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Sex-specific effects of methylenetetrahydrofolate reductase polymorphisms on schizophrenia with methylation changes

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ABSTRACT

Background: Methylenetetrahydrofolate reductase (MTHFR) is the critical enzyme in biotransformation. The polymorphism of *MTHFR* is a risk factor for schizophrenia. However, whether the *MTHFR* polymorphism is associated with schizophrenia disease phenotypes and what is the underlying mechanism of *MTHFR* polymorphism in schizophrenia is under-investigated. In this study, we aim to verify the correlation between *MTHFR* polymorphisms and clinical features of schizophrenia, while exploring the differential genomic methylation and disease related genes as the potential targets for schizophrenia.

Method: 242 patients of schizophrenia and 234 matched healthy controls were enrolled in this study. Polymorphisms of *MTHFR* from three sites (C677T, A1298C, G1793A) were examined by Taqman fluorescence probe method in leukocytes from all subjects. The positive and negative syndrome scale (PANSS), trail making test (TMT) and Clinical Global Impression (CGI) were checked on patients. Genomic methylation was tested and analyzed in fields of differential methylation positions (DMPs) and enrichment of genes that are potentially related to schizophrenia.

Results: Schizophrenic patients showed higher frequency of *MTHFR* polymorphisms at both single and multiple sites than healthy controls. Our data also showed that *MTHFR* C677T and multiple-site polymorphisms were positively correlated with PANSS positive rating, not negative score in male schizophrenia patients. Furthermore, while a significant reduction of global DNA methylation level was observed in schizophrenic patients, we also identified several genes which differentiated between schizophrenia and healthy controls at methylation levels.

Conclusions: This is a pilot study revealing that *MTHFR* polymorphisms at both single and multiple sites are related to the risk of schizophrenia and positive symptom of the disease. The risk of *MTHFR* polymorphism in schizophrenia and the clinical symptoms was only significant in male patients. While the sex-specific risk of MTHFR in schizophrenia is new and the reasons remain unanswered. Our methylation analysis suggested that there was significant hypomethylation of genomic DNA in schizophrenia patients with no sex difference. The correlation between *MTHFR* polymorphism and schizophrenia may attribute to the change of DNA methylation level, and some certain genes could be potential research objects for MTHFR effects on schizophrenia.

1. Introduction

Methylenetetrahydrofolate reductase is the enzyme catalyzing the reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrafolate (5-MTHF) and participates in one carbon metabolism, the process of which contains supply of methyl group for epigenetic modulation [1,2]. There were 14 common or rare single nucleotide polymorphisms of *MTHFR* have been reported to influence the expression of gene or damage MTHFR activity [3], in which C677T (rs1801133) and A1298C (rs1801131) were the most reported to be closely related to the activity of enzyme. For these two sites, each copy of the 677T or 1298C allele respectively causes a 35% or 17% reduction in MTHFR activity [4,5]. Another widely reported polymorphic site of *MTHFR* is G1793A (rs2274976), which was associated with diseases including various of cancers, cardiovascular diseases and male infertility, and implied a potential effect on MTHFR activity responsible for 5-MTHF reaction [6–9].

The polymorphism of *MTHFR* was reported to increase with more frequency of mutant genotypes or alleles in schizophrenic patients [10,11]. There were also several studies reporting the aggravation of schizophrenia symptoms with the increase of *MTHFR* polymorphism [12–14]. For the detailed process, apart from individual effects on neurodevelopment, the *MTHFR* polymorphism could lead to the damage

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of enzyme activity and repress the reaction of 5-MTHF generation. Consequently, 5-MTHF is deficiency in one carbon metabolism and there would be a shortage of methyl group supply that may directly produce a repressive effect on downstream epigenetic modulation [15]. In schizophrenia, the male schizophrenic patients usually have an earlier onset age, more severe symptoms and a poorer disease course [16,17]. There were also studies reporting the effects of sex hormones on pathophysiology of schizophrenia [18]. Then whether there are sex-specific effects on correlation between *MTHFR* polymorphism and schizophrenia is necessary to explore.

As MTHFR related methyl group supply could be damaged by polymorphic sites of the gene and the methyl group is a requisite for methylation modification of DNA in downstream, the methylation related process may exert potential influence on correlation between MTHFR and schizophrenia. With steric hindrance silence effects of methylation on gene expression [19–21], it is possible for one carbon metabolism that exerts effects on the schizophrenia by mean of methylation modification. There were studies on some schizophrenia related genes that have been identified as differential methylated in schizophrenic patients, including reelin (*RELN*), brain-derived neurotrophic factor (*BDNF*), catechol-*O*-methyltransferase (*COMT*), serotonin (*HTR1A*, *HTR2A*, *SLC6A4*) and glutamate (*GMR*), despite findings were not all consistent at all the sites [22,23]. There was also study reporting that genomic DNA methylation level decreased significantly in schizophrenic subjects compared with controls [24], which supported the correlation between potential association of DNA methylation and schizophrenia. Meanwhile association between *MTHFR* polymorphism and methylation was also hinted. Simonetta et al. reported hypomethylation of genome in heathy subjects with TT genotype of *MTHFR* C677T compared with those with CC genotype [25]. To work out the detailed mechanism of MTHFR-schizophrenia association, related genes enriched with DMPs are potential targets to study. Mostly these genes and their involved factors in different research levels, including macroscopical organisms, cellular and molecular processes may assist to elucidate the pathogenesis of schizophrenia in part.

We intended to study the *MTHFR* polymorphisms related to efficiency of MTHFR catalysis in both single and multiple sites. The frequency difference between schizophrenia and controls, and divergent clinical features along with different *MTHFR* polymorphism of schizophrenic patients were explored. Besides single polymorphism in each site, we also accumulated risk allele number on the three sites to explore the role of multiple polymorphisms of *MTHFR* in schizophrenia. Subjects with TT genotype of *MTHFR* C677T that damage the enzyme activity most and severe clinical symptoms were selected to test the genomic methylation level and potential schizophrenia related genes of certain biological processes through enrichment analysis of DMPs. The related methylation change is potentially the mechanism of effects from *MTHFR* polymorphisms on schizophrenia and some potential genes enriched with DMPs may undertake the subsequent study of verification and mechanism.

2. Materials and methods

The study was approved by Ethics Committee of Beijing Anding Hospital and was in accordance with the latest version of the Declaration of Helsinki. Participants were recruited from inpatients and outpatients of Beijing Anding Hospital and signed the informed consent voluntarily.

2.1. Subjects

We included 234 healthy controls without symptoms or family history of psychiatric disease from resource library of Beijing Anding Hospital, the sex ratio and age of whom made no difference from those of enrolled schizophrenic patients. 242 chronic schizophrenia patients of Chinese Han population from Beijing Anding Hospital were enrolled. The diagnosis process was made by two experienced psychiatrists according to Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV). Files of enrolled patients were completed by trained raters who were blind to results of gene detection and methylation assay, and consisted of individual information, disease state, previous history, family history, onset age and duration of disease, treatment condition and ratings of PANSS, CGI, TMT. Apart from clinical features collection, blood samples of enrolled patients and controls were acquired for the isolation of leukocytes and genomic DNA extraction. The flow diagram of participants recruitment and related research process are shown in Fig. 1. Detailed inclusion criteria and exclusion criteria were shown in Table 1.

2.2. MTHFR SNP assay

DNA was extracted from leukocyte by Miniprep System for test of genotypes including sites of C677T, A1298C and G1793A of *MTHFR*. All genotypes demonstrated Hardy Weinberg equilibrium. Genotyping was conducted by the Taqman fluorescence probe and ABI PRISM 7500 Sequence Detection System. The probes were obtained from commercial products of Thermo Fisher. Alleles were detected in the method of Taqman fluorescence probe and PCR genotyping reported previously [12].

For multiple-site *MTHFR* polymorphisms, each subject had a total risk allele number by accumulating mutant allele number in *MTHFR* sites of C677T, A1298C and G1793A. We compared the different frequencies of total risk allele numbers between patients and controls to determine the association of *MTHFR* polymorphisms and schizophrenia, while exploring the correlation between *MTHFR* polymorphisms and clinical features of schizophrenia.

2.3. DNA methylation assay

16 patients of schizophrenia with TT genotype of *MTHFR* C677T and PANSS rating >60, together with 16 age and sex-matched controls were chosen to conduct assay of genomic DNA methylation. Approximately, 500 ng of genomic DNA from each sample was used for sodium bisulfite conversion using the DNA methylation Gold Kit (Zymo Research, USA) following the manufacturer's standard protocol. Genome-wide DNA methylation was assessed using the Illumina Infinium Human Methylation 850K BeadChip (Illumina Inc., USA) according to manufacturer's instructions. The array data was analyzed using ChAMP package in R software for deriving the methylation level. The methylation status of all the probes was denoted as β value, which is the ratio of the methylated probe intensity to the overall probe intensity. CpG sites having $|\Delta\beta| \geq 0.20$ between patients and controls and adjusted p value ≤ 0.05 was considered as differentially methylated site. A CpG position was considered hypermethylated if $\Delta\beta \geq 0.20$ or hypomethylated if $\Delta\beta \leq -0.20$. Average β value of promoters and CpG islands were compared between patients and control groups.

2.4. Statistical analysis

The data and diagrams were processed and made by GraphPad Prism 6.0c, SPSS statistics 24, Adobe Illustrator CC 2017, Adobe Photoshop CC 2017 and R software. We used χ^2 test to determine the differences in genotype or allele frequencies of *MTHFR* polymorphisms between patients and controls, as well as one-way ANOVA and simple linear regression to explore association of single and multiple *MTHFR* polymorphisms and clinical features of schizophrenia respectively. In methylation analysis, DMPs were screened by linear regression and empirical Bayes to calculate the p value and adjusted p value (FDR) after Benjamin & Hochberg multiple test. One-way ANOVA and enrichment analysis were used for genomic DNA methylation difference and gene ontology (GO) analysis. The multiple testing correction including Bonferroni and false discovery rate (FDR) were conducted as post hoc tests. The latter process also contained tests of hypergeometric

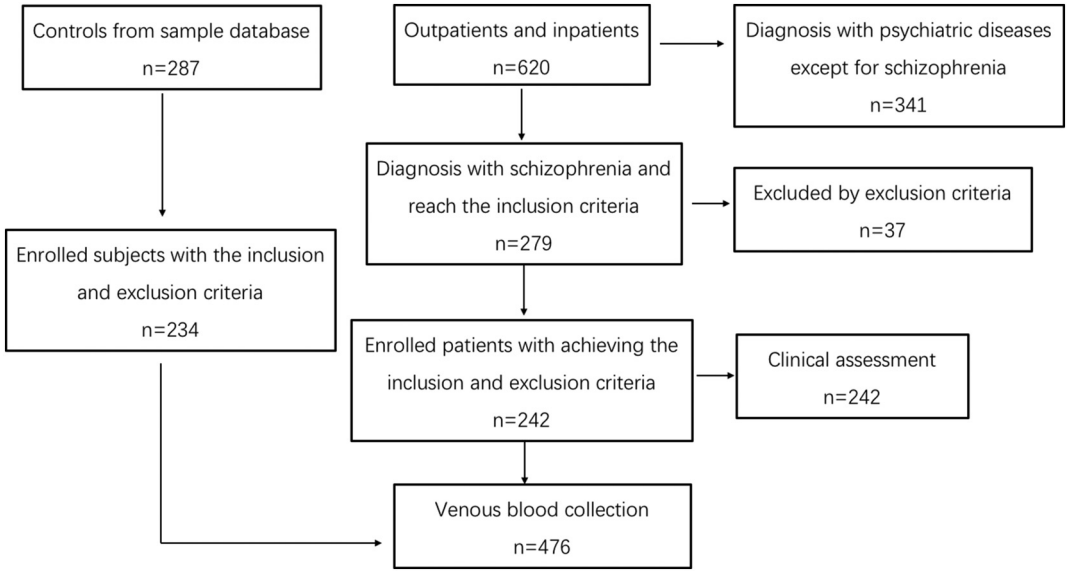

Fig. 1. Flow diagram of the participant recruitment and research process.

distribution and Fisher, while correlated terms were acquired from the database of GO (http://www.geneontology.org).

3. Results

3.1. Distribution of MTHFR genotypes in patients and controls

In demographic data including sex and age, there was no significant difference between patients and controls (Table 2). As shown in Table 3, the *MTHFR* genotypes distributed in Hardy Weinberg equilibrium for both patient and control groups ($p > 0.05$). There was linkage disequilibrium between C677T and A1298C sites ($R^2 = 0.20$, $D' = 1$). As with the non-random association of alleles at different loci, the C677T and A1298C may have interactive effects on mutual allele or genotype frequencies. Then we respectively focused on single and multiple sites of *MTHFR* polymorphisms in schizophrenic and control subjects. The statistical power of the enrolled samples was 0.85, 0.82 and 0.90 for the polymorphisms of C677T, A1298C and G1793A respectively.

3.1.1. Sex-specific single-site polymorphism in schizophrenic patients

For C677T, the frequency of TT genotype in schizophrenia group was significantly higher than that of controls, with a 1.54-fold times higher in T allele. After sex stratification, only male schizophrenic patients expressed a significant higher frequency of TT genotype or T allele than the male controls, but not in females. For A1298C and G1793A, neither of them made significant difference on genotype or allele frequency between patient and control groups (Table 3).

3.1.2. Sex-specific multi-site polymorphisms in schizophrenic patients

To elucidate the cumulative effect of multiple sites of *MTHFR* polymorphisms, we analyzed the total risk allele number of *MTHFR* C677T, A1298C and G1793A. Using multi-site polymorphisms model with a total risk allele number from 0 to 6, the actual interval was from 0 to 3. For both populations, the total risk allele number was mainly distributed in 0 to 2 group, while the controls were concentrated on 0 to 2 relatively in average and the patients mainly on 1 to 2. Our data showed a significant higher number of total risk allele in schizophrenic patients than that in controls. Interestingly, the significant difference of multi-site polymorphisms of *MTHFR* between schizophrenic patients and controls were still in male subjects after sex stratification analysis (Table 4).

Table 1
Inclusion criteria and exclusion criteria.

Controls	Patients
Inclusion: Age ≤ 60; Han population; Normal intelligence; Right handedness; No brain trauma and substance abuse history.	
No psychiatric, nervous system and severe somatic disease or related family history; Age, gender, education and live environment matched with patient group.	DSM-IV diagnostic criteria, No nervous system and severe somatic disease or related family history.
Exclusions: Age > 60; Nervous system and severe somatic disease or related family history; Pregnancy or lactation women; Food or drug allergic history; Blood donation or collection <3 months before the study; Subjects with other factors that is inadaptable.	
Psychiatric disease history; Antipsychotics, antidepressant, sedative hypnotics or mood stabilizer history.	Psychiatric disease except for schizophrenia; Patients status not appropriate to join, including emotional instability, violence or suicide tendency and so on.

Table 2
Demographic characteristic of controls and patients.

	CON			SCZ		
	Total	Male	Female	Total	Male	Female
Number[a]	234	135	99	242	119	123
Mean age[b]	27.6 ± 9.1	24.6 ± 7.5	32.0 ± 6.2	29.2 ± 11.6	26.4 ± 7.7	31.6 ± 12.0
Onset age	–	–	–	22.5 ± 7.8	21.0 ± 5.2	22.9 ± 8.3
Duration[c]	–	–	–	109.6 ± 88.4	105.3 ± 70.6	111.6 ± 95.1
Medication[d]	–	–	–	12.8 ± 6.9	10.3 ± 7.8	14.2 ± 5.9
Medication[e]	–	–	–	14.2 ± 2.3	13.6 ± 2.6	14.8 ± 2.1

Note. CON = Controls; SCZ = Schizophrenia. The quantitative data is shown in mean ± SD.

[a] No significant difference on sex frequency between schizophrenic patients and controls.

[b] No significant difference on mean age between schizophrenic patients and controls.

[c] Count in months.

[d] Medications of the patients before attendance. Medication dosage adopts olanzapine equivalents conversion.

[e] Medications of the patients after attendance.

Table 3
MTHFR polymorphisms between schizophrenia and controls.

MTHFR	C677T					A1298C					G1793A				
	CC	CT	TT	C	T	AA	AC	CC	A	C	GG	GA	AA	G	A
CON															
Total	71	113	50	255	213	171	58	5	400	68	221	13	0	455	13
Male	45	64	26	154	116	104	29	2	247	33	130	5	0	265	5
Female	26	49	24	101	97	67	29	3	163	35	91	8	0	190	8
HWE			0.69					0.97					0.66		
SCZ															
Total	45	122	75	212	272	174	63	5	411	73	218	24	0	460	24
Male	25	53	41	103	135	90	27	2	207	31	109	10	0	228	10
Female	20	69	34	109	137	84	36	3	204	42	109	14	0	232	14
HWE			0.71					0.80					0.36		

MTHFR	C677T	A1298C	G1793A
χ^2_{Total}[a]	11.04	10.87	0.10	0.06	4.85	3.03
χ^2_{Male}[b]	9.14	9.58	0.07	0.18	2.51	2.44
χ^2_{Female}[c]	3.34	1.98	0.07	0.03	0.67	0.63
p_{total} (q_{total})	0.004 (0.012)		0.9521 (0.9521)		0.0756 (0.1137)	
	0.001 (0.003)		0.8103 (0.8103)		0.0818 (0.1227)	
p_{male} (q_{male})	0.0104 (0.0312)		0.9636 (0.9636)		0.1128 (0.1692)	
	0.002 (0.006)		0.6692 (0.6692)		0.1185 (0.1778)	
p_{female} (q_{female})	0.1882 (0.5646)		0.9637 (0.9637)		0.4132 (0.6198)	
	0.1598 (0.4794)		0.8674 (0.8674)		0.4256 (0.6384)	
OR[d]	1.54 (1.19–1.98)		1.05 (0.73–1.49)		1.83 (0.92–3.63)	

Note. CON = Controls; SCZ = Schizophrenia; HWE = Hardy Weinberg Equilibrium; OR = Odds ratio.
[a] Comparison in genotype and allele frequencies of three polymorphisms between patients and controls.
[b] Comparison in genotype and allele frequencies of three polymorphisms between male patients and controls.
[c] Comparison in genotype and allele frequencies of three polymorphisms between female patients and controls.
[d] Odds ratio was counted for relative risk of mutant allele of each site.

3.1.3. Haplotype of two-site analysis in double heterozygotes and mutant homozygote in one-site

For there was a significant difference on *MTHFR* polymorphisms at both single and multiple-site and linkage disequilibrium existed between C677T and A1298C, we studied the site effect by haplotype analysis in double heterozygotes and mutant homozygote in one-site. Existing literatures have referred to the alteration of polymorphisms including C677T and A1298C. As reported before [5], for C677T and A1298C haplotypes, either site consisted of homozygous mutant alleles would exactly accompanied with the homozygous wild genotype of the other site, while there is no mutant homozygote of G1793A in our research. Therefore, for the mutant homozygotes, their haplotype only included TT/AA or CC/CC. In previous, we presented the analysis of single- and multi-site polymorphisms. As the total risk allele number of enrolled subjects ranged from 0 to 3, it was necessary to distinguish divergent haplotypes in groups with the same multiple risk allele number. Apart from 0- or 1-risk-allele subjects studied in the single-site analysis, or 3-risk-allele ones whose amounts was too limited, we selected subjects with 2 risk alleles to explore. We compared the distribution of three types of double heterozygotes and two types of one-site mutant homozygotes in six chi-square tests respectively between patients and controls. The method is as follows: Different haplotypes of the three polymorphic sites including C677T, A1298C and G1793A were classified, which consisted of double-heterozygotes type of CT/AC/GG, CT/AA/GA and CC/AC/GA, as well as one mutant homozygote with another site of wild-type homozygote including CC/CC/GG and TT/AA/GG. The above two types of zygote were compared respectively based on different haplotypes, thus six groups of chi-square tests were conducted to

Table 4
Total risk allele number of *MTHFR* in schizophrenia and controls.

MTHFR risk allele No.	0	1	2	3
CON				
Total	47	93	84	10
Male	32	55	45	3
Female	15	38	39	7
SCZ				
Total	26	83	114	19
Male	16	41	53	9
Female	10	42	61	10
χ^2_{Total}[a] 13.82	χ^2_{Male}[b] 10.06		χ^2_{Female}[c] 4.02	
p_{Total} (q_{Tatol}) = 0.0032 (0.0096)	p_{Male} (q_{Male}) = 0.0181 (0.02715)		p_{Female} (q_{Female}) = 0.2591 (0.2591)	

Note. CON = Controls; SCZ = Schizophrenia.
[a] Comparison in total risk allele number of *MTHFR* between patients and controls.
[b] Comparison in total risk allele number of *MTHFR* between male patients and controls.
[c] Comparison in total risk allele number of *MTHFR* between female patients and controls.

Table 5
Haplotype analysis in schizophrenia and controls.

Haplotype of dual sites	Double heterozygosity			Mutant homozygote	
	CT/AC/GG (677/1298)	CT/AA/GA (677/1793)	CC/AC/GA (1298/1793)	TT/AA/GG (677/1298)	CC/CC/GG (677/1298)
CON	39	8	10	49	4
SCZ	48	16	18	74	4
χ^2	0.52	0.36	0.16		
	0.08	0.71	0.53		
p ($p > 0.05$, do not need to calculate FDR)	0.47[a]	0.55[b]	0.69[c]		
	0.78[d]	0.40[e]	0.46[f]		

Note. CON = Controls; SCZ = Schizophrenia.
[a] Comparison in subjects with haplotype of CT/AC/GG and TT/AA/GG between patients and controls.
[b] Comparison in subjects with haplotype of CT/AA/GA and TT/AA/GG between patients and controls.
[c] Comparison in subjects with haplotype of CC/AC/GA and TT/AA/GG between patients and controls.
[d] Comparison in subjects with haplotype of CT/AC/GG and CC/CC/GG between patients and controls.
[e] Comparison in subjects with haplotype of CT/AA/GA and CC/CC/GG between patients and controls.
[f] Comparison in subjects with haplotype of CC/AC/GA and CC/CC/GG between patients and controls.

explore the potential site effects in schizophrenia. In our results of each comparison, there was no significant difference in each pair between schizophrenic patients and controls (Table 5), which revealed that there was no significant haplotype effect. To elucidate comprehensively, we collected the *MTHFR* gene information by the Haploview software for a supplement (Supplemental Fig. 1).

3.2. Analysis of clinical features

3.2.1. Single-site polymorphism

As previous, C677T, A1298C and G1793A were included in this analysis. One-way ANOVA analysis indicated that A1298C or G1793A polymorphism had no significant association with clinical features of schizophrenic patients. For C677T, the TT genotype associated with a significant increase in the positive score of PANSS. After sex stratification, similar difference still existed in male patients but not females (Fig. 2). For other clinical features and C677T polymorphism, no significant correlation was found.

3.2.2. Multi-site polymorphisms

To illustrate the cumulative effects of *MTHFR* polymorphisms on clinical features of schizophrenia, we assigned multi-site polymorphisms model into simple linear regression analysis with clinical features of schizophrenia. As a result, with the increase of total risk allele number, the positive score of PANSS increased markedly as more risk alleles appear on polymorphic sites of *MTHFR*. After sex stratification, similar difference still existed in male patients but not females (Fig. 3). No significant correlation was found between other clinical features and total risk allele number of patients.

3.3. DNA methylation

3.3.1. Genomic methylation level

There was significant difference on DMPs between schizophrenic patients and controls by analysis of one way ANOVA and heatmap (Fig. 4A), which indicated that the patients possessed more hypomethylated positions than controls. The demographics, PANSS and C677T genotype of enrolled subjects were shown in the table below the heatmap. The genomic DNA methylation level of the two groups was also significantly different. The β value of the patient and control groups were 0.5821 ± 0.2048 and 0.6792 ± 0.1698 (mean ± SD) respectively (Fig. 4B), which revealed that the genomic DNA methylation level of patient group was significantly lower than that of controls. In subgroup analysis, the results in male or female group were similar with that of total subjects, both of which indicated a significant genomic hypomethylation in schizophrenic patients. No sex-specific effect was found in genomic methylation analysis.

To explain and excluded possible factors that may influence the methylation level, we conducted simple linear regression analysis between antipsychotics and methylation, age and genomic methylation, as well as multiple linear regression with independent variable including antipsychotics and age, and dependent variable as methylation. The results revealed that there was no significant correlation between these two factors and methylation (Supplemental Table 1 and Supplemental Fig. 2). Based on our data, it seems like the divergent methylation level in schizophrenia may be attributed to *MTHFR* in some independent manners.

3.3.2. Differential methylation positions

We firstly screened and filtrated probes according to the following excluding rules: non-CpG, SNP related, location in X or Y chromosome, multi-hit. On the standard of $\Delta\beta \geq 0.10$, there were totally 2757 DMPs between patients and controls were eventually identified, which consisted of 1987 positions with hypomethylation and 770 positions of hypermethylation in schizophrenic patients respectively. The DMPs are also exhibited in the heatmap. Among genes with significant enrichment of DMPs, we divided them into two groups by relative methylation level between patients and controls. For patients, there were respectively 15 and 8 genes enriched by DMPs of hypo- and hypermethylation respectively after analysis (Fig. 4C). Furthermore, we also added several schizophrenia risk genes as additional study of genomic methylation, which includes *COMT*, *NRG1*, *SLA6A4* and *BDNF*. The results revealed that there were trends of hypomethylation in all four genes in schizophrenic patients, and the methylation level of *SLA6A4* decreased significantly in patients compared to healthy controls (Supplemental Fig. 3).

3.3.3. GO analysis

The database of GO conducts a standardized description in distinct research levels and it is just a simple annotation on gene product. The enrichment on biological processes, cellular components and molecular functions of genes with DMPs could be acquired by GO enrichment analysis. For GO analysis of our study, there were >900 terms containing genes with significant enrichment of DMPs, including multicellular organism development, system development and so on. The most significant enrichment terms were shown in Fig. 5. Combined with the former data, we screened the GO terms that contained the genes with DMP enrichment in our study and labelled them on the top of columns. The genes, related expression product and GO terms may support the further mechanism research of schizophrenia.

4. Discussion

Based on literature review, it was known that *MTHFR* polymorphism of C677T increased significantly in schizophrenia patients [26–28], while it is still unknown whether there were changes of *MTHFR* polymorphisms of multiple sites or haplotype. Then in our study, significant difference was found in *MTHFR* polymorphisms between schizophrenic patients and controls. For single site analysis, there was higher frequency of mutant alleles or homozygotes only in *MTHFR* C677T site in

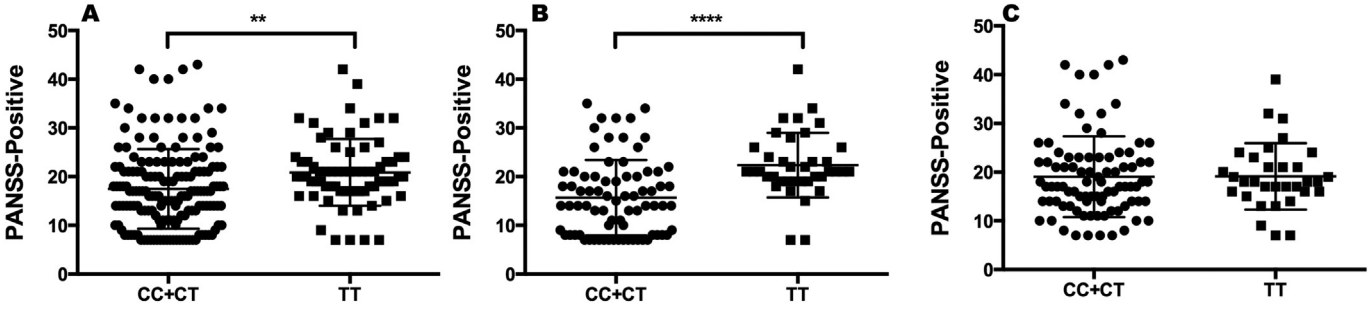

Fig. 2. PANSS positive ratings in one-way ANOVA analysis. A. PANSS positive ratings in all patients of different genotype. F = 3.04, $^{**}p = 0.0026$ (q = 0.0039). B. PANSS positive ratings in male patients of different genotype. F = 4.55, $^{****}p < 0.0001$ (q = 0.0003). C. PANSS positive ratings in female patients of different genotype. F = 0.03, $p = 0.970$ (q = 0.970). Dots or squares represent the rating of PANSS positive in patients with different genotypes. Short bars in the end and middle of segment mark the upper or lower quartile and mean value respectively. X axis means different populations grouped by genotypes and Y axis means PANSS positive ratings.

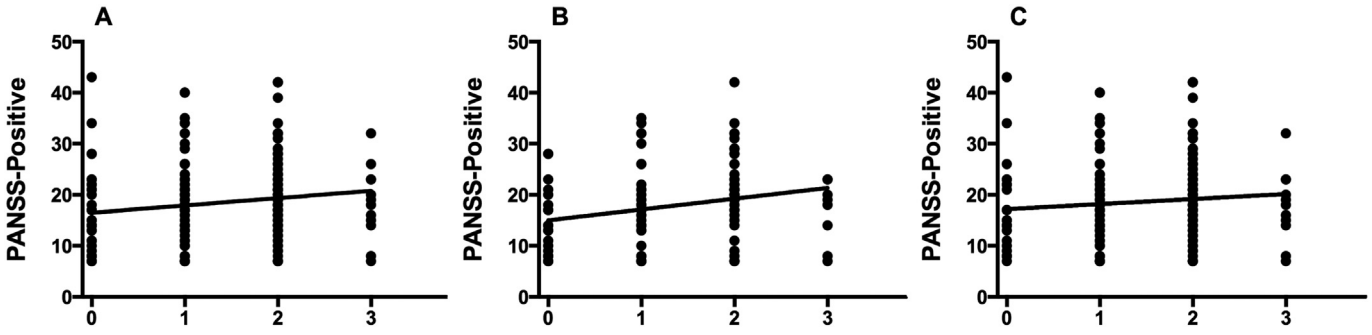

Fig. 3. PANSS positive ratings in single linear regression. A. PANSS positive ratings and total risk allele number of *MTHFR* in all patients. Y = 1.431× + 16.49, $p = 0.020$ (q = 0.030). B. PANSS positive ratings and total risk allele number of *MTHFR* in male patients. Y = 2.109× + 15.02, $p = 0.015$ (q = 0.030). C. PANSS positive ratings and total risk allele number of *MTHFR* in female patients. Y = 0.9915× + 17.19, $p = 0.171$ (q = 0.171). X axis means population amounts of different risk allele numbers respectively and Y axis means PANSS positive ratings.

schizophrenic patients compared with controls. For multiple sites analysis, the patients of schizophrenia markedly carried more risk alleles of the three *MTHFR* sites than controls. Both results of single and multiple sites revealed an association between *MTHFR* polymorphisms and schizophrenia, which implied a potential effect on risk of schizophrenia by the alteration of *MTHFR* polymorphisms. No significant difference was found in haplotype analysis between different haplotype groups. The result that C677T polymorphism increased in schizophrenia is consistent with the previous study and as to the cumulative effects of *MTHFR* multiple-site polymorphisms on schizophrenia, it could be a comprehensive expression of MTHFR activity damage. Apart from the research data, we also searched for the GWAS data in GWAS Catalog (Supplemental Table 2). There is a tight correlation between *MTHFR* C677T polymorphism and folate or homocysteine levels. The increase of *MTHFR* C677T polymorphism could significantly decrease the folate level and raise the homocysteine level in serum. Although the GWAS data on *MTHFR* and schizophrenia is deficient, the correlations of *MTHFR* with folate and homocysteine could indirectly support the effects on schizophrenia from *MTHFR* polymorphisms and methylation alteration through potential pathway of one carbon metabolism.

Apart from genotype or allele frequency change in schizophrenia, previous studies also reported the correlation between *MTHFR* polymorphisms and clinical features in schizophrenic patients. For single-site research, it was known that there was a positive correlation between the increase of *MTHFR* C677T polymorphism and aggravating in negative symptoms or executive function damage of schizophrenia [12,13], and the onset of schizophrenic patients got earlier with the increase in number of the mutant T allele in C677T site [14]. For multiple-

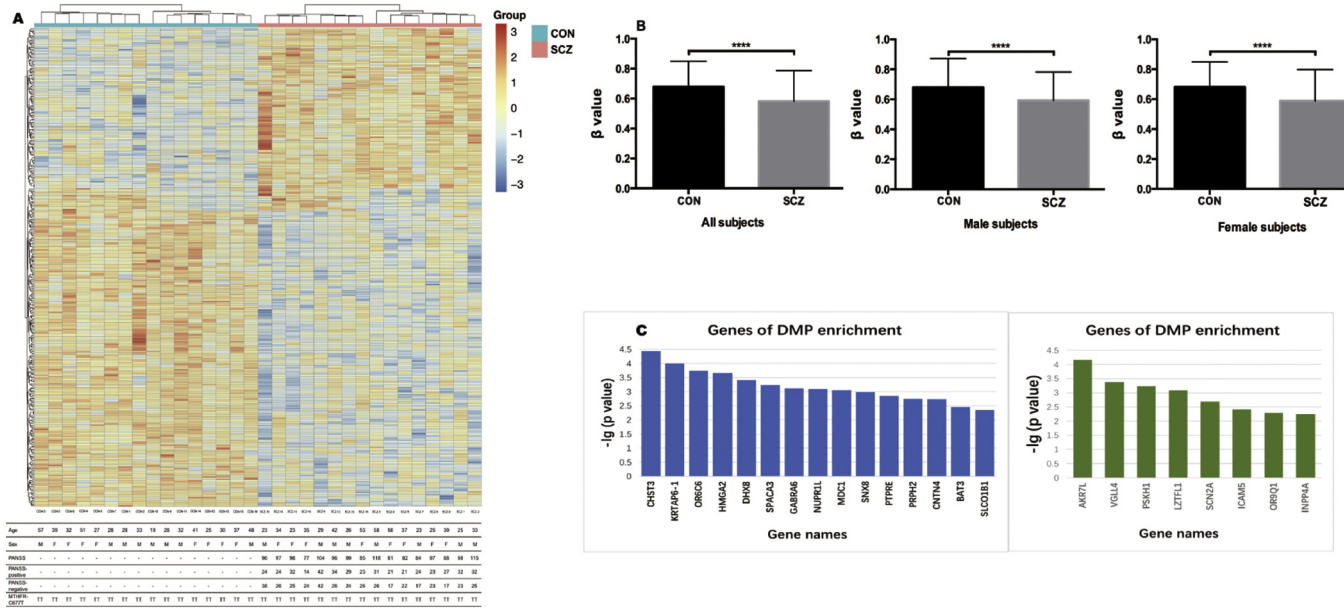

Fig. 4. Genomic methylation in schizophrenia patients and controls. A. Heatmap. Each box represents β value relative to standardized conversion (z-score) integrating all β value of samples for that one position. The mean value of each position after standardization is 0. Each row means a position with differential methylation between schizophrenia patients and controls, and each column means an individual enrolled. The demographics, PANSS and C677T genotype of subjects with methylation assay are listed below the heatmap. B. Genomic methylation level. The methylation level was measured in β value. The figures show subjects of schizophrenia with a hypomethylated pattern compared to controls in all, male and female populations respectively. Short bars mark the standard deviation. $F_{all} = 13.17$, $F_{male} = 11.27$, $F_{female} = 12.25$, $^{****}p < 0.0001$ (q < 0.0001). C. Genes of DMP enrichment. The left figure shows the genes enriched by DMPs with hypomethylation of patients than controls. The right figure shows the genes enriched by DMPs with hypermethylation of patients than controls. *CHST3*: Carbohydrate sulfotransferase 3, *KRTAP6-1*: Keratin associated protein 6-1, *OR6C6*: Olfactory receptor family 6 subfamily C member 6, *HMGA2*: High mobility group AT-hook protein 2, *DHX8*: ATP-dependent RNA helicase DHX8/PRP22, *SPACA3*: Sperm acrosome associated 3, *GABRA6*: Gamma-aminobutyric acid type A receptor α6 subunit, *NUPR1L*: Nuclear transcriptional regulator 1-like protein, *MDC1*: Mediator of DNA damage checkpoint protein 1, *SNX8*: Sorting nexin-8, *PTPRE*: Protein tyrosine phosphatase, receptor type E, *PRPH2*: Peripherin-2, *CNTN4*: Contactin 4, *BAT3*: (BAG6) large proline-rich protein BAG6, *SLCO1B1*: Solute carrier organic anion transporter family, member 1B, *AKR7L*: Aldo-keto reductase family 7 like protein, *VGLL4*: Vestigial like family member 4, *PSKH1*: Protein serine kinase H1, *LZTFL1*: Leucine zipper transcription factor-like protein 1, *SCN2A*: Sodium voltage-gated channel alpha subunit 2, *ICAM5*: Intercellular adhesion molecule 5, *OR9Q1*: Olfactory receptor family 9 subfamily Q member 1, *INPP4A*: Inositol polyphosphate-4-phosphatase.

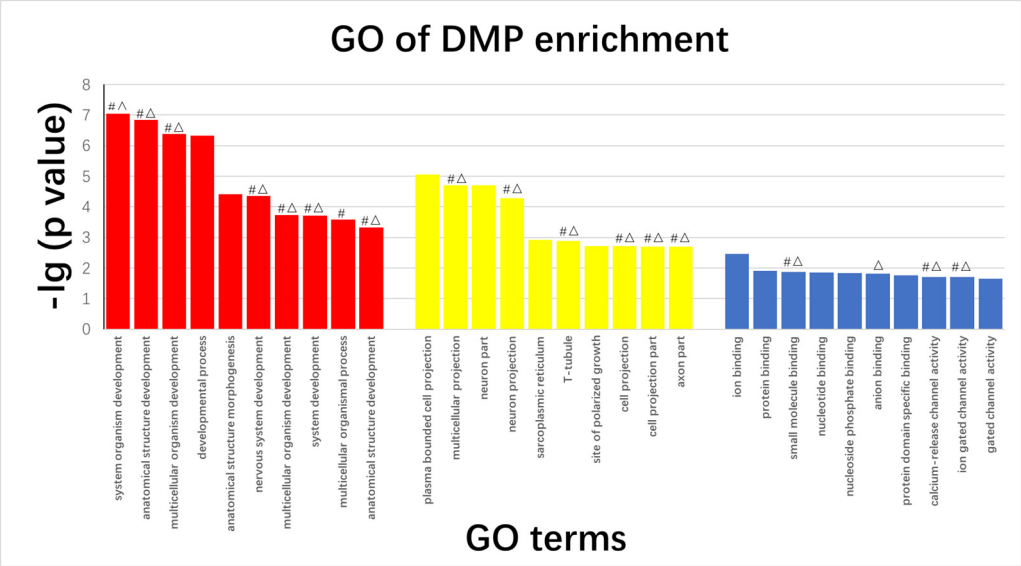

Fig. 5. GO histograms of significant enrichment. Screened by classic fisher test and 10 items with the most significance in each category of GO are exhibited. The larger the vertical coordinate is, the more significant the enrichment of the item is. X axis means different items with significant enrichment of GO; Y axis means $-\log_{10}$ (adjusted *p* value of DMPs). BP: biological processes, CC: cellular components, MF: molecular functions. #: The term related genes of DMP enrichment that is hypomethylated in schizophrenic patients. △: The term related genes of DMP enrichment that is hypermethylated in schizophrenic patients.

site research, there was also a positive correlation between the increase in total risk allele number of one carbon metabolism related genes and aggravating in negative symptoms of schizophrenia [29]. However, research of the multiple-site polymorphisms of *MTHFR* itself is lacking. In our study, the results showed that it was positive symptoms of schizophrenic patients that aggravated significantly with the increase of C677T or multi-site polymorphisms. Summarily, although inconsistent with previous study, our results supported the aggravating effects of *MTHFR* polymorphisms on schizophrenia. To elucidate, it may result from our enrolled patients in relapse condition, who were characterized by typical positive symptoms, while the negative symptoms was relatively not obvious to present typically.

In subgroup analysis of sex, the previous study revealed finitely. It was reported that the higher level of *MTHFR* C677T polymorphism in schizophrenic patients appeared in male subjects but not females [27]. But it was unknown for the sex effects on polymorphism divergence as well as for multiple-site polymorphisms on clinical features. Our study indicated that in male subjects but not females, there was a strong sex-specific significant higher degree of *MTHFR* C677T polymorphism in schizophrenia compared with controls, which is consistent with the previous study. From the result, an obvious sex effect could be achieved in *MTHFR* function. For the unreported contents, multiple-site study showed higher level of polymorphisms in schizophrenic patients only in male subjects rather than females. While in clinical feature research, it was also in male patients but not females that severity of positive symptoms aggravated with the increase of single-site C677T or multiple-site polymorphisms. In this case, it may be inferred that *MTHFR* effects may be influenced by sex factors including hormonal differences and genetics between male and female. To illustrate, some ideas of sex hormone may work well, as estrogen may play a role of protection in female schizophrenic patients through impact on neurodevelopment and social maturation. In another aspect, testosterone is a kind of male sex hormone, which could elevate the adverse illness course compared with estrogen [18]. Apart from sex hormone, other molecular factors like sex chromosomes and sex-dependent or sex-specific genetic risk were also involved in disease divergence of schizophrenia between different sexes [30].

While the detailed mechanism study of *MTHFR* effects on schizophrenia is still incomplete, there was some potential effects on pathogenesis of schizophrenia through *MTHFR* correlated process as reported before [12,26]. With more risk allele number among genotype of *MTHFR* C677T, A1298C and G1793A, the effect of risk increase in schizophrenia mainly attributes to the enzyme activity decrease and damage of related pathway catalyzed by this enzyme. C677T and A1298C was the most reported in psychiatric diseases including schizophrenia, major depression, bipolar disorder and autism [27,31,32], both polymorphisms of which were correlated with MTHFR activity. MTHFR is critical for metabolism of one carbon process and promote the methyl group supply in methylation modification of downstream. Since the DNA methylation could affect the related gene expression, it could be the mechanism acting on the association between *MTHFR* polymorphisms and schizophrenia. For DNA methylation, there are two kinds of assays on overall and partial extents, defined as genomic DNA methylation and specific gene region methylation respectively. Both forms of methylation level have been reported to make some certain degree of alteration in schizophrenic subjects [22,24], and genomic methylation level tend to be lower in healthy subjects with TT genotype of *MTHFR* C677T than that with CC [25]. But no study has reported the potential mechanism through the methylation alteration between *MTHFR* polymorphisms and schizophrenia. In our study, the genomic methylation level in patients is markedly lower than that of controls and more hypomethylated positions were found in patient group. Originating from DNA methylation levels measured at CpG islands in the region of promoter and adjacent sites, this result is consistent with some previous studies testing genomic DNA methylation [24]. While no sex-specific difference in genomic methylation level between the two groups was found. At this point, we regarded the *MTHFR* polymorphisms and clinical phenotypes of schizophrenic patients as one unity to explore the potential mechanisms since there was correlation between these two factors. The lower methylation level of schizophrenic patients compared with controls implied a potential role of methylation alteration caused by *MTHFR* polymorphisms, accompanied by schizophrenia with more severe symptoms in patients of TT genotype, while no attack of disease happened to TT in controls. The previously related sex-specific difference of *MTHFR* related effects on schizophrenia could not attribute to genomic methylation difference, while the sex related results may be influenced by factors including sex hormones, sex chromosomes or divergent levels of metabolites in one carbon metabolism.

Since significant methylation divergence appeared in the subjects of TT genotype and MTHFR activity could be damaged seriously by this

variant and supply of methyl group would be repressed after MTHFR inactivation, *MTHFR* polymorphism is one of the most critical factors that lead to DNA hypomethylation in schizophrenic patients. On basis of epigenetic change, the related modification of gene expression influenced by methylation change could further participate in some processes that may contribute to pathogenesis of schizophrenia. That's the potential pathway between *MTHFR* and schizophrenia while methylation alteration is the direct working point of *MTHFR*. Therefore, approximately 3000 gene positions were found to exhibit differential methylation levels between schizophrenic patients and controls, which contributed to their differential genomic DNA methylation levels. We selected the genes with the most significant DMPs and separate them by relative magnitudes of methylation between patients and controls. There were 15 genes most enriched with DMPs of hypomethylation and 8 genes most enriched with DMPs of hypermethylation in patient group. The genes and related products may contribute to the *MTHFR* related schizophrenia risk and symptoms. It was the potential genes enriched with the hypomethylated positions in schizophrenic patients that transit the effects of *MTHFR* related methylation change. While for the schizophrenia-related gene methylation assay, there was significant hypomethylation level of *SLC6A4* in schizophrenic patients, which may suggest the potential role of methylation alteration in this pathological status. As a gene of serotonin transporter, it is also a potential role to influence the transmitter pathway in schizophrenia.

Besides the analysis of genes with DMPs, the possible factors in this genomic methylation were explored by GO analysis. It was feasible to select the potential biological processes, cellular components, and molecular functions, which would be correlated with schizophrenia or possibly act as a biomarker for diagnosis or treatment. We selected the terms of GO that contains the previous genes with DMPs enrichment and label them, which could be a setpoint for subsequent research. In the molecular function including some ion channel related terms, there may be some effects on the differential positive symptoms in different genotypes. Previous studies have reported that sodium nitroprusside could improve the positive symptoms of schizophrenia in humans and rats, and one hypothesis was the effect on sodium ion channel [33,34]. Another study reported the calcium alteration in patients of panic disorder, revealing potential effects on psychotic symptoms through related ion and channels [35]. Summarily, additional studies that intend to explore the schizophrenia related genes with DMPs enrichment in certain biological processes or pathways are necessary, which may be available for research in pathogenesis and potential biomarker in diagnosis and treatment of schizophrenia.

There are several limitations to be recognized. First, the cohort size is relatively small and larger sample size is beneficial for the study of factors related to *MTHFR* in one carbon metabolism process. Second, more genetic changes of *MTHFR* that may reduce the enzyme activity need to be discovered and studied, each of which could result in activity alteration. Finally, for the potential genes with enrichment of DMPs, subsequent studies on related genes or terms are necessary to explore their distinct effects on schizophrenia. In future studies, the previous issues should be noticed and intensive researches are indispensable.

5. Conclusions

This is a pilot study revealing that *MTHFR* polymorphisms at both single and multiple sites are related to the risk of schizophrenia and positive symptom of the disease. Sex-specific effects exist in the correlation between *MTHFR* polymorphisms and schizophrenia. The risk of *MTHFR* polymorphisms in schizophrenia and the clinical symptoms was only significant in male patients but not females. While the sex-specific risk of *MTHFR* in schizophrenia is new and the reasons remain unanswered. Our methylation analysis suggested that there was significant hypomethylation of genomic DNA in schizophrenic patients with no sex difference. The correlation between *MTHFR* polymorphism and schizophrenia may attribute to the change of DNA methylation level, and some certain genes could be potential research objects for *MTHFR* effects on schizophrenia.

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Authors' contributions

LW and YL managed in the literature searches, designed the study, undertook the sample processing, interpreted the statistical analyses and wrote the first draft of the manuscript. GZ, ML and CW contributed to blood sample collection, subject and clinical data administration. RL instructed in the study approach and supervised the statistical analyses and their interpretations. All authors have contributed to and approved the final manuscript.

Declaration of competing interest

None.

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