length [F (1,16) = 1.46, p = 0.244] did not differ significantly between the ARMS subjects who later developed schizophrenia (n = 5) and those who did not (n = 17). These results did not change even when only antipsychotic-naïve ARMS subjects were included in the analyses. In the ARMS subjects, left olfactory sulcus depth was negatively correlated with the severity of positive formal thought disorder ($r = -0.709$, $p = 0.001$), but no significant correlations were found between the sulcus measures and other clinical variables.

To our knowledge, this is the first report to demonstrate altered depth of the olfactory sulcus in clinical high-risk subjects for psychosis. The olfactory sulcus, which appears in the fetal forebrain at around 16 weeks gestation (Chi et al., 1977), relates to olfactory function in healthy subjects and is usually deeper on the right hemisphere in association with functional lateralization in the olfactory system (Hummel et al., 2003). The present and our previous (Takahashi et al., 2013) MRI findings of olfactory sulcus are thus consistent with the observation of olfactory deficits in schizophrenia (Cohen et al., 2012) as well as high-risk subjects for developing psychosis (Brewer et al., 2003; Turetsky et al., 2012). Interestingly, Brewer et al. (2003) suggested that olfactory identification impairment was a premorbid marker of future conversion to schizophrenia. Although we failed to find the relation between the olfactory sulcus morphology and future transition, the left sulcus depth was associated with prodromal symptomatology. This lateralized finding might be partly consistent with the notion that the olfactory sulcus depth only in the left hemisphere, which is hypothesized to have greater susceptibility to plasticity as compared with right sulcus depth, correlates to olfactory function in healthy subjects (Hummel et al., 2003). Further studies should evaluate the sulcus morphology and its relation to olfactory functioning and clinical features in a larger well-defined high-risk cohort.

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13 March 2013

特集　早期介入の実際と工夫

富山県における早期介入活動の実際と工夫

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村中　泰子[1)]，倉知　正佳[1)]，水上　祐子[1,2)]，数川　悟[2)]，鈴木　道雄[1)]

富山県精神保健福祉センター（こころのリスク相談）と富山大学（こころのリスク外来）とが協働する早期介入活動（CAST）の現状を報告した．2006年10月から2012年3月までの間に同サービスを活用し，アット・リスク精神状態（ARMS）と診断された利用者の統合失調症への移行率は約23%であった．リスク外来における治療継続者の半分以上に対して抗精神病薬が用いられていた．非定型抗精神病薬の早期投与により，認知機能の正常化と良好な社会的転帰を得た自験例を示した．また，事象関連電位であるミスマッチ陰性電位によるARMSにおける精神病発症予測の可能性について論じた．社会機能を含む長期予後の改善を目標とした早期介入の実践が，今後求められよう．

＜索引用語：アット・リスク精神状態，前駆期，統合失調症，早期治療，ミスマッチ陰性電位＞

はじめに

富山県では，富山大学と県の精神保健福祉センター（心の健康センター）との共同事業として，精神病ハイリスク者を対象としたサービス活動を2006年10月から行っている．Consultation and Support Service in Toyama（CAST）（図1）とネーミングされたその概要や関連する治療法の考察については，これまでも専門誌上で発信してきた[10,11,13,14]．CASTの目的は，①アット・リスク精神状態（at risk mental state：ARMS）が疑われる思春期・青年期の若者や家族に専門家による相談，診断，治療の機会を提供すること．②すでに精神病を発症している患者に対し，エビデンスに基づいた医療をできるだけ早期に提供すること（精神病未治療期間の短縮）．③統合失調症の発症リスクの生物学的基盤を解明すること．④統合失調症前駆状態を対象とした，精緻な診断および治療法を開発すること．に集約される[11,14]．本稿では，富山県における早期介入活動の実際と工夫についての現状を報告する．

Ⅰ．CAST活動の現状

2012年3月までのCAST利用者122名の内訳を図2に示す．こころのリスク相談の利用者は75名，こころのリスク外来の受診者は77名であった．リスク相談来訪者のうち，ARMSあるいは統合失調症を疑われた計29名が「こころのリスク外

http://www.med.u-toyama.ac.jp/neuropsychiatry/index-kokoro.html

図1　富山県における早期介入活動：Consultation and Support Service in Toyama（CAST）のホームページ

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2）富山県心の健康センター

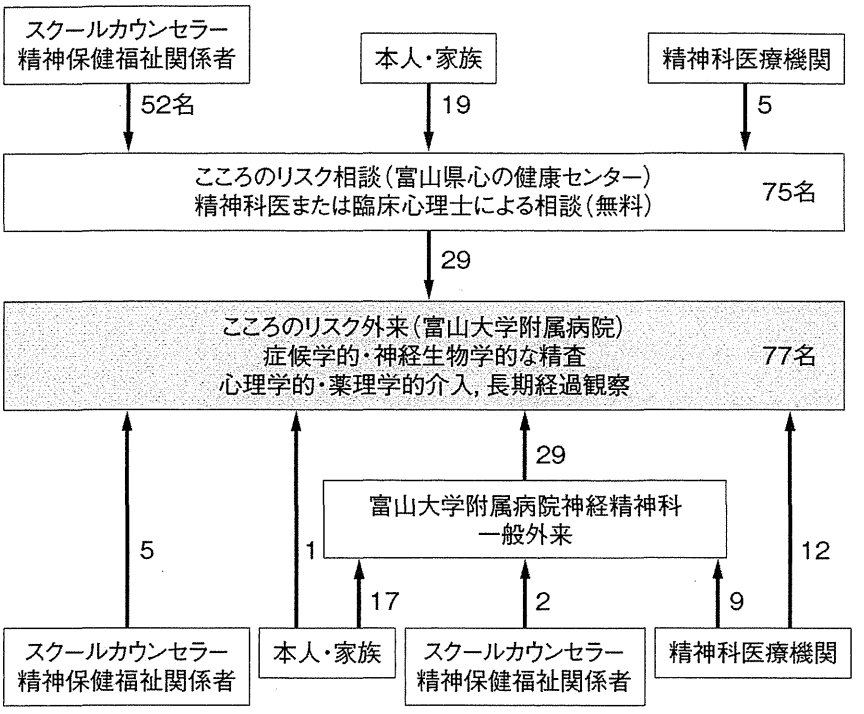

図2　2006年10月から2012年3月までのCAST利用者122名の内訳

来」を受診した．リスク相談には，スクールカウンセラーや心の健康センターなどの精神保健福祉関係者からの紹介が多かった．一方「こころのリスク外来」は，富山大学附属病院神経精神科の一般外来や，他の精神科医療機関から直接紹介された者が多数を占めた．

次に，ARMSの判定基準を満たす者が，DSM-IV-TRによりどのように分類されるかを調査した．表1のごとく，DSM-IV-TRのいずれの診断基準も満たさずARMSの症状のみを示す場合が最も多かった．一方，気分障害，不安障害，あるいはパーソナリティ障害の診断基準を満たす者も認めた．以上より，初期にARMSと判断される場合でも，後に統合失調症（精神病圏疾患）以外に発展しうる場合があると考えられた．また，44名中10名が統合失調症に移行した（リスク外来初診日から移行までの期間，0.5ヵ月～2年）．移行率は23%前後であり，これまでの報告[9]と一致した．

Ⅱ．運用上の工夫

われわれの活動における工夫の1つに，図3のようなチエックリスト[11]の使用が挙げられる．同リストは主に，リスク相談の段階におけるスクリーニングの補助として有用と思われる．

また，State-Trait Anxiety inventory（STAI）を用いた不安状態の評価も行っている．図4に示すように，リスク外来を受診する患者は，STAIで評価される特性不安が一般に高い．一方，統合失調症あるいは関連疾患と診断される場合は，ARMSよりも特性不安はかえって低くなる（図4）．これらの所見が早期介入における治療法の選択などに有用であるかは，今後検討を要する．

Ⅲ．治療の実際

表2にARMSに対する治療内容および経過を示す．2012年3月31日時点で治療を継続している者は15名，転院7名，終結11名，中断11名であった．治療継続者のうち，12名が薬物療法と支持的精神療法，3名が薬物療法に認知行動療法を組み合わせた治療を受けていた．比較的少量の抗精神病薬を投与されていた者は9名であった．以上より，当リスク外来では，治療継続者の半分以上に対し抗精神病薬が用いられていることになる．

ARMSに対する抗精神病薬使用については，一

表1 ARMSの判定基準を満たした患者のDSM-IV-TRによる診断

診断名	人数（計44名）	統合失調症へ移行（計10名）
ARMS症状のみ（DSM-IV-TRで分類不能）	10	4
特定不能の精神病性障害	3	2
気分障害		
大うつ病性障害	4	1
気分変調性障害	3	
特定不能のうつ病性障害	1	
不安障害		
社交不安障害	5	1
強迫性障害*	6	1
全般性不安障害	1	
適応障害	3	
パーソナリティ障害		
統合失調型パーソナリティ障害*	7	1
スキゾイドパーソナリティ障害	1	
不明（評価の途中で通院を中断）	1	

* I軸で強迫性障害，II軸で統合失調型パーソナリティ障害の診断がついた者

□自分の考えではない考えが浮かんでくる．どうでもよいことが頭に出てきて疲れる．
□人の話を聞くと遠まわしに自分のことを言っている気がする．
□最近，理由もなく誰かに嫌がらせをされている気がする．
……
□精神科的な病気じゃないか心配だ．

図3 リスク相談来所時に用いるチェック項目
（文献11）より改変引用）

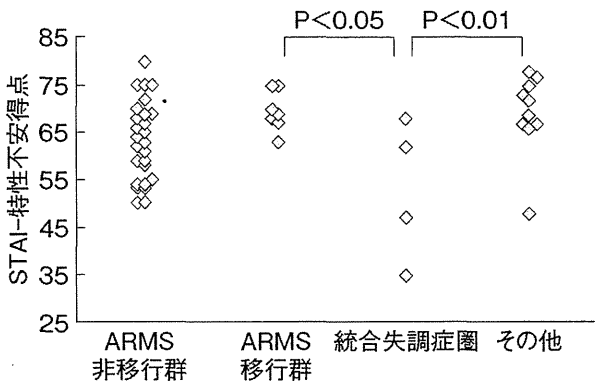

図4 State-Trait Anxiety inventory（STAI）によるリスク外来受診時における不安の評価
（P値は分散分析により算出）

般に慎重な意見が多い．例えば，国際早期精神病学会（IEPA）のガイドライン[6]では，“DSM-IV/ICD-10の精神病性障害の基準に合致しなければ，通常は抗精神病薬投与の適応ではない”と勧告されている．同ガイドラインは一方で，“低用量の非定型抗精神病薬（atypical antipsychotic drugs：AAPDs）をARMSに試験的に用いるべきである場合”として，①急速に悪化している，②重度の自殺リスクが存在し，抑うつの治療が無効，または，③攻撃性や敵意が増大し，他者へのリスクがあるとき，などを挙げている．

ここで，AAPDsの1つであるペロスピロンの早期投与により，認知機能の正常化と良好な社会的転帰を得た筆者らの自験例を示す[3]（図5）．

症例は15歳（高校2年生）男性で，奇妙な動作，考えがまとまらない，勉強に集中できないなどを主訴に，家族の勧めでこころのリスク相談および当リスク外来を受診した．初診時には明らかな幻覚妄想は認めず，思考の解体と機能低下，注

表 2　ARMS の治療内容および経過

	継続 (N=15)	転院 (N=7)	終結 (N=11)	中断 (N=11)
薬物療法＋支持的精神療法	12	7	4	3
抗精神病薬*	7	3	1	2
	(112.6)	(325.5)	(150)	(200)
その他	5	4	3	1
薬物療法＋認知行動療法	3	—	—	1
抗精神病薬*	2	—	—	—
	(408.5)			
その他	1	—	—	1
認知行動療法のみ	—	—	1	1
支持的精神療法のみ	—	—	4	4
検査のみ	—	—	2	2

*( )＝クロルプロマジン換算量の平均（mg/日）

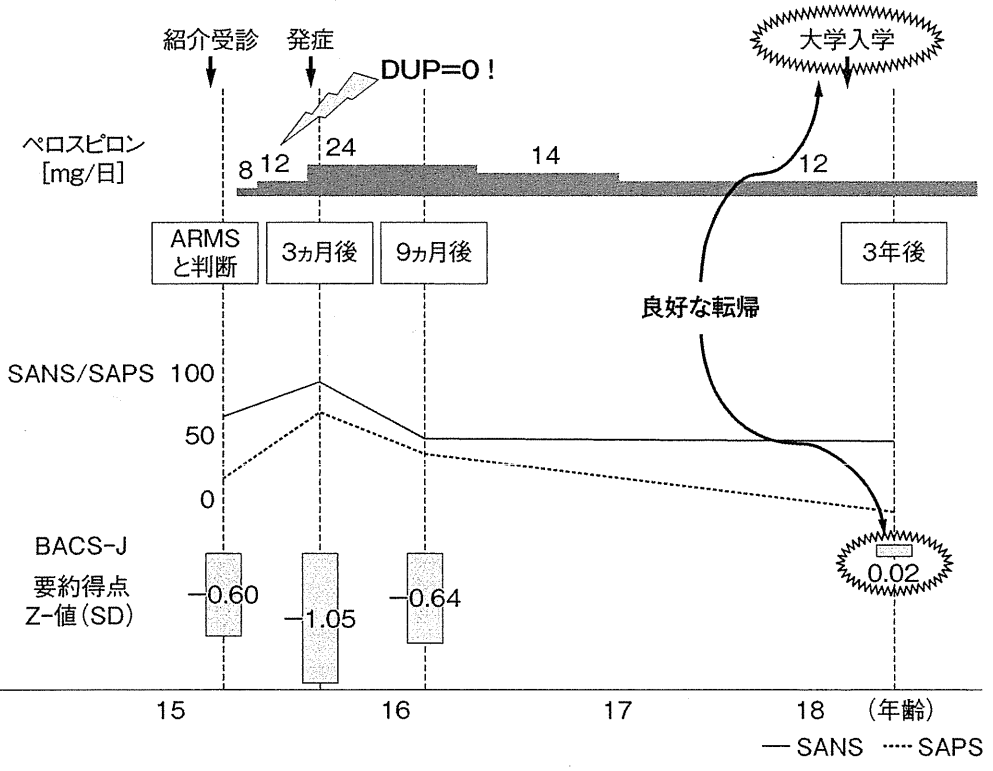

図 5　非定型抗精神病薬を用いた早期介入により，認知機能の正常化および良好な機能転帰を得た統合失調症の 1 例

リスク外来初診時，集中力困難，意欲減退など本人自ら苦痛を訴えた．ARMS が疑われ，さらに精神病症状（SANS/SAPS で測定）が急速に悪化している状態と考えられたため，ペロスピロンの投与を開始した．3 ヵ月後より，週 2〜3 回の幻聴や被害妄想が出現し，自我障害の体験も増加した．この時点で統合失調症と診断され，ペロスピロンが増量された．その後，陽性症状が徐々に軽快し，物事に集中できるようになった．治療開始 9 ヵ月後には精神病症状が改善していた．治療期間中は通学を続け，国立大学に現役で合格した．治療開始 3 年後には陽性症状は消失した．統合失調症認知機能評価尺度（BACS-J）で測定される認知機能は，初診時に低下していた．精神病が発症したペロスピロン投与開始 3 ヵ月後に増悪していたが，9 ヵ月後には発症前のレベルに回復した．3 年後には，健常者レベルまで回復（正常化）した（文献 3）より改変引用）．

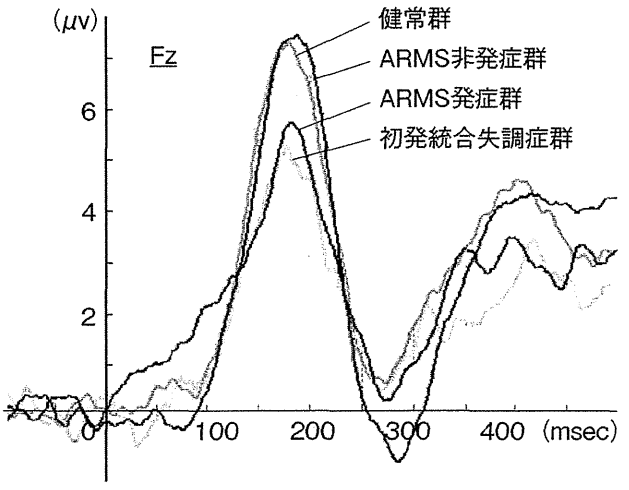

図6 ミスマッチ陰性電位（MMN）による精神病発症予測
図は前頭部中央（Fz）誘導におけるMMNの波形を示す．ARMS患者のうち，後に統合失調症へ移行する群（AMRS発症群）では，初発統合失調症群と同様の振幅の減少を発症前にすでに認める．一方，非移行群（ARMS非発症群）の振幅は，健常者と同等の振幅を示した（文献5）より改変引用）．

意集中困難などの症状が主体であった．統合失調症前駆期にあり，かつ発症が逼迫している状態と判断され，速やかに少量のペロスピロン投与による治療が開始された．3ヵ月後には幻聴，自我障害を伴う明らかな幻覚妄想状態を呈し（統合失調症発症），認知機能障害も精神症状と並行して一時的に増悪した．ペロスピロン増量による精神症状の軽快に伴い認知機能障害も改善し，3年後には健常レベルにまで改善した．この時点では陽性症状は消失しており，QOL・機能レベルも良好であった[3]（図5）．

Ⅳ．ミスマッチ陰性電位（MMN）

ARMSの病態生理および精神病発症予測に関する簡便な指標として，P300やMMNなどに代表される事象関連電位が注目される[1,5,7,12]．このうちMMNは，被検者が他に注意を向けた状況で音を聞かせるなどの刺激を与え，逸脱した音を提示したときに自動的に生じる脳波の波形から標準音を提示したときの波形を差し引いたものである．同電位は上側頭回や前頭葉皮質の機能を反映し，統合失調症では振幅が低下する[5,8]．ARMS患者でもMMNの振幅が減少していることが最近の研究において示されている[2,5,7]．筆者らも，後に精神病を発症するARMS患者において，発症前にミスマッチ陰性電位の振幅が減少していることを確認し（図6），神経心理学的所見との関連を検討した[5]．

おわりに

精神病の前駆期が疑われARMSと診断された者が，後に精神病を発症する確率は半分以下であり[9]，気分障害や不安障害など統合失調症以外の精神疾患に発展することもある．最近は，ARMS患者の社会機能を中心とした長期予後の改善に力が入れられている[14]．このような動向に留意しつつ，援助希求のあるハイリスク者への早期介入を実践することを，われわれは目指している[15,16]．

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Early Intervention Practice in Toyama Prefecture : Efforts to Improve the Clinical Service

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We report our activity in the Consultation and Support Service in Toyama (CAST), a clinical service provided by the collaboration of Toyama Prefectural Mental Health Center and University Hospital of Toyama(UHT). About 23% of users diagnosed with at-risk mental state (ARMS), during October 2006 until March 2012, transitioned to overt schizophrenia. More than half of the subjects who continued to visit the specialized clinic in UHT were treated with anti-psychotic drugs. We encountered a case of schizophrenia in which early treatment with an atypical psychotic drug was effective in normalizing cognitive function and achieving a good social consequence. The ability of mismatch negativity, an event-related potential, to predict progression to psychosis in subjects with ARMS is discussed. Further efforts should be directed towards improving long-term outcomes, such as social function, for users of the CAST.

<Authors' abstract>

<**Key words** : at-risk mental state, prodrome, schizophrenia, early treatment, mismatch negativity>

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ORIGINAL PAPER

DNA Methylation Signatures of Peripheral Leukocytes in Schizophrenia

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Abstract Schizophrenia (SCZ) is a complex psychiatric disease with a lifetime morbidity rate of 0.5–1.0 %. To date, aberrant DNA methylation in SCZ has been reported in several studies. However, no comprehensive studies using medication-free subjects with SCZ have been conducted. In addition, most of these studies have been limited to the analysis of the CpG sites in CpG islands (CGIs) in the gene promoter regions, so little is known about the DNA methylation signatures across the whole genome in SCZ. Genome-wide DNA methylation profiling (485,764 CpG sites) of peripheral leukocytes was conducted in the first set of samples (24 medication-free patients with SCZ and 23 non-psychiatric controls) using Infinium HumanMethylation450 Beadchips. Second, a monozygotic twin study was performed using three pairs of monozygotic twins that were discordant for SCZ. Finally, the data from these two independent cohorts were compared. A total of 234 differentially methylated CpG sites that were common between these two cohorts were identified. Of the 234 CpG sites, 153 sites (65.4 %) were located in the CGIs and in the regions flanking CGIs (CGI: 40.6 %; CGI shore: 13.3 %; CGI shelf: 11.5 %). Of the 95 differently methylated CpG sites in the CGIs, most of them were located in the promoter regions (promoter: 75.8 %; gene body: 14.7 %; 3′-UTR: 2.1 %). Aberrant DNA methylation in SCZ was identified at numerous loci across the whole genome in peripheral leukocytes using two independent sets of samples. These findings support the notion that altered DNA methylation could be involved in the pathophysiology of SCZ.

Keywords Schizophrenia · DNA methylation · Epigenetic · Microarray · Monozygotic twins

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Introduction

Schizophrenia (SCZ) is a mental disease characterized by auditory hallucinations, delusional ideas, and cognitive impairments. Its reported lifetime morbidity risk is 7.2 per 1,000 (Bhugra et al. 2005). SCZ is a complex disorder that results from genetic and environmental etiological influences, and its heritability is estimated to exceed 80 % (Sullivan et al. 2003). Although candidate gene approaches, genome-wide association studies, and copy number variant

studies have been carried out for SCZ (Harrison and Weinberger 2005; International Schizophrenia Consortium 2008; Purcell et al. 2009; Rees et al. 2011; Shi et al. 2009; Stefansson et al. 2009), the effects of each individual genetic factor are not large.

Epigenetics is defined as the study of mitotically or meiotically heritable variations in gene function that cannot be explained by changes in DNA sequence (Petronis et al. 2000). The 41–65 % concordance rate of SCZ in monozygotic twins, non-Mendelian inheritance, the presence of sporadic cases, sexual dimorphism, and parental origin effects suggest that epigenetic components are involved in the etiology of SCZ (Cardno and Gottesman 2000). DNA methylation, which is the addition of a methyl group to the cytosine in a CpG dinucleotide, is a major epigenetic mechanism, and attention to its role in SCZ has recently increased. To date, aberrant DNA methylation in SCZ has been reported in several studies (Abdolmaleky et al. 2006; Carrard et al. 2011; Chen et al. 2011; Dempster et al. 2011; Grayson et al. 2005; Iwamoto et al. 2005; Melas et al. 2012; Mill et al. 2008; Nohesara et al. 2011). Although antipsychotic drugs are known to influence DNA methylation (Dong et al. 2008; Melas et al. 2012; Mill et al. 2008; Shimabukuro et al. 2006; Tremolizzo et al. 2005), no comprehensive studies using medication-free subjects with SCZ have been conducted. In addition, most of previous studies have been limited to the analysis of the CpG sites in CpG islands (CGIs) in the gene promoter regions, so little is known about the DNA methylation signatures across the whole genome in SCZ.

In this study, first, genome-wide DNA methylation profiling (485,764 CpG dinucleotides) of peripheral leukocytes both in the first set of samples (24 medication-free SCZ patients and 23 non-psychiatric controls) and in the second set of samples (3 pairs of monozygotic twins discordant for SCZ) was conducted. Then, the data from these two independent cohorts were compared, and common changes in DNA methylation between the cohorts were detected.

Materials and Methods

Subjects

For the first set of samples, twenty-four medication-free patients with SCZ (11 males and 13 females, mean age: 30.9 ± 10.5 y) were recruited from Tokushima and Kochi University Hospitals in Japan. The diagnosis of SCZ was made by at least two experienced psychiatrists according to DSM-IV criteria on the basis of extensive clinical interviews and a review of medical records. None of the patients with SCZ had any psychiatric comorbidity. Among the twenty-four patients, sixteen of patients had no history of taking antipsychotics, and of the other eight patients, seven had not taken any antipsychotics for at least 2 months. Twenty-three control subjects (10 males and 13 females, mean age: 31.9 ± 9.7 year) were selected from volunteers who were recruited from hospital staff, students, and company employees documented to be free from psychiatric problems, a past history of mental illness, and medications. For the second set of samples, three pairs of monozygotic twins discordant for SCZ were recruited from Nagasaki University Hospital. All of the twins were males, and their mean age was 52.7 ± 10.4 year. These three pairs of twins were also reported in a previous study (Ono et al. 2010). All affected individuals among the twins were treated with various psychotic drugs. Demographic data of all samples analyzed in this study are presented in Supplementary Table S1. All subjects who participated in this study were of unrelated Japanese origin and signed written informed consent approved by the institutional ethics committees of the University of Tokushima Graduate School, Kochi Medical School, and Nagasaki University Graduate School of Biomedical Science to participate in this study.

DNA Methylation Methods

Genomic DNA was extracted from peripheral blood using the phenol–chloroform method. Bisulfite conversion of 500 ng genomic DNA was performed using the EZ DNA methylation kit (Zymo Research). DNA methylation level was assessed according to the manufacturer's instructions using Infinium® HumanMethylation450 Beadchips (Illumina Inc.). The technical schemes, the accuracy, and the high reproducibility of this array have been described in previous papers (Bibikova et al. 2011; Dedeurwaerder et al. 2011; Sandoval et al. 2011). Quantitative measurements of DNA methylation were determined for 485,764 CpG dinucleotides, which covered 99 % of the RefSeq genes and were distributed across the whole gene regions, including promoter, gene body, and 3′-untranslated regions (UTRs). They also covered 96 % of CGIs from the UCSC database with additional coverage in CGI shores (0–2 kb from CGI) and CGI shelves (2–4 kb from CGI). Detailed information on the contents of the array is available in the Infinium HumanMethylation450 User Guide and HumanMethylation450 manifest (www.illumina.com) and in recent papers (Bibikova et al. 2011; Sandoval et al. 2011). DNA methylation data were analyzed with the methylation analysis module within the BeadStudio software (Illumina Inc.). DNA methylation status of the CpG sites was calculated as the ratio of the signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value, known as β, ranges from 0 (completely unmethylated) to 1 (fully methylated). For intra-chip normalization of probe intensities, colored balance and back

ground corrections in every set of twelve samples from the same chip were performed using internal control probes. X chromosome CpG sites in the CGIs in the *AR* gene in this array as well as the internal control probes were checked to validate the DNA methylation measurements, as done in a previous study (Siegmund et al. 2007), and large sex differences were observed at all of these CpG sites (Supplementary Figure S1).

Statistical Methods

In the first set of samples, surrogate variable analysis (Leek and Storey 2007) was used to identify CpG loci showing significant differences in DNA methylation between medication-free patients with SCZ and the controls. This analysis is useful in clinical studies, where a large number of clinical variables, including known and unknown factors, have a complicated joint impact on microarray data, as applied in previous studies (Colantuoni et al. 2011; Numata et al. 2012). A false discovery rate (FDR) correction was applied at the 0.05 level for multiple testing. In the second set of samples, a paired t test was used to assess the significance of DNA methylation differences between the affected and unaffected twin subjects. P values < 0.05 and average DNA methylation differences between two groups >0.01 were considered significant differential methylation.

Results

Diagnostic Differences in DNA Methylation Between Medication-Free Patients With SCZ and Controls

DNA methylation levels were compared between 24 medication-free patients with SCZ and 23 control subjects using Infinium® HumanMethylation450 BeadChips. Of 485,764 CpG sites, significant diagnostic differences in DNA methylation were observed at 10,747 CpG sites at FDR 5 % correction. The top 100-ranking differentially methylated CpG sites are shown in Supplementary Table S2.

DNA Methylation Differences in Monozygotic Twins Discordant for SCZ

Genome-wide DNA methylation profiling of three pairs of monozygotic twins that were discordant for SCZ using the same Illumina methylation arrays was also conducted. Of 485,764 CpG sites, significant diagnostic differences in DNA methylation were observed at 15,872 CpG sites ($P < 0.05$ and average $\Delta\beta > 0.01$). The top 100 ranking differentially methylated CpG sites are shown in Supplementary Table S3. In addition, a list of the CpG sites that showed a $\Delta\beta > 0.3$ within each individual twin pair is shown in Supplementary Table S4.

Common Changes in DNA Methylation in SCZ Between the Two Independent Cohorts

The data from these two independent cohorts were compared, and a total of 234 differentially methylated CpG sites that were common between the cohorts were identified. Of these 234 CpG sites, 215 sites (92.4 %) demonstrated higher DNA methylation in SCZ compared to controls. When these 234 differentially methylated CpG sites were classified into four categories (CGI, CGI shore, CGI shelf, and others) according to the CpG content in the genes, 153 sites (65.4 %) were located in the CGIs and in the regions flanking CGIs (CGI shore and CGI shelf) (Supplementary Table S5). Although the proportions of CpG sites in the CGIs and in the regions flanking CGIs in this array were respectively 31 and 33 %, fewer changes in

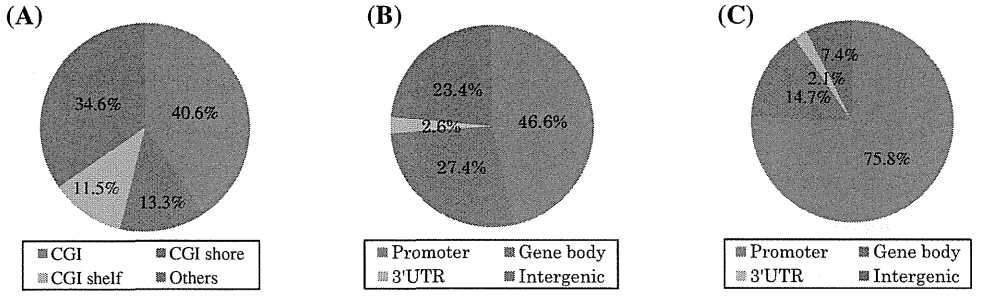

Fig. 1 The percentage of the CpG sites associated with schizophrenia (SCZ) between the two cohorts. **a** The percentage of the CpG sites associated with SCZ according to their CpG contents in the genes. Of the 234 CpG sites significantly associated with SCZ, 95 sites (40.6 %) were located in the CGIs, 31 sites (13.3 %) in CGI shores, and 27 sites (11.5 %) in CGI shelves. **b** The percentage of the CpG sites associated with SCZ according to their location in the genes. Of the 234 CpG sites significantly associated with SCZ, 109 sites (46.6 %) were located in the promoter regions, 64 sites (27.4 %) in gene bodies, and 6 sites (2.6 %) in 3′-UTRs. **c** The percentage of the CpG sites in the CGIs associated with SCZ according to their location in the genes. Of the 95 CpG sites significantly associated with SCZ, 72 sites (75.8 %) were located in the promoter regions, 14 sites (14.7 %) in gene bodies, and 2 sites (2.1 %) in 3′-UTRs

DNA methylation in SCZ in the regions flanking CGIs were observed than in the CGIs. Ninety five sites (40.6 %) were located in the CGIs, 31 sites (13.3 %) in CGI shores, and 27 sites (11.5 %) in CGI shelves (Fig. 1a). When these 234 differentially methylated CpG sites were classified into four different categories (promoter region, gene body, 3′-UTR, and intergenic region) according to their location in the genes, 109 sites (46.6 %) were located in the promoter regions, 64 sites (27.4 %) in gene bodies, and 6 sites (2.6 %) in 3′-UTRs (Fig. 1b). Of the 95 differentially methylated CpG sites in the CGIs, most of them were located in the promoter regions. Seventy two sites (75.8 %) were located in the promoter regions, 14 sites (14.7 %) in gene bodies, and 2 sites (2.1 %) in 3′-UTRs (Fig. 1c). Examples include two differentially methylated CpG sites in the CGIs in the promoter regions in the *B3GAT2* and *HDAC4* genes, which have been implicated in SCZ (Kähler et al. 2011; Kim et al. 2010) (Fig. 2).

Discussion

In this study, first, genome-wide DNA methylation profiling of peripheral leukocytes was conducted in the first set of samples (24 medication-free patients with SCZ and 23 non-psychiatric controls) using Infinium HumanMethylation450 Beadchips. To our knowledge, this study is the first to use medication-free samples with SCZ for DNA methylation profiling. Second, a monozygotic twin study was performed using three pairs of monozygotic twins that were discordant for SCZ. Although DNA methylation is associated with genotypic variants (Numata et al. 2012), a twin study is a useful method for investigating DNA methylation differences between disease phenotypes without the influence of genetic discordance. In fact, this approach has been applied successfully to identify epigenetic differences in complex diseases, such as autoimmune disease, type-1 diabetes, psoriasis, and bipolar disorder (Gervin et al. 2012; Javierre et al. 2010; Kuratomi et al. 2008; Rakyan et al. 2011). Finally, a total of 234 differentially methylated CpG sites that were common between the cohorts were identified.

The present study demonstrated that altered DNA methylation in SCZ occurred at CpG sites not only in the CGIs but also in CGI shores and CGI shelves. As shown in Fig. 1, aberrant DNA methylation in SCZ was mostly observed at CpG sites in the CGIs (40.6 %). Interestingly, of the 95 differently methylated CpG sites in the CGIs, most of them were located in the promoter regions (75.8 %). Among these 72 differentially methylated CpG sites in the CGIs in the promoter regions, several genes, such as *B3GAT2, HDAC4, and DGKI,* have been implicated in SCZ (Kähler et al. 2011; Kim et al. 2010; Moskvina et al. 2009). When we compared to previous methylation studies using peripheral blood samples (Carrard et al. 2011; Chen et al. 2011; Melas et al. 2012), we could not replicate altered DNA methylation changes in SCZ in the *COMT, HTR1A,* and *MAOA* genes. The lack of replications between studies may be due to differences in sample size, CpG sites examined, and the demographical features of samples (age, sex, race, medications, clinical subtypes, or illness severity). In particular, antipsychotic drugs are well known to influence DNA methylation (Dong et al. 2008; Melas et al. 2012; Mill et al. 2008; Shimabukuro et al. 2006; Tremolizzo et al. 2005). Irizarry et al. demonstrated that altered DNA methylation in cancer occurred in CGI shores rather than in the CGIs, and DNA methylation changes in CGI shores were strongly related to gene expression (Irizarry et al. 2009). In the present study, 15 differentially methylated CpG sites in the regions

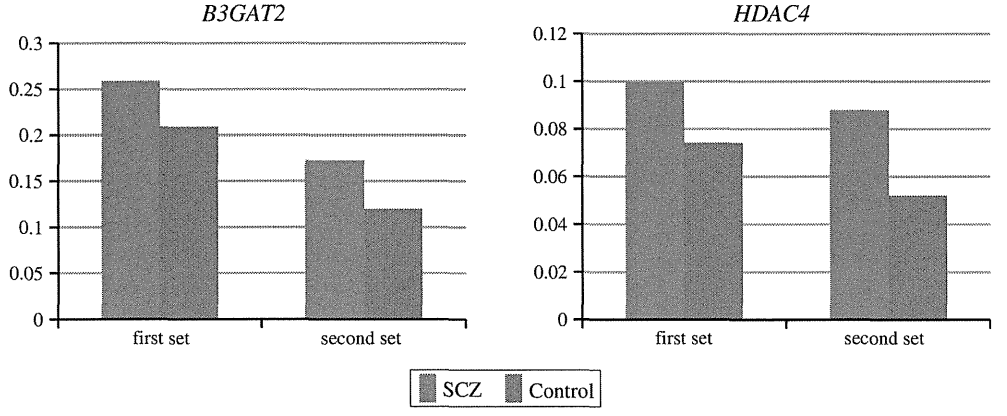

Fig. 2 DNA methylation signatures of two genes (*B3GAT2* and *HDAC4*). DNA methylation levels are shown on the *y-axis*. Patients with SCZ are shown in blue, and the controls are shown in red. The CpG sites of *B3GAT2* (cg19273746) and *HDAC4* (cg15142485) demonstrated significant differences in DNA methylation between SCZ and controls both in the first set of samples (24 medication-free SCZ patients and 23 controls) and in the second set of samples (3 pairs of monozygotic twins discordant for SCZ)

flanking CGIs in the promoter regions were identified, and several genes, such as *PCM1* and *INSIG2,* have been implicated in SCZ (Datta et al. 2010; Gurling et al. 2006; Lett et al. 2011). However, we did not observe more variable DNA methylation changes in SCZ in the regions flanking CGIs than in the CGIs. This observation is consistent with the findings of Deaton et al.'s report in the immune system (Deaton et al. 2011).

The present study also demonstrated that altered DNA methylation in SCZ occurred at CpG sites not only in the promoter regions but also in gene bodies. The role of DNA methylation in gene bodies is still unclear. Shann et al. demonstrated the correlation between intragenic hypomethylation and gene silencing in cancer cell lines (Shann et al. 2008), and Ball et al. demonstrated that gene body DNA methylation in highly expressed genes is a consistent phenomenon in human cells (Ball et al. 2009). Recently, it became apparent that CGIs in gene bodies act as alternative promoters (Illingworth et al. 2010; Maunakea et al. 2010) and that tissue-specific or cell type-specific CGI methylation is prevalent in gene bodies (Deaton et al. 2011; Maunakea et al. 2010). In the present study, 14 differentially methylated CpG sites in the CGIs in the gene bodies were identified. *GFRA2* is one such gene of interest. The *GFRA2* protein is a cell-surface receptor for *GDNF* and neurturin, and *GDNF* is a neurotrophic factor of dopaminergic neurons. The variants in this gene have been associated with tardive dyskinesia in patients with SCZ and antipsychotic responses in SCZ (Lavedan et al. 2009; Souza et al. 2010a, b).

There are several limitations to the present study. First, the sample size was not large. Replication studies will be needed in larger samples, including chronic patients with SCZ who are taking psychotic drugs. Second, the analyzed CpG sites were limited in number, although the 450 K microarray is one of the most powerful and cost-effective tools currently available for assessing methylation changes. Third, we demonstrated DNA methylation signatures of only peripheral leukocytes, not brain tissues. However, DNA methylation changes in major psychosis in the brain were also found in peripheral samples in particular genes (Kaminsky et al. 2012; Kuratomi et al. 2008). Hypermethylation of the *RAI1* gene in SCZ in our study was also observed in a previous comprehensive DNA methylation study using post-mortem brain tissues (Mill et al. 2008). Finally, it is not possible to differentiate methylation from 5-hydroxymethylation of cytosine, which also plays a critical role in gene regulation (Bhutani et al. 2011).

In summary, aberrant DNA methylation in SCZ was identified at numerous CpG sites across the whole genome in peripheral leukocytes using two independent sets of samples. Of the differently methylated CpG sites in the CGIs, most of them were located in the promoter regions. These findings support the hypothesis that altered DNA methylation could be involved in the pathophysiology of SCZ.

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

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Keywords: homocysteine, epigenetics, DNA methylation, schizophrenia, psychosis, 450K, microarray

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

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

Introduction

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

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

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

Results

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

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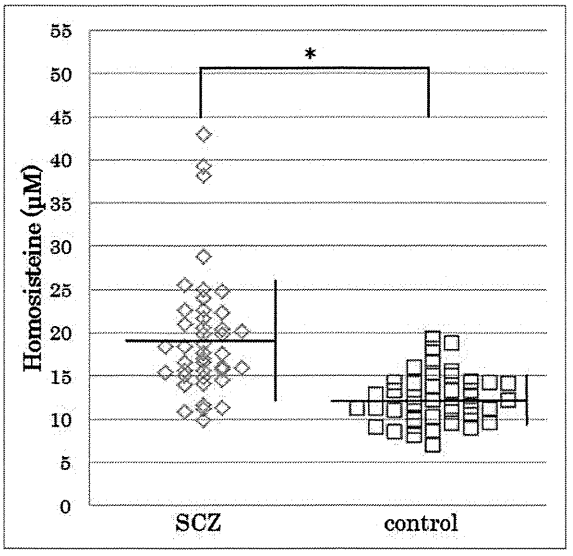

Figure 1. Plasma total homocysteine levels of patients with schizophrenia and controls. Blue dots represent plasma total homocysteine levels of patients with SCZ. Red dots represent plasma total homocysteine levels of controls. The mean plasma total homocysteine level in patients with SCZ (n = 42) was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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Materials and Methods

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

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

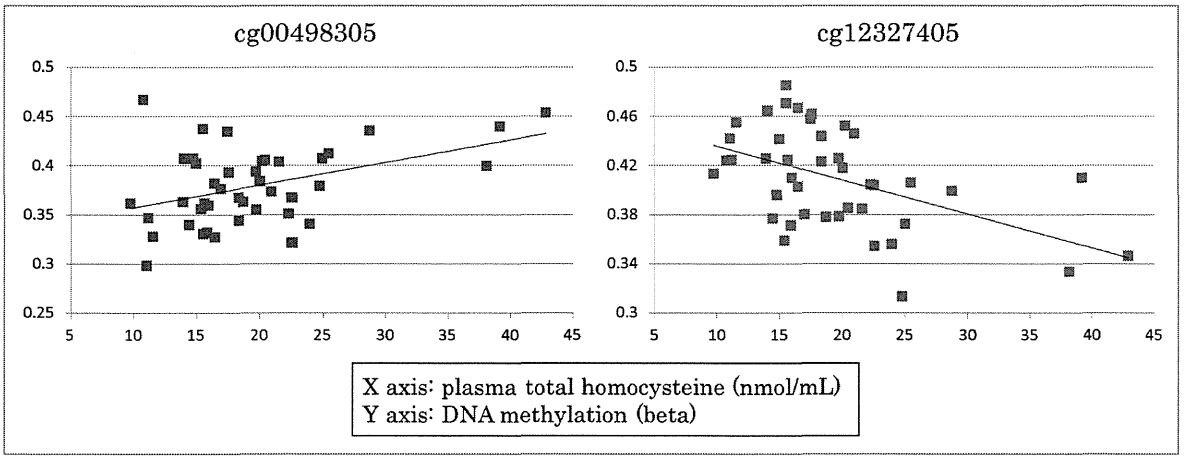

Figure 2. Two CpG sites in the *SLC18A2* (cg00498305) and *GNAL* (cg12327405) genes, which have been implicated in SCZ. A significant positive correlation of plasma total homocysteine with DNA methylation was observed at cg00498305 located in the CGI shore in the promoter region of the *SLC18A2* gene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

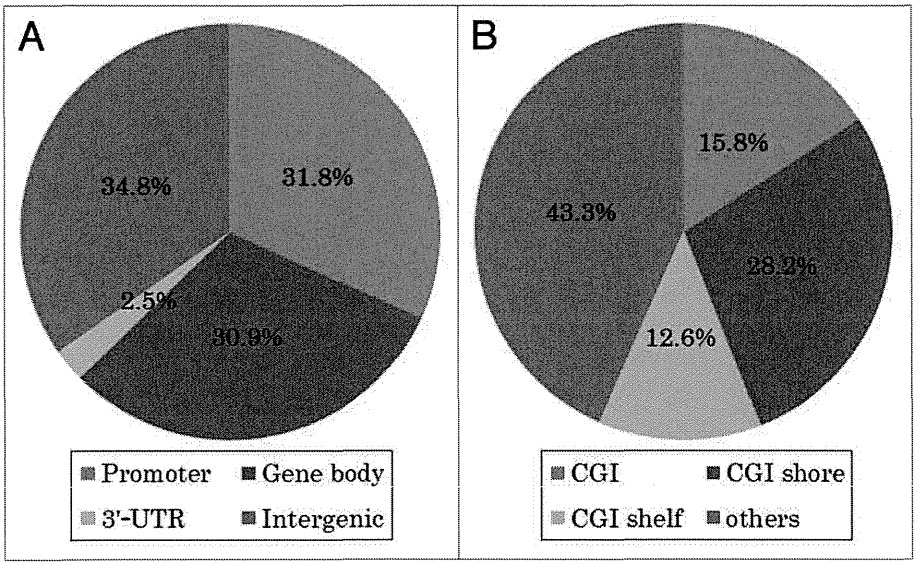

Figure 3. Percentages of 1,338 CpG sites at which plasma total homocysteine and DNA methylation were significantly correlated. (**A**) Of the 1,338 CpG sites, 425 (31.8%) were located in promoter regions, 414 (30.9%) were located in gene bodies and 34 (2.5%) were located in 3'-UTRs. (**B**) Of the 1,338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

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

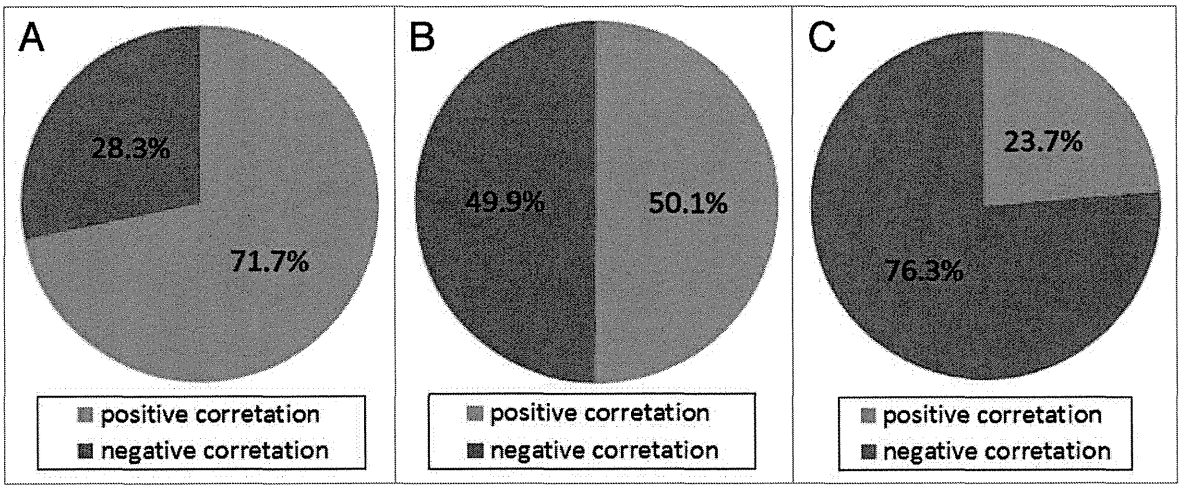

Figure 4. Percentage of CpG sites with positive correlations, located in CGIs, CGI shores and CGI shelves. (**A**) Of the 212 CpG sites located in the CGIs, 152 (71.7%) showed positive correlations between plasma total homocysteine and DNA methylation. (**B**) Of the 377 CpG sites located in the CGI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

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were dislosed.

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Supplemental Materials

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

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