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Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

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BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Estimation of prevalence of imprinting disorders

The number of patients, who were diagnosed by genetic and cytogenetic testing and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. The 95% confidence interval (CI) was calculated as previously described (Wakai *et al.*, 1997). The prevalence was determined, based on the population of Japan in 2009 (127 510 000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation

Genomic DNA was obtained from blood or buccal mucosal cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al.*, 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients or their parents' consent.

Bisulfite-treatment PCR including the SNPs

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo *et al.*, 1997; Smith *et al.*, 2003; Kobayashi *et al.*, 2006, 2009; Wood *et al.*, 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed ~50% methylation (Supplementary data, Fig. S1). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (*LINE1*) and *Alu* repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi *et al.*, 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients conceived naturally using Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7%). The total number of cases was calculated using a second-stage survey ensuring the exclusion of duplicates (Table I). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). From these figures (and using the 2009 population of Japan: 127 510 000) we estimated the prevalence of these syndromes to be 1 in 287 000, 1 in 134 000, 1 in 62 000 and 1 in 392 000, respectively, for BWS, AS, PWS and SRS. Further details are given in Supplementary data, Table SII and Supplementary data, Fig. S2.

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/ total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence in situ hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome; AS, Angelman syndrome; PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64–0.98% of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (Table I, Supplementary data, Table SIII). The numbers of patients with PWS and AS we identified was low; however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed *H19* and *KCNQ1OT1 (LIT1)* gDMRs, for SRS we assayed the *H19* gDMR and for PWS and AS we assayed the *SNRPN* gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of *H19* at chromosome 11 is known to be a frequent occurrence in SRS (Bliek *et al.*, 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a

role in this syndrome (OMIM 180860). We first identified SNPs in the previously reported 22 human DMRs using genomic DNA isolated from human sperm and blood from unaffected individuals, which could then be used in bisulfite-PCR methylation assays to assign methylation to the parental allele. We next collected a total of 15 SRS samples, including previously collected samples (ART: 2, naturally conceived: 4), which had DNA methylation errors at the paternal gDMR at *H19*. Five of these were born from ART and 10 were from natural conceptions. We analyzed and compared the DNA methylation status of the 3 other paternal gDMRs and the 19 maternal gDMRs (Supplementary data, Fig. S3, Table, Supplementary data, Table SIV). In four out of the five ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These four cases showed a mixture of hyper- and hypomethylation with mosaic (partial) patterns. In contrast, only 3 of the 10 naturally conceived patients showed DNA methylation errors at loci other than *H19* gDMR.

To determine whether DNA methylation errors occurred in patients at a broader level in the genomes, we assessed the methylation profiles of the non-imprinted *LINE1* and *Alu* elements. We examined a total of 28 CpG sites in a 413-bp fragment of *LINE1* and 12 CpG sites in a 152-bp fragment of *Alu* (Supplementary data, Table SIV), and no significant differences were found in the methylation ratios between patients conceived by ART and naturally.

The abnormal methylation pattern in BWS patients with epimutations

In BWS, hypermethylation of *H19* or hypomethylation of *KCNQ1OT1(LIT1)* at human chromosome 11 are both frequently reported (Choufani *et al.*, 2010). We collected seven BWS samples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and six from naturally conceived patients (Supplementary data, Fig. S3, Table II, Supplementary data, Table SIV). In the one ART (ICSI) case, we identified four additionally gDMR methylation errors, again present at both maternally and paternally methylated gDMRs and with mixed hyper- and hypomethylation patterns. Furthermore, the methylation error at the *NESPAS* DMR was mosaic in this patient. One of the six naturally BWS cases had similar changes. Although we had only one BWS case conceived by ART, widespread methylation errors were similar to those for the DNA methylation error pattern in SRS.

Phenotypic differences between ART patients and those conceived naturally

The increased frequency of DNA methylation errors at other loci in the ART cases suggested that the BWS and SRS cases born after ART might exhibit additional phenotypic characteristics. However, when we compared in detail the clinical features from both categories of conception (Supplementary data, Table SV), we found no major differences between ART and naturally conceived patients with BWS and SRS.

Discussion

Our key finding from this study was a possible association between ART and the imprinting disorders, BWS and SRS. We did not find a similar association with PWS and AS but our numbers were quite low in this study and a larger due to the questionnaire return rate and relative rarity of the diseases, international study will be required to reach definitive conclusions. Furthermore, factors such as PCR and/or cloning bias in the bisulfite method and correction for changing rate of ART over time must be considered when analyzing any results.

In addition to the possible association between ART and BWS/SRS, we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART shown by us and others is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable. The process of ART exposes the developing epigenome to many external influences, which have been shown to influence the proper establishment and maintenance of genomic imprints, including hormone stimulation (Sato *et al.*, 2007), *in vitro* culturing (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003), cryopreservation (Emiliani *et al.*, 2000; Honda *et al.*, 2001) and the timing of embryo transfer (Shimizu *et al.*, 2004; Miura and Niikawa, 2005). Furthermore, we and others have also shown that some infertile males, particularly those with oligozoospermia, carry pre-existing imprinting errors in their sperm (Marques *et al.*, 2004; Kobayashi *et al.*, 2007; Marques *et al.*, 2008) which might account for the association between ART and imprinting disorders.

Imprinting syndromes and their association with ART

We report the first Japanese nationwide epidemiological study to examine four well-known imprinting diseases and their possible association with ART. We found that the frequency of ART use in both BWS and SRS was higher than anticipated based on the nationwide frequency of ART use at the time when these patients were born. Several other reports have raised concerns that children conceived by ART have an increased risk of disorders (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Lim and Maher, 2009). However, the association is not clear in every study (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). The studies reporting an association were mainly from case reports or case series whereas the studies where no association was reported were cohort studies. Therefore, the differences in the epidemiological analytical methods might accounts for the disparity in findings.

Owing to the rare nature of the imprinting syndromes, statistical analysis is challenging. In addition, the diagnosis of imprinting diseases is not always clear cut. Many of the syndromes have a broad clinical spectrum, different molecular pathogenesis, and the infant has to have reached a certain age before these diseases become clinically detectable. It is therefore likely that some children with these diseases are not recorded with the specific diagnosis code for these syndromes. Nonetheless, in this study we were examining the relationship between ART and the imprinting syndromes and these confounding factors are likely to apply equally to both groups.

Both BWS and SRS occurred after ART but our numbers for PWS and AS were low, precluding any definitive conclusion for these two disorders. However, while most cases of BWS and SRS are caused by an epimutation, epimutations are very rare in PWS and AS (only 1–4%) and ART would not be expected to increase chromosome 15

Table II Abnormal methylation in patients with SRS and BWS.

Case	ART	Abnormal methylation			
SRS					
SRS-1	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated	PEG10 hypermethylated (mosaic)	GRB10 hypermethylated; ZNF597 hypomethylated
SRS 2	IVF-ET	H19 hypomethylated (mosaic)			
SRS 3	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated (mosaic)		
SRS 4	IVF-ET	H19 hypomethylated	GRB10 hypermethylated		
SRS 5	IVF-ET	H19 hypomethylated (mosaic)	INPP5F hypermethylated		
SRS 6		H19 hypomethylated			
SRS-7		H19 hypomethylated (mosaic)	ZNF597 hypermethylated (mosaic)	ZNF331 hypomethylated (mosaic)	
SRS 8		H19 hypomethylated			
SRS 9		H19 hypomethylated (mosaic)			
SRS-10		H19 hypomethylated			
SRS-11		H19 hypomethylated (mosaic)	PEG1 hypermethylated		
SRS-12		H19 hypomethylated			
SRS-13		H19 hypomethylated (mosaic)	FAM50B hypomethylated		
SRS 14		H19 hypomethylated			
SRS 15		H19 hypomethylated			
BWS					
BWS-1	ICSI	LIT1 hypomethylated	ZDBF2 hypermethylated	PEG1 hypermethylated	NESPAS hypomethylated (mosaic)
BWS-2		LIT1 hypomethylated			
BWS-3		LIT1 hypomethylated			
BWS-4		LIT1 hypomethylated			
BWS-5		LIT1 hypomethylated			
BWS-6		LIT1 hypomethylated	ZDBF2 hypomethylated	ZNF331 hypomethylated (mosaic)	
BWS-7		LIT1 hypomethylated			

ET, embryo transfer. Summary of the abnormal methylation patterns in the ART conceived and naturally conceived patients with Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) with epimutations. Numbers in parentheses show the results of the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gDMRs in all patients with SRS and BWS examined are presented in Supplementary data, Table SIV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

deletions or uniparental disomy, consistent with our findings. Prior to this investigation, there was some evidence for an increased prevalence of BWS after ART but less evidence for an increased prevalence of SRS, with five SRS patients reported linked to ART (Svensson *et al.*, 2005; Bliek *et al.*, 2006; Kagami *et al.*, 2007; Galli-Tsinopoulou *et al.*, 2008). Our population wide study provides evidence to suggest that both BWS and SRS occur more frequently after ART in the Japanese population.

Mechanisms of epimutation in the patients conceived by ART

By performing a comprehensive survey of all the known gDMRs in a number of patients with BWS and SRS, we found that multiple loci were more likely to be affected in ART cases than those conceived naturally. Lim *et al.* (2009) have reported a similarly increased frequency of multiple errors after ART, with 37.5% of patients conceived with ART and 6.4% of naturally conceived patients displaying abnormal methylation at additional imprinted loci. However, while Bliek *et al.* (2009) reported alterations in multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of *KCNQ1OT1*(*LIT1*) ICR, only 1 of the cases with multiple alterations was born after ART. Similarly, Rossignol *et al.* (2006) reported that 3 of 11 (27%) ART-conceived patients and 7 of 29 (24%) naturally conceived patients displayed abnormal methylation at additional loci. In these four earlier studies, not all gDMRs were assayed and it may be that by doing so, these incongruities will be resolved.

The pattern of cellular mosaicism we observed in some patients suggested that the imprinting defects occurred after fertilization rather than in the gamete as DNA methylation alterations arising in the gamete would be anticipated to be present in every somatic cell. This suggested the possibility that the DNA methylation errors occurred as a consequence of impaired maintenance of the germline imprints rather than a failure to establish these imprints in the germline or a loss of these imprints in the sperm or oocytes *in vitro*. Furthermore, some patients conceived by ART with SRS and BWS showed

alterations at both maternally and paternally methylated gDMRs suggesting that the defects were not limited to one parental germline. The mechanisms controlling the protection of imprinted loci against demethylation early in the development remain unclear. Our data suggested that this protection may fail in ART resulting in the tissue-specific loss of imprints, though it remains unclear if this ever occurs naturally. Potential factors involved could include the culture conditions for the newly fertilized oocyte and the length of exposure to specific media or growth factors, as part of the ART procedure. Some of the naturally conceived patients also had abnormal methylation at both maternally and paternally methylated gDMRs, which were in some cases mosaic. This could indicate that fertility issues arise as a consequence of pre-existing mutations in factors required to protect and maintain imprints early in life and it may therefore be possible to identify genetic mutations in these factors in this group of patients.

Clinical features

In our large scale epidemiological study, we found differences in the frequency of some classic features of SRS and BWS between patients conceived by ART and those conceived naturally. We found that 7/7 (100%) ART conceived SRS patients showed body asymmetry, whereas only 30/54 (55.5%) who were conceived naturally possessed this feature. Similarly in BWS, earlobe creases were present in 4/7 (57.1%) ART conceived cases and 44/89 (49.4%) naturally conceived, bulging eyes in 3/7 (42.8%) versus 21/89 (23.6%), exomphalos in 6/7 (85.7%) versus 61/89 (68.5%) and nephromegaly in 2/7 (28.6%) versus 18/89 (20.2%), respectively. It is therefore possible that the dysregulation of the additional genes does modify the typical SRS and BWS phenotypes (Azzi *et al.*, 2010). BWS patients with multiple hypermethylation sites have been reported with complex clinical phenotypes (Bliek *et al.*, 2009) and a recently recognized BWS-like syndrome involving overgrowth with severe developmental delay was reported after IVF/ICSI (Shah *et al.*, 2006).

In our study patients with diagnosed imprinting disorders that presented with defects at additional loci (i.e. other than the domain responsible for that disorder) did not display additional phenotypes not normally reported in BWS or SRS. Since we were effectively selecting for classic cases of BWS and SRS in the first instance, it is possible that there are individuals born through ART showing entirely novel or confounding phenotypes that were not identified in our survey. Alternatively, as many of the alterations we observed showed a mosaic pattern, it is possible that mosaic individuals have more subtle phenotypes. In light of this new information on mosaicism, we may be able to use our knowledge of the individual's epigenotype to uncover these subtle changes.

This study, and the work of our colleagues, highlights the pressing need to conduct long-term international studies on ART treatment and the prevalence of imprinting disorders, particularly as the use of ART is increasing worldwide. It remains to be seen if other very rare epigenetic disorders will also have a possible association with the use of ART. Furthermore, it is not yet known what other pathologies might be influenced by ART. For example, in addition to general growth abnormalities, many imprint methylation errors also lead to the occurrence of various cancers (Okamoto *et al.*, 1997; Cui *et al.*, 1998). Further molecular studies will be required to understand the pathogenesis of these associations, and also to identify preventative methods to reduce the risk of occurrence of these syndromes following ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.H., H.O., N.M., F.S. and A.S. performed the DNA methylation analyses. M.K., K.N. and H.S. collected the samples of the patients. K.N. did the statistical analyses. H.H., M.V.D.P., R.M.J. and T.A. wrote this manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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PRKAR1A Mutation Affecting cAMP-Mediated G Protein-Coupled Receptor Signaling in a Patient with Acrodysostosis and Hormone Resistance

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Context: Acrodysostosis is a rare autosomal dominant disorder characterized by short stature, peculiar facial appearance with nasal hypoplasia, and short metacarpotarsals and phalanges with cone-shaped epiphyses. Recently, mutations of *PRKAR1A* and *PDE4D* downstream of *GNAS* on the cAMP-mediated G protein-coupled receptor (GPCR) signaling cascade have been identified in acrodysostosis with and without hormone resistance, although functional studies have been performed only for p.R368X of *PRKAR1A*.

Objective: Our objective was to report a novel *PRKAR1A* mutation and its functional consequence in a Japanese female patient with acrodysostosis and hormone resistance.

Patient: This patient had acrodysostosis-compatible clinical features such as short stature and brachydactyly and mildly elevated serum PTH and TSH values.

Results: Although no abnormality was detected in *GNAS* and *PDE4D*, a novel *de novo* heterozygous missense mutation (p.T239A) was identified at the cAMP-binding domain A of *PRKAR1A*. Western blot analysis using primary antibodies for the phosphorylated cAMP-responsive element (CRE)-binding protein showed markedly reduced CRE-binding protein phosphorylation in the forskolin-stimulated lymphoblastoid cell lines of this patient. CRE-luciferase reporter assays indicated significantly impaired response of protein kinase A to cAMP in the HEK293 cells expressing the mutant p.T239A protein.

Conclusions: The results indicate that acrodysostosis with hormone resistance is caused by a heterozygous mutation at the cAMP-binding domain A of *PRKAR1A* because of impaired cAMP-mediated GPCR signaling. Because *GNAS*, *PRKAR1A*, and *PDE4D* are involved in the GPCR signal transduction cascade and have some different characters, this would explain the phenotypic similarity and difference in patients with *GNAS*, *PRKAR1A*, and *PDE4D* mutations. (***J Clin Endocrinol Metab* 97: E1808–E1813, 2012**)

Acrodysostosis is a rare autosomal dominant disorder characterized by short stature, peculiar facial appearance with nasal hypoplasia, short metacarpotarsals and phalanges with cone-shaped epiphyses, and variable degrees of mental retardation (1, 2). Recent studies have shown that acrodysostosis is caused by mutations of *PRKAR1A* (protein

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Abbreviations: AHO, Albright's hereditary osteodystrophy; CRE, cAMP-responsive element; CREB, CRE-binding; DMR, differentially methylated regions; GNAS, stimulatory G protein α-subunit; GPCR, G protein-coupled receptor; PDE4D, phosphodiesterase 4D, cAMP-specific; PHP-Ia, pseudohypoparathyroidism type Ia; PKA, protein kinase A; PRKAR1A, protein kinase, cAMP-dependent, regulatory type 1, α; RIα, type 1α regulatory subunit.

kinase, cAMP-dependent, regulatory type 1,α) and *PDE4D* (phosphodiesterase 4D, cAMP-specific) involved in the cAMP-mediated G protein-coupled receptor (GPCR) signaling cascade (3–5). *PRKAR1A* consists of 11 exons and encodes type 1α regulatory subunit (RIα) of protein kinase A (PKA) with a dimerization domain, an inhibitory site, and two cAMP-binding domains A and B (6). The PKA holoenzyme is a tetramer consisting of two regulatory subunits and two catalytic subunits, and cooperative binding of two cAMP molecules to each regulatory subunit leads to the dissociation of the catalytic subunits from the regulatory subunits (7). The regulatory subunit-associated catalytic subunits remain inactive, whereas the free catalytic subunits released from the regulatory subunits can phosphorylate a variety of substrate proteins including the cAMP-responsive element (CRE)-binding (CREB) protein (7, 8). It is likely, therefore, that the *PRKAR1A* mutations hinder the cAMP-mediated dissociation of the catalytic subunits from the regulatory subunits, thereby leading to reduced PKA signaling (3). *PDE4D* comprises 15 exons and encodes cAMP-dependent phosphodiesterase 4D (PDE4D) that regulates intracellular cAMP concentrations by converting cAMP to AMP (9). Thus, the *PDE4D* mutations appear to result in desensitization to cAMP because of persistently elevated intracellular cAMP concentrations, thereby affecting the cAMP-mediated GPCR signaling cascade (4). However, functional studies have been performed only for the *PRKAR1A* p.R368X mutation that resides on the last exon and is predicted to escape nonsense-mediated mRNA decay (3), whereas protein modeling analysis argues for the pathological consequences of the remaining *PRKAR1A* and *PDE4D* mutations (4, 5).

Notably, nine of 10 *PRKAR1A* mutation-positive patients and two of seven *PDE4D* mutation-positive patients identified to date exhibit resistance to PTH and/or TSH. Such clinical findings, *i.e.* acrodysostosis plus hormone resistance, overlap with those of pseudohypoparathyroidism type Ia (PHP-Ia), because PHP-Ia is associated with Albright's hereditary osteodystrophy (AHO) reminiscent of acrodysostosis and resistance to several hormones such as PTH and TSH. Indeed, although acrodysostosis and AHO have been classified as different skeletal disorders (2), it is often difficult to distinguish between acrodysostosis and AHO on the basis of clinical and radiological findings (10). Consistent with such phenotypic similarities, PHP-Ia is primarily caused by heterozygous loss-of-function mutations of *GNAS* (the stimulatory G protein α-subunit) (11) that resides in the upstream of *PRKAR1A* and *PDE4D* on the cAMP-mediated GPCR signaling cascade.

Here, we report on a novel *de novo PRKAR1A* mutation and its functional consequence in a patient with acrodysostosis and hormone resistance and discuss phenotypic findings in patients with *PRKAR1A*, *PDE4D*, and *GNAS* mutations.

Patients and Methods

Case report

This Japanese female patient was born to nonconsanguineous parents at 38 wk of gestation. At birth, her length was 46.5 cm (−0.75 SD) and her weight 1.81 kg (−2.8 SD). Neonatal screening tests were normal. Her gross motor milestones were somewhat delayed, with sitting alone without support at 10 months and walking alone at 21 months of age. Her stature remained below −2.0 SD of the mean.

At 3 yr and 10 months of age, she was referred to us because of short stature. Her height was 86.9 cm (−3.1 SD) and her weight 10.6 kg (−2.3 SD). She exhibited round face, nasal hypoplasia, anteverted nostrils, severe brachydactyly of the hands, and mild developmental retardation, and hand roentgenograms showed generalized shortening of the tubular bones with cone shaped epiphyses (Fig. 1A). Brain computerized tomography showed neither sc nor intracranial calcifications. Biochemical and endocrine studies revealed 1) increased serum PTH and plasma cAMP values and normal serum calcium, phosphate, and vitamin D values; 2) decreased urine calcium/creatinine ratio and normal percent tubular reabsorption of phosphate; 3) slightly elevated serum TSH value and normal free T_4 value; 4) age-appropriate serum LH and FSH values; and 5) normal GH response to GHRH stimulation (Table 1). Thus, she was suspected as having acrodysostosis with mild resistance to PTH and TSH.

The parents showed neither brachydactyly nor abnormal endocrine findings (Table 1), although the mother had short stature (144 cm, −2.6 SD).

Molecular and functional studies

We performed 1) direct sequencing for coding exons and their splice sites of *PRKAR1A*, *PDE4D*, and *GNAS*; 2) methylation analysis for four differentially methylated regions (DMR) around *GNAS*; 3) parental testing by microsatellite genotyping; 4) conservation of a substituted amino acid; 5) the forskolin-induced PKA activity of lymphoblastoid cell lines in terms of the phosphorylation status of the CREB protein using Western blot analysis; and 6) forskolin-induced PKA activity using HEK293 cells expressing the wild-type and the mutant proteins. The primers used in this study are shown in Supplemental Table 1, and the detailed methods are described in Supplemental Methods (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Results

Analysis of *PRKAR1A*, *PDE4D*, and *GNAS*

No pathological mutation was found for *PDE4D* and *GNAS*, nor was an aberrant methylation pattern detected for the DMR around *GNAS*. By contrast, a novel heterozygous missense mutation (c.715A→G; p.T239A)

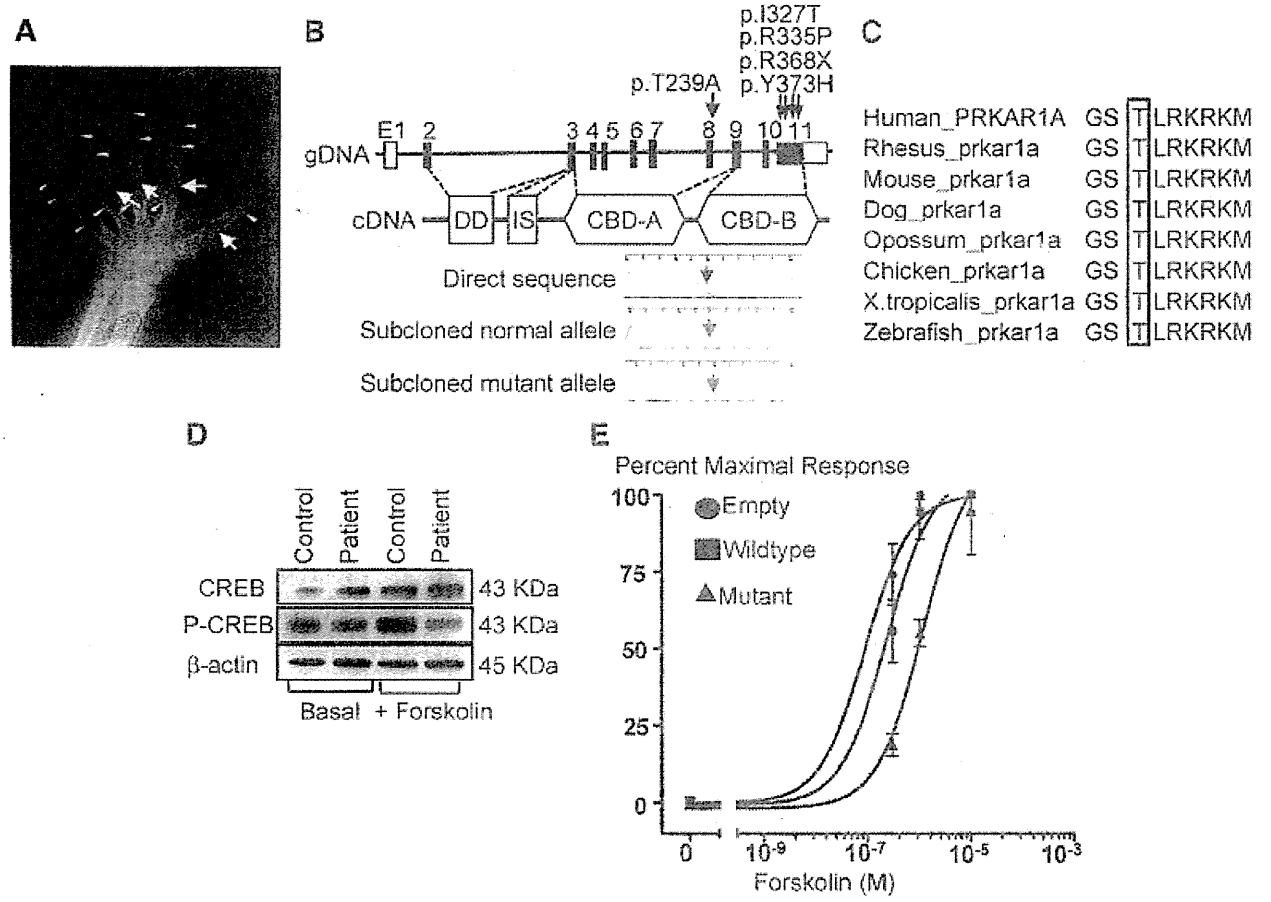

FIG. 1. Representative clinical and experimental findings of this patient. A, Radiograph of the left hand at 3 yr and 10 months of age. Note the shortening of all tubular bones with cone-shaped epiphyses (*arrows*) and early infusions (*arrowheads*). B, The structure of *PRKAR1A* and the position of the mutations identified. The *black and white boxes* on genomic DNA (gDNA) denote the coding regions on exons 2–11 and the untranslated regions, respectively. *PRKAR1A* encodes a dimerization domain (DD), an inhibitory site (IS), and two cAMP-binding domains A and B (CBD-A and -B). A missense mutation (p.T239A) was identified on exon 8 for the cAMP-binding domain A of this patient, whereas the previously described one nonsense and three missense mutations have been found on exon 11 for cAMP-binding domain B (3–5). C, Amino acid sequence of PRKAR1A. Note that the T239 residue is well conserved among species. D, Representative results of Western blot analysis for lymphoblastoid cell lines of the patient and a control subject. The cells were collected before (basal) and after stimulation with 10 μM forskolin. The samples were probed with antibodies for phospho-CREB protein (Ser 133) (P-CREB) and CREB protein, together with those for β-actin used as an internal control. E, Transactivating activities of the wild-type and the mutant PRKAR1A for the CRE-luc reporter. HEK293 cells were transfected with an empty expression vector or with vectors containing either the wild-type PRKAR1A or the p.T239A mutant. Samples were treated with various concentrations of forskolin. The values (percentages to the maximal CRE-luciferase activity) are expressed as the mean ± SE. Curves are fitted with sigmoidal dose-response models. In cells expressing the mutant protein, forskolin induced a concentration-dependent increase in CRE-luciferase activity, yet with a shift to the right in the dose-response curve. The EC_{50} values were significantly higher in the cells expressing the mutant protein than those expressing the wild-type protein ($P < 0.001$). The results are obtained from three independent experiments.

was identified on exon 8 at the cAMP-binding site A of *PRKAR1A* (Fig. 1B). This mutation was absent from her parents and 100 Japanese control subjects.

Parental testing

Microsatellite genotyping data were consistent with paternity as well as maternity of the parents (Supplemental Table 2).

Functional characterization of the mutant *PRKAR1A*

The T239 residue was well conserved among species (Fig. 1C). Protein modeling analysis indicated that the p.T239A resulted in loss of the hydrogen bond between the M236 and the T239 residues and in aberration of the random coils in the mutant PRKAR1A protein, although there was no gross conformational alteration affecting α-helices and β-strands (Supplemental Fig. 1). Western blot analysis indicated obviously reduced forskolin-induced CREB protein phosphorylation in the presence of the apparently normal amount of CREB protein in the lymphoblastoid cell line of this patient (Fig. 1D). Similarly, forskolin-induced PKA activity was significantly lower in the HEK293 cells expressing the mutant PRKAR1A protein than in those expressing the wild-type protein (Fig. 1E).

TABLE 1. Clinical and laboratory data of the patient and her parents

	Patient	Father	Mother[a]
Age (yr)	3 10/12	31	30
Height (cm) (SDS)	**86.9** (−3.1)	177 (+1.1)	**144** (−2.6)
Weight (kg) (SDS)	**10.9** (−2.3)	**89 (+2.5)**	45[b]
Blood			
Intact PTH (pg/ml)	**128** (10–65)	42 (10–65)	23 (10–65)
Calcium (mg/dl)	9.4 (8.5–10.2)	9.4 (8.5–10.2)	9.0 (8.5–10.2)
Phosphate (mg/dl)	5.6 (3.5–5.9)	3.2 (2.4–4.3)	3.6 (2.4–4.3)
25-Hydroxyvitamin D (ng/ml)	26 (7–41)	NA	NA
TSH (mU/liter)	**7.2** (0.5–5.0)	1.7 (0.5–5.0)	1.2 (0.5–5.0)
Free T_4 (ng/ml)	1.2 (0.9–1.6)	1.3 (0.9–1.6)	1.1 (0.9–1.6)
LH (IU/liter)	<0.1 (<0.7)	4.1 (0.8–5.7)	0.17 (1.8–10.2)[c]
FSH (IU/liter)	0.9 (0.6–5.3)	6.6 (2.0–8.3)	0.07 (3.0–14.7)[c]
GH (ng/ml) stimulated[d]	19.5 (>9)	NA	NA
cAMP (pmol/ml)	**39.6** (6.4–20.8)	NA	NA
Urine			
Calcium/creatinine ratio	**0.04** (0.13–0.25)	NA	NA
% TRP	91 (81.3–93.3)	NA	NA

The values in *parentheses* indicate the SD score (SDS) for heights and weights and the age-and sex-matched reference blood and urine hormone and laboratory data. The conversion factors to the SI unit are as follows: intact PTH, 1.0 (nanograms per liter); serum calcium, 0.25 (millimoles per liter); serum phosphate, 0.3229 (millimoles per liter); 25-hydroxyvitamin D, 2.496 (nanomoles per liter); free T_4, 12.9 (picomoles per liter); GH, 1.0 (micrograms per liter); and cAMP, 1.0 (nanomoles per liter). Hormone values have been evaluated by the age- and sex-matched Japanese reference data; abnormal data are in *bold*. NA, Not available; TRP, tubular reabsorption of phosphate.

[a] During the second trimester of pregnancy.

[b] Not assessed because of pregnancy.

[c] Low LH/FSH values are consistent with pregnant status of the mother (18).

[d] Blood sampling during the provocation tests were done at 0, 30, 60, 90, and 120 min after GHRH stimulation (1 μg/kg).

Discussion

We identified a novel *de novo* heterozygous *PRKAR1A* mutation in a patient with acrodysostosis and mild resistance to PTH and TSH. In this regard, several findings are noteworthy. First, although the phenotypic findings of this patient are similar to those of PHP-Ia, the severe skeletal lesion would be regarded as acrodysostosis rather than AHO. Consistent with this, a mutation was identified in *PRKAR1A* rather than *GNAS*. Second, the p.T239A was present at the cAMP-binding domain A, in contrast to the previously reported *PRKAR1A* mutations that were invariably located at the cAMP-binding domain B (3–5). In this regard, because cAMP binds first to the binding domain B and then to the binding domain A, it has been suggested that the binding domain B acts as the gatekeeper of the PKA activation, and that the binding domain A is relatively inaccessible to cAMP (8). Despite such a hierarchical phenomenon, this study indicates that mutations at the cAMP-binding domains A and B lead to a similar clinical phenotype. Third, functional analyses showed obviously reduced PKA signaling of the mutant PRKAR1A protein. Thus, the p.T239A mutation appears to impair the dissociation of the catalytic subunits from the RIα regulatory subunits, thereby leading to the reduced GPCR signaling, as has been stated by the functional studies for the p.R368X mutation (3). Although the underlying factors remains to be elucidated, loss of the hydrogen bond and aberration of the random coils in the mutant PRKAR1A protein may be relevant to this functional alteration.

To date, *GNAS*, *PRKAR1A*, and *PDE4D* mutations have been identified in patients with overlapping skeletal and endocrine phenotypes (3–5). It appears, however, that *GNAS* abnormalities usually lead to relatively mild skeletal phenotype and clinically discernible hormone resistance, whereas *PRKAR1A* and *PDE4D* mutations usually result in relatively severe skeletal lesion and mild or absent hormone resistance. In this regard, it is predicted in patients with maternally derived *GNAS* mutations that normally functioning *GNAS* is absent from several tissues including renal proximal tubules where *GNAS* is paternally imprinted and is present in a single copy in other tissues including skeletal tissues where *GNAS* is biparentally expressed (11, 12). By contrast, it is likely in patients with *PRKAR1A* and *PDE4D* mutations that normally functioning *PRKAR1A* and *PDE4D* are present in a single copy in all the tissues because of the absence of DMR around these genes (13). Such a difference in the functional gene dosage in several *GNAS*-imprinted tissues may more or less be relevant to the prevalent hormone resistance in *GNAS* mutations. Furthermore, because there are four genes encoding the regulatory subunits of PKA (RIa, RIb,

RIIa, and RIIb) and three genes encoding the catalytic subunits (14), such redundancy would also be related to the apparently mild hormone resistance in *PRKAR1A* mutations. However, these factors are unlikely to account for the apparently severe skeletal lesion in *PRKAR1A* and *PDE4D* mutations. Furthermore, although aberrant signaling via PTHrP receptor belonging to the GPCR families may play an important role in the development of skeletal lesions in *PRKAR1A* mutations, this perturbation is also relevant to the occurrence of skeletal lesions in *GNAS* abnormalities (15). Thus, there may be a hitherto unknown factor involved in the development of severe skeletal phenotype in *PRKAR1A* and *PDE4D* mutations. Notably, hormone resistance is apparently infrequent in acrodysostosis (2). It remains to be clarified whether acrodysostosis with and without hormone resistance may represent genetically heterogeneous conditions, and whether hormone resistance may have been overlooked or remained at a subclinical level in a certain fraction of patients with *PRKAR1A* and *PDE4D* mutations.

For *PRKAR1A*, more than 100 different mutations have been identified in Carney complex with multiple neoplasias and lentiginosis (16). Because most mutations reported in Carney complex are frameshift, nonsense, and splice mutations that are predicted to undergo nonsense-mediated mRNA decay and cause *PRKAR1A* haploinsufficiency, they would result in the increased amount of the free-lying intracellular catalytic subunits, leading to excessive PKA signaling in target tissues (16, 17). Furthermore, other types of mutations have also been identified in Carney complex, such as missense mutations at the cAMP-binding domain A (*e.g.* p.D183Y and p.A213D) and an in-frame deletion of 53 amino acids from the binding domain A (c.708 + 1 g→t) (16). Thus, in conjunction with the results of this study, we presume that *PRKAR1A* mutations can cause a mirror image of disorders in terms of the PKA activity, *i.e.* Carney complex resulting from defective association between the regulatory and the catalytic subunits and acrodysostosis with hormone resistance ascribed to impaired dissociation between the two subunits.

In summary, we identified a heterozygous *PRKAR1A* mutation affecting cAMP-mediated GPCR signaling in a patient with acrodysostosis with hormone resistance. Additional studies will permit a better clarification of the underlying causes in acrodysostosis with and without hormone resistance.

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Paternal uniparental disomy 14 and related disorders

Placental gene expression analyses and histological examinations

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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]

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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.

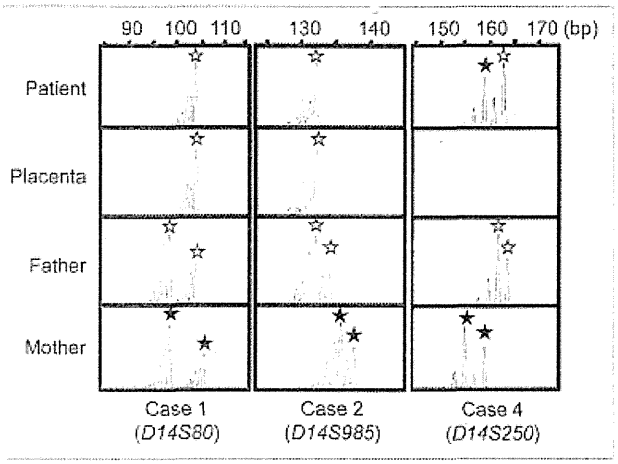

Figure 1. Representative results of microsatellite analysis, using leukocyte genomic DNA samples of the patient and the parents and placental genomic DNA samples. In cases 1 and 2, one of the two paternal peaks is inherited by the patients and the placentas, and no trace of maternal peaks is identified. 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.

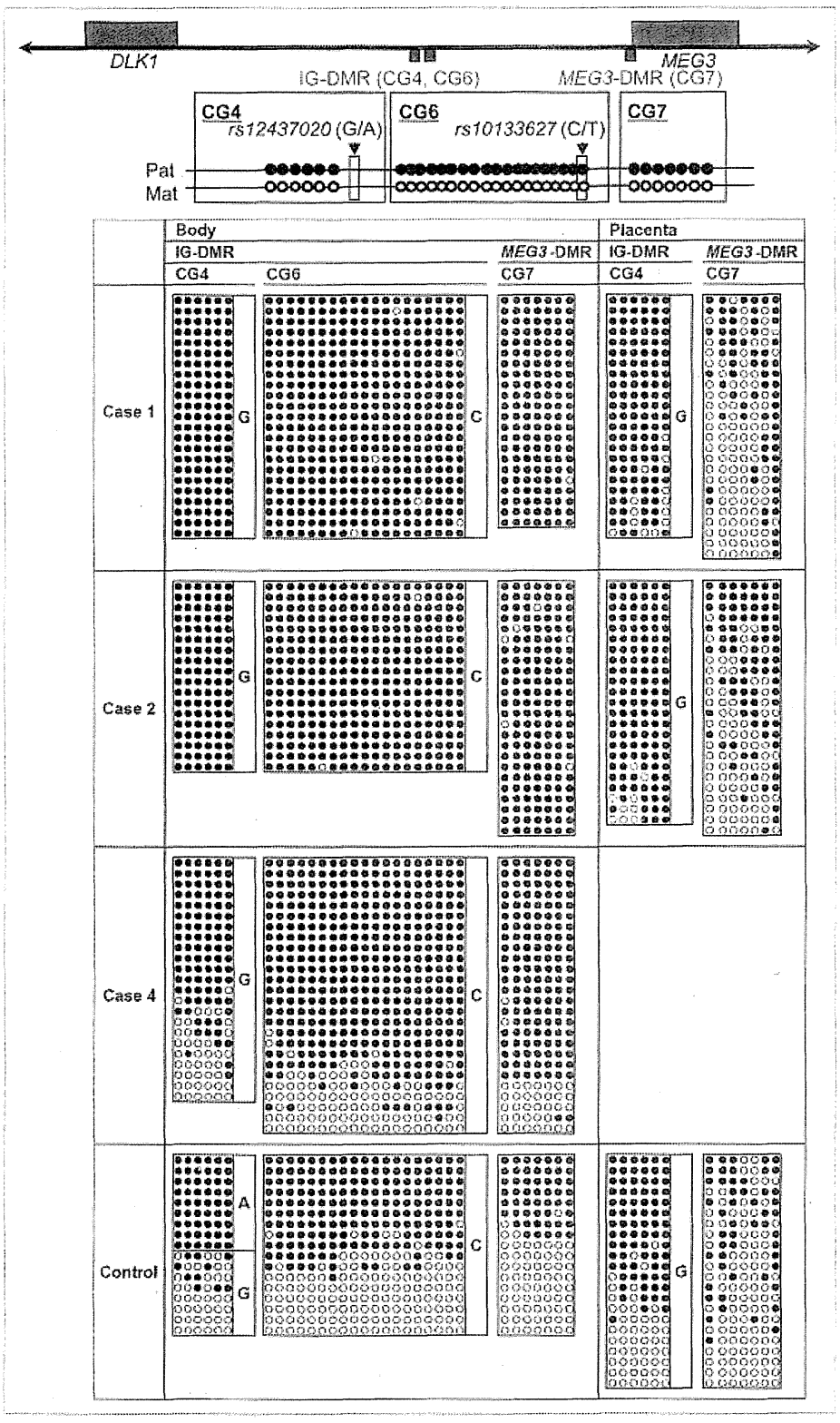

Figure 2. For figure legend, see page 1144.

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.

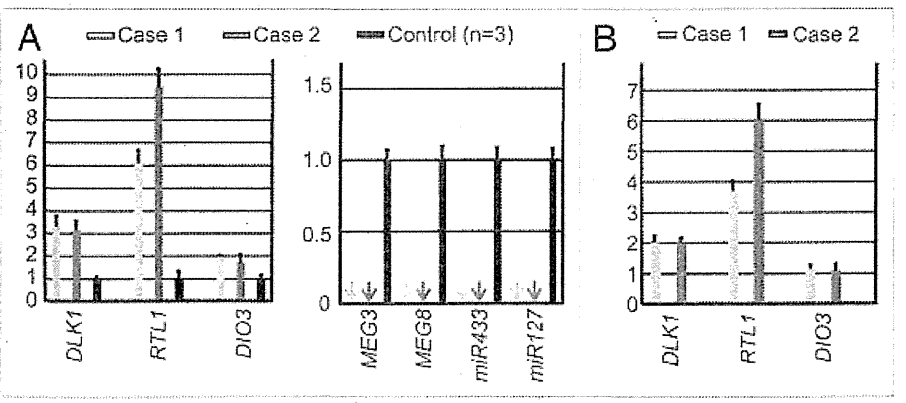

Figure 3. Quantitative real-time PCR analysis using placental samples. For a control, a pooled RNA sample consisting of an equal amount of total RNA extracted from three fresh control placentas was utilized. (**A**) Relative mRNA expression levels for *DLK1*, *RTL1*, and *DIO3* against *GAPDH* (mean ± 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*

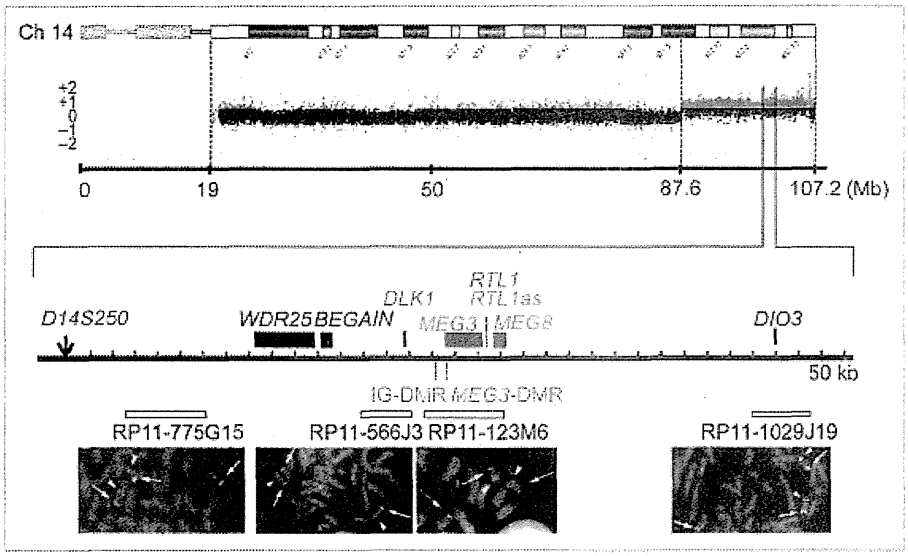

Figure 4. Array CGH and FISH analysis for the distal chromosome 14 region in case 4. In CGH analysis, the black, the red, and the green dots denote signals indicative of the normal, the increased (> +0.5), and the decreased (< −1.0) copy numbers, respectively. In FISH analysis, red signals (arrows) are 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