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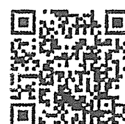

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# Two-Step Biochemical Differential Diagnosis of Classic 21-Hydroxylase Deficiency and Cytochrome P450 Oxidoreductase Deficiency in Japanese Infants by GC-MS Measurement of Urinary Pregnanetriolone/ Tetrahydroxycortisone Ratio and 11β-Hydroxyandrosterone

Yuhei Koyama,[1,2] Keiko Homma,[3] Maki Fukami,[4] Masayuki Miwa,[5] Kazushige Ikeda,[5] Tsutomu Ogata,[4] Tomonobu Hasegawa,[5*] and Mitsuru Murata[1]

**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.

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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,

[1] Department of Laboratory Medicine and [5] Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan; [2] Mitsubishi Chemical Medience Co., Tokyo, Japan; [3] Keio University Hospital Central Clinical Laboratories, Tokyo, Japan; [4] Department of Endocrinology and Metabolism, National Center for Child Health and Development, Tokyo, Japan.
* Address correspondence to this author at: Department of Pediatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan; Fax +81-3-5379-1978; e-mail thaseg@a6.keio.jp.
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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.



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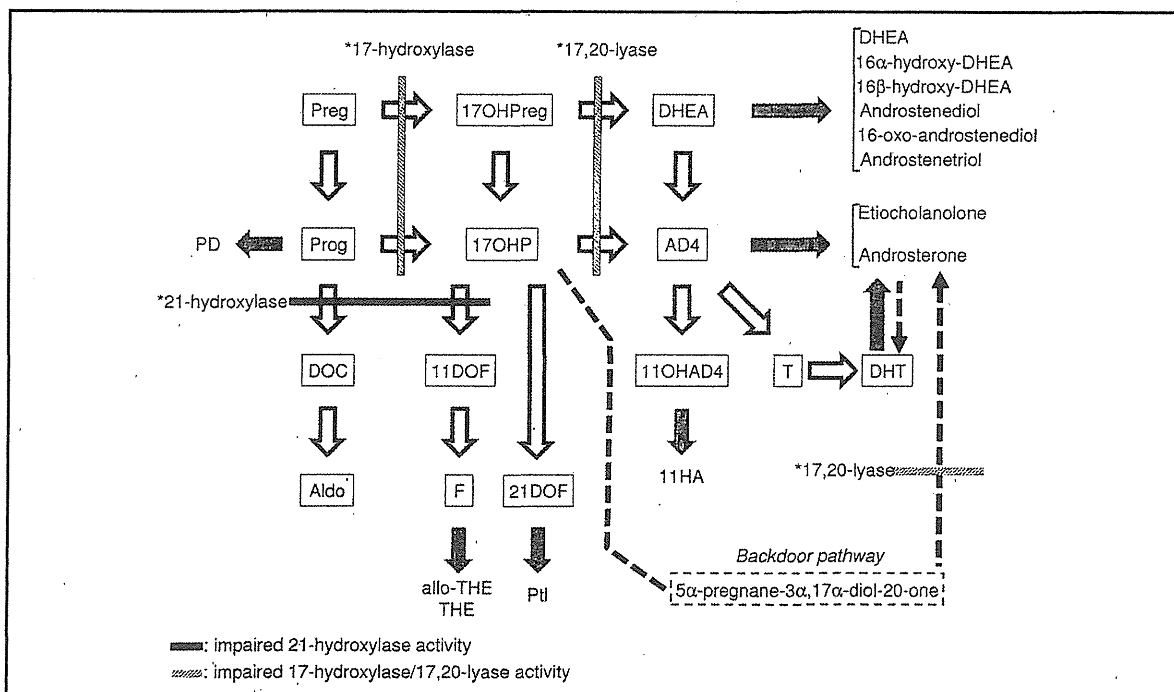


**Fig. 1. Steroid metabolic map. *: enzymes that work with POR.**
Preg, Pregnenolone; Prog, progesterone; DOC, deoxycorticosterone; Aldo, aldosterone; 17OHPreg, 17α-hydroxypregnenolone; 11DOF, 11-deoxycortisol; F, cortisol; T, testosterone; DHT, dihydrotestosterone. Open arrow, steroid synthesis; closed 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[d] | 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.

ments [ELISA assay (direct or extraction method) or RIA], and (*c*) consistently good overall general condition. Any subjects with abnormal physical findings were excluded. The control infants were without neurologic and endocrinologic abnormalities, and none of the subjects received antenatal or perinatal glucocorticoid before urine sampling. Table 1 shows characteristics of the study subjects.

Spot urine samples were randomly collected for the study and kept at −20 °C until analysis. We studied each infant for urinary steroid profile by GC-MS/selected ion monitoring (GC-MS-SIM), as reported (*8*) with minor modification. In brief, 0.05- to 0.2-mL urine samples were subjected enzymatic hydrolysis and organic solvent extraction and methyloxime-trimethylsilyl derivatized, and the derivative was subjected to GC-MS-SIM analysis. GC-MS-SIM analysis was performed on an HP5890II GC with an HP-Ultra1 fused silica column (25 m × 0.2 nm × 0.33 μm) coupled to an HP5971MS (Agilent Technologies). We quantified each steroid with stigmasterol as the internal standard. The turnaround time of the assay is 2 days.

We measured Ptl and the cortisol metabolites 5α-tetrahydrocortisone and 5β-tetrahydrocortisone (sum of these metabolites termed THEs) and calculated the ratio of Ptl to the cortisol metabolites (Ptl/THEs) (*5, 9*) to differentiate C21OHD and PORD from TH17OHP and controls. Ptl was considered to be equal to 0.001 mg/g creatinine for calculation in infants whose Ptl was under the detection limit (<0.001 mg/g creatinine). We measured metabolites of 3 steroids, namely dehydroepiandrosterone (DHEA), androstenedione (AD4), and 11β-hydroxyandrostenedione (11OHAD4), and calculated the sum of DHEA metabolites (DHEA, androstenediol, 16α-hydroxy-DHEA, 16β-hydroxy-DHEA, 16-oxo-androstenediol, and androstenetriol), the sum of AD4 metabolites (androsterone and etiocholanolone) (*8, 10*), and the 11OHAD4 metabolite (11HA). (11β-Hydroxyetiocholanolone could not be measured in newborns, as described (*8*).) The above sum of DHEA metabolites have been reported to include approximately 70% of DHEA metabolites in newborns (*11*). The above sum of AD4 metabolites are defined as androgen metabolites because, whereas these 2 steroids are metabolites of AD4, they are also the metabolites of testosterone and dihydrotestosterone in boys. Androgen metabolites are analyzed separately by sex, since male androgen metabolites in this age group are increased from testicular-derived androsterone and etiocholanolone.

We measured urinary creatinine by IATRO-LQ CRE (A)II (Mitsubishi Chemical Medience Co.) and expressed urinary steroid concentration relative to urinary creatinine (mg/g creatinine).

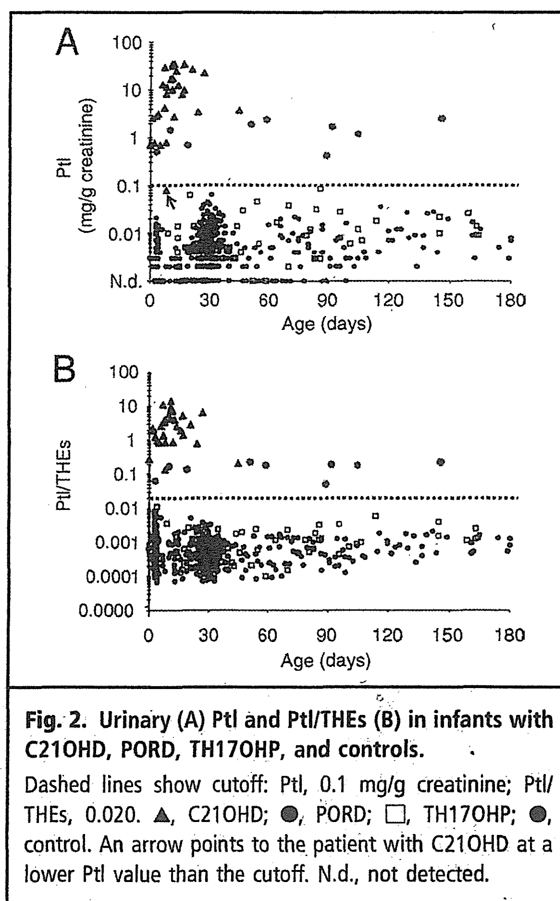


**Fig. 2. Urinary (A) Ptl and Ptl/THEs (B) in infants with C21OHD, PORD, TH17OHP, and controls.**

Dashed lines show cutoff: Ptl, 0.1 mg/g creatinine; Ptl/THEs, 0.020. ▲, C21OHD; ●, PORD; □, TH17OHP; ●, control. An arrow points to the patient with C21OHD at a lower Ptl value than the cutoff. N.d., not detected.

Statistical analysis of DHEA, androgen, and 11OHAD4 metabolites was carried out by Mann–Whitney *U*-test between C21OHD and PORD. A *P* value of <0.05 was considered statistically significant.

## Results

#### DIFFERENTIATION OF C21OHD AND PORD FROM TH17OHP AND CONTROLS

Fig. 2 shows the results of urinary Ptl and Ptl/THEs. Ptl [median (range) mg/g creatinine] was 10 (0.079–36) in C21OHD, 1.5 (0.42–2.6) in PORD, 0.006 (<0.001–0.086) in TH17OHP, and <0.001 (<0.001–0.064) in controls. Ptl/THEs was 2.5 (0.14–15) in C21OHD, 0.18 (0.051–0.23) in PORD, 0.00083 (0.00010–0.011) in TH17OHP, and 0.00038 (0.000068–0.0083) in controls. Ptl differentiated C21OHD and PORD from TH17OHP and controls with 96.9% (95% CI 91.6%–97.4%) sensitivity and 100% (99.8%–100%) specificity with cutoff 0.1 mg/g creatinine. All patients with TH17OHP and controls showed Ptl concentrations below the cutoff. An 8-day-old patient with C21OHD whose birth weight was 1628 g had a lower Ptl value

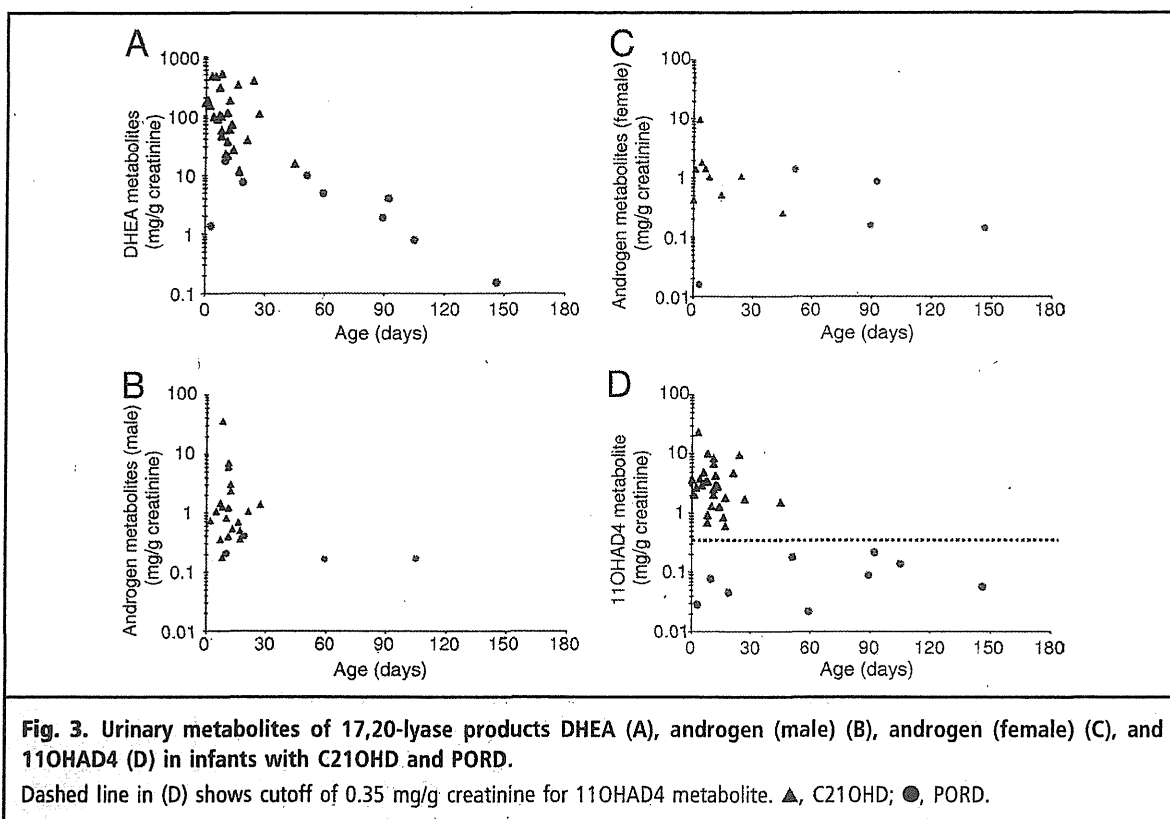


**Fig. 3. Urinary metabolites of 17,20-lyase products DHEA (A), androgen (male) (B), androgen (female) (C), and 11OHAD4 (D) in infants with C21OHD and PORD.**
Dashed line in (D) shows cutoff of 0.35 mg/g creatinine for 11OHAD4 metabolite. ▲, C21OHD; ●, PORD.

than the cutoff (Fig. 2A, arrow). Ptl/THEs differentiated C21OHD and PORD from TH17OHP and control with 100% (95.1%–100%) diagnostic sensitivity and 100% (99.8%–100%) diagnostic specificity with the 0.020 cutoff.

#### DISCRIMINATION BETWEEN C21OHD AND PORD

Fig. 3 shows the results of urinary metabolites of 17,20-lyase products (mg/g creatinine) in C21OHD and PORD. DHEA metabolites were 97 (11–505) in C21OHD and 4.0 (0.15–17) in PORD. Androgen metabolites were, for boys, 1.1 (0.18–36) in C21OHD and 0.19 (0.17–0.41) in PORD, and for girls, 1.1 (0.26–9.9) in C21OHD and 0.16 (0.016–1.4) in PORD. 11OHAD4 metabolite was 3.0 (0.61–24) in C21OHD and 0.077 (0.022–0.22) in PORD. All metabolites of 17,20-lyase products showed significant differences between C21OHD and PORD (DHEA metabolites, $P < 0.001$; androgen metabolites (male), $P = 0.006$; androgen metabolites (female), $P = 0.039$; 11OHAD4 metabolite, $P < 0.001$). 11OHAD4 metabolite (11HA) discriminated between C21OHD and PORD with 100% (94.2%–100%) diagnostic sensitivity and 100% (81.4%–100%) diagnostic specificity with the 0.35 mg/g creatinine cutoff. Urinary DHEA and androgen metabolites showed overlap between C21OHD and PORD.

## Discussion

We established a 2-step biochemical differential diagnosis for C21OHD and PORD by urinary steroid profile. First, by using a specific cutoff of the ratio of Ptl to THEs, we were able to differentiate C21OHD and PORD from TH17OHP and controls. Second, by using a specific cutoff of 11HA, we were able to distinguish between C21OHD and PORD. Although a distinctive steroid excretion pattern in C21OHD and PORD had been reported *(3, 5, 6)*, no clear cutoff of urinary steroid metabolites was reported in any ages to discriminate between C21OHD and PORD. To the best of our knowledge, this is the first report of a cutoff for biochemical differential diagnosis of C21OHD and PORD in infants. As for TH17OHP, an adequate observation period and timing for repeat blood test are as yet unknown. It is difficult to differentiate between C21OHD and PORD by current standard hormonal testing and clinical observation *(3, 4)*. This 2-step method can diagnose TH17OHP, C21OHD, and PORD by 1 as-

say with a noninvasive spot urine sample while infants have increased concentrations of serum 17OHP.

Increased Ptl and Ptl/THEs in C21OHD and PORD must reflect the impaired activity of 21-hydroxylase. We showed that a specific cutoff of Ptl/THEs differentiated C21OHD and PORD from TH17OHP and controls with no overlap. In patients with C21OHD, impaired activity of 21-hydroxylase led to an increase in the precursor steroid 17OHP. As a result, Ptl, which is a metabolite of 21DOF, was increased. Because the activity of 21-hydroxylase in patients with PORD was impaired by less electron supply from POR, increased Ptl was reasonable.

Decreased 11HA in PORD must reflect the impaired activity of 17,20-lyase. The reason is unclear why the urinary 11OHAD4 metabolite was the only 1 among other metabolites of 17,20-lyase products that showed a difference between C21OHD and PORD, but the reason is probably that 11OHAD4 is of purely adrenal origin. DHEA metabolites showed tiny overlap between C21OHD and PORD that may derive from unexpectedly low DHEA metabolites in 3 patients with C21OHD. On the 1 hand, androgen metabolites showed a clear overlap that can be explained by androgen production in the backdoor pathway (Fig. 1, dashed arrow), which is proven in the tammar wallaby *(12)* and postulated in the human newborn *(13)*. Androsterone can be derived not only from AD4 and dihydrotestosterone in the conventional pathway but also from 5$\alpha$-pregnane-3$\alpha$,17$\alpha$-diol-20-one in the backdoor pathway *(12, 13)*. In PORD, androsterone could increase during early infancy *(13)*, resulting in overlap in androgen metabolites.

One may argue that PD, the ratio of PD to 17,20-lyase metabolites (e.g., 11HA, DHEA metabolites), or the ratio of 17$\alpha$-hydroxypregnenolone metabolite to DHEA metabolites *(13)* is discriminatory (Fig. 1). Unfortunately, the measurement of PD is sometimes problematic in our GC-MS method in newborns and we cannot calculate the concentration or the ratio. We have reported that the ratio of 17$\alpha$-hydroxypregnenolone metabolites to DHEA metabolites discriminated 22 patients with PORD from healthy infants *(13)*, whereas this ratio could not discriminate C21OHD and PORD completely in this cohort (data not shown).

This study had 4 limitations. First, nonclassic 21OHD was not included in study subjects. Nonclassic 21OHD is known to have lower blood 17OHP than C21OHD *(14, 15)*. Thus, it is conceivable that these individuals have lower Ptl/THEs and 11HA than C21OHD. Further studies are required to determine if the same cutoff can discriminate between nonclassic 21OHD and PORD. Second, the total number of affected infants, and specifically the number of very-low-birthweight infants (only 2), was limited in this study. Infants born with very low birthweight should have less hepatic enzyme activities such as 5$\beta$-reductase, 3$\alpha$-hydroxysteroid dehydrogenase, and 20$\alpha$-hydroxysteroid dehydrogenase, leading to less conversion of 21DOF to Ptl. Indeed, 1 patient with C21OHD who was born with low birthweight had a lower Ptl value than the cutoff. The third limitation is the random time at which urine samples were collected, although it is known that steroid metabolism starts to show diurnal variation around 2–3 months of age *(16)*. We unintentionally proved that we could use the urine sample whenever it is collected. As for the fourth limitation, our data in Japanese infants may not apply to other ethnic populations. Enzymatic residual activity in PORD has been reported to differ depending on genotype *(17)*. The common mutation was R457H in Japanese PORD *(7, 18)* and A287P in whites *(19)*. R457H has 1%–3% supporting activity for 17$\alpha$-hydroxylase and virtually no activity for 17,20-lyase compared to wild type, whereas A287P has 40% activity for 17$\alpha$-hydroxylase and about 20% for 17,20-lyase *(17, 19, 20)*. In fact, all subjects with PORD were Japanese with homozygous or heterozygous R457H mutation (Table 2). For PORD cohorts with higher 17,20-lyase activity (i.e., A287P), 11HA might not be as useful. Thus other cutoffs may be required for non-Japanese.

In conclusion, we demonstrated a 2-step biochemical differential diagnosis for C21OHD and PORD by urinary steroid metabolites such as Ptl, THEs, and 11HA. We believe that this 2-step biochemical diagnosis would be valuable for Japanese infants whose clinical differential diagnosis is difficult.

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**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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# Screening of *MAMLD1* Mutations in 70 Children with 46,XY DSD: Identification and Functional Analysis of Two New Mutations

**Nicolas Kalfa[1,2], Maki Fukami[3], Pascal Philibert[1], Francoise Audran[1], Catherine Pienkowski[4], Jacques Weill[5], Graziella Pinto[6], Sylvie Manouvrier[7], Michel Polak[6], Totsumo Ogata[3], Charles Sultan[1,2,8]***

**1** Service d'Hormonologie, Hôpital Lapeyronie, CHU de Montpellier et UM1, Montpellier, France, **2** Service de Chirurgie et Urologie Pédiatrique, Hôpital Lapeyronie, CHU de Montpellier et UM1, Montpellier, France, **3** Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo, Japan, **4** Unité d'Endocrinologie Pédiatrique, Hôpital des Enfants, CHU de Toulouse, Toulouse, France, **5** Clinique de Pédiatrie, Hôpital Jeanne de Flandre, CHU de Lille, Lille, France, **6** Unité d'Endocrinologie Pédiatrique, Hôpital Necker Enfants Malades, APHP, Paris, France, **7** Service de Génétique Clinique, Hôpital Jeanne de Flandre, CHU de Lille, Lille, France, **8** Unité d'Endocrinologie et Gynécologie Pédiatriques, Service de Pédiatrie, Hôpital Arnaud de Villeneuve et UM1, CHU de Montpellier, Montpellier, France

## Abstract

More than 50% of children with severe 46,XY disorders of sex development (DSD) do not have a definitive etiological diagnosis. Besides gonadal dysgenesis, defects in androgen biosynthesis, and abnormalities in androgen sensitivity, the Mastermind-like domain containing 1 (*MAMLD1*) gene, which was identified as critical for the development of male genitalia, may be implicated. The present study investigated whether *MAMLD1* is implicated in cases of severe 46,XY DSD and whether routine sequencing of *MAMLD1* should be performed in these patients. Seventy children with severe non-syndromic 46,XY DSD of unknown etiology were studied. One hundred and fifty healthy individuals were included as controls. Direct sequencing of the *MAMLD1, AR, SRD5A2* and *NR5A1* genes was performed. The transactivation function of the variant *MAMLD1* proteins was quantified by the luciferase method. Two new mutations were identified: p.S143X (c.428C>A) in a patient with scrotal hypospadias with microphallus and p.P384L (c.1151C>T) in a patient with penile hypospadias with microphallus. The *in vitro* functional study confirmed no residual transactivating function of the p.S143X mutant and a significantly reduced transactivation function of the p.P384L protein ($p = 0.0032$). The p.P359S, p.N662S and p.H347Q variants are also reported with particularly high frequency of the p.359T- p.662G haplotype in the DSD patients. Severe undervirilization in XY newborns can reveal mutations of *MAMLD1*. *MAMLD1* should be routinely sequenced in these patients with otherwise normal *AR, SRD5A2* and *NR5A1* genes.

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* E-mail: c-sultan@chu-montpellier.fr

## Introduction

The disorders of sex development (DSD) comprise a variety of anomalies defined by congenital conditions in which chromosomal, gonadal, or anatomical sex is atypical. The prevalence of the 46,XY disorders of sex development (46,XY DSD) is difficult to determine with accuracy because of the heterogeneity in the clinical presentation and the etiologies. The estimated incidence of severe 46,XY DSD with uncertain sex is 2.2 per 10,000 births [1], and for a minor form of 46,XY DSD with isolated and non-severe hypospadias, the incidence is estimated at 1 in 250–400 births [2]. Two independent surveillance systems in the United States, the nationwide Birth Defects Monitoring Program (BDMP) and the Metropolitan Atlanta Congenital Defects Program (MACDP), reported a near doubling in the hypospadias rate in comparison with the immediately preceding decades [3]. Although recent studies have questioned this reported rise and provide conflicting data [4,5], the elucidation of the pathophysiology of these genital malformations remains challenging.

The etiologies of 46,XY DSD are usually gonadal dysgenesis (defect in *SRY* and downstream genes such as *SOX9, WT1, NR5A1* [6,7], etc.), defects in androgen biosynthesis and, more frequently, abnormalities in androgen sensitivity. Unfortunately, more than 50% of children with severe 46,XY DSD presenting with uncertain sex do not have a definitive clinical diagnosis [8]. For instance, an *AR* gene defect is identified in less than 10% of the cases [9].

In addition to these well classified causes, a recent candidate gene was identified as critical for the development of male genitalia: the Mastermind-like domain containing 1 (*MAMLD1*) gene (formerly *CXorf6*). This gene was discovered during studies to find the gene responsible for X-linked myotubular myopathy, *MTM1*, which maps to proximal Xq28 [10]: *MAMLD1* was observed to be deleted in patients with both the myopathy and external genital malformations [10,11,12]. Polymorphisms of *MAMLD1* have been reported in patients with isolated hypospadias, the less severe form of 46,XY DSD, but these variants usually

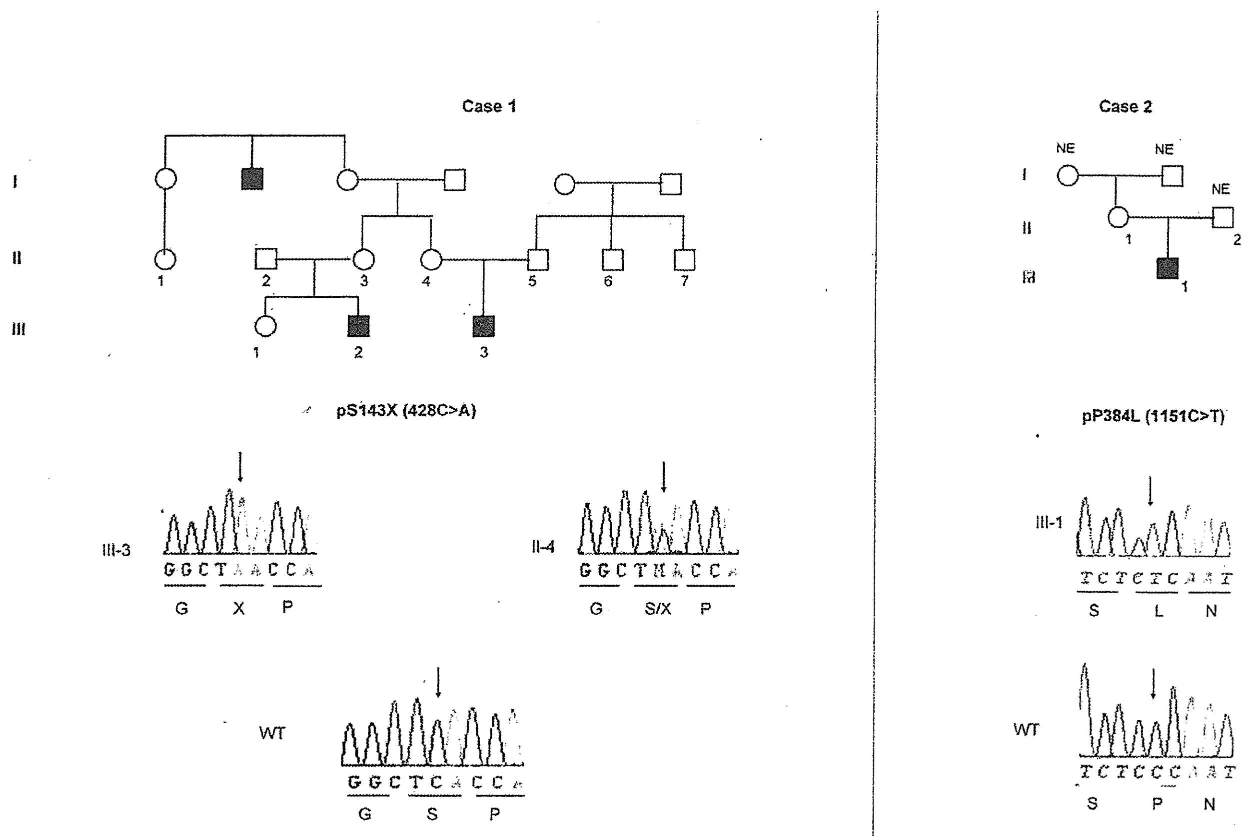


**Figure 1. Electrochromatograms and pedigrees of the three patients with *MAMLD1* mutations.** The black squares indicate patients with posterior hypospadias. All mutant sequences were controlled by wildtype (WT) DNA. Regarding case 1's family, only the members III-3 and II-4 were genotyped, as the other members in the pedigree declined genetic testing.
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do not affect the transactivation of the protein [13,14]. Conversely, severe 46,XY DSD with uncertain sex has been sparsely studied. To date, only one study has focused on these patients: Fukami et al. identified three nonsense mutations in four individuals from a group of 166 patients [15]. The aim of the present study was to determine whether *MAMLD1* is frequently implicated in newborns and children with severe 46,XY DSD with uncertain sex and whether *MAMLD1* should be routinely sequenced in these patients.

## Materials and Methods

### Patients and controls

Two hundred and twenty individuals were included in this study. Seventy children presented with non-syndromic 46,XY DSD of unknown etiology. According to the Quigley classification [16], 8 patients exhibited a stage 2 phenotype; 32 patients, stage 3; 20 patients, stage 4; 5 patients, stage 5; and 5 patients, stage 6. One hundred and fifty healthy individuals were included as controls. Controls were chosen among patients without urinary, genital, or endocrine disease, or any other congenital malformation. For instance, patients with acute appendicitis or operated on for circumcision without phimosis were included. This study was approved by the Institutional Review Board (CPP-Montpellier, ID RCB No. 2008-A00781-54). Written consent was obtained from the parents, carers or guardians on behalf of the participating minors. When a mutation was identified, other family members were examined if possible. The patients and controls were Caucasian.

### DNA extraction

DNA was extracted from peripheral blood using a QIAamp DNA blood minikit (Qiagen, Courtaboeuf, France).

### Mutational analysis of MAMLD1

Direct sequencing of *MAMLD1* coding exons and their flanking splice sites was performed in all patients and controls using primers as previously described [17]. The 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) was used. Sequencing reactions were repeated twice with at least two different PCR products. The DNA sequences were compared with the sequences of normal controls and the reference genomes from the ensembl.org database (Ensembl: ENSG00000013619) and the genebank database (MIM: 300120, NCBI Gene ID: 10046). It is notable that the number of the cDNA and amino acids has been changed recently because of the recognition of a novel *MAMLD1* start codon. This report describes *MAMLD1* cDNA and amino acids according to the new system.

### Molecular analysis of androgen sensitivity

A molecular analysis of the androgen receptor (*AR*) and 5 alpha reductase type 2 (*SRD5A2*) genes was performed in all patients.

**Table 1.** Clinical and hormonal data of patients with mutated *MAMLD1*.

| Patient | Case 1 | Case 2 |
|---|---|---|
| *MAMLD1* mutation | pS143X | pP384L |
| Previous medical history | None | Maternal diabetes |
| Genital phenotype | | |
| Urethral meatus | Scrotal | Penile posterior |
| Age at exam (yr,mo) | 0,0 | 0,0 |
| Microphallus | Yes, 20 mm | Yes, 20 mm with cuvature |
| Testis position | Intra-scrotal | Intra-scrotal |
| Testis size (normal = 1–2 ml) | Normal | Normal |
| Scrotal appearance | Ventral transposition, Bifid Scrotum | Bifid Scrotum |
| Renal and urinary tract structure | Normal | Normal |
| Extragenital phenotype | Normal | Normal |
| Growth | | |
| Birth height, cm (SDS) | 51 (+0) | 50.5 (+0) |
| Birth weight, Kg (SDS) | 3.540 (+0) | 3.750 (+0.5) |
| Serum hormone level | | |
| Time of measurment (yr,mo) | 0,0 | 0,3 |
| Testosterone (ng/ml) (1–3 ng/ml) | 1.78 | <0.07 |
| LH (UI/l) (1–12 UI/l) | 10 | 0.3 |
| FSH (UI/l) (1–10 UI/l) | 0.8 | 0.8 |
| AMH | 336 ng/ml | 19 ng/ml* |
| Inhibin | NA | <15 ng/ml* |

SD: standard deviation. ND: not determined. NA: not available. DHT: dihydrotestosterone. DHEA: dihydroepiandrsosterone. Parentheses indicate the standard deviation for height and weight and the normal range for hormone serum levels. Testes of 1–2 ml can be regarded as normal, as recently reported by Shibata et al. [34].
*It is notable that anti-mullerian hormone and inhibin were lowered in one case. *MAMLD1* is indeed reported to be expressed in Sertoli cells, as well [15].
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Exons 1–8 of the *AR* gene were amplified by PCR using sets of primers and reactions previously described [18]. Molecular analysis of the *SRD5A2* gene (exons 1–5) was performed as previously reported [19]. PCRs were verified for correct length on agarose gel, purified with Qiaquick PCR columns (Qiagen), and sequenced with the ABI Prism Big Dye terminator sequencing kit. *NR5A1*was sequenced in 46,XY DSD children with low plasma testosterone as previously published [6,20].

## Homology study

Ensembl.org detected the putative homologs of the human *MAMLD1* gene and alignments were made with the ClustalW software at http://www.ebi.ac.uk/Tools/msa/clustalw2/.

## Structure prediction

The potential impact of variants was first predicted using X *in-silico* tools for secondary structure, tertiary structure and prediction of the consequences of amino acid changes.

The secondary structure for wildtype and variants was predicted using JPred software [21] (http://www.compbio.dundee.ac.uk/www-jpred/). The relative accessibility of amino acids was studied with Netsurf software [22] (http://www.cbs.dtu.dk/services/NetSurfP/). The three-dimensional structure was predicted by the Protein Homology/analogY Recognition Engine (PhyreEngine) from the Structural Bioinformatics Group, Imperial College, London, at http:www.sbg.bio.ic.ac.uk/phyrew/. This tool can detect remote homologous proteins with similar tertiary structures, based on multiple sequence profiles with structure-based profiles [23].

The functional consequences of amino acid changes were predicted using four algorithms. Polyphen (Harvard, USA) [24,25], Panther [26], Sift (University of British Columbia) [27] and SNP-3D (University of Maryland) [28] were used, respectively, at http://genetics.bwh.harvard.edu/pph/, http://www.pantherdb.org/tools/csnpScoreForm.jsp., http://sift.jcvi.org/, and http://www.snps3d.org/modules.php?name=Search&op=advanced%20search. These algorithms are based on the alignment of orthologous and/or paralogous protein sequences and/or structural constraints.

## Transactivation analysis of MAMLD1

The transactivation function of the variant MAMLD1 proteins was analyzed by the luciferase method [29]. We used the previously reported luciferase reporter vector containing the promoter sequence of mouse hairy/enhancer of split 3 (*Hes3*) (−2,715∼+261 bp) [30] and expression vectors containing cDNAs for wildtype *MAMLD1*, p.S143X and p.P384L [29]. Mouse Leydig tumor (MLTC1) cells (ATCC, CRL-2065) seeded in 12-well dishes ($0.5–1.0\times10^5$ cells/well) were transiently transfected using Lipofectamine 2000 (Invitrogen) with 0.6 µg of luciferase reporter vector and 0.6 µg of expression vector for wildtype or variant *MAMLD1*, together with 20 ng of pRL-CMV vector (Promega) used as an internal control. As a control for the expression vectors, an empty counterpart vector was transfected. Luciferase assays performed with a Lumat LB9507 (Berthold) 48 hours after transfection were repeated three times.

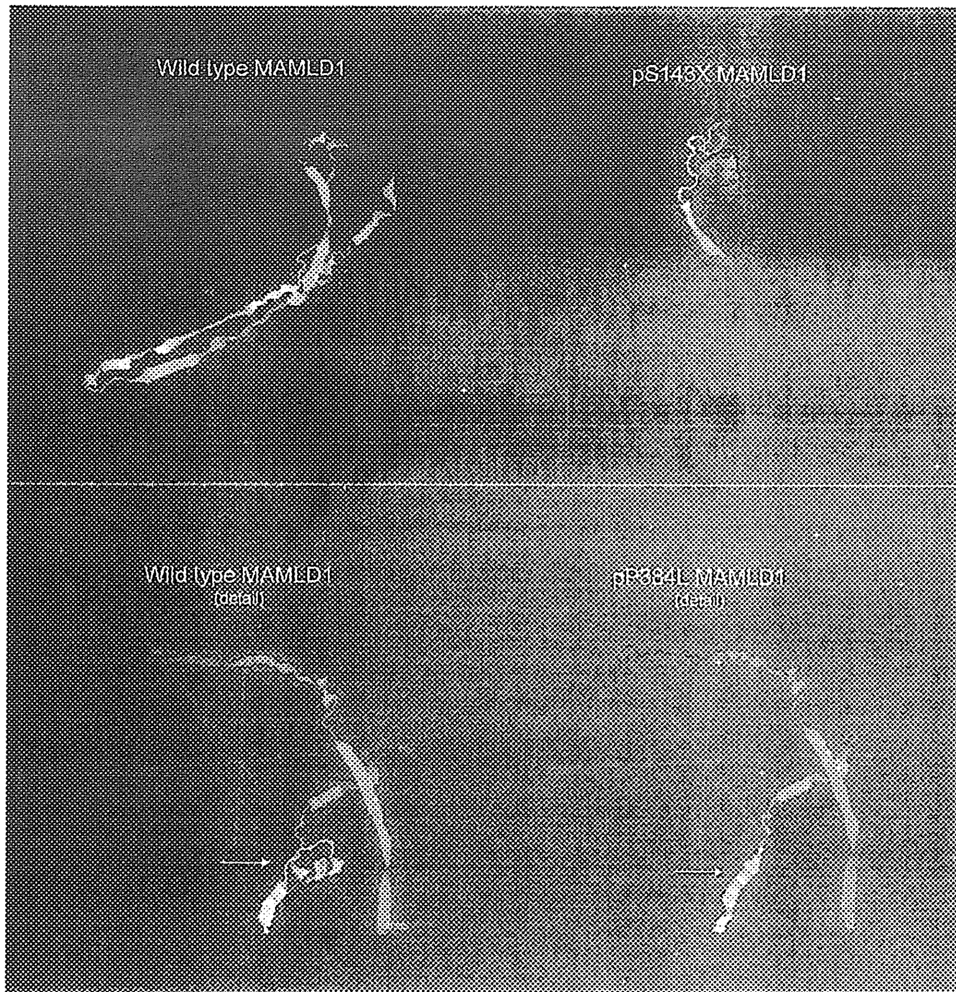


**Figure 2. Tertiary structure prediction of the wildtype protein (left column) and with the mutants.** 3D structure was predicted at Protein Homology/analogY Recognition Engine (PhyreEngine) from the Structural Bioinformatics Group, Imperial College, London, at http:www.sbg.bio.ic.ac.uk/phyre~/. The plain arrows show the changes in the shape of the protein between the wildtype and p.P384L.
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## Statistical methods

Haplotype frequencies were compared between cases and controls using the $\chi^2$ test and the Fisher test on SPSS 16.0 software. The odds ratio (OR) was also considered with the logit confidence intervals method: $OR-CI = e^{LN(OR)\pm 1.96\left(\frac{1}{A}+\frac{1}{B}+\frac{1}{C}+\frac{1}{D}\right)^{0.5}}$. Hapmap and ensembl.org were used to exclude linkage disequilibrium. Regarding the transactivation analysis of *MAMLD1*, the results are expressed using the mean and SD, and statistical significance was determined by the *t*-test.

# Results

## Mutations of MAMLD1 and functional analyses

Among the 70 newborns and children with 46,XY DSD, two new mutations were identified in two unrelated patients: p.S143X (c.428C>A) and p.P384L (c.1151C>T) (Fig. 1). The clinical and genetic data are summarized in Table 1. None of these mutations was noted in the control group. The sequences of the *AR, SRD5A2* and *NR5A1* genes were normal in these patients.

a- The p.S143X mutation was predicted to cause a short and truncated protein. The *in silico* prediction showed profoundly modified amino acid accessibility and 3D structure. Relative surface accessibility and absolute surface accessibility of the last amino acid changed from 0.248 to 0.834 and from 29.124 to 97.721, respectively. PhyreEngine predicted the loss of any functional site without a residual consensus sequence (no homologous sequence over 5% through whole genome) (Fig. 2). The *in vitro* functional study confirmed no residual transactivating function of the mutant (Fig. 3). Interestingly, a maternal uncle and a maternal cousin of the index case both exhibited severe hypospadias (not available for genetic testing). The mother was indeed heterozygous for the mutation (Fig. 1).

b- The p.P384L mutation was found in a patient with posterior penile hypospadias and microphallus. No cryptrochidism was noted. The secondary structure was predicted to be changed in the next four amino acids. The relative and absolute accessibilities of the amino acid were modified from 0.27 to 0.35 and from 39.07 to 65.25, respectively. The 3D structure prediction of the mutated protein was significantly changed (Fig. 2). All four *in silico*

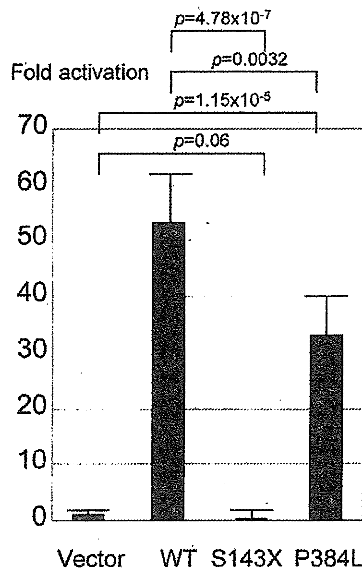


**Figure 3. Transactivation function of the variants of the MAMLD1 protein analyzed by the luciferase method.** The activity is evaluated for pHes3-luc vector.
doi:10.1371/journal.pone.0032505.g003

algorithms predicted affected protein function (Table 2) with a conserved amino acid throughout species (Table 3). Functional studies confirmed the significantly reduced transactivation function of the p.P384L protein with 60% residual activity when compared with the wildtype protein, $p = 0.0032$ (Fig. 3).

### Polymorphisms of MAMLD1

We identified three polymorphisms of *MAMLD1* in our series: p.P359S (c.1075C>T, rs41313406), p.N662S (c.1985A>G, rs2073043) and p.H347Q (c.1041C>A, rs62641609). Regarding the p.P359S and p.N662S polymorphisms, 14 patients exhibited double polymorphisms (S-S haplotype) and five had the p.N359S polymorphism. The phenotypes of the patients with the S-S haplotype were as follows: penile posterior hypospadias and cryptorchidism in three cases, hypospadias and microphallus in five cases (anterior n = 1, penile posterior n = 2 and scrotal hypospadias n = 2), and cryptorchidism and microphallus in six cases (bilateral cryptorchidism n = 5, unilateral cryptorchidism n = 1). Using hapmap and ensembl.org, no linkage disequilibrium was found for these two variants. In previous studies, we and others found that the S-S haplotype was present in only 6/150 controls (4.0%) and 23/360 controls (6.4%) [13,14]. By combining the published series for controls (matched patients and controls), we determined that the incidence of the S-S haplotype was higher in the DSD patients (20%, n = 70 vs. 6%, n = 510, $p = 0.0003$) (OR = 3.86, CI from 1.94 to 7.70, $p = 0.05$). Haplotypes and their relative frequencies in each group of patients are summarized in Table 4.

The p.H347Q variant, previously reported as a polymorphism especially in sub-Saharan populations (rs62641609, http://www.ensembl.org/Homo_sapiens/Variation/Summary?r=X:149638386-149639386;v=rs62641609;vdb=variation;vf=16740729), was identified in a patient with posterior hypospadias and microphallus (25 mm length at birth).

**Table 2.** Prediction of affected protein function using four algorithms.

| Algorithm | pP384L |
| --- | --- |
| Polyphen | Probably damaging |
| | score = 0.961 (sensitivity: 0.71; specificity: 0.93) |
| Sift | Affect protein function |
| | Sift score = 0.04 |
| Panther | Probability of deleterious effect = 0.42 |
| | (subPSEC score = −2.7) |
| SNPS3D | Deleterious |
| | (svm score = −1.75) |

References and online access are indicated in the text. Mathematical calculation of the significance of each score is available online.
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## Discussion

*MAMLD1* is a good candidate to explore in patients with unexplained 46,XY DSD, as it has been shown to be expressed in fetal Leydig cells around the critical period for sex development [15]. The transient knockdown of *MAMLD1* mRNA expression results in significantly reduced testosterone production in mouse Leydig tumor cells [29]. *MAMLD1* is further coexpressed with steroidogenic factor (*NR5A1*), which regulates the transcription of genes involved in sex development, and an *NR5A1* target site was found within the *MAMLD1* gene [29,31]. *MAMLD1* thus seems to have an important role in modulating testosterone production during sex development and is involved in the 46,XY disorders of sex development [32].

Regarding the minor forms of 46,XY DSD with isolated and non-severe hypospadias, mutational studies of *MAMLD1* have identified several polymorphisms in this gene. We reported the following variants in patients with isolated hypospadias: p.P359S, p.V505A, p.N662S and p.604ins3Q [13,17], all of which were recently confirmed as polymorphisms [14]. The p.Q602K mutation was also found in one patient with posterior hypospadias and was predicted to affect the splicing process. An association between isolated hypospadias and the rare haplotype p.P359S-p.N662S is also suspected [13,14].

**Table 3.** Homology study showed that this amino acid was highly conserved through species for the c.1041C>A and c.1151C>T mutations.

| | |
| --- | --- |
| Patient | MSSNTLSGSTLRGS**L**NALLSSMTSSSNAAL |
| Human-MAMLD1 | MSSNTLSGSTLRGS**P**NALLSSMTSSSNAAL |
| Pig | MSSSSLPGSTLHGS**P**GALLSSGAPSSSSAL |
| Horse | MSSSNLPGSTLQGS**P**NALLSSMVSGSSAAL |
| Chimpanzee | MSSNTLSGSTLRGS**P**NALLSSMTSSSNAAL |
| Mouse | MSSSSLSGSAVQSS**P**NALLSSMAPSSNASL |
| Rabbit | MAPHSLPGSSLQGS**P**NALLSSMAPNSSGAL |
| Dog | MASSNLPGSSFQAS**P**NALLASMASASSAGL |
| Cat | MASGNLPGSAFQGS**P**NALLASMASGSSAAL |

doi:10.1371/journal.pone.0032505.t003

**Table 4.** Incidence of exonic polymorphisms p.P359S and p.N662S, and relative haplotypes in normal controls and 46,XY DSD patients.

| Haplotype 359–662 | Patients, n = 70 | Controls, n = 510 | Fisher, p value | OR | OR confidence interval (p = 0.05) |
|---|---|---|---|---|---|
| p.359C- p.662A | 72.9% (n = 51) | 90.6% (n = 462) | p = 0.0001 | 0.28 | 0.15–0.51 |
| p.359T- p.662A | 0% | 1.5% (n = 8) | p = 0.60 | 0.42 | 0.02–7.35 |
| p.359C- p.662G | 7.1% (n = 5) | 0.8% (n = 9) | p = 0.02 | 4.28 | 1.39–13.17 |
| p.359T- p.662G (S-S polymorphism) | 20% (n = 14) | 6% (n = 31) | p = 0.0003 | 3.86 | 1.94–7.70 |

Controls are combined with the published series (matched for ethnicity of patients and controls) [13] [14]. The $\chi$-square test was performed. When combining all patients with the p.662G polymorphism whatever the p.359 allele, this p.662G was significantly more frequent in 46,XY DSD patients: 27.1% (n = 19) vs. 6.8% (n = 40), $p = 0.0001$.
doi:10.1371/journal.pone.0032505.t004

Regarding severe 46,XY DSD with uncertain sex, only one published paper to date has reported three *MAMLD1* mutations (p.E124X, p.Q197X and p.R653X) [15]. It is precisely in this situation of severe genital malformation that the diagnosis of the causative mechanism is of clinical interest for medical treatment (hormone substitution, pubertal follow-up). In order to determine whether this report was an exceptional observation or of practical clinical interest, we screened 70 patients with severe 46,XY DSD of unknown origin. We identified two new mutations of *MAMLD1* in patients with severe hypospadias and microphallus (1 stop codon and 1 missense mutation). These mutations were associated with a severe phenotype, and reduced (p.P384L) or abolished (p.S143X) transactivation function was found in two cases. 46,XY DSD with normal *AR, SRD5A2* and *NR5A1*gene sequences can thus reveal a mutation of *MAMLD1*. This finding suggests a new diagnostic investigation for these patients and may be helpful in genetic counselling if a mutation is identified. It also provides new insight into the pathophysiology of DSD. Indeed, in the family of the child bearing the p.S143X mutation, the mother was heterozygous and two other males on the maternal side of the family exhibited a consistent phenotype. Unfortunately, the family declined any further investigation.

The mechanisms by which these mutations with reduced transactivation induce DSD are still under investigation. As noted above, several studies have provided strong evidence of *MAMLD1* implication in fetal sex development through modulation of testosterone production at the time of sex differentiation. The plasma testosterone measured in one of our cases was indeed lowered but it was normal in the other one, as previously reported in patients with nonsense mutations [15]. Plasma testosterone evaluation is thus not systematically helpful in orienting the diagnosis of DSD since mutations of the genes implicated in testosterone production - such as *MAMLD1* and *NR5A1* - have been reported in 46,XY DSD patients with normal plasma testosterone. These findings, along with the absence of correlation between the *in vitro* functional analysis and the biological and clinical phenotype, suggest that the genital malformation is primarily related to a transient prenatal testicular (Leydig cell) dysfunction and the resulting compromised testosterone production around the critical period of sex differentiation [33]. In the postnatal period, the mouse homolog of *MAMLD1* was indeed reported to be weakly expressed in the testis at one week of age and the expression was faint thereafter.

We also report a high incidence of the rare haplotype p.P359S-p.N662S in our series. The p.P359S (which was designated p.P286S in the previous report) variant was first reported in a patient with hypospadias but it was absent in his brother and nephew with the same phenotype [15]. The p.N662S (which was designated p.P589S in the previous report) variant was found in hypospadiac patients but was also reported in a normal population, although with low incidence [15]. We and others have found that the S-S haplotype is associated with a minor form of DSD, i.e., isolated hypospadias [14], but the *in vitro* functional study of the p.P359S-p.N662S *MAMLD1* variant was inconclusive with unchanged transactivation function [13]. In the present study, we show that the combination of these alleles was present in as much as 15% of patients with severe 46,XY DSD. This is significantly higher than in the controls [combining the series, 15% (n = 70) vs. 10.7% (n = 510), $p = 0.0003$]. Again, a transient testosterone production failure during prenatal development may have contributed to the undervirilization of the external genitalia, but how this haplotype can be present in normal, mild and severe phenotypes remains to be elucidated.

Severe undervirilization in XY newborns can reveal mutations of *MAMLD1*. *MAMLD1* should be routinely sequenced in these patients with otherwise normal *AR, SRD5A2* and *NR5A1* genes.

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## Author Contributions

Conceived and designed the experiments: NK MF CS TO PP. Performed the experiments: NK MF PP FA. Analyzed the data: NK MF PP FA CP JW GP SM MP. Contributed reagents/materials/analysis tools: CP JW GP SM MP. Wrote the paper: NK TO CS PP FA.

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# Gradual Loss of ACTH Due to a Novel Mutation in *LHX4*: Comprehensive Mutation Screening in Japanese Patients with Congenital Hypopituitarism

**Masaki Takagi[1,2], Tomohiro Ishii[1], Mikako Inokuchi[1], Naoko Amano[1], Satoshi Narumi[1], Yumi Asakura[3], Koji Muroya[3], Yukihiro Hasegawa[2], Masanori Adachi[3], Tomonobu Hasegawa[1]***

**1** Department of Pediatrics, Keio University School of Medicine Tokyo, Japan, **2** Department of Endocrinology and Metabolism, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan, **3** Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Kanagawa, Japan

## Abstract

Mutations in transcription factors genes, which are well regulated spatially and temporally in the pituitary gland, result in congenital hypopituitarism (CH) in humans. The prevalence of CH attributable to transcription factor mutations appears to be rare and varies among populations. This study aimed to define the prevalence of CH in terms of nine CH-associated genes among Japanese patients. We enrolled 91 Japanese CH patients for DNA sequencing of *POU1F1, PROP1, HESX1, LHX3, LHX4, SOX2, SOX3, OTX2*, and *GLI2*. Additionally, gene copy numbers for *POU1F1, PROP1, HESX1, LHX3*, and *LHX4* were examined by multiplex ligation-dependent probe amplification. The gene regulatory properties of mutant LHX4 proteins were characterized *in vitro*. We identified two novel heterozygous *LHX4* mutations, namely c.249-1G>A, p.V75I, and one common *POU1F1* mutation, p.R271W. The patient harboring the c.249-1G>A mutation exhibited isolated growth hormone deficiency at diagnosis and a gradual loss of ACTH, whereas the patient with the p.V75I mutation exhibited multiple pituitary hormone deficiency. *In vitro* experiments showed that both *LHX4* mutations were associated with an impairment of the transactivation capacities of *POU1F1* and*αGSU*, without any dominant-negative effects. The total mutation prevalence in Japanese CH patients was 3.3%. This study is the first to describe, a gradual loss of ACTH in a patient carrying an *LHX4* mutation. Careful monitoring of hypothalamic–pituitary -adrenal function is recommended for CH patients with *LHX4* mutations.

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* E-mail: thaseg@a6.keio.jp

## Introduction

The proliferation and terminal differentiation of the anterior pituitary gland is strongly influenced by the precise spatial and temporal expression of transcription factors [1–3]. Mutations in these transcription factors often result in various types of congenital hypopituitarism (CH) [1–3]. Although previous studies have shown that these transcriptional factor mutations are rare among CH patients and that the mutation prevalence varies among populations, only a few genetic screening studies have been conducted. Graaff *et al.* identified a single patient with a *POU1F1* mutation from a study population of 79 multiple pituitary hormone deficiency (MPHD) patients (1.2%) in The Netherlands [4], and Dateki *et al.* reported one patient harboring an *LHX4* gross deletion from a cohort of 71 MPHD patients (1.4%) in Japan [5]. On the other hand, Reynaud *et al.* reported a mutation prevalence of 13.3% in a study population of 165 MPHD patients from the international GENHYPOPIT network [6]. Approximately 90% of the mutations identified in this report were *PROP1* common mutations (149delGA and 296delGA). Although the 296delGA mutation represents a mutational hot spot within the *PROP1* gene rather than a common founder mutation [7], studies from other ethnic groups often report a low prevalence of *PROP1* mutations [8,9].

This study aimed to determine the prevalence of transcription factor mutations in Japanese CH patients with PCR-based sequencing of nine CH-associated genes, namely *POU1F1, PROP1, HESX1, LHX3, LHX4, SOX2, SOX3, OTX2*, and *GLI2*. Additionally, we examined the gene copy numbers of *POU1F1, PROP1, HESX1, LHX3*, and *LHX4* by multiplex ligation-dependent probe amplification (MLPA).

## Materials and Methods

### Subjects

This study population consisted of 91 patients with GH-treated CH. The inclusion criteria were as follows: 1) short statue with severe GH deficiency (GH peak $<$ 3 ng/mL) confirmed by hypoglycemic provocation test, and 2) anterior pituitary hypoplasia as detected by brain magnetic resonance imaging (MRI). We excluded any CH patients of known cause, such as a brain tumor or brain surgery from this study. Patients or parents of patients under 18 years of age gave their written informed consent to

**Table 1.** Endocrine phenotype of 91 probands screened for 9 genes.

| | No. (%) with deficiencies of | | | |
|---|---|---|---|---|
| | GH | TSH | ACTH | LH/FSH |
| IGHD (n = 14) | 14(100) | | | |
| MPHD (n = 77) | 77(100) | 61(79) | 34(44) | 19(24) |

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participate in this study, which was approved by the Institutional Review Board of Keio University School of Medicine and the Institutional Review Board of Kanagawa Children's Medical Center.

## Endocrinological investigations

Hormonal assays were performed using several commercial RIA kits, and normal values for each center were taken into account. The results of biochemical investigations at diagnosis were recorded including basal free thyroxine (fT4), TSH, cortisol and ACTH levels, their peaks in response to pituitary stimulation tests. The patients were evaluated for serum GH level after two consecutive classical provocative tests (with arginine or insulin). GH peaks <6 ng/mL after stimuli support a diagnosis of GHD. GH peak < 3 ng/mL by hypoglycemic provocation test define severe GHD. A diagnosis of TSH deficiency was made if serum fT4 concentration was under the normal level (fT4 < 1.0 ng/dL) with inadequate low serum TSH concentration. Cortisol peaks <17 μg/dL by hypoglycemic provocation tests define ACTH deficiency. FSH–LH deficiency was diagnosed on the basis of delayed or absent pubertal development and inadequate increase in serum FSH and LH in response to LHRH.

## Imaging investigations

MRI included T1 and T2 weighted high-resolution pituitary imaging through the hypothalamo-pituitary axis (T1 sagittal 3-mm slices, T1 and T2 coronal 3-mm slices). Details noted included the size of the anterior pituitary, position of the posterior pituitary signal, presence and morphology of the optic nerves, optic chiasm, pituitary stalk, septum pellucidum, and corpus callosum.

## Mutation screening

For all patients, regardless the phenotype/pituitary MRI findings, we analyzed all coding exons and flanking introns of *POU1F1, PROP1, HESX1, LHX3, LHX4, OTX2, SOX2, SOX3*, and *GLI2* by PCR-based sequencing. We screened for deletion/duplication involving *POU1F1, PROP1, HESX1, LHX3*, and *LHX4* by MLPA analyses (SALSA MLPA KIT P216; MRC-Holland, Amsterdam, The Netherlands). We tested any detected sequence variations against 150 Japanese control subjects.

## RT-PCR

For mRNA analysis of the *LHX4* c.249-1G>A mutation, total RNA was extracted from Epstein-Barr virus-transformed lymphocytes derived from the propositus of pedigree 1. The cDNA produced from reverse transcription of RNA was subjected to PCR amplification using primers encompassing exons 2 to 4, and were subsequently processed for direct sequencing.

## Functional studies

We performed functional studies on the two novel *LHX4* mutations (p.R84X and p.V75I). To generate LHX4 expression vectors, LHX4 cDNA was cloned into pCMV-myc and pEGFP-N1 (Clontech, Palo Alto, CA). We introduced the two mutations by site-directed mutagenesis, using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Otsu, Japan). The luciferase reporter vectors were constructed by inserting the promoter sequences of *POU1F1* (*PIT1*),*αGSU* into a pGL3 basic vector (Promega, Madison, WI). A transactivation assay was performed using dual-luciferase reporter assay system (Promega) on COS7 and GH3 cells. For western blot analyses, we harvested COS7 cells transfected with the myc-tagged LHX4. Western blotting was performed with a mouse anti-myc monoclonal antibody (Invitrogen, Carlsbad, CA). For subcellular localization analyses, we visualized and photographed COS7 cells transfected with GFP-tagged LHX4 using a Leica TCS-SP5 laser scanning confocal microscope (Leica, Exton, PA). The sequences of the biotin-labeled doublestranded oligonucleotide used as probe in the EMSA experiment was 5′-GTATGAATCATTAATTGA-CAACATATTTTC-3′, as described previously [10]. The probes were detected with the Lightshift chemiluminescent EMSA kit (Pierce) according to the manufacturer's instruction.

# Results

## Patient details

Of the 91 patients, on the basis of hormonal deficiencies, 14 were determined to have isolated GH deficiency (IGHD), whereas 77 were MPHD. Detailed endocrine phenotype was available in all of the 91patients (Table 1). Results of the MRI scans were available in all patients with IGHD and MPHD. Details regarding the structural abnormalities of the hypothalamo-pituitary axis on neuroimaging in the probands are shown in Table 2. Among 77 MPHD patients, 12 were diagnosed as Septo-optic dysplasia.

## Mutation screening

We identified two novel heterozygous *LHX4* mutations, namely c.249-1G>A, expected to cause exon skipping, and c.223G>A (p.V75I), and one common heterozygous *POU1F1* mutation,

**Table 2.** Results of MR scans of probands screened for 9 genes.

| | Morphology of | | | | | | |
|---|---|---|---|---|---|---|---|
| | Anterior pituitary | Posterior pituitary | | | Stalk | | |
| | Hypoplasia | Normal | Ectopic | Absent | Normal | Invisible | Thin |
| IGHD (n = 14) | 14 | 5 | 9 | 0 | 4 | 5 | 5 |
| MPHD (n = 77) | 77 | 24 | 51 | 2 | 23 | 25 | 29 |
| Total (n = 91) | 91 | 29 | 60 | 2 | 27 | 30 | 34 |

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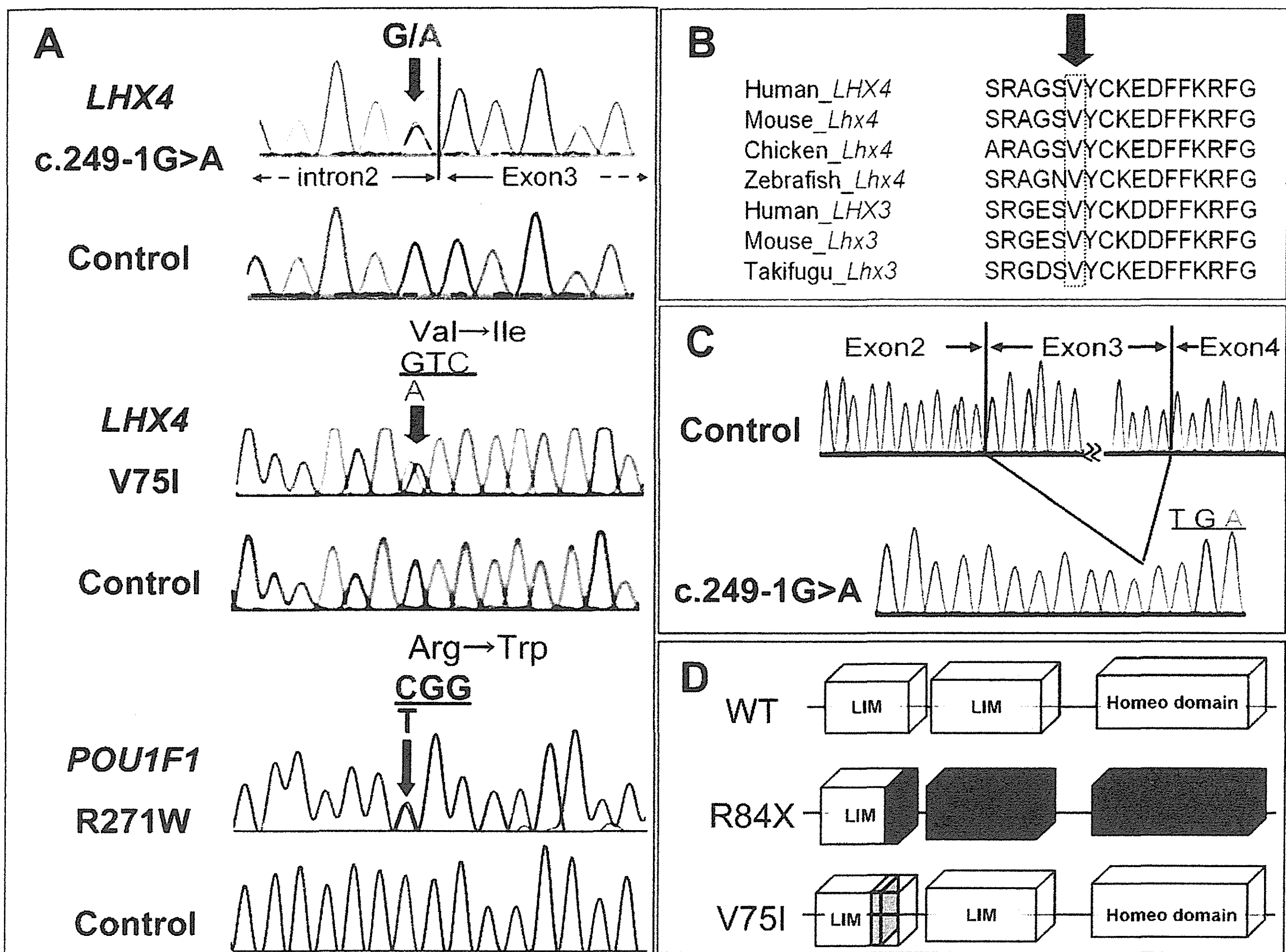


**Figure 1. Identification of sequence variations of *LHX4* and *POU1F1*.** *A*, Partial sequences of PCR products of the patients are shown. The upper chromatogram represents a heterozygous G to A substitution in the splice acceptor site of exon3. The middle chromatogram represents a heterozygous substitution of isoleucine (ATC) in place of valine (GTC) at codon 75. The arrow indicates the mutated nucleotide. The lower chromatogram represents a heterozygous substitution of tryptophan (TGG) in place of arginine (CGG) at codon 271. The arrow indicates the mutated nucleotide. *B*, Homology study showed valine at codon 75 is highly conserved through species in LHX4 and LHX3. C, Identification of exon3 skipping in the LHX4 cDNA derived from propositus of pedigree 1. LHX4 transcript with a deleted exon 3 creates a premature stop codon at the beginning of the remaining exon 4 (p.R84X). *D*, Schematic diagrams of the LHX4 protein. LHX4 cDNA encodes two LIM domains and one homeodomain. LHX4 with a p.R84X mutation results in the deletion of one of the two LIM domains and the entire homeodomain. Val75 is located within the first LIM domain.
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c.811C>T (p.R271W) [11] (FIG. 1A). The V75 in LHX4 is evolutionarily highly conserved (FIG. 1B), and these two *LHX4* mutations were not detected in any of the 150 healthy Japanese controls. We detected no gross or exon-level deletions/duplications using the MLPA analyses. For 14 IGHD patients, we additionally analyzed all coding exons and flanking introns of *GH1*, and *GHRHR* by PCR-based sequencing and MLPA (SALSA MLPA KIT P216 included all exons of *GH1* and *GHRHR*), failing to detect any sequence variation.

## RT-PCR

The RT-PCR generated a product of smaller size than that obtained from a control sample. Sequencing revealed that it corresponded to a LHX4 transcript skipping exon 3 (FIG. 1C). If translated, this abnormal transcript would generate a protein lacking one of the two LIM domains (LD) and the entire homeodomain (HD), p.R84X (FIG. 1D).

## Clinical phenotypes of the mutation carriers

**Pedigree 1: *LHX4* c.249-1G>A (FIG. 2A).** The propositus was a 16-year-old Japanese female, who was born at 39 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 51.0 cm (1.2 SD) and weight 3.3 kg (0.6 SD). She was referred to us at 5 years of age because of short stature. Her height was 92.4 cm (-3.6 SD). Endocrine studies indicated that the patient had IGHD (Table 3). Brain MRI showed anterior pituitary hypoplasia, with a visible but thin stalk, and an ectopic posterior pituitary gland (EPP). No other central nervous system abnormalities were visualized. Recombinant human GH therapy was started at age 6. Her growth was responded well to GH replacement. Although she had no definite episode of adrenal insufficiency, longitudinal data showed that her blood cortisol peak, after stimulation by hypoglycemia with insulin tolerance tests, decreased gradually with age (20.5, 17.5, 16.4, and 10.0 μg/dL, at ages of 5, 13, 14, and 15 years, respectively, Ref. >17 μg/

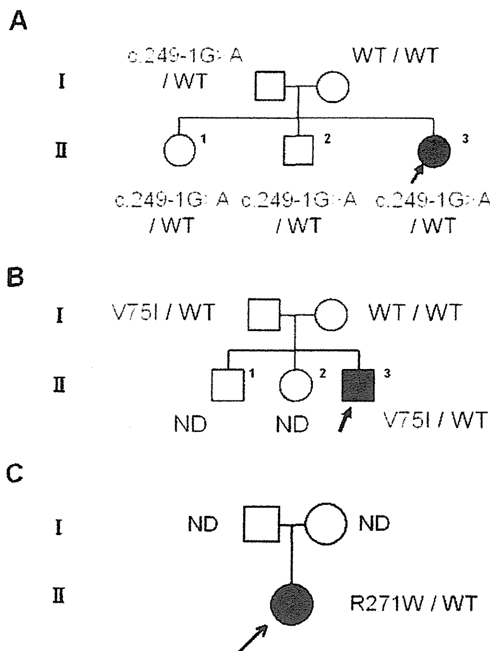


**Figure 2. The pedigrees of the affected families.** *A–C*, Pedigrees of families 1–3. Arrow indicates the propositus. ND: not determined.
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dL [12]), indicating of a gradual loss of ACTH. Follow-up MRI showed no changes as compared with the initial finding.

The father of the patient was 153.0 cm (-2.9 SD) tall, and the mother was 160.8 cm (0.5SD) tall. The elder brother and sister of the patient, both reached normal adult heights of 171.7 cm (0.2 SD) and 152.1 cm (-1.3 SD), respectively. Genetic analyses showed that the propositus, siblings and father carried the heterozygous *LHX4* c.249-1G>A mutation. No family members had any baseline hormonal abnormalities (Table 4).

**Pedigree 2: *LHX4* p.V75I (FIG. 2B).** The propositus was a 13-year-old Japanese male born at 41 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, his length was 51.0 cm (1.0 SD) and weight 3.3 kg (0.7 SD). He was referred to us at 3 months of age because of a micropenis and bilateral cryptorchidism. He had undetectable plasma testosterone and LH levels, indicating hypogonadotropic hypogonadism. Severe growth failure was observed at the age of 11 months. Hormonal data revealed GH and TSH deficiencies in addition to tentative gonadotropin deficiency (Table 5). Brain MRI exhibited anterior pituitary hypoplasia, poorly developed sella turcica, visible but thin stalk, and EPP. No other central nervous system abnormalities were visualized. Replacement therapy with thyroxine and recombinant human GH was started at the age of 1 year. The patient responded well to GH replacement. At the age of 13 years, he showed small intrascrotal testes (1 ml), no pubic hair (P1), and a microphallus with low concentration of basal testosterone (0.05 ng/mL Ref: 2.0–7.5).

**Table 3.** Endocrinological findings in Propositus of pedigree 1.

| | | **5yr** | | | **15yr** | | | **Reference** | |
|---|---|---|---|---|---|---|---|---|---|
| | **Stimulus** | **Basal** | | **Peak** | **Basal** | | **Peak** | **Basal** | **Peak** |
| GH (ng/ml) | Insulin | 2.7 | → | 2.9 | 1.8 | → | 2.6 | | >6 |
| TSH (mIU/ml) | TRH | 2.88 | → | 10.01 | 0.78 | → | 7.42 | | 10–35 |
| LH (mIU/ml) | LHRH | <0.2 | → | 2.8 | 6.7 | → | 21.2 | <0.1[a] | 1.93–4.73 [a] |
| | | | | | | | | <0.10–2.65[b] | 6.69–22.51 [b] |
| FSH (mIU/ml) | LHRH | 0.5 | → | 15.5 | 7.0 | → | 9.6 | 0.64–3.03[a] | 13.15–46.95[a] |
| | | | | | | | | 1.81–7.31[b] | 8.58–17.62[b] |
| PRL (ng/ml) | TRH | 10.4 | → | 19.7 | 5.7 | → | 28.1 | 1.7–15.4 | increase 2 times |
| ACTH (pg/ml) | Insulin | 44 | → | 46 | 7.3 | → | 14.9 | 9.8–27.3 | 28–130.5 |
| Cortisol (μg/dl) | Insulin | 19.1 | → | 20.5 | 7.5 | → | 10.0 | | >19.8[c] |
| | | | | | | | | | >17.0[d] |
| IGF-1 (ng/ml) | | 70.1 | | | 241 | | | 74–230[e] | |
| | | | | | | | | 262–510[f] | |
| Free T4 (ng/dl) | | 1.1 | | | 1.0 | | | 1.0–1.95 | |
| Free T3 (pg/ml) | | 4.2 | | | 2.1 | | | 2.23–5.30 | |
| Estradiol (pg/ml) | | | | | 28 | | | 12.3–170[g] | |

The conversion factors to the SI unit are as follows: GH 1.0 (μg/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), TSH 1.0 (mIU/liter), prolactin 1.0 (μg/liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), free T3, 1.54 (pmol/liter), and estradiol 3.671 (pmol/liter).
[a]Reference data of pre-pubertal Japanese girls [22]
[b]Reference data of pubertal (Tanner 2–3) Japanese girls [22]
[c]Reference data of UK children (younger than 10 years) [23]
[d]Reference data of UK children (older than 10 years) [23]
[e]Reference data of Japanese girls (5–7 years old) [24]
[f]Reference data of Japanese girls (15–17 years old) [24]
[g]Reference data of Japanese girls (15 years old) [25]
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**Table 4.** Endocrinological findings (baseline) in Family members of pedigree 1.

| | Father | Mother | Brother | Sister | Reference (Adult) |
|---|---|---|---|---|---|
| GH (ng/ml) | 0.7 | 3.2 | 0.5 | 0.4 | 0–23 |
| IGF-1 (ng/ml) | 110.0 | 156.0 | 357.0 | 276.0 | Male: 41–369 |
| | | | | | Female: 73–542 |
| TSH (μU/ml) | 0.77 | 1.60 | 0.50 | 0.94 | 0.3–3.50 |
| Free T4 (ng/dl) | 1.1 | 1.1 | 1.4 | 1.3 | 1.09–2.55 |
| Free T3 (pg/ml) | 2.5 | 2.6 | 3.1 | 3.1 | 3.23–5.11 |
| LH (mIU/ml) | 4.8 | 7.4 | 2.1 | 6.9 | Male: 2.2–8.4 |
| | | | | | Female: 1.4–15[a] |
| FSH (mIU/ml) | 2.9 | 4.3 | 2.3 | 7.9 | Male: 1.8–12 |
| | | | | | Female: 3–10[a] |
| PRL (ng/ml) | 11.2 | 11.2 | 7.8 | 5.5 | Male: 1.5–9.7 |
| | | | | | Female: 1.4–14.6 |
| ACTH (pg/ml) | 14 | 12 | 15 | 20 | 7.2–63.3 |
| Cortisol (μg/dl) | 8.2 | 6.3 | 10.3 | 10.3 | 7.6–21.4 |
| Estradiol (pg/ml) | | 397 | | 23 | Female: 11–230[a] |
| Testosterone (ng/ml) | 5.19 | | 5.56 | | Male: 2.01–7.50 |

[a]Follicular phase

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The patient's father was 160.5 cm (-1.8 SD) tall. Genetic analyses showed that the propositus and father carried the same heterozygous *LHX4* p.V75I mutation. No other family member was available for genetic studies. Evaluation of the hormonal data for the father was refused.

**Pedigree 3: *POU1F1* p.R271W (FIG. 2C).** The propositus was a 28-year-old Japanese female, who was born at 37 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 48.0 cm (-0.2 SD) and weight 2.6 kg (-1.0 SD). She was referred to us at 2 years of age because of severe short stature (-4.5 SD). Endocrine studies indicated that the patient had complete GH and PRL deficiencies and partial TSH deficiency (free T4 0.8 ng/dl, Ref. >1.0, with inadequate low TSH). Brain MRI at the age of 7 years exhibited anterior pituitary hypoplasia, normal stalk, and normal posterior pituitary gland. No other

**Table 5.** Endocrinological findings in Propositus of pedigree 2.

| | | 11month | | | 8yr | | | Reference | |
|---|---|---|---|---|---|---|---|---|---|
| | Stimulus | Basal | | Peak | Basal | | Peak | Basal | Peak |
| GH (ng/ml) | Insulin | 1.1 | → | 0.9 | 0.6 | → | 0.6 | | >6 |
| TSH (mIU/ml) | TRH | 0.56 | → | 6.81 | 2.00 | → | 10.81 | | 10-35 |
| LH (mIU/ml) | LHRH | 0.3 | → | 0.8 | 0.2 | → | 2.3 | <0.1[a] | <0.10-4.29 [a] |
| FSH (mIU/ml) | LHRH | 2.1 | → | 2.6 | 1.5 | → | 7.4 | 0.46-1.43[a] | 5.38-11.67[a] |
| Testosterone (ng/ml) | HCG | | | | <0.05 | | 0.17 | | >1.2[a] |
| PRL (ng/ml) | TRH | 5.6 | → | 10.1 | 7.7 | → | 13.0 | 1.7-15.4 | increase 2 times |
| ACTH (pg/ml) | Insulin | 44 | → | 170 | 44 | → | 50 | 9.8-27.3 | 28-130.5 |
| Cortisol (μg/dl) | Insulin | 31.0 | → | 38.4 | 13.4 | → | 17.2 | 5-20 | >19.8[b] |
| IGF-1 (ng/ml) | | 6.9 | | | 157 | | | 18-150 [c] | |
| | | | | | | | | 50-356[d] | |
| Free T4 (ng/dl) | | 1.1 | | | 1.1 | | | 1.01-1.95 | |
| Free T3 (pg/ml) | | 4.4 | | | 3.9 | | | 2.23-5.30 | |

The conversion factors to the SI unit are as follows: GH 1.0 (μg/liter), TSH 1.0 (mIU/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), testosterone, 0.035 (nmol/liter), prolactin 1.0 (μg/liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), and free T3, 1.54 (pmol/liter).
[a]Reference data of pre-pubertal Japanese boys (younger than 10 years) [22]
[b]Reference data of UK children (younger than 10 years) [23]
[c]Reference data of Japanese boys (younger than 1 years old) [24]
[d]Reference data of Japanese boys (7-9 years old) [24]

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