

Table 1
Demographic characteristics of subjects.

Group	Age (years) (mean ± S.D.)	Genotype	HAM-D score (mean ± S.D.)		ETI score (mean ± S.D.)	IR (%) (mean ± S.D.)
			Before	After 6 weeks		
Control (N = 50, 27M/23F)	40.3 ± 10.5	LL: 2, SL: 16, SS: 32				
Major depression (N = 50, 27M/23F)	40.3 ± 10.3	SL: 16, SS: 34	20.6 ± 4.8	8.1 ± 6.6	5.0 ± 3.8	60.2 ± 31.3

M: males, F: females, ETI: Early Trauma Inventory, IR: improvement ratio.
HAM-D: Hamilton Rating Scale for Depression.

2.2. Selection of genomic regions of the SLC6A4 gene for DNA methylation analysis

We chose the CpG island of the SLC6A4 gene as a target for analysis. The target region around the SLC6A4 gene was selected based on a previous report (Olsson et al., 2010). The sequence of the CpG island was identified using the UCSC genome database (<http://genome.ucsc.edu/>) (chr 17: 28562388–28563186). PCR primers were designed using Epidesigner software (<http://www.epidesigner.com/>). The schema for the target region used for analysis and the primers used for PCR are shown in Fig. 1.

2.3. Genotyping to generate S- and L-fragments

The sequence of 5-HTTLPR was identified using the UCSC genome database (chr 17: 28564123–28564473) (Fig. 1). PCR primers were designed using Primer 3 software (<http://frodo.wi.mit.edu/primer3/input.htm>). PCR was performed in a total volume of 5 μ L solution containing 10 ng DNA, 2 \times Gflex[®] PCR Buffer, 1 mM MgCl₂, 200 μ M dNTPs, 0.2 μ M of each primer, and 0.125 U of Tks Gflex[®] DNA polymerase (Takara, Tokyo, Japan) for 30 cycles (98 °C for 10 s, 60 °C for 15 s, and 68 °C for 30 s). The PCR products were then analyzed by 2% agarose gel stained with ethidium bromide. The distributions of genotypes in controls and patients with MD were in Hardy–Weinberg equilibrium (Healthy controls: $\chi^2 = 0$; Patients: $\chi^2 = 1.84$) (Table 1).

2.4. DNA methylation analysis by MassARRAY

All samples were collected between 11:00 AM and 1:00 PM, prior to the participants' lunch. Blood samples (5 mL) were collected and placed in vacuum tubes containing heparin sodium and stored at –80 °C. Genomic DNA was isolated using DNeasy[®] Blood and Tissue Kits (Qiagen, Hilden, Germany). Genomic DNA (1 μ g) was converted with sodium bisulfite using EZ DNA methylation kits (Zymo Research, Orange, CA). The concentration of bisulfite-converted DNA was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA); 10 ng of converted DNA was used for PCR. PCR was performed in a total volume of 5 μ L containing 1 μ M of each primer, 200 μ M

dNTP, 0.2 U HotStar Taq DNA polymerase (Qiagen), 2 mM MgCl₂, and 10 \times PCR buffer (Sequenom, Inc., San Diego, CA, USA). One of two primers in the PCR amplification of the target regions was tagged with a T7 promoter sequence: CAGTAATACGACTCACTA-TAGGGAGAAGGCT. This included a GGG transcription start and an 8-bp insert (AGAAGGCT) on the 5' end. The reaction mixture was preactivated for 4 min at 95 °C. DNA was amplified for 45 cycles (95 °C for 20 s, 56 °C for 30 s and 72 °C for 60 s) and the reaction was then continued at 72 °C for 3 min.

Unincorporated dNTPs were dephosphorylated by adding 1.7 μ L DNase free water and 0.3 U shrimp alkaline phosphatase (SAP) (Sequenom). The reaction was incubated at 37 °C for 20 min and SAP was inactivated for 5 min at 85 °C. Subsequently, PCR products (2 μ L) were incubated for 3 h at 37 °C with 5 μ L of Transcleave mix (Sequenom) for concurrent in vitro transcription and base-specific cleavage.

The resultant 10- to 20-nL products were spotted onto silicon matrix-preloaded chips (SpectroCHIP; Sequenom) using a MassARRAY nanodispenser (Sequenom), and analyzed using a MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF-MS) (Sequenom).

The spectra's methylation ratios were calculated using EpiTYPER software v1.0 (Sequenom). Triplicate independent analyses from bisulfite-converted DNA samples were undertaken. The method yielded quantitative results for each of the sequence-defined analytic units referred to as CpG units. These methods divided 81 CpG sites in the CpG island into 41 CpG units.

After obtaining the data, initial quality control was performed as follows. Poor-quality and non-valuable data for the quantitative methylation of each CpG unit measured by MALDI-TOF-MS were excluded. Ten CpG units were excluded because their molecular weights were outside the measurable range. In subsequent analyses, data for 2 CpG units were excluded as they were less than 80% of all samples. Data were available for 29 of 41 CpG units in the CpG island and for all samples.

2.5. Statistical analysis

For analysis of the DNA methylation profile, measurements after QC were combined in a data matrix, which was used in a 2-dimensional hierarchical clustering analysis with the "R" software package for statistical computing (available at CRAN, <http://cran.r-project.org/>). 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 Mann–Whitney *U* test was used to examine differences in the methylation rates of each CpG unit between healthy controls and patients. The Wilcoxon signed-rank test was used to examine differences in the methylation rates of each CpG unit between patients before and after 6 weeks of antidepressant treatment. The significance of each statistical test by Bonferroni correction was set at $P < 0.0017$.

The Spearman rank correlation test was used to examine the correlation between the methylation rate of each CpG unit and total HAM-D scores, total ETISR-SF scores, and improvement ratio (IR:

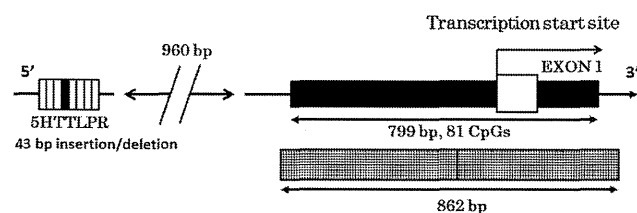


Fig. 1. Schematic representation of the SLC6A4 gene promoter region. Two primers covering the SLC6A4 transcription start site, exon I, part of intron I and the CpG island were used for the DNA methylation study. The target region consists of 862 bp including 81 CpGs. 5-HTTLPR is located 960 bp upstream of the CpG island.

(HAM-D before treatment – HAM-D after 6 weeks treatment)/HAM-D before treatment) in patients. The *T*-test was used for subsequent analyses of significance. Significance was set at $P < 0.05$.

3. Results

3.1. DNA methylation profiles in the CpG island of the SLC6A4 gene among all participants

Two-way hierarchical clustering analysis of DMR was undertaken to classify samples and CpG units into clusters according to their similarity, and a dendrogram was used to visualize the results. DNA methylation profiles at the CpG island in the SLC6A4 gene of all subjects are shown in heat map format (Supplemental Fig. 1). At any height of the dendrogram acquired from clustering analysis, it was impossible to distinguish between unmedicated patients and healthy controls (Supplemental Fig. 1).

Next, we compared the methylation rates of each CpG unit between unmedicated patients and healthy controls. There was no significant difference between unmedicated patients and healthy controls at any CpG unit (Table 2).

3.2. The influence of SLC6A4 genotyping for classifying participants

SLC6A4 genotyping analysis demonstrated that 18 healthy controls had *l/l* or *l/s* alleles (an *l* allele) and 32 healthy controls had *s/s* alleles. Similarly, while 16 patients had an *l* allele, 34 patients had *s/s* alleles (Table 1). Two-way hierarchical clustering analysis of DNA methylation profiles could not distinguish samples into any groups associated with diagnosis and/or allele (all participants with an *l* allele vs those with *s/s* alleles, healthy controls with an *l* allele

vs those with *s/s* alleles, or patients with an *l* allele vs those with *s/s* alleles) (Supplemental Fig. 2).

We next compared the methylation rates of each CpG unit among all participants by two-factor ANOVA using genotypes (*l* allele and *s/s* alleles) and diagnoses (healthy controls and patients) as fixed factors. Significance after Bonferroni correction was set at $P < 0.0017$. There was no significant effect of genotype on diagnosis. This result indicated that 5-HTTLPR did not affect DMR.

3.3. Correlations between DMR and total HAM-D scores, and DMR and total ETISR-SF scores

Next, we examined correlations between the methylation rates for each CpG unit of the CpG island of the SLC6A4 gene and the severity of depressive symptoms as well as the number of early adversities (Table 3). Regarding the severity of MD, there was a significant positive correlation between the methylation rate for CpG 76 and total HAM-D score ($|r| = 0.30$, $P = 0.03$, power = 0.57). Regarding early adversity, while there was a significant negative correlation between the methylation rate of CpG 3 and total ETISR-SF score ($|r| = 0.32$, $P = 0.02$, power = 0.63), a significant positive correlation was found between the methylation rate for CpG 76 and total ETISR-SF score ($|r| = 0.32$, $P = 0.02$, power = 0.63).

3.4. DNA methylation profiles in the CpG island of the SLC6A4 gene before and after treatment (unmedicated vs medicated patients)

To evaluate the effect of 6-week antidepressant treatment on DNA methylation profiles in the CpG island of the SLC6A4 gene, two-way hierarchical clustering analysis was performed (Supplemental Fig. 3). At any height of the dendrogram acquired

Table 2

The DNA methylation rates of each CpG unit at the CpG island of the SLC6A4 gene in healthy controls and patients with major depression.

	Control	Depression	Depression (6W)	Statistical analysis	
	Rate (%) (mean ± S.E.M)	Rate (%) (mean ± S.E.M)	Rate (%) (mean ± S.E.M)	Con vs Dep (P-value)	Dep vs D6W (P-value)
CpG 1	7.7 ± 0.4	8.4 ± 0.4	7.7 ± 0.5	0.20	0.68
CpG 3	5.1 ± 0.2	4.9 ± 0.3	6.1 ± 0.3	0.24	0.0004*
CpG 4	4.1 ± 0.3	4.0 ± 0.3	5.7 ± 0.6	0.84	0.03
CpG 10	4.1 ± 0.3	4.0 ± 0.3	5.7 ± 0.6	0.84	0.03
CpG 11	24.2 ± 0.7	24.0 ± 0.6	25.2 ± 0.8	0.50	0.95
CpG 24,25	5.7 ± 0.2	5.2 ± 0.2	4.6 ± 0.2	0.09	0.08
CpG 26,27,28	3.8 ± 0.2	3.7 ± 0.2	3.6 ± 0.2	0.79	0.28
CpG 29	4.6 ± 0.4	4.3 ± 0.7	6.3 ± 1.3	0.23	0.69
CpG 30	5.5 ± 0.9	5.9 ± 0.3	11.8 ± 3.2	0.58	0.17
CpG 31,32	1.6 ± 0.2	4.2 ± 0.2	2.0 ± 0.3	0.69	0.28
CpG 33,34,35,36	4.3 ± 0.2	7.3 ± 0.4	4.1 ± 0.2	0.10	0.29
CpG 37	6.6 ± 0.2	5.9 ± 0.3	5.5 ± 0.3	0.007	0.94
CpG 39,40	4.4 ± 0.1	4.2 ± 0.2	3.8 ± 0.2	0.21	0.10
CpG 41,42	7.3 ± 0.4	7.3 ± 0.4	8.0 ± 0.6	0.73	0.44
CpG 49,50,51,52	6.0 ± 0.4	5.8 ± 0.3	6.5 ± 0.5	0.72	0.13
CpG 55	14.5 ± 0.3	15.0 ± 0.3	15.2 ± 0.4	0.24	0.34
CpG 56	1.3 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	0.51	0.77
CpG 57,58	20.4 ± 0.5	20.8 ± 0.4	21.5 ± 0.5	0.43	0.23
CpG 62	19.9 ± 1.0	20.3 ± 1.0	19.2 ± 1.6	0.59	0.44
CpG 65,66	20.4 ± 0.5	20.8 ± 0.4	21.5 ± 0.5	0.43	0.23
CpG 67,68	24.1 ± 0.7	23.2 ± 0.8	23.8 ± 0.6	0.61	0.67
CpG 70	33.8 ± 1.9	35.2 ± 2.2	34.7 ± 2.7	0.92	0.18
CpG 71,72,73	13.8 ± 0.5	14.6 ± 0.4	13.5 ± 0.4	0.29	0.16
CpG 74	15.4 ± 0.7	16.1 ± 0.7	15.6 ± 0.9	0.63	0.99
CpG 75	49.1 ± 1.8	51.1 ± 1.8	49.3 ± 2.6	0.56	0.20
CpG 76	15.9 ± 0.5	16.3 ± 0.7	16.6 ± 0.7	0.91	0.56
CpG 78	3.3 ± 0.3	3.7 ± 0.4	2.8 ± 0.3	0.80	0.50
CpG 79	9.8 ± 0.8	11.2 ± 0.9	9.8 ± 0.8	0.14	0.59
CpG 80,81	32.3 ± 0.7	33.6 ± 0.8	34.1 ± 1.0	0.38	0.99

Control (Con): healthy controls, depression (Dep): unmedicated patients with MD, Depression6W (D6W); medicated patients with MD.

Statistical analysis: Mann–Whitney *U* test (healthy subjects and unmedicated patients with MD); Wilcoxon ranked signed test (unmedicated patients with MD and medicated patients with MD).

The mean methylation rate and *P*-value are shown.

The asterisks (*) behind of scores indicate statistically significant *P*-value. Significance was set at $P < 0.0017$.

Table 3

Relationships of the DNA methylation rates at the CpG island of the SLC6A4 gene with clinical characteristics in patients with major depression.

	Correlation coefficient		
	HAM-D	ETI	IR
CpG 1	0.26	-0.15	0.08
CpG 3	-0.03	-0.32*	0.36*
CpG 4	0.02	0.008	-0.01
CpG 10	0.02	0.008	-0.01
CpG 11	-0.24	0.25	-0.06
CpG 24,25	-0.09	0.08	-0.008
CpG 26,27,28	0.17	0.05	-0.004
CpG 29	0.11	-0.01	-0.12
CpG 30	-0.16	-0.12	0.15
CpG 31,32	-0.17	0.09	-0.10
CpG 33,34,35,36	0.03	0.14	-0.17
CpG 37	0.06	-0.04	0.18
CpG 39,40	-0.23	0.06	0.11
CpG 41,42	-0.21	-0.19	-0.03
CpG 49,50,51,52	-0.07	0.28	0.07
CpG 55	0.13	0.19	0.05
CpG 56	-0.26	0.18	0.04
CpG 57,58	0.19	0.03	0.12
CpG 62	0.24	0.10	0.05
CpG 65,66	0.19	0.03	0.12
CpG 67,68	0.15	-0.003	0.03
CpG 70	-0.15	-0.17	-0.10
CpG 71,72,73	0.04	0.05	-0.14
CpG 74	0.16	0.19	0.13
CpG 75	-0.07	-0.06	-0.23
CpG 76	0.30*	0.32*	-0.07
CpG 78	0.03	0.17	0.02
CpG 79	0.21	0.06	-0.20
CpG 80,81	0.13	0.11	0.14

ETI: Early Trauma Inventory, IR: improvement ratio, HAM-D: Hamilton Rating Scale for Depression.

Statistical analysis: Spearman rank correlation test.

The correlations between the methylation rates and total HAM-D scores, total ETI scores and IR are shown.

The asterisks (*) behind of several correlations indicate significantly correlations which have statistically significant *P*-values in subsequent analyses by *t*-test.

Significance was set at *P* < 0.05.

from clustering analysis, it was impossible to distinguish between unmedicated patients and medicated patients (Supplemental Fig. 3). We also compared the methylation rates for each CpG unit between unmedicated and medicated patients (Table 2). A significant difference in the methylation rates for CpG 3 ($|Z| = 3.47$, $P = 0.0004$, power = 0.77) was found between unmedicated and medicated patients.

3.5. Relationship between DMR and improvement ratios in response to 6-week antidepressant treatment

To evaluate the potential of DMR as a predictor of treatment response, we analyzed the relationship between pre-treatment methylation rates for each CpG unit and therapeutic responses. The therapeutic responses to antidepressants (paroxetine, fluvoxamine, or milnaciprane) for 6 weeks were defined based on IRs. Correlation analysis was conducted using the IR and the pre-treatment methylation rates for each CpG unit. The pre-treatment methylation rate of CpG 3 showed a significant positive correlation with IRs in MD patients ($|r| = 0.36$, $P = 0.02$, power = 0.64, Table 3).

Two-way hierarchical clustering analysis was performed using samples from patients with an IR of 50% or more, and patients with an IR of less than 50% (Supplemental Fig. 4). At any height of the dendrogram acquired from clustering analysis, it was impossible to classify patients into 2 groups in agreement with the IRs (Supplemental Fig. 4).

Subsequently, we compared the pre-treatment methylation rate of CpG 3 between these 2 groups because it was only changed

significantly after antidepressant treatment. The pre-treatment methylation rate of CpG 3 correlated with IRs, and a significant difference was observed ($|U| = 1.98$, $P = 0.047$, power = 0.33).

We determined the relationship between IR and the methylation change of CpG 3 before and after treatment. However, there was no significant correlation (data not shown). Furthermore, we did not identify a significant correlation between IR and ETI (data not shown).

4. Discussion

In the present study, we could not distinguish between healthy controls and unmedicated patients with MD, or between unmedicated and medicated patients with MD based on the results of two-dimensional hierarchical clustering analysis using DNA methylation rates of CpG units in the CpG island of the SLC6A4 gene. Two-way hierarchical clustering analysis and two-factor ANOVA using genotypes (*l* allele and *s/s* alleles) and diagnoses (healthy controls and patients with MD) as fixed factors revealed that 5-HTTLPR itself did not affect DMR. Comparisons of DNA methylation rates for each CpG unit indicated that analysis of the pre-treatment methylation rate of CpG 3 might be useful for determining the therapeutic responses to antidepressants. In addition, correlation analysis using the DNA methylation rate for each CpG unit revealed that DNA methylation rates of several CpG sites were associated with characteristics of patients with MD: CpG 3 and CpG 76 for early adversity in MD patients, and CpG 76 for severity of MD symptoms. We also analyzed the data with respect to the therapeutic response. Although we could not distinguish between patients with high and low therapeutic responses by clustering analysis using all the DNA methylation rates, correlation analysis revealed the pre-treatment DNA methylation rate of CpG 3 correlated with the therapeutic response and an individual comparison of CpG 3 revealed that the DNA methylation rate of CpG 3 was significantly different.

Many epigenetic changes are reversible in response to both external and internal stimuli, indicating a mechanism for interactions of the genome with the environment. Notably, various factors associated with complex diseases, for example nutrition, chemical exposure, and psychosocial stress, have been correlated with epigenetic changes, particularly DNA methylation (Feil and Fraga, 2011). Such findings integrate epigenetic research with population-based epidemiological research investigating not only diagnosis methods (Mill and Heijmans, 2013), but also drug responses and the effect of therapeutic drugs (Duarte, 2013). We recently reported that classification based on DNA methylation profiles of the CpG island in the promoter of exon I of the BDNF gene could be a valuable diagnostic biomarker for MD (Fuchikami et al., 2011). In addition, D'Addario et al. (2013) reported the possible association of changes in DNA methylation of the BDNF promoter with the onset of and/or susceptibility to bipolar disorder, or with mood-stabilizing compounds and antidepressants. In contrast with results of two previous studies, the present study suggested possible associations of DNA methylation of the SLC6A4 gene with the diagnosis of MD, antidepressant treatment, early adversity, and therapeutic responses only when analyses based on data for each specific CpG unit were used, but not when using analyses of all DMR.

In general, an association between DNA methylation and transcriptional activity is considered an important marker of functional relevance (Stranger et al., 2007; Dimas et al., 2009).

With regard to the SLC6A4 gene, Philibert and colleagues reported a significant association between SLC6A4 promoter methylation levels and mRNA levels using peripheral blood from patients with depression (Philibert et al., 2007). However, it is becoming evident that the relationship between DNA methylation

and gene expression is not always tightly linked. In terms of biomarker identification, the fact that methylation is closely associated with a specific pathological condition is thought to be important, even if methylation does not induce gene silencing (Ushijima, 2005). 5-HTT is associated with pathological conditions and the treatment of MD (Lesch and Gutknecht, 2005; Murphy et al., 2008). In this context, the present study only analyzed DNA methylation but not gene expression levels. Future studies examining DNA methylation in the promoter of the SLC6A4 gene together with gene expression will be needed to reveal the functional relevance of DNA methylation of this gene in the pathophysiology of MD.

Thus, although the biological significance of changes in DNA methylation rates for specific CpG units of the SLC6A4 gene is not known, the results of the present study suggest that such information may provide important clinical information regarding MD.

Previous reports described the possibility of a joint effect of 5-HTT methylation and 5-HTTLPR *s* allele carriage on the risk for depression (Olsson et al., 2010) and the effect of CpG islands on DNA methylation by distantly-located SNPs in a fraction of genes (Bell et al., 2011; Soto-Ramirez et al., 2013). Therefore, we evaluated the influence of the 5-HTTLPR allele on DMR. Contrary to the results of Olsson et al. (2010), we found no significant effect on the DNA methylation rates of any CpG unit by genotype. The discrepancy between these studies may be attributed to differences in the tissues (buccal cells vs blood cells) and analytic methods used (Olsson divided the CpG island of the SLC6A4 gene into sub-regions according to co-variation in methylation levels between CpG units).

Changes in the DNA methylation rate of CpG 3 were the most intriguing finding of this study. The DNA methylation rate of CpG 3 correlated negatively with ETISR-SF, and was significantly changed after antidepressant treatment, but correlated positively with therapeutic responses. Several studies have reported that increased DNA methylation of several CpG units within the SLC6A4 gene was associated with early adversity (Ouellet-Morin et al., 2013) and antidepressant treatment (Kang et al., 2013). Although those studies investigated different and smaller regions of the SLC6A4 gene for DNA methylation analyses compared with the present study, they suggested the versatility of DNA methylation of specific CpG units of the SLC6A4 gene induced by environmental factors. In addition, the high prevalence of early adversity in treatment-resistant depressed patients was reported recently (Tunnard et al., 2013). Although the function of CpG 3 methylation of the SLC6A4 gene is unknown, the sequence of changes in the methylation rates at this region (negative correlation with early adversity and positive correlation with response rates) indicates the possible involvement of this factor in the mechanism of resistance to antidepressant treatment.

In summary, analyses using DNA methylation rates for specific CpG units of the SLC6A4 gene, but not analyses based on DNA methylation rates of all CpG units, may be a potential biomarker for the diagnosis, severity of symptoms, early adversity, and history of antidepressant treatment in patients with MD. Notably, the pre-treatment DNA methylation rate for CpG 3 may be associated with antidepressant treatment responses. In addition, the DNA methylation rate for CpG 3 indicated possible involvement in the mechanism of treatment resistance associated with early adversity. Further studies examining the function of these CpG units will help to establish more sophisticated methods for the diagnosis and treatment of MD.

Contributors

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Conflict of interest

The research was conducted in the absence of any commercial or financial relationships.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jpsychires.2014.02.002>.

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