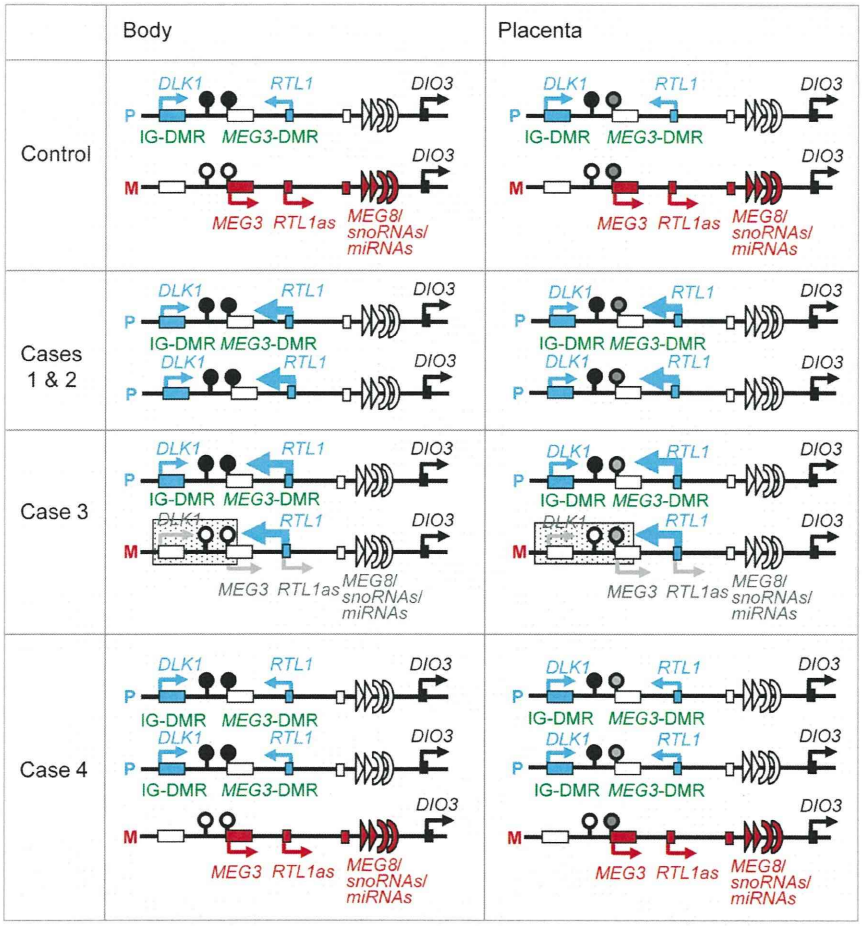

Figure 6. Schematic representation of the chromosome 14q32.2 imprinted region in a control subject, cases 1 and 2 with upd(14)pat, case 3 with a microdeletion (indicated by stippled rectangles), and case 4 with two copies of the imprinted region of paternal origin and a single copy of the imprinted region of maternal origin. This figure has been constructed using the present results and the previous data.[2,3] P, paternally derived chromosome; M, maternally derived chromosome. Filled and open circles represent hypermethylated and hypomethylated DMRs, respectively; since the *MEG3*-DMR is grossly hypomethylated and regarded as non-DMR in the placenta, it is painted in gray. *PEGs* (*DLK1* and *RTL1*) are shown in blue, *MEGs* (*MEG3*, *RTL1as*, *MEG8*, *snoRNAs* and *miRNAs*) in red, a probably non-imprinted gene (*DIO3*) in black, and non-expressed genes in white. Thick arrows for *RTL1* in cases 1–3 represent increased *RTL1* expression that is ascribed to loss of functional microRNA-containing *RTL1as* as a repressor for *RTL1*.

the *MEG3*-DMR, and FISH analyses for the 14q32.2 region were performed as described previously.[2,3] For FISH analysis of 17p13.3, a 17p sub-telomere probe and an RP11–411G7 probe for the 17p13.3 region were utilized, together with a CEP17 probe for the 17p11.1 region utilized as an internal control. The 17p sub-telomere probe was detected according to the manufacture's protocol, the RP11–411G7 probe was labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the CEP17 control probe was labeled with biotin and detected by avidin conjugated to fluorescein isothiocyanate. Quantitative real-time PCR analysis was performed on an ABI PRISM 7000 (Applied Biosystems) using TaqMan real-time PCR probe primer mixture for the following genes (assay No: Hs00171584 for *DLK1*, Hs00292028 for *MEG3*, Hs00419701 for *MEG8* and Hs00704811 for *DIO3*; assay ID: 001028 for *miR433* and 000452 for *miR127*). For *RTL1*, q-PCR analysis was performed with a forward primer hybridized to the sequence of *RTL1* and a reverse primer hybridized to the adaptor sequence. Fifty nanongrams of cDNA in a 50 μl reaction mixture contacting 2× KOD FX buffer (Toyobo), 2.0 mM dNTP mixture (Toyobo), KOD FX (Toyobo), SYBR Green I (Invitrogen), and primer set for *RTL1* were subjected to the ABI PRISM 7000. Data were normalized against *GAPDH* (catalog No: 4326317E) for *DLK1*, *MEG3*, *MEG8*, *RTL1*, and *DIO3*, and against *RNU48* (assay ID: 0010006) for *microRNAs*. The expression studies were performed three times for each sample. Oligoarray CGH was performed using 1× 1M format Human Genome Array (Catalog No G4447A) (Agilent Technologies).

Histopathological analysis. Placental samples were fixed with 20% buffered formaldehyde at room temperature and embedded in paraffin wax according to standard protocols for LM examinations. Then, sections of 3 μm thick were stained with hematoxylin-eosin. For EM examinations, fresh placental tissues were fixed with phosphate-buffered 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon 812 (catalog No. R3245, TAAB). Semithin sections were stained with 1% methylene blue, and ultrathin sections were double-stained with uranyl acetate and lead citrate. Subsequently, they were examined with a Ninhon Denshi JEM-1230 electron microscope.

For IHC analysis, sections of 3 μm thick were prepared by the same methods utilized for the LM examinations, and were examined with rabbit anti human DLK1 polyclonal antibody at 1:100 dilutions (catalog No 10636-1-AP, ProteinTech Group), rabbit anti human RTL1 polyclonal antibody at 1:200 dilutions, and rabbit anti human DIO3 polyclonal antibody at 1:50 dilutions (catalog No ab102926, abcam); anti human RTL1 polyclonal antibody was produced by immunizing rabbits with the synthesized RTL1 peptide (NH2-RGFPRDPSTESG-COOH) in this study. Sections were dewaxed in xylene and rehydrated through graded ethanol series and, subsequently, incubated in 10% citrate buffer (pH 6.0) for 40 min in a 98°C water bath, for antigen retrieval. Endogenous peroxidase activity was quenched with 1% H_2O_2 and 100% methanol for 20 min. To prevent non-specific background staining, sections are incubated with Protein Block Serum-Free (Dako corporation) for 10 min at room temperature. Then, sections were incubated overnight with primary antibody at 4°C

and, subsequently, treated with the labeled polymer prepared by combining amino acid polymers with peroxidase and anti-rabbit polyclonal antibody (Histofine Simple Stain MAX PO MULTI, Nichirei). Peroxidase activities were visualized by diaminobenzidine staining, and the nuclei were stained with hematoxylin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/21937

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ARTICLE

Relative frequency of underlying genetic causes for the development of UPD(14)pat-like phenotype

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Paternal uniparental disomy 14 (UPD(14)pat) results in a unique constellation of clinical features, and a similar phenotypic constellation is also caused by microdeletions involving the *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and/or the *MEG3*-DMR and by epimutations (hypermethylations) affecting the DMRs. However, relative frequency of such underlying genetic causes remains to be clarified, as well as that of underlying mechanisms of UPD(14)pat, that is, trisomy rescue (TR), gamete complementation (GC), monosomy rescue (MR), and post-fertilization mitotic error (PE). To examine this matter, we sequentially performed methylation analysis, microsatellite analysis, fluorescence *in situ* hybridization, and array-based comparative genomic hybridization in 26 patients with UPD(14)pat-like phenotype. Consequently, we identified UPD(14)pat in 17 patients (65.4%), microdeletions of different patterns in 5 patients (19.2%), and epimutations in 4 patients (15.4%). Furthermore, UPD(14)pat was found to be generated through TR or GC in 5 patients (29.4%), MR or PE in 11 patients (64.7%), and PE in 1 patient (5.9%). Advanced maternal age at childbirth (≥35 years) was predominantly observed in the MR/PE subtype. The results imply that the relative frequency of underlying genetic causes for the development of UPD(14)pat-like phenotype is different from that of other imprinting disorders, and that advanced maternal age at childbirth as a predisposing factor for the generation of nullisomic oocytes through non-disjunction at meiosis 1 may be involved in the development of MR-mediated UPD(14)pat.
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Keywords: genetic cause; maternal age effect; monosomy rescue; UPD(14)pat subtype

INTRODUCTION

Human chromosome 14q32.2 carries a ~1.2 Mb imprinted region with the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the post-fertilization-derived secondary *MEG3*-DMR, together with multiple imprinted genes.[1,2] Both DMRs are methylated after paternal transmission and unmethylated after maternal transmission in the body, whereas in the placenta the IG-DMR alone remains as a DMR and the *MEG3*-DMR is rather hypomethylated irrespective of the parental origin.[2,3] Furthermore, it has been shown that the unmethylated IG-DMR and *MEG3*-DMR of maternal origin function as the imprinting centers in the placenta and the body, respectively, and that the IG-DMR acts as an upstream regulator for the methylation pattern of the *MEG3*-DMR in the body but not in the placenta.[3]

As a result of the presence of the imprinted region, paternal uniparental disomy 14 (UPD(14)pat) (OMIM #608149) causes a unique constellation of body and placental phenotypes such as characteristic face, bell-shaped small thorax, abdominal wall defect, polyhydramnios, and placentomegaly.[2,4,5] Furthermore, consistent with the essential role of the DMRs in the imprinting regulation, microdeletions and epimutations affecting the IG-DMR or both DMRs of maternal origin result in UPD(14)pat-like phenotype in both the body and the placenta, whereas a microdeletion involving the maternally inherited *MEG3*-DMR alone leads to UPD(14)pat-like phenotype in the body, but not in the placenta.[2,3]

Of the three underlying genetic causes for UPD(14)pat-like phenotype (UPD(14)pat, microdeletions, and epimutations), UPD(14)pat is primarily generated by four mechanisms, that is, trisomy rescue (TR), gamete complementation (GC), monosomy rescue (MR), and post-fertilization mitotic error (PE).[6] TR refers to a condition in which chromosome 14 of maternal origin is lost from a zygote with trisomy 14 formed by fertilization between a disomic sperm and a normal oocyte. GC results from fertilization of a disomic sperm with a nullisomic oocyte. MR refers to a condition in which chromosome 14 of paternal origin is replicated in a zygote with monosomy 14 formed by fertilization between a normal sperm and a nullisomic oocyte. PE is an event after formation of a normal zygote. In this regard, a nullisomic oocyte specific to GC and MR is produced by non-disjunction at meiosis 1 (M1) or meiosis 2 (M2), and non-disjunction at M1 is known to increase with maternal age, probably because of a long-term (10–50 years) meiotic arrest at prophase 1.[7]

However, relative frequency of the genetic causes for UPD(14)pat-like phenotype remains to be determined, as well as that of underlying mechanisms for the generation of UPD(14)pat. Here, we report our data on this matter, and discuss the difference in the relative frequency

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among imprinted disorders and the possible maternal age effect on the relative frequency.

PATIENTS AND METHODS

Patients

This study comprised 26 patients with UPD(14)pat-like phenotype (9 male patients and 17 female patients) (Table 1). Of the 26 patients, 18 patients have been reported previously; they consisted of nine sporadic patients with full UPD(14)pat,[4,5] one sporadic patient with segmental UPD(14)pat,[4] the proband of sibling cases and four sporadic patients with different patterns of microdeletions involving the unmethylated DMRs of maternal origin,[2,3] and three patients with epimutations (hypermethylations) of the two normally unmethylated DMRs of maternal origin.[2] The remaining eight patients were new sporadic cases.

Phenotypic findings of the 26 patients are summarized in Supplementary Table 1; detailed clinical features of patients 6 and 16–25 are as described previously,[2–4] and those of the eight new patients 3, 5, 10–14, and 26 are shown in Supplementary Table 2, together with those of patients 1, 2, 4, 7–9, and 15 in whom detailed phenotypes were not described in the previous report.[5] All the 26 patients were identified shortly after birth because of the unique bell-shaped thorax with coat-hanger appearance of the ribs on roentgenograms obtained because of asphyxia. Subsequent clinical analysis revealed that 25 of the 26 patients exhibited both body and placental UPD(14)pat-like phenotype, whereas the remaining one previously reported patient (patient 22) manifested body, but not placental, UPD(14)pat-like phenotype.[3] The karyotype was found to be normal in 25 patients, although cytogenetic analysis was not performed in one previously reported patient who died of respiratory failure at 2 h of age (patient 6).[4] One patient (patient 15) was conceived by *in vitro* fertilization-embryo transfer.[5] This study was approved by the Institute Review Board Committee at the National Center for Child Health and Development, and performed after obtaining written informed consent.

Analysis of underlying genetic causes in patients with UPD(14)pat-like phenotype

We sequentially performed methylation analysis, microsatellite analysis, and fluorescence *in situ* hybridization (FISH), using leukocyte genomic DNA samples and lymphocyte metaphase spreads of all the 26 patients with UPD(14)pat-like phenotype. The detailed methods were as reported previously.[2,3] In brief, methylation analysis was performed for the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7 and the CTCF-biding sites C and D) by combined bisulfite restriction analysis and bisulfite sequencing. Microsatellite analysis was performed for multiple loci on chromosome 14, by determining the sizes of PCR products obtained with fluorescently labeled forward primers and unlabeled reverse primers. FISH analysis was carried out for the IG-DMR and the *MEG3*-DMR using 5104-bp and 5182-bp long PCR products, respectively, together with the RP11-566I2 probe for 14q12 utilized as an internal control.

In this study, furthermore, oligonucleotide array-based comparative genomic hybridization (CGH) was also performed for the imprinted region of non-UPD(14)pat patients, using a custom-build oligo-microarray containing 12 600 probes for 14q32.2–q32.3 encompassing the imprinted region and ~10 000 reference probes for other chromosomal region (4×180K format, Design ID 032112) (Agilent Technologies, Palo Alto, CA, USA). The procedure was as described in the manufacturer's instructions.

Analysis of subtypes in patients with UPD(14)pat

UPD(14)pat subtype was determined by microsatellite analysis.[8,9] In brief, heterodisomy for at least one locus was regarded as indicative of TR- or GC-mediated UPD(14)pat (TR/GC subtype), whereas isodisomy for all the informative microsatellite loci was interpreted as indicative of MR- or PE-mediated UPD(14)pat (MR/PE subtype) (for details, see Supplementary Figure S1). Here, while heterodisomy and isodisomy for a pericentromeric region in the TR/GC subtype imply a disomic sperm generation through M1

Table 1 Summary of patients examined in this study

Patient	*Genetic cause*	*UPD(14)pat subtype*	*Maternal age at childbirth (years)*	*Paternal age at childbirth (years)*	*Remark*	*Reference*
1	UPD(14)pat	TR/GC [M1]	31	35		5
2	UPD(14)pat	TR/GC [M1]	28	29		5
3	UPD(14)pat	TR/GC [M1]	29	38		This report
4	UPD(14)pat	TR/GC [M1]	36	41		5
5	UPD(14)pat	TR/GC [M2]	30	30		This report
6	UPD(14)pat	MR/PE	42	Unknown		4,5
7	UPD(14)pat	MR/PE	31	28		5
8	UPD(14)pat	MR/PE	32	33		5
9	UPD(14)pat	MR/PE	26	35		5
10	UPD(14)pat	MR/PE	38	38		This report
11	UPD(14)pat	MR/PE	26	32		This report
12	UPD(14)pat	MR/PE	41	36		This report
13	UPD(14)pat	MR/PE	30	28		This report
14	UPD(14)pat	MR/PE	39	34		This report
15	UPD(14)pat	MR/PE	42	37	Born after IVF-ET	5
16	UPD(14)pat	MR/PE	36	36		4,5
17	UPD(14)pat-seg.	PE	27	24	Segmental isodisomy	4,5
18	Microdeletion		31	34		2
19	Microdeletion		33	36		2
20	Microdeletion		28	27		2
21	Microdeletion		27	37	IG-DMR alone	3
22	Microdeletion		25	25	*MEG3*-DMR alone	3
23	Epimutation		35	36		2
24	Epimutation		28	26		2
25	Epimutation		27	30		2
26	Epimutation		33	33		This report

Abbreviation: IVF-ET, *in vivo* fertilization-embryo transfer using parental gametes.
The microdeletions in patients 18–22 are different in size.

and M2 non-disjunction respectively,[9] such discrimination between M1 and M2 non-disjunctions is impossible for the development of a nullisomic oocyte. Furthermore, it is usually impossible to discriminate between TR and GC, although the presence of trisomic cells is specific to TR. Similarly, it is also usually impossible to discriminate between MR and PE, although identification of segmental isodisomy or mosaicism is unique to PE (PE subtype).

Analysis of parental ages

We examined parental ages at childbirth in patients of different underlying causes and different UPD(14)pat subtypes. Statistical significance of the relative frequency was examined by the Fisher's exact probability test, and that of the median age by the Mann–Whitney's *U*-test. $P<0.05$ was considered significant.

RESULTS

Analysis of underlying causes in patients with UPD(14)pat-like phenotype

For the eight new sporadic patients, methylation analysis invariably revealed hypermethylation of both DMRs, and microsatellite analysis showed UPD(14)pat in seven patients and biparentally inherited homologs of chromosome 14 in the remaining one patient (patient 26). FISH analysis for patient 26 identified two signals for the two DMRs, and subsequently performed array CGH analysis showed no evidence for genomic rearrangements (Supplementary Figure S2). Thus, patient 26 was assessed to have an epimutation affecting the two DMRs. Furthermore, the results of array CGH analysis confirmed the presence of microdeletions in patients 18–21 and the absence of a discernible microdeletion in patients 23–25 (Supplementary Figure S2) (array CGH analysis was not performed in patient 22 with a 4303-bp microdeletion[3] because of the lack of DNA sample available). Thus, together with our previous data, all the 26 patients with UPD(14)pat-like phenotype had genetic alteration involving the imprinted region on chromosome14q32.2.

Consequently, the 26 patients with UPD(14)pat-like phenotype were classified as follows: (1) 16 sporadic patients with full UPD(14)pat and 1 sporadic patient with segmental UPD(14)pat (UPD(14)pat group); (2) the proband of the sibling cases and two sporadic patients with different patterns of microdeletions involving the two DMRs, one sporadic patient with a microdeletion involving the IG-DMR alone in whom the *MEG3*-DMR was epimutated, and one patient with a microdeletion involving the *MEG3*-DMR alone (deletion group); and (3) four patients with epimutations (hypermethylations) of both DMRs (epimutation group) (Figure 1 and Table 1).

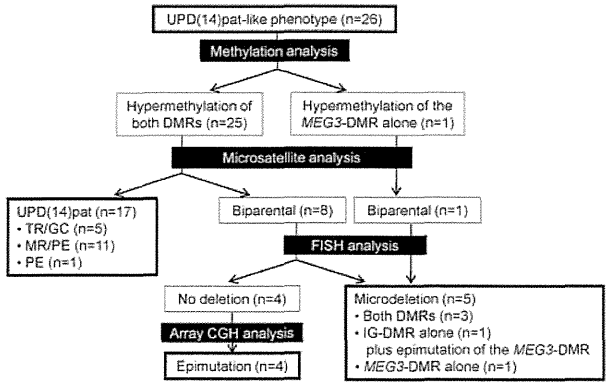

Figure 1 Classification of 26 patients with UPD(14)pat-like phenotype.

Analysis of subtypes in patients with UPD(14)pat

Heterozygosity for at least one locus indicative of TR/GC subtype was identified in five patients (patients 1–5), and the disomic pattern of pericentromeric region indicated M1 non-disjunction in patients 1–4 and M2 non-disjunction in patient 5. Full isodisomy consistent with MR/PE subtype was detected in 11 patients (patients 6–16), and segmental isodisomy unique to PE subtype was revealed in 1 patient (patient 17) (Table 1, Figure 1, and Supplementary Figure S3).

Analysis of parental ages

The distribution of parental ages at childbirth is shown in Figure 2. The advanced maternal age at childbirth ($\geq$35 years) was predominantly observed in the MR/PE subtype of UPD(14)pat. Furthermore, while the relative frequency of aged mothers ($\geq$35 years) did not show a significant difference between the MR/PE subtype of UPD(14)pat (6/11) and (i) other subtypes of UPD(14)pat (1/6) (P=0.159), (ii) deletion group (0/5) (P=0.057), and (iii) epimutation group (1/4) (P=0.338), it was significantly different between the MR/PE subtype and the sum of other subtypes of UPD(14)pat, deletion group, and epimutation group (2/15) (P=0.034). Similarly, while the median maternal age did not show a significant difference between the MR/PE subtype of UPD(14)pat (36 years) *vs* (i) other subtypes of UPD(14)pat (29.5 years) (P=0.118), (ii) deletion type (28 years) (P=0.088), and (iii) epimutation type (30.5 years) (P=0.295), it was significantly different between the MR/PE subtype of UPD(14)pat and the sum of other subtypes of UPD(14)pat, deletion group, and epimutation group (29 years) (P=0.045).

The paternal ages were similar irrespective of the genetic causes and the UPD(14)pat subtypes. In addition, the median paternal age was comparable between the TR/GC subtype of UPD(14)pat that postulates the production of a disomic sperm (35.0 years) and the sum of other subtypes of UPD(14)pat, deletion group, and epimutation group that assumes the production of a normal sperm (33.5 years) (P=0.322).

DISCUSSION

This study revealed that the UPD(14)pat-like phenotype was caused by UPD(14)pat in 65.4% of patients, by microdeletions in 19.2% of patients, and by epimutations in 15.4% of patients. Although the relative frequency of underlying genetic factors for the development of UPD(14)pat-like phenotype has been reported previously,[10] most data are derived from our previous publications. Thus, the present results are regarded as the updated and extended data on the relative frequency. For the relative frequency, it is notable that 25 of the 26 patients were confirmed to have normal karyotype, although chromosome analysis was not performed in patient 6. Thus, while Robertsonian translocations involving chromosome 14 is known to be a

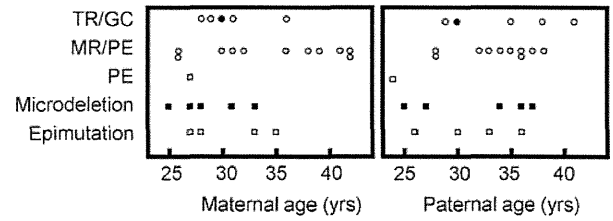

Figure 2 The distribution of parental ages at childbirth according to the underlying genetic causes for the development of UPD(14)pat-like phenotype and UPD(14)pat subtypes. Of the five plots for the TR/GC subtype, open and black circles indicate the TR/GC subtype due to non-disjunction at paternal M1 and M2, respectively.

predisposing factor for the occurrence of UPD(14)pat,[11–16] such a possible chromosomal effect has been excluded in nearly all patients examined in this study.

The relative frequency of underlying causes has also been reported in other imprinting disorders.[8,17–19] The data are summarized in Table 2 (a similar summary has also been reported recently by Hoffmann *et al*).[10] In particular, the results in patients with normal karyotype are available in Prader–Willi syndrome (PWS).[8] Furthermore, PWS is also known to be caused by UPD, microdeletions, and epimutations affecting a single imprinting region,[8,19] although Silver–Russell syndrome and Beckwith–Wiedemann syndrome (BWS) can result from perturbation of at least two imprinted regions,[17,18] and BWS and Angelman syndrome can occur as a single gene disorder.[17,19] Thus, it is notable that the relative frequency of underlying causes is quite different between patients with UPD(14)pat-like phenotype and those with PWS.[8,19] This would primarily be due to the presence of low copy repeats flanking the imprinted region on chromosome 15, because chromosomal deletions are prone to occur in regions harboring such repeat sequences.[20] Indeed, two types of microdeletions mediated by such low copy repeats account for a vast majority of microdeletions in patients with PWS,[21] whereas the microdeletions identified in patients with UPD(14)pat-like phenotype are different to each other. This would explain why microdeletions are less frequent and UPD and epimutations are more frequent in patients with UPD(14)pat-like phenotype than in those with PWS.

Advanced maternal age at childbirth was predominantly observed in the MR/PE subtype. This may imply the relevance of advanced maternal age to the development of MR-mediated UPD(14)pat, because the generation of nullisomic oocytes through M1 non-disjunction is a maternal age-dependent phenomenon.[22] Although no paternal age effect was observed, this is consistent with the previous data indicating no association of advanced paternal age with a meiotic error.[23] For the maternal age effect, however, several matters should be pointed out: (1) the number of analyzed patients is small, although it is very difficult to collect a large number of patients in this extremely rare disorder; (2) of the MR/PE subtype, the advanced maternal age is a risk factor for the generation of MR-mediated UPD(14)pat, but not for the development of PE-mediated UPD(14)pat; (3) it is impossible to discriminate between maternal age-dependent M1 non-disjunction and maternal age-independent M2 non-disjunction in the MR and GC subtypes (however, GC must be extremely rare, because it requires the concomitant occurrence of a nullisomic oocyte and a disomic sperm); (4) of the TR/GC subtype, the advanced maternal age is a risk factor for the generation of GC-mediated UPD(14)pat, but not for the development of TR-mediated UPD(14)pat; and (5) if a cryptic recombination(s) might remain undetected in some patients with apparently full isodisomy, this argues that such patients actually have TR- or GC-mediated UPD(14)pat rather than MR- or PE-mediated UPD(14)pat. Thus, further studies are required to examine the maternal age effect on the generation of MR-mediated UPD(14)pat. In addition, while a relationship is unlikely to exist between advanced maternal age and microdeletions and epimutations, this notion would also await further investigations.

Such a maternal age effect is also expected in the TR/GC subtype maternal UPDs after M1 non-disjunction, because the generation of disomic oocytes through M1 non-disjunction is also a maternal age-dependent phenomenon.[7] Indeed, such a maternal age effect has been shown for PWS patients with normal karyotype; the maternal age at childbirth was significantly higher in patients with heterodisomy for a very pericentromeric region indicative of TR/GC subtype UPD(15)mat after M1 non-disjunction than in those with other genetic causes.[8,9] For various chromosomes other than chromosome 15, furthermore, since maternal age at childbirth is higher in patients with maternal heterodisomy than in those with maternal isodisomy,[24] this would also argue for maternal age effect on the development of maternal UPDs. However, in the previous studies on maternal UPDs other than UPD(15)mat, the available data are quite insufficient to assess the maternal age effect. For example, although a relatively large number of patients with UPD(14)mat phenotype have been reported in the literature (reviewed in reference Hoffmann *et al*),[10] we could identify only six UPD(14)mat patients with normal karyotype in whom maternal age at childbirth was documented and microsatellite analysis was performed.[25–30] Furthermore, the microsatellite data are insufficient to identify the subtype of UPD(14)mat and to distinguish between M1 and M2 non-disjunction in the TR/GC subtype. Thus, while the maternal age at childbirth may be advanced in five patients with apparently TR/GC-mediated UPD(14)mat (27, 35, 37, 41, and 44 years)[25–27,29,30] (the maternal age at childbirth in the remaining one

Table 2 Relative frequency of genetic mechanisms in imprinting disorders

	UPD(14)pat-like phenotype	*BWS*	*SRS*	*AS*	*PWS*
Uniparental disomy	65.4%	16%	10%	3–5%	25% (25%)
	UPD(14)pat	UPD(11)pat (mosaic)	UPD(7)mat	UPD(15)pat	UPD(15)mat
Cryptic deletion	19.2%	Rare	—	70%	70% (72%)
Cryptic duplication	—	—	Rare	—	—
Epimutation					
Hypermethylation	15.4%	9%	—	—	2–5% (2%)
Affected DMR	IG-DMR/*MEG3*-DMR	*H19*-DMR	—	—	*SNRPN*-DMR
Hypomethylation	—	44%	>38%	2–5%	—
Affected DMR		KvDMR1	*H19*-DMR	*SNRPN*-DMR	
Gene mutation	—	5%	—	10-15%	—
Mutated gene		*CDKN1C*		*UBE3A*	
Unknown		25%	>40%	10%	
Reference	This study	17	18	19	8, 19

Abbreviations: AS, Angelman syndrome; BWS, Beckwith–Wiedemann syndrome; PWS, Prader–Willi syndrome; SRS, Silver–Russell syndrome.
Patients with abnormal karyotypes are included in BWS and AS, and not included in SRS. In PWS, the data including patients with abnormal karyotypes are shown, and those from patients with normal karyotype alone are depicted in parentheses.

patient with apparently MR/PE-mediated UPD(14)mat is 40 years),[28] the notion of a maternal age effect awaits further investigations for UPD(14)mat.

Finally, it appears to be worth pointing out that methylation analysis invariably revealed hypermethylated DMR(s) in all the 26 patients who were initially ascertained because of bell-shaped thorax with coat-hanger appearance of the ribs. This indicates that methylation analysis of the DMRs can be utilized for a screening of this condition, and that the constellation of clinical features in the UPD(14)pat-like phenotype, especially the bell-shaped thorax with coat-hanger appearance of the ribs, is highly unique to patients with UPD(14)pat-like phenotype.

In summary, this study confirms the relative frequency of underlying genetic causes for the UPD(14)pat phenotype and reveals the relative frequency of UPD(14)pat subtypes. Furthermore, the results emphasize the difference in the relative frequency of underlying genetic causes among imprinted disorders, and may support a possible maternal age effect on the generation of the nullisomic oocyte mediated UPD(14)pat. Further studies will permit a more precise assessment on these matters.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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PLOS ONE

Molecular and Clinical Studies in 138 Japanese Patients with Silver-Russell Syndrome

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Abstract

Background: Recent studies have revealed relative frequency and characteristic phenotype of two major causative factors for Silver-Russell syndrome (SRS), i.e. epimutation of the *H19*-differentially methylated region (DMR) and uniparental maternal disomy 7 (upd(7)mat), as well as multilocus methylation abnormalities and positive correlation between methylation index and body and placental sizes in *H19*-DMR epimutation. Furthermore, rare genomic alterations have been found in a few of patients with idiopathic SRS. Here, we performed molecular and clinical findings in 138 Japanese SRS patients, and examined these matters.

Methodology/Principal Findings: We identified *H19*-DMR epimutation in cases 1–43 (group 1), upd(7)mat in cases 44–52 (group 2), and neither *H19*-DMR epimutation nor upd(7)mat in cases 53–138 (group 3). Multilocus analysis revealed hyper- or hypomethylated DMRs in 2.4% of examined DMRs in group 1; in particular, an extremely hypomethylated *ARHI*-DMR was identified in case 13. Oligonucleotide array comparative genomic hybridization identified a ~3.86 Mb deletion at chromosome 17q24 in case 73. Epigenotype-phenotype analysis revealed that group 1 had more reduced birth length and weight, more preserved birth occipitofrontal circumference (OFC), more frequent body asymmetry and brachydactyly, and less frequent speech delay than group 2. The degree of placental hypoplasia was similar between the two groups. In group 1, the methylation index for the *H19*-DMR was positively correlated with birth length and weight, present height and weight, and placental weight, but with neither birth nor present OFC.

Conclusions/Significance: The results are grossly consistent with the previously reported data, although the frequency of epimutations is lower in the Japanese SRS patients than in the Western European SRS patients. Furthermore, the results provide useful information regarding placental hypoplasia in SRS, clinical phenotypes of the hypomethylated *ARHI*-DMR, and underlying causative factors for idiopathic SRS.

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Introduction

Silver-Russell syndrome (SRS) is a rare congenital developmental disorder characterized by pre- and postnatal growth failure, relative macrocephaly, triangular face, hemihypotrophy, and fifth-finger clinodactyly [1]. Recent studies have shown that epimutation (hypomethylation) of the paternally derived differentially methylated region (DMR) in the upstream of *H19* (*H19*-DMR) on chromosome 11p15.5 and maternal uniparental disomy for chromosome 7 (upd(7)mat) account for ~45% and 5–10% of SRS patients, respectively [1,2]. In this regard, phenotypic assessment has suggested that birth length and weight are more reduced and characteristic body features are more frequent in patients with *H19*-DMR epimutation than in those with upd(7)mat, whereas developmental delay tends to be more

frequent in patients with upd(7)mat than in those with *H19*-DMR epimutation [3,4]. Furthermore, consistent with the notion that imprinted genes play an essential role in placental growth and development [5], placental hypoplasia has been found in both *H19*-DMR epimutation and upd(7)mat [4,6], although comparison of placental weight has not been performed between *H19*-DMR hypomethylation and upd(7)mat. In addition, multilocus hypo- or hypermethylation and positive correlation between methylation index (MI, the ratio of methylated clones) and body and placental sizes have been reported in patients with *H19*-DMR epimutation [4,7–9], and several types of rare genomic alterations have been identified in a few of SRS patients [1,10–12].

Here, we report on molecular and clinical findings in 138 Japanese SRS patients, and discuss on the results obtained in this study.

Patients and Methods

Ethics statement

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. The parents of the affected children and the adult patients who can express an intention by themselves have given written informed consent to participate in this study and to publish their molecular and clinical data.

Patients

This study consisted of 138 Japanese patients (66 males and 72 females) with SRS phenotype aged 0–30 years (median 4.1 years), including 64 previously reported patients (20 patients with variable degrees of *H19*-DMR epimutation, three patients with upd(7)mat, one patient with 46,XY/46,XY,upd(7)mat mosaicism in whom upd(7)mat cells accounted for 91–92% of leukocytes and salivary cells and for 11% of placental tissue, and 40 patients of unknown cause) [4,6,13]. The 138 patients had a normal karyotype in all the ≥50 lymphocytes examined, and satisfied the selection criteria proposed by Netchine et al. [14], i.e., birth length and/or birth weight ≤ −2 standard deviation score (SDS) for gestational age as a mandatory criteria plus at least three of the following five features: (i) postnatal short stature (≤ −2 SDS) at 2 year of age or at the nearest measure available, (ii) relative macrocephaly at birth, i.e., SDS for birth length or birth weight minus SDS for birth occipitofrontal circumference (OFC) ≤ −1.5, (iii) prominent forehead during early childhood, (iv) body asymmetry, and (v) feeding difficulties during early childhood. Birth and present length/height, weight, and OFC were assessed by the gestational/postnatal age- and sex-matched Japanese reference data from the Ministry of Health, Labor, and Welfare and the Ministry of Education, Science, Sports and Culture. Placental weight was assessed by the gestational age-matched Japanese reference data [15]. Clinical features were evaluated by clinicians at different hospitals who participated in this study, using the same clinical datasheet. The SRS patients were classified into three groups by the molecular studies, i.e., those with *H19*-DMR hypomethylation (epimutation) (group 1), those with upd(7)mat (group 2), and the remaining patients (group 3).

Primers and samples

Primers utilized in this study are shown in Table S1. Leukocyte genomic DNA samples were examined in this study.

Methylation analysis

We performed pyrosequencing analysis for the *H19*-DMR encompassing the 6th CTCF (CCCTC-binding factor) binding site that functions as the primary regulator for the monoallelic *IGF2* and *H19* expressions [16–18], using bisulfite treated leukocyte genomic DNA samples of all the 138 patients. The procedure was as described in the manufacturer's instructions (Qiagen, Valencia, CA, USA). In brief, a 279 bp region was PCR-amplified with a primer set (PyF and PyR) for both methylated and unmethylated clones, and a sequence primer (SP) was hybridized to a single-stranded PCR products. Subsequently, the MIs were obtained for four CpG dinucleotides (CG5–CG7 and CG9), using PyroMark Q24 (Qiagen) (the MI for CG8 was not obtained, because the "C" residue of CG8 constitutes a C/T SNP) (Figure 1A). The PyF/PyR and SP were designed by PyroMark Assay Design Software Ver2.0. While the PyF sequence contains a SNP (*rs11564736*) with a mean minor allele frequency of 5% in multiple populations, the minor allele frequency is 0% in the Japanese as well as in the Asian populations (http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db = core;r = 11:2020801–2021801;v = rs11564736;vdb = variation;vf = 7864021). Thus, we utilized this PyF.

We also carried out combined bisulfite restriction analysis (COBRA) for the *H19*-DMR. The methods were as described previously [4]. In short, a 435 bp region was PCR-amplified with a primer set (CoF and CoR) that hybridize to both methylated and unmethylated clones, and MIs were obtained for two CpG dinucleotides (CG5 and CG16) after digestion of the PCR products with methylated allele-specific restriction enzymes (*Hpy*188I and *Afl*III) (Figure 1A).

Thus, we could examine CG5 by both pyrosequencing and COBRA. While we also attempted to analyze CG16 by both methods, it was impossible to design an SP for the analysis of CG16 (although we could design an SP between CG11 and CG12, clear methylation data were not obtained for CG16, probably because of the distance between the SP and CG16).

In addition, we performed COBRA for the KvDMR1 in all the 138 patients (Figure S1A) because of the possibility that epimutation of the KvDMR1 could lead to SRS phenotype via some mechanism(s) such as overexpression of a negative growth regulator *CDKN1C* [19], and for multiple DMRs on various chromosomes in patients in whom relatively large amount of DNA samples were available, as reported previously [4,20,21]. To define the reference ranges of MIs (minimum ~ maximum), 50 control subjects were similarly studied with permission.

To screen upd(7)mat, PCR amplification was performed for the *MEST*-DMR on chromosome 7q32.2 in all the 138 patients, using methylated and unmethylated allele-specific PCR primer sets, as reported previously [6] (Figure 2A). In addition, bisulfite sequencing and direct sequencing for the primer binding sites for the *ARHI*-DMR analysis were performed in a patient (case 13) with an extremely low MI for the *ARHI*-DMR.

Microsatellite analysis

Microsatellite analysis was performed for four loci within a ~4.5 Mb telomeric 11p region (*D11S2071*, *D11S922*, *D11S1318*, and *D11S988*) in patients with hypomethylated *H19*-DMR, to examine the possibility of upd(11p)mat involving the *H19*-DMR. Microsatellite analysis was also carried out for nine loci widely dispersed on chromosome 7 (Table S2) in patients with abnormal methylation patterns of the *MEST*-DMR, to examine the possibility of upd(7)mat and to infer the underlying causes for upd(7)mat, i.e., trisomy rescue, gamete complementation, monosomy rescue, and post-fertilization mitotic error [22]. The methods have been reported previously [4,6].

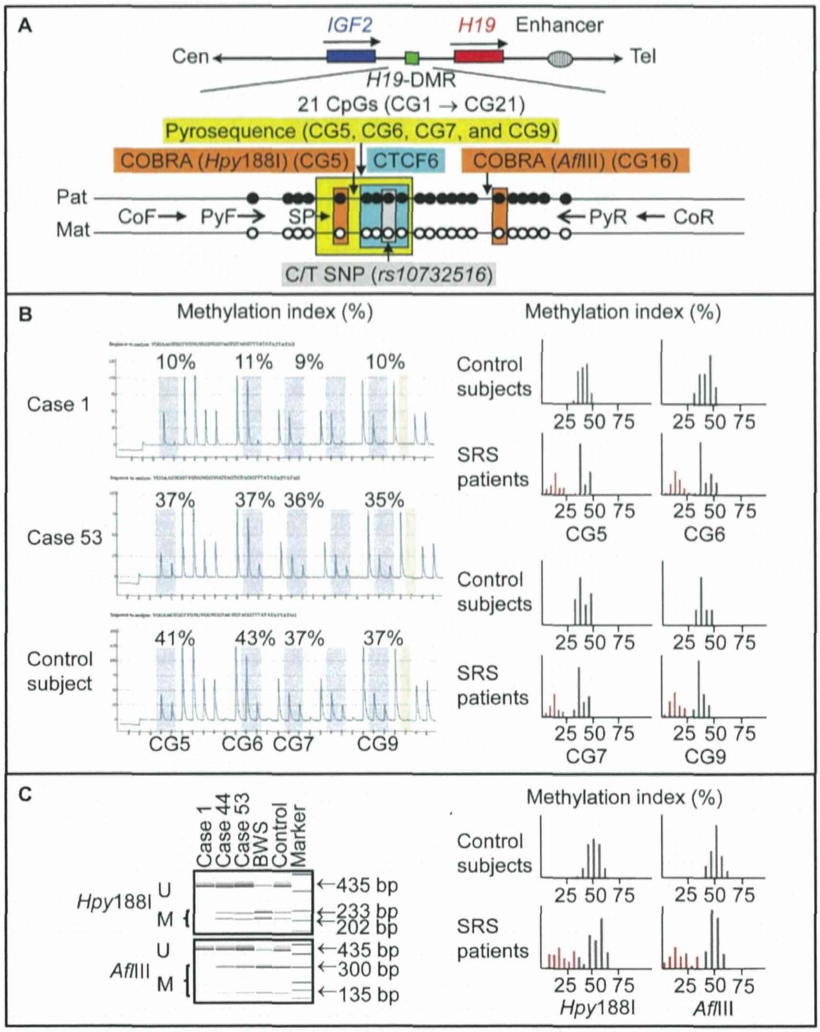

Figure 1. Methylation analysis of the *H19*-DMR, using bisulfite-treated genomic DNA. A. Schematic representation of a segment encompassing 21 CpG dinucleotides (CG1→CG21) within the *H19*-DMR. The cytosine residues at the CpG dinucleotides are usually methylated after paternal transmission (filled circles) and unmethylated after maternal transmission (open circles). The CTCF binding site 6 (CTCF6) is indicated with a blue rectangle; the cytosine residue at CG8 constitutes a C/T SNP (indicated with a gray rectangle). For pyrosequencing analysis, a 279 bp segment was PCR-amplified with PyF & PyR primers, and a sequence primer (SP) was hybridized to a single-stranded PCR products. Subsequently, the MIs were obtained for four CpG dinucleotides (CG5–CG7 and CG9) (indicated with a yellow rectangle). For COBRA, a 435 bp region was PCR-amplified with CoF & CoR primers, and the PCR product was digested with methylated allele-specific restriction enzymes to examine the methylation pattern of CG5 ands CG16 (the PCR products is digested with *Hpy*188I when the cytosine residue at CG5 is methylated and with *Afl*III when the cytosine residue at CG16 is methylated) (indicated with orange rectangles). *IGF2* is a paternally expressed gene, and *H19* is a maternally expressed gene. The stippled ellipse indicates the enhancer for *IGF2* and *H19*. B. Pyrosequencing data. Left part: Representative results indicating the MIs for CG5– CG7 and CG9. CG5– CG7 and CG9 are hypomethylated in case 1, and similarly methylated between case 53 and a control subject. Right part: Histograms showing the distribution of the MIs (the horizontal axis: the methylation index; and the vertical axis: the patient number). Forty-three SRS patients with low MIs are shown in red. C. COBRA data. Left part: Representative findings of PCR products loaded onto a DNA 1000 LabChip (Agilent, Santa Clara, CA, USA) after digestion with *Hpy*188I or *Afl*III. U: unmethylated clone specific bands; M: methylated clone specific bands; and BWS: Beckwith-Wiedemann syndrome patient with upd(11p15)pat. Right part: Histograms showing the distribution of the MIs.
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Oligoarray comparative genomic hybridization (CGH)

We performed oligoarray CGH in the 138 SRS patients, using a genomewide 4×180K Agilent platform catalog array and a custom-build high density oligoarray for the 11p15.5, 7p12.2, 12q14, and 17q24 regions where rare copy number variants have been identified in several SRS patients [1,10–12] as well as for the 7q32–qter region involved in the segmental upd(7)mat in four SRS patients [23–25]. The custom-build high density oligoarray contained 3,214 probes for 7p12.2, 434 probes for 7q32, 23,162 probes for 12q14, and 39,518 probes for 17q24, together with ~10,000 reference probes for other chromosomal region (Agilent Technologies, Palo Alto, CA, USA). The procedure was as described in the manufacturer's instructions.

Statistical analysis

After examining normality by χ^2 test, the variables following the normal distribution were expressed as the mean±SD, and those not following the normal distribution were expressed with the