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ORIGINAL ARTICLE

Aberrant DNA methylation associated with bipolar disorder identified from discordant monozygotic twins

G Kuratomi[1,2], K Iwamoto[1], M Bundo[1], I Kusumi[3], N Kato[2], N Iwata[4], N Ozaki[5] and T Kato[1]

[1]*Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Hirosawa, Wako, Saitama, Japan;* [2]*Department of Neuropsychiatry, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan;* [3]*Department of Psychiatry, Hokkaido University Graduate School of Medicine, Kita-ku, Sapporo, Hokkaido, Japan;* [4]*Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi, Japan and* [5]*Department of Psychiatry, Nagoya University Faculty of Medicine, Nagoya, Aichi, Japan*

To search DNA methylation difference between monozygotic twins discordant for bipolar disorder, we applied a comprehensive genome scan method, methylation-sensitive representational difference analysis (MS-RDA) to lymphoblastoid cells derived from the twins. MS-RDA isolated 10 DNA fragments derived from 5′ region of known genes/ESTs. Among these 10 regions, four regions showed DNA methylation differences between bipolar twin and control co-twin confirmed by bisulfite sequencing. We performed a case–control study of DNA methylation status of these four regions by pyrosequencing. Two regions, upstream regions of spermine synthase (*SMS*) and peptidylprolyl isomerase E-like (*PPIEL*) (CN265253), showed aberrant DNA methylation status in bipolar disorder. *SMS*, a gene on X chromosome, showed significantly higher DNA methylation level in female patients with bipolar disorder compared with control females. However, there was no difference of mRNA expression. In *PPIEL*, DNA methylation level was significantly lower in patients with bipolar II disorder than in controls. The expression level of *PPIEL* was significantly higher in bipolar II disorder than in controls. We found strong inverse correlation between gene expression and DNA methylation levels of *PPIEL*. These results suggest that altered DNA methylation statuses of *PPIEL* might have some significance in pathophysiology of bipolar disorder.
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Keywords: bipolar disorder; DNA methylation; epigenetics; gene expression; CpG island; lymphoblastoid

Introduction

Bipolar disorder is a mental disorder characterized by recurrent manic and depressive episodes affecting about 1% of the population. In spite of extensive studies, cause of bipolar disorder is unknown yet. Twin, adoption, family and linkage studies suggested that bipolar disorder is a complex disease caused by multiple genetic, environmental, or epigenetic risk factors.[1]

Epigenetics is defined as (the study of) mitotically or meiotically heritable variations in gene function that cannot be explained by changes in DNA sequence.[2] Among epigenetic mechanisms, methylation of the cytosine residue of the DNA molecule has been well studied. Methylation of CpG islands controls gene expression, genomic imprinting and X-chromosome inactivation. Abnormality of DNA methylation status is involved in various pathological process.[3–5]

Several lines of evidence suggested that epigenetics is relevant to bipolar disorder. Complex non-Mendelian inheritance, especially parent-of-origin effect, of bipolar disorder suggests the involvement of altered status of genomic imprinting and skewed X-chromosome inactivation.[6] Pharmacological studies also showed the possible role of DNA methylation in pathophysiology of bipolar disorder. Valproate, one of mood stabilizers, is known to be a histone deacetylase inhibitor.[7] Histone acethylation is coupled with DNA methylation and plays a role in the epigenetic regulation of gene expression. On the other hand, *S*-adenosyl methionine (SAM) is known to be effective for bipolar depression.[8] SAM supplies methyl residue in DNA methylation reaction, and enhance DNA methylation *in vitro*.[9] These data may suggest that epigenetic gene regulations may be relevant to the pathophysiology of bipolar disorder. Considering that DNA methylation may be involved in bipolar disorder, genome-wide screening for genes that is aberrantly methylated or demethylated in bipolar disorder is important.

Correspondence: Dr T Kato, Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.
E-mail: kato@brain.riken.jp
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In twin studies of bipolar disorder, the concordance rate in monozygotic twins (>65%) is much higher than in dizygotic twins (>14%). However, some monozygotic twins are discordant with respect to bipolar disorder. Such discordance between monozygotic twins could be arisen from epigenetic difference.[10] In this study, we focused on a pair of monozygotic twins discordant for bipolar disorder, which was reported in the previous report.[11] We previously searched for gene expression differences between these monozygotic twins. We found that *XBP1* and its downstream gene, *HSPA5*, are commonly downregulated in the affected twins.[12] However, we could not identify differences of DNA sequence or DNA methylation of *XBP1* between the twins. Expression differences of *XBP1* might be caused by differential DNA methylation status and expression level of other genes, such as upstream genes of *XBP1*.

In this study, to comprehensively scan DNA methylation difference between the genomes of twins, we applied MS-RDA.[13] MS-RDA was developed by Ushijima *et al.*[13] and has been successfully used to identify the differences of DNA methylation status. We applied the MS-RDA method to lymphoblastoid cells derived from monozygotic twins discordant for bipolar disorder.

Materials and methods

Subjects

For MS-RDA, we examined the pair of monozygotic twins 49-year-old males discordant with respect to bipolar disorder. They were reported in a previous study.[12] The zygosity was determined by traditional phenotype markers such as blood types and HLA, micro-satellite markers and the extensive single nucleotide polymorphism (SNP) genotyping by DNA microarray (Human Mapping 50K Xba Array, Affymetrix, Santa Clara, CA, USA). For real-time quantitative RT-PCR and pyrosequencing, we examined 16 unrelated patients with bipolar I disorder (BPI) (10 men and six women, 51.2±12.6 (mean±s.d.) years old), seven unrelated patients with bipolar II disorder (BPII) (one men and six women, 62.7±9.2 years old) and 18 unrelated control subjects (12 men and six women, 45.6±12.0 years old). They were the subjects from our previous study.[14] The patients were treated with various psychotropic drugs. Three of 16 BPI patients and none of seven BPII patients were treated with valproate.

For the additional analysis, an independent set of lymphoblastoid cells derived from 14 patients with bipolar II disorder in the Fujita Health University Hospital (four women and 10 men, 39.0±13.5 (average±s.d.) years old) was used.

Consensus diagnosis was made by two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association, 1994) criteria using the non-structured interview and scrutinizing medical records. Control subjects were assessed by a psychiatrist after non-structured interview, and they had no major physical or mental disorders affecting social functioning. Subjects with a history of major mental disorders, and a family history of major mental disorders within first-degree relatives were excluded from the study. All the subjects were Japanese.

Written informed consent was obtained from all the subjects. This study was approved by the ethics committees of RIKEN and participating institutions.

Lymphoblastoid cells culture

Lymphocytes from peripheral blood were transformed by Epstein–Barr virus (EBV) using standard techniques as described before.[15] Briefly, lymphocytes were separated from peripheral blood and cultured with RPMI 1640 Medium (Sigma-Aldrich, St Louis, MO, USA) containing 20% fetal bovine serum (FBS) (GIBCO, Carlsbad, Ca, USA), Penicillin and streptomycin (50 μg/ml each) (GIBCO) and supernatant of B95-8 cell culture inducted by Epstein–Barr virus. These cells were passaged every week until the cell lines were established. The cells were passaged three times a week using similar medium except for the addition of 10% FBS. The cells were kept frozen until the experiment. The blood samples of the twins were obtained on the same day, and these samples were similarly handled. The lymphoblastoid cells for the case–control analysis were used after the same number of reculture.

DNA extraction

For MS-RDA, we used genomic DNA extracted from lymphoblastoid cells of monozygotic twins discordant with respect to bipolar disorder. For pyrosequencing, we used genomic DNA extracted from lymphoblastoid cell samples that RNA was used for the real-time quantitative RT-PCR. DNA was prepared by extraction with phenol and chloroform followed by ethanol precipitation. For the analysis of peripheral blood leukocytes, the red blood cells were lysed with hypotonic buffer and genomic DNA was extracted from the pellet.

Outline of MS-RDA

MS-RDA was performed as described previously.[13] In MS-RDA, two genomes, tester and driver, were digested with *Hpa*II, a methylation sensitive restriction enzyme. *Hpa*II recognizes and digests 5′-CCGG-3′, but, is blocked by methylation of the internal C residue. After digestion, an adapter is ligated, and the entire restriction product is amplified by PCR. The PCR product is enriched with fragments originated from hypomethylated genomic regions that can be digested by HpaII. Thus, the amount of PCR product, in tester and driver, represents the DNA methylation status in each genome. After the first adapter is removed, a new adapter is ligated, only for the tester. The tester is mixed with the excess amount of driver product without the adapter. When there is no counterpart of a tester fragment in the driver, PCR

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 *Hpa*II 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 *Hpa*II 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)pipecazine-*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′-ACCGACGTCGACTATCCATGAAA-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′; phosphatidylinositol-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′-AATCAAAAAACAATTCA 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′.

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 μl of streptavidin-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) and 44 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 40 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), 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

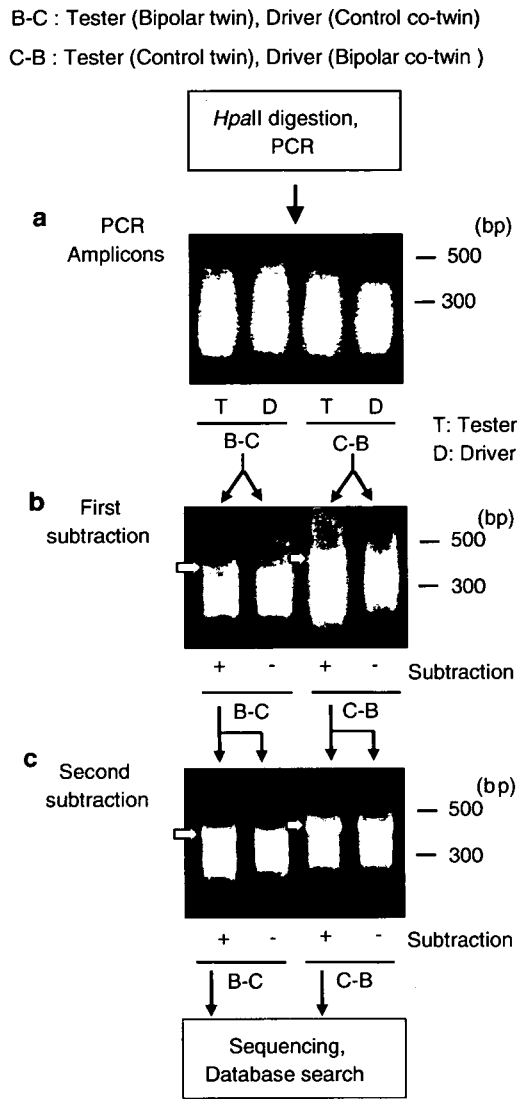


Figure 1 Results of the MS-RDA. (**a**) PCR amplicons. PCR amplicons were prepared from *Hpa*II digests of genomes derived from lymphoblastoid cells of twins. We used bipolar twin genome as a tester genome (T) and control co-twin genome as a driver genome (D) (series B-C; Figure 1a lanes 1 and 2), and 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

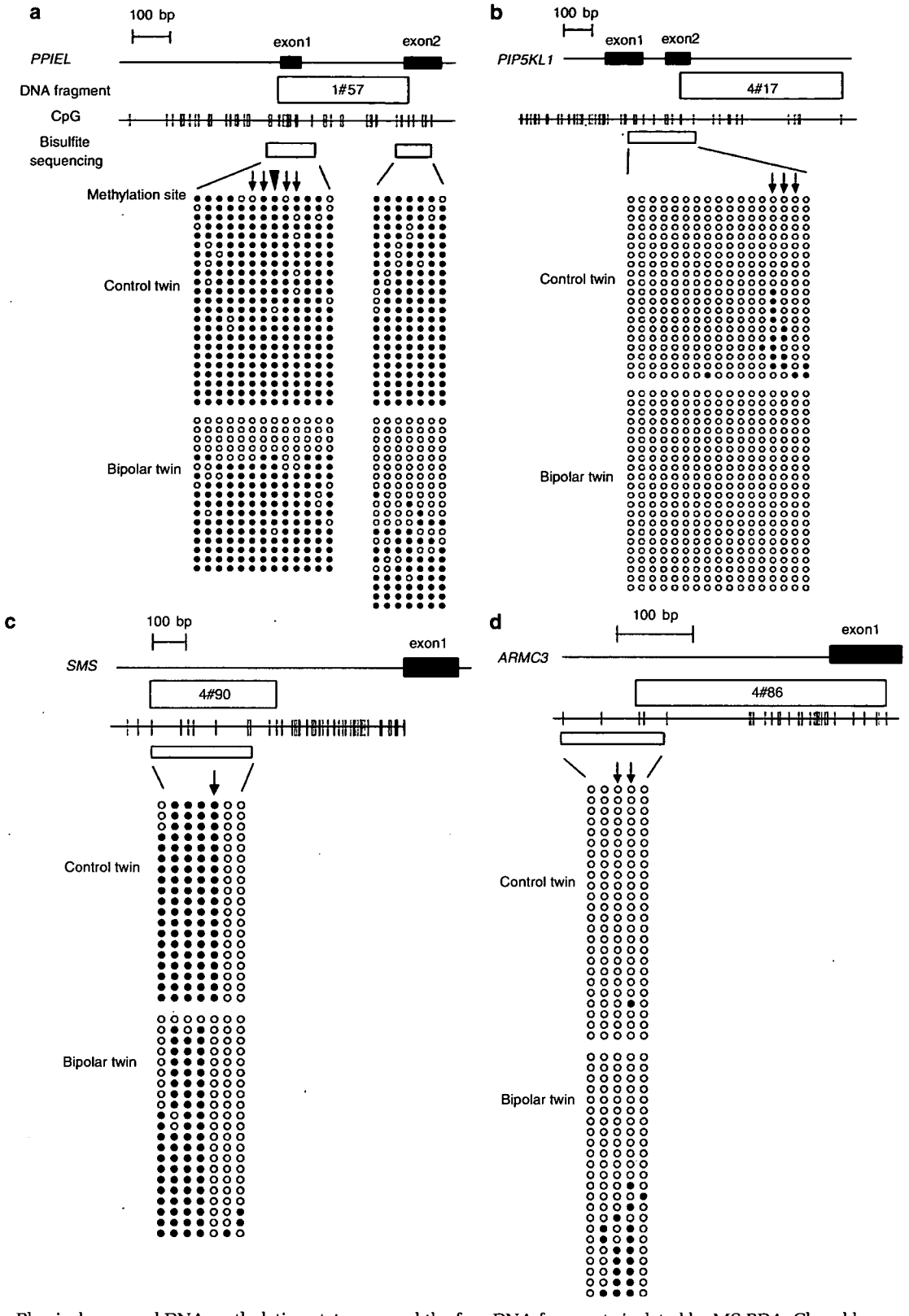


Figure 2 Physical map and DNA methylation status around the four DNA fragments isolated by MS-RDA. Closed boxes represent known exons. Open boxes represent the DNA fragments isolated by MS-RDA. CpG site is represented by tick marks. After bisulfite modification, the regions flanked by the DNA fragments (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.

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 ($58.3\pm11.5\%$; mean$\pm$s.d.) was significantly higher than that of six control females ($35.4\pm23.7\%$, $P=0.03$) (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 ($51.8\pm9.1\%$) than that in controls ($70.2\pm10.3\%$, $P=0.001$) (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*, $1.3\times10^{-3}\pm4.3\times10^{-4}$; *PPIEL/CFL1*, $8.8\times10^{-3}\pm2.5\times10^{-3}$) than that in controls (*PPIEL/GAPDH*, $6.9\times10^{-4}\pm3.6\times10^{-4}$; *PPIEL/CFL1*, $4.6\times10^{-3}\pm2.6\times10^{-3}$). 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*

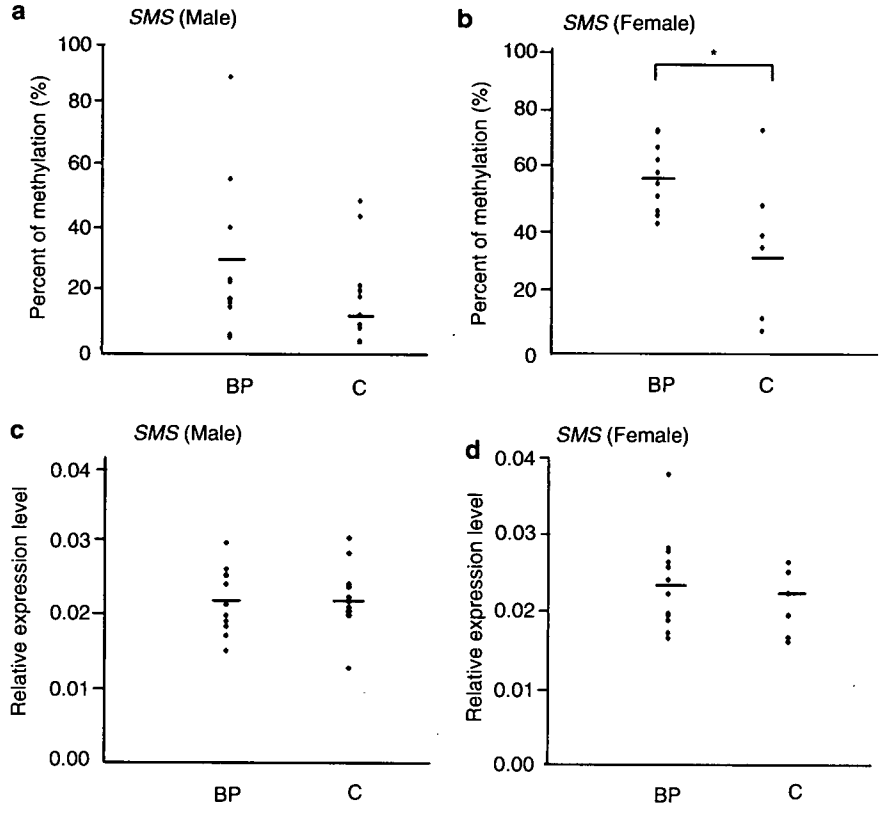
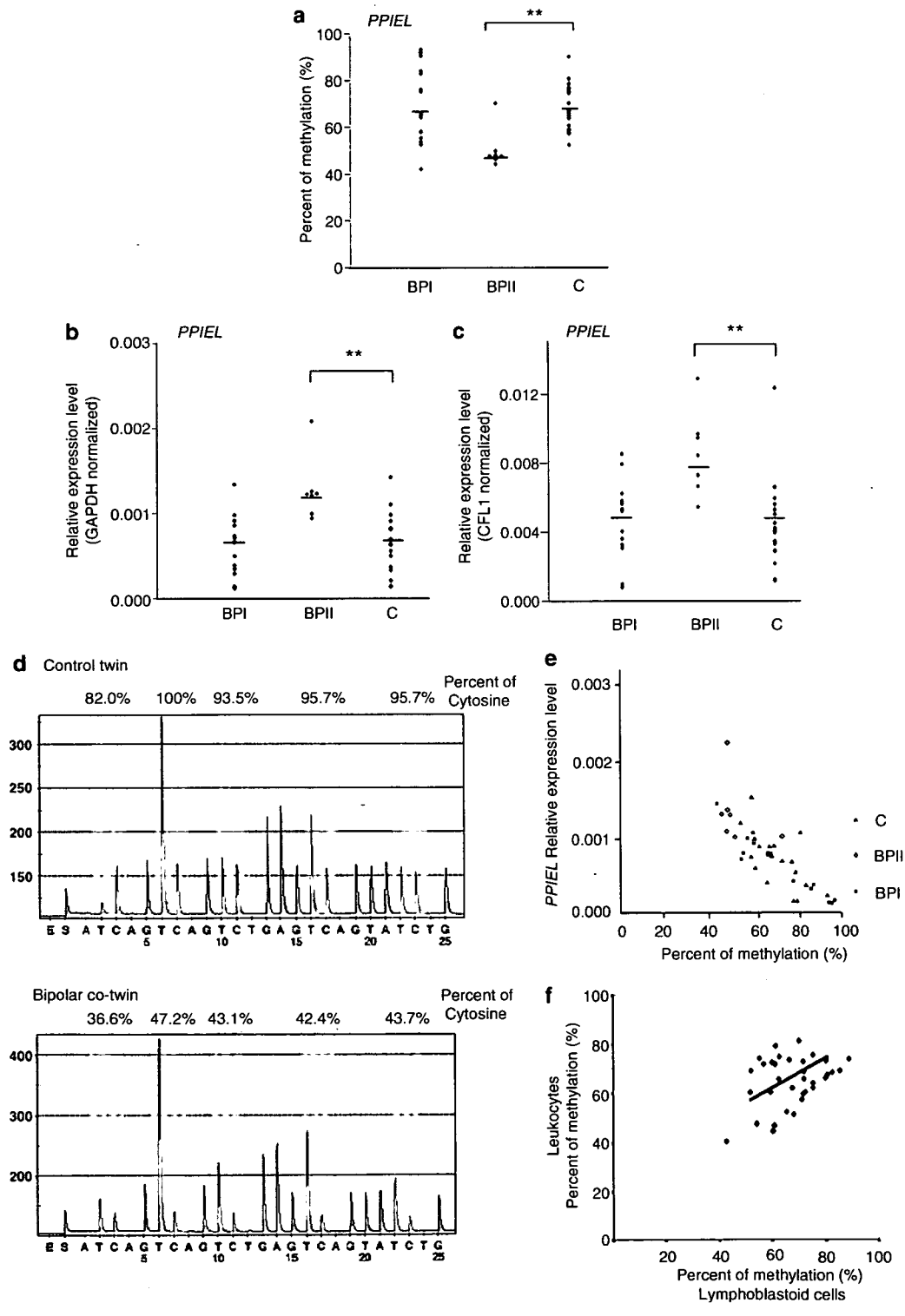


Figure 3 DNA methylation status and expression level of *SMS*. (**a**) DNA methylation status of *SMS* in males. Eleven male patients with bipolar disorder and 12 control male subjects were analyzed by pyrosequencing. Mean ± s.d. were 27.4 ± 26.0% for male patients with bipolar disorder and 15.0 ± 16.5% 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 ($5.2\times10^{-4}\pm1.3\times10^{-4}$)



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

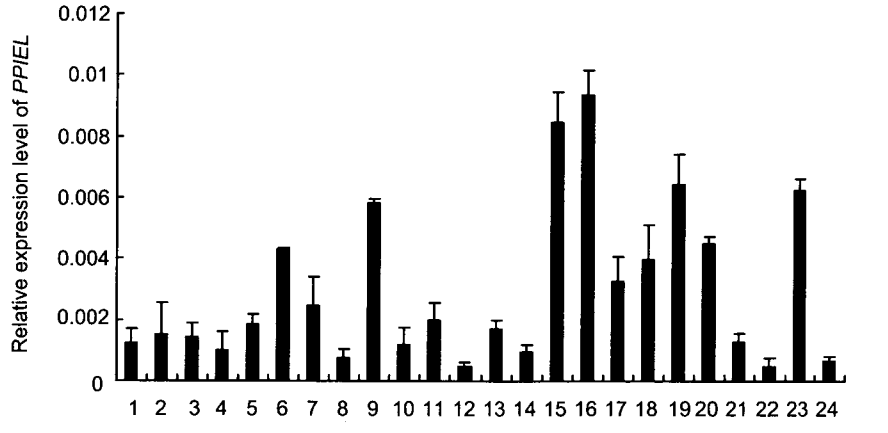


Figure 5 Expression level of *PPIEL* in human brain tissue. Expression level of *PPIEL* in human brain tissue was analyzed by quantitative RT-PCR. For cDNA samples derived from human brain tissue, Human Brain Parts Tissue Scan Real-Time (ORIGENE) was used. Lane 1, frontal lobe; lane 2, temporal lobe; 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

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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Population-Based Database of Multiples in Childhood of Ishikawa Prefecture, Japan

Syuichi Ooki

Department of Health Science, Ishikawa Prefectural Nursing University, Ishikawa, Japan

A new type of population-based database of multiples in childhood at the prefecture level was initiated in 2004 in Ishikawa Prefecture, Japan. We conducted an exhaustive search for demographic information concerning families with multiples, family support provided by governmental and medical institutions by mailed questionnaire, and at the same time tried to organize a human network to support such families. This registry aims not only to aid research on human genetics and maternal and child health, but also to contribute to the development of welfare programs for families with multiples.

The rate of multiple births has been increasing in Japan, as in other developed countries, since 1975 (Imaizumi & Nonaka, 1997). Currently more than 1% of all births are multiples. Therefore, there is an increasing need for appropriate information to be provided to parents and nursing staff on the growth and development of multiples, tips on childbearing, and social resources for families. However, little information is available, in part because the absence of a population-based registry of multiples makes it difficult to gather growth data on multiples, especially after birth (Ooki, 2006; Ooki & Asaka, 2005; Ooki & Yokoyama, 2004).

Studies on maternal and child health for families with multiples have a very short history. The majority of twin registries throughout the world seem to have been constructed primarily for genetic studies, as can be seen in the special issue of the *Twin Research* published in 2002, though several of these registries (Derom et al., 2002; Glinianaia et al., 2002; Strassberg et al., 2002) focus on maternal and child health for families with multiples. It appears to be very difficult to achieve a high participation rate from families with very young children in Japan, particularly if researchers perform only a genetic twin study with no feedback for the participants. The nurturing of multiples entails a higher burden physically, mentally and economically than that of singletons, and participants in twin studies surely expect appropriate information from twin researchers to facilitate the healthy growth of their twins. Offering information useful for the nurturing of multiples would be a strong incentive for the parents to participate in such studies.

Given this background, the construction of a population-based database of multiples in childhood at the prefectural level began in 2004. The goals of the registry are to contribute to the development of welfare programs for families with multiples as well as to co-ordinate research useful for both human genetics and maternal and child health.

Strategies for the Collection of Data on Multiples in Japan

There are four main types of data included in studies on multiples in Japan. First, vital statistics can be obtained without access to personal information concerning individuals (Imaizumi & Nonaka, 1997; Kato, 2004; Minakami et al., 1999). Second, data from large hospitals have been used in the field of obstetrics, primarily for the purpose of managing high-risk pregnancies. The collection of obstetric data on multiples is relatively easy with the trade-off of selection bias in favor of high-risk infants. Third, there is a volunteer-based database of multiples, which includes, for example, data from mothers belonging to associations for parents of multiples; it covers the gap between vital statistics and hospital data (Ooki & Asaka, 2005). It contains more detailed information on the condition of multiples after birth; both vital statistics and hospital data have difficulty addressing this. Fourth, the Basic Resident Registration of municipalities in Japan, which is open to public reading, can be used. This registration reflects the whole population of each area, provides family-based information on name, sex and birth date, and serves as a possible source for complete recruitment for twin research. Nevertheless, this method has certain weaknesses. Some municipalities demand very high fees for using the registration. For this reason, considerable funding is necessary for research. Additionally, electronic data are not available, requiring arduous transcription by

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Address for correspondence: Syuichi Ooki, Department of Health Science, Ishikawa Prefectural Nursing University, Tsu7-1 Nakanuma, Kahoku, Ishikawa, 929-1212, Japan. E-mail: sooki@kj8.so-net.ne.jp

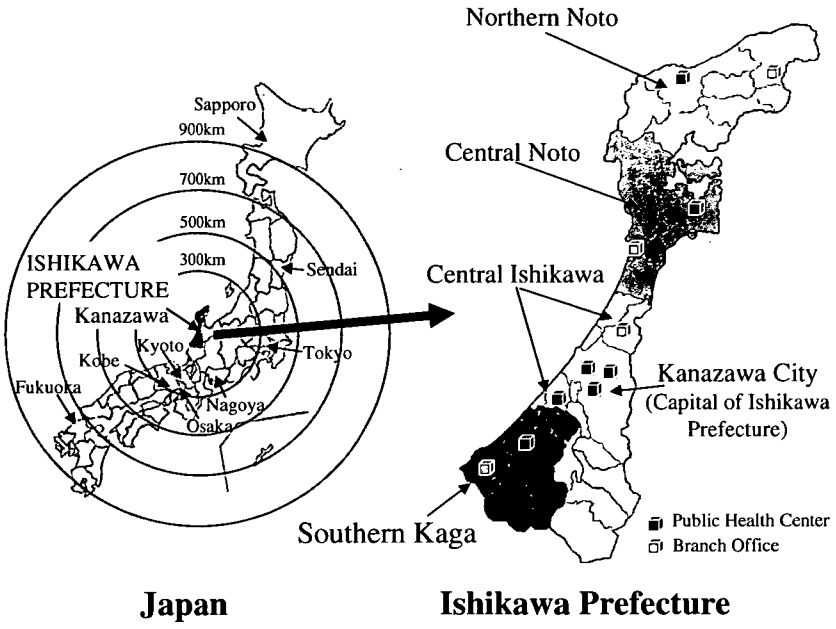

Figure 1
Position of Ishikawa Prefecture in Japan and its five districts.

hand. Moreover, some municipalities have recently begun restricting the availability of the registration data in keeping with new policies to ensure the protection of personal information.

These strategies each have their own strengths and limitations. Considering these, it was decided to construct a population-based registry of multiples.

Ishikawa Prefecture

Ishikawa Prefecture is located in the middle of the Hokuriku region of Honshu Island in Japan, as shown in Figure 1. The prefecture is long and narrow, from the south-west to the north-east. This area was governed by a very strong lord in the feudal age. This former feudalism may be reflected in attitudes toward patriarchy, sterility, child nurturing and multiple births in some districts. Kanazawa city, the prefectural capital, is now the center of the Hokuriku area, whereas Noto area is less-populated and accessibility is not good.

When this project was initiated in 2004, the total number of municipalities in this prefecture was 39 (nine cities, including Kanazawa city, 24 towns and six villages). The population is currently approximately 1,170,000, which is about 1% of the total Japanese population, and Kanazawa City has a population of approximately 450,000 (about 40%). Both the total population and birth rate of this prefecture have been gradually decreasing, while the percentage of people over 65 years of age has been increasing and is now about 20%. The birthrates of this prefecture over the past 10 years have been slightly lower than those of Japan as a whole.

Maternal and Child Health Administration in Japan

Japan consists of 47 prefectures, the basic unit of local government, and about 1800 municipalities (cities, towns and villages). National government policies for the health of mothers and children are planned and administered by the Maternal and Child Health Division of the Ministry of Health, Labor and Welfare. This division sets goals related to maternal and child health policies, performs research projects, eliminates regional differences in maternal and child health levels, and provides technical assistance to local public organizations and other associations (Maternal and Child Health in Japan 2004; Mothers' and Children's Health and Welfare Association, 2004).

Public health centers were operating in approximately 535 locations in 2006; additionally, the government offices of cities, towns and villages operate other municipal public health centers. These health centers administer independent policies together with the policies and administrative functions delegated or transferred by the Ministry of Health, Labor and Welfare. At present, most of the functions of Maternal and Child Health administration have been transferred from the prefectural level to the municipal level. Typically, a single prefecture has several public health centers, which serve several municipalities within their catchment area. Public health centers establish communication and coordination between municipalities with respect to maternal and child health projects in cities, towns and villages, give guidance and advice to municipalities on technical matters, and provide expert maternal and child health services. Each public health

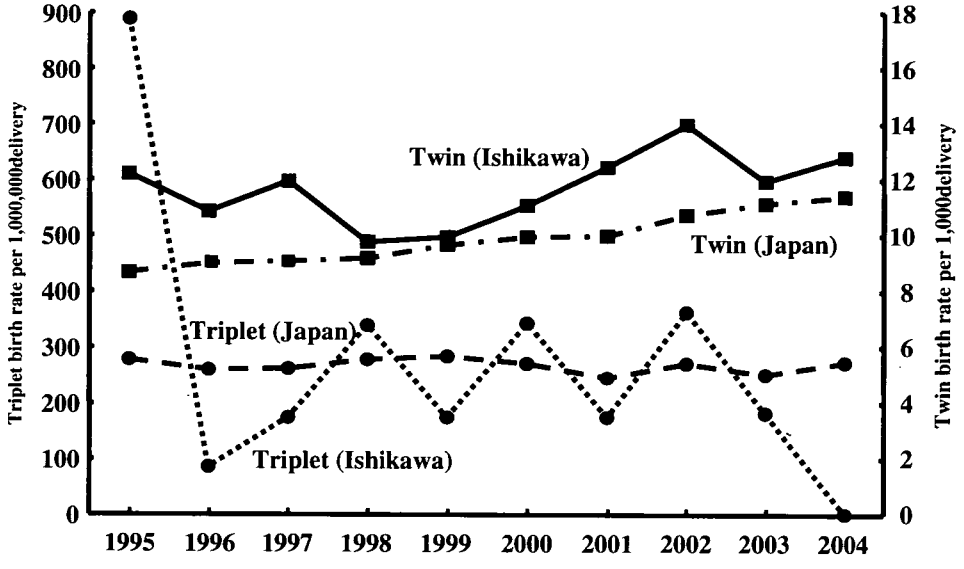

Figure 2

Secular trends of multiple birth rate of Ishikawa Prefecture based on the Japanese vital statistics from the Ministry of Health, Labor and Welfare.

center may also have a local public health center, the branch office, if necessary. In other cases, the city defined by law as the 'core city' of the prefecture may also have its own public health center. As shown in Figure 1, Ishikawa Prefecture has four prefectural public health centers, each with a branch office, and Kanazawa City, the core city, has three public health centers.

Vital Statistics About the Multiple Birthrate in Ishikawa Prefecture

To obtain an initial outline of total multiple births, we analyzed secular trends of multiple births in Ishikawa Prefecture based on the Japanese vital statistics from the Ministry of Health, Labor and Welfare. The multiple birthrate was defined as the number of deliveries divided by the total number of births, including stillbirth (Imaizumi & Nonaka, 1997).

Secular trends in the multiple birthrate of Ishikawa Prefecture between 1995 and 2004 based on the Japanese vital statistics are shown in Figure 2, which compares the statistics of Ishikawa Prefecture with those of Japan as a whole. The twin birthrate of this prefecture is consistently higher than that of Japan as a whole, appearing five times in the last 10 years among the 10 prefectures showing the highest twinning rates. The number of multiple births is between 116 and 159 deliveries each year after 1995. This number makes exhaustive identification of newborn multiples and a construction of the population-based registry possible.

Social Support by Governmental and Medical Institutions in Ishikawa Prefecture

Sources of support for families with multiples and information provided as support by governmental and medical institutions were compiled exhaustively from a mailed questionnaire in June 2004. Recipients were all public health centers in the prefecture, a municipal public health center, and obstetric and pediatric hospitals and clinics in Ishikawa Prefecture. According to the Japanese Medical Service Law, a medical institution with 20 or more beds is defined as a hospital (a medical center), and others are defined as a clinic. The number of surveyed institutions totaled 417, consisting of 49 governmental and 368 medical institutions. The number of support associations available to the parents of multiples in this prefecture was ascertained through governmental and medical institutions as well as through personally obtained information, and their activity was investigated.

The results of the questionnaire on support for families with multiples are summarized in Table 1. The response rate was greater than 90% with respect to governmental institutions of health centers, and around 60 to 70% with respect to medical institutions, with the exception of a very low response rate concerning pediatric clinics. This response rate in itself seemed to reflect the interest in or relationship with the multiples shown by the institutions in question. The primary obstetric and pediatric hospital in this prefecture answered the questionnaire; however, these results must be interpreted carefully since the response rate of medical institutions varied and was much lower than that of governmental institutions. Moreover, the results are summarized irrespective of the catchment area, so do not necessarily accurately reflect the imbalance of the multiple birthrate according to the area. Certainly several municipalities have no or very few families with multiples. No deliveries

Table 1

Social Support Provided by Governmental and Medical Institutions for Families with Multiples in Ishikawa Prefecture

Governmental institution			Medical institution				
	City/town health center	Prefectural health center		NICU	Obstetric hospital	Obstetric clinic	Pediatric hospital	Pediatric clinic
Response rate	92% (35/38)	91% (10/11)		65% (11/17)	71% (20/28)	58% (32/55)	58% (25/43)	34% (77/225)
			Relationship with multiples	100% (11/11)	90% (18/20)	25% (8/32)	42% (10/24)	34% (26/77)
Specialized family visit for families with multiples	3% (1/35)	33% (3/9)						
Specialized parenting class for families with multiples or expectant mothers of multiples	3% (1/35)	33% (3/9)	Specialized parenting class for families with multiples or expectant mothers of multiples		12% (2/17)	14% (1/7)		
			Advice or support for the multiple birth delivery or child bearing	73% (8/11)	85% (17/20)	27% (7/26)	25% (6/24)	11% (8/76)
Advice or support for associations for mothers of multiples	9% (3/35)	11% (1/9)	Advice or support for associations for mothers of multiples	75% (6/8)	59% (10/17)	57% (4/7)	50% (3/6)	0% (0/8)
			Access to the governmental public health nurses regarding multiples	63% (5/8)	29% (5/17)	57% (4/7)	50% (3/6)	25% (2/8)

Note: NICU = Neonatal intensive care unit.

of multiples are handled at local or small obstetric hospitals or clinics.

The three most important problem areas according to governmental and medical staff in supporting families with multiples were the following (combined results from both governmental and medical institutions; multiple answers permitted): the lack of knowledge on multiples among the professional supporters themselves (28% = 36/128), the lack of information on the families with multiples or on the multiple birth itself (20% = 26/128), and insufficient social resources available to families with multiples (20% = 25/128).

The number of multiple births in Ishikawa Prefecture, according to the five public health center catchment areas, are shown in Table 2. Multiple-birth deliveries were highly concentrated in the district at several higher-level medical institutions, that is, those that have a neonatal intensive care unit and deal with high-risk pregnancies, as expected. The medical institution handling approximately 75% of the multiple pregnancies of every year was specified, assuming that all pregnant women with a residential certificate in Ishikawa Prefecture use the medical institution of the prefecture.

There are 11 associations for parents of multiples, six in Kanazawa, three in Central Ishikawa, and two in Kaga, which vary in the size of their membership from about 10 to 200 families. The families of approximately 30% to 40% of multiples under 6 years of age are estimated to belong to some kind of association.

The present findings and outline of social support organizations available to families with multiples in Ishikawa Prefecture were important in the development of a closely focused case report including a detailed interview about, among other topics, parenting classes specialized for families with multiples or expectant mothers of multiples, and in the construction of an effective human network.

Construction of the Human Network

We tried to organize a human network to support families with multiples alongside the demographic research and questionnaire survey. Our network was constructed with the help of the relationships between families with multiples, support groups for child rearing, governmental and medical institutions, and research institute such as the prefectural university. The Health and Welfare Bureau of Ishikawa Prefecture provided assistance in a positive way: several intensive meetings were held for the purpose of exchanging information between members of associations for the parents of multiples, the medical staff of large hospitals, public health nurses, midwives and twin researchers. Workshops and other events were also held periodically, about two or three times per year, in both the local and central districts of Ishikawa Prefecture. The workshop program included professional lectures on multiples and meetings where the parents of multiples could meet with each other and exchange experiences. We vigorously presented information to the mass media, including television stations, newspaper publishing companies, and local bulletins and newsletters. The local mass media was found to be quite effective in advertising our program. Moreover, past research results on multiples were rewritten so as to be easily understood, and were provided in a brochure presented to participants in the workshop, family support events, and so forth.

Table 2

Numbers of Multiple Births Reported by Medical Institutions According to the Five Districts of Ishikawa Prefecture

District of the medical institution	1999	2000	2001	2002	2003	Reported number of multiple births
Northern Noto	1	4	4	1	3	13
Central Noto	5	12	6	11	6	40
Kanazawa City	51	47	71	74	63	306
Central Ishikawa	13	13	24	21	27	98
Southern Kaga	10	12	11	10	6	49
Reported total	80	88	116	117	105	506
Vital statistics	116	133	145	159	134	687
Estimated %*	69.0	66.2	80.0	73.6	78.4	73.7

Note: District of the medical institution does not necessarily mean the district of residence of families with multiples.

*Estimated % was calculated by dividing reported total number of multiple births in each year by the total number of multiple births in each year reported by vital statistics.

Finally, the 'Ishikawa Network of Support for Families with Multiples' was founded in July, 2005. This network comprises a wide rage of members, including families with multiples, maternal and child health authorities of the municipalities, and medical and research institutions. Its aims are to hold workshops, family support events and parenting classes specialized for families with multiples, to facilitate the exchange of information and discussion on maternal and child health policies, and to promote research on multiple births. Twin research includes studies in a wide range of fields that contribute to human genetics and maternal and child health.

The leaflet produced by this network is available in every public health center, municipal health center and obstetric and pediatric institution, as well as in other places where it would come to the attention of expectant mothers or parents of multiples. All expectant mothers who have submitted a notification of pregnancy receive a Maternal and Health Handbook, issued by the municipalities of Japan. If a woman receives more than one handbook, this indicates that she is an expectant mother of multiples. It is important to introduce the network to the families of multiples. Public health nurses, who introduce the network during their home visits, accomplish this effectively, especially in rural areas. In some cases, mothers with experience in nurturing multiples also visit maternity hospitals.

Perspective

The current work is the first attempt in Japan to construct a population-based database of multiples at the prefectural level, especially targeted to young children. Our strategies were appropriate for Japanese maternal and child health policies. The population of Ishikawa Prefecture is small compared to that of other major prefectures such as Tokyo, Kanagawa and Osaka. Recently, the number of multiple births in Ishikawa Prefecture has been approximately 100 to 150 every year, which makes an exhaustive study of multiples possible. We focus particularly on research in the field of human genetics, the so-called twin study method, and maternal and child health. In addition, our project stresses the importance of the health and welfare of families with multiples. This strategy is welcomed by the many participants and other involved parties, and seems to be highly cost effective. The project has only just begun; we hope it will become fruitful, allowing our approach to be applied to other small prefectures in Japan.

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