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Plasma total homocysteine is associated with DNA methylation in patients with schizophrenia

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Abbreviations: SCZ, schizophrenia; CGI, CpG island; UTR, untranslated region; VMAT2, vesicular transporter type2

Schizophrenia (SCZ) is a devastating psychiatric disorder with a median lifetime prevalence rate of 0.7–0.8%. Elevated plasma total homocysteine has been suggested as a risk factor for SCZ, and various biological effects of hyperhomocysteinemia have been proposed to be relevant to the pathophysiology of SCZ. As increased attention is paid to aberrant DNA methylation in SCZ, homocysteine is attracting additional interest as a potential key substance. Homocysteine is formed in the methionine cycle, which is involved in one-carbon methyl group-transfer metabolism, and it acts as a methyl donor when it is converted to S-adenosyl-methionine. To date, no studies have examined the relationship between homocysteine and genome-wide DNA methylation in SCZ. We examined the relationship between plasma total homocysteine and DNA methylation patterns in the peripheral leukocytes of patients with SCZ (n = 42) using a quantitative high-resolution DNA methylation array (485,764 CpG sites). Significant homocysteine-related changes in DNA methylation were observed at 1,338 CpG sites that were located across whole gene regions, including promoters, gene bodies and 3'-untranslated regions. Of the 1,338 sites, 758 sites (56.6%) were located in the CpG islands (CGIs) and in the regions flanking CGIs (CGI: 15.8%; CGI shore: 28.2%; CGI shelf: 12.6%), and positive correlations between plasma total homocysteine and DNA methylation were observed predominantly at CpG sites in the CGIs. Our results suggest that homocysteine might play a role in the pathogenesis of SCZ via a molecular mechanism that involves alterations to DNA methylation.

Introduction

Schizophrenia (SCZ) is a devastating psychiatric disorder with a median lifetime prevalence rate of 0.7–0.8%.[1] Elevated plasma total homocysteine has been suggested as a risk factor for SCZ,[2,3] and hyperhomocysteinemia has been proposed to contribute to the pathophysiology of SCZ via various biological effects, such as a partial antagonist of the glutamate site of the N-methyl-D-aspartate receptor,[4] the interferer of oxygen delivery by damaging placental vasculature,[2] DNA damage and cell cytotoxicity,[5] neuronal apoptosis[6] and mitochondrial nitric oxide accumulation.[7]

Recently, accumulating evidence has shown that DNA methylation is also implicated in SCZ.[8-27] As more attention is paid to DNA methylation, homocysteine has been recognized as a potentially key substance. Homocysteine is formed during the methionine cycle, is involved in one-carbon methyl group-transfer metabolism and acts as a methyl donor when it is converted to S-adenosyl-methionine. Several studies have reported an association between hyperhomocysteinemia and aberrant DNA methylation in several diseases, including atherosclerosis, osteoporosis, uremia and alcoholism.[28-31] Furthermore, Fryer and colleagues reported a significant correlation between cord blood-plasma total homocysteine and DNA methylation at numerous CpG sites.[32] These studies led us to hypothesize that hyperhomocysteinemia in SCZ might have an impact on the DNA methylation levels in specific genes. However, to date, there are no reports that examine the relationship between homocysteine and genome-wide DNA methylation in SCZ.

To gain further insight into the pathogenic mechanisms that underlie hyperhomocysteinemia in SCZ, we examined the relationship between plasma total homocysteine and DNA methylation patterns in the peripheral leukocytes of patients with SCZ by using a quantitative high-resolution DNA methylation array.

Results

Differences in plasma total homocysteine between patients with SCZ and controls. The mean plasma total homocysteine

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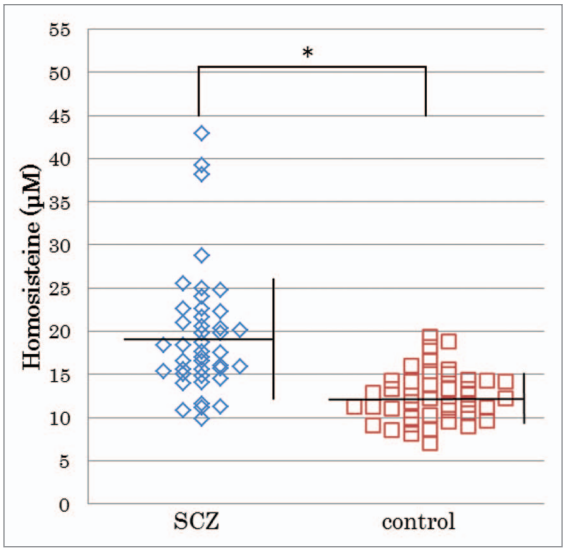

Figure 1. Plasma total homocysteine levels of patients with schizophrenia and controls. Blue dots represent plasma total homocysteine levels of patients with SCZ. Red dots represent plasma total homocysteine levels of controls. The mean plasma total homocysteine level in patients with SCZ (n = 42) was 19.5 ± 7.2 nmol/mL (mean ± SD), and the level in the control subjects (n = 42) was 12.4 ± 2.9 nmol/mL (mean ± SD). The plasma total homocysteine levels of the patient group were significant higher than those of the control group (Mann–Whitney U test, $p < 0.0001$).

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Relationship between plasma total homocysteine and genome-wide DNA methylation patterns in patients with SCZ. Of the 164,657 CpG sites analyzed, significant plasma total homocysteine-related changes in DNA methylation were observed at 1,338 sites ($p < 0.01$). The top 10-ranking CpG sites significantly associated with plasma total homocysteine are shown in **Table 1**, and the top 100-ranking CpG sites are shown in **Table S1**. Examples include two CpG sites in the *SLC18A2* and the *GNAL* genes (**Fig. 2**). Both of these genes have been implicated in SCZ.[33-36] When these 1,338 CpG sites were classified into four different categories according to their location in the genes [promoter, gene body, 3'-untranslated regions (UTRs) and intergenic region], 425 sites (31.8%) were located in the promoter regions, 414 sites (30.9%) in gene bodies and 34 sites (2.5%) in 3'-UTRs (**Fig. 3A**). When these 1,338 CpG sites were classified into four categories according to the CpG content in the genes [CpG island (CGI), CGI shore, CGI shelf, and others], 212 sites (15.8%) were located in the CGIs, 377 sites (28.2%) in CGI shores, and 169 sites (12.6%) in CGI shelves (**Fig. 3B**; **Table S2**). Of the significant 212 CpG sites in the CGIs, 74 sites (34.9%) were located in the promoter regions.

Of the 1,338 significant CpG sites, positive correlations of plasma total homocysteine with DNA methylation were observed at 580 sites (43.3%), and negative correlations were observed at 758 sites (56.7%). Positive correlations were found predominantly at CpG sites in the CGIs. The percentage of the CpG sites with positive correlations, which were located in the CGIs, CGI shores, and CGI shelves, were 71.7%, 50.1% and 23.7%, respectively (**Fig. 4**).

Discussion

In this study, we demonstrated that patients with SCZ had significantly elevated plasma total homocysteine levels compared with the controls' levels, and this result is consistent with the results of a previous meta-analysis.[3] We also performed a genome-wide DNA methylation profiling of the peripheral leukocytes in the same subjects with SCZ, and examined the relationship between plasma total homocysteine and DNA methylation patterns. We identified plasma total homocysteine-related changes in DNA methylation at numerous CpG sites. To our knowledge, this is the first study to examine the relationship between plasma total homocysteine and genome-wide DNA methylation in SCZ.

The present study demonstrated that significant correlations between plasma total homocysteine and DNA methylation were observed at CpG sites not only in the promoter regions but also in the gene bodies, and 3'-UTRs. Thus, plasma total homocysteine might affect DNA methylation across whole gene regions. Furthermore, plasma total homocysteine was significantly correlated with DNA methylation at CpG sites not only in the CGIs but also in CGI shores and CGI shelves. Consistent with a previous genome-wide DNA methylation study using cord blood-plasma total homocysteine,[32] both positive and negative correlations between plasma total homocysteine and DNA methylation were observed in this study. Notably, the proportions of the CpG sites with positive correlations differed among these three categories (CGI: 71.7%; CGI shore: 50.1%; and CGI shelf: 23.7%). These results suggest that plasma total homocysteine might influence DNA methylation depending on CpG densities.

To date, only one study has examined an association between homocysteine and DNA methylation in patients with SCZ: Bromberg and colleagues measured plasma total homocysteine and global blood DNA methylation in patients with SCZ by using a modification of the radiolabeled [^{3}H]dCTP-extension assay, and they failed to find a significant association.[10] This result suggests that DNA methylation must be analyzed at a gene-specific level in studies of SCZ. When we focused on specific genes that demonstrated significant correlations in our study, several genes of these genes, such as *SLC18A2, GNAL*, *KCNH2* and *NTNG2*, have been implicated in SCZ. *SLC18A2* encodes the vesicular transporter type2 (*VMAT2*), which transports monoamines into the synaptic vesicles.[37] Genetic variations of this gene have been associated with SCZ and cognitive functioning in patients with psychotic disorder.[34,36,38] *GNAL* encodes guanine nucleotide-binding protein G subunit α, and altered expression of this gene in the brain is associated with functional changes of dopamine D1 receptor.[33] This gene is located in the region of chromosome 18p11.2, and this region has been implicated in susceptibility to bipolar disorder and SCZ.[33,35,39] *KCNH2* is a member of a family that provides instructions for making potassium channels and

Table 1. The top 10-ranking of CpG sites significant associated with plasma homocysteine

Ranking	Probe ID	Minimum β value across samples	Maximum β value across samples	Standardized coefficient of plasma total homocysteine	P-value of plasma total homo-cysteine	Coefficient of age	P-value of age	Coefficient of CP equiva-lent dose	P-value of CP equiva-lent dose	Chromo-some	Position*	UCSC RefGene name	UCSC RefGene group	Relation to UCSC CpG island
1	cg04579505	0.123	0.272	0.765	5.98E−07	2.10.E−02	2.80.E−01	−5.39.E−04	2.49.E−02	16	67261564	*LRRC29*	Promoter	CGI shore
2	cg01546563	0.132	0.302	0.701	8.66E−06	7.59.E−02	6.71.E−04	−7.42.E−04	4.63.E−03	8	11567189	*GATA4*	Gene body	CGI shore
3	cg12423733	0.107	0.375	0.707	1.12E−05	2.22.E−02	2.97.E−01	−4.30.E−04	9.64.E−02	6	29454623	*MAS1L*	Promoter	Others
4	cg03004330	0.126	0.366	0.695	1.53E−05	6.04.E−02	6.66.E−03	−7.19.E−04	7.19.E−03	10	13934438	*FRMD4A*	Gene body	CGI shore
5	cg08607821	0.825	0.897	−0.697	2.06E−05	−7.35.E−02	1.55.E−03	2.05.E−04	4.35.E−01	13	24915164	—	Intergenic	CGI shore
6	cg04364311	0.671	0.824	−0.682	3.07E−05	−7.71.E−02	1.01.E−03	2.33.E−04	3.76.E−01	3	101231003	*SENP7*	Gene body	CGI shore
7	cg23158877	0.131	0.431	−0.685	3.24E−05	−6.68.E−02	4.01.E−03	4.66.E−04	8.42.E−02	11	86012876	*C11orf73*	Promoter	Others
8	cg05360577	0.134	0.279	0.672	3.62E−05	7.58.E−02	1.17.E−03	−5.40.E−04	4.43.E−02	11	17717629	—	Intergenic	CGI
9	cg24606762	0.101	0.391	0.674	4.10E−05	4.10.E−02	6.80.E−02	−5.92.E−04	3.01.E−02	20	61806972	—	Intergenic	CGI
10	cg11653336	0.756	0.894	−0.675	4.30E−05	−2.80.E−02	2.10.E−01	−1.22.E−05	9.63.E−01	12	133465188	*CHFR*	Promoter	CGI shore

*Positions refer to Genome Research Consortium human genome build 37 (GRCh37)/UCSC human genome 19 (hg19).

that modulates neuronal firing. Altered *KCNH2* expression in the hippocampus in SCZ, and a genetic association of this gene with SCZ and SCZ-related neuropsychological deficits in healthy subjects have been reported.[40,41] The *NTNG2* gene plays a role in synaptic formation and maintenance.[42,43] Altered the *NTNG2* gene expression in postmortem brains in SCZ and the genetic associations of this gene with SCZ have been reported.[44]

There are several limitations to the present study. First, the sample size was not large and the risk of potential false-positive results due to multiple testing must be considered. Replication studies with larger samples are necessary. Second, the number of CpG sites that have been analyzed was limited, although the 450K microarray is one of the most powerful tools currently available for assessing DNA methylation changes. Third, the subjects analyzed were chronic patients with SCZ who were receiving treatment with various antipsychotic medications. Antipsychotic drugs are known to influence DNA methylation status.[19,20,45,46] Fourth, some genetic variants, clinical symptoms, and other components of the methionine cycle, such as S-adenosyl-methionine, folic acid, and vitamin B, might be involved in variations of DNA methylation and plasma total homocysteine.[21,47-52] Finally, hyperhomocysteinemia has been identified as an independent risk factor for several neurological disorders, such as depression and dementia, in addition to SCZ,[3,53-55] so further disease-specific DNA methylation analysis will be necessary.

In summary, significant correlations between plasma total homocysteine and DNA methylation were identified at numerous CpG sites in patients with SCZ, and these results suggest that homocysteine might play a role in the pathogenesis of SCZ via a molecular mechanism involving alterations to DNA methylation.

Materials and Methods

Subjects. Forty-two male patients with SCZ (mean age: 51.8 ± 6.7 y) were recruited from Tokushima and Kochi University Hospitals in Japan. The diagnosis of SCZ was made according to DSM-IV criteria by at least two expert psychiatrists on the basis of extensive clinical interviews and a review of medical records. None of the patients had any psychiatric comorbidity or cardiovascular diseases. All patients were treated with various antipsychotic drugs. The mean chlorpromazine equivalent dose was 829.2 ± 498.2 mg/d. Forty-two male control subjects, well matched for age (mean age: 51.9 ± 5.5 y), were selected from volunteers who were recruited from hospital staff, students, and company employees documented to be free from psychiatric problems, past histories of mental illness and medications, including vitamin supplements. All subjects who participated in this study were of unrelated Japanese origin. All subjects signed written informed consent approved by the institutional ethics committees of the University of Tokushima Graduate School and Kochi Medical School.

Plasma total homocysteine analysis. Plasma total homocysteine levels were measured by high performance liquid chromatography. Homocysteine was labeled with 4-fluoro-7-sulfamoylbenzofurazan and detected by a fluorescent detector according to the method of previous studies.[10]

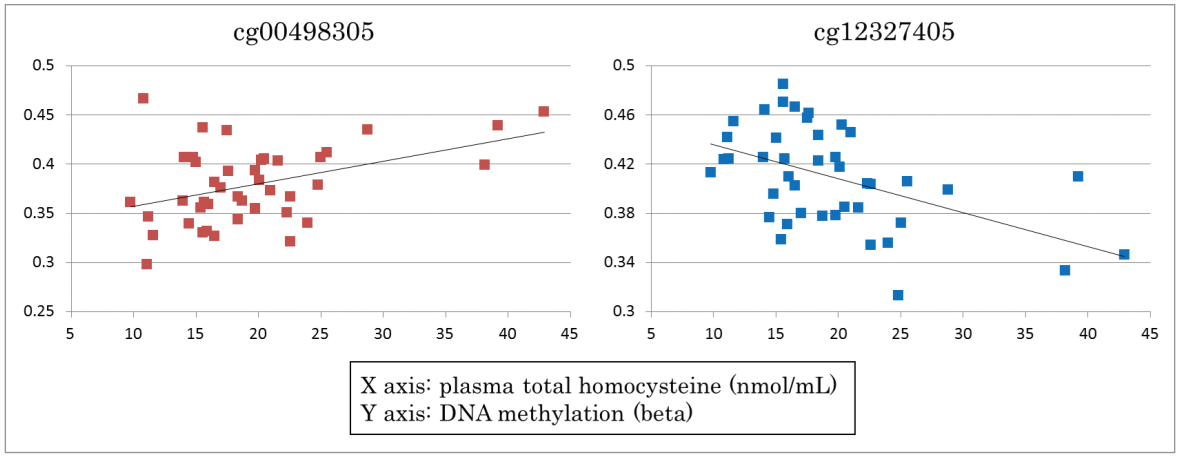

Figure 2. Two CpG sites in the *SLC18A2* (cg00498305) and *GNAL* (cg12327405) genes, which have been implicated in SCZ. A significant positive correlation of plasma total homocysteine with DNA methylation was observed at cg00498305 located in the CGI shore in the promoter region of the *SLC18A2* gene (p = 1.67E−03). A significant negative correlation of plasma total homocysteine with DNA methylation was observed at cg12327405 in the CGI in the promoter region of the *GNAL* gene (p = 2.85E−04). [X-axis: plasma total homocysteine (nmol/mL); Y-axis: DNA methylation (β)].

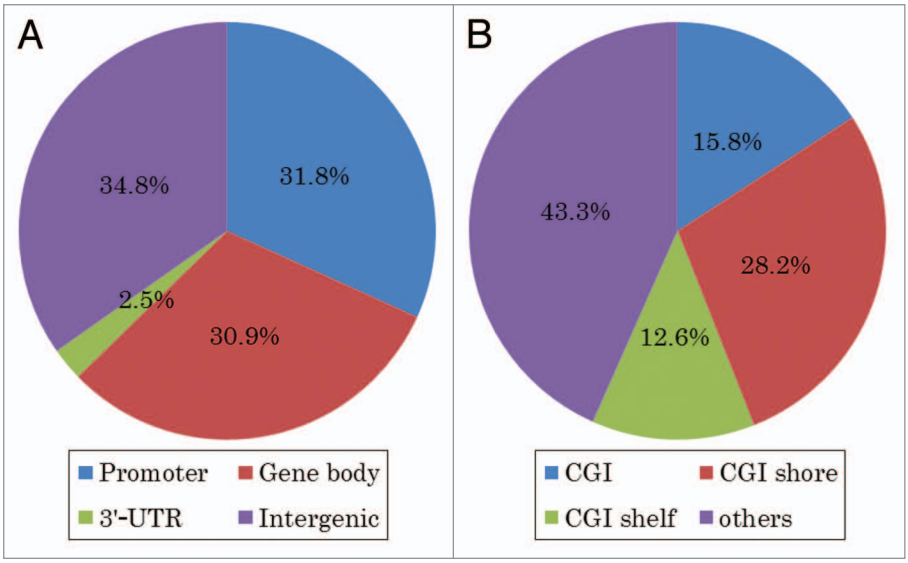

Figure 3. Percentages of 1,338 CpG sites at which plasma total homocysteine and DNA methylation were significantly correlated. (**A**) Of the 1,338 CpG sites, 425 (31.8%) were located in promoter regions, 414 (30.9%) were located in gene bodies and 34 (2.5%) were located in 3'-UTRs. (**B**) Of the 1,338 CpG sites, 212 (15.8%) were located in CGIs, 377 (28.2%) were located in CGI shores and 169 (12.6%) were located in CGI shelves.

DNA methylation methods. Genomic DNA was extracted from peripheral blood using the phenol-chloroform method. Bisulfite conversion of 500 ng of genomic DNA was performed with the EZ DNA methylation kit (Zymo Research). DNA methylation level was assessed with Infinium® HumanMethylation450 BeadChips (Illumina Inc.) according to the manufacturer's instructions. The technical schemes, accuracy, and high reproducibility of this array have been described in previous papers.[56-58] Quantitative measurements of DNA methylation were determined for 485,764 CpG dinucleotides that covered 99% of the RefSeq genes and were distributed across whole gene regions, including promoters, gene bodies, and 3'-UTRs. The arrays also covered 96% of the CGIs from the UCSC database with additional coverage in CGI shores (0–2 kb from CGI) and CGI shelves (2–4 kb from CGI). Detailed information on the contents of the array is available in the Infinium HumanMethylation450 User Guide, HumanMethylation450 manifest (www.illumina.com) and recent papers.[56,58] DNA methylation data was analyzed using the methylation analysis module within the BeadStudio software (Illumina Inc.). DNA methylation status of the CpG sites was calculated as the ratio of the signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value, known as β, ranges from 0 (completely unmethylated) to 1 (fully methylated). For intra-chip normalization of the probe intensities, colored balance and background corrections in every set of 12 samples from the same chip were performed using internal control probes. X chromosome CpG sites in the CGIs in the *AR* gene as well as the internal control probes were checked to validate the DNA methylation measurements, as in a previous study.[59] Of the 485,764 CpG sites, the loci that have β-values of < 0.1 or > 0.9 were eliminated, as in previous studies.[32,60] The loci that are potentially confoundable with single nucleotide polymorphisms with a minor allele frequency of > 0.1 in the HapMap-JPT population were also removed because DNA methylation is associated with genotypic variants.[61] The final data set includes 164,657 CpG sites.

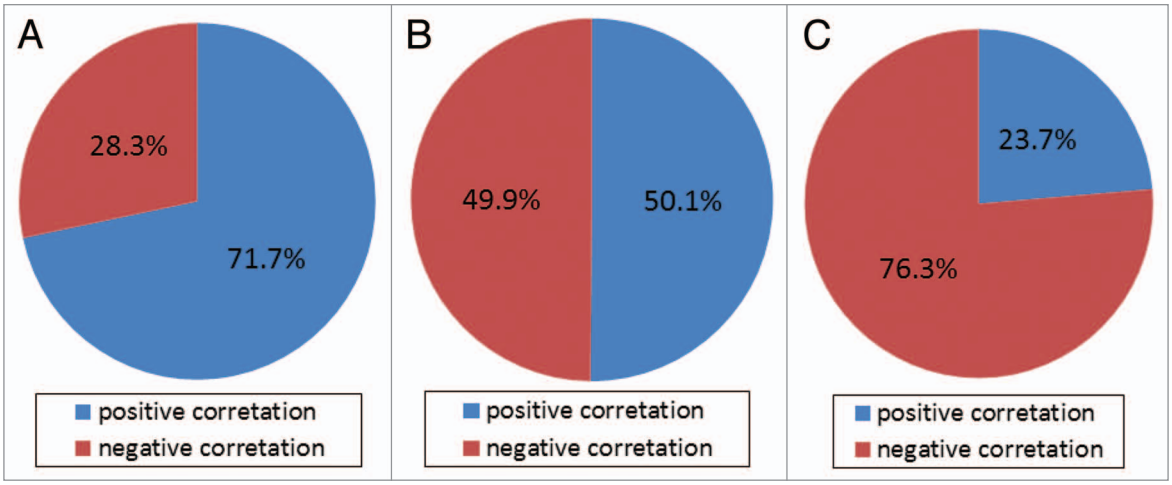

Figure 4. Percentage of CpG sites with positive correlations, located in CGIs, CGI shores and CGI shelves. (**A**) Of the 212 CpG sites located in the CGIs, 152 (71.7%) showed positive correlations between plasma total homocysteine and DNA methylation. (**B**) Of the 377 CpG sites located in the CGI shores, 189 (50.1%) showed positive correlations between plasma total homocysteine and DNA methylation. (**C**) Of the 169 CpG sites located in the CGI shelves, 40 (23.7%) showed positive correlations between plasma total homocysteine and DNA methylation.

Statistical methods. Differences in plasma total homocysteine levels between the two groups were examined using a Mann–Whitney U test. The influences of plasma total homocysteine on DNA methylation was examined with a multiple linear regression analysis adjusted for age and chlorpromazine equivalent dose as potential confounders, after standardizing DNA methylation β and plasma total homocysteine values with Z-scores across the samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were dislosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/24621

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