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ORIGINAL ARTICLE

Alterations in DNA methylation rates of brain-derived neurotrophic factor in patients with schizophrenia

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KEYWORDS
Brain-derived neurotrophic factor;
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Abstract
Background and objectives: Schizophrenia (SZ) is one of the most disabling mental illness and the elucidation of diagnostic and therapeutic biomarkers are required. Recent studies investigating the brain morphology, the gene expression profile, and the genetic epidemiology have suggested the involvement of Brain-derived neurotrophic factor (BDNF) and its epigenetic regulation in the pathophysiology of SZ. The current study was conducted to determine the association of DNA methylation of the BDNF gene with the diagnosis or with the characteristics of patients with SZ.
Methods: We analyzed genomic DNA from peripheral blood of 22 patients with SZ and 22 healthy subjects. The DNA methylation rates (DMRs) of the CpG island at the promoter of exón I of the BDNF gene were measured using EpiTYPER® and the MassARRAY® system (Agena Biosciences). We examined the validity of the methylation profiles as a diagnostic biomarker for SZ by clustering analyses, differences in DMRs between patients and healthy controls, and the relationship between DMRs and patient characteristics.
Results: The clustering analysis failed to distinguish between healthy controls and patients with SZ, though the DMRs of 4 CpG units were significantly different between these two groups. Whereas the DMR of one CpG (CpG 28) was significantly correlated with the amount of daily antipsychotics, there was no influence of age, severity, or duration of illness on the DMRs of the BDNF gene.

Abbreviations: BDNF, brain-derived neurotrophic factor; BPRS, brief psychiatric rating scale; DMRs, DNA methylation rates; SZ, Schizophrenia; YLD, years lived with disability.

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Conclusion: Despite the small number of subjects, our results suggest the involvement of the changes in DMRs of the BDNF gene in the pathophysiology of SZ.

Introduction

According to Global Burden of Disease Study 2013 Collaborators, schizophrenia (SZ) is the twelfth leading contributor to years lived with disability (YLD) in 1990 and was the eleventh leading contributor in 2013.[1] In addition, since several studies have demonstrated a significant increase in the risk of premature mortality in patients with SZ,[2] SZ is widely recognized as one of the most disabling mental illnesses. Despite advances in elucidating the pathophysiology of SZ, these findings indicate that there is an urgent need for more effective treatments for the disease.

Regarding the pathophysiology of SZ, numerous brain imaging studies using magnetic resonance imaging (MRI) and volumetric analyses have recently demonstrated abnormalities in various region of the brain of SZ patients, including the superior temporal gyrus, insula, and hippocampus.[3] A meta-analysis of structural MRI studies in SZ patients reported progressive structural brain abnormalities, such as a decrease in whole brain volume, based on longitudinal volumetric analyses.[4] In addition, histological studies using postmortem human brain tissues have demonstrated synaptic abnormalities in SZ such as decreased spine density, spine number, or dendrite length.[5] Thus, although the precise mechanism of the morphological changes in patients with SZ is still largely unknown, it has been hypothesized that progressive degeneration in the brain is involved in the pathophysiology of the disease.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is closely involved in synaptic plasticity, spine development, and synaptogenesis in the central nervous system.[6] Several studies have reported decreased levels of BDNF mRNA or BDNF immunoreactivity in postmortem brain tissue of patients with SZ.[7]

Similarly, it is well known that the blood levels of BDNF in patients with SZ are significantly lower than those in healthy subjects.[8] In particular, Rizos et al.[9] demonstrated a significant positive correlation between serum levels of BDNF and hippocampal volume in drug-naïve SZ patients at the first psychotic episode. Based on these findings, it is conceivable that dysregulation of BDNF gene expression, including transcription or translation, may be closely involved in the pathophysiology of SZ.

With respect to gene transcription, it has recently been revealed that epigenetic regulation of chromatin structure, such as histone modification and cytosine methylation, play a critical role in the transcription of various genes. Several genome-wide DNA methylation studies using human brain tissues and blood from patients with SZ have suggested that alterations in DNA methylation might be associated with the pathophysiology of SZ,[10] and the profiles of DNA methylation of some genes might be a good biomarker for SZ.[11] Additional strongly supportive evidence for the involvement of epigenetic dysregulation in the pathophysiology of SZ is the concordance rate for SZ in homozygotic twins, which is approximately 50%.[12] This finding indicates that environmental influences, at least in part, contribute to the pathophysiology of SZ. In fact, Kordi-Tamandani and associates[13] found BDNF IV promoter hypomethylation by the use of methylation-specific PCR, but Ikegame et al.[14] found BDNF I promoter hypermethylation based on pyrosequencing analysis in patients with SZ. Although the reason for this discrepancy is unknown, it is likely that the different methods analyzing the small region of the promoter may be involved.

In this study, the wider range of DNA methylation rates (DMRs) of the CpG island at the promoter of exon I (CpG I) of the BDNF gene were measured in blood from 22 patients with SZ and 22 healthy subjects using a EpiTYPER® and the MassARRAY® System (Agena Biosciences, San Diego, CA) to determine whether DNA methylation profiles in patients with SZ could serve as an epigenetic biomarker for the diagnosis of SZ. We analyzed the CpG island at the promoter of first exon which play an important role in proximal promoter activity and preinitiation.[15] Additionally, the importance of the altered DNA methylation in this region had been reported in several psychiatric disorders.[16] Among several methods for assessment of cytosine methylation, we selected this system because of its potency to accurately and simultaneously quantify multiple CpG positions.[17] In addition, we measured the DMR of each CpG within CpG I of the BDNF gene to examine whether the DMRs in patients with SZ differ from those of healthy controls, and to determine whether a relationship exists between DMRs and the characteristics of patients with SZ.

Methods

Subjects

A total of 22 patients with SZ and 22 healthy controls participated in this study. Demographic characteristics of the participants are shown in Table 1. All subjects were Japanese males who had been diagnosed by at least two trained psychiatrists according to DSM-IV criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. The severity of SZ was evaluated using the Brief Psychiatric Rating Scale. All patients had no history of current or past substance-related disorder or physical diagnoses. Healthy controls were recruited by advertisement and all were free of current or past psychiatric or physical diagnoses and had no first-degree relatives with psychiatric disorders. Blood samples were collected at Hiroshima University Hospital.

Table 1 Demographic and clinical characteristics of subjects.

Group	Age (Years) (Mean ± S.D.)	Duration of illness (Years) (Mean ± S.D.)	Dosage of daily antipsychotics (mg) (mean ± S.D.)	BPRS (mean ± S.D.)
SZ (N = 22)	36.4 ± 11.8	14.7 ± 13.0	753 ± 851	61.5 ± 16.2
Control (N = 22)	41.1 ± 13.4			

All participants were male Japanese.
Dosage of daily antipsychotics was represented by chlorpromazine equivalent dose.
BRPS: Brief Psychiatric Rating Scale score.

This study was approved by the ethics committees of Hiroshima university School of Medicine. All subjects received a description of the study and gave written informed consent.

Selection of genomic regions of the BDNF gene for methylation analysis

In general, the state of the promoter upstream of the first exon is considered to greatly influence gene transcription via its proximal promoter activity or its role in preinitiation. In addition, altered DNA methylation of CpG sites within the CpG island at the promoter of exon I of the BDNF gene have been reported in major depression[18] and in SZ.[14] In light of these data, we chose the CpG island of the BDNF gene upstream of exon I (CpG I) as the target region for DNA methylation analysis. Methylation primers were designed as previously described.[18] Briefly, the sequence of CpG I was identified by the use of the UCSC genome browser (http://genome.ucsc.edu/), and primers were designed using Epidesigner software (http://www.epidesigner.com/). The locations of target region and 4 primers in the BDNF gene are schematically demonstrated in Fig. 1.

DNA methylation analysis by EpiTYPER® and the MassARRAY® system

All blood samples were collected between 11:00 AM and 1:00 PM. Blood samples (5 mL) were collected and placed in vacuum tubes containing heparin sodium and stored at −80 °C until the day of analysis. DNA methylation analysis were performed as previously described.[18] Briefly, Genomic DNA was isolated using DNeasy® Blood & Tissue Kits (Qiagen, Hilden, Germany), bisulfite modification was performed using an EZ DNA methylation kit (Zymo Research, Orange, CA). DNA methylation was quantified by EpiTYPER® and the MassARRAY® system (Agena Biosciences) according to the manufacturer's instructions. These steps produced quantitative data for each of the sequence-defined analytic units referred to as CpG units, which contain either 1 individual CpG site or an aggregate of downstream CpG sites. Ultimately, 33 CpG units of CpG I were used for the analyses. Methylation levels of 33 CpG units were triplicately measured for each sample.

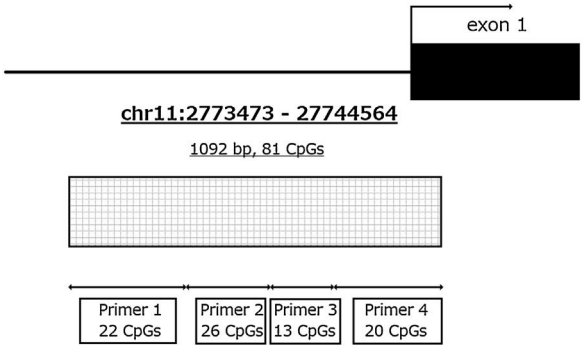

Figure 1 Schema of CpGs and primers used for DNA methylation analyses.
The target region used for methylation analysis consists of 1092 bp including 81 CpGs upstream of exon I.

Statistical analysis

We used ''R'' software package for statistical computing in all analyses. For statistical analysis, the daily dosage of antipsychotics was converted to chlorpromazine equivalent doses. Spearman rank correlation test was used to evaluate the correlation of the DMRs of each CpG unit with the age of the subjects. In addition, to analyze the correlations of DMRs with dosage of antipsychotics, with BPRS scores, and with duration of illness in patients with SZ, we computed semi-partial Spearman correlations to control for age as covariate. Significance with Bonferroni correction was set at $P < 0.0015$. Two-way hierarchical clustering analysis was performed using the DMRs of CpG I. except for the DMR of CpG 28 to investigate whether we could divide samples concordant with diagnosis using the methylation profile. Hierarchical clustering analyses were performed using hclust in the R cluster package, with Euclidean metric and complete linkage. Samples with closer methylation patterns were closely clustered. The difference in the DMRs of each CpG units between healthy controls and patients was analyzed by analysis of covariance (ANCOVA; age as covariate) followed by Bonferroni multiple comparison correction, and P-values were set at $P < 0.0015$.

Results

All the DMRs of all samples are shown in Table 2.

Table 2 The DMRs of each CpG units at the CpG island of BNDF exon I in schizophrenic patients and control.

	Control Rate (%) (Mean ± S.E.M.)	SZ Rate (%) (Mean ± S.E.M.)	ANCOVA P-value
CpG_1,7	9.4 ± 0.6	5.1 ± 0.6	<0.0001*
CpG_2	5.6 ± 0.5	3.5 ± 0.7	0.021
CpG_3,6	8.1 ± 0.5	7.5 ± 0.9	0.54
CpG_4	23.3 ± 3.2	31.7 ± 3.2	0.064
CpG_5	2.3 ± 0.3	1.8 ± 0.4	0.32
CpG_8,9	10.2 ± 0.4	13.2 ± 0.5	<0.0001*
CpG_14	7.3 ± 0.6	12.6 ± 1.8	0.010
CpG_15	9.8 ± 0.4	7.0 ± 0.8	0.0051
CpG_17	6.4 ± 0.5	3.3 ± 0.7	0.0008*
CpG_18	2.5 ± 0.4	0.8 ± 0.2	0.0004*
CpG_19,20,21	5.1 ± 0.5	3.3 ± 0.4	0.0054
CpG_22	13.5 ± 1.2	22.2 ± 3.9	0.038
CpG_23	3.8 ± 0.4	3.4 ± 0.6	0.57
CpG_24	1.4 ± 0.1	1.4 ± 0.2	0.82
CpG_25,26,27	3.7 ± 0.2	3.3 ± 0.2	0.23
CpG_28	3.0 ± 0.2	3.0 ± 0.3	0.84
CpG_29,30,31	2.2 ± 0.1	1.8 ± 0.2	0.034
CpG_32	11.7 ± 1.4	13.5 ± 0.9	0.27
CpG_33,34	7.8 ± 0.4	10.2 ± 1.0	0.030
CpG_36	5.6 ± 1.0	4.3 ± 0.9	0.36
CpG_37	4.7 ± 0.3	5.4 ± 0.4	0.25
CpG_47	3.0 ± 0.3	3.1 ± 0.3	0.80
CpG_48	3.7 ± 0.2	4.9 ± 0.6	0.055
CpG_50,51	3.7 ± 0.3	4.7 ± 0.3	0.018
CpG_52	3.0 ± 0.4	2.4 ± 0.3	0.20
CpG_59	7.3 ± 1.5	5.4 ± 0.5	0.26
CpG_61	4.8 ± 0.4	4.6 ± 0.6	0.75
CpG_63,77	2.2 ± 0.4	1.8 ± 0.4	0.44
CpG_71	21.9 ± 3.6	28.9 ± 4.7	0.28
CpG_72,73	4.1 ± 0.4	3.9 ± 0.4	0.77
CpG_74,75	3.8 ± 0.4	2.6 ± 0.2	0.011
CpG_78	16.7 ± 2.5	11.6 ± 1.8	0.11
CpG_80,81	6.0 ± 0.4	6.0 ± 0.5	0.98

The asterisks (*) behind of scores indicate statistically significant P-values.
Significance was set at $P < 0.0015$.

The influences of age, and the dosage of daily antipsychotics on the DMRs of the BDNF gene

Prior to evaluating the potential of BDNF DMRs for the diagnosis of SZ, we first examined the influence of other factors (age, dosage of daily antipsychotics) on BDNF DMRs.

The influence of age or of dosage of daily antipsychotics was examined by analyzing the correlation with BDNF DMRs (Table 3). Although we could not find the significant correlation of the DMRs for any CpG units with age in the present study, to assess DMRs and variables of interest, we controlled for age as covariant because of the well- known influence of age associated DNA methylation.[19] There was a significant negative correlation between the DMR of CpG 28 and dosage of daily antipsychotics ($|r| = 0.71$, $P = 0.00022$).

Consequently, the DMR of CpG 28 was excluded from subsequent analyses.

DNA methylation profile in CpG I of the BDNF gene among patients with SZ

To evaluate the potential of BDNF CpG I DNA methylation for diagnosis SZ diagnosis, we performed two-way hierarchical clustering analysis using DMRs of CpG units at CpG I to classify samples and CpG units into clusters according to their similarity, and dendrograms were used to visualize the results. The DNA methylation profile at BDNF CpG I of all samples are shown in heat map format (Fig. 2). We could not distinguish between patients with SZ and healthy controls at any height in the dendrogram.

We next analyzed the difference in DMRs of each CpG unit between healthy controls and patients with SZ (Table 2). The DMRs of 4 CpG units (CpG1/7, CpG8/9, CpG17, CpG18) out of 33 CpG units in BDNF CpG I were significantly different between the 2 groups.

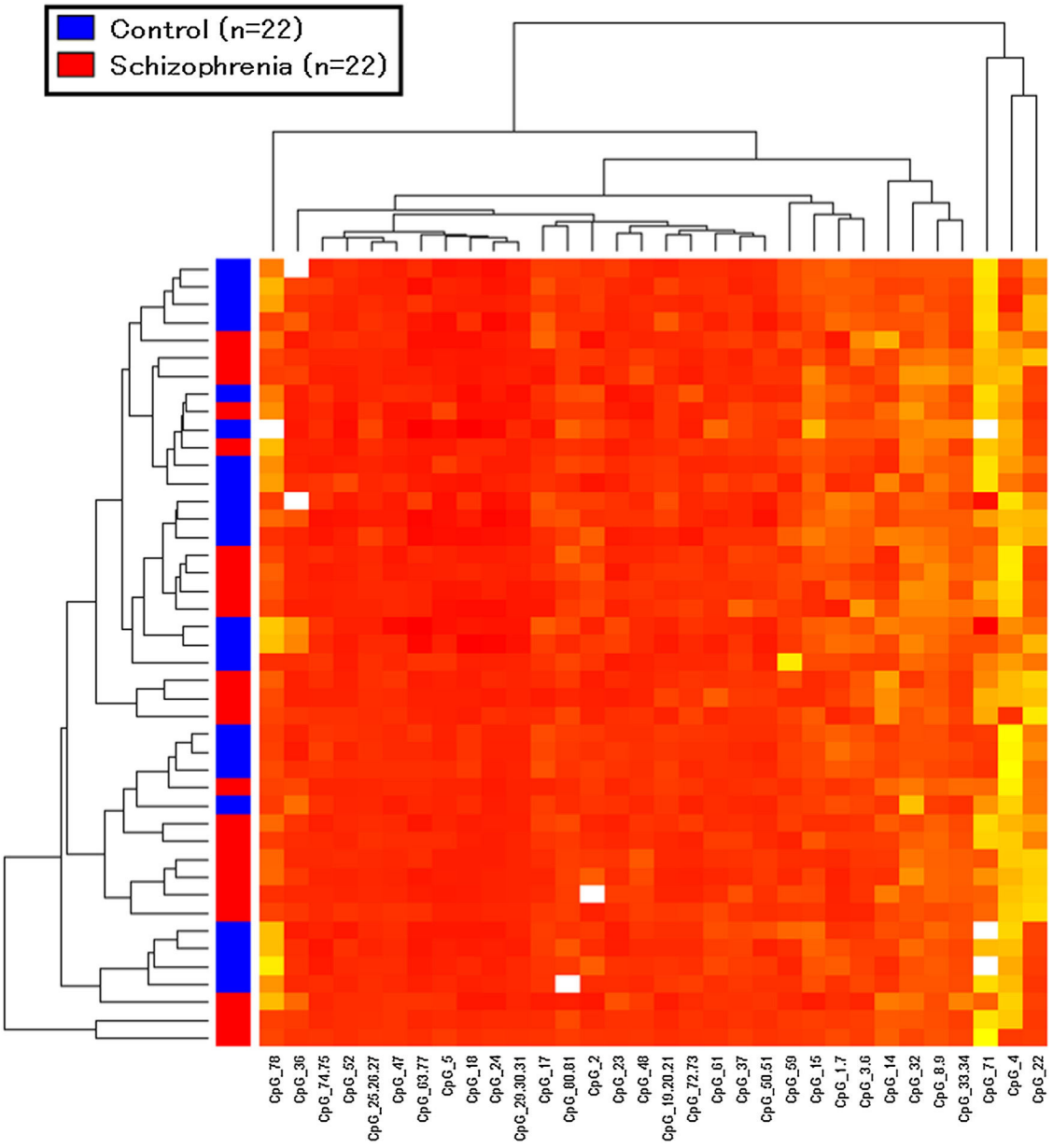

Figure 2 Hierarchic cluster analysis of subjects and their methylation profiles at CpG I of the BDNF gene.
Two-way hierarchic cluster analysis of 44 samples (rows) and DNA methylation of CpG units at CpG I of the BDNF gene (columns) are shown. DNA methylation values are depicted by a pseudocolor scale as methylation rate increases from red (nonmethylated) to yellow (methylated). White denotes data of poor quality, and such data points were excluded before clustering. Samples are color-coded according to the diagnoses of samples (depicted in legend at upper left).

Correlations between DMRs of each CpG unit and BPRS scores, and between DMRs and duration of illness

We subsequently examined correlations between the DMRs of each CpG unit at CpG I of the BDNF gene and severity of SZ (BPRS) as well as the duration of illness. None of the DMRs correlated with BPRS scores or duration of illness (data not shown).

Discussion

The results of the present study demonstrated that the 2-dimensional hierarchical clustering analysis using the DMRs of 33 CpG units within CpG I of the BDNF gene failed to distinguish healthy controls and patients with SZ, although the DMRs of 4 CpG units (CpG1/7, CpG8/9, CpG17, CpG18) were significantly different between these 2 groups. Whereas the DMR of 1 CpG (CpG 28) was significantly correlated with the

Table 3 Correlations of the DMRs at the CpG island of BNDF exon I with a between the DMRs and dosage of daily antipsychotics in patients with SZ.

	Correlation coefficient	P-value
CpG_1,7	0.28	0.21
CpG_2	0.21	0.35
CpG_3,6	−0.048	0.83
CpG_4	−0.41	0.061
CpG_5	0.31	0.16
CpG_8,9	−0.079	0.73
CpG_14	−0.009	0.97
CpG_15	0.002	0.99
CpG_17	−0.46	0.031
CpG_18	0.19	0.39
CpG_19,20,21	0.16	0.47
CpG_22	−0.21	0.36
CpG_23	−0.33	0.14
CpG_24	−0.32	0.14
CpG_25,26,27	−0.11	0.63
CpG_28	−0.71	0.00022 **
CpG_29,30,31	−0.30	0.17
CpG_32	0.24	0.28
CpG_33,34	−0.025	0.91
CpG_36	0.11	0.63
CpG_37	−0.40	0.068
CpG_47	−0.45	0.037
CpG_48	−0.081	0.72
CpG_50,51	0.12	0.59
CpG_52	−0.38	0.079
CpG_59	−0.31	0.17
CpG_61	−0.13	0.56
CpG_63,77	−0.052	0.82
CpG_71	0.009	0.97
CpG_72,73	−0.19	0.40
CpG_74,75	−0.21	0.34
CpG_78	0.057	0.80
CpG_80,81	−0.52	0.013

Correlation coefficient and P-value by semi-partial correlation coefficients procedure is shown.
The asterisks (*) behind of scores indicate statistically significant p-values.
Significance was set at $P < 0.0015$.

amount of daily antipsychotics, there was no influence of age, severity, or duration of SZ on the DMRs of 33 units within CpG I of the BDNF gene.

To our knowledge, only a few studies have investigated DMRs of the BDNF gene in peripheral blood from patients with SZ. For example, Kordi-Tamandani and associates[13] found differential methylation profiles at promoter IV of the BDNF gene between healthy controls and patients with SZ according to methylation-specific PCR. Ikegame et al.[14] reported a significantly increased DMR of CpG 72 in CpG I of the BDNF gene in patients with SZ based on pyrosequencing analysis. Çöpoğlu et al.[20] reported no differences in DMRs around the promoters of exon I and IV in SZ patients compared with healthy controls as determined by methylation-specific PCR. Although the reason for the differences in findings among these 3 studies are unknown, different analytic methods used for DNA methylation and analyses of the different regions of the BDNF gene may account for the discrepancy.

Çöpoğlu and associates reported that the mean duration of illness in SZ patients with hemi-methylation around CpG I of the BDNF gene was significantly longer tan those without methylation.[20] However, none of 33 CpGs showed a significant correlation with the duration of illness in this study. Although the reason for the discordant findings from these 2 studies is unknown, differences in the number of CpG sites analyzed and the method used for measurement of DMRs may account for the discrepancy. In addition, the patients in both studies received various types of antipsychotics including first-generation and second-generation antipsychotics. In this context, it cannot be ruled out that administration of different types and dosages of antipsychotics may affect the DMRs of the BDNF gene and subsequently lead to a discrepant relationship between disease duration and DMRs of the BDNF gene in these studies.

In line with the influence of antipsychotics on DMRs, it was reported that administration of antipsychotics affected the DMRs of several genes in mouse prefrontal cortex.[21,22] The influence of various antipsychotics, including haloperidol (a selective D2 receptor antagonist), clozapine (a 5HT2a-preferring receptor antagonist), risperidone (D2 and 5HT2A receptor antagonists), olanzapine (a 5HT2a-preferring receptor antagonist), and sulpiride (a D2/D3 receptor antagonist), on DMRs was examined in mouse brain. Increased promoter methylation of the GAD67 and RELN genes by 7 day-treatment with methionine was reversed by clozapine, olanzapine, sulpiride and quetiapine but not by haloperidol or risperidone. Melka et al.[23] investigated the effect of olanzapine on genome-wide DNA methylation in rat hippocampus, cerebellum, and liver, and found widespread changes in gene-specific DNA methylation (including the change of the BDNF gene in hippocampus) among all 3 tissues studied. In the present study, because we have examined the effect of only the chlorpromazine equivalent dosage of antipsychotics on the DMRs of the BDNF gene and found a significant effect on only 1 CpG site, it cannot be concluded that long-term administration of antipsychotics regulates the DMRs of the various genes.

There are several limitations of the present study that should be noted. First, although differences in DNA methylation depending on leukocyte subtypes have been reported,[24] our methylation data were derived from whole blood containing various leukocyte subtypes. Second, we could not measure the blood levels of BDNF mRNA in this study. The associations between epigenetic changes in the promoters of the BDNF gene such as promoter I and IV, and changes in BDNF mRNA expression, have been thoroughly studied in cellular and animal models.[16] Future study examining the DMRs in CpG I and levels of exon I mRNA of the BDNF gene simultaneously is needed to elucidate the functional significance of changes in DNA methylation in the pathophysiology of SZ. Third, participants in the current study were all Japanese males. Although it is not known whether there are differences in DNA methylation of the BDNF gene among races, interracial differences in global genomic DNA methylation in peripheral blood had been reported.[25,26] In addition, 2 previous studies[14,27] reported sex-dependent differences in DNA methylation of BDNF gene. Fourth, we lack the data of smoking status and body mass index of participants, which are

reported to affect DNA methylation.[28–30] Lastly, the human BDNF gene has multiple functional promoters, and the association of the alteration of DNA methylation levels within other BDNF promoters and the pathophysiology of SZ had been reported.[16] Thus, further studies using a much larger number of participants should be performed to elucidate the influence of race/ethnicity/gender/lifestyle on DMRs of the multiple promoters with the measurement of the expression of multiple promoters within the BDNF gene.

In summary, we were unable to develop a new epigenetic biomarker for the diagnosis or severity of illness in patients with SZ using profiles of DMRs within CpG I of the BDNF gene. However, 4 of 33 CpG units within CpG I were differentially methylated in patients with SZ. Further studies examining alterations in DMRs of the BDNF gene in patients with SZ before and after treatment with antipsychotics may shed light on the epigenetic pathophysiology of SZ.

Ethical considerations

This study was approved by the ethics committees of Hiroshima university School of Medicine. All subjects received a description of the study and gave written informed consent.

Conflict of interest

The authors have no conflict of interest to declare.

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