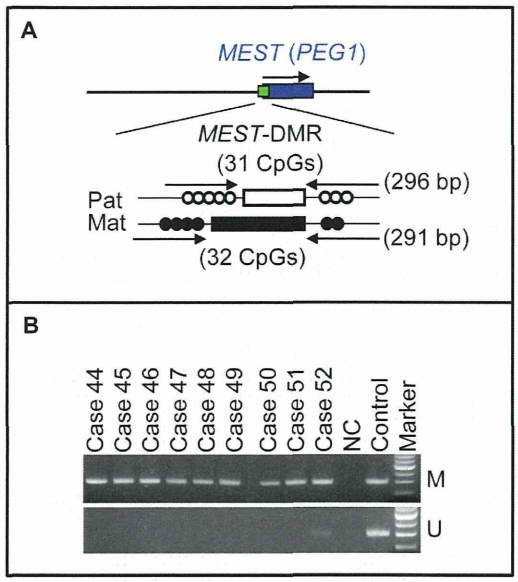

Figure 2. Methylated and unmethylated allele-specific PCR analysis for the *MEST*-DMR. A. Schematic representation of the *MEST*-DMR. The cytosine residues at the CpG dinucleotides are usually unmethylated after paternal transmission (open circles) and methylated after maternal transmission (filled circles). The PCR primers have been designed to hybridize either methylated or unmethylated clones. B. The results of methylation analysis with methylated and unmethylated allele-specific primers.
doi:10.1371/journal.pone.0060105.g002

median and range. Statistical significance of the mean was analyzed by Student's *t*-test or Welch's *t*-test after comparing the variances by *F* test, that of the median by Mann-Whitney's *U*-test, that of the frequency by Fisher's exact probability test, and that of the correlation by Pearson's correlation coefficient after confirming the normality of the variables. $P<0.05$ was considered significant.

Results

Identification of *H19*-DMR hypomethylation

Representative findings are shown in Figure 1B and 1C, and the MIs are summarized in Table 1. Overall, the MIs obtained by the pyrosequencing analysis tended to be lower and distributed more narrowly than those obtained by the COBRA. Despite such difference, the MIs obtained by the pyrosequencing analysis for CG5–CG7 and CG9 and by the COBRA for CG5 and CG16 were invariably below the normal range in the same 43 patients (cases 1–43) (group 1). By contrast, the MIs were almost invariably within the normal range in the remaining 95 patients, while the MIs obtained by the pyrosequencing analysis slightly (1–2%) exceeded the normal range in the same three patients (cases 136–138).

In the 43 cases of group 1, microsatellite analysis for four loci at the telomeric 11p region excluded maternal upd in 14 cases in whom parental DNA samples were available; in the remaining 29 cases, microsatellite analysis identified two alleles for at least one locus, excluding maternal uniparental isodisomy for this region. Furthermore, oligoarray CGH for the chromosome 11p15.5 region showed no copy number alteration such as duplication of maternally derived *H19*-DMR and deletion of paternally derived *H19*-DMR. For the KvDMR1, the MIs of the 138 patients remained within the reference range (Fig. S1B and C).

Table 1. The methylation indices (%) for the *H19*-DMR.

	Cases 1–43	Cases 44–138	Control subjects
Pyrosequencing analysis			
CG5	4–24	35–50	33–48
CG6	5–26	36–53	34–51
CG7	4–24	35–49	30–47
CG9	5–23	34–48	30–46
COBRA			
CG5 (*Hpy*188I)	3.3–35.1	37.8–60.8	36.2–58.5
CG16 (*Afl*III)	4.1–35.0	43.0–59.4	38.7–60.0

The position of examined CpG dinucleotides (CG5–7, CG9, and CG16) is shown in Figure 1A.
COBRA: combined bisulfite restriction analysis.
doi:10.1371/journal.pone.0060105.t001

Identification of upd(7)mat

Methylation analysis for the *MEST*-DMR revealed that unmethylated bands were absent from eight patients and remained faint in a single patient (cases 44–52) (group 2) (Figure 2B). Subsequent microsatellite analysis confirmed upd(7)mat in the eight patients and mosaic upd(7)mat in the remaining one patient, and indicated trisomy rescue or gamete complementation type upd(7)mat in cases 44–48, monosomy rescue or post-fertilization mitotic error type upd(7)mat in cases 49–51, and post-fertilization mitotic error type mosaic upd(7)mat in case 52 (Table S2).

Multiple DMR analysis

We examined 17 autosomal DMRs other than the *H19*-DMR in 14 patients in group 1, four patients in group 2, and 20 patients in group 3, and the *XIST*-DMR in eight female patients in group 1, one female patient in group 2, and five female patients in group 3 (Table S3). The MIs outside the reference ranges were identified in five of 14 examined cases (35.7%) and six of a total of 246 examined DMRs (2.4%) in group 1. In particular, a single case with the mean MI value of 23 obtained by the pyrosequencing analysis for CG5–CG7 and CG9 had an extremely low MI for the *ARHI*-DMR (case 13 of group 1). This extreme hypomethylation was confirmed by bisulfite sequencing, and direct sequencing showed normal sequences of the primer-binding sites, thereby excluding the possibility that such an extremely low MI could be due to insufficient primer hybridization because of the presence of a nucleotide variation within the primer-binding sites (Figure 3). Furthermore, no copy number variation involving the *ARHI*-DMR was identified by CGH analysis using a genomewide catalog array. Consistent with upd(7)mat, three DMRs on chromosome 7 were extremely hypermethylated in four examined cases of group 2. Only a single DMR was mildly hypermethylated in a total of 345 examined DMRs in group 3. The abnormal MIs, except for those for the *H19*-DMR in group 1 and for the three DMRs on chromosome 7 in group 2, were confirmed by three times experiments.

Oligonucleotide array CGH

A ~3.86 Mb deletion at chromosome 17q24 was identified in a single patient (case 73 of group 3) (Figure 4).

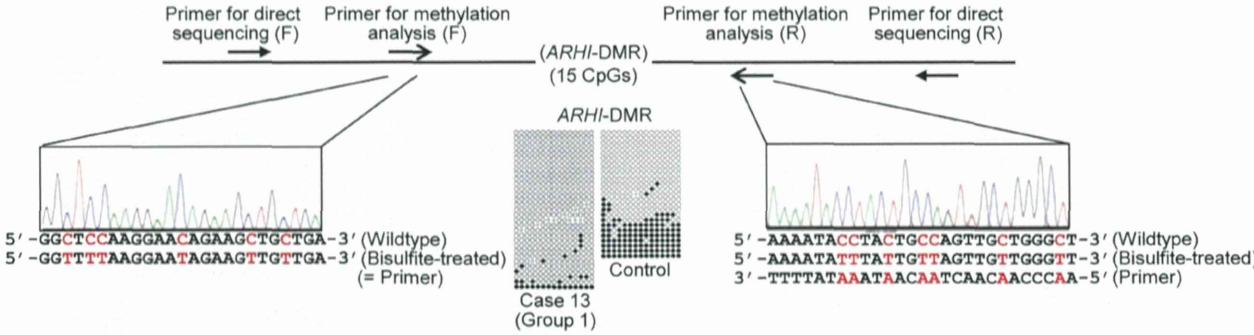

Figure 3. Analysis of the *ARHI*-DMR in case 13. For bisulfite sequencing, each line indicates a single clone, and each circle denotes a CpG dinucleotide; the cytosine residues at the CpG dinucleotides are usually unmethylated after paternal transmission (open circles) and methylated after maternal transmission (filled circles). Electrochromatograms delineate the sequences of the primer binding sites utilized for the methylation analysis.
doi:10.1371/journal.pone.0060105.g003

Epigenotype-phenotype analysis

Clinical findings of SRS patients in groups 1–3 are summarized in Table 2. All the patients met the mandatory criteria, and most patients in each group had severely reduced birth length and weight (both≤−2 SDS). For the five clinical features utilized as scoring system criteria, while 23.2% of patients in group 1 and 22.2% of patients in group 2 exhibited all the five features, there was no patient in group 3 who was positive for all the five features. By contrast, while 39.5% of patients in group 1 and 33.3% of patients in group 2 manifested just three of the five features, 77.6% of patients in group 3 were positive for just three features. In particular, the frequencies of relative macrocephaly at birth and body asymmetry were low in group 3, while those of the remaining three scoring system criteria including prominent forehead during early childhood were similar among groups 1–3.

Phenotypic comparison between groups 1 and 2 revealed that birth length and weight were more reduced and birth OFC was more preserved in group 1 than in group 2, despite comparable gestational age. In the postnatal life, present height and weight became similar between the two groups, whereas present OFC became significantly smaller in group 1 than in group 2. Body asymmetry and brachydactyly were more frequent and speech delay was less frequent in group 1 than in group 2. Placental weight was similar between the two groups, and became more similar after excluding case 52 with mosaic upd(7)mat (see legends for Table 2). Parental age at childbirth was also similar between the two groups. In group 2, placental weight was grossly similar among examined cases, as was parental age at childbirth (see legends for Table 2).

Case 13 with an extremely low MI for the *ARHI*-DMR and case 73 with a cryptic deletion at chromosome 17q24 had no specific phenotype other than SRS-like phenotype (Table S4). However, of the five clinical features utilized as scoring system criteria, all the five features were exhibited by case 13 and just three features were

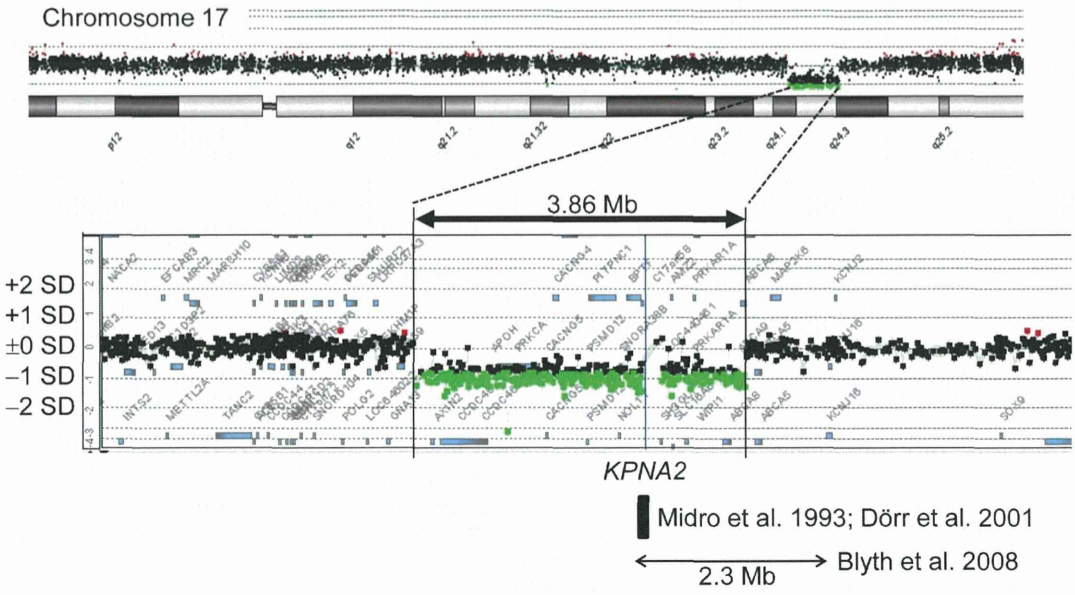

Figure 4. Oligonucleotide array CGH in case 73, showing a ~3.86 Mb deletion at chromosome 17q24. The black, the red, and the green dots denote signals indicative of the normal, the increased(>+0.5), and the decreased (< −1.0) copy numbers, respectively. The horizontal bar with arrowheads indicates a ~2.3 Mb deletion identified in a patient with Carney complex and SRS-like phenotype [44], and the black square represent a ~65 kb segment harboring the breakpoint of a *de novo* translocation 46,XY,t(1;17)(q24;q23–q24) identified in a patient with SRS phenotype [45,46].
doi:10.1371/journal.pone.0060105.g004

Table 2. Phenotypic comparison in three groups of patients with Silver-Russell syndrome.

	H19-DMR hypomethylation	Upd(7)mat	Unknown	*P*-value		
	(Group 1)	(Group 2)	(Group 3)	G1 vs. G2	G1 vs. G3	G2 vs. G3
Patient number	43 (31.2%)	9 (6.5%)	85 (62.0%)			
Mandatory criteria	43/43 (100%)	9/9 (100%)	85/85 (100%)	1.000	1.000	1.000
Scoring system criteria (5/5)	10/43 (23.2%)	2/9 (22.2%)	0/85 (0.00%)	0.965	**1.52×10^{-4}**	**2.58×10^{-2}**
Scoring system criteria (4/5)	16/43 (37.2%)	4/9 (44.4%)	19/85 (22.4%)	0.792	**1.45×10^{-2}**	0.145
Scoring system criteria (3/5)	17/43 (39.5%)	3/9 (33.3%)	66/85 (77.6%)	0.821	**7.17×10^{-4}**	0.161
Gestational age (weeks:days)	38:0 (34:3~40:0) (n = 36)	38:0 (34:4~40:0) (n = 9)	37:6 (27:1~41:4) (n = 65)	0.877	0.120	0.450
BL (SDS)	−4.13±2.01 (n = 31)	−3.18±1.16 (n = 9)	−2.93±1.43 (n = 60)	**2.67×10^{-2}**	**6.69×10^{-5}**	0.619
BW (SDS)	−3.50±0.85 (n = 42)	−2.90±0.64 (n = 9)	−2.71±1.14 (n = 64)	**3.28×10^{-2}**	**5.87×10^{-4}**	0.640
BL≤−2 SDS and/or BW≤−2 SDS*	43/43 (100%)	9/9 (100%)	85/85 (100%)	1.000	1.000	1.000
BL≤−2 SDS and BW≤−2 SDS	39/43 (90.7%)	7/9 (77.8%)	76/85 (89.4%)	0.474	0.821	0.304
BOFC (SDS)	−0.54±1.22 (n = 29)	−1.44±0.47 (n = 9)	−1.92±1.09 (n = 48)	**3.74×10^{-2}**	**1.52×10^{-6}**	0.202
BL (SDS) − BOFC (SDS)	−3.70±2.02 (n = 27)	−1.73±1.20 (n = 9)	−0.943±1.48 (n = 43)	**1.02×10^{-2}**	**3.40×10^{-9}**	0.111
BW (SDS) − BOFC (SDS)	−3.21±1.20 (n = 27)	−1.53±0.57 (n = 9)	−1.04±1.55 (n = 48)	0.326	**7.38×10^{-9}**	0.331
Relative macrocephaly at birth† BL or BW (SDS) − BOFC (SDS)≤−1.5	29/29 (100%)	7/9 (77.8%)	16/45 (35.6%)	0.341	**3.67×10^{-8}**	**2.05×10^{-2}**
Present age (years:months)	4.1 (0:6~30:6) (n = 31)	4.8 (2:4~25:2) (n = 9)	4.3 (0:1~18:6) (n = 60)	0.437	0.813	0.335
PH (SDS)	−3.58±1.65 (n = 35)	−3.77±1.13 (n = 9)	−3.17±1.50 (n = 61)	0.757	0.218	0.253
PH≤−2 SDS (≥2 years)†	29/35 (82.5%)	8/9 (88.9%)	52/61 (85.2%)	0.760	0.758	0.772
PW (SDS)	−3.15±1.16 (n = 32)	−2.77±0.76 (n = 9)	−2.77±1.34 (n = 59)	0.362	0.144	0.968
POFC (SDS)	−1.16±1.18 (n = 21)	−0.01±0.91 (n = 9)	−1.81±1.57 (n = 35)	**2.01×10^{-3}**	0.107	**3.08×10^{-3}**
PH (SDS) − POFC (SDS)	−2.47±1.63 (n = 16)	−3.62±1.38 (n = 8)	−1.55±1.82 (n = 35)	0.103	**4.39×10^{-2}**	**1.64×10^{-2}**
PW (SDS) − POFC (SDS)	−2.84±1.31 (n = 21)	−2.69±1.36 (n = 9)	−1.08±1.71 (n = 35)	0.782	**2.54×10^{-2}**	**1.90×10^{-4}**
Relative macrocephaly at present PH or PW (SDS) − POFC (SDS)≤−1.5	20/21 (95.2%)	8/8 (100%)	29/43 (67.4%)	0.223	**4.77×10^{-3}**	0.156
Triangular face during early childhood	42/43 (97.7%)	8/9 (88.9%)	65/65 (100%)	0.442	0.0773	**5.98×10^{-3}**
Prominent forehead during early childhood†	31/37 (83.8%)	7/9 (100%)	41/53 (77.4%)	0.200	0.456	0.978
Ear anomalies	14/35 (40.0%)	3/9 (33.3%)	15/55 (27.3%)	0.717	0.290	0.823
Irregular teeth	12/26 (46.2%)	4/9 (44.4%)	12/45 (26.7%)	0.930	0.0968	0.291
Body asymmetry†	30/37 (81.1%)	3/9 (33.3%)	19/59 (32.2%)	**4.77×10^{-3}**	**3.51×10^{-6}**	0.947
Clinodactyly	29/37 (78.4%)	5/9 (55.6%)	50/58 (86.2%)	0.167	0.323	**2.68×10^{-2}**
Brachydactyly	30/38 (78.9%)	2/9 (22.2%)	34/56 (60.7%)	1.16×10^{-3}	0.0642	**3.24×10^{-2}**
Syndactyly	3/36 (8.3%)	0/9 (0.00%)	3/52 (5.77%)	0.375	0.641	0.464
Simian crease	4/26 (15.4%)	2/7 (28.6%)	6/49 (12.2%)	0.429	0.705	0.252
Muscular hypotonia	17/32 (53.1%)	5/9 (55.6%)	12/50 (24.0%)	0.898	**7.49×10^{-3}**	0.0564
Developmental delay	18/37 (48.6%)	6/9 (66.7%)	25/54 (46.3%)	0.337	0.826	0.262
Speech delay	8/31 (25.8%)	6/9 (66.7%)	18/43 (41.9%)	**2.55×10^{-2}**	0.156	0.179
Feeding difficulty†	16/34 (47.1%)	6/9 (66.7%)	25/51 (49.0%)	0.301	0.860	0.333
Placental weight (SDS)	−2.10±0.74 (n = 14)	−1.72 ± 0.74 (n = 6)[a]	−1.02±0.86 (n = 18)	0.312	**4.12×10^{-3}**	**8.24×10^{-3}**
Paternal age at childbirth (years:months)	32:0 (19:0~52:0) (n = 24)	35:0 (27:0~48:0) (n = 9)	32:0 (25:0~46:0) (n = 45)	0.223	1.00	0.105
Maternal age at childbirth (years:months)	32:0 (19:0~43:0) (n = 25)	33:0 (25:0~42:0) (n = 9)[b]	30:0 (22:0~43:0) (n = 46)	0.275	0.765	0.117

BL: birth length; BW: birth weight; BOFC: birth occipitofrontal circumference; PH: present height; PW: present weight; POFC: present occipitofrontal circumference, and SDS: standard deviation score.

For body features, the denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature.

*Mandatory criteria and †five clinical features utilized as selection criteria for Silver-Russell syndrome proposed by Netchine et al. [14].

Significant *P*-values(<0.05) are boldfaced.

[a]Placental weight SDS is −1.68, −2.55, −2.24, −1.12, −2.14 and −0.60 in case 46, 47, 49, 50, 51 and 52, respectively; the placental weight SDS is −1.95±0.57 in five cases except for case 52 with mosaic upd(7)mat.

[b]Maternal childbearing age is 32, 32, 33, 42, 32, 34, 33, 25 and 36 years in case 44–52, respectively.

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manifested by case 73. In addition, cases 136–138 with slightly elevated MIs for CG5–CG7 and CG9, and cases with multilocus methylation abnormalities, had no particular phenotype other than SRS-compatible clinical features.

Correlation analysis

In group 1, the mean value of the MIs for CG5–CG7 and CG9 obtained by pyrosequencing analysis was positively correlated with the birth length and weight, the present height and weight, and the placental weight, but with neither the birth nor the present OFC (Table 3). Such correlations with the growth parameters were grossly similar but somewhat different for the MIs obtained by COBRA (Table S5). Furthermore, the placental weight was positively correlated with the birth weight and length, but not with the birth OFC. Such positive correlations were not found in groups 2 and 3.

Discussion

The present study identified hypomethylation of the *H19*-DMR and upd(7)mat in 31.2% and 6.5% of 138 Japanese SRS patients, respectively. In this regard, the normal KvDMR1 methylation patterns indicate that the aberrant methylation in 43 cases of group 1 is confined to the *H19*-DMR. Furthermore, oligoarray CGH excludes copy number variants involving the *H19*-DMR, and microsatellite analysis argues against segmental maternal isodisomy that could be produced by post-fertilization mitotic error [26]. These findings imply that the *H19*-DMR hypomethylation is due to epimutation (hypomethylation of the normally methylated *H19*-DMR of paternal origin).

The frequency of epimutations detected in this study is lower than that reported in Western European SRS patients [1,2,14], although the frequency of upd(7)mat is grossly similar between the two populations [2,11,14,27,28]. In this context, it is noteworthy that, of the five scoring system criteria, the frequencies of relative macrocephaly at birth and body asymmetry were low in group 3, while those of the remaining three scoring system criteria were similar among groups 1–3. Since relative macrocephaly and body asymmetry are characteristic of *H19*-DMR epimutation, the lack of these two features in a substantial fraction of cases in group 3 would primarily explain the low frequency of *H19*-DMR epimutations in this study. In group 3, furthermore, the low prevalence of relative macrocephaly at birth appears to be discordant with the high prevalence of prominent forehead during early childhood. Since relative macrocephaly was evaluated by an objective method (SDS for birth length or birth weight minus SDS for birth OFC≤−1.5) and prominent forehead was assessed by a subjective impression of different clinicians, it is recommended to utilize relative macrocephaly as a more important and reliable feature in the scoring system than prominent forehead. In addition, the difference in the ethnic group might also be relevant to the low frequency of *H19*-DMR epimutations in this study.

Table 3. Correlation analyses in patients with *H19*-DMR hypomethylations.

Parameter 1	Parameter 2	*r*	*P*-value
Methylation index (%)* vs.	Birth length (SDS)	0.647	**6.70×10^{-3}**
	Birth weight (SDS)	0.590	**7.80×10^{-3}**
	Birth OFC (SDS)	0.190	0.498
	Present height (SDS)	0.612	**5.33×10^{-3}**
	Present weight (SDS)	0.605	**7.81×10^{-3}**
	Present OFC (SDS)	−0.166	0.647
	Placental weight (SDS)	0.809	**8.30×10^{-3}**
Placental weight (SDS) vs.	Birth weight (SDS)	0.717	**8.64×10^{-3}**
	Birth length (SDS)	0.636	**2.63×10^{-2}**
	Birth OFC (SDS)	0.400	0.198

SDS: standard deviation score; and OFC: occipitofrontal circumference.
*The mean value of MIs for CG5, CG6, CG7, and CG9 obtained by pyrosequencing analysis.
Significant *P*-values(<0.05) are boldfaced.
doi:10.1371/journal.pone.0060105.t003

Epigenotype-phenotype correlations in this study are grossly similar to those previously reported in Western European SRS patients [1–3]. Cases 1–43 in group 1 with *H19*-DMR epimutation had more reduced birth weight and length, more preserved birth OFC and more reduced present OFC, more frequent body features, and less frequent speech delay than case 44–52 in group 2 with upd(7)mat, although the difference in the prevalence of somatic features appears to be less remarkable in this study than in the previous studies [3,4]. This provides further support for the presence of relatively characteristic clinical features in *H19*-DMR epimutation and upd(7)mat [1–3]. In this context, previous studies have indicated biallelic *IGF2* expression in the human fetal choroid plexus, cerebellum, and brain, and monoallelic *IGF2* expression in the adult brain, while the precise brain tissue(s) with such a unique expression pattern remains to be clarified [29,30,31]. This may explain why the birth OFC is well preserved and the present OFC is reduced in group 1. However, since the difference in present OFC between groups 1 and 2 is not necessarily significant in the previous studies [32], the postnatal OFC growth awaits further investigations.

Placental weight was similarly reduced in groups 1 and 2. Thus, placental weight is unlikely to represent an indicator for the discrimination between the two groups, although the present data provide further support for imprinted genes being involved in placental growth, with growth-promoting effects of *PEGs* and growth-suppressing effects of *MEGs* [5,6]. It should be pointed out, however, that the placental hypoplasia could be due to some other genetic or environmental factor(s). In particular, while placental weight was apparently similar among cases of group 2, possible confined placental mosaicism [33,34] with trisomy for chromosome 7 may have exerted some effects on placental growth in cases with trisomy rescue type upd(7)mat.

Correlation analysis would imply that the *IGF2* expression level, as reflected by the MI of the *H19*-DMR, plays a critical role in the determination of pre- and postnatal body (stature and weight) and placental growth in patients with *H19*-DMR epimutation. Since the placental weight was positively correlated with the birth length and weight, the reduced *IGF2* expression level appears to have a similar effect on the body and the placental growth. Furthermore, the lack of correlations between the MI and birth and present OFC and between placental weight and birth OFC would be compatible with the above mentioned *IGF2* expression pattern in the central nervous system [29]. Although the MI would also reflect the *H19* expression level, this would not have a major growth effect. It has been implicated that *H19* functions as a tumor suppressor [35,36].

Multilocus analysis revealed co-existing hyper- and hypomethylated DMRs predominantly in cases of group 1, with frequencies of 35.7% of examined patients and 2.4% of examined DMRs. The results are grossly consistent with the previous data indicating that co-existing abnormal methylation patterns of DMRs are almost exclusively identified in patients with *H19*-DMR epimutation with frequencies of 9.5~30.0% of analyzed patients and 1.8~5.2% of a

total of analyzed DMRs [7–9]. Notably, the co-existing methylation abnormalities were predominantly observed as mild hypermethylations of maternally methylated DMRs and were restricted to a single DMR or two DMRs in patients with multilocus abnormalities. Such findings are obviously inexplicable not only by assuming a *ZFP57* mutation that is known to cause severely abnormal methylation patterns of multiple DMRs or a *ZAC1* mutation that may affect methylation patterns of multiple DMRs [37–39], but also by assuming defective maintenance of methylation in the postzygotic period [7]. Thus, some factor(s) susceptible to the co-occurrence of hypomethylation of the *H19*-DMR and hypermethylation of other DMR(s) might be operating during a gametogenic or postzygotic period in cases with *H19*-DMR epimutation.

The patients with multilocus methylation abnormalities had no specific clinical features other than SRS-compatible phenotype. Previous studies have also indicated grossly similar SRS-like phenotype between patients with monolocus and multilocus hypomethylations [7], although patients with multilocus hypomethylation occasionally have apparently severe clinical phenotype [7]. These findings would argue for the notion that the *H19*-DMR epimutation has an (epi)dominant clinical effect. Indeed, *H19*-DMR hypomethylation has led to SRS-like phenotype in a patient with parthenogenetic chimerism/mosaicism [21], whereas *H19*-DMR hypermethylation has resulted in Beckwith-Wiedemann syndrome-like phenotype in patients with androgenetic mosaicism [40].

An extremely hypomethylated *ARHI*-DMR was found in case 13. In this regard, it is known that *ARHI* with a potentially cell growth suppressor function is normally expressed from paternally inherited chromosome with unmethylated *ARHI*-DMR [41]. Indeed, hypermethylation of the *ARHI*-DMR, which is predicted to result in reduced expression of *ARHI*, has been identified as a tumorigenic factor for several cancers with an enhanced cell growth function [42,43]. Thus, it is possible that hypomethylation of the *ARHI*-DMR has led to overexpression of *ARHI*, contributing to the development of typical SRS phenotype in the presence of a low but relatively preserved MI of the *H19*-DMR in case 13.

Oligonucleotide array CGH identified a ~3.86 Mb deletion at chromosome 17q24 in case 73 of group 3. This provides further support for the presence of rare copy number variants in several SRS patients and the relevance of non-imprinted gene(s) to the development of SRS [10]. Interestingly, the microdeletion overlap with that identified in a patient with Carney complex and SRS-like features [44], and the overlapping region encompasses a ~65 kb segment defining the breakpoint of a *de novo* reciprocal translocation involving 17q23–q24 in a patient with SRS-like phenotype (Figure 4) [45,46]. Furthermore, the translocation breakage has affected *KPNA2* involved in the nuclear transport of proteins [46–48]. Thus, *KPNA2* has been regarded as a candidate gene for SRS, although mutation analysis of *KPNA2* has failed to detect a disease-causing mutation in SRS patients [49].

Lastly, it would be worth discussing on the comparison between pyrosequencing analysis and COBRA. Since the same 43 patients were found to have low MIs by both analyses, this implies that both methods can be utilized as a diagnostic tool. While the distribution of the MIs was somewhat different between the two methods, this would primarily be due to the difference in the employed methods such as the hybridization efficiency of utilized primers. Importantly, pyrosequencing analysis was capable of studying plural CpG dinucleotides at the CTCF6 binding site, whereas COBRA examined only single CpG dinucleotides outside the CTCF6 binding site. Thus, the MIs obtained by pyrosequencing analysis would be more accurate than those obtained by COBRA in terms of *IGF2* expression levels, and this would underlie the reasonable correlations of MIs yielded by pyrosequencing analysis with body and placental growth parameters.

In summary, the present study provides useful information for the definition of molecular and clinical findings in SRS. However, several matters still remain to be elucidated, including underlying mechanisms in SRS patients with no *H19*-DMR epimutation or upd(7)mat and the DMR(s) and imprinted gene(s) responsible for the development of SRS in patients with upd(7)mat. Furthermore, while advanced maternal age at childbirth has been shown to be a predisposing factor for the development of upd(15)mat because of increased non-disjunction at meiosis 1 [50], such studies remain fragmentary for upd(7)mat, primarily because of the relative paucity of upd(7)mat. Further studies will permit a better characterization of SRS.

Supporting Information

Figure S1 Methylation analysis of the KvDMR1 using COBRA. A. Schematic representation of the KvDMR1. A 326 bp region harboring 24 CpG dinucleotides was studied. The cytosine residues at the CpG dinucleotides are usually methylated after paternal transmission (filled circles) and unmethylated after maternal transmission (open circles); after bisulfite treatment, this region is digested with *Hpy*188I when the cytosine at the 5th CpG dinucleotide (indicated with a green rectangle) is methylated and with *Eco*I when the cytosines at the 22nd CpG dinucleotide (indicated with a pink rectangle) is methylated. *KCNQ1OT1* is a paternally expressed gene, and *KCNQ1* and *CDKN1C* are maternally expressed genes. B. Representative COBRA results. U: unmethylated clone specific bands; M: methylated clone specific bands; and BWS: Beckwith-Wiedemann syndrome patient with upd(11p15)pat. C. Histograms showing the distribution of the MIs (the horizontal axis: the methylation index; and the vertical axis: the patient number).
(TIF)

Table S1 Primers utilized in the methylation analysis and microsatellite analysis.
(XLS)

Table S2 The results of microsatellite analysis.
(XLSX)

Table S3 Methylation indices for multiple differetially methylated resions (DMRs) obtained by COBRA in 38 patients with Silver-Russell syndrome.
(XLSX)

Table S4 Clinical findings in two unique patients.
(DOC)

Table S5 Correlation analyses in patients with H19-DMR hypomethylations.
(DOC)

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Author Contributions

Conceived and designed the experiments: TF KY TO. Performed the experiments: TF KN CT S. Sano K. Matsubara MK KY. Analyzed the data: TF KN KH KY. Contributed reagents/materials/analysis tools: SM TN TH RH YM K. Muroya TK CN S. Sato TO. Wrote the paper: TO.

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

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

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

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

Conclusions Upd(14)mat and epimutation of 14q32.2 represent clinically discernible phenotypes and should be designated "upd(14)mat syndrome." This syndrome demonstrates a PWS-like phenotype particularly during infancy. The *MEG3* methylation test can detect upd(14)mat syndrome defects and should therefore be performed for all undiagnosed infants with hypotonia. *(J Pediatr 2009;155:900-3).*

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

Methods

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

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

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

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

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

Results

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

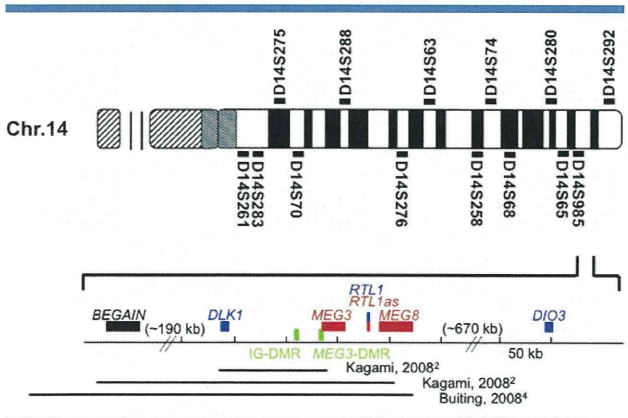

Figure 1. Schematic map of the 14q32.2 imprinted region. Loci on chromosome 14 represent markers used for microsatellite polymorphism analysis. Paternally expressed genes are shown in *blue*, maternally expressed genes in *red*, and nonimprinted genes are shown in *black*. Differentially methylated regions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

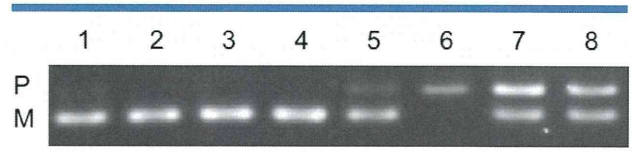

Figure 2. *MEG3* methylation test. *P*, Paternal methylated signal; *M*, maternal unmethylated signal; *1-5*, cases 1-5, respectively; *6*, paternal uniparental disomy 14; *7*, patient with PWS; *8*, normal control. Cases 1-4 show only the maternal unmethylated signal, and case 5 shows a faint paternal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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

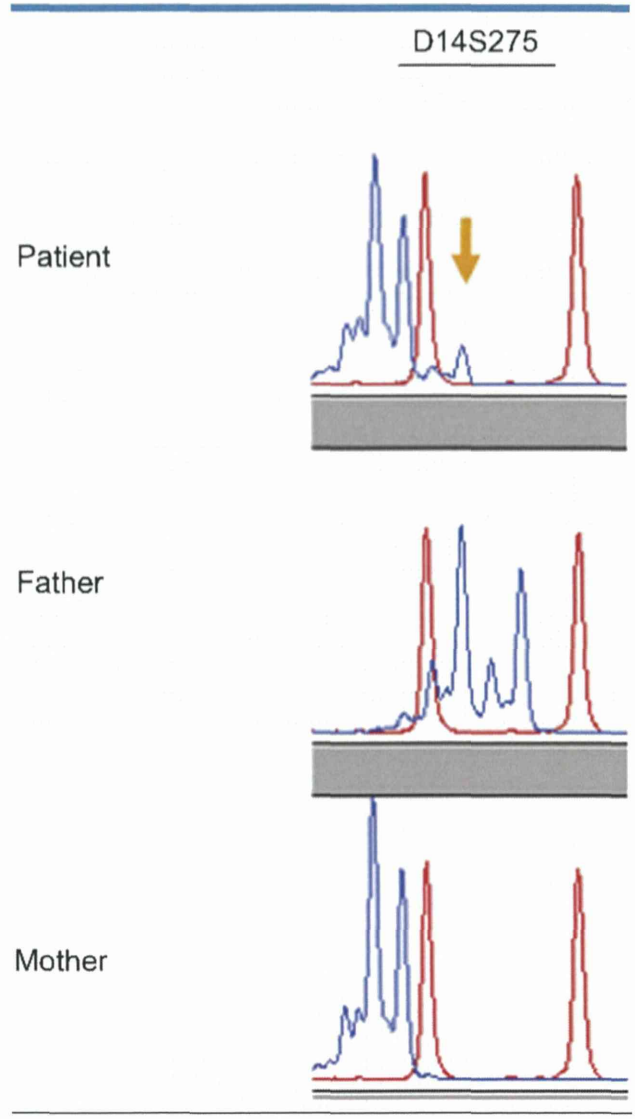

Figure 3. Microsatellite polymorphism analysis at D14S275 for the family of case 5. The patient demonstrates 3 peaks (146, 148, 152 bp), 2 (146, 148 bp) of which are transmitted from the mother, but 1 small peak (152 bp) indicated by the *arrow* is transmitted from the father. *Red peaks* depict size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

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SHORT REPORT

Epimutation (hypomethylation) affecting the chromosome 14q32.2 imprinted region in a girl with upd(14)mat-like phenotype

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Maternal uniparental disomy for chromosome 14 (upd(14)mat) causes clinically discernible features such as pre- and/or postnatal growth failure, hypotonia, obesity, small hands, and early onset of puberty. The monoallelic expression patterns at the 14q32.2 imprinted region are tightly related to methylation status of the *DLK1–MEG3* intergenic differential methylation region (DMR) and the *MEG3*-DMR that are severely hypermethylated after paternal transmission and grossly hypomethylated after maternal transmission. We examined this imprinted region in a 2 2/12-year-old Japanese patient who was born with a normal birth size (length, +0.2 SD; weight, −0.5 SD) and showed postnatal growth failure (height, −3.1 SD; weight, −3.4 SD), hypotonia, frontal bossing, micrognathia, and small hands. Methylation analysis, genotyping analysis, and deletion analysis were performed with blood samples of the patient and the parents, showing that the DMRs of this patient were grossly hypomethylated in the absence of upd(14)mat and deletion of the DMRs. The results indicate the occurrence of an epimutation (hypomethylation) affecting the normally methylated DMRs of paternal origin, and imply that epimutations should be examined in patients with upd(14)mat-like phenotype.
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Keywords: epimutation; growth failure; imprinting; differentially methylated region; upd(14)mat

Introduction

Maternal uniparental disomy for chromosome 14 (upd(14)mat) results in clinically discernible features such as pre- and postnatal growth failure, hypotonia, obesity, small hands, and early onset of puberty.[1] Phenotypic development is consistent with chromosome 14q32.2 region harboring several paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* and maternally expressed genes (*MEGs*) such as *MEG3* (alias *GTL2*), *RTL1as* (*RTL1* antisense), and *MEG8*.[2,3] The parent-of-origin-specific monoallelic expression patterns are tightly related to methylation status of differential methylation regions (DMRs).[4] For the 14q32.2 imprinted region, the previous studies have identified the intergenic DMR (IG-DMR) between *DLK1* and *MEG3* and the *MEG3*-DMR that are severely hypermethylated after paternal transmission and grossly hypomethylated after maternal transmission.[5–7] In particular, the germline-derived IG-DMR plays a pivotal role in the imprinting regulation, because methylation pattern of the secondary *MEG3*-DMR is dependent on that of the IG-DMR.[8]

The upd(14)mat-like phenotype has also been exhibited by non-disomic patients. Temple *et al*[9] described a patient

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with upd(14)mat-like phenotype and an epimutation (hypomethylation) of the normally methylated DMR of paternal origin. Kagami *et al*[5] reported three patients with upd(14)mat-like phenotype and microdeletions affecting the 14q32.2 imprinted region including the DMRs of paternal origin. In this regard, the IG-DMR deletion from the paternally derived chromosome has no effect on the imprinting status, although that from the maternally derived chromosome results in a maternal to paternal epigenotypic alteration.[5,7] Thus, simple genotype–phenotype correlations can be applied for the three patients with the microdeletions, implying that loss of paternally derived *DLK1* and *RTL1* constitutes primary additive underlying factors for the development of upd(14)mat-like phenotype, although the perturbation of other imprinted genes could also have some effects.[5]

Here, we report an epimutation identified in a patient with upd(14)mat-like phenotype.

Patient and methods

Case report

This Japanese girl was born at 41 weeks of gestation after natural conception, with a history of mild oligohydramnios in the third trimester. At birth, her length was 50.0 cm (+0.2 SD), her weight 3.03 kg (−0.5 SD), and her head circumference 34.5 cm (+0.8 SD). The non-consanguineous parents were clinically normal, and the height was 161.0 cm (−1.7 SD) for the father and 154.5 cm (−0.7 SD) for the mother.

At 5 months of age, she was referred to us, because she was unable to control her head. Physical examination revealed generalized hypotonia without palsy and abnormal tendon reflex, and several somatic features such as frontal bossing, micrognathia, and small hands (Supplementary Figure 1). In addition, her length became below −2 SD of the mean from 10 months of age, while hypotonia was gradually ameliorated. She controlled her head at 7 months of age, sat without support at 11 month, and walked without support at 19 months. Repeatedly performed biochemical studies for hypotonia and growth failure were normal, as were skeletal roentgenograms and brain magnetic resonance imaging. The karyotype was 46XX in all the 30 lymphocytes examined. With a provisional diagnosis of Prader–Willi syndrome (PWS) that is primarily based on hypotonia and growth deficiency, fluorescence *in situ* hybridization (FISH) analysis for *SNRPN* and methylation analysis for the DMR at the *SNRPN* promoter region were performed,[10] showing normal findings. In addition, hypomethylation of the *H19*-DMR and upd(7)mat, which can cause growth failure, were also excluded by previous methods.[11,12] On the last examination at 2 2/12 years of age, her height was 76.1 cm (−3.1 SD), her weight 7.9 kg (−3.4 SD), and her head circumference 44.9 cm (−1.9 SD). Her mental development appeared age appropriate.

Methylation analysis

This study was approved by the Institutional Review Board Committees at Hokkaido University Hospital and National Center for Child Health and Development. After obtaining written informed consent, we examined the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (Figure 1a), using bisulfite-treated leukocyte genomic DNA. For the IG-DMR, bisulfite sequencing was performed as reported previously,[5] and the SNPs (*rs12437020* for CG4 and *rs10133627* for CG6) were also genotyped. For the *MEG3*-DMR, PCR amplification was performed with methylated and unmethylated allele-specific primers, as described previously.[5,6] A hitherto unreported upd(14)mat patient and the previously reported upd(14)pat patient[5] were similarly analyzed with permission.

Genotyping analysis

We performed microsatellite analysis for 16 loci on chromosome 14 and SNP analysis for 39 loci around the DMRs (Supplementary Table 1). The primers used were as reported previously.[5]

Deletion analysis

Lymphocyte metaphase spreads were hybridized with a long and accurate (LA)-PCR product encompassing the IG-DMR and that spanning the *MEG3*-DMR (Figure 1a), together with an RP11-566I2 probe for 14q12 used as an internal control. Furthermore, the two LA-PCR products were also obtained from the patient and a control subject, and subjected to fragment size comparisons after restriction enzyme digestions, to detect a possible tiny deletion in the patient. The detailed methods for the deletion analysis have been reported previously.[5]

Results

Methylation analysis

The results are shown in Figure 1b. For the IG-DMR, CG4 and CG6 were grossly hypomethylated in the patient and the upd(14)mat patient, severely methylated in the upd(14)pat patient, and delineated in apparently mosaic patterns in the parents. In addition, the CG4 SNP typing indicated parental origin-dependent methylation patterns in the parents, and heterodisomy for the this region in the upd(14)mat patient. The CG6 SNP typing data were not informative. For the *MEG3*-DMR, PCR products were obtained with an unmethylated allele-specific primer pair alone in the patient and the upd(14)mat patient, with a methylated allele-specific primer pair alone in the upd(14)pat patient, and with both primer pairs in the parents.

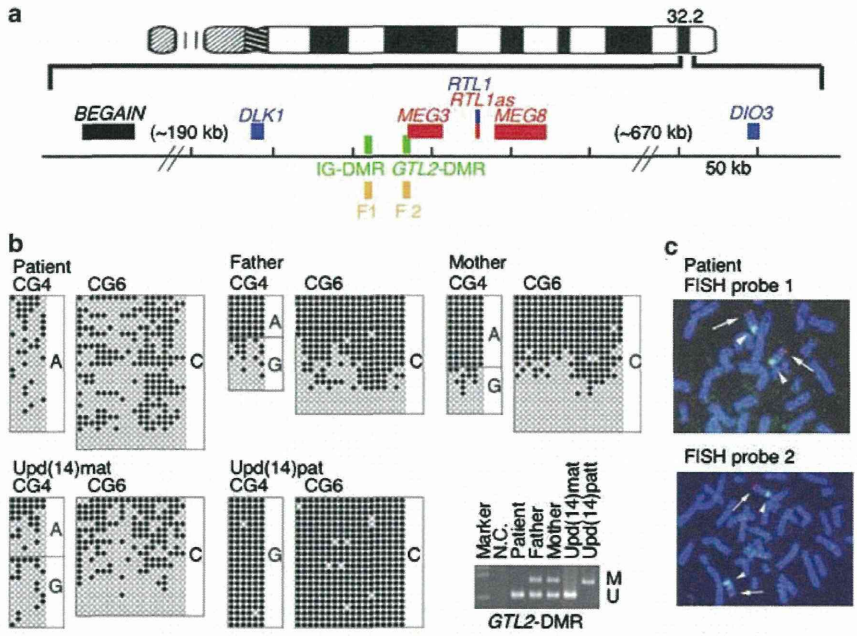

Figure 1 Summary of the molecular studies. (**a**) The regional physical map of the human chromosome 14q32.2 imprinted region. *PEGs* are shown in blue and *MEGs* in red; although it remains to be clarified whether *DIO3* is a *PEG*, mouse *Dio3* is known to be preferentially but not exclusively expressed from a paternally derived chromosome in embryos.[13] *WDR25* and *BEGAIN* appear biparentally expressed genes. The IG-DMR and the *MEG3*-DMR are depicted in green, and the FISH probes 1 and 2 covering the DMRs indicated in orange. The physical distance is ~190 kb between *BEGAIN* and *DLK1*, ~170 kb between *DLK1* and *MEG8*, and ~670 kb between *MEG8* and *DIO3*. (**b**) Methylation patterns of the IG-DMR (CG4 and CG6) and the *MEG3*-DMR. Bisulfite sequencing has been performed for CG4 and CG6. Each line indicates a single clone and each circle denotes a CpG island; filled and open circles represent methylated and unmethylated cytosines, respectively. The SNP typing data for CG4 and CG6 are also shown. Methylated (M) and unmethylated (U) allele-specific PCR amplification has been performed for the *MEG3*-DMR. NC: negative control. (**c**) FISH analysis using FISH probe 1 (F1) for the IG-DMR and FISH probe 2 (F2) for the *MEG3*-DMR. The red signals (arrows) have been detected by the two FISH probes and the green signals (arrowheads) have been identified by an RP11-566I2 probe for 14q12 used as an internal control.

Genotyping analysis

Microsatellite analysis demonstrated biparental origin of the two chromosome 14 homologs, and SNP analysis indicated lack of a segmental upd(14)mat around the DMRs (Supplementary Table 1).

Deletion analysis

FISH probes 1 and 2 detected two signals in the patient (Figure 1c). The fragment size comparison after enzyme digestions showed no abnormal bands suggestive of a tiny deletion in the patient.

Discussion

This patient had hypomethylated DMRs in the absence of discernible maternal disomy affecting the DMRs or loss of the paternally derived DMRs. This implies the occurrence of an epimutation (hypomethylation) affecting the normally methylated DMRs of paternal origin. To our knowledge, such an epimutation (hypomethylation) has previously been identified only in a patient reported by Temple *et al*.[9] Actually, the DMR examined in that patient appears to be a part of the *MEG3*-DMR rather than the IG-DMR on the basis of its position. It is likely, however, that the IG-DMR is also hypomethylated in that patient, because the *MEG3*-DMR can stay hypomethylated only in the presence of the hypomethylated IG-DMR.[8]

Clinical features of the two patients with epimutation are summarized in Table 1, together with those of upd(14)mat patients. Notably, clinical features are grossly similar in epimutation patients and upd(14)mat patients. Although our patient had no prenatal growth failure, lack of prenatal and/or postnatal growth failure has been described in several upd(14)mat patients,[14–16] and this would be due to body growth being a multifactorial trait subject to multiple genetic and environmental factors.[17] In this regard, it has been reported that clinical features are comparable between patients with paternal upd(14) and those with epimutations (hypermethylation) affecting the normally hypomethylated DMRs of maternal origin.[5] Taken together, the methylation patterns of the DMRs appear to be closely related to the expression patterns of virtually all the imprinted genes on 14q32.2.

It is noteworthy that the patient was initially suspected as having PWS. Indeed, growth deficiency, hypotonia, and small hands are shared by upd(14)mat and PWS,[18,19] and

Table 1 Clinical phenotypes in patients with epimutations and upd(14)mat

	Epimutations		*Upd(14)mat*
	This report	*Temple* et al[9]	*(n = 35)*[a]
Age	2 2–12 years	10 7–12 years	0–30 years
Sex	Female	Male	M:F = 17:18
Premature delivery	–	–	10/25
Prenatal growth failure	–	+	24/27
Postnatal growth failure	+	+	26/32
Somatic features	+	+	23/35[b]
Frontal bossing	+	+	9/9
High arched palate	–	+	7/9
Micrognathia	+	–	5/5
Small hands	+	+	24/27
Scoliosis	–	+	5/19
Others			
Hypotonia	+	+	25/28
Obesity	–	–	14/34
Early onset of puberty	Unknown	Borderline	14/16
Mental retardation	–	–	10/27
Thyroid dysfunction	–	–	ND

ND: not described.
In the column summarizing the clinical features of 35 patients with upd(14)mat, the denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature; thus, the differences between the denominators and the numerators denote the number of patients evaluated to be negative for that feature.
[a]Patients with maternal uniparental disomy for chromosome 14 reported in the literature, several upd(14)mat patients with no phenotypic description have not been included. The references for the 35 upd(14)mat patients are summarized in Kagami *et al.*[5]
[b]The ratio of patients with at least one somatic feature.

upd(14)mat has occasionally been identified in patients referred for molecular examination of PWS.[19,20] Thus, upd(14)mat and epimutations should be considered in patients with PWS-like phenotype.[18,19]

In summary, we observed an epimutation (hypomethylation) of the paternally derived DMRs in a patient with upd(14)mat-like phenotype. Further studies will identify epimutations in patients with upd(14)mat-like phenotype, thereby contributing to clarify the relevance of epimutations in human imprinted disorders.

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Disclosure

The authors have reported no conflicts of interest.

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

RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Prenatal Genetic Testing for a Microdeletion at Chromosome 14q32.2 Imprinted Region Leading to UPD(14)pat-like Phenotype

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TO THE EDITOR:

Human chromosome 14q32.2 imprinted region carries several paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* and maternally expressed genes (*MEGs*) such as *MEG3* (alias, *GTL2*) and *RTL1as* (*RTL1 antisense*), together with the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR (Fig. 1) [da Rocha et al., 2008; Kagami et al., 2008a]. Consistent with this, paternal uniparental disomy 14 (UPD(14) results in a unique phenotype characterized by facial abnormality, small bell-shaped thorax, abdominal wall defects, placentomegaly, and polyhydramnios [Kagami et al., 2005, 2008a,b]. In this regard, we have recently reported that heterozygous microdeletions and epimutations (hypermethylations) affecting unmethylated DMR (s) of maternal origin also lead to UPD(14)pat-like phenotype [Kagami et al., 2008a, 2010, 2012]. Indeed, after studying 26 patients with UPD(14)pat-like phenotype, we identified UPD (14)pat in 17 patients (65.4%), microdeletions in 5 patients (19.2%), and epimutations in 4 patients (15.4%) [Kagami et al., 2012]. Importantly, although there is no report describing recurrence of UPD(14)pat and epimutation in familial members with a normal karyotype, microdeletions can be transmitted recurrently from mothers with the same heterozygous microdeletions to offsprings [Kagami et al., 2008a]. Here, we report on our experience of a prenatal genetic testing for a microdeletion at the chromosome 14q32.2 imprinted region.

A 33-year-old Japanese woman came to us with her husband seeking for prenatal diagnosis of a fetus at 9 weeks of gestation. The first child and the mother have been reported previously as cases 3 and 11 of Family B in Kagami et al. [2008a]. In brief, the child had upd(14)pat-like phenotype and a maternally derived 411,354 bp microdeletion involving *WDR25*, *BEGAIN*, *DLK1*, *MEG3*, *RTL1/RTL1as*, and *MEG8* (Fig. 1). The mother had UPD(14)mat-like phenotype and the same microdeletion on the paternally derived chromosome 14. The parents hoped to deliver the fetus at a local hospital if there is no microdeletion or at our hospital with a neonatal intensive care unit if a microdeletion is identified.

After thorough consultation, we performed trans-abdominal chorionic villus sampling (CVS) at 12 weeks of gestation. Immediately after the sampling, fluorescence in situ hybridization was carried out with an RP11-566J3 probe detecting a segment within

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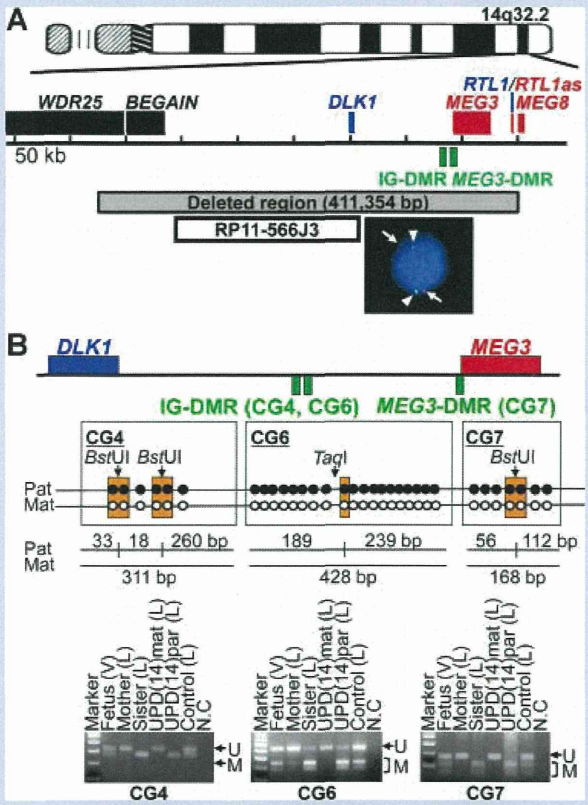

FIG. 1. Summary of the molecular studies. A: The physical map of the 14q32.2 imprinted region and the FISH finding of the fetus. *PEGs* are shown in blue, *MEGs* in red, and the IG-DMR and the *MEG3*-DMR in green. The gray rectangle indicates the 411,354 bp microdeletion identified in the first child and the mother, and the white rectangle denotes the region detected by the RP11-566J3 BAC probe. The FISH analysis reveals two red signals (arrows) identified by the RP11-566J3 BAC probe and two green signals (arrowheads) detected by the RP11-566I2 BAC probe for 14q12 utilized as an internal control. B: The methylation analysis for the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) by COBRA. The black and white circles indicate methylated and unmethylated cytosines at the CpG dinucleotides, respectively. Pat: paternally derived chromosome; and Mat: maternally derived chromosome. PCR products for CG4 (311 bp) are digested with *Bst*UI into three fragments (33, 18, and 260 bp) when cytosines at the first and the second CpG dinucleotides and the fourth and fifth CpG dinucleotides (indicated with orange rectangles) are methylated. The PCR products for CG6 (428 bp) are digested with *Taq*I into two fragments (189 and 239 bp) when the cytosine at the 9th CpG dinucleotide (indicated with an orange rectangle) is methylated. The PCR products of CG7 (168 bp) are digested with *Bst*UI into two fragments (56 and 112 bp) when the cytosines at the fourth and fifth CpG dinucleotides (indicated with orange rectangle) are methylated. Both methylated (M)- and unmethylated (U)-specific bands are identified in the chorionic villus sample. V, villi; L, leukocytes; and N.C, negative control.

the deleted region of the first child and the mother, delineating two signals on villus cell interphase spreads (Fig. 1). Next combined bisulfite restriction analysis (COBRA) was performed for the IG-DMR and the *MEG3*-DMR using villus cell genomic DNA, identifying both methylated- and unmethylated allele-specific bands (Fig. 1B). These findings clearly excluded the presence of a microdeletion in the fetus by 14 weeks of gestation. Subsequent pregnant course was uneventful, and a phenotypically normal infant was delivered at term by a caesarean section.

To our knowledge, this is the first report describing a prenatal genetic testing for a familial microdeletion affecting the chromosome 14q32.2 imprinted region. Although such a genetic testing is possible only when an accurate genetic diagnosis has been made for the proband, it permitted the precise diagnosis before the second to the third trimester when characteristic UPD(14)pat-like features such as bell-shaped small thorax with coat hanger appearance and polyhydroamnios become detectable by ultrasonographic studies [Suzumori et al., 2010; Yamanaka et al., 2010]. Such an early prenatal diagnosis, though it is associated with a certain risk such as CVS-induced abortion, provides critical information for the clinical management. When a microdeletion is excluded as shown in this case, this releases the parents from the anxiety of having an affected fetus and allows for a standard follow-up during pregnancy. By contrast, when a microdeletion is identified, this will allow for appropriate management during pregnancy (e.g., amnioreduction to mitigate the risk of threatened premature delivery) and pertinent therapeutic interventions for the infant (e.g., respiratory management). Thus, prenatal genetic diagnosis appears to be beneficial for the fetus and the parents, when it is performed at appropriate institutes where a multidisciplinary team including a genetic counselor is available.

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