CLINICAL REPORT

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Growth Hormone Secretion and its Effect on Height in Pediatric Patients With Different Genotypes of Prader–Willi Syndrome

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INTRODUCTION

Prader–Willi syndrome (PWS) is a pleiotropic congenital disorder [Prader et al., 1956] that includes neonatal hypotonia, hypogonadism, developmental delay, childhood-onset obesity, short stature, and behavioral abnormalities [Goldstone et al., 2008]. There have been multiple studies investigating growth hormone (GH) secretion in patients with PWS [Corrias et al., 2000; Grugni et al., 2006].

Although it was not initially clear whether GH secretion was diminished in these patients, recent studies established impaired GH secretion in patients with PWS, including low insulin like growth factor 1 (IGF-1) levels and favorable responses to GH treatment [Eiholzer et al., 2000]. Whether the degree of this GH deficiency depends on the genetic subtypes (deletion or uniparental disomy, UPD) is not known. Grugni et al. [2009] reported that GH secretion in adult patients with UPD was lower than that of those with deletions. However, they did not compare their response to GH treatment. Moreover, to our knowledge, there have been no studies to evaluate GH secretion and response to GH treatment in different genetic subtypes in children with PWS. We therefore investigated whether there were any differences in GH secretion and the response to GH treatment according to the difference of genotypes in children with PWS.

MATERIALS AND METHODS

Patients

Seventy-six Japanese patients with PWS followed at our University Hospital were included in this study. All patients had genetically confirmed PWS by methylation study, fluorescence in situ hybridization (FISH), and/or analysis of microsatellite makers and were prepubertal (Tanner breast stage 1, age <12 years for girls; testicular volume <4 ml, age <14 years for boys) at the initiation of GH. Medical records were reviewed and data including genotype, birth length, birth weight, age, height, body mass index (BMI: Weight (kg)/height (m) 2), serum IGF-1 level, and insulin-like growth factor binding protein-3 (IGFBP-3) level at the initiation of GH treatment were obtained (Table I).

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TABLE I. Baseline Characteristics of Subjects

	Deletion	UPD	
N of prepubertal PWS	55(M35, F20)	21(M12, F9)	
Birth length	47.6 ± 2.4 cm	45.3 ± 5.2 cm	$P = 0.045$
Birth weight	2496 ± 379 g	2378 ± 435 g	$P = 0.37$
Age at start of GH (yr)	3.5y (8m–13.5y)	3.6y (10m–11y)	
BMI at start of GH (%)	17.2 ± 4.7	17.3 ± 3.0	$P = 0.97$
Height SDS_{PWS} at start	−0.52 ± 1.10	−0.72 ± 0.62	$P = 0.46$
Height SDS_{NOR} at start	−2.68 ± 1.53	−2.86 ± 1.0	$P = 0.64$
IGF-I at start (ng/ml)	85.1 ± 81.5	62.2 ± 63.7	$P = 0.30$
IGFBP3 at start (μg/ml)	1.34 ± 0.52	1.24 ± 0.68	$P = 0.56$

Height SDSPWS: Height SDS score for Japanese patients with PWS.
Height SDSNOR: Height SDS score in Japanese normal children.

Fifty-five (35 males) patients with deletions and 21 (12 males) patients with UPD were enrolled. The birth weight, age, height, BMI, serum IGF-1 level, and IGFBP-3 level at the initiation of GH therapy were similar between the two groups, whereas the birth length was lower in the UPD group than in the deletion group ($P = 0.045$).

The study protocol was reviewed and approved by the Institutional Review Board of Dokkyo Medical University following the World Medical Association Declaration of Helsinki. All the participants and/or guardians provided written informed consent to participate.

Endocrine Protocol

The GH stimulation tests were performed with arginine (0.5 g/kg) intravenously or insulin (0.1 U/kg) intravenously prior to the initiation of GH treatment. Eleven patients in the deletion group and four patients in the UPD group were excluded from this study because data about GH stimulation tests were unavailable. These excluded patients had been already on GH treatment in other facilities before being referred to us. The rest of the patients underwent ariginine stimulation only (deletion, n = 3; UPD, n = 1), insulin stimulation only (deletion, n = 1; UPD, n = 0), or both arginine and insulin stimulation (deletion, n = 40; UPD, n = 16). The GH responses were evaluated by GH peak levels. To evaluate the efficacy of GH, we used the Japanese PWS height standard deviation score (SDS) and BMI [Nagai et al., 2000]. All the patients were treated with recombinant human GH (Genotropin, Pfizer, New York, NY) 0.245 mg/ kg/week subcutaneously.

Analysis

We compared GH peak levels in response to arginine and/or insulin stimulation tests in patients with deletions to that of patients with UPD. Then we compared efficacy of GH treatment between those groups by evaluating the yearly change in height SDSs and BMI. We analyzed the data for the first 3 years because previous studies showed that the efficacy of GH treatment on height is generally most prominent during early period after the initiation of growth hormone [Eiholzer and l'Allemand, 2000; Obata et al., 2003]. We used paired *t*-test, Student *t*-test, Welch *t*-test, and Mann–Whitney's U-test for statistical analyses.

RESULTS

Although responses to arginine stimulation were similar (peak GH level: 9.40 ± 6.8 ng/ml, deletion; 6.32 ± 4.60 ng/ml, UPD; $P = 0.092$), responses to insulin stimulation test were significantly lower in the UPD group than in the deletion group (peak GH level: 11.1 ± 8.6 ng/ml, deletion; 3.6 ± 2.2 ng/ml, UPD; $P = 0.0013$, Table II). Yearly improvements in height SDS were not significantly different between the groups (first year SDS: 0.47 ± 0.47, deletion; 0.68 ± 0.26, UPD; $P = 0.14$, Table III). However, it was significantly different in the two groups when compared within the subgroups satisfying the Japanese criteria (peak GH response to at least two different provocation tests <6 ng/ml) of GH deficiency (0.42 ± 0.26, deletion; 0.70 ± 0.21, UPD; $P = 0.0044$).

DISCUSSION

The aim of this study was to investigate whether there were differences in the GH secretion and in the clinical response to the GH replacement therapy in children with PWS caused by deletions compared to those with UPD.The GH response to insulin stimulation test was significantly lower in the UPD group than in the deletion group. Grugni et al. [2009] reported that GH rise by GHRH + arginine provocation was significantly higher in adults with deletions compared to adults with UPD. Our result in pediatric patients was consistent with this adult study. We demonstrated

TABLE II. Peak GH Level by Stimulation Tests

	Deletion	UPD	
Arginine stimulation (n = 60, ng/ml)	9.4 ± 6.8	6.3 ± 4.6	$P = 0.092$
Insulin stimulation (n = 57, ng/ml)	11.1 ± 8.6	3.6 ± 2.2	$P = 0.0013$

TABLE III. Response to GH Treatment

	Deletion	UPD	
Height $\Delta SDS_{PWS}1$ (n = 54)	0.47 ± 0.47	0.68 ± 0.26	$P = 0.14$
Height $\Delta SDS_{PWS}2$ (n = 38)	0.090 ± 0.85	0.25 ± 0.37	$P = 0.56$
Height $\Delta SDS_{PWS}3$ (n = 31)	0.091 ± 0.34	0.054 ± 0.23	$P = 0.79$

Height ΔSDSPWS1: Yearly change in Japanese PWS height standard deviation score in the first year.
Height ΔSDSPWS2: Yearly change in Japanese PWS height standard deviation score in the second year.
Height ΔSDSPWS3: Yearly change in Japanese PWS height standard deviation score in the third year.

that the GH secretion ability in the UPD group was lower than in the deletion group in pediatric patients.

We could not demonstrate a similar between-subgroup difference by arginine provocation test. The GH level in response to the insulin and arginine provocative tests are among the most frequently used parameters to assess GH secretion. However, since their effects of stimulating the secretion of GH are distinct, it is possible that the power of this study was not large enough to detect the difference in the arginine study.

The peak GH level by insulin stimulation in deletion group was at the low-normal range in our study (11.1 ± 8.6 ng/ml). A previous study also showed that GH response to insulin and arginine was low-normal to blunted in PWS and not necessarily low [Hoybye et al., 2002]. We did not use area under the curve as a parameter to assess GH response to provocation tests in this study. Koppeschaar et al., 2004 examined whether there was any advantage of using area under the curve instead of peak value of GH. They showed that they were both similarly effective for diagnosis of adult GH deficiency.

If there is a genotype-dependent difference in the degree of GH deficiency, it is assumed that there might also be a genotype-dependent difference in the response to the GH treatment.

Therefore, we also evaluated the effect of GH on height gain in children with PWS. We hypothesized that GH efficacy was more prominent in the UPD group. However, our study showed no significant difference in yearly improvements in height SDS between the deletion and UPD group at least for the first 3 years.

It is not clear why patients with PWS due to a deletion showed similar yearly growth rate compared with those with UPD even though the GH provocative response was different. Carrascosa et al. [2011] examined the relationship of the GH provocative test to growth on GH therapy and found no significant relationship in prepubertal short children. Previous studies suggested that there may be considerable heterogeneity in GH secretion in patients with PWS [Burman et al., 2001; Grugni et al., 2009]. Indeed, the overall comparison of the deletion and UPD groups in our study did not show a significant difference in the response to GH therapy. However, when compared within subpopulation of patients showing overt GH deficiency alone, the UPD group showed significantly greater response to GH therapy than deletion group. Moreover, polymorphism in the GH receptors might play a role in the heterogeneous responses to the GH treatment. Park et al. [2011] reported that patients with PWS and an exon-3 deletion polymorphism (d3, Database of Genomic Variants ID: Variation_64191) in the GH receptor gene had significantly greater height SDSs and higher IGF-I level before GH treatment. Further study with a larger number of patients is needed to compare the response to GH treatment among genotypes in the subgroups stratified by factors including severity of GH deficiency and receptor polymorphism.

In conclusion, the secretion of GH in the pediatric patients with UPD group was significantly lower than that in those with deletion group. However, yearly improvements in height SDS by GH treatment were not significantly different between the deletion and UPD group.

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RESEARCH LETTER

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Mosaic upd(7)mat in a Patient With Silver–Russell Syndrome

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TO THE EDITOR:

Silver–Russell syndrome (SRS) is a congenital developmental disorder characterized by pre- and post-natal growth failure, relative macrocephaly, triangular face, hemihypotrophy, and 5th finger clinodactyly [Russell, 1954; Silver et al., 1953]. Recent studies have shown that hypomethylation (epimutation) of the paternally derived differentially methylated region (DMR) in the upstream of *H19* (*H19*-DMR) on chromosome 11p15 and maternal uniparental disomy for chromosome 7 (upd(7)mat) account for ~45% and ~5–10% of SRS patients, respectively [Eggermann, 2010; Binder et al., 2011]. Furthermore, consistent with the involvement of imprinted genes in both body and placental growth [for review, Coan et al., 2005], epimutations of the *H19*-DMR and upd(7)mat are known to result in placental hypoplasia [Yamazawa et al., 2008a,b]. Here, we report on a Japanese boy with mosaic upd(7)mat who was identified through genetic screenings in 120 patients with SRS-like phenotype.

This Japanese boy was conceived naturally to a 41-year-old father and a 36-year-old mother. The parents were non-consanguineous and healthy. The paternal height was 165 cm (−0.9 SD), and the maternal height 155 cm (−0.6 SD).

At 35 weeks of gestation, he was delivered by a cesarean because of fetal distress. At birth, his length was 37.4 cm (−3.1 SD), his weight 1.28 kg (−3.1 SD), and his head circumference 29.0 cm (−1.3 SD). The placenta weighed 400 g (−0.6 SD [Kagami et al., 2008]). Shortly after birth, he was found to have ventricular septal defect, hydronephrosis, and abnormal external genitalia (hypospadias, bifid scrotum, and bilateral cryptorchidism). He received orchidopexy at $1^{10}/_{12}$ years of age and genitoplasty at $2^{4}/_{12}$ years of age. He exhibited feeding difficulty and speech delay.

At $5^{1}/_{12}$ years of age, he was referred because of short stature. His height was 87.9 cm (−4.3 SD), weight was 10.4 kg (−2.9 SD), and his head circumference 49.0 cm (−0.7 SD). Physical examination showed relative macrocephaly, abnormal teeth, 5th finger clinodactyly, and underdeveloped muscles. There was no hemihypotrophy. Endocrine studies for short stature yielded normal results, as did radiological examinations. His karyotype was 46,XY in all the 50 lymphocytes examined. He was clinically diagnosed as having SRS, and molecular studies were performed after obtaining the approval from the Institutional Review Board Committee at National Center for Child Health and Development and the written informed consent from the parents.

We first performed methylation analysis of the *MEST*-DMR on chromosome 7q32.2 using leukocyte genomic DNA by the previously described methods [Yamazawa et al., 2008b], because this patient showed relatively mild SRS-phenotype with speech delay

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and feeding difficulty characteristic of upd(7)mat [Hitchins et al., 2001; Kotzot, 2008]. The methylation analysis showed a major peak for methylated clones and a minor peak for unmethylated clones in this patient (Fig. 1A). We also examined the *H19*-DMR and other multiple DMRs on various chromosomes by the bio-COBRA (combined bisulfite restriction analysis) method, as reported previously [Yamazawa et al., 2010]. The *GRB10*-DMR on chromosome 7p12.1 and the *PEG10*-DMR on chromosome 7q21.3 exhibited skewed methylation patterns consistent with the predominance of maternally derived clones, as did the *MEST*-DMR (Fig. 1B). By

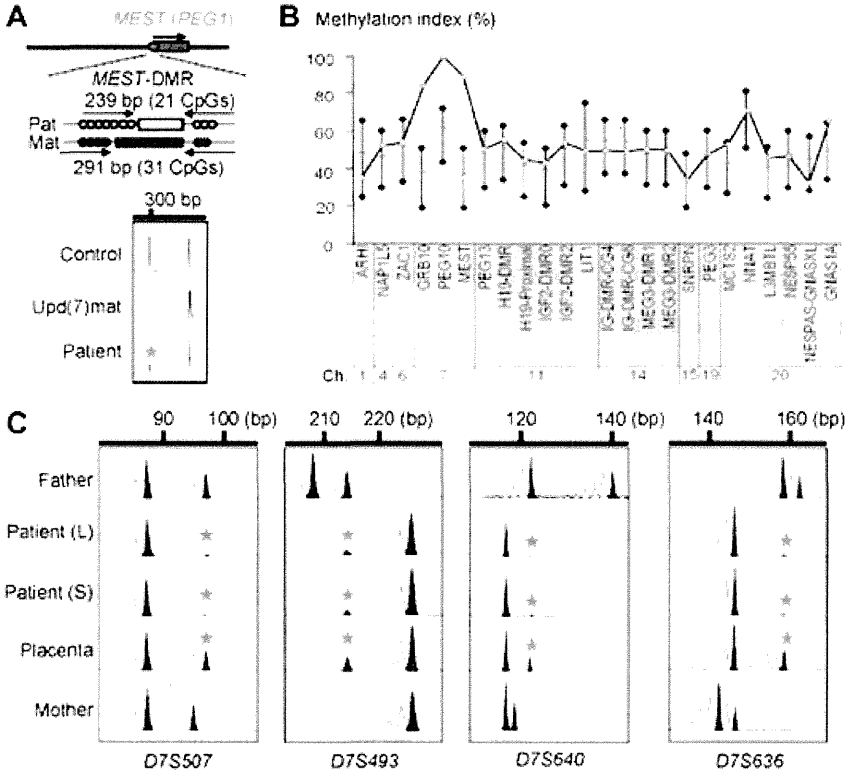

FIG. 1. Representative molecular results. A: Methylation analysis for the *MEST*-DMR. The methylated and unmethylated allele-specific primers were designed to yield PCR products of different sizes, and the PCR products were visualized on the 2100 Bioanalyzer (Agilent, Santa Clara, CA). Both methylated and unmethylated alleles are amplified in a control subject, and the methylated allele only is identified in a previously reported patient with upd(7)mat [Yamazawa et al., 2008b]. In this patient, a major peak for the methylated allele and a minor peak for the unmethylated allele (a red asterisk) are delineated. B: Methylation indices of 24 DMRs examined by the bio-COBRA. The PCR products were digested with methylation sensitive restriction enzymes, and the methylation indices (the ratios of methylated clones) were calculated using peak heights of digested and undigested fragments on the 2100 Bioanalyzer using 2100 expert software. The black vertical bars indicate the reference data in 20 normal control subjects (maximum – minimum). The DMRs highlighted in blue and pink are methylated after paternal and maternal transmissions, respectively. C: Microsatellite analysis. Major peaks of maternal origin and minor peaks of paternal origin (red asterisks) are identified in this patient. The minor peaks of paternal origin are more obvious in the placenta than in the leukocytes (L) and salivary cells (S). Calculation of the mosaic ratio using the *D7S507* data, under the assumption of no trisomic cells. For this locus, the patient is considered to be heterozygous with the major 87 bp peak of maternal origin and a minor 97 bp peak of paternal origin. The father is also heterozygous with the two peaks of the same sizes, and the area under curve (AUC) is larger for the short 87 bp peak than for the long 97 bp peak. This unequal amplification is consistent with short products being more easily amplified than long products. In this patient, the AUC ratio between the major 87 bp peak and the minor 97 bp peak is obtained as 1.0:0.043 for leukocytes, 1.0:0.044 for salivary cells, and 1.0:0.803 in placental tissue, after compensation of the unequal amplification between the two peaks using the paternal data. Here, let "XL" represent the frequency of the upid(7)mat cells in leukocytes (thus, (1 – XL) denotes the frequency of normal cells in leukocytes). Then, the paternally derived 97 bp peak is generated by one paternally derived chromosome in the normal cells, that is, (1 – XL), and the maternally derived 87 bp peak is formed by the products from two maternally derived homologous chromosomes in the upid(7)mat cells and one maternally derived chromosome in the normal cells, that is, {2XL + (1 – XL)} = (XL + 1). Thus, the AUC ratio between the two peaks is represented as (XL + 1):(1 – XL) = 1.0:0.043, and "XL" is calculated as 0.92 (92%). Similarly, when "XS" and "XP" represent the frequency of the upid(7)mat cells in salivary cells and placental tissue, respectively, "XS" is obtained as 0.91 (91%) and "XP" as 0.11 (11%). Furthermore, when "XB" represents the frequency of the upid(7)mat cells in buccal epithelium cells, "XB" is obtained as 0.91 (91%), on the basis of the previous report that salivary cells comprises ~40% of buccal epithelium cells and ~60% of leukocytes [Thiede et al., 2000].

TABLE I. The Results of Microsatellite Analysis

Locus	Position	Father	Patient (L)	Patient (S)	Placenta	Mother	Assessment
D7S517	7p22.2	254/258	(254)/258	(254)/258	(254)/258	256/258	Maternal Iso-D[a]/biparental
D7S507	7p15–21	87/97	87/(97)	87/(97)	87/(97)	87/95	Maternal Iso-D[a]/biparental
D7S493	7p15.3	208/214	(214)/226	(214)/226	(214)/226	226	Maternal D[b]/biparental
D7S484	7p14–15	96/100	(96)/98	(96)/98	(96)/98	98/100	Maternal Iso-D/biparental
D7S502	7q11.12	298	294/(298)	294/(298)	294/(298)	294/304	Maternal Iso-D/biparental
D7S669	7q11.2	116/126	(116)/124	(116)/124	(116)/124	124	Maternal D[b]/biparental
D7S515	7q21–22	169/173	171/(173)	171/(173)	171/(173)	169/171	Maternal Iso-D/biparental
D7S640	7q21.1–31.2	122/140	116/(122)	116/(122)	116/(122)	116/118	Maternal Iso-D/biparental
D7S684	7q34	169/179	177/(179)	177/(179)	177/(179)	177/179	Not informative
D7S636	7q35–36	158/162	146/(158)	146/(158)	146/(158)	142/146	Maternal Iso-D/biparental
D7S798	7q36	73/79	(79)/83	(79)/83	(79)/83	73/83	Maternal Iso-D/biparental

L, leukocytes; S, salivary cells; D, disomy.
The Arabic numbers denote the PCR product sizes in bp.
The minor peaks are indicated in parentheses.
[a]On the basis of the results of other informative loci, the major peaks are considered to be of maternal origin.
[b]Because of the maternal homozygosity, disomic status (isodisomy or heterodisomy) is unknown for these loci.

contrast, other DMRs including the *H19*-DMR showed normal methylation patterns.

We next performed microsatellite analysis for 11 loci on various parts of chromosome 7, using genomic DNA from leukocytes of the patient and the parents, from salivary cells of the patient, and from formalin-fixed and paraffin-embedded placental tissue. Major peaks consistent with maternal uniparental isodisomy and minor peaks of paternal origin were unequivocally identified for *D7S484*, *D7S502*, *D7S515*, *D7S640*, *D7S636*, and *D7S798*; furthermore, similar patterns were also detected for *D7S517*, *D7S507*, *D7S669*, and *D7S493*, although the results were not informative for *D7S684* (Fig. 1C and Table I). The minor peaks of paternal origin were similar between leukocytes and salivary cells and more evident in placental tissue. These findings, together with the normal karyotype in lymphocytes, indicated mosaic full maternal isodisomy for chromosome 7 (upid(7)mat) in this patient. Furthermore, since such a condition is frequently associated with mosaicism for trisomy 7 [Petit et al., 2011], we performed fluorescence in situ hybridization (FISH) analysis for stocked lymphocyte pellets, using a CEP7 probe for *D7Z1* (Abbott Laboratories, Abbott Park, IL). The FISH analysis identified two normal signals in 995 of 1,000 interphase nuclei examined, with no trace of trisomic nuclei; while a single signal was delineated in the remaining five nuclei, this was regarded as a false-positive finding. Thus, assuming no trisomic cells, the frequency of the full upid(7)mat cells was calculated as 92% in leukocytes, using the results of *D7S507* (Fig. 1C). In addition, similarly assuming no trisomic cells in other tissues, the frequency of the full upid(7)mat cells was calculated as 91% salivary cells (and in buccal cells) and 11% in placental tissue, although we could not perform FISH analysis in buccal cells and placental cells.

These results imply that this patient had an abnormal cell lineage with full upid(7)mat and a normal cell lineage with biparentally inherited chromosome 7 homologs at least in lymphocytes, and these had no trisomy 7. It is likely that mitotic non-disjunction and subsequent trisomy rescue (loss of the paternally derived chromosome 7 from a trisomic cell) took place in the post-zygotic developmental stage, resulting in the production of the mosaic full upid(7)mat (Fig. 2). While full upid(7)mat can also be produced by monosomy rescue (duplication of a single maternally derived chromosome 7 in a zygote), this mechanism is predicted to cause non-mosaic rather than mosaic upid(7)mat [Miozzo et al., 2001]. Although it remains to be clarified why trisomic cells mediating the production of full upid(7)mat cells were apparently absent in lymphocytes of this patient, there might be a negative selection against lymphocytes with trisomy 7.

However, the presence or absence of demonstrable trisomic cells was studied only in lymphocytes. In this regard, trisomic cells have been identified more frequently in skin fibroblasts and amniocytes than in blood cells in patients with mosaic trisomy 7 [Chen et al., 2010; Petit et al., 2011], and they are usually more frequently detected in the placental tissue than in the body tissue, as has been demonstrated by confined placental trisomy [Kalousek et al., 1991]. These findings would argue for the possible presence of trisomic cells in several tissues including placenta of this patient.

The full upid(7)mat cells were assessed to account for the majority of the leukocytes and salivary cells (buccal cells) and the minority of the placental tissue, under the assumption of no

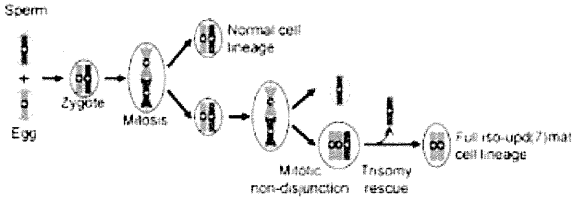

FIG. 2. Schematic representation of the generation of the mosaic upid(7)mat. The maternally and paternally derived chromosome 7 homologs are shown in red and blue, respectively. In this figure, mitotic non-disjunction is assumed at the second mitosis.

trisomic cells. In this regard, if trisomic cells may be present in a certain fraction of buccal cells and placental tissue, the full upid(7)mat cells would still account for a relatively major fraction of buccal cells and a relatively minor fraction of the placental cells. While such a variation in the frequency of the full upid(7)mat cells among different tissues would primarily be a stochastic event, it should be pointed out that human genetic studies are usually performed for leukocytes. Indeed, if the upid(7)mat cells were barely present in leukocytes, the mosaic upid(7)mat would not have been detected. Such a bias in human studies would more or less be relevant to the relative predominance of the full upid(7)mat cells in leukocytes.

Two findings are noteworthy with regard to clinical features of this patient. First, this patient had relatively mild SRS phenotype with speech delay and feeding difficulty. Since such clinical features are grossly consistent with those of patients with upd(7)mat [Hitchins et al., 2001; Kotzot, 2008], it is inferred that the upid(7)mat cells accounted for a considerable fraction of body cells relevant to the development of SRS phenotype. Second, the placental size remained within the normal range. This would be consistent with the relative paucity of the upid(7)mat cells in the placenta.

In summary, we observed mosaic upid(7)mat in a patient with SRS. Further studies will identify mosaic upd(7)mat with or without demonstrable trisomy 7 in patients with relatively mild SRS-like phenotype.

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Original Article

46, XY gonadal dysgenesis: new *SRY* point mutation in two siblings with paternal germ line mosaicism

Stoppa-Vaucher S, Ayabe T, Paquette J, Patey N, Francoeur D, Vuissoz J-M, Deladoëy J, Samuels ME, Ogata T, Deal CL. 46, XY gonadal dysgenesis: new *SRY* point mutation in two siblings with paternal germ line mosaicism.
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Familial recurrence risks are poorly understood in cases of *de novo* mutations. In the event of parental germ line mosaicism, recurrence risks can be higher than generally appreciated, with implications for genetic counseling and clinical practice. In the course of treating a female with pubertal delay and hypergonadotropic hypogonadism, we identified a new missense mutation in the *SRY* gene, leading to somatic feminization of this karyotypically normal XY individual. We tested a younger sister despite a normal onset of puberty, who also possessed an XY karyotype and the same *SRY* mutation. Imaging studies in the sister revealed an ovarian tumor, which was removed. DNA from the father's blood possessed the wild type *SRY* sequence, and paternity testing was consistent with the given family structure. A brother was 46, XY with a wild type *SRY* sequence strongly suggesting paternal Y-chromosome germline mosaicism for the mutation. In disorders of sexual development (DSDs), early diagnosis is critical for optimal psychological development of the affected patients. In this case, preventive karyotypic screening allowed early diagnosis of a gonadal tumor in the sibling prior to the age of normal puberty. Our results suggest that cytological or molecular diagnosis should be applied for siblings of an affected DSD individual.

Conflict of interest

The authors have no conflict of interest of any nature.

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Key words: dysgerminoma – genetic counseling – germ line mosaicism – gonadoblastoma – SRY point mutation – 46, XY complete gonadal dysgenesis – 46, XY DSD

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46, XY complete gonadal dysgenesis (CGD) is characterized by a 46, XY karyotype, normal female external genitalia, completely undeveloped ('streak') gonads, no sperm production, and presence of normal Müllerian structures. Although many different genes are required for both male and female sex determination, the Y chromosome plays a primary role through the Y-linked gene *SRY*. This gene is critical for testis

determination, and its absence results in the development of a female gonad in chromosomally male embryos (1). Human SRY is a 204 residue nuclear protein comprising three domains: a central HMG-box domain, which is highly conserved among mammalian species (approximately 80 residues in length), surrounded by N- and C-terminal domains (2). Mutations of the *SRY* gene [henceforth referred to as *SRY* (−)] are the cause of 46, XY CGD in approximately 10–15% of 46, XY disorders of sex development (DSD) (3). Mutations in the *SRY* gene include point, frameshift and deletion mutations, with the majority located within the HMG-box affecting DNA binding. The majority of the mutations described to date are singletons although 15 familial cases have been described which show vertical transmission and/or recurrence in siblings.

We report a novel point mutation within the SRY HMG-box domain shared by two sisters, which markedly lowers DNA binding activity. Only one sister was symptomatic at the time of molecular diagnosis; and we tested the second female sibling as a preventive measure despite signs of normal female puberty. Importantly, the younger sister with 46, XY CGD had developed a mixed ovarian tumor whereas her older sister showed no evidence of neoplasia at the time of gonadectomy. Another sister was 46, XX and phenotypically normal. Their father and a 46, XY phenotypically normal brother do not carry the mutation in leukocyte DNA, suggesting paternal germline mosaicism. We review the literature on familial *SRY* mutations and the implications of germline mosaicism for recurrence rate calculations and genetic counseling.

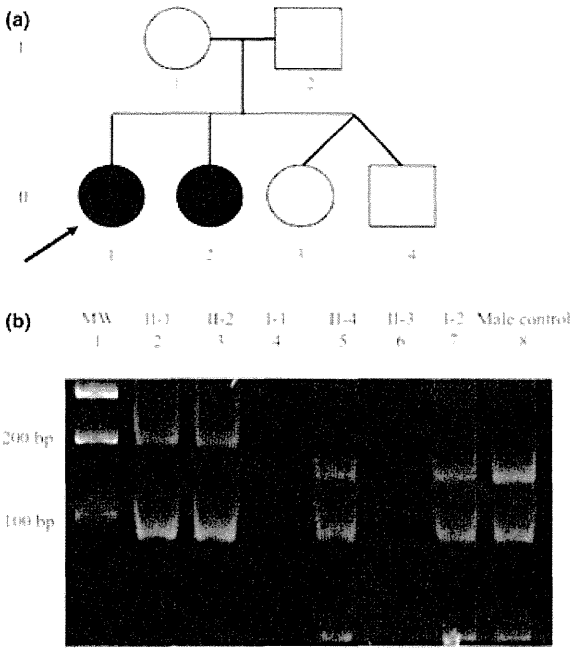

Fig. 1. (a) Pedigree of the family with the two sisters 46, XY complete gonadal dysgenesis (CGD) (II-1 and II-2) and their father who is carrying a germ line mosaic mutation of *SRY* (I-2). An arrow indicates the proband. The full circle symbol represents the feminized 'XY' children. (b) Restriction enzyme analysis (HPY 188I) of single exon of *SRY* gene. The mutation removes a restriction enzyme site so that three bands are found in normal healthy boys (I-2, II-4 and normal control man), two bands are found in the girls with 46, XY CGD (II-1, II-2) and no bands in the two healthy girls (I-1, II-3). Lane 1, molecular weight marker; Lanes 2 and 3, *SRY* mutant in both affected siblings; Lanes 4 and 6, *SRY* negative DNA from the mother and unaffected sister; Lanes 5, 7 and 8, *SRY* positive DNA in the father, the brother and the normal male control.

Materials and methods

Subjects

Patient 1

The proband, a 16-year-old healthy adolescent, was referred because of pubertal delay with hypergonadotropic hypogonadism (FSH 74 IU/l, $n = 12–30$, ovulatory phase; LH 17 IU/l, $n = 40–200$, ovulatory phase). The patient had begun estrogen replacement therapy 12 months before her first visit to our clinic. Family history revealed a 14-year-old prepubertal sister and a twin brother and sister aged 11 years, all in good health (Fig. 1a). Her unrelated, Caucasian parents had no endocrine disorder or history of delayed puberty but her mother had received clomiphen citrate before the twin pregnancy. On physical examination, the patient's weight was 75 kg (90th–97th), her height was 174 cm (97th) and no dysmorphisms were noted. The Tanner stage was B3-P3 with normal female external genitalia. Imaging revealed a bone age delay of 3 years and a prepubertal uterus with small tubular structures suggestive of Fallopian tubes, but no ovaries were found on pelvic ultrasound. Bone mineral density revealed osteopenia (Z score = −2.4). Anti-ovarian antibodies were negative. The karyotype was 46, XY and a FISH analysis confirmed the homogenous presence of the SRY locus in all cells examined. The patient underwent laparoscopic removal of streak gonads; pelvic exploration confirmed the presence of Müllerian structures. Following completion of pubertal induction, the patient was cycled with an oral contraceptive and Vitamin D and calcium was introduced.

Patient 2

This 14-year-old sister of the proband was seen for screening, following the diagnosis of her sister. On physical examination, her weight was 70 kg (90th–97th), her height was 170 cm (90th). She had adipomastia, but Tanner stage 2 breasts and pubic hair. Her external genitalia were that of a normal female. She was also found to have hypergonadotropic hypogonadism (FSH: 66 IU/L), $n = 12–30$; LH: 13 IU/L, $n = 40–200$; Estradiol: <7; 3 pmol/l despite breast budding, and a normal female level of testosterone (0.64 nmol/l) and DHEAS (3.35 µmol/l) as well as normal thyroid function test (TSH: 2.10 mU/l). The karyotype was also 46, XY without mosaicism. Imaging studies revealed the presence of a right adnexal mass of 46 × 42 × 29 mm, and tumor markers were positive (βHCG: 14 IU/L, $n = <5$; afp: 1.4 µg/l, $n < 10$). Pathological examination of the surgically removed

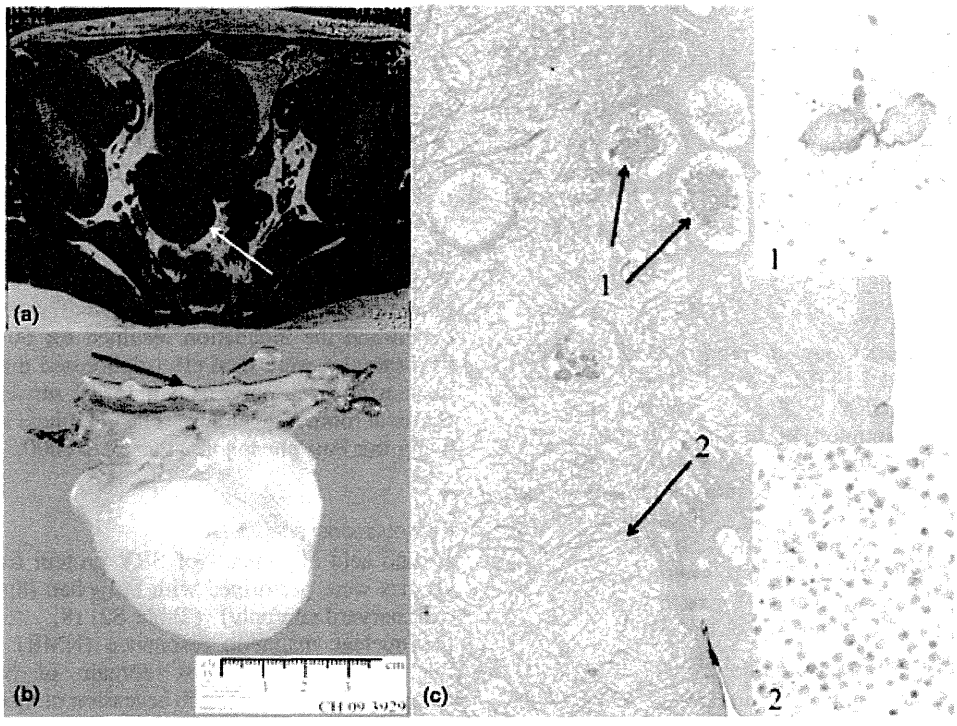

Fig. 2. Investigation of patient II-2: (**a**) Hypointense mass of 46 × 42 × 29 mm on cross section T1-weighted magnetic resonance imaging (arrow); (**b**) macroscopic picture of the right removed gonad. An arrow indicates the fallopian tube; (**c**) histopathologic section, H&E staining, magnification ×25. Region 1. Seminiferous tubules with hyaline contents corresponding to a gonadoblastoma (magnification ×630) (arrow 1). Region 2. High cellularity and anarchic architecture corresponding to a dysgerminoma (magnification ×630) (arrow 2).

gonads revealed a mixed ovarian tumor showing multiple islands of dysgerminoma and less numerous foci of gonadoblastoma surrounded by Sertoli cells; the left adnex showed no evidence of tumor and consisted of a highly fibrous ovarian structure with no demonstrable follicles (Fig. 2).

Methods

SRY mutation analysis with Hpy188I

Initial mutation analysis of the SRY coding region was performed by Gendia (Antwerp, Belgium), testing DNA from the proband's peripheral blood leukocytes. Following a preliminary report of a point mutation in the SRY HMG box, mutation status was confirmed by restriction fragment length polymorphism (RFLP) analysis performed at Saint-Justine Hospital; family members were likewise screened by RFLP at Sainte-Justine Hospital. Polymerase chain reaction (PCR) amplification of a fragment of SRY exon 1 was performed with primers, PCR conditions and minor modifications to those described in the study of Semerci et al. (4). Platinum Taq DNA polymerase (Invitrogen Corporation, Burlington, ON, Canada) was used with the following cycling parameters: initial denaturation at 94°C for 5 min followed by 30 cycles of: denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s. The PCR products (270 bp) was then subjected to digestion with Hpy188I in recommended buffer (New England Biolabs Inc, Pickering, ON, Canada) and the fragments analyzed on 7% polyacrylamide mini-gel. The normal allele (A allele) leads to 4 fragments of 132, 88, 45 and 4 bp whereas the mutation (C allele) destroys 1 restriction site and produces 3 fragments of respectively 177, 88 and 4 bp.

Markers used for paternity testing

Microsatellite and single nucleotide polymorphism (SNP) markers used for paternity testing, along with their chromosomal location and reference for PCR amplification, can be found in Table S1, Supporting information.

Plasmid construction

The pcDNA3.1(+) plasmid vector (Invitrogen) containing a *BamHI* and *EcoRI* cloning site was used for *in vitro* SRY synthesis. The SRY DNA insert was generated using both wild type and mutant DNA from peripheral blood leukocytes; the forward primer sequence (5′-GCGGATCCgcacctttcaattttgtcgc-3′) contained a *BamHI* restriction site and the *SRY*-39 to -20 site. The reverse primer sequence (5′-GCG AATTCTACAGCTTTGTCCAGTGGC-3′) contained an *Eco*RI restriction site and the SRY TAG site.

The PCR amplification was performed in a 50-μL reaction volume containing PrimeSTAR Buffer (10 mM Tris–HCl(pH 8.2), 20 mM NaCl, 0.02 mM EDTA,

0.2 mM DTT, 1 mM $MgCl_2$), 200 µM each of dNTP (dATP, dCTP, dGTP, dTTP), 15 pmol of oligonucleotide primers, 65 ng of DNA, and 1.25 IU Taq DNA Polymerase (TaKaRa:R010A). PCR thermal cycling conditions were: a 2-min predenaturation period at 94°C and 35 cycles of the following: 98°C for 10 s, 60°C for 5 s, and 72°C for 60 s. The SRY cDNA sequence was 663 nt and both the wild type (pcDNA3.1(+)-wt) and mutant vectors (pcDNA3.1(+)-E89A) were verified by sequencing.

In vitro translation

The TNT® T7 Quick Coupled Transcription/Translation Systems (Promega:L1170) was used to synthesize ^{35}S-methionine-labeled SRY in a reaction mix containing [^{35}S]-methionine (Perkin Elmer, Waltham, MA; NEG709A), TNT Quick Master Mix and 1.0 µg of the expression vector in a final reaction volume of 50 µl. The mixture was incubated at 30°C for 90 min, and the labeled wild type and mutant SRY proteins were analyzed by 12% SDS-PAGE; autoradiography showed a single, 30 kD band as expected for both proteins.

Preparation of oligonucleotide probe

The SRY-binding sequence upstream of *SOX9* (AAC AAT) (5) was incorporated into a ^{32}P-labeled oligonucleotide probe as previously described by Shahid et al. (6). The oligonucleotides used were: F;5′-GGGT TAACGT AACAAT GAATCTGGTAGTA-3′, R;5′-GT ACTACCAGATTC ATTGTT ACGTTAACC-3′.

Electrophoretic mobility shift assay

The ability of the SRY wt and mutant proteins to bind to DNA was assessed in a binding reaction containing increasing amount of wild type or mutant proteins in the binding buffer described in the study by van de Wetering et al. (7). After a pre-incubation of 20 min on ice of the proteins in binding buffer, the probe was added and the incubation resumed for 60 min on ice. The samples were then electrophoresed through a non-denaturing 12% polyacrylamide gel run in 0.5× TBE buffer at room temperature. The gel image was analyzed using the Fuji phosphoimager FLA-7000.

Computational analysis

Amino acid alignments of SRY protein from different species were performed with Polyphen (http://genetics.bwh.harvard.edu/pph/) (Table S2) (8). A picture of the nuclear magnetic resonance (NMR) structure of the wild-type HMG-Box domain of SRY protein complexed with DNA (pdb accession number 1J46) was designed with the PyMOL Molecular Graphic System (http://www.pymol.org/) (Fig. 3b).

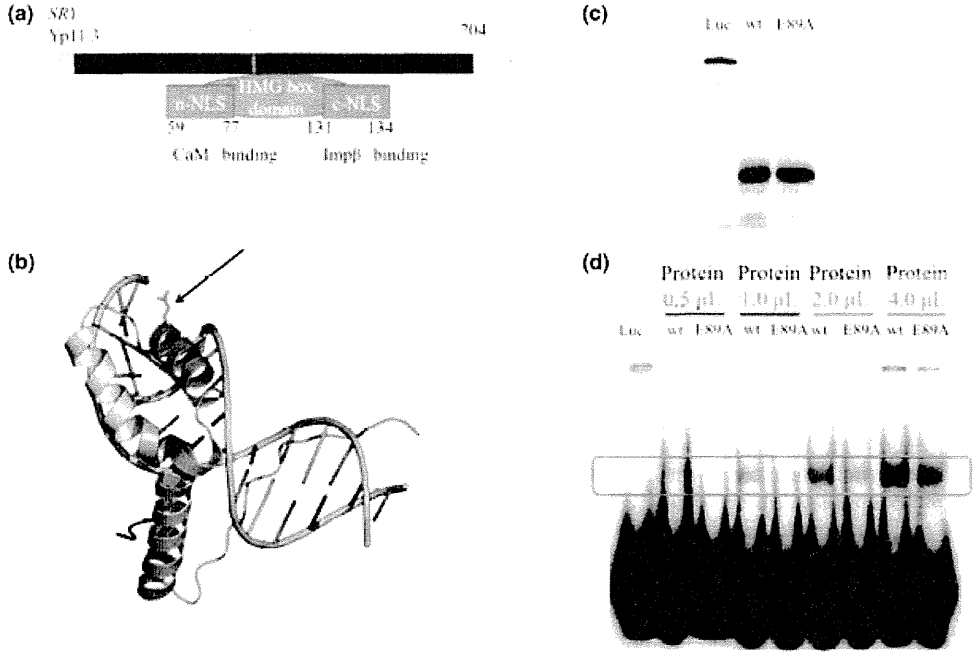

Fig. 3. Novel *SRY* mutation. (**a**) Schematic of the SRY protein showing the location of the DNA binding domain (HMG box) in relation to the identified missense mutation, which causes a glutamic acid (E) to alanine (A) change in amino acid 89. (**b**) Nuclear magnetic resonance structure of the wild-type HMG-Box domain of the SRY protein complexed with DNA (pdb accession number 1J46). The side chain of the involved amino acid is depicted (Glu89) (arrow). The picture was designed with the PyMOL Molecular Graphic System (http://www.pymol.org/). (**c**) Synthesize SRY protein in vitro. An autoradiograph of *in vitro*-translated 35S-labeled SRY-wt, SRY-E89A proteins analyzed by 12% SDS-PAGE, showing a single 30-kDa band. Luc, luciferase (negative control). (**d**) Electrophoretic Mobility Shift Assay. As explained in Materials and Methods, increasing concentrations of wt or recombinant SRY protein were added to a ^{32}P-labeled oligonucleotide probe encompassing the SRY binding region of SOX9. Lane 1 shows a luciferase control. The binding capacity of the wt protein surpasses that of the mutant protein at all concentrations tested.

Results

Chromosome analysis (performed in a peripheral hospital and based on examination of 20 metaphases) from peripheral cultured blood lymphocytes revealed a 46, XY karyotype in the proband, a phenotypic female (see pedigree, Fig. 1a). Sequence analysis of the *SRY* gene in the proband showed a single point mutation c.266A>C (p.Glu89Ala) which was confirmed by an RFLP assay designed to distinguish the wild type and mutant alleles at this position in the gene. This missense mutation is located in the DNA binding domain of the protein, and the substitution of an uncharged amino acid for one with a positive charge suggests that protein structure in this region is perturbed; Polyphen analysis supported this prediction and also showed that Glu89 in SRY HMG-Box domain is highly conserved across species (Table S2) (8) (Fig 3a,b). The variant has not been reported among unaffected males nor in the known SRY mutation databases (Patrick Willems, Gendia, personal communication), nor in the Human Genome Mutation Database (HGMD).

To determine the DNA binding characteristics of the E89A variant, we performed an electrophoretic mobility shift assay using *in vitro* synthesized wild type or mutant protein, and an oligonucleotide probe containing the *SOX9* promoter SRY binding sites. As seen in Fig. 3d, the mutant protein displayed markedly decreased binding activity at all protein concentrations employed. These data suggest that the E89A variant has a pathogenic effect on protein function and is not simply a rare neutral polymorphism.

By the RFLP assay, neither of the proband's parents carried the *SRY* mutation, based on analysis of blood leukocyte-derived DNA (Fig. 1b). The father showed no unusual phenotype characteristics with regard to primary and secondary sexual traits, suggesting that the father might be a germ line mosaic for the mutation. Correct paternity was confirmed by genotyping eight highly polymorphic microsatellites and three SNPs on six different chromosomes; informative loci are shown in the Table S1. All markers were consistent with the given family structure.

Based on the current standard of practice, there would normally be no special reason to perform any genetic tests on the proband's siblings (one brother, two sisters). However, we chose to verify the karyotype (examination of 30 metaphases) of all three siblings, and unexpectedly one sister was also 46, XY despite a clinical examination that suggested breast budding compatible with a Tanner stage B2-3. By our RFLP assay, we determined that the proband's nominal sister was also carrying the *SRY* mutation, while the brother carried a normal SRY gene sequence (Fig. 1b, lanes 7). The youngest sister had a normal 46, XX karyotype. On further examination, the 46, XY *SRY* (−) sister was found to have a right side ovary tumor showing multiple islands of dysgerminoma and less numerous foci of gonadoblastoma surrounded by Sertoli cells (Fig. 2). The left ovary was not tumorous. Bilateral ovaries were removed surgically from both 46, XY sisters, who are now receiving hormone therapy to allow normal secondary sexual development.

Discussion

We have identified a novel point mutation p.Glu89Ala within the HMG box of the *SRY* gene shared by two 46, XY phenotypically female 'sisters'. Functional studies support that this variant is a pathogenic mutation and not a neutral polymorphism, as the mutation decreases the SRY DNA-binding capacity in a well validated *in vitro* assay. Their father is wild type for *SRY* sequence as is a 46, XY phenotypically normal brother. The brother's (dizygotic) twin sister has a normal 46, XX karyotype.

Previously reported familial cases of 46, XY CGD as a result of *SRY* mutation are summarized in Table 1. There are 16 different mutations within 16 such families, but only one family had evidence of paternal germ cell mosaicism as in our family. However in that study correct paternity was not confirmed explicitly (9).

Most aspects of phenotype of the two 46, XY sisters described here are typical of patients with 46, XY CGD. However, the younger sister exhibited a seemingly normal onset of puberty, which is atypical in patients with SRY mutations. In retrospect, although the low estradiol levels compatible with breast budding (Tanner B2) are currently unmeasurable by our clinically available assay (lower limit 73 pmol/l), we suspect that the ovarian tumor may have secreted sufficient estrogens to initiate puberty. Notably, as seen in Table 1, five of the 46, XY SRY^{mut} females exhibiting breast development at diagnosis (and not yet on estrogen therapy), four out of five were found to have an ovarian tumor as in our younger patient. Villanueva et al. (10) reported the case of a 17-year-old girl with 46, XY CGD and a breast development corresponding to Tanner stage V. She was found to have bilateral gonadoblastoma and right dysgerminoma. The estrogen levels in that patient, although persistently in the range of follicular phase concentrations, were higher than those of postmenopausal women and apparently were high enough to ensure breast development but not menarche. As her estrogen and androgen levels dropped markedly postoperatively, her dysgenetic gonads were obviously the source of sex steroids. Barakat et al. reported a 16-year-old girl with 46, XY CGD and spontaneous breast development that occurred at 13 years followed by menarche at 14 years. She was referred for secondary amenorrhea and clitoromegaly 2 years later, and was found to have bilateral gonadoblastoma. The authors showed that both gonads produced estradiol in culture (11). The low estradiol levels in our patient's younger sibling despite her spontaneous breast development could also be explained by conversion of dehydroepiandrosterone to estrogen in mammary tissue as suggested by Cantu et al. (12), although the Tanner B3 breasts and the absence of the finding in the older affected sister argue against this.

Tumor risk in patients with 46, XY CGD has been estimated at 30%, but may be as high as 50–70%

Table 1. Familial cases of 46, XY females with SRY mutations

References	Case number	Mutation	Type	SRY domain	*In vitro* analysis	Age at Dx	Tanner	Reason for referral	Gonadal tumor	Father's Lk DNA
(23)	1	P125L	Point	HMG box	Yes	18 years	B3 on E_2 therapy, pubic hair sparse	Amenorrhea	Dg R	Mosaicism
	2, sibling of 1					14 years and 11 months	B2, pubic hair sparse	Amenorrhea	No	
	3, half sibling of 1					4 years and 6 months	B1	Screening	No	
	4	S91G	Point	HMG box	Yes	11 years and 9 months	B1P1	Clitoromegaly	Gb unil	Mosaicism
(24)	5	Y17X	Stop	N-terminal domain	No	25 years	B1P1	NA	Gb L	Not performed
	6, sibling of 5					20 years	B1P1	NA	Gb	
(25)	7	Y127F	Point	HMG box	Yes	16 years	B4P4	Amenorrhea	Gb + Dg	Mutation
(26)	8	S3L	Point	N-terminal domain	No	17 years and 6 months	B1P4	Amenorrhea	No	Mutation
(27)	9[a]	60X	Single bp del (stop)	HMG box	No	17 years	B3P4	Abdominal pain	Gb L + Dg R	Mosaicism
	10, sibling of 9					9 years	NA	Screening	Gb bilat	
(28)	11	Q97X	Point (stop)	HMG box	No	Birth	NA	Amniocentesis 46, XY in a girl	No	Mosaicism
	12, sibling of 11					16 years	Prepubertal	Screening and pubertal delay	Gb bilat	
(29)	13	S76R	Missense	HMG box	No	20 years	Prepubertal	Amenorrhea and pubertal delay	NA	Mutation
(30)	14[b]	F109S	Point	HMG box	Yes	17 years and 28 years	B3	Amenorrhea	Gb R	Mutation
(31)	15[c]	I90M	Point	HMG box	No	NA	NA	Pubertal delay	NA	Mutation
(32)	16[d]	F67V	Point	HMG box	No	17 years	NA	Amenorrhea	Dg bilat	Mosaicism
	17					8 years	NA	Screening	Dg unil	
(33)	18[e]	V60L	Point	HMG box	No	17 years	NA	Amenorrhea	No	Mutation
	19, sibling of 18					20 years	NA	Amenorrhea and pubertal delay	No	
	20, paternal aunt of 18					36 years	NA	Amenorrhea/Gb	Gb L	

Table 1. Continued

References	Case number	Mutation	Type	SRY domain	*In vitro* analysis	Age at Dx	Tanner	Reason for referral	Gonadal tumor	Father's Lk DNA
(34)	21[f]	R30I	Missense	N-terminal domain	Yes	18 years	Prepubertal	Screening and pubertal delay	No	Mutation
(35)	22	L116S	Missense	HMG box	Yes	16 years	Tanner I	Pubertal delay	No	Mosaicism[g]
	23, half-sibling of 22					16/35 years	NA	Primary amenorrhea	Gb bilat	
(9)	24		*SRY* deletion		No	19 years	Poorly developed breast (clitoromegaly)	Amenorrhea	No	Normal
	25, sibling of 24					18 years	Undeveloped breast	Amenorrhea	Gb bilat	
Our case	26	E89A	Missense	HMG box	Yes	16 years	B3 on E_2 therapy, P3	Amenorrhea and pubertal delay	No	Normal
	27, sibling of 26					14 years	B2P3	Screening	Gb/ Dg R	

B, breast development; bilat, bilateral; DG, dysgerminoma; Dx, diagnosis; E_2, estrogen, Gb, gonadoblastoma; L, left; Lk, leukocytes; NA, not available; No, no tumor found; P, pubic hair; R, right; unil, unilateral.

[a]Father with testicular dysgenesis syndrome (cryptorchidism, seminoma in this testis diagnosed at 39 years, severe hypospadias, oligoasthenozoospermia).

[b]46, XY mutation positive in normal brother and uncle.

[c]46, XY mutation positive in normal fertile brother.

[d]Mosaicism in father's sperm.

[e]46, XY mutation positive in normal paternal uncle.

[f]46, XY mutation positive in two brothers with ambiguous genitalia diagnosed at 8 and 10 days, and in three normal brothers. 46, XY mutation positive in a paternal aunt with ambiguity.

[g]DNA from periarticular inflammatory tissue of the deceased father.

in the third decade of life, and 80% at 40 years. Patients may present with either gonadoblastoma or the more invasive dysgerminoma (13). The presence of at least a part of the Y chromosome is necessary for development of a gonadoblastoma in a dysgenetic gonad. This so-called gonadoblastoma locus (*GBY*) on Yp11.1 contains a potential oncogene named testis specific protein Y-encoded (TSPY) involved in cell cycle regulation (14–16). The *TSPY* gene is expressed in both fetal and adult testes, and it has been hypothesized that it regulates the normal proliferation of both embryonic prespermatogonia/gonocytes and adult spermatogonial cells (15,17).

The finding of germ cell mosaicism in two of the 16 familial examples of SRY mutations has significant implications for genetic counseling. There are multiple genetic diseases (>60) in which confined germline mosaicism has been observed (18). Although recurrence risk calculations are problematic, Duchenne muscular dystrophy has one of the highest risks of transmission by leukocyte mutation negative females, estimated at between 14% and 20%. There are two possibilities for such a high recurrence. One is that the mutation occurs very early before the formation of the germ cells and is therefore present in both somatic and germinal cells, although this might be expected to cause a mutation in 100% of the germ cells, rather than mosaicism. Alternatively, a mutation may occur following germline-soma divergence but early in the germinal lineage. The examination of several different tissues (buccal, smear, muscle, or hair follicle) may discriminate between these alternatives, although in the case of 46, XY CGD, molecular analyses of single paternal sperm would be the ideal test to determine probably recurrence risk (19). However, sperm are subject to clonal expansions, in some cases with a suggestion of deleterious mutations actually providing a competitive advantage in the testis, thus it is probably impossible to provide families with an accurate recurrence risk generally (20–22).

In conclusion, our identification of a novel point mutation in the *SRY* gene shared by two 46, XY CGD sisters revealed probable paternal germline mosaicism. Functional analysis indicates that this mutation decreases the DNA-binding activity of the SRY protein. More importantly, this family illustrates that upon identification of the proband, even sisters with pubertal onset should be screened cytologically and/or for *SRY* mutations, because even in the case of germ cell mosaicism a high recurrence risk is possible. In fact, pubertal onset in a mutation-bearing patient may herald the presence of a gonadal tumor. It is important to note that preventive screening of siblings, in families with one sporadic 46, XY feminized case, is not necessarily the current standard of practice. Our decision to screen both seemingly unaffected sisters, leading to the identification in one of a mutation requiring hormonal treatment, and a tumor requiring surgical excision, had major impact to improve the feminized sister's long-term quality of life.

Supporting Information

The following Supporting information is available for this article:

Table S1. Paternity confirmation.

Table S2. Amino acid alignments across species in the regions flanking E89 (red box) performed with Polyphen (http://genetics.bwh.harvard.edu/pph/).

Additional Supporting information may be found in the online version of this article.

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Two-Step Biochemical Differential Diagnosis of Classic 21-Hydroxylase Deficiency and Cytochrome P450 Oxidoreductase Deficiency in Japanese Infants by GC-MS Measurement of Urinary Pregnanetriolone/Tetrahydroxycortisone Ratio and 11β-Hydroxyandrosterone

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BACKGROUND: The clinical differential diagnosis of classic 21-hydroxylase deficiency (C21OHD) and cytochrome P450 oxidoreductase deficiency (PORD) is sometimes difficult, since both deficiencies can have similar phenotypes and high blood concentrations of 17α-hydroxyprogesterone (17OHP). The objective of this study was to identify biochemical markers for the differential diagnosis of C21OHD, PORD, and transient hyper 17α-hydroxyprogesteronemia (TH17OHP) in Japanese newborns. We established a 2-step biochemical differential diagnosis of C21OHD and PORD.

METHODS: We recruited 29 infants with C21OHD, 9 with PORD, 67 with TH17OHP, and 1341 control infants. All were Japanese and between 0 and 180 days old; none received glucocorticoid treatment before urine sampling. We measured urinary pregnanetriolone (Ptl), the cortisol metabolites 5α- and 5β-tetrahydrocortisone (sum of these metabolites termed THEs), and metabolites of 3 steroids, namely dehydroepiandrosterone, androstenedione (AD4), and 11β-hydroxyandrostenedione (11OHAD4) by GC-MS.

RESULTS: At a cutoff of 0.020, the ratio of Ptl to THEs differentiated C21OHD and PORD from TH17OHP and controls with no overlap. Among metabolites of DHEA, AD4, and 11OHAD4, only 11β-hydroxyandrosterone (11HA), a metabolite of 11OHAD4, showed no overlap between C21OHD and PORD at a cutoff of 0.35 mg/g creatinine.

CONCLUSIONS: A specific cutoff for the ratio of Ptl to THEs can differentiate C21OHD and PORD from TH17OHP and controls. Additionally, the use of a specific cutoff of 11HA can distinguish between C21OHD and PORD.

Classic 21-hydroxylase deficiency (C21OHD)[6] is the most common form of congenital adrenal hyperplasia, which is transmitted as an autosomal recessive trait. C21OHD is caused by mutations of *CYP21A2* (cytochrome P450, family 21, subfamily A, polypeptide 2)[7] encoding 21-hydroxylase, which catalyzes steroid hydroxylation at C21 (Fig. 1) *(1)*. C21OHD shows adrenal insufficiency, disorders of sex development in 46,XX, and increased serum 17α-hydroxyprogesterone (17OHP). Cytochrome P450 oxidoreductase (POR) deficiency (PORD) is a recently established form of congenital adrenal hyperplasia that is also transmitted as an autosomal recessive trait. PORD is caused by mutations of *POR* encoding POR, which transfers electrons to microsomal P450 enzymes such as 17-hydroxylase/17,20-lyase, 21-hydroxylase, and aromatase (Fig. 1) *(2)*. PORD shows adrenal dysfunction,

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[6] Nonstandard abbreviations: C21OHD, classic 21-hydroxylase deficiency; 17OHP, 17α-hydroxyprogesterone; POR, cytochrome P450 oxidoreductase; PORD, POR deficiency; TH17OHP, transient hyper 17OHPnemia; Ptl, pregnanetriolone; 21DOF, 21-deoxycortisol; 11HA, 11β-hydroxyandrosterone; PD, pregnanediol; GC-MS-SIM, GC-MS/selected ion monitoring; THE, tetrahydrocortisone; DHEA, dehydroepiandrosterone; AD4, androstenedione; 11OHAD4, 11β-hydroxyandrostenedione.
[7] Human genes: *CYP21A2*, cytochrome P450, family 21, subfamily A, polypeptide 2; *POR*, P450 (cytochrome) oxidoreductase.

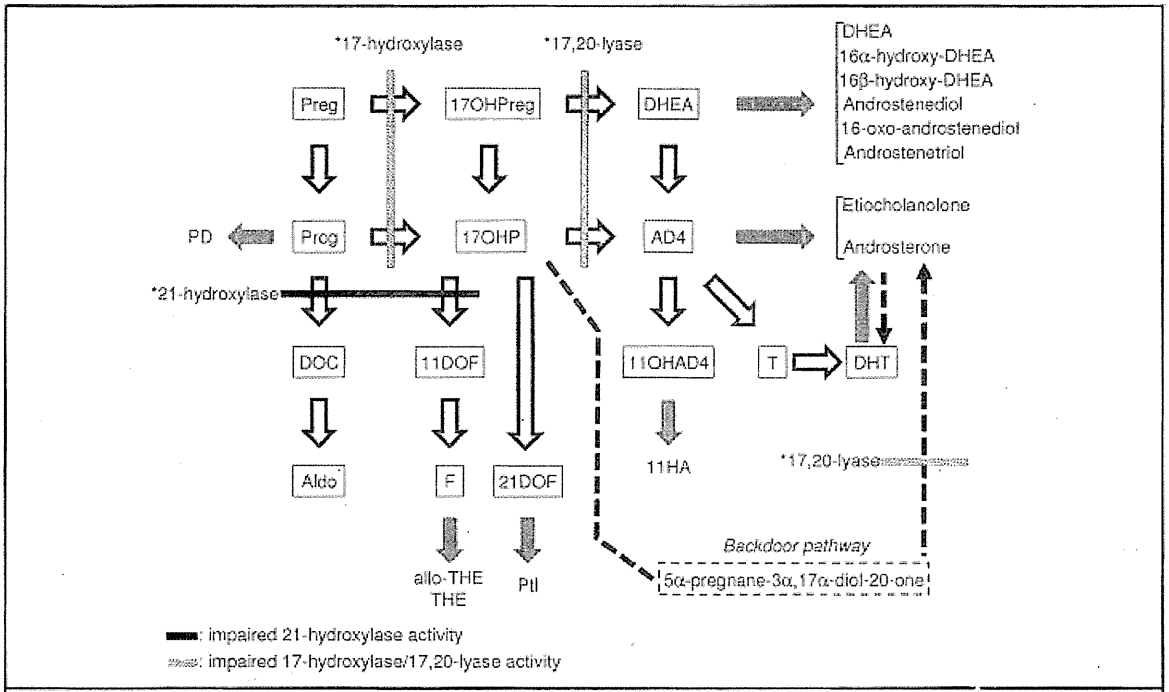

Fig. 1. Steroid metabolic map. *: enzymes that work with POR.

Preg, Pregnenolone; Prog, progesterone; DOC, deoxycorticosterone; Aldo, aldosterone; 17OHPreg, 17α-hydroxypregnenolone; 11DOF, 11-deoxycortisol; F, cortisol; T, testosterone; DHT, dihydrotestosterone. Open arrow, steroid synthesis; closed arrow, steroid metabolism; dashed arrow, backdoor pathway; open square, steroids in blood. Note that both 21-hydroxylase and 17-hydroxylase/17,20-lyase activities are reduced in PORD whereas only 21-hydroxylase is reduced in C21OHD.

disorders of sex development in 46,XX and 46,XY, skeletal dysplasia, maternal virilization during pregnancy, and increased serum 17OHP. In addition to increased concentrations of 17OHP, clinical manifestations of C21OHD and PORD can be similar, leading to difficulty in differential diagnosis *(3, 4)*. We previously reported the biochemical differential diagnosis of C21OHD from transient hyper 17OHPnemia (TH17OHP) and controls in term and preterm neonates by measuring urinary pregnanetriolone (Ptl), which was a final metabolite of 21-deoxycortisol (21DOF) *(5)*. Shackleton et al. *(6)* reported biochemical differential diagnosis of PORD from controls by a distinctive steroid excretion pattern, namely low urinary metabolites of cortisol and androgens and high metabolites of pregnenolone and progesterone. We reported that PORD had high urinary Ptl concentrations and that the ratio of 11β-hydroxyandrosterone (11HA) to pregnanediol (PD) could differentiate PORD from C21OHD in 3 infants between the ages of 1 and 3 months *(3)*. However, no cutoff for urinary steroid metabolites has been reported at any age for distinguishing between C21OHD and PORD. In our laboratory, the measurement of PD in newborns is sometimes problematic owing to unknown interferences, and we have not been able to calculate the ratio of 11HA to PD for the differential diagnosis of C21OHD and PORD.

The objective of this study was to identify biochemical markers for the differential diagnosis of C21OHD, PORD, and TH17OHP and to set the cutoff in Japanese infants <6 months old, the period during which most patients with C21OHD or PORD are diagnosed *(7)*. We paid attention to 21-hydroxylase and 17,20-lyase activities since, theoretically, the former enzymatic activity is impaired in both C21OHD and PORD and the latter is impaired in PORD but not C21OHD.

Materials and Methods

All legal guardians gave written informed consent, and the study was approved by the institutional review board committee at Keio University Hospital. We recruited 29 infants with C21OHD, 9 with PORD, 67 with TH17OHP, and 1341 control infants from 2000 through 2009 at Keio University Hospital and 45 other hospitals throughout Japan (Table 1). All infants were

Table 1. Characteristics of the study subjects.

	C21OHD	PORD	TH17OHP	Control
n	29	9	67	1341
Sex, M/F	20/9	4/5	44/23	759/582
Median gestational age, weeks (range)	38 (31–41)	40 (37–41)	37 (26–41)	38 (22–42)
Median age at analysis, days (range)	11 (0–45)	59 (3–146)	35 (3–164)	4 (0–180)
Median birth weight, g (range)	3060 (1464–4030)	2818 (2330–3066)	2670 (895–4980)	2878 (442–4506)

Japanese, with ages between 0–180 days. The diagnosis of C21OHD and PORD was confirmed by *CYP21A2* and *POR* gene analysis, respectively (Table 2). The diagnosis of TH17OHP was made in the neonates fulfilling all the following criteria; (*a*) 17OHP concentration in the dried blood spot mass screening program in Japan (direct ELISA assay), (*b*) blood 17OHP concentration confirmed to be normal by repeated measure-

Table 2. Genotypes of 29 subjects with C21OHD and 9 with PORD.

Patients	Sex	Gestational age, weeks	Copy 1	Copy 2
C21OHD				
1, 2, 3	M	36, 36, 40	Del or conv[a]	Del or conv
4, 5	M	38, 39	Del or conv	R356W
6	M	38	Del or conv	E6 cluster
7	M	40	Del or conv	L307+T
8, 9	M	38, 40	Del or conv	I2 splicing
10	M	41	Del or conv	I172N
11	M	38	Del or conv	I78 mol/L
12	M	38	Δ8bp[a]	Q318X
13, 14	M	38, 39	R356W	I2 splicing
15	M	38	E6 cluster	I2 splicing
16	M	38	I2 splicing	I2 splicing
17, 18	M	35, 39	I2 splicing	I172N
19	M	35	I172N	I172N
20	M	39	Del or conv, I172N	Δ8bp, I2 splicing
21, 22	F	38, 40	Del or conv	Del or conv
23	F	31	Del or conv	Δ8bp
24	F	39	Del or conv	I172N
25	F	38	R356W	R356W
26, 27	F	37, 38	I2 splicing	I2 splicing
28, 29	F	35, 39	I2 splicing	I172N
PORD				
1,2	M	38, 40	R457H	R457H
3	M	37	R457H	Q555fsX611
4	M	40	R457H	A462 S463insIA
5, 6, 7, 8	F	37, 39, 40, 40	R457H	R457H
9	F	41	R457H	E580Q

[a] Del or conv, deletion or large gene conversion; E6 cluster, cluster of mutations (I236D, V237E, M239K) in exon 6; I2 splicing, intron 2 −13 A/C>G; Δ8bp, 8-bp deletion in exon 3.