Regular Article

Investigating DNA Methylation of SHATI/NAT8L Promoter Sites in Blood of Unmedicated Patients with Major Depressive Disorder

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Major depressive disorder (MDD) is one of the most common psychiatric diseases. However, early detection and diagnosis of MDD is difficult, largely because there is no known biomarker or objective diagnostic examination, and its diagnosis is instead based on a clinical interview. The aim of this study was to develop a novel diagnostic tool using DNA methylation as a blood biomarker. We sought to determine whether unmedicated patients with MDD showed significant differences in DNA methylation in the promoter region of the SHATI/N-acetyltransferase 8 like (SHATI/NAT8L) gene compared to healthy controls. Sixty participants with MDD were recruited from all over Japan. They were diagnosed and assessed by at least two trained psychiatrists according to DSM-5 criteria. DNA was extracted from peripheral blood. We then assessed DNA methylation of the SHATI/NAT8L promoter regions in patients with MDD by pyrosequencing. Methylation levels of the SHATI/NAT8L promoter region at CpG sites in peripheral blood from unmedicated patients were significantly higher than in healthy controls. In contrast, medicated patients with MDD showed significantly lower methylation levels in the same region compared to healthy controls. Since previous studies of DNA methylation in MDD only assessed medicated patients, the methylation status of the SHATI/NAT8L promoter region in unmedicated patients presented herein may prove useful for the diagnosis of MDD. To our knowledge, this is the first attempt to measure methylation of the SHATI/NAT8L gene in drug-naïve patients with psychiatric diseases. Based on our findings, methylation of SHATI/NAT8L DNA might be a diagnostic biomarker of MDD.

Key words major depressive disorder; diagnostic biomarker; SHATI; DNA methylation; unmedicated patient

INTRODUCTION

The prevalence of psychiatric diseases, such as major depressive disorder (MDD), schizophrenia, and bipolar disorder, is increasing worldwide. Of these conditions, MDD is one of the most commonly resistant to treatment.1 For example, antidepressants are associated with a relatively low response rate in 70–80% of patients.2–4 The reliability of psychiatric disease diagnoses is an issue of concern, largely because there is no known biomarker or objective diagnostic examination, and physicians make a diagnosis based on the International Classification of Disease-10 by WHO or the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) by the American Psychological Association. As a new approach to diagnosing mental disorders based on the dimensions of observable behavior and neurobiological measures, the Research Domain Criteria (RDoC) project was initiated.5 Elucidation of biomarkers for MDD based on neurobiological measures, such as the RDoC concept, are expected to effect earlier intervention and treatment,5 and consequently, improve the QOL of patients, families, and society as a whole.

The SHATI/N-acetyltransferase 8 like (SHATI/NAT8L) shows N-acetyl transfer activity, which encodes a protein synthesizing N-acetylaspartate (NAA) from aspartate and acetyl-CoA. NAA is biosynthesized to N-acetylaspartylglutamate (NAAG) by condensation with glutamic acid by NAAG synthetase.7 The levels of both NAA and NAAG have been reported to be decreased in the hippocampus of postmortem brains of patients with psychiatric diseases such as depression.8 Given this evidence, SHATI/NAT8L is likely associated with MDD. In this study, we focused on SHATI/NAT8L as a biomarker for psychiatric diseases. We have previously reported that brain levels of Shati/Nat8l mRNA changed in a mouse model of depression (mice exposed to forced swim stress or chronic social defeat stress).9,10

Given that epigenetic changes are known to be involved in psychiatric diseases,11–13 attempts to find biomarkers using SHATI/NAT8L are worthwhile. It has been reported that methylation of SHATI/NAT8L is altered in the promoter region in a mouse model of schizophrenia, possibly providing a biomarker.14 Methylation of DNA is a chemical reaction carried out by DNA methyltransferase (DNMT), which adds a carbon
atom to the fifth position of the pyrimidine ring of cytosine. Methylation mainly occurs at CpG sites, a nucleotide sequence in which guanine appears next to cytosine. Regions containing a high amount of CpG dinucleotide repeats are known as CpG islands.\(^{15}\) The alteration of methylation of CpG islands is thought to be involved in the regulation of gene expression in many diseases.\(^{16}\) However, gene expression can be influenced by epigenetic changes caused by medication.\(^{17}\) Thus, when searching for peripheral biomarkers, the effect of medication should be carefully considered.\(^{18}\) Specifically, a history of medication should be controlled for in the comparison of patients and healthy control subjects.

In the present study, we collected DNA samples from the blood of unmedicated patients from all over Japan. The extent of methylation of the SHAT1/NAT8L gene was examined. NAA regulated by the SHAT1/NAT8L is used as a biomarker of MDD in specific human brain regions using proton magnetic resonance spectroscopy (\(\text{H-MRS}\)).\(^{19}\) However, methylation of SHAT1/NAT8L from blood might be a biomarker that can be used more easily than using MRS. To our knowledge, this is the first attempt to measure methylation of the SHAT1/NAT8L gene in drug-naïve patients with psychiatric diseases.

**MATERIALS AND METHODS**

**Study Design and Subjects** DNA samples were obtained from 60 patients diagnosed with MDD. Of these, 20 patients (male:12, female:8) from the National Center of Neurology and Psychiatry (NCNP) Biobank were untreated; the other 40 patients (male: 14, female: 26) from Osaka University were treated. All patients had undergone a structured interview using the Mini-International Neuropsychiatric Interview (M.I.N.I.), modified in Japanese, and were diagnosed with MDD by trained psychologists or psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV). Untreated patients had never taken psychotropic drugs such as antidepressants, antipsychotics, or mood-stabilizers. In treated patients, trained psychiatrists confirmed that patients had taken antidepressants, antipsychotics, or mood-stabilizers by checking medicine notebooks and medical records. Patient’s age is 12–60 years. 69 healthy volunteers aged 12–60 years were recruited from the NCNP Biobank and Osaka University as controls. In unmedicated patients, we obtained blood samples at initial diagnosis. In medicated patients, we collected blood samples from the COCORO consortium at the University of Osaka (Permit number 423) and the COCRORO consortium at the University of Osaka (Permit number A2018-009) and the COCRORO consortium at the University of Osaka (Permit number 423) were used. All participants provided written informed consent.

**Bisulfite Conversion** Genomic DNA was used in bisulfite reactions, in which unmethylated cytosine residues were converted to uracil residues, using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer’s guidelines and published methods.\(^{19}\) DNA Protect Buffer, bisulfite solution, and DNA (200 ng) were mixed and incubated under the cycle conditions recommended by the manufacturer using a TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa, Shiga, Japan). The bisulfite conversion thermal cycler conditions were as follows: 1 cycle at 95°C for 5 min, 1 cycle at 60°C for 10 min, 1 cycle at 95°C for 5 min, and 1 cycle at 60°C for 10 min. Converted DNA was then purified and eluted with elution buffer.

**PyroMark PCR** To prepare a single-stranded PCR product for use in the subsequent pyrosequencing procedure, one PCR primer must be labeled with biotin at its 5’ mark. PCR primers were designed by PyroMark Assay Design Software 2.0 (www.qiagen.com). PyroMark PCR was performed using the PyroMark PCR Kit (Qiagen) according to the manufacturer’s guidelines and published methods.\(^{20}\) PCR Master Mix, CoralLoad Concentrate, primers, ribonuclease (RNase)-free water, and converted DNA (20 ng) were briefly mixed. The reaction was performed under the recommended cycle conditions with a TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa): 1 cycle at 95°C for 15 min; 45 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 10 min. The PCR primer sequences used were as follows: 5’-GGAGTT ATGGGAGATTAAAGATTA-3’ and 5’-AAAAAAAAC AATACTCTTAACAAGATACCC-3’ as primers for the CpG sites from the transcription start site (TSS); −1714, −1700, −1696 base pairs (bp) (up and down), 5’-GGGTAATTGTTGAGAAGGTTT-3’ and 5’-CCCCCTCAATATCTAAAACCC-3’ as primers for the CpG sites from TSS; −1532, −1509, −1492, −1482, and 1480 bp (up and down). Primers were designed using PyroMark Assay Design Software 2.0 (Qiagen). The conditions for designing primers were as follows: %GC >55, CpG Is length: 300–4000 bp, and observed/expected CpG >0.65.

**Pyrosequencing** Pyrosequencing was performed according to the PyroMark Q24 User Manual and previously

| Sample          | n  | Age (years)   | Sex (male/female) |
|-----------------|----|---------------|-------------------|
| Control         | 68 | 47.29 ± 1.94  | 30/38             |
| MDD Unmedicated | 20 | 40.75 ± 2.58  | 12/8              |
| MDD Medicated   | 40 | 55.18 ± 1.00  | 14/26             |

Data are expressed as mean ± standard errors of the means (S.E.M.).
published reports. Streptavidin beads (GE Healthcare, Buckinghamshire, U.K.), PyroMark binding buffer (Qiagen), PCR product, and water were mixed and then agitated for 10 min using a mixer at 1400 rpm. The sequencing primer was diluted to 0.3 µM in annealing buffer (Qiagen), and the solution was added to each PyroMark Q24 Plate (Qiagen). PCR products were separated, denatured, washed, and added to the sequencing primer in annealing buffer using the PyroMark Q24 Vacuum Workstation (Qiagen). The primer was annealed by heating to 80°C for 2 min and then cooling to room temperature (25°C). PyroMark Gold Q96 reagents (Qiagen), which were used for the reaction and the PCR product plate, were set and analyzed by PyroMark Q24 device (Qiagen). The following primers were designed by PyroMark Assay Design Software 2.0 (www.qiagen.com): 5’-AGA GAT ATT TGA GTA TAG GGT TTT AGT-3’ as the primer for CpG sites from TSS: −1714, −1700, −1696, −1532, −1509, −1492, −1482, and −1480. These primers were also designed using PyroMark Assay Design Software 2.0 (Qiagen).

Statistical Analysis All data are shown as the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using one-way ANOVA, followed by the Bonferroni’s post-hoc test (Prism version 5). The level of statistical significance was set at p < 0.05. Cohen’s-d was used as a measure of the effect size. Cohen defined effect sizes as “small, d = 0.2,” “medium, d = 0.5,” and “large, d = 0.8.” Receiver operating characteristic (ROC) curve analysis was used to determine the utility of the biomarker for MDD using pyrosequencing in predicting group status. The best possible cut-off value in the ROC curve was identified with the highest Youden index.

RESULTS Methyltion Rates Is Increased in Unmedicated Patients with MDD but Not Medicated Patients with MDD We analyzed the methylation rates at CpG sites of the SHATI/NAT8L promoter in peripheral blood collected from unmedicated patients with MDD. We focused on the CpG sites −1714, −1700, −1696, −1532, −1509, −1492, −1482, and −1480 bp (8CpGs) from TSS where the DNA methylation pattern has been shown to change in patients with psychiatric disorder. As shown in Table 2, methylation rate in peripheral blood from unmedicated patients with MDD was significantly increased at CpG sites −1700 (df = 84, p = 0.023, t = −2.49), −1532 (df = 84, p = 0.025, t = −2.64), −1492 (df = 80, p = 0.035, t = −3.23) and −1482 (df = 72, p < 0.001, t = −2.78) bp compared to healthy controls. The effect size at CpG site −1700 (Cohen’s-d = 0.67) and −1532 (Cohen’s-d = 0.70) is medium, and −1492 (Cohen’s-d = 0.87) and −1482 (Cohen’s-d = 0.82) was large. In the ROC analysis, it is shown that the utility of the biomarker for MDD using pyrosequencing have modest sensitivity (0.733) and modest specificity (0.954) at CpG site −1482 (area under the curve (AUC) = 0.868) (Table 3).

Next, we also measured the methylation rates at CpG sites of the SHATI/NAT8L promoter in peripheral blood collected from medicated patients with MDD. We found that the increased methylation rates in the unmedicated patient with MDD were significantly decreased by medication at CpG sites −1714, −1700, −1532, −1509, −1492, −1482, and −1480. These primers were also designed using PyroMark Assay Design Software 2.0 (Qiagen).

Table 2. Methylation Rates of SHATI/NAT8L DNA in Peripheral Blood from Unmedicated and Medicated Patients with MDD

| CpG site (position from the transcription start site) | Control Unmedicated | MDD Unmedicated | MDD Medicated | Cohen’s-d effect size |
|---------------------------------------------------|---------------------|-----------------|---------------|----------------------|
| −1714                                             | 78.23 ± 0.29        | 78.77 ± 0.56    | 78.40 ± 0.34  | 0.23                 | 0.16               |
| −1700                                             | 49.78 ± 0.28        | 51.47 ± 0.82*   | 50.97 ± 0.30  | 0.67                 | 0.20               |
| −1696                                             | 31.77 ± 0.42        | 32.82 ± 0.50    | 33.81 ± 0.30  | 0.26                 | 0.50               |
| −1532                                             | 33.51 ± 0.36        | 36.52 ± 0.75*   | 34.08 ± 0.41**| 0.70                 | 0.89               |
| −1509                                             | 34.70 ± 0.30        | 36.63 ± 0.52    | 35.33 ± 0.24* | 0.18                 | 0.75               |
| −1492                                             | 42.58 ± 0.28        | 44.63 ± 0.53*   | 41.88 ± 0.67**| 0.87                 | 0.75               |
| −1482                                             | 48.00 ± 0.36        | 52.71 ± 0.72*** | 47.65 ± 0.49***| 0.82                 | 0.88               |
| −1480                                             | 31.04 ± 0.28        | 31.78 ± 0.72    | 31.33 ± 0.47  | 0.33                 | 0.16               |

Data are expressed as mean ± S.E.M. Significance is set at *p < 0.05, **p < 0.005 vs. control, †p < 0.05, ††p < 0.01, †††p < 0.005 vs. unmedicated.

Table 3. ROC Curve Analysis in Unmedicated Patients with MDD Compared with Controls

| CpG site (position from the transcription start site) | Cut-off value | Sensitivity | Specificity | AUC   |
|---------------------------------------------------|---------------|-------------|-------------|-------|
| −1714                                             | >78.51        | 0.550       | 0.574       | 0.552 |
| −1700                                             | >51.45        | 0.438       | 0.754       | 0.642 |
| −1696                                             | >33.56        | 0.600       | 0.557       | 0.578 |
| −1532                                             | >36.39        | 0.474       | 0.866       | 0.655 |
| −1509                                             | >36.27        | 0.722       | 0.523       | 0.586 |
| −1492                                             | >43.04        | 0.772       | 0.619       | 0.734 |
| −1482                                             | >51.25        | 0.733       | 0.954       | 0.868 |
| −1480                                             | >34.43        | 0.250       | 0.969       | 0.569 |

ROC: receiver operating characteristic.
Methylation levels of DNA from blood may be useful for the diagnosis of MDD. It has been reported that methylation of the BDNF gene is lower in the blood of patients with MDD. A combination of biomarkers is best for reliable diagnosis. In the present study, because medical treatment may induce epigenetic changes, we investigated methylation levels of DNA from unmedicated and medicated patients. Methylation levels of the SHATINAT8L promoter region at CpG sites in peripheral blood from unmedicated patients were significantly higher than those of healthy controls. However, medicated patients with MDD showed no significant difference in methylation levels in the same region compared to healthy controls, and showed significantly lower methylation levels compared to unmedicated patients. These findings suggest that SHATINAT8L methylation status could be a diagnostic marker in unmedicated patients.

Methylation of promoter-proximal DNA is involved in suppressing gene expression. On the other hand, in the absence of methylation, gene expression is facilitated. Our findings suggest that SHATINAT8L levels are decreased in the blood because DNA methylation is increased. NAA and NAAG have been reported to be decreased in postmortem brains of patients with depression, which is consistent with decreased expression of the SHATINAT8L gene responsible for NAA and NAAG synthesis in the blood. However, some studies have reported that epigenetic regulation in the brain and peripheral blood may have anti-parallel relationship. In fact, Shati/Nat8l mRNA is increased in the striatum in a mouse model of depression. Two possibilities are considered in this deference relationship of expression between in blood and brain: The first possibility is that altered expression of SHATINAT8L is region- or tissue-specific. The altered region of the brain in the mouse model of depression is the dorsal striatum, which differs from reports of regions where NAA is decreasing in human patients with depression, as there are no reports about the function of SHATINAT8L in the dorsal striatum in depression. The second possible reason is due to species differences. Methylation-related enzymes, such as DNMT1, are decreased in patients with depression, but DNMT1 is increased in a mouse model of depression in same region, resulting in making the differences of gene expression between in human and animals. Therefore, decreased levels of SHATINAT8L in the blood in humans is not considered inconsistent with increased levels in the dorsal striatum in a mouse model of depression. In our study, we wanted to emphasize that we do not need to focus on the brain to diagnose human psychiatric disease, we can readily assess DNA methylation from blood for this purpose.

Methylation-related enzymes such as DNA demethylases (TET enzymes), DNMT, and Methyl-CpG binding protein (MeCP) are involved in inducing methylation changes. TET enzymes oxidize methylated cytosine and remove methyl groups. It has been reported that TET1 levels are higher in patients with psychiatric diseases than in healthy controls. DNMT1 is an enzyme that methylates cytosine and is reportedly decreased in patients with depression. These two proteins are essential for DNA methylation and demethylation and are thought to affect gene expression. MeCP2 is a protein that binds only to methylated CpG sites and suppresses gene transcription. The expression changes of methylation-related enzymes may need to be considered to confirm that SHATINAT8L methylation is a useful blood diagnostic marker, resulting in greater reliability. However, it is presently virtually impossible to investigate these changes in the human brain.

Next, we examined the effect of treatment with antidepressant drugs on DNA methylation. In our study, methylation rates in medicated patients were lower than those in unmedicated patients. It has been reported that treatment with fluoxetine, a selective serotonin reuptake inhibitor (SSRI), reverses DNA methylation via phosphorylation of MeCP2, and regulates transcription in mice. In addition, tricyclic antidepressant drugs, such as amitriptyline and imipramine, and the SSRI paroxetine, decrease DNMT activity. Therefore, medication might affect methylation-related enzymes and alter methylation levels. However, these studies were performed in the brain, not in blood. Understanding these mechanisms of DNA methylation regulation in the blood is very important. Future studies should validate this possibility by measuring the activity of these enzymes in the blood.

Recently, magnetic resonance spectroscopy (MRS) has enabled the determination of the concentration of neurotransmitters and changes in various metabolites. It might be possible to analyze NAA and NAAG via MRS. However, the MRS apparatus is not yet widely available and cannot be used at all hospitals. Thus, diagnosis of psychiatric disorders from blood samples would be more practical than diagnosis by MRS.

Previous studies have reported that age and/or gender affect DNA methylation. Here, we did not observe a correlation or difference between DNA methylation of the SHATINAT8L promoter region and gender or age. This suggests that SHATINAT8L methylation levels can be used as a diagnostic marker for any patient. To be used as a reliable and accurate diagnostic biomarker, the standard values for each age and gender must be determined in future studies.

Our findings suggest that increased methylation of SHATINAT8L at the promoter-proximal CpG site, especially −1482 bp from the transcription start site, could be a diagnostic marker for MDD in unmedicated patients. We also showed that antidepressant medication causes epigenetic changes at this locus. This suggests the importance of using unmedicated patient subjects when searching for blood biomarkers in diagnosing MDD. In addition, SHATINAT8L methylation level might serve as a biomarker as well as an indicator of therapeutic response in patients with MDD. By establishing SHATINAT8L methylation in blood as a biomarker of MDD, earlier intervention and treatment would be expected. The prevalence of MDD is increasing each year, thus, the need for reliable biomarkers to diagnose MDD is increasing. The conformation of SHATINAT8L methylation level as a new diagnostic marker for MDD will be beneficial for many
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Author Contributions The study was designed HM and AN. HM, KU, MI and YK performed the assessment of DNA methylation. HM, YY, KO, RH, SY, YG and TS collected the samples. HM, KU and TS drafted the manuscript. AN provided critical revision of the manuscript for important intellectual content. All authors read and gave approval of the final manuscript.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA, Warden D, Ritz L, Norquist G, Howland RH, Lebowitz B, McGrath PJ, Shores-Wilson K, Biggs MM, Balasubramani GK, Fava M. Evaluation of outcomes with citalopram for MDD using measurement-based care in STAR*D: implications for clinical practice. Am. J. Psychiatry. 163, 28–40 (2006).

2) Jiang C, Lin WJ, Sadahiro M, Labonté B, Menard C, Pfau ML, Tammenga CA, Turecki G, Nestler EJ, Russo SJ, Salton SR. VGF function in MDD and antidepressant efficacy. Mol. Psychiatry, 23, 1632–1642 (2018).

3) Keller MB, Gelens J, Hirschfeld RM, Rush AJ, Thase ME, Koees JH, Markowitz JC, Fawcett JA, Koran LM, Klein DN, Russell JM, Korrin SG, McCullough JP, Davis SM, Harrison WM. The treatment of chronic MDD, part 2: a double-blind, randomized trial of sertraline and imipramine. J. Clin. Psychiatry, 59, 598–607 (1998).

4) Keller MB, McCullough JP, Klein DN, Arnow B, Dunner DL, Gelenberg AJ, Markowitz JC, Nemeroff CB, Russell JM, Thase ME, Trivedi MH, Zajecka J. A comparison of nefazodone, the cognitive behavioral-analysis system of psychotherapy, and their combination for the treatment of chronic MDD. N. Engl. J. Med., 342, 1462–1470 (2000).

5) Kaufman J, Gelernter J, Hudziak JJ, Tyrra AR, Coplan JD. The Research Domain Criteria (RDoC) project and studies of risk and resilience in maltreated children. J. Am. Acad. Child Adolesc. Psychiatry, 54, 617–625 (2015).

6) Edwards J, Jackson HI, Pattisson PE. Emotion recognition via facial expression and affective prosody in schizophrenia: a methodological review. Clin. Psychol. Rev., 22, 789–832 (2002).

7) Becker I, Lodder J, Gieselmann V, Eckhardt M. Molecular characterization of N-acetylaspartylglutamate synthetase. J. Biol. Chem., 285, 29156–29164 (2010).

8) Reynolds LM, Reynolds GP. Differential regional N-acetylaspartate deficits in postmortem brain in schizophrenia, bipolar disorder and major depressive disorder. J. Psychiatr. Res., 45, 54–59 (2011).

9) Miyamoto Y, Iegaki N, Fu K, Ishikawa Y, Sumi K, Azuma S, Uno K, Muramatsu SI, Nitta A. Striatal N-acetylaspartate synthetase SHAT1/NAT8L regulates MDD-like behaviors via mGluR3-mediated serotonergic suppression in mice. Int. J. Neuropsychopharmacol., 20, 1027–1035 (2017).

10) Nitta A, Noike H, Sumi K, Miyasaki H, Tanaka T, Takaoka K, Nagakura M, Iegaki N, Kaji J, Miyamoto Y, Muramatsu SI, Uno K. SHAT1/NAT8L and N-acetylaspartate (NAA) have important roles in regulating nicotinic acetylcholine receptors in neuronal and psychiatric diseases in animal models and humans. Nicotinic Acetylcholine Receptor Signaling in Neuroprotection. Springer, Singapore, pp. 89–111 (2018).

11) Petronis A. The origin of schizophrenia: genetic thesis, epigenetic antithesis, and resolving synthesis. Biol. Psychiatry, 55, 965–970 (2004).

12) Uchida S, Yamagata H, Seki T, Watanabe Y. Epigenetic mechanisms of major MDD: targeting neuronal plasticity. Psychiatri Clin. Neurosci., 72, 212–227 (2018).

13) Kular L, Kular S. Epigenetics applied to psychiatry: clinical opportunities and future challenges. Psychiatry Clin. Neurosci., 72, 195–211 (2018).

14) Uno K, Kikuchi I, Iwata M, Uehara T, Matsuoka T, Sumiyoshi T, Miyamoto Y, Jinno H, Takada T, Furukawa-Hibi Y, Nabeshima T, Miyamoto Y, Nitta A. Decreased DNA methylation in the SHAT1/NAT8L promoter in both patients with Schizophrenia and a methamphetamine-induced murine model of schizophrenia-like phenotype. PLOS ONE, 11, e0157959 (2016).

15) Bird AP. Cpg-rich islands and the function of DNA methylation. Nature, 321, 209–213 (1986).

16) Bird AP. DNA methylation and gene expression. Genes Dev., 16, 6–21 (2002).

17) Jin HJ, Pei L, Li YN, Zheng H, Yang S, Wan Y, Mao L, Xia YP, He QW, Li M, Yue ZY, Hu B. Alleviative effects of fluoxetine on depressive-like behaviors by epigenetic regulation of BDNF gene transcription in mouse model of post-stroke MDD. Sci. Rep., 7, 14926 (2017).

18) Kageyama Y, Kasahara T, Morishita H, Mataga N, Deguchi Y, Tani M, Kuroda K, Hattori K, Yoshida S, Inoue K, Kato T. Search for plasma biomarkers in drug-free patients with bipolar disorder and schizophrenia using metabolome analysis. Psychiatry Clin. Neurosci., 71, 115–123 (2017).

19) Kim DC, Kim KU, Kim YZ. Prognostic role of methylation status of the MGMT promoter determined quantitatively by pyrosequencing in glioblastoma patients. J. Korean Neurosurg. Soc., 59, 26–36 (2016).

20) Delaney C, Garg SK, Yung R. Analysis of DNA Methylation by Pyrosequencing. Methods Mol. Biol., 1133, 299–304 (2015).

21) Gillio-Tos A, Fiano V, Grasso C, Trevisan M, Gori S, Mongia A, De Marco L, Ronco G. Assessment of viral methylation levels for high risk HPV types by newly designed consensus primers PCR and pyrosequencing. PLOS ONE, 13, e0194619 (2018).

22) Fuchikami M, Morinobu S, Segawa M, Okamoto Y, Yamawaki S, Kuroda K, Hattori K, Yoshida S, Inoue K, Kato T. Search for DNA methylation profiles of the brain-derived neurotrophic factor (BDNF) gene as a potent diagnostic biomarker in major MDD. Sci. Rep., 6, e23881 (2016).

23) Bird AP. DNA methylation patterns and epigenetic memory. Genes Dev., 16, 6–21 (2002).

24) Nabil Fikri RM, Norlelawati AT, Nour El-Huda AR, Hanisah MN, Kartini A, Norsidah K, Nor Zamzila A, Reeln (RELN) DNA methylation in the peripheral blood of schizophrenia. J. Psychiatr. Res., 88, 28–37 (2017).

25) Auta J, Smith RC, Dong E, Tueling P, Sershen H, Boules S, Lajtha A, Davis J, Guidotti A. DNA-methylation gene network dysregulation in peripheral blood lymphocytes of schizophrenia patients. Schizophr. Res., 150, 312–318 (2013).

26) Uno K, Miyanshi H, Sodeyama K, Fujiwara T, Miyazaki T, Mura-
matsu SI, Nitta A. Vulnerability to depressive behavior induced by overexpression of striatal SHAT1/NATS1 via the serotonergic neuronal pathway in mice. *Behav. Brain Res.*, 376, 112227 (2019).

27) Higuchi F, Uchida S, Yamagata H, Otsuki K, Hobarra T, Abe N, Shibata T, Watanabe Y. State-dependent changes in the expression of DNA methyltransferases in mood disorder patients. *J. Psychiatr. Res.*, 45, 1295–1300 (2011).

28) Williams K, Christensen J, Helin K. DNA methylation: TET proteins-guards of Cpg islands? *EMBO Rep.*, 13, 28–35 (2012).

29) Gruenbaum Y, Cedar H, Razin A. Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature*, 295, 620–622 (1982).

30) Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell*, 64, 1123–1134 (1991).

31) Dong E, Gavin DP, Chen Y, Davis J. Upregulation of TET1 and downregulation of APOBEC3A and APOBEC3C in the parietal cortex of psychotic patients. *Transl. Psychiatry*, 2, e159 (2012).

32) Meehan RR, Lewis JD, Bird AP. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res.*, 20, 5085–5092 (1992).

33) Park SW, Seo MK, Lee JG, Hien LT, Kim YH. Effects of maternal separation and antidepressant drug on epigenetic regulation of the brain-derived neurotrophic factor exon I promoter in the adult rat hippocampus. *Psychiatry Clin. Neurosci.*, 72, 255–265 (2018).

34) Zhang L, Sui RB. Proton magnetic resonance spectroscopy study on the metabolism changes of cerebellum in patients with post-stroke MDD. *Cell. Physiol. Biochem.*, 41, 1393–1402 (2017).

35) Zannas AS. Epigenetics as a key link between psychosocial stress and aging: concepts, evidence, mechanisms. *Dialogues Clin. Neurosci.*, 21, 389–396 (2019).

36) Yousefi P, Huen K, Davé V, Barcellos L, Eskenazi B, Holland N. Sex differences in DNA methylation assessed by 450K BeadChip in newborns. *BMC Genomics*, 16, 911 (2015).

37) Lepine JP, Briley M. The increasing burden of MDD. *Neuropsychiatr. Dis. Treat.*, 7 (Suppl. 1), 3–7 (2011).

38) Andersen I, Thielen K, Bech P, Nygaard E, Diderichsen F. Increasing prevalence of MDD from 2000 to 2006. *Scand. J. Public Health*, 39, 857–863 (2011).

39) Mata DA, Ramos MA, Bansal N, Khan R, Guille C, Di Angelantonio E, Sen S. Prevalence of MDD and depressive symptoms among resident physicians: a systematic review and meta-analysis. *JAMA*, 314, 2373–2383 (2015).