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(squalene monooxigenase) involved in cholesterologenesis and CYP17A1 (17α-hydroxylase and 17,20 lyase), CYP21A2 (21-hydroxylase), and CYP19A1 (aromatase) involved in steroidogenesis (3, 4).

PORD has been identified in multiple patients (4). Mutations are diverse, including missense, nonsense, frameshift, and splice site mutations (4). Notably, however, A287P is the most common mutation in Caucasian patients, and R457H is the most prevalent founder mutation in Japanese patients (1–8). In addition, there is no patient with two apparently null mutations, suggesting that absence of a residual POR activity is incompatible with life (4–6). Clinical features are also variable, with a wide range of expressivity and penetrance. Indeed, ABS-compatible skeletal features and DSD are severely manifested by some patients and apparently absent in other patients (4–6). In addition, adrenal crisis remains relatively rare (4, 6), and maternal virilization is not a consistent feature (5, 6, 9).

To date, however, several critical matters remain to be clarified. First, although about 12% of patients have one apparently normal *POR* allele (4), it is uncertain whether such patients represent manifesting heterozygotes or have hidden aberrations in nonexamined region(s) (4–10). Second, the underlying factors for the clinical diversity remain to be determined, although variable supporting activities of different POR mutants for target enzymes would have a certain role (5, 11, 12). Third, pubertal development and longitudinal growth have poorly been investigated.

To examine these matters, we analyzed the *POR* gene in affected patients and performed genotype-phenotype correlations in terms of the dosage effect of the R457H mutant.

Patients and Methods

Patients

This study consisted of 35 Japanese patients aged 0.1–23.8 yr (16 patients with 46,XY and 19 patients with 46,XX), including previously reported 23 cases (6, 8, 9) (Table 1). Of the 35 patients, 25 were sporadic cases and the remaining 10 were familial cases from families A–D. Twenty-three sporadic cases and four probands (cases 10, 15, 30, and 35) were ascertained by skeletal features and/or DSD, two sporadic cases (cases 1 and 5) by newborn mass screening for 21-hydroxylase deficiency, and the remaining six cases by familial studies.

Molecular analysis

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. The primers used in this study are shown in supplementary Table 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org. After taking written informed consent, peripheral blood samples were obtained from all the patients and the parents of 19 sporadic cases and two familial cases (families A and C). Subsequently, genomic DNA samples were subjected to direct sequencing for the *POR* exons 1–16, together with their flanking splice sites. To confirm a heterozygous mutation, the corresponding PCR products were subcloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and the two alleles were sequenced separately.

When lymphoblastoid cell lines were available, fluorescent *in situ* hybridization (FISH) analysis was performed with two long PCR products spanning exons 4–7 (probe 1) and exons 8–12 (probe 2). The two probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin. A spectrum green-labeled probe for *D7Z1* (CEP7) (Abbott, Abbott Park, IL) was used as an internal control. For a case with a probable microdeletion, RT-PCR was performed with a variety of primers, to determine the deletion size. Furthermore, to examine the occurrence of transcription failure in cases with apparent heterozygosity and that of the nonsense-mediated mRNA decay (NMD) in cases with premature truncation mutations, the lymphoblastoid cell lines available were incubated for 8 h with and without an NMD inhibitor cycloheximide (CHX; 100 μg/ml; Sigma, St. Louis, MO), and direct sequencing was performed for RT-PCR products (13, 14).

In addition to disease-causing mutations, we also examined the presence or absence of a common A503V variant that has been shown to have a mildly decreased supporting activity at least for CYP17A1 (~60%) (15), to investigate whether the A503V variant can function as a modifier of the clinical phenotype. To examine whether the A503V variant resides on the same allele carrying R457H, PCR products encompassing both the 457th and 503rd codons were subcloned and subjected to direct sequencing.

Clinical assessment

Skeletal features were assessed by bone survey. Adrenal function was evaluated by basal and ACTH-stimulated blood hormone values [250 μg/m² (maximum 250 μg) bolus iv; blood sampling at 0 and 60 min] and by urine steroid profiles determined by the gas chromatography/mass spectrometry using first morning urine samples in cases aged older than 6 months (16) (several urine steroid metabolites cannot be measured precisely during the first 6 months of age due to interference of unknown steroids derived from the fetal adrenocortex). DSD was clinically evaluated, as was pubertal development in boys aged older than 14.3 yr (mean +2 SD age for pubic stage 2) and in girls aged older than 12.8 yr (mean +2 SD age for breast stage 2) (17). When possible, basal blood pituitary-gonadal hormone values were also obtained as well as human chorionic gonadotropin (hCG)-stimulated testosterone (T) values (3000 IU/m² per dose im for 3 consecutive days; blood sampling on d 1 and 4). In addition, clinical records were surveyed for the data of 17-hydroxyprogesterone (17-OHP) values at the newborn mass screening, adrenal crisis, maternal virilization during pregnancy, polycystic ovary (PCO) in female cases, and body measurement.

Penile length, clitoral size, Tanner stage, testis size, age of menarche, and statural growth were assessed by age- and sex-matched Japanese reference data (17–20), as were hormone values (21–23). Because urine steroid metabolites (Ms) expressed in a logarithm scale grossly followed the normal distribution and showed marked change with age in control

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TABLE 1. Summary of molecular analyses

Patients				*POR* mutations	
Case	Karyotype	Age (yr)	Inheritance	Nucleotide changes[a]	Aminoacid changes
Group A: homozygotes for R457H					
1	46,XY	5.0	Sporadic	1370G>A/1370G>A	R457H/R457H
2	46,XY	23.8	Familial-A	1370G>A/1370G>A	R457H/R457H
3	46,XY	22.6	Familial-A	1370G>A/1370G>A	R457H/R457H
4	46,XY	6.7	Sporadic	1370G>A/1370G>A	R457H/R457H
5	46,XY	0.4	Sporadic	1370G>A/1370G>A	R457H/R457H
6	46,XX	0.4	Sporadic	1370G>A/1370G>A	R457H/R457H
7	46,XX	0.4	Sporadic	1370G>A/1370G>A	R457H/R457H
8	46,XX	2.0	Sporadic	1370G>A/1370G>A	R457H/R457H
9	46,XX	14.1	Sporadic	1370G>A/1370G>A	R457H/R457H
10	46,XX	15.0	Familial-A (P)	1370G>A/1370G>A	R457H/R457H
11	46,XX	3.0	Sporadic	1370G>A/1370G>A	R457H/R457H
12	46,XX	0.2	Sporadic	1370G>A/1370G>A	R457H/R457H
13	46,XX	0.1	Sporadic	1370G>A/1370G>A	R457H/R457H
14	46,XX	18.0	Sporadic	1370G>A/1370G>A	R457H/R457H
Group B: compound heterozygotes for R457H and an apparently null mutation					
15	46,XY	16.8	Familial-B (P)	1370G>A/601C>T	R457H/Q201X
16	46,XY	5.1	Familial-B	1370G>A/601C>T	R457H/Q201X
17	46,XY	14.8	Sporadic	1370G>A/1329-1330insC	R457H/I444fsX449
18	46,XY	17.5	Sporadic	1370G>A/(5A>G)	R457H/Non-transcribed (G5G)[b]
19	46,XY	25.1	Sporadic	1370G>A/143delG	R457H/R48fsX63
20	46,XY	0.2	Sporadic	1370G>A/1665delG	R457H/Q555fsX612
21	46,XY	13.1	Sporadic	1370G>A/(−)[c]	R457H/DeltaExons 2–13[d]
22	46,XX	9.0	Sporadic	1370G>A/IVS7+1G>A	R457H/IVS7+1G>A
23	46,XX	14.8	Sporadic	1370G>A/1698-1699insC	R457H/Y567fsX574
24	46,XX	13.2	Sporadic	1370G>A/1329-1330insC	R457H/I444fsX449
25	46,XX	12.9	Familial-B	1370G>A/601C>T	R457H/Q201X
26	46,XX	6.6	Sporadic	1370G>A/(−)[c]	R457H/Non-transcribed[b]
27	46,XX	4.2	Sporadic	1370G>A/(−)[c]	R457H/Non-transcribed[b]
28	46,XX	17.0	Sporadic	1370G>A/1329-1330insC	R457H/I444fsX449
Group C: other compound heterozygotes					
29	46,XY	0.4	Sporadic	1370G>A/1386-1387insATCGCC	R457H/A462-S463insIA
30	46,XY	23.5	Familial-C (P)	1370G>A/1835-1858del[e]	R457H/L612-W620delinsR
31	46,XY	18.0	Familial-C	1370G>A/1835-1858del[e]	R457H/L612-W620delinsR
32	46,XY	17.9	Familial-D	1733A>G/1329-1330insC	Y578C/I444fsX449
33	46,XX	0.8	Sporadic	1370G>A/1738G>C	R457H/E580Q
34	46,XX	0.7	Sporadic	1370G>A/1042-1044delGTC	R457H/348delV
35	46,XX	0.5	Familial-D (P)	1733A>G/1329-1330insC	Y578C/I444fsX449

The genomic position corresponding to each mutation based on NC_000007.12 sequence at the National Center for Biotechnology Information database (Bethesda, MD) is as follows: R457H, 75452433G>A; Q201X, 75448386C>T; I444fsX449, 75452391-2insC; G5G, 75421261A>G; R48fsX63, 75421389delG; Q555fsX612, 75453099delG; IVS7 + 1G>A, 75448861G>A; Y567fsX574, 75453205-6insC; A462-S463insIA, 75452349-50insATCGCC; L612-W620delinsR, 75453432-55delTAAAGCAAGACCGAGAGCACCTGT; Y578C, 75453237A>G; E580Q, 75453245G>C; and 348delV, 75451086-88delGTC. Cases 1–3, 6–10, 15–18, 22–26, 29–33, and 35 have been reported previously (6, 8, 9), and the remaining 12 cases were first examined in this study. P, Proband.

[a] The A of the ATG encoding the initiator methionine residue of the predicted translation product is denoted position +1.

[b] The allele with G5G and the apparently normal alleles are not trasncribed into mRNA.

[c] The (−) symbol indicates the absence of a recognizable mutation on the exonic sequences.

[d] An intragenic microdeletion involving exons 2–13.

[e] 1835-1858delTAAAGCAAGACCGAGAGCACCTGT.

subjects of both sexes (854 males and 909 females), the M data of the patients were expressed as the SD score to allow for the comparison among patients of different sexes and ages.

Statistical analysis

Statistical significance of the frequency of clinical features was analyzed by the Fisher's exact probability test, and that of the median of nonpaired and paired variables was examined by the Mann-Whitney's *U* test and the Wilcoxon signed-rank test, respectively. $P < 0.05$ was considered significant.

Results

POR mutations

The results are summarized in Table 1. Direct sequencing revealed 12 types of mutations and one silent substitution (G5G) (Fig. 1A), with R457H being identified in 40 of the 58 alleles (~70%) in 25 sporadic cases and four probands of families A–D. Of the 12 mutations, R48fsX63, Q555fsX612, and 348delV were first identified in this study. These mutations were absent in 100 control subjects.

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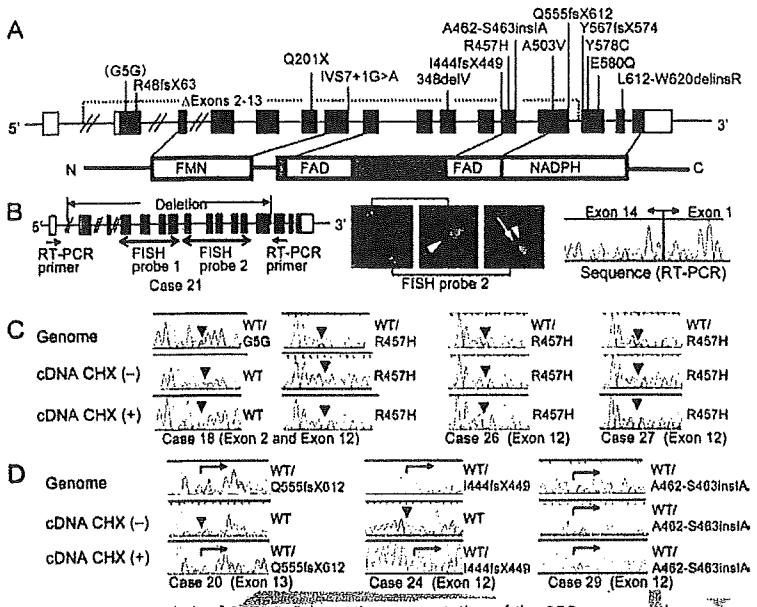

FIG. 1. Mutation analysis of *POR*. A, Schematic representation of the *POR* gene and the positions of identified mutations. The Japanese founder mutation R457H is shown in *red*, other disease-causing mutations in *black*, and the common A503V variant in *blue*. *Upper diagram*, The genomic structure comprising 16 exons. The *black* and *white boxes* denote the coding and the untranslated regions, respectively. *Lower diagram*, The protein structure consisting of the cofactor binding domains (FMN, flavin mononucleotide; FAD, flavin-adenine dinucleotide; and NADPH: nicotinamide-adenine dinucleotide phosphate, reduced) and the connecting domain (*stippled area*). B, FISH and RT-PCR sequencing analyses in case 21. *Left diagram*, The positions of the two FISH probes and those of the primers for RT-PCR. *Middle diagram*, FISH findings showing two signals for *D7Z1* (*arrowheads*) and a single signal for *POR* (*arrow*) delineated by the FISH probe 2. *Right diagram*, RT-PCR sequencing indicating the fusion between exons 1 and 14 (the deletion of exons 2–13). C, Transcription failure in cases 18, 26, and 27. Although heterozygosity for R457H is delineated for the genomic DNA, RT-PCR sequencing indicates absent expression of the wild-type (WT) alleles in the three cases. Similarly, although heterozygosity for G5G is shown for the genomic DNA of case 18, RT-PCR sequencing reveals no expression of the G5G allele. Such lack of transcripts is not recovered by CHX. D, Nonsense-mediated mRNA decay in cases 20 and 24 but not case 29. Although heterozygosity for the mutations is shown for the genomic DNA, RT-PCR sequencing delineates the WT alleles only before CHX treatment and the heterozygosity after CHX treatment in cases 20 and 24. The NMD is not observed in case 29.

Fifteen cases were apparently homozygous for R457H, and hemizygosity was excluded in 14 of the 15 cases by parental analysis indicating heterozygosity for R457H in both parents (cases 1–3, 6–11, and 13) and by FISH analysis with two FISH probes (cases 4, 5, 12, and 14). Notably, however, FISH analysis delineated a heterozygous microdeletion in case 21, and RT-PCR sequencing analysis revealed loss of exons 2–13 in this case (Fig. 1B). The mother was heterozygous for R457H, and the father was heterozygous for the intragenic microdeletion.

Three cases were apparently heterozygous for R457H (cases 18, 26, and 27), although case 18 also had G5G. However, RT-PCR sequencing analysis using lymphoblastoid cell lines showed nearly complete absence of mRNA derived from the apparently normal alleles in the three cases (Fig. 1C). The mRNA remained undetected after CHX treatment, indicating transcription failure.

Of the 11 other types of mutations, the nonsense and four frameshift mutations (Q201X, R48fsX63, I444fsX449, Q555fsX612, and Y567fsX574) leading to premature termination and the conserved splice donor site mutation (IVS7+1G>A) appeared to be null mutations, whereas the remaining five mutations (Y578C, E580Q, 348delV, A462-S463insIA, and L612-W620delinsR) were unknown for residual activities. Indeed, RT-PCR sequencing analysis performed before and after CHX treatment in three cases with available lymphoblastoid cell lines demonstrated that the alleles carrying Q555fsX612 and I444fsX449 underwent NMD, whereas the allele harboring A462-S463insIA escaped NMD (Fig. 1D).

The common A503V variant was absent from cases of group A and was identified in four cases of group B (cases 22, 23, 26, and 27) and four cases of group C (cases 29–31, and 34). The eight cases with A503V were all compound heterozygotes with R457H and another mutation, and direct sequencing for subcloned PCR products encompassing both 457th and 503rd codons revealed lack of coexistence of R457H and A503V. Thus, it was indicated that the A503V variant was absent from all of the 47 alleles carrying R457H and was present on alleles carrying IVS7+1G>A, Y567fsX574, A462-S463insIA, L612-W620delinsR, and 348delV and on the two nontranscribed alleles.

Classification of the patients

On the basis of the above results, the 35 cases were classified into three groups: group A, homozygotes for R457H (cases 1–14); group B, compound heterozygotes for R457H and one apparently null mutation (cases 15–28); and group C, other types of compound heterozygotes (cases 29–35) (Table 1). The residual POR activity was predicted to be higher in group A than group B, although it was unknown for group C. In addition, group B was subclassified into A503V-positive cases (cases 22, 23, 26, and 27) and negative cases (cases 15–21, 24, 25, and 28).

Clinical features

The prevalence of each clinical feature in groups A–C is summarized in Table 2, together with its comparison between groups A and B. The sex ratio was similar between groups A and B, as was the median age. T2,AQ:D

ABS-compatible skeletal features were definitely more prevalent in group B than group A (Table 2 and supplementary Fig. 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). In particular, severe brachycephaly, elbow joint synostosis, and choanal stenosis were exclusively identified in group B.

Adrenal steroidogenic dysfunction was biochemically identified in all cases, with some difference between groups A and B. Blood ACTH was normal or elevated at the baseline, 17-OHP was normal or elevated at the baseline and above the normal range after ACTH stimulation, and cortisol was normal at the baseline but barely responded to ACTH stimulation (Fig. 2A). F2 Significant difference between groups A and B was identified for basal 17-OHP value ($P = 0.044$) and basal and ACTH-stimulated cortisol values ($P = 0.018$ and $P = 0.022$). Urine Ms of progesterone and 17-OHP were elevated, whereas those of an-

TABLE 2. The prevalence of each clinical feature in groups A–C and its comparison between groups A and B

	Group A (n = 14)	Group B (n = 14)	Group C (n = 7)	Groups A *vs.* B (*P* value)
Sex (male:female)	5:9	7:7	4:3	0.35
Age (median, range, yr)	4.0 (0.1–23.8)	13.1 (0.2–17.5)	0.8 (0.4–23.5)	0.19
Skeletal features				
Any skeletal feature	7/14	14/14	7/7	0.0029
Brachycephaly (overt)	0/14	14/14	6/7[a]	0.000000025
Elbow joint synostosis[b]	0/14	7/14	4/7	0.0029
Arachnodactyly (overt)	5/14	14/14	7/7	0.048
Choanal stenosis	0/14	5/14	1/7	0.020
Joint contracture	7/14	14/14	7/7	0.0029
Adrenal dysfunction				
Adrenal crisis	0/14	4/14	1/7[c]	0.049
Detection by mass screening[d]	5/8	3/8	2/4	0.31
46,XY DSD				
Any genital feature at birth	1/5[e]	3/7[f]	3/4	0.42
Hypospadias	0/5	2/7	1/4	0.32
Cryptorchidism	0/5	3/7	2/4	0.16
Micropenis	1/5	2/7	3/4	0.64
46,XX DSD				
Any genital feature at birth	9/9[e]	7/7[f]	3/3	1.0
Clitoromegaly	8/9	5/7	3/3	0.40
Labial fusion	8/9	5/7	2/3	0.40
Common urogenital sinus	2/9	2/7	0/3	0.61
Maternal virilization	8/14	5/14	4/7	0.22
Pubertal failure 46,XY				
Delayed (>2 SD) or no pubertal sign	0/2[g]	3/4[h]	2/3	0.20
Small testis (<2 SD)	0/2	2/4	1/3	0.40
Primary hypogonadism	0/2	2/2	3/3	0.17
Pubertal failure 46,XX				
Delayed (>2 SD) or no pubertal sign	3/3[g]	4/4[h]		1.0
Delayed (>2 SD) or no menses	0/2[i]	2/2		0.17
Primary hypogonadism	3/3	3/3		1.0
Polycystic ovary	4/9	3/6	1/3	0.62

The denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature; thus, the differences between the denominators and numerators denote the number of patients evaluated to be negative for that feature.

[a] Severe craniosynostosis is absent in case 33 with two missense mutations.

[b] Humeroradial, humeroulnar, or radioulnar synostosis.

[c] Adrenal crisis has been manifested by case 35 with Y578C and I444fsX449.

[d] The measurement of 17-OHP in the mass screening for 21-hydroxylase deficiency has been performed since 1988 in Japan.

[e,f] DSD is more frequent in 46,XX cases than 46,XY cases in groups A ($P = 0.0050$) and B ($P = 0.035$).

[g,h] The *P* values between 46,XY and 46,XX cases are 0.19 for group A and 0.50 for group B.

[i] Elevated gonadotropins (LH and/or FSH) and/or decreased T or E_2, as compared with age- and sex-matched reference data.

[j] Only a few vaginal spottings.

drostenedione, 11-deoxycortisol, cortisol, and aldosterone grossly remained within the normal range (Fig. 2B). The M ratio indicating 17α-hydroxylase activity remained almost normal, consistent with the elevation of both substrates and products, whereas the M ratios indicating 17,20 lyase and 21-hydroxylase activities were grossly decreased. Significant difference between groups A and B was identified for MS of progesterone ($P = 0.044$), those of 17-OHP ($P = 0.022$), those of aldosterone ($P = 0.0084$), and M ratio indicating 17,20 lyase activity ($P = 0.011$). Adrenal crisis was observed only in group B with a significant difference between groups A and B, whereas the detection frequency of elevated 17-OHP in mass screening was similar between groups A and B (Table 2).

DSD was more prevalent in 46,XX cases than 46,XY cases in both groups A and B (Table 2, footnote, and supplementary Fig. 2). 46,XY DSD in group A was micropenis in one case, and that in group B included more severe phenotypes. By contrast, 46,XX DSD was invariably identified in both groups A and B. Maternal virilization during pregnancy was often found in groups A and B with a similar prevalence. Serum T of case 20, aged 0.2 yr in group B, was 6.5 and 7.6 nmol/liter (1.9 and 2.2 ng/ml) before and after hCG stimulation, respectively.

Pubertal development was apparently normal in two 46,XY cases of group A and one of four 46,XY cases in group B and was invariably affected in 46,XX cases in both groups A and B (Table 2). In family A of group A, cases 2 and 3 exhibited full pubertal development with testis volume of 20 ml, whereas case 10 had obvious pubertal failure with Tanner B2 stage. T value of case 18, aged 17.5 yr in group B, was low at the baseline (0.7 nmol/liter,

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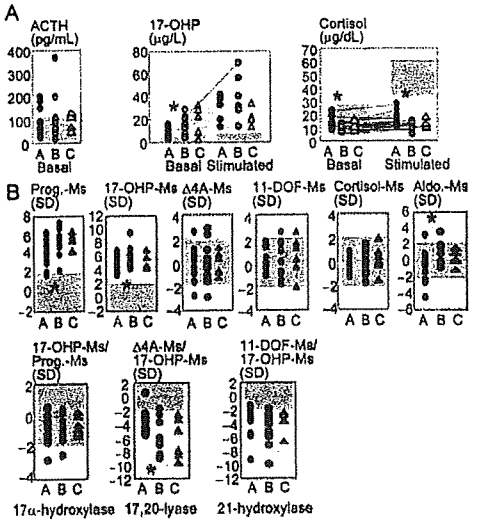

FIG. 2. Adrenal steroidogenic dysfunctions in groups A–C. *Light blue areas* represent the normal ranges. *Red asterisks* indicate the presence of significant differences between groups A and B. A, Basal and ACTH-stimulated blood hormone values. B, Basal urine steroid M values. Prog, Progesterone; Δ4A, androstenedione; 11DOF, 11-deoxycortisol; Aldo, aldosterone.

0.2 ng/ml) and poorly responded to hCG stimulation (1.0 nmol/liter, 0.3 ng/ml). PCO was observed in infantile or pubertal cases with a similar frequency between groups A and B, and cases 22 and 24 had ovarian torsion. Notably, bilateral ovarian cysts of case 10 markedly reduced in size after treatment with estradiol (E_2) (supplementary Fig. 2).

Long-term growth patterns were obtained in eight cases (Fig. 3). Whereas childhood heights tended to be high in both groups A and B, pubertal growth was different between the two groups. Cases in group A lacked obvious pubertal growth spurt but continued to grow for a long term, attaining tall adult heights, whereas those in group B showed rather compromised pubertal growth with worsening of scoliosis (supplementary Fig. 1).

There was no phenotypic difference between A503V-positive and -negative cases of group B (supplementary Table 2). In addition, the phenotypes in group C were grossly similar to those in group B (Table 2). In particular, craniosynostosis was identified in all cases except for case 33 with R457H and E580Q, and adrenal crisis was manifested by case 35 with Y578C and I444fsX449.

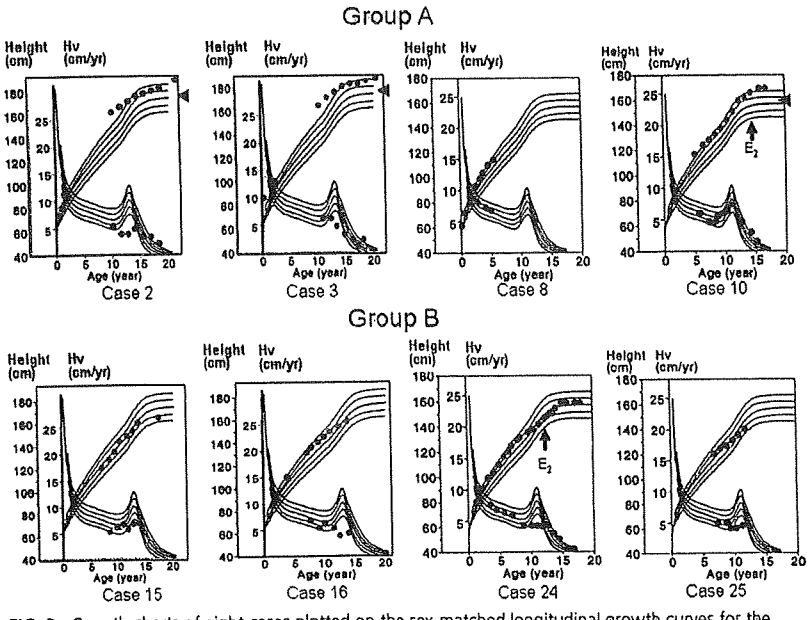

FIG. 3. Growth charts of eight cases plotted on the sex-matched longitudinal growth curves for the normal Japanese children (+2 SD, +1 SD, the mean, −1 SD, and −2 SD). The *triangles* in cases 2, 3, and 10 represent the target heights. Cases 10 and 24 are placed on E_2 replacement therapy. Hv, Height velocity.

Discussion

Molecular studies

Detailed molecular studies were performed in this study, providing two notable findings. First, all 35 cases were found to be homozygotes or compound heterozygotes for *POR* mutations including intragenic microdeletion and transcription failure. Because the microdeletion was found in case 21 with apparent R457H homozygosity, such a microdeletion might be hidden in the previously reported patients with apparent homozygosity (1, 5). Similarly, because transcription failure was invariably identified in cases 18, 26, and 27 with apparent heterozygosity, it may also underlie in the previously reported patients with apparent heterozygosity (4, 5, 10). In this regard, it is likely that the three cases carry a mutation in a hitherto unidentified *cis*-regulatory sequence(s) for the transcription of *POR*, as has been reported for several genes (24).

Second, RT-PCR sequence analysis indicated the occurrence of NMD in the two frameshift mutations (I444fsX449 and Q555fsX612). In this context, all the premature termination codons caused by the nonsense and the four frameshift mutations satisfy the positional conditions for the occurrence of NMD that functions as an mRNA surveillance mechanism to prevent the formation of aberrant proteins (13, 14). Thus, it is likely that the remaining three mutations (Q201X, R48fsX63, and Y567fsX574) are also null mutations subject to NMD *in vivo*.

Genotype-phenotype correlations

Genotype-phenotype correlations also provide several informative findings. Skeletal features were clearly different between groups A and B. Because cholesterol production in skeletal tissues is carried out in a simple one way manner (Fig. 4), this would explain why the skeletal phenotype is obviously dependent on the R457H dosage, reflecting the residual activity. It is likely that the threshold level for the development of severe skeletal phenotypes resides between a single copy and two copies of the R457H residual activity.

Adrenal steroidogenic dysfunction was grossly similar between groups A and B, although it was somewhat milder in group A than group B. Such a relatively minor role of R457H dosage in adrenal steroidogenesis

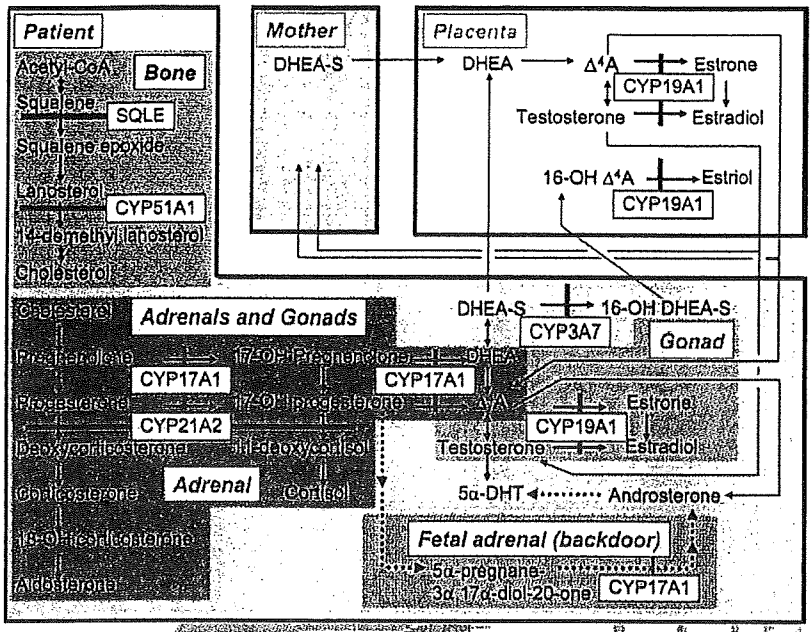

FIG. 4. Simplified schematic representation indicating impaired cholesterologenesis and steroidogenesis in PORD. DHEA, Dehydroepiandrosterone; DHEA-S, DHEA sulfate; Δ^4A, androstenedione; DHT, dihydrotestosterone; SQLE, CYP51A1, CYP17A1, CYP21A2, CYP19A1, and CYP3A7 are POR-dependent enzymes. The important Ms only are shown, and the reaction steps in which some Ms are omitted are indicated by two tandem arrows. Note that the amount of estriol synthesized in the placenta far exceeds the total amount of estrone and E_2 (~10 times) (34).

may primarily be due to the complexity of steroidogenesis in PORD (Fig. 4). For example, both production and degradation of 17-OHP are carried out by POR-dependent enzymes, and such enzymatic reactions would depend on the R457H dosage and the differential supporting activity of the R457H protein for target enzymes as well as the amount of substrates and products. Furthermore, the basal cortisol values imply that the baseline steroidogenic capacity can grossly be sustained, even in group B. Indeed, whereas basal blood 17-OHP values were significantly higher in group B than group A, some of them remained within the normal range, and several cases of both groups were not detected in neonatal mass screening. Nevertheless, the R457H dosage would have important clinical relevance, because the ACTH-stimulated blood cortisol was drastically reduced especially in group B, and adrenal crisis was observed only in group B. Furthermore, because 17,20 lyase activity alone was significantly different between groups A and B (Fig. 2B), this would provide further support for the previous finding that 17,20 lyase activity is the most sensitive index of defective POR activity (5, 15).

46,XY DSD was not so remarkable, whereas 46,XX DSD was invariably identified. This suggests a mildly reduced androgen production in genetic males and a definitely excessive androgen production in genetic females. In this context, there are three androgen sources during the fetal life in PORD, *i.e.* the fetal testis, backdoor pathway, and placenta (3, 4, 9, 25, 26) (Fig. 4). For fetal testicular T production specific to 46,XY cases, placental hCG-stimulated T production around the critical period for sex development would be more compromised in group B than group A because testicular T production is performed in a simple one-way manner, as in cholesterologenesis. Furthermore, because T responses to hCG stimulation were reduced, at least in the two examined cases of group B, this implies the compromised maximum T production capacity. By contrast, the backdoor- and placenta-derived androgen productions common to both 46,XY and 46,XX cases may be similar between groups A and B: 1) whereas 17-OHP as the source metabolite for the backdoor pathway is higher in group B than group A, the supporting activity for fetal adrenal CYP17A1 involved in the backdoor pathway would be lower in group B than group A; and 2) whereas fetal adrenal derived dehydroepiandrosterone as the source metabolite for placental androgens would be lower in group B than group A (4, 9, 25), the residual supporting activity for placental CYP19A1 would be lower in group B than group A. Thus, the total amount of androgens would be relatively well preserved in 46,XY cases with a mild difference in the fetal testis-derived T between groups A and B and invariably and similarly increased in 46,XX cases of both groups A and B. Furthermore, this notion explains why maternal virilization during pregnancy was similar between groups A and B because it is primarily due to androgens of the placental origin rather than the fetal gonadal or the backdoor origin (3, 4, 25).

Assessment of pubertal development was possible in a limited number of patients. However, pubertal development appeared to differ between groups A and B and between 46,XY and 46,XX cases. In this regard, T and E_2 biosynthesis during puberty is also performed in a simple one-way manner, and T production is mediated by CYP17A1 and E_2 production is mediated by both CYP17A1 and CYP19A1 (Fig. 4). Thus, gonadal steroid production would depend on the R457H dosage, with T production being less compromised than E_2 production. In addition, our observation suggests the frequent occurrence of PCO in infancy and puberty when gonadotropins are physiologically elevated (27) and the beneficial effect of estrogen replacement therapy in the amelioration of PCO.

Evaluation of growth pattern also remained fragmentary. However, two implications are possible. First, the intrinsic skeletal abnormalities may be relevant to the growth pattern. Indeed, relative tall stature in childhood may be compatible with the elongation of long bones as indicated by arachnodactyly and dolichostenomelia, and worsening of scoliosis during puberty in group B would also be consistent with the low POR activity (supplementary Fig. 1). Second, the spontaneous pubertal growth pattern of cases 2 and 3 without scoliosis is considered to represent a mild form of that of male patients with aromatase deficiency (28, 29). Such a qualitatively similar but quantitatively different pubertal growth pattern would be explained by assuming a drastically attenuated but not abolished *in vivo* supporting function of the R457H protein for aromatase.

Lastly, clinical features were similar between A503V-positive and -negative cases in group B. However, this would not argue

against a possible phenotypic effect of mildly hypomorphic A503V, because A503V of the four cases in group B was present on the alleles carrying apparently null mutations. Thus, it remains unknown whether A503V can modify phenotypic features in PORD, although the previous study argues against a modifying effect of A503V on clinical phenotypes in 21-hydroxylase deficiency (30). Furthermore, because A503V was absent from all of 47 alleles carrying R457H, this would provide further support for the previous notion that R457H is a founder mutation accompanied by a specific haplotype (6, 7). Thus, whereas A503V was identified in only eight of the 70 alleles (11.4%) in this study, this frequency is obviously biased by the high prevalence of R457H in Japanese patients. Rather, the frequency of A503V in R457H-negative alleles suggests that the prevalence of A503V is considerably high in the Japanese population, as reported in other populations (from 19.1% in African American to 36.7% in Chinese American) (15).

Remarks and conclusion

It should be pointed out that the results are totally based on the studies of Japanese patients. In this regard, A287P is common in Caucasian patients (4, 5), and clinical studies in 10 A287P-positive patients including three homozygotes (five with 46,XY and five with 46,XX) have suggested phenotypic similarities and differences between R457H-positive patients and A287P-positive patients: 1) skeletal phenotype is usually obvious and appears to be grossly dependent on the A287P dosage; 2) 46,XY DSD is variable and is apparently independent of the A287P dosage; 3) 46,XX DSD is also variable and absent in one A287P homozygote and one of four compound heterozygotes with A287P; and 4) maternal virilization during pregnancy is not described (1, 2, 5, 31, 32). Thus, skeletal phenotype would be explained by assuming that both R457H and A287P have drastically lost supporting activities for CYP51A1 and/or SQLE involved in cholesterologenesis, although functional studies have not been performed. Furthermore, clinical features relevant to steroidogenic dysfunction would be grossly consistent with the previous *in vitro* functional data. It has been reported that R457H yields only 1–3% supporting activities for 17α-hydroxylase and aromatase, and virtually no activity for 17,20 lyase, whereas A287P provides supporting activities of about 40% for 17α-hydroxylase, about 20% for 17,20 lyase, about 70% for 21-hydroxylase, and about 100% for aromatase (1, 5, 11, 33). Thus, the relative activities of frontdoor and backdoor pathways would be different largely between R457H-positive and A287P-positive patients, and placental T production would remain minor, if any, in A287P-positive patients. Collectively, the Japanese data would not apply simply to other populations.

In conclusion, the present study in Japanese patients argues against the heterozygote manifestation and suggests that the residual POR activity reflected by the R457H dosage constitutes the underlying factor for the clinical variability in some features but not other features, probably because of the simplicity and the complexity of the POR-dependent metabolic pathways relevant to each phenotype. Further studies including genotype-phenotype analyses in various ethnic groups will permit a better clarification of the molecular and clinical characteristics of PORD.

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Letter to the Editor

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Nomenclature for alleles of the cytochrome P450 oxidoreductase gene

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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-alpha-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 b_5, 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 al[7] recently reported that upd(14)mat was detected in 4 of 33 patients who were suspected to have PWS and raised the question that upd(14)mat could be present in patients with PWS-like phenotype. Thus we examined patients who presented with PWS-like phenotype, but in whom PWS had been excluded.

Methods

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

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

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

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

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

Results

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

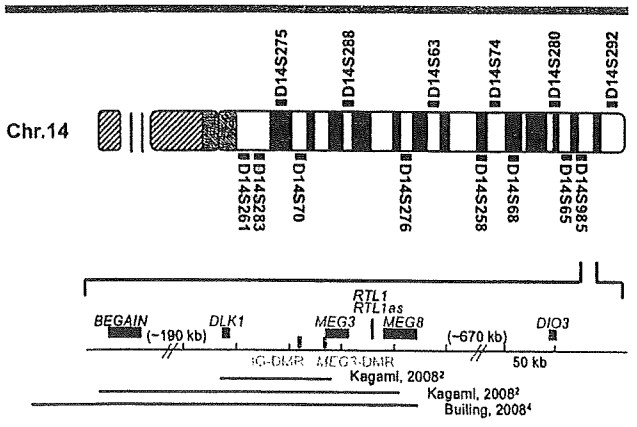

Figure 1. Schematic map of the 14q32.2 imprinted region. Loci on chromosome 14 represent markers used for microsatellite polymorphism analysis. Paternally expressed genes are shown in *blue*, maternally expressed genes in *red*, and nonimprinted genes are shown in *black*. Differentially methylated regions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

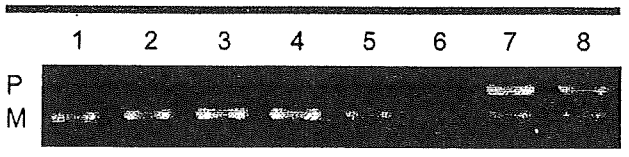

Figure 2. *MEG3* methylation test. *P*, Paternal methylated signal; *M*, maternal unmethylated signal; *1-5*, cases 1-5, respectively; *6*, paternal uniparental disomy 14; *7*, patient with PWS; *8*, normal control. Cases 1-4 show only the maternal unmethylated signal, and case 5 shows a faint paternal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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

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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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

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TABLE I. 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								
Gastational age (weeks)	25	42	38	38	39	40	42	38
Birth weight (g)	625	2490	2620	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	T443fsX468	M113T	E148X	P170L	−	−	−

NM, non mineralized; DSD, disorders of sex development.
[a]Deceased 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 I, 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

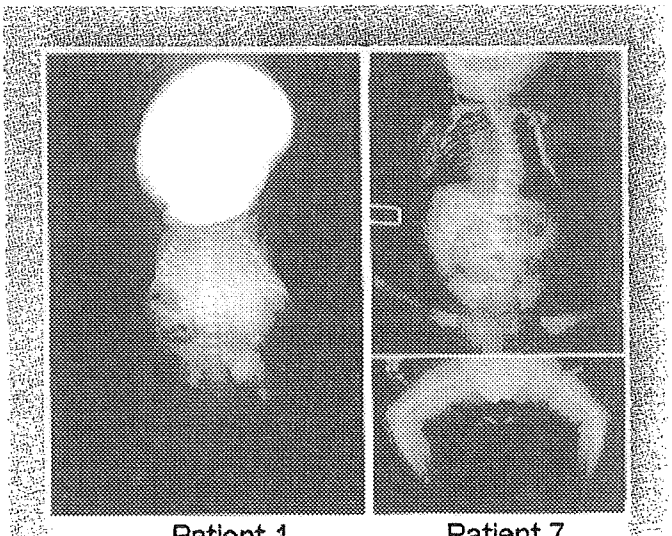

FIG. 1. Representative roentgenograms indicating CD in patient 1 at birth and ACD in patient 7 at 3 months of age.

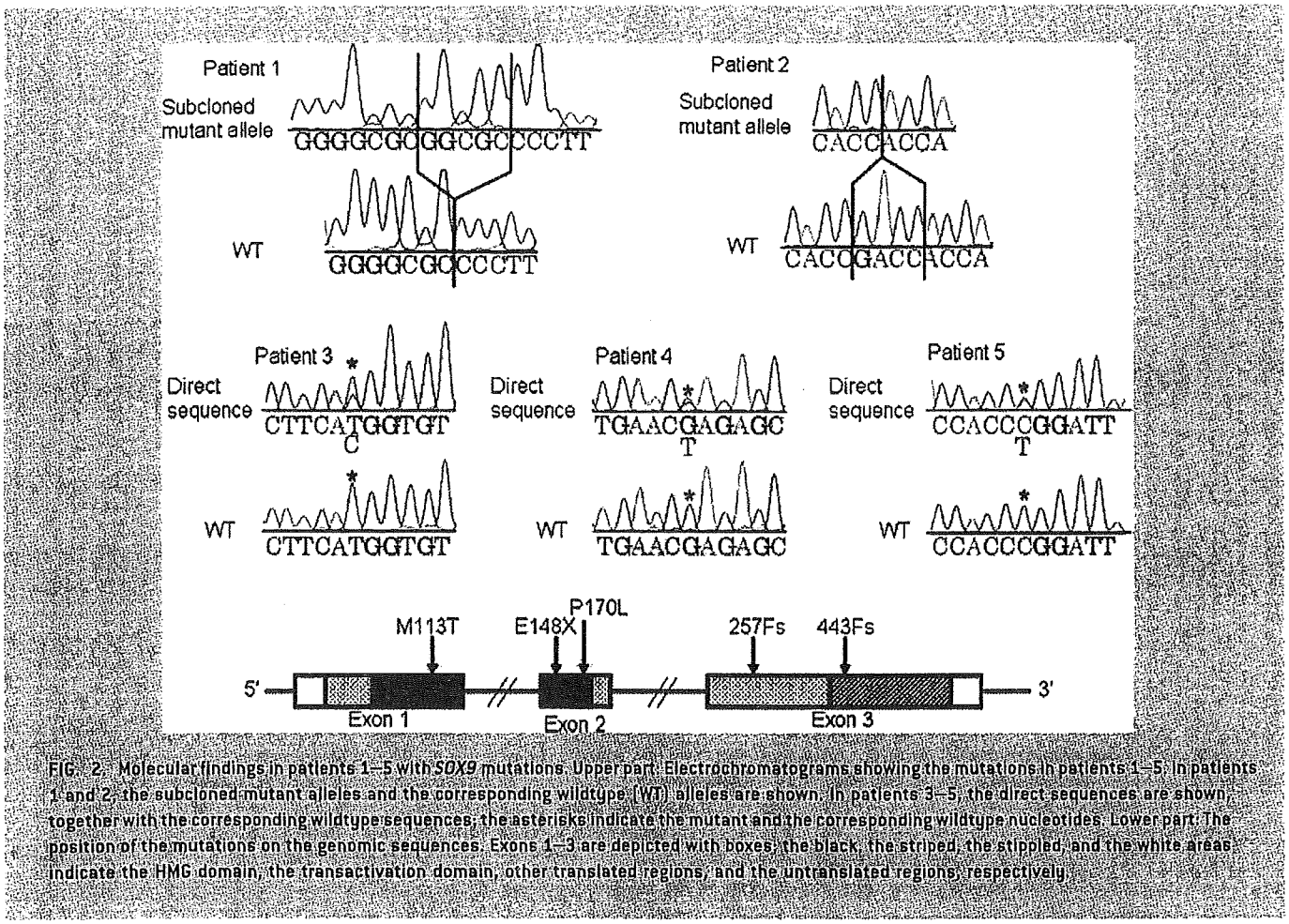

FIG. 2. Molecular findings in patients 1–5 with *SOX9* mutations. Upper part: Electrochromatograms showing the mutations in patients 1–5. In patients 1 and 2, the subcloned mutant alleles and the corresponding wildtype (WT) alleles are shown. In patients 3–5, the direct sequences are shown, together 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 immunoenzymometric assay 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

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