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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References

1. **Wilson LC, Oude Luttikhuis ME, Baraitser M, Kingston HM, Trembath RC** 1997 Normal erythrocyte membrane Gsα bioactivity in two unrelated patients with acrodysostosis. J Med Genet 34:133–136
2. **Graham Jr JM, Krakow D, Tolo VT, Smith AK, Lachman RS** 2001 Radiographic findings and Gs-α bioactivity studies and mutation screening in acrodysostosis indicate a different etiology from pseudohypoparathyroidism. Pediatr Radiol 31:2–9
3. **Linglart A, Menguy C, Couvineau A, Auzan C, Gunes Y, Cancel M, Motte E, Pinto G, Chanson P, Bougnères P, Clauser E, Silve C** 2011 Recurrent PRKAR1A mutation in acrodysostosis with hormone resistance. N Engl J Med 364:2218–2226
4. **Lee H, Graham Jr JM, Rimoin DL, Lachman RS, Krejci P, Tompson SW, Nelson SF, Krakow D, Cohn DH** 2012 Exome sequencing identifies PDE4D mutations in acrodysostosis. Am J Hum Genet 90:746–751
5. **Michot C, Le Goff C, Goldenberg A, Abhyankar A, Klein C, Kinning E, Guerrot AM, Flahaut P, Duncombe A, Baujat G, Lyonnet S, Thalassinos C, Nitschke P, Casanova JL, Le Merrer M, Munnich A, Cormier-Daire V** 2012 Exome sequencing identifies PDE4D mutations as another cause of acrodysostosis. Am J Hum Genet 90:740–745
6. **Scott JD** 1991 Cyclic nucleotide-dependent protein kinases. Pharmacol Ther 50:123–145
7. **Taskén K, Skålhegg BS, Solberg R, Andersson KB, Taylor SS, Lea T, Blomhoff HK, Jahnsen T, Hansson V** 1993 Novel isozymes of cAMP-dependent protein kinase exist in human cells due to formation of RIα-RIβ heterodimeric complexes. J Biol Chem 268:21276–21283
8. **Kim C, Xuong NH, Taylor SS** 2005 Crystal structure of a complex between the catalytic and regulatory (RIα) subunits of PKA. Science 307:690–696
9. **Verghese MW, McConnell RT, Lenhard JM, Hamacher L, Jin SL** 1995 Regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. Mol Pharmacol 47:1164–1171
10. **Ablow RC, Hsia YE, Brandt IK** 1977 Acrodysostosis coinciding with pseudohypoparathyroidism and pseudo-pseudohypoparathyroidism. AJR Am J Roentgenol 128:95–99

11. **Weinstein LS, Yu S, Warner DR, Liu J** 2001 Endocrine manifestations of stimulatory G protein α-subunit mutations and the role of genomic imprinting. Endocr Rev 22:675–705
12. **Mantovani G, Bondioni S, Locatelli M, Pedroni C, Lania AG, Ferrante E, Filopanti M, Beck-Peccoz P, Spada A** 2004 Biallelic expression of the Gsα gene in human bone and adipose tissue. J Clin Endocrinol Metab 89:6316–6319
13. **He H, Olesnanik K, Nagy R, Liyanarachchi S, Prasad ML, Stratakis CA, Kloos RT, de la Chapelle A** 2005 Allelic variation in gene expression in thyroid tissue. Thyroid 15:660–667
14. **Foss KB, Landmark B, Skålhegg BS, Taskén K, Jellum E, Hansson V, Jahnsen T** 1994 Characterization of in-vitro-translated human regulatory and catalytic subunits of cAMP-dependent protein kinases. Eur J Biochem 220:217–223
15. **Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Jüppner H, Segre GV, Kronenberg HM** 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273:663–666
16. **Horvath A, Bertherat J, Groussin L, Guillaud-Bataille M, Tsang K, Cazabat L, Libé R, Remmers E, René-Corail F, Faucz FR, Clauser E, Calender A, Bertagna X, Carney JA, Stratakis CA** 2010 Mutations and polymorphisms in the gene encoding regulatory subunit type 1-α of protein kinase A (PRKAR1A): an update. Hum Mutat 31:369–379
17. **Robinson-White A, Meoli E, Stergiopoulos S, Horvath A, Boikos S, Bossis I, Stratakis CA** 2006 PRKAR1A mutations and protein kinase A interactions with other signaling pathways in the adrenal cortex. J Clin Endocrinol Metab 91:2380–2388
18. **Kronenberg HM, Melmed S, Polonsky KS, Larsen PR** 2008 Endocrine change in pregnancy. In: Braunstein GD, ed. Williams textbook of endocrinology. 11th ed. Philadelphia: Saunders Elsevier; 743

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ARTICLE

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

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

INTRODUCTION

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

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

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

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

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

PATIENTS AND METHODS

Patients

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

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

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

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

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

Analysis of subtypes in patients with UPD(14)pat

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

Table 1 Summary of patients examined in this study

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

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

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

Analysis of parental ages

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

RESULTS

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

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

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

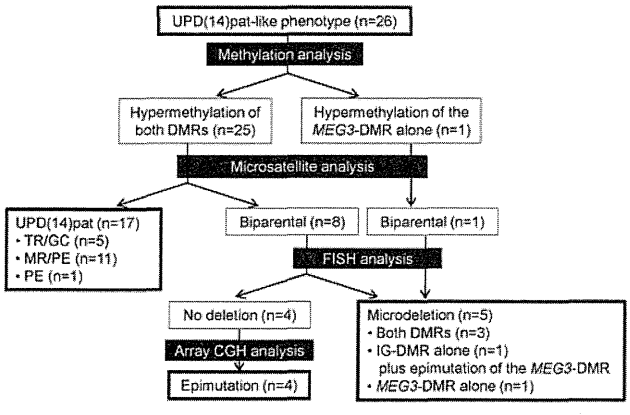

Figure 1 Classification of 26 patients with UPD(14)pat-like phenotype.

Analysis of subtypes in patients with UPD(14)pat

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

Analysis of parental ages

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

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

DISCUSSION

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

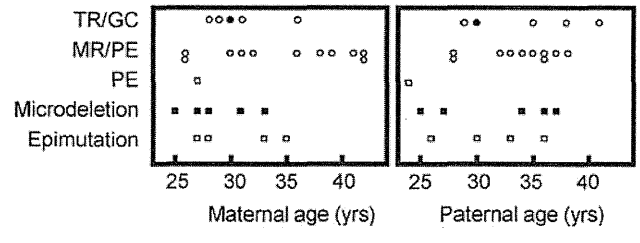

Figure 2 The distribution of parental ages at childbirth according to the underlying genetic causes for the development of UPD(14)pat-like phenotype and UPD(14)pat subtypes. Of the five plots for the TR/GC subtype, open and black circles indicate the TR/GC subtype due to non-disjunction at paternal M1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

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

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

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

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

Table 2 Relative frequency of genetic mechanisms in imprinting disorders

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

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

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

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

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 da Rocha ST, Edwards CA, Ito M, Ogata T, Ferguson-Smith AC: Genomic imprinting at the mammalian Dlk1-Dio3 domain. *Trends Genet* 2008; **24**: 306–316.
2 Kagami M, Sekita Y, Nishimura G *et al*: Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-like phenotypes. *Nat Genet* 2008; **40**: 237–242.
3 Kagami M, O'Sullivan MJ, Green AJ *et al*: The IG-DMR and the MEG3-DMR at human chromosome 14q32.2: hierarchical interaction and distinct functional properties as imprinting control centers. *PLoS Genet* 2010; **6**: e1000992.
4 Kagami M, Nishimura G, Okuyama T *et al*: Segmental and full paternal isodisomy for chromosome 14 in three patients: narrowing the critical region and implication for the clinical features. *Am J Med Genet A* 2005; **138A**: 127–132.
5 Kagami M, Yamazawa K, Matsubara K, Matsuo N, Ogata T: Placentomegaly in paternal uniparental disomy for human chromosome 14. *Placenta* 2008; **29**: 760–761.
6 Shaffer LG, Agan N, Goldberg JD, Ledbetter DH, Longshore JW, Cassidy SB: American College of Medical Genetics statement of diagnostic testing for uniparental disomy. *Genet Med* 2001; **3**: 206–211.
7 Jones KT: Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age. *Hum Reprod Update* 2008; **14**: 143–158.
8 Matsubara K, Murakami N, Nagai T, Ogata T: Maternal age effect on the development of Prader-Willi syndrome resulting from upd(15)mat through meiosis 1 errors. *J Hum Genet* 2011; **56**: 566–571.
9 Robinson WP, Christian SL, Kuchinka BD *et al*: Somatic segregation errors predominantly contribute to the gain or loss of a paternal chromosome leading to uniparental disomy for chromosome 15. *Clin Genet* 2000; **57**: 349–358.
10 Hoffmann K, Heller R: Uniparental disomies 7 and 14. *Best Pract Res Clin Endocrinol Metab* 2011; **25**: 77–100.
11 Wang JC, Passage MB, Yen PH, Shapiro LJ, Mohandas TK: Uniparental heterodisomy for chromosome 14 in a phenotypically abnormal familial balanced 13/14 Robertsonian translocation carrier. *Am J Hum Genet* 1991; **48**: 1069–1074.
12 Papenhausen PR, Mueller OT, Johnson VP, Sutcliffe M, Diamond TM, Kousseff BG: Uniparental isodisomy of chromosome 14 in two cases: an abnormal child and a normal adult. *Am J Med Genet* 1995; **59**: 271–275.
13 Cotter PD, Kaffe S, McCurdy LD, Jhaveri M, Willner JP, Hirschhorn K: Paternal uniparental disomy for chromosome 14: a case report and review. *Am J Med Genet* 1997; **70**: 74–79.
14 Yano S, Li L, Owen S, Wu S, Tran T: A further delineation of the paternal uniparental disomy (UPD14): the fifth reported liveborn case. *Am J Hum Genet* 2001; **69** (Suppl): A739.
15 Kurosawa K, Sasaki H, Sato Y *et al*: Paternal UPD14 is responsible for a distinctive malformation complex. *Am J Med Genet A* 2002; **110**: 268–272.
16 McGowan KD, Weiser JJ, Horwitz J *et al*: The importance of investigating for uniparental disomy in prenatally identified balanced acrocentric rearrangements. *Prenat Diagn* 2002; **22**: 141–143.
17 Sasaki K, Soejima H, Higashimoto K *et al*: Japanese and North American/European patients with Beckwith-Wiedemann syndrome have different frequencies of some epigenetic and genetic alterations. *Eur J Hum Genet* 2007; **15**: 1205–1210.
18 Eggermann T: Epigenetic regulation of growth: lessons from Silver-Russell syndrome. *Endocr Dev* 2009; **14**: 10–19.
19 Gurrieri F, Accadia M: Genetic imprinting: the paradigm of Prader-Willi and Angelman syndromes. *Endocr Dev* 2009; **14**: 20–28.
20 Pujana MA, Nadal M, Guitart M, Armengol L, Gratacos M, Estivill X: Human chromosome 15q11-q14 regions of rearrangements contain clusters of LCR15 duplicons. *Eur J Hum Genet* 2002; **10**: 26–35.
21 Varela MC, Kok F, Setian N, Kim CA, Koiffmann CP: Impact of molecular mechanisms, including deletion size, on Prader-Willi syndrome phenotype: study of 75 patients. *Clin Genet* 2005; **67**: 47–52.
22 Pellestor F, Andreo B, Anahory T, Hamamah S: The occurrence of aneuploidy in human: lessons from the cytogenetic studies of human oocytes. *Eur J Med Genet* 2006; **49**: 103–116.
23 Sloter E, Nath J, Eskenazi B, Wyrobek AJ: Effects of male age on the frequencies of germinal and heritable chromosomal abnormalities in humans and rodents. *Fertil Steril* 2004; **81**: 925–943.
24 Kotzot D: Advanced parental age in maternal uniparental disomy (UPD): implications for the mechanism of formation. *Eur J Hum Genet* 2004; **12**: 343–346.
25 Fokstuen S, Ginsburg C, Zachmann M, Schinzel A: Maternal uniparental disomy 14 as a cause of intrauterine growth retardation and early onset of puberty. *J Pediatr* 1999; **134**: 689–695.
26 Hordijk R, Wierenga H, Scheffer H, Leegte B, Hofstra RM, Stolte-Dijkstra I: Maternal uniparental disomy for chromosome 14 in a boy with a normal karyotype. *J Med Genet* 1999; **36**: 782–785.
27 Sanlaville D, Aubry MC, Dumez Y *et al*: Maternal uniparental heterodisomy of chromosome 14: chromosomal mechanism and clinical follow up. *J Med Genet* 2000; **37**: 525–528.
28 Towner DR, Shaffer LG, Yang SP, Walgenbach DD: Confined placental mosaicism for trisomy 14 and maternal uniparental disomy in association with elevated second trimester maternal serum human chorionic gonadotrophin and third trimester fetal growth restriction. *Prenat Diagn* 2001; **21**: 395–398.
29 Aretz S, Raff R, Woelfle J *et al*: Maternal uniparental disomy 14 in a 15-year-old boy with normal karyotype and no evidence of precocious puberty. *Am J Med Genet A* 2005; **135**: 336–338.
30 Mitter D, Buiting K, von Eggeling F *et al*: Is there a higher incidence of maternal uniparental disomy 14 [upd(14)mat]? Detection of 10 new patients by methylation-specific PCR. *Am J Med Genet A* 2006; **140**: 2039–2049.

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MAMLD1 and 46,XY Disorders of Sex Development

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Abstract

MAMLD1 (mastermind-like domain containing 1) is a recently discovered causative gene for 46,XY disorders of sex development (DSD), with hypospadias as the salient clinical phenotype. To date, microdeletions involving *MAMLD1* have been identified in six patients, and definitive mutations (nonsense and frameshift mutations that are predicted to undergo nonsense mediated mRNA decay [NMD]) have been found in six patients. In addition, specific *MAMLD1* cSNP(s) and haplotype may constitute a susceptibility factor for hypospadias. Furthermore, in vitro studies have revealed that (1) the mouse homolog is expressed in fetal Sertoli and Leydig cells around the critical period for sex development; (2) transient *Mamld1* knockdown results in significantly reduced testosterone production primarily because of compromised 17α-hydroxylation and *Cyp17a1* expression in Murine Leydig tumor cells; (3) MAMLD1 localizes to the nuclear bodies and transactivates the promoter activity of a non-canonical Notch target gene hairy/enhancer of split 3, without demonstrable DNA-binding capacity; and (4) *MAMLD1* is regulated by steroidogenic factor 1 (SF1). These findings suggest that the *MAMLD1* mutations cause 46,XY DSD primarily because of compromised testosterone production around the critical period for sex development. Further studies will provide useful information for the molecular network involved in fetal testosterone production.

Keywords
- *MAMLD1*
- 46,XY DSD
- hypospadias
- testosterone

MAMLD1 (mastermind-like domain containing, 1), previously known as *CXORF6* (chromosome X open reading frame 6), is a recently discovered gene for 46,XY disorders of sex development (DSD) with abnormal external genitalia, especially hypospadias.[1] After the first report describing *MAMLD1* mutations in human 46,XY DSD, a remarkable progress has been made for *MAMLD1*. Here, we summarize the current knowledge about *MAMLD1*, including some hitherto unreported data.

Cloning of *CXORF6* as a Candidate Gene for 46, XY DSD

A gene for 46,XY DSD has been postulated around *MTM1* for myotubular myopathy on Xq28. Indeed, since genital development is normal in patients with intragenic *MTM1* mutations and invariably abnormal in six patients with microdeletions involving *MTM1* (patients 1–6 in ►**Table 1**),[2–5] this suggests that a gene for sex development resides in the vicinity of *MAM1*, and that loss or disruption of the putative sex development gene results in 46,XY DSD as a consequence of contiguous gene deletion syndrome.

In 1997, Laporte et al[6] identified a protein coding gene *CXORF6* from a 430-kb region deleted in two sporadic cases with myotubular myopathy and 46,XY DSD[2] (►**Fig. 1**). *CXORF6* consists of seven exons, and harbors a protein coding sequence on exons 3–6 that is predicted to produce two proteins of 701 and 660 amino acids because of in-frame alternative splicing with and without exon 4. Furthermore, subsequent studies have shown that *MAMLD1* is located

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Table 1 Genital findings in Patients with MAMLD1 Deletions or Mutations

Patient	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12
Deletion/Mutation	Deletion	Deletion	Deletion	Deletion	Deletion	Deletion	p.E124X	p.E124X	p.Q197X	p.R653X	p.E109fs121X	p.E109fs121X
Inheritance	Sporadic	Sporadic	Familial	Familial	Familial	Sporadic	Familial	Familial	Sporadic	Sporadic	Sporadic	Sporadic
Age at examination	Neonate	Neonate	Neonate	Neonate	Fetus	..	4 months	1 month	2 years	1 month	1 year	1 2/12 year
Ambiguity	Yes	No	No	No	No	No	No	No	No	No	No	No
Hypospadias	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
(Type)	Severe	Penoscrotal	Glandular	Penile	Penile	N.D.	Penoscrotal	Penoscrotal	Penoscrotal	Penoscrotal	Penile	Penoscrotal
Micropenis	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	No	No	Yes	Yes	No	No
Cryptorchidism	Yes (B)	N.D.	Yes (R)	Yes (B)	N.D.	N.D.	Yes (B)	No	No	Yes (B)	Yes (B)	No
Scrotal abnormalities	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	Yes.	Yes	Yes	Yes	No	No
Other findings	Vaginal pouch											

Patients 1 & 2: cases 474 and 441 in Hu et al[2]; patient 3–5: cases III-1, III-2, and III-3 in Barsch et al[4]; patient 6: case CNM86 in Biancalana et al[5]; patients 7–10: cases 1–4 in Fukami et al[1]; and patients 11 and 12: cases 2 and 3 in Kalfa et al[11] N.D.: not described; B: bilateral; and R: right.

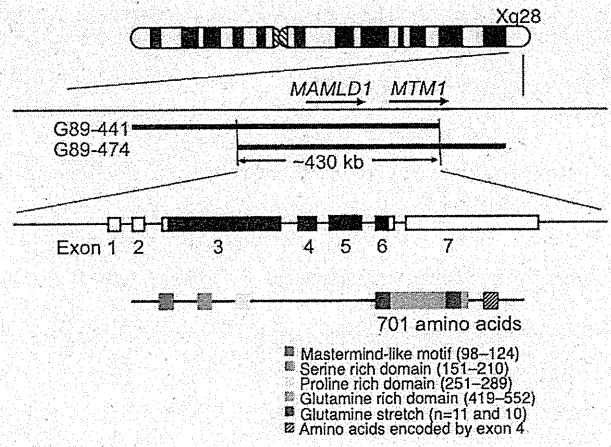

Figure 1 Positional cloning and the structure of *MAMLD1*. *MAMLD1* (*CXORF6*) has been isolated from a ~430 kb region commonly deleted in two patients with 46,XY DSD and myotubular myopathy (G89–441 and G89–474).[2] The horizontal bars indicate the deleted segments that involve *MAMLD1* and *MTM1* for myotubular myopathy. *MAMLD1* comprised 7 exons; the black and the white boxes represent the coding regions and the untranslated regions, respectively. MAMLD1 protein harbors mastermind-like domain and other characteristic domains.

within the smallest region of overlap in all patients with myotubular myopathy and 46,XY DSD,[7] and no other candidate gene for 46,XY DSD has been identified within the commonly deleted region. These findings imply that *MAMLD1* is an excellent candidate gene for 46,XY DSD.

MAMLD1 Mutations in 46,XY DSD Patients

The first evidence for *MAMLD1* being the causative gene for 46,XY DSD came from our group.[1] We performed direct sequencing for the coding exons 3–6 and their flanking splice sites of *MAMLD1* in 117 Japanese patients with various types of 46,XY DSD including 56 patients with hypospadias (16 with glandular type, 16 with penile type, 20 with pen scrotal type, and 4 with perineal type) associated with other external genital abnormalities, as well as in 49 European and Chinese patients with various types of abnormal genitalia ranging from hypospadias to feminized genitalia. Consequently, three nonsense mutations were identified in Japanese patients with hypospadias and other external abnormalities: p.E124X on exon 3 in two maternally related half brothers, p.Q197X on exon 3 in a sporadic patient, and p.R653X on exon 5 in a sporadic patient (patients 7–10 in ►**Table 1**).[1] The mothers of families A and C were heterozygous for the mutations, although the mother of family B was not studied.

The three nonsense mutations satisfy the conditions for the occurrence of nonsense mediated mRNA decay (NMD).[8] Consistent with this, RT-PCR from leukocytes indicated drastically reduced transcripts for the three nonsense mutations.[1] Furthermore, the NMD was prevented by the NMD inhibitor cycloheximide, providing further support for the occurrence of NMD in the three nonsense mutations. The occurrence of NMD was also demonstrated in the carrier mothers.[9] Thus, although the NMD has not been confirmed in the testicular

tissue, the results indicate that the three nonsense mutations are actually pathologic disease-causing mutations.

The occurrence of NMD would explain the apparently discordant genital phenotype between the patient with p.R653X and the Japanese patient with a microdeletion involving *MTM1* reported by Tsai et al.[10] In contrast to the p.R653X, the microdeletion resulted in the generation of a fusion gene between exons 1 to 4 of *MAMLD1* and exons 3 to 16 of *MTMR1* (locus order: *MAMLD1–MTM1–MTMR1*) that escaped NMD and was expressed at least in the muscle. Thus, although both the cases retained *MAMLD1* exons 1 to 4 and were missing *MAMLD1* exons 5 to 7, the patient with p.R653X had 46,XY DSD because of NMD, and the patient with the microdeletion had apparently normal genital development because of its positive expression.

Subsequently, Kalfa et al[11] have identified p.E109fs121X (c.325delG) that is predicted to undergo NMD in two of 41 41 patients with hypospadias of variable degrees (patients 11 and 12 in ►**Table 1**). Furthermore, several mutations confirmed by functional studies have been identified to date (our unpublished observation).

Additional substitutions have also been identified in patients with 46,XY DSD. First, Kalfa et al[11] identified p.V432A and p.531ins3Q (expansion of the second polyglutamine domain from 10 to 13) in single sporadic patients. However, both variants were detected in normal individuals by subsequent examination.[12] In addition, we performed functional studies for p.V432A using Hes3 (see below), and found apparently normal transcriptional activity (►**Fig. 2**). Second, Chen et al[12] identified p.Q529K, which could affect splicing, in a patient with severe hypospadias. However, no functional studies have been performed for p.Q529K. Third, Brandao et al[13] detected p.H432Q, which interestingly appears to have an increased rather than a decreased function, in 4 of 50 patients with 46,XY DSD. However, this substitution is registered as a polymorphism at present, indicating the presence of this substitution in apparently normal individuals. Thus, there is no direct evidence for these substitutions being pathologic mutations. Rather, these substitutions appear to be variations rather than mutations. Nevertheless, p.531ins3Q, which may affect the three-dimensional protein structure, could function as a susceptibility factor, as has been shown for the polyglutamine expansion in exon 1 of *AR* for androgen receptor.[14]

Taken together, it is obvious that *MAMLD1* is a causative gene for 46,XY DSD with hypospadias as a salient phenotype, because of the identification of nonsense mutations and a frameshift mutation that should be subject to NMD. Furthermore, it might be possible that the identified substitutions may function as susceptibility factors.

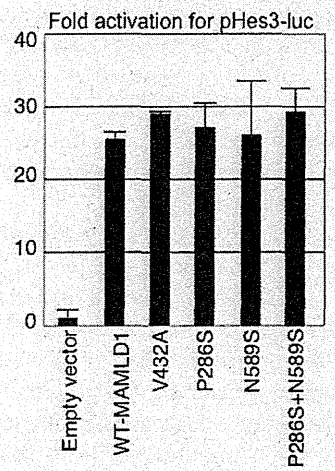

Figure 2 Functional studies for *MAMLD1* substitutions. The p.V432A, p.P286S, p.N589S, and p.P286S–p.N589S (S–S haplotype) have normal transactivating activities for the promoter of a non-canonical Notch target Hes3.

Phenotypes in Affected Patients

Genital findings in patients with microdeletions involving *MAMLD1* and in those with definitive intragenic *MAMLD1* mutations are shown in ►**Table 1**. Although detailed phenotypes are not examined in patients with microdeletions encompassing *MAMLD1*, affected patients almost invariably have hypospadias of variable degrees and often exhibit other genital features such as micropenis, cryptorchidism, and abnormal scrotum. Furthermore, patient 1 manifests rather ambiguous genitalia with virginal pouch, and patient 12 exhibits apparently isolated hypospadias phenotype. Thus, the phenotypic spectrum of *MAMLD1* mutations appears to be somewhat variable, with hypospadias as the core genital abnormality.

Detailed endocrine data are available in patients 7 to 10 in ►**Table 1**.[1] Serum testosterone was sufficiently high during the mini-puberty period, and response was well to human chorionic gonadotropin (hCG) stimulation during infancy to early childhood. This implies that *MAMLD1* mutations exert their deleterious effects primarily in the fetal period, as supported by the *Mamld1* expression pattern in the fetal and postnatal testes (see below). However, our long-term follow-up examinations have revealed that patients with *MAMLD1* mutations exhibit primary gonadal dysfunction in late childhood (our unpublished observation). This is consistent with weak but detectable *Mamld1* expression in the postnatal testis (our unpublished observation), and suggests deterioration in testicular function with age.

Expression Patterns of *MAMLD1*|*Mamld1*

In the human, PCR-based screening for cDNA samples has revealed ubiquitous expression of *MAMLD1* including fetal testis, with two in-frame splice variants, a major form with exon 4 and a minor form without exon 4.[1] Furthermore, RT-PCR analysis using human fetal testis has shown clear and gradually increasing expression of *MAMLD1* during the second trimester.[15]

More detailed expression studies have been performed in the mouse.[1] In situ hybridization (ISH) analysis has shown that, in the fetal testis, *Mamld1* is weakly expressed in the internal region at E11.5, and clearly expressed in Sertoli cells and in a small number of Leydig cells at E12.5. At E14.5, *Mamld1* is still clearly expressed in Sertoli cells and in the

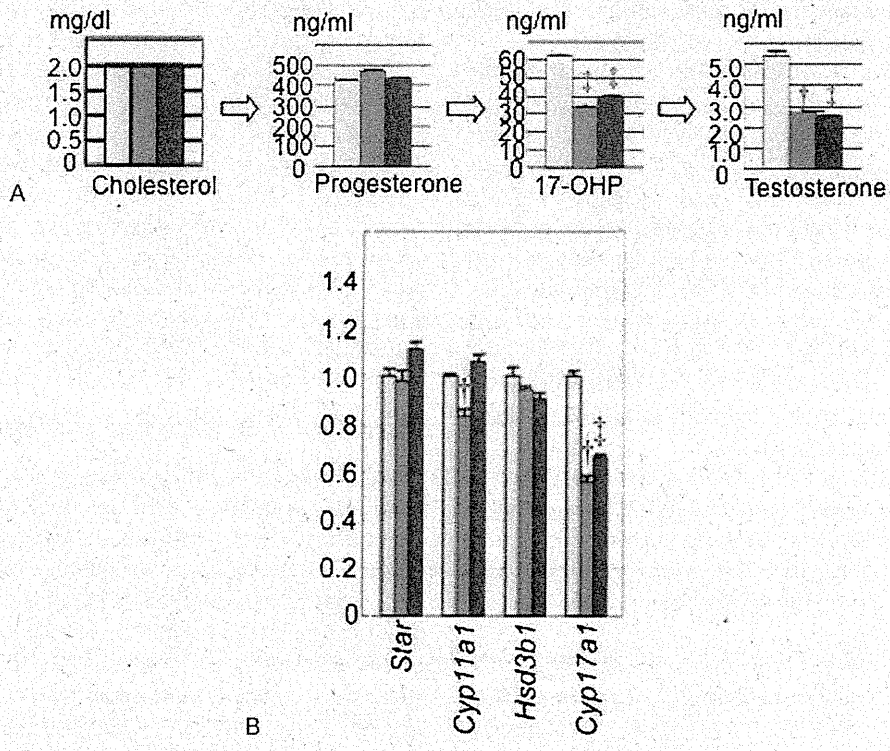

Figure 3 Representative data of the *Mamld1* knockdown experiments using MLTCs. Endogenous *Mamld1* expression has been markedly reduced to 10–15% by knockdown with si-RNAs. Shown are steroid metabolite concentrations in culture media and endogenous *Mamld1* expression levels in MLTCs. The white bars indicate the data obtained from MLTCs transfected with non-targeting RNA, and the light gray and the dark gray bars indicate the data obtained from MLTCs transfected with two different siRNAs.†: $P < 0.01$; and ‡: $P < 0.001$. (1) Representative steroid metabolite concentrations in culture media. (2) Real-time RT-PCR analysis for steroidogenic enzymes.

majority of Leydig cells. Such cell-type specific expression patterns were confirmed by co-localization of *Mamld1* mRNA and Nr5a1 (alias, steroidogenic factor 1 [SF1] or Ad4bp) protein as the marker for Sertoli and Leydig cells.[16,17] In the fetal ovary, *Mamld1* is expressed in a small number of somatic cells primarily at the boundary to the mesonephros at E11.5 and E12.5, and weakly expressed in a small number of somatic cells in the internal region at E14.5. In extragonadal tissues at E12.5, *Mamld1* is clearly expressed in the Müllerian ducts, forebrain, somite, neural tube, and pancreas, and weakly expressed in the external genital region. However, *Mamld1* expression is absent in the adrenals.

ISH analysis has revealed that, in the postnatal testis, *Mamld1* expression is weakly identified within the cords until one week of age and becomes faint thereafter; however, RT-PCT analysis still detects clear expression of *Mamld1* in the postnatal testis (our unpublished observation). In the ovary, *Mamld1* expression is barely detected until 2 weeks of age and clearly identified in granulosa cells at the perifollicular regions of most of Graafian follicles at 3 and 8 weeks of age.

Relevance of *Mamld1* to Testosterone Production

The above data imply that MAMLD1 is involved in the testosterone production in the critical period for sex development during fetal life, and that *MAMLD1* deletions/mutations cause hypospadias primarily because of compromised testosterone production around the critical period for sex development. In this context, there are two major possibilities how *MAMLD1* mutations lead to compromised testosterone production: (1) compromised steroidogenic activity in Leydig cells; and (2) reduced proliferation of Leydig cells. To test which of the two possibilities is more relevant, we performed knockdown analysis with two different siRNAs for *Mamld1*, using mouse Leydig tumor cells (MLTCs).[18] MLTCs are known to have the capacity to produce testosterone primarily via Δ^4-pathway, although the amount of testosterone production remains small primarily because of low 17α-hydroxylase and Hsd17b3 activities.[19] MLTCs are also known to retain responsiveness to hCG.[19–21]

Representative data of the steroidogenic activity are shown in ►**Fig. 3**; the data were obtained at 48 hours after the incubation of siRNA-transfected and non-transfected MLTCs followed by stimulation with hCG (for details, see reference[18]). The concentrations of pregnenolone and progesterone remained comparable between the culture media with siRNA-transfected MLTCs and those with nontargeted MLTCs, whereas the concentrations of 17-OH pregnenolone, 17-OH progesterone, dehydroepiandrosterone, androstenedione, and testosterone were significantly lower (~50–60%) in the culture media with siRNA-transfected MLTCs than in those with non-targeted MLTCs. Furthermore, comparison of

the steroid metabolite concentrations in the media with non-targeted MLTCs confirmed the Δ^4-pathway dominant testosterone production, markedly low 17α-hydroxylase activity and well-preserved 17/20 lyase activity for both Δ^4- and Δ^5-pathways, and extremely low Hsd17b3 activity in MLTCs. These results indicated that *Mamld1* knockdown further reduced 17α-hydroxylase activity that was originally low in MLTCs. Consistent with these findings, real-time RT-PCR and microarray analyses showed significantly decreased *Cyp17a1* expression (~70%) in siRNA-transfected MLTCs. The siRNAs knockdown did not affect the expressions of *Nr5a1* (Sf1), *Star*, *Por*, and *Insl3*. The assessment of *Hsb17b3* was impossible because of its extremely low expression. By contrast, the proliferation capacity, which was examined for 120 hours, was comparable between siRNA-transfected MLTCs and non-transfected MLTCs.

These results imply that *MAMLD1* is involved in testosterone production via augmenting CYP17A1 (17α-hydroxylase) activity. In this regard, it is noteworthy that *Mamld1* is clearly expressed in fetal Leydig and Sertoli cells and is barely expressed in adrenal cells,[1,7] and that 17α-hydroxylase activity is indispensable for testosterone production in Leydig cells.[22] Thus, it appears likely that *Mamld1* enhances *Cyp17a1* expression primarily in Leydig cells, permitting the production of a sufficient amount of testosterone for male sex development. In addition, since the expressions of other genes involved in testosterone production and insulin-like 3 biosynthesis were not clearly affected in siRNA-transfected MLTCs,[18] this would argue against the possibility that *Mamld1* knockdown causes a global dysfunction of MLTCs, resulting in testosterone hyposecretion.

However, a straightforward explanation appears to be difficult between impaired 17α-hydroxylase activity and reduced *Cyp17a1* expression. Indeed, 17/20 lyase activity was well preserved in siRNA-transfected MLTCs, although the same Cyp17a1 enzyme is utilized for both17α-hydroxylase and 17/20 lyase reactions.[22] In addition, defective 17α-hydroxylase activity occurred in the presence of ~70% of *Cyp17a1* expression, despite 17α-hydroxylase deficiency being an autosomal recessive disease in which 50% of enzyme reduction has no major effect on the steroid metabolism.[22] In this context, it is notable that MLTCs originally have a markedly low 17α-hydroxylase activity and a well preserved 17/20 lyase activity for both Δ^4- and Δ^5-pathways.[19] Such a unique property of MLTCs may be relevant to the preferential impairment of 17α-hydroxylase activity in siRNA-transfected MLTCs. Thus, although the in vitro data strongly argue for a positive role of *MAMLD1* in testosterone production, further studies are necessary to examine the precise in vivo function of *MAMLD1*.

Transcriptional Regulation of *MAMLD1*/*Mamld1* by *NR5A1*/*Nr5a1*

Mouse *Mamld1* is coexpressed with *Nr5a1* (Sf1), and *NR5A1*/*Nr5a1* is known to regulate the transcription of a vast array of genes involved in sex development, by binding to specific DNA sequences.[16,17] This implies that *MAMLD1*/*Mamld1* is also controlled by *NR5A1*/*Nr5a1*. Consistent with this notion, human *MAMLD1* harbors a putative NR5A1 binding sequence "CCAAGGTCA" at intron 2 upstream of the coding region, and mouse *Mamld1* also carries a putative Nr5a1 binding site at intron 1 upstream of the coding region.[9] Furthermore, we performed DNA binding and luciferase assays, showing that NR5A1 protein binds to the putative target sequence and exerts a transactivation function.[9] These findings argue for the possibility that *MAMLD1*/*Mamld1* expression is regulated by *NR5A1*/*Nr5a1*.

In Vitro Function of MAMLD1 Protein

MAMLD1 protein has a unique structure with homology to that of mastermind like 2 (MAML2) protein (►**Fig. 1**).[9] In particular, both MAMLD1 and MAML2 contain a unique amino acid sequence to which we designate mastermind-like (MAML) motif. The MAML motif was well conserved among MAMLD1 orthologs identified in frog, bird, and mammals. In addition, glutamine-, proline-, and serine-rich domains reside on MAMLD1.

MAML2 is a non-DNA binding transcriptional co-activator in Notch signaling that plays an important role in cell differentiation in multiple tissues by exerting either inductive or inhibiting effects according to the context of the cells.[23–25] Upon ligand-receptor interaction, Notch intracellular domain (N-ICD) is translocated from the cell surface to the nucleus and interacts with a DNA-binding transcription factor, recombination signal binding protein-J (RBP-J), to activate target genes like hairy/enhancer of split 1 (*Hes1*) and *Hes5*.[26] In this canonical Notch signaling process, MAML2 forms a ternary complex with N-ICD and RBP-J at nuclear bodies, enhancing the transcription of the Notch target genes.[23,24,27–29] In addition to such canonical Notch target genes, recent studies have shown that *Hes3* can be induced by stimulation with a Notch ligand, via a STAT3 mediated pathway.[30] This finding, together with lack of *Hes3* induction by N-ICD,[25] implies that *Hes3* represents a target gene of a non-canonical Notch signaling.

Thus, we have performed functional studies of wildtype MAMLD1 in terms of Notch signaling, thereby revealing several findings.[9] First, MAMLD1 is distributed in a speckled pattern and co-localized with the MAML2 protein in the nuclear bodies. Second MAMLD1, as well as MAML2, is unlikely to have a DNA-binding capacity. Third, although MAMLD1 is incapable of enhancing the promoter activities of the canonical Notch target genes *Hes1* and *Hes5* with the RBP-J binding site, MAMLD1 transactivates the promoter activity of the non-canonical Notch target gene *Hes3* without the RBP-J binding site. These findings imply that MAML2 and MAMLD1 may have derived from a common ancestor and evolved as a co-activator for the canonical and the non-canonical Notch signaling.

We have also performed similar functional studies for the three nonsense mutants identified in patients 7 to 10 (p.E124X, p.Q197X, and p.R653X) and missense variants (p.P286S, p.Q507R, and p.N589S).[9] The p.E124X and p.Q197X proteins, though they localize to the nucleus, are incapable of

localizing to nuclear bodies and have no transactivation function for Hes3, whereas the p.R653X protein as well as the three variant proteins localize to the nuclear bodies and retained nearly normal transactivating activities. This suggests that the p.E124X and p.Q197X proteins have no transactivation function primarily because of the failure in localizing to the nuclear bodies, and that the p.R653X protein, when it is artificially produced, has a normal transactivating activity. Thus, if not all the mRNAs with nonsense mutations are subject to NMD,[8] this would permit the production of functional protein for p.R653X, but not for p.E124X and p.Q197X. In addition, the transactivation function has been shown to be significantly reduced in a p.L103P protein (an artificially constructed variant affecting the MAML motif) and normal in the ΔExon 4.[9] This implies the importance of the MAML motif, and the biological equivalence between exon 4 positive and negative *MAMLD1*.

MAMLD1 Variations as a Susceptibility Factor for Hypospadias

It is possible that single nucleotide polymorphisms (SNPs), especially those in the promoter region and the cDNA sequence (cSNPs), and haplotypes (a combination of SNPs) of disease-causing genes constitute susceptibility factors of the corresponding diseases. In this regard, *MAMLD1* harbors several cSNPs, and the p.P286S and p.N589S cSNPs are fairly common in Caucasian populations, although they remain rare in the Japanese population.[1,12] In this regard, Chen et al[12] have revealed that the p.P286S allele, the p.N589S allele, and the p.P286S–p.N 589S haplotype (*S–S* haplotype) are more frequent in patients with hypospadias than in control males. While functional studies using the non-canonical Notch target Hes3 showed normal transactivation function for p.P286S allele, the p.N589S allele, and the 286S–589S haplotype (►**Fig. 2**), it is known that a substitution exerts differential effects on different promoters.[31] Thus, it remains possible that a specific SNP(s) or haplotype(s) may form a susceptibility factor for the development of hypospadias and other forms of 46,XY DSD.

Implications for Primary Ovarian Insufficiency

MAMLD1 may also have a certain role in the ovarian development. This notion is primarily based on two findings: (1) murine *Mamld1* is clearly identified in granulosa cells at the perifollicular regions of most of Graafian follicles at 3 and 8 weeks of age; and (2) a female with a heterozygous microdeletion involving *MAMLD1*, who gave birth to a boy with the same microdeletion and 46,XY DSD, has exhibited ovarian dysfunction from her late teens,[10] although ovarian dysfunction has not been identified in other obligatory carrier females. These findings may suggest that *MAMLD1* is involved in the normal ovarian development, and that rather exceptional females with a heterozygous *MAMLD1* mutation/deletion and skewered inactivation of the X chromosome carrying the normal allele manifest primary ovarian dysfunction. In this context, we examined a total of 78 females with primary ovarian insufficiency and 46,XX karyotype, and identified p.P494S and p.428delQ (shortening of the first polyglutamine domain from 11 to 10) (our unpublished observation). However, functional studies using Hes3 system showed apparently normal transcription activities for the two variants. Thus, further studies are necessary to reveal the relevance of *MAMLD1* to ovarian development.

Conclusions

MAMLD1 is a causative gene for 46,XY DSD with hypospadias as the salient clinical phenotype. It appears to play a supportive role in the testosterone production around the critical period for sex development. Interestingly, *Mamld1* knockout mice exhibit normal genital findings and reproductive functions, although they manifest metabolic syndrome (our unpublished observation). Further studies will permit to reveal the frequency of *MAMLD1* mutations in 46,XY DSD and the in vivo function of *MAMLD1*.

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References

1 Fukami M, Wada Y, Miyabayashi K, et al. CXorf6 is a causative gene for hypospadias. Nat Genet 2006;38(12):1369–1371
2 Hu LJ, Laporte J, Kress W, et al. Deletions in Xq28 in two boys with myotubular myopathy and abnormal genital development define a new contiguous gene syndrome in a 430 kb region. Hum Mol Genet 1996;5(1):139–143
3 Laporte J, Guiraud-Chaumeil C, Vincent MC, et al; ENMC International Consortium on Myotubular Myopathy. European Neuro-Muscular Center. Mutations in the MTM1 gene implicated in X-linked myotubular myopathy. Hum Mol Genet 1997;6(9):1505–1511
4 Bartsch O, Kress W, Wagner A, Seemanova E. The novel contiguous gene syndrome of myotubular myopathy (MTM1), male hypogenitalism and deletion in Xq28:report of the first familial case. Cytogenet Cell Genet 1999;85(3–4):310–314
5 Biancalana V, Caron O, Gallati S, et al. Characterisation of mutations in 77 patients with X-linked myotubular myopathy, including a family with a very mild phenotype. Hum Genet 2003;112 (2):135–142
6 Laporte J, Kioschis P, Hu LJ, et al. Cloning and characterization of an alternatively spliced gene in proximal Xq28 deleted in two patients with intersexual genitalia and myotubular myopathy. Genomics 1997;41(3):458–462
7 Ogata T, Laporte J, Fukami M. MAMLD1 (CXorf6): a new gene involved in hypospadias. Horm Res 2009;71(5):245–252
8 Kuzmiak HA, Maquat LE. Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. Trends Mol Med 2006;12(7):306–316

9 Fukami M, Wada Y, Okada M, et al. Mastermind-like domain-containing 1 (MAMLD1 or CXorf6) transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence. J Biol Chem 2008;283(9):5525–5532
10 Tsai TC, Horinouchi H, Noguchi S, et al. Characterization of MTM1 mutations in 31 Japanese families with myotubular myopathy, including a patient carrying 240 kb deletion in Xq28 without male hypogenitalism. Neuromuscul Disord 2005;15(3):245–252
11 Kalfa N, Liu B, Klein O, et al. Mutations of CXorf6 are associated with a range of severities of hypospadias. Eur J Endocrinol 2008;159(4):453–458
12 Chen Y, Thai HT, Lundin J, et al. Mutational study of the MAMLD1-gene in hypospadias. Eur J Med Genet 2010;53(3):122–126
13 Brandão MP, Costa EM, Fukami M, et al. MAMLD1 (mastermind-like domain containing 1) homozygous gain-of-function missense mutation causing 46,XX disorder of sex development in a virilized female. Adv Exp Med Biol 2011;707:129–131
14 Zitzmann M. The role of the CAG repeat androgen receptor polymorphism in andrology. Front Horm Res 2009;37:52–61
15 O'Shaughnessy PJ, Baker PJ, Monteiro A, Cassie S, Bhattacharya S, Fowler PA. Developmental changes in human fetal testicular cell numbers and messenger ribonucleic acid levels during the second trimester. J Clin Endocrinol Metab 2007;92(12):4792–4801
16 Morohashi KI, Omura T. Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J 1996;10(14):1569–1577
17 Parker KL, Schimmer BP. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 1997;18 (3):361–377
18 Nakamura M, Fukami M, Sugawa F, Miyado M, Nonomura K, Ogata T. Mamld1 knockdown reduces testosterone production and Cyp17a1 expression in mouse Leydig tumor cells. PLoS ONE 2011;6(4):e19123
19 Panesar NS, Chan KW, Ho CS. Mouse Leydig tumor cells produce C-19 steroids, including testosterone. Steroids 2003;68(3):245–251
20 Ascoli M, Puett D. Gonadotropin binding and stimulation of steroidogenesis in Leydig tumor cells. Proc Natl Acad Sci U S A 1978;75(1):99–102
21 Rebois RV. Establishment of gonadotropin-responsive murine leydig tumor cell line. J Cell Biol 1982;94(1):70–76
22 Achermann JC, Hughes IA. Disorders of sex development. In: Kronenberg HM, Melmed M, Polonsky KS, Larsen PR, eds. Williams textbook of endocrinology. 11th editions. Philadelphia: Saunders; 2008:783–848
23 Lin SE, Oyama T, Nagase T, Harigaya K, Kitagawa M. Identification of new human mastermind proteins defines a family that consists of positive regulators for notch signaling. J Biol Chem 2002;277 (52):50612–50620
24 Wu L, Sun T, Kobayashi K, Gao P, Griffin JD. Identification of a family of mastermind-like transcriptional coactivators for mammalian notch receptors. Mol Cell Biol 2002;22(21):7688–7700
25 Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284 (5415):770–776
26 Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 2003;194 (3):237–255
27 Nam Y, Sliz P, Song L, Aster JC, Blacklow SC. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Cell 2006;124(5):973–983
28 Wilson JJ, Kovall RA. Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. Cell 2006;124(5):985–996
29 Tonon G, Modi S, Wu L, et al. t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. Nat Genet 2003;33(2):208–213
30 Androutsellis-Theotokis A, Leker RR, Soldner F, et al. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 2006;442(7104):823–826
31 Ito M, Achermann JC, Jameson JL. A naturally occurring steroidogenic factor-1 mutation exhibits differential binding and activation of target genes. J Biol Chem 2000;275(41):31708–31714

RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Tissue-Limited Ring Chromosome 18 Mosaicism as a Cause of Pitt–Hopkins Syndrome

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TO THE EDITOR:

We wish to congratulate the authors on their detailed review of Pitt–Hopkins syndrome (PTHS, [OMIM 610954]) published in a recent issue of this journal [Marangi et al., 2011]. PTHS represents a rare disorder with characteristic facial gestalt, episodic hyperventilation, and severe developmental delay with absent speech [Pitt and Hopkins, 1978]. Haploinsufficiency of transcription factor 4 (TCF4) gene at chromosome 18q21.2 is responsible for PTHS [Brockschmidt et al., 2007; Zweier et al., 2007]. The comprehensive review by Marangi et al. illustrated that TCF4 point mutations, balanced translocations spanning the TCF4 locus, and even very large 18q deletions can result in the distinctive PTHS phenotype as long as the TCF4 locus is deleted. Here, we wish to demonstrate that ring (18) mosaicism represents yet another mechanism leading to the classic PTHS phenotype.

The propositus was born to unrelated Japanese parents. Her family history was non-contributory, with one older sister who is developing normally. The propositus was born at 40 and 1/7 weeks gestation via normal spontaneous vaginal delivery. Her birth weight was 2,805 g, and her head circumference was 32.3 cm. Soon after birth, she exhibited frequent episodes of projective vomiting caused by severe gastroesophageal reflux. Subsequently, she developed recurrent aspiration pneumonia requiring multiple hospital admissions. A magnetic resonance imaging of the brain at the age of 23 months revealed "delayed myelination" consistent with an age of 15 months but no other major structural abnormalities. A G-band chromosome analysis performed at that time was reportedly normal. She was first presented to us at the age of 3 years because of severe developmental delays. Her weight was 9.8 kg (−2.1 SD), height 83.8 cm (−2.1 SD), and head circumference 44.4 cm (−3.2 SD). Upon examination, she had a happy disposition with unexpected laughing, clapping of her hands and absent speech, microcephaly, global hypotonia, scoliosis, a short neck and syndactyly, and bilateral single palmar creases. Her finger pads were not prominent. She had characteristic facial features, that is, midfacial hypoplasia, a short philtrum, separated incisors, fleshy ears, downward slanting eyes, a pointed chin, macroglossia, and a prominent lower lip (Fig. 1). There was no apparent brachycephaly (Fig. 1). The serum IgA level was within the normal range, that is, 95 mg/dl [age reference 60–354 mg/dl]. She started to have generalized seizures at the age of 3 years. She is currently 11 years old and is unable to sit without support or to communicate verbally. She has been severely constipated requiring a daily enema. She exhibits episodic apnea-hyperpnea, often provoked by emotional excitement.

A FISH analysis using a BAC probe spanning the TCF4 locus (RP11-1079G18) was performed using a buccal swab specimen, and a mosaic deletion in 97.7% of the cells was revealed (Fig. 2a). An extensive chromosomal G-band analysis of a peripheral blood sample showed 5 ring (18) cells out of the 110 cells that were studied, yielding an average prevalence of one abnormal copy in 22 cells (Fig. 2b). An array comparative genomic hybridization (CGH)

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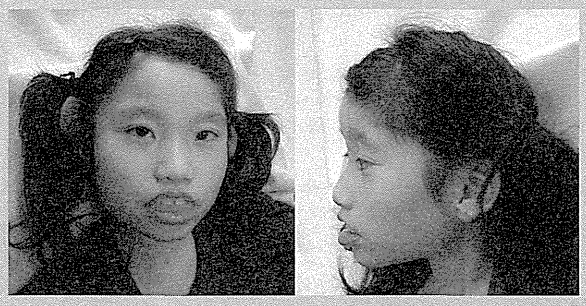

FIG. 1. Facial appearance of the propositus. Note midfacial hypoplasia, a short philtrum, separated incisors, fleshy ears, downward slanting eyes, a pointed chin, macroglossia, and a prominent lower lip.

analysis of her peripheral blood that was performed concurrently with the FISH analysis revealed a 29.12-Mb deletion from position 46,962,563 to 76,083,258 in 18q21.2-q23 and a 1.5-Mb deletion from position 108,560 to 1,617,028 in 18p11.32. The mosaic ratio estimated from an array CGH analysis was 26% according to the method described by Cheung et al. [2007].

The patient reported herein suggests that a ring (18) mosaicism can cause PTHS. A mosaic ring chromosome has not been recognized as a cause of PTHS, although there have been two PTHS patients due to mosaic deletion of TCF4 [Giurgea et al., 2008; Stavropoulos et al., 2010]. It was difficult to ascertain whether PTHS has been overlooked in patients with ring (18) mosaicism, since cytogenetic confirmation of TCF4 involvement has never performed in reported patients with ring (18) mosaicism [Lo-Castro et al., 2011].

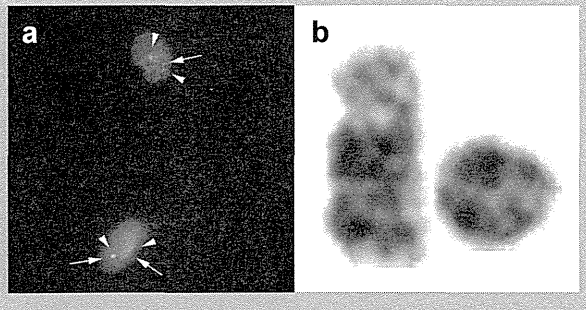

FIG. 2. Cytogenetic analyses of the propositus. a: A interphase FISH analysis of buccal swab specimen using TCF 4 probe (arrows, red) and CEP 18 probe (arrowheads, green) demonstrated 87 cells with one TCF4 probe (top) and two cells with two TCF4 probes (bottom) out of 89 cells that were studied. b: A G-band analysis of a peripheral blood sample revealed presence of ring chromosome 18 (right) in addition to normal chromosome 18 (left), consistent with ring (18) mosaicism.

In retrospect, reaching a correct diagnosis in the propositus was not a straightforward process: a pointed chin in the presence of autism, epilepsy, and a happy disposition with unexpected laughing was suggestive of Angelman syndrome [MIM 105830], whereas hand clapping in the presence of autism and epilepsy was reminiscent of Rett syndrome [MIM 312750], especially in females. Indeed, we had to perform a round of methylation studies and a UBE3A mutation analysis for Angelman syndrome as well as MECP2 mutation analysis before reaching a correct diagnosis. Because PTHS is gradually gaining recognition as differential diagnosis of Angelman syndrome and Rett syndrome among pediatric neurologists, we did perform a FISH for TCF4 on peripheral blood cells, but the test was interpreted as negative apparently because of the extremely low level of mosaicism of the ring (18). Her diagnosis would have been easily missed without performing a FISH using a buccal mucosa sample or an array CGH.

Indeed, the large discrepancy in the level of mosaicism in two aspects of the propositus hindered the diagnosis: first, there was a discrepancy between the mosaic ratio in the peripheral blood obtained from a G-band analysis (5/110) and that estimated from an array CGH analysis (26%). This discrepancy reflects the preferential selection of normal cells (46, XX cells) during the PHA stimulation of T cells required in the G-banding analysis. The array CGH analysis, which does not depend on the PHA stimulation process, is not subject to such an artifactual bias in the assessment of the mosaic rate [Ballif et al., 2006]. Not unexpectedly, in retrospect, the initial G-band analysis failed to detect the presence of cells with ring (18) chromosomes. Second, there was a discrepancy between the mosaic ratio in the peripheral blood evaluated using the array CGH analysis and that obtained from an interphase FISH study using the buccal smear. Since buccal smear FISH is not subject to the selection bias discussed above, the difference in the mosaic rate likely reflects a true difference in the level of mosaicism among tissues (i.e., peripheral blood vs. buccal cells). It is not clear whether the high percentage of abnormal cells in the buccal smear represents the situation in her central nervous system. Given her profound neurological disability, we suspect that her brain tissue may contain a very high percentage of abnormal cells. The situation is quite comparable to Pallister–Killian syndrome (tetrasomy 12p mosaicism), in which a diagnosis is dependent on a FISH study of non-blood tissues [Manasse et al., 2000] or an array CGH study of the blood [Theisen et al., 2009].

Clearly, the haploinsufficiency of deleted genes on chromosome 18q other than TCF4 has contributed to the phenotype of the propositus. The propositus exhibited macroglossia and delayed myelination. Macroglossia has been described in patients with Beckwith–Wiedemann like phenotype and 18q deletion [Brewer et al., 1998; Lirussi et al., 2007]. However, the propositus did not have other features of Beckwith–Wiedemann syndrome, such as overgrowth. The initial brain MRI of the propositus reported delayed myelination, which could be attributable to the deletion of myelin basic protein (MBP, OMIM #159430) located in 18q23 [Popko et al., 1987]. Although severe mental retardation with autistic features can be seen both in 18q deletion syndrome and in PTHS, episodic hyperventilation observed in the propositus is a distinctive feature of PTHS [Ouvrier, 2008]. On the other hand,

some commonly observed features of PTHS were not apparent. The propositus did not have characteristic facial features such as a pointed nasal tip, flaring nostrils, or brachycephaly, which are known features of PTHS [Zweier et al., 2007]. Moreover, Lehalle et al. [2011] reported that several individuals with PTHS have prominent finger pads, which were not apparent in the propositus. The absence of these features might be a consequence of the haploinsufficiency of genes other than TCF4.

In summary, an extensive and thorough investigation of the TCF4 locus, including that on a mosaic ring (18), should be performed in patients with a high clinical suspicion of PTHS.

REFERENCES

Ballif BC, Rorem EA, Sundin K, Lincicum M, Gaskin S, Coppinger J, Kashork CD, Shaffer LG, Bejjani BA. 2006. Detection of low-level mosaicism by array CGH in routine diagnostic specimens. Am J Med Genet Part A 140A:2757–2767.

Brewer CM, Lam WW, Hayward C, Grace E, Maher ER, FitzPatrick DR. 1998. Beckwith-Wiedemann syndrome in a child with chromosome 18q deletion. J Med Genet 35:162–164.

Brockschmidt A, Todt U, Ryu S, Hoischen A, Landwehr C, Birnbaum S, Frenck W, Radlwimmer B, Lichter P, Engels H, et al. 2007. Severe mental retardation with breathing abnormalities (Pitt-Hopkins syndrome) is caused by haploinsufficiency of the neuronal bHLH transcription factor TCF4. Hum Mol Genet 16:1488–1494.

Cheung SW, Shaw CA, Scott DA, Patel A, Sahoo T, Bacino CA, Pursley A, Li J, Erickson R, Gropman AL, et al. 2007. Microarray-based CGH detects chromosomal mosaicism not revealed by conventional cytogenetics. Am J Med Genet Part A 143A:1679–1686.

Giurgea I, Missirian C, Cacciagli P, Whalen S, Fredriksen T, Gaillon T, Rankin J, Mathieu-Dramard M, Morin G, Martin-Coignard D, et al. 2008. TCF4 deletions in Pitt-Hopkins Syndrome. Hum Mutat 29: E242–E251.

Lehalle D, Williams C, Siu VM, Clayton-Smith J. 2011. Fetal pads as a clue to the diagnosis of Pitt-Hopkins syndrome. Am J Med Genet Part A 155A:1685–1689.

Lirussi F, Jonard L, Gaston V, Sanlaville D, Kooy RF, Winnepenninckx B, Maher ER, Fitzpatrick DR, Gicquel C, Portnoi MF, et al. 2007. Beckwith-Wiedemann-like macroglossia and 18q23 haploinsufficiency. Am J Med Genet Part A 143A:2796–2803.

Lo-Castro A, El-Malhany N, Galasso C, Verrotti A, Nardone AM, Postorivo D, Palmieri C, Curatolo P. 2011. De novo mosaic ring chromosome 18 in a child with mental retardation, epilepsy and immunological problems. Eur J Med Genet 54:329–332.

Manasse BF, Lekgate N, Pfaffenzeller WM, de Ravel TJ. 2000. The Pallister-Killian syndrome is reliably diagnosed by FISH on buccal mucosa. Clin Dysmorphol 9:163–165.

Marangi G, Ricciardi S, Orteschi D, Lattante S, Murdolo M, Dallapiccola B, Biscione C, Lecce R, Chiurazzi P, Romano C, et al. 2011. The Pitt-Hopkins syndrome: Report of 16 new patients and clinical diagnostic criteria. Am J Med Genet Part A 155A:1536–1545.

Ouvrier R. 2008. Hyperventilation and the Pitt-Hopkins syndrome. Dev Med Child Neurol 50:481.

Pitt D, Hopkins I. 1978. A syndrome of mental retardation, wide mouth and intermittent overbreathing. Aust Paediatr J 14:182–184.

Popko B, Puckett C, Lai E, Shine HD, Readhead C, Takahashi N, Hunt SW III, Sidman RL, Hood L. 1987. Myelin deficient mice: Expression of myelin basic protein and generation of mice with varying levels of myelin. Cell 48:713–721.

Stavropoulos DJ, MacGregor DL, Yoon G. 2010. Mosaic microdeletion 18q21 as a cause of mental retardation. Eur J Med Genet 53:396–399.

Theisen A, Rosenfeld JA, Farrell SA, Harris CJ, Wetzel HH, Torchia BA, Bejjani BA, Ballif BC, Shaffer LG. 2009. aCGH detects partial tetrasomy of 12p in blood from Pallister-Killian syndrome cases without invasive skin biopsy. Am J Med Genet Part A 149A:914–918.

Zweier C, Peippo MM, Hoyer J, Sousa S, Bottani A, Clayton-Smith J, Reardon W, Saraiva J, Cabral A, Gohring I, et al. 2007. Haploinsufficiency of TCF4 causes syndromal mental retardation with intermittent hyperventilation (Pitt-Hopkins syndrome). Am J Hum Genet 80:994–1001.

RESEARCH ARTICLE

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Clinical Phenotype and Candidate Genes for the 5q31.3 Microdeletion Syndrome

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Array-based technologies have led to the identification of many novel microdeletion and microduplication syndromes demonstrating multiple congenital anomalies and intellectual disability (MCA/ID). We have used chromosomal microarray analysis for the evaluation of patients with MCA/ID and/or neonatal hypotonia. Three overlapping de novo microdeletions at 5q31.3 with the shortest region of overlap (SRO) of 370 kb were detected in three unrelated patients. These patients showed similar clinical features including severe neonatal hypotonia, neonatal feeding difficulties, respiratory distress, characteristic facial features, and severe developmental delay. These features are consistent with the 5q31.3 microdeletion syndrome originally proposed by Shimojima et al., providing further evidence that this syndrome is clinically discernible. The 370 kb SRO encompasses only four RefSeq genes including neuregulin 2 (*NRG2*) and purine-rich element binding protein *A* (*PURA*). *NRG2* is one of the members of the neuregulin family related to neuronal and glial cell growth and differentiation, thus making *NRG2* a good candidate for the observed phenotype. Moreover, *PURA* is also a good candidate because *Pura*-deficient mice demonstrate postnatal neurological manifestations.

Key words: 5q31.3 deletion syndrome; neonatal hypotonia; neuregulin 2; purine-rich element-binding protein *A*

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Am J Med Genet Part A.

INTRODUCTION

Recently developed array-based technology has identified many novel microdeletion and microduplication syndromes demonstrating multiple congenital anomalies and intellectual disability (MCA/ID) [Li and Andersson, 2009]. Because of a high detection ratio compared to conventional cytogenetic technology, array-based analysis has been suggested as the first-tier cytogenetic diagnostic test for patients with MCA/ID [Miller et al., 2010]. However, the detailed clinical presentation and/or candidate genes responsible for the respective phenotypes in many such newly detected syndromes remain to be elucidated.

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Several constitutional microdeletions involving 5q31 have been reported [Mosca et al., 2007; Shimojima et al., 2011]. Recently, Shimojima et al. reported two patients with overlapping microdeletions at 5q31.3. Their condition was named the 5q31.3 microdeletion syndrome because the patients showed clinical features including severe developmental delay, distinctive facial features, and delayed myelination [Shimojima et al., 2011]. We have identified and characterized three additional patients with microdeletions at 5q31.3 that overlap with the deleted regions observed in the patients reported by Shimojima et al. [2011]. These patients exhibited strikingly similar clinical features to those observed in the patients reported by Shimojima et al. The genotype–phenotype correlation of these patients narrowed the shortest region of overlap (SRO) of 5q31.3 microdeletion syndrome in this study.

One patient was initially deposited in the DECIPHER database (Database of Chromosomal Inbalances and Phenotype in Humans using Ensemble Resources, https://decipher.sanger.ac.uk), and the corresponding DECIPHER number is given.

PATIENT AND METHOD

Clinical Report

Patient 1. This 6-year-old Japanese boy was born at 40 weeks and 6 days to healthy parents without perinatal events. Birth weight was 3,888 g (>97th centile), length 52.5 cm (>97th centile) and head circumference 37 cm (>97th centile). Soon after birth he was noted to have hypotonia and was admitted to a neonatal intensive care unit (NICU). His early course was relevant for mild respiratory distress and severe feeding difficulties prompting tube feedings. At 3 months of age, he was suspected to have Prader–Willi syndrome (PWS) because of neonatal hypotonia, developmental delay and feeding difficulties, but normal DNA methylation studies excluded this diagnosis [Kubota et al., 1997]. In the neonatal period, his face appeared hypotonic. He did not have limbs or genital

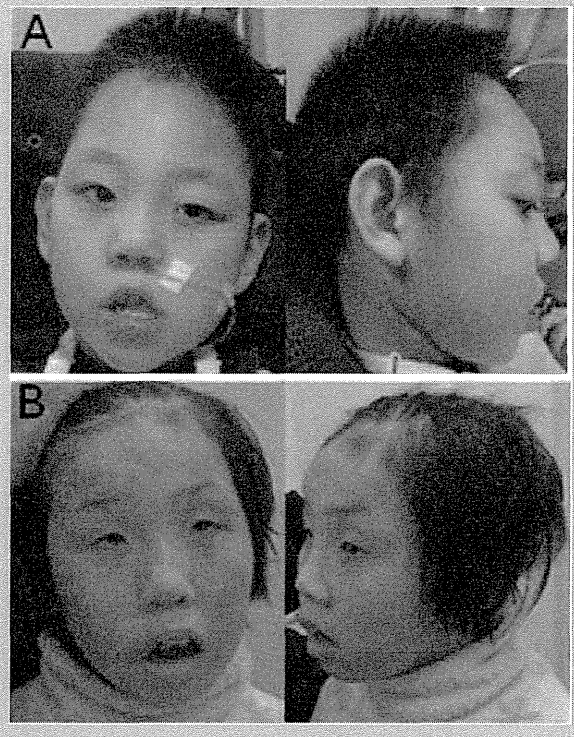

FIG. 2. Facial appearance of Patient 1 (A) and Patient 2 (B). A narrow forehead with prominence of the metopic region, lateral sparseness of the eyebrows, hypertelorism, depressed nasal bridge, open-tented mouth, marked philtrum, and micrognathia are noted.

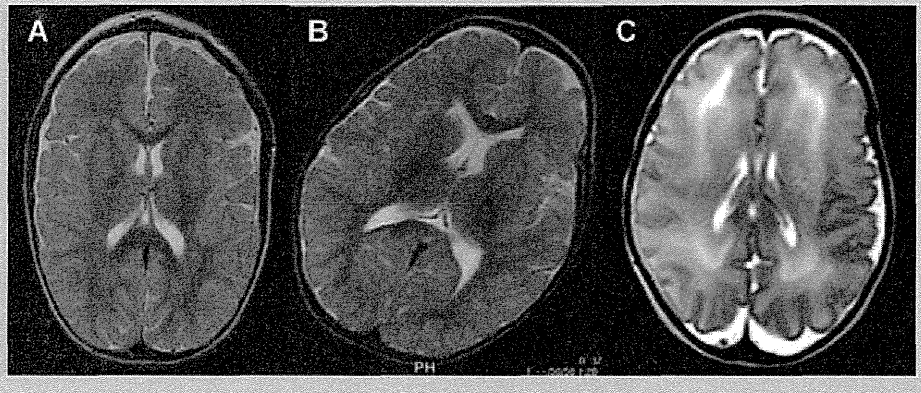

FIG. 1. Brain MRI (T2 weighted axial images at the level of basal ganglia) of Patient 1 at 4 years and 7 months (A), Patient 2 at 1 year and 10 months (B), and Patient 3 at 10 days of age. (A) Mild temporal lobe atrophy is shown, but myelination delay is not evident. (B) Incomplete myelination at the anterior limb internal capsule indicates mild myelination delay. (C) A simplified frontal gyral pattern and diffuse mild T2 weighted high signal intensity in the white matter are demonstrated.

abnormalities. His motor development was severely delayed. He acquired head control and rolled over at 2 years of age. He developed tonic seizures during sleep at the age of 4 years. Electroencephalogram (EEG) demonstrated multifocal spike foci. The use of valproic acid and nitrazepam successfully controlled his epilepsy. MRI of the brain performed at 4 years and 7 months of age, showed mild temporal lobe volume loss but no other structural anomalies. Myelination delay was not evident (Fig. 1A). At 6 years of age, he was unable to sit alone. His weight, height and head circumference were 17.6 kg (25th centile), 110 cm (25th centile), and 50.7 cm (50 centile), respectively. He still required tube feedings. His mental development was profoundly delayed with no meaningful words. He presented with facial dysmorphic features that include a narrow forehead with prominence of the metopic region, lateral sparseness of the eyebrows, hypertelorism, depressed nasal bridge, high palate, open-tented mouth, marked philtrum, and micrognathia (Fig. 2A).

Patient 2. This 12-year-old Japanese girl was born at 42 weeks and 2 days to healthy parents. Delivery was uneventful and she did not have neonatal asphyxia. Birth weight was 3,372 g (90th centile), length 55.0 cm (>97th centile) and head circumference 34.3 cm (75th centile). At day 3, she was noted to have hypotonia and feeding difficulties and tube feedings were initiated on day 7. She developed respiratory distress at day 10 and she was transferred to NICU for oxygen therapy. Her condition gradually improved, and oxygen therapy was stopped in a month, but tube feedings continued for additional 50 days. Her development was significantly delayed and she acquired head control at 6 months, and was able to walk without support at 6 years. MRI at 1 year and 10 months did not show structural abnormalities, but demonstrated mild myelination delay (Fig. 1B). She had no meaningful words at 12 years indicating severe intellectual disability. She had dysmorphic facies with narrow forhead and prominence of the metopic region, lateral sparseness of the eyebrows, hypertelorism, downslanting palpebral fissures, depressed nasal bridge, open and tented mouth, high palate, marked philtrum, and micrognathia (Fig. 2B). She did not have short stature, limb, or genital abnormalities. She did not have epilepsy. EEG at 9 years did not show epileptics discharges, while background activity was relatively slow for her age.

Patient 3 (DECIPHER #248784). This 15-month-old boy was born at 37 weeks and 1 day of gestational age with a birthweight of 3,150 g to a then 33-year-old female via spontaneous vaginal delivery. The pregnancy was complicated by type 1 diabetes mellitus and a history of group B streptococcus infection. Mild respiratory distress was seen after delivery. He then developed multiple episodes of apnea and was noted to have severe axial hypotonia. He also showed feeding difficulties due to laryngomalacia. He was evaluated by the Genetic Service at 34 days of age because of severe axial hypotonia, neonatal feeding difficulties and hypoventilation. Clinical examination revealed his weight, length, and head circumference to be 3,945 g (50th centile), 54 cm (75th centile), and 38 cm (90th centile), respectively. His facial features revealed a narrow forehead, prominence of the metopic region, hypertelorism, depressed nasal bridge, high palate, open-tented mouth, marked philtrum, and micrognathia. His physical examination revelead undescended right testis, axial hypotonia and diminished reflexes in all four extremities. His brain MRI at 10 days of age showed a simplified frontal gyral pattern and diffuse mild T2 weighted high signal intensity in the white matter (Fig. 1C). At his last neurological clinic visit at 15 months of age he had significant central hypotonia but his deep tendon reflexes were present and equal bilaterally. The patient was also found to have hyperekplexia. An EEG video monitoring study done at the same age was mildly abnormal due to diffuse background slowing without asymmetry between hemispheres. The described clinical events captured during this recording (eye deviation upward to the left at rest or during photic stimulation, startle response, and blank staring spells) did not have an EEG correlation suggesting the diagnosis of non-epileptic spells. He was found to have moderate global developmental delay but he continued to make developmental progress. He achieved head control, rolled both ways, lifted his head up and was able to do prop sitting. He cooed and smiled but had no intelligible words.

Microarray Analysis

Genome-wide copy number changes were investigated using the Genome-Wide Human SNP Nsp/Sty Array Kit 5.0 for Patient 1 and Patient 2 (Affymetrix, Santa Clara, CA) or Agilent platform (Baylor

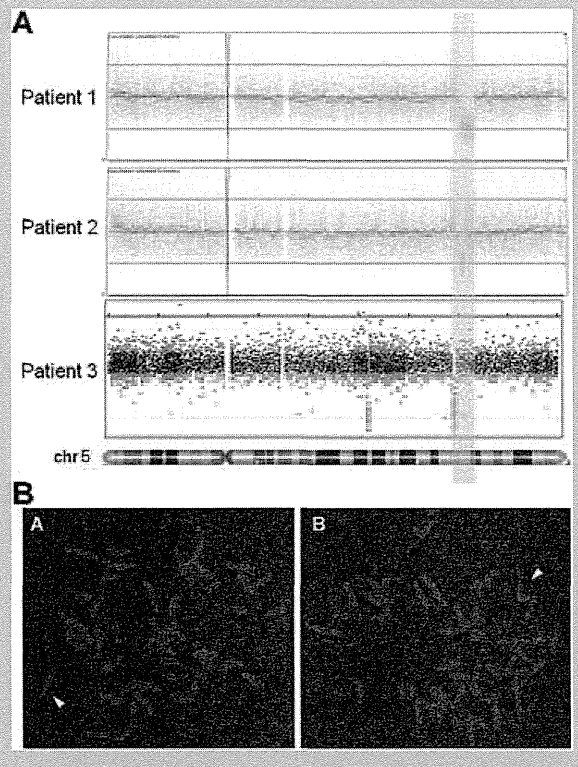

FIG. 3. A: DNA array analysis of three patients with microdeletion encompassing 5q31.2-q31.3. B: FISH analysis using RP11-277D10 BAC clone (green) in 5q31.3 and RP11-701H24 BAC clone (red) in 15q11.2 confirmed 5q31.3 microdeletion in Patient 1 (A) and Patient 2 (B). Arrowhead indicates the deletion on chromosome 5. The FISH photograph was not available for Patient 3.