and with the disease allele frequency of 0.001. At loci with a positive LOD score by the GeneChip genotyping, possibly disease-associated haplotypes were constructed using SNP calls.

When SNP information was not informative, microsatellite markers were used for genotyping. Microsatellite markers used were referred to the National Center for Biotechnology Information (NCBI) database. One each of primer pairs for the markers was labeled with FAM, HEX, or NED (Supplementary Table 1), and PCR was performed in a 10 μl mixture containing 5 ng genomic DNA; 0.25 U ExTaq DNA polymerase HS-version (TAKARA Bio Inc., Kyoto, Japan); 200 μM dNTP; 0.5 μM primer; 1× ExTaq buffer on the T1 Thermocycler (Biometra, Goettingen, Germany). PCR products were separated on Genetic Analyzer 3130xl (AppliedBiosystems), and genotyping was carried out using GeneMapper software (AppliedBiosystems). At the regions where the affected individuals have a disease-associated haplotype, two-point LOD score was calculated by MLINK program (included in FASTLINK software version 4.0P) [14].

2.5. Mutation analysis

Some genes located within candidate regions identified by the linkage analysis were selected for further mutation analysis. A few other genes, albeit outside the regions, were also subjected to mutation analysis. Primer pairs for such genes were designed using Primer3-web 0.3.0 (http://frodo.wi.mit.edu/primer3/input.htm), according to their genomic sequences retrieved from the University of California, Santa Cruz (UCSC) Genome Browser Home (http://genome.cse.ucsc.edu/). PCR was carried out in a 15 μl reaction mixture containing 5 ng DNA; 0.25 U ExTaq DNA polymerase HS version; 200 mM dNTP; 0.5 μM each primer; 1× ExTaq buffer on the T1 Thermocycler. PCR products were subjected to direct sequencing, using BigDye Terminator v3.1 Cycle sequencing Kit (AppliedBiosystems) and Genetic Analyzer 3130xl. Electropherograms of sequences were aligned with ATGC software (GENETYX Corp., Tokyo, Japan) to inspect base alterations.

2.6. Search for genomic aberration

To search for copy number change within the candidate loci identified by linkage analysis, we used Affymetrix® Genome-Wide Human SNP Array 5.0 (920,568 probes; Affymetrix). Genomic DNA extracted from white blood cell of proband was processed according to manufacture's protocol. Intensity data from each probes were obtained from Affymetrix® Genotyping Console 3.0 as a CEL files. Unpaired copy number analysis of whole genome was carried out using Partek Genomics Suite (Partek, MO, USA) and regions with copy number change were determined by Hidden Markov Model at default settings.

3. Results

3.1. Linkage and haplotype analyses

The mean SNP call rate was 92.49% in 11 fingernail DNA samples, compared to 98.11% in a blood DNA sample from the proband. Incorrect SNP calls may result in seemingly inconsistent parent–child transmissions, but the call rates obtained are actually enough for further studies. We thus advanced to calculate LOD scores using these data.

The linkage analysis using MERLIN software revealed 18 regions with positive LOD scores (>0.00). Of the 18 regions, 14 with the following conditions were excluded: those without any functional full-length RefSeq genes; those in small size (<200 kb); and those in which some affected members did not have a common haplotype. Consequently, four loci, 5p13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.33–q13.42, remained as possibly linked regions (Figs. 1 and 3).

We then genotyped with microsatellite markers and calculated two-point LOD scores, considering the affected, unaffected, and the unknown family members. We confirmed three of the four candidate loci. They were a 48-Mb region between markers rs1366265 and rs1373965 at 5p13.2–q14.1, a 6-Mb region between rs850819

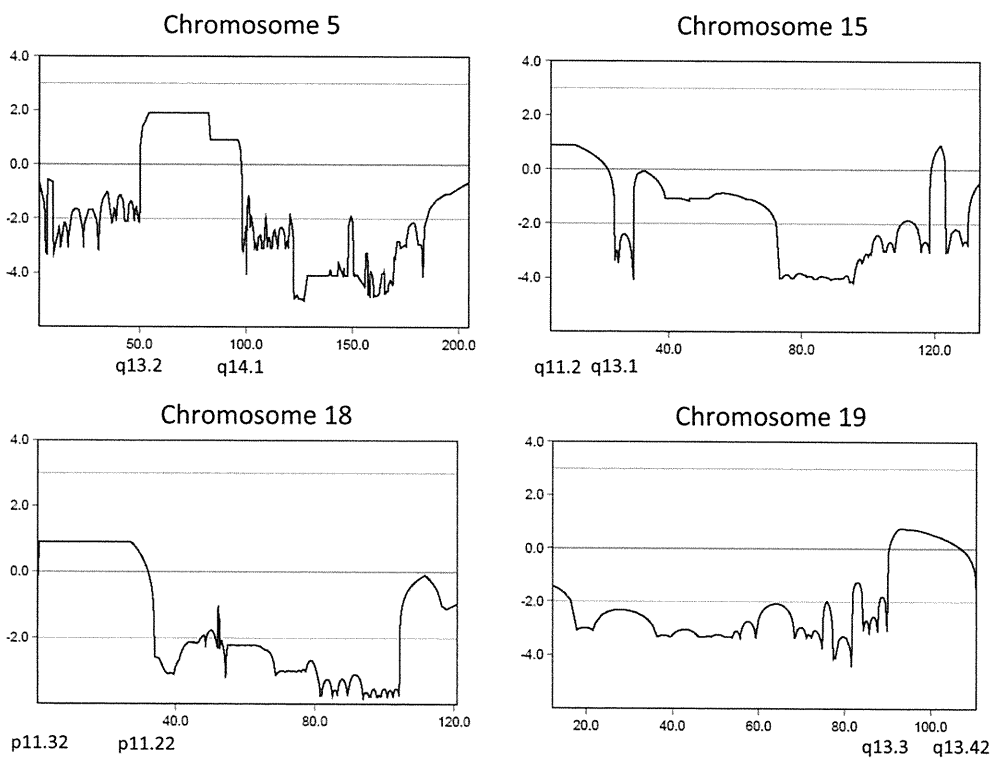

Fig. 3. Multipoint LOD scores calculated by MERLIN in four chromosomal regions, 5q13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.3–q13.42.

and rs818089 at 15q11.2–q13.1, both giving the maximum two-point LOD score of 1.632 ($\theta = 0$), and a 9-Mb region between rs486633 and rs1942150 at 18p11.32–p11.22 with the maximum LOD score of 0.851 ($\theta = 0$) (Table 1). As a possibly disease-associated haplotype on 19q13.33–q13.42 was transmitted to two definitively unaffected individuals (III-6 and IV-1), chromosome 19 was ruled out from the candidacy (Table 1, Fig. 1).

3.2. Mutation analysis of candidate genes

Within the 48-Mb region at 5p13.2–q14.1, there are about 200 RefSeq genes. Ten (*MAP3K1*, *DAB2*, *OCLN*, *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EDFLAM*, *ERBB2IP*, and *PIK3R1*) from these genes were focused and selected as candidates for brain AVM, since they concern development or maintenance of vessels, are associated with other heritable vascular disorders such as HHT, or are expressed in the brain with AVM [8,22,24]. Mutation analyses in these 10 genes revealed no pathologic mutation in the proband, although other affected members were not examined because of insufficient amount of their DNA. Although the genes endoglin isoform 1 precursor (*ENG*), activin A receptor type II like 1 (*ALK1*) and RAS p21 protein activator 1 (*RASA1*) are not located in the candidate region, we investigated whether any of them are involved in the etiology of AVM in the family as a partial symptom of HHT or AVM-CM. Direct sequencing of these three genes failed to show any causative variants.

Copy number analysis of proband revealed one increased copy number loci at 12q and decreased at 2p, 3q, 4q, 6p, 7q and 22q (data not shown). But all these alterations were reported previously as copy number polymorphisms (http://projects.tcag.ca/variation/) and out of our candidate loci. In addition, neither deletions nor microdeletions were detected at 9q34.11 of *ENG*, 12q13.13 of *ALK1* and 5q14.3 of *RASA1*.

4. Discussion

We have reported a family consisting of two affected members with brain AVM, one with pulmonary AVM and one with both brain and pulmonary AVM. The condition in this family met the criteria of familial brain AVM and seems to be inherited in an autosomal dominant mode. We tried to assign the location of a putative disease-gene by linkage analysis and search for mutations by subsequent candidate gene approach.

The linkage analysis of the family revealed three candidate regions (5p13.2–q14.1, 15q11.2–q13.1, and 18p11.32–p11.22) with relatively high LOD scores of 1.632, 1.632 and 0.851, respectively (Table 1). However, neither region was conclusive. This insufficient mapping may have arisen from the small pedigree size, and/or from incomplete ascertainment of affected members, e.g., probable existence of asymptomatic affected persons among the "unknown" members. Indeed, as for a candidate locus at 5p13.2–q14.1, the proband's maternal grandmother (I-2) and son (IV-3) had a haplotype common to the three affected members (Fig. 1), but they were fallen into the "unknown" individuals. If DNA from IV-2 was available and if MRI examinations of VI-3 and I-2 were carried out, we would have obtained more definitive results. As we performed linkage analysis using high-density SNP genotyping, 14 small regions not containing RefSeq genes or miRNAs showed a positive LOD score. It is possible that an unidentified transcribed RNA in one of these regions could cause familial AVM, but these regions are candidate loci with a lower priority than those containing known genes. Thus, the three regions have remained at present as the equally possible loci for AVM. The three regions do not overlap with a previously reported candidate locus of familial brain AVM, i.e., 6p25 [11], and do not contain genes responsible for syndromic AVM (heritable disorders involving AVM) or cerebral cavernous malformations, such as *ENG* [17], *ALK1* [12], *RASA1* [3,6,20,21,26], and *PTEN* [23], *KRIT1* [13], *MGC407* [5], *PDCD10* [2].

We then searched for mutations in 10 genes within 5p13.2–q14.1, among which *MAP3K1*, *DAB2* and *OCLN* encode proteins playing roles in the TGF-β signaling pathway, and *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EGFLAM*, *ERBB2IP* and *PIK3R1* were those expressed in brain AVM tissues by previous microarray analysis [8,22,24]. Nevertheless, no pathologic mutation was found in any of them. Because the presence of both brain AVM and pulmonary AVM in this pedigree is reminiscent of Hereditary Hemorrhagic Telangiectasia, we analyzed *ENG* and *ALK1* for mutations and genomic aberrations, which may cause HHT1 and HHT2 respectively [12,17]. The proband did not have any mutations in the coding exons or intron/exon boundaries of either gene, nor any genomic aberrations at those loci. We also analyzed *RASA1* because this may cause CM-AVM, which is characterized by multiple CM and AVM [3,6,20,21,26]. No causative mutation or genomic aberration was detected in the proband. Although other genes, such as *KRIT1*, *MGC407* and *PDCD10*, have been shown to cause slow-flow lesions i.e., cerebral cavernous malformation [2,5,13], they were not investigated in the present study, because the clinical manifestations in our family did not meet the criteria for these diseases.

Participation of family members and compliance with guidelines for human genome researches are critical to conduct a linkage analysis. Whole-blood samples cannot occasionally be available in some family members because of their far domicile. In such the case, fingernail DNA is useful, since clipped fingernails can be mailed in a usual way, and stored long at a room temperature, as indicated previously [16,19]. The present study is the first experience to adopt fingernail DNA to genome-wide high-density SNP microarray analysis. The performance obtained from fingernail DNA was sufficient, showing all SNP call rates of >86%. According to the manufacture's protocol, samples with an SNP call rate of <85% should further be evaluated before including the data in downstream analysis. Incorrect SNP calls may make serious problems in linkage analysis. For instance, SNPs with parent–child transmission inconsistency may be omitted, leading to a reduced LOD score.

In conclusion, we have assigned the familial AVM locus to three alternative regions, 5p13.2–q13.2, 15q11.2–q13.1 and 18p11.32–p11.22, by a genome-wide, high-density, SNP-based

Table 1
Two-point LOD scores for brain AVM at various loci.

Locus	Recombination fraction (θ)					
	0.00	0.01	0.02	0.03	0.04	0.05
AVM5pl17xAC	0.032	0.030	0.029	0.027	0.026	0.025
D5S418	0.551	0.535	0.518	0.501	0.484	0.467
AVMch5p25xAC	1.334	1.301	1.268	1.234	1.201	1.167
AVM5pr18xGT	0.511	0.491	0.472	0.452	0.433	0.414
AVMch5c27xGT	1.630	1.597	1.564	1.531	1.497	1.463
AVM5c18xAC	1.373	1.344	1.314	1.285	1.255	1.225
D5S407	1.632	1.599	1.566	1.532	1.499	1.465
D5S647	1.154	1.121	1.089	1.056	1.023	0.991
AVM5qr19xCA	0.810	0.790	0.769	0.748	0.727	0.706
D15S1021	0.171	0.164	0.157	0.150	0.143	0.137
D15S128	0.876	0.858	0.841	0.823	0.805	0.787
D15S986	0.812	0.791	0.770	0.749	0.728	0.707
D15S975	0.400	0.387	0.374	0.361	0.348	0.335
D15S1002	1.330	1.298	1.266	1.234	1.202	1.170
D15S1019	1.632	1.599	1.566	1.532	1.499	1.465
D18S59	0.199	0.214	0.225	0.234	0.241	0.246
D18S459	0.142	0.136	0.131	0.125	0.120	0.114
D18S1132	0.677	0.663	0.650	0.636	0.623	0.609
D18S452	0.851	0.832	0.813	0.794	0.774	0.755
D18S471	0.240	0.231	0.222	0.214	0.205	0.197
D19S927	−0.302	−0.277	−0.254	−0.234	−0.216	−0.200
D19S418	−2.655	−2.453	−2.257	−2.078	−1.919	−1.778
D19S605	−0.648	−0.574	−0.512	−0.460	−0.414	−0.374

linkage analysis with fingernail DNA in an AVM family. However, mutation analyses of some genes in the regions failed to identify any pathological changes.

Funding

This research was supported in part by Grants-in-Aid for Scientific Research (Nos. 17019055 and 19390095) from the Ministry of Education, Sports, Culture, Science and Technology of Japan, and by SORST from Japan Science and Technology Agency (JST). K.Y. was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan.

Competing interests

There are no competing interests.

Acknowledgments

We are grateful to the family members for their participation in this research. We also thank Ms Yasuko Noguchi, Ms Miho Ooga, and Ms Chisa Hayashida for their technical assistance.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmg.2010.06.007.

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Failure to Confirm CNVs as of Aetiological Significance in Twin Pairs Discordant for Schizophrenia

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Copy number variations (CNVs) are common structural variations in the human genome that strongly affect genomic diversity and can play a role in the development of several diseases, including neurodevelopmental disorders. Recent reports indicate that monozygotic twins can show differential CNV profiles. We searched CNVs in monozygotic twins discordant for schizophrenia to identify susceptible loci for schizophrenia. Three pairs of monozygotic twins discordant for schizophrenia were subjected to analysis. Genomic DNA samples were extracted from peripheral blood lymphocytes. We adopted the Affymetrix Genome-Wide Human SNP (Single Nucleotide Polymorphism) Array 6.0 to detect copy number discordance using Partek Genomics Suite 6.5 beta. In three twin pairs, however, validations by quantitative PCR and DNA sequencing revealed that none of the regions had any discordance between the three twin pairs. Our results support the hypothesis that epigenetic changes or fluctuation in developmental process triggered by environmental factors mainly contribute to the pathogenesis of schizophrenia. Schizophrenia caused by strong genetics factors including copy number alteration or gene mutation may be a small subset of the clinical population.

Keywords: CNVs, schizophrenia, genotype, monozygotic twin, epigenetic change

Schizophrenia is a chronic, debilitating psychiatric illness with a 1% worldwide prevalence. Genetic studies have shown that schizophrenia has a high heritability, with strong genetic factors involved in its etiology. Twin studies have played an important role in the elucidation of the genetic factors underlying neurodevelopmental disorders. Several twin studies have revealed that the concordance rate between monozygotic twins is 41–79% for schizophrenia, whereas the concordance rate between dizygotic twins is 0–14% (Shih et al., 2004; Kakiuchi et al., 2008). The higher concordance rate in monozygotic rather than dizygotic twins for schizophrenia suggests the contribution of genetic factors. Phenotypically discordant monozygotic twins are especially interesting resources for genetic studies, and twin studies could facilitate the identification of the causative genes of phenotypes. Kondo et al. (2002) reported that a nonsense mutation in *IRF6*, which is a causative gene for Van der Woude syndrome, was found in one affected individual in monozygotic phenotypically discordant twins. In relation to neurodevelopmental disorders, Bruder et al. (2008) reported that discordant monozygotic twins with parkinsonism showed different copy number variation (CNV) profiles.

CNVs are the most prevalent type of structural variations in the human genome that largely contribute to genomic diversity. Redon et al. (2006) and Carter et al. (2007) showed that as much as 12% of the human genome and thousands of genes are variable in copy number. A great number of CNVs may not be pathogenic but simply contribute to human genome diversity not related to phenotype. Meanwhile, some CNVs have been proven a significant factor related to disease susceptibility. Recent studies reported that CNVs contribute to genetic vulnerability factors and can play an important role in the etiology of several neurodevelopmental disorders (Sebat et al., 2007; 2009). Xu et al. (2008) found that de novo copy number mutations were about eight times more frequent in patients with sporadic schizophrenia. Numerous copy number analyses in schizophrenia revealed that genes that were disrupted by CNVs, which include *TBX1*, *ERBB4*, *SLC1A3*, *RAPGEF4*, *CIT*, *NRXN1*, and 16p11.2 region, were candidate genes and regions for schizophrenia (Cook et al., 2008; McCarthy et al., 2009; Merikangas et al., 2009; Walsh et al., 2008); however, most of these are rare copy number variants and the contribution of those genes to schizophrenia is restricted to a tiny part of etiologies.

Received 8 June, 2010; accepted 30 July, 2010.

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To date, numerous causative genes for schizophrenia have been identified; however, because of genetic heterogeneity, there is still a long path to the elucidation of the pathogenesis of schizophrenia. To identify causative genes for schizophrenia, we have utilized the Affymetrix Genome-Wide Human SNP Array 6.0 in three pairs of monozygotic twins discordant for schizophrenia. Here, we describe the results of CNV and genotype profiles in three pairs of monozygotic twins.

Methods

Subjects

Three pairs of monozygotic twins discordant for schizophrenia participated in this study. Ten years had passed after the onset of schizophrenia in the affected individuals in all twin pairs. All of the twins were male, and their mean age was 53 years old. Two well-trained psychiatrists diagnosed the twins by structured clinical interview, and all affected individuals corresponded to the DSM-IV-TR criteria for the undifferentiated type of schizophrenia.

DNA Microarrays

Ten ml of peripheral blood samples was collected after obtaining written informed consent, and genomic DNA was extracted from blood lymphocytes using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Experimental procedures were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

DNA microarray experiments were performed using Affymetrix Genome-Wide Human SNP Array 6.0 (SNP Array 6.0) (Affymetrix, Santa Clara, CA, USA). We performed a paired analysis for loss of heterozygosity (LOH) and an unpaired analysis for copy number analysis using control individuals' data. All of the computer analyses were performed using Genotyping Console (Affymetrix) and Genomics Suite version 6.5 beta software (Partek, St. Louis, MO, USA). Genomic copy number data were analyzed with Partek Genomics Suite software using a segmentation algorithm with stringent p value cutoff.

Quantification of Genome Copy Number

We performed real-time quantitative PCR using an intercalating dye, SYTO13 (Molecular Probes, Eugene, OR, USA), which is an alternative to SYBR green I, or using Universal Probe Library (Roche Diagnostics, Mannheim, Germany) to verify copy number changes suggested by the microarray analyses. Primers and probes were designed using the website software Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com). Real-time PCR amplification was run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). All samples were measured in tetraplicates.

DNA Sequencing

To verify the SNP genotypes obtained by SNP Array 6.0, we performed direct sequencing of PCR-amplified genomic DNA fragments including SNPs that showed discordant allele calls in each twin pair. The amplified fragments were directly sequenced after purification with exonuclease I and NTPhosTM Thermolabile Phosphatase (Epicentre, Madison, WI, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit and run on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed using Variant Reporter (Applied Biosystems) and ATGC version 6.0 (Software Development, Tokyo, Japan).

Results

Microarray Analysis Results

Quality control (QC) data obtained from the SNP Array 6.0 are summarized in Table 1. The call rate and contrast QC in SNP Array 6.0 data were > 95% and > 1.50, respectively, for all samples, and both values indicated experiments using the SNP Array 6.0 were done well.

Copy number analysis of microarray data using Partek Genomics Suite showed some deleted or amplified regions in each twin pair (data not shown). Regions with discordant genotyping between twins from microarray data are summarized in Table 2.

Unpaired analysis of 6 individuals in comparison with ethnically-matched normal controls (HapMap-JPT) revealed that an approximately 3 kb region within the *SLC25A37* gene was deleted in two of the three schizophrenia twin pairs, 11A/B and 31A/B. The deleted region (chromosome 8:23460969–23463786) was not registered in the Database of Genomic Variants (http://projects.tcag.ca/variation/).

Quantitative PCR Results

We verified the copy number state by real-time PCR of the regions with discordant copy number, including genes, by paired analysis using SNP Array 6.0. Primers were designed for the middle position of the regions.

Table 1

Summary of Twin Samples and Affymetrix GeneChip Genotyping Results

Samples	Sex	Phenotype	SNP call rate	Contrast QC*
11A / B	Male	Schizophrenia/unaffected	99.444 / 99.516	2.38 / 2.48
21A / B	Male	Schizophrenia/unaffected	98.974 / 99.175	1.88 / 2.22
31A / B	Male	Schizophrenia/unaffected	99.199 / 99.179	2.26 / 1.60

Note: *Contrast QC (Quality Control) is per sample Quality Control test metric for SNP Array 6.0 intensity data. In high-quality data sets, the Contrast QC metric is higher than the 0.4 threshold according to user manual provided by the manufacturer.

Table 2

List of Loss of Heterozygosity Regions Derived from Microarray Data

chr. #	Physical position		Twin #	Validated SNPs	Overlapping genes
	Start	End			
1	4309356	4465925	11A / B	rs7521665, rs4654438	LOC284661
	45006976	45050681	31A / B	rs6676749	BEST4, PLK3, RPS8, SNORD38A, SNORD38B, SNORD46, SNORD55
	170792582	170870563	31A / B	rs2472550	Region overlaps with 70.55% of C1orf9
2	50182138	50311147	31A / B	rs1452762, rs6712119	Contained within NRXN1
	142093343	142097262	31A / B	rs355581	Contained within LRP1B
3	3693732	3821526	31A / B	rs7613060, rs769806	Region overlaps with 4.23% of LRRN1
	123371895	123393318	31A / B	rs1501900	Region overlaps with 37.81% of CASR
4	24093201	24162064	31A / B	rs4697063	Region overlaps with 34.68% of DHX15
	81368193	81418990	11A / B	rs10518238, rs1458046	Region overlaps with 24.07% of FGF5
	101451872	101646851	31A / B	rs3756037	Region overlaps with 57.10% of EMCN
	109080142	109167540	31A / B	rs4395588	Region overlaps with 15.93% of CYP2U1 and 42.51% of HADH
	126258785	126764905	31A / B	rs7660602	FAT4
5	38382422	38389445	11A / B	rs9292705	Contained within EGFLAM
	166816487	166823787	31A / B	rs17068499	Contained within ODZ2
6	35297977	35376388	31A / B	rs3800385	ZNF76, region overlaps with 3.59% of DEF6 and 36.49% of SCUBE3
	119363250	119468737	31A / B	rs6913082	Contained within FAM184A and 74.19% of FAM184A
8	86383578	87077669	31A / B	rs1845891, rs1553015, rs6605618	CA1, CA2, CA3, REXO1L1, REXO1L2P
9	207826	208183	31A / B	rs10964703	Contained within DOCK8
	3900136	3920251	31A / B	rs630219	Contained within GLIS3
	7154039	7156090	31A / B	rs1556100	Contained within KDM4C
	112777053	112781741	31A / B	rs3758281, rs16915618	Contained within LPAR1
10	68497020	68657339	31A / B	rs10822972	Contained within CTNNA3, region overlaps with 21.12% of LRRTM3
	100181485	100219522	31A / B	rs11599112	Region overlaps with 28.02% of HPSE2 and 39.99% of HPS1
11	8896463	9040536	11A / B	rs4929922	C11orf17, region overlaps with 29.17% of SCUBE2
	19449860	19466526	31A / B	rs11820210	Contained within NAV2
12	21894811	21895465	31A / B	rs4148673	Contained within ABCC9
	33716220	36801139	31A / B	rs11052835, rs2387324	ALG10
	38818800	39404433	21A / B	rs7132869	LRRK2, region overlaps with 5.43% of CNTN1
	63692809	63739310	11A / B	rs4964104	Region overlaps with 18.58% of WIF1
	69385261	69392041	31A / B	rs10879183	Contained within PTPRR
	77123022	77139445	31A / B	rs9971904	Region overlaps with 48.10% of NAV3
	120088239	120155175	31A / B	rs25643	Region overlaps with 29.88% of P2RX7 and 34.55% of P2RX4
13	102227169	102252370	31A / B	rs9514058	KDELC1, region overlaps with 11.79% of BIVM
16	13150832	13161027	31A / B	rs4781419	Contained within SHISA9
22	24570234	24607029	31A / B	rs6004793	Contained within MYO18B
	36847351	36893417	31A / B	rs2076116	Contained within PLA2G6

Note: Chr. # means the number of chromosome.

Quantitative PCR was performed for a total of 120 regions. However, we could not reconfirm the differences between twins in all 120 tested regions. In addition, quantitative PCR within the *SLC25A37* gene revealed no loss or gain of the genome in comparison with ethnically matched control individuals.

Sequencing Results

DNA sequencing was performed for a total of 37 regions surrounding SNPs that had shown discordant genotype calls from microarray analysis within twin pairs. We selected one or more SNP(s) called discordant genotype in each LOH region. Sequencing revealed all of the SNPs were concordant between twin pairs (data not shown).

Discussion

In this study, we analyzed genomic alterations, CNVs and genotypes, in three pairs of monozygotic twins discordant for schizophrenia. None of the regions of copy number difference between twins shown by SNP Array 6.0 were reverified by quantitative PCR, and none of the genotype discordance was reverified by sequencing. Additionally, no novel CNVs was detected in the identified CNVs between twins. To our knowledge, this is the first report verifying the data from high-density and high-resolution DNA microarrays by quantitative PCR and DNA sequencing. Our results indicate that genomic alterations including CNVs and gene mutations contribute minimally to etiologies of

schizophrenia. The large genome-wide study by The Wellcome Trust Case Control Consortium (WTCCC) revealed CNVs is not main cause of bipolar disorder, which is one of the neurodevelopmental disorders (WTCCC, 2010). This report have a different concept from our study because our study aimed to find copy number alteration as a single gene disorder, however, WTCCC report could not discover the CNV contributing to the bipolar disorder. Our results may support the hypothesis that epigenetic changes (Roth et al., 2009), which can influence the expression of genes without affecting the DNA sequence, mainly contribute to the pathogenesis of schizophrenia.

SNP Array 6.0 allows us to detect different genotype or copy number neutral LOH regions. In our twin comparison, copy number neutral LOH would indicate segmental uniparental disomy (UPD) in twin pairs. Actually, UPD of the paternal allele at 11p15 in the affected twin caused discordance for hemihypertrophy in monozygotic twins (West et al., 2003). Furthermore, recent studies revealed that UPD was associated with schizophrenia. UPD on chromosome 1 and 5q32-qter in a patient with schizophrenia has been described in 2004 and 2006, respectively (Abecasis et al., 2004; Seal et al., 2006). But no genotype difference between twins was confirmed in this study.

Here, we presented no genomic discordance between twins; hereinafter, we will discuss some speculation about the relation between genetic factors and phenotypic discordance. First, it is possible that mosaicism at specific tissues (i.e., brain) because of postzygotic genomic rearrangements causes discordant phenotypes between monozygotic twins. Although we used DNA samples extracted from peripheral blood cells in this study, mosaic genomic rearrangement could be detected in brain. It is clear that the ideal source for studies of neurodevelopmental disorders is brain tissue. Nonetheless, it is practically impossible to harvest the brain tissue of twins (Kato et al., 2005). Olfactory sensory neurons have recently been shown to be easily accessible neuronal cells that have been useful for studies on schizophrenia (Arnold et al., 2001), enabling the study of neuronal cell character including genotype and copy number state. Second, it is possible that smaller-scale genomic aberrations below detection sensitivity influence the discordant phenotype of monozygotic twins. SNP Array 6.0 is one of the highest resolution platforms commercially available and allows us to identify CNVs much smaller than 10 kb. However, McCarroll et al. (2008) showed that the detection rate using the SNP Array 6.0 sharply diminished for CNVs <4 kb. To increase sensitivity, the use of many more detection probes is needed, and more than one experimental platform should be performed in future studies.

Bruder et al. (2009) successfully detected many copy number changes in peripheral blood using a Bacterial Artificial Chromosome (BAC)-array at mosaic state (~20%) in nine monozygotic twins discordant for parkinsonism. All of the nine pairs had copy number discordancy in their reports. Because their results suggested copy number change could be found in the mosaic state, tissue-specific mosaicism is a possible explanation for psychiatric disorders. We may have overlooked copy number change in a mosaic state in peripheral blood with the use of the SNP Array 6.0 instead of the BAC-array because the SNP Array 6.0 is a powerful tool to identify small regions with copy number change but is not suitable to detect copy number in a mosaic state.

It seems most likely that epigenetic changes between monozygotic twins influence the phenotypic discordance of monozygotic twins. Several recent studies indicate that epigenetic changes contribute to the etiology of schizophrenia. Rett syndrome and Fragile X syndrome are neurodevelopmental disorders caused by a single gene defect and dysregulation of DNA methylation very early in life (Amir et al., 1999; Das et al., 1997). Kaminsky et al. (2009) have shown that differences in DNA methylation profiles increase in monozygotic twins along with aging. Because the onset of schizophrenia is later than Rett syndrome and Fragile X syndrome, it is possible that cumulative epigenetic modifications could be one cause of schizophrenia development. Furthermore, a recent study by Roth et al. (2009) suggested that DNA methylation and histone modification triggered by influence of environmental factors is responsible for the difference in onset age between these disorders. Akbarian et al. (2005) indicated that histone modification contributes to the pathogenesis of prefrontal dysfunction in patients with schizophrenia based on the finding that the level of H3-(methyl)arginine 17 in patients with schizophrenia exceeded control values by 30%. Thus, genome-wide DNA methylation and genome-wide histone modification studies for monozygotic twins discordant for phenotypes may be promising techniques in future twin studies. In fact, Baranzini et al. (2010) reported genomic sequence and epigenetics (methyl-cytosine) analysis of monozygotic twin discordant for multiple sclerosis using next generation sequencer. They could not find reproducible differences between twins, but these comprehensive analyses including genome and epigenome sequence are just started. As Crow (2002) discussed, it is important to analyze the genetic and epigenetic influence to the species-specific characteristics. Comprehensive genetic and epigenetic analysis of discordant monozygotic twins will be advanced using next generation technologies.

In summary, we did not detect genomic alterations including CNVs and gene mutations between twins discordant for phenotype. Our results indicate that schizophrenia caused by genomic alterations may be a small subset of the clinical population and may support the hypothesis that epigenetic mechanisms triggered by the influence of environmental factors are associated with the etiology of schizophrenia. Experimental investigations of epigenetic mechanisms

such as expression analysis, methylation site sequence and histone modification studies using DNA samples extracted from olfactory sensory neurons are needed to identify the differences responsible for discordant phenotypes in future studies.

Acknowledgments

We are grateful to the subjects and their families for their participation in this research. We especially thank Ms Miho Ooga and Ms Chisa Hayashida for their technical assistance. K.Y. was supported partly by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare and partly by grants from the Takeda Scientific Foundation and the Naito Foundation.

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Journal of Human Genetics (2011) 56, 91–93

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SHORT COMMUNICATION

Androgenetic/biparental mosaicism in a girl with Beckwith–Wiedemann syndrome-like and upd(14)pat-like phenotypes

Kazuki Yamazawa[1,5], Kazuhiko Nakabayashi[2], Kentaro Matsuoka[3], Keiko Masubara[1], Kenichiro Hata[2], Reiko Horikawa[4] and Tsutomu Ogata[1]

This report describes androgenetic/biparental mosaicism in a 4-year-old Japanese girl with Beckwith–Wiedemann syndrome (BWS)-like and paternal uniparental disomy 14 (upd(14)pat)-like phenotypes. We performed methylation analysis for 18 differentially methylated regions on various chromosomes, genome-wide microsatellite analysis for a total of 90 loci and expression analysis of *SNRPN* in leukocytes. Consequently, she was found to have an androgenetic 46,XX cell lineage and a normal 46,XX cell lineage, with the frequency of the androgenetic cells being roughly calculated as 91% in leukocytes, 70% in tongue tissues and 79% in tonsil tissues. It is likely that, after a normal fertilization between an ovum and a sperm, the paternally derived pronucleus alone, but not the maternally derived pronucleus, underwent a mitotic division, resulting both in the generation of the androgenetic cell lineage by endoreplication of one blastomere containing a paternally derived pronucleus and in the formation of the normal cell lineage by union of paternally and maternally derived pronuclei. It appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of BWS-like and upd(14)pat-like phenotypes, but not for the occurrence of other imprinting disorders or recessive Mendelian disorders.
Journal of Human Genetics (2011) **56,** 91–93; doi:10.1038/jhg.2010.142; published online 11 November 2010

Keywords: androgenesis; Beckwith–Wiedemann syndrome; mosaicism; upd(14)pat

INTRODUCTION

A pure androgenetic human with paternal uniparental disomy for all chromosomes is incompatible with life because of genomic imprinting.[1,2] However, a human with an androgenetic cell lineage could be viable in the presence of a normal cell lineage. Indeed, an androgenetic cell lineage has been identified in six liveborn individuals with variable phenotypes.[3–7] All the androgenetic cell lineages have a 46,XX karyotype, and this is consistent with the lethality of an androgenetic 46,YY cell lineage.

Here, we report on a girl with androgenetic/biparental mosaicism, and discuss the underlying factors for the phenotypic development.

CASE REPORT

This patient was conceived naturally to non-consanguineous and healthy parents. At 24 weeks gestation, the mother was referred to us because of threatened premature delivery. Ultrasound studies showed Beckwith–Wiedemann syndrome (BWS)-like features,[8] such as macroglossia, organomegaly and umbilical hernia, together with polyhydramnios and placentomegaly. The mother repeatedly received amnioreduction and tocolysis.

She was delivered by an emergency cesarean section because of preterm rupture of membranes at 34 weeks of gestation. Her birth weight was 3730 g (+4.8 s.d. for gestational age), and her length 45.6 cm (+0.7 s.d.). The placenta weighed 1040 g (+7.3 s.d.).[9] She was admitted to a neonatal intensive care unit due to asphyxia. Physical examination confirmed a BWS-like phenotype. Notably, chest roentgenograms delineated mild bell-shaped thorax characteristic of paternal uniparental disomy 14 (upd(14)pat),[10] although coat hanger appearance of the ribs indicative of upd(14)pat was absent (Supplementary Figure 1). She was placed on mechanical ventilation for 2 months, and received tracheostomy, glossectomy and tonsillectomy in her infancy, due to upper airway obstruction. She also had several clinical features occasionally reported in BWS[8] (Supplementary Table 1). Her karyotype was 46,XX in all the 50 lymphocytes analyzed. On the last examination at 4 years of age, she showed postnatal growth failure and severe developmental retardation.

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Received 9 September 2010; revised 18 October 2010; accepted 22 October 2010; published online 11 November 2010

MOLECULAR STUDIES

This study was approved by the Institutional Review Board Committee at the National Center for Child health and Development, and performed after obtaining informed consent.

Methylation analysis

We first performed bisulfite sequencing for the *H19*-DMR (differentially methylated region) and KvDMR1 as a screening of BWS[11,12] and that for the IG-DMR and the *MEG3*-DMR as a screening of upd(14)pat,[10] using leukocyte genomic DNA. Paternally derived clones were predominantly identified for the four DMRs examined (Figure 1a). We next performed combined bisulfite restriction analysis for multiple DMRs, as reported previously.[13] All the autosomal DMRs exhibited markedly skewed methylation patterns consistent with predominance of paternally inherited clones, whereas the *XIST*-DMR on the X chromosome showed a normal methylation pattern (Figure 1a).

Genome-wide microsatellite analysis

Microsatellite analysis was performed for 90 loci with high heterozygosities in the Japanese population.[14] Major peaks consistent with paternal uniparental isodisomy and minor peaks of maternal origin were identified for at least one locus on each chromosome, with the minor peaks of maternal origin being more obvious in tongue and tonsil tissues than in leukocytes (Figure 1b and Supplementary Table 2). There were no loci with three or four peaks indicative of chimerism. The frequency of the androgenetic cells was calculated as 91% in leukocytes, 70% in tongue cells and 79% in tonsil cells, although the estimation apparently was a rough one (for details, see Supplementary Methods).

Expression analysis

We examined *SNRPN* expression, because *SNRPN* showed strong expression in leukocytes (for details, see Supplementary Data). *SNRPN* expression was almost doubled in the leukocytes of this patient (Figure 1c).

DISCUSSION

These results suggest that this patient had an androgenetic 46,XX cell lineage and a normal 46,XX cell lineage. In this regard, both the androgenetic and the biparental cell lineages appear to have derived from a single sperm and a single ovum, because a single haploid genome of paternal origin and that of maternal origin were identified in this patient by genome-wide microsatellite analysis. Thus, it is likely that after a normal fertilization between an ovum and a sperm, the paternally derived pronucleus alone, but not the maternally derived pronucleus, underwent a mitotic division, resulting both in the generation of the androgenetic cell lineage by endoreplication of

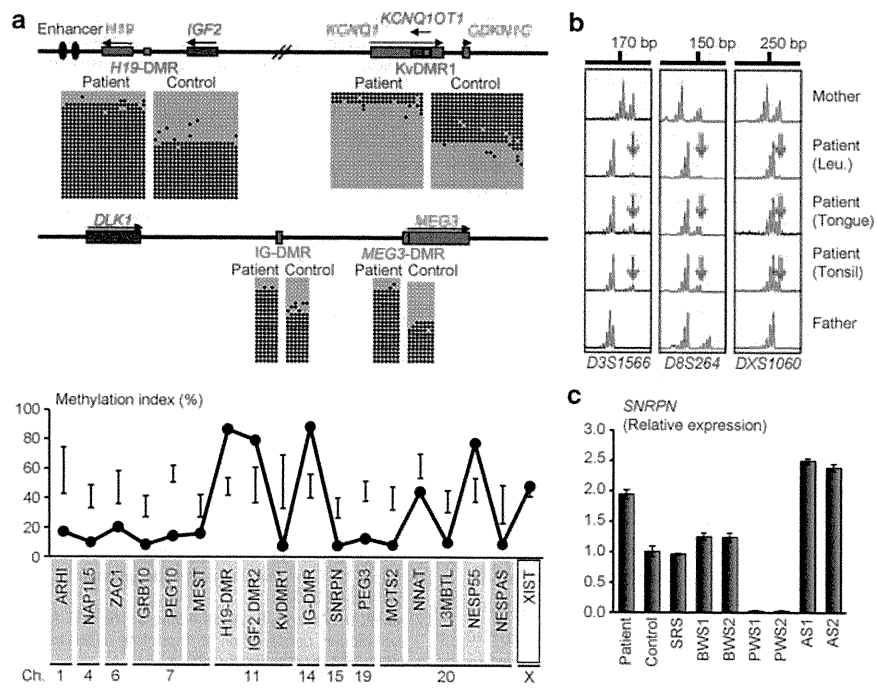

Figure 1 Representative molecular results. (**a**) Methylation analysis. Upper part: Bisulfite sequencing data for the *H19*-DMR and the KvDMR1 on 11p15.5, and those for the IG-DMR and the *MEG3*-DMR on 14q32.2. Each line indicates a single clone, and each circle denotes a CpG dinucleotide; filled and open circles represent methylated and unmethylated cytosines, respectively. Paternally expressed genes are shown in blue, maternally expressed gene in red, and the DMRs in green. The *H19*-DMR, the IG-DMR, and the *MEG3*-DMR are usually methylated after paternal transmission and unmethylated after maternal transmission, whereas the KvDMR1 is usually unmethylated after paternal transmission and methylated after maternal transmission.[10,11] Lower part: Methylation indices (the ratios of methylated clones) obtained from the COBRA analyses for the 18 DMRs. The DMRs highlighted in blue and pink are methylated after paternal and maternal transmissions, respectively. The black vertical bars indicate the reference data (maximum – minimum) in leukocyte genomic DNA of 20 normal control subjects (the *XIST*-DMR data are obtained from 16 control females). (**b**) Representative microsatellite analysis. Major peaks of paternal origin and minor peaks of maternal origin (red arrows) have been identified in this patient. The minor peaks of maternal origin are more obvious in tongue and tonsil tissues than in leukocytes (Leu.). (**c**) Relative expression level (mean ± s.d.) of *SNRPN*. The data are normalized against *TBP*. SRS: an SRS patient with an epimutation (hypomethylation) of the *H19*-DMR; BWS1: a BWS patient with an epimutation (hypermethylation) of the *H19*-DMR; BWS2: a BWS patient with upd(11)pat; PWS1: a Prader-Willi syndrome (PWS) patient with upd(15)mat; PWS2: a PWS patient with an epimutation (hypermethylation) of the *SNRPN*-DMR; AS1: an Angelman syndrome (AS) patient with upd(15)pat; and AS2: an AS patient with an epimutation (hypomethylation) of the *SNRPN*-DMR. The data were obtained using an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

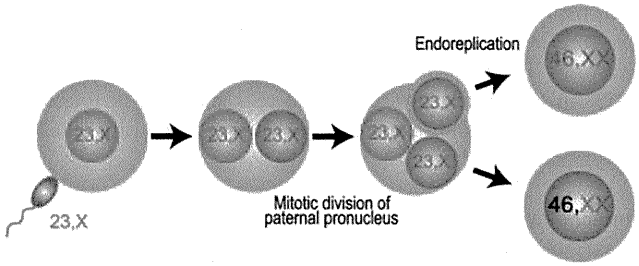

Figure 2 Schematic representation of the generation of the androgenetic/biparental mosaicism. Polar bodies are not shown.

one blastomere containing a paternally derived pronucleus and in the formation of the normal cell lineage by union of paternally and maternally derived pronuclei (Figure 2). This model has been proposed for androgenetic/biparental mosaicism generated after fertilization between a single ovum and a single sperm.[5,15,16] The normal methylation pattern of the *XIST*-DMR is explained by assuming that the two X chromosomes in the androgenetic cell lineage undergo random X-inactivation, as in the normal cell lineage. Furthermore, the results of microsatellite analysis imply that the androgenetic cells were more prevalent in leukocytes than in tongue and tonsil tissues.

A somatic androgenetic cell lineage has been identified in seven liveborn patients including this patient (Supplementary Table 1).[3–7] In this context, leukocytes are preferentially utilized for genetic analyses in human patients, and detailed examinations such as analyses of plural DMRs are necessary to detect an androgenetic cell lineage. Thus, the hitherto identified patients would be limited to those who had androgenetic cells as a predominant cell lineage in leukocytes probably because of a stochastic event and received detailed molecular studies. If so, an androgenetic cell lineage may not be so rare, and could be revealed by detailed analyses as well as examinations of additional tissues in patients with relatively complex phenotypes, as observed in the present patient.

Phenotypic features in androgenetic/biparental mosaicism would be determined by several factors. They include (1) the ratio of two cell lineages in various tissues/organs, (2) the number of imprinted domains relevant to specific features (for example, dysregulation of the imprinted domains on 11p15.5 and 14q32.2 is involved in placentomegaly[9,17]), (3) the degree of clinical effects of dysregulated imprinted domains (an (epi)dominant effect has been assumed for the 11p15.5 imprinted domains[18]), (4) expression levels of imprinted genes in androgenetic cells (although *SNRPN* expression of this patient was consistent with androgenetic cells being predominant in leukocytes, complicated expression patterns have been identified for several imprinted genes in both androgenetic and parthenogenetic fetal mice, probably because of perturbed *cis*- and *trans*-acting regulatory mechanisms[19]) and (5) unmasking of possible paternally inherited recessive mutation(s) in androgenetic cells. Thus, in this patient, it appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of BWS-like and upd(14)pat-like body and placental phenotypes, but remained below the threshold level for the occurrence of other imprinting disorders or recessive Mendelian disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Health, Labor, and Welfare, and the Ministry of Education, Science, Sports and Culture.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

Parthenogenetic chimaerism/mosaicism with a Silver-Russell syndrome-like phenotype

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Received 20 March 2010
Revised 6 May 2010
Accepted 8 May 2010
Published Online First 3 August 2010

ABSTRACT

Introduction We report a 34-year-old Japanese female with a Silver-Russell syndrome (SRS)-like phenotype and a mosaic Turner syndrome karyotype (45,X/46,XX).

Methods/Results Molecular studies including methylation analysis of 17 differentially methylated regions (DMRs) on the autosomes and the *XIST*-DMR on the X chromosome and genome-wide microsatellite analysis for 96 autosomal loci and 30 X chromosomal loci revealed that the 46,XX cell lineage was accompanied by maternal uniparental isodisomy for all chromosomes (upid(AC)mat), whereas the 45,X cell lineage was associated with biparentally derived autosomes and a maternally derived X chromosome. The frequency of the 46,XX upid(AC)mat cells was calculated as 84% in leukocytes, 56% in salivary cells, and 18% in buccal epithelial cells.

Discussion The results imply that a parthenogenetic activation took place around the time of fertilisation of a sperm missing a sex chromosome, resulting in the generation of the upid(AC)mat 46,XX cell lineage by endoreplication of one blastomere containing a female pronucleus and the 45,X cell lineage by union of male and female pronuclei. It is likely that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of SRS phenotype, but not for the occurrence of other imprinting disorders or recessive Mendelian disorders.

Although a mammal with maternal uniparental disomy for all chromosomes (upd(AC)mat) is incompatible with life because of genomic imprinting,[1] a mammal with a upd(AC)mat cell lineage could be viable in the presence of a co-existing normal cell lineage. In the human, Strain *et al*[2] have reported 46,XX peripheral blood cells with maternal uniparental isodisomy for all chromosomes (upid(AC)mat) in a 1.2-year-old phenotypically male patient with aggressive behaviour, hemifacial hypoplasia and normal birth weight. Because of the 46,XX disorders of sex development, detailed molecular studies were performed, revealing the presence of a normal 46,XY cell lineage in a vast majority of skin fibroblasts and a upid(AC)mat 46,XX cell lineage in nearly all blood cells. In addition, although the data are insufficient to draw a definitive conclusion, Horike *et al*[3] have also identified 46,XX peripheral blood cells with possible upd(AC)mat in a phenotypically male patient through methylation analyses for plural differentially methylated regions (DMRs) in 11 patients with Silver–Russell syndrome (SRS)-like phenotype. This patient was found to have a normal 46,XY cell lineage and a triploid 69,XXY cell lineage in skin fibroblasts.

However, such patients with a upd(AC)mat cell lineage remain extremely rare, and there is no report describing a human with such a cell lineage in the absence of a normal cell lineage. Here, we report a female patient with a upid(AC)mat 46,XX cell lineage and a non-upd 45,X cell lineage who was identified through genetic screenings of 103 patients with SRS-like phenotype.

MATERIALS AND METHODS

Case report

This Japanese female patient was conceived naturally and born at 40 weeks of gestation by a normal vaginal delivery. At birth, her length was 44.0 cm (−3.1 SD), her weight 2.1 kg (−2.9 SD) and her occipitofrontal head circumference (OFC) 30.5 cm (−2.3 SD). The parents and the younger brother were clinically normal (the father died from a traffic accident).

At 2 years of age, she was referred to us because of growth failure. Her height was 77.7 cm (−2.5 SD), her weight 8.45 kg (−2.6 SD) and her OFC 43.5 cm (−2.5 SD). Physical examination revealed several SRS-like somatic features such as triangular face, right hemihypoplasia and bilateral fifth finger clinodactyly. She also had developmental retardation, with a developmental quotient of 56. Endocrine studies for short stature were normal as were radiological studies. Cytogenetic analysis using lymphocytes indicated a low-grade mosaic Turner syndrome (TS) karyotype, 45,X[3]/46,XX[47]. Thus, a screening of TS phenotype[4] was performed, detecting horseshoe kidney but no body surface features or cardiovascular lesion. Chromosome analysis was repeated at 6 and 32 years of age using lymphocytes, revealing a 45,X[8]/46,XX[92] karyotype and a 45,X[12]/46,XX[88] karyotype, respectively. On the last examination at 34 years of age, her height was 125.0 cm (−6.2 SD), her weight 37.5 kg (−2.0 SD) and her OFC 51.2 cm (−2.8 SD). She was engaged in a simple work and was able to get on her daily life for herself.

Sample preparation

This study was approved by the Institutional Review Board Committees at National Center for Child health and Development. After obtaining written informed consent, genomic DNA was extracted from leukocytes of the patient, the mother and the brother and from salivary cells, which comprise ~40% of buccal epithelial cells and ~60% of leukocytes,[5] of the patient. Lymphocyte metaphase spreads and leukocyte RNA were also

obtained from the patient. Leukocytes of healthy adults and patients with imprinting disorders were utilised for controls.

Primers and probes

The primers utilised in this study are summarised in supplementary methods and supplementary tables 1–3.

DMR analyses

We first performed bio-combined bisulfite restriction analysis (COBRA)[6] and bisulfite sequencing of the *H19*-DMR (A) on chromosome 11p15.5 by the previously described methods[7] and methylation-sensitive PCR analysis of the *MEST*-DMR (A) on chromosome 7q32.2 by the previously described methods[8] with minor modifications (the methylated and unmethylated allele-specific primers were designed to yield PCR products of different sizes, and the PCR products were visualised on the 2100 Bioanalyzer (Agilent, Santa Clara, California, USA)). This was because hypomethylation (epimutation) of the normally methylated *H19*-DMR of paternal origin and maternal uniparental disomy 7 are known to account for 35–65% and 5–10% of SRS patients, respectively.[9 10] In addition, fluorescence in situ hybridisation (FISH) analysis was performed with a ~84-kb RP5-998N23 probe containing the *H19*-DMR (BACPAC Resources Center, Oakland, California, USA). We also examined multiple other DMRs by bio-COBRA. The ratio of methylated clones (the methylation index) was calculated using peak heights of digested and undigested fragments on the 2100 Bioanalyzer using 2100 expert software.

Genome-wide microsatellite analysis

Microsatellite analysis was performed for 96 autosomal loci and 30 X chromosomal loci. The segment encompassing each locus was PCR-amplified, and the PCR product size was determined on the ABI PRISM 310 autosequencer using GeneScan software (Applied Biosystems, Foster City, California, USA).

PCR analysis for Y chromosomal loci

Standard PCR was performed for six Y chromosomal loci. The PCR products were electrophoresed using the 2100 Bioanalyzer.

Expression analysis

Quantitative real-time reverse transcriptase PCR analysis was performed for three paternally expressed genes (*IGF2*, *SNRPN* and *ZAC1*) and four maternally expressed genes (*H19*, *MEG3*, *PHLDA2* and *CDKN1C*) that are known to be variably (usually weakly) expressed in leukocytes (UniGene, http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene), using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). *TBP* and *GAPDH* were utilised as internal controls.

RESULTS

DMR analyses

In leukocytes, the bio-COBRA indicated severely hypomethylated *H19*-DMR, and bisulfite sequencing combined with *rs2251375* SNP typing for 30 clones revealed maternal origin of 29 hypomethylated clones and non-maternal (paternal) origin of a single methylated clone in this patient (figure 1A). Thus, the marked hypomethylation of the *H19*-DMR was caused by predominance of maternally derived clones rather than hypomethylation of the *H19*-DMR of paternal origin. FISH analysis for 100 lymphocyte metaphase spreads excluded an apparent deletion of the paternally derived *H19*-DMR or duplication of the maternally derived *H19*-DMR (Supplementary figure 1). Methylation-sensitive PCR amplification for the *MEST*-DMR delineated a major peak for the methylated allele and a minor peak for the unmethylated allele (figure 1B). This also indicated the predominance of maternally derived clones and the co-existence of a minor portion of paternally derived clones. Furthermore, autosomal DMRs invariably exhibited markedly abnormal methylation patterns consistent with predominance of maternally inherited DMRs, whereas the methylation index of the *XIST*-DMR on the X chromosome remained within the female reference range (figure 1C). The abnormal methylation patterns were less obvious in salivary cells (thus, in buccal epithelial cells) than in leukocytes, except for the methylation index for the *XIST*-DMR that mildly exceeded the female reference range (figure 1A–C).

Microsatellite analysis

Major peaks consistent with maternal uniparental isodisomy and minor peaks of non-maternal (paternal) origin were identified for at least one locus on each autosome, with the minor peaks of non-maternal origin being more obvious in salivary cells than in leukocytes (figure 1D and supplementary table 4). Furthermore, the frequency of the upid(AC)mat cells was calculated as 84% in leukocytes, 56% in salivary cells and 18% in epithelial buccal cells, using the area under curves for the maternally and the non-maternally inherited peaks (supplementary note). Such minor peaks of non-maternal origin were not detected for all the 30 X chromosomal loci examined.

PCR analysis for Y chromosomal loci

PCR amplification failed to detect any trace of Y chromosome-specific bands in leukocytes and salivary cells (Supplementary figure 2).

Expression analysis

Expression analysis using control leukocytes indicated that, of the seven examined genes, *SNRPN* expression alone was strong enough to allow for a precise assessment (Supplementary figure 3). *SNRPN* expression was extremely low in this patient (figure 1E).

DISCUSSION

These results imply that this patient had a upid(AC)mat 46,XX cell lineage and a non-upd 45,X cell lineage. Indeed, methylation patterns of the *XIST*-DMR is explained by assuming that the two X chromosomes in the upid(AC)mat cells undergo random X-inactivation and that 45,X cells with the methylated *XIST*-DMR on a single active X chromosome[11] are relatively prevalent in buccal epithelial cells. Furthermore, lack of non-maternally derived minor peaks for microsatellite loci on the X chromosome is explained by assuming that the two X chromosomes in the upid(AC)mat cells and the single X chromosome in the 45,X cells are derived from a common X chromosome of maternal origin, with no paternally derived sex chromosome. It is likely, therefore, that a parthenogenetic activation took place around the time of fertilisation of a sperm missing a sex chromosome, resulting in the generation of the 46,XX cell lineage with upid(AC)mat by endoreplication (the replication of DNA without the subsequent completion of mitosis) of one blastomere containing a female pronucleus and the 45,X cell lineage with biparentally derived autosomes and a maternally derived X chromosome by union of male and female pronuclei (figure 2), although it is also possible that a paternally derived sex chromosome was present in the sperm but was lost from the normal

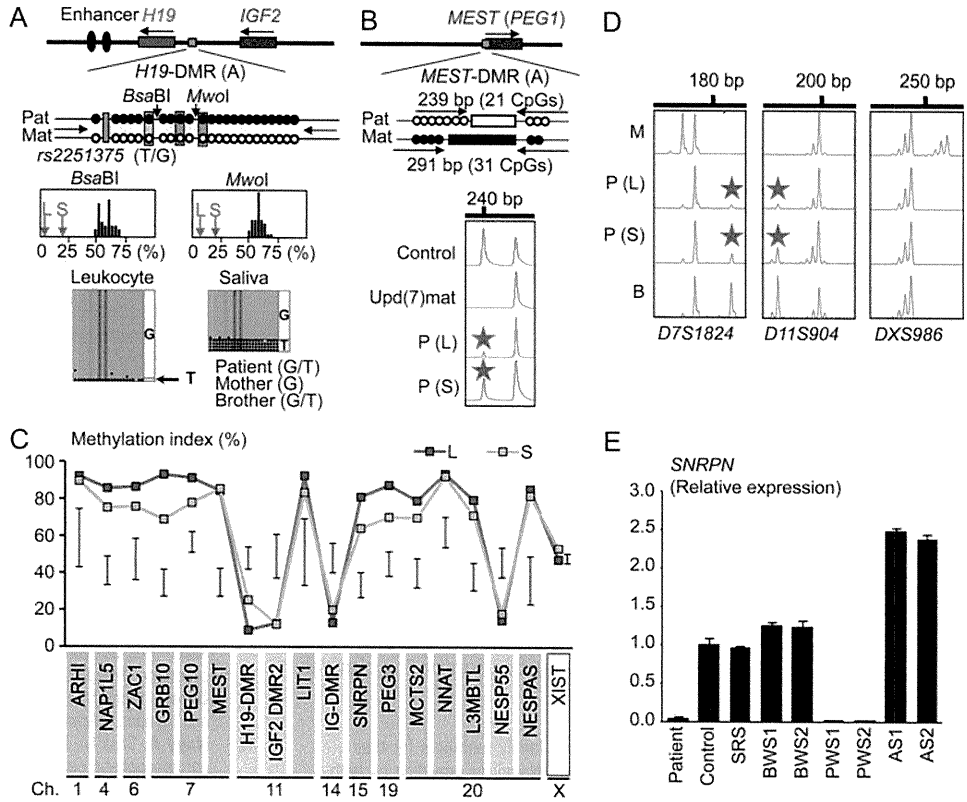

Figure 1 Representative molecular results. Pat, paternally derived allele; Mat, maternally derived allele; P, patient; M, mother; B, brother; L, leukocytes; and S, salivary cells. Filled and open circles in A and B represent methylated and unmethylated cytosine residues at the CpG dinucleotides, respectively. A. Methylation patterns of the *H19*-DMR (A) harbouring 23 CpG dinucleotides and the T/G SNP (*rs2251375*) (a grey box). The PCR products are digested with *Bsa*BI when the cytosine at the sixth CpG dinucleotide (highlighted in yellow) is methylated and with *Mwo*I when the two cytosines at the ninth and the 11th CpG dinucleotides (highlighted in orange) are methylated. For the bio-COBRA data, the black histograms represent the distribution of methylation indices (%) in 50 control participants, and L and S denote the methylation indices for leukocytes and salivary cells of this patient, respectively. For the bisulfite sequencing data, each line indicates a single clone. B. Methylated and unmethylated allele-specific PCR analysis for the *MEST*-DMR (A). In a control participant, the PCR products for methylated and unmethylated alleles are delineated, and the unequal amplification is consistent with a short product being more easily amplified than a long product. In a previously reported patient with upd(7)mat,[8] the methylated allele only is amplified. In this patient, major peaks for the methylated allele and minor peaks for the unmethylated allele (red asterisks) are detected. C. Methylation patterns for the 18 DMRs examined. The DMRs highlighted in blue and pink are methylated after paternal and maternal transmissions, respectively. The black vertical bars indicate the reference data (maximum–minimum) in 20 normal control participants, using leukocyte genomic DNA (for the *XIST*-DMR, 16 female data are shown). D. Representative microsatellite analysis. Minor peaks (red asterisks) have been identified for *D7S1824* and *D11S904* but not for *DXS986* of the patient. Since the peaks for *D7S1824* and *D11S904* are absent in the mother and clearly present in the brother, they are assessed to be of paternal origin. E. Relative expression level (mean ± SD) of *SNRPN* on chromosome 15. The data have been normalised against *TBP*. SRS, an SRS patient with an epimutation (hypomethylation) of the *H19*-DMR; BWS1, a BWS patient with an epimutation (hypermethylation) of the *H19*-DMR; BWS2, a BWS patient with upd(11)pat; PWS1, a PWS patient with upd(15)mat; PWS2, a PWS patient with an epimutation (hypermethylation) of the *SNRPN*-DMR; AS1, an Angelman syndrome (AS) patient with upd(15)pat; and AS2, an AS patient with an epimutation (hypomethylation) of the *SNRPN*-DMR.

cell lineage at the very early developmental stage. Hence, in a strict sense, this patient is neither a chimera resulting from the fusion of two different zygotes nor a mosaic caused by a mitotic error of a single zygote. In this regard, a triploid cell stage is assumed in the generation of a upid(AC)mat cell lineage, and such triploid cells may have been detected in skin fibroblasts of the patient reported by Horike *et al*.[3]

The upid(AC)mat cells accounted for the majority of leukocytes even in adulthood of this patient, despite global negative selective pressure.[12 13] This phenomenon, though intriguing, would not be unexpected in human studies because leukocytes are usually utilised for genetic analyses. Rather, if the upid(AC)mat cells were barely present in leukocytes, they would not have been detected. It is likely, therefore, that upid(AC)mat cells have occupied a relatively large portion of the definitive haematopoietic tissues primarily as a stochastic event. Furthermore, parthenogenetic chimera mouse studies have revealed that parthenogenetic cells are found at a relatively high frequency in some tissues/organs including blood and are barely identified in other tissues/organs such as skeletal muscle and liver.[13] Such a possible tissue-specific selection in favour of the preservation of parthenogenetic cells in the definitive haematopoietic tissues may also be relevant to the predominance of the upid(AC)mat cells in leukocytes. In addition, a reduced growth potential of 45,X cells[14] may also have contributed to the skewed ratio of the two cell lineages.

Clinical features of this patient would be determined by several factors. They include: (1) the ratio of two cell lineages in various tissues/organs, (2) the number of imprinted regions or DMRs relevant to the development of specific imprinting disorders (eg, plural regions/DMRs on chromosomes 7 and 11 for SRS[9 10] and a single region/DMR on chromosome 15 for Prader–Willi syndrome (PWS)),[15] (3) the degree of clinical effects of dysregulated imprinted regions/DMRs (an (epi)dominant effect has been

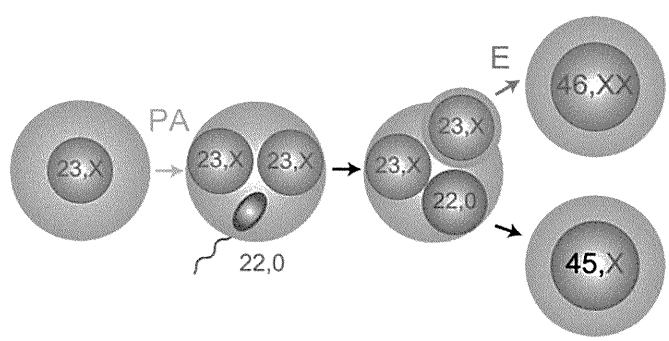

Figure 2 Schematic representation of the generation of the upid(AC)mat 46,XX cell lineage and the non-upd 45,X cell lineage. Polar bodies are not shown. PA, parthenogenetic activation; and E, endoreplication of one blastomere containing a female pronucleus.

assumed for the 11p15.5 imprinted regions including the *IGF2–H19* domain on the basis of SRS or Beckwith–Wiedemann syndrome (BWS) phenotype in patients with multilocus hypomethylation[16] and BWS-like phenotype in patients with a upid (AC)pat cell lineage,[17] a mirror image of a upid(AC)mat cell lineage), (4) expression levels of imprinted genes in upid(AC)mat cells (although *SNRPN* expression of this patient was consistent with upid(AC)mat cells being predominant in leukocytes, complicated expression patterns have been identified for several imprinted genes in androgenetic and parthenogenetic fetal mice, probably because of perturbed *cis*- and *trans*-acting regulatory mechanisms)[18] and (5) unmasking of possible maternally inherited recessive mutation(s) in upid(AC)mat cells.[19] Collectively, it appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of SRS phenotype and horseshoe kidney characteristic of TS[4] but remained below the threshold level for the occurrence of other imprinting disorders or recessive Mendelian disorders.

In summary, we identified a upid(AC)mat 46,XX cell lineage in a woman with an SRS-like phenotype and a 45,X cell lineage accompanied by autosomal haploid sets of biparental origin. This report will facilitate further identification of patients with a upid(AC)mat cell lineage and better clarification of the clinical phenotypes in such patients.

Acknowledgements We thank the patient and her family members for their participation in this study. We also thank Dr. Toshiro Nagai for providing us with blood samples of patients with Prader–Willi syndrome.

Funding This work was supported by grants from the Ministry of Health, Labor, and Welfare and from the Ministry of Education, Science, Sports and Culture.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Review Board Committees at National Center for Child health and Development.

Contributors Drs Kazuki Yamazawa (first author) and Kazuhiko Nakabayashi (second author) contributed equally to this work.

Provenance and peer review Not commissioned; externally peer reviewed.

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産婦人科の実際　第59巻 第12号　(2010年11月1日 発行)

産婦人科の実際

特集　産科医療における遺伝学

産科とエピジェネティクス

秦　健一郎

金原出版株式会社

Vol. 59 No. 12 2010

特集 産科医療における遺伝学

産科とエピジェネティクス

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DNAメチル化をはじめとするエピジェネティックな遺伝子発現制御は，ヒトの発生に必須の機構である。特に，DNAメチル化によって制御されるゲノムインプリンティングは，胎盤と胎児の発生分化に深く関与している。エピジェネティックな制御機構はまた，長期間遺伝子発現の変化が固定されるような現象の分子基盤となりうることも示唆されている。ヒト疾患のエピジェネティクス研究はまだ端緒についたばかりであるが，われわれの研究をはじめ，産科疾患とエピジェネティックな異常の関連を示す解析結果が得られつつあり，今後の展開が待たれる。

はじめに

ヒトゲノムプロジェクトが2003年に終了し，約30億塩基対のゲノム配列標準データが公開され，マイクロアレイによる網羅的発現解析や一塩基多型解析などのハイスループットな解析手法が格段の進歩を遂げた。ヒト疾患の遺伝子解析はもはや，特殊な研究ではなくなった。一方で，「エピジェネティクス」も含め，遺伝子配列が判明しただけでは理解できない生命現象が数多く残されており，ヒト疾患との関連が注目されている。本稿では，産科領域にかかわるエピジェネティクスについての知見を俯瞰するとともに，われわれの行っている研究を簡潔に紹介する。

I. エピジェネティクスの背景

エピジェネティックな生命現象の典型例として，図1にDNAのメチル化を挙げる。DNAを構成する四つの塩基G，C，T，Aのうち，シトシンの5位の炭素にメチル基（CH_3）がくっつくという簡単な化学的修飾により，そのシトシンを含むゲノム領域の機能は原則として抑制される。例えば，ある遺伝子のプロモーター領域が何らかの理由でメチル化されると，その遺伝子の発現が抑制されてしまうことがある（図1の遺伝子A）。しかも，いったんメチル化されると，細胞分裂後も安定して娘細胞に伝達され，遺伝子の発現が抑制され続ける場合がある。このような現象は，点変異や欠失による遺伝子の機能喪失と見分けがつかないが，遺伝子配列に変化を伴わないという点が決定的に異なる。遺伝子配列に変化を伴わないため，その後DNAメチル化が何らかの理由で消失すると，それまで失われていたかにみえていた遺伝子機能が復活する。

上記で示した例は，遺伝子配列の変化を伴わずに遺伝子機能が変化し，しかも細胞分裂を経て安定して受け継がれるため，「遺伝子配列を介在しない遺伝情報」ととらえることができる。このような現象を，従来のgenetics（遺伝学）

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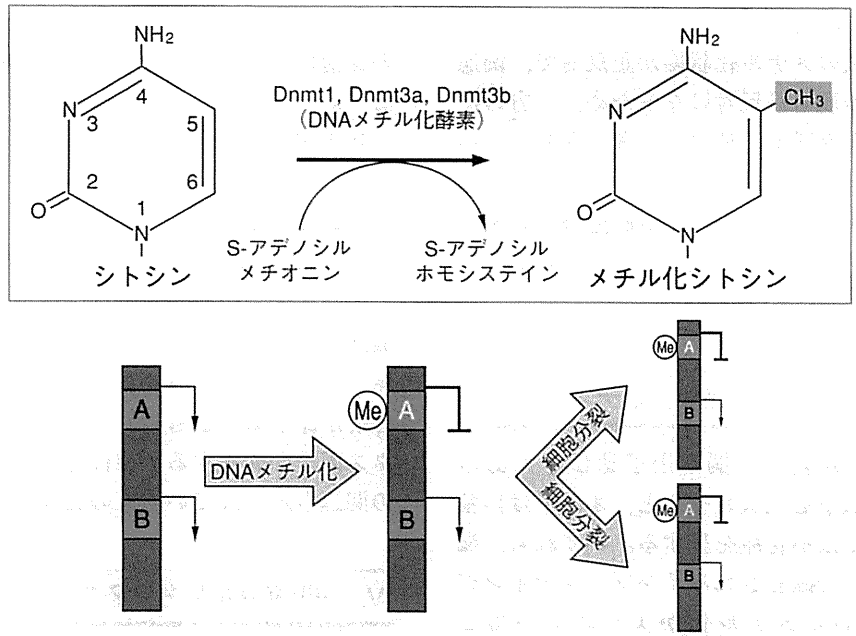

図1　DNAのシトシンのメチル化によるエピジェネティックな遺伝子発現制御

という言葉に対し，epi-という接頭語を付け，epigenetics（エピジェネティクス）と呼ぶ。適切な日本語訳が存在しないため，本稿では以降エピジェネティクスと表記する。

II. エピジェネティクスと個体発生

DNAメチル化は，エピジェネティックな生命現象のなかでも比較的詳細の解明が進んでおり，しかもDNAメチル化によって制御されるゲノムインプリンティング現象は，産科領域と深い関連がある。

経験的に，ヒトが単為発生しないことはよく知られているが，1980年代に行われた一連の巧妙な遺伝学的あるいは発生工学的な実験により，その理由の一端が示された。染色体の一部あるいは全部が片親由来である二倍体胚を作製したところ，正常な発生には，父と母それぞれに由来するゲノム両方が必要であることがわかった[1)2)]。実は，一部の遺伝子群（数百個と推定されている）は，父由来と母由来でDNAメチル化状態が正反対になっており，親の由来を区別することができる。そしてこれらの遺伝子は，通常の遺伝子発現とは異なり，厳密にどちらかの親由来の遺伝子だけが発現する（片親性発現する）。例えば単為発生が起こると，二倍体であってもすべてが母親由来の遺伝子なので，これらの遺伝子の発現量は正常時と比較してゼロまたは二倍と大きく乱れてしまい，単為発生胚は発生異常を呈する。このようなDNAメチル化状態の違いは，親世代の精子と卵子の形成過程で確立され，受精後も生涯不変である（図2の破線）。DNAメチル化によって親の由来情報が「刷り込まれ」ていることから，このような片親性発現をゲノムインプリンティングと呼ぶ。

ゲノムインプリンティングは，胎盤を有する哺乳類に特有のエピジェネティックな現象である。非常に興味深いことに，インプリンティングが破綻すると，多くの場合胎児発育異常を伴う。例えば，インプリンティング異常疾患であるSilver-Russell症候群の一部は，インプリンティング遺伝子*H19*領域の低DNAメチル化状態により発症するが，FGR（IUGR）が必発である。その逆に，Beckwith-Wiedemann症候群の一部は，同じ*H19*領域が高メチル化状態に

なっており，胎児は過形成・過成長を伴う。あるゲノム領域のメチル化状態が正反対で，関連する遺伝子発現も正反対になるため，一方の症候群では発育が悪く，他方は大きくなる，という因果関係が矛盾なく説明できる。ゲノムインプリンティングが，実際にヒト胎児発育に関与していることを示唆する典型的なモデルである。

III. 生殖細胞のエピジェネティクス

DNA メチル化酵素関連因子遺伝子である *Dnmt3L* 遺伝子を欠失させると，オスでは無精子症，メスでは不育症を呈する。いずれも，配偶子形成過程で確立されるインプリンティング遺伝子の DNA メチル化が失われていること[3)4)]，また，少なくともオス生殖細胞では，ある種の反復配列領域の DNA メチル化も失われることが示されている[5)6)]。ゲノムインプリンティングに必要なメチル化が，配偶子形成過程で「刷り込まれない」ために，*Dnmt3L* 遺伝子ホモ変異メスの卵子は一見正常に受精して着床するものの，主に胎盤の形成異常を原因として，すべて妊娠中期に致死となってしまう。

ヒストンのメチル化酵素である *G9a* 遺伝子（DNA は，ヒストンに巻きついて折りたたまれ，高次構造を構築している。ヒストンのアセチル化やメチル化は，高次構造の変化を介して遺伝子発現を制御するため，エピジェネティックな機構の一つと考えられている）を，生殖細胞で働かなくすると，やはりオスでは無精子症を呈し，メスでは著しい成熟生殖細胞の減少をきたす[7)]。

近年，蛋白質に翻訳されずに RNA のまま機能している non-coding RNA 分子（非翻訳 RNA 分子）の存在が注目を集めている。これらの分子は，DNA やヒストンの修飾と協調しながら，エピジェネティックな遺伝子発現制御に関与している。なかでも生殖細胞には，低分子の特殊な non-coding RNA 分子が存在し，レトロトランスポゾン遺伝子の DNA メチル化による抑制に関与していると考えられる。これらの機構に異常のあるマウスは，乏精子症を呈する[8)9)]。

ヒトでも，乏精子症の精子を解析すると，DNA メチル化異常が見つかってくることが報告されている[10)11)]。今のところ，このようなヒト症例で前述の *Dnmt3L* などのエピジェネティクス関連因子遺伝子を調べても，明らかな機能欠失変異は見つからない。今後，生殖細胞発生分化を制御するエピジェネティクス機構の理解が深まるとともに，未知のエピジェネティクス因子が介在する可能性も含め，ヒト症例との関連も示されていくものと期待される。

IV. 初期胚発生におけるエピジェネティクス

主にモデル生物を使った解析から，生殖発生過程における DNA メチル化状態の大まかな変動が明らかになっている。図 2 で示したように，配偶子形成過程と，受精後着床から原腸陥入期にかけて，ゲノム全域にわたり，大きな脱メチル化と再メチル化の波が 2 回観察される。インプリンティング遺伝子の DNA メチル化は，配偶子形成過程においてのみ消去され，自身の性に応じた DNA メチル化状態が構築される（図 2 の破線）。それ以外のゲノム領域の DNA メチル化状態は，受精後から胚盤胞期にかけて全体的に低 DNA メチル化状態となる。エピジェネティックな抑制が外れることで，その後の分化能を獲得していくと考えられている（図 2 の実線）。いわゆる ES 細胞（胚性幹細胞）は，低 DNA メチル化状態である胚盤胞期の内部細胞塊から樹立される。ただし，インプリンティング遺伝子の DNA メチル化状態は，詳細未知の機構によりこの脱メチル化過程を免れる（図 2 の破線）。その後，分化に応じて各細胞系譜・組織に特異的な DNA メチル化状態が獲得され，最終分化後はその状態が維持される（図 2 の実線と波線）。*PGC7/Stella* 遺伝子産物は，卵子に存在し，初期発生に必要な母性因子として知られている。同遺伝子を欠失したマウス卵