The evolving role of whole-exome sequencing in the management of disorders of sex development

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Abstract

Objective: Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal and anatomical sex is atypical. Despite wide laboratory and imaging investigations, the etiology of DSD is unknown in over 50% of patients.

Methods: We evaluated the etiology of DSD by whole-exome sequencing (WES) at a mean age of 10 years in nine patients for whom extensive evaluation, including hormonal, imaging and candidate gene approaches, had not identified an etiology.

Results: The eight 46,XY patients presented with micropenis, cryptorchidism and hypospadias at birth and the 46,XX patient presented with labia majora fusion. In seven patients (78%), pathogenic variants were identified for RXFP2, HSD17B3, WT1, BMP4, POR, CHD7 and SIN3A. In two patients, no causative variants were found. Mutations in three genes were reported previously with different phenotypes: an 11-year-old boy with a novel de novo variant in BMP4; such variants are mainly associated with microphthalmia and in few cases with external genitalia anomalies in males, supporting the role of BMP4 in the development of male external genitalia; a 12-year-old boy with a known pathogenic variant in RXFP2, encoding insulin-like 3 hormone receptor, and previously reported in adult men with cryptorchidism; an 8-year-old boy with syndromic DSD had a de novo deletion in SIN3A.

Conclusions: Our findings of molecular etiologies for DSD in 78% of our patients indicate a major role for WES in early DSD diagnosis and management—and highlights the importance of rapid molecular diagnosis in early infancy for sex of rearing decisions.
Introduction

Disorders of sex development (DSD) are classified as a congenital discrepancy between external genitalia, and gonadal and chromosomal sex (1). The prevalence of DSD, including hypospadias, is estimated at 5 out of 1000 newborns (2), with 75% of affected individuals having 46,XY karyotype (3). The current classification of DSD includes four categories 46,XY, 46,XX, sex chromosome DSD (1) and syndromic DSD. Syndromic DSD are conditions associated with congenital malformations in addition to the atypical genitalia. These may be due to monogenic defects, biochemical abnormalities of steroid synthesis, or microdeletions, duplications or unbalanced rearrangements (4).

The investigation of infants with ambiguous genitalia is challenging because determining the molecular etiology can, in some children, be crucial for reaching sex of rearing decisions. The etiology of 46,XY DSD is unknown in more than 50% of cases, despite extensive laboratory and imaging investigations, and the diagnosis is often deferred to the second decade of life (5). The evaluation of an infant with ambiguous genitalia includes clinical examination, karyotyping, and laboratory and imaging evaluations. Until the last decade, targeted gene sequencing was used to identify the genetic etiology of patients with DSD. However, in addition to being expensive and time-consuming, in many cases, this approach failed to identify the etiology. Therefore, it is currently recommended only when clinical and hormonal assessments point to a specific gene (5). Today, high-throughput sequencing (HTS) panels of genes involved in sex determination and differentiation are available. More than 60 genes have been described in association with DSD (6, 7). The recent availability of whole-exome sequencing (WES), mainly for research purposes, has led to improved accuracy of diagnosis of DSD patients, identifying causal variants in more than 50% of cases, as well as novel genes causing DSD (8). Here, we report a cohort of nine children with DSD, in which wide laboratory, imaging, and targeted hypothesis-driven
sequencing investigations failed to identify the etiology of DSD. Use of WES in a research setting identified the genetic etiologies in seven (78%) of these patients.

Materials and methods

Patients

The cohort consisted of nine patients with atypical genitalia for whom extensive laboratory, imaging, and initial genetic assessments failed to identify the etiology of DSD. Excluded from the cohort were patients with ambiguous genitalia whose genetic etiology was identified by sequencing of candidate genes. All patients were followed in our clinic every 6 months.

Biochemical analysis

Hormonal levels were obtained at referral, during follow-up and at the last visit. At diagnosis, all patients were assessed for baseline hormone profile, and LHRH stimulation test (100 µg LHRH, with blood sampling at baseline, 30, 60, and 90 min) and ACTH stimulation test (250 µg Synacthen, with blood sampling at baseline and 60 min) were performed. hCG stimulation test (100 IU/kg Pregnyl, with blood sampling at baseline and 72 h) was performed in patients with 46,XY DSD. Repeat LHRH stimulation test was performed at the time of the study. LH, FSH, testosterone, TSH, FT₄, and cortisol were measured by direct automated chemiluminescent immunoradiometric assay using the ADVIA Centaur immunoassay system (Bayer Corporation, Tarrytown, NY). 17-OHP was measured by enzyme immunoassay (IBL International GmbH, Hamburg, Germany), and androstenedione was measured by chemiluminescent enzyme immunoassay (IMMULITE 2000, Siemens, Gwynedd, UK). Urinary glucocorticoid level was determined by gas chromatography–mass spectrometry (GCMS) to exclude adrenal enzyme deficiency.
Genetic analyses

This study was approved by the Ethics Committee of Ha’Emek Medical Center and by the Genetics Committee of the Israeli Ministry of Health. Blood samples were collected after the parents signed the appropriate consent form. Genomic DNA was extracted from peripheral mononuclear cells using the Blood Amp Kit (QIAGEN Inc., Valencia, CA). Targeted gene-by-gene sequencing was performed either when a candidate gene was suspected based on the phenotype and hormonal results that indicated a specific etiology, or based on the availability of specific gene testing in the genetics laboratory. Sanger sequencing of the coding exons and untranslated regions was used to identify pathogenic variants in candidate genes AR, NR5A1, SRD5A2, CHD7, LHR, WT1, GPR54 and DHCR7. The specific variant p.R80Q in the HSD17B3 gene, to which most cases of 17βHSD deficiency are attributed in the Israeli-Arab population, was analyzed when clinical and hormonal findings suggested deficiency of this enzyme (9).

Exon enrichment was performed using Agilent SureSelect Human All Exon V4. Paired-end sequencing was performed on the Illumina HiSeq2000 platform with an average sequencing coverage of 50X as described elsewhere (10, 11). Details of the exome sequencing procedures are listed in the supplementary materials and potentially pathogenic variants were verified by Sanger sequencing (Supplementary Data 1). The samples were analyzed as trios.

Results

Of the nine recruited patients, two were from consanguineous families (cases 2 and 5). Ultrasonography scan of the fetus during pregnancy identified a female phenotype in four out of seven patients, whereas karyotyping after birth revealed the 46,XY genotype in all of them. Median age at presentation was 21 days (range 7–455). All male genotype patients presented
with severe atypical genitilia, including all or part of the following: cryptorchidism, hypospadias, small testicular volume, and bifid scrotum (Table 1). Only one patient presented with atypical female phenotype (case 5). Additional organ anomalies were found in six out of the nine patients (Table 1). Median age at last visit was 12.6 years, and pubertal stages at last visit are summarized in Table 1. ACTH stimulation test revealed normal cortisol and 17-OHP responses in all subjects apart from case 5, in whom elevated peak 17-OHP concomitant with low peak cortisol suggested the diagnosis of congenital adrenal hyperplasia (Table 2). LHRH stimulation tests performed at presentation revealed an elevated LH peak in seven patients and an elevated FSH peak in four patients. hCG stimulation test revealed a variable rise in testosterone response after 72 h, but the results were inconclusive for a specific etiology (Table 2). Urinary GCMS profile was normal in all patients except for case 5, in whom the ratio between the adrenal metabolites indicated deficiency of oxidoreductase. Repeat LHRH stimulation tests were performed at the median age of 12 years (Table 2), revealing elevated peak LH in three patients and elevated peak FSH in four patients, consistent with the diagnosis of primary testicular failure. This wide hormonal investigation did not lead to a specific individual's etiology for DSD.

Eight patients had 46,XY karyotype, and only one had 46,XX karyotype. Targeted gene-by-gene approach and sequencing of specific candidate genes were negative for pathogenic variants in all patients. Patients underwent WES at the median age of 10 years, revealing three previously described pathogenic variants and four novel variants that constitute strong candidates for explaining the etiology of DSD (Table 3). In the other two patients, variants that could explain the phenotype were not observed. Case 1 had the previously reported variant c.664A>C, p.T222P in LGR8, also known as RXFP2, inherited from his mother (9). Case 2 had a novel homozygous autosomal recessive variant of HSD17B3 resulting in 17βHSD deficiency (c.673G>A, p.V225M), previously described by our group (10). Case 3 had a novel de novo splice-site variant of Wilms Tumor 1 gene (WT1), c.1433-3C>G. Case 4 had a novel de novo missense variant of BMP4, c.209G>T, p.R70L. Case 5 had the previously described homozygous variant of the cytochrome P450
oxidoreductase gene (POR) (11). Case 6, who had syndromic DSD, had a previously reported
*de novo* autosomal dominant variant of *CHD7* causing Charge syndrome, c.1480C>T,
p.R494T (12) and case 8, who also had syndromic DSD, carried a *de novo* deletion,
c.2809_2810del, p.K937QfsTer2 of *SIN3A* (SIN3 transcription regulator family member A).

**Detailed description of the patients**

**Case 1**: The proband, born to unrelated healthy parents, was referred to our clinic at
the age of 10 days for investigation of atypical genitalia. His karyotype was 46,XY. Hormonal
analysis indicated an elevated LH peak following LHRH stimulation and normal basal and
hCG-stimulated testosterone values. He underwent bilateral orchiopexy at the age of 17
months. Sequencing of four different candidate genes for pathogenic variants, and for the
common pathogenic variant of *HSD17B3* in the Israeli-Arab population, p.R80Q, was
negative. WES identified a missense variant of *RXFP2*, which is maternally inherited and has
been previously described in association with testicular maldescent (12). At the age of 13
years, he had a pubertal stage of Tanner P3 with short penile length and testicular volume of 4
mL. Peak LH and FSH following LHRH stimulation were exaggerated, indicating primary
testicular insufficiency

**Case 2**: The proband, female phenotype baby was referred due to palpable masses in
both inguinal canals at the age of 3 months. Cleft soft palate was found in physical
examination. Her karyotype was 46,XY. Laboratory evaluation revealed elevated peak LH
following LHRH stimulation. A low basal testosterone: androstenedione ratio of 0.39 (normal
range >0.8), suggested 17βHSD deficiency; however, sequencing of the common Israeli-Arab
population variant p.R80Q, which was only available at that time, was negative for the
variant. Sequencing of candidate genes, including *SRD5A2, LHR, AR* and *GPR54*, was
negative for pathogenic variants. WES performed at the age of 8 years revealed a novel
missense variant of the *HSD17B3* gene previously reported by us (13). The parents were
heterozygous for the identified mutation. At the age of 14.3 years, he had a pubertal stage of Tanner P3, G3, and elevated basal LH and FSH.

**Case 3:** The patient was reviewed at our institute at the age of 7 days for assessment of atypical genitalia. He had normal kidneys and absence of Mullerian duct remnant on ultrasonographic imaging. His karyotype was 46,XY. A candidate gene approach excluded pathogenic variants in **SRD5A2**, **AR** and **NR5A1**. WES performed at the age of 11 years identified a novel *de novo* splice-site variant of the **WT1** gene (c.1433-3C>G). At the age of 12.3 years, he had pubertal stage Tanner P3, G1, and elevated basal and peak LH and FSH. Annually repeated ultrasonographic imaging demonstrated normal kidneys with no abnormal findings.

**Case 4:** The proband was first seen in our clinic at the age of 12 days due to atypical genitalia. He was born to unrelated parents after in-vitro fertilization twin pregnancy. Prenatal ultrasound demonstrated a female fetus. In addition, he had a subaortic membrane and atrial septal defect. No other anomalies were found. His karyotype was 46,XY. A hormonal evaluation indicated normal gonadotropin and testosterone levels. Sequencing of the **SRD5A2**, **AR**, and **WT1** genes did not reveal pathogenic variants. WES revealed a *de novo* missense variant of **BMP4** predicted as pathogenic. This variant is absent from all public single-nucleotide polymorphism databases. At the age of 12.6 years, he had pubertal stage Tanner P3, penile length of 3.5 cm and testicular volume of 4 mL.

**Case 5:** The proband was born to first-cousin parents and first seen in our clinic at the age of 30 days with fusion of the labia majora and small vaginal–urethral orifice. Her karyotype was 46,XX. Results of ACTH stimulation test suggested congenital adrenal hyperplasia due to 21-hydroxylase deficiency. However, sequencing of **CYP21A** did not detect a pathogenic variant. GCMS was consistent with POR deficiency, but sequencing of the **POR** gene revealed no pathological variant. At the age of 11 years, using WES, a homozygous missense variant of **POR**, previously described in an Israeli-Bedouin family, was identified (14). The parents were heterozygous for the identified mutation. At the age of 11.8
years, she had no signs of puberty with low peak LH response to LHRH stimulation test and estrogen supplementation therapy was initiated.

Case 6: The 46,XY proband was born to unrelated parents and was referred to us at the age of 25 days due to atypical genitalia. In addition, he had dysmorphic facial features, severe hypotonia, right-sided aortic arch and conductive hearing impairment. Brain MRI demonstrated hypoplastic pons with mild fourth-ventricle dilatation. His clinical characteristics suggested syndromic DSD. LHRH and ACTH stimulation tests were within the normal range, but he had low peak testosterone values following hCG stimulation. The candidate gene approach revealed no pathogenic variants in \textit{DHCR7}, \textit{NR5A1} or \textit{WT1}. WES performed at 3.5 years of age revealed a previously described \textit{de novo} and heterozygous missense variant (c.1480C>T, p.R494T) of the \textit{CHD7} gene (15). The proband was later diagnosed with severe mental retardation and autism. At the age of 8 years, he had small penile length and nonpalpable testes.

Case 8. The 46,XY proband was referred to our clinic due to micropenis at the age of 1.1 years. His parents were unrelated. In addition, he had mental retardation, hydrocephalous, attention deficit hyperactivity disorder (ADHD), convulsive disorder, cardiac anomalies, short stature with growth hormone deficiency, and autism. No other anomalies were found. WES performed at the age of 12.75 years identified \textit{de novo} pathogenic deletion of 2 nucleotides in \textit{SIN3A}, c.2809_2810del (p.K937QfsTer2). At the age of 8 years, he had severe micropenis and a testicular volume of 1 mL.

Discussion

Using WES, we identified pathogenic variants that explained the phenotype of DSD in 78% of our cohort. Patients underwent WES at a mean age of 10 years, following lack of success with traditional diagnostic strategies (16), including wide hormonal assessments, imaging, and targeted gene sequencing, in finding the etiology of DSD. These traditional approaches have been found to identify the etiology of DSD in only 20% of cases, and a specific
diagnosis is often deferred to the second decade of life (6, 17–19). Recently, HTS panels have been used for the diagnosis of DSD (6, 9,–20,21). An international study including 326 patients with DSD identified its etiologies in 43% of them using a HTS panel of 64 known genes (9). Use of the HTS panel reduced costs, enabled an earlier specific diagnosis and facilitated clinical management. However, these panels cover only genes that are known to be involved in sex development and determination. In contrast, WES theoretically sequences all genes in the human genome and as new genes causing DSD are discovered, the patient datasets can be reanalyzed for pathogenic variants that were not previously recognized. Since WES generates a wealth of genetic data, it has been recommended that the molecular results, together with the clinical and hormonal findings, be interpreted by a multidisciplinary team that includes clinicians and medical geneticists (5). Here, using WES, we identified seven causative variants (four novel and three previously reported) that explained the etiology of DSD.

A de novo missense variant, c.209G>T, p.R70L, in the BMP4 gene was identified in case 4, an 11.3-year-old 46,XY male who presented at the age of 12 days with atypical external genitalia. BMP4 is a member of a large cytokine family related to the transforming growth factor beta proteins. BMP4 heterozygous loss-of-function variants (MIM 112262) have been described in association with autosomal dominant microphthalmia with brain and digital anomalies (MCOPS6), a syndrome that is characterized by ocular, digital and brain anomalies, cleft lip and palate, and renal malformations (22, 23). In mice, Bmp4 is expressed in both the mesenchyme and the urethral epithelium and it is essential for outgrowth of the genital tubercle (24). Mice lacking Bmp4 show hypoplasia of the genital tubercle together with reduced expression of other outgrowth factors, including Wnt5a, Hoxd13 and p63 (25). The development of male external genitalia consists of two phases. The first is development of the genital tubercle, which is regulated by BMP proteins, including BMP4, and the second is from 8 weeks onward, when the gonads have differentiated into testes in 46,XY individuals and the hormone-dependent phase begins, when testosterone causes elongation of the genital tubercle and the urethral groove terminates (24). Consistent with observations in mice lacking
Bmp4, variants in the human BMP4 gene have been reported in a few cases associated with hypospadias (6, 26). Eight missense variants were identified in Chinese patients with hypospadias by direct sequence analysis of BMP4 and BMP7 (26). Furthermore, HTS performed in 70 patients with variable DSD phenotypes revealed three heterozygous missense variants of BMP4 that were predicted to be damaging (20). Two cases had an additional SRD5A2 variant on one allele, suggesting dysgenic or polygenic inheritance. Interestingly, variants in BMP4 have been reported in association with combined pituitary hormone deficiency, suggesting that BMP4 participates in an early stage of pituitary development by inducing the formation of Rathke's pouch (27–29). Other than a subaortic membrane, atrial septal defect, severe hypospadias, and cryptorchidism, the boy had no other anomalies. This case highlights the role of BMP4 in external genital development.

Case 1 had a previously described heterozygous T222P variant in the RXFP2 gene. Insulin-like 3 hormone and its receptor Rxfp2 have been shown to play an important role in testicular descent in mice, with mice lacking Rxfp2 having an abdominal testis (30). The T222P variant has been reported in adult males with cryptorchidism attributed to reduced activity of the RXFP2 protein caused by poor membrane expression of the mutant receptor (12). However, other authors have called into question the role of RXFP2 variants in XY DSD, since no association between T222P or RXFP2 variant and male cryptorchidism has been reported (31, 32).

In case 8, with syndromic DSD, a de novo pathogenic deletion in SIN3A, c.2809_2810del (p.K937QfsTer2), was identified. Heterozygous variants in the SIN3A gene (MIM 607776) are associated with Witteveen–Kolk syndrome and are characterized by neurological disorders, including developmental delay, microcephaly, intellectual disability, and autism spectrum disorders (33). Other variable features include characteristic facial dysmorphia (broad forehead, long face, downsloping palpebral fissures, depressed nasal bridge, large fleshy ears, long and smooth philtrum, small mouth, and pointed chin), short stature, microcephaly, joint hypermotility, and small hands and feet. Male genital abnormalities were reported in four cases (34), and genetic inactivation of Sin3A in the
germline of XY mice leads to sterility resulting from early apoptotic death and a Sertoli-cell only phenotype (35). Case 8 carried a de novo, heterozygous loss-of-function variant and had features typical of Witteveen–Kolk syndrome, including intellectual disability, hydrocephalus, ADHD, convulsive disorder, cardiac anomalies, short stature with growth hormone deficiency, and autism. This variant is predicted to result in a truncated protein that will be recognized by the nonsense-mediated decay surveillance complexes and degraded.

Case 3 had a novel de novo splice-site variant of WT1. Mutations of the WT1 gene (OMIM 607102) are associated with Denys–Drash syndrome, presenting with renal failure and high risk for Wilms tumor, and Frasier syndrome exhibiting nephrotic syndrome with a high risk for gonadoblastoma. In our case, primary testicular failure was observed in the patient at the age of 12 years, but with no renal anomalies.

Case 5 had a mutation that had been previously reported in an Israeli-Bedouin family (14). This POR mutation (OMIM 124015) has been reported in association with Antley–Bixler syndrome displaying genital atypia, disordered steroidogenesis and skeletal anomalies. In our case, the patient presented with labia majora fusion and primary adrenal insufficiency in infancy. At the age of 12 years, she had no signs of puberty, indicating absence of gonadal steroid secretion.

Case 6 had a de novo heterozygous missense variant of the CHD7 gene (OMIM 608892), exhibiting syndromic DSD with severe mental retardation and autism. CHD7 has been reported as one of the genes causing syndromic DSD (15).

The sex of rearing decision is crucial for an individual's future, and takes into account many factors, including cultural background, future fertility, degree of virilization, potential adult sexual function, surgical intervention, life-long replacement therapy, and future malignancies. However, the main parameter to be considered is the likely gender identity in adulthood, which is strongly dependent on the specific DSD etiology (5, 36). In case 2, a female infant presented to our clinic at the age of 90 days with bilateral palpable masses. Genetic evaluation revealed a 46,XY karyotype. Hormonal evaluation and the candidate gene approach did not identify the etiology. It was only at the age of 8 years that WES identified a
homozygous missense mutation of the \textit{HSD17B3} gene. Knowing the etiology at infancy might have led to a different decision regarding sex of rearing in this case, highlighting the importance of WES in early molecular diagnosis of DSD and its important implications for the sex of rearing decision. Although WES identified the etiology of DSD in 78\% of the cohort, 22\% of the patients still remained with no diagnosis. Future directions to improve the accuracy of DSD diagnosis, might include using whole-genome sequencing, and improving bioinformatics methods by using available, rapid functional assays to prove causality of the identified variants (6). Moreover, repeat genetic testing is warranted because new genes are being discovered all the time.

\textbf{Conclusions}

Our findings of molecular etiologies for DSD in 78\% of our patients indicate a major role for WES in early DSD diagnosis and management, and highlight the importance of rapid molecular diagnosis in early infancy for sex of rearing decisions.

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\textbf{Conflict of interest}

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data Availability Statement

Data sharing not applicable

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| No. | Origin            | Consanguinity | Prenatal ultrasound phenotype | Age† (days) | Phenotype† | Karyotype | Age‡ (years) | Phenotype‡ | Others                                                              |
|-----|-------------------|---------------|-------------------------------|-------------|------------|-----------|-------------|------------|----------------------------------------------------------------------|
| 1   | Arab-Muslim       | No            | Female                        | 10          | Bilateral UDT, micropenis, hypospadias G4 | 46,XY    | 13.5        | P3         | 5.5         | 4                ADHD, learning difficulties                            |
| 2   | Arab-Muslim       | Yes           | Female                        | 90          | Labioscrotal folds, clitoromegaly, single orifice | 46,XY    | 14.3        | P3         | 7           | 5-6             Cleft soft palate                                    |
| 3   | Arab-Muslim       | No            | NA                            | 7           | Bilateral UDT, micropenis, hypospadias G4        | 46,XY    | 13.1        | P3         | 5           | Rt. 2 Lt. NP None                                                |
| 4   | Arab-Muslim       | No            | Female                        | 12          | Rt. UDT, hypospadias G4, bifid scrotum           | 46,XY    | 12.6        | P3         | 3.5         | 4                Subaortic membrane, ASD, recurrent UTI, mild sensorineural hearing impairment |
| 5   | Arab-Muslim       | Yes           | Female                        | 30          | Fusion of labia majora, small vaginal-urethral orifice | 46,XX    | 11.8        | P1         | B1          | None                                                      |
| 6   | Druze             | No            | Male (micropenis)              | 25          | NP testis, micropenis, hypospadias G4            | 46,XY    | 8.4         | P1         | 3.3         | NP                Autism, mental retardation, heart anomalies, deafness, dysmorphism |
| 7   | Arab-Muslim       | No            | Female                        | 14          | Hypoplastic bifid scrotum, micropenis & chorde, small TV | 46,XY    | 12.1        | P2         | 2.5         | 5                None                                                   |
| 8   | Jewish-Morocco    | No            | Male                          | 455         | Bilateral UDT, micropenis                        | 46,XY    | 8.1         | P1         | 2.5         | 1                ADHD, autism, mental retardation, convulsive disorder, hydrocephalus, SOD, ASD, GHD   |
| 9   | Jewish-Morocco    | No            | Male                          | 21          | Bilateral UDT, micropenis, hypospadias G4        | 46,XY    | 13.9        | P3         | 3           | 5                Learning and behavioral difficulties                   |
†At diagnosis.
‡At last visit.

TV, testicular volume; PH, pubic hair (Tanner stage); UDT, undescended testis; ADHD, attention deficit hyperactivity disorder; SOD, septo-optic dysplasia; ASD, atrial septal defect; GHD, GH deficiency; UTI, urinary tract infection; NP, nonpalpable; G4, grade 4; NA, not available.
| No. | Age \(^\dagger\) (days) | LHRH test\(^\dagger\) | hCG test | Age\(^\dagger\) (years) | LHRH test\(^\dagger\) | T (ng/ml) | GCMS | Other tests |
|-----|------------------|--------------------|----------|-----------------|--------------------|----------|------|-------------|
|     | LH (mIU/L) | LH peak (mIU/L) | FSH (mIU/L) | FSH peak (mIU/L) | T (ng/ml) | T\(^\dagger\) (ng/ml) | LH (mIU/L) | LH peak (mIU/L) | FSH (mIU/L) | FSH peak (mIU/L) |
| 1   | 14   | 2.8   | 46   | <0.4 | 3.9   | 2.59   | 4.65 | 13   | 2.8   | 29.4 | 8.5   | 21.4 | 2.87 | N |
| 2   | 90   | 7.2   | 52   | 0.57 | 1.46 | 0.7   | 2.13 | 14.9 | 14.3 | ND   | 43.2 | ND   | 3.5  | N |
| 3   | 7    | 2.0   | 13.6 | 5.1  | 19.7 | 1.1   | 10.8 | 12.3 | 6.4   | 66   | 74   | 160  | 0.6  | N |
| 4   | 224  | <0.5  | 5.3  | 1.1  | 5.3  | 0.24  | 3.35 | 12.6 | 2.5   | ND   | 5.66 | ND   | 1.58 | N |
| 5   | 30   | ND    | ND   | ND   | ND   | ND    | ND   | ND   | 11.8  | <0.07 | 1.5  | 3.5  | 14.3 | <0.24 N |
| 6   | 25   | <0.5  | 6.5  | 1.4  | 20.4 | <0.1  | 1.9  | 8.4  | <0.07 | ND   | 0.8  | ND   | 0.14 | N |
| 7   | 14   | 19.7  | 86   | 8.7  | 19.8 | 1.76  | 3.2  | 12.7 | 0.85  | ND   | 3.06 | ND   | 0.5  | N |
| 8   | 455  | <0.07 | ND   | 1.35 | ND   | <0.1  | ND   | 8.7  | <0.07 | ND   | 1.5  | ND   | <0.07 ND |
| 9   | 21   | 2.3   | 39.6 | 5.99 | 33.1 | 1.25  | 1.69 | 12.0 | 1.5   | 27.6 | 11.2 | 28.4 | 1.2  | N |

Normal ranges\(^\dagger\) <0.3-2.5 | 1.3-3.8 | <0.5-2.2 | 2.6-6.3 | <0.03 | 3 times basal | <0.3-2.5 | 1.3-3.8 | <0.5-2.2 | 2.6-6.3 | 2.3-8.65\(^\dagger\)†

\(^\dagger\) At diagnosis

\(^\dagger\) Normal range for pre-pubertal male

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At last visit

After 72 h

Normal range for adult male

Bold faced numbers represent values above the normal ranges

T, testosterone; ND, not determined; N, normal, T:A, testosterone:androstenedione ratio
**TABLE 3** Molecular findings in all patients

| No | Karyotype | EMS | Targeted gene approach | Age | Gene (Transcript ID) | DNA | Protein | Inheritance | Type of mutation (ACMG Classification) | Non-pathogenic variants |
|----|-----------|-----|------------------------|-----|----------------------|-----|---------|------------|----------------------------------------|------------------------|
| 1  | 46,XY     | 5   | DHCR7, SRD5A2, AR, NR5A1, GPR54, R80Q mutation of HSD17B3 | 12.8 | RXFP2 (ENST00000307765.5) | c.664A>C | p.T222P | AD | Missense – previously described | PROKR2 (c.809G>A, p.R270H) |
| 2  | 46,XY     | 6   | SRD5A2, LHR, AR, R80Q mutation of HSD17B3 | 8.0  | HSD17B3 (ENST00000375263.3) | c.673G>A | p.V225M | AR | Missense – novel. (Pathogenic) |
| 3  | 46,XY     | 5.5 | SRD5A2, AR, NR5A1 | 11.0 | WT1 (ENST00000333235 1.3) | c.1433-3C>G | | AD | Splice – novel de-novo (pathogenic) |
| 4  | 46,XY     | 4   | SRD5A2, AR, WT1 | 11.3 | BMP4 (ENST00000245 451.4) | c.809G>T | p.R70L | AD | Missense – novel de-novo (pathogenic) |
| 5  | 46,XX     | _   | CYP21A, POR | 11.0 | POR (ENST00000461988.1) | c.1615G>A | p.G539R | AR | Missense – previously described (pathogenic) |
| 6  | 46,XY     | 5   | NR5A1, WT1, DHCR7 | 3.5  | CHD7 (ENST00000423902.2) | c.1480C>T | p.R494T | AD | Nonsense – previously described de-novo (Pathogenic) |
| 7  | 46,XY     | 8   | SRD5A2, NR5A1, AR | 11.0 | | | | | No pathological variants |
| 8  | 46,XY     | 8.5 | SRD5A2, AR, LHR | 12.75 | SIN3A (ENST00000394947.3) | c.2809_281del | p.K937QfsTer2 | AD | Del-novel de-novo (Pathogenic) |
| 9  | 46,XY     | 6   | ND | 8.0  | | | | | VUS |

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Mean (range) 10 (3.5-12.8)

†At the time that WES was performed.

AD, Autosomal dominant; AR, Autosomal recessive; ND, not done; VUS, variants of unknown significance, EMS, external masculinization score (37)
Supplement 1.

WES

Exon enrichment was performed using Agilent SureSelect Human All Exon V4. Paired-end sequencing was performed on the Illumina HiSeq2000 platform with an average sequencing coverage of 50x as described elsewhere. Read files were generated from the sequencing platform via the manufacturer’s proprietary software. Reads were mapped using the Burrows–Wheeler Aligner, and local realignment of the mapped reads around potential insertion/deletion (indel) sites was carried out with GATK version 1.6. Single-nucleotide polymorphism (SNP) and indel variants were called using the GATK Unified Genotyper for each sample. SNP novelty was determined against dbSNP build 138 (www.ncbi.nlm.nih.gov/projects/SNP/). Datasets were filtered for novel or rare (MAF < 0.01) variants. Novel and rare variants were analyzed by a range of web-based bioinformatics tools using the Ensembl SNP Effect Predictor (http://www.ensembl.org/homo sapiens/userdata/uploadvariations). All variants were screened manually against Human Gene Mutation Database Professional [Biobase] (http://www.biobase-international.com/product/hgmd). In-silico analysis was performed to determine the potential pathogenicity of the variants using Polyphen (http://genetics.bwh.harvard.edu/pph), and SIFT (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html) online tools that predict the effects of human variants on protein function. We focused our analyses on non-synonymous coding, nonsense, and splice-site variants, filtering out all known common variations contained in dbSNP 138 and the 1000 Genomes Project (http://www.1000genomes.org/). Variants were also screened for novelty against the GnomAD variant server.