product can be amplified from the tester. If there is counterpart in the driver, PCR amplification is suppressed by the driver products, because they can hybridize with the tester fragment, but cannot be amplified by PCR because of lack of adapter sequence. By repeating this procedure twice, DNA fragments derived from hypomethylated region only in the tester genome were selectively amplified.[16]

MS-RDA

Driver and tester genomes of 10 μg each were digested with 100 units of HpaII overnight. After phenol and chloroform extraction followed by ethanol precipitation, 1 μg of digestion product was ligated to 500 pmol of RHpa adapter by 800 units of T4 DNA Ligase (New England Biolabs (NEB), Berkley, MA, USA). RHpa adapter was prepared by annealing two oligonucleotides, RHpa24 and RHpa11. The ligation product was amplified by 25 cycles of PCR with RHpa24 oligonucleotide as primer as reported by Lisitsyn et al.[16]

The RHpa adapter of the tester and driver amplicons was removed by digestion with HpaII and separation with gel filtration chromatography (CHROMA SPIN + TE-200 Columns; Clontech Laboratories Inc., Mountain View, CA, USA). The JHpa adapter (500 pmol), which was prepared by annealing JHpa24 and JHpa11, was ligated to 200 ng of the tester amplicon with T4 DNA ligase. A 200 ng of the tester DNA with the JHpa adapter at its ends was mixed with 20 μg of the driver DNA. The DNA mixture was purified by phenol and chloroform extraction and ethanol precipitation and dissolved in 4 μl of 3 × EE buffer (3 mM EDTA/3 mM N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid), pH 8.0), denatured at 96°C for 10 min and reannealed at 67°C overnight in the presence of 1 M NaCl. One-tenth of the reannealed product was amplified by PCR with the JHpa24 oligonucleotide as a primer for 10 cycles. DNA fragments linearly amplified, existing as single-stranded DNA, were digested with 100 units of Mung-Bean Nuclease, and the remaining double-stranded DNA was again amplified by PCR for 20 cycles with JHpa24 oligonucleotide.

The second cycle of competitive hybridization was performed by switching JHpa adapter to an NHpa adapter, which was prepared by annealing NHpa24 and NHpa11. A total of 20 ng of the product of the first cycle was mixed with 20 μg of driver DNA. Denaturing, reannealing and selective amplification of the self-annealed product were performed as for the first cycle. PCR products were purified by MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA) and TA cloned using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Single bacterial colonies were subject to sequencing analysis. In total, 92 independent clones were analyzed. Using the obtained sequences, a homology search was performed with the BLAST program at a GenBank Web site.

Primers were as follows: RHpa24, 5'-AGCACTCTC CAGCCTCTCACCGAC-3'; RHpa11, 5'-CGGTCGGTGAG -3'; JHpa24, 5'-ACCGACGTCGACTATCCATGAAC-C-3';

JHpa11, 5'-CGGTTTCATGG-3'; NHpa24, 5'-AGGCAA CTGTGCTATCCGAG-GGAC-3'; NHpa11, 5'-CGGTCC CTCGG-3'.

Bisulfite sequencing

After denaturation, 1 μg of genomic DNA was treated with 3.6 M sodium bisulfite. The reaction was performed at 55°C overnight. Genomic DNA was then purified with a wizard DNA clean-up system (Promega, Madison, WI, USA) and eluted with 50 μl of water. We typically used 1 μl of bisulfite-modified DNA for PCR. The CpG islands[17] were obtained through the UCSC (University of California, SantaCruz, CA, USA) Genome Browser (http://www.genome.ucsc. edu/index). Primer pairs were determined using Meth Primer software.[18] Primer pairs were as follows: PPIEL-1, 5'-TAAATTTATTTTTGGATTTAGAGTA-3' and 5'-ACAAACTCCACAACTCTAAT-CCATT-3'; PPIEL-2, 5'-TTTAGTTTAATTTTGGTATTGTTTG-3' and 5'-ATCTA-AAAAAAATATCCTTATTTCC-3'; phosphatidyl-inositol-4-phosphate 5-kinase-like 1 (PIP5KL1), 5'-GGG GGTTTAAATTTGTTTAGGTTAT-3' and 5'-CCCTTCC AAAAT-ACACAATCTAC; spermine synthase (SMS), 5'-AGTGATGGAGGAGTTTTGTTAG-ATA-3' and 5'-C CCCAAACCAAAACCCCTCTTATTT-3'; Armadillo repeat containing 3 (ARMC3), 5'-AGGGTTATGAGAAGT TTTGTGGAAA-3' and 5'-AATCAAAAAAACAATTCA ACCTCAAT-3'. PCR products were purified by MinElute PCR Purification Kit, and TA cloned using TOPO TA cloning kit. Single-bacterial colonies were subject to sequencing analysis.

RNA extraction and quantitative RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen). After the DNase I treatment, 5 μg of total RNA was used for cDNA synthesis by oligo(dT) 12–18 primer and SuperScript II Reverse Transcriptase (RT) (Invitrogen).

Real-time quantitative RT-PCR using SYBR/GREEN I dye (Applied Biosystems, Foster city, CA, USA) was performed with ABI7900 (Applied Biosystems). After the denaturation at 95°C for 5 min, the PCR conditions were 95°C for 15 s and 60°C for 1 min for 50 cycles. The comparative C_t method was employed for quantification of transcripts according to the manufacture's protocol (User Bulletin #2, Applied Biosystems). Each sample was quantified in duplicate. Each experiment was repeated at least three times. Amplification of the single product was confirmed by monitoring the dissociation curve and by 3% agarose gel electrophoresis. Expression level of peptidylprolyl isomerase E-like (PPIEL) was also examined using commercially available cDNA (human brain parts tissue scan real-time, ORIGENE, Rockville, MD, USA. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cofilin 1 (CFL1) for normalization. Primer pairs for GAPDH and CFL1 have been shown previously.[19] Primer pairs used for qRT-PCR are as follows: PPIEL, 5'-TCGTGCCCATGACCACAG AG-3' and 5'-CGGTGGAAGCTGCTTCCCTT-3; SMS, 5'-TCCAATCTCCACGTCTCCAGAA-3' and 5'-TGTCA GATTGACACAGTT-CCCCTG-3'.

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Statistical analysis

Statistical analysis was performed using SPSS 11.0J software (SPSS Japan, Tokyo, Japan). Mann–Whitney *U*-test was employed for the analysis of expression levels and DNA methylation levels. Spearman's correlation coefficient was employed to examine the correlation between age and expression levels. One way analysis of variance (ANOVA) with the cofactors of age and sex was used to examine the effect of diagnosis controlling the confounding variables. $P < 0.05$ was considered significant.

Pyrosequencing

The PCR product of bisulfite-modified DNA was used for pyrosequencing analysis according to the manufacturer's standard protocol (Biotage, Uppsala, Sweden). Briefly, $4 \mu l$ of streptavidin-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) and $44 \mu l$ of binding buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, 0.1% Tween-20 at pH 7.6) were mixed with $40 \mu l$ 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 $50 \mu l$ 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), heated to 90°C for 2 min and cooled to room temperature. Sequencing reactions were performed with a PSQ 96 SNP Reagent Kit (Biotage) according to the manufacturer's instructions. The percentage of methylation were calculated from the raw data using the allele quantification algorithm of the software provided by the manufacturer (PSQ96MA2.1.1 software, Biotage).[20] Each experiment was performed at least three times. The primer pairs and the analyzed sequences are as follows: PCR primer for *PPIEL*, 5'-TGAGAGGATTTTGTTTTGTTTTATAT-3' and Bio-5'-ACCCCTTCTT-TCCTTAATCTAATAC-3'; sequencing primer for *PPIEL*, 5'-GGAGGTAGTTATT-TTGTT TTTAG-3'; reading sequence for *PPIEL*, T/CGTTTTT/CGTT/CGGA-AGTTT/CGTATT/CGT; PCR primer for *SMS*, 5'-AGTGATGGAGGAGTTTTGTTA-GATA-3' and Bio-5'-CCCCAAACCAAAACCCCTCTTATTT-3'; sequencing primer for *SMS*, 5'-TAGATTTTTGTTAATAA TGG-3'; reading sequencing for *SMS*, TAATAATTT/CGA.

Accession numbers

The DDBJ/EMBL/GenBank accession numbers of the sequences discussed in this paper are as follows: *PIP5KL1*, BC042184; *SMS*, BC009898; *ARMC*, BC039312; *PPIEL*, CN265253.

Results

Isolation of differentially methylated DNA fragments by MS-RDA

Two independent series of MS-RDA was performed using genomic DNA extracted from lymphoblastoid cells of monozygotic twins discordant with respect to bipolar disorder. To search hypomethylated region in the patient with bipolar disorder, we used genome derived from bipolar twin as the tester genome and genome derived from control co-twin as the driver genome. PCR amplicons were prepared from *Hpa*II digests of these two genomes (Figure 1a lanes 1 and 2). As a result of the first cycle subtraction, one band was visible specifically in the subtracted products (Figure 1b lane 1 arrow). After second cycle subtraction, this band was visible more clearly (Figure 1c lane 1 arrow). After the two rounds of subtraction, products were cloned into TOPO vector and sequenced. A total of 51 DNA fragments were obtained. We obtained 21 DNA fragments derived from ribosomal DNA and four DNA fragments derived from mitochondrial DNA. This is in accordance with the initial paper of MS-RDA, showing that MS-RDA tends to amplify high copy number DNA, such as mitochondrial DNA, EB virus DNA, ribosomal DNA, LINE/SINE and repeat sequence.[13] These fragments were regarded as false positives. By sequencing and BLAST search at a Genbank web site, 26 nonredundant DNA fragments derived from human chromosome were obtained (Table 1).

To search hypermethylated region in the bipolar patient, we also performed the other MS-RDA reaction, using the genome derived from control twin as the tester genome and genome derived from bipolar co-twin as the driver genome (Figure 1a lanes 3 and 4). As a result of this MS-RDA, a total of 41 DNA fragments were obtained. We obtained one DNA fragment derived from human chromosomal DNA, 34 DNA fragments derived from EB virus DNA, and six fragments derived from mitochondrial DNA (Table 1).

As a result of two independent series of MS-RDA, eight DNA fragments were derived from CGI located near the 5' region of known genes/ESTs, and two DNA fragments were derived from exon 1 of known genes/ESTs. We focused on these 10 DNA fragments and further analyzed. In total of 10 DNA fragments, nine fragments were derived from putative hypomethylated region in the patient, and one fragment was derived from putative hypermethylated region in the patient. For genomic region around these 10 DNA fragments, we performed bisulfite sequencing to examine actual DNA methylation status (Figure 2). In four genomic regions, differences of DNA methylation status between bipolar twin and control co-twin were confirmed by bisulfite sequencing (Table 2). In other six regions, we did not detect any difference of DNA methylation status.

Of four aberrant methylation regions, three were hypomethylated regions in the patient, while one was hypermethylated region in the patient. Fragment 1#57

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B-C : Tester (Bipolar twin), Driver (Control co-twin)

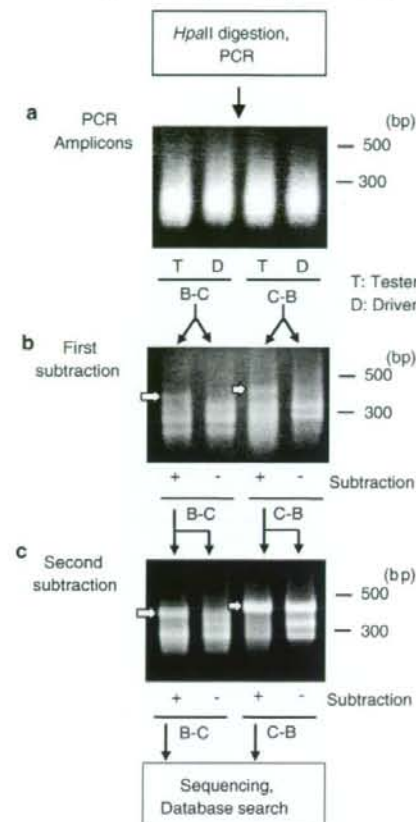C-B : Tester (Control twin), Driver (Bipolar co-twin)

Figure 1 Results of the MS-RDA. (a) PCR amplicons. PCR amplicons were prepared from *Hpa*II digests of genomes derived from lymphoblastoid cells of twins. We used bipolar twin genome as a tester genome (T) and control co-twin genome as a driver genome (D) (series B-C; Figure 1a lanes 1 and 2), and vice versa (series C-B; Figure 1a lanes 3 and 4). (b) The results of the first cycle subtraction. We performed first subtraction of driver genome from tester genome (Subtraction +). As a control, we also performed PCR of tester genome without first subtraction (Subtraction −). After first subtraction, one band was visible specifically in the subtracted products in both series, B-C (Figure 1b, lane 1, arrow) and C-B (Figure 1b, lane 3, arrow). These bands were visible more clearly after the second rounds of subtraction (Figure 1c, lanes 1 and 3). (c) The results of the second cycle subtraction. We performed second subtraction of driver genome from resultant of first subtraction (Subtraction +). As a control, we also performed PCR of resultant first subtraction without second subtraction (Subtraction −). After the two rounds of subtraction, products were cloned into TOPO vector and sequenced.

Table 1 Summary of the clones isolated from MS-RDA

	Tester (bipolar twin) Driver (control co-twin)	Tester (control twin) Driver (bipolar co-twin)	Total
Mitochondrial DNA	4	6	10
Ribosomal DNA	21	0	21
EB virus DNA	0	34	34
CpG island	7	1	8
Exon 1	2	0	2
Intron	11	0	11
LINE/SINE	4	0	4
Repeat sequence	2	0	2
Total	51	41	92

was derived from putative promoter region of an EST, CN265253 (Figure 2a). A homology search showed that CN265253 mRNA had high homology with peptidyl-prolyl *cis–trans* isomerase E (*PPIE*), or cyclophilin E. Because this gene is a partial duplication of *PPIE* and not the ortholog of mouse *Ppie* (see below), we named it as *PPIEL* (peptidylprolyl isomerase E-like). DNA methylation status of putative promoter region of *PPIEL* was analyzed by bisulfite sequencing. These regions were almost completely methylated in control twin. In contrast, these regions were globally hypomethylated in bipolar co-twin. Fragment 4#17 was derived from exon2 of the phosphatidylinositol-4-phosphate 5-kinase-like 1 (*PIP5KL1*) gene (Figure 2b). This region was partially methylated in control twin, and completely demethylated in bipolar co-twin. Fragment 4#90 was derived from 5′ region of the *SMS* gene (Figure 2c). Between control twin and bipolar co-twin, DNA methylation status of this region was approximately the same excluding one CpG site (Figure 2c arrow). This CpG site was completely methylated in control twin. In contrast, this site was completely unmethylated in bipolar co-twin. Fragment 4#86 was derived from 5′ region of the Armadillo repeat containing 3 (*ARMC3*) gene (Figure 2d). This region was unmethylated in control twin and partially methylated in bipolar co-twin.

Case–control studies of DNA methylation status in four candidate genes
To examine whether or not these four aberrant DNA methylation statuses are seen in bipolar disorder in general, DNA methylation status was analyzed by pyrosequencing in the DNA derived from lymphoblastoid cells of 16 bipolar I disorder, seven bipolar II disorder and 18 control samples (Table 1). Analyzed CpG sites by pyroseqencing were indicated by arrows and an arrowhead in Figure 1. We found significant differences in methylation statuses of *PPIEL* and *SMS*, and these genes were further analyzed. *PIP5KL1* and
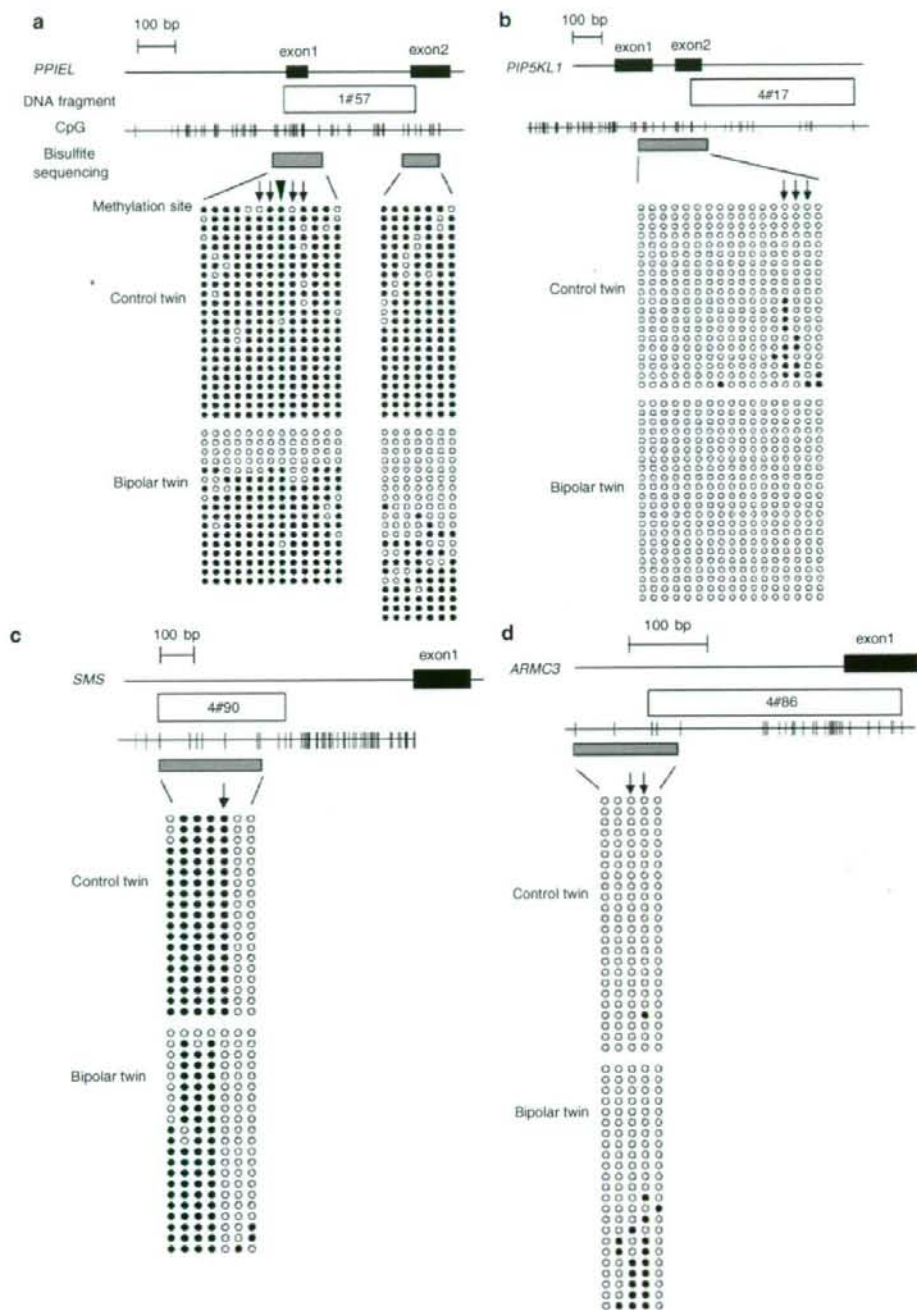
Figure 2 Physical map and DNA methylation status around the four DNA fragments isolated by MS-RDA. Closed boxes represent known exons. Open boxes represent the DNA fragments isolated by MS-RDA. CpG site is represented by tick marks. After bisulfite modification, the regions flanked by the DNA fragments (gray boxes) were amplified and sequenced. The region examined by the bisulfite sequencing included the CCGG site at both the ends of the PCR fragments obtained by MS-RDA. Open and closed circles show unmethylated and methylated cytosines, respectively. Arrow and arrowhead show the positions analyzed by pyrosequencing.

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Table 2 Candidate DNA fragments isolated by MS-RDA

No.	Clone name	Length (bp)	Chromosome	Accession no. of flanking genes/EST	Symbols of flanking genes	Full names of flanking genes	Case–control studies[a] P < 0.05
1	1#57[b]	469	1p34.3	AK092606	PPIEL	Peptidylprolyl isomerase E-like	Yes (BPII)
2	4#17[b]	412	9q34.11	BC042184	PIP5KL1	Phosphatidylinositol-4-phosphate 5-kinase-like 1	No
3	4#90[b]	324	Xp22.11	BC009898	SMS	Spermine synthase	Yes
4	4#86[c]	347	10p12.2	BC039312	ARMC3	Armadillo repeat containing three	No

[a]DNA methylation status were examined by pyrosequencing in 18 BPI samples, seven BPII samples and 18 control samples. P-values were calculated by Mann–Whitney U-test.
[b]These DNA fragments were derived from MS-RDA using bipolar sample as the tester genome and control sample as the driver genome.
[c]This DNA fragment was derived from MS-RDA using control sample as the tester genome and bipolar sample as the driver genome.

ARMC3 were not methylated or only weakly methylated, in consistent with the results in twins. No significant differences between bipolar disorder and control samples were observed in DNA methylation statuses of PIP5KL1 (BPI, $P = 0.72$; BPII, $P = 0.59$) and ARMC3 (BPI, $P = 0.94$; BPII, $P = 0.21$).

DNA methylation status and expression level of SMS in bipolar disorder
The DNA methylation status of SMS was analyzed by pyrosequencing in the DNA of lymphoblastoid cells. Because SMS is located on X chromosome, one allele is assumed to be methylated due to X-chromosome inactivation. Thus, DNA methylation status was separately compared in male and female patients. Owing to small number of patients in each gender, bipolar I disorder and bipolar II disorder were combined in this analysis.

Average DNA methylation level of SMS in 12 female patients with bipolar disorder ($58.3 \pm 11.5\%$; mean $\pm$ s.d.) was significantly higher than that of six control females ($35.4 \pm 23.7\%$, $P = 0.03$) (Figure 3b). This finding was basically similar even when a patient treated with valproate, which potentially affect histone acetylation and subsequently DNA methylation, is excluded ($P = 0.05$). No significant difference between patients with bipolar disorder and controls were observed in males (11 patients with bipolar disorder and 12 controls, $P = 0.14$), although there was non-significant but similar tendency to female patients (Figure 3a). We found no significant correlations between age of control subjects and DNA methylation level of SMS in female patients ($R = -0.476$, $P = 0.233$, $n = 6$).

We also examined expression level of SMS using real-time quantitative RT-PCR. No significant differences between patients with bipolar disorder and controls were observed in male ($P = 0.21$) or female ($P = 0.43$) patients (Figure 3c and d). The expression level of SMS was not correlated with DNA methylation status both in all male subjects ($R = 0.26$, $P = 0.13$) and all female subjects ($R = 0.35$, $P = 0.12$).

DNA methylation status and expression level of PPIEL in bipolar disorder
We analyzed DNA methylation status of five CpG sites in putative promoter region of PPIEL by pyrosequencing. The DNA methylation statuses of these all five CpG sites were significantly lower ($P < 0.05$) in patients with bipolar II disorder than that in controls. One-way ANOVA with cofactors of age and sex also showed significant effect of diagnosis ($P < 0.05$). Since these five sites showed similar results, we show the data of one site in the middle (Figure 2a arrowhead) as representative of five sites. The average DNA methylation status analyzed by pyrosequencing was significantly lower in patients with bipolar II disorder ($51.8 \pm 9.1\%$) than that in controls ($70.2 \pm 10.3\%$, $P = 0.001$) (Figure 4a). This result was not affected by valproate because none of patients with bipolar II disorder was treated with valproate. Patients with bipolar I disorder were not significantly different from controls ($P = 0.96$). No significant correlations were found between age in control subjects and DNA methylation level of PPIEL ($R = -0.16$, $P = 0.53$). There was no significant difference in the DNA methylation level of PPIEL with regard to gender in control subjects ($P = 0.96$).

We analyzed expression level of PPIEL using real-time quantitative RT-PCR normalized by GAPDH (Figure 4b) and CFL1 (Figure 4c). The expression level of PPIEL was significantly ($P = 0.001$) higher in patients with bipolar II disorder (PPIEL/GAPDH, $1.3 \times 10^{-3} \pm 4.3 \times 10^{-4}$; PPIEL/CFL1, $8.8 \times 10^{-3} \pm 2.5 \times 10^{-3}$) than that in controls (PPIEL/GAPDH, $6.9 \times 10^{-4} \pm 3.6 \times 10^{-4}$; PPIEL/CFL1, $4.6 \times 10^{-3} \pm 2.6 \times 10^{-3}$). No significant correlations were found between age in control subjects and DNA methylation level of PPIEL ($R = 0.21$, $P = 0.41$). There was no significant difference in the expression level of PPIEL

a *SMS* (Male)

b *SMS* (Female)

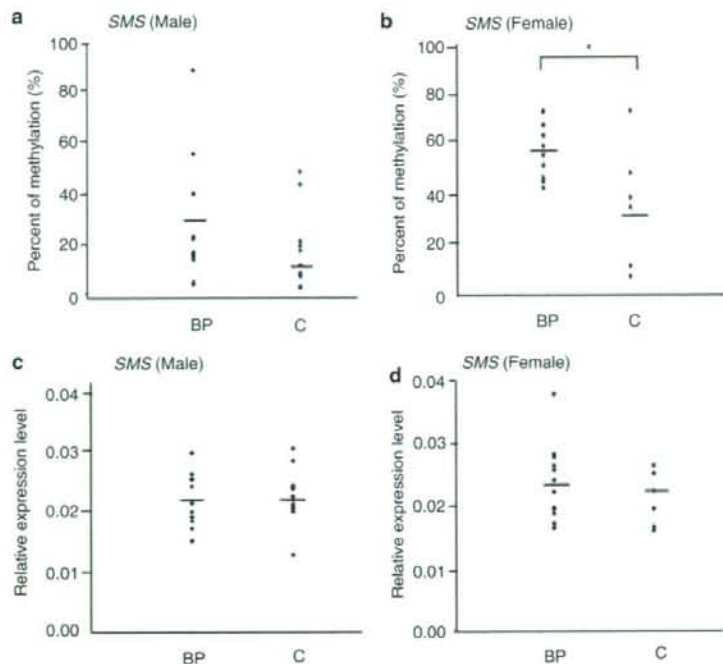**c** *SMS* (Male)

d *SMS* (Female)

Figure 3 DNA methylation status and expression level of *SMS*. (a) DNA methylation status of *SMS* in males. Eleven male patients with bipolar disorder and 12 control male subjects were analyzed by pyrosequencing. Mean ± s.d. were 27.4 ± 26.0% for male patients with bipolar disorder and 15.0 ± 16.5% for control males, respectively. Closed diamond represents percent of methylated allele (%) in each sample. Bar represents the average of percent of methylated allele in a group. (b) DNA methylation status of *SMS* in females. Twelve females with bipolar disorder and six control females were analyzed by pyrosequencing. The average DNA methylation level was significantly ($P = 0.03$) higher in female patients with bipolar disorder (58.3 ± 11.5%, mean ± s.d.) than that in control females (35.4 ± 23.7%). (c) The expression level of *SMS* in males. Eleven male patients with bipolar disorder and 12 control males were analyzed by real-time quantitative RT-PCR. The quantity level of mRNA of each gene was normalized with that of *GAPDH*. The average expression levels in male patients with bipolar disorder and control males were 0.022 ± 0.004 (*SMS/GAPDH*) and 0.022 ± 0.005, respectively. Closed diamond represents expression level in each sample. Bar represents the average of expression levels in a group. (d) The expression level of *SMS* in females. Twelve female patients with bipolar disorder and six female controls were analyzed by quantitative real-time PCR. The average expression levels in female patiens with bipolar disorder and control females were 0.024 ± 0.006 and 0.021 ± 0.004, respectively.

Figure 4 DNA methylation status and expression level of *PPIEL*. (a) The DNA methylation status of *PPIEL*. Sixteen patients with bipolar I disorder, seven patients with BPII disorder and 18 controls were analyzed by pyrosequencing. The average DNA methylation level of *PPIEL* was significantly ($P = 0.001$) lower in patients with bipolar II disorder (51.8 ± 9.1%, mean ± s.d.) than that in controls (70.2 ± 10.3%). (b, c) The expression level of *PPIEL* in 16 patients with bipolar I disorder, seven patients with bipolar II disorder and 18 controls. The quantity level of mRNA of each gene was normalized with that of *GAPDH* (b) or *CFL1* (c). Expression levels of *PPIEL* were significantly ($P = 0.001$) higher in patients with bipolar II disorder (*PPIEL/GAPDH*, $1.2 \times 10^{-3} \pm 4.3 \times 10^{-4}$; *PPIEL/CFL1*, $8.8 \times 10^{-3} \pm 2.5 \times 10^{-3}$) than that in controls (*PPIEL /GAPDH*, $6.9 \times 10^{-4} \pm 3.6 \times 10^{-4}$; *PPIEL/CFL1*, $4.6 \times 10^{-3} \pm 2.3 \times 10^{-3}$). (d) DNA methylation status of *PPIEL* in monozygotic twins discordant for bipolar disorder. Pyrograms of control twin (upper) and bipolar co-twin (lower) were shown. Shaded bars encompassing T/C pairs, derived from unmethylated C/methylated C by sodium bisulfite treatment. (e) Correlation between DNA methylation status and expression level of *PPIEL*. Correlation of expression levels and DNA methylation status in 16 patients with bipolar I disorder (square), seven patients with bipolar II disorder (diamond) and 18 controls (triangle) were analyzed. Expression levels of *PPIEL* were inversely correlated with DNA methylation status ($P < 0.001$, $R = -0.81$). (f) Correlation between DNA methylation status in lymphoblastoid cells and peripheral leukocytes. DNA methylation status of *PPIEL* in lymphoblastoid cells and peripheral leukocytes from seven patients were compared. DNA methylation statuses of five CpG sites of *PPIEL* (Figure 2, four arrows and an arrowhead) were analyzed by pyrosequencing. DNA methylation statuses of *PPIEL* in peripheral leukocytes were significantly correlated with those in lymphoblastoid cells ($P = 0.02$, $R = 0.41$, Pearson's correlation coefficient).

with regard to gender ($P = 0.50$) or valproate treatment (*CFL1* normalized, $P = 0.25$).

We also analyzed the DNA methylation status and expression of lymphoblastoid DNA of monozygotic twin discordant for bipolar disorder by pyrosequencing and real-time quantitative RT-PCR.

The DNA methylation status of bipolar twin (35–45%) was lower than that of control co-twin (80–100%), similarly to the results obtained by bisulfite sequencing (Figure 4d). The expression level of *PPIEL* normalized with *GAPDH* was much higher in bipolar twin ($5.2 \times 10^{-4} \pm 1.3 \times 10^{-4}$)
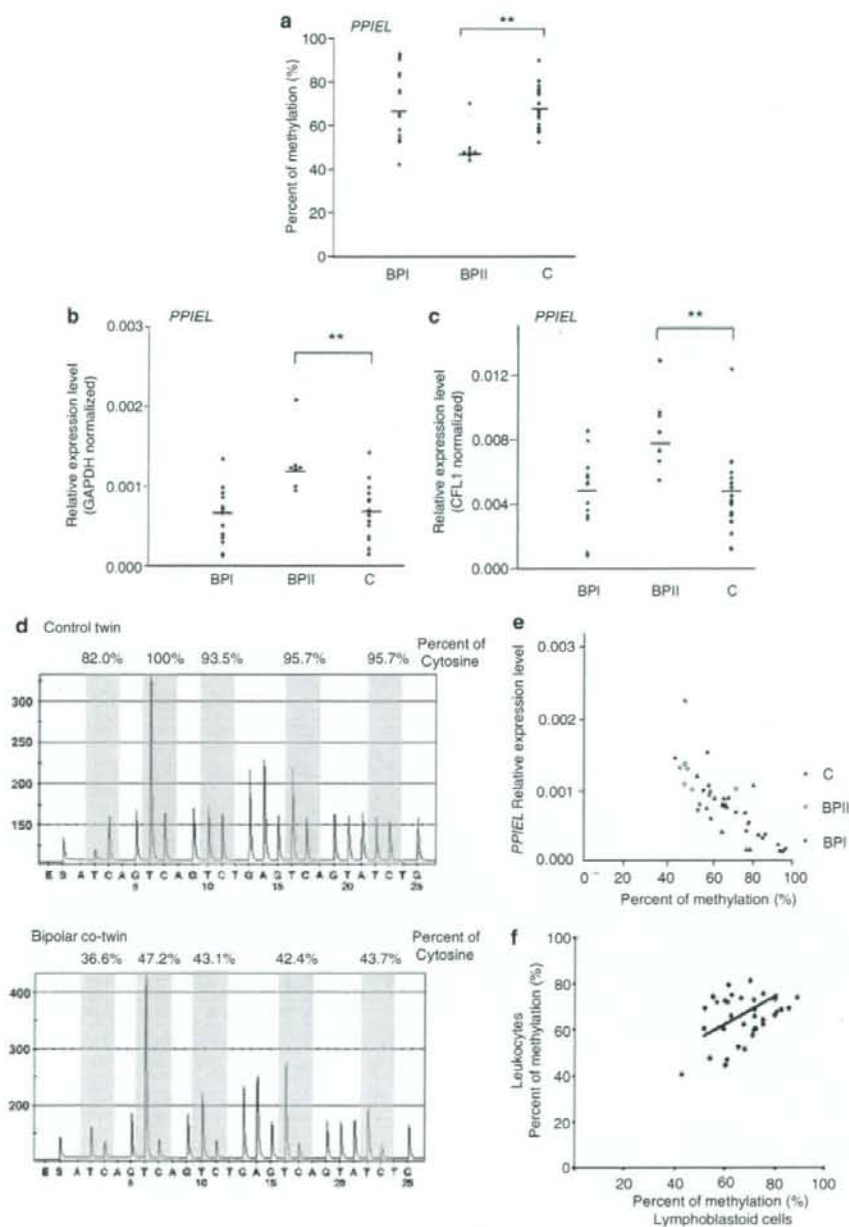
than that in control co-twin $(2.7 \times 10^{-6} \pm 3.2 \times 10^{-6})$.

In all samples of bipolar I disorder, bipolar II disorder and controls, expression levels of *PPIEL* were inversely correlated with its DNA methylation level ($P < 0.001$, $R = -0.81$) near the 5′ region of the *PPIEL* exon 1 (Figure 4e). Expression levels were weakly, but inversely correlated with DNA methylation level near the exon 2 ($P < 0.001$, $R = -0.54$).

Because the number of patients with bipolar II disorder in the initial sample set is small ($N = 7$), we also examined 14 additional patients with bipolar II disorder (Fujita samples). We re-analyzed the DNA methylation status of *PPIEL* by pyrosequencing in the original samples and Fujita samples. The initial finding of lower DNA methylation level in bipolar II disorder ($N = 7$) compared with controls ($N = 18$) was replicated (controls: $n = 18$, $80.0 \pm 11.9\%$ (mean $\pm$ s.d.); BPII: $n = 7$, $59.1 \pm 10.2\%$, $P = 0.001$). The Fujita sample (14 patients with bipolar II disorder) also showed significantly lower DNA methylation level ($68.0 \pm 12.3\%$, $P = 0.008$) compared with controls (Supplementary Figure 1).

To test whether altered DNA methylation status of *PPIEL* is due to EBV transformation, we tested the relationship between the DNA methylation status in lymphoblastoid cells and that in peripheral leukocytes. When DNA methylation status of *PPIEL* was examined by pyrosequencing in seven healthy subjects whose leukocyte DNA is also available, methylation levels of five CpG sites in lymphoblastoid cells and those in peripheral leukocytes were significantly correlated with each other ($P = 0.02$, $R = 0.41$) (Figure 4f).

Expression profiles of PPIEL in the human brain
If *PPIEL* is not expressed in the brain, pathophysiological significance of hypomethylation of this gene might be questioned. We could not visualize the distribution of *PPIEL* mRNA by *in situ* hybridization because this gene is not found in rodents. Instead, we examined the expression level of *PPIEL* in the human brain by quantitative RT-PCR using commercially available cDNA. The quantity level of mRNA of each gene was normalized with that of *GAPDH*. As a result, we found that *PPIEL* is ubiquitously expressed in the brain (Figure 5). The expression is highest in the pituitary gland and substantia nigra, but relatively lower in the frontal cortex and hippocampus.

Discussion

DNA methylation difference between twins
In this study, we searched for DNA methylation difference between a pair of monozygotic twins discordant for bipolar disorder, and identified four genes with altered DNA methylation status between the twins. Because a recent study showed that DNA methylation difference in lymphocytes can be found between healthy monozygotic twins and it increases with aging, it is not easy to directly associate this finding with the discordant phenotype.[21] Thus, we performed a case–control analysis to search for the pathophysiological significance of this finding. We excluded a pathophysiological significance of two genes, *PIP5KL1* and *ARMC3*, because there was no difference of DNA methylation status between patients with bipolar disorders and controls, and we studied the possible significance of other two genes further.

DNA methylation status of *SMS* at Xp22.11 was significantly different between female patients with bipolar disorder and female controls. However, lack of relationship between mRNA expression level and DNA methylation status, and no difference of mRNA
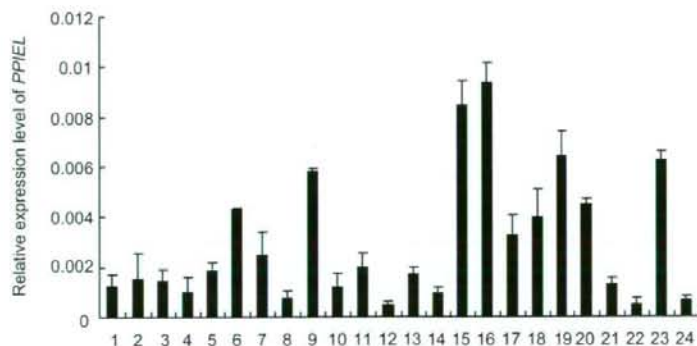

Figure 5 Expression level of *PPIEL* in human brain tissue. Expression level of *PPIEL* in human brain tissue was analyzed by quantitative RT-PCR. For cDNA samples derived from human brain tissue, Human Brain Parts Tissue Scan Real-Time (ORIGENE) was used. Lane 1, frontal lobe; lane 2, temporal lobe; lane 3, occipital lobe; lane 4, parietal lobe; lane 5, paracentral gyrus; lane 6, postcentral gyrus; lane 7, olfactory bulb; lane 8, thalamus; lane 9, corpus callosum; lane 10, hypothalamus; lane 11, amygdala; lane 12, hippocampus; lane 13, caudate; lane 14, putamen; lane 15, substantia nigra; lane 16, pituitary gland; lane 17, cerebellum grey; lane 18, cerebellum white; lane 19, cerebellum vermis; lane 20, nucleus accumbens; lane 21, pons; lane 22, medulla; lane 23, spinal cord; lane 24, choroid plexus. The level of mRNA of each gene was normalized with that of *GAPDH*.

levels between patients and controls, did not support the functional significance of the DNA methylation status of *SMS*. The DNA methylation difference initially detected in the twins can be attributed to only one CpG site, rather than global DNA methylation difference. In addition, the direction of DNA methylation difference observed in the case–control study was in the opposite direction to the finding in twins. These findings altogether do not support the role of altered DNA methylation status of *SMS* in pathophysiology of bipolar disorder.

On the other hand, in the case of *PPIEL*, both DNA methylation status and mRNA expression level were different between patients with bipolar II disorder and controls. The DNA methylation status was well correlated with mRNA expression level, and the DNA methylation difference found between twins is global DNA methylation status of several CpG sites, rather than a single CpG site. These findings support the possible functional significance of the DNA methylation at this site. The observed difference of DNA methylation status was not attributed to the difference of confounding factors such as age, gender and valproate treatment, although other confounding factors cannot be totally ruled out.

Because the number of samples of bipolar II disorder was small, we also examined an additional sample set (Fujita samples). Reduced DNA methylation status of *PPIEL* in bipolar II disorder was confirmed also in this sample set. However, we should cautiously interpret this finding because no control samples from Fujita Health University Hospital were available.

Pathophysiological significance of hypomethylation of PPIEL

PPIEL is neighboring the *BMP8A* and *OXCT2P* genes. This region of human genome on 1p34.3 (BO1 locus) has the other region having high homology, carrying *BMP8B* and *OXCT2* genes (BO2 locus).[22] This is supposed to be generated by segmental duplication that occurred before the primate–rodent split. However, the region containing *PPIEL* has been lost in mice. Thus, *PPIEL* is a primate-specific gene. *PPIEL* is predicted to encode a protein of 96 amino acids. Although the putative open reading frame is shorter than *PPIE*, predicted amino-acid sequence of *PPIEL* partially maintains the peptidyl-prolyl *cis–trans* isomerase (PPI) domain. This gene has not been well characterized yet, and there is no clear evidence that it is translated into protein.

The function of PPI domain is to convert the stereological structure of proline assembled in a polypeptide.[23] PPI is known as a target of immunosuppressants such as cyclosporin A. In the case of cyclosporin A, its inhibition of PPI activity impairs proper folding of calcineurin, which causes inhibition of immunoreaction in T cells.[23] The function of PPIs depends on the target molecule, and it is difficult to identify the function of *PPIEL* at this stage. *PPIEL* was highly expressed in the pituitary gland and substantia nigra, but relatively lower in the frontal cortex and hippocampus. This suggests that *PPIEL* does not play a role in folding of ubiquitous molecules, but might be involved in specific neuronal function, such as dopamine neurotransmission or neuroendocrine systems.

Methodological considerations

It is not known how comprehensively the MS-RDA method can detect the DNA methylation difference. It is evident that this method can cause both false-negative and false-positive findings. With regard to false negatives, the fragments that can be detected by this method depend on the types of restriction enzyme used. Thus, some of DNA methylation differences between twins might have been missed. With regard to false positives, many false-positive clones were detected as shown in Table 1. Even among the 10 genomic regions around CpG island or exon 1 detected by MS-RDA, DNA methylation difference for six regions could not be validated by bisulfite sequencing method. In spite of these inherent problems, practical usefulness of MS-RDA as a method to screen aberrantly methylated genomic regions has been shown.[13] Indeed, new cancer-related aberrant DNA methylation has been discovered by this method.

Although we used lymphoblastoid cells in this study, the ideal source of DNA methylation analysis is undoubtedly the brain tissue. Indeed, several lines of evidence suggest the relationship between schizophrenia and aberrant DNA methylation of the reeling promoter causing its downregulation in the brain.[24,25] We also reported that CpG island of *SOX10* was highly methylated in the brains of patients with schizophrenia, correlating with reduced expression of SOX10.[26] However, it is practically impossible to use brain sample in the study of discordant twin.

Although the symptoms of bipolar disorder should be arisen from brain dysfunction, intermediate phenotype, such as abnormalities in Ca^{2+} levels,[27,28] altered endoplasmic reticulum stress response[12] or altered inositol level,[29] have also been observed in lymphoblastoid cells. Thus, use of lymphoblastoid cells for epigenetic analysis may be validated at least in the study of bipolar disorder.

In addition, peripheral blood cells have already been used for epigenetic analysis of schizophrenia. Tsujita et al.[30] applied RLGS (restriction landmark genome scanning) method to analyze epigenetic difference between a pair of monozygotic twins discordant for schizophrenia, and identified differences between twins. McDonald et al.[31] used MS-RDA method to analyze a pair of monozygotic twins discordant for schizophrenia, but could not identify any difference. Petronis et al.[32] examined the DNA methylation status of the promoter of dopamine D2 receptor gene, and found that epigenetic distance is larger in a pair of discordant monozygotic twins compared with healthy monozygotic twins. Pathophysiological significance of the DNA methylation

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differences suggested in the previous studies is not well characterized yet, and thus usefulness of DNA methylation analysis in peripheral blood cells in mental disorders is still controversial. However, aberrant DNA methylation of an imprinted gene, KCNQ1OT1, identified in fibroblasts could also be identified in lymphocytes in monozygotic twins discordant for Beckwith Wiedemann syndrome.[33] Thus, at least aberrant imprinting status may be detectable even in peripheral blood cells. Otherwise, DNA methylation difference detected in this study might reflect the alteration of DNA methylation status occurred in the early developmental stage. In any case, the molecular mechanism of altered DNA methylation of PPIEL should be closely examined in the future.

In this study, we used lymphoblastoid cells transformed by EBV, rather than fresh lymphocytes, which potentially affect the results. By using lymphoblastoid cell lines, effects of medication can be eliminated by culturing the cells for more than 1 month after blood sampling, and cellular heterogeneity can be minimized. However, it cannot be ruled out that the effect of drugs lasts after in vitro culture for a long time. Although effect of valproate, which is reported to affect DNA methylation status, can be excluded in this study, effects of other drugs cannot be totally excluded.

It is suggested that EBV may alter the DNA methylation status of some genes,[34] but it has not been well characterized how and to what extent the EBV alters DNA methylation status. To exclude the effect of EBV, we analyzed methylation status of PPIEL in leukocytes of this pair of discordant twins. Similar to lymphoblastoid cells, bisulfite sequencing revealed that unmethylated allele of PPIEL was more frequently seen in affected twin (32%) compared with healthy co-twin (14%). Further, we found that the DNA methylation status in the lymphoblastoid cells was significantly correlated with that in peripheral leukocytes. These results support that hypomethylation of PPIEL observed in the lymphoblastoid cells of patients was not artificially caused by EBV but reflects the intrinsic inter-individual variability of DNA methylation status.

It should be noted that the number of subjects in this study is too small to draw a definite conclusion. Especially, the number of initial samples of patients with bipolar II disorder is very small ($n=7$) and the second sample set is not large enough ($n=14$). Thus, the results should be interpreted with caution. Nevertheless, this is the first study of altered DNA methylation status in bipolar disorder, which suggested a possible role of altered DNA methylation status in the pathophysiology of bipolar disorder.

Conclusion

In summary, we identified DNA methylation difference between monozygotic twins discordant for bipolar disorder using MS-RDA method. Hypomethylation of PPIEL found in the affected twin was confirmed in a case–control study. DNA methylation status of PPIEL was well correlated with mRNA expression levels.

These results suggest the possible role of PPIEL, in the pathophysiology of bipolar disorder, although it is not known whether it is the cause of the disease or the secondary or compensatory changes induced by the disease. This finding should be tested in a larger patient population, before it is applied to diagnostic testing or other clinical applications.

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Association analysis of nuclear receptor Rev-erb alpha gene (*NR1D1*) with mood disorders in the Japanese population

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ABSTRACT

Several investigations have suggested that alterations in circadian rhythms may lay the foundation for the development of mood disorder (bipolar disorder and major depressive disorder). Recently, the nuclear receptor Rev-erb alpha was reported to be related to circadian rhythms, and was shown to be involved in the biological action of lithium *in vitro*. These evidences indicate that the nuclear receptor Rev-erb alpha gene (*NR1D1*) is a good candidate gene for the pathogenesis of mood disorders. To evaluate the association between *NR1D1* and mood disorders, we conducted a case–control study of Japanese samples (147 bipolar patients, 322 major depressive disorder patients and 360 controls) with three tagging SNPs selected by HapMap database. One SNP showed an association with bipolar disorder in females. After Bonferroni correction for multiple testing, however, this significance disappeared. No significant association was found with major depressive disorder. In conclusion, our findings suggest that *NR1D1* does not play a major role in the pathophysiology of mood disorders in the Japanese population.
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1. Introduction

Many circadian disruptions are believed to occur in mood disorders such as major depressive disorder and bipolar disorder (Kato, 2007; Mansour et al., 2005; McClung, 2007a,b; Wirz-Justice, 2006). They include diurnal mood fluctuation, early morning awakening, and shortened REM sleep latency in depressive phase. Severe sleep–wake rhythm disturbance is also often observed in mood disorder patients (Boivin, 2000). Circadian sleep disorders such as delayed sleep phase syndrome that are often associated with mood symptoms (Dagan et al., 1998), can be treated with bright light, which has a strong effect in resetting circadian rhythms (Rosenthal et al., 1990). Recently, both seasonal and non-seasonal affective disorders have been treated with light, the mechanism for which is believed to be attributable to circadian properties (Tuunainen et al., 2004). Moreover, many studies have suggested that some circadian related genes, such as *CLOCK* and *PER3*, are involved in both circadian rhythm sleep disorders (Ebisawa et al., 2001; Iwase

et al., 2002; Jones et al., 2007; Pedrazzoli et al., 2007; Takano et al., 2004) and mood disorders (Johansson et al., 2003; Mansour et al., 2006; Shi et al., 2008). These facts suggest a close relationship between circadian rhythms and mood disorders, and so genes associated with the molecular clock mechanism are good candidates for the etiology of mood disorders.

Recently, Rev-erb alpha and glycogen synthase kinase-3 β (GSK-3β), which are known to be important circadian components (Yin et al., 2006), have been discussed in relation with lithium. Lithium is one of the most well-known mood stabilizers in the pharmacotherapy of mood disorder, and is also known to have circadian properties from *in vitro* and animal studies (Beaulieu et al., 2004; Gould et al., 2004; Sinha et al., 2005). Several studies have revealed that lithium affects circadian rhythms through GSK-3β (Beaulieu et al., 2004; Gould et al., 2004; Sinha et al., 2005). Yin et al. (2006) showed that Rev-erb alpha is a target of GSK-3β kinase activity, needed to mediate this effect to regulate circadian rhythms. Moreover, GSK-3β was also reported to be the target of valproic acid, another important mood stabilizer (Li et al., 2002). Although several studies have investigated the association between GSK-3β gene (*GSK3B*) and mood disorders, the results have been rather inconsistent (Benedetti et al., 2004a,b; Lee et al., 2006; Michelon et al., 2006; Nishiguchi et al., 2006; Serretti et al., 2008; Szczepankiewicz et al., 2006).

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Considering the above, the nuclear receptor Rev-erb alpha gene (*NR1D1*) seems a good candidate gene for the pathogenesis of mood disorders. *NR1D1* (OMIM *602408, 8 exons in this genomic region spanning 8.73 kb) is located on 17q11, which was shown to be a susceptibility region for BP (Dick et al., 2003; Ewald et al., 2005; Verheyen et al., 1999). To evaluate the association between *NR1D1* and mood disorders, we conducted a case–control study of Japanese samples (147 BP patients, 322 MDD patients and 360 controls).

2. Materials and methods

2.1. Subjects

The subjects in the association analysis were 147 BP patients (75 males and 72 females: 93 patients with bipolar I disorder and 54 patients with bipolar II disorder; mean age ± standard deviation (S.D.) 47.8 ± 14.6 years), 322 MDD patients (156 males and 166 females; mean age ± standard deviation 47.1 ± 15.9 years) and 360 healthy controls (201 males and 159 females; 35.9 ± 14.7 years). All subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. The patients were diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. None had severe medical complications such as cirrhosis, renal failure and heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls, which included hospital staff, their families and medical students.

The study was described to subjects and written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University School of Medicine.

2.2. SNP selection and LD evaluation

We first consulted the HapMap database (release#20/phase II, Jan 2006, www.hapmap.org, population: Japanese Tokyo, minor allele frequencies (MAFs) of more than 0.05) and included 5 SNPs covering *NR1D1* (5′-flanking regions including about 750 bp from the initial exon and about 1 kb downstream (3′) from the last exon: HapMap database contig number chr17: 35501880.35510616). Then three 'tagging (tag) SNPs' were selected with the criteria of r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger), in Haploview (Barrett et al., 2005), three 'tag SNPs' (SNP2: rs939347, SNP4: rs2071427, SNP5: rs3744805) were selected for the following association analysis.

2.3. SNP genotyping

We used TaqMan assays (Applied Biosystems, Inc., Foster City, CA) for all SNPs. One allelic probe was labeled with FAM dye and the other with the fluorescent VIC dye. The plates were heated for 2 min at 50 °C and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 58 °C for 1 min. The three SNPs of the *NR1D1* were characterized as follows. The primers used were forward 5′-TGCCGGCTCCGCAGCGCTGCGGGGT-3′ and reverse 5′-GCGAATCTGGAGCTCCCGGTGCAAA-3′ in SNP2, forward 5′-AAAGGT-GACCTGCCTGCCACACCTC-3′ and reverse 5′-GGCAAGCACCTAGATTGGTCTGCCA-3′ in SNP4 and forward 5′-CAGAGCCATGACCACTACCCCGCCC-3′ and reverse 5′-CACCACCCGCCTCTCCATCTCAGTA-3′ in SNP5. Detailed information is available on request.

2.4. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan).

Marker-trait association analysis was used to evaluate allele- and genotype-wise association with the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan), and haplotype-wise association analysis was evaluated with a likelihood ratio test using the COCAPHASE2.403 program (Dudbridge, 2003). Bonferroni's correction was used to control inflation of the type I error rate. Power calculation was performed using two statistical programs prepared by Ohashi et al. (2001) (Ohashi et al.'s software) or Purcell et al. (2003) (Genetic Power Calculator; GPC). The significance level for all statistical tests was 0.05.

3. Results

The LD structure as determined from the HapMap database can be seen in Fig. 1. Genotype frequencies of all SNPs were in HWE. We detected no significant associations with mood disorders in the
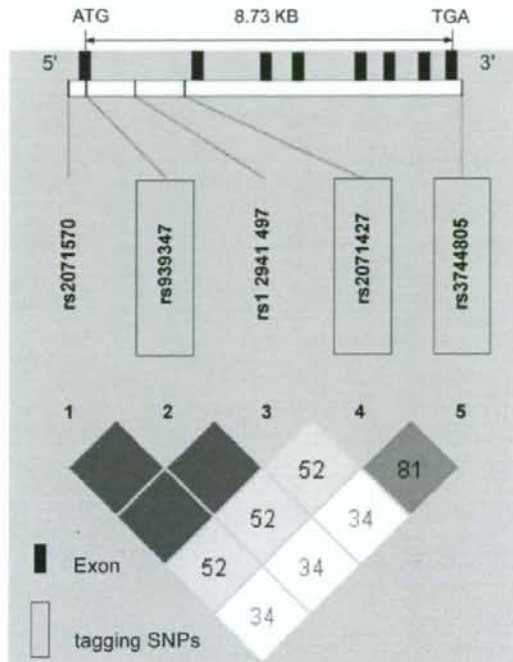

Fig. 1. LD evaluation and tagging SNPs in *NR1D1*. ATG is the start codon and TGA is the stop codon. Vertical bars represent exons. Tagging SNPs selected from the HapMap database are represented by black boxes. Color scheme was based on D′ value (this result represents complete LD between SNP1, SNP2 and SNP3, and these SNPs constitute one block. On the other hand, we detected only loose LD between SNP4 and SNP5). Other information can be seen at the Haploview website.

allele/genotype-wise analysis (Table 1), or the haplotype analysis (BP: $P = 0.607$ and MDD: $P = 0.235$). It is known that there are sex differences in not only the pathophysiology of mood disorders (Baron, 1981; Currier et al., 2006; Faraone et al., 1987) but also in circadian rhythm (Lehnkering and Siegmund, 2007). In addition, Szczepankiewicz et al. (2006) reported an association between a diagnosis of BP II in females and the *GSK3B*, dividing the subjects by clinical diagnosis and sex. Therefore, we performed an explorative analysis of subjects divided by clinical diagnosis (except MDD) or sex. A significant association was found between one tag SNP (SNP2) and BP in females in a genotype-wise analysis ($P = 0.0236$); however, this might have resulted from type I error due to multiple testing ($P = 0.142$ after Bonferroni's correction). No association was detected between other SNPs and any subgroup or either sex (Tables 2 and 3).

We obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.47–1.49 (Ohashi et al.'s software) and 1.56–1.59 (GCP) in BP and 1.36–1.37 (Ohashi et al.'s software) and 1.31–1.33 (GCP) in MDD, under a multiplicative model of inheritance (Ohashi et al., 2001; Purcell et al., 2003).

4. Discussion

We analyzed the genetic association between *NR1D1* and mood disorders in the Japanese population. No association was found between *NR1D1* and mood disorders by allele/genotype-wise or haplotype-wise analysis. In the explorative analysis, however, a

Table 1
Tag SNPs and association analysis of NR1D1

SNP ID[a]	Phenotype[b]	MAF[c]	N	Genotype distribution[d]			P-value			
				M/M	M/m	m/m	HWE[e]	Genotype	Allele	
SNP2 (rs939347, A > G)	Controls	0.483	360	92	188	80	0.387			
	MDD	0.489	322	78	173	71	0.178	0.907	0.831	
	BP	0.490	147	38	68	41	0.367	0.462	0.852	
SNP4 (rs2071427, G > A)	Controls	0.468	360	103	177	80	0.811			
	MDD	0.478	322	90	156	76	0.600	0.911	0.706	
	BP	0.473	147	42	71	34	0.706	0.873	0.891	
SNP5 (rs3744805, A > G)	Controls	0.489	360	96	176	88	0.680			
	MDD	0.497	322	80	164	78	0.738	0.833	0.768	
	BP	0.524	147	29	82	36	0.152	0.222	0.313	

[a] Major allele > minor allele.
[b] MDD: major depressive disorder, BP: bipolar disorder.
[c] MAF: minor allele frequency.
[d] M: major allele, m: minor allele.
[e] Hardy–Weinberg equilibrium.

Table 2
Explorative analysis of gender subgroups

SNP ID[a]	Phenotype[b]	MAF[c]	N	Genotype distribution[d]			P-value[f]			Corrected P-value[g]
				M/M	M/m	m/m	HWE[e]	Genotype	Allele	Genotype
SNP2 (rs939347, A > G)	Controls, male	0.480	201	55	99	47	0.850			
	MDD, male	0.497	156	37	83	36	0.423	0.696	0.658	
	BP, male	0.467	75	20	40	15	0.536	0.791	0.779	
	Controls, female	0.488	159	37	89	33	0.130			
	MDD, female	0.487	166	41	90	35	0.269	0.942	0.889	
	BP, female	0.556	72	18	28	26	0.0714	**0.0236**	0.175	0.142
SNP4 (rs2071427, G > A)	Controls, male	0.470	201	59	95	47	0.467			
	MDD, male	0.487	156	46	68	42	0.111	0.704	0.651	
	BP, male	0.44	75	22	40	13	0.476	0.514	0.527	
	Controls, female	0.465	159	44	82	33	0.646			
	MDD, female	0.470	166	44	88	34	0.409	0.963	0.909	
	BP, female	0.507	72	20	31	21	0.239	0.327	0.408	
SNP4 (rs3744805, A > G)	Controls, male	0.478	201	53	104	44	0.601			
	MDD, male	0.519	156	39	72	45	0.345	0.313	0.270	
	BP, male	0.507	75	16	42	17	0.298	0.685	0.543	
	Controls, female	0.503	159	43	72	44	0.234			
	MDD, female	0.476	166	41	92	33	0.153	0.142	0.487	
	BP, female	0.542	72	13	40	19	0.313	0.252	0.443	

[a] Major allele > minor allele.
[b] MDD: major depressive disorder, BP: bipolar disorder.
[c] MAF: minor allele frequency.
[d] M: major allele, m: minor allele.
[e] Hardy–Weinberg equilibrium.
[f] Bold represents significant P-value.
[g] Calculated using Bonferroni's correction.

significant association of one tag SNP (SNP2) was found with BP in females in a genotype-wise analysis ($P = 0.0236$). Bonferroni's correction increased this P value to 0.142, suggesting it might have resulted from type I error due to multiple testing.

Szczepankiewicz et al. (2006) reported an association between a diagnosis of BP II in females and the GSK3B, which is closely linked with NR1D1. Some gender differences are seen in mood disorders, with the prevalence of major depressive disorder being higher in females. Hormonal variations in females, such as during the menstrual cycle, pregnancy and menopause, affect the onset and course of mood disorders, and it is possible that the etiology of mood disorders differs somewhat in females and males. Although our findings failed to show a clear association between NR1D1 and mood disorders in the Japanese population and, additional study of

circadian related genes with larger female samples may be warranted.

One recent study has reported no association between NR1D1 and BP (Shi et al., 2008). However, other clock genes that have been reported to be significantly associated with BP are CLOCK, ARNTL, PER3 and TIMELESS (Kato, 2007; McClung, 2007a,b). One of the biological action by lithium treatment has been reported to affect the expression of clock genes mediated by Rev-erb alpha in vitro (Yin et al., 2006). Because these clock genes, including NR1D1 and GSK3B, are related to each other, the pharmacogenomics of BP (lithium response) and gene–gene interactions among clock genes will also need to be investigated in the future.

A few points of caution should be noted in interpreting our results. First, the lack of association may be due to biased samples,

Table 3
Explorative analysis of clinical subgroups

SNPID[a]	Phenotype[b]	MAF[c]	N	Genotype distribution[d]			P-value		
				M/M	M/m	m/m	HWE[e]	Genotype	Allele
SNP2 (rs939347, A > G)	Controls	0.483	360	92	188	80	0.387		
	BPI	0.522	93	24	41	28	0.261	0.235	0.353
	BPII	0.491	54	14	27	13	0.998	0.842	0.886
SNP4 (rs2071427, G > A)	Controls	0.468	360	103	177	80	0.811		
	BPI	0.473	93	29	40	24	0.186	0.558	0.902
	BPII	0.472	54	13	31	10	0.265	0.528	0.936
SNP5 (rs3744805, A > G)	Controls	0.489	360	96	176	88	0.680		
	BPI	0.532	93	18	51	24	0.328	0.342	0.292
	BPII	0.509	54	11	31	12	0.275	0.473	0.693

[a] Major allele > minor allele.
[b] BPI: bipolar I disorder, BPII: bipolar II disorder.
[c] MAF: minor allele frequency.
[d] M: major allele, m: minor allele.
[e] Hardy–Weinberg equilibrium.

such as unmatched aged samples, or small sample sizes, especially in BP samples. On average, the controls are much younger than the patients. This means that a number of young controls may go on to develop one these disorders, most likely MDD, since the incidence of major depression is as high as 5% or more. Also, our control subjects did not undergo structured interviews. Hashimoto et al. (2005) referred to the problem of sampling bias with mood disorders; for example, in some patients a diagnosis of major depressive disorder may become bipolar disorder in the future. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association of such extremely rare variants (e.g. MAF <0.01) from the viewpoint of power. Replication studies using larger samples or a family-based association approach will be required for conclusive results (Neale and Sham, 2004).

In conclusion, our results suggest that NR1D1 probably does not play a major role in mood disorders in the Japanese population.

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BIOLOGICAL PSYCHIATRY - ORIGINAL ARTICLE

Genetic association analysis of tagging SNPs in alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptor genes (*CHRNA4* and *CHRNB2*) with schizophrenia in the Japanese population

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Abstract Several lines of evidence suggest that nicotinic cholinergic dysfunction may contribute to the cognitive impairments in schizophrenia. The majority of high affinity nicotine binding sites in the human brain have been implicated in heteropentameric alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptors; therefore, these two neuronal nicotinic acetylcholine receptors genes (*CHRNA4* and *CHRNB2*) are considered to be attractive candidate genes for the pathophysiology of schizophrenia. To represent these two genes in a gene-wide manner, we first evaluated the linkage disequilibrium structure using our own control samples. Thirteen SNPs (7 SNPs for *CHRNA4* and 5 SNPs for *CHRNB2*) were selected as tagging SNPs. Using these tagging SNPs, we then conducted genetic association analysis of case-control samples (738 schizophrenia and 753 controls) in the Japanese population. No significant association was detected in the allele/genotype-wise or haplotype-wise analysis. Our results suggest that *CHRNA4* and *CHRNB2* do not play a major role in Japanese schizophrenia.

Introduction

Cognitive impairments in areas such as attention, executive function, language and memory, for which there is not much hope of recovery with treatment, have been implicated as endophenotypes for schizophrenia (Green 1996), and such impairments may be partially mediated by nicotinic acetylcholine receptors (Levin and Simon 1998). A recent study has reported an association of such cognitive impairments with abnormalities in the neuronal network in the prefrontal cortex, superior temporal gyrus and cerebellum in schizophrenics (Bonilha et al. 2008). The smoking rate of schizophrenics is much higher than that of healthy individuals with lifetime history (Hughes et al. 1986). Schizophrenics may smoke to compensate for their cognitive dysfunctions, since nicotine has cognitive-enhancing properties (Kumari and Postma 2005).

The majority of high affinity nicotine binding sites in the brain consist of heteropentameric alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptors (nAChRs) (Flores et al. 1997). Several lines of evidence support an association between abnormalities in alpha4 and beta2 subunits and schizophrenia. Firstly, beta2 subunits in the ventral tegmental area in mutant mice showed alternation of dopamine release in the nucleus accumbens and changes in cognitive functions such as navigation and exploratory behaviour compared with wild mice (Maskos et al. 2005). Alpha4 subunit mutant mice also showed increased anxiety-like behaviour and a reduction of substantia nigra dopaminergic neurons on ageing (Labarca et al. 2001; Ross et al. 2000). These animal study results

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may reflect dopamine and neurodevelopmental hypotheses of the pathophysiology of schizophrenia, and these behaviors may be major symptoms of schizophrenia (Lang et al. 2007). Secondly, a postmortem study reported fewer alpha4/beta2 subunits in the hippocampi of schizophrenics (Freedman et al. 1995). Thirdly, De Luca et al. reported that the alpha4 gene (*CHRNA4*) and beta2 subunit gene (*CHRNB2*) showed genetic interactions with schizophrenia in a family-based association study in the Canadian population (De Luca et al. 2006), and *CHRNA4* and *CHRNB2* were also associated with smoking among schizophrenics (Faraone et al. 2004; Voineskos et al. 2007).

Therefore, we conducted a genetic association study of *CHRNA4* (located on 20q13) and *CHRNB2* (located on 1q21) with schizophrenia in the Japanese population. In this study, we applied the "gene-wide" approach recommended by Ikeda to consider the population differences (Ikeda et al. 2008), first evaluating the linkage disequilibrium (LD) structure of these genes and selecting tagging SNPs ("tag SNPs"). These tag SNPs were then used to present the LD properties of the gene in the Japanese population in the following association analysis.

Materials and methods

Subjects

The subjects in the association analysis were 738 schizophrenia patients (395 males and 343 females; mean age ± standard deviation (SD) 47.4 ± 16.6 years; age of onset 26.0 ± 9.55 years) and 753 healthy controls (326 males and 427 females; 37.3 ± 14.3 years). Patients were grouped according to the following DSM-IV subtypes of schizophrenia: Paranoid Type ($n = 216$), Disorganized Type ($n = 221$), Catatonic Type ($n = 29$), Residual Type ($n = 142$), Undifferentiated Type ($n = 130$). All healthy controls are identical to those in our previous paper (Kishi et al. 2008). The subjects for LD evaluation were 96 controls, who were also among the sample used in the association analysis. All subjects were unrelated to each other and ethnically Japanese. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews that included current and past psychiatric history. None had serious medical complications such as cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls that included hospital staff, their families and medical students.

After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University School of Medicine.

SNP selection and LD evaluation

Methods for selection of tagging SNPs were described in our previous paper (Kishi et al. 2008). Briefly, we first consulted the HapMap database (release#20/phaseII, Jan 2006, www.hapmap.org) to select the tag SNPs; however, no dense marker sets were listed in HapMap (2SNPs and 5SNPs in *CHRNA4* and *CHRNB2*, respectively). Therefore, we accessed another information source, the JSNP database (Haga et al. 2002; Hirakawa et al. 2002), and picked up 11 SNPs to evaluate the denser LD structure of *CHRNA4* (we tried to select SNPs with minor allele frequencies (MAFs) of more than 0.05 to increase the power).

At first, we genotyped all these SNPs using our own 96 control samples to evaluate the LD structure in the Japanese population. We then selected tag SNPs with the criteria of r^2 threshold greater than 0.8 in "pair-wise tagging only" mode using the "Tagger" program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger) in Haploview 3.2 for the following association analysis (Barrett et al. 2005; Gabriel et al. 2002).

SNP genotyping

For rapid genotyping of SNPs, we used TaqMan assays (Applied Biosystems) and direct sequencing (Table 1). Detailed information, including primer sequences and reaction conditions, is available on request.

Statistical analysis

The genotypic deviation from Hardy-Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan).

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses), and the log-likelihood test (haplotype-wise analysis; the haplotype frequencies were estimated with the expectation-maximization algorithm) (SAS/Genetics, release 8.2). In this haplotype-wise analysis, the information of the "LD block" (criteria based on 95% confidential intervals on the D' values) in the LD evaluation step were used.

Power calculation was performed using a statistical program prepared by Ohashi et al. (2001). The significance level for all statistical tests was 0.05.

Table 1 tag SNPs and association analysis of *CHRNA4* and *CHRNB2*

| Gene symbol | SNP ID[a] | Phenotype | MAF | Genotype deviation | | | | P value | | |
				N	M/M	M/m	m/m	HWE	Genotype	Allele
CHRNA4	SNP A-1 rs755203	SCZ	0.418	732	250	352	130	0.752	0.972	0.809
		CON	0.414	747	259	358	130	0.742		
	SNP A-2 rs2273506	SCZ	0.105	732	585	141	6	0.431	0.124	0.0542
		CON	0.127	747	564	176	7	0.0939		
	SNP A-6 rs2273504	SCZ	0.465	732	214	355	163	0.493	0.741	0.560
		CON	0.454	747	232	351	164	0.152		
	SNP A-9 rs1044396	SCZ	0.287	732	379	286	67	0.222	0.572	0.476
		CON	0.275	747	393	297	57	0.932		
	SNP A-10 rs1044397	SCZ	0.390	731	278	336	117	0.360	0.826	0.539
		CON	0.401	747	273	349	125	0.454		
	SNP A-12 rs2236196	SCZ	0.116	731	573	147	11	0.656	0.167	0.309
		CON	0.128	747	563	177	7	0.0874		
	SNP A-13 rs4522666	SCZ	0.422	732	240	366	126	0.501	0.567	0.650
		CON	0.430	747	247	357	143	0.490		
CHRNB2	SNP B-1 rs4845652	SCZ	0.116	735	579	141	15	0.0697	0.401	0.194
		CON	0.101	749	607	132	10	0.359		
	SNP B-2 rs2072658	SCZ	0.201	738	467	246	25	0.283	0.930	0.772
		CON	0.205	747	466	256	25	0.155		
	SNP B-3 rs2072659	SCZ	0.143	734	542	174	18	0.370	0.623	0.606
		CON	0.136	751	559	179	13	0.759		
	SNP B-4 rs2072660	SCZ	0.252	735	410	279	46	0.873	0.585	0.352
		CON	0.238	753	440	268	45	0.622		
	SNP B-5 rs3811450	SCZ	0.119	736	576	145	15	0.106	0.218	0.981
		CON	0.119	751	580	163	8	0.354		

P value for association of SNPs in *CHRNA4* and *CHRNB2* with schizophrenia

[a] tag SNPs

SCZ schizophrenia, *CON* controls, *MAF* minor allele frequency of 96 controls, *N* number, *M* major allele, *m* minor allele, *HWE* Hardy–Weinberg equilibrium

Results

For the initial LD evaluation, 13 SNPs (2SNPs from HapMap, 11SNPs from JSNP) and five SNPs (5SNPs from HapMap) for *CHRNA4* and *CHRNB2*, respectively, were genotyped for 96 controls. The LD structure results can be seen in our previous paper (Kishi et al. 2008). Seven and five SNPs were then selected as tag SNPs for each gene (Kishi et al. 2008). The genotypic distributions of all SNPs were in HWE.

No significant associations were found between any tag SNPs in *CHRNA4* and *CHRNB2* and Japanese schizophrenia in the allele/genotype-wise analysis (Table 1) or in the haplotype analysis (*CHRNA4* Block I-P: 0.502, Block II-P: 0.432, *CHRNB2* Block I-P: 0.564). To further investigate these associations, we included an explorative analysis of gender effects, because recent studies showed

that genetic factors underlying nicotine addiction probably play a different role in female and male smokers (Feng et al. 2004; Greenbaum et al. 2006; Li et al., 2005). This suggests the existence of sex-specific genetic components in nicotine use disorders or these genes. In the present analysis, we found that SNP A-10 and B-4 were significantly associated with male and female schizophrenics, respectively (SNP A-10 and male schizophrenia P: 0.0389, SNP B-4 and female schizophrenia P: 0.0262). However, these results were no longer statistically significant after Bonferroni correction (SNP A-10 and male schizophrenia P: 0.545, SNP B-4 and female schizophrenia P: 0.262).

In the power analysis, we obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.23–1.36 and 1.26–1.35 for *CHRNA4* and *CHRNB2*, respectively, under a multiplicative model of inheritance.

Discussion

In this study, we found no significant association between two major cholinergic receptor genes, *CHRNA4* and *CHRNB2*, and schizophrenia in the Japanese population.

Although nominal significant associations between two SNPs (A-10 and B-4) and subgroups divided by gender were detected in the explorative analyses, these associations might have been the result of type I error due to multiple testing.

A recent family-based association study showed gene–gene interactions between *CHRNA4* and *CHRNB2* (De Luca et al. 2006). Therefore, we applied the recently recommended strategy of "gene-based" association analysis for the purpose of detecting susceptibility genes for schizophrenia (Neale and Sham 2004), and conducted a case-control association analysis by selecting the tag SNPs. As in the original study, we did not detect an association between these genes and schizophrenia. To evaluate the interactions with each SNP in these genes, we then analyzed the gene–gene interactions with the use of the Multifactor Dimensionality Reduction (MDR) method (Hahn et al. 2003). This analysis, however, revealed no interactions with schizophrenia (data not shown).

Voineskos et al. showed a significant association of rs3746372 in *CHRNA4* with heavy smoking in schizophrenia (Voineskos et al. 2007). The rs3746372 is located upstream 39593 bp from the initial exon and rs3746372. In this study, we selected tag SNPs in an association analysis after performing a LD evaluation that covered *CHRNA4*, including the promoter region, using our control samples. Although we confirmed LD between the SNPs selected in this study and rs3746372 according to the HapMap database, this LD was not found to be tight. Since we thought that rs3746372 might not be included in *CHRNA4*, we did not perform an association analysis of this SNP.

A very recent study reported that two functional SNPs (rs6122429 and rs2236196) in CHRNA4 were associated with luciferase activity (Winterer et al. 2007), and the question of whether these SNPs were associated in our schizophrenic samples should be examined. Of these SNPs, rs2236196 was included in this study, but rs6122429 was not. To evaluate whether our tag SNPs represent this rs6122429, located in the 5′ flanking region of CHRNA4, we genotyped rs6122429 using our 96 control samples. We found that this SNP was in LD with our SNP1 ($r^2 = 0.85$), and thus speculate that rs6122429 is not associated with schizophrenia in the Japanese population.

Our study is reasonable in terms of its design (selecting tag SNPs to represent each gene) and sample size large enough to gain high power. However, a couple of limitations should be noted. First, our samples were not age- or gender-matched. Although we included subgroup analyses divided by gender, careful interpretation is needed with respect to the association of schizophrenia itself. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association for a rare variant (e.g. MAF < 0.05). A larger sample size will be required for conclusive results in mutation search and association analysis. Lastly, several investigations have suggested that alternation of nAChRs may reflect the cognitive dysfunction and nicotine dependence associated with schizophrenia (Faraone et al. 2004; Levin and Simon 1998; Voineskos et al. 2007). Moreover, because the heritability of nicotine dependence and schizophrenia is reported to be about 40–70% (Li et al. 2003; Maes et al. 2004; Swan et al. 1990) and 80% (Cannon et al. 1998), respectively, we considered that the influence of genetic factors was about the same. Therefore, further study will be required to investigate the relationship between these genes and cognitive function or/and high smoking rate in schizophrenia, because we did not have information on smoking history in our samples or evaluate cognitive function.

In conclusion, our results suggest that *CHRNA4* and *CHRNB2* do not play a major role in schizophrenia in the Japanese population.

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