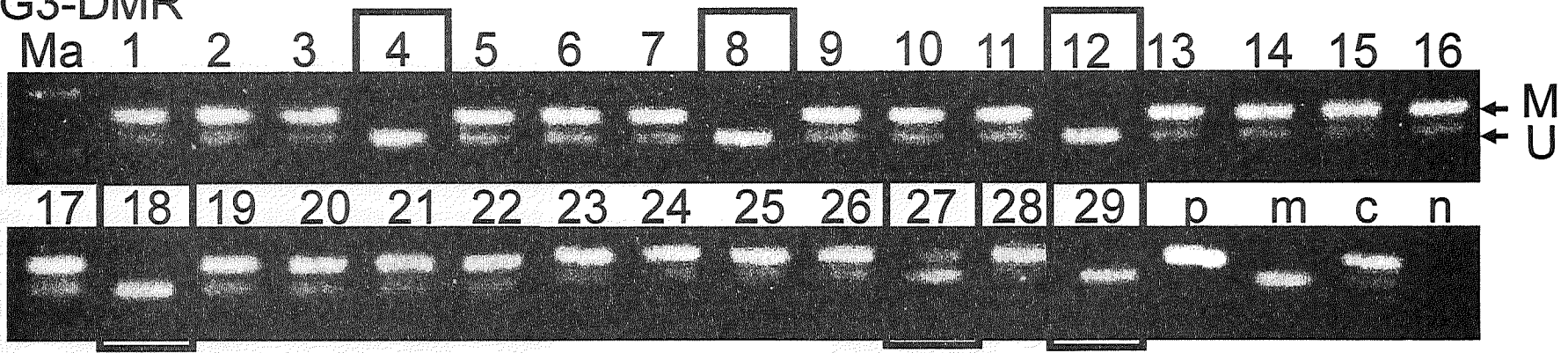
MEG3-DMR



Ma: Marker, p: upd(14)pat, m: upd(14)mat, c: control

M: メチル化アレル特異的プライマーPCR産物　U: 非メチル化アレル特異的プライマーPCR産物

IG-DMR

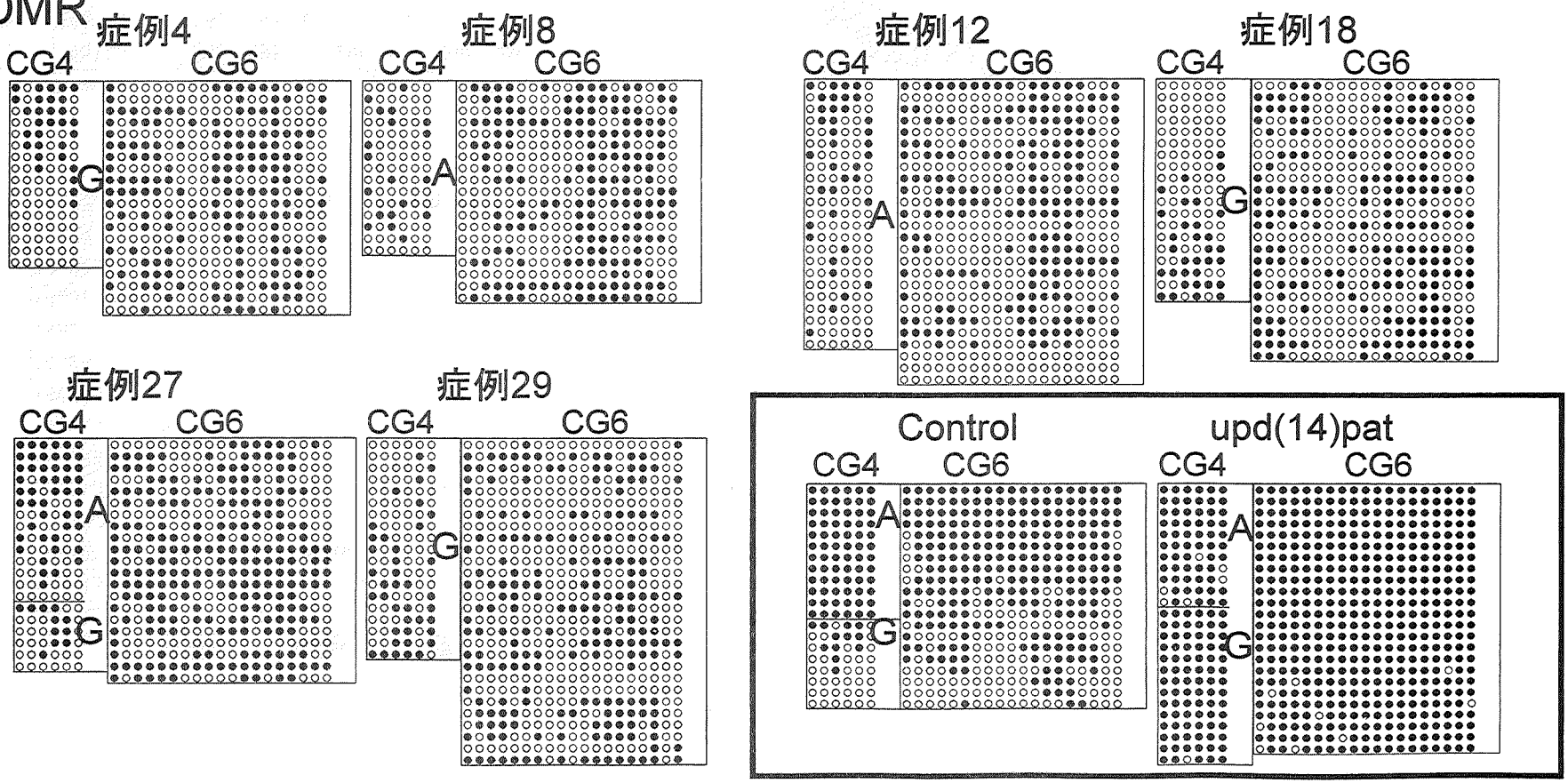


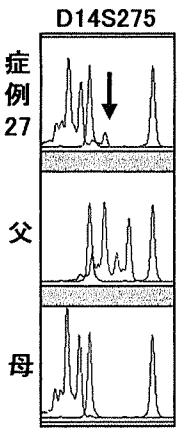
図3

A

Marker	Region	症例4	症例8	症例18	症例29
D14S261	14q11.2	–	–	–	hetero (mar)
D14S283	14q11.2	–	–	–	– (mar)
D14S275	14q12	iso	hetero	–	– (mar)
D14S70	14q13.1	–	–	–	–
D14S288	14q21.2	–	–	iso	iso
D14S276	14q22.3	hetero	–	–	
D14S63	14q23.2	hetero	hetero	iso	hetero
D14S258	14q24.2	–	–	iso	hetero
D14S74	14q24.3	–	hetero	iso	hetero
D14S68	14q31.3	–	hetero	–	–
D14S280	14q32.12	–	–	hetero	hetero
D14S65	14q32.2	iso	hetero	–	–
D14S985	14q32.2	iso	–	iso	hetero
D14S292	14q32.33	iso	hetero	hetero	hetero

B

Marker	Region	症例27				父		母	
D14S261	14q11.2	275	297	297	i	275	299	297	273
D14S283	14q11.2	139	139	139	i	139	137	139	147
D14S275	14q12	152	146	148	h	152	156	146	148
D14S70	14q13.1	103	101	101		103	101	101	101
D14S288	14q21.2	196	190	204	h	196	188	190	204
D14S276	14q22.3	244	246	246		244	242	246	246
D14S63	14q23.2	193	187	189	h	193	187	187	189
D14S258	14q24.2	198	196	196		198	200	196	196
D14S74	14q24.3	305	299	301	h	305	299	299	301
D14S68	14q31.3	323	321	321		323	323	321	321
D14S280	14q32.12	241	243	247	h	241	245	243	247
D14S65	14q32.2	147	135	147	h	147	147	135	147
D14S985	14q32.2	247	247	249	h	247	253	247	249
D14S292	14q32.33	89	87	89	h	89	89	87	89



C

Marker	Region	症例12			父		母	
D14S261	14q11.2	298	298		298	274	298	298
D14S283	14q11.2	149	147	b	149	139	147	137
D14S275	14q12	146	146		146	156	146	146
D14S70	14q13.1	102	100	b	102	102	100	104
D14S288	14q21.2	201	191	b	201	203	191	207
D14S276	14q22.3	241			241	239		247
D14S63	14q23.2	187	187		187	187	187	187
D14S258	14q24.2	206	204		206	196	204	202
D14S74	14q24.3	299	313	b	299	260	313	303
D14S68	14q31.3	323	323		323	323	323	323
D14S280	14q32.12	248	246	b	248	248	246	246
D14S65	14q32.2	135	141		135	135	141	135
D14S985	14q32.2	255	255		255	251	255	257
D14S292	14q32.33	84	86		84	86	86	86

D

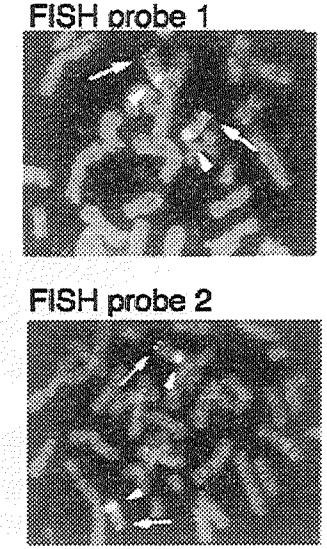


図4

図5

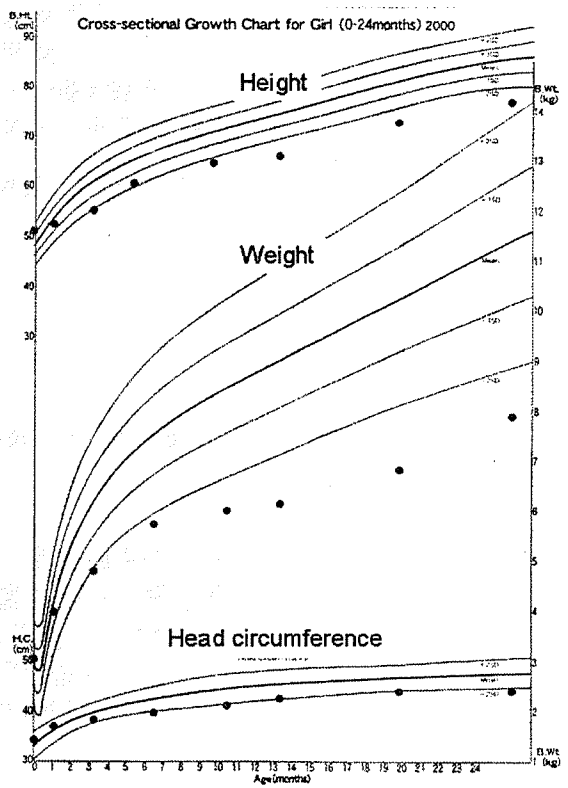

Cross-sectional Growth Chart for Girl (0-24months) 2000
Height
Weight
Head circumference
Age (months)

図6

臨床症状

Prader-Willi症候群 (PWS)

新生児期から乳児期
- 低出生体重児、成長障害
- 著しい筋緊張低下（哺乳障害）
- 短小陰茎、陰嚢低形成
- 発達遅延
- 小さい手足
- 特徴的顔貌 （平坦な顔、テント上の上口唇、薄い上口唇、三角口）

幼児期
- 低身長、小さな手足、アーモンド様の目、過食、肥満、精神発育遅延など

思春期から成人
- 低身長、小さな手足、側湾、骨粗しょう症、思春期遅延など

14番染色体母性片親性ダイソミー（upd(14)mat)

- 低出生体重児、成長障害
- 筋緊張低下（哺乳障害）
- 運動発達遅延
- 軽度精神発達遅延
- 小さな手足
- 特徴的顔貌 (前額部突出、短い人中、小顎、下向きの口角　など)

RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics A

Prenatal Findings of Paternal Uniparental Disomy 14: Report of Four Patients

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TO THE EDITOR:

A common finding in neonates with paternal uniparental disomy for chromosome 14 (upd(14)pat) is the abnormal curvature of the ribs that is referred to as a "coat-hanger" appearance. The radiographs of the ribs together with other clinical findings usually lead to correct diagnosis. In the fetus, however, it is difficult to detect this deformity by ultrasonography or other clinical findings associated with upd(14)pat.

We encountered four patients with upd(14)pat at our hospital and followed them during the prenatal and postnatal periods. In one case, it was possible to visualize the typical deformation of the ribs prenatally by X-ray photos, which led to a suspicion of upd(14)pat and allowed us to prepare for postnatal management. Here we present the prenatal findings in our series of upd(14)pat.

All four cases were referred to our hospital, the Kanagawa Children's Medical Center, a tertiary care referral center, from 1999 to 2007. Clinical information is summarized in Table I. Cases 1 and 4 have been partly reported [Kurosawa et al., 2002; Ozawa et al., 2009]. In all four cases polyhydramnios was present prenatally, and all required serial amnioreductions. A small thorax was recognized in all. A fetal omphalocele was detected in two (Cases 1 and 4); however the shapes of these omphaloceles were not typical. Case 1 had a large omphalocele that included massive Wharton's jelly (Fig. 1a). Case 4 showed an omphalocele with a wide base of the hernia which led us to suspect diastasis recti. In contrast to a typical omphalocele, no constriction at the transverse view was observed at the base of the herniated part (Fig. 1b). As upd(14)pat was suspected in Case 3, we attempted to visualize the fetal thorax at 27 weeks of gestation using X-ray. However, due to the fetal position the results were inconclusive. Deformity of the extremities was detected by ultrasonography, but an MRI examination at 32 weeks of gestation did not show any additional findings. In Case 4, an X-ray photo taken at 33 weeks of gestation revealed the "coat-hanger appearance" of the fetal ribs which suggested upd(14)pat (Fig. 2a). This finding led us to inform the parents of the suspected diagnosis and prepare for postnatal care. In this case, an MRI performed at 31 weeks of gestation did not show the distinctive deformity of the thorax probably because it was used to evaluate the atypical shape of the omphalocele. This MRI could not clearly demonstrate the margin of the omphalocele arising from the abdominal wall in the sagital view of the fetal trunk. Because of polyhydramnios, an enlarged abdominal circumference with an atypical omphalocele and the suspicion of a narrow thorax, we suspected upd(14)pat and tried to visualize the deformity of the fetal ribs using three-dimensional ultrasonography (which was inconclusive) and X-ray, which showed the deformity.

Postnatally all infants were born preterm (32–36 weeks of gestation) and required mechanical ventilation at birth (Table I). The birth weights were larger than average for the gestational age. The placental weights were also larger than average for the birth weight and above +2 SD of the mean in three cases. All cases showed a "coat-hanger appearance" of the ribs, and Figure 2 shows the

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TABLE I. Clinical Findings of Four Cases

	Case 1[a]	Case 2	Case 3	Case 4[a]
Maternal age	25	27	31	28
Paternal age	26	30	35	29
Fetal findings				
Ultrasonographic findings	Polyhydramnios, small thorax, omphalocele	Polyhydramnios, bell-shaped small thorax, skin edema of the head and neck, Small stomach[b], hepatomegaly, enlarged kidneys, slightly short femur	Polyhydramnios, small thorax, large abdomen, small stomach[b], radial hypoplasia, deformity of the foot	Polyhydramnios, small thorax, omphalocele, slightly short limbs, slightly enlarged lateral ventricles
Required amnioreduction	5 times after 25 weeks	6 times after 29 weeks	4 times after 26 weeks	3 times after 24 weeks
Amniotic fluid karyotyping	46, XX	46, XX	Not performed	46, XX
Mode of delivery	CS	VD	VD	CS
	Rupture of the membrane, Breech presentation (foot)			Arrest of labor
Neonatal outcome				
Gestational week at birth	32 weeks and 3 days	35 weeks and 3 days	34 weeks and 2 days	36 weeks and 3 days
Sex	Female	Female	Male	Female
Birth weight	2,213 g	2,930 g	2,508 g	3,372 g
Mechanical ventilation	Yes (for 1 year and 4 months)	Yes (for 3 years and 9 months and continuing)	Yes	Yes (for 5 days)
Placental weight (g)	470	710	830	970
DNA analysis	upd(14)pat	Epimutation	upd(14)pat	upd(14)pat
Survival	>8 years old	>3 years old	Died at 117 days	>1 year old
Developmental delay	+	+		+

CS, cesarean delivery; VD, vaginal delivery.

[a]Partly reported in Kurosawa et al. [2002] and Ozawa et al. [2009].

[b]It is hard to visualize the stomach pouch.

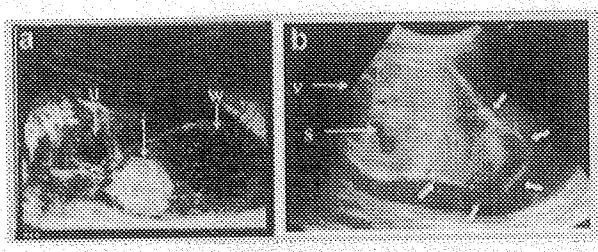

FIG. 1. Transverse view of the fetal abdomen by ultrasonography. a: At 19 weeks of gestation in Case 1. u, Urinary bladder; i, intestine; w, Wharton's jelly. The omphalocele containing the small intestine and massive Wharton's jelly. b: At 33 weeks of gestation in Case 4. v, Vertebra; s, stomach. Short arrows are showing the wide based omphalocele.

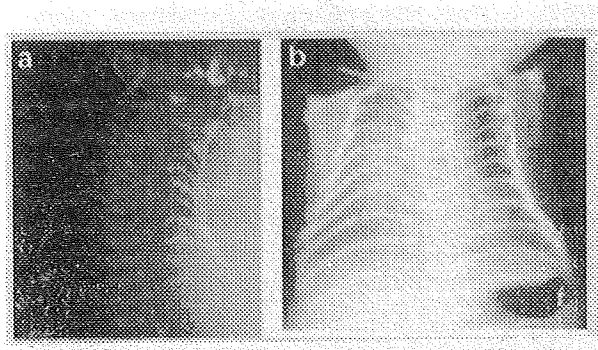

FIG. 2. Prenatal and postnatal chest X-ray photo of Case 4. a: 33weeks of gestation. b: After birth.

comparison of prenatal and postnatal X-rays in Case 4. Surgical repair of the omphalocele was successfully performed on day 1 in Cases 1 and 4 with both having herniation containing only the small intestine. All cases survived the neonatal period. Case 3 died at 177 days old due to hepatic failure. Case 1, a female, had turned 8 years old, and her physical health was good. Mental retardation and developmental delay were recognized in all living cases with varying degrees of severity.

The DNA analyses (kindly performed by Dr. Tsutomu Ogata and Dr. Masayo Kagami, Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, Tokyo.) demonstrated isodisomy of chromosome 14 was present in Cases 1, 3, and 4, and an epimutation of the 14q32.2 region in Case 2.

While neonates with upd(14)pat have some distinctive features, these are difficult to detect prenatally. Mattes et al. [2007] reviewed 19 cases of upd(14)pat including one overlapping case with this report (Case 1) [Kurosawa et al., 2002]. Together with our other three cases, a total of 22 cases have been reported. Polyhydramnios was mentioned in 20 cases. Therefore, when presented with polyhydramnios that requires serial amnioreductions, we recommend evaluation of the shape of both the thorax and abdomen of the fetus using medical imaging techniques in addition to ultrasonography. This is especially important when atypical omphalocele is present. The X-ray, which can be performed with less radiation compare to a three-dimensional or helical CT, is a simple method as long as the fetal position is suitable to visualize the distinctive shape of the thorax and once fetal ossification becomes detectable.

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Maternal Uniparental Disomy 14 Syndrome Demonstrates Prader-Willi Syndrome-Like Phenotype

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Objective To delineate the significance of maternal uniparental disomy 14 (upd(14)mat) and related disorders in patients with a Prader-Willi syndrome (PWS)–like phenotype.

Study design We examined 78 patients with PWS-like phenotype who lacked molecular defects for PWS. The *MEG3* methylation test followed by microsatellite polymorphism analysis of chromosome 14 was performed to detect upd(14)mat or other related abnormalities affecting the 14q32.2-imprinted region.

Results We identified 4 patients with upd(14)mat and 1 patient with an epimutation in the 14q32.2 imprinted region. Of the 4 patients with upd(14)mat, 3 had full upd(14)mat and 1 was mosaic.

Conclusions Upd(14)mat and epimutation of 14q32.2 represent clinically discernible phenotypes and should be designated "upd(14)mat syndrome." This syndrome demonstrates a PWS-like phenotype particularly during infancy. The *MEG3* methylation test can detect upd(14)mat syndrome defects and should therefore be performed for all undiagnosed infants with hypotonia. *(J Pediatr 2009;■:■-■).*

Maternal uniparental disomy 14 (upd(14)mat) is characterized by prenatal and postnatal growth retardation, neonatal hypotonia, small hands and feet, feeding difficulty, and precocious puberty.[1] Chromosome 14q32.2 contains several imprinted genes, and loss of expression of paternally expressed genes including *DLK1* and *RTL1* is believed to be responsible for upd(14)mat phenotype.[2] Thus far, 5 patients with epimutations and 4 patients with a microdeletion affecting the 14q32.2 imprinted region have been reported to have upd(14)mat-like phenotype.[2-4] Paternal uniparental disomy 14 (upd(14)pat) shows a distinct and much more severe phenotype characterized by facial abnormality, bell-shaped thorax and abdominal wall defects.[1] Initially, upd(14)mat was identified in patients with Robertsonian translocations involving chromosome 14, but increasing numbers of patients with a normal karyotype have been recognized.[1,5] Because maternal uniparental disomy 15 is responsible for the condition in more than 20% of patients with Prader-Willi syndrome (PWS), of which the overall prevalence is more than 1 in 15000 births,[6] one could suspect that upd(14)mat is underestimated. Phenotype of upd(14)mat is known to resemble that of PWS, which is characterized by neonatal hypotonia, small hands and feet, mental retardation, and hyperphagia resulting in obesity beyond infancy. Mitter et al[7] recently reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS and raised the question that upd(14)mat could be present in patients with PWS-like phenotype. Thus we examined patients who presented with PWS-like phenotype, but in whom PWS had been excluded.

Methods

The median age of the 78 patients enrolled in the study was 18.5 months, and the range was 1.4 to 324 months. Sex ratio was 1:1. All patients demonstrated PWS-like phenotype including hypotonia during infancy. We initially performed the *SNURF-SNRPN* DNA methylation test, and normal methylation results excluded the diagnosis of PWS.[8]

This study was approved by the Institutional Review Board Committees at Hokkaido University Graduate School of Medicine and National Center for Child Health and Development. The parents of the patients gave written informed consent.

DNA methylation status at the promoter region of imprinted *MEG3*, located in 14q32.2, was examined (**Figure 1**). Genomic DNA was extracted from leukocytes and treated with sodium bisulfite, and methylated allele– and unmethylated allele–specific primers were used to polymerase chain reaction amplify each allele, as described previously.[9] If aberrant DNA methylation was identified,

PWS	Prader-Willi syndrome
Upd(14)mat	Maternal uniparental disomy 14
Upd(14)pat	Paternal uniparental disomy 14

From the Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo (K.H., S.S.), the Department of Endocrinology and Metabolism (M.Kagami, T.O.), the Division of Clinical Genetics and Molecular Medicine (T.T.), and the Department of Pediatric Neurology (M. Kubota), National Research Institute for Child Health and Development, Tokyo, the Division of Medical Genetics, Kanagawa Children's Medical Center, Yokohama (K.K.), the Department of Pediatrics, Yamagata University School of Medicine, Yamagata (M. Kato), the Department of Pediatrics, Obihiro Kosei Hospital, Obihiro (K.U.), and the Department of Pediatrics, Nishi-Niigata Chuo National Hospital, Niigata (J.T.), Japan

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we carried out microsatellite polymorphism analysis for 16 loci on chromosome 14 (ABI PRISM Linkage Mapping Set v2.5; Applied Biosystems, Foster City, California) with DNA from the patients and their parents (**Figure 1**). Polymerase chain reaction products were analyzed on an ABI310 automatic capillary genetic analyzer and with GeneMapper software (Applied Biosystems). If aberrant DNA methylation was identified but the patient demonstrated biparental origin of the chromosome 14s, we further examined the chromosomes for DNA methylation state, parental origin, and microdeletion in 14q32.2, as described previously.[2, 3]

Results

We identified abnormal hypomethylation at the *MEG3* promoter in 5 of 78 patients (**Figure 2**). Almost complete lack of methylation was found in 4 patients (case 1 to 4), but 1 patient (case 5) demonstrated faint methylation. Polymorphism studies demonstrated that 3 (cases 2 to 4) of the 4 patients with complete lack of *MEG3* promoter methylation had complete upd(14)mat, but 1 patient (case 1) had inherited both parental alleles (**Table I**; available at www.jpeds.com). We further examined the DNA methylation state and microdeletion or segmental upd at 14q32.3, and concluded that this patient (case 1) had an epimutation. The detailed data have been reported previously.[3] The patient (case 5) with faint *MEG3* methylation was demonstrated to have 2 maternal alleles, as well as 1 paternal allele with lower signal intensity. This indicated mosaicism of upd(14)mat (80%) and a normal karyotype (20%) (**Figure 3**; available at www.jpeds.com).

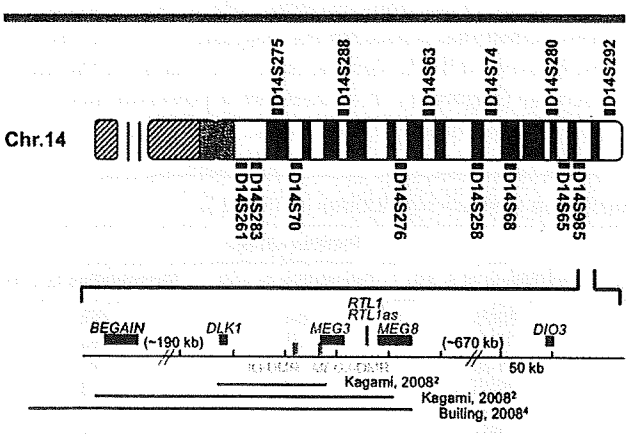

Figure 1. Schematic map of the 14q32.2 imprinted region. Loci on chromosome 14 represent markers used for microsatellite polymorphism analysis. Paternally expressed genes are shown in *blue*, maternally expressed genes in *red*, and nonimprinted genes are shown in *black*. Differentially methylated regions (DMRs) are shown in *green*. *IG-DMR*, Intergenic DMR. Reported microdeletions are demonstrated as *horizontal bars*.

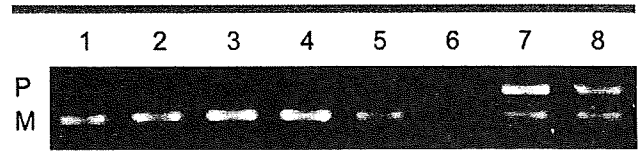

Figure 2. *MEG3* methylation test. *P*, Paternal methylated signal; *M*, maternal unmethylated signal; *1-5*, cases 1-5, respectively; *6*, paternal uniparental disomy 14; *7*, patient with PWS; *8*, normal control. Cases 1-4 show only the maternal unmethylated signal, and case 5 shows a faint paternal methylated signal.

The profiles of the patients with upd(14)mat or an epimutation are shown in **Table II**. We compared clinical features in these patients (**Table III**). All patients were referred to us during infancy because of hypotonia and motor developmental delay. Small hands and feet were also present in all patients. Prenatal growth retardation was present in all but 1 patient (case 1) who was later shown to have an epimutation. However, this patient had development of postnatal growth retardation, which was present in all patients. Premature onset of puberty was not evaluated in this study because the patients were too young. Apparent intellectual delay was only present in the patient who had upd(14)mat mosaicism (case 5). The clinical features of the patients with epimutation or with mosaic upd(14)mat were not distinct from those of the patients with full upd(14)mat.

Discussion

We detected 5 patients with upd(14)mat or epimutation at the 14q32.2-imprinted region in 78 subjects who had initially been suspected to have PWS. Mitter et al[7] reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS. However, Cox et al[10] reported that they did not find any upd(14)mat in 35 patients suspected to have PWS. Our study suggests that a significant number of patients with upd(14)mat are suspected to have PWS during infancy. To clarify how upd(14)mat and PWS share clinical features, we examined the clinical manifestations of our patients with upd(14)mat or an epimutation. All patients showed neonatal hypotonia and were referred to us during infancy. Feeding difficulty in the neonatal period and small hands and feet were also common to these patients and resembled features of PWS. It is noteworthy that all patients were referred during infancy, suggesting that upd(14)mat and PWS resemble each other, particularly during this period. Therefore upd(14)mat and related disorders, as well as PWS, should be important differential diagnoses for infants with hypotonia and feeding difficulty. Distinct features for upd(14)mat included less-specific facial characteristics, constant prenatal growth failure, and better intellectual development. Precocious puberty is not present in PWS; however, this was not evaluated in this study because the patients were not

Table II. Profiles of the patients with upd(14)mat and epimutation of 14q32.2

	Case 1	Case 2	Case 3	Case 4	Case 5
Molecular class	Epimutation	Upd(14)mat	Upd(14)mat	Upd(14)mat	Upd(14)mat (mosaic)
Age	2 y 2 m	4 y 2 m	2 y 7 m	1 y 9 m	3 y 4 m
Sex	Female	Male	Female	Female	Female
Karyotype	46,XX	46,XY	46,XX	46,XX	46,XX
Gestational age	41 w 5d	36 w 1 d	37 w 3 d	40 w 4 d	36 w
Birth weight g (SD)	3034 (0)	1955 (−2.6)	1680 (−3.3)	1858 (−2.8)	1434 (−3.9)
Birth length cm (SD)	50 (+0.7)	45.7 (−1.5)	40 (−4.0)	45 (−1.6)	39 (−3.9)
Birth OFC cm (SD)	Unknown	32 (−1.0)	30.4 (−2.0)	32 (−0.8)	30 (−2.2)
Present height cm (SD)	76.1 (−3.1)	89.5 (−2.8)	79 (−2.7)	72.5 (−3.4)	77.8 (−4.5)
Present weight kg (SD)	8.18 (−2.4)	11.6 (−2.1)	8.4 (−2.8)	6.4 (−3.7)	8.84 (−3.3)
Present OFC cm (SD)	45.2 (−1.5)	51.0 (+0.5)	48 (0)	44 (−1.8)	46.0 (−1.6)

old enough to demonstrate this feature. It is possible that when the patients get older, the clinical features of upd(14)mat may become more distinct from those of PWS.

We detected an epimutation in the 14q32.2-imprinted region, as well as upd(14)mat. The clinical features of the patient with the epimutation were grossly similar to those of patients with upd(14)mat. Thus far 5 patients with an epimutation in the paternal allele, including our patient, have been identified.[4,11] These patients exhibit clinical features indistinguishable from those with full upd(14)mat. Our patient with an epimutation demonstrated normal birth weight, but previously reported patients with an epimutation have shown intrauterine growth retardation. [4,11] Therefore normal birth weight is not a specific feature related to epimutation.

One of the patients with upd(14)mat was mosaic for upd(14)mat and normal karyotype. It is not easy to understand the pathogenesis of such a mosaic, but similar mosaicism of chromosome 15 has been reported.[12] Mosaicism for upd(15)mat and normal cell lines has been found in a patient with the PWS phenotype.[12] Similarly, our patient with mosaic upd(14)mat demonstrated typical clinical features of upd(14)mat. This could be explained by the small proportion of normal cell lines (less than 20%), or it could be that the level of mosaicism is different in each tissue. It is possible that the proportion of normal cells may be lower in the brain, which is most responsible for the phenotype of upd(14)mat.

As is clear in our series of patients, upd(14)mat phenotype can be caused by an epimutation of 14q32.2. Recently, Kagami et al[2] reported a microdeletion in 14q32.2 associated with a similar phenotype (**Figure 1**). Buiting et al[4] also reported a patient with a 1Mb deletion at 14q32.2 (**Figure 1**). Therefore upd(14)mat phenotype is associated with not only upd(14)mat but an epimutation or small deletion. This genetic complexity is similar to that of PWS. PWS is caused by paternal deletion of 15q11-q13, maternal uniparental disomy of chromosome 15, and epimutation (imprinting defect). A new name such as upd(14)mat syndrome would be appropriate to represent the entire upd(14)mat clinical features represented by upd(14)mat, epimutation of 14q32.2 and microdeletion in 14q32.2. Alternatively, Buiting et al[4] suggested the term, "Temple syndrome," because upd(14)mat was first described by Dr. I. K. Temple in 1991, who subsequently described an epimutation in 2007.[4,5,11]

Finally, it should be emphasized that the *MEG3* methylation test could detect not only upd(14)mat but an epimutation and small deletions involving *MEG3*.This is because the *MEG3* DMR that is used for the diagnostic DNA methylation test is involved in the shortest region of overlap of the microdeletions (**Figure 1**). It is therefore a powerful method for screening patients with upd(14)mat syndrome.

Table III. Clinical features in patients with upd(14)mat, epimutation and microdeletions of 14q32.2

	Present study					Previous studies		
	Case 1	Case 2	Case 3	Case 4	Case 5	Upd(14)mat (n = 35)	Epimutation (n = 4)	Microdeletion (n = 4)
Premature delivery	−	−	−	−	−	10/25	0/4	0/3
Prenatal growth failure	−	+	+	+	+	24/27	4/4	3/3
Postnatal growth failure	+	+	+	+	+	26/32	3/4	3/3
Somatic features	+	+	+	+	+	23/35	4/4	3/3
Frontal bossing	+	+	+	+	−	9/9		
High arched palate	−	+	+		+	7/9		
Micrognathia	+	+	−	+	+	5/5		
Small hands	+	+	+	+	+	24/27	4/4	3/3
Scoliosis	−	−	−	−	−	5/19		
Others								
Hypotonia	+	+	+	+	+	25/28	4/4	1/1
Obesity	−	−	−	−	−	14/34	3/4	1/4
Early onset of puberty	NA	NA	NA	NA	NA	14/16	3/4	2/3
Mental retardation	−	−	−	−	+	10/27	2/4	1/4

NA, Not applicable.
Previous studies are based on references 2, 3 and 4.

Upd(14)mat syndrome demonstrates PWS-like phenotype during infancy, and it should be considered when seeing a patient with hypotonia. The *MEG3* methylation test should be performed to identify this syndrome. ■

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Table I. Microsatellite polymorphism analyses for chromosome 14 in 6 families with aberrant MEG3 methylation

		Case 1 family			Case 2 family			Case 3 family			Case 4 family			Case 5 family		
Locus	Region	Patient	Father	Mother	Patient	Father	Mother	Patient	Father	Mother	Patient	Father	Mother	Patient	Father	Mother
D14S261	14q11.2	298, 298	274, 298	298, 298	297, 297	297, 299	297, 297	298, 298	296, 298	298, 298	297, 297	297, 297	275, 297	275, 297, 297	275, 299	273, 297
D14S283	14q11.2	147, 149	139, 149	137, 147	139, 139	137, 137	139, 139	137, 149	133, 137	137, 149	150, 150	142, 150	140, 150	139, 139	137, 139	139, 147
D14S275	14q12	146, 146	146, 156	146, 146	149, 149	145, 145	149, 151	148, 152	146, 146	148, 152	155, 155	149, 155	149, 155	146, 148, 152	152, 156	146, 148
D14S70	14q13.1	100, 102	102, 102	100, 104	101, 101	101, 103	101, 101	103, 103	99, 101	103, 103	104, 104	104, 106	104, 104	101, 101, 103	101, 103	101, 101
D14S288	14q21.2	191, 201	201, 203	191, 207	201, 201	203, 203	201, 201	193, 193	193, 203	193, 193	195, 195	213, 215	195, 197	190, 196, 204	188, 196	190, 204
D14S276	14q22.3	241, —	239, 241	247, —	242, 244	244, 246	242, 244	244, 244	242, 244	244, 244	245, 245	241, 241	245, 245	244, 246, 246	242, 244	246, 246
D14S63	14q23.2	187, 187	187, 187	187, 187	187, 193	183, 189	187, 193	183, 187	189, 191	183, 187	191, 191	185, 195	191, 195	187, 189, 193	187, 193	187, 189
D14S258	14q24.2	204, 206	196, 206	202, 204	196, 196	198, 202	196, 196	196, 196	200, 202	196, 196	202, 202	204, 204	202, 204	196, 196, 198	198, 200	196, 196
D14S74	14q24.3	299, 313	260, 299	303, 313	303, 303	303, 305	303, 303	299, 303	299, 301	299, 303	295, 295	305, 313	295, 301	299, 301, 305	299, 305	299, 301
D14S68	14q31.3	323, 323	323, 323	323, 323	321, 321	323, 323	321, 321	321, 323	323, 323	321, 323	323, 323	325, 325	321, 323	321, 321, 323	323, 323	321, 321
D14S280	14q32.12	246, 248	248, 248	246, 246	243, 243	243, 245	243, 243	247, 247	243, 247	247, 247	248, 248	244, 244	242, 248	241, 243, 247	241, 245	243, 247
D14S65	14q32.2	135, 141	135, 135	135, 141	145, 145	135, 149	135, 145	135, 147	137, 145	135, 147	150, 150	150, 150	150, 150	135, 147	147, 147	135, 147
D14S985	14q32.2	255, 255	251, 255	255, 257	250, 250	246, 254	250, 254	247, 247	249, 249	247, 247	248, 248	246, 248	248, 254	247, 249	247, 253	247, 249
D14S292	14q32.33	84, 86	84, 86	86, 86	92, 92	86, 88	88, 92	85, 87	83, 85	85, 87	92, 92	86, 92	88, 92	87, 89	89, 89	87, 89

Figure 3. Microsatellite polymorphism analysis at D14S275 for the family of case 5. The patient demonstrates 3 peaks (146, 148, 152 bp), 2 (146, 148 bp) of which are transmitted from the mother, but 1 small peak (152 bp) indicated by the *arrow* is transmitted from the father. *Red peaks* depict size markers.

The IG-DMR and the *MEG3*-DMR at Human Chromosome 14q32.2: Hierarchical Interaction and Distinct Functional Properties as Imprinting Control Centers

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Running head: Imprinting Control Centers at Human 14q32.2

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Abstract

Human chromosome 14q32.2 harbors the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR, together with multiple imprinted genes. Although previous studies in cases with microdeletions and epimutations affecting both DMRs and paternal/maternal uniparental disomy 14 (upd(14)pat/mat)-like phenotypes argue for a critical regulatory function of the two DMRs for the 14q32.2 imprinted region, the precise role of individual DMR remains to be clarified. We studied an infant with upd(14)pat body and placental phenotypes (patient 1) and a neonate with upd(14)pat body, but no placental, phenotype (patient 2). Structural analysis showed a heterozygous 8,558 bp microdeletion involving the IG-DMR alone in patient 1, and a heterozygous 4,303 bp microdeletion involving the *MEG3*-DMR alone in patient 2. Methylation and expression analyses revealed that loss of the hypomethylated IG-DMR of maternal origin in patient 1 was associated with epimutation (hypermethylation) of the *MEG3*-DMR in the body and caused paternalization of the imprinted region in examined body and placental tissues, whereas loss of the hypomethylated *MEG3*-DMR of maternal origin in patient 2 permitted normal methylation pattern of the IG-DMR and resulted in maternal to paternal epigenotypic alteration in examined body tissues. These results, together with the finding that the IG-DMR remains as a DMR and the *MEG3*-DMR exhibits a non-DMR in the placenta, imply that the IG-DMR and the *MEG3*-DMR function as imprinting control centers in the placenta and the body, respectively, with a hierarchical interaction for the methylation pattern in the body.

Author Summary

Human chromosome 14q32.2 imprinted region harbors the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR, together with multiple imprinted genes. Consistent with this, paternal and maternal uniparental disomy 14 causes distinct phenotypes. Here, we show 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, and that the IG-DMR and the *MEG3*-DMR function as imprinting control centers in the placenta and the body, respectively. To our knowledge, this is the first study demonstrating not only different roles between the primary and the secondary DMRs at a single imprinted region, but also an essential regulatory function for the secondary DMR. Thus, the results provide significant advance in the clarification of underlying mechanisms involved in the imprinting regulation at the 14q32.2 region and the phenotypic development in uniparental disomy 14.

Introduction

Human chromosome 14q32.2 carries a cluster of protein-coding paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* and non-coding maternally expressed genes (*MEGs*) such as *MEG3* (alias, *GTL2*), *RTL1as* (*RTL1* antisense), *MEG8*, *snoRNAs*, and *microRNAs* [1,2]. Consistent with this, paternal uniparental disomy 14 (upd(14)pat) results in a unique phenotype characterized by facial abnormality, small bell-shaped thorax, abdominal wall defects, placentomegaly, and polyhydramnios [2,3], and maternal uniparental disomy 14 (upd(14)mat) leads to less-characteristic but clinically discernible features including growth failure [2,4].

The 14q32.2 imprinted region also harbors two differentially methylated regions (DMRs), i.e., the germline-derived primary *DLK1-MEG3* intergenic DMR (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR [1,2]. Both DMRs are hypermethylated after paternal transmission and hypomethylated 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 [1,2]. Furthermore, previous studies in cases with upd(14)pat/mat-like phenotypes have revealed that epimutations (hypermethylation) and microdeletions affecting both DMRs of maternal origin cause paternalization of the 14q32.2 imprinted region, and that epimutations (hypomethylation) affecting both DMRs of paternal origin cause maternalization of the 14q32.2 imprinted region, while microdeletions involving the DMRs of paternal origin have no effect on the imprinting status [2,5–8]. These findings, together with the notion that parent-of-origin specific expression patterns of imprinted genes are primarily dependent on the methylation status of the DMRs [9], argue for a critical regulatory function of the two DMRs for the 14q32.2 imprinted region, with possible different effects between the body and the placenta.

However, the precise role of individual DMR remains to be clarified. Here, we report that the IG-DMR and the *MEG3*-DMR show a hierarchical interaction for the methylation pattern in the body, and function as imprinting control centers in the placenta and the body, respectively. To our knowledge, this is the first study demonstrating not only different roles between the primary and secondary DMRs at a single imprinted region, but also an essential regulatory function for the secondary DMR.

Results

Clinical reports

We studied an infant with upd(14)pat body and placental phenotypes (patient 1) and a neonate with upd(14)pat body, but no placental, phenotype (patient 2) (Figure 1). Detailed clinical features of patients 1 and 2 are shown in Table 1. In brief, patient 1 was delivered by a caesarean section at 33 weeks of gestation due to progressive polyhydramnios despite amnioreduction at 28 and 30 weeks of gestation, whereas patient 2 was born at 28 weeks of gestation by a vaginal delivery due to progressive labor without discernible polyhydramnios. Placentomegaly was observed in patient 1 but not in patient 2. Patients 1 and 2 were found to have characteristic face, small bell-shaped thorax with coat hanger appearance of the ribs, and omphalocele. Patient 1 received surgical treatment for omphalocele immediately after birth and mechanical ventilation for several months. At present, she is 5.5 months of age, and still requires intensive care including oxygen administration and tube feeding. Patient 2 died at four days of age due to massive intracranial hemorrhage, while receiving intensive care including mechanical ventilation. The mother of patient 1 had several non-specific clinical features such as short stature and obesity. The father of patient 1 and the parents of patient 2 were clinically normal.

Sample preparation

We isolated genomic DNA (gDNA) and transcripts (*mRNAs*, *snoRNAs*, and *microRNAs*) from fresh leukocytes of patients 1 and the parents of patients 1 and 2, from fresh skin fibroblasts of patient 2, and from formalin-fixed and paraffin-embedded placental samples of patient 1 and similarly treated pituitary and adrenal samples of patient 2 (although multiple body tissues were available in patient 2, useful gDNA and transcript samples were not obtained from other tissues probably due to drastic post-mortem degradation). We also made metaphase spreads from leukocytes and skin fibroblasts. For comparison, we obtained control samples from fresh normal adult leukocytes, neonatal skin fibroblasts, and placenta at 38 weeks of gestation, and from fresh leukocytes of upd(14)pat/mat patients and formalin-fixed and paraffin-embedded placenta of a upd(14)pat patient [2,3].

Structural analysis of the imprinted region

We first examined the structure of the 14q32.2 imprinted region (Figure 2). Upd(14) was excluded in patients 1 and 2 as well as in the mother of patient 1 by microsatellite analysis (Table S1), and FISH analysis for the two DMRs identified a familial heterozygous deletion encompassing the IG-DMR alone in patient 1 and her mother and a *de novo* heterozygous deletion encompassing the *MEG3*-DMR alone in patient 2 (Figure 2). The microdeletions were further localized by SNP genotyping for 70 loci (Table S1) and quantitative real-time PCR (q-PCR) analysis for four regions around the DMRs (Figure S1A), and serial direct sequencing for the long PCR products harboring the deletion junctions successfully identified the fusion points of the microdeletions in patient 1 and her mother and in patient 2 (Figure 2). According to the NT_026437 sequence data at the NCBI Database (Genome Build 36.3) (http://preview.ncbi.nlm.nih.gov/guide/), the deletion size was 8,558 bp (82,270,449–82,279,006 bp) for the microdeletion in patient 1 and her mother, and 4,303 bp (82,290,978–82,295,280 bp) for the microdeletion in patient 2. The microdeletion in patient 2 also involved the 5' part of *MEG3* and five of the seven putative CTCF binding sites A–G [11], and was accompanied by insertion of a 66 bp sequence duplicated from *MEG3* intron 5 (82,299,727–82,299,792 bp on NT_026437). Direct sequencing of the exonic or transcribed regions detected no mutation in *DLK1*, *MEG3*, and *RTL1*, although several cDNA polymorphisms (cSNPs) were identified (Table S1). Oligoarray comparative genomic hybridization identified no other discernible structural abnormality (Figure S1B).

Methylation analysis of the two DMRs and the seven putative CTCF binding sites

We next studied methylation patterns of the previously reported IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) [2], using bisulfite treated gDNA samples. Bisulfite sequencing and combined bisulfite restriction analysis using body samples revealed a hypermethylated IG-DMR and *MEG3*-DMR in patient 1, a hypomethylated IG-DMR and differentially methylated *MEG3*-DMR in the mother of patient 1, and a differentially methylated IG-DMR and hypermethylated *MEG3*-DMR in patient 2, and bisulfite sequencing

using placental samples showed a hypermethylated IG-DMR and rather hypomethylated *MEG3*-DMR in patient 1 (Figure 3A,B).

We also examined methylation patterns of the seven putative CTCF binding sites by bisulfite sequencing. The sites C and D alone exhibited DMRs in the body and were rather hypomethylated in the placenta (Figure 3A,C), as observed in CG7. Furthermore, to identify an informative SNP(s) pattern for allele-specific bisulfite sequencing, we examined a 349 bp region encompassing the site C and a 356 bp region encompassing the site D as well as a 300 bp region spanning the previously reported three SNPs near the site D, in 120 control subjects, the cases with upd(14)pat/mat, and patients 1 and 2 and their parents. Consequently, an informative polymorphism was identified for a novel G/A SNP near the site D in only a single control subject, and the parent-of-origin specific methylation pattern was confirmed (Figure 3D). No informative SNP was found in the examined region around the site C, and no other informative SNP was identified in the two examined regions around the site D, with the previously known three SNPs being present in a homozygous condition in all the subjects analyzed.

Expression analysis of the imprinted genes

Finally, we performed expression analyses, using standard reverse transcriptase (RT)-PCR and/or q-PCR analysis for multiple imprinted genes in this region (Figure 4A–C). For leukocytes, weak expression was detected for *MEG3* and *SNORD114-29* in a control subject and the mother of patient 1 but not in patient 1. For skin fibroblasts, although all *MEGs* but no *PEGs* were expressed in control subjects, neither *MEGs* nor *PEGs* were expressed in patient 2. For placentas, although all imprinted genes were expressed in control subjects, *PEGs* only were expressed in patient 1. For the pituitary and adrenal of patient 2, *DLK1* expression alone was identified.

Expression pattern analyses using informative cSNPs revealed monoallelic *MEG3* expression in the leukocytes of the mother of patient 1 (Figure 4D), and biparental *RTL1* expression in the placenta of patient 1 (no informative cSNP was detected for *DLK1*) and biparental *DLK1* expression in the pituitary and adrenal of patient 2 (*RTL1* was not expressed in

the pituitary and adrenal) (Figure 4E), as well as maternal *MEG3* expression in the control leukocytes and paternal *RTL1* expression in the control placentas (Figure S2). Although we also attempted q-PCR analysis, precise assessment was impossible for *MEG3* in the mother of patient 1 because of faint expression level in leukocytes and for *RTL1* in patient 1 and *DLK1* in patient 2 because of poor quality of mRNAs obtained from formalin-fixed and paraffin-embedded tissues.

Discussion

The data of the present study are summarized in Figure 5. Parental origin of the microdeletion positive chromosomes is based on the methylation patterns of the preserved DMRs in patients 1 and 2 and the mother of patient 1 as well as maternal transmission in patient 1. Loss of the hypomethylated IG-DMR of maternal origin in patient 1 was associated with epimutation (hypermethylation) of the *MEG3*-DMR in the body and caused paternalization of the imprinted region and typical upd(14)pat body and placental phenotypes, whereas loss of the hypomethylated *MEG3*-DMR of maternal origin in patient 2 permitted normal methylation pattern of the IG-DMR in the body and resulted in maternal to paternal epigenotypic alteration and typical upd(14)pat body, but no placental, phenotype. In this regard, while a 66 bp segment was inserted in patient 2, this segment contains no known regulatory sequence [16] or evolutionarily conserved element [17] (also examined with a VISTA program, http://genome.lbl.gov/vista/index.shtml). Similarly, while no control samples were available for pituitary and adrenal, the previous study in human subjects has shown paternal *DLK1* expression in adrenal as well as monoallelic *DLK1* and *MEG3* expressions in various tissues [16]. Furthermore, the present and the previous studies [2] indicate that this region is imprinted in the placenta as well as in the body. Thus, these results, in conjunction with the finding that the IG-DMR remains as a DMR and the *MEG3*-DMR exhibits a non-DMR in the placenta [2], imply the following: (1) the IG-DMR functions hierarchically as an upstream regulator for the methylation pattern of the *MEG3*-DMR on the maternally inherited chromosome in the body, but not in the placenta; (2) the hypomethylated *MEG3*-DMR functions as an essential imprinting regulator for both *PEGs* and *MEGs* in the body; and (3) in the placenta, the