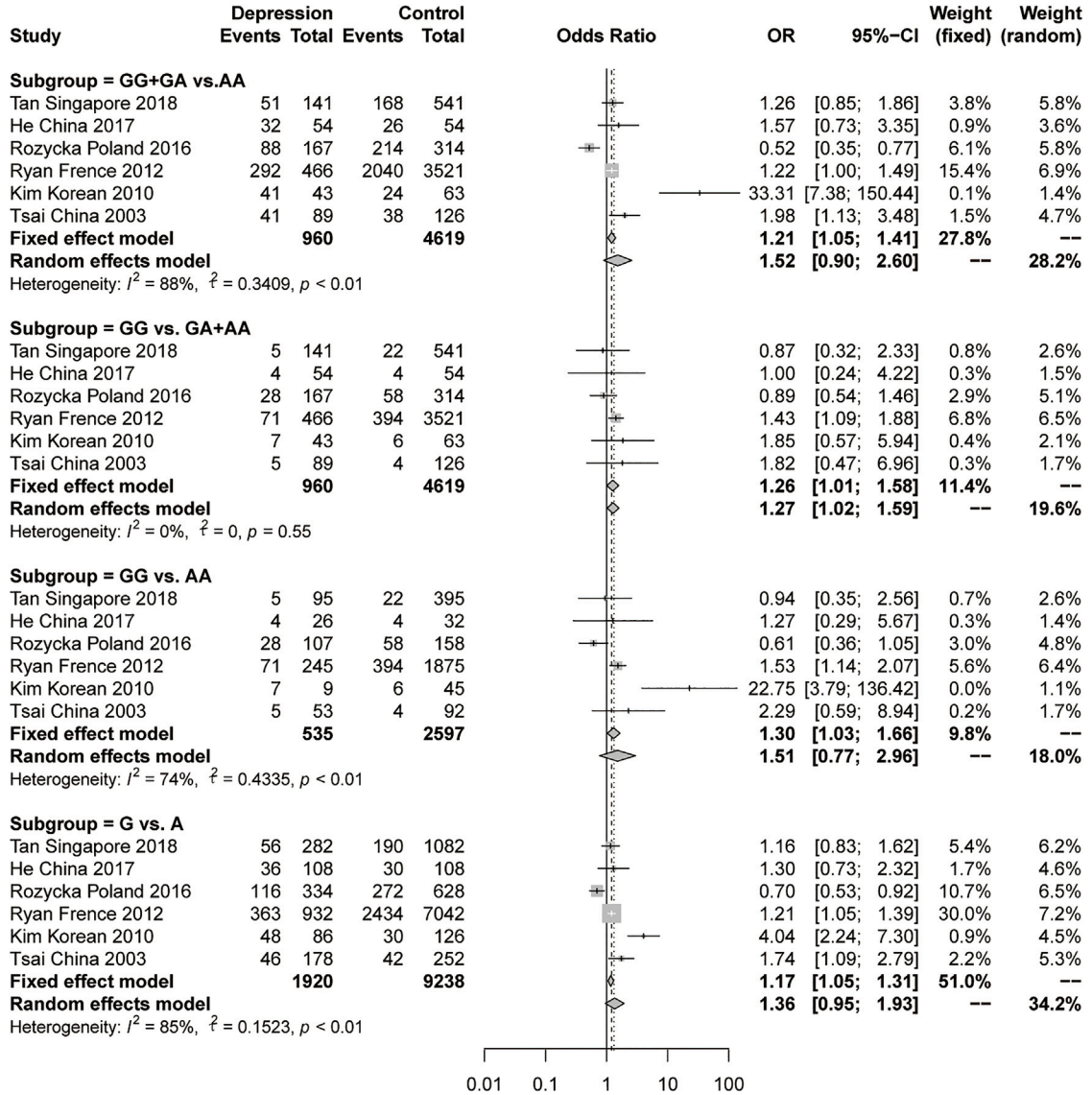

FIGURE 3 | Forest plot of studies comparing the distribution of the rs9340799 polymorphism of ERα between women with depression and controls.

analysis. Significant differences were observed in the dominant model (GG + GA vs AA, OR = 2.53, 95% CI = 1.10–5.83, p = 0.0287, I^2 = 84%, p < 0.01) and allelic model (G vs A, OR = 1.76, 95% CI = 1.05–2.93, p = 0.0307, I^2 = 78%, p < 0.01) under the random-effects model, but still with a large heterogeneity (**Figure 4A**). The sensitivity analysis was then performed, and the results showed that omitting Kim's study could dramatically decrease the heterogeneity to 0% in all four models. A significant difference persisted in the dominant models (GG + GA vs AA, OR = 1.47, 95% CI = 1.10–1.98, p = 0.0096, I^2 = 0%, p = 0.43) and allelic model (G vs A, OR = 1.33, 95% CI: 1.04–1.69, p = 0.0236, I^2 = 0%, p = 0.39) under the fixed-effect model, but the same was not true of the recessive model or the additive model (**Figure 4B**). There was no significant difference in the Caucasian subgroup, with a pooled sample size of 633 female depression patients and 3835 controls (**Supplementary Figure S1**).

There were 5 studies genotyped rs1256049 in the ERβ gene in 388 female depression patients and 504 controls. There were no significant differences according to any of the four genetic models in the initial analysis (forest plots are shown in **Supplementary Figure S2**). Sensitivity analysis was performed to trace the sources of heterogeneity because of the high heterogeneity in the dominant model (AA+ GA vs GG, I^2 = 59%, p = 0.05) and allelic model (A vs G, I^2 = 56%, p = 0.06). The most effective reduction in heterogeneity (to 14% and 15%, respectively) emerged when Kang's study was omitted (Kang et al., 2015). Then, significant differences were observed in the dominant model (AA+ GA vs GG, OR = 1.62, 95% CI: 1.19–2.21, p = 0.0024, fixed-effects model) and

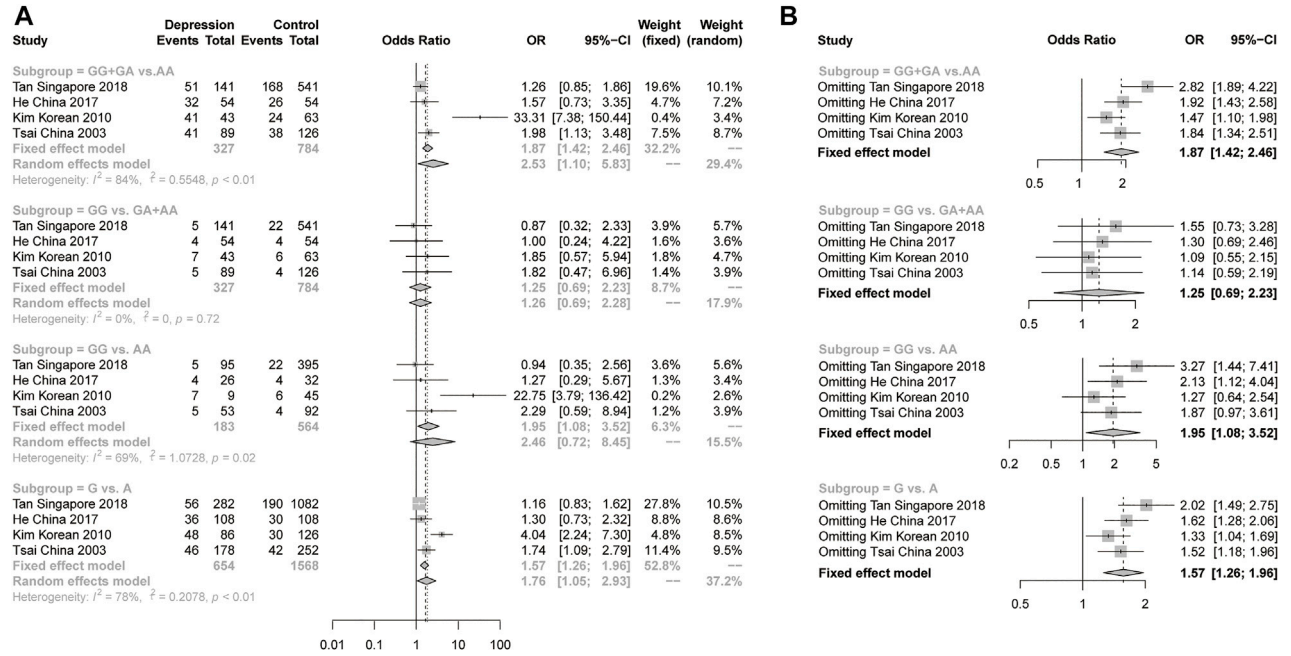

FIGURE 4 | (A), Forest plot of studies comparing the distribution of the rs9340799 polymorphism of ERα between women with depression and controls in the Asian subgroup. **(B)**, The sensitivity analysis of the studies pooled in the meta-analysis of rs9340799 polymorphism and women with depression in Asian subgroup by omitting one study at a time.

the allelic model (A vs G, OR = 1.35, 95% CI: 1.07–1.72, p = 0.012, fixed-effects model) (**Figure 5**). There was no significant difference in the recessive model or the additive model of rs1256049.

The meta-analysis failed to find an association between depression susceptibility and the rs4986938 polymorphism (**Supplementary Figure S3**) in women cohort. The shape of Begg's funnel plots and the results of Egger's (Egger et al., 1997) and Peters' (Peters et al., 2006) regression tests showed no publication bias in any of the analyses (**Supplementary Figure S4**, **Supplementary Figure S5**, **Supplementary Figure S6**, **Supplementary Figure S7**, **Supplementary Table S1**).

4 DISCUSSION

According to recent studies, ERα and ERβ polymorphisms are believed to be closely involved in sex-specific clinical symptoms and outcomes in women with depression (Hernández-Hernández et al., 2019). This suggests that investigation of ER genes and their functions might be important for understanding the pathophysiological mechanism of sex-specific depression risk. The present meta-analysis aggregated a large, population-based sample of female depression patients and controls to examine associations between polymorphisms in ER genes and sex-specific depression. A total of 10 case–control studies involving 1323 female depression patients and 5051 controls were pooled in the meta-analysis.

The rs2234693 polymorphism is located in the first intron of ERα and has been reported to impact the response of the ERα gene to estrogen by altering transcription factor binding (Herrington et al., 2002). Many reports have revealed that the rs2234693 polymorphism is a susceptibility factor for the onset of depression in women. The results from our meta-analysis provide evidence that rs2234693 of ERα was significantly associated with the risk of depression in women in four genetic models. There was a consistent tendency for the C allele of rs2234693 to be associated with an increased risk in women with depression in the pooled studies. Tsai et al. (Tsai et al., 2003) first reported the significant association of rs2234693 in female depression patients and was then identified in the Chinese population by He's study (He et al., 2017). Tan's (Tan et al., 2018) and Ryan's (Ryan et al., 2012) studies also showed a higher frequency of the C allele in the depression group than in the control group. Moreover, a significant difference remained in the subgroup of Asians. The preponderance of the evidence confirms an association between the rs2234693 polymorphism and depression in women.

The ERα rs9340799 polymorphism has been widely studied, but conflicting results have been reported, and high heterogeneity was found in our meta-analysis. The variations and heterogeneity are particularly relevant in genetic heterogeneity in ethnicity. Therefore, subgroup analyses based on ethnicity were conducted in the present meta-analysis. In the Asian subgroup specifically, the G allele showed a tendency to be a risk factor for depression in women, and significant ORs were reported in the dominant model and the allelic model of rs9340799, albeit with high heterogeneity. The high heterogeneity might derive from the unstable allelic frequency, which was induced by the limitation of the sample size in individual studies. For instance, in the patient

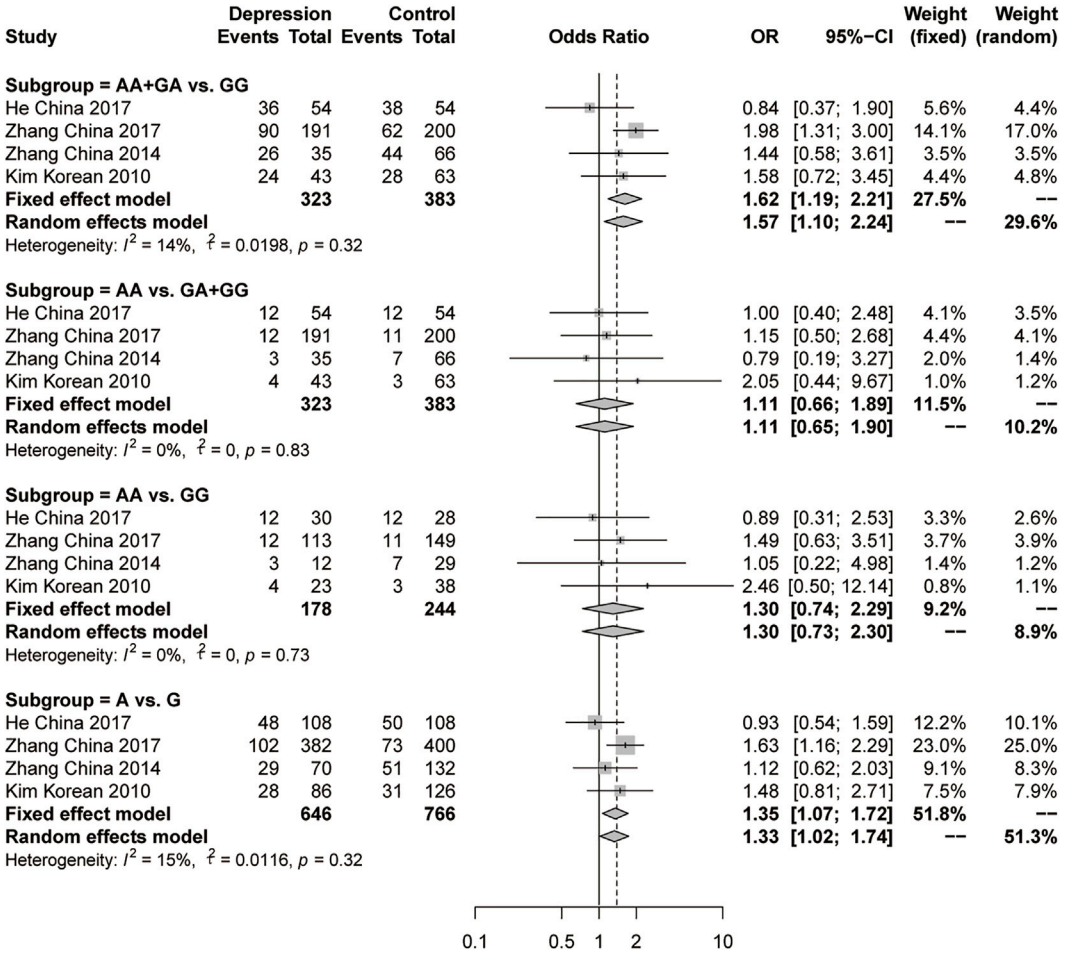

FIGURE 5 | Forest plot of studies comparing the distribution of the rs1256049 polymorphism of ERβ between women with depression and controls after the omission of Kang's study.

group of a Korean sample with 43 female depression patients and 63 controls, Kim et al. (Kim et al., 2010) noted a higher frequency of the G allele than other studies. Omitting Kim's study could effectively reduce the heterogeneity (I^2 = 0%). The results were still significant in the pooled sample from the three remaining studies. In the Caucasian subgroup, we failed to find any significant difference in the pooled data from two studies. Collectively, the G allele of the rs9340799 polymorphism is a potential risk allele for depression in Asian women.

rs1256049 is a silent mutation in exon 5 of ERβ gene. Zhang et al. (Zhang et al., 2017) reported T allele of rs1256049 was susceptible to major depressive disorder with a relatively larger sample from the Asian group. But the other studies failed to identify the results. The controversial results might drive by the limitation of the sample size which could lead to insufficient statistical power in detecting the difference. In our meta-analysis, significant differences were observed only in the dominant model and the allelic model. The sample size limitation also exited in the recessive model and the additive model of rs1256049. That is, the small sample size led to the extremely low frequency in the AA genotype in the recessive model and the additive model. The association between rs1256049 and depression needs to be identified in further study with larger sample size.

The limitations in this meta-analysis should be noted. For example, because of the limited effective number of samples, our meta-analysis was unable to test the relationship of ERα and ERβ polymorphisms with depression susceptibility in the Caucasian subgroup. Additionally, our meta-analysis pooled samples of women with depression in the gestational, menopausal, perinatal and other periods. Further subgroup analysis should be carried out based on these different periods marked by distinct hormonal fluctuations, such analysis may help to clarify the role of hormones in depression and contribute to effective treatment options.

5 CONCLUSION

In conclusion, our meta-analysis yielded evidence that the ERα polymorphism rs2234693 is associated with susceptibility to depression in women. Additionally, the ERα rs9340799 polymorphism is a potential risk factor for depression under the dominant and allelic models in Asian women. Further studies

with larger sample sizes are still needed to support this conclusion.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors. This study was registered in PROSPERO (registration no. CRD 42022324847).

AUTHOR CONTRIBUTIONS

HH and JY conceived and designed the experiments and revised the manuscript. WW, YL, and DL were involved in data collection and analysis. CL and MX drafted the manuscript. CL and MX contributed equally to this work. All authors were involved in the revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.936296/full#supplementary-material

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EDITED BY
Weihua Yue,
Peking University Sixth Hospital, China

REVIEWED BY
Paola Magioncalda,
Taipei Medical University, Taiwan
Jiansong Zhou,
Second Xiangya Hospital, Central South University, China
Hao Yan,
Peking University, China

*CORRESPONDENCE
Tao Li,
litaozjusc@zju.edu.cn

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Identifying transdiagnostic biological subtypes across schizophrenia, bipolar disorder, and major depressive disorder based on lipidomics profiles

Shiwan Tao[1], Yamin Zhang[2,3], Qiang Wang[1], Chunxia Qiao[1], Wei Deng[2,3], Sugai Liang[2,3], Jinxue Wei[1], Wei Wei[2,3], Hua Yu[2,3], Xiaojing Li[2,3], Mingli Li[1], Wanjun Guo[2,3], Xiaohong Ma[1], Liansheng Zhao[1] and Tao Li[2,3,4]*

[1]Mental Health Center and Psychiatric Laboratory, West China Hospital of Sichuan University, Chengdu, Sichuan, China, [2]Department of Neurobiology, Affiliated Mental Health Center & Hangzhou Seventh People's Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China, [3]NHC and CAMS Key Laboratory of Medical Neurobiology, MOE Frontier Science Center for Brain Science and Brain-Machine Integration, School of Brain Science and Brain Medicine, Zhejiang University, Hangzhou, Zhejiang, China, [4]Guangdong-Hong Kong-Macao Greater Bay Area Center for Brain Science and Brain-Inspired Intelligence, Guangzhou, China

Emerging evidence has demonstrated overlapping biological abnormalities underlying schizophrenia (SCZ), bipolar disorder (BP), and major depressive disorder (MDD); these overlapping abnormalities help explain the high heterogeneity and the similarity of patients within and among diagnostic categories. This study aimed to identify transdiagnostic subtypes of these psychiatric disorders based on lipidomics abnormalities. We performed discriminant analysis to identify lipids that classified patients (N = 349, 112 with SCZ, 132 with BP, and 105 with MDD) and healthy controls (N = 198). Ten lipids that mainly regulate energy metabolism, inflammation, oxidative stress, and fatty acylation of proteins were identified. We found two subtypes (named Cluster 1 and Cluster 2 subtypes) across patients with SCZ, BP, and MDD by consensus clustering analysis based on the above 10 lipids. The distribution of clinical diagnosis, functional impairment measured by Global Assessment of Functioning (GAF) scales, and brain white matter abnormalities measured by fractional anisotropy (FA) and radial diffusivity (RD) differed in the two subtypes. Patients within the Cluster 2 subtype were mainly SCZ and BP patients and featured significantly elevated RD along the genu of corpus callosum (GCC) region and lower GAF scores than patients within the Cluster 1 subtype. The SCZ and BP patients within the Cluster 2 subtype shared similar biological patterns; that is, these patients had comparable brain white matter abnormalities and functional impairment, which is consistent with previous studies. Our findings indicate that peripheral lipid abnormalities might help identify homogeneous transdiagnostic subtypes across psychiatric disorders.

KEYWORDS

schizophrenia, bipolar disorder, major depressive disorder, lipidomics profile, brain whiter matter

1 Introduction

Schizophrenia (SCZ), bipolar disorder (BP), and major depressive disorder (MDD) are three common psychiatric disorders with a heavy disease burden (Vigo et al., 2016). In clinical practice, it is an issue that the boundaries among the various diagnoses are not clearly distinct from each other. Patients with different diagnoses usually present baffling similarities to each other, such as the "with psychotic features" item of BP and MDD diagnoses and the emotional dysfunctions in SCZ. This is partly because rather than diagnostic objective criteria or biological markers, the current psychiatric diagnosis nosology relies on descriptive information elicited from self-report history and clinical observation (Scadding, 1996; Craddock and Mynors-Wallis, 2014; Heckers and Kendler, 2020). This hampers the diagnostic accuracy of these psychiatric disorders.

Increasing evidence has demonstrated that there are overlapping biological characteristics across SCZ, BP, and MDD, such as genetic risk factors (Cross-Disorder Group of the Psychiatric Genomics, 2013; Cross-Disorder Group of the Psychiatric Genomics et al., 2013; Ruderfer et al., 2014; Cross-Disorder Group of the Psychiatric Genomics Consortium. Electronic address and Cross-Disorder Group of the Psychiatric Genomics, 2019; Andlauer et al., 2021), brain structure and functional abnormalities (Meda et al., 2014; Godwin et al., 2018; Kelly et al., 2018; Li et al., 2018; Favre et al., 2019; van Velzen et al., 2020) and cognitive impairment (Czobor et al., 2007; Reichenberg et al., 2009; Millan et al., 2012; Barch and Sheffield, 2014; Reilly and Sweeney, 2014; Tamminga et al., 2014). All of these findings imply that the diagnostic classes are not distinct entities, and the descriptive diagnosis nosology has fundamental flaws. Therefore, it is necessary to identify natural biological homogeneous subtypes across different psychiatric disorders.

Lipid metabolites are downstream biochemical end products that are more close to phenotypes than genomics and proteomics. As an essential part of systems biology, lipidomics could comprehensively illuminate the lipid metabolic profile of individuals and identify changes related to phenotype (Patti et al., 2012; Zhao et al., 2014). Plasma lipid alterations, therefore, are sensitive and specific to several observed risk factors for psychiatric disorders, including genetic variations, brain white matter (WM) structural abnormalities, and oxidative stress and inflammation. For example, the ABCD1 gene mutation caused very long-chain fatty acid accumulation in brain WM, which led to psychiatric symptoms (Kitchin et al., 1987; Kemp et al., 2016). Plasma lipids, such as triglyceride, were also reported to be associated with brain WM microstructural changes and axonal degeneration (Iriondo et al., 2021). Notably, derived from peripheral essential omega-6 and omega-3 polyunsaturated fatty acids, lipid-derived mediators serve as pro/anti-inflammatory mediators regulating brain inflammation (Laye et al., 2018). Brain tissues are susceptible to oxidative stress due to their high oxygen consumption and unsaturated fatty acid enrichment, which have been reported to be associated with SCZ, BP, and MDD (Salim, 2017; Cobley et al., 2018).

Lipidomics has recently been developed as a powerful tool to investigate the natural characteristics of SCZ, BP, and MDD. The peripheral lipidomics profile alterations of these psychiatric disorders have been pervasively characterized using ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) technology and have served as promising biomarkers for early diagnosis and clinical outcome prediction (Oresic et al., 2011; Brunkhorst-Kanaan et al., 2019; Zhou et al., 2019; Bot et al., 2020; Hussain et al., 2020; Zhuo et al., 2020; Dickens et al., 2021). Therefore, taking advantage of lipidomics analysis may help identify biologically homogeneous subtypes across these psychiatric disorders. In this study, first, we investigated the peripheral lipidomics profile abnormalities between psychiatric patients (with SCZ, BP, and MDD) and healthy controls (HCs) by discriminant analysis, and identify the most contributory lipids for classification. Then, based on these identified lipids, we further investigated the potential subtypes across SCZ, BP, and MDD by consensus clustering analysis. To comprehensively profile the differences in these potential biological subtypes, we further described and compared the brain WM microstructure and clinical features of these subtypes.

2 Methods

2.1 Participants

All participants, who were right-handed Han Chinese and aged 16–55 years old, were interviewed by at least two trained psychiatrists using the Structured Clinical Interview of the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR)—Patient Version (SCID-P). A total of 547 participants (112 patients with SCZ, 132 with BP, 105 with MDD, and 198 HCs) were

recruited from West China Hospital of Sichuan University between 2014 and 2019. The inclusion criteria for patients were as follows: 1) fulfilment of one of the DSM-IV-TR criteria for SCZ, BP, or MDD; 2) Han Chinese; 3) right-handed; 4) education achievement of more than 6 years; and 5) scores on Wechsler's intelligence test equal to or higher than 70. The exclusion criteria for patients were as follows: 1) comorbidity with other DSM-IV-TR axis I or axis II disorders (such as alcohol and substance abuse); 2) presence of organic brain diseases, neurological diseases or somatic diseases undergoing drug treatment (such as diabetes); 3) any history of head trauma; 4) any physical therapies, such as electroconvulsive therapy, undergone within the past 6 months before magnetic resonance imaging (MRI) scan; 5) any contraindication to perform MRI scan; 6) pregnant or breastfeeding; and 7) Wechsler's intelligence test scores less than 70. In this study, all SCZ patients were first-episode and drug-naïve. There were 77 MDD patients and 63 BP patients who were drug-naïve, and 28 MDD and 69 BP patients who were not drug-naïve but had at least a two-week wash-out period.

HCs were enrolled via online and local advertisements. They were screened for any mental disorder by the SCID—Non-Patient Version (SCID-NP). The exclusion criteria for HCs were similar to those for patients. Moreover, HCs with first-degree relatives with DSM-IV-TR axis I or II disorders were excluded.

2.2 Ethical principles

This study abided by the guidelines of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of West China Hospital, Sichuan University. After the study procedure had been fully explained, written informed consent was obtained from all participants and their guardians if participants were less than 18 years old.

2.3 Clinical assessment

We used the Global Assessment of Functioning (GAF) scale to evaluate functional impairment in all patients. The Positive and Negative Syndrome Scale (PANSS), the Young Mania Rating Scale (YMRS), the Hamilton Anxiety Scale (HAMA), and the Hamilton Depression Scale (HAMD) were used to assess symptom severity in patients as appropriate. Clinical features, including onset age, total duration of illness period (TDP), current duration of illness period (CDP), duration of untreated period (DUP), current episode state, BP I or II subtype for BP, and the number of episodes for MDD, were also documented.

2.4 Lipidomics data acquisition and preprocessing

Peripheral blood was collected in EDTA tubes from all participants on the same day they were enrolled in this study. Lipid extraction, UHPLC–MS/MS analysis, and lipid qualitative and quantitative identification are described in the Supplementary Methods. A total of 7212 lipid features in the positive polarity model and 4,898 lipid features in the negative polarity model were obtained. The lipidomic data were preprocessed by the "statTarget" (version 1.22.0) (Luan et al., 2018) and "MetaboAnalystR" (version 3.0.3) (Pang et al., 2020) packages in R software (version 4.1.0). We performed preprocessing steps as follows: 1) drift signal correction using the quality control-based robust locally estimated scatterplot smoothing (LOESS) signal correction (QC-RLSC) algorithm (Dunn et al., 2011); 2) a quality assurance procedure to remove metabolic features with relative standard deviation (RSD) >20%, which was calculated for all QC samples (Dunn et al., 2011); 3) log2 transformation and ComBat batch effect correction (Johnson et al., 2007); and 4) interquartile range (IQR) data filtering. The quality control results of the lipidomics data are described and depicted in Supplementary Figure S1. After the preprocessing steps, a total of 1,164 lipids remained for discriminant analysis.

2.5 DTI data acquisition and preprocessing

Brain WM microstructural abnormalities were measured by fractional anisotropy (FA) and radial diffusivity (RD) using diffusion tensor imaging (DTI) scans. The FA indicates the underlying characteristics of white matter microstructure, such as the directionality of axonal fibres, diameter, and density (Basser, 1995; Basser and Pierpaoli, 1996). RD is considered an indicator of myelin sheath thickness, reflecting myelin damage (Song et al., 2002; Song et al., 2005). Altered FA or RD in some regions indicated the brain white matter microstructures abnormalities here. The DTI scan parameters are described in the Supplementary Methods. Raw images were processed by MRIcroN (http://www.mricro.com), DTIPrep, and FMRIB Software Library (FSL) (version 5.0.8). The imaging format was converted by MRIcroN, and then the imaging quality was checked by DTIPrep (translation <2 mm, rotation <0.5 mm). Individual images that met the quality control criteria were included for subsequent procedures (6 samples were excluded after checking the imaging quality). The preprocessing steps included motion and eddy current correction, gradient direction reorientation, and brain mask estimation to remove the nonbrain spaces. After calculating diffusion tensor metrics, normalization and linear/nonlinear registration were also performed to allow comparison across participants. Brain regions of interest (ROIs) were defined by the JHU-ICBM-DTI-81 WM labels atlas ($n = 48$). The z scores

of the mean FA and RD in each ROI were calculated for further statistical analysis.

2.6 Statistical analysis

2.6.1 Demographic characteristics

Demographic characteristics including age, sex, educational attainment (years), and body mass index (BMI) of different groups (psychiatric patients and HCs) were compared using the independent test or chi-square test. BMI was calculated as weight divided by height squared (kg/m^2). All the analyses above were calculated in R software.

2.6.2 Discriminant analyses for patients and HCs based on lipidomics data

Preprocessed lipids were further analysed using the "mixOmics" (version 6.16.3) package in R software (Rohart et al., 2017). The data were centred on zero mean and unit variance (auto scaling). Principal component analysis (PCA) was used to check the homogeneity of the samples and determine whether QC samples were tightly clustered together. After removing the outliers, we developed a sparse partial least square-discriminant analysis (sPLS-DA) model, a supervised machine learning analysis, to identify the lipids that contributed most to the classification of psychiatric and HC groups. Parameter tuning processes were performed using the *tune* function to determine the optimal parameters. The performance of the tuning sPLS-DA model obtained with a balanced error rate (BER) was estimated with 7-fold cross validation and repeated 1,000 times. The optimal parameters, including the number of components and variables, were selected when the tuning model had a low classification error rate. The performance of the optimal sPLS-DA model was estimated by using the *perf* function, with 7-fold cross validation repeated 1,000 times. Evaluated indexes included BER and overall classification error rate (prediction distances were calculated by max, centroids, and Mahalanobis distance) and areas under the receiver operating curve (AUCs). We also performed univariate analysis and two-sample Wilcoxon rank-sum tests to complement the multivariate analysis, followed by false discovery rate (FDR) adjustment. The most important variables (lipids) for differentiating the psychiatric and HC groups satisfied the following cut-off criteria: 1) AUCs of the sPLS-DA model >0.8, 2) variable importance in projection (VIP) scores >1, 3) occurrence frequency of the lipids >0.8 after performing 1,000 times of cross validation, and 4) p value <0.05 after FDR adjustment. A total of 10 significantly altered lipids met all the above criteria to differentiate between patients and HCs.

2.6.3 Identifying lipid-based subtypes utilizing consensus cluster

We developed an unsupervised cluster model to investigate the potential subtypes within the group of psychiatric patients using the data of 10 identified lipids. Consensus partitioning was performed and summarized by the "cola" package (Gu et al., 2021). Features for consensus partitioning were calculated by four methods: standard deviation (SD), median absolute deviation (MAD), coefficient of variation (CV), and ability to correlate to other rows (ATC). Partitioning methods included hierarchical clustering (hclust), k-means clustering (kmeans), partitioning around medoids (pam), and spherical k-means clustering (skmeans). The partitioning step was repeatedly executed 50 times for each partitioning method. The mean silhouette score and concordance were calculated to evaluate the cluster models and select the optimal number (k) of subtypes. The SD-skmeans model generated an optimal k of 2.

2.6.4 The differences between lipid-based subtypes across multiple-level data

We compared the differences between the two subtypes in terms of clinical features (including the global functional impairment measured by GAF scale scores; symptoms severity measured by PANSS scores, YMRS scores, HAMA scores, and HAMD scores in patients as appropriate; onset age; TDP; CDP; and DUP), and brain WM microstructural alterations (measured by FA and RD) in R software. A two-sample t test was performed to compare the difference in GAF scores and ROI-based FA and RD data between the subtypes (followed by FDR adjustment). The 48 WM regional FA and RD effect sizes of subtypes (Cohen's d) were also calculated.

3 Results

3.1 Demographic characteristics

We removed 7 individuals (3 patients and 4 HCs) after the lipidomic data quality control process (Supplementary Figure S1). The demographic characteristics of the remaining 346 psychiatric patients and 194 HCs are described in Table 1. There were no significant differences between patients and HCs in terms of age, sex, or BMI. The mean educational attainment years of participants in the HC group were higher than those of participants in the psychiatric group ($p < 0.001$).

3.2 Discriminant analyses for patients and HCs

3.2.1 Choosing optimal parameters from the tuning model

The performance of tuning the sPLS-DA model is displayed inFigure 1A. The balanced classification error rates were decreased when more components were added to the model. In the tuning model, the first two components (composed of 2 lipid features selected from the first component and 20 lipid features selected from the second component) were sufficient to achieve good performance (error rate = 0.046 ± 0.005, 7-fold cross validation repeated 1,000 times).

TABLE 1 Comparison of demographic characteristics between the psychiatric patient and healthy control groups.

Variables	Patients (n = 346)	HC (n = 194)	x^2/t-statistic	p value
Sex[a] (male/female)	141/205	66/128	2.11	0.15
Age[b]	24.86 ± 8.32	25.22 ± 8.22	0.48	0.63
Educational Attainment[b] (years)	13.29 ± 2.79	15.20 ± 2.44	8.27	<0.001***
BMI[b]	21.04 ± 3.01	20.93 ± 2.60	−0.42	0.68

[a]The p *value* was obtained by the chi-square test.
[b]The p *value* was obtained by the two-sample t test.
*p < 0.05; **p < 0.01; ***p < 0.001.
Age, sex and BMI data are presented as the mean ± standard deviation. BMI was calculated as weight divided by height squared (kg/m^2).
HC, healthy control; BMI, body mass index.

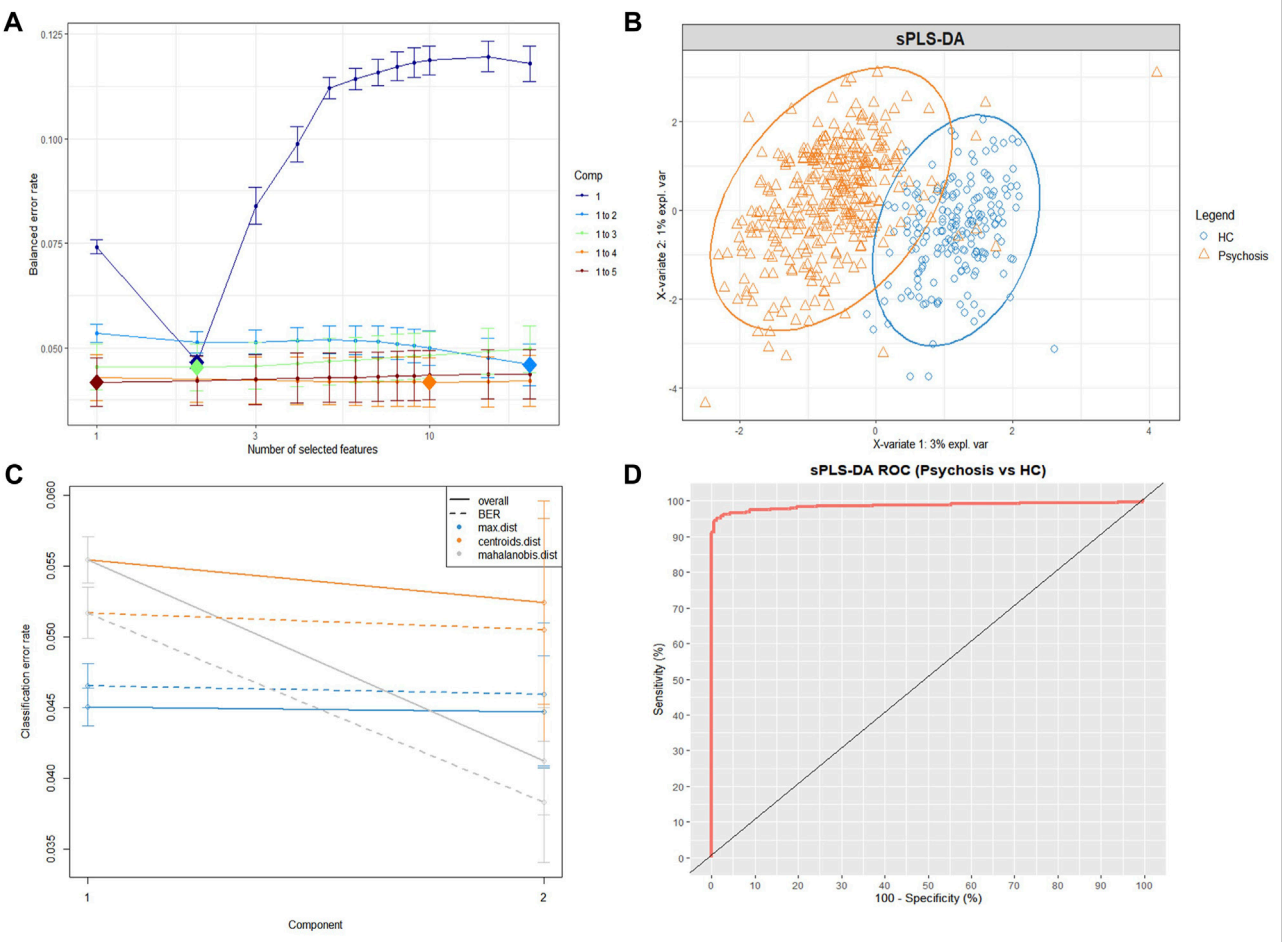

FIGURE 1
The sPLS-DA model for differentiating psychiatric patients and healthy controls using lipidomic data. **(A)** Balanced error rates (BERs) decreased when more components were added to the tuning sPLS-DA model. Here, the first 2 components (light blue line) were sufficient to achieve good performance (error rate = 0.046 ± 0.005, 1,000×7-fold cross-validation), and the optimal features of each component are indicated as a diamond plot. **(B)** The sPLS-DA sample plot with ellipse circles indicating the 95% confidence interval. The first two components of the sPLS-DA model differentiating the psychiatric patient group (orange triangle) from the HC group (blue circle). **(C)** Classification performance per component (overall and BER) for three prediction distances using repeated cross-validation (1,000×7-fold). All the classification error rates were lower than 0.06. **(D)** The ROC curve of the sPLS-DA model, and the AUC = 0.986. *sPLS-DA*, sparse partial least squares discriminant analysis; *ROC*, receiver operating curve; *AUC*, area under the receiver operating curve; *HC*, healthy control group; *Psychosis*, psychiatric patient group.

TABLE 2 Identified differential lipids for classifying psychiatric patients and healthy controls.

Lipids	Classification	Formula	Molecular weight	VIP		Freq	Trend[a]
				Comp 1	Comp 2		
9,12-Octadecadienal	Fatty acyls/Fatty aldehydes	$C_{18}H_{32}O$	264.2455	33.38	31.50	1.00	↑***
20-oxo-22,23,24,25,26,27-hexanorvitamin D3	Sterol lipids/Vitamin D3 like derivatives	$C_{21}H_{30}O_2$	314.2248	6.92	6.53	1.00	↓***
10-nitro-9Z,12Z-octadecadienoic acid	Fatty acyls/Nitro fatty acids	$C_{18}H_{31}NO_4$	325.2255	0.00	9.52	1.00	↓***
DGTS 16:0/18:1	Other	$C_{44}H_{83}NO_7$	737.6169	0.00	2.71	1.00	↑***
4-amino-3-methylbutanoic acid	γ-Aminobutyric acid analogue	$C_5H_{11}NO_2$	117.0791	0.00	2.07	0.93	↓***
Cyclopentaneoctanoic acid	Fatty acyls/Unsaturated fatty acids	$C_{17}H_{26}O_5$	310.1781	0.00	1.70	0.94	↑***
OxPC 16:0-18:1+2O	Other	$C_{42}H_{82}NO_{10}P$	791.5690	0.00	1.59	0.95	↑***
Caprylic acid	Fatty acyls/Straight chain fatty acids	$C_8H_{16}O_2$	144.1152	0.00	1.37	0.97	↑***
Hexadecandioic acid	Fatty acyls/Dicarboxylic acids	$C_{16}H_{30}O_4$	286.2145	0.00	1.30	0.93	↑***
12-Tridecynoic acid	Fatty acyls/Unsaturated fatty acids	$C_{13}H_{22}O_2$	210.1621	0.00	1.12	0.89	↑***

[a]Up arrow (↑) indicates an upregulated trend in psychiatric patients compared with healthy controls; down arrow (↓) indicates a downregulated trend in psychiatric patients compared with healthy controls.

***p value < 0.001, adjusted by false discovery rate (FDR) adjustment.

VIP, variable importance in projection; *Comp1*, first component of the classification model; *Comp2*, second component of the classification model; *Freq*, lipid occurrence frequency when performing 1,000 times cross-validation; *DGTS*, diacylglyceryl- N,N,N- trimethylhomoserine; *OxPC*, [2-[(Z)-12,13-dihydroxyoctadec-9-enoyl]oxy-3-hexadecanoyloxypropyl] 2-(trimethylazaniumyl)ethyl phosphate.

3.2.2 Identifying contributing lipids for the classification of psychiatric patients and HCs

Figure 1B displays the sPLS-DA sample plot. The first two components accurately distinguished psychiatric patients from HCs. Figure 1C displays the BER and overall error rates of the two components for three prediction distances (7-fold cross validation, repeated 1,000 times). All classification error rates were less than 0.06 (details in Supplementary Table S1). The receiver operating curve (ROC), as an additional measure that helped reflect the performance of the sPLS-DA model, is depicted in Figure 1D, and the AUC = 0.986. The low classification error rates and high AUC indicate that the previous tuning process led to a final sPLS-DA model that achieved good performance. According to the cut-off criteria mentioned in the methods, 10 lipids were selected (Table 2).

3.3 Consensus cluster analysis within the group of psychiatric patients

Consensus clustering was performed among the psychiatric patients. The skmeans model generated stable partitions compared to other methods, especially when combined with SD (details in Supplementary Table S2). The confident samples with silhouette scores >0.5 (N = 319) were classified into two stable subtypes named the Cluster 1 and Cluster 2 subtypes (mean silhouette = 0.8; concordance = 0.9). The consensus heatmap (Figure 2A) provides a visual representation of how consistent two samples were in the same subtype. The PCA plot (Figure 2B) also confirmed that the two subtypes were separate from each other.

3.4 The differences in lipid-based subtypes across multiple-level data

3.4.1 Demographic characteristics of the two subtypes

There were no significant differences between the two lipid-based subtypes in terms of demographic characteristics (age, sex, educational attainment, and BMI) (Table 3).

3.4.2 The differences in clinical features of the two subtypes

Cluster 1 included 179 patients (52.65%), and Cluster 2 included 140 patients (41.18%). The clinical diagnosis distribution varied between the two subtypes (x^2 = 65.81, p < 0.001) (Table 3). Cluster 1 consisted of 33 (18%) patients with SCZ, 59 (33%) patients with BP and 87 (49%) patients with MDD, and Cluster 2 consisted of 65 (46%) patients with SCZ, 64 (46%) patients with BP and 11 (8%) patients with MDD (Figure 3A). A higher proportion of patients with MDD was in Cluster 1 (89%) than in Cluster 2 (11%). In contrast, more patients with SCZ were allocated to Cluster 2 (66%) than to Cluster 1 (34%). Patients with BP were uniformly distributed in Cluster 1 (52%) and Cluster 2 (48%) (Figure 3B). In regard to clinical features, Cluster 2 patients (50.17 ± 13.83) showed significantly lower GAF scores than Cluster 1 patients (54.96 ± 13.08) (t = 2.94, p = 0.0036) (Table 3). The clinical features of schizophrenia did not show any difference between the two clusters (Supplementary Table S3); the distribution of bipolar I and bipolar II disorder differed in the two clusters

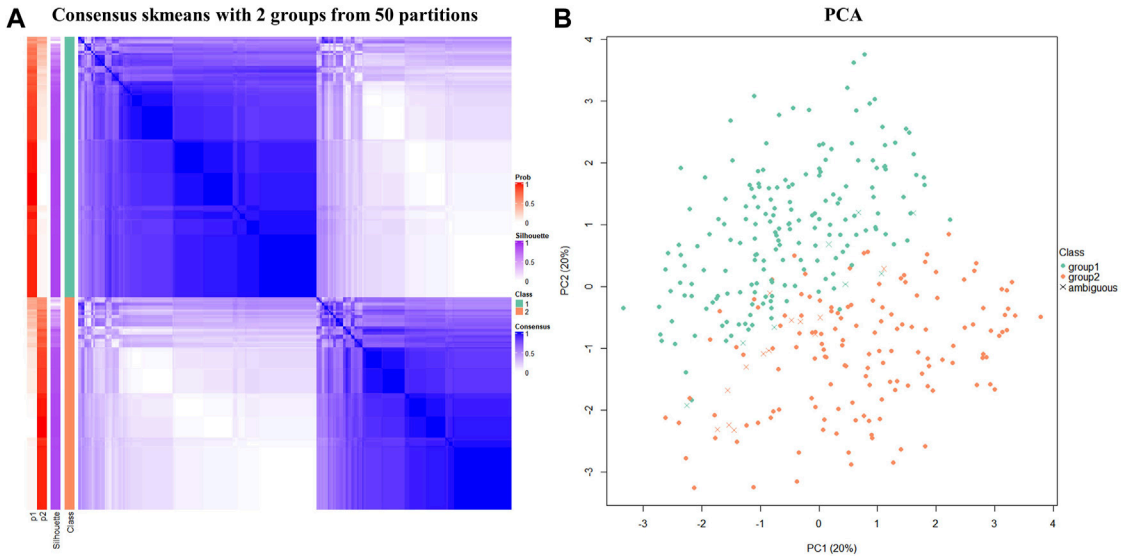

FIGURE 2
The consensus clustering analysis identified two subgroups within psychiatric patients. **(A)** The consensus matrix heatmap plot visualized the stability of the two subtypes. The "p1" and "p2" labels refer to the probability of the sample being classified into "class 1" and "class 2," respectively, after clustering was repeated 50 times. "Prob" refers to the calculated probability of the sample being classified into the corresponding subgroup. The "consensus" legend refers to how consistent two samples were in the same subgroup. **(B)** The PCA plot confirmed that there were two subgroups of psychiatric patients. The confident samples (silhouette score >0.5) are classified into two subgroups obviously separated from each other, and the ambiguous samples (silhouette score <0.5) are indicated by crosses on the plot.

TABLE 3 Comparison of demographic characteristics and functional impairment assessment between the lipid-based subgroups.

Variables	Cluster 1	Cluster 2	x^2/*t*-statistic	*p value*
	(*n* = 179)	(*n* = 140)		
Demographic characteristic				
Sex[a] (male/female)	64/115	62/78	2.05	0.15
Age[b]	25.11 ± 8.45	24.21 ± 7.73	0.98	0.33
Educational Attainment[b] (years)	13.15 ± 2.92	13.40 ± 2.60	−0.82	0.41
BMI[b]	20.82 ± 2.84	21.24 ± 3.28	−1.22	0.22
Clinical diagnosis distribution[a]				
SCZ	33	65	65.81	<0.001***
BP	59	64		
MDD	87	11		
Clinical assessment[b]	(*n* = 156)	(*n* = 123)		
GAF scale scores	54.96 ± 13.08	50.17 ± 13.83	2.94	0.0036**

[a]The *p* value was obtained by the chi-square test.
[b]The *p* value was obtained by the independent two-sample *t* test.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Age, sex, BMI and GAF scale scores are presented as the mean ± standard deviation. BMI was calculated as weight divided by height squared (kg/m^2). HC, healthy control; BMI, body mass index; GAF, Global Assessment of Functioning Scale.

(x^2 = 4.87, p = 0.027), and a higher proportion of bipolar I patients occurred in Cluster 2 (61%) than in Cluster 1 (39%) (Supplementary Table S4). In addition, the HAMA scores of MDD patients with a Cluster 2 (11.36 ± 5.73) subtype were significantly lower than those with a Cluster 1 subtype (16.07 ± 5.61) (t = 2.56, p = 0.024) (Supplementary Table

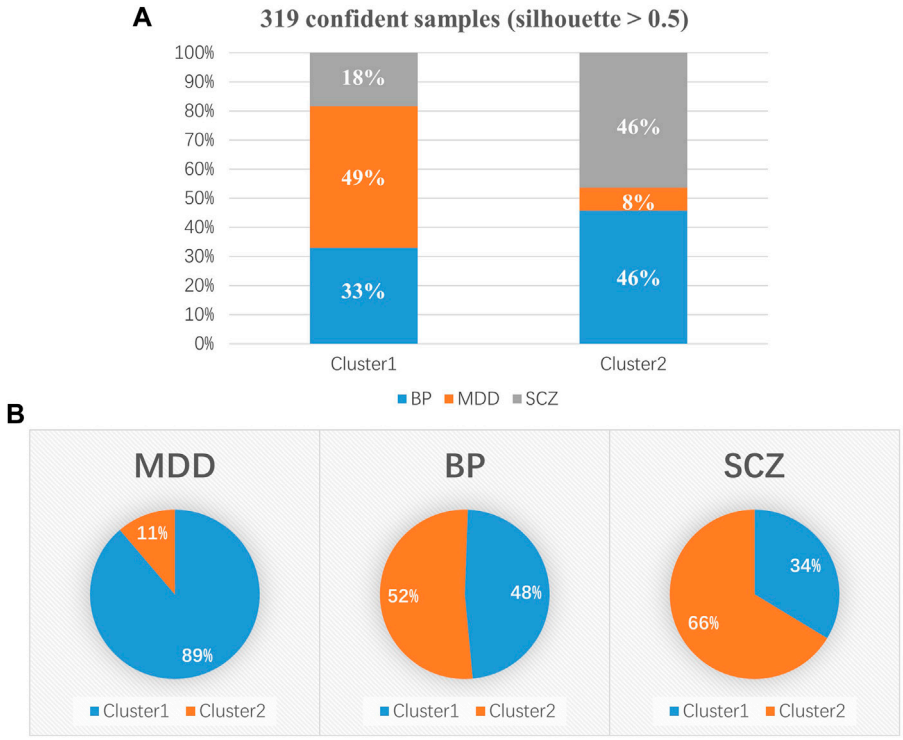

FIGURE 3
Distribution of clinical diagnoses in the two subgroups. **(A)** Cluster 1 included 179 psychiatric patients, consisting of 33 (18%) patients with SCZ, 59 (33%) patients with BP and 87 (49%) patients with MDD, and Cluster 2 included 140 psychiatric patients. **(B)** A higher proportion of MDD patients was present in Cluster 1 (89%) than in Cluster 2 (11%). There were more patients with SCZ in Cluster 2 (66%) than in Cluster 1 (34%). Patients with BP were uniformly distributed in Cluster 1 (52%) and Cluster 2 (48%). SCZ, schizophrenia; BP, bipolar disorder; MDD, depressive disorder. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

S5). Table 4 provides a general summary schema to summarize the comparison results of the clinical features of SCZ, BP and MDD allocated to the two subtypes.

3.4.3 Brain white matter alterations between the two subtypes

After FDR adjustment, patients in the Cluster 2 group showed significantly increased RD (1.169 ± 0.768) compared to those in the Cluster 1 group (0.857 ± 0.771) (Cohen's d = 0.405; $t = -3.591$; $p.adj = 0.018$), mainly along the genu of corpus callosum (GCC) (Figure 4; Supplementary Table S6). Patients within the Cluster 2 subtype showed trends of decreased FA along the fornix (including the column and body of the fornix) ($p = 0.018$, Cohen's d = 0.266) and right posterior thalamic radiation ($p = 0.032$, Cohen's d = 0.243) and increased FA mainly along the left hippocampus region ($p = 0.044$, Cohen's d = 0.226) compared to patients within the Cluster 1 subtype, although significance did not survive FDR adjustment (Supplementary Table S7). Supplementary Table S8 provides the association of identified lipids and brain WM alterations in psychiatric patients.

4 Discussion

In this study, discriminant analysis identified 10 disease-specific lipids that contribute to the classification of psychiatric patients (including patients with SCZ, BP, and MDD) and HCs. We further found two lipid-based subtypes (named the Cluster 1 and Cluster 2 subtypes) within the psychiatric patients utilizing cluster analysis. The two subtypes differed in clinical features and brain WM abnormalities. The clinical diagnosis distribution significantly differed in the two subtypes: patients with BP were uniformly distributed in the two subtypes, but a higher proportion of patients with MDD (89%) was noted in Cluster 1, and a higher proportion of patients with SCZ (66%) was noted in Cluster 2. Patients in Cluster 2 showed significantly lower GAF scores than those in Cluster 1. Moreover, the patients within Cluster 2 showed significantly increased RD in the GCC, decreased FA trends in the fornix and posterior thalamic radiation, and increased FA trend in the hippocampus.

Patients within the Cluster 2 subtype mainly consisted of those with SCZ and BP (a total of 92%). Previous studies have indicated that SCZ and BP are characterized by similar biological

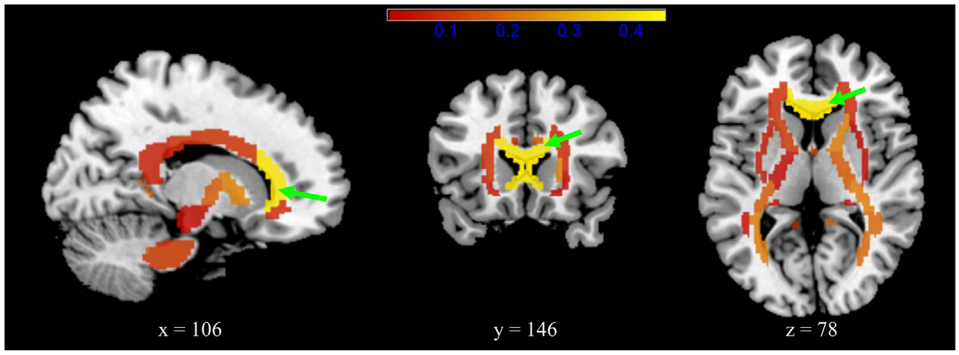

FIGURE 4
Radial diffusivity (RD) differences between the two subgroups for 48 white matter brain regions that represent the major fasciculi. The colour bar (red–yellow) indicates the mean effect size of the group (Cohen's d). The genu of the corpus callosum (green arrow) showed significantly increased RD in patients in the Cluster 2 subgroup (Cohen's d = 0.405; *p.adj* = 0.018).

TABLE 4 Comparison of clinical features of SCZ, BP and MDD patients between the lipid-based subgroups.

Variables[a]	SCZ	BP	MDD
PANSS scale	–		
YMRS scale		–	
HAMA scale		–	+
HAMD scale		–	–
Maternal gestation	–		
Full-term/preterm pregnant period	–		
Full-term normal/caesarean delivery	–		
Bipolar I/II subtype		+	
Psychotic feature		–	
Onset age	–	–	–
TDP (month)	–	–	–
CDP (month)		–	–
DUP (month)		–	–
Current episode state		–	
Depressive episodes			–

[a]+ indicates a significant difference in SCZ, BP, and MDD patients between the lipid-based subgroups in the corresponding item; – indicates there are no significant differences.
PANSS, positive and negative syndrome scale; YMRS, young mania rating scale; HAMA, hamilton anxiety scale; HAMD, hamilton depression scale; TDP, total duration of illness period; CDP, current duration of illness period; DUP, duration of untreated period.

patterns, such as high genetic correlation (Cross-Disorder Group of the Psychiatric Genomics et al., 2013) and comparable WM abnormalities. A large-scale meta-analysis has reported that patients with SCZ/BP (but not in MDD) shared limbic system (such as the fornix) abnormalities (Koshiyama et al., 2020), and posterior thalamic contraction (Mamah et al., 2016). In this study, the interesting constituent ratio of clinical diagnosis, and the decreased FA trend along the fornix and posterior thalamic region in patients within the Cluster 2 subtype were supported and consistent with previous findings. We also observed that bipolar I and bipolar II disorder distributions differed between the two subtypes. This finding is consistent with previous studies that found biological heterogeneity between bipolar I and bipolar II disorder (Charney et al., 2017; Huang et al., 2022). HAMA scores of MDD patients differed in the two subtypes. However, considering that only 11 MDD patients were allocated to Cluster 2, the small sample size may not satisfy the statistical power. Other clinical features of SCZ, BP and MDD showed no differences between the two lipid-based subtypes, which support that there are mismatch boundaries between biological subtypes and clinical diagnosis based on descriptive data. The differential findings of the two subtypes support the high similarity among SCZ and BP patients.

In addition to the lipid-based biological pattern, Cluster 2 patients also presented differential WM abnormalities measured by RD mainly along the GCC region and lower GAF scores. Brain WM abnormalities in the corpus callosum have been widely and consistently reported across several psychiatric disorders by meta-analyses, especially in the GCC of patients with SCZ (Kelly et al., 2018; Koshiyama et al., 2018; Favre et al., 2019; van Velzen et al., 2020). Lower GAF scores indicate severer psychological, social and occupational functioning impairment. The GCC is the bend of the anterior corpus callosum; thus, it facilitates prefrontal interhemispheric connectivity and relates to social competence, planning and memory performance, etc. (Paul et al., 2007). We speculate that the greater functioning impairment of patients within the Cluster 2 subtype are potentially the consequences of structural abnormalities in the GCC. As RD is a specific index reflecting the demyelination or morphology abnormalities of fibre tracts (Song et al., 2002; Song et al., 2005), the significantly elevated RD in the GCC region of Cluster 2 patients might reflect greater brain WM lesions here. In summary, these findings indicate that the lipid-based subtypes across psychiatric disorders also showed differential multiple-level biological characteristics.

The 10 identified lipids from the sPLS-DA model relate to several abnormal physiological processes, including inflammation and oxidative stress, brain structural or functional abnormality regulation, and metabolic deterioration. 10-Nitro-9Z,12Z-octadecadienoic acid (nitrolinoleic acid, LNO_2) is rich in human plasma and red cell membranes. It acts as a lipid-derived mediator in activating antioxidant signalling pathways (Kalyanaraman, 2004; Koutoulogenis and Kokotos, 2021). LNO_2 also exhibits robust cell signalling activities as an anti-inflammatory (Coles et al., 2002; Schopfer et al., 2005; Wright et al., 2006; Koutoulogenis and Kokotos, 2021). In this study, decreased plasma LNO_2 might indicate the vulnerable anti-inflammatory status of psychiatric patients. In addition, there are several other identified lipids associated with inflammation and oxidative stress. Of note, change in hexadecanedioic acid level was reported to be related to inflammatory status, and it contributed to the classification of SCZ and HCs in previous studies (Cui et al., 2020; Qian et al., 2021). The diacylglyceryl-N,N,N-trimethylhomoserine (DGTS)16:0/18:1 level is considered a biomarker reflecting low oxidative stability among wheat varieties (Wei et al., 2021). A meta-analysis has revealed that first-episode psychiatric patients exhibited a proinflammatory and vulnerable antioxidant status (Fraguas et al., 2019). Recently, a proposed hypothesis illuminated immune/inflammatory-mediated alteration of brain WM in the limbic system as the main pathophysiological mechanism of psychiatric disorders (Magioncalda and Martino, 2022). In this study, DGTS 16:0/18:1 and hexadecandioic acid positively related to RD of GCC and FA of left hippocampus. These above-altered lipids might indicate inflammation and oxidative stress imbalance in psychiatric patients.

Although there is insufficient evidence, previous studies have implicated that the altered 12-tridecynoic acid and 4-amino-3-methylbutanoic acid levels might be related to the regulation of brain structural abnormalities. The Wnt signalling pathways are important in modulating synapse growth and synaptic plasticity in humans, and altered Wnt signalling was documented in patients with SCZ and BP (Tabares-Seisdedos and Rubenstein, 2009; Hoseth et al., 2018). 12-Tridecynoic acid is one of the lipids that participates in the fatty acylation/deacylation of Wnt proteins (Gao and Hannoush, 2014; Torres et al., 2019), which are necessary for their biofunction (Willert et al., 2003; Rios-Esteves et al., 2014). However, there is no direct evidence linking the changes in fatty acid levels to Wnt protein activation. In this study, the effect of increased 12-tridecynoic acid levels on the brain structure is unknown, and further studies could perhaps investigate the relationship between them by evaluating Wnt signalling pathways. 4-Amino-3-methylbutanoic acid is a 3-substituted γ-aminobutyric acid (GABA) analogue with greater affinity for GABA receptors in the human brain (Nicholson et al., 1979). Moreover, it could raise GABA levels by increasing L-glutamic acid decarboxylase (GAD) activity in the mouse brain and produce an anticonvulsant effect (Silverman et al., 1991; Taylor et al., 1992). However, there is also no evidence linking decreased 4-amino-3-methylbutanoic acid concentrations with the function of central GABAergic neurons, which requires more research. These peripheral lipid alterations may provide clues and broaden our understanding of the mechanisms underlying brain structure abnormalities, which is one of the main pathogenic mechanisms of psychiatric disorders.

Unhealthy dietary and behaviour patterns have recently been noted as risk factors for the metabolic deterioration of patients with SCZ, BP, and MDD (Beyer and Payne, 2016; Vancampfort et al., 2017). A previous randomized crossover trial reported that the high fiber consumption dietary intervention decreased plasma 9,12-octadecadienal level. It is considered to be involved in mediating the positive effect of a healthy diet on maintaining satiety and preventing obesity (Lankinen et al., 2011). In this study, increased 9,12-octadecadienal level in psychiatric patients might reflect the unhealthy dietary pattern (such as low consumption of fiber and fruit) of these patients (Dipasquale et al., 2013). Caprylic acid is important in regulating food intake behaviour by esterifying ghrelin, which is a key peptide hormone with orexigenic biofunction (Kojima et al., 1999; Kojima and Kangawa, 2002; Delporte, 2013). Previous randomized controlled trial studies have reported that ingestion of caprylic acid helps stimulate food intake behaviour, and has been used to treat anorexia nervosa (Kawai et al., 2017) and cachectic patients (Ashitani et al., 2009). Disordered eating behaviours are common among SCZ, BP (such as binge eating, food cravings, and night eating), and MDD (emotional and external eating) patients, which were occurred in the initial onset and cannot all be attributed to the side effects of drug treatment (Paans et al., 2018; Stogios et al., 2020; Sankaranarayanan et al., 2021). In this study, the increased caprylic acid level might provide clues about the disordered eating behaviour among psychiatric patients. Apart from attention to dietary patterns and disordered eating behaviour, an unhealthy behaviour pattern is another important risk factor for metabolic deterioration. Of note, 20-oxo-22,23,24,25,26,27-hexanorvitamin D3 is only synthesized by skin tissue in humans through ultraviolet B (UVB) induced physicochemical processes (Slominski et al., 2012). Since UVB is essential in the synthetic process, it is reasonable to infer that the significantly decreased 20-oxo-22,23,24,25,26,27-hexanorvitamin D3 levels in psychiatric patients may be attributed to lower sunlight exposure, which is associated with unhealthy behavioural patterns (such as sedentary behaviour). A previous meta-analysis reported that patients with SCZ, BP and MDD have significant sedentary behaviour (average 476 min per day) during waking hours and low activity (38.4 min per day) (Vancampfort et al., 2017). Above all, the alterations in 9,12-octadecadienal, caprylic acid, and 20-oxo-22,23,24,25,26,27-hexanorvitamin D3 levels might reflect the unhealthy dietary and behavioural pattern of the mechanism that underlying metabolic deterioration of psychiatric patients.

There are some limitations to this study. First, as antipsychotic drugs affect lipid metabolism, we tried our best to recruit drug-naïve patients. In this study, all the recruited schizophrenia patients were first-episode and drug-naïve, however, drug-naïve bipolar disorder

patients were hard to recruit due to the diagnosis delay and high misdiagnosis rate (Culpepper, 2014; Fritz et al., 2017). We recruited 63 (47.8%) drug-naïve BP patients and set at least 2 weeks wash-out period for other BP patients (the median current duration of illness period was 2 months). When it comes to MDD patients, the main treatment strategies are SSRI/SNRI drugs, even so, we recruited 77 (77.3%) drug-naïve patients and also set at least 2 weeks wash-out period for other MDD patients. Then, because sample collection at a single center with lower variability may restrict the generalization of these findings. We will conduct independent sample validation in the future study to make these findings more robust and convincing. Moreover, the biological functions of some identified lipids are attractive, such as LNO_2 and caprylic acid. Although previous studies have evidenced their biological function in psychiatric patients, further studies could better elucidate the effects of these lipids on psychiatric diseases. For example, adding inflammatory factors examination, and the questionnaire about the dietary and behaviour patterns.

5 Conclusion

In conclusion, our findings suggested that peripheral blood lipidomic profile alterations could help identify homogeneous transdiagnostic subtypes across psychiatric disorders consisting of SCZ, BP and MDD. One of the subtypes that mainly consisted of patients with SCZ and BP represented more severe brain WM abnormalities and functional impairments. It is suggested that lipid-based subtypes might help identify patients with differential biological characterizations.

Data availability statement

The datasets presented in this article are not readily available because According to the Regulations of the People's Republic of China on the Administration of Human Genetic Resources, which came into effect on 1 July 2019, those who provide or open to use human genetic resources information to foreign organizations, individuals and institutions established or actually controlled by them shall submit the information to the administrative department of science and technology under The State Council for the record and submit backup information. The plasma sample analyzed in this article are also within the limits of this regulation. Requests to access the datasets should be directed to litaozjusc@zju.edu.cn.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of West China Hospital, Sichuan University. Written informed consent to participate in this study was provided by all participants. For participants who were less than 18 years old, their legal guardian/next of kin also provided written informed consent.

Author contributions

ST, YZ, and QW designed the analysis strategies. CQ, WD, HY, XL, ML, WG, and XM contributed to clinical data collection and assessment. JW and LZ contributed to lipidomics data acquisition. SL and WW contributed to guiding the imaging data analysis. ST performed the statistical analysis. ST and YZ interpreted the results and wrote the manuscript. TL overviewed the whole experiment and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.969575/full#supplementary-material

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EDITED BY
Cunyou Zhao,
Southern Medical University, China

REVIEWED BY
Ming Li,
Kunming Institute of Zoology (CAS), China
Wen Wang,
University of Minnesota Twin Cities, United States

*CORRESPONDENCE
Jingjing Zhao,
jingjing.zhao@snnu.edu.cn

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A genome-wide association study identified one variant associated with static spatial working memory in Chinese population

Liming Zhang, Zijian Zhu, Qing Yang and Jingjing Zhao*

School of Psychology, Shaanxi Normal University, and Shaanxi Provincial Key Research Center of Child Mental and Behavioral Health, Xi'an, China

Spatial working memory (SWM) is a kind of memory that temporarily preserves spatial information (the location or order of objects, etc.). Individuals with mental disorders tend to show worse performance in SWM task. This study investigated the genetic basis of two subtypes of SWM, static spatial working memory (SSWM) and dynamic spatial working memory (DSWM) in humans, using quantitative genomic analyses. A total of **451** Chinese students were tested on their magnitudes of SSWM and DSWM. A genome-wide association study (GWAS) was performed. Two SNPs (top SNP: rs80263879, $p = 1.6 \times 10^{-9}$, gene: *epoxide hydrolase 2*, *EPHX2*) reaching genome-wide significance for SSWM were identified. There is a high linkage disequilibrium between these two SNPs. The data of expression quantitative trait locus (eQTL) showed that different genotypes of rs80263879 and rs72478903 made significant differences in the expression of *EPHX2* gene in the spinal cord ($p = 0.022$, $p = 0.048$). Enrichment analysis identified a gene set significantly associated with DSWM. Overall, our study discovered a candidate genetic locus and gene set for the genetics of the SWM.

KEYWORDS

GWAS, spatial working memory, single nucleotide polymorphisms (SNPs), gene, experiment

Introduction

Spatial working memory (SWM) is an important component of working memory, it can produce, operate and maintain visual images, related to spatial position, motion, etc. SWM can be divided into static spatial working memory (SSWM) and dynamic spatial working memory (DSWM) (Pickering, Gathercole, Hall, & Lloyd, 2001; Cocchi et al., 2007). In the SSWM task, spatial information is usually processed once; in the DSWM task, spatial information has to be processed continuously. SWM reflects the ability to temporarily store spatial information (Goodale & Milner, 1992), varing widely across individuals. In particular, individuals with mental disorders, such as autism spectrum disorder (ASD) or

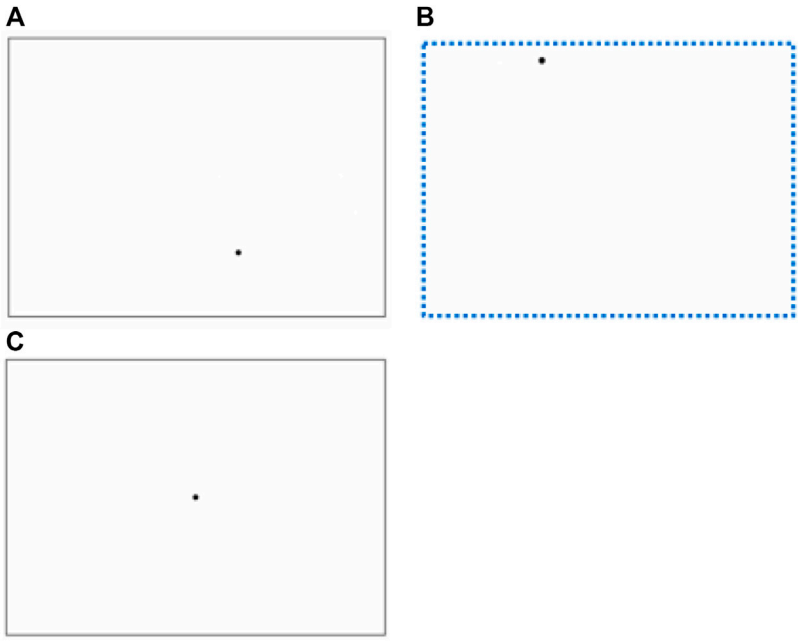

FIGURE 1
Stimuli in experiment. The above three figures reveal 'Stimuli in the learning stage of SSWM task' **(A)**, 'Stimuli in the learning stage of DSWM task' **(B)** (There are no dashed tracks in the formal experiment), 'Stimuli in the test stage of SSWM and DSWM task' **(C)**.

schizophrenia, have varying degrees of impairment in SWM (Jiang et al., 2015; Minor and Park, 1999). The reason may be that the development process of SWM in patients with mental diseases from their 10th to 20th years of age has stagnated (Song et al., 2013).

What are the factors causing the individual differences of SWM ability? First, there is a research evidence that environmental factors (educational level, age, etc.) influence SWM ability, these environmental factors were significantly correlated with SWM ability in healthy subjects, but not in schizophrenic subjects (Stratta et al., 2001). Second, studies revealed that genetic factors influenced SWM ability in schizophrenic patients. For instance, researchers divided schizophrenic patients into different categories according to different genotypes of a specific gene (catechol-O-methyltransferase) (Miskowiak et al., 2017). Different groups of subjects performed differently in SWM task. However, so far, there is no genetic research on SWM in the general population and at the genome-wide level.

In this study, we did a GWAS among high school students and college students to reveal the molecular mechanism of SWM. We analyzed two SWM subtypes (i.e., SSWM and DSWM). We investigated the function of loci significantly associated with the phenotype. We also performed enrichment analysis to see if phenotypes were affected by the joint effects of multiple loci or genes. Our results showed that SWM has a specific genetic mechanism.

Methods

Participants

Cohorts consisted of college students recruited from the Shaanxi Normal University and Xi'an Jiaotong University in Northwest of China, and senior high school students recruited from Sichuan LuXian No.2 High School in Southwest of China and Xi'an No. 1 middle school in Northwest of China. There were 544 participants in total, with 100% of Han population. The average age is 17 years old (standard deviation, SD = 1.32), with 54% of female. Each participant had no history of mental illness, and none of the subjects reported psychiatric or visual illness. All subjects had normal visual acuity or corrected visual acuity.

The experimental procedure was approved by the evaluation committee of Shaanxi Normal University. The subjects were clear about the purpose of the experiment. They signed informed consent before the experiment.

Behavioral assay

Stimuli. Three stimuli of SWM were created, Figures 1A,C for the SSWM task, Figures 1B,C for the DSWM task. Each SWM stimulus was composed of a rectangular box and a dot, and was presented on a white backgroud. Figures 1A,B are the learning stage stimuli, Figure 1C is the test stage stimuli.

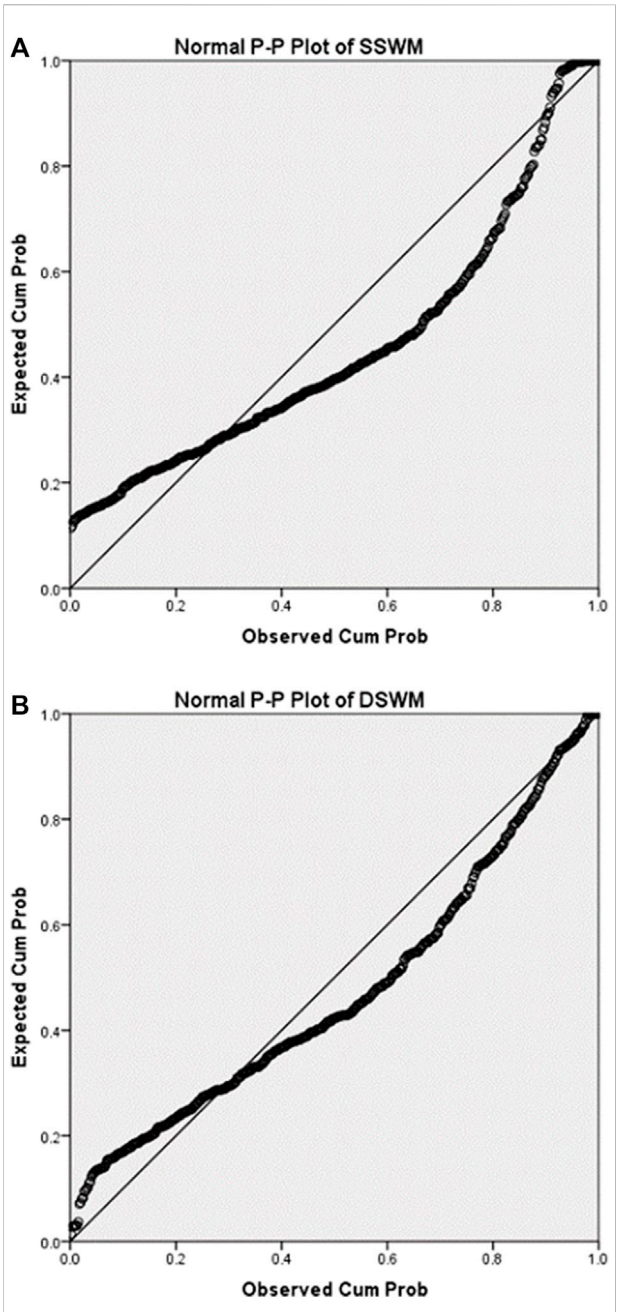

FIGURE 2
P-P plots for phenotypes. The above two figures summarise 'consistency between the cumulative proportion of data of DSWM and the cumulative proportion of normal distribution' **(A)**, 'consistency between the cumulative proportion of data of SSWM and the cumulative proportion of normal distribution' **(B)**. The closer the scatter distribution is to the diagonal, the more the data conforms to the normal distribution.

The stimuli were generated and presented by MATLAB and its toolbox (PsychToolbox). The size of the LCD screen of the stimulating computer is 23.8 inches and the refresh rate is 60 Hz. The viewing distance was kept at 40 cm using a chin rest. Luminance for all participants remained unchanged.

Procedure. SSWM task: The experiment is based on Gordon's spatial positioning test (Gordon, 1986). Specific procedure was as follows. At first, a rectangular box with a certain size was randomly presented at different positions on the computer screen, and at the same time, a dot with a certain size was randomly presented at different position of the rectangular. After a few seconds, these stimulus disappeared automatically, as shown in Figure 1A; then a rectangular box and a dot at its center with the same size appeared on the screen, as shown in the Figure 1C. The participants' task was to move the dot in the center of the rectangular box to the position in the rectangular box where the dot just disappeared through the direction key on the keyboard. The position of the rectangular box on the screen is different in the learning stage and the testing stage, so as to avoid the subjects completing the task by remembering the coordinate position of the point in the whole screen.

The specific operation of DSWM task is that a dot randomly appeared in the upper left area of the screen and completed the following movements in sequence at a fixed speed: a centimeters from left to right → b centimeters from top to bottom → a centimeters from right to left → b centimeters from bottom to top. This dot moved to its original place and then disappeared. The motion trajectory of dot finally formed a rectangle box with the same size as that in the SSWM task. After the dot disappeared, another dot appeared in the disappeared motion track (Figure 1B). After the latter dot disappeared, participants completed the same task as the SSWM experiment.

This task has a total of 50 attempts and takes a total of 40 min. The measurement index of this task is the distance deviation between the position of the last dot and the position of the initial dot, that is, how many screen pixels are apart.

Genotype quality control and imputation

DNA was extracted from saliva samples of 520 participants, and individuals were genotyped using Illumina Asian screening array (650K) by Beijing Compass Biotechnology (N1 = 288) and Genergy Biotechnology (N2 = 232). The quality control of two samples from different companies is exactly the same. Briefly, SNPs were filtered out if they showed a variant call rate <0.9, a minor allele frequency (MAF) < 0.05, a hardy-weinberg equilibrium (HWE) < 10^{-5}. Individuals were filtered out if they showed a missing genotype data (mind) < **0.10** (3 people from sample1 were removed, 1 people from sample2 were removed), unexpected duplicates or probable relatives (PI-HAT>0.20) (Anderson et al., 2010; Chang et al., 2015).

For imputation, autosomal variants were aligned to the 1000G genomes phase 1v3 reference panel. Imputation was performed using Michigan imputation Server 4.0 in 5 Mb chunks with 500 kb buffers (Das et al., 2016), filtering out variants that were monomorphic in the Genome Asia Pilot

TABLE 1 Correlations for sociodemographic variables (gender and grade) and phenotypes (SSWM and DSWM).

	SSWM	DSWM
Gender	-0.103*(a)	-0.015(a)
Grade	-0.432**(b)	-0.067(b)

* means $p < 0.05$, ** means $p < 0.01$, a means Pearson's r, b means Spearman's rho.

(GAsP). Chunks with 51% genotyped variants or concordance rate <0.92 were fused with neighboring chunks and re-imputed. Imputed variants were filtered out for rsq <0.60, MAF<0.05, mind<0.1, HWE<10^{-5} using Plink (v1.90).

After imputation, we merged two gene samples, and the merged sample left 516 individuals and 4 196 499 SNPs.

Genome-wide association analyses

After completing the above operations, **451** individuals have genetic data, phenotypic data and covariate data at the same time. Genome-wide association analyses were performed using Plink 1.90 (Rentería et al., 2013), fitting an additive model to the linear regression model with adjustment for sex, grade, and the first two principle components of population structure (Chang et al., 2015). Manhattan plots and Quantile-quantile plots were generated using the ggplot2 package in R. We used locuszoom to generate regional association plots (Pruim et al., 2010). The sign of genome-wide significance is $p < 5 \times 10^{-8}$.

Bioinformatics analysis

In order to examine whether the discovered rs80263879 and rs72478903 influnce gene expression, we checked the expression quantitative trait locus (eQTL) for these two loci in the Genotype-Tissue Expression (GTEx) Cohort (https://www.gtexportal.org/home/) (GTEx Consortium, 2020). Analysis of eQTL can be with reference to the study of Ramasamy et al. (Ramasamy et al., 2014).

Gene- and pathway-based enrichment tests

Gene-based and pathway-based enrichment tests for SSWM and DSWM were conducted by MAGMA (de Leeuw et al., 2015). Gene-based analyses is based on GWAS data. Every SNP is located on the protein-coding gene according to NCBI37.3. Genes were analyzed after internal quality control. We adopted default internal quality control steps and internal quality control values of magma. A total of 17,225 genes were involved in the analysis, so the threshold of significance was set at 2.90×10^{-6} ($p = 0.05/17{,}225$).

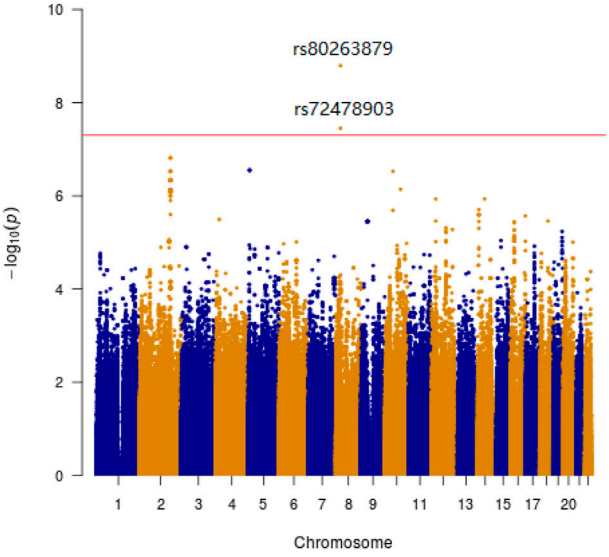

FIGURE 3
Manhattan plot showing genome-wide association analysis for SSWM. X-axis represents the position of SNPs on the chromosome, y-axis represents the significance level of association analysis. The horizontal red line represents the genome-wide significance level threshold (5×10^{-8}).

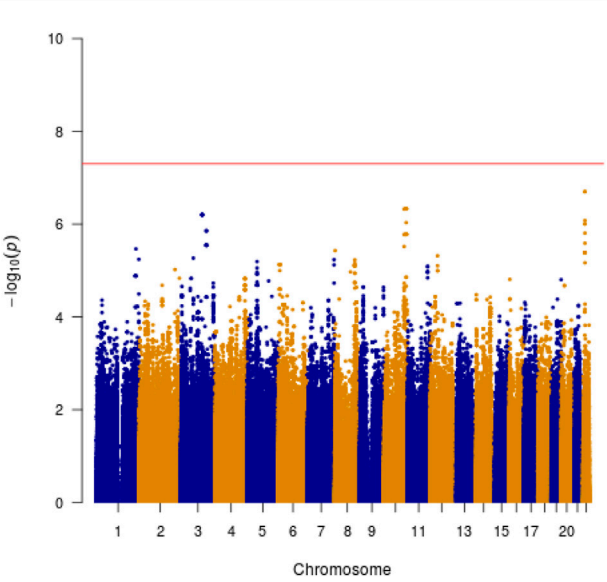

FIGURE 4
Manhattan plot showing genome-wide association analysis for DSWM.

TABLE 2 Two lead single-variant associations detected in the GWAS analyses.

SNP	CHR:BP	ALLEL1	Test	NMISS	BETA	STAT	P
rs80263879	8:27389631	A	ADD	188	19.73	6.358	1.6×10^{-9}
rs72478903	8:27400068	A	ADD	188	17.82	5.759	3.5×10^{-8}

Pathway-based analyses are derived from the results of gene-based analyses using a competitive gene-set analysis (Gerring et al., 2019). Orginal pathway were from Molecular Signatures Database website (MSigDB, c2.all.v7.0.entrez). A total of 5 497 pathways were involved in the analysis, so the threshold of significance was set at 9.10×10^{-6} ($p = 0.05/5\ 497$).

Results

Characteristics of behavioral data

After phenotypic data were collected, we used P-P plot to draw the consistency of the cummulative proportion of SSWM (Figure 2A) and DSWM (Figure 2B) with the cumulative proportion of normal distribution. P-P plot shows that the distributions of the two phenotypes are a little skewed. Then, we did correlation analysis between phenotypes and sociodemographic variables (gender and grade) (Table 1). Results showed that there were significant correlations between SSWM task and sociodemographic variables.

Genome-wide study of single-marker association

We identified two genome-wide significant loci associated with SSWM (rs80263879, $p = 1.6 \times 10^{-9}$; rs72478903, $p = 3.5 \times 10^{-8}$). These two loci have strong linkage disequilibrium. And these two SNPs are all located within the gene *EPHX2*. We have not identified any SNP significantly associated with DSWM. However, rs80263879 revealed weak association with DSWM ($p = 4.5 \times 10^{-2}$).

Manhattan plots were adopted to reveal the results of association analysis for SSWM (Figure 3) and DSWM (Figure 4). Due to the quality control of the two samples, the specific number of people at each locus is somewhat different. More details for significant locus were reported in Table 2. We used quantile-quantile plots for SSWM (Figure 5A) and DSWM (Figure 5B) to reveal the rationality of their respective analysis models. The genomic inflation factors λ of quantile-quantile plots was 1.023 for SSWM and 1.013 for DSWM, confirming little influence of population stratification. Regional association plot of the significant locus are revealed in Figure 6.

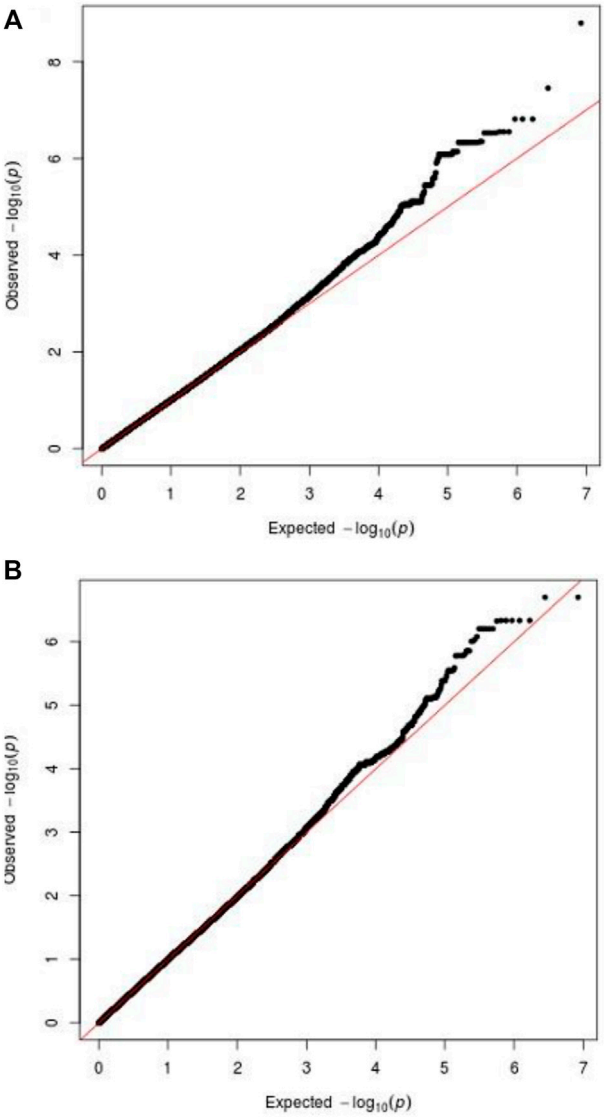

FIGURE 5
Q-Q plot for phenotypes. The above two figures show 'Q-Q plot for SSWM' **(A)**, 'Q-Q plot for DSWM' **(B)**.

Genetic mechanism of rs80263879 and rs72478903

Two genome-wide significant loci we identified are located in gene *EPHX2*. According to the data of eQTL from GTEx, both loci

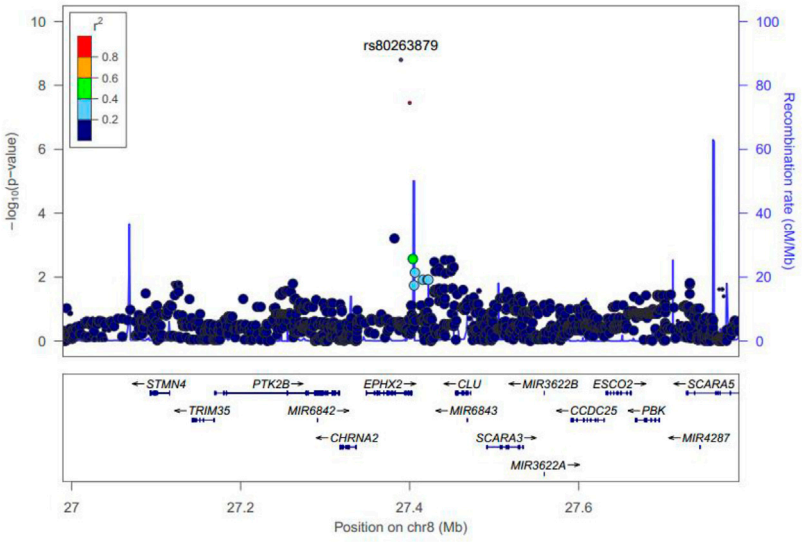

FIGURE 6
Regional plot of rs80263879 for SSWM. The most significant locus was marked violet. r^2 is the LD value of rs80263879 and any other locus.

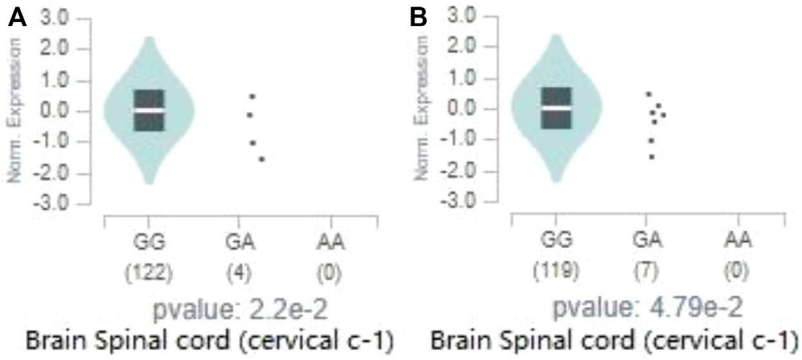

FIGURE 7
Effect of significant loci on the expression of *EPHX2*. The above two figures reveal 'expression amount of *EPHX2* on different genotypes of rs80263879 in spinal cord (cervical c-1)'**(A)**, 'expression amount of *EPHX2* on different genotypes of rs72478903 in spinal cord (cervical c-1)'**(B)** from Genotype-Tissue Expression (GTEx) Cohort.

have a significant regulatory effect on the expression of *EPHX2* in the spinal cord (cervical c-1) (rs80263879, $p = 2.2 \times 10^{-2}$; rs72478903, $p = 4.8 \times 10^{-2}$). Figure 7A reveals that the major allele G of rs80263879 is associated with increased *EPHX2* expression in spinal cord (cervical c-1). Results of eQTL in other 12 brain regions of rs80263879 are revealed in Supplementary Figure S1. Similarly, Figure 7B reveals that the major allele G of rs72478903 is associated with increased *EPHX2* expression in **spinal cord (cervical c-1).** Results of eQTL in other 12 brain regions of rs72478903 are revealed in Supplementary Figure S2.

Gene- and pathway-based associations

In the pathway-based enrichment analysis, we found a pathway (Standard name: GRAHAM_CML_QUIESCENT_VS._NORMAL_DIVIDING_UP) significantly enriched with DSWM after Bonferroni correction ($\beta = 0.51$, se = 0.11, $p = 1.36 \times 10^{-6} < 9.10 \times 10^{-6}$). Genes on the pathway in our data are shown in Supplementary Table S1. Genes of the pathway were found to be up-regulated in certain leukemia-derived quescent hemopoietic stem cells expressing CD34 in contrast with dividing

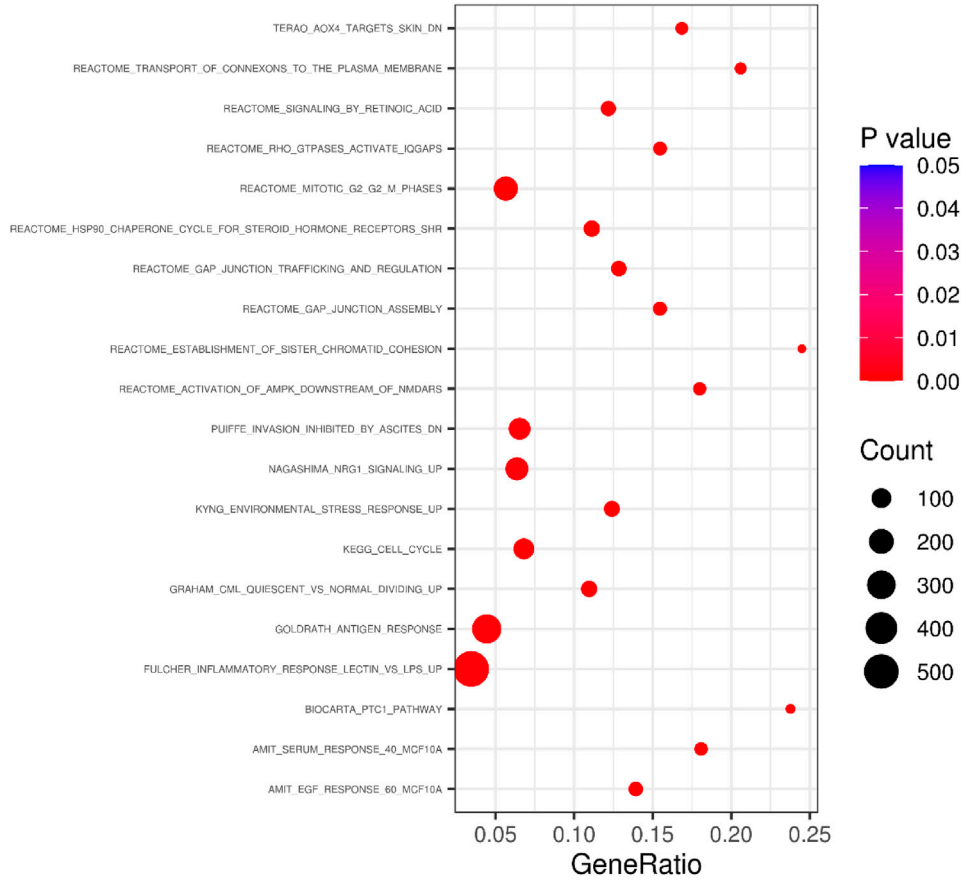

FIGURE 8
Bubble plot summarising the results of the pathway-based enrichment tests for DSWM. The plot reveals 20 pathways with the lowest *p* values.

cells supplied by the normal population (Graham, Vass, Holyoake, & Graham, 2010). Bubble plot of the 20 pathways with the lowest *p* values was shown in Figure 8, and full results for pathway-based enrichment analysis were shown in Supplementary Table S2.

Discussion

This study is the first genome-wide association study on spatial working memory in the general population. We reported two genome-wide significant loci (rs80263879 and rs72478903) associated with static spatial working memory. These two loci have strong linkage disequilibrium. We also found that rs80263879 and rs72478903 affect the expression of *EPHX2* in the spine. In addition, we identified a gene set significantly associated with dynamic spatial working memory. These results provide evidence for the genetic basis of spatial working memory.

Spatial working memory is an important ability in people's daily life, in the field of aerospace for predicting flight performance (Tirre & Raoufl 1998), and in representing an endophenotype of mental disorders such as schizophrenia. Spatial working memory has also been suggested to be influenced by genetic factors. Indeed, researchers have conducted genome-wide association analysis in schizophrenic patients and controls, and identified some loci associated with spatial working memory (Ren et al., 2015). However, so far, no genome-wide association analysis of spatial working memory has been conducted in general population. Here, our study report the first-hand evidence for the genetic basis of spatial working memory in a population of Chinese young adults.

We identified two genome-wide significant SNPs with strong LD among each other associated with static spatial working memory. These two loci are located within the gene *EPHX2*. *EPHX2* is associated with familial hypercholesterolemia (Pillai, Shah, Reddy, Ashavaid, & Vishwanathan, 2022). This was the

first time that it had been found to be related to spatial working memory. Spatial working memory is usually impaired among mental disorders such as schizophrenia and ASD (Jiang et al., 2015). Expression of *EPHX2* mRNA from schizophrenia and ASD have been found higher than that of controls (Ma et al., 2019). Therefore, we speculate that EPHX2 may play its role in mental disorders through spatial working memory. However, this speculation requires further empirical evidence for validation.

Different genotypes of rs80263879 and rs72478903 make gene *EPHX2* express in different degrees in the spinal cord, indicating that the spinal cord may be associated with SWM. Research on mice revealed that spinal cord injury can significantly block the expression of dopamine receptors in the frontal lobe, thus damaging SWM (Kheyrkhah et al., 2020). However, effects of these loci on regulating the expression of the gene *EPHX2* are weak, and number of subjects with "GA" genotype is small. Therefore, more samples are needed to verify this regulatory effect in the future.

A gene set significantly associated with dynamic spatial working memory was identified in enrichment analysis. The expression of genes in this gene set is positively regulated in quiescent CD34 + cells from some leukemia. CD34 + cells can differentiate into endothelial progenitor cells, which can be conducive to the repair of patients' myocardium (Kim et al., 2016).

In conclusion, we identified two loci significantly associated with static spatial working memory, which are located on the gene *EPHX2* and regulate the expression of this gene. We also identified a gene set associated with dynamic spatial working memory. Our research deepens the understanding of the genetic basis of spatial working memory and can provide reference for the treatment of mental diseases to some extent.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found in the GWA catalog http://ftp.ebi.ac.uk/pub/databases/gwas/summary_statistics/ GCST90133001-GCST90134000, GCST90133162, and GCST90133163.

Ethics statement

The studies involving human participants were reviewed and approved by the evaluation committee of Shaanxi Normal University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

LZ is responsible for the main work, ZZ guides the experimental design, QY assists data analysis and JZ gives comprehensive guidance.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.915275/full#supplementary-material

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