

Pyrosequencing analysis of *IRS1* methylation levels in schizophrenia with tardive dyskinesia

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Abstract. Tardive dyskinesia (TD) is a serious side effect of certain antipsychotic medications that are used to treat schizophrenia (SCZ) and other mental illnesses. The methylation status of the insulin receptor substrate 1 (*IRS1*) gene is reportedly associated with SCZ; however, no study, to the best of the authors' knowledge, has focused on the quantitative DNA methylation levels of the *IRS1* gene using pyrosequencing in SCZ with or without TD. The present study aimed to quantify DNA methylation levels of 4 CpG sites in the *IRS1* gene using a Chinese sample including SCZ patients with TD and without TD (NTD) and healthy controls (HCs). The general linear model (GLM) was used to detect DNA methylation levels among the 3 proposed groups (TD vs. NTD vs. HC). Mean DNA methylation levels of 4 CpG sites demonstrated normal distribution. Pearson's correlation analysis did not reveal any significant correlations between the DNA methylation levels of the 4 CpG sites and the severity of SCZ. GLM revealed significant differences between the 3 groups for CpG site 1 and the average of the 4 CpG sites ($P=0.0001$ and $P=0.0126$, respectively). Furthermore, the TD, NTD and TD + NTD groups demonstrated lower methylation levels in CpG site 1 ($P=0.0003$, $P<0.0001$ and $P<0.0001$, respectively) and the average of 4 CpG sites ($P=0.0176$, $P=0.0063$ and $P=0.003$,

respectively) compared with the HC group. The results revealed that both NTD and TD patients had significantly decreased DNA methylation levels compared with healthy controls, which indicated a significant association between the DNA methylation levels of the *IRS1* gene with SCZ and TD.

Introduction

Schizophrenia (SCZ) has a strong genetic component with a heritability of up to 80% (1,2). Tardive dyskinesia (TD) is presented by repetitive and jerking movements in the face, neck and tongue and is considered to be a serious side effect associated with the use of specific antipsychotic medications that are used to treat SCZ and other mental illnesses. TD may demonstrate a comorbidity of 20-30% with SCZ (3,4). Recent studies have reported that complex interactions of genetic, environmental and epigenetic factors play important roles in SCZ and TD (4-7). DNA methylation is an epigenetic mechanism that influences CG dinucleotides by adding a methyl group (CH₃) to them. Previously, several studies have reported the association between DNA methylation and SCZ (8-12) and TD (13,14).

Insulin resistance has reportedly been associated with atherosclerosis and type 2 diabetes (15). The insulin receptor substrate 1 (*IRS1*) gene is located at 2q36.3 (16-18) and it may be involved in the regulation of critical metabolic and signaling and secretion pathways (19-22). A genome wide association study in a French sample showed that one single nucleotide polymorphism (SNP) rs2943641, within 500 kb upstream of the *IRS1* gene, was associated with type 2 diabetes, insulin resistance and hyperinsulinemia (23). Furthermore, the *IRS1* gene DNA methylation was associated with high body mass index (BMI) scores and obesity (24,25). However, there was no significant association between the methylation of the *IRS1* gene and type 2 diabetes (26). Gunnell *et al* (27) attempted to evaluate the SNP rs1801278 in the *IRS1* gene with SCZ but could not identify any significant association. Recently, *IRS1* gene methylation has been found to be associated with SCZ (9,28). Pyrosequencing provides a quantitative analysis of DNA methylation levels (29-31) and has been employed

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to detect DNA methylation levels of the *IRS1* gene in type 2 diabetes (26) and human islets of type 2 diabetes (32).

A pilot study using methylated DNA immunoprecipitation coupled with next-generation sequencing (our unpublished data) revealed that the *IRS1* gene was hypomethylated in TD compared with SCZ groups. The present study aimed to quantify DNA methylation levels of the *IRS1* gene via pyrosequencing to examine the association of the *IRS1* gene DNA methylation levels with SCZ or TD. The general linear model (GLM) was used to examine the differences in the DNA methylation levels among diagnostic groups.

Materials and methods

Participants. The present study recruited 10 SCZ patients with TD and 10 without TD (NTD) from the Beijing Huilongguan Hospital (China) between January 2016 and June 2017. SCZ was diagnosed using the Diagnostic and Statistical Manual of Mental Disorders version IV (DSM-IV) (33) and TD was confirmed by two well-trained psychiatrists with extensive clinical experiences, based on the criteria of Schooler and Kane (34). The 20 SCZ patients were administered with one of the following: Risperidone, paliperidone or olanzapine. The TD patients were typically between 18 and 40 years of age and demonstrated an abnormal involuntary movement scale (AIMS) score that was >3 in at least one part or >2 in two or more parts (35). The same criteria were used for NTD patients with AIMS=0. The exclusion criteria included: Patients with severe physical or organic encephalopathy, drug or alcohol abuse history (except tobacco), pregnant or lactating women, patients administered with neurotrophic agents or free radical metabolism drugs within 12 weeks prior to participation, and other mental illnesses demonstrating a diagnosis of DSM-IV Axis I (14). The severity of TD symptoms was assessed using AIMS, with an inter-rater correlation coefficient (ICC)>0.80. The patients' psychotic symptoms were evaluated using the positive and negative syndrome scale (PANSS) (36), with an ICC>0.85, which was maintained for the PANSS total score after the scale training. A total of 10 healthy controls (HCs) matched for age, sex and education were subsequently recruited from the local community. Ethical approval was received from the Ethics Review Board of Beijing Huilongguan Hospital, China, and written informed consent was obtained from all participants or their guardians.

DNA extraction, bisulfite treatment and pyrosequencing. Fasting venous blood (5 ml) from a forearm vein was obtained from each participant at 7:00 a.m. the next morning after the day of clinical assessment. DNA was extracted from the above blood samples using a standard genomic DNA sample kit (Illumina, Inc.), according to the manufacturer's protocol. DNA concentration and purity were detected by NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Inc.) and the integrity was tested using 1% agarose gel electrophoresis (14). Subsequently, 500 ng of each sample was treated with bisulfite, by employing the Epitect Bisulfite Kit (Qiagen GmbH), according to the manufacturer's protocol. Parts of the CpG islands in the promoter region within the *IRS1* gene were amplified with the help of PCR assays. DNA fragments were typically amplified

using the PyroMark PCR kit (Qiagen GmbH), according to the manufacturer's protocol, from 2 μ l bisulfite-treated genomic DNA. Sample preparation and pyrosequencing reactions were subsequently carried out using the PyroMark Q96 ID (Qiagen GmbH). The pyrosequencing assays were performed for all the study samples on both Pyro Mark Q24 MDx and PyroMarkQ96 ID, while using Pyro Mark Gold reagents (Qiagen GmbH). The Pyro Mark Assay Design software version 2.0 (Qiagen GmbH) was used to generate the primers for the *IRS1*, targeting four CpGs in the gene promoter (Table I). Percentage of each CpG site and the mean methylation percentage of the 4 CpGs quantitatively revealed the methylation levels.

Statistical analysis. The χ^2 test was used to detect gender differences among TD, NTD and HC groups. Pearson's correlation analysis was concurrently used to examine the correlations among methylation levels among the 4 CpG sites, average level of the 4 CpG sites and severity of SCZ. Differences among the continuous variables including age and education were evaluated by Fisher's F test in the GLM. Normality of DNA methylation levels was tested by the Shapiro-Wilk test. Differences of DNA methylation levels among the 3 groups, TD, NTD and HC, for individual CpG sites and the mean of 4 CpGs were detected using GLM. All the analyses were performed using SAS version 9.4 software (SAS Institute, Inc.). The PROC POWER statement in SAS was used to compute power in the present study. P<0.05 was considered to indicate a statistically significant difference.

Results

Demographics and clinical characteristics. The demographic factors in the TD, NTD and HC groups are presented in Table II. No statistical significances were observed in age (P=0.910), sex (P=1.00) and educational levels (P=0.832) among the 3 groups. There were no significant differences between TD and NTD groups in the disease duration and treatment, drug dose quantized using CPZ equivalents or drug types. Additionally, no significant difference was observed between TD and NTD groups in the PANSS total, positive, negative or general scores (Table III).

Normality test. The average DNA methylation levels of the 4 CpG sites followed normal distribution (Fig. 1) based on the Shapiro-Wilk test (P>0.150).

Correlation analyses. Table IV demonstrated that no significant correlations were found among the individual methylation levels of the 4 CpG sites (all P-values >0.05); however, CpG sites 1 and 4 demonstrated a strong correlation to the mean value of the 4 CpG sites (P<0.0001). Furthermore, there were no obvious correlations among CpG sites and the severity of SCZ measured by PANSS scores.

GLM analyses. The linear GLM revealed significant differences with regard to the CpG site 1 and the mean value of the 4 CpG sites (P=0.0001 and P=0.0126, respectively; Table V and Fig. 2), among the 3 groups. Furthermore, the HC group demonstrated higher methylation levels in the CpG site 1

Table I. Primer sequences used in the pyrosequencing analysis.

Primer type	Primer sequence	CpG Sites	Position 5'-3'
Forward	5'-AGTGGTTATAGAGTTTGTATGTTTATTAGT-3'	4	146-174
Reverse	5'-CCTAAAACCCAAAAACCTAAATCA-3'		294-271
Sequencing	5'-GTTTGTATGTTTATTAGTTGTAGTA-3'		158-181

Table II. Descriptive characteristics of patients and controls.

Variable	TD group (n=10)	NTD group (n=10)	HC group (n=10)	P-value
Male/female	5/5	5/5	5/5	1.000
Age (years)	31.6±11.8	33.8±11.0	33.2±11.9	0.910
Education (years)	10.6±3.2	10.9±2.6	11.4±3.3	0.832
Duration of disease (years)	11.1±11.1	10.2±9.2	n/a	1.000
Duration of treatment (months)	25.6±22.1	24.5±26.1	n/a	0.570
CPZ equivalents (mg)	662.1±431.4	498.4±275.4	n/a	0.474
Drug type				0.584
1 typical antipsychotic	1	0	n/a	
1 atypical antipsychotic	6	7	n/a	
2 atypical antipsychotics	3	3	n/a	

TD, schizophrenia patients with tardive dyskinesia; NTD, schizophrenia patients without tardive dyskinesia; HC, healthy controls. P-values are based on χ^2 test for categorical variable and generalized linear model for continuous variable.

Table III. Clinical parameters of TD and NTD groups.

Variable	TD group (n=10)	NTD group (n=10)	P-value
PANSS total score	74.0±21.6	73.4±9.7	0.633
PANSS			
Positive	17.0±7.2	18.7±4.6	0.489
Negative	23.6±8.5	18.4±6.2	0.184
General	33.6±8.6	35.2±7.0	0.458
Abnormal involuntary movement scale	13.4±5.3	0	<0.0001

TD, schizophrenia patients with tardive dyskinesia; NTD, schizophrenia patients without tardive dyskinesia; PANSS, positive and negative syndrome scale. P-values are based on t-test.

compared with those in TD, NTD and TD + NTD (P=0.0003, P<0.0001 and P<0.0001, respectively) and the average of 4 CpG sites (P=0.0176, P=0.0063 and P=0.003, respectively).

Discussion

To the best of our knowledge, this is the first study to quantitatively analyze DNA methylation using pyrosequencing and determine the *IRS1* gene promoter methylation levels of the 4 CpG sites among the TD, NTD and HC groups. GLM analyses revealed lower methylation levels in CpG site 1 and the mean value of the 4 CpG sites of the TD, NTD and TD + NTD groups compared with the control group.

The methylation status of the *IRS1* gene has been found to be associated with SCZ through post-mortem analysis of human brain tissue from 24 patients with SCZ and 24 unaffected controls using the Illumina Infinium HumanMethylation450 Bead Chip (9). Another study using blood samples and Illumina HumanMethylation450 BeadChip reported that *IRS1* was associated with SCZ (28). The present study used pyrosequencing to reveal that the DNA methylation level in SCZ patients (NTD group) was significantly lower compared with healthy controls with regard to the CpG site 1 and average values of the 4 CpG sites in a Chinese sample (Table V). Furthermore, it was observed that the TD group demonstrated significantly lower

Table IV. Pearson correlation coefficients among methylation levels and clinical characters.

Variable	IRS1 Site 1	IRS1 Site 2	IRS1 Site 3	IRS1 Site 4	IRS1 Average	PANSST	PANSSP	PANSSN	PANSSG
IRS1 Site 1	1.000	-0.345	0.299	0.285	0.821 ^a	-0.323	-0.499	-0.219	-0.121
IRS1 Site 2		1.000	-0.055	-0.222	-0.276	-0.092	0.013	0.122	0.037
IRS1 Site 3			1.000	0.076	0.310	0.198	0.013	0.266	0.111
IRS1 Site 4				1.000	0.770 ^a	0.059	-0.052	-0.051	0.153
IRS1 Average					1.000	-0.160	-0.366	-0.128	0.008
PANSST						1.000	0.521 ^b	0.832 ^a	0.836 ^a
PANSSP							1.000	0.184	0.177
PANSSN								1.000	0.623 ^c
PANSSG									1.000

^aP<0.0001, ^bP <0.05, ^cP<0.01. IRS1, insulin receptor substrate 1; PANSS, positive and negative syndrome scale; PANSST, the total score; PANSSP, positive score; PANSSN, negative score; PANSSG, general psychopathology score.

Table V. General linear model analysis of DNA methylation levels.

Group	Mean/F/t/P-value	Site 1	Site 2	Site 3	Site 4	Average
TD	Mean ± standard deviation	84.3±3.8	100.0±0.0	100.0±0.0	86.9±1.3	92.8±1.0
NTD		84.0±3.4	99.7±0.7	99.8±0.7	86.8±2.9	92.6±1.4
HC		90.2±1.4	99.8±0.7	100.0±0.0	87.8±5.6	94.4±1.6
TD vs. NTD vs HC	F value	13.14	0.51	0.95	0.14	5.20
	P-value	0.0001	0.6052	0.4011	0.8659	0.0126
TD vs. HC	t value	-4.22	0.92	0.0	-0.42	-2.53
	P-value	0.0003	0.3677	1.0000	0.6758	0.0176
NTD vs. HC	t value	-4.59	0.07	-1.20	0.06	-2.97
	P-value	<0.0001	0.9433	0.2403	0.9524	0.0063
TD + NTD vs. HC	t value	-5.21	0.56	-0.72	-0.54	-3.26
	P-value	<0.0001	0.5822	0.4762	0.5903	0.0030
TD vs. NTD	t value	0.25	0.85	1.17	-0.50	0.36
	P-value	0.8072	0.4048	0.2527	0.6237	0.7220

TD, schizophrenia patients with tardive dyskinesia; NTD, schizophrenia patients without tardive dyskinesia; HC, healthy controls. F value is based on GLM for comparing three groups and t value is based on GLM for comparing two groups.

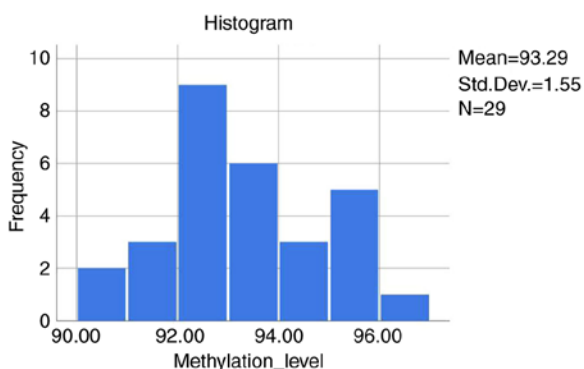


Figure 1. Normality test for the average methylation level for 4 CpG sites.

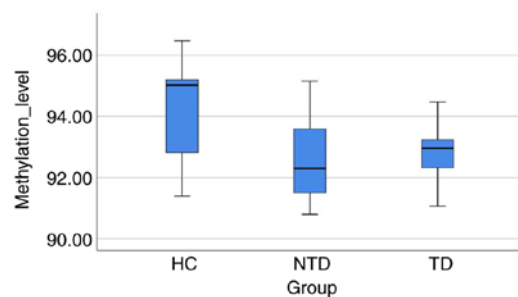


Figure 2. F-test based on GLM analysis of the average methylation levels of 4 CpG sites for comparing three groups. HC, healthy controls; NTD, schizophrenia patients without tardive dyskinesia; TD, schizophrenia patients with tardive dyskinesia.

DNA methylation levels compared with healthy controls (Table V).

Studies have previously indicated that insulin may act as a metabolic signal and the *IRS1* gene may influence body

weight control and glucose homeostasis (37-39). Furthermore, several studies have also reported associations of *IRSI* polymorphisms with cancer, diabetes, glucose levels and obesity (40-44). Furthermore, insulin/insulin receptor signaling and the insulin-like growth factor (IGF) pathway may have different functions in the central nervous system in brain development, blood glucose regulation, dendritic growth and neuronal apoptosis (37,45-48). However, Che *et al* (47) was unable to identify any association between the *IRSI* gene and epilepsy in a Chinese sample but suggested that the genes associated with the insulin signaling pathway may affect the therapeutic response of temporal lobe epilepsy. Previous epidemiologic studies have demonstrated that SCZ and other mental illnesses may increase the risk of developing type 2 diabetes and other metabolic disorders (49,50). The co-morbidity of type 2 diabetes and SCZ may partially be due to concurrent biological susceptibility of these two conditions (51,52). As an example, the *TCF7L2* gene is associated with both type 2 diabetes and type 2 diabetes with SCZ or the schizoaffective disorder observed in African American patients (50), while the rs7903146 in the *TCF7L2* gene is associated with SCZ (53). Furthermore, insulin-like growth factor II mRNA-binding protein 2 gene (*IGF2BP2*) may be associated with SCZ (54). Additionally, insulin signaling, among other type 2 diabetes-related pathways, could act as a bridge between SCZ and type 2 diabetes (55). However, one previous study could not associate rs1801278 in the *IRSI* gene with SCZ (27). Therefore, the present study provided further evidence of the involvement of *IRSI* gene with regard to the development of SCZ or TD (Table IV).

A previous study examined the DNA methylation levels of three CpG sites in the *IRSI* gene with type 2 diabetes using pyrosequencing (26); however, there was no significant difference in the methylation levels of the 3 CpG sites in the *IRSI* gene between the controls and type 2 diabetes patients, which suggested that the DNA methylation levels of the *IRSI* gene did not play a major role in the occurrence of type 2 diabetes (26). The present study revealed that both TD and NTD groups had significantly lower methylation levels in the CpG site 1 and mean values of the 4 CpG sites in the *IRSI* gene compared with healthy controls (Table V). One primary difference may be that there were 3 CpG sites in the above type 2 diabetes study (26), while there were 4 in the present study; however, it is possible that these CpG sites may be different. Notably, the present study found numerous similarities, which included the fact that DNA methylation levels in the diabetes groups were slightly lower compared with those in the non-diabetes groups, specifically in the CpG sites 1 and 2 in the entire sample. Furthermore, in the above type 2 diabetes study, a similar trend was noted among the groups of men and women, despite the fact that the differences did not reach a 5% significant level (26).

Previous studies have suggested a role of *IRSI* in cognitive impairment and Alzheimer's disease (56-59). The present study and the previous reports related to the role of *IRSI* gene methylation in SCZ (9,28) may provide adequate evidence to demonstrate that SCZ and Alzheimer's disease may share a common network of dysregulation (60). Furthermore, epidemiologic studies have reported a possible association between the insulin resistance of type 2 diabetes mellitus and increased incidence of Alzheimer's disease (61,62). As a matter of fact,

insulin resistance results in a diminished glucose uptake in similar regions of the brain in Alzheimer's disease and type 2 diabetes mellitus (58,59). Therefore, the *IRSI* gene may have a pleiotropic effect on Alzheimer's disease, type 2 diabetes and SCZ.

There are a number of strengths in the current study. First, this is the first study to conduct a quantitative analysis of DNA methylation levels of the *IRSI* gene in SCZ and TD, while previous studies focused on the methylation status in SCZ (9,28). Second, the present study applied a GLM to examine the methylation levels of the 4 CpG sites within the *IRSI* gene among TD, NTD and HC groups. Third, the present study tested the normality of the average methylation levels of the 4 CpG sites and observed that the average DNA methylation levels of these sites followed normal distribution. Although correlation analysis was performed, significant correlations among the methylation percentages of the four CpG sites with the severity of SCZ was not observed. However, the present study has certain limitations: First, a peripheral blood sample was used in view of the difficulty in obtaining brain tissues to study the disorders of the central nervous system. Second, the sample size of the three groups in the methylation study was relatively small because the prevalence of TD in general population is typically low. Based on the PROC POWER determined in SAS 9.4, the power level was 63.3% with 30 individuals on comparing overall means in DNA methylation levels for the four CpG sites; however, it was possible to increase the power up to 99.1% while testing the CpG site 1. Third, the present study examined and included a limited number of sites associated with *IRSI* gene methylation (only 4 sites), which highlighted the importance of increasing the number of sites in future investigations. Additionally, there may be variations between medications, which could not have been detected by the present study.

In conclusion, pyrosequencing demonstrated that the DNA methylation levels of the *IRSI* gene in TD and NTD groups were significantly lower compared with the healthy control group. However, the DNA methylation levels in TD did not demonstrate any significant differences compared with those in the NTD group. This is the first study to compare CpG methylation levels of TD and NTD with healthy controls and the findings demonstrated adequate evidence of the possible roles of *IRSI*-associated DNA methylation in SCZ and TD. In the future, it will be worthy to detect age and gender effects using a large sample and perform the functional study of these 4 CpG sites of the *IRSI* gene to evaluate the role of this gene in the pathogenesis of SCZ and TD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

YLL, FY and YT planned and managed the project. YLL, JH, ZW, FY and ST were involved in designing the study and collecting the data. YLL, PZ, ZW and ST performed recruitment and clinical assessment. KW, PZ, YLL, YL, YKL and YT conducted statistical analyses, interpreted the results, searched the literatures and wrote parts of the manuscript. All authors performed a final review of the manuscript and all authors approved the submission of this manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Review Board of Beijing Huilongguan Hospital (China). Written informed consent was obtained from all participants enrolled in our study and/or from their guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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