MEDICAL PRACTICE

Diagnosis of 5alpha-reductase 2 deficiency: a local experience

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5Alpha-reductase 2 deficiency is an autosomal recessive disorder characterised by lack of masculinisation in XY individuals due to failure to convert testosterone to dihydrotestosterone, the bioactive androgen. Traditionally, the testosterone-to-dihydrotestosterone ratio is used to diagnose this condition but interpreting these results is not always straightforward, thus they may be inconclusive. On the contrary, urinary steroid profiling unambiguously demonstrates a significantly reduced excretion of 5alpha-reduced steroid metabolites compared to their 5beta counterparts. This analytical technique can also simultaneously confirm or rule out other causes of ambiguous genitalia due to steroidogenic defects. Making a DNA-based diagnosis by studying the *SRD5A2* gene has become increasingly popular. Here, we report six Chinese patients from different families who were all diagnosed with 5alpha-reductase 2 deficiency based on urinary steroid profile findings and mutational analysis of the *SRD5A2* gene. R227Q was the most commonly identified mutation in these patients. Management of sexual development disorders is also discussed.

Key words
Dihydrotestosterone; Genitalia; Mutation; Testosterone 5-alpha-reductase

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Introduction

5α-Reductase 2 is an enzyme consisting of 254 amino acids and is encoded by the *SRD5A2* gene. It is a membrane-bound NADPH-dependent type of enzyme, catalysing the reduction of the $\Delta^{4,5}$ double bond in a variety of steroid substrates.[1] 5α-Reductase 2 deficiency is an autosomal recessive disorder characterised by under-virilisation of the male external genitalia at birth due to failure to synthesise dihydrotestosterone (DHT), which is the bioactive androgen. Patients may present with an almost complete female phenotype or isolated defects including hypospadias, bifid scrotum, micropenis, urogenital sinus opening on the perineum, or a combination of these defects.[2] The diagnosis is made either in infancy or at puberty when there is virilisation of the external genitalia in patients who have been raised as females but are genetically male. A normal-to-high male level of serum testosterone (T), low level of DHT, and an elevated T/DHT ratio are the biochemical hallmarks.

Urinary steroid profiling (USP) has established clinical applications for the investigation of a wide range of defects in the steroidogenic pathways.[3-5] Gas chromatography (GC) or gas chromatography–mass spectrometry (GC-MS), which are the major analytical techniques used in this test, provide both qualitative and quantitative information on a wide spectrum of steroid metabolites in a single analysis. Data gained from USP are especially valuable when the relevant hormone or enzyme assays are not readily available, or when pulsatile secretion, diurnal rhythm, the presence of cross reactants or variations in binding proteins might confound the interpretation of spot serum samples. Patients with 5α-reductase 2 deficiency characteristically show reduced ratios of 5α- to 5β-reduced metabolites of C19 and C21 steroids.[2] In this article, we are going to review cases of patients who presented with ambiguous genitalia and were diagnosed with 5α-reductase 2 deficiency based on the characteristic USP findings. Mutational analyses of the *SRD5A2* gene supported this diagnosis.

Case studies

Patient 1 was born at term and was the second child in the family. At birth, he was found to have a micropenis with a stretched penile length of 0.8 cm and width of 0.5 cm. There was no hypospadias and both testes were palpable in the scrotum and were of normal size. The family history was unremarkable and his parents were non-consanguineous. A chromosomal study revealed a karyotype of 46,XY. A few days after birth, his serum luteinising hormone (LH) was 2.6 IU/L, follicle-stimulating hormone (FSH) was 1.3 IU/L, T was 6.8 nmol/L (reference interval [RI], 3.0-12.0), and growth hormone was 48.4 mIU/L. No specific diagnosis was made at that time. His penile length increased to 1.5 cm after three doses of T propionate 25 mg administered intramuscularly, 4-weekly. At 7 years of age, his

stretched penile length was 3 cm and the width was 1 cm. His USP showed that the 5α-tetrahydrocortisol (5α-THF), which is the major 5α-reduced steroid metabolite, was very low (15 µg/day; RI, 273-1570; Fig 1a). Its 5β-counterpart, tetrahydrocortisol (THF), was 310 µg/day (RI, 202-725). The 5α-THF/THF ratio was 0.05 (RI, 0.43-5.49; Table), which was diagnostic of 5α-reductase 2 deficiency. A family study found his father had an unremarkable urinary steroid excretion pattern, but the 5α-THF/THF ratio was borderline low in his mother (0.24; RI, 0.30-1.90). His 12-year-old brother's USP results were normal (Table; Fig 1b). A mutational analysis of the *SRD5A2* gene showed the patient is heterozygous for a mutation 164T>C, changing codon 55 from CTG to CCG, ie L55P (Fig 2a). He is also heterozygous for a known mutation, R227Q (Fig 2b). His father is a L55P carrier, and his mother is a R227Q carrier. No mutations were detected in his brother's *SRD5A2* gene.

Patient 2 was a 26-year-old woman. She was the first child in the family and was born at term with ambiguous genitalia. Her parents were not related. Examination at birth noted a phallus 1 cm in length. There was a single urogenital sinus opening. Gonads were palpable bilaterally in the labioscrotal folds. A chromosomal study revealed a 46,XY karyotype. The serum LH was 1.7 IU/L and the FSH level was lower than 0.3 IU/L. A human chorionic gonadotropin (HCG) stimulation test showed a satisfactory rise in T, from 1.2 nmol/L to 31.2 nmol/L. No uterus could be seen on ultrasonography and a genitogram revealed a male type urethra with a prostatic utricle. Partial androgen insensitivity syndrome (AIS) or 5α-reductase 2 deficiency was suspected but the exact cause was unknown. Nevertheless, the patient was raised as a girl. A bilateral gonadectomy, recessive cliteroplasty, urethroplasty, and vaginoplasty were performed and hormonal replacement therapy using oestrogen was started at around puberty. A USP performed recently showed the level of 5α-reduced steroid metabolites was significantly lower than their 5β counterparts, with the 5α-THF/THF ratio being 0.03 (Table), compatible with a homozygous 5α-reductase 2 deficiency. Mutational analysis of the *SRD5A2* gene showed that she is homozygous for the mutation G203S (Fig 2c), which is a known mutation causing 5α-reductase 2 deficiency.

Patient 3 was born at full term with a birth weight of 3.4 kg. He was the only child of the family, the parents were non-consanguineous, and there was no family history of any significant endocrine disorders. He was brought to the clinic for assessment of micropenis at the age of 2 years. His stretched penile length at that time was 2.8 cm, without hypospadias. Both testes were palpable in the scrotum with normal rugae. There were no dysmorphic features or abnormal pigmentation and physical examination of his other systems was unremarkable. A LH-releasing hormone stimulation test showed a baseline LH lower than 0.5 IU/L, FSH 1.0 IU/L, and T lower than 0.4 nmol/L. There was a greater than 3-fold rise in T to 5.5 nmol/L after HCG stimulation, indicating a normal

5α還原酶2型缺陷症的診斷：本地經驗

5α還原酶2型缺陷症是一種常染色體隱性遺傳病，由於XY染色體組型的人不能把男性激素睾酮轉化成生物活性雄激素的二氫睾酮，所以缺乏男性化的特徵。雖然傳統上會用睾酮—二氫睾酮的比例來確定此症，但因往往不能直接解釋結果，也不能作出定論。相反，尿中類固醇檢測利用與同組的5β數據比較，清楚顯示5α還原酶類固醇代謝物顯著減少。此分析技術同時可以確定或排除因類固醇激素合成的缺陷，而產生的外生殖器性別不清的原因。通過DNA實驗研究*SRD5A2*基因愈見普遍。本文報告來自不同家庭的六位華籍病人，透過尿中類固醇檢測結果及*SRD5A2*基因的突變分析，證實他們患有5α還原酶2型缺陷症。在所有病人中都分析出R227Q突變。本文並討論如何處理性發育障礙的病。

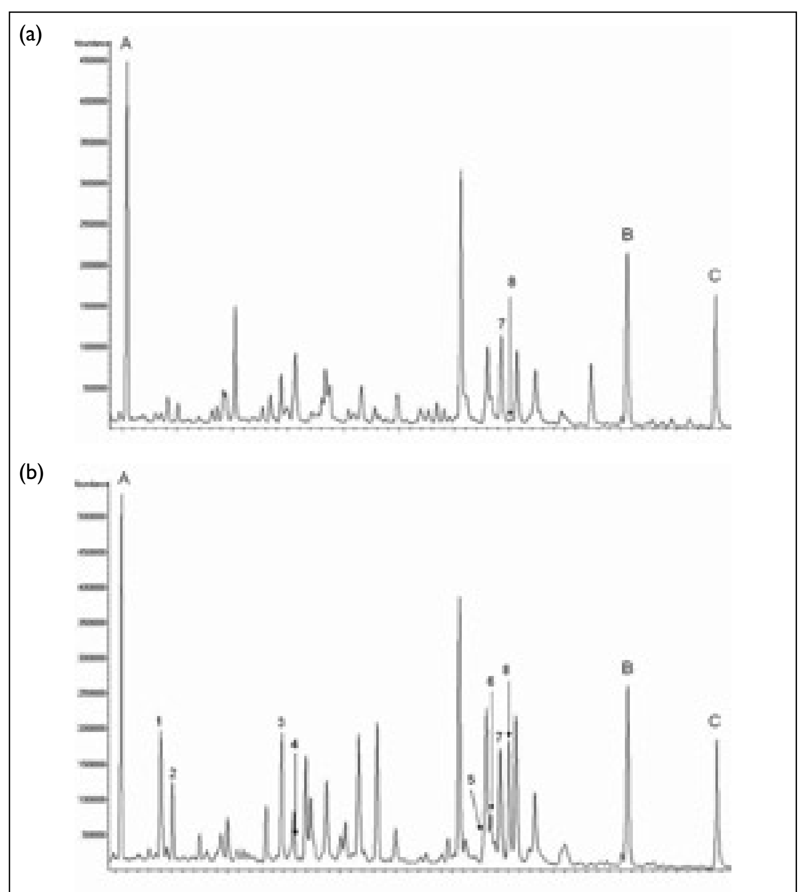

FIG 1. Gas chromatograms of urinary steroid profiling of (a) patient 1 and (b) his brother

A, 5α-androstane-3α,17α-diol; B, stigmasterol; C, cholesteryl butyrate. A, B, and C are internal standards. 1, androsterone; 2, aetiocholanolone; 3, 11-hydroxyandrosterone; 4, 11-hydroxyaetiocholanolone; 5, tetrahydrocorticosterone; 6, 5α-tetrahydrocorticosterone; 7, tetrahydrocortisol; 8, 5α-tetrahydrocortisol. Excretion of steroids 1 to 6 was too low to be identified in patient 1; only steroids 7 and 8 were indicated

TABLE. 5α- and 5β-Reduced steroid metabolite ratios in the patients and the family members*

Patient/family member	Age when USP was performed (years)	5α- and 5β-reduced steroid metabolite ratios in USP				*SRD5A2* Genotype
		A/Ae	11OHA/11OHAe	5α-THB/THB	5α-THF/THF	
Reference intervals						
Male						
<6 years		0.52-3.90	4.92-40.00	2.12-6.58	1.10-3.64	
6-10 years		0.47-5.58	0.45-51.57	0.72-4.83	0.43-5.49	
11-17 years		1.03-2.99	1.12-35.46	0.69-4.20	0.73-2.66	
>17 years		0.67-2.96	1.09-38.45	0.84-3.50	0.48-2.53	
Female						
>17 years		0.44-2.20	1.18-28.03	0.88-2.88	0.30-1.90	
Patient 1	7	0.19†	0.50	0.22†	0.05†	L55P/R227Q
Father of patient 1	42	0.96	2.19	1.64	0.75	L55P/wild-type
Mother of patient 1	39	0.37†	1.86	0.68†	0.24†	R227Q/wild-type
Elder brother of patient 1	12	2.03	2.55	1.96	1.57	Wild-type/wild-type
Patient 2	26	0.19†	1.68	0.22†	0.03†	G203S/G203S
Patient 3	5	0.24†	2.79†	0.20†	0.04†	Q6X/R227Q
Father of patient 3	NA	ND	ND	ND	ND	R227Q/wild-type
Mother of patient 3	NA	ND	ND	ND	ND	Q6X/wild-type
Patient 4	17	0.23†	1.85	0.27†	0.02†	R227Q/R227Q
Elder brother of patient 4	NA	ND	ND	ND	ND	R227Q/wild-type
Patient 5	4	0.11†	0.90†	0.20†	0.00†	V10G/R227Q
Father of patient 5	NA	ND	ND	ND	ND	R227Q/wild-type
Mother of patient 5	NA	ND	ND	ND	ND	V10G/wild-type
Patient 6[6]	8	0.05†	0.14†	0.21†	0.02†	R227Q/R227Q

* USP denotes urinary steroid profiling; A androsterone; Ae aetiocholanolone; 11OHA 11-hydroxyandrosterone; 11OHAe 11-hydroxyaetiocholanolone; THB tetrahydrocorticosterone; THF tetrahydrocortisol; NA not available; and ND not done

† Abnormal parameters

Leydig cell response. A chromosomal study indicated a 46,XY karyotype. His USP revealed a significantly low 5α-THF/THF ratio (Table), and this was diagnostic of 5α-reductase 2 deficiency. A mutational analysis of the *SRD5A2* gene showed Q6X and R227Q (Fig 2d) mutations and family analysis revealed his father is a R227Q mutation carrier and his mother a carrier of the Q6X mutation.

Patient 4 was born at full term and was the family's second child. He was referred to us for assessment of micropenis at the age of 17 years, with a stretched penile length of 6.5 cm and breadth of 2.5 cm. He reached puberty at the age of 14. Both testes were palpable in the scrotum and were of normal size, 15 mL on the right side and 20 mL on the left side. There were normal rugae and pigmentation and no hypospadias. His axillary hair was normal and his pubic hair was in Tanner Stage 4. His growth was normal with a body height at the 90th percentile and weight at the 50th percentile. Examination of other systems was unremarkable. His elder brother had good past health and normal pubertal development. His mother suffered from thyroid disease, controlled with medications. The parents were non-consanguineous. His serum LH was 6.6 IU/L, FSH was 7.1 IU/L, and T was 14.3 nmol/L. His USP showed that the 5α-reduced steroid metabolites were much lower compared with their 5β counterparts, with a 5α-THF/THF ratio of 0.02 (Table). Mutational analysis of the *SRD5A2* gene identified a homozygous R227Q mutation. His elder brother is also a carrier of this mutation.

Patient 5 was born at 41 weeks by normal spontaneous delivery with a birth weight of 2.95 kg, and good Apgar scores of 8 (1 min) and 9 (5 min). He was the family's second child. His elder sister was 6 years old and in good health. His parents are non-consanguineous and there was no family history of significant illness. He was noted to have hypospadias shortly after birth and was assessed by a paediatrician who found him to be a normal-looking baby with no dysmorphic features. All growth parameters were normal. He had penoscrotal hypospadias with chordee and the penile length was 2 cm. Both gonads were palpable in the scrotum. All other systems were normal. A chromosomal analysis showed a 46,XY karyotype. His spot growth hormone was 21.2 mIU/L, LH and FSH were 2.7 and 2.4 IU/L respectively. His HCG stimulation test showed a rise of T from 3 to

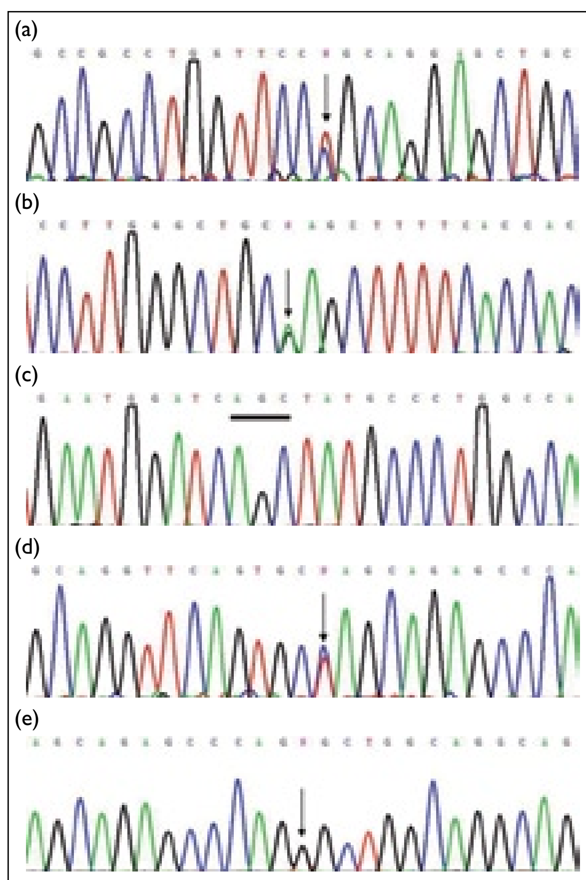

FIG 2. Electropherograms of segments of the *SRD5A2* gene showing the mutation sites in different patients. The heterozygous mutation sites are indicated by arrows and denoted by the letter N. All in sense direction
(a) Heterozygous L55P (CTG→CCG) in patient 1; (b) heterozygous R227Q (CGA→CAA) in patient 1; (c) homozygous G203S (GGC→AGC) in patient 2 (codon underlined); (d) heterozygous Q6X (CAG→TAG) in patient 3; (e) heterozygous V10G (GTG→GGG) in patient 5

10 nmol/L, signifying functioning testicular tissue. A two-stage hypospadias repair was done at 3 and 4 years of age. Further endocrine tests were done at 4.5 years and his cortisol response upon short synacthen stimulation was normal. His USP showed a low 5α-THF/THF ratio (Table), which was diagnostic of 5α-reductase 2 deficiency. A mutation analysis of the *SRD5A2* gene revealed he was heterozygous for R227Q. He was also heterozygous for a novel missense mutation V10G, with a base change in codon 10 from GTG to GGG (Fig 2e). His father was a R227Q carrier and his mother a carrier of V10G.

The clinical history, USP findings, and mutational analysis of patient 6 have been published earlier.[6]

Urinary steroid profiling

The USP was carried out on 24-hour urine collections using the analytical methods described by our group earlier.[7]

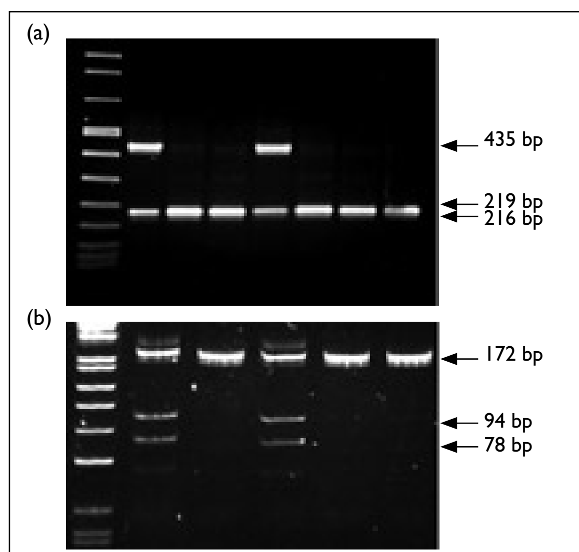

FIG 3. Restriction enzyme analysis for (a) L55P mutation in patient 1 and his family members, and (b) V10G mutations in patient 5 and his family members
(a) Digestion of the polymerase chain reaction (PCR) products of exon 1 by PstI and electrophoresis on a 2% agarose gel. Lane 1, markers; lane 2, father; lane 3, mother; lane 4, brother; lane 5, patient 1; lanes 6 to 8, control subjects. The PstI site is abolished by the L55P mutation (wild-type: 216 + 219 bp; mutant: 435 bp), showing the father and the patient are heterozygous for this mutation. (b) Digestion of the PCR products of exon 1 by BstNI and electrophoresis on a 10% polyacrylamide gel. Lane 1, markers; lane 2, patient 5; lane 3, father; lane 4, mother; lanes 5 and 6, control subjects. The BstNI site is created by the V10G mutation (wild-type: 172 bp; mutant: 78 + 94 bp), showing the mother and the patient are heterozygous for this mutation

BOX. Differential diagnoses of sex development disorders in an XY individual

Defects in testicular development
Examples:
Denys-Drash syndrome (mutation in *WT1* gene)
WAGR syndrome
XY complete gonadal dysgenesis (Swyer syndrome)
Ovotesticular
Deficiency in androgen synthesis
Examples:
Leydig cell aplasia/hypoplasia
Smith-Lemli-Opitz syndrome
Congenital lipoid adrenal hyperplasia due to steroidogenic acute regulatory protein deficiency
Cholesterol side-chain cleavage (P450scc) deficiency
3β-hydroxysteroid dehydrogenase deficiency
17α-hydroxylase/17,20-lyase deficiency
P450 oxidoreductase deficiency
17β-hydroxysteroid dehydrogenase deficiency
Persistent Müllerian duct syndrome
5α-reductase 2 deficiency
Defects in androgen action
Complete androgen insensitivity syndrome
Partial androgen insensitivity syndrome

Mutational analysis of the *SRD5A2* gene

Mutational analysis of the *SRD5A2* gene was carried out on DNA extracted from the peripheral blood of the patients and their family members after

obtaining informed and written consent. Genomic DNA was extracted from peripheral whole blood using a QIAamp DNA blood kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Exons 1 to 5 and the flanking introns of the *SRD5A2* gene were amplified by polymerase chain reaction (PCR) using the following primers (5′ to 3′): E1F, GGCCGCGCTCTCTTCTGG; E1R, CTGCCTCCTTGGCGTTCCT; E2F, GCCTGTGATATACATCCTCCTG; E2R, AGGTGAGGGAGGGGAAGATG; E3F, CTTTCTGCCACGTCTTAGGA; E3R, CATTCGTGCCCTCACTGTCC; E4F, TATGACTATGGAGGGAGCCT; E4R, GCCCAGCAAGTCAGAATATG; E5F, CAAGAAATAGGCTGTGGGAAGG; E5R, GCAGACACCACTCAGAATCC. The PCR conditions were as follows: one cycle of 94°C for 12 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 45 s, and an extension at 72°C for 45 s. The reaction mixture of final volume 25 µL contained 100 ng DNA, 1× PCR buffer (Applied Biosystems, Foster City, CA, US), 2.0 mmol/L $MgCl_2$, 0.2 µmol/L dNTP, 12.5 pmol of each primer, and 0.625 U AmpliTaq Gold DNA polymerase. DNA sequencing was performed as described previously by Chan et al.[8] Novel mutations were confirmed by performing a restriction enzyme analysis on 50 healthy control subjects to exclude polymorphism (Fig 3).

Discussion

5α-Reductase 2 deficiency is one of the major differential diagnoses in the investigation of ambiguous genitalia in a genotypic male. The other differential diagnoses are listed in the Box. Despite the well-established underlying biochemical defects, the diagnosis of 5α-reductase 2 deficiency is never as straightforward as it seems. Due to the synthesis of DHT by 5α-reductase 1 or the synthesis of DHT by residual activity of the type 2 mutant enzyme, patients with 5α-reductase 2 deficiency never have undetectable DHT, and may have levels falling within the low normal range, making the T/DHT ratio unremarkable.[9] Moreover, patients with partial AIS may give a T/DHT ratio mimicking 5α-reductase 2 deficiency due to under-development of DHT-dependent genital tissues.[10] An additional hurdle for our local clinicians is that DHT assays are not available in public hospital laboratories in Hong Kong. To overcome these problems, we made use of locally available tests, which are USP and mutational analysis of the *SRD5A2* gene, to diagnose 5α-reductase 2 deficiency. Urinary steroid profiling has the advantage of measuring the entire steroid metabolite spectrum. In patients with 5α-reductase 2 deficiency, USP demonstrates extremely low levels of 5α-reduced metabolites unambiguously, as compared to their 5β counterparts, a feature seen in all our patients. There are three other pairs of 5α- and 5β-reduced steroid metabolites in the USP which can assist with the diagnosis of 5α-reductase 2 deficiency, namely androsterone (A)/aetiocholanolone (Ae), 11-hydroxyandrosterone (11OHA)/11-hydroxyaetiocholanolone (11OHAe), and 5α-tetrahydrocorticosterone (5α-THB)/tetrahydrocorticosterone (THB). The use of metabolite ratios helps to magnify the impact of the enzyme deficiency, although in our experience, 11OHA/11OHAe is the least sensitive pair. Urinary steroid profiling is useful even after orchidectomy and in patients with AIS since these steroid metabolites are mainly produced in the liver rather than the gonads or genital skin.[11]

Steroid metabolism is very different in neonates from that in children and adults due to the presence of the foetal zone of the adrenal cortex.[12] Before 3 months of age, cortisone and its metabolites (11-oxo containing compounds) are the major corticosteroids produced by the adrenals due to the high activity of 11-β hydroxysteroid dehydrogenase (HSD).[13] It is technically demanding to detect the trace amounts of cortisol and its metabolites even when using GC/MS in selected ion monitoring mode.[14] Therefore, it is not an ideal period for making a diagnosis of 5α-reductase 2 deficiency using USP. A sample collected at age 3 months or older is more likely to contain the relevant steroid metabolites, as this is the time when the adrenal foetal zone has almost regressed completely.

In addition to detecting a 5α-reductase 2 deficiency, the USP can also help to rule out other adrenal steroidogenic defects causing ambiguous genitalia, including 3β-HSD, 17α-hydroxylase and 17,20-lyase deficiencies in genotypic males, and 3β-HSD, 21-hydroxylase and 11β-hydroxylase deficiencies in genotypic females.[3-5] Nevertheless, due to the lack of a characteristic steroid excretion pattern in 17β-HSD deficiency and AIS, the USP is not useful for diagnosing these two conditions. The diagnosis of 17β-HSD deficiency requires an elevated androstenedione to T ratio. This ratio is characteristically exaggerated after HCG stimulation.[15] In AIS, the T level is usually elevated at puberty, as is oestrogen due to extensive aromatisation, resulting in gynaecomastia. Luteinising hormone is also elevated, possibly due to resistance at the hypothalamic-pituitary level.[16] Mutational analysis of the *17HSD3* and *AR* genes assist with confirming the diagnoses of these two conditions, respectively.

Mutation at codon 55 of the *SRD5A2* gene leading to 5α-reductase 2 deficiency has been reported previously.[9] Nevertheless, in that report, leucine was substituted by glutamine instead of by proline as in our patient. Therefore, the L55P mutation we identified is a newly reported mutation for the *SRD5A2* gene.

Previous kinetic studies on 5α-reductase 2 have shown that mutations in the C-terminal half of the protein affect the ability of the enzyme to bind its cofactor, NADPH, whereas those at both the N- and C-terminals affect T binding.[17] In patient 5, the two mutations are located near both terminals. Although we have not carried out an in-vitro study to measure the mutant enzyme activity, the novel mutation V10G together with the mutation R227Q, which is commonly found in Asian patients with 5α-reductase 2 deficiency[6,18-20] and is present in all our patients except for patient 2, appeared to have a significantly detrimental effect on the normal functioning of the enzyme. This was reflected by patient 5's profile; he had the lowest values in all the four pairs of 5α- to 5β-reduced metabolite ratios.

Ambiguous genitalia in a baby demands immediate clinical assessment and laboratory evaluation. Gender assignment is determined by factors including gonadal and genital development, surgical options, fertility, family and cultural expectations. Besides genetic and hormonal factors, gender role behaviour and gender identity are influenced by psychological, social, cultural, and family dynamics. Most patients with 5α-reductase 2 deficiency live as males, even those initially raised as females.[21,22] Virilisation occurs at puberty and they are potentially fertile. Treatment with percutaneous DHT increases the size of the phallus in infants and children with 5α-reductase 2 deficiency.

In conclusion, the diagnosis and management of children with sexual development disorders requires a multidisciplinary team approach. Urinary steroid profiling is a recommended investigative tool for these children. Genetic studies can be used as confirmation tests, offering the additional benefits of family screening and genetic counselling.

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