Risperidone-induced changes in DNA methylation in peripheral blood from first-episode schizophrenia patients parallel changes in neuroimaging and cognitive phenotypes

Maolin Hu\textsuperscript{a,b,1}, Yan Xia\textsuperscript{c,d,1}, Xiaofen Zong\textsuperscript{a,b,1}, John A. Sweeney\textsuperscript{e,f}, Jeffrey R. Bishop\textsuperscript{g}, Yanhui Liao\textsuperscript{h}, Gina Giase\textsuperscript{i}, Bingshan Li\textsuperscript{j}, Leah H. Rubin\textsuperscript{k}, Yunpeng Wang\textsuperscript{l}, Zongchang Li\textsuperscript{a,m}, Ying He\textsuperscript{a}, Xiaogang Chen\textsuperscript{a,n,n,n}, Chunyu Liu\textsuperscript{m,n}, Chao Chen\textsuperscript{a,n,n,n}, Jinsong Tang\textsuperscript{a,n}.

\textsuperscript{a} Department of Psychiatry, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China
\textsuperscript{b} Department of Psychiatry, Renmin Hospital of Wuhan University, Wuhan, Hubei, China
\textsuperscript{c} Analytic and Translational Genetics Unit, Massachusetts General Hospital, Boston, MA, United States
\textsuperscript{d} Broad Institute of MIT and Harvard, Cambridge, MA, United States
\textsuperscript{e} Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati, Cincinnati, Ohio, United States
\textsuperscript{f} Huaxi MR Research Center, Department of Radiology, Sichuan University, Chengdu, China
\textsuperscript{g} Department of Experimental and Clinical Pharmacology and Department of Psychiatry, University of Minnesota, Minneapolis, MN, United States
\textsuperscript{h} Department of Psychiatry, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China
\textsuperscript{i} Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, United States
\textsuperscript{j} Genetics Institute, Vanderbilt University School of Medicine, Nashville, TN, United States
\textsuperscript{k} Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, United States
\textsuperscript{l} Center for Lifespan Changes in Brain and Cognition (LCBC), Department of Psychology, University of Oslo, 0317 Oslo, Norway
\textsuperscript{m} National Health Institute, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China
\textsuperscript{n} National Clinical Research Center on Mental Disorders, Changsha, Hunan, China
\textsuperscript{o} National Technology Institute on Mental Disorders, Changsha, Hunan, China
\textsuperscript{p} Hunan Key Laboratory of Psychiatry and Mental Health, Changsha, Hunan, China
\textsuperscript{q} Department of Psychiatry, SUNY Upstate Medical University, Syracuse, NY, United States
\textsuperscript{r} National Clinical Research Center on Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan, China
\textsuperscript{s} Department of Psychiatry, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China
\textsuperscript{t} Department of Psychology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310016, China; Key Laboratory of Medical Neurobiology of Zhejiang Province, Hangzhou, China

ARTICLE INFO

Keywords:
- Biomarker
- DNA methylation
- Risperidone treatment
- First-episode schizophrenia

ABSTRACT

Background: Second generation antipsychotics such as risperidone are first-line pharmacotherapy treatment choices for schizophrenia. However, our ability to reliably predict and monitor treatment reaction is impeded by the lack of relevant biomarkers. As a biomarker for the susceptibility of schizophrenia and clozapine treatment response, DNA methylation (DNAm) has been studied, but the impact of antipsychotics on DNAm has not been explored in drug-naïve patients.

Objective: The aim of the present study was to examine changes of DNAm after short-term antipsychotic therapy in first-episode drug-naïve schizophrenia (FES) to identify the beneficial and adverse effect of risperidone on DNAm and their relation to treatment outcome.

Methods: Thirty-eight never treated schizophrenia patients and 38 demographically matched individuals (healthy controls) were assessed at baseline and at 8-week follow-up with symptom ratings, and cognitive and functional imaging procedures, at which time a blood draw for DNAm studies was performed. During the 8-week period, patients received treatment with risperidone monotherapy. An independent data set was used as replication.

Results: We identified brain related pathways enriched in 4,888 FES-associated CpG sites relative to controls. Risperidone administration in patients altered DNAm in 5,979 CpG sites relative to baseline. Significant group differences were observed in 2,578 CpG sites.

Conclusions: This study is the first to explore the impact of risperidone monotherapy on DNAm in drug-naïve FES patients. These changes might reflect beneficial and adverse effects of risperidone on DNAm.
1. Introduction

The majority of individuals with schizophrenia (SCZ) are treated with second-generation antipsychotic drugs such as risperidone. Risperidone has receptor affinities for dopamine, serotonin, and other neurotransmitter receptors (Miyamoto et al., 2005) and effectively treats acute psychosis and reduces risk for relapse (Tiihonen et al., 2017). Although risperidone and other antipsychotics bind to target receptors within hours of administration, clinical efficacy can take weeks, perhaps due to slower acting complex biological changes, including changes in gene expression (Thomas, 2006).

Accumulating evidence indicates that gene expression regulation, including those related to DNA methylation (DNAm), may be relevant to the clinical effects of antipsychotic drugs (Chong et al., 2002; Langlois et al., 2001; Thomas et al., 2003). Previous studies have shown that second generation antipsychotics can affect DNAm (Dong et al., 2016; Guidotti and Grayson, 2014; Melka et al., 2013, 2014a, 2014b; Melka et al., 2015; Murata et al., 2014; Sugawara et al., 2015; Tang et al., 2014). For example, olanzapine alters DNAm in the brains of mice (Melka et al., 2013) and clozapine alters DNAm in human peripheral leukocytes (Kinoshita et al., 2017). Clinical trials in this field primarily involve chronically treated individuals with long histories of illness and varied drug treatments, so that treatment related methylation changes are not easily identified. Thus, studying drug-naive individuals experiencing a first episode of SCZ (FES) before and after monotherapy treatment may produce insights into both disease-associated DNAm alterations (via pretreatment case control comparisons) and understanding of effects of antipsychotics on DNAm (via comparison of patients before and after treatment. FES has not been investigated previously in a pharmacoepigenetic study of this nature.

In the present study, the first goal was to identify disease-related DNAm differences in antipsychotic naive individuals with SCZ. Second, we examined the effect of acute antipsychotic treatment on DNAm. We hypothesized that disease-related DNAm changes occur before treatment, and that acute antipsychotic therapy would normalize at least some of the DNAm. We then investigated whether DNAm changes after acute risperidone monotherapy are related to changes in brain structure, cognition, and symptom severity (Fig. 1 A).

2. Methods

2.1. Participants

We recruited 80 participants included 42 right-handed Chinese Han patients with FES and 38 demographically-matched healthy controls. The patients were recruited from October 2012 to January 2014 at Henan Province Mental Health Center, the Second Affiliated Hospital of Xinxiang Medical University, Henan, China. Two attending psychiatrists diagnosed the participants using the Structured Clinical Interview for DSM-IV-TR, patient version (SCID-I/P). All patients with FES had an
onset of psychotic symptoms less than one-year before study participation, and had received no prior antipsychotic treatment. Healthy controls had no history of Axis I or II disorders (Structured Clinical Interview for DSM-IV Axis I Disorders—Non-Patient Edition (SCID-I/NP); Structured Clinical Interview for DSM-IV Axis II Disorders), nor any known first-degree family history of significant psychiatric illness. Individuals with neurological disease, systemic disease or substance abuse disorders (except tobacco cigarette smoking) were excluded from this study. Eight patients and eight healthy controls were regular cigarette smokers. Among all participants, there were no regular alcohol users. Furthermore, the alcohol and tobacco use were not interrupted by the treatment. All participants provided written informed consent and were given the option to withdraw from the study at any time. One patient dropped out of the baseline DTI scan and dropped out of the follow-up MRI scan. Three more patients opted out of the follow-up MRI scans. As a result, the following study included 38 patients (25 men; 13 women; mean age, 25.0 years; age range, 18–37) with complete information and 38 healthy controls (25 men; 13 women; mean age, 24.8 years; age range 18–32) (Table 1).

We also obtained a replication data set involving participants from a prior study involving similar entry criteria (untreated first episode SCZ), treatment (4–6 weeks risperidone), and phenotyping (Bishop et al., 2015). This sample included four controls (2 men, 2 women; 2 African American, 1 Hispanic and 1 White; age range, 27–30) and three FESs (1 men, 2 women; 2 African American, 1 Hispanic; age range, 18–40) (Supplementary Table S1D).

2.2. Clinical measures

FES patients were treated with risperidone monotherapy at a dosage of 4 to 6 mg/day for eight weeks, without the addition of other psychotropic medications. Patients were treated with benzhexol as needed for extrapyramidal symptoms. Nineteen patients took at least one dose of lorazepam (range 1–2 mg) during the 8-weeks of risperidone treatment. Patients were not allowed to take lorazepam within the week before the second MRI scan. Symptom severity and cognition were assessed, functional imaging studies were conducted, and a blood draw were collected at baseline and again after 8 weeks of treatment. We collected the same cognition and imaging assessments, and a blood draw for the controls at baseline. All procedures were approved by the research ethics committee of the Second Xiangya Hospital and the Second Affiliated Hospital of Xinhua Medical University.

Symptom severity was evaluated using the Positive and Negative Syndrome Scale (PANSS) (Aboraya and Nasrallah, 2016). Participants completed neuropsychological tests for evaluating cognitive function including: Stroop Color Word Test (SCWT), Wisconsin Card Sorting Test (WCST), Trail Making Test (TMT), Verbal Fluency Test (VFT), and Digit Span Distraction Test (DSDT) (Hazar et al., 2016; Jensen and Rohwer, 1966; Kohli and Kaur, 2006). Magnetic resonance imaging (MRI) scans were performed on all participants using a 3T MRI scanner (Siemens Magnetom Verio 3.0T MRI, Erlangen, Germany) with a 16-channel head coil. Resting-state fMRI data were collected, and quantified using the fractional amplitude of low-frequency fluctuations (fALFF) and regional homogeneity (ReHo) metrics. Detailed scanning parameters and analysis methods for fMRI data are provided in previous reports (Geoffroy et al., 2016; Hu et al., 2016).

Phenotypic variables (MRI and Cognition) used in tests for correlation with treatment-related DNA changes were from brain regions or cognitive measures that changed significantly between pre- and post-treatment in the SCZ cohort or were significantly different between patient and control cohorts before treatment (Supplementary Table S1A-S1C). On this basis, for spontaneous brain activity, we included fALFF in bilateral putamen and right caudate, and ReHo in the right caudate and left putamen. For cognitive function, we included SCWT, WSC, and TMT. Full statistical analyses examining these phenotypic changes in relation to DNA changes are shown in Supplementary Table S1A, S1B, and S1C.

2.3. Quantification and analysis of DNA methylation

Whole genome methylation status was examined in 114 samples. DNA (500 ng) was isolated using QiAamp DNA Blood Mini Kit (Qiagen; Germantown, MD) and treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research; Irvine, CA). DNA was quantified using Infinium® Human Methylation 450 K BeadChip (Illumina Inc.; San Diego, CA).

2.3.1. Preprocessing and quality control of DNA methylation data

Most analyses were performed in R (version 3.3.1). Raw intensity files were preprocessed and quantile-normalized using the Bioconductor package ChAMP, version 2.0.1 (Morris et al., 2014). Proportions of methylation values (Illumina “Beta” scale) were calculated. After which, BIMIQ (Teschendorff et al., 2013) was used to adjust for type II bias. Probes were removed according to the following criteria: (1) detection p-value above 0.01 in one or more samples; (2) bead counts less than three in at least 5% of samples; (3) having common SNPs as identified in Zhou et al. (2017); (4) aligning to multiple locations as identified in Zhou et al. (2017); and (5) identified in Naem et al. (2014), which are not high quality probes; and (6) located in sex chromosomes. There were 164,684 probes remaining for differential methylation analysis. Batch and positional effect of each chip was adjusted using the ComBat empirical Bayesian approach (Johnson et al., 2007; Jiao et al., 2018). The reference-based method was used to calculate blood cell type proportions (Horvath, 2013). We used linear regression to regress out effects of cell type proportion, age, sex, smoking status, and drinking status for each probe.

2.3.2. Power analysis

With a sample size of 76 for the risperidone-association analysis, which is subjected to paired t-test (pre vs post treatment), we had 80% power to detect 12% mean methylation changes at P < 1.00e-6 adjusting for genome-wide significance. For the case control analysis of 76 samples, there was 80% power to detect 13% mean methylation changes at P < 1.00e-6. The power analysis was based on the power simulations across a range of sample sizes and effect sizes according to the calculation of Saffari et al. (2018).

2.3.3. Identification of differentially methylated positions and genomic regions

To identify differentially methylated positions (DMPs), we
conducted three epigenome-wide association analyses (EWAS): the FES association analysis on pre-treatment FES versus controls as PreFES-EWAS, the risperidone association analysis on pre-treatment FES versus post-treatment FES as risperidone-EWAS, and the post-treatment FES association analysis on post-treatment FES versus control as PostFES-EWAS (Fig. 1 A). The DMPs in the three association analyses were named PreFES-DMP, risperidone-DMP, and PostFES-DMP separately. A linear regression model in Limma package (Ritchie et al., 2015) was used for testing the PreFES-DMP and PostFES-DMP at each CpG site. Paired t-tests were used to test for risperidone-DMP between pre- and post-treatment samples in the patients. For multiple testing correction, we used the threshold of 1.00e-6 as recommended by Saffari et al. (2018) and Rukyan et al. (2011) on a estimation based on Bonferroni correction. We reported the DMPs with nominal ($p < 0.05$) and genome-wide ($p < 1.00e-6$) significance levels.

To identify differentially methylated regions (DMRs) in association with treatment response and SCZ trait, we used the mCSEA (Methylated CpGs Set Enrichment Analysis) (Martorell-Marugan et al., 2019) in R (version 3.5). We ran the mCSEA analysis for pre-treatment FES versus post-treatment FES (paired test), pre-treatment FES versus control samples, and post-treatment FES versus control samples. We ranked all evaluated CpG sites after quality control with the differential statistics above. Then we used the mCSEA test function to search promoter and gene body DMRs. We specified five CpGs as the minimum amount per region and performed 10,000 permutations to calculate P value. The P value was then adjusted by the Benjamini and Hochberg procedure to correct for multiple hypothesis testing.

2.3.4. Estimation of risperidone-induced changes in DNA methylation

To estimate DNA changes related to beneficial and adverse effects of risperidone, we compared the direction of effect size between two analyses: PreFES-EWAS and risperidone-EWAS (Fig. 1 B). In PreFES-EWAS, we defined up-regulated CpG sites as CpG sites that have higher methylation levels in patients than in controls, while the down-regulated CpG sites were defined as lower methylation levels in patients than in controls. In the Risperidone-EWAS, the up-regulated CpG sites were defined as CpG sites with higher methylation levels in post-treatment patients than in pre-treatment patients, while the down-regulated CpG sites had lower methylation levels in post-treatment patients than in pre-treatment patients.

Based on the two comparisons we divided the CpG sites into two groups: the CpG sites with consistent up/down regulation, and CpG sites with contrasting up/down regulation. Any changes in methylation in the contrasting CpG sites was interpreted to represent a beneficial effect, which means that treatment shifted abnormal level of methylation toward normal values. For example, before treatment a CpG site is down regulated in patients; whereas after treatment, the methylation levels rise making it closer to normal. In the same way, the changes in CpG sites with consistent up/down regulation could represent adverse effect, which means that treatment shifted methylation further away from normal control values.

To characterize the degree of beneficial effect and adverse effect evident in the CpG sites, we estimated the percentages of CpG sites with beneficial effect and adverse effect among the following categories: All CpG sites used in the analyses were classified into beneficial and adverse effects according to the comparison between treatment-associated changes and FES-control differences (Fig. 1 b). Then, PreFES-DMPs, risperidone-DMPs, and postFES-DMPs were fit into the categories of beneficial and adverse effects to calculate their relative proportions.

2.3.5. Over-representation analysis

To evaluate the enrichment of DMP genes in SCZ GWAS fine-mapping genes using the up to date GWAS of SCZ from PGC3 (Trubetskoy et al., 2022). SCZ related candidate genes were also obtained from SZDB (http://www.szdb.org/) (Wu et al., 2017) and NPdenovo (http://www.wzgenomics.cn/NPdenovo/) (Li et al., 2016). A two-sided Fisher’s exact test was used to estimate the enrichment. Gene ontology and KEGG pathway analyses were performed using missMethyl package, which consider the number of CpG islands corresponding to each gene (Phipson et al., 2016).

2.3.6. Replication

In the replication dataset, blood samples were drawn at pre- and then post-treatment for each individual. DNA was quantified using Infinium Human Methylation 27 BeadChip (Illumina, CA) at the Northwestern University Core facility. This data was used to replicate the results of PreFES-EWAS, risperidone-EWAS, PostFES-EWAS, and the degree of beneficial effect and adverse effect.

2.4. Construction of the methylation-phenotype network

To estimate parallel DNA changes and phenotype, we constructed a methylation-phenotype network by correlating the changes of DNA methylation and changes of phenotypic variables. We choose the CpG sites with normalized effect to construct the network analysis. The normalized CpG sites were those with beneficial effect and overlapped between PreFES-DMPs and risperidone-DMPs, excluding the PostFES-DMPs. All the phenotypic variables used were listed in the Supplementary Table S1 based on previous publications (Geoffroy et al., 2016; Hu et al., 2016). The relationships between pairs of variables (for example, methylation change of a CpG site and change of PANSS scores) were examined using Spearman rank correlation tests. Associations with an absolute correlation coefficient larger than 0.3 and a p-value smaller than 0.05 were plotted in the network, and these variables were treated as edges in the graph (Fig. 2). The graph was made using Cytoscape (Shannon et al., 2003) v3.5.0 (http://cytoscape.org/) software. The false discovery rate (FDR) procedure was used to correct for multiple comparisons. Correlations with a FDR corrected $p < 0.05$ were considered significant.

3. Results

3.1. PreFES-DMPs enriched in neuronal function-related pathway

After quality control, 164,684 probes were left for further analysis. 4888 CpG sites were differentially methylated between pretreatment FEES and controls, named as PreFES-DMPs ($t$-test, $p < 0.05$). As shown in a Manhattan plot (Fig. 2 A), none exceeded genome-wide significance with $p < 1.00e-6$. Most of PreFES-DMPs (3782 out of 4888, 77.37%) showed hypomethylation in FES patients. Some top signals with several DMPs were located in specific genes, such as cg12407791 ($p = 2.03e-5$) and cg07010633 ($p = 1.22e-4$) at UNCI3D (Table 2). The PreFES-DMPs were enriched in genes related to neurogenesis (FDR $< 1.68e−12$), generation of neurons (FDR $< 1.68e−12$), and central nervous system development (FDR $= 4.04e-10$) (Table 3).

Using the replication dataset, 637 out of the 4888 PreFES-DMPs were measured, and six PreFES-DMPs were replicated ($p < 0.05$). These include cg08063724 at the first exon of MTCLI, cg17366294 at promoter of C4orf37, cg05119831 at gene body of PPRN2, cg0933411 at the promoter of DL1G, cg17145652 at 5’UTR of RNFI170 and cg24765079 at body of CDH1. Comparing with previously published SCZ-associated methylation results, eleven PreFES-DMPs reported by the largest EWAS in SCZ (1714 individuals) (Hannon et al., 2016) were replicated ($p < 0.05$) in the discovery data examined herein.

The PreFES-DMPs were enriched in the 108 loci associated with SCZ risk by GWAS,(Schizophrenia Working Group of the Psychiatric Genomics, 2014) Among the 108 GWAS loci, 64 contained 770 CpG sites. In these 770 CpG sites, 77 were PreFES-DMPs. Replication tests showed that the GWAS loci had significant enrichment of the PreFES-DMPs.
Among the 628 potential causal genes from the fine-mapping of the SCZ GWAS (Trubetskoy et al., 2022), 447 genes were studied. There were 130 genes were PreFES-DMPs. Enrichment tests showed that the GWAS fine-mapping genes had significant enrichment of the PreFES-DMPs genes (enrichment odds ratio = 1.4, Fisher’s Exact test p = 5.34e-12).

For the region level analysis, 3419 regions of the gene body and 8229 regions of promoter were generated from the 164,684 CpG sites tested after quality control. In the pre-treatment FES association test no region reached P.adj < 0.05, but 341 gene body DMRs and 858 promoter DMRs had changes of nominal p < 0.05.

3.2. Risperidone-DMPs and DMRs enriched in neural signaling pathways

One risperidone-DMP, cg08778598 at the gene body of SDHAP3, was differentially methylated in the pre- and post-treatment comparison (P = 4.84e-7) (Fig. 2 B). Methylation was increased by 3% after risperidone treatment at this CpG site. There were 5979 risperidone-DMPs with nominally significant (p < 0.05) differences between pre- and post-treatment. Of these, 3483 (58.25%) had increased methylation levels after treatment while 2496 CpG sites were decreased.

Using the replication data, 517 CpG sites of the 5979 were detected, and 11 were replicated. These included cg09991975 at the promoter of JOSD1, cg26309951 at 5’UTR of MORF4L2, cg21376883 at body ACTN2, cg01598046 at the promoter of TRAIP, cg00066816 at the promoter of IL12B, cg17617223 at the promoter of ZER1, cg15432938 at the promoter of FRAT1, cg15875120 at the promoter of FAM188A, cg22560190 at the promoter of CNTN1, cg12100791 at the promoter of PYCARD, and cg15928446 at the promoter of PRR14.

Pathway analysis revealed 5979 risperidone-DMPs enriched in brain-related KEGG pathways such as axon guidance (FDR = 1.60e-4), Wnt signaling pathway (FDR = 1.20e-3), MAPK signaling pathway (FDR = 1.20e-3) and calcium signaling pathway (FDR = 4.84e-3) (Table 3).

None of the DMRs reached P.adj < 0.05 for the risperidone-induced differentially methylation region analysis within either gene body or promoter regions, but 210 DMRs were detected in gene body and 555 DMRs in promoter regions with nominal significance p < 0.05. These DMRs involved genes enriched for the calcium signaling pathway (FDR = 0.0118) and long-term depression (FDR = 0.0413). Of the above Entrez genes, the greatest changes were at sites CACNA1A, RYR1, NOS1, LTB4R2, and PTGER3.

3.3. PostFES-DMPs showed a trend of enrichment in metabolic pathways

Through comparing between FES and healthy controls at post-treatment (Fig. 2 C), we identified 6760 PostFES-DMPs with nominal p < 0.05. Similar to PreFES-associated DMPs, most PostFES-DMPs (4034 out of 6760, 59.67%) showed hypomethylation in post-treatment FES.

Using replication data, 3777 of the 6760 PostFES-DMPs were measured and 8 PostFES-DMPs were replicated with p < 0.05. Pathway analysis did not find any KEGG pathways enriched in the PostFES-DMPs.
Similar with FES-associated CpG sites, we found that 85.31% out of the beneficial effect, while only 14.68% showed adverse effect (Fig. 3A). We next explored whether DNAm changes of normalized sites were correlated with changes in spontaneous brain activity related with PANSS reductions (Fig. S2); in 129 sites, DNAm changes accounted with at least one phenotype (absolute correlation coefficient \( r > 0.3 \), \( p < 0.05 \), Supplementary Fig. S1). From the perspective of different phenotypic domains, DNAm changes after treatment in 96 sites correlated with the 27,578 detected CpG sites showed beneficial effect, whereas 35.71% CpG sites showed adverse effects.

### Table 2

| Probe information | Statistical Analysis | t value/ paired t value | P value | \( \Delta^a \) |
|-------------------|----------------------|-------------------------|---------|-----------|
| Probe information |                      |                         |         |           |
| cg13407791 17     | 73,824,354            | UNC13D Body             | -4.556  | 2.03E-05  |
| cg12070285 6      | 74,072,447            | C00R21 1stExon          | -4.469  | 3.32E-05  |
| cg00401265 X      | 78,003,113            | LPAR4 Promoter          | 4.349   | 4.31E-05  |
| cg13918808 X      | 13,062,651            | FAM9C 5'UTR             | 4.272   | 6.03E-05  |
| cg15150970 X      | 25,473,529            | DNMT3A 5'UTR            | -4.247  | 6.57E-05  |
| cg12500147 19     | 1,287,256             | CNR2 5'UTR              | -4.169  | 8.23E-05  |
| cg25289658 X      | 135,278,646           | FHL1 5'UTR              | -4.105  | 1.03E-04  |
| cg03596919 X      | 51,150,887            | CXorf67 1stExon         | 4.101   | 1.12E-04  |
| cg07010633 17     | 73,824,396            | UNC13D Body             | -4.067  | 1.22E-04  |
| cg13728834 9      | 35,846,324            | TME4M8 Body             | -4.035  | 1.32E-04  |

### 3.4. Risperidone-induced beneficial and adverse effects in DNAm

By comparing the direction of effect size between pre-treatment FEP association analysis (PreFES vs control) and treatment association analysis (PostFES vs Pre-FES), we found that 63.02% of the 164,684 CpG sites showed beneficial effect, whereas 36.98% CpG sites showed adverse effect (Fig. 1B). For the 4888 PreFES-DMRs, 85.32% showed beneficial effect, while only 14.68% showed adverse effect (Fig. 3A). Using FDR \( p < 0.05 \), but found several pathways with \( \Delta = 2.12e-3 \), insulin signaling pathway (FDR \( p = 2.41e-2 \), and TGF-beta signaling pathway (FDR \( p = 2.92e-2 \)).

The mean difference between patients and controls, for FES associated differential methylation, is the mean methylation value of FES minus CTLs. For risperidone-induced methylation change, it is the mean methylation value of post-treatment minus pre-treatment.

#### 3.5. Methylation-phenotype network reflecting how changes in methylation are related to changes in phenotypes after therapy

In the DNAm sites with beneficial effect, we identified 580 normalized sites, i.e. sites that after treatment no longer had significant differences relative to healthy controls. These 580 sites mapped to 568 unique genes, including 113 SCZ-related candidate genes from SZDB (http://www.szdb.org/) and NPdenovo (http://www.wzgenomics.cn/NPdenovo), such as MAN2A1 (Supplementary Table S2).

We next explored whether DNA methylation changes were correlated with phenotypic changes in symptom severity, cognitive function, and brain physiology measures obtained using fMRI. Although the correlation between the changes of DNAm with phenotype changes did not survive the multiple testing, these analyses using nominal thresholds revealed that 284 out of the 580 normalized sites correlated with changes in spontaneous brain activity.
found in fMRI (Supplementary Fig. S3) (absolute Spearman correlation coefficient $> 0.3$, $p < 0.05$). Two CpG sites (cg09175724 and cg19248041) correlated with five phenotypic variables, nine CpG sites correlated with four phenotypic variables, 25 CpG sites correlated with three phenotypic variables, and 61 CpG sites correlated with two phenotypic variables. For example, cg09175724 at the 5'UTR of CDC42EP2, correlated with reduction in PANSS-Total score assessing global clinical psychopathology (correlation coefficient $=0.40$, $p = 1.30e-2$), PANSS-Positive symptom score (correlation coefficient $=0.57$, $p = 2.07e-4$), and regional reductions in brain neurophysiology reflected in fALFF measures from resting state fMRI in both left (correlation coefficient $=-0.35$, $p = 2.93e-2$) and right putamen (correlation coefficient $=-0.39$, $p = 1.30e-2$).

DNAm of one CpG site (cg25535999) at the gene body of NR3C1, which encodes the glucocorticoid receptor, was normalized by treatment, and changes in that DNAm correlated with PANSS-G, PANSS-T changes and cognitive improvement measured by the SCWT. Another CpG site, cg25114611 at the promoter of FKBP5 (TSS1500) was also normalized after treatment, and those changes correlated with changes of nodal degree of anterior cingulate and paracingulate gyrus (ACG, left and right) and in nodal efficiency of right ACG. Normalization in 11 CpG sites in calcium pathway genes correlated with multiple phenotypic changes. For example, cg06204009 at the promoter of ATP2B3 and cg17119907 at the 5'UTR of NOS1 were normalized after treatment and correlated with the PANSS-G changes. In the calcium pathway, normalization in the CpG site cg26571093 at the gene body of CACNA1H was correlated with changes in resting state brain function within the caudate nucleus (left and right) and with cognitive improvement measured by the SCWT (Supplementary Table S3).

### 4. Discussion

This is the first study of treatment-naïve individuals with FES who were assessed and then followed after receiving risperidone monotherapy to examine methylation changes in peripheral blood and their relation to changes in clinically relevant phenotypes after treatment with risperidone. Through comprehensive comparisons between pre- and post-treatment FES samples of patients, and controls samples, treatment-related DNAm changes were identified along with hundreds of normalized CpG sites – many of which parallel brain or behavioral phenotypic changes.

Limiting the present study to a previously untreated FES population has important advantages, notably that it avoided the potentially confounding effects of chronic illness and prior drug treatment on methylation, providing a better opportunity to assess antipsychotic treatment effects on gene methylation. This is important for both of our aims: (1) identifying disease-related methylation changes without treatment effects, and (2) investigating treatment effects of acute antipsychotic use on DNAm. Although the sample size is small due to the challenges collecting such samples, the findings in regard to these two aims are well supported by previous publications of various study design: The over-representation of FES-associated CpG sites within the SCZ-related GWAS loci is consistent with the largest previous SCZ GWAS study (Hannon et al., 2016). Regarding treatment effects, some of the associated genes are pivotal for neuronal function (e.g., HDAC6), while some have been previously been associated with SCZ by GWASs (e.g., MAN2A1, CNTN4, MEF2C), and others are involved in de novo mutations that have been related to SCZ (e.g., NCKAP1, CAPRIN1, DNMT3A).

Several of the genes have been implicated in the pathophysiology of SCZ (Bhandari et al., 2016) with functions related to long-term synaptic depression and disease-associated calcium signaling pathways. Notably, the use of risperidone monotherapy, consistent drug exposure period, and consistent dosage enhanced the validity of the pre- and post-treatment comparisons. Furthermore, having healthy controls as a reference allowed us to distinguish beneficial effects from adverse effects by equating changes in DNAm with consistent up/down regulation.
More than 80% of observed risperidone induced DNAm changes were toward the methylation levels of healthy individuals. Impressively, this pattern was reasonably well replicated in an independent data set. We note that our consideration of beneficial changes defined as shifts toward healthy control values that eliminated significant group difference is biologically informative, whether these changes are beneficial and adverse from a broader systems biology or clinical perspective need to be evaluated in other ways. This is broadly consistent with evidence that antipsychotic drugs are primarily beneficial in this population (Tiihonen, 2016), and provide novel mechanistic insights into mechanisms of drug action.

For the first time, to our knowledge, we linked post-antipsychotic methylation changes with changes in psychiatric symptoms as well as neuropsychological and neurophysiological abnormalities in SCZ. We related the methylation-phenotype network identified in this study to brain changes for two reasons. First, methylation status and methylation changes induced by the drug in blood cells correlate closely with methylation in the brain (Aberg et al., 2014, 2013). Second, while previous studies in SCZ associated methylation with changes in cognition and brain as assessed by MRI (Rubin et al., 2016), we also linked post-antipsychotic methylation changes with changes in psychiatric symptoms. The overlap of DNAm and established SCZ risk loci is of significant clinical relevance. Studies of methylation in blood cells may provide an important approach for understanding SCZ pathophysiology and drug treatment effectiveness, by understanding how treatment related methylation changes might normalize protein production in genes where genotype is related to illness expression.

Although the conclusions are supported statistically and were to a degree validated in an independent dataset, our both discovery and replicate data sets were not large. Limited statistical power may have reduced sensitivity to detect smaller changes, and may also increase false positive findings. Thus, our results need to be validated in a larger FES cohort.

Future studies should identify the time course of methylation changes after therapy with several important clinical implications, including possible early detection of treatment responders and non-responders which is not possible on clinical evaluation. Rapid onset of methylation change may be a useful index of future treatment response and therefore guide earlier changes in drug therapy for treatment non-responders. Variability in timing or extent of methylation changes across individuals might account for variability in the speed and extent of treatment effects. Methylation changes that diminish or persist after longer term treatment might be related to course of illness, risk for relapse, etc. Studies for developing new drugs based on EWAS studies should use drug naïve individuals instead of treated individuals. The majority of post-treatment methylation changes in patients appeared to be adverse effects which might otherwise be mistaken for illness related features. This pattern is consistent with the interpretation that the differences between the treated patients and controls are more likely drug induced rather than disease induced methylation changes. The prior treatment may have already made some beneficial changes in treated patients so that starting a new treatment might generate additional effects that could be hard to detect. Finally, we note that while correlations of our observed methylation changes with changes in psychopathology, cognition and brain physiology suggest a brain- and disease-relevance of our methylation changes observed in blood samples, the degree to which all observed changes in blood are relevant to the brain remain to be determined. note that the cause of changes over time in methylation remains to be determined through further animal model and cell lines studies are needed.

**Funding and disclosure**

This work was supported by grants from the National Natural Science Foundation of China (81871057 and 82171495 to J.T;81871056 and 81473611 to J.H.; 82022024 and 31907527 to C.C.; 81901357 to X.C.; 81471361 to X.C.; 82022024 and 31907527 to C.C.; 81901357 to X.Z), the National Key Research and Development Program of China (2022YFA0103700 to J.T;2021YFE0191400 to X.C ; 2016YFC130600 to C.C.), NIH grants 1 U01 MH110340-01, 1R01ES024988 (to C.L.), MH083888 (to J.R.B.), Wuhan Science and Technology Bureau grant (201706062010169 to M. H.), the University of Cincinnati Schizophrenia Program Fund (to J.A.S.), and Central South University Graduate Project grant (502221702 to Y.X.). Dr. Sweeney is supported by a teaching award from Sichuan University and consults to VeraSci. Other authors have nothing to disclose.

**Author statement**

Dr Hu, Dr Zong and Ms Xia had full access to all the data. Ms Xia takes responsibility for the integrity of the data and the accuracy of the data analysis. Dr. J. Tang, Dr X. Chen, Dr Liu and Dr C. Chen supervised this work and contributed equally as principal investigators.

**Study concept and design:** Jinsong Tang, Xiaogang Chen, Chunyu Liu, Chao Chen

**Acquisition data:** Maolin Hu, Xiaofen Zong, Jinsong Tang, Jeffrey R Bishop, John A Sweeney, Zongchang Li, Ying He, Yanhui Liao.

**Analysis and interpretation of data:** Yan Xia, Chao Chen, John Sweeney, Chunyu Liu, Leah Rubin, Binghsan Li, Jinsong Tang, Yanhui
Liao, Xiaogang Chen.

Drafting of the manuscript: Yan Xia.

Critical revision of the manuscript for important intellectual content: Yan Xia, John Sweeney, Jinsong Tang, Chunyu Liu, Chao Chen, Yanhui Liao, Yunpeng Wang, Gina Giase, Jeffrey Bishop, Leah Rubin.

Statistical analysis: Yan Xia, Chao Chen, Bingsha Li, Jinsong Tang

Obtained funding: Jinsong Tang, Xiaogang Chen, Chao Chen, Chunyu Liu, MaoLin Hu, Yan Xia, Jeffrey Bishop, Xiaofen Zong.

Study supervision: Jinsong Tang, Xiaogang Chen, Chao Chen, Chunyu Liu.

Supplementary materials

Fig. S1. Overall methylation-phenotype network.

Pink line means the correlation between the two nodes is negative, gray line means the correlation is positive. The width of the line represents the absolute value of correlation coefficient.

Fig. S2. Methylation-phenotype network for PANSS scores. Fig. S3. Methylation-phenotype network for fMRI.

Table S1. description of brain function variables and replication data. (A) brain activity variables; (B) brain topological network variables; (C) cognitive function variables; (D) replication data set.

Table S2. Normalized genes overlapped with schizophrenia related genes.

Table S3. Correlation of methylation in calcium genes with brain function variables.

Declaration of Competing Interest

No potential conflict of interest was reported by the authors. None.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81871057 and 82171484 and 81471361 to X.C.; 82020204 and 31907052 to C.C.; 81901350 to X.Z.), the National Key Research and Development Program of China (2022YFE0103700 to JT; 2021YFE0191400 to X.C.; 2016YFC1306000 to C.C.), NIH grants 1 U01 MH103340-01, R01ES024988 (to C.L.), MH083888 (to J.R.B.), Wuhan Science and Technology Bureau grant (20170602010169 to M.H.), the University of Cincinnati Schizophrenia Program Fund (to J.A.S.), and Central South University Graduate Project grant (S2221702 to X.Y.).

Dr Hu, Dr Zong and Ms Xia had full access to all the data. Ms Xia takes responsibility for the integrity of the data and the accuracy of the data analysis. Dr. J. Tang, Dr X. Chen, Dr Liu and Dr C. Chen supervised this work and contributed equally as principal investigators.

Study concept and design: Jinsong Tang, Xiaogang Chen, Chunyu Liu, Chao Chen.

Acquisition data: MaoLin Hu, Xiaofen Zong, Jinsong Tang, Jeffrey R Bishop, John A Sweeney, Zongchang Li, Ying He, Yanhui Liao.

Analysis and interpretation of data: Yan Xia, Chao Chen, Sweeney, Chunyu Liu, Leah Rubin, Bingshan Li, Jinsong Tang, Yanhui Liao, Chao Chen, Xiaofen Zong.

Study supervision: Jinsong Tang, Xiaogang Chen, Chao Chen, Chunyu Liu.

Dr. Sweeney is supported by a teaching award from Sichuan University and consults to VeraSci. Other authors have nothing to disclose.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jpsychres.2022.114789.

References

Aberg, K.A., McClay, J.L., Norella, S., Clark, S., Kumar, G., Chen, W., Khachane, A.N., Xie, L., Hudson, A., Gao, G., Harada, A., Holtman, C.M., Sullivan, P.F., Magnusson, P. K., van den Oord, E.J., 2014. Methylene-wide association study of schizophrenia: identifying blood biomarker signatures of environmental insults. JAMA Psychiatry 71 (3), 255–264.

Aberg, K.A., Xie, L.Y., McClay, J.L., Norella, S., Vunck, S., Snider, S., Beardsley, P.M., van den Oord, E.J., 2013. Testing two models describing how methylene-wide studies in blood are informative for psychiatric conditions. Epigenomics 5 (4), 367–377.

Aharoni, A., Nassrallah, H.A., 2016. Perspectives on the Positive and Negative Syndrome Scale (PANSS): use, misuse, drawbacks, and a new alternative for schizophrenia research. Ann. Clin. Psychiatry 28 (2), 125–131.

Bhandari, A., Voineskos, D., Daskalaki, Z.J., Rajji, T.K., Blumberger, D.M., 2016. A review of impaired neural plasticity in schizophrenia investigated with non-invasive brain stimulation. Front. Psychiatry 7, 45.

Bishop, J.R., Reilly, J.L., Harris, M.S., Patel, S.R., Kittles, R., Badner, J.A., Prasad, K.M., Nimgaonkar, V.L., Keshavan, M.S., Sweeney, J.A., 2015. Pharmacogenetic associations of the type-3 metabotropic glutamate receptor (GRM3) gene with working memory and clinical symptom response to antipsychotics in first-episode schizophrenia. Psychopharmacology (Berl.) 232 (1), 145–154.

Chong, V.Z., Young, I.T., Mishra, R.K., 2002. cDNA array reveals differential gene expression following chronic neuroleptic administration: implications of synapsin II in haloperidol treatment. J. Neurochem. 82 (6), 1533–1539.

Dong, E., Tueting, P., Matriciano, F., Grayson, D.R., Guidotti, A., 2016. Behavioral and molecular neurogenetic alterations in prematurely stressed mice: relevance for the study of chromatin remodeling properties of antipsychotic drugs. Transl. Psychiatry 6, e711.

Geoffroy, P.A., Etain, B., Lajenf, M., Zerdazi, E.H., Brichant-Petitjean, C., Hellbrunner, U., Hou, L., Degardin, F., Rietschel, M., McMahon, P.J., Schulze, T.G., Jamain, S., Marie-Claire, B., Bellisseur, F., 2016. Circadian gene expression and lithium response in bipolar disorder: associations with PPARCIA (PGC-1alpha) and BORA. Genes Brain Behav. 15 (7), 660–668.

Guidotti, A., Grayson, D.R., 2014. DNA methylation and demethylation as targets for antipsychotic therapy. Dialog. Clin. Neurosci. 16 (3), 419-429.

Hannon, E., Dempster, E., Viana, J., Burrage, J., Smith, A.R., Macdonald, R., Clift, D., Mustard, C., Breen, G., Therman, S., Kaprio, J., Toulopoulou, T., Hulshoff Pol, H.E., Bohlken, M.M., Kahn, R.S., Nenadic, I., Hultman, C.M., Murray, R.M., Coller, D.A., Bass, N., Gurling, H., McQuillin, A., Schalkwyk, L., Mill, J., 2016. An integrated genetic-epigenetic analysis of schizophrenia: evidence for co-localization of genetic associations and differential DNA methylation. Genome Biol. 17 (1), 176.

Hazin, L., Leite, G., Oliveira, R.M., Alencar, J.C., Fichman, H.H., Marques, P.D., de Mello, C.B., 2016. Brazilian normative data on letter and category fluency tasks: effects of gender, age, and geopolitical region. Front. Psychol. 7, 684.

Horvath, S., 2013. DNA methylation age of human tissues and cell types. Genome Biol. 14 (10), R115.

Hu, M., Zong, X., Zheng, J., Mann, J.J., Li, Z., Pantazatos, S.P., Li, Y., Liao, Y., He, Y., Zhou, J., Sang, D., Zhao, H., Tang, J., Chen, H., Lv, L., Chen, X., 2016. Risperidone-induced topological alterations of anatomical brain network in first-episode drug-naive schizophrenia patients: a longitudinal diffusion tensor imaging study. Psychol. Med. 46 (12), 2549–2560.

Jensen, A.R., Rohwer Jr, W.D., 1966. The Stroop color-word test: a review. Acta Psychol. (Amst) 25 (1), 36–93.

Jiao, C, Zhang, C, Dai, R, Xia, Y, Wang, K, Giase, G, Chen, C, Liu, C, 2018. Positional effects revealed in Illumina methylation array and the impact on analysis. Epigenomics 10 (5), 643–659.

Johnson, W.E., Li, C., Cabrinovic, A., 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8 (1), 118-127.

Kohista, M., Numata, S., Tajima, A., Yamamori, H., Yasuda, Y., Fujimoto, M., Watanabe, S., Umehara, H., Shimodera, S., Nakazawa, T., Kikuchi, M., Nakaya, A., Hashimoto, H., Imoto, I., Hashimoto, O., Tsuchiya, T., 2017. Effect of clozapine on DNA methylation in peripheral leukocytes from patients with treatment-resistant schizophrenia. Int. J. Mol. Sci. 18 (3).
M. Hu et al. Psychiatry Research 317 (2022) 114789

10

Thomas, E.A., George, R.C., Danielson, P.E., Nelson, P.A., Warren, A.J., Lo, D., Tiihonen, J., 2016. Real-world effectiveness of antipsychotics. Acta Psychiatr. Scand. 134 (5), 371–379.

Tiihonen, J., Mitterforser-Rutz, E., Majak, M., Mehtala, J., Hoti, F., Jedenius, E., Morris, T.J., Butcher, L.M., Feber, A., Teschendorff, A.E., Chakravarthy, A.R., Rakyan, V.K., Down, T.A., Balding, D.J., Beck, S., 2013. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 29 (2), 649–655.

Tiihonen, J., Mittendorfer-Rutz, E., Majak, M., Mehtala, J., Hoti, F., Jedenius, E., Morris, T.J., Butcher, L.M., Feber, A., Teschendorff, A.E., Chakravarthy, A.R., Rakyan, V.K., Down, T.A., Balding, D.J., Beck, S., 2011. Epigenome-wide association studies for common human diseases. Nat. Rev. Genet. 12 (8), 529–541.

Tiihonen, J., Nendz transforming a schizophrenia-related locus. Nature 604 (7858), 508–513.

Tiihonen, J., Nendz transforming a schizophrenia-related locus. Nature 604 (7858), 508–513.

Tiihonen, J., Nendz transforming a schizophrenia-related locus. Nature 604 (7858), 508–513.

Tiihonen, J., Nendz transforming a schizophrenia-related locus. Nature 604 (7858), 508–513.