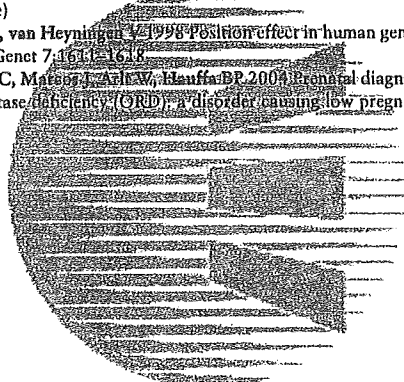


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Letter to the Editor

Pharmacogenetics and Genomics 2009, 19:565–566

Nomenclature for alleles of the cytochrome P450 oxidoreductase gene

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Received 18 February 2009 Accepted 26 February 2009

Recent focus on the cytochrome P450 oxidoreductase (*POR*) gene has resulted in the discovery of numerous new polymorphic alleles. Many of these were found [1–6] because of their association with steroidogenic disorders and congenital skeletal malformations resembling the phenotype of Antley-Bixler syndrome [7], whereas other alleles have been found as a consequence of sequencing the *POR* gene in normal unrelated individuals [8,9]. The association of *POR* variants with clinical phenotypes is the result of *POR* serving as the major electron donor for cytochrome P450 (*CYP*) enzymes with important endogenous functions in hormone biosynthesis. Consequently, defective *POR* alleles can be the cause of abnormal glucocorticoid, mineralocorticoid, and sex steroid synthesis [10], thus leading to a form of congenital adrenal hyperplasia. In addition, *POR* deficiency can cause skeletal defects, the mechanism of which is yet unknown but has been suggested to result from impaired sterol synthesis [11] because of decreased electron flow from *POR* to lanosterol 14- α -demethylase (*CYP51A1*) and squalene monooxygenase (*SQLE*). In addition, as *POR* is

equally important as an electron donor to *CYP* enzymes involved in the metabolism of drugs, *POR* variants may affect drug bioavailability. The effect of *POR* mutations on the activity of some drug-metabolizing *CYP* enzymes has been documented *in vitro* [12–14], but not yet *in vivo*. In addition, *POR* is an electron donor for heme oxygenase, cytochrome *b₅*, and several additional small molecules that can be directly metabolized by *POR* without *CYP* enzymes. Thus, an increasing focus on the importance of *POR* in drug response and adverse drug reactions is to be expected.

Until now, no systematic guidelines have been proposed for the naming of *POR* alleles. To standardize *POR* allelic nomenclature, the Human *CYP* Allele Nomenclature Chair and Committee have taken the initiative to devise a system for the designation of *POR* alleles that follows the guidelines for *CYP* allelic star (*CYP**) nomenclature (<http://www.cypalleles.ki.se/criteria.htm>). The *POR* allele nomenclature web page (<http://www.cypalleles.ki.se/por.htm>) was launched in September 2008, listing 35 different alleles. On this *POR* web page, the alleles are presented together with their corresponding nucleotide and amino acid changes, and the phenotypic consequences observed by *in vitro* and *in vivo* studies. Among the more important *POR* variants are *POR**2 and *5 (Arg457His and Ala287Pro, respectively), the former being the most frequent mutation in Japanese and Chinese *POR*-deficient patients [5,15], whereas the latter is the *POR* mutation most frequently found in Caucasians. Alleles with frameshift mutations (*POR**9, *10, and *20–24), deletions, insertions, and several of the alleles that result in amino acid substitutions are also associated with *in vivo* phenotypes, as is a splice defect in the *POR**3 allele.

To maintain a common nomenclature system within the field, fellow scientists investigating *POR* polymorphisms are highly recommended to submit novel *POR* allelic variants to the Human *CYP* Allele Nomenclature Committee (<http://www.cypalleles.ki.se/criteria.htm>) by contacting the Webmaster for designation and reservation of novel *POR* allele names.

The authors of this Letter, a number of whom have identified the novel *POR* alleles, are supportive of this new nomenclature system, and will use this system in their future work.

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

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

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

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

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

Maternal uniparental disomy 14 (upd(14)mat) is characterized by prenatal and postnatal growth retardation, neonatal hypotonia, small hands and feet, feeding difficulty, and precocious puberty.¹ Chromosome 14q32.2 contains several imprinted genes, and loss of expression of paternally expressed genes including *DLK1* and *RTL1* is believed to be responsible for upd(14)mat phenotype.² Thus far, 5 patients with epimutations and 4 patients with a microdeletion affecting the 14q32.2 imprinted region have been reported to have upd(14)mat-like phenotype.²⁻⁴ Paternal uniparental disomy 14 (upd(14)pat) shows a distinct and much more severe phenotype characterized by facial abnormality, bell-shaped thorax and abdominal wall defects.¹ Initially, upd(14)mat was identified in patients with Robertsonian translocations involving chromosome 14, but increasing numbers of patients with a normal karyotype have been recognized.^{1,5} Because maternal uniparental disomy 15 is responsible for the condition in more than 20% of patients with Prader-Willi syndrome (PWS), of which the overall prevalence is more than 1 in 15000 births,⁶ one could suspect that upd(14)mat is underestimated. Phenotype of upd(14)mat is known to resemble that of PWS, which is characterized by neonatal hypotonia, small hands and feet, mental retardation, and hyperphagia resulting in obesity beyond infancy. Mitter et al⁷ recently reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS and raised the question that upd(14)mat could be present in patients with PWS-like phenotype. Thus we examined patients who presented with PWS-like phenotype, but in whom PWS had been excluded.

Methods

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

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

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

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This work was partially supported by a grant from the Ministry of Education, Science and Culture of Japan. The authors declare no conflicts of interest.

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

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

Results

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

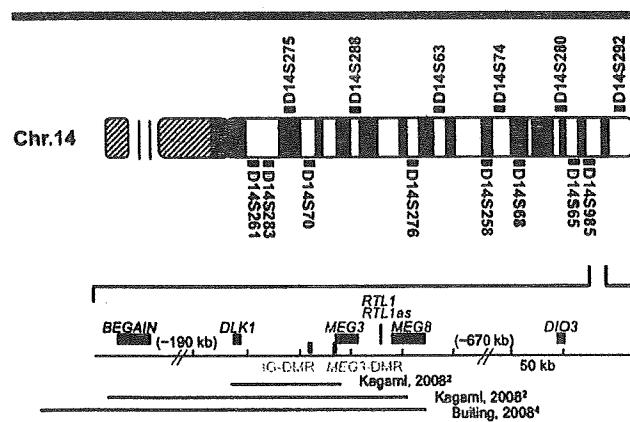


Figure 1. Schematic map of the 14q32.2 imprinted region. Loci on chromosome 14 represent markers used for microsatellite polymorphism analysis. Paternally expressed genes are shown in blue, maternally expressed genes in red, and nonimprinted genes are shown in black. Differentially methylated regions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

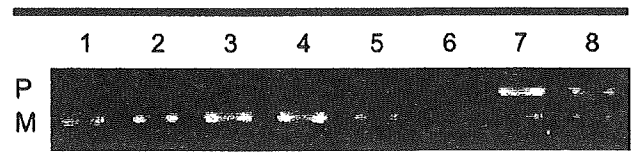


Figure 2. *MEG3* methylation test. P, Paternal methylated signal; M, maternal unmethylated signal; 1-5, cases 1-5, respectively; 6, paternal uniparental disomy 14; 7, patient with PWS; 8, normal control. Cases 1-4 show only the maternal unmethylated signal, and case 5 shows a faint paternal methylated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

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

Discussion

We detected 5 patients with upd(14)mat or epimutation at the 14q32.2-imprinted region in 78 subjects who had initially been suspected to have PWS. Mitter et al⁷ reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS. However, Cox et al¹⁰ reported that they did not find any upd(14)mat in 35 patients suspected to have PWS. Our study suggests that a significant number of patients with upd(14)mat are suspected to have PWS during infancy. To clarify how upd(14)mat and PWS share clinical features, we examined the clinical manifestations of our patients with upd(14)mat or an epimutation. All patients showed neonatal hypotonia and were referred to us during infancy. Feeding difficulty in the neonatal period and small hands and feet were also common to these patients and resembled features of PWS. It is noteworthy that all patients were referred during infancy, suggesting that upd(14)mat and PWS resemble each other, particularly during this period. Therefore upd(14)mat and related disorders, as well as PWS, should be important differential diagnoses for infants with hypotonia and feeding difficulty. Distinct features for upd(14)mat included less-specific facial characteristics, constant prenatal growth failure, and better intellectual development. Precocious puberty is not present in PWS; however, this was not evaluated in this study because the patients were not

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

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

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

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

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

brain, which is most responsible for the phenotype of upd(14)mat.

As is clear in our series of patients, upd(14)mat phenotype can be caused by an epimutation of 14q32.2. Recently, Kagami et al² reported a microdeletion in 14q32.2 associated with a similar phenotype (Figure 1). Buiting et al⁴ also reported a patient with a 1Mb deletion at 14q32.2 (Figure 1). Therefore upd(14)mat phenotype is associated with not only upd(14)mat but an epimutation or small deletion. This genetic complexity is similar to that of PWS. PWS is caused by paternal deletion of 15q11-q13, maternal uniparental disomy of chromosome 15, and epimutation (imprinting defect). A new name such as upd(14)mat syndrome would be appropriate to represent the entire upd(14)mat clinical features represented by upd(14)mat, epimutation of 14q32.2 and microdeletion in 14q32.2. Alternatively, Buiting et al⁴ suggested the term, "Temple syndrome," because upd(14)mat was first described by Dr. I. K. Temple in 1991, who subsequently described an epimutation in 2007.^{4,5,11}

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

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

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

NA, Not applicable.

Previous studies are based on references 2, 3 and 4.

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

The authors thank Dr. T. Ariga for critical reading of the manuscript.

Submitted for publication Mar 20, 2009; last revision received May 6, 2009; accepted Jun 22, 2009.

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Table 1. Microsatellite polymorphism analyses for chromosome 14 in 6 families with aberrant MEG3 methylation

Locus	Region	Case 1 family			Case 2 family			Case 3 family			Case 4 family			Case 5 family		
		Patient	Father	Mother	Patient	Father	Mother	Patient	Father	Mother	Patient	Father	Mother	Patient	Father	Mother
D14S261	14q11.2	298, 298	274, 298	298, 298	297, 297	297, 297	298, 298	296, 298	298, 298	296, 298	297, 297	297, 297	275, 297	275, 299	273, 297	
D14S283	14q11.2	147, 149	139, 149	137, 147	139, 139	137, 137	137, 149	133, 137	137, 149	137, 149	150, 150	142, 150	137, 139	137, 139	139, 147	
D14S275	14q12	146, 146	146, 156	146, 146	149, 149	145, 145	148, 152	146, 146	148, 152	148, 152	155, 155	149, 155	146, 148, 152	152, 156	146, 148	
D14S70	14q13.1	100, 102	102, 102	100, 104	101, 101	101, 103	103, 103	99, 101	103, 103	103, 103	104, 104	104, 106	101, 101, 103	101, 103	101, 101	
D14S288	14q21.2	191, 201	201, 203	191, 207	201, 201	203, 203	193, 193	193, 203	193, 193	193, 203	195, 195	213, 215	190, 196, 204	188, 196	190, 204	
D14S276	14q22.3	241, —	239, 241	247, —	242, 244	244, 246	242, 244	242, 244	244, 244	242, 244	245, 245	241, 241	244, 246, 246	242, 244	246, 246	
D14S63	14q23.2	187, 187	187, 187	187, 187	187, 193	183, 187	183, 187	189, 191	183, 187	183, 187	191, 191	185, 195	187, 189, 193	187, 193	187, 189	
D14S298	14q24.2	204, 206	196, 206	202, 204	196, 196	198, 202	196, 196	200, 202	196, 196	200, 202	202, 202	204, 204	196, 196, 198	198, 200	196, 196	
D14S74	14q24.3	299, 313	260, 299	303, 313	303, 303	303, 305	299, 303	299, 301	299, 303	299, 303	295, 295	305, 313	299, 301, 305	299, 305	299, 301	
D14S68	14q31.3	323, 323	323, 323	323, 323	321, 321	323, 323	321, 323	323, 323	321, 323	323, 323	323, 323	325, 325	321, 321, 323	323, 323	321, 321	
D14S280	14q32.12	246, 248	248, 248	246, 248	243, 243	243, 245	243, 243	243, 247	247, 247	243, 247	248, 248	244, 244	241, 243, 247	241, 245	243, 247	
D14S65	14q32.2	135, 141	135, 141	135, 141	145, 145	135, 149	135, 145	135, 147	135, 147	137, 145	150, 150	150, 150	147, 147	147, 147	135, 147	
D14S985	14q32.2	255, 255	251, 255	255, 257	250, 250	246, 254	250, 254	247, 247	247, 247	249, 249	248, 248	246, 248	247, 249	247, 253	247, 249	
D14S292	14q32.33	84, 86	84, 86	86, 86	92, 92	86, 88	86, 92	83, 85	85, 87	83, 85	92, 92	86, 92	87, 89	89, 89	87, 89	

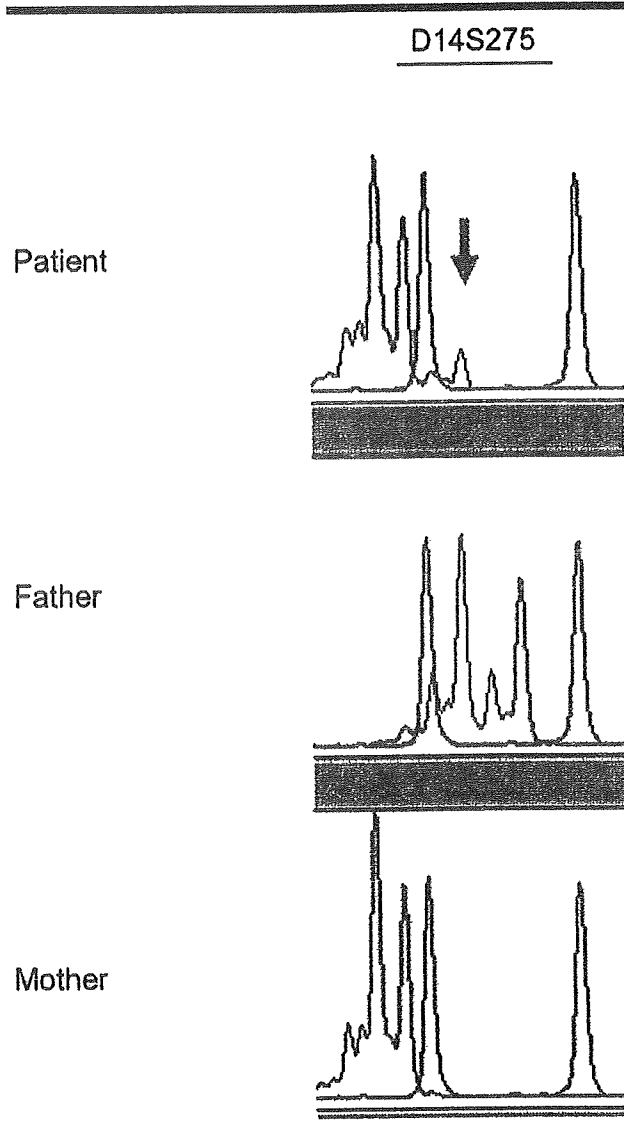


Figure 3. Microsatellite polymorphism analysis at D14S275 for the family of case 5. The patient demonstrates 3 peaks (146, 148, 152 bp), 2 (146, 148 bp) of which are transmitted from the mother, but 1 small peak (152 bp) indicated by the arrow is transmitted from the father. Red peaks depict size markers.



Mutation Analysis of *SOX9* and Single Copy Number Variant Analysis of the Upstream Region in Eight Patients With Campomelic Dysplasia and Acampomelic Campomelic Dysplasia

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Received 21 August 2009; Accepted 16 July 2009

TO THE EDITOR:

Campomelic dysplasia (CD; OMIM 114290) is a rare skeletal disorder characterized by hypoplastic scapulae, 11 pairs of ribs, pelvic abnormalities, and bowing of the lower limb bones [Maroteaux et al., 1971]. Affected patients often die shortly after birth due to respiratory distress, and roughly two-thirds of affected genetic males have disorders of sex development (DSD) due to dysgenetic testes [Mansour et al., 1995]. Acampomelic campomelic dysplasia (ACD) is associated with similar but milder skeletal features and lacks long bone curvature [MacPherson et al., 1989].

SOX9 on chromosome 17q24 is a member of SRY-related gene family [Harley et al., 2003]. It encodes a 509-amino acid protein that harbors a high mobility group (HMG) domain with a DNA-binding capacity and a proline/glutamine/serine-rich domain with a transactivation function [Harley et al., 2003]. Furthermore, putative *cis*-control elements have been mapped within the 1 Mb region upstream of *SOX9* [Hill-Harfe et al., 2005].

To date, it has been shown that both CD and ACD can be caused by heterozygous intragenic *SOX9* mutations or chromosomal aberrations (translocations, inversions, or deletions) affecting *SOX9* or the putative enhancer region [Pfeifer et al., 1999; Thong et al., 2000; Moog et al., 2001; Harley et al., 2003; Pop et al., 2004; Leipoldt et al., 2007]. However, the frequency and the type of mutations and chromosomal aberrations are quite different

How to Cite this Article:

Wada Y, Nishimura G, Nagai T, Sawai H, Yoshikata M, Miyagawa S, Hanita T, Sato S, Hasegawa T, Ishikawa S, Ogata T. 2009. Mutation analysis of *SOX9* and single copy number variant analysis of the upstream region in eight patients with campomelic dysplasia and acampomelic campomelic dysplasia.

Am J Med Genet Part A 149A:2882–2885.

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Published online 16 November 2009 in Wiley InterScience (www.interscience.wiley.com)
DOI 10.1002/ajmg.a.33107

TABLE 1. Clinical and Molecular Findings in Patients Examined in This Study

	Campomelic dysplasia				Acampomelic campomelic dysplasia			
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
Patient								
Gestational age (weeks)	25	42	38	38	39	40	42	38
Birth weight (g)	625	2490	2670	2060	3400	2700	2680	2306
Present age (y.m)	Stillbirth	0:11	[0:5] ^a	[1:5] ^a	11:6	19:8	3:2	3:9
Karyotype	46,XY	46,XX	46,XX	46,XX	46,XY	46,XY	46,XX	46,XX
Phenotype								
Cleft palate	-	-	+	-	+	+	+	+
Micrognathia	+	+	+	+	+	-	-	+
Scapular hypoplasia	+	+	+	+	+	+	+	+
Tibial bowing	+	+	+	+	-	-	-	-
Femoral bowing	+	+	+	+	-	-	-	-
11 pairs of ribs	-	+	+	+	-	+	+	+
Small thoracic cage	+	+	+	+	+	+	+	-
NM thoracic pedicles	+	+	+	+	-	-	+	+
Scoliosis	-	-	-	-	+	+	+	-
Narrow iliac wings	+	+	+	+	+	+	+	+
Clubfeet	+	+	+	+	-	-	-	+
46,XY/DSD	+	-	-	-	-	-	-	-
Mutation								
cDNA	771 772insGGCGC	1330 1333delGACC	T338C	G442T	C509T	-	-	-
Amino acids	G257fsX296	T443fsX458	M113I	E148X	P170L	-	-	-

NM, non-mineralized; DSD, disorders of sex development.
^aDeceased at 5 months and 1 year and 5 months, respectively.

between CD and ACD. CD is predominantly caused by nonsense or frameshift mutations or by chromosomal aberrations disrupting *SOX9*, although missense mutations and chromosomal aberrations impairing the enhancer region are also occasionally identified. By contrast, ACD is almost exclusively caused by missense mutations or by chromosomal aberrations affecting the enhancer region. Thus, while missense mutations are exclusively identified within the HMG box in both CD and ACD [Kwok et al., 1995; Cameron and Sinclair, 1997; Meyer et al., 1997; Hageman et al., 1998; Moog et al., 2001; Thong et al., 2000], these findings imply that severe mutations usually result in CD whereas mild mutations usually lead to ACD.

However, the underlying causes remain to be determined in several patients, especially those with ACD, and such patients may have hidden perturbation in the putative enhancer region. Thus, we performed mutation analysis of *SOX9* in eight patients with CD or ACD and single copy number variant (CNV) analysis [Redon et al., 2006] of the upstream region in *SOX9* mutation negative patients.

Clinical features of the eight patients are summarized in Table 1, and representative roentgenograms are shown in Figure 1. Patients 1–4 showed CD-compatible severe clinical features, whereas patients 5–8 exhibited relatively mild ACD-compatible clinical features. In addition, patient 1 ended in a stillbirth, and patients 3 and 4 died of respiratory insufficiency during infancy, although patient 2 aged 11 months was alive. By contrast, patients 5–8 have survived a relatively long period. Among genetic males, patient 1 exhibited DSD with nearly complete female external genitalia, while patients 5 and 6 showed male external genitalia.

We first performed mutation analysis of *SOX9*. This study was approved by the Institutional Review Board Committees at National Center for Child Health and Development, and performed after obtaining written informed consent. Genomic DNA samples

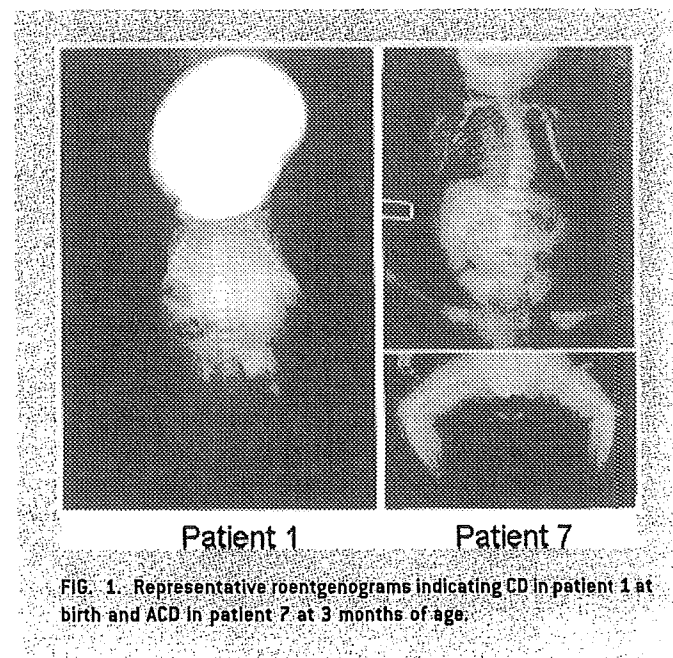


FIG. 1. Representative roentgenograms indicating CD in patient 1 at birth and ACD in patient 7 at 3 months of age.

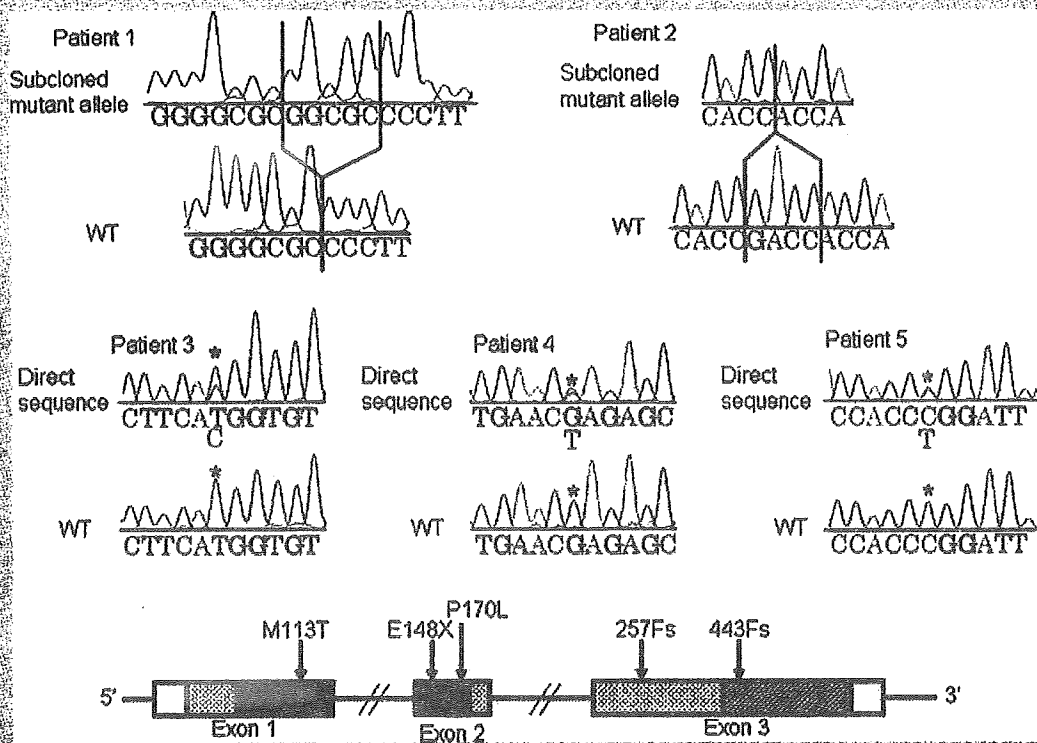


FIG. 2. Molecular findings in patients 1–5 with *SOX9* mutations. Upper part: Electrochromatograms showing the mutations in patients 1–5. In patients 1 and 2, the subcloned mutant alleles and the corresponding wildtype (WT) alleles are shown. In patients 3–5, the direct sequences are shown, together with the corresponding wildtype sequences; the asterisks indicate the mutant and the corresponding wildtype nucleotides. Lower part: The position of the mutations on the genomic sequences. Exons 1–3 are depicted with boxes; the black, the striped, the stippled, and the white areas indicate the HMG domain, the transactivation domain, other translated regions, and the untranslated regions, respectively.

extracted from cord blood cells (patient 1) or peripheral blood cells (patients 2–8) were amplified by PCR for all the three coding exons and were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA) (the primer sequences are available on request). To confirm frameshift mutations, the corresponding PCR products were subcloned with TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and normal and mutant alleles were sequenced separately.

Consequently, we identified a novel heterozygous 5-bp insertion mutation at exon 3 that is predicted to cause a frameshift at the 257th glycine codon and resultant termination at the 296th codon (G257fsX296) in patient 1, a novel heterozygous 4-bp deletion mutation at exon 3 that is predicted to cause a frameshift at the 443rd threonine codon and resultant termination at the 468th codon (T443fsX468) in patient 2, a novel heterozygous missense mutation at exon 1 (M113T) in patient 3, a recurrent heterozygous nonsense mutation at exon 2 (E148X) in patient 4, and a novel heterozygous missense mutation at exon 2 (P170L) in patient 5 (Fig. 2). The two missense mutations resided within the HMG. The mutations of patients 1–4 were absent in their parents. In addition, while mutation analysis was refused by the parents of patient 5, the P170L missense mutation was absent in 200 control subjects. No mutations were identified in patients 6–8.

Then, to examine for a small deletion, we carried out the whole genome CNV analysis in patients 6–8 and their parents, using custom high density oligonucleotide microarray based on Affymetrix platform [Redon et al., 2006]. In brief, 25 bp oligonucleotide probes are designed on 1,330,354 *Nsp* I restriction fragments with average and median spacing of 2,271 and 776 bp. The experimental protocol is the same as the Affymetrix 500K arrays. Ninety microgram of target was hybridized overnight to the arrays [Fujii et al., 2007]. The signal intensity ratio of the sample to reference was calculated by Genome Imbalance Map Algorithm [Ishikawa et al., 2005], using NA10851 HapMap DNA samples from Coriell Cell Repositories (Camden, NJ) as the reference samples. Consequently, no deletion was indicated in the whole genome including the 5' region of *SOX9* in patients 6–8.

The results are primarily consistent with the previous data. Three of four patients with CD died during fetal life or infancy, whereas patients 5–8 with ACD survived into childhood or puberty. 46,XY with DSD was observed in patient 1 with CD but not in patients 5 and 6 with ACD. Similarly, truncating mutations of *SOX9* were identified in patients 1–3 with CD, together with a missense mutation in patient 4 with CD, whereas only one missense mutation was found in patients with ACD.

We could not detect a microdeletion in patients 6–8 with ACD in whom no intragenic mutations were identified. Although the underlying causes remain to be clarified in patients 6–8, there are several possible explanations for the development of ACD in patients 6–8. First, a mutation(s) may exist in the unexamined intronic or the downstream region. Second, a tiny deletion may remain undetected. Third, there may be a mutation in some gene(s) other than *SOX9*. Further studies will identify underlying mechanisms involved in the development of ACD in *SOX9* mutation negative patients.

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CLINICAL STUDY

An immunologically anomalous but considerably bioactive GH produced by a novel *GH1* mutation (p.D116E)

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Abstract

Context: Although GH values measured by an immunoassay usually reflect GH bioactivities, discrepancy exists between immunoactivity and bioactivity in a rare condition known as 'bioinactive GH'.

Objective: To report an immunologically anomalous but considerably bioactive GH.

Methods: We performed mutational and functional analyses of *GH1* in a 7-year-old Japanese boy with short stature (−3.0 s.d.) in whom serum GH values measured with a Tosoh immunoassay kit were all undetectable in three provocation tests, whereas urine GH value measured with a Hitachi immunoassay kit was within the normal range. Serum IGF-1 was at a low-normal range, and IGF-binding protein-3 was below the normal range.

Results: Mutation analysis showed a missense GH produced by a novel *GH1* mutation (p.D116E) of paternal origin and a frameshift mutation (p.Q68fsX106) of maternal origin. Genotype–phenotype correlations in this family and *in vitro* functional studies indicated that the p.D116E-GH was immeasurable with the Tosoh kit but was measurable, though maybe not precise, with a Daiichi kit, and had a reduced *in vivo* bioactivity. The p.Q68fsX106 yielded no GH protein.

Conclusions: The results suggest that the p.D116E affects the GH epitope primarily recognized by the Tosoh kit but not by the Hitachi or the Daiichi kits, thereby producing an immunologically anomalous but considerably bioactive GH. The presence of such a hormone discordant for immunoactivity and bioactivity should be kept in mind, to allow for an appropriate assessment of endocrine data.

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Introduction

GH measurement by an immunoassay is indispensable for the diagnosis of GH deficiency. Indeed, GH provocation tests are almost invariably performed in children with short stature (1), and measured serum GH values usually reflect GH bioactivities. However, in a rare condition known as 'bioinactive GH', discrepancy exists between measured GH values and GH bioactivities (2–4). Thus, this condition is associated with low insulin-like growth factor-1 (IGF-1) values, short stature, and good responses to GH therapy, in the presence of apparently normal to mildly elevated serum GH values.

Here, we report an immunologically anomalous but considerably bioactive GH identified in a patient with short stature.

Patient and methods

Case report

This Japanese boy was born to non-consanguineous parents at 39 weeks of gestation after an uncomplicated

pregnancy and delivery. At birth, his length was 50.0 cm (+0.6 s.d.) and his weight was 2.97 kg (+0.2 s.d.).

At 7 years and 1 month of age, he was referred to us because of proportionate short stature (Fig. 1). Endocrine and auxological data are summarized in Tables 1 and 2. Notably, serum GH values measured with a Tosoh immunoassay kit (Tosoh, Tokyo, Japan) were all undetectable during insulin, clonidine, and GH-releasing hormone provocation tests, whereas urine GH value measured with a Hitachi chemiluminescence enzyme immunoassay kit (Hitachi Chemical) was within the normal range. Serum IGF-1 value was at a low-normal range, and IGF-binding protein-3 (IGFBP-3) was below the normal range. Other pituitary hormones and thyroid hormones were normal. Since these endocrine and auxological data satisfied the criteria for GH therapy in Japan (the criteria in children aged ≥ 5 years: height, below −2.5 s.d.; peak GH value, below 6.0 ng/ml at least in two provocation tests; and serum IGF-1 value, below 200 ng/ml) (5), recombinant human GH therapy (0.175 mg/kg per week) was started at 7 years and

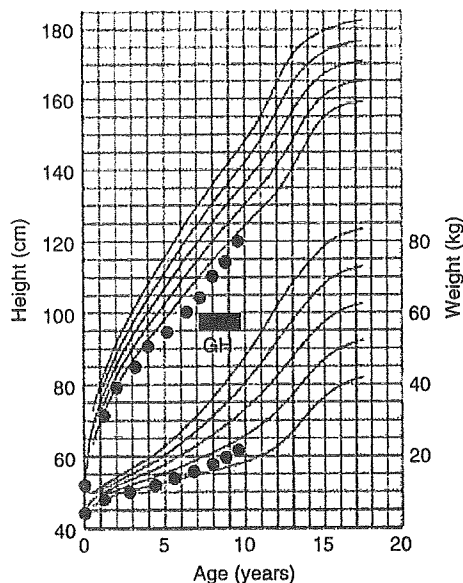


Figure 1 Growth charts of this patient (black circles) plotted on the Japanese sex-matched standard growth curves (+2 s.d., +1 s.d., the mean, -1 s.d., and -2 s.d.). The period of GH therapy is indicated.

3 months after consultation with the parents, but the responsiveness to this therapy was not so remarkable (Fig. 1 and Table 2).

Clinical data of the family members are summarized in Table 2. Since endocrine data of the sister and the mother were examined after the investigations of the patient, basal GH values were measured with the Tosoh kit and a Daiichi IRMA kit (Radio Isotope, Tokyo, Japan; endocrine data were not available in the father). In addition, the Daiichi kit was also applied to measure the basal GH in stocked serum samples of the patient, although the serum samples during the provocation

tests at 7 years and 1 month of age were not preserved. Notably, the basal GH values of the patient and the sister were obviously different between the two kits, and the GH values in the sister did not show a simple 1:2 ratio between the two kits. The sister and the father had low but normal heights, and the sister had normal endocrine data. The mother had normal clinical findings.

Mutation analysis

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. After obtaining written informed consent, leukocyte genomic DNA samples of this patient, the sister, and the parents were amplified by PCR for the coding exons 1–5 and their flanking splice sites of *GH1*, and the PCR products were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA, USA). To confirm a heterozygous mutation, the corresponding PCR products were subcloned with a TOPO TA cloning kit (Invitrogen), and wildtype (WT) and mutant (MT) alleles were sequenced separately. The primers used are shown in Table 3, and the primer positions are depicted in Fig. 2A.

Expression analysis

WT-*GH1* from a normal subject and MT-*GH1* from this patient were PCR-amplified with primers GH-1F and GH-5R (Fig. 2A; Table 3) using genomic DNA samples, and the PCR products were subcloned into pCR2.1 plasmid using the TOPO TA cloning kit. Then, *GH1* gene fragments were cleaved from the plasmid DNA with *EcoRI* and ligated to the *EcoRI* site of an expression vector pRK5. The expression vectors (8 µg) were transiently transfected to SDR-P-1D5 cells obtained from the GH-deficient spontaneous dwarf rat (6),

Table 1 Endocrine studies at 7 years of age.

	Stimulus (dosage)	Patient		Reference values	
		Baseline	Peak	Baseline	Peak
Serum					
GH (ng/ml) ^a	Insulin (0.1 U/kg)	<0.1	<0.1	0.1–20.5	>6.0
	Clonidine (0.1 mg/m ²)	<0.1	<0.1	0.1–20.5	>6.0
	GHRH (1 µg/kg)	<0.1	<0.1	0.1–20.5	>6.0
LH (mIU/ml)	GnRH (100 µg/m ²)	<0.2	5.0	0.0–1.4	0.4–6.0
FSH (mIU/ml)	GnRH (100 µg/m ²)	1.5	19.2	0.6–4.0	6.3–15.6
ACTH (pg/ml)	Insulin (0.1 U/kg)	26.1	179	7.2–22.1	>50
TSH (µU/ml)		1.8		0.44–4.1	
Free T ₄ (ng/dl)		1.2		1.03–2.0	
Free T ₃ (pg/ml)		3.5		2.40–4.68	
Urine					
GH (pg/mg.cr) ^b		17		>7.0	

Reference values indicate the normal ranges in age-matched Japanese boys (26, 27). Blood sampling during the provocation tests: 0, 15, 30, 60, 90, and 120 mins. IGF-1, insulin-like growth factor-1; T₄, thyroxine; T₃, tri-iodothyronine; and GHRH, growth hormone releasing hormone.

^aMeasured with a Tosoh immunoassay kit.

^bMeasured with a Hitachi immunoassay kit.

Table 2 Summary of clinical data of the family members.

	Patient	Sister	Father	Mother
Age (years)	7 1/12 ^a	9 6/12 ^b	6 2/12	39
Height (cm)	104.5	120.3	105.2	163.0
Height SDS ^c	-3.0	-2.4	-1.8	-1.5
Bone age (years) ^d	3 3/12	5 8/12	NE	NE
GH (ng/ml; Tosoh kit) ^e	<0.1	<0.1 ^f	5.23	NE
GH (ng/ml; Daiichi kit) ^e	1.5	2.86 ^f	8.35	NE
	(0.1-20.5)	(0.1-19.5)	(0.1-21.0)	-
IGF-1 (ng/ml)	93	140	234	NE
	(63-339)	(87-405)	(61-372)	-
IGFBP-3 (ng/ml)	1.36	2.29	2.36	NE
	(1.76-3.38)	(1.99-3.41)	(1.66-2.91)	-
				(0.1-3.7)
				236
				(109-265)
				2.79
				(1.99-3.19)

IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor-binding protein-3; and NE, not examined. The basal hormone values are shown; the values in parentheses represent age- and sex-matched Japanese reference data (26, 27).

^aBefore GH therapy.

^bOn GH therapy.

^cAssessed by the age- and sex-matched Japanese reference data (28).

^dEvaluated by the TW-2 method standardized for Japanese (29).

^eRecombinant GH standard (WHO International Reference Preparation 98/574) has been utilized for the calibration of both kits.

^fSince blood sample was obtained at 15 h after the GH injection, these values would primarily, if not totally, represent endogenous GH values.

using Gene Pulser Electroporation System (Bio-Rad Laboratories). The transfected cells were incubated for 48 h in a plate with a diameter of 10 cm, and GH in the culture media was measured with the Tosoh and the Daiichi kits. This analysis was performed for three independent experiments. Furthermore, western blotting was performed for the culture media using Rabbit polyclonal GH antibodies (Abs) and anti-Rabbit IgG conjugated with alkaline phosphatase (Promega).

Bioassay

A cell proliferation bioassay was performed for WT-GH and MT-GH, using mouse pro-B cell lymphoma cells that express GH receptor (Ba/F3-hGHR cells) (7). The detailed protocol has been reported previously (8). In brief, WT-GH and MT-GH were prepared in solutions at concentrations of 5, 10, and 20 ng/ml that were determined with the Daiichi kit. Each GH solution of 25 µl was added to 200 µl of Ba/F3-hGHR cell suspension (1×10^5 cells/ml), and the mixture was

incubated for 48 h at 37 °C. At the end of the incubation, a colorimetric end point was obtained by an eluted stain bioassay (9), and a bioactive response was determined with a kinetic microplate reader (Molecular Devices, Menlo Park, CA, USA) using optical densities at the test wavelength of 550 nm and a reference wavelength of 650 nm to correct for differential scattering. The experiments were performed in quadruplicate. Statistical significance was examined by Student's *t*-test.

Protein modeling analysis

The protein conformation was analyzed by Esy-Pred3D (10).

Results

Mutation analysis

Two novel mutations were identified in the patient, a 2 bp deletion at exon 3 (c.280-281delCA) that is predicted to cause a frameshift at the 68th codon for glutamine and resultant termination at the 106th codon (p.Q68fsX106) and a missense mutation at exon 4 (c.426C>G) that is predicted to result in a substitution of aspartic acid with glutamic acid at the 116th codon of GH produced by a novel GH1 mutation (p.D116E; Fig. 2B). The father and the sister were heterozygous for the p.D116E, and the mother was heterozygous for the p.Q68fsX106 mutation (Fig. 2C).

Functional studies

Expression analysis showed that the p.D116E-GH in the three different culture media was immeasurable with the Tosoh kit but was clearly measurable with the Daiichi kit, and that the p.Q68fsX106-GH was

Table 3 Primers utilized in this study.

Primer	Forward Reverse	AT (C) PS (bp)
< Mutation analysis >		
GH-1F	ACAGGTGGGGTCAACAGTGG	60
GH-1R	CCAGGGACCAGGAGCTTTCT	303
GH-2F	CAATCTCAGAAAGCTCCTGG	60
GH-2R	AGCTCCTTAGTCTCCTCCTC	374
GH-3/4F	AGATGAGCACACGCTGAGTG	62
GH-3/4R	AAGGTGAGTTCTCTGGGTC	584
GH-5F	AGGCCTTTCTCTACACCCTG	60
GH-5R	AGAAGGACACCTAGTCAGAC	435
< Expression analysis >		
GH-1F	ACAGGTGGGGTCAACAGTGG	60
GH-5R	AGAAGGACACCTAGTCAGAC	1727

AT, annealing temperature; and PS, product size.

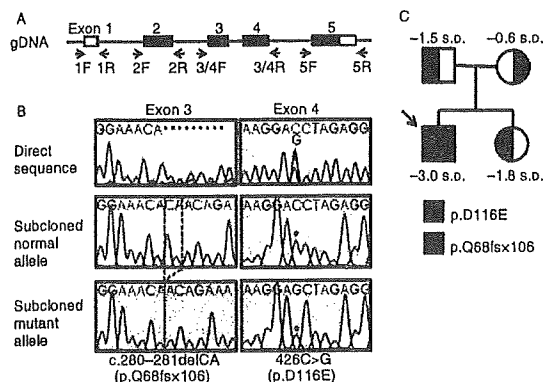


Figure 2 (A) Schematic representation of *GH1*. The black and white boxes on genomic DNA (gDNA) denote the coding regions on exons 1–5 and the UTRs respectively. The arrows indicate the position of the primers utilized in this study. (B) Mutation analysis of *GH1* in this patient. The electrochromatograms delineate the c.280–281delCA (p.Q68fsX106) mutation in exon 3 (left) and the c.426C>G (p.D116E) mutation in exon 4 (right). The mutations have been indicated by the direct sequencing, and confirmed by the subsequently performed sequencing of the subcloned normal and mutant alleles. (C) Pedigree of the family. The height SDS is shown for each family member; for the patient the s.d. before GH therapy is indicated. Of the two mutations identified in this patient, p.D116E is of paternal origin and p.Q68fsX106 is of maternal origin. The sister is heterozygous for the p.D116E mutation.

undetectable by both of the kits (Table 4). Western blot analysis delineated a 22 kDa band for the p.D116E-GH as well as for the WT-GH (Fig. 3A), and a similar band intensity was identified when 3 ng of the p.D116E-GH measured with the Daiichi kit (13 μ l of culture media of experiment 3 in Table 4) and 5 ng of WT-GH were utilized. For the p.Q68fsX106-GH, no band was identified for the same amount of culture media (13 μ l). Bioassay revealed that the bioactivity was similar between the WT-GH and the p.D116E-GH ($P=0.069, 0.066, \text{ and } 0.127$ at GH concentrations of 5, 10, and 20 ng/ml based on the Daiichi kit respectively; Fig. 3B). Protein-modeling analysis indicated a normal conformation of the p.D116E-GH (Fig. 3C).

Discussion

This patient had apparently complete GH deficiency and two novel compound *GH1* mutations (p.D116E and p.Q68fsX106). However, his growth pattern including normal birth length, the relatively mild postnatal growth failure, and the poor response to GH therapy is not typical for congenital GH deficiency (11, 12), and the urine GH and serum IGF-1 and IGFBP-3 values indicate a hidden GH activity. Consistent with this, the p.D116E-GH was immeasurable with the Tosoh kit but was measurable with the Daiichi kit, and had an apparently normal *in vitro* biological function. In this regard, the three kits employed in this study utilize two monoclonal

Table 4 GH values in the culture media (ng/ml).

Experiment	p.D116E		p.Q68fsX106	
	Tosoh kit	Daiichi kit	Tosoh kit	Daiichi kit
1	<0.1	90	<0.1	<0.1
2	<0.1	107	<0.1	<0.1
3	<0.1	232	<0.1	<0.1

Abs for GH, one against an epitope within the 22 kDa GH-specific residues (32–46 amino acids) and the other against an epitope specific to each kit. The Hitachi kit detects an epitope at the N-terminal region, while the epitope specifically recognized by the Tosoh and Daiichi kits is unknown. Thus, while the p.Q68fsX106 appears to be an amorphic mutation that is incapable of producing GH probably because of nonsense-mediated mRNA decay (13), it is likely that the p.D116E affects the GH epitope primarily recognized by the Tosoh kit but not by the Hitachi or the Daiichi kit, thereby producing a possible immunologically anomalous but biologically active GH. This notion would also explain why the basal serum GH values measured with the Tosoh kit were obviously lower than those measured with the Daiichi kit in the patient and the sister with p.D116E.

It remains to be determined, however, whether the p.D116E-GH has a normal biological function *in vivo*. Although the *in vitro* bioassay indicated an apparently normal function for the p.D116E-GH, it is known that

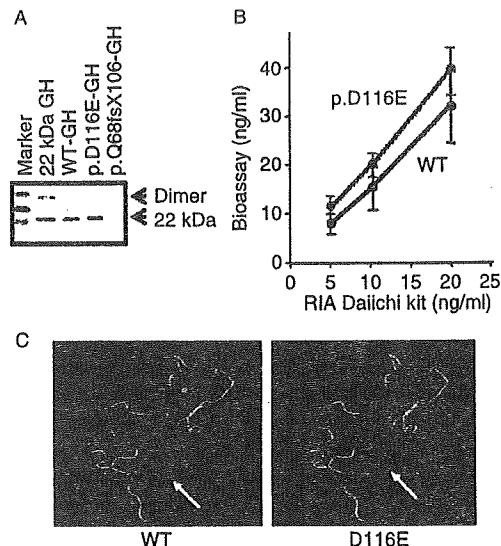


Figure 3 (A) Western blot analysis, showing the presence of 22 kDa WT-GH and p.D116E-GH. The standard 22 kDa human GH is used as an internal control (B). Note that a similar band intensity is delineated for 3 ng of the p.D116E-GH and 5 ng of WT-GH measured with the Daiichi kit. (B) Bioassay of the WT-GH and the p.D116E-GH, using Ba/F3-hGH receptor cells. The results are expressed using the mean and the s.d. (C) Ribbon diagrams of the GH proteins. The white arrows indicate the 116 residue for WT-GH and the p.D116E-GH.

the results obtained with artificially constructed cell lines do not necessarily reflect the *in vivo* biological effects of hGH MTs (8, 14). Indeed, the difference in the GH value between the two kits in the sister and the relationship between the GH value and the band intensity in the western blotting may imply that the p.D116E-GH was not measured precisely even with the Daiichi kit, so that a relatively large amount of the p.D116E-GH was probably utilized in the *in vitro* bioassay, compensating for a possible hypofunction of the p.D116E-GH. Furthermore, since the previously described p.D116A-GH harboring a missense mutation within the GH receptor-binding site 2 has a 5.7-fold lower affinity to the GH receptor than the WT-GH (15), this would argue for a functional importance of the D116 residue and implicate a similar functional alteration of the p.D116E-GH. In addition, although *GH1* missense mutations reported to date are relatively rare (16), GH missense MTs, including those within or near the GH receptor binding site 2, frequently have a reduced or altered biological activity (2, 4, 17–21).

In this regard, comparison of clinical data between the patient with functional hemizygoty for the p.D116E and the mother with functional hemizygoty for the WT *GH1* would suggest that the p.D116E-GH has a reduced, though not abolished, *in vivo* bioactivity (Table 2). In support of this, most individuals with heterozygous *GH1* deletions have normal stature (22) as observed in the mother, while this patient had short stature. It may also be possible that the p.D116E-GH is less secreted from the pituitary into the circulation when compared with an intact GH protein, although the clinical findings of the father and the sister heterozygous for the p.D116E would argue against the possibility that the p.D116E-GH exerts an obvious dominant negative effect (Table 2). However, since short stature is a highly heterogeneous phenotype subject to multiple genetic and environmental factors (23, 24), some factors other than the *GH1* mutations may be involved in the development of short stature in this patient. In addition, there may be an ascertainment bias, because GH-related studies are almost exclusively performed in individuals with short stature. Further studies will permit to clarify the *in vivo* biological function of the p.D116E-GH and its relevance to the development of short stature.

Such an immunologically anomalous and biologically active hormone has been reported previously. It is known that the common LH variant (V-LH) with two completely linked Trp8Arg and Ile15Thr substitutions in the LH β -subunit is immunologically undetectable when a MAB recognizing an epitope present in the intact LH α/β dimer is utilized, but is measurable when two monoclonal Abs recognizing specific sites in the LH β subunit are utilized (25). Notably, the V-LH appears to have somewhat weaker bioactivity than the WT-LH, and is often associated with the primary ovarian dysfunction in the Japanese population (25).

Nevertheless, elevated LH values characteristic of primary ovarian dysfunction cannot be identified without applying the method using two monoclonal Abs, although FSH values are definitely increased. Thus, when a discrepancy is present between values of a specific hormone and other biochemical data or clinical findings, it is recommended to measure the specific hormone with a different kit, to avoid the misdiagnosis of hormone deficiency.

In summary, we identified an immunologically anomalous but considerably bioactive GH produced by p.D116E mutation. Indeed, such abnormalities along the GH/IGF-1 axis may also be identified by performing GH-related endocrine studies in children with short stature. The presence of such an apparently immeasurable but bioactive hormone, as well as a measurable but bioinactive hormone, should be kept in mind, to allow for an appropriate assessment of endocrine data.

Declaration of interest

The authors declare no conflict of interest.

Funding

This research was supported by Grants for Child Health and Development and Research on Children and Families from the Ministry of Health, Labor, and Welfare.

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Received 19 May 2009

Accepted 19 May 2009

MAMLD1 (CXorf6): A New Gene Involved in Hypospadias

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Key Words

MAMLD1 · CXorf6 · Hypospadias · Testosterone

Abstract

MAMLD1 (mastermind-like domain containing 1), previously known as *CXorf6* (chromosome X open reading frame 6), has been shown to be a causative gene for hypospadias. This is primarily based on the identification of nonsense mutations (E124X, Q197X, and R653X), which undergo nonsense-mediated mRNA decay, in patients with penoscrotal hypospadias. Subsequent studies have shown that (1) the mouse homolog is transiently expressed in fetal Sertoli and Leydig cells around the critical period of sex development; (2) transient knockdown of *Mamld1* results in significantly reduced testosterone production in murine Leydig tumor cells; (3) MAMLD1 protein shares homology to mastermind-like 2 (MAML2) protein that functions as a co-activator in canonical Notch signaling; (4) MAMLD1 localizes to the nuclear bodies and transactivates the promoter activity of a non-canonical Notch target gene hairy/enhancer of split 3 (*Hes3*), rather than the canonical Notch target genes such as *Hes1* and *Hes5*, without demonstrable DNA-binding capacity, and (5) *MAMLD1* is regulated by steroidogenic factor 1. These findings suggest that the *MAMLD1* mutations cause hypospadias primarily because of compromised testosterone production around the critical period of sex development, and provide useful information for the molecular network involved in fetal testosterone production.

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Introduction

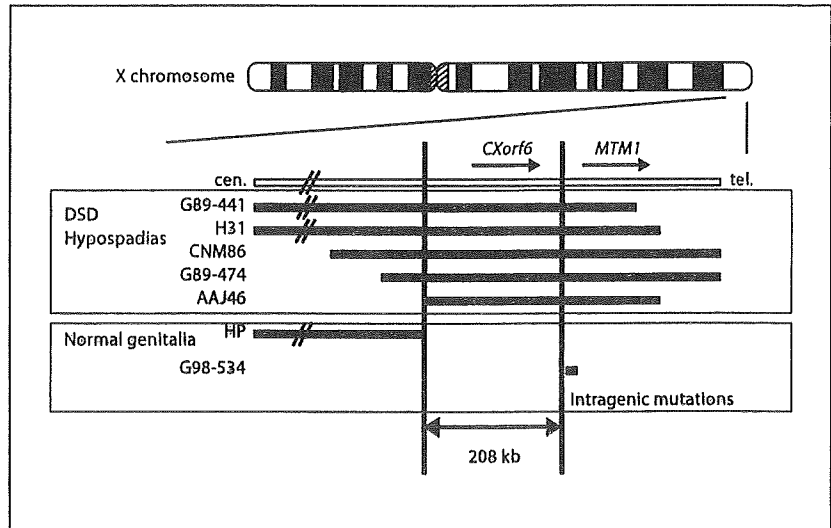
Hypospadias is defined by the urethral opening on the ventral side of the penis, and is classified into mild glandular or penile type and severe penoscrotal or perineal type [1]. It is a mild form of 46,XY disorders of sex development (DSD), and affects ~0.5% of male newborns [2]. Hypospadias is primarily caused by compromised androgen effects, and appears as an isolated anomaly or in association with other genital anomalies such as micropenis and cryptorchidism. To date, while mutation analyses have been performed for multiple genes involved in androgen effects such as *SRD5A2* for 5 α -reductase and *AR* for androgen receptor, pathologic mutations have been identified only in a very small portion of patients [2]. This would be consistent with hypospadias being a highly heterogeneous condition subject to multiple genetic and environmental factors. Indeed, several candidate genes such as *ATF3*, *FKBP52*, *FGFR2*, *FGF8*, *FGF10*, and *BMP7* have been identified, and multiple susceptibility factors for hypospadias have been found in several genes such as *ESR1*, *ESR2*, and *SRD5A2* [3–7].

We have recently shown that *CXorf6* (chromosome X open reading frame 6) is a novel gene for hypospadias [8], and coined a new gene symbol *MAMLD1* (mastermind-like domain containing 1) on the basis of its characteristic protein structure [9]. Here, we review the current knowledge about *MAMLD1*.

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Fig. 1. Identification of *CXorf6* as a candidate DSD gene on Xq28 by deletion mapping. The horizontal bars indicate the deleted segments in each case. Of the 7 male patients with microdeletions around *MTM1* for myotubular myopathy, 5 show 46,XY DSD (primarily hypospadias), whereas 2 patients have normal genitalia as do patients with intragenic *MTM1* mutations. The smallest overlapping deleted region is roughly 208 kb in physical length, and contains *CXorf6* as a sole gene within the critical region.



Cloning of a Candidate Gene for 46,XY DSD

A gene for 46,XY DSD has been postulated around *MTM1* for myotubular myopathy on Xq28, on the basis of the finding that genital development is normal in patients with intragenic *MTM1* mutations, and invariably abnormal in 6 patients with microdeletions involving *MTM1* [10–13]. The 6 patients consist of 3 sporadic and 3 familial cases, and 5 of them have glandular, penile, or penoscrotal hypospadias and the remaining 1 patient exhibits ambiguous genitalia [10–12]. These findings suggest that a gene for 46,XY DSD, especially that for hypospadias, resides in the vicinity of *MTM1*, and that loss or disruption of the gene results in the development of 46,XY DSD as consequence of a contiguous gene deletion syndrome.

In 1997, Laporte et al. [14] identified *MAMLD1* (named *CXorf6* at that time) from a 430-kb region deleted in 2 sporadic cases with myotubular myopathy and 46,XY DSD [12]. *MAMLD1* comprises at least 7 exons, and harbors an open reading frame on exons 3–6 that is predicted to produce 2 proteins of 701 and 660 amino acids as a result of in-frame alternative splicing with and without exon 4. Furthermore, subsequent studies have shown loss of *MAMLD1* in all patients with myotubular myopathy and 46,XY DSD (fig. 1), 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, especially hypospadias.

MAMLD1 Mutations in Hypospadiac Patients

We performed direct sequencing for the coding exons 3–6 and their flanking splice sites of *MAMLD1* in 166 patients with various types of DSD or abnormal external genitalia. They consisted of 117 Japanese patients (113 sporadic cases and 4 probands of familial cases), 45 European patients (39 sporadic cases and 6 probands of familial cases), and 4 Chinese patients (4 probands of familial cases). The 117 Japanese patients comprised: 19 cases with gonadal dysgenesis (10 with complete type and 9 with incomplete type) with no demonstrable mutation in the known or candidate sex development genes *SRY*, *DMRT1*, *SFI*, and *LHX9* [2]; 2 cases with 46,XY DSD of unknown cause; 56 cases with hypospadias (16 with glandular type, 16 with penile type, 20 with penoscrotal type, and 4 with perineal type), and 40 cases with isolated cryptorchidism (33 with unilateral inguinal or abdominal type and 7 with bilateral inguinal type). All the Japanese patients had a normal male karyotype and lacked extragenital features except for short stature in 6 cases, mental retardation in 3 cases, and multiple congenital anomalies in 2 cases. Thus, most patients exhibited abnormal external genitalia as the sole recognizable abnormality. The 49 European and Chinese patients had various types of abnormal genitalia, ranging from hypospadias to feminized genitalia (detailed phenotypes are unknown).

Consequently, 3 nonsense mutations were identified in Japanese patients with hypospadias: E124X in mater-