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Case Report

Ambiguous Genitalia Due to 3β-Hydroxysteroid Dehydrogenase Type 2 Deficiency: Clinical, Genetic, and Functional Characterization of Two Novel *HSD3B2* Variants

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Abstract

3β-Hydroxysteroid dehydrogenase 2 deficiency (3βHSD2D) is a rare form of congenital adrenal hyperplasia (CAH) with variable clinical presentation. We describe a 46, XY child with ambiguous genitalia and CAH without apparent adrenal insufficiency due to 2 novel heterozygous variants in the *HSD3B2* gene (c.779C > T/p.Pro260Leu and c.307 + 1G > A/p.Gly103Asp,fs29X). The disease-causing effect of the novel variants was assessed by genetic and functional studies informing on positive genotype-phenotype correlation. Sex registration was female, and no gender dysphoria has been noted until the present age of 7 years, but psychological assessments have been difficult with a concomitant diagnosis of autism spectrum disorder. Virilization that already progresses prepubertally through peripheral conversion of androgen precursors by 3β-hydroxysteroid dehydrogenase 1 will pose an increasing challenge during puberty.

Key Words: congenital adrenal hyperplasia, CAH, difference of sexual development, DSD, 3β-hydroxysteroid dehydrogenase 2 deficiency, ambiguous genitalia

Abbreviations: 3βHSD2D, 3β-hydroxysteroid dehydrogenase 2 deficiency; 17OHP4, 17-hydroxyprogesterone; A4, androstenedione; AMH, anti-müllerian hormone; CAH, congenital adrenal hyperplasia; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; DSD, difference of sexual development; GC-MS, gas chromatography–mass spectrometry; hCG, human chorionic gonadotropin; LC-MS, liquid chromatography–mass spectrometry; T, testosterone; WT, wild-type.

Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders affecting steroid biosynthesis [1, 2]. Very rare causes of CAH include 3β-hydroxysteroid dehydrogenase 2 deficiency (3βHSD2D, OMIM 613890) [1]. In early steps of adrenal and gonadal steroidogenesis, 3βHSD2 converts precursors into metabolites of the mineralocorticoid, glucocorticoid, and sex steroid pathways (Fig. 1). Variants in the *HSD3B2* gene lead to a variable degree of 3βHSD2D with mild to severe clinical signs of CAH and sexual ambiguity [3, 4]. Precursors accumulating through 3βHSD2D reach peripheral tissues, where type 1 3βHSD (3βHSD1/*HSD3B1*) is expressed and able to convert these to bioactive androgens (see Fig. 1).

At birth, 3βHSD2D may manifest with a difference of sexual development (DSD) both in 46, XX and 46, XY individuals [1, 3, 4]. 46, XY individuals have incomplete male genital development (hypospadias, ambiguous genitalia) due to lower production of testosterone (T) and dihydrotestosterone (DHT). 46, XX individuals have normal or mildly virilized external genitalia (clitoromegaly, labial fusion) because of increased dehydroepiandrosterone (DHEA) being converted to T by 3βHSD1 peripherally. According to the severity of 3βHSD2D, adrenal insufficiency manifests with or without salt loss (hypoaldosteronism) or adrenal crisis (hypocortisolism).

Here, we describe the clinical presentation, steroid biochemistry, and molecular genetics of 3βHSD2D in a patient harboring 2 novel *HSD3B2* variants. Additionally, we provide functional data for novel variants and discuss challenges in patient care.

Case Presentation

A 10-day-old, term-born child of African/South American nonconsanguineous parents was referred to the multiprofessional DSD team because of ambiguous genitalia and slightly increased 17-hydroxyprogesterone (17OHP4) in newborn screening. Family and pregnancy history, birth, and neonatal adaptation were unremarkable, except for maternal insulin therapy for

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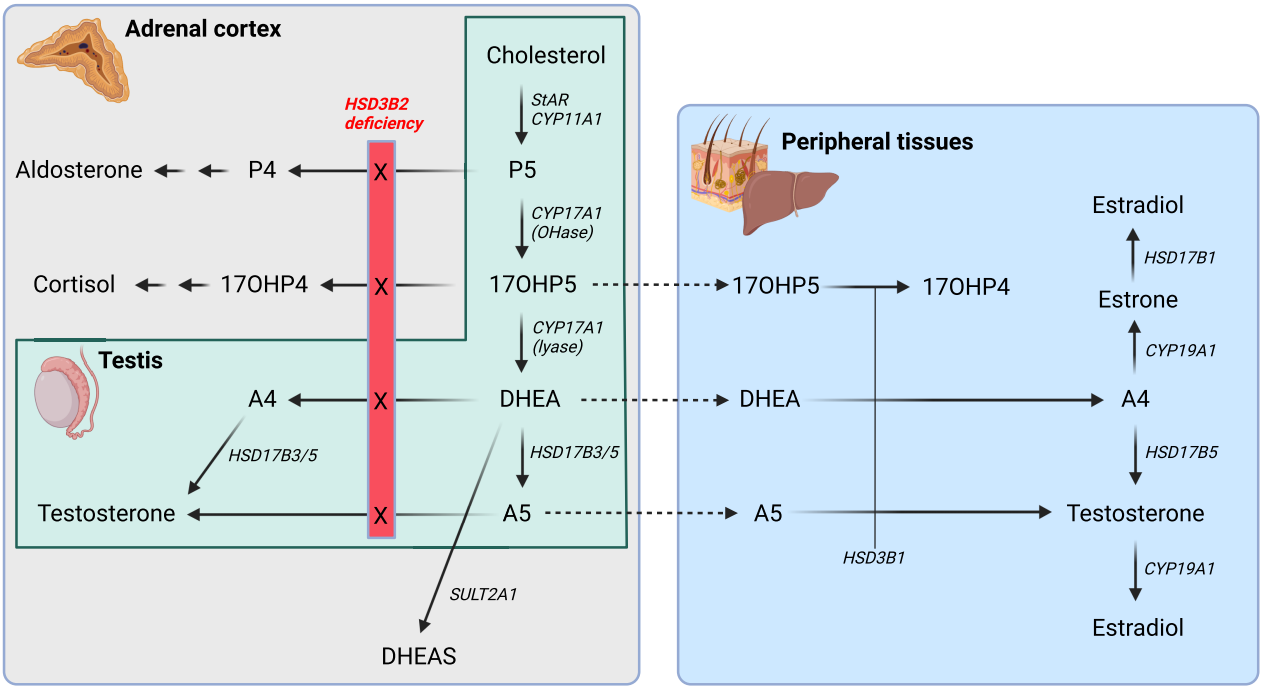

Figure 1. Scheme for steroid biosynthesis pathways in the adrenal cortex, testis, and peripheral tissues. In 3βHSD2 deficiency, conversion of Δ5 steroids (pregnenolone [P5], 17-hydroxypregnenolone [17OHP5], dehydroepiandrosterone [DHEA]) to corresponding Δ4 steroids (progesterone [P4], 17-hydroxyprogesterone [17OHP4], androstenedione [A4]) is affected; but unaffected 3βHSD1 activity may convert secreted precursors in the periphery.

gestational diabetes. At birth, a midwife and a pediatrician recommended female sex registration. At referral, primary investigation revealed an External Genital Score of 6.5/12 with a 2.2-cm genital tubercle, scrotal hypospadias, and labioscrotal gonads [5]. Normally structured testes and no uterus were described by ultrasound. Karyotype was 46, XY. Laboratory investigations showed electrolytes, cortisol, T, DHEA, and antimüllerian hormone (AMH) levels in the normal male range, but increased dehydroepiandrosterone sulfate (DHEAS), androstenedione (A4) and 17OHP4 levels. At age 1 month, ACTH and 17OHP4 were normal, while 17-hydroxypregnenolone (17OHP5) and DHEA were increased (Table 1). Human chorionic gonadotropin (hCG) test showed a low T level and a normal T/DHT ratio. At age 3 months, sodium, potassium, adrenocorticotropin (ACTH), and T were normal, thus excluding a severe form of salt-wasting CAH. Serum and urine steroid profiling using liquid and gas chromatography–mass spectrometry (LC-MS, GC-MS) revealed no specific diagnosis. The girl needed a herniotomy at age 2 months and was diagnosed with an autism spectrum disorder at age 4 years. During the first 5 years, the phallus grew up to 4.5 cm and the child developed premature adrenarche with pubarche, relative tall stature, and bone age advancement of +1.8 years related to the male standard. At age 5 years, electrolytes were normal and plasma high-resolution LC-MS steroid findings showed normal cortisol and 17HOP4, but high DHEA and DHEAS, and low aldosterone levels. Still without a specific diagnosis, further diagnostic workup was performed.

Diagnostic Assessment

Biochemical Assessments at Age 5 Years

Baseline serum aldosterone levels were very low. Electrolytes, ACTH, T, and DHT were normal, and DHEA and DHEAS were elevated (see Table 1). ACTH test (0.25 mg Synacthen/1.73 m^2) showed normal stimulation of cortisol and progesterone metabolites; hence, common steroid synthesis disorders were excluded, but the DHEA/A4 ratio was significantly increased, before and after stimulation, indicating reduced 3βHSD2 activity [7].

Genetic Analysis, American College of Medical Genetics and Genomics Classification, and In Vitro Studies

Genetic workup on genomic DNA extracted from the patient's leukocytes (whole-exome sequencing with customized set of genes analyzed) discovered 2 novel heterozygous variants in the *HSD3B2* gene (NM000198.4): a missense c.779C > T variant in exon 4 (p.Pro260Leu) and a donor splice site c.307 + 1G > A (rs776761493) variant in intron 3 (Fig. 2). According to American College of Medical Genetics and Genomics classification [9], these novel variants were likely pathogenic. To confirm their disease-causing effect, we tested both variants using established experimental approaches [10, 11]. For the missense c.779C > T *HSD3B2* variant, we transfected nonsteroidogenic HEK293T cells with wild-type (WT) and variant *HSD3B2* and tested enzyme activities for conversion of pregnenolone to progesterone, 17OHP5 to 17OHP4, and DHEA to 4A. We found partial inactivity of 3βHSD2 supported steroid conversions for the novel variant compared to WT for all 3 tested reactions (Fig. 3). For the splice-site variant c.307 + 1G > A, we performed a minigene experiment with an expression vector including the human genomic sequence of WT and variant *HSD3B2* covering exon 3, intron 3, and exon 4 (Fig. 4). WT and variant *HSD3B2* minigenes were transfected into HEK293T cells, and sequencing

Table 1. Main biochemical findings in a child with a 46, XY difference of sexual development due to 3β-hydroxysteroid dehydrogenase 2 deficiency

		Age and analytical matrix and method							
		4 d	10 d	1 mo	3 mo	5 y		6 y	7 y
		Newborn screening	Plasma, filter paper dried blood	Plasma, 72 h > hCG test	Plasma	Plasma (basal)	Plasma (60-min post Synacthen)	Plasma	Plasma
Analyte	**Unit**	**IA**	**IA**	**GC-MS**	**IA**	**LC-HRMS**	**LC-HRMS**	**LC-HRMS**	**LC-HRMS**
17OHP4	nmol/L	**27** ↑[a] (<20)	**21** ↑[a] (<20)	2.3[b] (1.5-30)		2.2 (<2.75)	16.8		1.43 (<2.75)
Progesterone	nmol/L		**22.7** ↑[c] (0.7-3.5)			0.12 (0.05-0.83)	1.34		0.15 (0.05-0.83)
17OHP5	nmol/L			**64.4** ↑[b] (0.3-9.5)		ND			ND
17OHP5/17OHP4 ratio				**28** ↑ (5.1 ± 5)[d]					
DHEA	nmol/L		9.3[e] (1.0-30.0)	**20.1** ↑[b] (2.1-8.3)		**10.8** ↑ (1.0-3.8)	**23** ↑		**7.1** (1.0-6.4)
DHEAS	nmol/L		**3.0** ↑[c,f] (0.1-1.3)			**5.3** ↑ (0.35-1.96)	**5.7** ↑	**6.0** ↑ 0.35-1.96)	3.2 (0.35-5.25)
A4	nmol/L		**11.7** ↑[c] (0.4-3.3)	1.0[b] (0.7-2.4)	**2.9** ↑[c] (0.1-1.9)	0.5 (0.15-1.5)	0.9		0.5 (0.15-0.59)
DHEA/A4 ratio			0.79[d] (8.4 ± 9.7)	**19.9** ↑[d] (8.4 ± 9.7)		**20.8** ↑[d] (2.3 ± 2.0)	**25.6** ↑[d] (12.0 ± 4.3)		**14.2** ↑[d] (3.5 ± 1.2)
Testosterone	nmol/L		1.9[c] (0.5-12.6)	**5.2** ↓[b] (2.8-11; > 6.93 after hCG)	0.79[c] (0.1-3.1)	0.13 (0.1-0.9)	0.18	0.14 (0.05-0.35)	0.09 (0.05-0.35)
DHT	nmol/L			1.96[b] (0.2-2.8)	0.39[c] (<0.8)	0.08 (<0.15)	0.13		**0.153** ↑ (<0.15)
Testosterone/DHT ratio				2.65 (<30)	2.0	1.65	1.38		0.59
ACTH	pmol/L				4.1[c] (2-11)	6.7[c] (2-11)		6.2[c] (2-11)	3.5[c] (2-11)
Cortisol	nmol/L		123[c] (31-519)			171 (83-579)	588 (>500)	176 (83-579)	**585** (83-579)
Renin	mU/L							**47.0** ↑[g] (2.8-39.9)	11.3[g] (2.8-39.9)
Aldosterone	nmol/L					**0.09**↓ (0.14-2.22)	0.19 (0.14-2.22)	**0.05**↓ (0.14-2.22)	**0.01**↓ (0.14-2.22)
Sodium	mmol/L		136[b] (135-145)		136[b] (135-145)	139[b] (135-145)		139[b] (135-145)	141[b] (135-145)
Potassium	mmol/L		**5.4**[b] (3.5-5.0)		4.3[b] (3.5-5.0)	4.1[b] (3.5-5.0)		3.6[b] (3.5-5.0)	3.8[b] (3.5-5.0)

(*continued*)

Table 1. Continued

		Age and analytical matrix and method							
		4 d	10 d	1 mo	3 mo	5 y		6 y	7 y
		Newborn screening	Plasma, filter paper dried blood	Plasma, 72 h > hCG test	Plasma	Plasma (basal)	Plasma (60-min post Synacthen)	Plasma	Plasma
Analyte	**Unit**	**IA**	**IA**	**GC-MS**	**IA**	**LC-HRMS**	**LC-HRMS**	**LC-HRMS**	**LC-HRMS**
AMH	pmol/L		349[i] (193-1074)			**149↓**[i] (221-1063)		**190↓**[i] (221-1063)	336[i] (35-1032)
Urine			10 d GC-MS	1 mo GC-MS				6 y GC-HRMS	
16OH-DHEA	µg/ mmol-Crea		3071 (94-7898)	4925[j] (94-7898)				**3587 ↑** (1-46)	
Pregnenetriol (5PT)	µg/ mmol-Crea		11.9 (7-156)	**302 ↑**[j] (7-156)				**3450 ↑** (2-36)	

Steroids were measured using LC/GC-HRMS except 17OHP4 in newborn screening by IA and during the first 3 months of life by IA or GC/MS, as indicated in the footnotes. ACTH, renin, sodium, potassium, and AMH were measured by methods in a routine laboratory setting as indicated in the notes. Reference ranges at corresponding ages are presented in parentheses and values outside the reference range are highlighted with bold. To obtain conventional units, multiply by 0.314 for progesterone in ng/mL; 31.7 for 17OHP5 in ng/dL; 33.0 for 17OHP4 in ng/dL; 28.8 for DHEA in ng/dL; 37.7 for DHEAS in µg/dL; 28.7 for A4 in ng/dL; 28.8 for testosterone in ng/dL; 29.1 for DHT in ng/dL; 4.54 for ACTH in pg/mL; 0.036 for cortisol in µg/dL; 0.36 for aldosterone in ng/mL; 1 for sodium and potassium in mEq/L; 0.14 for AMH in ng/mL.

Abbreviations: 17OHP4, 17-hydroxyprogesterone; 17OHP5, 17-hydroxypregnenolone; A4, androstenedione; ACTH, adrenocorticotropin; AMH, anti-müllerian hormone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; GC, gas chromatography; hCG, human chorionic gonadotropin; HRMS, high-resolution mass spectrometry; IA, immunoassay; LC, liquid chromatography; MS, mass spectrometry; ND, not detected.

[a]Immunofluorometric assay (DELFIA, Wallac Inc).

[b]GC-MS plasma steroid profile: Sánchez-Guijo et al [6].

[c]Chemiluminescence-Immunoassay (Immulite, Siemens Healthineers AG).

[d]Age-related reference ranges of ratios of steroids by radioimmunoassay after celite chromatographic purification: Lutfallah et al [7].

[e]ELISA Assay (Eagle Bioscience).

[f]DHEAS measured on day 24 of life.

[g]Immunochemiluminometric assay (Quest Diagnostics).

[h]Flame photometry (Eppendorf EFOX, blood gas analysis).

[i]Gen II enzyme linked immunosorbent assay (ELISA) (Beckman Couter).

[j]GC-MS urine steroid profile: Heckmann et al [8].

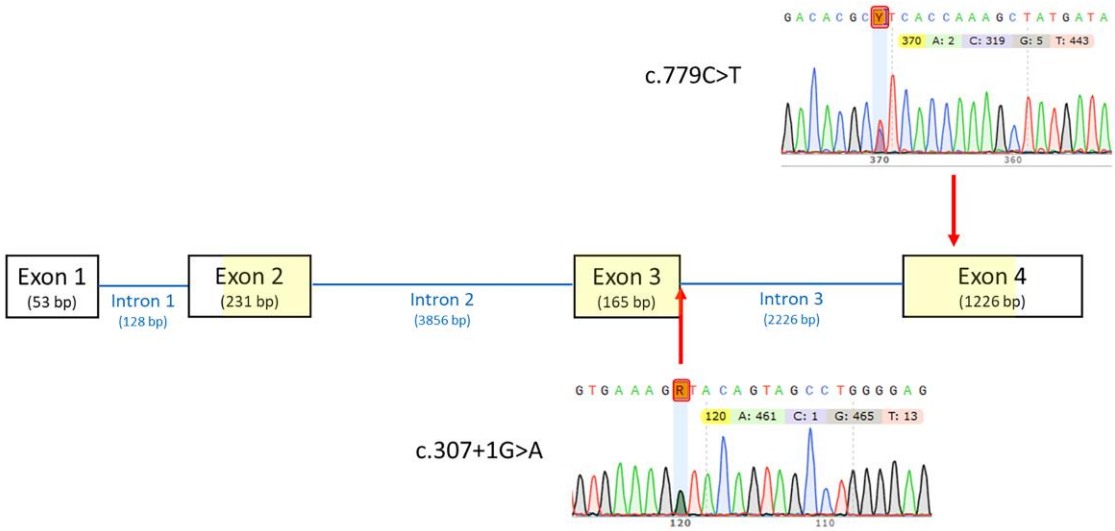

Figure 2. Genetic analysis of the *HSD3B2* gene (Chr 1:119, 415, 150-119, 423, 034) revealing the heterozygous variants (c.779C > T and c.307 + 1G > A) in the studied patient (boxes and arrows). The *HSD3B2* gene consist of 4 exons, of which 3 are coding (highlighted in yellow). Whole-exome sequencing of patient DNA was performed using TWIST comprehensive exome (TWIST bioscience) on a NovaSeq 6000 sequencing system (Illumina). The following genes were subsequently analyzed: *AAAS, ABCD1, AIRE, AMH, AMHR2, AR, ARX, ATRX, BMP4, BMP7, CDKN1C, CHD7, CTU2, CUL4B, CYB5A, CYP11A1, CYP11B1, CYP17A1, CYP19A1, CYP21A2, DHCR7, DHH, DHX37, GATA4, GPX1, HOXA13, HOXA4, HSD17B3, HSD3B2, LHCGR, MAMLD1, MAP3K1, MC2R, MCM4, MRAP, MYRF, NNT, NR0B1, NR2F2, NR3C1, NR5A1, POLE, POR, PPP1R12A, PRDX3, RPL10, RSPO1, SAMD9, SGPL1, SOX10, SOX9, SRD5A2, SRY, STAR, TOE1, TSPYL1, TXNRD2, WT1,* and *WTAP*.

of the transcripts revealed that the variant *HSD3B2* shifted splicing into intron 3, thereby leading to a frameshift with stop codon after 29 amino acids (p.Gly103Asp,fs29X).

Treatment

After being diagnosed with 3βHSD2D (see Table 1), the child was started on hydrocortisone (10 mg/m^2/d) and fludrocortisone (0.05 mg/d) treatment at age 5 years.

Outcome and Follow-up

Under hydrocortisone and fludrocortisone treatment, growth velocity normalized, while androgen precursor levels from the Δ5 pathway (DHEA, DHEAS) remained slightly elevated (see Table 1). At 7 years, her height was 130.6 cm (+1.76 SD) and weight 32.3 kg (+2.12 SD; female Swiss/World Health Organization references). Tanner pubertal stage was P3, G3 (with a stretched phallus length of 6 cm), with some axillary hair, but no gynecomastia. Her blood pressure was normal (109/75 mm Hg). By ultrasound, the right gonad was located inguinolabioscrotally, 1 mL, and the left gonad labioscrotally, 0.9 mL, both of normal testicular structure. By male standards [14], bone age was advanced by +2.5 years and projected adult height by Bayley and Pinneau was 178.6 ± 5 cm, which was in accordance with parental-related adult height for males (170 cm). Laboratory values showed a less elevated DHEA and DHEAS with normal values for electrolytes, ACTH, renin, and T, and slightly elevated DHT and cortisol levels (see Table 1). Due to the additional diagnosis of autism with a delay of cognitive and language development by more than 2 years, intensive psychosocial support was provided for the whole family. Gender issues have been a very sensitive topic to discuss, and specialized health care professionals of the DSD team were counseling the family since birth. So far, the girl has not expressed any signs of gender dysphoria and considerations of gender reassignment have been postponed until shared decision-making might become feasible.

Discussion

3βHSD2D, a rare form of CAH caused by genetic variants in the *HSD3B2* gene, has variable clinical presentation related to residual enzyme activity, ranging from severe DSD with life-threatening early cortisol deficiency and salt-wasting crisis to nonclassic forms with normal glucocorticoid production and a milder effect on androgen production [1, 3, 4]. The presented case had ambiguous genitalia at birth without adrenal insufficiency but abnormal 17OHP4 neonatal screening, and was assigned "female" sex after birth, before consulting with a specialized DSD team; finally, at age 6 years, after several attempts, the girl received a specific diagnosis of 46, XY DSD with androgen deficiency due to a nonclassic CAH caused by novel, compound heterozygous *HSD3B2* variants. The diagnosis was confirmed biochemically and by genetic analysis; the disease-causing mechanism of novel *HSD3B2* variants was assessed by functional studies.

Diagnosis of 3βHSD2D can be challenging for several reasons. First, a similar phenotype of nonsyndromic DSD/ambiguous genitalia at birth without (apparent) hypocortisolism may also be caused by other monogenic disorders of steroidogenesis (Table 2). Second, 3βHSD2D manifests itself with a broad phenotype with or without ambiguous genitalia at birth and adrenal insufficiency, or only later in life. Third, the steroid metabolome of 3βHSD2D may not be easily recognized because marker steroids are often not measured as part of the routine diagnostics for CAH, and these steroid levels change according to adrenal and gonadal developmental at birth, in the first year of life, and with puberty; thus age- and sex-specific

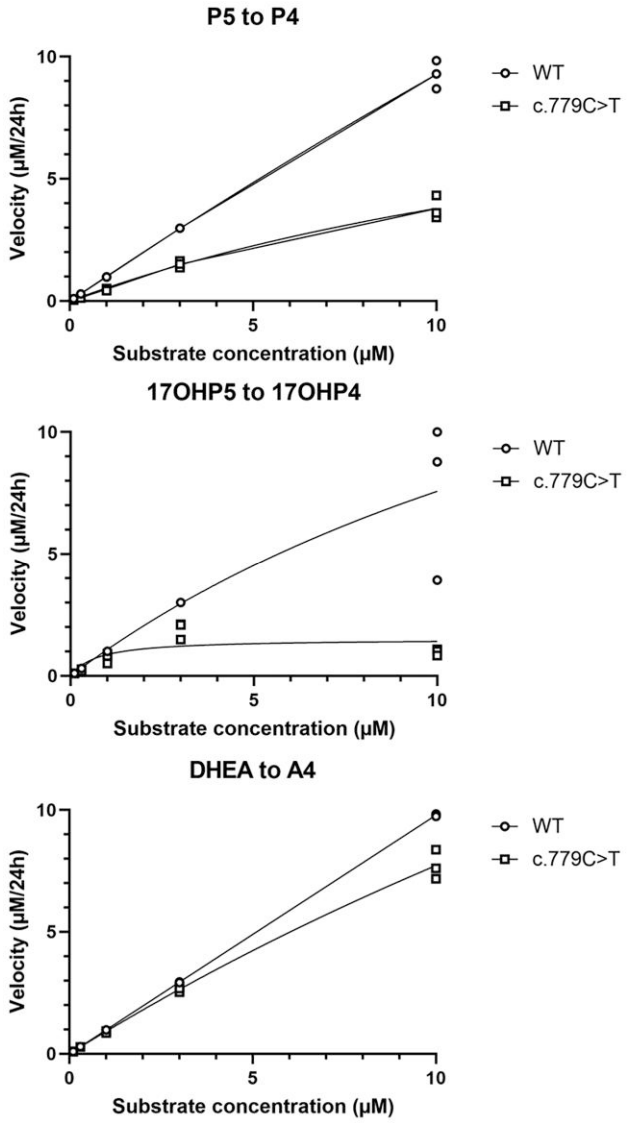

Figure 3. Functional characterization of the *HSD3B2* variant p.(Pro260Leu) *in vitro*. Nonsteroidogenic HEK293T cells were transiently transfected (Lipofectamine 2000; 1 µg of DNA/well in 6-well plates) with wild-type (WT) and variant *HSD3B2* expression vectors for 48 hours. Cell medium was enriched with a 3βHSD2 precursor steroid mixture in Dulbecco's modified Eagle's medium in 5 different concentrations. The activity of transfected cells to convert either pregnenolone (P5) to progesterone (P4), 17-hydroxypregnenolone (17OHP5) to 17-hydroxyprogesterone (17OHP4), or dehydroepiandrosterone (DHEA) to androstenedione (A4) at given substrate concentrations (x-axis) was assessed by measuring steroids in the supernatants after 24 hours' incubation by high-resolution LC-MS [12]. Conversion is given as µM/24 hours. Graphs were produced by GraphPad Prism from data of 3 independent experiments performed in triplicates. Equal expression of the WT and variant HSD3B2 was confirmed by RT-qPCR (data not shown; primer sequences are available on request).

steroid normative values measured by GC/LC-MS profiling are essential for the biochemical workup [15, 16].

At birth, steroid levels reflect the fetal-placental-maternal unit comprising the androgen producing gonads (eg, testes) and fetal adrenals that involute in the first weeks after birth and are replaced by the definitive adrenal cortex [17]. Both organs are largely inactive in the production of sex steroids/androgen precursors during infancy and become active again only at adrenarche and puberty. However, with 3βHSD2D, steroid precursors accumulate in active steroid organs, reach the circulation, and can be converted to bioactive androgens and estrogens in the placenta or in peripheral tissues by unaffected 3βHSD1 activity (see Fig. 1) [18]. Therefore, 3BHSD2D may cause a positive neonatal CAH screening showing transiently elevated 17OHP4 levels. Aided by peripheral 3βHSD1 activity, the same alternate pathway (see Fig. 1) also leads to excess androgen/sex hormone production resulting in premature pubarche or atypical precocious puberty, virilization, gynecomastia or polycystic ovaries, while the gonadal sex hormone biosynthesis is lost or diminished [1, 3, 4].

To diagnose 3βHSD2D biochemically, comprehensive steroid profiling using GC/LC-MS methods is essential, but not always sufficient. Measurements for calculating product/precursor (Δ4/Δ5) ratios of 3βHSD2 activities are informative, and serum 17OHP5 levels greater than 100 nmol/L (either basal or after ACTH stimulation) have been reported to be the single best marker for 3βHSD2D [1, 3], but Δ5 steroids such as pregnenolone or 17OHP5 may be hard to detect and quantify and are therefore often not included in the LC-MS methods; 17OHP4 is not a specific marker for 3βHSD2D as it can be low, normal, or high, depending on peripheral 3βHSD1 activity [4]. The differential diagnosis of elevated 17OHP4 in neonatal screening includes steroid biosynthesis defects due to mutations of *CYP21A2*, *CYP11B1*, *CYP17A1*, *POR*, and *HSD3B2*. Therefore, comprehensive steroid profiling using LC/GC-MS methods instead of single steroid measurements with immunoassays is recommended for diagnosing steroid disorders [15]. But even with these methods, measurements and data interpretation can be tricky, especially with partial enzyme activities, as illustrated by the presented case of 3βHSD2D. In such cases, a genetic workup early in the course can inform on the specific diagnosis and related consequences [19].

The combination of 2 heterozygous gene variants, 1 with partial active and 1 with loss-of-function, is a typical profile for a nonclassic CAH in which the phenotype often reflects the activity of the less affected variant. We confirmed this particular genotype-phenotype profile of 2 novel *HSD3B2* variants by *in vitro* studies. Our 46, XY patient had ambiguous genitalia at birth prompting female sex assignment, and prepubertal virilization with penile growth and signs of premature adrenarche by age 5 years. Thus, 3βHSD2D should be kept in mind as a very rare cause of premature adrenarche due to nonclassic CAH [20], especially if clinical signs include significant growth advancement and very early pubarche. Similarly, signs of androgen excess and polycystic ovaries in 46, XX individuals later in life may be caused by milder forms of nonclassic, late-onset 3βHSD2D [21]. By contrast, our 46, XY girl will likely become further virilized with pubertal stimulation of testicular steroidogenesis (eg, hypothalamic-pituitary-gonadal axis activation) and peripheral metabolism of accumulated precursors to androgens and estrogens, also leading to gynecomastia [22].

In the reported case, sex registration at birth was "female" before an interdisciplinary DSD team was consulted and specific investigations were performed and discussed with the parents. This is certainly not recommended [23]. The possibility of gender dysphoria and necessity for sex

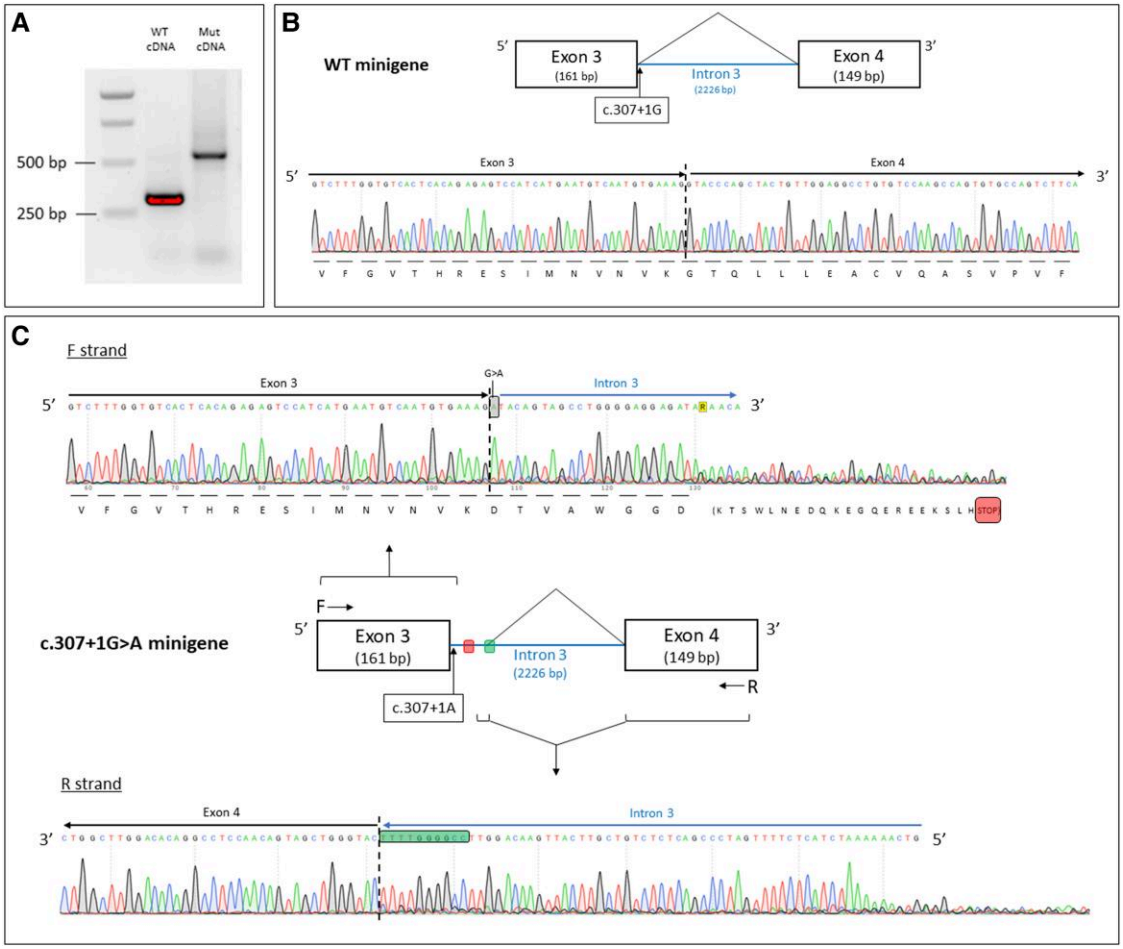

Figure 4. Study of the HSD3B2 c.307 + 1g > a variant by minigene experiment. Human *HSD3B2* wild-type (WT) minigene was designed to cover the end of the third and beginning of the fourth exon, and their in-between intron 3; it was cloned into pcDNA3 (GenScript). The mutant (Mut) c.307 + 1G > A minigene was then generated using site-directed mutagenesis following the QuikChange protocol by Stratagene (Agilent Technologies Inc). Correct sequences of plasmids were confirmed by direct sequencing (MicroSynth AG). HEK293T cells were transiently transfected with the WT and Mut minigenes (Lipofectamine 2000, 1 µg of DNA/well in 6-well plates). Total RNA was extracted and RT-PCR for *HSD3B2* cDNA was performed. A shows the complementary DNA (cDNA) products in agarose gel. WT cDNA fragments were approximately 300 bp corresponding to the length of exons 3 and 4 in the WT minigene. Mut cDNA fragments were approximately 500 to 600 bp, indicating aberrant splicing. Both WT and Mut cDNA fragments were also subjected to direct sequencing (MicroSynth AG). B, Arrangement of the WT *HSD3B2* minigene leading to normal splicing as seen in chromatogram of the WT cDNA fragment shown in A. C, Arrangement of the c.307 + 1G > A HSD3B2 minigene and chromatograms (separately with forward and reverse primers) of the mutant cDNA fragment shown in A. Forward strand of the c.307 + 1G > A fragment showed that this variant leads to continuation of the read frame into intron 3. Reverse strand of the sequenced c.307 + 1G > A shows that this variant leads to altered splicing in the predicted constitutive splicing donor site (in position c.307 + 214-233, highlighted with green boxes). Alternative splicing sites were predicted using the online tool Alternative Splice Site Predictor [13]. Variant in the donor splice site of intron 3 is highlighted by a gray box. Predicted STOP codon in c.307 + 1G > A variant is highlighted with red boxes, after 29 amino acids coded from the intronic site. Depicted in sequences of WT and c.307 + 1G > A are also their exon (black arrow) and intron (blue arrow) locations, exon-intron boundaries (dashed vertical line), and corresponding amino acid sequences with read frame 1.

reassignment has been raised by the DSD team caring for the family; however, so far, the girl has not expressed gender dysphoria, which may remain difficult to assess with a comorbidity of autism and developmental delay. We consider 3 possible scenarios for her future sex identity development: 1, She feels good with the natural course and an intersexual virilized female phenotype; 2, she would like to conserve the female gender and oppose virilization by hormonal (eg, gonadotropin-releasing hormone blockade and estrogen treatment) and surgical treatments (gonadectomy, genital surgery); 3, she requests male sex reassignment and medical interventions to support the male-typical phenotype including surgery (hypospadias repair) and T replacement (as needed).

Fertility data in 3βHSD2D are very limited but 46, XY patients seem to be infertile due to abnormal testis development and arrested spermatogenesis or azoospermia [4, 24]. Approximately 20% to 30% of 46, XY patients with 3βHSD2D have been described to have testicular adrenal rest tumors [4] but the risk for malignancies seems to be low [22].

In conclusion, we discuss the pitfalls and challenges in diagnosis and management of a 46, XY 3βHSD2D patient who presented at birth with ambiguous genitalia, without adrenal insufficiency, was registered with female sex, and was late diagnosed at age 5 years by steroid profiling and genetic analysis. Two novel *HSD3B2* variants were identified and functionally tested to confirm their disease-causing effect.

Table 2. Typical clinical and steroid hormone characteristics of 3β-hydroxysteroid dehydrogenase 2 deficiency (3βHSD2D) and some closely related monogenic disorders of steroidogenesis that may be challenging to distinguish from 3βHSD2D

Disorder	Gene	Enzyme function	17OHP4 level in newborn screening	Typical serum steroid profile	Typical clinical characteristics
3βHSD2 Deficiency	*HSD3B2*	Converts Δ5 steroids (P5, 17OHP5, DHEA) to corresponding Δ4 steroids (P4, 17OHP4, A4)	Low/normal/increased depending on the activity of 3βHSD type 1 in periphery	Increased Δ5 steroid, decreased Δ4 steroids, decreased Δ4/Δ5 ratio	• Variable degree from classic form with AI, salt-wasting, and hypokalemic hypertension (high renin) to mild or no effect on MC/GC synthesis in nonclassic forms • DSD in both sexes (46, XY more severe than 46, XX)
21-Hydroxylase deficiency	*CYP21A2*	Converts Δ4 progesterones (P4, 17OHP4) to intermediate metabolites of mineralocorticoid and glucocorticoid pathways	Increased	Increased Δ5 and Δ4 steroids and androgen excess, decreased GCs, variable effect on MC levels	• Variable from AI and salt-wasting in classic form to mild or no effect on MC/GC synthesis in nonclassic forms • 46,XX DSD in classic form; variable degree of adrenal androgen excess in non-classic form
11-Hydroxylase deficiency	*CYP11B1*	Converts 11DOC to CORT, S to F, and A4/T to intermediates in C11-oxyandrogen pathway	May be increased	Increased Δ5 and Δ4 steroids and androgen excess, decreased GC/MC levels after 11-hydroxylation step (F/CORT/ALDO)	• Variable level of AI but no salt-wasting, hypertension instead • 46, XX DSD in classic form; variable degree of adrenal androgen excess in nonclassic form
17-Hydroxylase (/lyase) deficiency	*CYP17A1*	Converts P5 to 17OHP5 and DHEA, and P4 to 17OHP4	Usually low to normal, but may be increased (isolated 17-lyase deficiency)	Elevated steroids in MC path (11DOC/CORT/ALDO), low androgen precursors. In rare case of isolated 17-lyase deficiency, only androgen production is affected	• Hypokalemic hypertension (low renin) due to MC excess • Usually no AI due to glucocorticoid effect from increased CORT • 46, XY DSD; no secondary sexual characteristics and pubertal development in both sexes
P450 Oxidoreductase deficiency	*POR*	Acts as electron donor to P450 enzymes, including 17-hydroxylase/lyase, 21-hydroxylase, and aromatase	Mildly increased	Increased progestogens (P5, P4, 17OHP4).	• Variable degree of GC deficiency, mostly subclinical; in most cases ranging from mild (PCOS-like) to severe form of Antley-Bixler syndrome • DSD and delayed puberty in both sexes
Cytochrome B5 deficiency	*CYB5B*	Acts as allosteric factor to facilitate the interaction of 17-lyase with P450 oxidoreductase	Normal	Isolated sex steroid deficiency	• No effect on MC/GC synthesis • 46, XY DSD and absence of puberty in 46, XX and 46, XY
17-Hydroxy steroid dehydrogenase deficiency	*HSD17B3*	Converts A4 to T	Normal	Decreased T/A4 ratio	• No effect on MC/GC synthesis • 46, XY DSD
5α-Reductase deficiency	*SRD5A2*	Converts T to DHT	Normal	Decreased DHT/T ratio	• No effect on MC/GC synthesis • 46, XY DSD
Aldo-keto-reductase 2/4 deficiency	*AKR1C2/4*	Converts intermediates in backdoor pathway from Δ4 progesterones (P4/17OHP4) to DHT	Unknown	Unknown	• No effect on MC/GC synthesis • 46, XY DSD

(*continued*)

Table 2. Continued

Disorder	Gene	Enzyme function	17OHP4 level in newborn screening	Typical serum steroid profile	Typical clinical characteristics
Steroid acute regulatory protein deficiency	*STAR*	Promotes entry of cholesterol to mitochondria for steroid biosynthesis	Low	Decreased steroid biosynthesis including MCs, GCs, and androgens	• AI in both sexes • 46, XY DSD/ambiguous genitalia at birth • Incomplete sexual development in both sexes or hypergonadotropic hypogonadism later in life
Steroidogenic factor 1	*NR5A1/ SF-1*	Orphan nuclear receptor regulating development and function of steroidogenic tissues	Low to normal	None	• AI with salt-wasting in very rare cases • Broad phenotype of 46, XY and 46, XX DSD

Reference no [1].

Abbreviations: 11DOC, 11-deoxycorticosterone; 17OHP4, 17α-hydroxyprogesterone; 17OHP5, 17α-hydroxypregnenolone; AI, adrenal insufficiency; ALDO, aldosterone; A4, androstenedione; CORT, corticosterone; DHT, dihydrotestosterone; DSD, difference in sexual development; F, cortisol; GC, glucocorticoid; MC, mineralocorticoid; P5, pregnenolone; P4, progesterone; S, 11-deoxycortisol; T, testosterone.

Learning Points

- A diagnosis of 3βHSD2 deficiency is challenging and any suspected CAH, in which no 21-hydroxylase deficiency is found, should be evaluated carefully with comprehensive steroid profiling using highly specific and sensitive methods (GC/LC-MS).
- If 3βHSD2D is suspected but steroid profiles are inconclusive, genetic analysis is recommended.
- Clinicians should be aware that in both 46, XX and 46, XY children with 3βHSD2 deficiency, adrenarche and puberty are often early and atypical. Spontaneous virilization may occur before and during pubertal development and may require sex hormone treatment.
- In a newborn with ambiguous genitalia, sex registration/assignment should be considered carefully and without time pressure in close collaboration with the family and an interdisciplinary DSD team as suggested in the "shared decision-making" process; psychosocial support for the DSD child and family should be installed at diagnosis and continued into adulthood.

Contributors

J.L. and K.S. performed functional assays. J.L. and T.d.T. performed steroid analyses. A.S. was involved in genetic analysis. D.A. was responsible for the treatment and diagnosis of the patient and, together with C.E.F., involved in the further diagnostics and editing of the manuscript. J.L. wrote the first draft of the manuscript. All authors reviewed and approved the final draft.

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Disclosures

The authors have nothing to disclose.

Informed Patient Consent for Publication

Signed informed consent obtained directly from the patient's relatives or guardians.

Data Availability Statement

Some or all data sets generated during and/or analyzed during this study are not publicly available but are available from the corresponding author on reasonable request.

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