Original Article

Methylation of the *MAOA* promoter is associated with schizophrenia

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Background: Earlier studies have shown that patients with schizophrenia have abnormalities in DNA methylation. Monoamine oxidase A (*MAOA*) has been extensively studied due to its biological role in neurological function. However, the relationship between the DNA methylation of the *MAOA* gene and schizophrenia is unclear. This study aims to elucidate the relationship between the methylation of the *MAOA* gene promoter and schizophrenia.

Methods: There were 151 individuals with schizophrenia (104 males and 47 females), which were diagnosed according to DSM-V, the DNA of peripheral blood of all samples was extracted and chemically modified with bisulfite. The promoter region of *MAOA* gene was sequenced by Methylation Target Technical Method (MethylTargetTM), and 247 controls (204 males and 43 females) included in the study. *MAOA* gene promoter methylation was compared between the case and control groups. Meanwhile, we measured DNA methylation in two regions of *MAOA* (*MAOA-2* and *MAOA-3*).

Results: In the male schizophrenia group (BM) and the male control group (DM), *MAOA*-2 and *MAOA*-3 methylation were positively associated with schizophrenia. In the female schizophrenia group (BF) and the female control group (DF), *MAOA*-2 methylation was associated with schizophrenia.

Conclusions: Although the role of gene methylation in the development of schizophrenia is still unclear, our findings suggest that DNA methylation of *MAOA* may contribute to the onset of schizophrenia.

Keywords: Methylation; Monoamine oxidase A (*MAOA*); schizophrenia; DNA

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Introduction

Schizophrenia is a chronic and severe mental illness with many clinical manifestations, negatively affecting thought, perception, emotion, and behavior (1,2). Schizophrenia shows much heterogeneity in the type, symptoms, and course of the disease. Although lifetime morbidity is as low as 1%, it can still lead to serious and negative consequences, including disability (3). Patients with schizophrenia are more violent compared to mentally healthy people or those with other mental disorders (4). Additionally, the quality of life for patients with schizophrenia is degraded while simultaneously imposing a heavy burden on families and society (5). Therefore, there is an urgent need to understand better the mechanisms underlying the pathophysiology of schizophrenia.

The development of molecular biological techniques has aided the discovery of polymorphic sites associated with schizophrenia. Monoamine oxidase A (*MAOA*) plays a crucial role in the metabolism of biogenic monoamines, including serotonin, dopamine, and epinephrine (6). *MAOA* and neurotransmitter abnormalities are closely related to the development of schizophrenia (7,8). The gene encoding *MAOA* is located on the X chromosome with a variable nucleotide repeat (VNTR), located 1.2-kb (Xp11.4–p11.3) upstream of the *MAOA* coding sequence and consists of

a 30-bp repeat sequence (9). Carrying 2 or 3 VNTRs is defined as having low *MAOA* activity, while greater than 3 is referred to as high *MAOA* activity (7). Some studies have revealed that individuals with low *MAOA* activity alleles have a higher risk of schizophrenia (10,11). However, another study showed that schizophrenia in women is associated with high *MAOA* activity (12). Therefore, the inconsistency of these results suggested the involvement of alternative molecular mechanisms that could influence gene expression and lead to the development of schizophrenia.

Epigenetic modifications, including methylation, are essential gene molecular regulatory mechanisms to allow genes to cope with environmental changes. DNA's methylation refers to the selective addition of a methyl group to the CpG nucleotide of methyltransferase-catalyzed DNA to form 5-methylcytosine (13). Studies have shown that DNA methylation can regulate gene expression in mental disorders by altering chromatin structure, DNA conformation, DNA stability, and DNA-protein interactions (14,15). DNA's methylation variation is an essential contributor to individual phenotypic, differences, and abnormalities in methylation that are closely related to schizophrenia, violent aggression, and other complex diseases. A DNA methylation analysis of 485,764 CpG sites across the genome revealed 234 sites displayed significantly different methylation in schizophrenia patients (16). This study supported the idea that DNA methylation could regulate the pathophysiological processes involved in schizophrenia. However, the relationship between DNA methylation of *MAOA* and the onset of schizophrenia has not been reported before.

In this study, we extracted the methylation status of the *MAOA* gene promoter in 151 patients with schizophrenia (104 males and 47 females) and 247 controls (204 males and 43 females). Our results found that methylation of *MAOA-2* and *MAOA-3* is associated with schizophrenia.

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Kunming Medical University (No: KY2019.57).

Samples selection criteria

We investigated methylation of the *MAOA* gene promoter in a total of 151 individuals with schizophrenia (104 males and 47 females) in the Yunnan Psychiatric Hospital, Yunnan, China. Criteria for admission of schizophrenia: (I) in line with DSM-V diagnostic criteria; (II) diagnosis was made by at least two psychiatrists independently; (III) Yunnan Han nationality, age 18–60. Exclusion criteria: (I) combined with brain organic diseases, brain trauma or serious physical diseases; (II) combined with nervous system diseases and other mental disorders; (III) pregnant or lactating women; (IV) other drug abuse history within 6 months. Accordingly, 247 unrelated subjects (204 males and 43 females) were also recruited as control subjects. Criteria for admission of control group: (I) the individuals whose gender, nationality and age match the above case group; (II) no genetic history; no history of major physical diseases. The control group also met the above exclusion criteria. Written informed consent was obtained from the patients themselves or their family members and guardians before enrollment.

DNA's methylation

Blood was obtained from all individuals, and genomic DNA was extracted from Ethylenediaminetetraacetic acid (EDTA) blood using a QIAamp DNA Blood Mini Kit (QIAGEN company, German). Primer 3 (http://primer 3.ut.ee//) was used to design primers after bisulfite disposed. The Polymerase chain reaction (PCR) primers for MAOA-2 were: Forward—5'-AAGTYGGGGGTATAATTGTTTAGGTT-3'; Reverse—5'-CTAAAACCCCRAAAACCACTCT-3'. The PCR primers for MAOA-3 were: Forward—5'-GGGGGAGTYGGGTATTGTG-3'; Reverse—5'-ACCCCCACCTCAATACCTAAC-3'.

Gel electrophoresis and NanoDrop analysis

Agarose gel electrophoresis detected genomic DNA. The electrophoresis bands were visible, with no clear degradation and no RNA contamination. Genomic DNA quality was quantified using a Nanodrop 2000 (NanoDrop Technologies, USA). Samples with concentrations greater than or equal to 20 ng/μL or a total of 1 μg with an OD260/OD280 DA ≥1.8 were used (this amount can be used in 10 PCR panels).

Design of primers and optimization of single-locus PCR conditions

Primers were designed and provided by Genesky Company

Table 1 Target DNA methylation sequence information

Target	Chr	Gene	mRNA	mRNA_Strand	TSS	Start	End	Length	Target_Strand	Distance2TSS
MAOA_2	X	*MAOA*	NM_000240	+	43514154	43514479	43514728	250	+	325
MAOA_3	X	*MAOA*	NM_000240	+	43514154	43515466	43515218	249	–	1064

Target, the name of the target fragment; Chr, chromosome; mRNA, RNA close to the product; mRNA_Strand, the direction of mRNA; TSS, the transcription initiation site of mRNA; Start, the initial position of the product on the reference genome; End, the termination position of the product on the reference genome; Length, the length of the product; Target_Strand, the direction of product; Distance2TSS, the distance between the product and the TSS.

(Shanghai, China). The human genome treated with bisulfite was selected and amplified as primer-template to obtain a clear single band for later experiments.

The optimization of multiple PCR primers

The primers optimized in the last step were mixed into a panel of multiple PCR primers. On the particular method of capillary electrophoresis, we determined whether each pair of primers in multiple systems could be amplified efficiently and specifically, and then adjusted to optimize the composition and concentration of primers for the multiple PCR panels. The optimized multiple PCR primer panel was used to conduct multiple PCR amplification with the transformed sample genome as the template. The Genesky Company conducted multiple PCR amplification (Shanghai, China).

Treatment of DNA with bisulfite

The samples were processed by EZ DNA Methylation-God Kit (ZYMO company, USA), to convert cytosine C without methylation of genomic DNA into uracil U (Bisulfite Conversion Efficiency is shown in *Figure S1*).

Specific tag sequences were added to the samples

Specific tag sequences compatible with the Illumina platform (Illumina, USA) were introduced to the end of the library by PCR amplification using primers with Index sequences. The PCR procedure with 11 cycles was used to reduce the nonspecific amplification as much as possible.

Quantitative and computer sequencing

The final methyl target sequencing library was obtained by mixing Index PCR products of all samples. The fragment length of the library was verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) (see *Figure S2*).

Results

The total level of MAOA methylation between schizophrenia patients and healthy control subjects is similar

By using methylation sequencing of target regions (*Table 1*), we investigated the total DNA methylation profile of *MAOA* in 151 patients with schizophrenia and 247 controls (*Table S1*). Principal component analysis (PCA; *Figure 1*) and hierarchical clustering (*Figure 2*) were employed to visualize the DNA methylation profile of *MAOA* in all samples. Neither PCA nor hierarchical clustering analysis could separate the schizophrenia patients and the control subjects as expected when investigating all *MAOA* methylation sites.

There are several differentially methylated sites in male and female schizophrenia patients

Although there was no significant difference between the total DNA methylation level of *MAOA* between schizophrenia patients and controls, we explored whether there was a difference in methylation at specific methylation sites on *MAOA*. Interestingly, we found several DNA methylation sites (*Table 2*) in the *MAOA* promoter differently methylated in schizophrenia when compared with healthy control samples.

There are several differentially methylated segments in male and female schizophrenia patients

Next, we investigated whether there were some DNA segments, which were differentially methylated between the schizophrenia patients and healthy controls. By calculating the mean methylation level of all CpG sites on *MAOA*, we found that the methylation of *MAOA-2* and *MAOA-3* was significantly altered between schizophrenics and controls

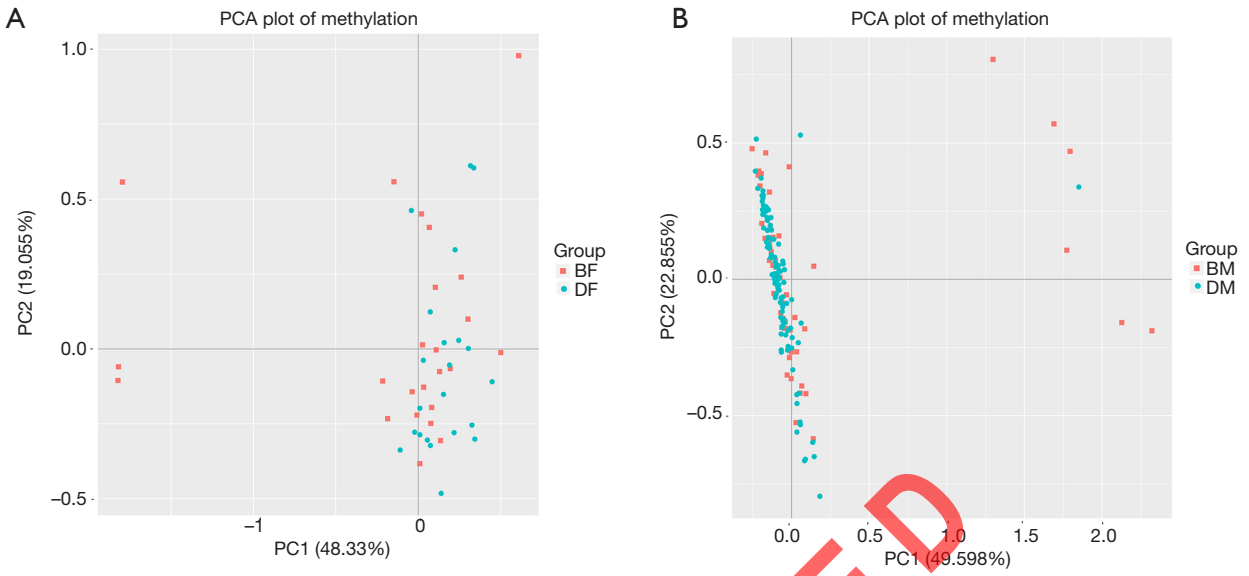

Figure 1 PCA analysis of all samples based on the methylation level of CpG locus in the amplicons in BF and DF (A) and in BM and DM (B). The sample groups were distinguished according to the color of PCA; The X and Y axes indicate the index that the best reflects the real species composition of samples, respectively.

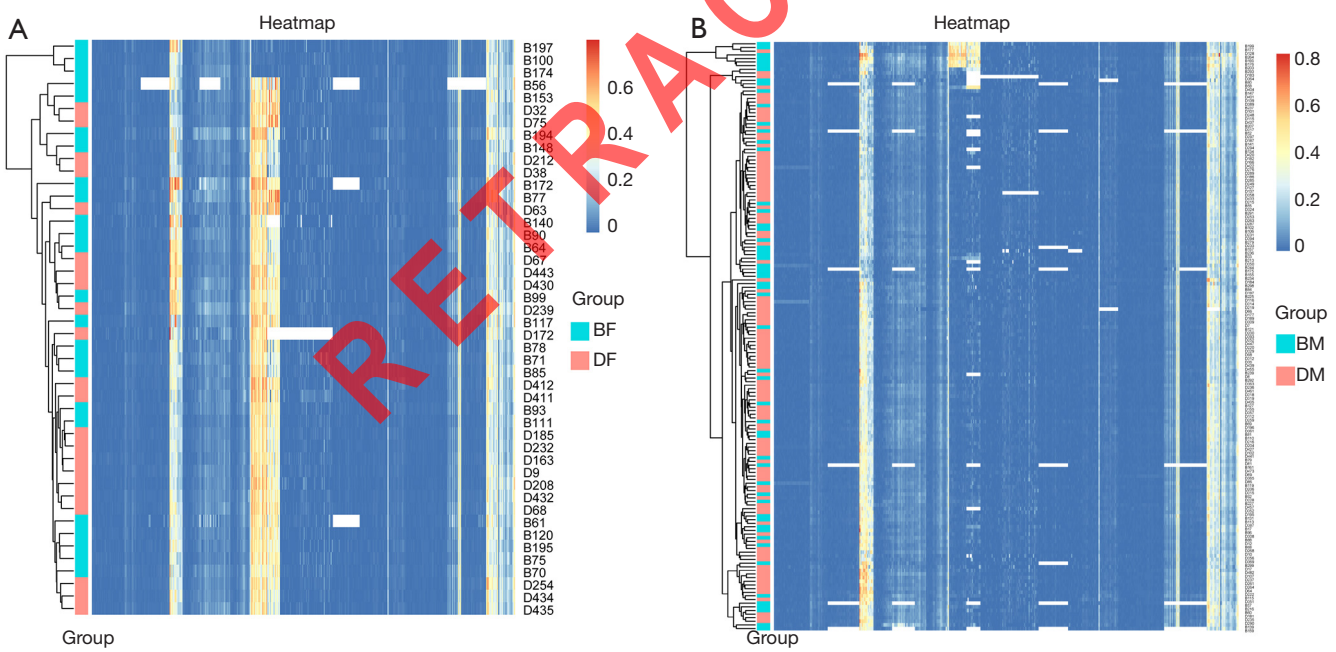

Figure 2 Cluster analysis of all samples based on the methylation level of CpG locus in the amplicons in BF and DF (A) and in BM and DM (B). Each cell of the heatmap indicates the relative methylation level of CpG sites in the corresponding row of samples, and the change of methylation level is reflected by the color gradient. A deeper blue indicates a lower methylation level, while deeper red indicates a higher methylation level. The similarity of the methylation level of samples is shown by the arrangement order of rows. The more adjacent rows are, the higher the overall similarity of the methylation level of samples they represent. The tertiary diagram on the left of the figure systematically describes this similarity.

Table 2 Different methylation sites between patients and healthy controls

Target	Position	Type	P value (*t*-test)	Q value (U test)	P value (Logistic)	OR (L95–U95) (Logistic)	Methyl diff
MAOA_2	28	CG	0.023	0.280	0.020	1.0470 (1.0074–1.0881)	0.056
MAOA_2	34	CG	0.024	0.047	0.021	1.0492 (1.0074–1.0927)	0.052
MAOA_2	50	CG	0.031	0.693	0.024	1.0527 (1.0068–1.1007)	0.045
MAOA_2	71	CG	0.021	0.463	0.018	1.0528 (1.0088–1.0987)	0.053
MAOA_2	80	CG	0.029	0.165	0.026	1.0532 (1.0063–1.1022)	0.043
MAOA_3	22	CG	0.027	0.020	0.049	1.0665 (1.0002–1.1372)	0.023
MAOA_3	27	CG	0.024	0.342	0.015	1.0646 (1.0122–1.1197)	0.039
MAOA_3	48	CG	0.012	0.021	0.008	1.0556 (1.0141–1.0987)	0.059
MAOA_3	54	CG	0.076	0.122	0.063	1.0479 (0.9974–1.1009)	0.026
MAOA_3	64	CG	0.030	0.055	0.020	1.0492 (1.0075–1.0926)	0.042

Target, the name of the amplicon of the methylation site; POS, the specific location of the methylation site at the amplicon; Type, the type of methylation site; P value (*t*-test), the *t*-test model is used to calculate the P value; P value (U-test), the U-test model is used to calculate the P value; P value (Logistic), the P value was calculated through the logistic regression model (10 samples as example).

Table 3 Differentially methylated segments between the male schizophrenia group and the control group

Target	P value (*t*-test)	P value (U test)	P value (Logistic)	OR (L95–U95) (Logistic)	Methyl diff
MAOA_2	0.023739	0.3617054	0.02045575	1.0564(1.0085-1.1066)	0.04674876
MAOA_3	0.0144194	0.05942464	0.009992034	1.0636(1.0148-1.1146)	0.045110497

Target, the name of the amplicon of the methylation site; P value (*t*-test), the *t*-test model is used to calculate the P value; P value (U-test): the U-test model is used to calculate the P value; P value (Logistic), the P value was calculated through the logistic regression model. Methyl diff, the difference degree of methylation between the two groups equals average methylation degree of the case group minus average methylation degree of the control group; normal distribution test W value/P value: Shapiro-Wilk's normal test was used. The larger the W value value (close to 1), and the P value is not significant, the data is considered to conform to normal distribution. 3–5,000 data is required. OR (L95–U95) (Logistic), Logistic regression OR value and 95% confidence interval.

in men (P<0.005; *Table 3*; *Figure 3*). Similarly, there was a significant difference in *MAOA-2* methylation between schizophrenics and controls in women (*Table 4*; *Figure 4*).

Differentially methylated haplotype identification

Furthermore, we conducted a methylation haplotype analysis to find differentially methylated haplotypes between schizophrenia and control patients. Finally, we discovered that some methylation haplotypes were significantly different between schizophrenia and control patients (*Table 5*).

Discussion

In recent years, the relationship between DNA methylation and schizophrenia has been extensively explored (17-19). Schizophrenia is a complicated disease, which might be influenced by epigenetic modifications, including methylation, and is vulnerable to environmental factors (20). This present study reveals that methylation of the *MAOA* gene promoter at the *MAOA-2* and *MAOA-3* methylation sites is linked to susceptibility to schizophrenia in both males and females.

It is reported that DNA methylation—especially *MAOA* methylation perhaps is in response to environmental influences, may also be a factor concerning these mental disorders. The presently observed trend is towards CpG-specific *MAOA* hypomethylation-due to increased gene expression and decreased serotonin or norepinephrine availability. *MAOA* methylation has been linked to many psychiatric disorders. Studies reveal that in panic disorder patients, patients exhibit lower *MAOA* methylation

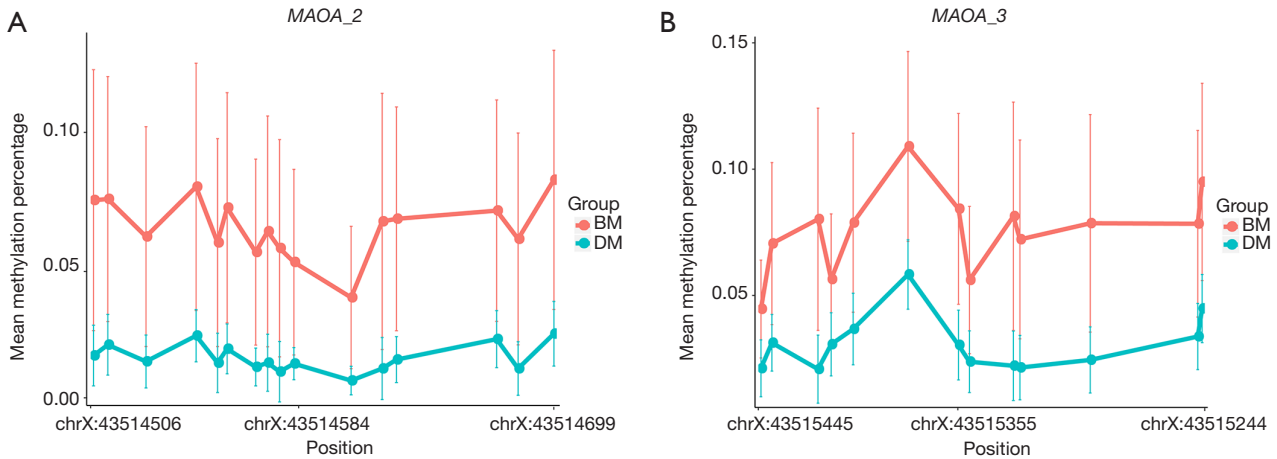

Figure 3 The mean methylation percentage (methyl/total CpG) at each location in BM and DM in *MAOA-2* (A) and *MAOA-3* (B). The bp position on the X chromosome is shown on the X-axis. The average methylation ratios of BM are colored with red, while the average methylation ratios of DM are colored with blue. BM, the male schizophrenia group; DM, the male control group.

Table 4 Differentially methylated segments between the female schizophrenia group and the control group, others were the same with *Table 3*

Target	P value (*t*-test)	P value (U test)	P value (Logistic)	OR (L95–U95) (Logistic)	Methyl diff
MAOA_2	0.040204	0.04084197	0.1366052	0.8981 (0.7796–1.0346)	-0.074072348
MAOA_3	0.134099	0.3756063	0.1524039	0.9584 (0.9041–1.0158)	-0.053151449

Target, the name of the amplicon of the methylation site; P value (*t*-test), the *t*-test model is used to calculate the P value; P value (U-test): the U-test model is used to calculate the P value; P value (Logistic), the P value was calculated through the logistic regression model. Methyl diff, the difference degree of methylation between the two groups equals average methylation degree of the case group minus average methylation degree of the control group; normal distribution test W value/P value: Shapiro-Wilk's normal test was used. The larger the W value value (close to 1), and the P value is not significant, the data is considered to conform to normal distribution. 3–5,000 data is required. OR (L95–U95) (Logistic), Logistic regression OR value and 95% confidence interval.

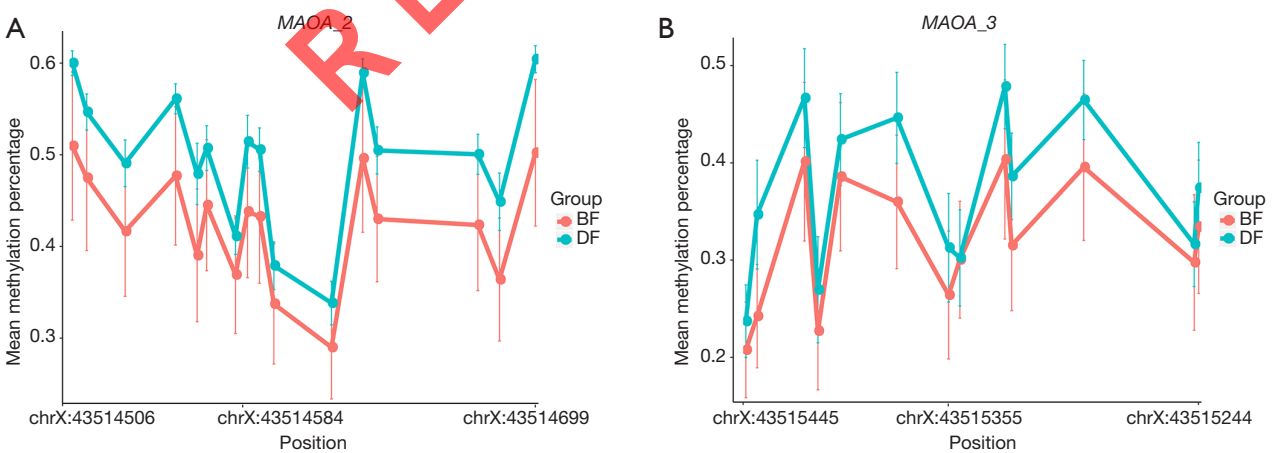

Figure 4 The mean methylation percentage (methyl/total CpG) at each location in BF and DF in *MAOA-2* (A) and *MAOA-3* (B). The bp position on the X chromosome is shown on the X-axis. The average methylation ratios of BM are colored with red, while the average methylation ratios of DM are colored with blue. BM, the male schizophrenia group; BF, the female schizophrenia group; DF, the female control group.

Table 5 The methylation haplotype analysis of the CpG site

Target	Haplotype	Depth	B100	B102
MAOA_2	TTTTTTTTTTTTTTT	148,377	0.790575916	0.869387755
MAOA_2	TTTTTTTTTTTTTTC	2,612	0.007853403	0.009329446
MAOA_2	TTTTTTTTTTTTCTT	2,343	0.007853403	0.009912536
MAOA_2	CCCCCCCCCCCCCCCC	2,129	0	0
MAOA_2	TCTTTTTTTTTTTTT	2,127	0.007853403	0.013411079
MAOA_3	TTTTTTTTTTTT	15,976	0.790575916	0.869387755
MAOA_3	TTTTTCTTTTTT	764	0.007853403	0.009329446
MAOA_3	TTTTTTTTTTTC	343	0.007853403	0.009912536
MAOA_3	TCTTTTTTTTTT	314	0	0
MAOA_3	TTTTCTTTTTTT	286	0.007853403	0.013411079

Target, the name of amplicon; Haplotype, the type of haplotype; Depth, the number of sequences supported for this haplotype (10 samples as an example).

compared to controls, and there is a negative correlation between baseline psychiatric disorder severity and *MAOA* methylation (21).

In addition to schizophrenia, DNA methylation of *MAOA* is also associated with other mental disorders and is gender-dependent. These results may be because the *MAOA* gene is found on the X chromosome. *MAOA* methylation status has been significantly associated with lifetime nicotine dependence and alcohol dependence in women, but not men (22). In a sample of female patients with acrophobia, *MAOA* methylation was significantly reduced in patients compared to controls, and treatment for this disorder could significantly enhance *MAOA* methylation (15). In male patients, hypermethylation of exon 1 and intron 1 of *MAOA* was associated with post traumatic stress disorder (PTSD) when compared to controls (22). Hypomethylation of the *MAOA* promoter region has also been significantly associated with smoking behavior in women (23). Our results also suggest a difference in *MAOA* methylation in male and female schizophrenia patients.

DNA's methylation of *MAOA* contributes to mental disorders, including schizophrenia, through the regulation of serotonin. In a pharmacokinetic study investigating 61 female patients with major depression, decreased methylation at two individual CpG sites in the *MAOA* promoter region was linked to a slower response to treatment with serotonin reuptake inhibitors over 6 weeks (24). Abnormalities in DNA methylation at the *MAOA* promoter may be associated with schizophrenia in males (25). Males with antisocial personality disorder (ASPD) showed hypermethylation in the *MAOA* promoter compared to healthy men. These findings were associated with whole-blood serotonin levels and reduced transcriptional activity *in vitro* (26). A prospective examination of associations between epigenome-wide methylation patterns in cord blood at birth and propensity to develop conduct disorders between the ages of 4–13 years identified a subthreshold association for increased methylation in the vicinity of the *MAOA* gene (27).

Limitation

In the process of sample collection, the number of female patients is significantly less than that of male patients, so that the number of female samples in this study is insufficient. This phenomenon has also attracted our attention, but the real reason for this phenomenon cannot be reasonably explained. We are considering whether the difference in the prevalence of schizophrenia between male and female patients is worthy of further study. Due to the limited number of female clinical samples used in the experiment, the general applicability of the results of this study is limited to a certain extent, so it is necessary to expand the sample size in future research.

Conclusions

Our study showed that the pathophysiology of schizophrenia might be mediated by DNA methylation

of the *MAOA* promoter. However, the role of DNA methylation in the progression of schizophrenia and its underlying molecular mechanisms are still unclear. In future studies, we will investigate the methylation of other genes related to the pathogenesis of schizophrenia and investigate the changes induced by *MAOA* methylation in greater detail.

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Footnote

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-4481

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-4481). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Kunming Medical University (No: KY2019.57). Written informed consent was obtained from all subjects in our study.

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Supplementary

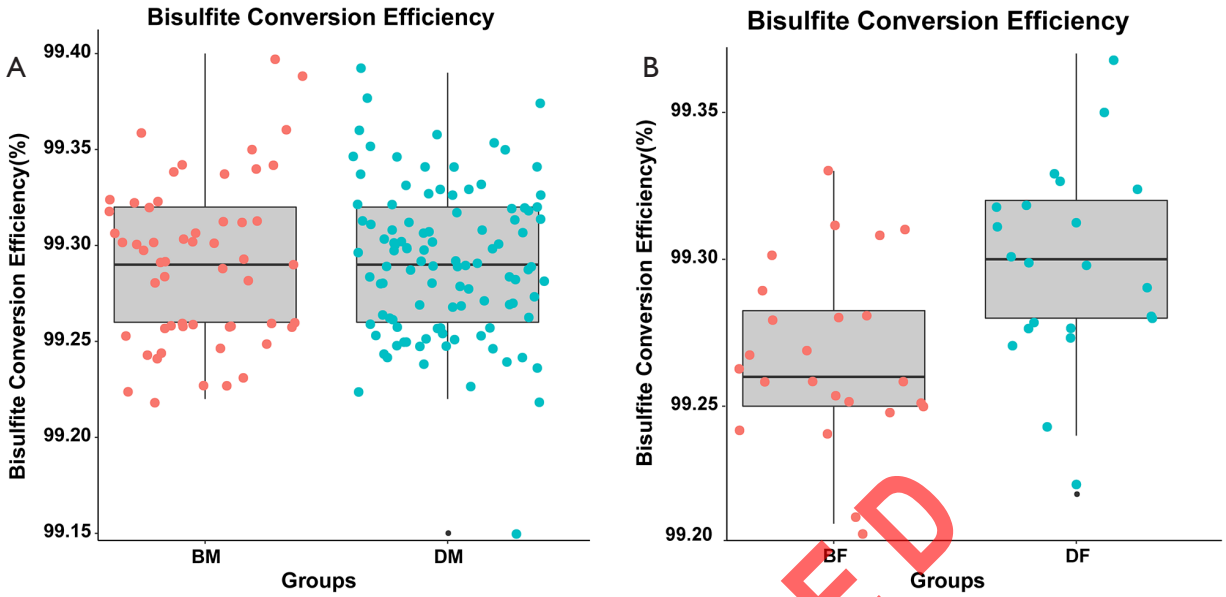

Figure S1 Bisulfite conversion efficiency in BM and DM (A) and in BF and DF (B). BM, the male schizophrenia group; BF, the female schizophrenia group; DF, the female control group.

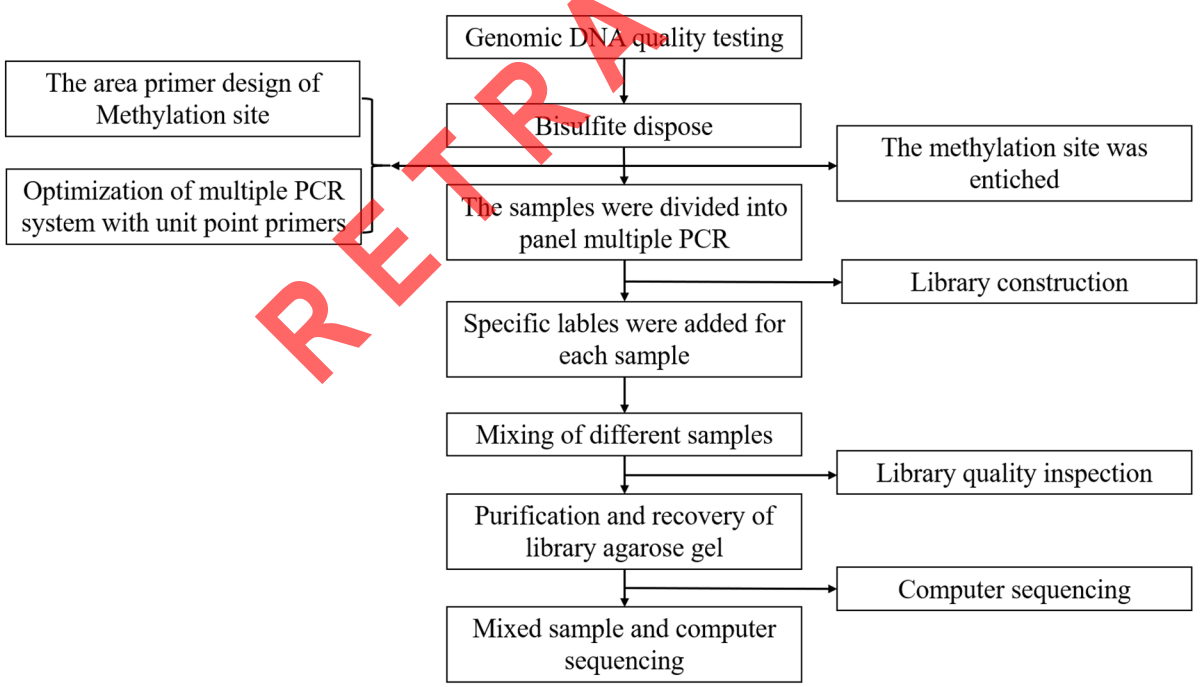

Figure S2 The flow chart.

Table S1 The DNA methylation levels of the CpG site in the amplicon

Target	Position	Chr	Genome position	Distance2TSS	Type	B100	B102
MAOA_2	28	X	43514506	352	CG	0.020	0.008
MAOA_2	34	X	43514512	358	CG	0.023	0.019
MAOA_2	50	X	43514528	374	CG	0.020	0.009
MAOA_2	71	X	43514549	395	CG	0.015	0.017
MAOA_2	80	X	43514558	404	CG	0.023	0.006
MAOA_3	22	X	43515445	1291	CG	0.016	0.026
MAOA_3	27	X	43515440	1286	CG	0.016	0.020
MAOA_3	48	X	43515419	1265	CG	0.024	0.005
MAOA_3	54	X	43515413	1259	CG	0.020	0.016
MAOA_3	64	X	43515403	1249	CG	0.028	0.016

Target, the name of amplicon; Position, the position of the methylation site on the amplicon; Chr, the location of the fragment in the chromosome; Distance 2 TSS: the relative distance of this site to the transcription start site in the reference genome and the negative sign indicates that this site is upstream of the transcription start site; Type, the type of methylation (CG stands for the methylation at the CG site, 2 samples as an example).