Research Square

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Alteration mRNA Expression Profile of Autophagy Related mTOR Pathway in Schizophrenic Patients with Olanzapine Treatment.

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Abstract

Background: mTOR signaling pathway involves in the pathogenesis of schizophrenia and the mechanism of extrapyramidal adverse reactions of antipsychotic drugs, which might mediate by mTOR-dependent autophagy impairment. This study aimed to examine the mRNA expression levels of Mammalian rapamycin target protein (mTOR) pathway genes in schizophrenia patients with olanzapine treatment, which is considered to be an mTOR inhibitor and autophagy inducer.

Methods: Thirty-two acute schizophrenia patients had been treated with olanzapine for four weeks (average dose 14.24 ± 4.35 mg/d), along with 32 healthy volunteers. Before and after olanzapine treatment, the Positive and Negative Syndrome Scale (PANSS) was used to evaluate the symptoms of schizophrenia patients, and the mRNA expression levels of mTOR pathway-related genes, including MTOR, RICTOR, RPTOR, and DEPTOR, were detected by real-time quantitative PCR with the fasting venous blood of all the samples.

Results: The MTOR and RICTOR mRNA expression levels of acute schizophrenia patients significantly decreased than them of healthy controls, and furtherly significantly decreased after four weeks of olanzapine treatment. While DEPTOR mRNA expression levels of acute schizophrenia patients had no significant difference with them of healthy controls, but significantly increased after treatment. And the mRNA expression levels of RPTOR had no significant difference in the three groups. The pairwise correlation of MTOR, DEPTOR, RPTOR, and RICTOR mRNA expression levels in acute schizophrenia patients and healthy controls showed significant correlationships. After olanzapine treatment, the correlationships of mRNA expression levels disappeared between DEPTOR and MTOR, and also between DEPTOR and RICTOR.

Conclusions: The results inferred the abnormalities of mTOR pathway, especially DEPTOR, might play important roles in autophagy mechanism of the pathophysiology in schizophrenia and olanzapine treatment.

Background

Schizophrenia is a serious chronic mental disorder, and its prevalence rate is about 1% of the population worldwide [1]. The pathogenesis of schizophrenia is not completely clear, and there are no reliable objective biological indicators for adjuvant diagnosis and treatment. Recent research has indicated that the mammalian rapamycin target protein (mTOR) pathway may be involved in schizophrenia pathogenesis.

mTOR is a highly conserved serine/threonine kinase with a molecular weight of 289 kDa [2]. mTOR, regulatory-associated protein of mTOR (RPTOR), and DEP domain-containing MTOR-interacting protein (DEPTOR) form the mTORC1 complex, which is mainly involved in cell growth, apoptosis, energy metabolism, and autophagy; meanwhile, mTOR, DEPTOR, and rapamycin-insensitive companion of mTOR (RICTOR) form the mTORC2 complex, which is mainly involved in the construction and survival of

cytoskeleton protein [3]. The main upstream signaling pathways of mTOR are the P13K/Akt pathway and the AMPK pathway. The major effector target proteins of the mTOR downstream pathway are 4E-BPl and S6K1, which are involved in protein translation initiation, gene transcription, and cell cycle regulation [4].

The mTOR signaling pathway might be involved in the pathogenesis of schizophrenia. Neurobiochemical studies have inferred that the mTOR signaling pathway is involved in the downstream signal transduction mechanism of genes related to schizophrenia, such as DRD2 [5], DISC1 [6], BDNF [7], NMDAR [8], 5-HTR [9], and Reelin [10]. Studies have shown that mice with disordered mTORC2 signaling in the brain exhibit changes in striatum DA-dependent behavior, such as increased basal ganglia-related activities, stereotyped counts, and significant enhancement of the psychomotor effects of amphetamine [11].

The mTOR pathway could change synaptic plasticity and affect cognitive functions such as learning and memory. Recent studies have supported that cognitive deficits in schizophrenic patients are related to synaptic plasticity, and schizophrenia has also been defined as a "synaptic disease" [12]. Tischmayer et al. discovered the importance of the mTOR signaling pathway to the long-term memory of Mongolian gerbils with a linear frequency modulation tone experiment [13]. Parsons et al. [14] found that direct infusion of rapamycin into the amygdala of rodents could prevent the phosphorylation of p70S6K, thus affecting the formation of fear memory. Dash et al. [15] also demonstrated the correlation of glucose levels, mTOR activation, and spatial memory formation in animal experiments. Furthermore, the close correlation between cell scaffold dynamics and mTOR signaling is the key to early axonal transport defects and synaptic transmission changes, which is a common pathological feature of schizophrenia [16].

The mTOR signaling pathway plays a role in the mechanism of extrapyramidal adverse reactions of antipsychotic drugs. Gene expression microarray analysis of first-episode schizophrenic patients before and after treatment with risperidone or paliperidone showed that the mTOR pathway was involved in extrapyramidal system reactions [17, 18]. It was also reported that the interaction of four SNP loci, rs1130214 (AKT1), rs456998 (FCHSD1), rs7211818 (Raptor), and rs1053639 (DDIT4), could predict the extrapyramidal response of schizophrenic patients after using antipsychotic drugs. The accuracy of prediction was 85%-88% in 243 schizophrenic patients treated with risperidone or other antipsychotic drugs [19].

In summary, the mTOR signaling pathway was involved in the downstream signal transduction mechanism of genes related to schizophrenia, in the alteration of synaptic plasticity and cognitive functions, and in the mechanism of extrapyramidal adverse reactions to antipsychotic drugs. However, so far, little is known about the gene expression profile of the mTOR pathway in schizophrenic patients. This study examined the expression levels of mTOR pathway genes in schizophrenia patients before and after olanzapine treatment, which is of great significance to further understand the pathological changes in mTOR pathway of schizophrenia.

Materials And Methods

Participations

A total of 45 schizophrenic inpatients with acute episodes were recruited from September 2017 to June 2018 at Wuxi Mental Health Center of Nanjing Medical University as the case group. There were 3 patients excluded later because of a revised diagnosis, and 10 patients fell off because they could not complete the evaluation and blood collection on the fourth weekend. At the time of admission, one deputy chief psychiatrist made identical diagnoses according to the diagnostic criteria of schizophrenia in the Diagnostic and Statistical Manual of Mental Illness, 4th Edition (DSM-IV) without other Axis 1 diagnoses, and the total PANSS score for each patient was over 90. The 46 healthy volunteers of the healthy control group were recruited from people undergoing health examinations at Wuxi Tongren Rehabilitation Hospital. All subjects included in this study were of Han nationality, aged between 18 and 60 years, and not menstruating, pregnant, or lactating if female; they had no serious physical diseases; no past history of substance abuse; no blood transfusion treatment in the past three months; and no electroconvulsive therapy, antipsychotics, emotional stabilizers, or antidepressants in the past three months.

Methods

Antipsychotics therapeutic protocol

Enrolled schizophrenia patients received olanzapine single-drug therapy. The initial dose of olanzapine was 5 mg/d, and the therapeutic dose was increased to 10–20 mg/d after one week. The average dose of olanzapine across all the patients was 14.24 ± 4.35 mg/d, and the observation period was 4 weeks. The treatment not combined with other antipsychotics, mood stabilizers, antidepressants, sedatives, or electroconvulsive therapy for any of the patients in the study.

Demographic And Clinical Data Collection

On the day of enrollment, demographic data and clinical data of schizophrenia patients were collected by one psychiatric doctor, who had passed the consistency training for a PANSS assessor at the Sixth Hospital of Peking University. The psychiatric symptoms of all schizophrenia patients were assessed at baseline and the fourth weekend of drug treatment.

Blood Specimen Collection

The fasting elbow venous blood of acute schizophrenia patients was collected at 6:30 a.m. on the day of enrollment and the fourth weekend of drug treatment. The healthy controls only had venous blood drawn on the day of admission under the same conditions. Blood samples were collected with a 2 ml PAXgene Blood RNA Tube (BD Biosciences, USA) and frozen at − 80°C.

Rna Extraction, Quality Control, And Reverse Transcription Reaction

The total RNA was extracted with TRIzol (Invitrogen, USA). Their concentration and purity were measured with NanoDrop ND-1000 (NanoDrop Technologies, USA), and the A260/A280 ratio ranged from 1.8 to 2.1. Formaldehyde denaturation gel electrophoresis was used to detect the integrity of RNA and to exclude DNA contamination. The first strand cDNAs were synthesized by reverse transcription reaction with SuperScript III Reverse Transcriptase (Invitrogen) and Oligo $(dT)_{18}$ on GeneAmp PCR System 9700 (ACROBiosystems, USA). The above experimental operations were carried out with reference to the corresponding protocol.

Real-time Quantitative Pcr

Real-time quantitative PCR was carried out with qPCR SYBR Green master mix (CloudSeq Co., USA), 2 μl cDNA (nearly 400 ng), 0.5 μl each of forward and reverse primers (10 μM) designed by Primer Premier 5.0 (all the primers are listed in Table 1, with GAPDH as the housekeeping gene), and a total reaction system up to 10 μl on a ViiA 7 Real-Time PCR System (ACROBiosystems). The PCR cycle conditions were as follows: pre-denaturing at 95°C for 10 min; 40 cycles of denaturing at 95°C for 10 s and annealing at 60°C for 60 s (collecting fluorescence); terminating at 95°C for 15 s. After the amplification reaction was completed, the temperature was raised from 60°C to 99°C (ramp rate = 0.05°C/s) to establish the melting curve of the PCR product. Delta-delta Ct was used to detect the relative mRNA expression.

Table 1
Primer sequence and PCR product length of housekeeping gene and target genes

Gene	Primer types	Primer sequence	Tm value(°C)	PCR product length(bp)
GAPDH	Forward	GGCCTCCAAGGAGTAAGACC	60.07	122
	Reverse	AGGGGAGATTCAGTGTGGTG	59.96	
MTOR	Forward	AGCCGGAATGAGGAAACC	60.01	232
	Reverse	CAAATCTGCCAATTCGGG	60.00	
DEPTOR	Forward	ATTGTTGGTGACGCGGTT	59.97	137
	Reverse	AGCCCGTTGACAGAGACG	59.98	
RICTOR	Forward	AAGGCCAAACAGCTCACG	59.98	230
	Reverse	ACTCCATGAGGGTGGCAA	60.05	
RPTOR	Forward	CAGGACTTGCTGGTGGCT	59.98	84
	Reverse	GCTGACGGGAGTGCAGTT	59.99	

Statistical Analyses

Data collation and analysis were performed with the SPSS 19.0 statistical software package. The sex and age data were compared between the case group and control group with the *t* test and χ^2 test. The Kolmogorov-Smirnov test showed that the expression-level data of four target genes conformed to the normal distribution. A paired-samples *t* test was used to compare the expression levels of the target genes of the case group before and after treatment, and an independent-samples *t* test was used to compare the expression levels of target genes between the control group and case group before treatment. The data were presented as the mean and standard deviation (+ *SD*), and the test level was α = 0.05 bilaterally.

Results

Comparison of demographic and clinical data between the case group and control group

A total of 32 schizophrenic patients completed the study, including 16 female patients and 16 male patients, with an average age of 37.53 ± 10.84 years. After admission, their average dose of olanzapine was 14.24 ± 4.35 mg/d. The 46 healthy volunteers of the healthy control group included 19 females and 27 males, with an average age of 36.78 ± 10.96 years. There was no significant difference in sex, age, marital status, or years of education between the case group and control group ($p > 0.05$; Table 2).

Table 2
Demographic and clinical data between case group and control group

	Case group (n = 45)	Control group (n = 46)	t/χ^2 value	*p* value
Age (years old)	38.20 ± 11.72	36.78 ± 10.96	0.596	0.553
Gender (male %)	48.9%	58.7%	0.530	0.467
Marital status (married %)	44.4%	52.1%	0.114	0.736
Years of Education (%)	-	-	3.031	0.695
1–6 years	8.9%	6.5%	-	-
7–9 years	40%	39.1%	-	-
10–12 years	17.8%	21.7%	-	-
13–15 years	15.6%	13.0%	-	-
Over 16 years	17.7%	19.5%	-	-
First-episode patients (%)	33.3%	-	NA	NA
Age of first onset (years old)	31.22 ± 11.26	-	NA	NA
Disease duration (years)	2.80 ± 1.91	-	NA	NA
Olanzapine dose (mg/d)	14.24 ± 4.35	-	NA	NA
PANSS total score before treatment	115.84 ± 15.64	-	NA	NA
PANSS total score after treatment	76.38 ± 14.72	-	NA	NA

Note: The data of sex, marital status and years of education were compared with χ^2 test, and the data of age was compared with t test. The data of sex, age, marital status and years of education had no significant difference between the case group and control group (p□0.05).

Comparison Of Mrna Expression Levels Of Mtor Pathway Genes

The comparison of mRNA expression levels of mTOR pathway genes showed that before olanzapine treatment, MTOR, RPTOR, and RICTOR mRNA expression levels were significantly lower in the case group than in the control group, while there was no significant difference in DEPTOR mRNA between them. After olanzapine treatment, the DEPTOR mRNA expression levels significantly increased in the case group, with no significant difference in the other three target genes (Table 3 and Fig. 1).

Table 3
Comparison of mRNA expression levels of mTOR pathway genes in control group and case group before and after olanzapine treatment. ($\bar{x}$+SD)

	Case group		Control group	χ^2	p	Multiple comparisons		
	Baseline (n = 45)	After treatment (n = 32)	(n = 46)			MD[a]	MD[b]	MD[c]
MTOR[a,b,c]	1.08 ± 0.59	0.51 ± 0.30	2.63 ± 1.78	67.624	0.000	0.568	2.123	1.556
DEPTOR[a,c]	1.72 ± 2.90	5.23 ± 2.49	0.75 ± 0.52	63.753	0.000	3.506	4.477	0.971
RPTOR [c]	2.30 ± 2.40	1.60 ± 1.13	2.35 ± 1.48	6.553	0.038	0.745	0.748	0.046
RICTOR[a,b,c]	1.06 ± 0.58	0.49 ± 0.29	3.53 ± 2.36	80.788	0.000	0.564	3.040	2.476

Note: Kolmogorov-Smirnov test and Levene variance homogeneity test showed that the expression level data of four target genes did not conform to normal distribution and were not uniform. Therefore, Kruskal Wallis non-parametric test was used to compare the expression levels of target genes between the control group and case group before and after treatment, and Tamhane test was used to multiple comparisons.

[a] Comparison of mRNA expression levels of target genes in case group before and after treatment had significant difference ($p < 0.05$);

[b] Comparison of mRNA expression levels of target genes between control group and case group before treatment had significant difference ($p < 0.05$);

[c] Comparison of mRNA expression levels of target genes between control group and case group after treatment had significant difference ($p < 0.05$).

Correlation analysis of mRNA expression levels of mTOR pathway genes

MTOR, DEPTOR, RPTOR, and RICTOR gene expression levels in patients with acute schizophrenia and the normal control group were significantly correlated in all three groups, with the MTOR and RICTOR gene expression levels having the highest correlation ($r = 0.987–1.000$). However, after 4 weeks of olanzapine treatment, the pairwise correlation of gene expressions level between DEPTOR and MTOR and between DEPTOR and RICTOR disappeared (Fig. 2).

Correlation analysis of mTOR pathway gene expression levels and demographic and clinical data

No correlations between the four target genes and demographic or clinical data, such as gender, age, marital status, education level, first episode or recurrence, course of disease, or PANSS score, were found in the health control group or case group before or after treatment.

Discussion

The mTOR signaling pathway exists in almost all the peripheral tissues and the central nervous system, and is involved in regulating protein synthesis, mitochondrial biogenesis, cell proliferation, cell survival, cell death [20, 21], and synaptic plasticity [12]. The abnormality of the mTOR pathway has attracted more and more attention, and has been found in many diseases, including cancer [22], obesity [23], type II diabetes mellitus [24], neurological and psychiatric diseases [25], neurodegeneration, and brain tumors. Recent studies have shown that many psychiatric drugs, including mood stabilizers and neurorelaxants, which are also autophagy-inducible factors, can regulate autophagy and play a therapeutic role in the mTOR pathway [26, 27].

To date, there have been relatively few studies on the relationship between expression levels of the mTOR pathway and the pathogenesis of schizophrenia. They have mostly been pre-clinical studies, with few studies involving schizophrenia patients. In this study, acute schizophrenic patients were recruited to study the expression levels of the MTOR, DEPTOR, RPTOR, and RICTOR genes of the mTOR pathway before and after olanzapine treatment and to explore their feasibility to be the biomarker of schizophrenia and olanzapine effect.

Previous studies have shown that mTOR catalytic subunits are essential for normal brain physiology and development. MTOR deficiency mice may exhibit telencephalon deficiency or early embryonic death [28, 29]. Overactivation of MTOR can also lead to cortical atrophy in the early embryonic stage and to cortical hypertrophy and severe epileptic seizures in late embryonic or postpartum mitotic neurons [30]. Merenlender-Wagner et al. have confirmed that MTOR-dependent autophagic dysfunction is accompanied by changes in the gene expression and protein level of microtubule-associated protein 6 (MAP6) in brain samples of postmortem schizophrenic patients and in schizophrenic mouse models [31, 32]. Zhou et al. found that the downregulation of DISC1 in the dentate gyrus of adult mice resulted in abnormal morphology and excitability of neural networks and a schizophrenia-like behavior phenotype, which could be rescued by rapamycin injection [33].

Few studies have researched MTOR gene expression in patients with mental disorders. Mostaid et al. found that mTOR mRNA expression levels were negatively correlated with the duration of illness in treatment-resistant schizophrenia patients, and clozapine exposure could decrease mTOR mRNA expression levels in an in vitro culture of PBMC cells from treatment-resistant schizophrenia patients [34]. Machado-Vieira et al. found decreased mTOR mRNA expression levels in 25 unmedicated depressed individuals with bipolar disorder, which showed no significant change after 6 weeks of lithium therapy [35]. Dong et al. suggested that prenatal stress induces decreased mTOR mRNA levels, which may be associated with anxiety-like and alcohol drinking behaviors in adulthood [36]. These above studies

suggested that there may be abnormal expression of the MTOR gene in schizophrenia and other psychiatric disorders. Our results found that MTOR gene expression levels were significantly lower in acute schizophrenia patients before treatment than in healthy controls and did not change significantly after olanzapine treatment.

DEPTOR has been reported to be an endogenous regulator of the mechanistic target of rapamycin complex 1 (mTORC1) and mTORC2. DEPTOR is widely expressed from the forebrain to the hindbrain, including the hippocampus, the mediobasal hypothalamus, and the circumventricular organs (CVOs) [37]. There have been relatively few studies on DEPTOR in mental disorders and no reports in schizophrenia. Fabbri and Serretti used the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD) genome-wide dataset to investigate the genetic predictors of long-term treatment outcomes and found that the DEPTOR gene, which is susceptible to antidepressant action, may affect the long-term treatment outcome of BD [38]. Davies et al. demonstrated the reduction of DEPTOR protein level in the precentral gyrus, postcentral gyrus, and occipital lobe of Alzheimer's disease (AD) patients, as well as a reduction of DEPTOR expression in late-onset AD compared to early-onset familial AD [39]. In our study, DEPTOR gene expression did not differ significantly between healthy controls and acute schizophrenia patients before treatment, but after 4 weeks of olanzapine treatment, the DEPTOR expression level significantly increased in schizophrenic patients.

RPTOR is an important component of mTORC1 and a regulatory protein of mTOR. RPTOR knockout mice showed decreased body weight, brain weight, and cortical thickness compared to other 7-week-old wild-type mice [40]. The research on RPTOR in mental disorders has focused on the prediction function of mTOR pathway-related genes in antipsychotic (AP)-induced extrapyramidal symptoms (EPS). Mas et al. analyzed gene–gene interactions in nine genes related to the mTOR pathway in order to develop genetic predictors of the appearance of EPS and identified a four-way interaction among rs1130214 (AKT1), rs456998 (FCHSD1), rs7211818 (Raptor), and rs1053639 (DDIT4) that correctly predicted AP-induced EPS in 97 of the 114 patients (85% accuracy). Then, they validated the predictive power of the four-way interaction in two independent cohorts and reported 86% and 88% accuracy, respectively [19]. Boloc et al. developed a pharmacogenetic predictor of AP-induced EPS based on two SNPs in the AKT1 gene (rs33925946 and rs1130214) and two SNPs in the RPTOR gene (rs3476568 and rs9915667) in 131 schizophrenia inpatients treated with risperidone. Their prediction model achieved 66% accuracy of AP-induced EPS in the discovery cohort and showed similar performance in replications of schizophrenia cohort treatment with risperidone or other Aps [41]. In this study, RPTOR gene expression levels were significantly lower in acute schizophrenia patients before treatment than in health controls and did not change significantly after olanzapine treatment.

RICTOR is a component of mTORC2. RICTOR KO animal experiments have shown that it plays an important role in the pathogenesis of schizophrenia. Dadalko et al. found that neuron-specific Rictor knockout mice exhibited altered striatal DA-dependent behaviors, such as increased basal locomotion and stereotypic counts and exaggerated response to the psychomotor effects of amphetamine [42]. Siuta et al. demonstrated that neuronal Rictor knockout mice showed impairments in neuronal Akt Ser473

phosphorylation, prepulse inhibition (PPI) deficits, hypodopaminergia in the rostral cortex, an increase in NE transporter (NET) expression and function, and schizophrenia-like behaviors [43]. Moreover, RICTOR is also associated with the pathological mechanisms of other mental disorders. Miyata et al. used ovariectomized (OVX) mice exposed to chronic mild stress to simulate depression during menopause to conduct studies of genome-wide gene expression in both the medial prefrontal cortex and blood cells. They found that RICTOR was the top-ranked regulator associated with the production of OVX-induced gene expression alterations in both tissues [44]. Eriguchi et al. used exome sequencing to identify novel risk loci of sporadic Tourette syndrome (TS) cases and found that rs140964083 (RICTOR) was a novel candidate factor for TS etiology [45]. In this study, the RICTOR gene expression level was significantly lower in acute schizophrenia patients before treatment than in healthy controls and did not change significantly after olanzapine treatment.

This study also found that MTOR, DEPTOR, RPTOR, and RICTOR gene expression levels were significantly pairwise correlated in acute schizophrenia patients and the normal control group, and MTOR pathway genes might interact and coordinate as a whole to play the biological role. However, after 4 weeks of olanzapine treatment, the pairwise correlations of gene expression levels between DEPTOR and MTOR and between DEPTOR and RICTOR disappeared. MTOR, DEPTOR, and RICTOR are the key components of the mTORC2 complex. These findings suggest that olanzapine may influence the mRNA expression of DEPTOR and the formation of the mTOR complex.

Conclusions

This study found that before olanzapine treatment, the mRNA expression levels of MTOR, RPTOR, and RICTOR were significantly lower in the case group than in the control group, while DEPTOR mRNA expression levels showed no significant difference between two groups. After olanzapine treatment, the DEPTOR mRNA expression levels significantly increased in the case group, with no significant difference in the other three target genes. This study also found that MTOR pathway genes might interact and coordinate as a whole to play the biological role in healthy controls and acute schizophrenia patients. However, after olanzapine treatment, the pairwise correlations between the gene expression levels of DEPTOR and MTOR and of DEPTOR and RICTOR disappeared. It can be inferred that olanzapine may influence the mRNA expression of DEPTOR and the formation of the mTOR complex. To date, the exact function of the DEPTOR gene in schizophrenia has not been fully elucidated. And the role of the mTOR pathway in the curative effect and adverse reactions of antipsychotic drugs had also been mentioned in above several researches.

This study also has some limitations. The sample size was small, and the shedding rate of acute schizophrenia patients was a little high. Additionally, the mTOR pathway genes are abundantly expressed in the body, and there is no clear evidence that the expression level of mTOR pathway genes in peripheral blood can reflect their function in the brain. But it is of great significance to further understand the pathological changes of mTOR pathway in schizophrenia, and to explore their feasibility to be the biomarker of schizophrenia.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki and was approved by the Ethics Committee of the Wuxi Mental Health Center. Prior to the study, the procedure was fully explained, and written informed consent was obtained from each subject or the first legal guardian if the patient had limited decision-making capacity.

Consent for publication

Not applicable.

Availability of data and materials

Data of this study are not publicity available as being a part of a broader project, which data are still analyzing, but are available from the corresponding author on reasonable request.

Competing interests

The authors report no potential competing interests.

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Authors' contributions

LL designed this manuscript. FC, SG and YG recruited the schizophrenia cases and health controls, collected the demographic data and rated clinical scales. CF and LL managed the literature searches, interpreted the data and prepared the manuscript. All authors contributed to and approved the final manuscript and reviewed it critically for important intellectual content.

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