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## The association of DNA methylation and brain volume in healthy individuals and schizophrenia patients

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### Abstract

Both methylation and brain volume patterns hold important biological information for the development and prognosis of schizophrenia (SZ). A combined study to probe the association between them provides a new perspective to understanding SZ. Genomic methylation of peripheral blood and regional brain volumes derived from magnate resonance imaging were analyzed using parallel independent component analyses in this study. Nine methylation components and five brain volumetric components were extracted for 94 SZ patients and 106 healthy controls. After controlling for age, sex, race, and substance use, a component comprised primarily of bilateral cerebellar volumes was significantly correlated to a methylation component from 14 CpG sites in 13 genes. Both patients and healthy controls demonstrated similar associations, but patients had significantly smaller cerebellar volumes and dysmethylation in the associated epigenetic component compared to controls. The 13 genes are enriched in cellular growth and proliferation with some genes involved in neuronal growth and cerebellum development (*GATA4*, *ADRA1D*, *EPHA3*, and *KCNK10*), and these genes are prominently associated with neurological and psychological disorders. Such findings suggest that the methylation pattern of the genes coding for cellular growth may influence the cerebellar development through regulating gene expression, and the alteration in the methylation of these genes in SZ patients may contribute to the cerebellar volume reduction observed in patients.

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### Contributors

The funding sources had no involvement in the study design, data analysis and interpretation of data, and in the writing of the manuscript.

### Conflict Of Interest

The authors of this manuscript declare no conflict of interest.

## Keywords

DNA methylation; brain volume; schizophrenia; multivariate association; cerebellum

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## 1. Introduction

Schizophrenia (SZ) has been considered the result of genetic predisposition and environmental insults. Epigenetics, by integrating both genetic and environmental factors, has been suggested to hold the promise of filling in the gaps in the study of SZ etiology (Nishioka et al., 2012; Thibaut, 2012). As one of the main epigenetic mechanisms, DNA methylation with the ability to regulate gene expression has shown its association with SZ. Studies of postmortem brain tissue have documented methylation alterations in SZ patients (Wockner et al., 2014), and studies of peripheral blood also lend support to the critical role of methylation in SZ etiology. For instance, DNA in blood has shown differential methylation patterns across SZ patients and controls (Carrard et al., 2011; Dempster et al., 2011; Walton et al., 2014), its global methylation was associated with early disease onset (Melas et al., 2012), and its methylation pattern of immune genes was related to SZ symptoms (Liu et al., 2014).

Volumetric association of specific brain regions with SZ consistently emerge in the literature, including reduced superior temporal, cingulate, hippocampal, prefrontal, and cerebellar volumes in patients relative to controls along with increased cerebral spinal fluid and enlarged lateral ventricles (Asami et al., 2012; Koo et al., 2008; Mitelman et al., 2009; Nakamura et al., 2007; Takahashi et al., 2010). Such brain structural alteration underlies clinical symptoms and cognitive deficits of patients, such as reduced superior temporal gyrus associated with auditory hallucination (Barta et al., 1990; Levitan et al., 1999) and dorsal lateral prefrontal cortex inefficacy influencing working memory function (Levitan et al., 1999). The interplay of genetics and environment is thought to cause brain structural variation (Geoffroy et al., 2013). One mega-study of genomic association with brain volumes identified SNPs associated with hippocampal volume in both diseased and healthy populations (Stein et al., 2012). Monozygotic twins with SZ showed structural differences from their normal co-twins (Suddath et al., 1990).

While both methylation and brain structure anomalies are associated with SZ, a combined approach exploring the relationship between these might prove more informative in the pursuit of novel perspectives to understanding such a complex illness. Specifically, methylation—synthesizing the effects of genetic predisposition and environmental factors—can potentially relate to brain volume dynamics in the development of SZ. In this study, we apply a parallel independent component analysis (pICA) (Liu and Calhoun, 2014; Liu et al., 2009) to genomic methylation data derived from whole blood and regional volumes across whole brain. Although methylation is tissue specific, methylation patterns of peripheral blood have been shown to be related to the patterns of brain tissues. (Davies et al., 2012; Nishioka et al., 2013; Wockner et al., 2014) Recently, Aberg et al. have proposed and tested two possible models to explain the relation between methylation patterns of blood and brain in context of psychiatric conditions: a ‘signature’ model where a disease causing factor

affects both brain and methylation in the blood, and a ‘mirror-site’ model where the methylation status in the blood is correlated with the corresponding diseasecausing site in the brain (Aberg et al., 2013). Given that both models could be effective, the focus of our study is to objectively assess the relationship between peripheral blood methylation and volumetric brain structure within a sample of chronic SZ patients and healthy controls.

## 2. Methods

### 2.1

Participants in this study were from the Mind Clinical Imaging Consortium, a collaborative effort of four research sites (Gollub et al., 2013). Institutional review board at each site approved the study and all participants provided written informed consent. All healthy participants were free of any medical, neurological or psychiatric illnesses, including any history of substance abuse. Patients were diagnosed as SZ, schizophreniform or schizoaffective disorder by the Structured Clinical Interview for DSM-IV-TR Disorders and the Comprehensive Assessment of Symptoms and History (Andreasen et al., 1992). Patients’ symptoms were assessed by the scale of the assessment of positive symptoms and the scale of the assessment of negative symptoms.(NC, 1983, 1984) Patients were also assessed for alcohol abuse/dependence by DSM-IV, and categorized into two groups: 1) abuse or dependence, 2) never abused or dependent. Their medication information (equivalent dosage of chlorpromazine (Gardner et al., 2010)) and age of illness onset were also collected. For a subset of participants nicotine use was measured by calculated pack years (Cullen et al., 2012). (See supplementary material (SM) 1 for more details) After quality control, data from 94 SZ patients and 106 healthy controls (HC) were studied. As listed in Table 1, no significant difference was observed in age and sex between patients and controls. Significantly more African Americans were enrolled as patients than controls.

### 2.2

DNA extracted from whole blood was assessed by the Illumina Infinium HumanMethylation27 Assay (27578 CpG sites) for each participant. A methylation value,  $\beta$ , represents the ratio of the methylated probe intensity to the total probe intensity, ranging from 0 (unmethylated) to 1 (fully methylated). A series of quality control measures were applied to remove bad samples and probes, correct the batch effect, and control for confounding factors of age, sex, race, and substance use (see details in SM 2). While the goal of this study is to help understand the methylation’s impact on brain volume alteration in SZ patients, we selected 906 CpG sites associated with diagnoses by a liberal threshold ( $p < 0.01$  uncorrected) in a linear regression model using age, sex, race, alcohol use and diagnosis as regressors (see SM 5 for the full list). The residual methylation values of these sites after correction for age, sex, race, and alcohol use were further analyzed for association with brain volume.

### 2.3

$T_1$ -weighted MRI images were acquired at four participating sites with three sites using 1.5T scanner and one site using 3T scanner. Details of scanning parameters and quality control, as seen in SM3, can be found (Segall et al., 2009). MRI images were processed using

FreeSurfer 4.5 (Fischl et al., 2002) to segment out brain regions. Volumes of 80 cortical and subcortical regions were then normalized with the total intracranial volume for each participant.

## 2.4

Preprocessed methylation and normalized brain volume data were analyzed using the pICA program implemented in the Fusion ICA Toolbox (<http://mialab.mrn.org/software/fit>); pICA is designed to extract independent components from the two modalities in parallel and simultaneously enhance the inter-modality correlations of pairs of components (one from each modality). As shown in the following equation, data (X) for each modality are decomposed into a set of underlying independent components (S) and their loading matrix (A). Maximization of entropy terms,  $H(Y)$ , guarantees the independency of components within each modality. Maximization of the correlation,  $\text{corr}(A_1, A_2)$ , results in pairs of components that share maximally the loading patterns in subjects. A full description of the algorithm can be found in previous studies (Liu et al., 2008; Liu et al., 2009). In this application components are a linear combination of methylation values of multiple genes or volumes of various brain regions, and the correlations between loadings measure how tightly the variation in the expression of the methylation component in subjects is related to the variation of the brain volume component in subjects.

$$X_1 = A_1 \cdot S_1; X_2 = A_2 \cdot S_2; \max\{H(Y_1) + H(Y_2) + \text{corr}(A_1, A_2)^2\};$$

pICA was used with the following settings: (a) nine methylation components and five brain volume components estimated by minimum description length (De Ridder et al., 2005), which provided the highest stability in subset evaluations, (b) correlation constraint threshold of 0.20, (c) 10 repeated runs. The significance of the identified methylation-brain volume association was corrected for  $9 \times 5$  multiple tests using Bonferroni correction. The stability of pICA results was evaluated by 10 subsets, each with 90% of samples and tested with pICA following the same settings above. We also performed a permutation test to assess the validity of identified methylation-brain volume association by permuting the samples in both methylation and brain volume datasets 1000 times.

To control for possible scanning site influence on brain volume, as well as age, sex, race, and diagnosis effect, we conducted an analysis of covariance (ANCOVA) test with the identified brain volume component (loadings) as the dependent variable, and independent variables including three dummy variables for scanning sites, or age, sex, race, or diagnosis, and the identified methylation component. For each identified component, we tested the SZ and HC group difference, correlation with medication, length of illness, age of onset, and symptoms (positive, negative, and disorganized symptoms).

## 2.5

Ingenuity pathways analysis (IPA) software (<http://www.ingenuity.com>) was used for pathway analysis of identified genes. IPA provides enrichment scores for canonical pathways, diseases and bio-functions, and networks defined in the Ingenuity® Knowledge Base.

## 2.6

A public gene expression omnibus (GEO) dataset, **GSE41037**, includes DNA methylation profiling of whole blood from 325 SZ patients and 394 healthy subjects, assessed by the Illumina Infinium Methylation27 Assay. Although no brain structural imaging data are available to validate the main findings on the relationship between methylation and brain structure, we would like to use **GSE41037** to verify the methylation differences in SZ. Without substance use information, a simple linear regression with diagnosis, age and sex was used to validate SZ association of the identified CpG sites in our data.

## 3. Results

The application of pICA on methylation and brain volume data yielded one significant component pairing with a correlation of  $-0.31$  ( $p=6.08\times 10^{-7}$ ) (see SM 4 for the complete components). As seen in Figure 1, the higher the methylation component, the smaller the brain volume component. The stability test revealed that this component pairing was present in all 10 subsets with an averaged correlation of  $-0.29\pm 0.04$ , and the permutation test yielded an empirical p-value of 0.012. When we controlled for diagnosis, the correlation remained significant ( $p=4.0\times 10^{-4}$ ), and no significant diagnosis effect was observed given the correlation was  $-0.28$  ( $p=0.004$ ) within the HC group and  $-0.22$  ( $p=0.04$ ) within the SZ group. Scanning sites did affect the brain volume component ( $p=4.41\times 10^{-5}$ ), but did not affect the methylation-brain volume correlation ( $p=5.26\times 10^{-6}$  after controlling for scanning sites). Similar results were obtained when controlling for age, sex and race. The correlation remained significant ( $p=4.27\times 10^{-6}$ ) and age showed significant influence on the brain volume component ( $p=0.04$ ) but not sex and race. We plotted the paired components in Figure 2, where the methylation component was mainly contributed to by 14 CpG sites (weight  $|z|>3$ ) in 13 genes (Table 2), and the brain volume component compromised left and right cerebellums with weights (z-score) of 5.94 and 6.41 respectively.

Left and right cerebellums both showed volume reduction in SZ patients compared to controls with effect sizes of 4.5% ( $p=2.1\times 10^{-3}$ ) and 4.4% ( $p=3.2\times 10^{-3}$ ) respectively. The whole component showed significantly lower values in patients ( $p=0.004$ ). Within the patient group, we found no significant correlation of the cerebellar component with medication, length of illness, age of onset or any symptoms. The associated methylation component revealed group differences ( $p=4.71\times 10^{-26}$ , biased by the pre-selection of CpG sites) with patients having significantly higher values (integrated values of the 14 CpG sites) than controls. No significant correlation between the methylation component and medication, length of illness, age of onset, or symptom scores was observed. Pathway analyses on the 13 genes of the component reported that the top genetic network implicated was cellular development, cellular growth and proliferation, and embryonic development derived from genes *GATA4*, *CDX1*, *MCCCI*, *ADRAID*, *EPHA3*, and *KCNK10*. The top affected diseases were neurological and psychological disorders ( $p=1.33\times 10^{-2}$ ), where genes *ADRAID*, *MCCCI*, *MCHR1*, *EPHA3*, and *STMN2* all showed involvement. Table 2 presents the location of the 14 identified CpG sites and their methylation differences between patients and controls. All 14 sites were hyper- or hypo-methylated in SZ patients in our data ( $p<0.01$  uncorrected), and five sites were also consistently, significantly ( $p<0.05$

uncorrected) hyper- or hypo-methylated in the validation data (highlighted in Table 2). Additionally, we also validated the 906 CpG sites selected in this study. We found 496 sites showed significant ( $p < 0.05$  uncorrected) SZ differences and were consistently hypomethylated in both datasets. The empirical p value of getting 496 confirmed group differentiating sites from 906 sites is less than  $1.0 \times 10^{-4}$ . Pathway analyses on the genes hosting the 496 CpG sites showed that the top canonical pathways affected were T-lymphocyte-mediated apoptosis of target cell, type I diabetes mellitus signaling, primary immunodeficiency signaling. And the top network was cellular growth, cell-mediated immune response.

#### 4. Discussion and conclusions

One methylation component was significantly associated to the cerebellum volume component, and the association was not attributable to age, sex, race or diagnosis. As shown in Figure 1, the healthy controls and SZ patients present comparable methylation-cerebellum volume relations, indicating the observed association is not driven by group differences, but likely reflects basic biological processes.

Pathway analyses revealed that the 13 genes are enriched in basic cellular development, particularly cellular growth and proliferation. Some of these genes have shown direct connections with cerebellum development or neuronal growth. For instance, EPH receptor A3 coded by gene *EPHA3* mediates neuronal developmental processes as one of the main axonal guidance molecules (Gibson and Ma, 2011; Saywell et al., 2014), and directly influences cerebellum formation (Saywell et al., 2014) and cerebral cortex size. (Depaepe et al., 2005) Animal studies have shown that the adrenergic alpha 1 receptor (ADRA1D encodes one subtype) reacting with noradrenaline is essential for normal cerebellar development and function (Herold et al., 2005; Podkletnova and Alho, 1998; Schambra et al., 2005). The expression level of GATA binding protein 4 (encoded by gene *GATA4*) in the brain regulates negatively astrocyte cell proliferation and positively apoptosis (Agnihotri et al., 2009). Other genes influence more generally cell function and development. The protein encoded by gene *KCNK10* (potassium channel, subfamily K, member 10) mediates excitability of various types of cells including neurons (Deng et al., 2009; Xiao et al., 2009). *MCCC1* (methylcrotonoyl-CoA carboxylase 1) encodes an enzyme that plays a key role in the breakdown of leucine in controlling protein synthesis and regulating cell metabolism in various cell types (Li et al., 2003; Yang et al., 2010).

All the 14 CpG sites but one locate near the transcription start site, most in the promoter regions or the 1<sup>st</sup> exon regions, and within CpG islands. Hypermethylation in the promoters is known to have silencing effects on gene expression (Bird and Wolffe, 1999), and so is the methylation in the 1<sup>st</sup> exon regions (Brenet et al., 2011). Most, perhaps all, CpG islands are sites of transcription initiation (Deaton and Bird, 2011), and methylation in CpG islands is equipped to regulate gene transcription. Therefore, the methylation pattern of the 14 sites most likely influences the corresponding genes' expression level and further has effects on the cellular function. Targeted studies with gene expression and protein function are necessary to confirm the effect of hypomethylation of these genes.

Although the methylation-cerebellum connection is not SZ specific, our results do show that patients have smaller cerebellar volumes and higher values in the methylation component compared to controls. Decreased cerebellar volumes in first episode SZ patients have been repeatedly reported with effect sizes of 5–10% (Bottmer et al., 2005; Thomann et al., 2009), suggesting that cerebellar volume reduction is not due to medication. Our data, although from chronic patients, showed very similar results and no medication effects. The previous studies also reported the decreased cerebellar volume is most associated with neurological soft signs (NSS), not with psychopathological symptoms. Cerebellum, being part of the fronto-thalamic-cerebellar circuitry, has also been hypothesized to affect cognition (Picard et al., 2008). Taken together, the functional impact of cerebellar reduction is perplexing, and more towards NSS or cognition than typical schizophrenic symptoms (Picard et al., 2008; Varambally et al., 2006), which is in line with our findings of no significant correlation with positive or negative symptoms.

The 14 CpG sites of the methylation component showed SZ differences in our data, and the differences in five sites were also confirmed in the cross-validation data. The discrepancy may be partially due to the sample size difference and confounding factors such as race (Liu et al., 2010), substance use (Nielsen et al., 2012), medication (Melka et al., 2014) and comorbidity (Xiao et al., 2014). Although the SZ differences of some genes cannot be confirmed, their association with cerebellar volumes is valid in our subset and permutation tests. Pathway analyses also showed that the top affected diseases by these genes are neurological and psychological disorders. Their association with SZ can be further strengthened by the fact that many drugs such as olanzapine, aripiprazole, risperidone, quetiapine are antagonist of adrenergic alpha 1 receptors for treatment of SZ and/or bipolar disorder.

Regarding methylation differences in SZ patients, 496 CpG sites showed normally hyper- or hypo-methylation in patients in both the discovery and validation datasets. Without correction for multiple comparisons, significance of each site is not warranted, but as a whole they carry valid information. Pathway analyses results highlight several immune response related pathways, consistent with a previous study showing methylation of immune system genes associated with SZ symptoms (Liu et al., 2014) and support the involvement of immune system in the pathology of SZ. The study of methylation associated with brain volume is related, but different from that focusing on SZ differences, as more neural developmental genes are identified for the association with cerebellar volumes.

The findings of this study need to be interpreted within the bounds of several limitations. First, the methylation is derived from whole blood, rather than brain tissue, which limits the ability to understand underlying mechanisms. We speculate that the methylation level of DNA in blood may indicate the methylation level of DNA in cerebellum cells and changes of methylation influence gene expression and further downstream cerebellum development. SZ patients carry altered methylation status which contributes to cerebellum reduction. This hypothesis needs to be further studied. Second, DNA methylation of whole blood reflects a mixture of various cells (Reinius et al., 2012), and we were not able to factor out potential effects of cell types and total white blood cell count variation. Third, the methylation values were assessed by Illumine bisulfite-based technique, which cannot differentiate between

cytosine methylation and hydroxymethylation. We were not able to verify methylation values of identified genes using a different, gene-specific DNA methylation assessment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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JL and VD designed this study. JL, PJ and JC analyzed the data. All authors contributed to the preparation of this manuscript.

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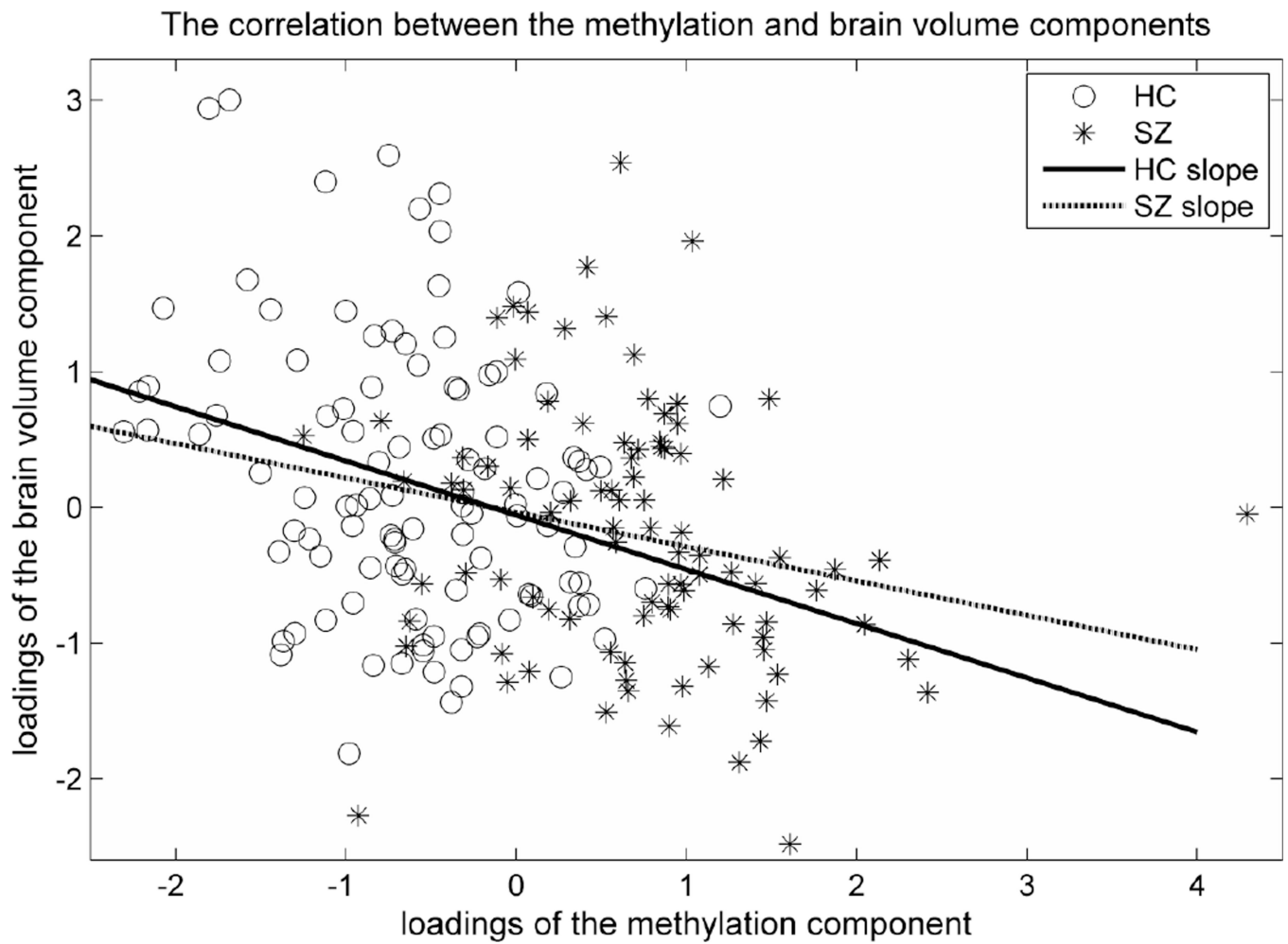
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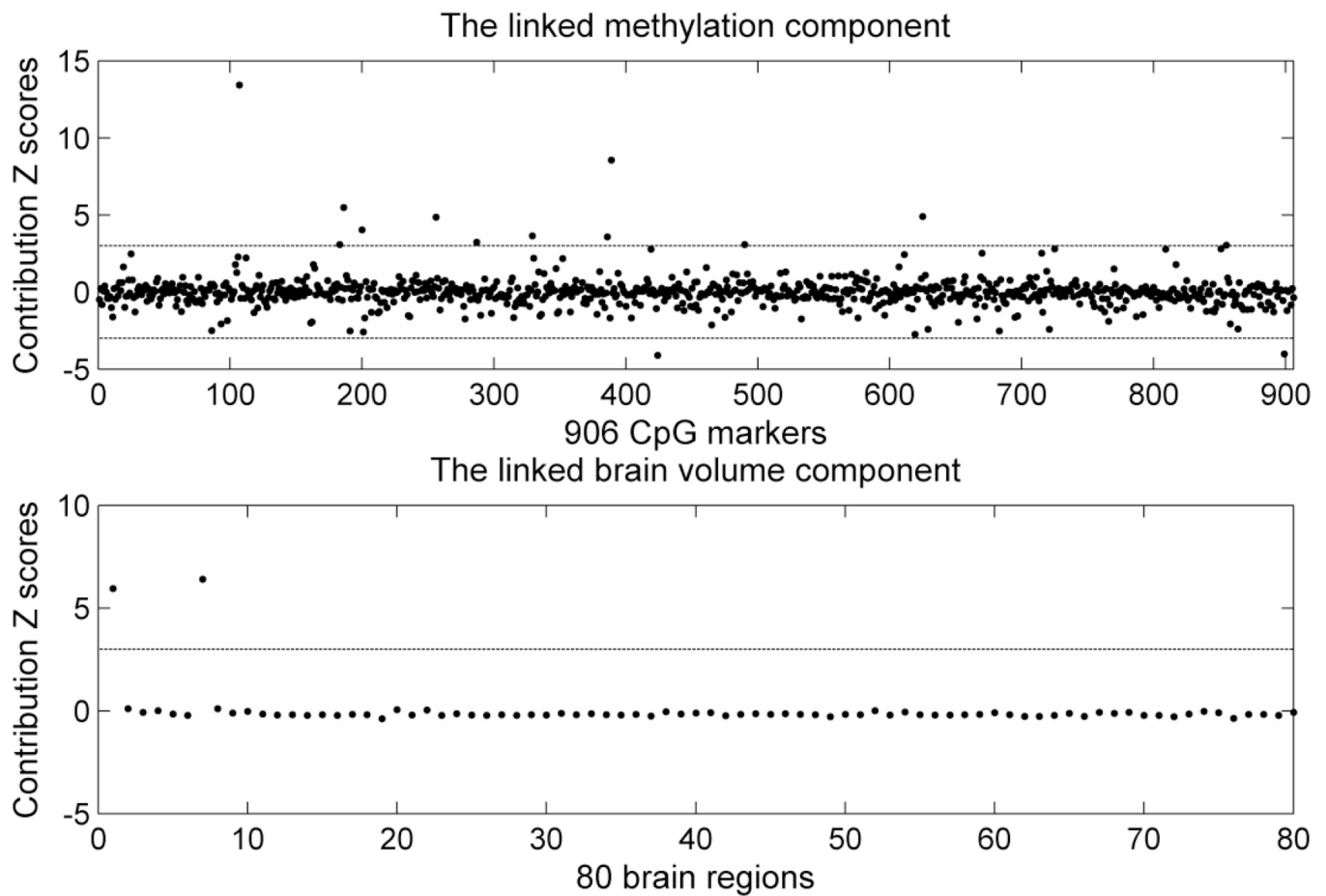
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**Figure 1.**

Loadings of the associated brain volume component and methylation component. Data for schizophrenia patients were plotted in solid black circles, while data for controls were plotted in hollow gray circles. Loadings are normalized as unitless values.



**Figure 2.** Contribution Z scores of each variable in the associated methylation and brain volume components. X axis plots variables: CpG markers for the methylation component and brain regions for the brain volume component. Y axis plots the Z scores. The lines indicate the  $|z| = 3$  threshold used to select top contributing variables. Only markers (regions) outside the threshold are considered to be effective to the corresponding components.

**Table 1**

Demographic information of participants

Group(N)	M/F	Age	Race (White/Black/Asian/others)	Alcohol depend/abuse	Pack_years (sample size N ; Mean $\pm$ SD)
<b>SZ(94)</b>	70/24	34.69 $\pm$ 10.45	69/17/4/4	31	N=45 ; pack_year: 8.2 $\pm$ 11.3
<b>HC(106)</b>	68/38	32.50 $\pm$ 10.96	95/3/4/4	0	N=55 ; pack_year:1.7 $\pm$ 4.9

Table 2

Information about the 14 CpG sites contributing to the methylation component

Gene	Target ID	D_TSS#	Gene region	CpG island&	Z Score	$\frac{\beta(SZ)}{\beta(HC)}$ *	P value*	$\frac{\beta(SZ)}{\beta(HC)}$ **	P value**
C1orf65	cg27202708	59	promoter	y	13.43	0.025	6.7E-03	-0.007	3.8E-01
GATA4	cg26020513	6640	intron	y	8.56	0.020	3.9E-03	-0.004	5.6E-01
<b>MCCC1</b>	<b>cg08395365</b>	<b>119</b>	<b>promoter</b>	<b>y</b>	<b>5.49</b>	<b>0.005</b>	<b>2.0E-04</b>	<b>0.027</b>	<b>4.0E-04</b>
<b>KCNK10</b>	<b>cg10935723</b>	<b>383</b>	<b>promoter</b>	<b>n</b>	<b>4.90</b>	<b>-0.009</b>	<b>2.4E-03</b>	<b>-0.016</b>	<b>5.3E-03</b>
CDX1	cg15452204	112	exon	y	4.86	0.004	4.6E-03	-0.001	9.3E-01
<b>NPDC1</b>	<b>cg26581729</b>	<b>863</b>	<b>intron</b>	<b>y</b>	<b>-4.09</b>	<b>-0.002</b>	<b>3.6E-03</b>	<b>-0.021</b>	<b>1.6E-03</b>
EPHA3	cg18055394	196	promoter	y	4.04	0.005	9.7E-03	-0.018	4.0E-04
<b>MCHRI</b>	<b>cg21342728</b>	<b>436</b>	<b>exon</b>	<b>n</b>	<b>-4.02</b>	<b>-0.003</b>	<b>6.5E-03</b>	<b>-0.021</b>	<b>7.0E-04</b>
TMEM176B	cg03964111	130	promoter	n	3.64	-0.005	4.4E-03	-0.003	6.5E-01
STMN2	cg23326689	81	exon	y	3.58	0.009	9.0E-04	-0.010	1.4E-02
RAET1L	cg07579404	114	promoter	y	3.24	0.006	3.0E-04	-0.000	8.8E-01
NALP6	cg08961832	615	intron	y	3.09	0.001	1.9E-03	-0.016	2.0E-02
<b>MCCC1</b>	<b>cg04991337</b>	<b>137</b>	<b>exon</b>	<b>y</b>	<b>3.08</b>	<b>0.002</b>	<b>9.8E-03</b>	<b>0.007</b>	<b>5.5E-03</b>
ADRA1D	cg09614401	650	exon	y	3.05	-0.006	5.0E-04	0.007	5.8E-02

# : Distance to Transcription Start Site in base pairs;

& : whether inside a CpG island, y: yes; n: no;

\* : SZ group mean  $\beta$  value minus HC group mean  $\beta$  value; these are results from the discovery data after age, sex, race, substance use corrected. Positive values indicate hyper-methylation in patients, and negative values indicate hypo-methylation in patients.

\*\* : these are results from the validation data after age and sex corrected. Positive values indicate hyper-methylation in patients, and negative values indicate hypo-methylation in patients.