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（精神障害分野）

気分障害の神経病理学に基づく分類を目指した脳病態の解明

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平成２３年度　総括研究報告書

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目　　次

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研究要旨：

　うつ病は、大きな社会負担、急増する患者数等から社会問題となっているが、双極スペクトラム、非定型うつ病、血管性うつ病など、多様な病型が存在し、その診療には混乱も生じている。本研究の目的は、遺伝要因、早期養育環境、ストレス、加齢、動脈硬化などのうつ病の危険因子によって引き起こされる脳病態を、死後脳研究、脳画像解析、疫学研究、および動物モデルにおける神経科学的解析などによって明らかにし、脳病態に応じた気分障害の分類を目指すことである。

　本研究の特色は、うつ病の危険因子による脳病態を多角的に明らかにすると共に、これを患者死後脳で確認することである。気分障害の疾患概念を病理学に基づいて再構築し、病型に応じた診断法、治療法を開発することができれば、精神医学が病理学に基づく医学へと進化する第一歩となる。現在混沌としている「うつ」が種々の脳疾患と心の悩みに明確に分類され、適切に対応されれば、自殺者数の減少、円滑な人事管理など、多くの波及効果が期待でき、国民の生活の質を向上させると期待される。

　平成23年度は、ミトコンドリアDNA異常を持つモデルマウスにおいて、視床の神経病理学的変化を、免疫組織化学的手法により検討すると共に、人における染色方法を確立した。

　また、脳卒中患者におけるMRI構造画像を用いた検討により、抑うつと関係のある脳領域として、新たに、橋を含む脳幹部、左大脳基底核、左前頭葉皮質が見いだされた。

　うつ病の原因に関わる脳部位を同定するため、免疫賦活によるうつ病モデルを検討し、視床下部において、ミクログリア活性化が遷延していることを見いだした。

　また、高齢者ブレインバンクで蓄積された症例の精神医学的診断を後方視的に行い、11例の大うつ病性障害患者を見いだした。これらの症例では、脳血管障害、神経原線維変化などの神経病理学的変化を認めた。

　これらの結果から、気分制御に関わる脳部位における種々の病変が気分障害の病態に関与していることが示唆された。

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A 研究目的
　社会生活障害を引き起こす疾患として最大の要因であるうつ病の患者数は近年急速に増加し、社会問題となっている。しかし、抗うつ薬によく反応するメランコリー型に加え、抗うつ薬により悪化する双極スペクトラム、抗うつ薬が奏効しにくく心理療法も重要となる非定型うつ病、難治化しやすい血管性うつ病など、治療抵抗性のうつ病が増加し、うつ病診療は混沌とした状況にある。面接に頼った現在の診断法には限界があり、現状の

打開には、神経病理学所見に基づいて疾患概念を再構築すると共に亜型分類を確立し、脳病態診断に基づいて治療を最適化する他ない。

うつ病の発症には遺伝、養育、ストレス、加齢など、種々の危険因子が関与する。

加藤らはミトコンドリアDNA異常が脳に蓄積する遺伝子改変マウスが双極性障害様の行動異常を示すことを見出した。

山下らは基底核に脳血管障害が存在するとうつ病の中でも意欲低下が優勢となることを見出し、血管性うつ病の神経学的基盤を示した。

神庭らはインターロイキン1(IL-1)が脳内モノアミン系に影響することや、インターフェロンがIL-1β上昇を介して海馬神経新生を低下させることを見出し、うつ病の神経免疫仮説を確立した。また久山町の65歳以上の全住民を対象としてうつ病の調査を行い、有病率を明らかにした。

村山らは1万名近い脳を有する高齢者ブレインバンクを構築し、千例以上の検討で、加齢に伴い徐々にLewy小体が蓄積し、脳機能障害が引き起こされることを示した。

上記の他多くの研究から、遺伝要因によるミトコンドリア機能障害、ストレスによる神経内分泌免疫学的変化、加齢に伴う脳血管障害および異常蛋白蓄積など、各危険因子による脳機能障害がうつ病を引き起こすと考えられる。本研究では、こうした脳病態を臨床研究および動物モデルを用いて明らかにすると共に、これを患者死後脳で検討する。

本計画の特徴は、うつ病の異種性の高さを前提とし、各危険因子と脳病態の関連を明らかにし、脳病態を臨床診断（亜型分類）と対応づけることにより、気分障害の神経病理学的な分類の基盤を固めることである。本研究によりうつ病の神経病理学的所見に基づく分類が可能となれば、診療やメンタルヘルス管理の最適化が可能となり、自殺者減少、生活の質の向上につながる。

B. 研究方法

動物実験では、ミトコンドリアDNA変異が脳内に蓄積するモデルマウスを用いて、ミトコンドリアDNA蓄積を指標として、気分障害類似の行動異常と関連する脳の異常を同定すると共に、こうした変化を可視化する技術を開発する。

また、うつ病の原因脳部位を探索するため、免疫賦活モデルを用いて、行動変化を観察すると共に、ミクログリア活性化の分布と時間経過を観察する。

脳卒中患者における、定量的 MRI を用いて、病変部位と症状の関連を検討する。

また、高齢者ブレインバンクの登録症例で、気分障害の調査を行うと共に、新たな剖検例について、神経病理学的検索を行う。

C.研究結果

ミトコンドリア DNA(mtDNA)合成酵素の変異を脳特異的に発現させた双極性障害モデルマウスにおいて開発した、mtDNA 欠失が蓄積した細胞を可視化する方法を用いて、mtDNA 由来蛋白質であるチトクローム酸化酵素(Cox)、および核DNA 由来蛋白質である、クエン酸脱水素酵素の抗体、および核を染色する DAPI を用いた三重染色を行い、モデルマウスにおける Cox 陰性細胞の分布を検討した。その結果、視床の一部に、Cox陰性細胞を認めた。

また、気分障害患者の死後脳で同様の検討を行うべく、ヒト死後脳切片を用いて、マウスと同様の三重染色を行う方法を確立し、検討を行った。その結果、視床で Cox 陰性細胞を認める症例を見いだした。

うつ病の原因となる脳部位を同定するため、マウスにリポポリサッカライド（LPS）を投与し、免疫賦活を行った。実験には、8〜10 週齢のBALB/c マウスを行い、LPS は 0.8 mg/kg を腹腔内投与した。Iba-1 に対する免疫組織化学によって、ミクログリアを可視化し、Image J を用いて形状・活性化を評価した。まず、行動を確認したところ、24 時間後においても、sickness behaviorが遷延していることが見いだされた。更に、ミクログリア活性化を調べたところ、視床下部弓状核から腹内側核にかけて、ミクログリアの活性化が遷延していることがわかった。

脳卒中患者における、定量的 MRI を用いて、病変部位と症状の関連を検討した研究では、症例数を増やし、最終的に、初発脳卒中患者 113 例（68.0±10.2 歳）における解析を行った。臨床評価としては、Hospital Anxiety and Depression scale (HADS),、Apathy score、認知機能検査等を行った。1.5T MRI を撮像し、マニュアルで病変をトレース後 VBM （voxel based morphometry)解析を行った。

その結果、抑うつ症状と有意な関連のある病変部位として、橋を含む脳幹部、左大脳基底核、左前頭葉皮質が見いだされた。

これまでに蓄積された、高齢者ブレインバンクサンプルに関し、神経病理学的所見とうつ病の有無に関する調査を行った。

高齢者ブレインバンクで剖検され、経病理学的所見を検討した 611 例中、「depression」の記載がある症例を、データーベースを用いて検索した。

その結果、精神科受診歴があり、うつ病、躁うつ病、または双極性障害と診断された者、22名が見いだされた。これらの症例について、経験ある精神科医（T.K.）がカルテを精査し、後方視的にDSM-IV基準を適応した。その結果、大うつ病性障害と診断される者は11名で、うち7名は反復性、4名は単一エピソードであった。うち10名は、脳血管障害、神経原線維変化などの病理学的所見を認めた。

これらの結果より、うつ病の中には、既知の神経病理学的変化に伴う場合があると考えられた。

D.考察

動物モデル研究では、気分障害の原因脳部位として、視床の関与が疑われた。また、人でも同様の病変を見いだした。

免疫賦活モデルを用いた検討では、視床下部の一部でミクログリア活性化が遷延することが見出された。

一方、脳卒中患者におけるMRIの研究では、抑うつ症状と縫線核病変の関連が示唆された。

また、老年期のうつ病患者における検討から、さまざまな神経病理学的過程が関与する可能性が示唆されたが、うつ病を引き起こす病理学的変化の性質はさまざまであり、病変の性質に加え、病変部位の検討が重要であると考えられた。

E.結論

これまでのうつ病研究では、抗うつ薬の作用機序などに注目される場合が多く、脳のどのような病理学的異常が生じるのかは、わかっていなかった。

本研究の結果、気分障害に気分制御にかかわるさまざまな脳部位における、脳血管病変、変性など、種々の病変が関与していることが示唆された。

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G.知的財産権の出願・登録状況
1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他

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村山繁雄,齊藤祐子.	認知症の病理学.	綜合臨床	60(9)	1805-1808,	2011

資　　料

Hypermethylation of serotonin transporter gene in bipolar disorder detected by epigenome analysis of discordant monozygotic twins

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Bipolar disorder (BD) is a severe mental disorder characterized by recurrent episodes of mania and depression. Serotonin transporter (HTT) is a target of antidepressants and is one of the strongest candidate molecules of mood disorder, however, genetic study showed equivocal results. Here, we performed promoter-wide DNA methylation analysis of lymphoblastoid cell lines (LCLs) derived from two pairs of monozygotic twins discordant for BD. To rule out the possible discordance of copy number variation (CNV) between twins, we performed CNV analysis and found the copy number profiles were nearly identical between the twin pairs except for immunoglobulin-related regions. Among the three genes we obtained as candidate regions showing distinct difference of DNA methylation between one of the two pairs, hypermethylation of SLC6A4, encoding HTT, in the bipolar twin was only confirmed by bisulfite sequencing. Then, promoter hypermethylation of SLC6A4 in LCLs of BD patients was confirmed in a case–control analysis. DNA methylation of SLC6A4 was significantly correlated with its mRNA expression level in individuals with the S/S genotype of HTTLPR, and mRNA expression level was lower in BD patients carrying the S/S genotype. Finally, DNA methylation of the same site was also higher in the postmortem brains of BD patients. This is the first study to report the role of epigenetic modification of SLC6A4 in BD using an unbiased approach, which provides an insight for its pathophysiology.
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Introduction

Mood disorders such as major depression and bipolar disorder (BD) cause severe social burdens and there is an urgent need to elucidate the pathophysiology of these disorders. Studying the mechanisms of action of psychotropic drugs has been a major strategy to understand the pathophysiology of mental disorders, and research has focused on serotonin transporter (HTT), the target molecule of antidepressants. Altered density of HTT in the brains of patients with mood disorders has been reported in postmortem samples[1] and by in vivo molecular imaging.[2–6] A serotonin-transporter-linked promoter region (HTTLPR) includes the functional polymorphism, in which the short (S) allele has lower promoter activity than the long (L) allele,[7] and it has been implicated in mood disorders.[1,8–10] The S allele was reported to be a risk factor for mood disorders, interacting with stress and/or early environment.[8] However, the results of subsequent studies are conflicting[11–13] and none of the recent genome-wide association studies supported the role of SLC6A4 in mood disorders.[14]

We have been searching for gene expression and epigenetic differences between monozygotic (MZ) twins discordant for BD to elucidate the molecular pathways

relevant to its pathophysiology.[15,16] Although high concordance rate of BD in MZ twins supports the contribution of genetic factor in BD, importantly, it is not 100%. Because MZ twins have been regarded as having identical genomes, these facts suggest the importance of environmental or epigenetic factor for the onset of mental disorders. In fact, considerable epigenomic differences between MZ twins have been reported by several groups.[17–22] On the other hand, there are also reports of genomic differences between MZ twins such as point mutations,[23–25] microsatellite repeat length[26] and copy number variations (CNVs).[27] Therefore, both genetic and epigenetic factors may have a role in the discordance of the onset of diseases between MZ twins.

Here, we performed promoter-wide DNA methylation analysis of lymphoblastoid cell lines (LCLs) derived from two pairs of MZ twins discordant for BD. To rule out the possible discordance of CNV between twins, we performed CNV analysis.

Materials and methods

Samples. We enrolled two pairs of MZ twins discordant with respect to BD (Supplementary Table S1). The twins

were 49-year-old males (pair 1, previously reported in Kusumi et al.[28]) and 42-year-old males (pair 2). Detailed clinical information was described in previous reports and their zygosities were determined by micro-satellite markers and single nucleotide polymorphisms.[15,16] We used their genomic DNA derived from LCLs for all analyses.

In the CNV analysis, we used a female sample (NA15500, Coriell Cell Repository, Camden, NJ, USA) as a reference and an unrelated healthy female sample for quality control of real-time PCR analysis. Methods of CNV analysis are shown in Supplementary Information.

To exclude the genomic regions in which DNA methylation is different between peripheral blood leukocytes (PBLs) and LCLs, we used four sets of PBLs and LCLs from the same individuals who were unrelated healthy Japanese males and females.[29] For the collection of PBLs, blood was collected into a 7 ml tube containing EDTA. Red blood cells were lysed by osmotic shock by adding 40 ml of red blood cell lysis buffer containing 10 mM Tris/HCl, pH 7.6, 6 mM $MgCl_2$ and 10 mM NaCl. After centrifugation, the pellet was used for DNA extraction.

For the case–control analysis, we examined 20 unrelated BD patients (12 males and 8 females, 38.8 ± 13.1 years old (mean $\pm$ s.d.)) and 20 unrelated control subjects (16 males and 4 females, 38.5 ± 8.9 years old) using their genomic DNA derived from LCLs. They were subjects from our previous report,[30] and all of them were Japanese. Written informed consent was obtained from all the subjects.

Postmortem prefrontal cortices (BA10) derived from BD patients ($N = 35$) and controls ($N = 35$) were provided by the Stanley Foundation Brain Collection (The Stanley Medical Research Institute, Chevy Chase, MD, USA). We were able to use 31 BD samples and 32 control samples for DNA methylation analysis.

This study was approved by the Research Ethics Committee of RIKEN.

Cell cultures. LCLs were previously established by transforming the lymphocytes with Epstein–Barr (EB) virus following standard protocol.[31] Briefly, lymphocytes were separated from peripheral blood and cultured with RPMI 1640 medium containing 20% fetal bovine serum (Gibco, Carlsbad, CA, USA), penicillin and streptomycin (50 µg ml^{-1} each) and supernatant of the B95-8 cell culture infected by the EB virus. After the cells showed stable growth, they were passaged two or three times a week using similar medium except for 10% fetal bovine serum. The cells were then kept frozen and recultured for the experiment. We used low-passage LCLs for case–control studies. Genomic DNA was extracted using Gentra Puregene Cell kit (Qiagen, Hilden, Germany).

DNA methylation analysis
Enrichment of methylated DNA. We used a MethylCollector Ultra Kit (Active Motif, Carlsbad, CA, USA), which can enrich methylated DNA fragments with as few as five methylated CpG sites. DNA fragments (100 ng) were sonicated to produce 100- to 300-bp fragments using a sonicator (Covaris, Woburn, MA, USA) and they were incubated with a His-tagged recombinant MBD2b/MBD3L1 protein complex.

After stringent washing, methylated DNA was eluted and purified using the MiniElute PCR purification kit (Qiagen).

Promoter tiling array. We used a GeneChip Human Promoter 1.0R array (Affymetrix, Santa Clara, CA, USA), which contains 4.6 million probes tiled to cover more than 25 500 human promoter regions. Sample preparation for the tiling array was performed according to the Affymetrix chromatin immunoprecipitation assay protocol provided by the manufacturer. Briefly, after amplification, DNA samples were labeled using a GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix), and they were then hybridized with Affymetrix GeneChip Human Promoter 1.0R arrays. The arrays were stained and washed using GeneChip Fluidics Station 450 and scanned with the GeneChip 3000 7G Scanner. We used fully CpG unmethylated DNA obtained by whole genome amplification as a reference sample.

Tiling array data analysis. For the detection of methylated regions (MRs), model-based analysis of tiling arrays (MAT) software was used.[32] By using MAT, the signals of each probe in each array can be standardized individually. Parameters used in the MAT analysis were as follows: bandwidth, 300; maximum gap, 300; minimum number of probes, 10; P value, 10^{-4}.

The filtering process was performed with R script (http://www.r-project.org) using the following steps (Supplementary Figure S4). Scripts are available upon request.

(1) Data of each twin pair were directly compared. The regions showing significant differences between the BD twin and the healthy co-twin were selected. Only those regions containing six or more CpG sites were further selected because the MethylCollector can collect only the DNA fragments that contain five or more CpG sites. The selected MRs were named BD (bipolar twin)-dominant MRs and C (healthy co-twin)-dominant MRs.

(2) The data of each twin were compared with those of a reference sample (that is, unmethylated DNA). Among the BD-dominant MRs, the regions showing the significantly methylated signal compared with a reference sample in BD twin but not in healthy co-twin were selected. The selected regions were named BD-specific MRs. C-specific MRs were determined vice versa. This step highlighted the methylation difference between twins and eliminates significant but subtle differences to maximize the chance for successful confirmation by other methods.

(3) The MRs overlapping with a CpG islands were selected. For validation of tiling array data, we arbitrarily selected 13 representative MRs for bisulfite sequencing.

Bisulfite conversion. Either 2 µg (for LCL samples) or 500 ng (for postmortem brain samples) of genomic DNA was converted for methylation sequencing using Epitect bisulfite kits (Qiagen), according to the manufacturer's standard protocol. Completion of sodium bisulfite conversion was confirmed during sequencing by ensuring that known lone cytosines were read as thymines.

Bisulfite sequencing. Primer pairs were determined using Meth Primer software.[33] PCR products were purified by MinElute PCR Purification Kit (Qiagen) or digested with thermostable β-agarase (NIPPON GENE, Tokyo, Japan) and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Single bacterial colonies were subjected to sequencing analysis. Each bisulfite sequencing data was quantified by quantification tool for methyaltion analysis.[34]

Filtering of tiling array data. To further narrow the number of MRs by setting more stringent criteria based on the results of bisulfite sequencing, we reset the threshold for filtering as a P value $<10^{-6}$ of direct comparison between each twin pair (Supplementary Figure S5, step 4).

Next, we excluded the regions that showed alteration of DNA methylation status before and after the transformation by EB virus. We previously performed the DNA methylation analysis using the same platform in four sets of PBLs and LCLs established from the same individuals and identified the regions that showed methylation only in LCLs or only in PBLs.[30] MRs that overlapped with those regions were excluded (Supplementary Figure S5, step 5).

Finally, from the candidate regions for BD-specific MRs, the regions that were detected as MRs in at least one of LCL were excluded (Supplementary Figure S4), because the regions methylated in LCLs of healthy individuals cannot be related to disease. With regard to the C-specific MRs, only the regions overlapping with common MRs in four LCLs were selected. Loss of methylation in patients can be regarded as related to disease only when methylation in that region is consistently seen in healthy individuals (Supplementary Figure S5, step 6).

Case–control analysis

Pyrosequencing. The PCR product of bisulfite-modified DNA was used for pyrosequencing analysis according to the manufacturer's standard protocol (Qiagen). Briefly, 4 μl of streptavidin-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) and 54 μl of binding buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, 0.1% Tween-20 at pH 7.6) were mixed with 50 μl of PCR product for 10 min at room temperature. The reaction mixture was placed onto a MultiScreen-HV, Clear Plate (Millipore, Billerica, MA, USA). After applying the vacuum, the beads were treated with a denaturation solution (0.2 N NaOH) for 1 min and washed twice with washing buffer (10 mM Tris-acetate at pH 7.6). The beads were then suspended with 50 μl of annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate at pH 7.6) containing 10 pmol of sequencing primer. The template-sequencing primer mixture was transferred onto a PSQ 96 Plate (Biotage, Uppsala, Sweden), heated to 90 °C for 2 min and cooled to room temperature. Sequencing reactions were performed with a PyroMark Gold Q96 Reagents Kit (Qiagen) according to the manufacturer's instructions. The percentages of methylation were calculated from the raw data using the allele quantification algorithm of the software provided by the manufacturer (PSQ96MA2.1.1 software, Qiagen).[35] The PCR primers used for bisulfite sequencing of the promoter region of *SLC6A4* were 5'-TTTTTAGTTGTTTG GGTATTTGTGTTA-3' (forward) and 5'-AAAACTTTACAAC CTCTTAAAAACCC-3' (reverse). The sequencing primers

were 5'-TTTTGTATAAAGTTATTTGT-3', 5'-AATATAAATTA TGGGTTGAA-3' and 5'-ATTTTTTTTTAAGGGGTTTTT-3', and the reading sequences were AT/CGT/CAT, AT/CGA AAGTAAGTAATTTTTTTTTAAAGT/CG and TAT/CGG.

Genotyping. Using genomic DNA derived from LCLs, we investigated the length polymorphism repeat (HTTLPR) in the promoter region of *SLC6A4*, which comprises a 44-bp insertion (long, L allele) or deletion (short, S allele), and one single nucleotide polymorphism in L allele (LA/LG), which was reported to be related to gene expression as well as HTTLPR genotype.[36]

For HTTLPR genotype analysis, amplification of genomic DNA was performed as initially described by Kaiser *et al.*,[37,38] with slight modifications. In a total volume of 10 μl, we used 40 ng DNA; 200 μM each of dATP, dCTP, and dTTP; 150 μM dGTP, 50 μM; 7-deaza-GTP (Roche, Mannheim, Germany); 1 μM of each primer; 10* buffer 1; and 2.8 units of mixed polymerase (Taq- and Tgo-polymerase) from the Expand Long-Range PCR System (Roche). Applied primer sequences were 5'-GCAACCTCCCAGCAACTCCCTGTA-3' (forward) and 5'-GAGGTGCAGGGGGATGCTGGAA-3' (reverse). The PCR program of both reactions consisted of an initial cycle of 2 min at 94 °C followed by 35 cycles each consisting of 10 s at 98 °C, 30 s at 68 °C and 30 s at 72 °C. After amplification, HTTLPR amplicons were separated by electrophoresis in 3% agarose gels after staining with ethidium bromide.

For LA/LG single nucleotide polymorphism analysis, each product of L allele in L/S genotype subjects was digested with thermostable β-agarase (NIPPON GENE) and subjected to sequencing analysis.

Real-time quantitative RT-PCR. Total RNA was extracted using Trizol (Invitrogen) and cleaned following the standard protocol. After DNase I treatment, 5 μg of total RNA was used for complementary DNA synthesis by oligo (dT) 12–18 primer and SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR using Taqman Universal PCR master mix, No AmpErase UNG (Applied Biosystems, a part of Life Technologies, Carlsbad, CA, USA), was performed with an ABI Prism 7900HT (Applied Biosystems). The PCR conditions were denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The comparative Ct method was used to quantify transcripts according to the manufacturer's protocol (User Bulletin 2, Applied Biosystems). Each sample was quantified in triplicate. Amplification of single products was confirmed by monitoring the dissociation curve. We used the expression level of actin beta (*ACTB*) as an internal control. The standard ABI primer probe sets were used (*SLC6A4*, Hs00984356; *ACTB*, Hs99999903). We performed statistical analysis using SPSS (SPSS Japan, Tokyo, Japan).

Results

Copy number differences between the twins. The copy number profiles were seemingly identical between both twin pairs (Supplementary Figure S1). To find subtle copy number differences between the twins, we detected their respective

copy number changes compared with a reference and calculated the fold change of each probe. In both twin pairs, the differences with fold changes of more than 1.2 were mostly found on chromosomes 2, 14 and 22, which contain immunoglobulin (Ig)-related regions (Supplementary Figure S2). We examined 40 regions including one Ig-related region by real time PCR analysis. Although the copy number change of Ig-related region was confirmed, none of other regions with copy number differences between the twins were confirmed by real-time PCR analysis (Supplementary Figure S3). This suggested that most of the apparent copy number differences detected by the array were false positives, except for Ig-related regions. The differences of the copy number in Ig-related regions between the twins are reasonable because a single B cell having a specific genome rearrangement of Ig-related gene[39] is amplified by EB virus transformation in LCLs.

DNA methylation differences between the twins. Promoter-wide DNA methylation profiles of the twins were examined by Affymetrix GeneChip Human Promoter 1.0R tilling arrays after methylated DNA was enriched using MBD2b and MBD3L1 conjugated beads. Fully unmethylated DNA obtained by whole genome amplification was used as a reference. Using this method, the DNA methylated status within the 10 kb range for each of more than 25 500 promoters could be analyzed. MAT[32] was used to identify MRs. By using MAT, the signals of each probe in each array can be standardized individually.

DNA methylation analysis by the promoter tiling array showed MRs specific to the bipolar twin (80 or 4, respectively) or those to the healthy co-twin (71 or 81, respectively) in each twin pair (Supplementary Figure S4). Among the 236 regions containing six or more CpG sites, 13 representative regions overlapping with CpG islands were analyzed by bisulfite sequencing to validate the tiling array data. Although the DNA methylation differences between the twins were confirmed in most regions, some differences with P values more than 10^{-6} could not be confirmed (Supplementary Figures S5 and S6). To narrow the number of candidate MRs, we applied more stringent criteria (P value $\leqslant 10^{-6}$) based on the results of bisulfite sequencing. We compared the DNA methylation status between four sets of PBLs and LCLs obtained from the same individuals and found that MRs in PBLs were largely conserved in LCLs but a part of the regions were methylated only in LCLs or only in PBLs.[30] Thus, we eliminated those 'variable' MRs during establishment of LCLs. Furthermore, the regions detected as MRs in at least one of the LCL were excluded from the BD-specific MRs, and only the regions overlapping with common MRs in four LCLs were selected with regard to the C-specific MRs (Supplementary Figure S4). We finally obtained three candidate MRs: *KIAA1530* on chromosome 4 as a hypermethylated region in the healthy co-twin, and *FANK1* (fibronectin type III and ankyrin repeat domains 1) on chromosome 10 and *SLC6A4*, encoding serotonin transporter on chromosome 17 (Supplementary Figure S7), as a hypermethylated region in the bipolar twin, only from pair 1.

These three regions were examined by bisulfite sequencing, and we could confirm the differential DNA methylation status between the twins only in the promoter region of *SLC6A4* (Figure 1b and Supplementary Figures S8 and S9).
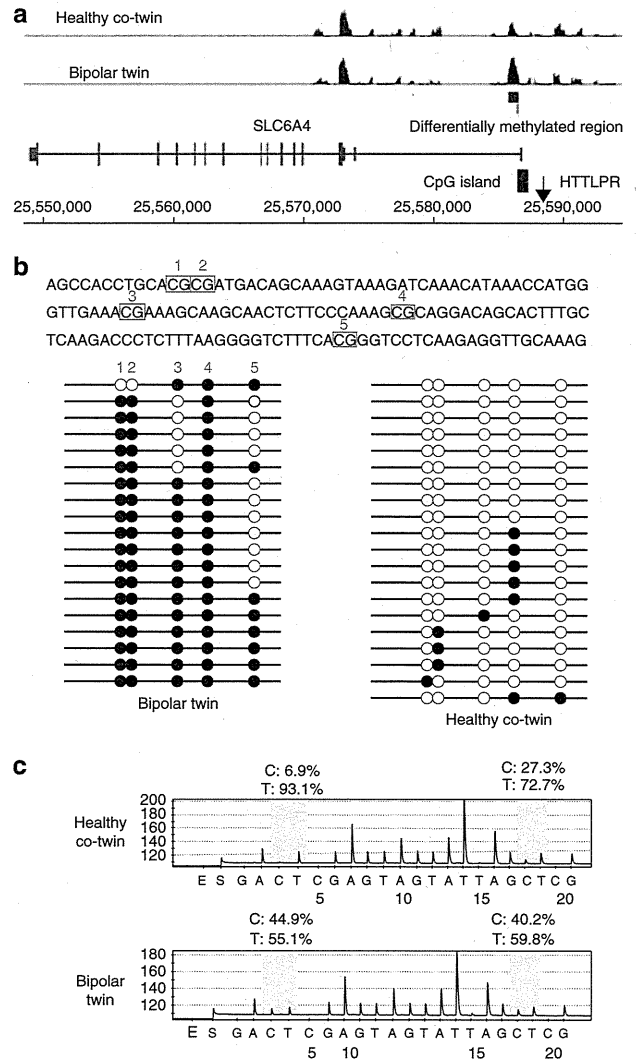

Figure 1 DNA methylation difference between twins in *SLC6A4*. (a) Results of comprehensive DNA methylation analysis of lymphoblastoid cell lines (LCLs) of a pair of monozygotic twins discordant for bipolar disorder using tiling arrays. The vertical axis represents the signal intensity, and the horizontal axis represents the base number on the chromosome 17 (NCBl36/hg18). Exon–intron structure of the *SLC6A4* is shown below the data of tiling arrays. The CpG island and the regions of HTTLPR are shown by a green square and an arrow, respectively. The region showing statistically significant methylation difference between the bipolar twin and the healthy co-twin, and the region examined by bisulfate sequencing are shown by a blue square and a red bar, respectively. (b) Results of bisulfite sequencing. The genomic region examined by bisulfite sequencing, which corresponds to the base numbers from 25 586 333 and 25 586 482, is shown above. The five CpG sites are surrounded by red squares. Black and white circles represent the methylated and unmethylated CpGs, respectively. Each raw shows the data of one clone. Five circles in one raw represent the five CpG sites shown above. This region is methylated in the bipolar twin but not in the healthy co-twin. (c) Representative results of pyrosequencing using independent samples. The results of two CpG sites (3 and 4) were shown by yellow shadows. Percentages of C and T mean fractions of methylation and unmethylation on each CpG site, respectively. Each fraction of methylation on other CpG sites of the bipolar twin and the healthy co-twin was 24.2 and 0 on CpG1, 28.8 and 0 on CpG2, and 9.3 and 4.3 on CpG5, respectively. The differences of DNA methylation in *SLC6A4* between the twins were confirmed using the other LCL samples of the twins recultured for this experiment.

This region located on the CpG island shore at the 2–3 kb downstream of HTTLPR was hypermethylated in the bipolar twin. We also examined the DNA methylation status of the twins using independent cell culture by pyrosequencing to rule out a possibility of cell culture-induced artificial or stochastic change (Figure 1c).

Case–control analysis for DNA methylation of LCLs in SLC6A4. Because there are DNA methylation changes even between healthy MZ twins, the existence of DNA methylation difference does not warrants the relationship with the disease. Thus, we next performed a case–control analysis using the LCLs of 20 patients with BD and 20 controls. The DNA methylation of the two CpG sites (CpG3 and CpG4) was significantly higher in BD patients than in controls ($P < 0.05$, Mann–Whitney U-test; Figure 2a). We also analyzed the expression level of SLC6A4 in LCLs by real-time quantitative RT-PCR. HTTLPR, including a single nucleotide polymorphism in L allele (LA/LG) that reportedly affects gene expression, was genotyped.[36] Because all

subjects with L allele, but one control subject with S/LG genotype, had LA allele, LA and LG alleles were not separately analyzed. Among the subjects with the S/S genotype, the mRNA expression level was significantly lower ($P < 0.05$, Mann–Whitney U-test; Figure 2b) and the DNA methylation level of CpG3 was significantly higher ($P < 0.05$; Mann–Whitney U-test; Figure 2c) in the BD patients compared with controls. The same trend was seen in CpG4 ($P = 0.059$, Mann–Whitney U-test). The DNA methylation level of CpG3 was significantly correlated with its mRNA expression level only in subjects with the S/S genotype ($r = -0.425$, $P = 0.043$, $n = 23$; Figure 2d) and not in those with the S/L genotype ($r = -0.262$, $P = 0.346$, $n = 15$; Figure 2e).

DNA methylation status of postmortem brain samples in SLC6A4. DNA methylation patterns in blood cells may not directly reflect the status of the brain. To test whether hypermethylation of SLC6A4 is also observed in the brain, we examined the DNA methylation status of these
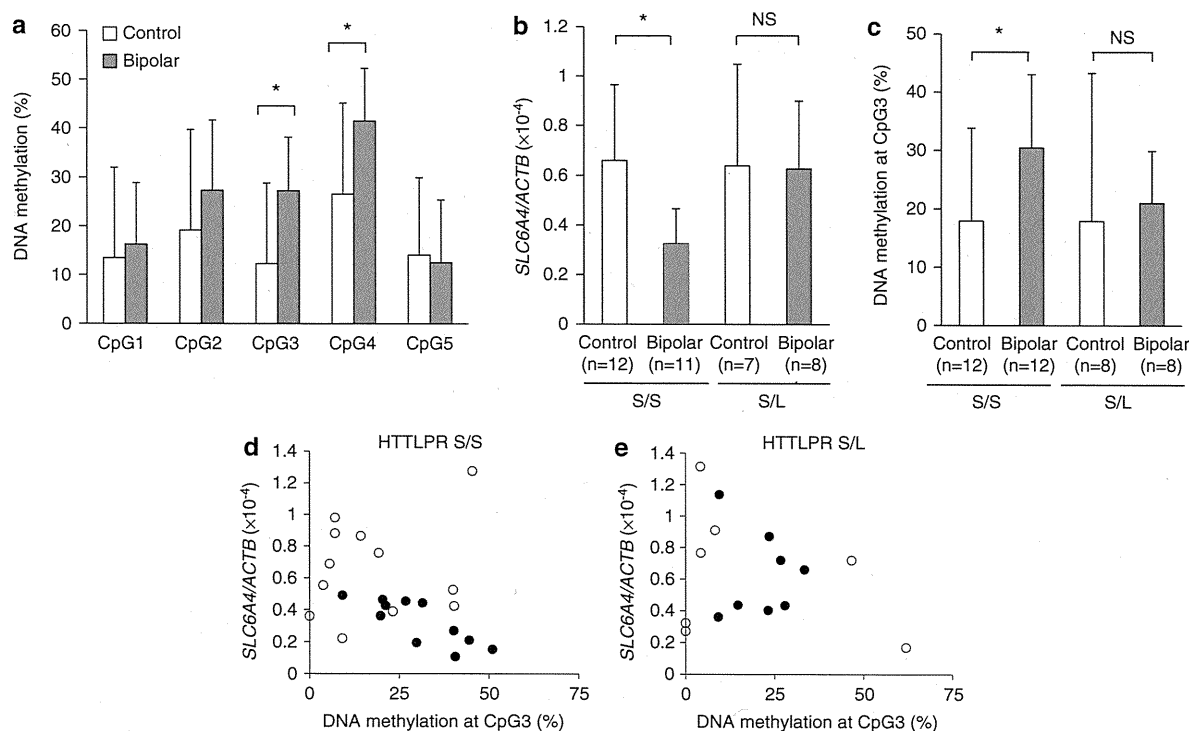

Figure 2 Case–control analysis of DNA methylation and gene expression in LCLs. (a) DNA methylation levels of five CpG sites shown in the Figure 1 in LCLs (bipolar disorder (BD), $n = 20$, controls, $n = 20$) using pyrosequencing. Two CpG sites (3 and 4) showed significantly higher DNA methylation in BD patients. Values indicate mean ± s.d. Asterisk indicates significance; $P = 0.008$ and $P = 0.035$ for CpG3 and CpG4, respectively, by Mann–Whitney U test. (b) Relationship between expression level of SLC6A4 and HTTLPR genotype (BD, $n = 19$, controls, $n = 19$). One datum in each diagnostic group was turned out to be at outlier by Smirnov–Grubbs test and removed from data analysis. Among the all samples ($n = 38$), the mRNA level of SLC6A4 tended to be lower in BD patients compared with controls ($P = 0.08$; Mann–Whitney U-test). The mRNA expression level was significantly lower in BD patients compared with controls only in subjects with the S/S genotype ($P = 0.007$; Mann–Whitney U-test) and not in those with the S/L genotype ($P = 0.867$; Mann–Whitney U-test). Values indicate mean ± s.d. An asterisk indicates significant difference ($P < 0.05$). NS, not statistically significant. (c) Relationship between the DNA methylation of CpG3 and HTTLPR genotype (BD, $n = 20$, controls, $n = 20$). The DNA methylation of the CpG3 site was significantly higher in BD patients compared with controls only in the subjects with the S/S genotype ($P = 0.020$, Mann–Whitney U-test) but not in those with the S/L genotype ($P = 0.105$, Mann–Whitney U-test). The DNA methylation of the CpG4 site also tended to be higher in BD patients compared with controls only in the subjects with the S/S genotype ($P = 0.052$, Mann–Whitney U-test) and not in those with the S/L genotype ($P = 0.279$, Mann–Whitney U-test). Values indicate mean ± s.d. (d, e) Correlation between the DNA methylation at the CpG3 site and mRNA expression level of SLC6A4 (BD, $n = 19$, controls, $n = 19$). There was a significant correlation in the subjects with the S/S genotype ($r = -0.425$, $P = 0.043$, $n = 23$); (d), whereas no significant correlation was found in the subjects with the S/L genotype ($r = -0.262$, $P = 0.346$, $n = 15$); (e). Closed circles represent patients with BD and open circles represent control subjects.
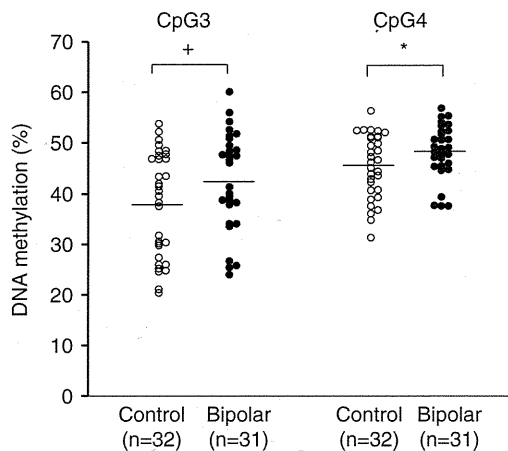
Figure 3 DNA methylation analysis of *SLC6A4* in the prefrontal cortices of bipolar disorder (BD) patients and control subjects. The CpG3 and CpG4, shown in the Figure 1, were analyzed by pyrosequencing. Closed circles represent patients with BD and open circles represent control subjects. Horizontal bars indicate averages. *$P<0.05$, $^+P<0.10$ (Mann–Whitney U-test, one-tailed). There was no significant difference of gender between the BD patients and controls. Age, postmortem interval (hours) and brain pH were not significantly correlated with the DNA methylation level of both CpG3 and CpG4. In the BD patients, there was no significant difference of the DNA methylation level of the two CpG sites between the subjects with and without antidepressant treatment.

two CpG sites in postmortem brains of 31 BD patients and 32 controls obtained from the Stanley Foundation Brain Collection. CpG4 showed a significantly higher DNA methylation level in BD patients compared with controls ($P<0.05$, Mann–Whitney U-test, one-tailed), and CpG3 also showed the same trend ($P=0.059$, Mann–Whitney U-test, one-tailed; Figure 3). There was no significant effect of confounding factors, such as gender, age, postmortem interval (hours), brain pH and administration of antidepressants, on the DNA methylation level of the two CpG sites.

Discussion

Multiple lines of evidence suggest the role of *SLC6A4* in BD. Although the results of serotonin transporter-binding potential in BD patients are conflicting,[2–6] one of them reported the low potential in the depressive state,[40] and less gene expression of serotonin transporter in postmortem brains of depressed suicide victims was also reported.[41] HTTLPR was initially reported to be associated with BD, though subsequent studies show conflicting results.[9,42,43] Gene-environment (G × E) interaction[14] and resulting epigenetic changes[44] might explain the inconsistent results of genetic studies in mood disorder.[14] In this study, we found a possible interaction between HTTLPR genotype and BD diagnosis on the DNA methylation and mRNA expression level of *SLC6A4*. DNA methylation level of the CpG island upstream of *SLC6A4* was reported to be associated with environmental factors[45,46] and possibly with HTTLPR genotype.[47] Weaver *et al.*[48] hypothesized that DNA methylation plays a role as an epigenetic mark of G × E interaction, and they suggested that hypermethylation of the promoter region of the glucocorticoid receptor gene associated with poor maternal care causes stress vulnerability. Hypermethylation of *SLC6A4* in BD may reflect epigenetic

effect in G × E interaction and contribute to the patho physiology of BD.

In this study, we performed promoter-wide DNA methylation analysis and detected hypermethylation of *SLC6A4* in the bipolar twin. The differences of methylation patterns between MZ twins may depend on age and difference of nurturing environment,[18] and a twin study showed that the variation of DNA methylation in *SLC6A4* is attributable to unique environmental factors rather than heritable factors.[49] It would be important to elucidate the origin and timing of the methylation change in *SLC6A4*[50] in the future study. In this study, only one of the two twins showed marked difference of DNA methylation of *SLC6A4*. Considering the heterogeneity of BD, it is not realistic to postulate that all patients with BD are caused by the same genetic or epigenetic factor. Thus, a close investigation in a large series of well-characterized discordant twins may provide a clue to understand the pathophysiology of BD.[51] In this study, the difference between the groups was relatively modest in case–control studies. There was a large inter-individual difference of DNA methylation at CpG3 in LCLs of patients with BD (9.2 to 51%), possibly reflecting the heterogeneity of BD. In these analyses, we used genomic DNA derived from LCLs cultured with drug-free medium to minimize the effect of medication on DNA methylation. Although none of BD patients were taking antidepressants at the blood sampling, it is difficult to know what the effects of medications and co-morbid illnesses are on the methylation signatures observed in these samples. Moreover, we only examined a portion of the *SLC6A4*. Although DNA methylation in LCLs is not the same as PBLs, MRs in PBLs are largely maintained in LCLs.[30] In this study, we excluded the MRs affected by EB virus transformation and finally detected the hypermethylation of *SLC6A4* in the brains of BD patients. We could only examine the methylation of *SLC6A4* in prefrontal cortices, but further analysis of other regions expressing high levels of HTT, such as dorsal raphe nucleus, would be interesting.

In summary, we detected hypermethylation of *SLC6A4* in BD, using an unbiased approach. DNA methylation of *SLC6A4* may be an epigenetic mark resulting from a G × E interaction that leads to the development of BD. Our findings add a new insight to elucidate the pathophysiology of mood disorder.

Conflict of interest

The authors declare no conflict of interest.

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Association of *ANK3* With Bipolar Disorder Confirmed in East Asia

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Results of genome-wide association studies (GWASs) for bipolar disorder (BD) have indicated *ANK3* as one of the most promising candidates for a susceptibility gene. In this study, we performed genetic association analysis of two single-nucleotide polymorphisms (SNPs) in *ANK3* (rs1938526 and rs10994336), whose genome-wide significant associations were reported in a previous meta-analysis of GWASs, using genotyping data of Korean and Japanese case–control samples and a part of data from a GWAS in Han-Chinese from Taiwan. The total number of participants was 2,212 cases (352 from Korea, 860 from Japan, and 1,000 from Taiwan) and 2,244 controls (349 from Korea, 895 from Japan, and 1,000 from Taiwan). We could not detect any significant difference of allele frequency in individual analyses using each of the three populations. However, when we combined the three data sets and performed a meta-analysis, rs1938526 showed nominally significant association ($P = 0.048$, odds ratio $= 1.09$). The overrepresented allele in BD was same as that reported in Caucasian GWASs. On the other hand, any significant association was not detected in rs10994336. This discrepancy between two SNPs may be explained by the different degree of linkage disequilibrium between Asian and Caucasian. These findings further supported the association between *ANK3* and BD, and also suggested the genomic region around rs1938526 as a common risk locus across ethnicities.
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Key words: GWAS; mood disorder; Asian; association study; replication

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INTRODUCTION

Recent genome-wide association studies (GWASs) for bipolar disorder (BD) have identified a number of new candidate genes. The *ANK3* gene, encoding ankyrin-G, is one of these newly found candidates. The ankyrin family proteins link the spectrin cytoskeleton to various membrane proteins, including ion channels [Bennett and Baines, 2001]. Increased content of a minor form of

312

ankyrin in erythrocyte membranes of BD patients treated with lithium was reported [Zhang and Meltzer, 1989]. Ankyrin-G was originally detected at the axonal initial segment and nodes of Ranvier [Kordeli et al., 1995]. In Purkinje cells derived from mutant mice with cerebellum-specific knockout of ankyrin-G, deficient ability to initiate action potentials and to support rapid and repetitive firing was observed [Zhou et al., 1998]. Thus, ankyrin-G plays a certain role in neuronal excitability.

Association between *ANK3* and BD was initially reported in the first GWAS using DNA pooling [Baum et al., 2008]. Afterwards, genome-wide significant associations in two single-nucleotide polymorphisms (SNPs) in *ANK3* (rs1938526 and rs10994336) were detected in a meta-analysis of GWASs for BD with 4,387 cases and 6,209 controls [Ferreira et al., 2008]. Schulze et al. [2009] confirmed the association reported in the first pooling-GWAS by individual genotyping and replicated the association of rs10994336 in an independent sample. Other GWASs also reported suggestive association signals in *ANK3* region [Scott et al., 2009; Smith et al., 2009; Lee et al., 2010]. According to these results, *ANK3* has attracted huge attentions in the field of genetic research for BD. However, except for the suggestive association reported in a GWAS of Han-Chinese in Taiwan [Lee et al., 2010], all of the previously reported associations were of Caucasian samples. Therefore, further analysis of *ANK3* in BD using samples with an ethnicity different from Caucasian is warranted to test the robustness of this association. In this study, we focused on the two SNPs in *ANK3*, whose genome-wide significant associations were observed in Ferreira et al. and evaluated their association with BD, using genotyping data of these two SNPs in case–control samples from Korea and Japan and a part of data from a GWAS in Taiwan. The total number of participants was 2,212 cases (352 from Korea, 860 from Japan and 1,000 from Taiwan) and 2,244 controls (Korea, 349, Japan, 895, and Taiwan, 1,000).

MATERIALS AND METHODS
Samples and Data

Korean case–control samples. A total 701 individuals of unrelated patient and control samples were included in this study, which consisted of 349 controls (173 men, 176 women; mean age = 25.9 ± 6.6 years) and 352 patients with BD (149 men, 203 women; mean age = 33.5 ± 12.0 years; 254 bipolar I disorder [BDI], 98 bipolar II disorder [BDII]). Participants were all ethnically Korean. All the patients met the diagnostic criteria of DSM-IV for BD. The patients were individually interviewed by trained researchers using the Korean-translated version of the Diagnostic Interview for Genetic Studies [DIGS, Joo et al., 2004]. Consensus diagnostic meetings with more than two psychiatrists were held regularly to evaluate the participants' final diagnoses and to rule out subjects with organic brain disease, alcohol or drug problems, or other general medical conditions possibly manifesting as psychiatric symptoms. Healthy controls were free from present, past, and family history (first-degree relatives) of psychiatric illness or substance abuse diagnoses. The study protocol was approved by the ethics committee of Seoul National University Hospital. Written informed consent was obtained from each patient prior to enrollment.

Japanese COSMO case–control samples. These samples were recruited through the COSMO (Collaborative Study of Mood Disorders) consortium [Iwayama et al., 2010], which comprises 860 unrelated bipolar patients (424 men, 436 women; mean age 50.2 ± 14.4 years; 584 BDI, 276 BDII) and 895 age- and sex-matched controls (445 men, 450 women; mean age 49.9 ± 13.5 years). All samples are of Japanese origin. The patients were diagnosed by at least two experienced psychiatrists according to the DSM-IV criteria for BDI or BDII on the basis of unstructured interviews and reviews of their medical records. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews. The present study was approved by the ethics committees of all participating institutes. All controls and patients gave informed written consent to participate in the study, after provision and explanation of study protocols and objectives.

GWAS data in Taiwan. Genotype data of two *ANK3* SNPs were obtained by a GWAS in Taiwan [Lee et al., 2010] and were kindly provided by Dr. Andrew Cheng (Institute of Biomedical Sciences, Academia Sinica, Taiwan). The samples consisted of 1,000 BDI patients (464 men, 536 women; mean age 42.8 ± 12.9 years) and 1,000 normal controls (502 men, 498 women; mean age 51.7 ± 17.8 years). All samples are of Han Chinese origin. Detailed clinical information and method of diagnosis is described in the previous paper [Lee et al., 2010].

Markers, genotyping, and data analysis. In this study, we genotyped following two SNP markers in *ANK3*: rs1938526 (A/G) and rs10994336 (C/T). In Ferreira et al., rs1938526, which was genotyped, was associated with BD with odds ratio (OR) of 1.395 and *P*-value of 1.3×10^{-8} and rs10994336, whose genotype was imputed, was associated with OR of 1.450 and *P*-value of 9.1×10^{-9}.

In Korean and Japanese samples, TaqMan assay (Applied Biosystems, Foster City, CA) was used for genotyping. In GWAS in Taiwan, genotypes were determined using the Illumina HumanHap550-Duo BeadChip (Illumina, San Diego, CA).

Deviation from Hardy–Weinberg equilibrium and linkage disequilibrium between the two SNPs were calculated using Haploview software v 4.2 [Barrett et al., 2005]. Association between markers and BD in each individual population was assessed using the chi-square test. A meta-analysis using Mantel–Haenszel model and evaluation of sample heterogeneity were performed on Review Manager computer software [RevMan, 2008]. The I^2 statistics were used for the assessment of heterogeneity between the samples [Higgins and Thompson 2004; Trikalinos et al., 2008]. Statistical power for detection was calculated by Genetic Power Calculator [http://pngu.mgh.harvard.edu/~purcell/gpc/, Purcell et al., 2003] with a multiplicative model and following parameters; risk allele frequency, 0.3642 for rs1938526 and 0.2699 for rs10994336, these values were calculated as the frequencies of the previously reported risk alleles in our control samples; number of cases, 2,212; number of controls, 2,244; prevalence, 0.01; OR, 1.1, or 1.2; α-level, 0.05.

RESULTS

The results of the case–control association analyses in each population and the meta-analysis were summarized in Table I. When genetic association between rs1938526 or rs10994336 and BD were