Table 2. Clinical and molecular findings in wild-type and PatDi(12) mice and mice with maternally inherited ΔIG-DMR and Δ*Gtl2*-DMR.

	Wildtype	PatDi(12)	ΔIG-DMR (~4.15 kb)[a]	Δ*Gtl2*-DMR (~10 kb)[b]
				Neomycin cassette (+)
<Body>				
Phenotype	Normal	Abnormal[c]	PatDi(12) phenotype[c]	Normal at birth
				Lethal by 4 weeks
Methylation pattern				
IG-DMR	Differential	Methylated	Methylated[d]	Differential
Gtl2-DMR	Differential	Methylated	Epimutated[e]	Methylated[d]
Expression pattern				
Pegs	Monoallelic	Increased (~2x)	Biparental	Grossly normal
			Increased (2x or 4.5x)[f]	
Megs	Monoallelic	Absent	Absent	Decreased (<0.2~0.5x)[g]
<Placenta>				
Phenotype	Normal	Placentomegaly	Apparently normal	Not determined
Methylation pattern				
IG-DMR	Differential	Methylated	Not determined	Not determined
Gtl2-DMR	Non-DMR	Non-DMR	Not determined	Not determined
Expression pattern				
Pegs	Monoallelic	Not determined	Increased (1.5~1.8x)[g]	Decreased (0.5~0.85x)[g]
Megs	Monoallelic	Not determined	Decreased (0.6~0.8x)[g]	Decreased (<0.1~1.0)[g]
Remark			Paternal transmission[h]	Paternal transmission[i]
				Biparental transmission[j]

a The deletion size is smaller than that of patient 1 and her mother in this study, especially at the centromeric region.
b The microdeletion also involves *Gtl2*, and the deletion size is larger than that of patient 2 in this study.
c Body phenotype includes bell-shaped thorax with rib anomalies, distended abdomen, and short and broad neck.
d Hemizygosity for the methylated DMR of paternal origin.
e Hypermethylation of the maternally derived DMR.
f 2x *Dlk1* and *Dio3* expression levels and 4.5x *Rtl1* expression level. The markedly elevated *Rtl1* expression level is ascribed to a synergic effect between activation of the usually silent *Rtl1* of maternal origin and loss of functional microRNA-containing *Rtl1as* as a repressor for *Rtl1* [26,36–38].
g The expression level is variable among examined tissues and examined genes.
h The ΔIG-DMR of paternal origin has permitted normal *Gtl2*-DMR methylation pattern, intact imprinting status, and normal phenotype in the body (no data on the placenta).
i The Δ*Gtl2*-DMR of paternal origin is accompanied by normal methylation pattern of the IG-DMR and variably reduced *Pegs* expression and increased *Megs* expression in the body, and has yielded severe growth retardation accompanied by perinatal lethality.
j The homozygous mutants have survived and developed into fertile adults, despite rather altered expression patterns of the imprinted genes.
doi:10.1371/journal.pgen.1000992.t002

DNase, cDNA samples for *DLK1*, *MEG3*, *MEG8*, and *snoRNAs* were prepared with oligo(dT) primers from 1 μg of RNA using Superscript III Reverse Transcriptase (Invitrogen), and those for *microRNAs* were synthesized from 300 ng of RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). For *RTL1*, cDNA samples were synthesized with *RTL1*-specific primers that do not amplify *RTL1as*. Control gDNA and cDNA samples were extracted from adult leukocytes and neonatal skin fibroblasts purchased from Takara Bio Inc. Japan, and from a fresh placenta of 38 weeks of gestation. Metaphase spreads were prepared from leukocytes and skin fibroblasts using colcemide (Invitrogen).

Structural analysis

Microsatellite analysis and SNP genotyping were performed as described previously [2]. For FISH analysis, metaphase spreads were hybridized with a 5,104 bp FISH-1 probe and a 5,182 bp FISH-2 probe produced by long PCR, together with an RP11-566I2 probe for 14q12 used as an internal control [2]. The FISH-1 and FISH-2 probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the RP11-566I2 probe was labeled with biotin and detected by avidin conjugated to fluorescein isothiocyanate. For quantitative real-time PCR analysis, the relative copy number to RNaseP (catalog No: 4316831, Applied Biosystems) was determined by the Taqman real-time PCR method using the probe-primer mix on an ABI PRISM 7000 (Applied Biosystems). To determine the breakpoints of microdeletions, sequence analysis was performed for long PCR products harboring the fusion points, using serial forward primers on the CEQ 8000 autosequencer (Beckman Coulter). Direct sequencing was also performed on the CEQ 8000 autosequencer. Oligoarray comparative genomic hybridization was performed with 1×244K Human Genome Array (catalog No: G4411B) (Agilent Technologies), according to the manufacturer's protocol.

Methylation analysis

Methylation analysis was performed for gDNA treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research). After PCR amplification using primer sets that hybridize both methylated and unmethylated clones because of lack of CpG

dinucleotides within the primer sequences, the PCR products were digested with appropriate restriction enzymes for combined bisulfite restriction analysis. For bisulfite sequencing, the PCR products were subcloned with TOPO TA Cloning Kit (Invitrogen) and subjected to direct sequencing on the CEQ 8000 auto-sequencer.

Expression analysis

Standard RT-PCR was performed for *DLK1*, *RTL1*, *MEG3*, *MEG8*, and *snoRNAs* using primers hybridizing to exonic or transcribed sequences, and one μl of PCR reaction solutions was loaded onto Gel-Dye Mix (Agilent). Taqman real-time PCR was carried out using the probe-primer mixtures (assay No: Hs00292028 for *MEG3* and Hs00419701 for *MEG8*; assay ID: 001028 for *miR433*, 000452 for *miR127*, 000568 for *miR379*, and 000477 for *miR154*) on the ABI PRISM 7000. Data were normalized against *GAPDH* (catalog No: 4326317E) for *MEG3* and *MEG8* and against *RNU48* (assay ID: 0010006) for the remaining *miRs*. The expression studies were performed three times for each sample.

To examine the imprinting status of *MEG3* in the leukocytes of the mother of patient 1, direct sequence data for informative cSNPs were compared between gDNA and cDNA. To analyze the imprinting status of *RTL1* in the placental sample of patient 1 and that of *DLK1* in the pituitary and adrenal samples of patient 2, RT-PCR products containing exonic cSNPs informative for the parental origin were subcloned with TOPO TA Cloning Kit, and multiple clones were subjected to direct sequencing on the CEQ 8000 autosequencer. Furthermore, *MEG3* expression pattern was examined using leukocyte gDNA and cDNA samples from multiple normal subjects and leukocyte gDNA samples from their mothers, and *RTL1* expression pattern was analyzed using gDNA and cDNA samples from multiple fresh normal placentas and leukocyte gDNA from the mothers.

Supporting Information

Figure S1 Structural analysis. (A) Quantitative real-time PCR analysis (q-PCR) for four regions (q-PCR-1-4) in patient 2. The q-PCR-1 and q-PCR-2 regions are present in two copies whereas q-PCR-3 and q-PCR-4 regions are present in a single copy in patient 2. The four regions are present in two copies in the parents and a control subject, in a single copy in the two previously reported patients with microdeletions involving the examined regions (Deletion-1 and Deletion-2 are case 2 and case 3 in Kagami et al. [2], respectively), and in three copies in a hitherto unreported case with 46,XX,der(17)t(14;17)(q32.2;p13)pat who have three copies of the 14q32.2 imprinted region. Since the microsatellite locus *D14S985* is present in two copies (Table S1) and the *MEG3*-DMR is deleted (Figure 2) in patient 2, this has served to localize the breakpoints. (B) Oligoarray comparative genomic hybridization for a ~1 Mb imprinted region. All the signals remain within the normal range (-1 SD ~ +1 SD) (shaded in light blue) in patients 1 and 2.
Found at: doi:10.1371/journal.pgen.1000992.s001 (1.17 MB TIF)

Figure S2 Expression analysis. (A) Maternal *MEG3* expression in the leukocytes of normal subjects. Genotyping has been performed for three cSNPs using genomic DNA (gDNA) and cDNA of leukocytes from control subjects and gDNA samples of their mothers, indicating that both maternally and non-maternally (paternally) derived alleles are delineated in the gDNA, whereas maternally inherited alleles alone are identified in cDNA. These three cSNPs have also been studied in the mother of patient 1 (Figure 5D). (B) Paternal *RTL1* expression in the placenta of a normal subject. Genotyping has been carried out for *RTL1* cSNP using gDNA and cDNA samples of a fresh placenta and gDNA sample from the mother, showing that both maternally and non-maternally (paternally) derived alleles are delineated in the gDNA, whereas a non-maternally (paternally) inherited allele alone is detected in cDNA. This cSNP has also been examined in the placenta of patient 1 (Figure 5E). Furthermore, the results confirm that the primers utilized in this study have amplified *RTL1*, but not *RTL1as*.
Found at: doi:10.1371/journal.pgen.1000992.s002 (0.39 MB TIF)

Figure S3 Schematic representation of the observed and predicted methylation and expression patterns in previously reported cases with upd(14)pat/mat-like phenotypes and in normal and upd(14)pat/mat subjects. For the explanations of the illustrations, see the legend for Figure 6. Previous studies have indicated that (1) Epimutation-1, Deletion-1, Deletion-2, and Deletion-3 lead to maternal to paternal epigenotypic alteration; (2) Epimutation-2 results in paternal to maternal epigenotypic alteration; and (3) Deletion-4 and Deletion-5 have no effect on the epigenotypic status [2,5–8,26]. (A) Cases with typical or mild upd(14)pat phenotype. Epimutation-1: Hypermethylation of the IG-DMR and the *MEG3*-DMR of maternal origin in the body, and that of the IG-DMR of maternal origin in the placenta (the *MEG3*-DMR is rather hypomethylated in the placenta) (cases 6–8 in Kagami et al. [2]). Deletion-1: Microdeletion involving *DLK1*, the two DMRs, and *MEG3* on the maternally inherited chromosome (case 2 in Kagami et al. [2]). Deletion-2: Microdeletion involving *DLK1*, the two DMRs, *MEG3*, *RTL1*, and *RTL1as* on the maternally inherited chromosome (cases 3 and 5 in Kagami et al. [2]). Deletion-3: Microdeletion involving the two DMRs, *MEG3*, *RTL1*, and *RTL1as* on the maternally inherited chromosome (case 4 in Kagami et al. [2]). These findings are explained by the following notions: (1) Epimutation (hypermethylation) of the normally hypomethylated IG-DMR of maternal origin directly results in paternalization of the imprinted region in the placenta and indirectly leads to paternalization of the imprinted region in the body via epimutation (hypermethylation) of the usually hypomethylated *MEG3*-DMR of maternal origin. Thus, the epimutation (hypermethylation) is predicted to have impaired the IG-DMR as the primary target, followed by the epimutation (hypermethylation) of the *MEG3*-DMR after fertilization; (2) Loss of the hypomethylated *MEG3*-DMR of maternal origin leads to paternalization of the imprinted region in the body; and (3) Loss of the hypomethylated IG-DMR of maternal origin results in paternalization of the imprinted region in the placenta. Furthermore, epigenotype-phenotype correlations imply that the severity of upd(14)pat phenotype is primarily determined by the *RTL1* expression dosage rather than the *DLK1* expression dosage [2]. (B) Cases with upd(14)mat-like phenotype. Epimutation-2: Hypomethylation of the IG-DMR and the *MEG3*-DMR of paternal origin (Temple et al. [5], Buiting et al. [6], Hosoki et al. [7], and Zechner et al. [8]). Deletion-4: Microdeletion involving *DLK1*, the two DMRs, and *MEG3* on the paternally inherited chromosome (cases 9 and 10 in Kagami et al. [2]). Deletion-5: Microdeletion involving *DLK1*, the two DMRs, *MEG3*, *RTL1*, and *RTL1as* on the paternally inherited chromosome (case 11 in Kagami et al. [2] and patient 3 in Buiting et al. [6]). These findings are consistent with the following notions: (1) Epimutation (hypomethylation) of the normally hypermethylated IG-DMR of paternal origin directly results in maternalization of the imprinted region in the placenta and indirectly leads to maternalization of the imprinted region in the body through epimutation (hypomethylation) of the usually hypermethylated *MEG3*-DMR of paternal origin. Thus, epimutation (hypomethylation) is predicted to have affected the IG-DMR

as the primary target, followed by the epimutation (hypomethylation) of the *MEG3*-DMR after fertilization; and (2) Loss of the hypermethylated DMRs of paternal origin has no effect on the imprinting status [2,26], so that upd(14)mat-like phenotype is primarily ascribed to the additive effects of loss of functional *DLK1* and *RTL1* from the paternally derived chromosome (the effects of loss of *DIO3* appears to be minor, if any [2,35]). Although the *MEGs* expression dosage is predicted to be normal in Deletion-4 and Deletion-5 and doubled in Epimutation-2 as well as in upd(14)mat, it remains to be determined whether the difference in the *MEGs* expression dosage has major clinical effects or not. (C) Normal and upd(14)pat/mat subjects.
Found at: doi:10.1371/journal.pgen.1000992.s003 (2.72 MB TIF)

Table S1 The results of microsatellite and SNP analyses.
Found at: doi:10.1371/journal.pgen.1000992.s004 (0.19 MB DOC)

Table S2 Clinical features in the mother of patient 1.
Found at: doi:10.1371/journal.pgen.1000992.s005 (0.09 MB DOC)

Table S3 Primers utilized in the present study.
Found at: doi:10.1371/journal.pgen.1000992.s006 (0.14 MB DOC)

Author Contributions

Conceived and designed the experiments: MK ACFS TO. Performed the experiments: MK MF KM FK. Analyzed the data: MK TO. Contributed reagents/materials/analysis tools: MJO AJG YW OA NM KM TO. Wrote the paper: TO.

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Epigenetics 7:10, 1142–1150; October 2012; © 2012 Landes Bioscience

Paternal uniparental disomy 14 and related disorders

Placental gene expression analyses and histological examinations

Masayo Kagami,[1] Kentaro Matsuoka,[2] Toshiro Nagai,[3] Michiko Yamanaka,[4] Kenji Kurosawa,[5] Nobuhiro Suzumori,[6] Yoichi Sekita,[7] Mami Miyado,[1] Keiko Matsubara,[1] Tomoko Fuke,[1] Fumiko Kato,[1,8] Maki Fukami[1] and Tsutomu Ogata[1,8,*]

[1]Department of Molecular Endocrinology; National Research Institute for Child Health and Development; Tokyo, Japan; [2]Departments of Pathology; National Center for Child Health and Development; Tokyo, Japan; [3]Department of Pediatrics; Dokkyo University School of Medicine; Koshigaya, Japan; [4]Department of Integrated Women's Health; St. Luke's International Hospital; Tokyo, Japan; [5]Division of Medical Genetics; Kanagawa Children's Medical Center; Yokohama, Japan; [6]Department of Obstetrics and Gynecology; Nagoya City University Graduate School of Medicine; Nagoya, Japan; [7]Department of Pathology; Graduate School of Medicine; Osaka University, Osaka, Japan; [8]Department of Pediatrics; Hamamatsu University School of Medicine; Hamamatsu, Japan

Keywords: Upd(14)pat, microdeletion, placenta, expression dosage, histopathology, imprinting

Abbreviations: *PEGs*, paternally expressed genes; *MEGs*, maternally expressed genes; DMRs, differentially methylated regions; IG-DMR, *DLK1-MEG3* intergenic DMR; *RTL1as*, *RTL1* antisense; upd(14)pat, paternal uniparental disomy 14; BWS, Beckwith-Wiedemann syndrome; q-PCR, quantitative real-time PCR; CGH, oligoarray comparative genomic hybridization; LM, light microscopic; EM, electron microscopic; IHC, immunohistochemical

Although recent studies in patients with paternal uniparental disomy 14 [upd(14)pat] and other conditions affecting the chromosome 14q32.2 imprinted region have successfully identified underlying epigenetic factors involved in the development of upd(14)pat phenotype, several matters, including regulatory mechanism(s) for *RTL1* expression, imprinting status of *DIO3* and placental histological characteristics, remain to be elucidated. We therefore performed molecular studies using fresh placental samples from two patients with upd(14)pat. We observed that *RTL1* expression level was about five times higher in the placental samples of the two patients than in control placental samples, whereas *DIO3* expression level was similar between the placental samples of the two patients and the control placental samples. We next performed histological studies using the above fresh placental samples and formalin-fixed and paraffin-embedded placental samples obtained from a patient with a maternally derived microdeletion involving *DLK1*, the-IG-DMR, the *MEG3*-DMR and *MEG3*. Terminal villi were associated with swollen vascular endothelial cells and hypertrophic pericytes, together with narrowed capillary lumens. DLK1, RTL1 and DIO3 proteins were specifically identified in vascular endothelial cells and pericytes, and the degree of protein staining was well correlated with the expression dosage of corresponding genes. These results suggest that *RTL1as*-encoded microRNA functions as a repressor of *RTL1* expression, and argue against *DIO3* being a paternally expressed gene. Furthermore, it is inferred that DLK1, DIO3 and, specially, RTL1 proteins, play a pivotal role in the development of vascular endothelial cells and pericytes.

Introduction

Human chromosome 14q32.2 region carries a cluster of imprinted genes including protein coding paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* (alias *PEG11*) and non-coding maternally expressed genes (*MEGs*) such as *MEG3* (alias *GTL2*) and *RTL1as* (*RTL1* antisense encoding microRNAs).[1,2] 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.[2] We have previously revealed that the hypomethylated IG-DMR and *MEG3*-DMR of maternal origin function as imprinting control centers in the placenta and the body, respectively, and that 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.[3]

*Correspondence to: Tsutomu Ogata; Email: tomogata@hama-med.ac.jp
Submitted: 06/21/12; Revised: 08/20/12; Accepted: 08/22/12
http://dx.doi.org/10.4161/epi.21937

Consistent with these findings, paternal uniparental disomy 14 [upd(14)pat] results in a unique phenotype characterized by facial abnormality, small bell-shaped thorax with coat hanger appearance of the ribs, abdominal wall defects, placentomegaly and polyhydramnios.[2,4] We have studied multiple patients with upd(14)pat and related conditions, such as epimutations of the maternally derived DMRs and various types of microdeletions involving the maternally inherited imprinted region, suggesting that markedly increased *RTL1* expression is the major underlying factor for the development of upd(14)pat-like phenotype.[2] The notion of excessive *RTL1* expression is primarily based on the following mouse data indicating a trans-acting repressor function of *Rtl1as*-encoded microRNAs for *Rtl1* expression: (1) targeted deletion of the maternally derived IG-DMR causes maternal to paternal epigenotypic switch of the imprinted region, with ~4.5 times rather than ~2 times of *Rtl1* expression as well as ~2 times of *Dlk1* expression and nearly absent *Megs* expression, in the presence of two functional copies of *Pegs* and no functional copy of *Megs*[5] and; (2) targeted deletion of the maternally derived *Rtl1as* results in 2.5–3.0 times of *Rtl1* expression, in the presence of a single functional copy of *Rtl1*.[6] Similarly, in the human, typical upd(14)pat phenotype is observed in patients with epimutations that are likely associated with markedly increased *RTL1* expression because of the combination of two functional copies of *RTL1* and no functional copy of *RTL1as*, whereas relatively mild upd(14)pat-like phenotype is found in patients with maternally inherited microdeletions involving *RTL1as* that are likely accompanied by moderately elevated *RTL1* expression because of the combination of a single functional copy of *RTL1* and no functional copy of *RTL1as*.[2]

Human imprinting disorders are usually associated with placental abnormalities. For example, Beckwith-Wiedemann syndrome (BWS) and upd(14)pat are associated with placentomegaly,[4,7] and Silver-Russell syndrome is accompanied by hypoplastic placenta.[8] Similarly, mouse imprinting aberrations also usually affect placental growth and development.[9] In agreement with this, virtually all the imprinted genes studied to date are expressed in the placenta and play a pivotal role in the placental growth and development,[10] although placental structure is more or less different between placental animals.[11]

However, several matters remain to be clarified in upd(14)pat and related conditions. For example, it is unknown whether human *RTL1* expression is actually elevated in the absence of functional *RTL1as*-encoded microRNAs. It is also unknown whether *DIO3* is a *PEG*, although mouse *Dio3* has been shown to undergo partial imprinting.[12] In this regard, while we examined fresh blood cells, cultured skin fibroblasts and formalin-fixed and paraffin-embedded placental and body samples obtained from patients with upd(14)pat-like phenotype, precise assessment of *RTL1* and *DIO3* expression levels was impossible because of extremely low *RTL1* and *DIO3* expression levels in fresh blood cells and cultured skin fibroblasts and poor quality of RNAs extracted from paraffin-embedded tissues.[2,3] In addition, while cSNP genotyping has demonstrated paternal *DLK1* and *RTL1* expression and maternal *MEG3* expression in the body and the placenta,[2,3] no informative cSNP data showing paternal *DIO3* expression have been obtained.[2,3] Furthermore, although standard light microscopic (LM) examinations have been performed using formalin-fixed and paraffin-embedded placental samples, fine placental histopathological studies, such as electron microscopic (EM) examinations and immunohistochemical (IHC) examinations, remain to be performed.

To examine these unresolved matters, fresh placental tissues are highly useful, because precise quantitative real-time PCR (q-PCR) analyses and EM studies can be performed with fresh placentas. Thus, we performed q-PCR analyses and EM studies, as well as IHC studies with RTL1 antibodies produced by ourselves and commercially available DLK1 and DIO3 antibodies, using fresh placental samples obtained from two previously reported patients with prenatally diagnosed upd(14)pat.[13,14] We also performed IHC studies using formalin-fixed and paraffin-embedded placental samples obtained from a previously reported patient with a microdeletion involving *DLK1*, but not *RTL1* and *DIO3*,[2] to compare the placental protein expression levels between upd(14)pat and the microdeletion. Furthermore, we also studied a hitherto unreported patient with an unbalanced translocation involving the 14q32.2 imprinted region, to obtain additional data regarding the *RTL1-RTL1as* interaction and the primary factor for the development of upd(14)pat phenotype.

Results

Patients and samples. This study consisted of three previously reported patients with typical body and placental upd(14)pat phenotype and a normal karyotype (cases 1–3),[2,13-15] and a new patient with various non-specific features and a 46,XX,der(17)t(14;17)(q31;p13) karyotype accompanied by three copies of the distal 14q region and a single copy of the terminal 17p region (case 4) . Clinical phenotypes of cases 1–4 are summarized in **Table S1**. In brief, cases 1 and 2 were suspected to have upd(14)pat phenotype including bell-shaped thorax by prenatal ultrasound studies performed for polyhydroamnios, and were confirmed to have upd(14)pat by microsatellite analysis after birth. Case 3 was found to have typical upd(14)pat phenotype during infancy and was shown to have a maternally derived microdeletion affecting the chromosome 14q32.2 imprinted region. Case 4 had growth failure, developmental delay, multiple non-specific anomalies, and omphalocele. There was no history of polyhydramnios or placentomegaly. Thus, except for omphalocele, case 4 had no upd(14)pat-like phenotype. The parental karyotype was normal, indicating a de novo occurrence of the unbalanced translocation.

We obtained fresh placental samples immediately after birth from prenatally diagnosed cases 1 and 2 for molecular studies using genomic DNA and RNA, and fresh leukocyte samples from cases 1, 2 and 4 and their parents for molecular studies using genomic DNA. The fresh placental samples of cases 1 and 2 were also utilized for histopathological examinations, together with formalin-fixed and paraffin-embedded placental samples of case 3. For controls, we obtained three fresh placentas at 37 weeks of gestation, and fresh leukocytes from three adult subjects; for molecular studies using placentas, we prepared pooled samples

consisting of an equal amount of DNA or RNA extracted from each placenta.

Molecular studies in cases 1 and 2. We performed microsatellite analysis for 19 loci on chromosome 14 and bisulfite sequencing for the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7), using placental and leukocyte genomic DNA samples; while microsatellite analysis had been performed for 15 loci in case 1 and 16 loci in case 2, only leukocyte genomic DNA samples were examined in the previous study.[15] Consequently, we identified two peaks for *D14S609* and single peaks for the remaining loci in case 1 (the combination of paternal heterodisomy and isodisomy), and single peaks for all the examined loci in case 2 (apparently full paternal isodisomy) (**Table S2**). Furthermore, no trace of maternally inherited peak was identified in both placental and leukocyte genomic DNA samples (**Fig. 1**). Bisulfite sequencing showed that both the IG-DMR and the *MEG3*-DMR were markedly hypermethylated in the leukocytes of cases 1 and 2, whereas in the placental samples the IG-DMR was obviously hypermethylated and the *MEG3*-DMR was grossly hypomethylated to an extent similar to that identified in control placentas (**Fig. 2**). Furthermore, q-PCR analysis for placental RNA samples revealed that *DLK1*, *RTL1*, and *DIO3* expression levels were 3.3 times, 6.1 times and 1.9 times higher in the placental samples of case 1 than in the control placental samples, respectively, and were 3.1 times, 9.4 times and 1.7 times higher in the placental samples of case 2 than in the control placental samples, respectively (**Fig. 3A**). By contrast, the expressions of all *MEGs* examined were virtually absent in the placental samples of cases 1 and 2. PCR products were sufficiently obtained after 30 cycles for the fresh placental as well as leukocyte samples, consistent with high quality of DNA and RNA obtained from fresh materials.

Molecular studies in case 3. Detailed molecular findings have already been reported previously.[2] In brief, microsatellite analysis revealed biparentally derived homologs of chromosome 14, and a deletion analysis demonstrated a maternally inherited 108,768 bp microdeletion involving *DLK1*, the IG-DMR, the *MEG3*-DMR, and *MEG3*, but not affecting *RTL1*/*RTL1as*. Since loss of the DMRs causes maternal to paternal epigenotypic alteration,[2] it is predicted that case 3 has a single functional copy of *DLK1* and two functional copies of *RTL1* and *DIO3*, as well as no functional copy of *RTL1as* and other *MEGs*. Bisulfite sequencing showed that both the IG-DMR and the *MEG3*-DMR were markedly hypermethylated in leukocytes, whereas in the formalin-fixed and paraffin-embedded placental samples the IG-DMR was obviously hypermethylated and the *MEG3*-DMR was comprised of roughly two-thirds of hypermethylated clones and roughly one-third of hypomethylated clones. In addition, RT-PCR analysis for such placental samples indicated positive *PEGs* (especially *RTL1*) expression and absent *MEGs* expression. For the formalin-fixed and paraffin-embedded placental samples, PCR products could be obtained only after 35 cycles, because of poor quality (severe degradation) of DNA and RNA.

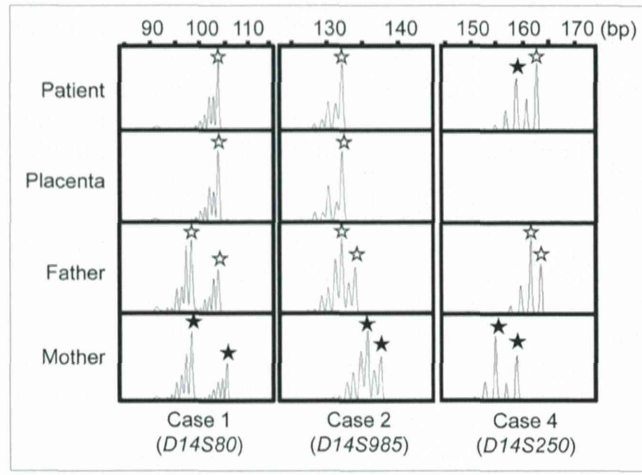

Figure 1. Representative results of microsatellite analysis, using leukocyte genomic DNA samples of the patient and the parents and placental genomic DNA samples. In cases 1 and 2, one of the two paternal peaks is inherited by the patients and the placentas, and no trace of maternal peaks is identified. In case 4, both paternally and maternally derived peaks are found in the patient, with the paternally derived long peak being larger than the maternally inherited short peak.

Molecular findings in case 4. We examined the presence or absence of the 14q32.2 imprinted region on the der(17) chromosome (**Fig. 4**). Oligoarray comparative genomic hybridization (CGH) indicated three copies of a ~19.6 Mb 14q31–qter region, and FISH analysis for four segments around the chromosome 14q32.2 imprinted region delineated positive signals on the der(17) chromosome as well as on the normal chromosome 14 homologs. This demonstrated the presence of the 14q32.2 imprinted region on the der(17) chromosome. In addition, similar oligoarray CGH and FISH analysis revealed loss of a ~455 kb region from the distal chromosome 17p (**Fig. S1**).

Thus, we investigated the parental origin of the translocated 14q distal region. Microsatellite analysis for *D14S250* and *D14S1007* on the translocated 14q distal region delineated biparentally derived two peaks, with paternally derived long PCR products showing larger peaks than maternally derived short PCR products (**Fig. 1**; **Table S2**). Since short products are usually more easily amplified than long products, this indicated paternal

Figure 2 (See opposite page). Bisulfite sequencing analysis of the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7), using leukocyte and placental genomic DNA samples. Filled and open circles indicate methylated and unmethylated cytosines at the CpG dinucleotides, respectively. Upper part: structure of CG4, CG6, and CG7. Pat, paternally derived chromosome; Mat, maternally derived chromosome. The PCR products for CG4 (311 bp) harbor 6 CpG dinucleotides and a G/A SNP (*rs12437020*), those for CG6 (428 bp) carry 19 CpG dinucleotides and a C/T SNP (*rs10133627*) and those for CG7 (168 bp) harbor 7 CpG dinucleotides. Lower part: the results of cases 1, 2, 4 and a control subject. Each horizontal line indicates a single subcloned allele. The control data represent the methylation patterns obtained with a leukocyte genomic DNA sample extracted from a single subject heterozygous for the G/A SNP (*rs12437020*) (body) and those obtained with a pooled DNA sample consisting of an equal amount of genomic DNA extracted from three control placentas homozygous for that SNP.

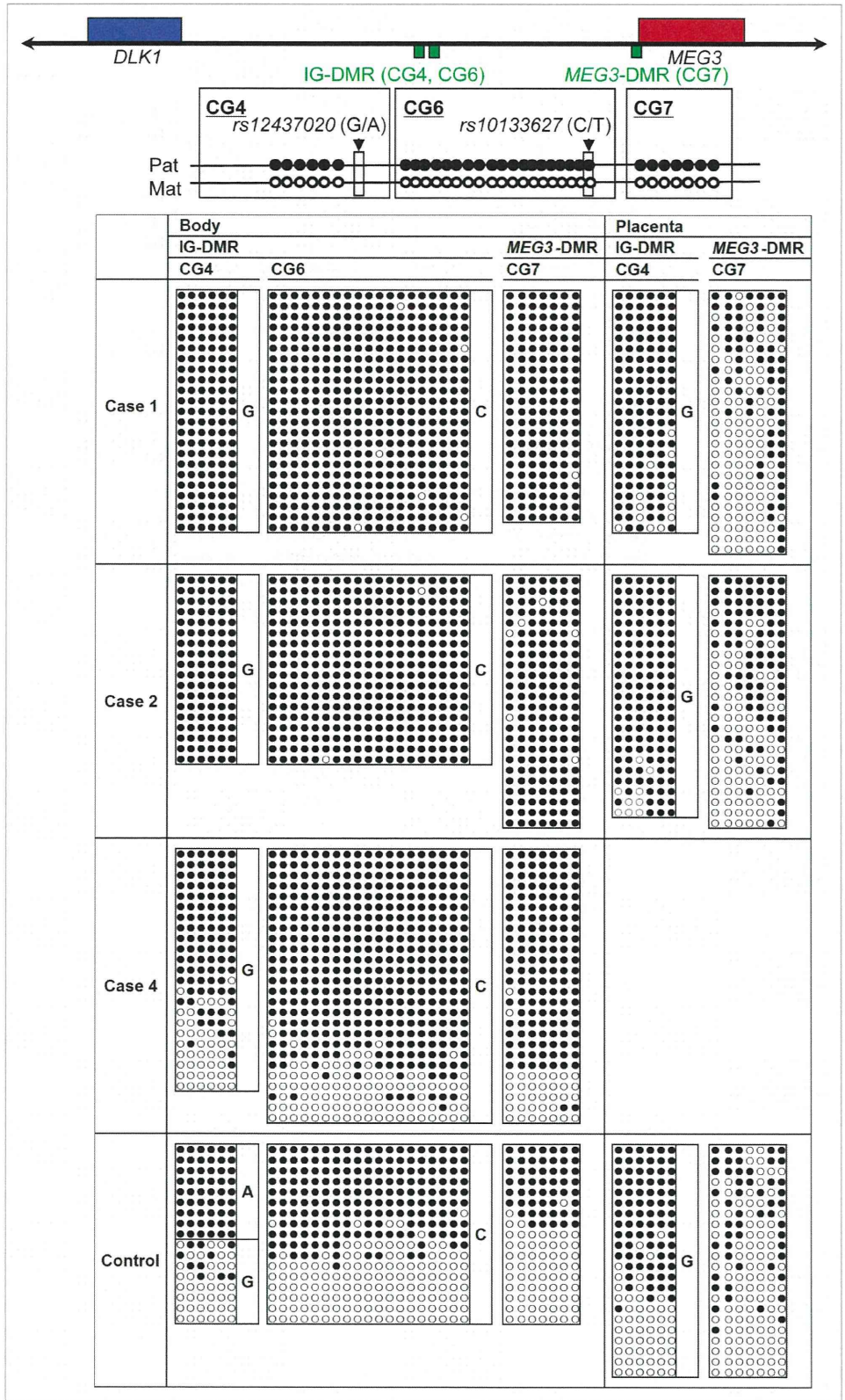

Figure 2. For figure legend, see page 1144.

origin of the der(17) chromosome harboring the chromosome14q32.2 imprinted region. Consistent with this, bisulfite sequencing showed moderate hypermethylation of the IG-DMR and the *MEG3*-DMR (Fig. 2).

Placental histopathological studies. We performed LM and EM studies, and IHC examinations (Fig. 5). LM examinations showed proliferated chorionic villi in cases 1–3. Capillary lumens were irregularly dilated with thickened endothelium in the stem to intermediate villi, but not in the terminal villi. Immature villi were present in case 3, probably because of 30 weeks of gestational age. Chorangioma was also identified in case 3. There was no villous chorangiosis, edematous change of villous stroma, or mesenchymal dysplasia characterized by grapelike vesicles in cases 1–3. Although the terminal villi exhibited no definitive abnormalities in the LM studies, EM examinations revealed swelling of vascular endothelial cells and hypertrophic change of pericytes in the terminal villi, together with narrowed capillary lumens, in cases 1 and 2.

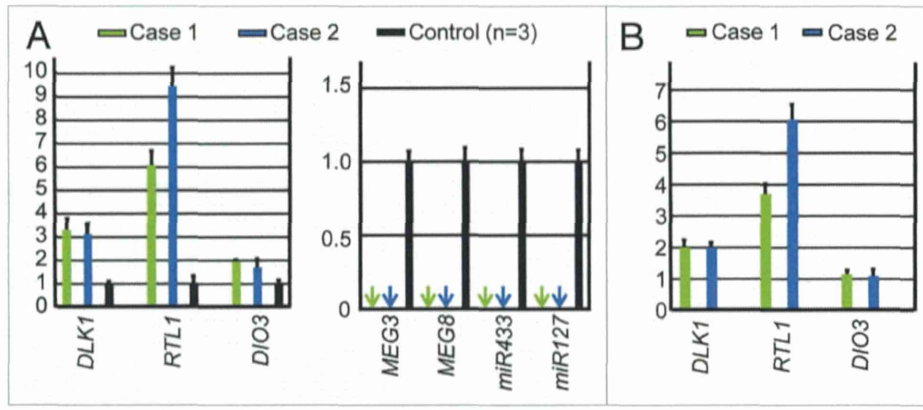

Figure 3. Quantitative real-time PCR analysis using placental samples. For a control, a pooled RNA sample consisting of an equal amount of total RNA extracted from three fresh control placentas was utilized. (**A**) Relative mRNA expression levels for *DLK1*, *RTL1*, and *DIO3* against *GAPDH* (mean ± SE) and lack of *MEGs* expression (indicated by arrows) (*miR433* and *miR127* are encoded by *RTL1as*) in the placental samples of cases 1 and 2. (**B**) Relative mRNA expression levels for *DLK1*, *RTL1*, and *DIO3* against *GAPDH* (mean ± SE), in the equal amount of expression positive placental cells (vascular endothelial cells and pericytes) of cases 1 and 2 (corrected for the difference in the relative proportion of expression positive cells between the placental samples of cases 1 and 2 and the control placental samples, on the assumption that the *DLK1* expression level is "simply doubled" in the expression positive placental cells of case 1 and 2).

IHC examinations identified RTL1, DLK1 and DIO3 protein expressions in the vascular endothelial cells and pericytes of chorionic villi, but not in the cytotrophoblasts, syncytiotrophoblasts, and stromal cells, in the placentas of cases 1–3 and in the control placenta. The PEGs protein expression level was variable in the control placenta, with moderate DLK1 expression, high RTL1 expression, and low DIO3 expression. Furthermore, DLK1 protein expression was apparently stronger in the placentas of cases 1 and 2 than in the placenta of case 3 and the control placenta, RTL1 protein expression was obviously stronger in the placentas of cases 1–3 than in the control placenta, and DIO3 protein expression was apparently similar between the placentas of cases 1–3 and the control placenta.

Discussion

We studied placental samples obtained from cases 1–3 with typical body and placental upd(14)pat phenotype. In this regard, the microsatellite data suggest that upd(14)pat with heterodisomic and isodisomic loci in case 1 was caused by trisomy rescue or gamete complementation, and that upd(14)pat with isodisomic loci alone in case 2 resulted from monosomy rescue or postzygotic mitotic error, although it is possible that heterodisomic locus/loci remained undetected in case 2.[15] Notably, there was no trace of a maternally inherited locus indicative of the presence of trisomic cells or normal cells with biparentally inherited chromosome 14 homologs in the placentas as well as in the leukocytes of cases 1 and 2. In addition, the microdeletion of case 3 has been shown to be inherited from the mother with the same microdeletion.[2] These findings imply that the placental tissues as well as the leukocytes of cases 1–3 almost exclusively, if not totally, consisted of cells with upd(14)pat or those with the microdeletion.

The q-PCR analysis was performed for the fresh placental samples of cases 1 and 2. In this context, two matters should be pointed out. First, the proportion of vascular endothelial cells and pericytes expressing *DLK1*, *RTL1*, and *DIO3* would be somewhat variable among samples, because only a small portion of the placenta was analyzed. This would be relevant to the some degree of difference in the expression levels between the placental samples of cases 1 and 2. Second, the relative proportion of vascular endothelial cells and pericytes expressing *DLK1*, *RTL1*, and *DIO3* would be higher in the placental samples of cases 1 and 2 than in the control placental samples, because the placentas of cases 1 and 2 were accompanied by proliferation of the chorionic villi with such expression positive cells. Thus, it would be inappropriate to perform a simple comparison of relative expression levels against *GAPDH* between the placental samples of cases 1 and 2 and the control placental samples. Indeed, although a complex regulatory mechanism(s), as implicated for the *RTL1* expression,[1,2] is unlikely to be operating for the *DLK1* expression, the relative *DLK1* expression level was 3.3 times and 3.1 times, not 2 times, higher in the placental samples of cases 1 and 2 than in the control placental samples, respectively (Fig. 3A). Assuming that *DLK1* expression level is simply doubled in expression positive cells of cases 1 and 2, it is predicted that the relative proportion of such expression positive cells is 1.65 times (3.3 ÷ 2.0) and 1.55 times (3.1 ÷ 2.0) larger in the placental samples of cases 1 and 2 than in the control placental samples, respectively. Thus, the expression level against *GAPDH*

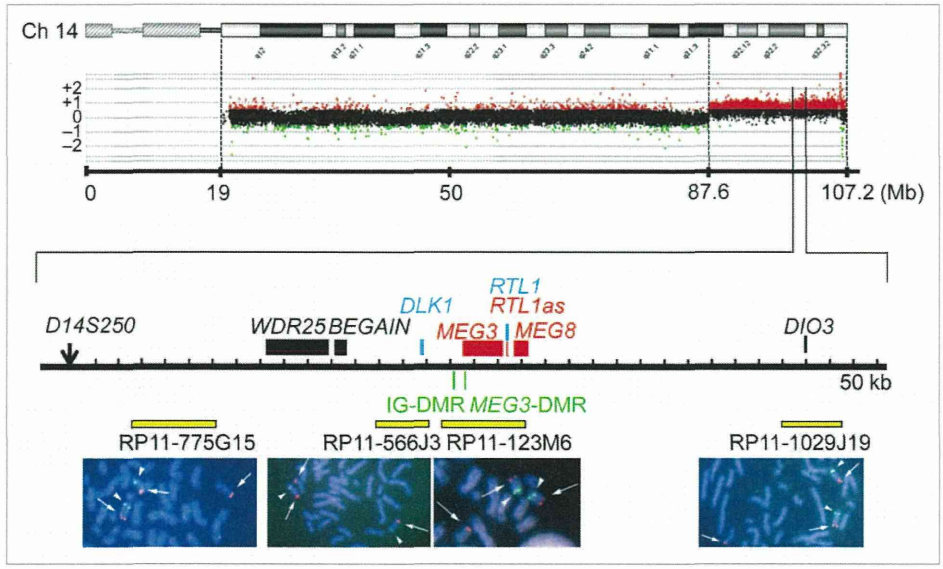

Figure 4. Array CGH and FISH analysis for the distal chromosome 14 region in case 4. In CGH analysis, the black, the red, and the green dots denote signals indicative of the normal, the increased (> +0.5), and the decreased (< −1.0) copy numbers, respectively. In FISH analysis, red signals (arrows) are derived from the probes detecting the various parts of the 14q32.2 imprinted region (the physical positions are indicated with yellow bars), and the green signals (arrowheads) are derived from an RP11–566I2 probe for 14q11.2 used as an internal control.

in the equal amount of expression positive cells is estimated as 3.69 times (6.1 ÷ 1.65) increased for *RTL1* and 1.15 times (1.9 ÷ 1.65) increased for *DIO3* in case 1, and as 6.06 times (9.4 ÷ 1.55) increased for *RTL1* and 1.09 times (1.7 ÷ 1.55) increased for *DIO3* in case 2 (**Fig. 3B**).

Thus, the expression data are summarized as follows (**Fig. 6**). First, it is inferred that the relative *RTL1* expression level is markedly (~5 times) increased in the expression positive cells of the placentas with upd(14)pat, as compared with the control placentas. This degree of elevation is grossly similar to that identified in the body of mice with the targeted deletion of the maternally derived IG-DMR (~4.5 times).[5] Such a markedly increased *RTL1* expression would be explained by assuming that *RTL1as*-encoded microRNAs (e.g., *miR433* and *miR127*) function as a repressor for *RTL1* expression through the RNAi mechanism, as has been indicated for the mouse *Rtl1-Rtl1as* interaction.[16,17] Second, it is unlikely that *DIO3* is solely expressed from the paternally inherited allele, although it remains to be determined whether *DIO3* undergoes partial imprinting like mouse *Dio3*[12] or completely escapes imprinting. In either case, the results would explain why patients with upd(14)pat and upd(14)mat lack clinically recognizable thyroid disorders,[2] although *DIO3* plays a critical role in the inactivation of thyroid hormones.[18]

This study provides further support for a critical role of excessive *RTL1* expression in the development of upd(14)pat phenotype (**Fig. 6**). Indeed, markedly (~5 times) increased *RTL1* expression is shared in common by cases 1–3 with typical upd(14)pat body and placental phenotype. In this context, it is notable that case 4 had no clinically recognizable upd(14)pat body and placental phenotype, except for omphalocele. This would imply that a single copy of *RTL1as* can almost reduce the *RTL1* expression dosage below the threshold level for the development of upd(14)pat phenotype by exerting a trans-acting repressor effect on the two functional copies of *RTL1*. By contrast, the relevance of *DLK1* to upd(14)pat phenotype is unlikely, because case 3 exhibited typical upd(14)pat phenotype in the presence of a single functional copy of *DLK1*, and case 4 showed no upd(14)pat phenotype except for omphalocele in the presence of two functional copies of *DLK1*. Similarly, if *DIO3* were more or less preferentially expressed from paternally inherited allele, the relevance of *DIO3* to upd(14)pat phenotype would also remain minor, if any. Case 4 had no upd(14)pat phenotype except for omphalocele in the presence of with two copies of *DIO3* of paternal origin. It should be pointed out, however, that the absence of *MEGs* expression may have a certain effect on the development of upd(14)pat phenotype.

The placental histological examinations revealed several informative findings. First, DLK1, RTL1, and DIO3 proteins were specifically identified in vascular endothelial cells and pericytes of chorionic villi in the control placenta, with RTL1 protein being most strongly expressed. These results, together with abnormal LM and EM findings of such cells in cases 1–3, suggest that these proteins, especially RTL1 protein, plays a pivotal role in the development of endothelial cells and pericytes. In this regard, it may be possible that the endothelial thickening and resultant narrowing the capillary lumens in the terminal villi have resulted in the dilatation of the stem to intermediate portions of the chorionic villi.

Second, the degree of protein staining was well correlated with the expression dosage of corresponding genes. In this regard, since characteristic macroscopic and microscopic placental features were identified in cases 1–3 who shared markedly elevated RTL1 protein expression, this is consistent with the notion that upd(14)pat phenotype is primarily caused by the markedly

elevated *RTL1* expression.[2] Indeed, DLK1 protein expression was not exaggerated in case 3 with typical upd(14)pat phenotype, and DIO3 protein expression was not enhanced in cases 1–3. It may be possible, however, that the abnormality of placental structures may have resulted in a difference in immunostaining without an actual change in gene expression. This point awaits further investigations.

Third, villous chorangiosis, stromal expansion, and mesenchymal dysplasia were not identified in the placental samples of cases 1–3, although such a lesion(s) may have existed in non-examined portions. Notably, such lesions are frequently observed in placentas of patients with BWS.[19-21] Thus, while both upd(14)pat and BWS are associated with placentomegaly and polyhydroamnios, characteristic histological findings appear to be different between upd(14)pat and BWS.

This study would also provide useful information on the methylation patterns of the *MEG3*-DMR in the placenta. Our previous studies using formalin-fixed and paraffin-embedded placental samples revealed that roughly two-thirds of clones were hypermethylated and the remaining roughly one-third of clones were hypomethylated in case 3 as well as in the previously reported patients with upd(14)pat (not cases 1 and 2) and epimutation (hypermethylation of the IG-DMR and the *MEG3*-DMR of maternal origin), and that roughly one-third of clones were hypermethylated and the remaining roughly two-thirds of clones were hypomethylated in control placental samples (see Fig. S2C in ref. 2). However, this study showed that the *MEG3*-DMR was grossly hypomethylated in the fresh placental samples of cases 1 and 2, with an extent similar to that identified in the fresh control placental samples. In this regard, it is notable that PCR products could be obtained only after 35 cycles for the formalin-fixed and paraffin-embedded placental samples and were sufficiently obtained after 30 cycles for the fresh placental samples. Thus, several specific clones may have been selectively amplified in the previous study. Furthermore, it may be possible that efficacy of bisulfite treatment (conversion of unmethylated cytosine into uracils and subsequently thymines) may be insufficient for the formalin-fixed and paraffin-embedded placental samples. Thus, it appears that the present data denote precise methylation patterns of the *MEG3*-DMR in the placenta.

In summary, the present study provides useful clues for the clarification of regulatory mechanism for the *RTL1* expression, imprinting status of *DIO3* and characteristic placental histological findings in patients with upd(14)pat and related conditions. Further studies will help improve our knowledge about upd(14)pat and related conditions.

Methods

Ethical approval. This study was approved by the Institutional Review Board Committees of each investigator, and performed after obtaining written informed consent.

Primers. Primers utilized in this study are summarized in Table S3.

Sample preparation for molecular studies. Genomic DNA samples were obtained from leukocytes using FlexiGene DNA Kit (Qiagen) and from placental samples using ISOGEN (Nippon Gene). Transcripts of *DLK1, MEG3, RTL1, MEG8* and *DIO3* were isolated with ISOGEN (Nippon Gene), and *microRNAs* were extracted with mirVana™ miRNA Isolation Kit (Ambion). After DNase treatment, cDNA samples for *DLK1, MEG3, MEG8* and *DIO3* were prepared with oligo(dT) primers from 1 μg of RNA using Superscript III Reverse Transcriptase (Invitrogen), and those of *microRNAs* were synthesized from 300 ng of RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). For *RTL1*, 3'-RACE was utilized to prevent amplification of *RTL1as*; cDNA was synthesized from 1 μg of RNA using Superscript III Reverse Transcriptase with a long primer hybridizing to poly A site and introducing the adaptor sequence. Lymphocyte metaphase spreads for FISH analysis were prepared from leukocytes using colcemide (Invitrogen).

Molecular studies. Microsatellite analysis for 19 loci on chromosome 14, methylation analysis for the IG-DMR and

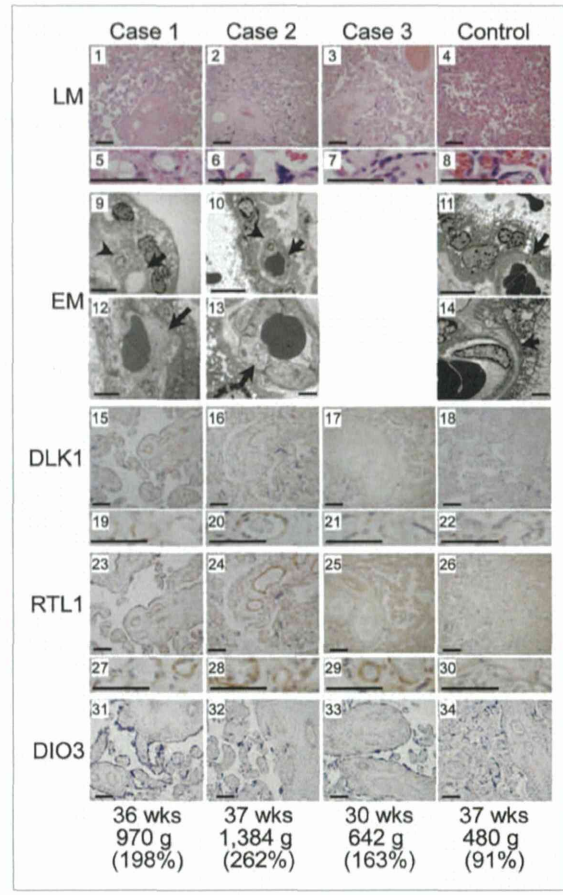

Figure 5. Histological examinations. LM, light microscopic examinations; EM, electron microscopic examinations; DLK1, RTL1 and DIO3, immunohistochemical examinations for the corresponding proteins. The arrows and arrowheads in the EM findings indicate endothelial cells and pericytes, respectively. Scale bars represent 100 μm for 1–4, 15–18, 23–26 and 31–34, 50 μm for 5–8, 19–22 and 27–30, 5 μm for 9–11 and 2 μm for 12–14. Gestational age, placental weight, and % placental weight assessed by the gestational age-matched Japanese references for placental weight[4,22] are described.