Effect of mood stabilizers on DNA methylation in human neuroblastoma cells

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Abstract

Unraveling the epigenetic status of neuronal cells in the brain is critical to our understanding of the pathophysiology of psychiatric disorders, which may reflect a complex interaction between genetic and environmental factors. Several epigenetic studies of mood disorders have been conducted with postmortem brains. However, proper interpretation of the results is hampered by our scant understanding of the effects of mood stabilizers on the epigenetic status of neuronal cells. We performed both comprehensive and gene-specific analyses to examine DNA methylation in human neuroblastoma SK-N-SH cells treated with three mood stabilizers: lithium, valproate and carbamazepine. Measurement of the level of DNA methylation of about 27 000 CpG sites revealed a profound epigenetic effect of lithium, compared with the two other mood stabilizers. In addition, we found that the mood stabilizers have common epigenetic targets and a propensity to increase DNA methylation. Gene-specific analysis involved detailed analysis of the methylation of promoter regions of SLC6A4 and BDNF, both of which have been reported to show altered DNA methylation in bipolar disorder patients or suicide victims, by extensive bisulfite sequencing. We did not observe significant changes in DNA methylation at BDNF promoter IV. However, we found that CpG sites of SLC6A4, which were hypermethylated in patients with bipolar disorder, were hypomethylated in the neuroblastoma cells treated with mood stabilizers. Our results will contribute to a better understanding of the epigenetic changes associated with mood disorders, and they also provide new insight into the mechanisms of action of mood stabilizers.

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Key words: BDNF, carbamazepine, lithium, serotonin transporter, valproate.

Introduction

Severe mood disorders, such as major depression (MD) and bipolar disorder (BD), are accompanied by heavy social burdens. Although a genetic predisposition has been identified in both MD patients and BD patients, the pathogenesis and pathophysiology of these disorders remain largely unclear (Kato, 2008). Large-scale, genome-wide association studies have identified candidate genes for both MD and BD (Psychiatric GWAS Consortium Steering Committee, 2009; Psychiatric GWAS Consortium Coordinating Committee, 2009; Hek et al., 2013). However, their effects are generally small in magnitude, and they do not fully account for the etiology of mood disorders. A complex interaction between genetic and environmental factors is believed to play a critical role in the pathophysiology of mood disorders. Because epigenetic status, such as DNA methylation and histone modifications, is affected by environmental insults, an understanding of the epigenetic status of neuronal cells in the brains of patients with mood disorders will provide important insights into the pathophysiology of these disorders (Kato et al., 2005; Mill and Petronis, 2007; Petronis, 2010).

Several epigenetic studies of mood disorders have been conducted with postmortem brains. The findings...
include hypermethylation of PRIMA1 in the prefrontal cortex (PFC) of MD subjects, detected by array screening (Sabunciyan et al., 2012); hypermethylation of BDNF, DBN1 and SLC6A4 in the PFC of BD subjects (Sugawara et al., 2011; Rao et al., 2012); hypomethylation of MB-COMT in the PFC of BD subjects (Abdolmaleky et al., 2006); hypomethylation of HCG9 in various brain regions of BD subjects (Kaminsky et al., 2012); and altered DNA methylation of genes related to glutamatergic neurotransmission, GABA neurotransmission and brain development in the PFC of BD subjects, detected by array screening (Mill et al., 2008). These reports clearly suggest that genes involved in important neuronal functions or implicated in the genetic studies manifested epigenetic alterations in the brain. Although these important studies have advanced our understanding of the pathophysiology of mood disorders, they clearly have limitations. In addition to small sample sizes and the potential confounding effect of cause of death, antemortem and postmortem conditions remain unclear (Iwamoto and Kato, 2009; Pidsley and Mill, 2011; Nishioka et al., 2012).

Medication status is one of the most significant variables affecting epigenetic status in the brain, because patients with psychiatric disorders generally take medication. In addition, valproate, a major mood stabilizer used for patients with BD, is known to act as a histone-deacetylase (HDAC) inhibitor (Gottlicher et al., 2001), and HDAC inhibitors activate gene expression. However, little is known about the epigenetic action of most of the drugs used for psychiatric diseases.

To gain new insight into the mechanisms of action of such drugs and a better understanding of the epigenetic changes reported in psychiatric patients, we have systematically tested the epigenetic effects of medications in a human neuroblastoma cell line. Here, we report the epigenetic effects of three mood stabilizers, namely lithium, valproate and carbamazepine, as revealed by both comprehensive and site-specific approaches. We separately cultured a neuroblastoma SK-N-SH cell line with the three mood stabilizers, using effective therapeutic concentrations. For the comprehensive approach, we examined changes in DNA methylation at about 27 000 CpG sites using an Illumina Infinium platform (Illumina Inc., USA). In addition, for the site-specific approach, we examined the DNA methylation status of the promoter regions of BDNF and SLC6A4 in detail by bisulfite sequencing.

Among the epigenetic candidates, promoter regions of both genes have been extensively examined for their role in not only mood disorders but also various other neuropsychiatric conditions. BDNF encodes a neurotrophin expressed in various brain regions (Murer et al., 2001) and plays an important role in neuronal functions, including differentiation and development of neurons and synaptic plasticity (Huang and Reichardt, 2001). We examined the DNA methylation status of the promoter IV region of BDNF. This region was hypermethylated in Wernicke's area in suicide victims (Keller et al., 2010) and had neuronal-activity-dependent changes in DNA methylation in cultured rodent brain cells (Chen et al., 2003; Martinovich et al., 2003).

SLC6A4 encodes a protein that transports synaptic serotonin into presynaptic neurons and is one of the most intensively studied genes in the field of psychiatry. We examined the downstream region of the promoter-associated CpG island, where hypermethylation was found in the PFC as well as in lymphoblastoid cell lines of patients with BD (Sugawara et al., 2011).

Methods

Cell culture

Cells from a human neuroblastoma cell line, SK-N-SH (American Type Culture Collection), were cultured for 8 d in Eagle's minimal essential medium containing 10% fetal bovine serum and supplemented with one of the three drugs. The medium was changed on days 2, 5 and 8. On day 9, the cells were retrieved to extract DNA. For the purpose of validating the bead array and bisulfite cloning methods, we prepared two independent cell cultures without drugs. Each of other drug concentrations was tested once.

Drugs

We used three mood stabilizers: valproic acid sodium salt (C4024), lithium chloride (L4408), and carbamazepine (C4024) (Sigma-Aldrich, USA). Valproate and lithium were directly dissolved into the medium. Carbamazepine was dissolved in dimethylsulfoxide (DMSO) before it was added to the medium. The concentration of each drug in the medium was based on the therapeutic concentrations for BD (Allen et al., 2006; Goodwin and Jamison, 2007; Nolen and Weisler, 2013). We prepared the minimum and maximum therapeutic concentrations: valproate, 0.3 and 0.6 mM; lithium, 0.6 and 1.2 mM; and carbamazepine, 0.05 and 0.1 mM.

DNA extraction and bisulfite modification

We used the standard phenol–chloroform protocols to extract DNA. Sodium bisulfite modification was
performed using an Epitect Bisulfite Kit (Qiagen, The Netherlands) for cloning and sequencing analysis and an EZ DNA Methylation Kit D500 (ZymoResearch, USA) for the Infinium HumanMethylation assay (Illumina Inc., USA).

**Illumina infinium methylation assay**

We quantified DNA methylation at 27,578 CpG sites using an Infinium HumanMethylation27 BeadChip, according to the manufacturer’s instructions. Briefly, a total of 500 ng of bisulfite-modified DNA was isothermally amplified and was then fragmented. Fragmented DNA was hybridized to an Infinium HumanMethylation27 BeadChip. A single-base extension reaction was performed to incorporate fluorescently labeled nucleotides. The Infinium platform yields DNA methylation level as a $\beta$ value, ranging from 0 (no methylation) to 1 (complete methylation). The $\beta$ value is calculated with the following equation:

$$\beta \text{ value} = \frac{\text{methylated intensity}}{\text{methylated intensity} + \text{unmethylated intensity} + 100}$$

where methylated intensity and unmethylated intensity are the signal intensities of methylated and unmethylated probes, respectively.

**Data analysis**

We calculated the difference in $\beta$ values between drug-treated cells and the corresponding control for each probe. For lithium and valproate treatments, the average $\beta$ values of two independent controls were used for difference calculation. For carbamazepine treatment, the $\beta$ value of treatment with DMSO alone was used for difference calculation. If the absolute difference was $>0.15$, the probe was identified as differentially methylated (Bibikova et al., 2006). A clustering analysis was performed with a CLC Genomics Workbench (CLC Bio, Denmark) while those treated with mood stabilizers were clustered together, those treated with mood stabilizers were clustered separately (Fig. 1c).

We then identified differentially methylated genes based on differences in $\beta$ values. Among the 14,475 genes covered by this platform, 345, 64 and 64 genes were hypermethylated and 138, 36 and 14 genes were hypomethylated in cells treated with lithium, valproate and carbamazepine, respectively. The numbers of these genes indicate that all three mood stabilizers had a propensity to increase rather than decrease DNA methylation. The Venn diagram shows that

**TA-cloning of bisulfite-PCR products and DNA sequencing analysis**

The sequences of bisulfite-PCR primers for SLC6A4 were 5'-TTTTTAGTTGTTGGGTATTGTGTTA-3' (forward) and 5'-AAAAACTTACAACCTTAAAAA-CCC-3' (reverse), according to a previous study (Sugawara et al., 2011). Those for BDNF were 5'-GGGGGAGGATTAATTGAGTTTTG-3' (forward) and 5'-CCCATACACAAAAACCTCAAATATTTC-3' (reverse), also according to a previous study (Keller et al., 2010). Agarose gel electrophoresis confirmed that amplified products were single-banded. The PCR products were then extracted from the agarose gel with a MinElute Gel Extraction Kit (Qiagen, The Netherlands). The extracted samples were then cloned into *E. coli* DH5α-competent cells with a TOPO TA Cloning Kit (Life Technologies, USA). At least 20 colonies per one experimental condition were used for colony PCR, and the colony-PCR products were subjected to DNA sequencing. Similar to the analysis of the bead array, DNA methylation levels of two independent controls were used for difference calculation for both lithium and valproate treatments. For carbamazepine treatment, DNA methylation level of treatment with DMSO alone was used for difference calculation. Results of bisulfite sequencing were visualized with QUMA software (Kumaki et al., 2008).

**Results**

**Alteration of DNA methylation by mood stabilizers**

We found very strong correlation in DNA methylation levels over a wide range of CpG sites ($R^2=0.992$, Pearson’s correlation) between two cell lines that were independently cultured without drugs (Fig. 1a). In contrast, the DNA methylation status of the lithium-treated cells clearly differed from that of the control ($R^2=0.960$) (Fig. 1b). Cells treated with the other two mood stabilizers also showed a wide range of epigenetic effects when compared with control cells ($R^2=0.977$ for valproate and $R^2=0.980$ for carbamazepine; data not shown). In an unbiased hierarchical clustering analysis using $\beta$ values of all CpG sites, two cell lines independently cultured without drugs and cell lines treated with DMSO alone were clustered together, while those treated with mood stabilizers were clustered separately (Fig. 1c).

We then identified differentially methylated genes based on differences in $\beta$ values. Among the 14,475 genes covered by this platform, 345, 64 and 64 genes were hypermethylated and 138, 36 and 14 genes were hypomethylated in cells treated with lithium, valproate and carbamazepine, respectively. The numbers of these genes indicate that all three mood stabilizers had a propensity to increase rather than decrease DNA methylation. The Venn diagram shows that
valproate and carbamazepine influenced similar genes, and lithium altered the methylation level of those genes and many others as well (Fig. 2).

Epigenetic changes in common to all three mood stabilizers included 41 hypermethylated and 11 hypomethylated genes. Clustering analysis of DNA methylation profiles for these 52 genes successfully discriminated between controls and drug-treated cells (Fig. 2b). Gene ontology analysis of these common alterations revealed that terms related to development are repeatedly included in the biological process category and that those encoding secreted molecules or working in the extracellular region are included in the cellular component category (Table 1). Results of gene ontology analysis of specifically valproate- or carbamazepine-associated changes in DNA methylation were very similar to those for the epigenetic changes common to all three drugs (data not shown). On the other hand, specifically lithium-associated changes in DNA methylation included a wide range of gene ontology terms. Importantly, they included terms related to neuronal functions such as cell–cell signaling, synaptic transmission, locomotion and neurotransmitter receptor activity (Supplementary Tables S1 and S2).

Site-specific analysis of SLC6A4 and BDNF

Significant hypermethylation was reported in the downstream region of the promoter-associated CpG island, called the CpG island shore (Irizarry et al., 2009), in SLC6A4 in postmortem brains of BD subjects (Sugawara et al., 2011) and in promoter IV of BDNF in postmortem brains of suicide victims (Keller et al., 2010). Based on these previous reports, we examined the same regions by means of bisulfite PCR and extensive cloning–sequencing analysis. To validate our experimental approach, we first conducted a comparison of DNA methylation levels of SLC6A4 in two cell lines independently cultured without drugs (Fig. 3a).

The DNA methylation levels of 5 CpG sites in two experiments showed a very strong correlation ($R^2=0.947$, Pearson’s correlation) and little variation (the average absolute difference of five CpG sites was 3.8%) (Fig. 3a), indicating the robustness of our assay. In this analysis, the cells were separately cultured with the three mood stabilizers, at a concentration equivalent to either the minimum or maximum blood level for therapeutic efficacy, and they underwent bisulfite sequencing (Fig. 3b). We compared the level of DNA methylation in drug-treated cells with that of
the average however, we found that the third and fourth CpG sites were hypomethylated by all mood stabilizers, except for valproate, which had a concentration of 0.3 mM (Fig. 3d).

Next we examined the DNA methylation status of BDNF promoter IV, also by means of bisulfite sequencing analysis. In contrast to the findings for SLC6A4, no significant changes were detected in drug-treated cells at the four CpG sites that showed significant hypermethylation in suicide victims (Keller et al., 2010) (Fig. 4).

In the bead array platform, there are four probes that measured the DNA methylation level of SLC6A4 (cg22584138 and cg05016953) or BDNF (cg27351358 and cg16257091). Among them, one probe for SLC6A4 (cg22584138) corresponds to the CpG1 site. Consistent with the bisulfite-cloning analysis, this site and other three probes showed no alterations of DNA methylation level by the drug treatment (Supplementary Table S3).

Discussion

We examined the epigenetic effects of three major mood stabilizers in a human neuroblastoma cell line by performing both comprehensive and gene-specific analyses with bisulfite sequencing. Our comprehensive analysis unexpectedly revealed a profound epigenetic effect of lithium compared with the two other mood stabilizers. In addition, we found that there was a significant overlap of the affected genes among the three mood stabilizers, suggesting that they have a common mechanism of epigenetic regulation. By gene ontology analysis, we found that epigenetic changes associated with mood disorders involved development-related genes and genes in the extracellular space. Genes with commonly altered methylation included those previously implicated in mood disorders. For example, expression of NF-κB inhibitor (NFKBIZ) was significantly up-regulated and was correlated with gray-matter volume of the caudate in mood disorders (Savitz et al., 2013). Down-regulation of PAX5 and MT1H in the postmortem brains of BD subjects has also been reported (Benes et al., 2007; Sequeira et al., 2012). In addition, peptidylglycine α-amidating mono-oxygenase (PAM), which encodes an enzyme for maturation of neuropeptides such as NPY and PACAP by amidation (Bradbury and Smyth, 1991), may be involved in mood regulation. Mice heterozygous for the Pam gene showed abnormalities in emotional responses (Gaier et al., 2010), and lithium treatment increased Pam expression in slices of rat cortex (Brandish et al., 2005). Other commonly altered genes included annexin 2 (ANXA2) and genes encoding its
interacting partner S100 proteins (S100A2 and S100A6). They have roles in cellular transport, the cytoskeleton and transduction of various signals. The drug-specific methylation changes will also provide important information to help in elucidating the mechanism of the action of these drugs, although these will include non-specific and less important changes.

The mood stabilizers showed a propensity to increase rather than decrease DNA methylation. This seems to contradict the well-known effect of valproate as an HDAC inhibitor. Valproate generally activates gene expression and has been reported to induce hypomethylation. We observed that the average β values of lithium and carbamazepine treatments were

<table>
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<th>Category</th>
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<th>Count</th>
<th>%</th>
<th>p value</th>
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The underlined genes are hypomethylated. Data are limited to the terms whose p values are <0.05 and gene counts are ≥6.
increased compared with those of controls (0.354 vs. 0.349 for lithium and 0.355 vs. 0.346 for carbamazepine). On the other hand, valproate treatment showed a decreased average β value compared with controls (0.345 vs. 0.349). These results suggest that each of the mood stabilizers has a different impact on global DNA methylation levels. DNA methylation analysis of SLC6A4 suggested that valproate decreased DNA methylation at the specific CpG sites implicated in BD and that both effects have important regulatory roles. By gene-specific analysis, we found hypomethylation of SLC6A4 CpG 3 and CpG 4 in the neuroblastoma cells treated with the minimum or maximum concentration of mood stabilizers. Remarkably, these two CpG sites were reported to be hypermethylated in the PFC of patients with BD (Sugawara et al., 2011), implying that the epigenetic effect of mood stabilizers on these CpG sites is associated with drug efficacy. On the other hand, the level of DNA methylation of promoter IV of BDNF was not significantly affected by the minimum or maximum concentration of mood stabilizers. These results suggest a site-specific preference of the effect of mood stabilizers and that there was little effect on BDNF promoter IV. Because regulation of BDNF transcription is very complex, with many alternative splice variants (Pruunsild et al., 2007), methylation levels of other BDNF promoters are worth testing.

The limitations of this study should be noted. First, it was based on in vitro experiments, so it is possible that the experimental conditions did not precisely reflect those of an in vivo brain. Second, concentration and duration of drug treatment may drastically affect results. Although we tested both minimum and maximum therapeutic concentrations of mood stabilizers in our gene-specific analysis, we did not test the duration of treatment. It should be also noted that we could not apply a rigorous statistical approach in this study. Therefore, independent experiments will be necessary to confirm the detailed findings.

Third, we did not elucidate the functional significance of DNA methylation changes in this study. Comprehensive gene expression analysis will help elucidate the consequences of changes in DNA methylation. It should be noted that we did not detect the gene expression level of SLC6A4 in this cell line by

![Fig. 3. Effect of mood stabilizers on DNA methylation of the downstream region of the promoter-associated CpG island of SLC6A4.](a) Comparison of two different control cultures of SK-N-SH lines (◊ and ■) that were cultured without drugs. Bisulfite sequencing showed strong reproducibility. (b) A typical example of bisulfite sequencing analysis. One row indicates the data derived from a single bacterial colony. Non-methylated and methylated cytosine are represented by O and ●, respectively. In each sample, at least 20 independent bacterial colonies were used for sequencing analysis. (c) Changes in DNA methylation of SLC6A4 induced by each of the three mood stabilizers. (d) Changes in DNA methylation of SLC6A4 by mood stabilizers, focusing on CpG3 and CpG4 from (c). Chromosomal locations of the CpGs analyzed are as follows: chr17:28562220, chr17:28562222, chr17:28562264, chr17:28562289 and chr17:28562334.
the standard Taqman probe-based assay (data not shown), suggesting that the functional significance of methylation changes in SLC6A4 cannot be determined on the basis of expression levels in this cell line.

Fourth, because the methods employed in this study were based on bisulfite modification, we could not discriminate between 5-methylcytosine and other cytosine modifications such as 5-hydroxymethylcytosine. Therefore, altered levels of DNA methylation in this study should be interpreted as the changes of status of the cytosine modification.

Despite these limitations, our results provide important insight into epigenetic regulation by mood stabilizers and will facilitate interpretation of DNA methylation changes reported in studies of mood disorders. In particular, CpG sites of SLC6A4 that were hypermethylated in BD subjects were hypomethylated in neuroblastoma cells treated with mood stabilizers, suggesting that epigenetic regulation by mood stabilizers is related to their therapeutic efficacy.

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Statement of Interest
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Supplementary material
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