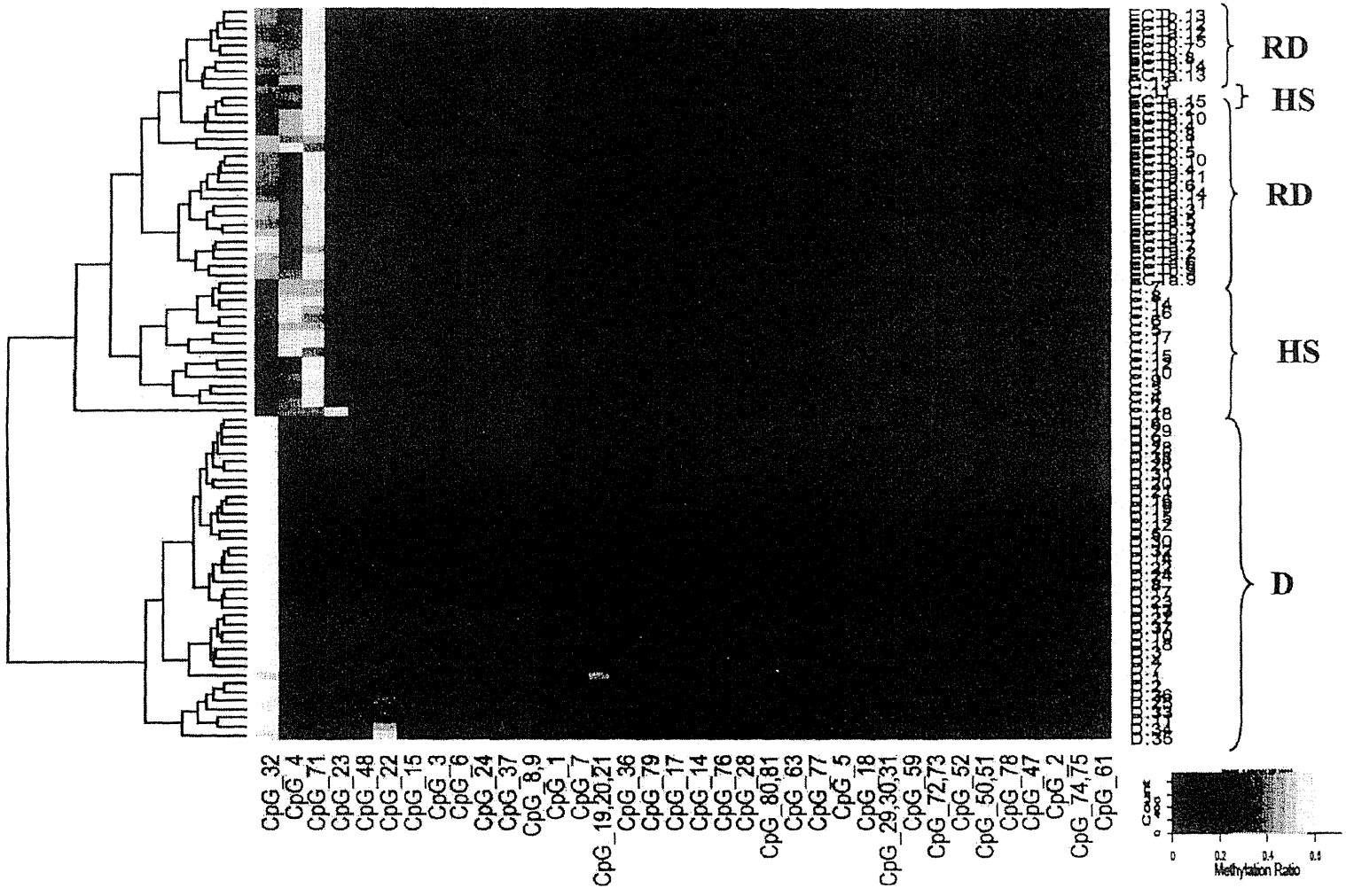

図 6-3-1　電気けいれん療法を受けた難治性大うつ病患者の BDNF 遺伝子 exon I プロモーターのメチル化プロフィール
（巻頭カラー viii 頁）　D: 未治療うつ病群（38 名），RD: 難治性うつ病群（15 名× 2），HS: 健康対照者群（18 名）

型群にメチル化率の亢進がみられ，高いメチル化率は気分安定薬と抗うつ薬による治療を受けている患者で特異的にみられていたと報告している。加えてこの研究ではlithiumおよびsodium valproateの服用がメチル化率の有意な低下と関連していることも報告しており，双極性障害の診断バイオマーカーとして遺伝子メチル化を考える場合には，薬物の作用にも注意が必要であることを提唱している。Dell'Ossoら[3]は大うつ病43例・双極性障害Ⅰ型61例・Ⅱ型50例を対象に解析を行っており，Ⅰ型に比べて大うつ病群およびⅡ型群でメチル化率の亢進を報告している。なかでも大うつ病群で高いメチル化率が報告され，D'Addarioら[2]の報告同様にlithiumやsodium valproateの服用は低メチル化と関連していることも報告している。Carlbergら[1]は大うつ病207名・双極性障害59名・健康者278名を対象にBDNF遺伝子exon Ⅰプロモーターのメチル化率を解析しており，大うつ病群でメチル化率の有意な亢進を報告している。加えてこの研究では高い大うつ病群でのメチル化率は抗うつ薬治療との有意に関連することを示している反面，筆者らの報告同様にうつ病症状の重症度とメチル化率の間には有意な相関はみられなかったことも報告している。

SLC6A4遺伝子によるうつ病および双極性障害のバイオマーカー開発（表6-3-2）

うつ病を対象にSLC6A4遺伝子のメチル化を解析したKangら[9]の報告は，健康者群とのメチル化の比較は行っておらず，むしろ抗うつ薬治療によるメチル化率の変化や，病状および養育環境を含めたストレス因に焦点を当てた研究である。この研究からはSLC6A4遺伝子exon Ⅰプロモーター上のメチル化率は，幼少期の不遇な養育環境と有意な相関を示すほか，うつ病の家族歴や現状でのストレス重症度などとも有意な相関を示していたが，治療による病状変化との間に関連はみられなかった。Zhaoら[26]はSLC6A4遺伝子exon Ⅰプロモーター上のメチル化を84組の一卵性双生児を対象に計測

表 6-3-2　大うつ病を対象とした SLC6A4 遺伝子メチル化研究

研究者	文献	年	解析部位（解析した CpG 数）	解析方法
Kang	9)	2013	-479 ～ -350（転写開始点から）7 CpGs	Pyrosequencing
Zhao	26)	2013	-213 ～ -69（転写開始点から）20 CpGs	Pyrosequencing
Okada	16)	2014	chr17: 28562360 - 28563221	MassARRAY
Domschke	4)	2014	chr: 28563286 - 28562652 9 CpGs	DS

DS : Direct sequencing

し，うつ病の重症度（Beck Depressive Inventory II）との関連を解析している。この研究では，双生児間の CpG メチル化率の違いはうつ病症状の違いと相関しており，メチル化率が 10% 増大するとうつ病スコアが 4.4 増大すると報告している。これらの研究結果からは，SLC6A4 遺伝子のメチル化がうつ病診断マーカーになるという結論は導かれなかった。これに対して筆者ら[16]は未治療うつ病 50 例と健康者 50 例を対象に，SLC6A4 遺伝子 exon I プロモーター上のメチル化を解析している。われわれの研究結果によると，BDNF 遺伝子とは異なりメチル化プロフィールそのものによるうつ病と健康者との分類はできず，2 群間で有意にメチル化率の異なる CpG も検出されなかった。ただし，抗うつ薬による治療効果と有意に相関する CpG 部位を発見しており，うつ病診断マーカーではなく抗うつ薬による治療反応性のマーカーになる可能性を提唱している。同様に SLC6A4 遺伝子 exon I プロモーター上のメチル化とうつ病治療効果を解析した Domschke ら[4]の研究では，低メチル化率は抗うつ薬による低い治療効果と相関していたことが報告されている。うつ病を対象とした研究ではないが，地域を対象とした疫学調査の中でうつ病の既往の有無で SLC6A4 遺伝子の exon I を囲む CpG アイランドのメチル化を解析した研究もみられるが，抑うつ症状とメチル化率の間に有意な相関は得られなかった[17]。この他にもうつ病の既往と SLC6A4 遺伝子のメチル化率をみた研究もあるが，この研究では平均メチル化率がう

つ病の既往で高くなる傾向を報告している[19]。このような現時点でのうつ病を対象としたSLC6A4遺伝子exon Ⅰプロモーター上のメチル化解析の結果は，うつ病の診断マーカーとしての可能性ではなく，むしろ，抗うつ薬による治療反応性との関連を示唆しており，Surrogate markerとしての可能性を提唱する結果となっている。同様にうつ病の診断バイオマーカーとは異なるが，うつ病発症の危険因子である虐待を含む幼少期の不遇な環境と，SLC6A4遺伝子のメチル化率との相関や被虐待児で高いメチル化率を示す報告もある[9,16,18]。

SLC6A4遺伝子exon Ⅰを含む領域のメチル化と，双極性障害の関連を解析した報告もみられる。Sugawaraら[22]は一卵性双生児間でのリンパ芽球由来のDNAを用いてSLC6A4遺伝子のメチル化率の比較を行っており，その結果は双極性障害患者でメチル化率の亢進を示していた。同時にこの研究では双極性障害患者の死後脳でも，同様のメチル化率の亢進を報告している。本研究の成果は，SLC6A4遺伝子exon Ⅰを含むCpGアイランドのショアに位置するCpGのメチル化率が，双極性障害の診断マーカーとなる可能性を提唱している。

その他の遺伝子メチル化によるうつ病および双極性障害のバイオマーカー開発の現状

BDNFやSLC6A4遺伝子以外にも，うつ病を対象としたメチル化研究の報告はみられる。Zillら[27]はAngiotensin converting enzyme（ACE）遺伝子の発現調節領域のメチル化を大うつ病81名と健康者81名を対象に解析し，うつ病群での有意なメチル化率の亢進を報告している。Melasら[14]は女性のうつ病群82名と健康者群92名を対象に唾液由来のDNAを用いて，Monoamine oxidase A遺伝子のexon Ⅰプロモーター領域のCpGのメチル化を計測し，うつ病群での有意な低下を報告している。うつ病の病態関連遺伝子のメチル化を標的とした研究とは異なり，Uddinら[23]はHuman

Methylation 27 BeadChip（Illumina）を用いたアレイでの，うつ病と関連する遺伝子メチル化の変化を解析している。この研究は lifetime depression を対象とした疫学調査を母集団とした研究であり，ゲノムワイドにメチル化率の変化をうつ病既往者 33 名と健康者 67 名で比較している。複数のメチル化率の低下している遺伝子が lifetime depression 群で見いだされているが，なかでも IL-6 遺伝子のメチル化の低下と IL-6 発現量との間の負の相関が報告されている。

双極性障害のメチル化研究でも，BDNF あるいは SLC6A4 遺伝子以外の遺伝子を対象とした研究がみられる。Ghadirivasfi ら[8] はセロトニン $_{2A}$（5-HT_{2A}）受容体遺伝子プロモーター領域のメチル化を計測しており，一塩基置換部位である T102C 多型部位のシトシンのメチル化率が双極性障害群および統合失調症群で健康者群と比べて有意に低下していた。その上に，患者群の第一親等でも同様に有意なメチル化の低下がみられていた。このような結果は 5-HT_{2A} 受容体のこの部位のメチル化の低下が，双極性障害の診断バイオマーカーではなく疾患 trait マーカーである可能性を示唆していると考えらえる。Nohesara ら[15] は自分たちで行った，双極性障害者や統合失調症者の死後脳での Membrane-bound catechol-O-methyltransferase（MB-COMT）遺伝子プロモーターのメチル化率低下が，末梢血でも反映されるか検証を行っている。この研究では MB-COMT 遺伝子プロモーターのメチル化率が健康者に比べて双極性障害群および統合失調症群では 50% まで低下していることを報告しているが，双極性障害と統合失調症を分類することは困難であったため，双極性障害に特異的な診断マーカーとは言えないと思われる。

マイクロ RNA を用いたうつ病・双極性障害のバイオマーカーの開発

miRNA はタンパク質に翻訳されないノンコーディング RNA で，約 25

kb 程度の短い RNA である。miRNA も DNA から二本鎖の pri-miRNA として転写され，Drosha によって pre-miRNA に切り出され，Exportin-5 によって核内から細胞質に運搬される。その後は Dicer によって一本鎖の miR となり，相補的塩基配列をもつ 3' 側の非翻訳領域に結合して，翻訳を阻害することや，mRNA の分解を導くことが報告されている。

Garbett ら[7] は 16 例の大うつ病者と健康者間で線維芽細胞由来の miRNA 量の比較を行い，38 種類の miRNA 発現量に有意な差のみられたことを報告している。Fan ら[5] は 723 種類の miRNA の比較が行える Affymetrix アレイを用いて，大うつ病群と健康者群の間で有意に発現が変化している 26 種類の miRNA を抽出している。この結果を基盤に real-time PCR 法を用いて，大うつ病 81 名と健康者 46 名を対象に 26 種類の miRNA 発現の差を検証したところ，5 種類の miRNA（miRNA-26b, miRNA-1972, miRNA-4485, miRNA-4498, miRNA-4743）の発現量に有意な亢進の得られたことが報告されている。

双極性障害に関する miRNA の研究では，Rong ら[20] が双極性障害躁状態患者 21 名と健康者 21 名を対象に miRNA-134 の発現を real-time PCR 法を用いて解析しており，未治療・治療 2, 4 週間後の miRNA-134 の発現量は，健康者と比べて有意な低下を示していた。miRRNA-134 発現量は躁症状評価と比べて負の相関を示しており，miRNA-134 の発現量が急性躁状態の診断バイオマーカーや気分安定薬による治療反応を推測する Surrogate marker となる可能性を提唱している。

このように miRNA を対象としたバイオマーカー研究は報告が少なく，アレイを用いた大規模な研究も行われているが，今後の多数の研究が必要と思われる。

まとめ

大うつ病および双極性気分障害に関するエピジェネティック・バイオマー

カーとして，DNA メチル化と miRNA を対象とした研究を筆者らの結果も合わせ紹介した。末梢血や唾液由来の DNA を用いたメチル化研究の現状をまとめると，BDNF 遺伝子 exon I プロモーター領域の CpG アイランドのメチル化は，うつ病の診断マーカーとなる可能性があり，今後の未治療うつ病多数例を対象とした研究による，マーカーとしてメチル化率を計測する CpG の部位の組み合わせの抽出が必要と思われる。その一方で SLC6A4 遺伝子の exon I を囲む領域のメチル化は，うつ病の診断マーカーとしての可能性は乏しく，むしろ，幼少期の不遇な養育環境やうつ病症状の重症度との関連を示唆するマーカーと考えられる。同時に SLC6A4 遺伝子のメチル化率は，抗うつ薬との治療反応性を推測する Surrogate marker としての役割が期待される状況である。双極性障害の DNA メチル化による診断バイオマーカーの開発については，lithium や sodium valproate によるメチル化率の低下が報告され，気分安定薬服用と未服用の患者では結果の異なることが予想される上に，うつ病と異なり未治療の双極性障害の診断の困難さも，このような研究の進展を妨げる要因と考えられる。

気分障害のみならず精神疾患の診断バイオマーカーの開発については，DNA メチル化も miRNA も同様であるが，健康者間での範囲や性差あるいは年齢による変化など，まだまだ基礎的な解析が不足していると思われる。特に DNA メチル化に関しては，メチル化解析を行う領域やメチル化率を計測する方法論の違いもある上に，未治療と治療中の対象者との違いも推測され，今後の大規模な研究による検証が期待されるところと思われる。

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Biological tests for major depressive disorder that involve leukocyte gene expression assays

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ABSTRACT

Background: Development of easy-to-use biological diagnostic tests for major depressive disorder (MDD) may facilitate MDD diagnosis and delivery of optimal treatment. Here, we examined leukocyte gene expression to develop a biological diagnostic test for MDD.
Methods: 25 drug-naive MDD patients (MDDs) and 25 age- and sex-matched healthy subjects (Controls) participated in a pilot study. A subsequent replication study involved 20 MDDs and 18 Controls. We used custom-made PCR array plates to examine mRNA levels of 40 candidate genes in leukocyte samples to assess whether any combination of these genes could be used to differentiate MDDs from Controls based on expression profiles.
Results: Among 40 candidate genes, we identified a set of seven genes (*PDGFC, SLC6A4, PDLIM5, ARHGAP24, PRNP, HDAC5, and IL1R2*), each of which had expression levels that differed significantly between MDD and Control samples in the pilot study. To identify genes whose expression best differentiated between MDDs and Controls, a linear discriminant function was developed to discriminate between MDDs and Controls based on the standardized values of gene expression after Z-score transformation. Ultimately, five genes (*PDGFC, SLC6A4, ARHGAP24, PRNP,* and *HDAC5*) were selected for a multi-assay diagnostic test. In the pilot study, this diagnostic test demonstrated sensitivity and specificity of 80% and 92%, respectively. The replication study yielded nearly identical results, sensitivity of 85% and specificity of 89%.
Conclusions: Using leukocyte gene expression profiles, we could differentiate MDDs from Controls with adequate sensitivity and specificity. Additional markers not yet identified might further improve the performance of this test.

1. Introduction

Major depressive disorder (MDD) is a highly prevalent and moderately heritable psychiatric disorder that causes major psychological, physical, and social impairments. Lifetime prevalence for MDD is estimated at about 16% or 17% and is definitely over 10%; women are affected twice as often as men (Kessler et al., 2003; Faravelli et al., 2013). Biological (Nestler et al., 2002), genetic (Levinson, 2006), and environmental factors (Caspi et al., 2003) can affect the onset of MDD, but MDD pathogenesis is largely unknown. Clusters of symptoms are currently used to diagnose MDD, and most care is delivered by general practitioners. Recent evidence indicates that diagnostic accuracy is highly variable in practice (Cepoiu et al., 2008). Undoubtedly, the development of easy-to-use biological diagnostic tests for MDD can radically improve diagnostic accuracy.

Quantitative profiling of leukocyte mRNA expression is an emerging and promising approach for assessing mental conditions. Psychological changes associated with depression clearly affect the hypothalamus-pituitary-adrenal (HPA) axis and the neuroendocrine, autonomic nervous, and immune systems (Connor and Leonard, 1998; Raison and Miller, 2003). Importantly, receptors

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for stress mediators are expressed in leukocytes; for example, neurotransmitter, hormone, growth factor, and cytokine receptors are found in leukocytes; additionally these cells produce various cytokines, including pro-inflammatory cytokines that stimulate the HPA axis directly (Arzt, 2001; Ohmori et al., 2005).

Recently, changes in leukocyte gene expression have been linked to MDD (Iga et al., 2008; Hepgul et al., 2013). However, expression of any one gene explains only a small proportion of the variance associated with depression. Combining measurements from individual markers into a single measurement often results in superior diagnostic test performance. The goal of present work was to develop and test the performance of a composite, multi-assay diagnostic test for MDD based on leukocyte gene expression profiles.

2. Methods and materials

2.1. Subjects

The protocol was approved and monitored by the Institutional Review Boards at each participating center. Written informed consent was obtained from each participant before any study procedures were performed.

For the pilot study, we enrolled 25 MDDs from four psychiatric hospitals in the Tokushima Prefecture of Japan: each MDD participant was experiencing a single or recurrent major depressive episode; 25 non-depressed healthy individuals were recruited from Tokushima University Hospital to serve as Controls. The replication study included 20 MDDs (14 drug-naïve patients and 6 medicated patients) from psychiatric hospitals in Tokushima and Kochi Prefectures and 18 Controls recruited from Tokushima University Hospital. Each of the six medicated patients was being treated with antidepressants, but none reached remission (HAM-D $\leq$ 7). The diagnosis of MDD was established according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria by at least two trained psychiatrists. None of the patients or controls in our study had any other medical disorder or any concomitant medication. Antidepressant-treated patients (n = 6) in the replication study had the antidepressants listed in Table 2. Exclusion criteria for MDDs included not having used non-steroidal anti-inflammatory agents, steroids or anticonvulsants within at least the 2 months before study initiation. Demographic data for participants in each study are shown in Tables 1 and 2.

2.2. Tissue processing, RNA purification, and sample preparation for real-time PCR analysis

PAX gene blood RNA tubes (Qiagen, Tokyo, Japan) and PAX gene Blood RNA kits (Qiagen, Tokyo, Japan) were used according to the manufacturer's recommendations to extract total RNA from peripheral leukocytes taken from whole blood samples. More specifically, PAX gene Blood RNA kits were used to purify total RNA from 2.5-ml samples of human whole blood collected in PAX gene Blood RNA tubes. RNA concentration and RNA integrity were analyzed with an Agilent 2100 Caliper LabChip Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA Integrity Number (RIN) values calculated using Agilent 2100 Caliper LabChip Bioanalyzer were sufficient for the samples to be used for real-time PCR analysis; mean RIN for all leukocyte samples was 7.12 ± 0.84. After assessing RNA quality and quantity, individual total RNA samples (2 μm each), random (N6) primers, and Quantiscript Reverse Transcriptase (Qiagen, Tokyo, Japan) were used to synthesize cDNAs.

Table 1
Demographic data of participants in the pilot study.

	MDD	Healthy subjects	p Value
N	25	25	
Male	7	9	
Female	18	16	0.54
Age	43.0 ± 14.0	40.4 ± 11.9	0.26
HAM-D score	22.4 ± 7.1		

Table 2
Demographic data of participants in the replication study.

	MDD (naïve)	Healthy subjects	p Value
N	20 (14)	18	
Male	6	5	
Female	14	13	0.60
Age	47.0 ± 11.9	44.8 ± 11.2	0.56
HAM-D score	20.8 ± 6.5		

Concomitant antidepressants: sertraline 50 mg/day (n = 1), sertraline 25 mg/day (n = 1), fluvoxamine 75 mg/day (n = 1), sertraline 25 mg/day (n = 1), sulpiride 50 mg/day (n = 1), mirtazapine 7.5 mg/day (n = 1).

2.3. PCR array procedure

A customized PCR array plate was used to examine gene expression levels (Fig. 1). This plate had 96 wells and could house a single unique genetic probe in each well. We could use this plate to simultaneously assess the expression level of multiple genes. The following criteria were used to select 40 candidate genes for the pilot study: 1) expression must be altered in the leukocytes of patients with MDD (Hobara et al., 2010; Iga et al., 2005, 2006, 2007a; Numata et al., 2009), 2) expression must be altered by lithium administration (Watanabe et al., 2014), and 3) the gene must be previously associated with the neurobiology of MDD and

Gene Symbol	1	2	3	4	5	6
A		NTF3	ARTN	PDLIM5	GLO1	REST
B	ARRB1	NR3C1	EMP1	NMUR1	HSPH1	PRNP
C	FASLG	IL1B	IL6	IFNG	TNF	HDAC2
D	HDAC5	PDE4B	TNFSF12	CREB1	SLC6A4	VEGFA
E	NSUN7	ARHGAP24	UBE2B	ANK3	IL1R2	PDGFC
F	IL12A	IL15	IL18	CXCL1	MDK	SOCS3
G	MPO	FOS	JUN	ATF2	STAT3	RN18S1
H	ABL1	GAPDH	HPRT1	ACTB	B2M	18S

Fig. 1. Specific criteria were used to select 40 candidate genes; mRNA levels of these candidates were examined simultaneously with custom-made PCR array plates. Expression of genes indicated in blue was lower in patients than in controls. Expression of genes indicated in red was higher in patients than in controls. Genes indicated in green were highly expressed in leukocytes. Based on previous findings, expression of genes indicated in yellow changed following lithium treatment. Genes indicated in gray are housekeeping genes. Abbreviations: NTF3: neurotrophin3, ARTN: artemin, PDLIM5: PDZ and LIM domain5, GLO1: glyoxalase1, REST: RE1-silencing transcription factor, ARRB1: arrestin beta1, NR3C1: nuclear receptor subfamily3 group C member1, EMP1: epithelial membrane protein1, NMUR1: neuromedin U receptor1, HSPH1: heat shock 105 kDa/110 kDa protein 1, PRNP: prion protein, FASLG: Fas ligand, IL1B: interleukin1 beta, IL6: interleukin6, IFNG: interferon gamma, TNF: tumor necrosis factor, HDAC2: histone deacetylase2, HDAC5: histone deacetylase5, PDE4B: phosphodiesterase4B cAMP-specific, TNFSF12: tumor necrosis factor superfamily menber12, CREB1: cAMP responsive element binding protein1, SLC6A4: solute carrier family6 member4, VEGFA: vascular endothelial growth factor A, NSUN7: NOP2/Sun domain family member7, ARHGAP24: Rho GTPase activating protein 24, UBE2B: ubiquitin-conjugating enzyme E2B, ANK3: ankyrin3 node of Ranvier, IL1R2 interleukin1 receptor type II, platelet derived growth factor C, IL12A: interleukin 12A, IL15: interleukin15, IL18: interleukin18, CXCL1: chemokine ligand1, MDK: midkine, SOCS3: suppressor of cytokine signaling3, MPO: myeloperoxidase, FOS: FBJ murine osteosarcoma viral oncogene homolog, JUN: jun proto-oncogene, ATF2: activating transcription factor2, STAT3: signal transducer and activator of transcription3, RN18S1: 18S ribosomal RNA, ABL1: c-abl oncogene1 non-receptor tyrosine kinase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, HPRT1: hypoxanthine phosphoribosyltransferase1, ACTB: actin beta, B2M beta-2-microglobulin, 18S: 18S ribosomal RNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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detectably expressed in human leukocytes. For gene expression analysis, real-time quantitative RT-PCR analysis was performed on an ABI 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Measurements of expression of each gene were conducted in duplicate. The ΔΔCt method was used to determine relative expression of each gene in MDDs and Controls samples. NormFinder, a statistical application, was used to evaluate the stability of reference genes (Andersen et al., 2004). *GAPDH* had the best stability score in both the pilot and the replication study and was used as the reference gene for both studies. Primers for each gene are listed in the Supplemental Table.

2.4. Development of the discrimination-score (D-score) and statistical analyses

A value, designated Ctx, was determined for each gene and was used to calculate the relative gene expression level (⊿Ctx) by normalization to the reference gene, *GAPDH*. All statistical analyses were performed with R ver3.1.1. Mann-Whiteney U tests ($p < 0.05$) identified seven genes whose expression differed significantly between MDDs and Controls. A linear discriminant function was developed for discrimination between MDDs and Controls based on standardized values of gene expression after Z-score transformation. The selection and optimization of the combination of explanatory variables were performed with a round-robin algorithm (as shown in Supplemental Table 2). Discriminant analyses and calculations of Wilks's lambda were performed for all 127 combinations of explanatory variables, where 2 to 7 variables (genes) were used per combination, and the sensitivity and specificity of each combination (n = 127) were investigated. For each number of explanatory variables (from 2 to 7 genes), the combination showing the lowest Wilks's lambda is listed in Table 5. Thus, the criterion for the selection of explanatory variables was that the combination showed the lowest Wilks' lambda for that number of explanatory variables (Table 5 and Supplemental Table 2). A low Wilks's lambda means that the within-groups sum of squares and product matrix were each small. Therefore, the optimized combination of genes with the lowest Wilks's lambda seemed to be a gene set that generally possessed low-expression variance, and each gene selected for the optimal combination tended to show relatively high expression levels and low variances, such as PDGFC and ARHGAP24 (as shown in Table 3).

Ultimately, a combination of five genes (*PDGFC*, *SLC6A4*, *ARHGAP24*, *PRNP*, and *HDAC5*) was selected based on discriminating capacity (Table 5). A Discrimination score (D-score) for each individual (MDD or Control) was calculated by multiplying coefficients of linear discriminants, obtained by the *lda()* function of the MASS package in R, to the standardized values of expression of the five selected genes. A D-score was calculated for each participant sample in the pilot and replication studies. The D-score indicates the probability of membership among the cases or among the controls for each individual. The highest membership probability for each case allowed for classification into one or the other diagnostic group.

A D-score was calculated for each sample in the pilot study as follows:

$$\text{D} - \text{score} = 0.7345871*\text{PDGFC} + 0.3783558*\text{SLC6A4} + 0.5009830*\text{ARHGAP24} - 0.7760468*\text{PRNP} + 0.4286675*\text{HDAC5}$$

A D-score was calculated for each sample in the replication study as follows:

$$\text{D} - \text{score} = 0.05191909*\text{PDGFC} + 0.87038335*\text{SLC6A4} + 0.49387808*\text{ARHGAP24} - 0.59542868*\text{PRNP} + 0.14893918*\text{HDAC5} + 0.03535955$$

Any subject whose D-score was above zero was identified as a "healthy subject", and those with a D-score below zero was identified as an "MDD patient". Indexes shown in Figs. 2 and 3 represent the orders of samples sorted by D-score.

Table 3
Delta Ct values of genes with significant differences in expression levels in the pilot study.

Gene	MDD patients(n = 25)	Healthy subjects(n = 25)	p Value
PDGFC	8.17 ± 0.59	8.73 ± 0.72	0.00247
SLC6A4	9.08 ± 1.05	9.78 ± 0.79	0.00397
PDLIM5	4.85 ± 0.54	5.26 ± 0.61	0.00849
ARHGAP24	6.47 ± 0.60	6.90 ± 0.52	0.01794
PRNP	6.59 ± 1.23	5.79 ± 0.61	0.01794
HDAC5	4.48 ± 0.50	4.91 ± 0.67	0.03360
IL1R2	2.85 ± 0.90	3.33 ± 0.59	0.03709

Table 4
Delta Ct values for each of 5 individual genes (bold) that constitute the Discriminant-score and five other genes with significant differences in expression levels in the replication study.

Gene	MDD patients(n = 20)	Healthy subjects(n = 18)	p Value
PDGFC	**7.13 ± 1.02**	**7.52 ± 0.88**	**0.176**
SLC6A4	**7.92 ± 1.10**	**9.20 ± 1.09**	**0.000499**
PDLIM5	3.97 ± 0.91	4.51 ± 0.56	0.149
ARHGAP24	**5.60 ± 0.60**	**6.25 ± 0.68**	**0.099**
PRNP	**6.13 ± 1.68**	**6.11 ± 0.88**	**0.654**
HDAC5	**3.31 ± 0.68**	**3.85 ± 0.66**	**0.0222**
IL1R2	1.85 ± 0.93	2.36 ± 0.70	0.0587
REST	0.86 ± 1.239	2.06 ± 0.773	0.00148
IFNG	10.47 ± 1.927	9.17 ± 1.585	0.0443
FOS	1.73 ± 0.996	2.54 ± 0.706	0.0282
STAT3	1.45 ± 1.079	2.39 ± 0.633	0.00433
IL18	8.16 ± 0.792	7.50 ± 0.470	0.00148

3. Results

Clinical and demographic characteristics of the subjects in the pilot and replication studies are presented in Tables 1 and 2, respectively. There were no significant differences in gender ratio or subject age between MDDs and Controls in either the pilot or the replication study.

3.1. Pilot study

A PCR array was used to examine gene expression in a primary cohort comprising 25 unmedicated MDDs and 25 matched Controls. Among the 40 genes examined, seven (*PDGFC*, *SLC6A4*, *PDLIM5*, *ARHGAP24*, *PRNP*, *HDAC5*, and *IL1R2*) had expression values that differed significantly between the MDDs and Controls. Expression values for each of these seven individual genes are reported in Table 3. The D-scores for each subject are graphically depicted in Fig. 2. The index in Fig. 2 represents the order of samples sorted by D-score. Of the 25 MDDs, 20 had an MDD-positive D-score test. Of 25 Controls, 2 had a MDD-positive D-score test. D-scores determined in the pilot study demonstrated sensitivity and specificity of 80% and 92%, respectively, in differentiating between MDDs and Controls. D-score was not significantly correlated with age or severity of illness (HAM-D score). Additionally, D-scores did not differ significantly between males and females.

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Table 5
Results of discriminant analysis involving 25 healthy subjects and 25 MDD patients in the pilot study.

Gene number	Candidate gene	Lowest Wilks' Lambda	Sensitivity	Specificity
2	PDGFC, PRNP	0.000213656	72	88
3	PDGFC, PRNP, ARHGAP24	0.000186772	80	84
4	PDGFC, PRNP, ARHGAP24, SLC6A4	0.0004431493	80	84
5	PDGFC, PRNP, ARHGAP24, SLC6A4, HDAC5	0.000762158	80	92
6	PDGFC, PRNP, ARHGAP24, SLC6A4, HDAC5, PDLIM5	0.001769471	80	92
7	PDGFC, PRNP, ARHGAP24, SLC6A4, HDAC5, PDLIM5, IL1R2	0.003991697	80	92

A combination of five gene expression values contributed to increases in sensitivity and specificity. According to this analysis, five genes (*PDGFC*, *SLC6A4*, *ARHGAP24*, *PRNP*, and *HDAC5*) were selected for calculation of the Discriminant-score.

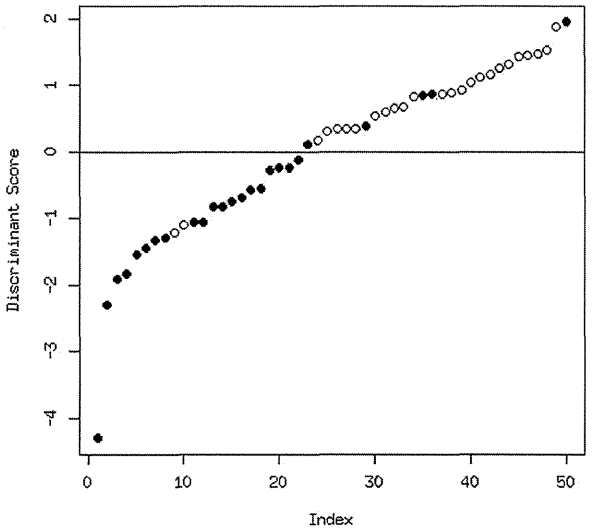

Fig. 2. D-scores from the pilot study (●: MDD patient, ○: healthy subject). The distribution of D-scores indicated a sensitivity and specificity of 80% and 92%, respectively, in differentiating between MDD patients and healthy subjects.

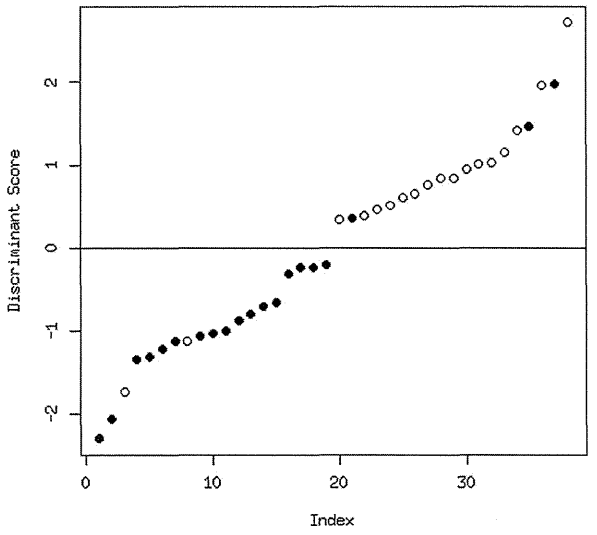

Fig. 3. D-scores from the replication study (●: MDD patients, ○: healthy subjects). The distribution of D-scores in the replication study indicated sensitivity and specificity of 85% and 89%, respectively, in differentiating between MDD patients and healthy subjects.

3.2. Replication study

Expression values for each of the five individual genes that constituted the D-score and for five other genes (*REST*, *IFNG*, *FOS*, *STAT3*, and IL18) that exhibited statistically significant findings in the replication study are listed in Table 4. D-scores for each subject are graphically depicted in Fig. 3. The index in Fig. 3 represents the order of samples sorted by D-score. D-scores in the replication study demonstrated sensitivity and specificity of 85% and 89%, respectively, in differentiating between MDDs and Controls. D-score was not significantly correlated with age or severity of illness (HAM-D score). D-scores did not differ significantly between males and females or between antidepressant-treated patients and antidepressant-naïve patients.

4. Discussion

We examined the diagnostic performance of a multi-assay, leukocyte gene expression-based test in a case-control population comprising patients with MDD and control subjects. Of 40 candidate genes, seven exhibited significantly different expression between MDDs and Controls in a pilot study involving a custom PCR array. A combination of five of these seven genes contributed to raising test sensitivity and specificity (Table 5). Based on this analysis, five genes (*PDGFC*, *SLC6A4*, *ARHGAP24*, *PRNP*, and *HDAC5*) were selected based on their discriminating capacity. In the pilot study, the application of the test resulted in overall sensitivity and specificity of 80% and 92%, respectively. In the replication study, overall sensitivity and specificity were nearly identical (85% and 89%, respectively) to those in the pilot study; these findings confirmed the findings of the pilot study. To our knowledge, this is the first leukocyte gene expression-based test for MDD that involves a customized PCR array plate and demonstrates confirmed sensitivity and specificity of approximately 80% or greater. In contrast, the combined dexamethasone–corticotrophin-releasing hormone test, which is an improvement on the traditional dexamethasone suppression test, performed at 61% sensitivity and 71% specificity in a recent study of MDDs (Watson et al., 2006).

Our current findings confirmed previous findings that the expression of some genes (e.g., *SLC6A4* and *HDAC5*) in leukocytes differs between patients with MDD and control subjects (Iga et al., 2005, 2007b). These genes may be promising markers for MDD diagnosis because similar findings have been reported from several independent laboratories (Hobara et al., 2010; Tsao et al., 2006; Belzeaux et al., 2010). *PDGFC* had the lowest P-value among the seven candidate genes identified in the pilot study. Recent studies have shown that PDGFC is both an angiogenic and a neuronal survival factor, and it appears to be an important component of neurovascular crosstalk (Lee et al., 2013). Although we could not confirm a previous findings, specifically that *VEGFA* mRNA levels were significantly higher in leukocytes of MDDs (Iga et al., 2007b; Shibata et al., 2013; Galecki et al., 2013), there was a trend

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indicating increases in *VEGFA* mRNA in MDDs in this study (P = 0.16 Mann-Whiteney U test). Therefore, genes that mediate neurovascular crosstalk such as *PDGFC* and *VEGFA* might be useful markers for MDD diagnosis. Interestingly, *PRNP* was the only gene among the five D-score-component genes that exhibited significantly lower expression in MDDs. *PRNP* encodes the prion protein, which has been implicated in various types of transmissible neurodegenerative spongiform encephalopathies. PRNP is a potentially important molecule influencing T-cell activation (Tsutsui et al., 2008; Ingram et al., 2009). Decreased *PRNP* expression in patients with MDD may indicate impaired T cell function, which may directly contribute to development of MDD (Miller, 2010).

Consistent with our proposal that a multi-assay-based test can perform better than single-assay biomarkers, considerable overall sensitivity and specificity were achieved even though consistent statistically significant differences between patients with MDD and healthy subjects were only demonstrated for two of the seven markers. Whether additional markers that are not yet identified can further improve the performance of this test is unknown. Accumulating evidence indicates that multi-assay-based tests actually performed better than single-assay biomarkers. For example, Spijker et al. showed that analysis of blood levels of seven lipopolysaccharide (LPS)-induced genes could be used as an endophenotype for MDD diagnosis (Spijker et al., 2010); the MDD score, which comprised data from the seven genes, could discriminate depressive patients from healthy controls with sensitivity of 76.9% and specificity of 71.8%. Although LPS stimulation required a 5- to 6-h incubation in the study, analysis of LPS-induced blood gene expression may overcome the normal noisiness associated with analyzing expression in basal blood (Whitney et al., 2003). Papakostas et al. used two independent samples of patients with MDD to examine a serum protein-based test for MDD. Serum levels of nine biomarkers were combined to yield an MDD-Score (Papakostas et al., 2013). In their pilot study, the test resulted in overall sensitivity and specificity of 92% and 81%, respectively. In their replication study, overall sensitivity and specificity were 91% and 81%, respectively. Although further research is needed to confirm the performance of these multi-assay-based tests, they seem to be equivalent to a serum protein-based test and may be potential as a clinical diagnostic test of MDD.

There are various advantages associated with measuring leukocyte gene expression for a diagnostic test. For example, mRNA is stable for long-term storage, and many genes can be examined quickly and simultaneously with only a small amount of blood when DNA microarrays or PCR arrays are used. Furthermore, such tests are simple to repeat as the disease progresses. In contrast, tests based on serum protein levels usually require days (not hours) to complete, and the medication and repeated blood sampling required for DST and Dex/CRH testing may be burdensome in clinical use.

There are several limitations to the present study that need to be taken into account. The influence of food or physical condition (e.g., body mass index, smoking, infection, and inflammation) on leukocyte gene expression was not examined. The present study was conducted with only adult patients; test specificity and sensitivity with very young, adolescent, or elderly patients were not examined. Furthermore, whether similar performance would have been observed for patients with MDD who had other, non-Japanese ethnic ancestry cannot be determined from the present study.

In conclusion, our findings indicated that a multi-assay MDD test based on expression of five genes in leucocytes yielded sensitivity and specificity of over 80% each in differentiating patients with MDD from non-depressed controls. Additionally, our findings present the possibility that the gene expression profiles of peripheral leukocytes may be useful for clinical diagnostic test for MDD. Further research is needed to confirm the performance of the test across various age groups and ethnic groups, to examine any additional diagnostic implications of the test, and to examine any prognostic implications of this test.

Contributors

T. Ohmori designed the study and S. Watanabe and J. Iga wrote the study protocol. K. Ishii undertook the statistical analysis. S. Numata and S. Shimodera and H. Fujita contributed for participant's recruitment and sample collection. S. Watanabe and J. Iga wrote all drafts of the manuscript and T. Ohmori carefully supervised and corrected the manuscript drafts. All authors approved the final manuscript.

Conflicts of interest

All authors reported no biomedical financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpsychires.2015.03.004.

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RESEARCH PAPER

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Blood diagnostic biomarkers for major depressive disorder using multiplex DNA methylation profiles: discovery and validation

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Aberrant DNA methylation in the blood of patients with major depressive disorder (MDD) has been reported in several previous studies. However, no comprehensive studies using medication-free subjects with MDD have been conducted. Furthermore, the majority of these previous studies has been limited to the analysis of the CpG sites in CpG islands (CGIs) in the gene promoter regions. The main aim of the present study is to identify DNA methylation markers that distinguish patients with MDD from non-psychiatric controls. Genome-wide DNA methylation profiling of peripheral leukocytes was conducted in two set of samples, a discovery set (20 medication-free patients with MDD and 19 controls) and a replication set (12 medication-free patients with MDD and 12 controls), using Infinium HumanMethylation450 BeadChips. Significant diagnostic differences in DNA methylation were observed at 363 CpG sites in the discovery set. All of these loci demonstrated lower DNA methylation in patients with MDD than in the controls, and most of them (85.7%) were located in the CGIs in the gene promoter regions. We were able to distinguish patients with MDD from the control subjects with high accuracy in the discriminant analysis using the top DNA methylation markers. We also validated these selected DNA methylation markers in the replication set. Our results indicate that multiplex DNA methylation markers may be useful for distinguishing patients with MDD from non-psychiatric controls.

Introduction

Major depressive disorder (MDD) is a common mood disorder with a lifetime prevalence rate of 16.6%.[1] According to the Global Burden of Disease Study 2010, MDD was not only one of the major leading causes of global disability-adjusted life years but also a contributor of burden allocated to suicide.[2,3] Clinical diagnosis of MDD is made on the basis of clinical symptoms that are exhibited by patients. However, an accurate diagnosis of MDD is difficult for clinicians,[4] and there are no established biomarkers to support the diagnosis of MDD.

Epigenetics is defined as the study of mitotically or meiotically heritable variations in gene function that cannot be explained by changes in DNA sequence.[5] DNA methylation, which is the transference of a methyl group to the 5-carbon position of the cytosine in a CpG dinucleotide, is one of the major mechanisms of epigenetic modifications. There is growing evidence for the lasting influence of a modified DNA methylation status that is established early in life.[6,7] Aberrant DNA methylation in the blood of patients with MDD and the associations of different DNA methylation patterns with the phenotypic discordance of MDD between twins have been reported.[8-11] Although the effects of antidepressants and mood stabilizers on DNA methylation, both of which are major medications for patients with MDD, have been reported,[12-14] no comprehensive studies using medication-free subjects with MDD have been conducted. Furthermore, most of the previous studies have been limited to the analysis of the CpG sites in CpG islands (CGIs) in the gene promoter regions.

The main aim of the present study is to identity DNA methylation markers that distinguish patients with MDD from non-psychiatric controls. We first conducted a genome-wide DNA methylation profiling (485,764 CpG dinucleotides) of peripheral leukocytes in a discovery set of samples (20 medication-free patients with MDD and 19 non-psychiatric controls), and intended to classify patients with MDD and the controls using

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the selected DNA methylation markers in the discriminate analysis. Furthermore, we examined whether these selected multiplex DNA methylation markers from the discovery set could be a reliable tool to discriminate between patients with MDD and non-psychiatric controls in another independent set of samples (12 medication-free patients with MDD and 12 non-psychiatric controls).

Results

Diagnostic differences in DNA methylation between medication-free patients with MDD and control subjects in the discovery set

DNA methylation levels were compared between 20 medication-free patients with MDD and 19 control subjects using Infinium® HumanMethylation450 BeadChips. Of 431,489 CpG sites, significant diagnostic differences in DNA methylation were observed at 363 CpG sites at FDR 5% correction (nominal $P < 4.15 \times 10^{-5}$) (**Supplementary Table S1**). Of these 363 CpG sites, all demonstrated lower DNA methylation in patients with MDD than in the controls (**Fig. 1**), and some of them, such as *CAPRIN1* (cell cycle associated protein 1),[15] *CITED2* (Cbp/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2),[16] *DGKH* (diacylglycerol kinase, eta),[17] *GSK3B* (glycogen synthase kinase 3 β),[18] and *SGK1* (serum/glucocorticoid regulated kinase 1),[19] have been associated with MDD. When these 363 differentially methylated CpG sites were classified into four categories (CGI, CGI shore, CGI shelf, and others) according to the CpG content in the genes, most of them (99.4%) were located in the CGIs. When these 363 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, 313 sites (86.2%) were located in the promoter regions, and 64 sites (9.1%) were in the gene bodies. When we performed functional annotation of genes that showed significant diagnostic differences in DNA methylation, significant gene-ontology terms included nucleus, nuclear lumen, nuclear part, and nucleoplasm at FDR 5% correction (nominal $P < 3.10 \times 10^{-5}$) (**Supplementary Table S2**). When we performed a discriminant analysis between patients with MDD and the control subjects based on the top 18 MDD-associated DNA methylation markers of the Mann-Whitney U test comparison results, which were detected in 100% subjects (**Supplementary Table S3**), we were able to segregate patients with MDD from the controls with a sensitivity of 100% and a specificity of 100% (**Fig. 2A**).

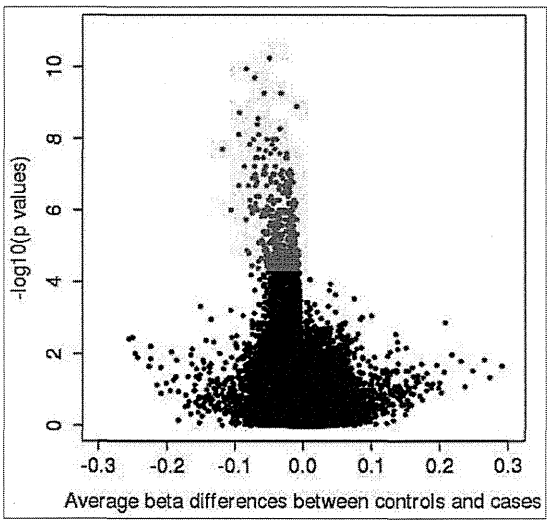

Figure 1. Diagnostic differences in DNA methylation. The X axis represents average β differences (Δβ value) between control subjects and patients with major depressive disorder (MDD). The Y axis represents $-\log_{10} P$ values. Each dot represents an individual CpG site (a total of 431,489 CpG sites). Red dots represent 363 CpG sites that showed significant diagnostic differences between patients with MDD and the controls at 5% FDR correction. Δβ value > 0 corresponds to higher DNA methylation in patients with MDD than in the controls, whereas Δβ value < 0 corresponds to lower DNA methylation in patients with MDD than in the controls. Of these 363 CpG sites, all of them demonstrated lower DNA methylation in patients with MDD than in the controls.

Validation of diagnostic DNA methylation markers for MDD in an independent set of samples

DNA methylation levels were measured in an independent replication cohort of 12 medication-free patients with MDD and 12 control subjects using the same Illumina DNA methylation arrays. Of the top 100 differentially methylated CpG sites in the discovery set, 84 sites were also found to be significant in the replication set of samples (Mann-Whitney U-test, $P < 0.05$). To assess whether a panel of selected DNA methylation markers identified in the discovery set was useful as a reliable tool to discriminate between patients with MDD and non-psychiatric controls, 17 out of 18 sites were used for a discriminant analysis in the replication set, because the DNA methylation level of one site (cg11338389) was not detected in 100% of the subjects in the replication set. We were also able to obtain a 100% sensitivity and 100% specificity in the replication set using these 17 multiplex markers (**Fig. 2B**).

DNA methylation and expression in the *GSK3B* gene

Among the CpG sites that demonstrated significant diagnostic differences in DNA methylation, *GSK3B* is one of the most interesting genes, because this gene has been implicated in not only MDD[18] but also in the therapeutic mechanism of antidepressants.[20] A significantly lower DNA methylation in patients with MDD than in the controls was observed at the CpG site (cg14472315) in the CGI in the promoter region of the *GSK3B* gene both in the discovery set and in the replication set (Mann-Whitney U-test, $P = 9.7 \times 10^{-6}$ and 1.4×10^{-4}, respectively). *GSK3B* mRNA expression level in blood was compared between 19 medication-free patients with MDD and 19 control subjects, and a significantly higher *GSK3B* expression level in patients with MDD than in the controls was observed (Patients: 0.20 ± 0.17; Controls: 0.10 ± 0.03; Student t test, $P = 5.0 \times 10^{-3}$), which is consistent with a previous human post-mortem brain study by Oh

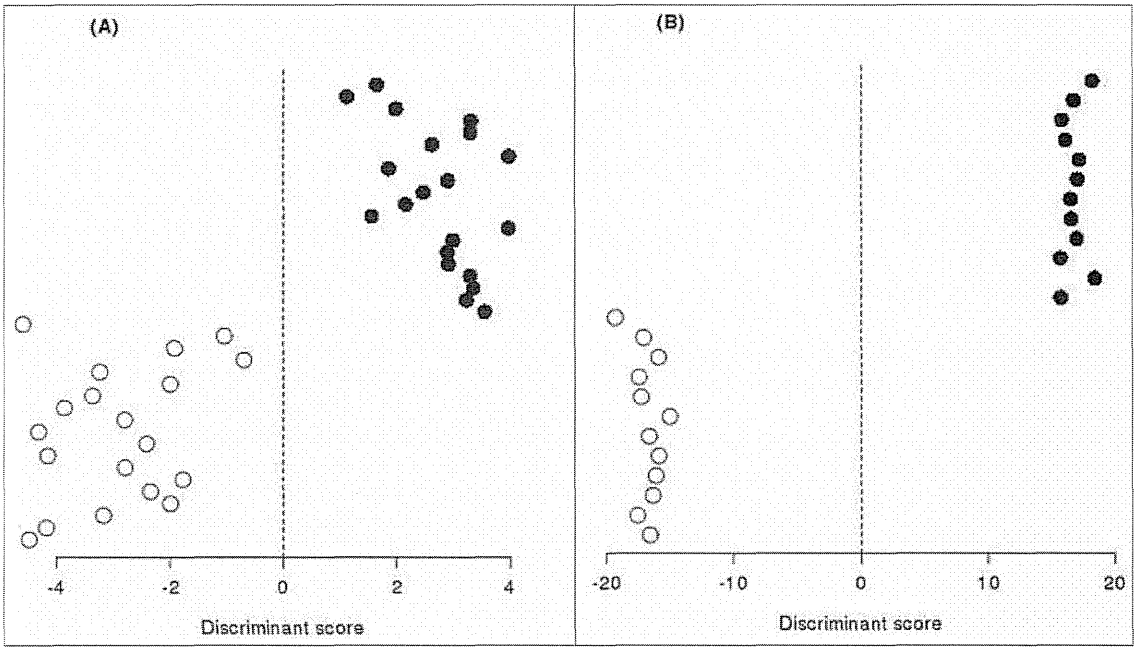

Figure 2. Plot of discriminate scores of patients with major depressive disorder and control subjects. The following color scale is used: black, major depressive disorder (MDD); white, control (CTRL). The x axis represents discriminant scores of samples. **(A)** Discriminant scores of 20 MDD patients and 19 CTRLs. We were able to segregate patients with MDD from the CTRLs using the top 18 MDD-associated DNA methylation markers of the Mann-Whitney U test comparison results in a discovery set of samples. **(B)** Discriminant scores of 12 MDD patients and 12 CTRLs. We were also able to obtain 100% sensitivity and 100% specificity using the same17 multiplex DNA methylation markers in a replication set of samples.

et al.[18] When we examined the relationship between the *GSK3B* promoter DNA methylation and expression in the combined samples (N = 38), we found a significant inverse correlation between them (r = −0.319; $P = 0.025$, one-tailed) (**Fig. 3**).

Discussion

In this study, we conducted a genome-wide DNA methylation profiling of the peripheral leukocytes of patients with MDD

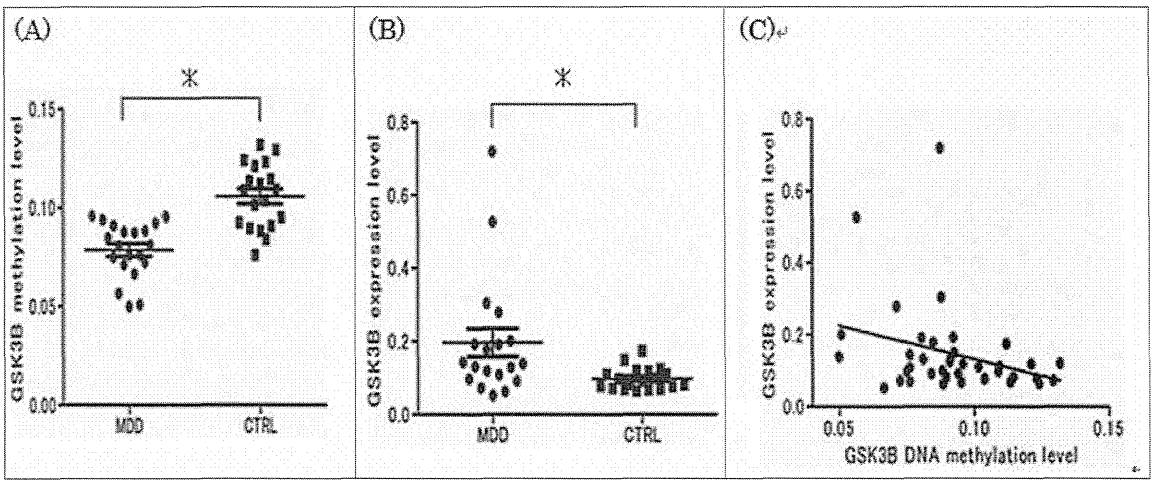

Figure 3. *GSK3B* DNA methylation and expression. **(A)** *GSK3B* DNA methylation. The average DNA methylation level of CpG site (cg14472315) in the CGI in the promoter region of the *GSK3B* gene was significantly lower in patients with major depressive disorder (MDD) than that in the controls (CTRLs) in a discovery set (MDD, n = 20, and CTRL, n 19) (0.079 ± 0.014 and 0.106 ± 0.016, respectively, Mann-Whitney U-test, $P = 9.7 \times 10^{-6}$). **(B)** *GSK3B* expression. The average expression level of the *GSK3B* gene was significantly higher in patients with MDD than that in the CTRLs in a discovery set (MDD, n = 19, and CTRL, n = 19) (0.197 ± 0.168 and 0.098 ± 0.03, respectively, Student t test, $P = 5.0 \times 10^{-3}$). **(C)** Relationship between the *GSK3B* promoter DNA methylation and expression. A significant inverse correlation between the *GSK3B* promoter DNA methylation and expression was observed in the combined samples (MDD, n = 19, and CTRL, n = 19) (r = −0.319; $P = 0.025$, one-tailed).

using Infinium HumanMethylation450 BeadChips. To our knowledge, this is the first comprehensive DNA methylation study of blood using medication-free patients with MDD.

First, we identified aberrant DNA methylation in MDD at 363 CpG sites in the discovery set, and demonstrated that altered DNA methylation in MDD was likely to show a pattern of DNA hypomethylation. This pattern was different from that of DNA hypermethylation in the medication-free patients with schizophrenia in our previous study,[21] suggesting that disease-specific DNA methylation changes may occur in blood.

Next, we selected top DNA methylation markers as potential diagnostic biomarkers from the discovery set of samples (20 medication-free patients with MDD and 19 control subjects), and validated these selected markers in an independent replication set of samples (12 medication-free patients with MDD and 12 control subjects) with 100% accuracy, suggesting that multiplex DNA methylation markers may be useful for distinguishing patients with MDD from non-psychiatric controls. To date, only one study has successfully distinguished between medication-free patients with MDD and healthy controls based on the DNA methylation profiles of CpG sites in the CGI at the *BDNF* gene promoter region.[9] Although Infinium HumanMethylation450 BeadChips covered this gene, the exact locations of CpG sites of this array were different from those of a previous *BDNF* study by Fuchikami et al. Several studies have attempted to differentiate patients with MDD from controls using multiplex biomarkers based on gene expression, plasma metabonomics, and serum assay.[15,22,23] The multiplex DNA methylation diagnostic markers for MDD that we identified in the present study exhibited a higher accuracy compared to the previously used blood biomarkers, and may therefore enable earlier and more suitable therapeutic intervention in patients with MDD.

Among these 313 differentially methylated CpG sites in the CGIs in the gene promoter regions, several genes, such as *DGKH* (cg00109274), *GSK3B* (cg14472315), and *SGK1* (cg06642177), have been implicated in MDD. DNA hypomethylation in MDD was observed in these 3 genes, and these results were confirmed in the independent replication cohort ($N = 24$, Mann-Whitney U test, $P < 0.05$). *DGKH* was originally identified as a susceptibility gene of bipolar disorder in the genome-wide association study by Baum et al.[24] Genetic variants of this gene have also been associated with MDD,[24] and *DGKH* plays an important role in the phosphatidylinositol pathway, which is thought to be involved in the action of lithium,[25,26] not only a primary therapeutic agent for bipolar disorder but also an augmentation agent of antidepressants for MDD. *GSK3B* is a fascinating enzyme that plays crucial roles in many signaling processes that are involved in key functions of the brain.[27] Extensive evidence suggests that *GSK3B* may be implicated in MDD. Genetic variants of this gene have been associated with MDD, the antidepressant response in patients with MDD, brain structural changes in patients with MDD, and clinical measures of MDD.[28-31] In addition, increased expression of this gene was observed in the brain of patients with MDD.[18] Moreover, *GSK3B* seems to be involved in the therapeutic mechanism of antidepressants and lithium. Treatment with the antidepressants fluoxetine and imipramine, as well as lithium, increased the level of phosphor-Ser9-*GSK3B* in mouse brain.[20,32] Furthermore, growing evidence indicated that *BDNF* also plays an important role in the pathophysiology of MDD,[33] and the phosphorylation of *GSK3B* mediated by *BDNF/PI3K/Akt* has been reported to be involved in the antidepressant action of the second generation antipsychotics, such as aripiprazol and olanzapine.[34,35] *SGK1* contributes to the regulation of transport, hormone release, neuroexcitability, inflammation, cell proliferation, and apoptosis.[36] There is evidence of the crucial involvement of the glucocorticoid receptor (GR) in the development of MDD.[37] *SGK1* is one of the GR target genes. Moreover, increased expression of this gene in the peripheral blood of drug-free patients with MDD has been reported.[19] In addition, *SGK* isoforms were involved in the signaling of *BDNF*.[38]

There are several limitations to the present study. First, cell-type heterogeneity in peripheral blood might affect DNA methylation patterns.[39] So, cell-type-specific studies may be needed. Second, the number of CpG sites analyzed was limited, and there was a selection bias of the analyzed CpG sites in this study. More comprehensive studies will be needed. Third, we compared only MDD patients with non-psychiatric controls. Further studies using patients with other psychiatric disorders will be needed to establish reliable markers which support the diagnosis of MDD. Fourth, we included several patients with MDD during their recurrent depressive episodes. So the medications during their previous episodes might affect the present DNA methylation status. Finally, although we used well-matched patients and controls with respect to age and gender, we did not take other confounding factors, such as smoking or body mass index, into consideration in our analysis.[40,41]

In summary, this study demonstrated that multiplex DNA methylation markers distinguished patients with MDD from the control subjects with high accuracy, and this result was replicated in an independent set of samples. These results suggest that multiplex DNA methylation markers may be useful for distinguishing patients with MDD from non-psychiatric controls.

Materials and Methods

Participants

For our discovery set of samples, 20 medication-free patients with MDD (2 males and 18 females, mean age: 44.2 ± 15.2 y) were recruited from Tokushima and Kochi University Hospitals in Japan. The diagnosis of MDD 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. The severity of MDD was evaluated using the Hamilton Rating Scale for Depression (HAM-D). Among the 20 patients, 17 had no history of antipsychotics, including antidepressant and mood stabilizers, and 2 had not taken any antipsychotics for at least six months, and one patient took Zolpidem. Nineteen control subjects (2 males and 17 females, mean age: 42.4 ± 12.3 y) were selected from volunteers who were recruited from hospital staff, students, and company employees documented to be free from