Exome Sequencing for the Diagnosis of 46,XY Disorders of Sex Development

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Context: Disorders of sex development (DSD) are clinical conditions where there is a discrepancy between the chromosomal sex and the phenotypic (gonadal or genital) sex of an individual. Such conditions can be stressful for patients and their families and have historically been difficult to diagnose, especially at the genetic level. In particular, for cases of 46,XY gonadal dysgenesis, once variants in *SRY* and *NR5A1* have been ruled out, there are few other single gene tests available.

Objective: We used exome sequencing followed by analysis with a list of all known human DSD-associated genes to investigate the underlying genetic etiology of 46,XY DSD patients who had not previously received a genetic diagnosis.

Design: Samples were either submitted to the research laboratory or submitted as clinical samples to the UCLA Clinical Genomic Center. Sequencing data were filtered using a list of genes known to be involved in DSD.

Results: We were able to identify a likely genetic diagnosis in more than a third of cases, including 22.5% with a pathogenic finding, an additional 12.5% with likely pathogenic findings, and 15% with variants of unknown clinical significance.

Conclusions: Early identification of the genetic cause of a DSD will in many cases streamline and direct the clinical management of the patient, with more focused endocrine and imaging studies and better-informed surgical decisions. Exome sequencing proved an efficient method toward such a goal in 46,XY DSD patients. **(*J Clin Endocrinol Metab* 100: E333–E344, 2015)**

Sex determination initiates when the bipotential gonad's genetic program determines the formation of either an ovary or testis. Subsequent differentiation of the internal and external genitalia is controlled by locally secreted and circulating sex hormones. Disruption of either determination or differentiation can lead to a disorder of sex development (DSD), ie, a discrepancy between an individual's chromosomal sex and phenotypic sex (1). Although an accurate genetic diagnosis and better understanding of genotype-phenotype correlations will offer a clearer prognosis to families, many DSD patients still do not receive a genetic diagnosis.

In 46,XY individuals, defects in testis determination often result in gonadal dysgenesis and can be caused by the loss of function of *SRY* (2) or *NR5A1* (3). However, variants in these genes only account for 10–15% of cases each, leaving most 46,XY gonadal dysgenesis cases undiagnosed at the genetic level. 46,XY DSDs caused by defects of differentiation are most often due to disruption of sex hormone synthesis or receptors, such as variants in the androgen receptor (AR) (4). They are often diagnosed clinically by detection of alterations in circulating hormone levels (5) but are not always explained by variants in known genes.

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Abbreviations: AIS, androgen insensitivity syndrome; AMH, anti-Müllerian hormone; AR, androgen receptor; DSD, disorder of sex development; INDEL, insertion and deletion; PMDS, persistent Müllerian duct syndrome; VUS, variants of uncertain clinical significance.

Table 1. List of DSD Genes in the Panel Used to Filter Exome-Sequencing Variants

Gene	Alternative Name	Coverage, %	Reported Associated Phenotype
Sex determination (gonadal dysgenesis, testicular and ovotesticular DSD)			
RSPO1	RSPONDIN	100	46,XX sex reversal and palmoplantar hyperkeratosis
SOX9	SRA1	100	46,XX sex reversal and campomelic dysplasia
SRY	TDF	100	46,XX testicular DSD and 46,XY ovarian DSD
CBX2	CDCA6	99	46,XY sex reversal
NR0B1	DAX1/AHCH	98	46,XY sex reversal
NR5A1	SF1	97	46,XY sex reversal; 46,XX premature ovarian failure
WWOX		95	46,XY gonadal dysgenesis
DMRT1	DMT1	93	46,XY gonadal dysgenesis
WNT4		92	46,XY DSD, 46,XY complete gonadal dysgenesis
MAP3K1	MEKK	89	46,XY sex reversal
DHH	HHG	85	46,XY partial or complete gonadal dysgenesis
SOX3	PHP	78	46,XX sex reversal
WT1	AWT1/WAGR	77	Wilms tumor-aniridia-genital anomalies-retardation syndrome
DMRT2		76	46,XY gonadal dysgenesis
GATA4		64	46,XY ambiguous genitalia
Sex differentiation (eg, steroid synthesis/receptors)			
AKR1C4	3-a-HSD, C11/CDR/DD4/HAKRA	100	46,XY DSD
AMHR2	MISR2	100	PMDS
ATRX	RAD54	100	α-Thalassemia X-linked intellectual disability syndrome
CYP11A1	P450SCC	100	CAH
CYP17A1		100	17-α-hydroxylase-deficient CAH
FGFR2		100	Apert syndrome
HSD17B3	SDR12C2	100	17-β hydroxysteroid dehydrogenase III deficiency
HSD3B2	SDR11E2	100	3-β-hydroxysteroid dehydrogenase-deficient CAH
POR		100	Cytochrome P450 oxidoreductase deficiency
SRD5A2		100	Steroid 5-α-reductase deficiency
STAR	StAR/STARD1	100	Cholesterol desmolase-deficient CAH
AR	AIS	95	Complete AIS/partial AIS
LHCGR	LCGR/LGR2/LHR/ULG5	92	Leydig cell hypoplasia
AKR1C2	BABP/DD/DD2/HAKRD/MCDR2	91	46,XY DSD
CYP21A2	CA21H/CAH1/CPS1	79	21-hydroxylase-deficient CAH
FOXL2	BPES	79	Blepharophimosis, ptosis, and epicanthus inversus
MAMLD1	CG1/F18/CXORF6	69	Hypospadias
AMH	MIS	59	PMDS
ARX	CT121/EIEE1/ISSX	50	X-linked lissencephaly with ambiguous genitalia (XLAG)
Central causes of hypogonadism			
ARL6	BBS3	100	Bardet-Biedl syndrome
BBS2		100	Bardet-Biedl syndrome
BBS5		100	Bardet-Biedl syndrome
BBS7	BBS2L1/FLJ10715	100	Bardet-Biedl syndrome
BBS9	B1/PTHB1	100	Bardet-Biedl syndrome

(*Continued*)

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Table 1. Continued

Gene	Alternative Name	Coverage, %	Reported Associated Phenotype
BBS10	FLJ23560	100	Bardet-Biedl syndrome
BBS12	FLJ35630/FLJ41559	100	Bardet-Biedl syndrome
CHD7	FLJ20357/FLJ20361/ KIAA1416	100	Kallmann syndrome, normosmic IGD, CHARGE syndrome
GNRH1	GNRH/GRH/LHRH	100	Isolated abnormality in GnRH secretion or response
GNRHR	LHRHR	100	Isolated abnormality in GnRH secretion or response
HESX1	ANF/RPX	100	Combined pituitary hormone deficiency
HFE	HLA-H	100	Hemochromatosis
LEP		100	Morbid obesity
MKKS	BBS6	100	Bardet-Biedl syndrome/McKusick-Kaufman syndrome
PROKR2	GPR73b/GPRg2/PKR2	100	IGD with anosmia (Kallmann syndrome) and normosmic IGD
PROP1		100	Combined pituitary hormone deficiency
TAC3	NKB/ZNEUROK1	100	Isolated abnormality in GnRH secretion or response
TACR3	Neurokinin β receptor/ NK3R	100	Isolated abnormality in GnRH secretion or response
TRIM32	BBS11	100	Bardet-Biedl syndrome
TTC8	BBS8	100	Bardet-Biedl syndrome/retinitis pigmentosa, autosomal recessive
BBS1		99	Bardet-Biedl syndrome
BBS4		99	Bardet-Biedl syndrome
FGFR1	BFGFR/CD331/CEK/FLG	98	Kallmann syndrome, normosmic IGD, and Pfeiffer syndrome
PCSK1	PC1/PC3/SPC3	98	Morbid obesity
KAL1	Anosmin-1/KALIG-1	95	IGD with anosmia (Kallmann syndrome)
LEPR	CD295/OBR	95	Morbid obesity
LHX3		87	Combined pituitary hormone deficiency
FGF8	AIGF	79	IGD with anosmia (Kallmann syndrome) and normosmic IGD
PROK2	BV8/KAL4/MIT1/PK2	76	IGD with anosmia (Kallmann syndrome) and normosmic IGD
KISS1R	AXOR12/HOT7T175	54	Isolated abnormality in GnRH secretion or response

Abbreviations: CAH, congenital adrenal hyperplasia; IGD, isolated GnRH deficiency.

Previously, we developed a targeted capture approach for 35 known DSD genes (6). This approach confirmed the genetic diagnosis in a known group of samples and identified a genetic cause in two of five previously undiagnosed patients. Here we have expanded this approach by using exome sequencing to capture almost all coding exons, followed by bioinformatic filtering using a comprehensive DSD gene list. The exome covers approximately 95% of RefSeq genes, thus covering most protein-coding sequence, which currently harbors 80–90% of known disease-causing variants (7). Therefore, all genes with any involvement in sex development can be analyzed concurrently, and new genes can be included in the analysis without having to reconfigure the sequencing pipeline or resequence the samples. We present data from a group of 40 46,XY DSD patients sequenced at the University of California-Los Angeles (UCLA) Clinical Genomic Center and analyzed using a gene list.

Materials and Methods

Samples were submitted to the UCLA research laboratory under an Institutional Review Board-approved protocol (no. 11-001775-AM-00007; Principal Investigator, E. Vilain) or to the UCLA Clinical Genomics Center. Exomes were captured using SureSelect All Exon 50 Mb capture kit (Agilent Technologies) and sequenced on a HiSeq2000 or HiSeq2500 (Illumina, Inc) as 50-bp or 100-bp paired-end runs. Base-calling was performed using Illumina's real-time analysis software. Sequence reads (QSEQ or FASTQ files) were aligned to the human reference genome (hg_g1k_b37 assembly) using Novoalign V2.07.13 (http://www.novocraft.com/main/page.php?s=novoalign). The output BAM file was sorted and merged, and PCR duplicates were removed using Picard (http://picard.sourceforge.net/). INDEL (insertion and deletion) realignment and recalibration were performed using the Genome Analysis Tool Kit (GATK) (http://www.broadinstitute.org/gatk/). Mean coverage was over 80 × for each sample, and approximately 93% of the RefSeq gene coding regions ± 2 bp was covered at 10 × or greater (individual gene coverage is indicated in Table 1). Single-nucleotide variants and small INDELs were called using GATK's Unified Genotyper, then recalibrated and filtered using GATK variant-quality score recalibration and variant filtration tools. Consanguinity analysis was performed by identifying regions >1 Mb of homozygosity using Linkdatagen (http://bioinf.wehi.edu.au/software/linkdatagen/) (8) and Plink software (http://pngu.mgh.harvard.edu/~purcell/plink/) (9). All high-quality variants were annotated using Variant Annotator X, a custom-designed variant effect predictor (10).

Variants with a minor allele frequency of <1% in the Exome Sequencing Project (ESP) of more than 6500 individuals were intersected with a DSD gene list to identify mutations in known

Table 2. Phenotypic Descriptions, Previous Clinical Findings, and Genetic Findings From Current Study

Sample ID	External Genitalia	Anatomy	Gonads
RDSD001	Female	No uterus, blind vagina	None found by ultrasound
RDSD002	Female	No records	Complete gonadal dysgenesis
RDSD003	Female	No uterus, blind vagina, hypoplastic labia, Fallopian tubes	No records
RDSD004	Female	No records	Gonadal dysgenesis
RDSD005	Female	No uterus, bilateral Fallopian tubes	Streak, with rete testis and seminiferous tubules
RDSD006	Female	No records	No records
RDSD007	Female	No records	Complete gonadal dysgenesis
RDSD008	Female	No uterus	No records
RDSD009	Female	No uterus, blind vagina, typical labia	Immature testis with Fallopian tubes on left; immature testis with vas on right
RDSD010	Female, enlarged clitoris	No records	No records
RDSD011	Female	Uterus and 1/3 vagina present	No records
RDSD012	Female	No records	No records
RDSD013	Female	Uterus and Fallopian tubes present	Bilateral inguinal streak
RDSD014	Female	No records	Complete gonadal dysgenesis
RDSD015	Female	No clitoromegaly, non-rugated labia, vagina	Small bilateral inguinal gonads, fibrous tissue with Sertoli cells
RDSD016	Female, enlarged clitoris	No uterus	Two testes
RDSD017	Female	Hypoplastic partial bicornuate uterus with cervix, Fallopian tubes	Bilateral streak gonads
RDSD018	Ambiguous (raised male)	Partial labioscrotal fusion, micropenis, penoscrotal hypospadias, urogenital sinus, Müllerian remnants	No palpable gonads
RDSD019	Ambiguous (raised female)	Uterus, cervix, Fallopian tube on left long urethra	Disorganized testicular tissue on left; normal testis on right
RDSD020	Ambiguous (raised female)	Vas and Müllerian structures	No records
RDSD021	Ambiguous (raised male)	Microphallus, posterior labioscrotal fusion	No records
RDSD022	Ambiguous	No records	No records
RDSD023	Ambiguous (raised male)	Microphallus, chordee, hypospadias, incompletely fused scrotum	Ovotestis with Fallopian tube and partial uterus; contralateral normal testis
RDSD024	Ambiguous (raised male)	UG sinus, cryptorchidy, "mild phallus," "unfold area of scrotum"	Left: immature testis, seminiferous tubules, epididymis; right: fibro-fatty connective tissue, ductal structures consistent with mesonephric ducts, possible vas
RDSD025	Male, micropenis	No records	Cryptorchidy
RDSD026	Male	See detail in legend[c]	No gonad on right; left, inguinal fibrotic and atrophic testis, no Sertoli; exuberant Leydig cell proliferation, in complex specimen
RDSD027	Male	Fallopian tubes and small uterus	Two abdominal testes, normal testicular tissue
CDSD028	Female	No uterus, no adnexa (U/S)	Inguinal (no other information)
CDSD029	Male	Bifid scrotum, penoscrotal transposition, penoscrotal hypospadias, no micropenis	Bilateral descended gonads, likely testes (normal male hormones)
CDSD030	Female, clitoromegaly	No posterior fusion, vaginal opening, no uterus (U/S)	Palpable in inguinal hernia, testes with no malignancies
CDSD031	Ambiguous (raised female)	Posterior labial fusion, no rugation, no clitoromegaly, UG sinus	Complete gonadal dysgenesis, abdominal, no oocytes, no seminiferous tubules
CDSD032	Female	Vagina, UG sinus, no uterus, no Fallopian tubes	Inguinal testes with calcifications and immature seminiferous tubules lacking spermatogonial development

(*Continued*)

Table 2. Continued

Sample ID	Additional Clinical Findings	Other Diagnostic Tests	Gene Identified
RDSD001	ASD, VSD, BAV, hydrocephalus, cerebellar hypoplasia, optic nerve hypoplasia, ear pits, blindness	aCGH; SLO[a]	*CHD7*
RDSD002	No records	No records	
RDSD003	Tall stature, breast Tanner 3	Familial, AR, SRY present	
RDSD004	No records	aCGH, TESCO[b]	
RDSD005	No records	Familial, aCGH, SRY present	*DHH*
RDSD006	Amelia	SRY, SOX9, WNT4, aCGH	
RDSD007	Adrenal rests	SRY, SF1	
RDSD008	Adrenal insufficiency, small adrenals	Consanguinity, aCGH, TESCO	*STAR*
RDSD009	Jejunal atresia (corrected) and microcephaly	aCGH, TESCO, SRY promoter	*AR*
RDSD010	No records	aCGH	
RDSD011	Short stature, dysmorphic features, failure to thrive	SRY present, aCGH	
RDSD012	Kidney disease	WT1	
RDSD013	Gonadal dysgenesis, normal uterus	SYR present, sequenced	
RDSD014	Presentation at age 15	No records	*MAP3K1*
RDSD015	Raised as male	AR, RHOA, SRY, DHH, SF1, MAP14, CXORF6, MAP3K1	*LHCGR*
RDSD016	Clinical diagnosis of AIS	No variants in AR	*NR5A1*
RDSD017	Tall stature, primary amenorrhea	No records	*MAP3K1*
RDSD018	No records	aCGH 311 kb duplication on chromosome 11 of unknown clinical significance	
RDSD019	No records	SRY present	*WT1*
RDSD020	Developmental delay, agenesis of corpus callosum, Dandy-Walker malformation	No Y chromosome abnormality, aCGH	
RDSD021	Hypotonia, congenital adrenal hypoplasia, dysmorphic features, cardiac defect	SRY present, aCGH, no 22q del	
RDSD022	Microcephaly, intestinal dysmotility, optic nerve hypoplasia	No records	
RDSD023	No records	Familial; normal male hormonal profile	*MAP3K1*
RDSD024	Denys-Drash syndrome, end-stage renal disease, bilateral nephrectomy before age 2, no Wilms tumor	SRY present	*WT1*
RDSD025	Hypoplastic adrenal gland, dysmorphic features, hypotonia, brain malformations	Consanguinity, aCGH	
RDSD026	PMDS	No records	*AMHR2*
RDSD027	PMDS	Elevated AMH; normal LH, FSH, T	*AMHR2*
CDSD028	No records	SRY present	*HSD17B3*
CDSD029	Normal renal U/S, normal EKG	No records	*CHD7*
CDSD030	Primary amenorrhea, elevated T, DHT, and T/DHT ratio	SRY present; AR	
CDSD031	Bilateral gonadectomy	SRY present	
CDSD032	Deafness, impaired cognition	No records	*NRP1*

(*Continued*)

Table 2. Continued

Sample ID	External Genitalia	Anatomy	Gonads
CDSD033	Female	No records	Bilateral hernias with palpable gonads, normal-appearing testes descended into labial folds after orchiopexy
CDSD034	Ambiguous (raised male)	Penoscrotal transposition, penoscrotal hypospadias, bifid scrotum	Likely testes (normal hormonal function and U/S) in upper scrotum requiring orchiopexy
CDSD035	Male	"Abnormal genitalia"	Undescended testes
CDSD036	Ambiguous (raised male)	Midshaft hypospadias, chordee	Bilateral descended testes
CDSD037	Male, micropenis		Vanishing testes
CDSD038	Ambiguous (raised male)	2-cm phallus, penoscrotal hypospadias, penoscrotal transposition, micropenis, chordee	Bilateral descended testes
CDSD039	Male, micropenis	No ovaries or uterus (U/S), 1-cm phallus, small scrotum	Vanishing testes; nubbin with no testicular tissue (laparoscopy)
CDSD040	Ambiguous (raised male)	Perineal hypospadias, 2-cm phallus, bifid scrotum, penoscrotal transposition, no Müllerian structures	Bilateral descended testes; no gonadal dysgenesis

Abbreviations: UG, urogenital; ASD, atrial septal defect; VSD, ventricular septal defect; BAV, bicuspic aortic valve; EKG, electrocardiogram; U/S, ultrasound; IUGR, intrauterine growth restriction; aCGH, array comparative genomic hybridization. aCGH was performed and did not detect any copy-number variants. Sex of rearing was indicated when it was discordant with external genitalia or external genitalia were ambiguous.

[a] SLO is the endocrine test for Smith-Lemli Opitz.

[b] TESCO, testis-specific enhancer of *SOX9*, was analyzed and no variant was detected.

[c] Hypoplastic vas, epididymis, rete testis, microcalcifications, portion of seminal vesicle and prostatic tissue, primitive Fallopian tube, vagina, endocervix, uterine structure.

DSD genes. The gene list (Table 1) was generated by combining the genes included in our capture panel (6) with a search of online databases such as OMIM, HGMD professional, and GeneTests using the key word "sex." HGMD contains information about genes and variants that have been identified in human disease, and findings from HGMD are considered "clinical genes." OMIM contains information from both human disease and animal models. Thus, these two databases are overlapping, but each contains information not in the other, so using both generates the longest list. This list was curated so that all genes included were published in at least one human case of DSD, and it is dynamic so it can be updated as soon as new findings are published. When parental samples were available, sequencing results were filtered to identify all de novo, homozygous, and compound heterozygous variants, even if the variants were not within the known DSD gene list.

Sanger sequencing using custom-designed primers was used to confirm the exome sequencing results for all research samples (except case RDSD005, where insufficient sample remained). As of May 2013, a UCLA Clinical Genomics Center retrospective data analysis showed that Sanger sequencing confirmation was unnecessary for single-nucleotide variants with high exome sequencing quality score (11). All INDELs are validated by Sanger sequencing.

Using American College of Medical Genetics and Genomics guidelines, we classified variants into five main categories: pathogenic, likely pathogenic, variants of uncertain clinical significance (VUS), likely benign, and benign (12). Premature termination codons and splice site variants are considered mutations by definition (12). Variants are also called pathogenic if previously reported in a similar clinical phenotype. Novel variants in genes related to the clinical phenotype that are predicted to be damaging are classified likely pathogenic. To assess the possible impact on protein structure and function, we used in silico algorithms SIFT (13), PolyPhen2 (14), and Condel (15). Unless otherwise stated, all novel variants discussed here were predicted pathogenic by all three algorithms. Single variants in apparently dominant conditions were only considered if present in the ESP at less than 0.1%.

Results from the Clinical Genomics Center are evaluated by the UCLA Genomic Data Board, a team of experts that meets weekly to analyze exome findings. The Board consists of the center's three directors, laboratory professionals, American Board of Medical Genetics and Genomics board-certified geneticists, genetic counselors, and clinicians including, if possible, the referring physician for each case. All variant calling is discussed and ultimately decided by this interdisciplinary group, a great strength of the UCLA Clinical Genomics Center.

Results

We report the results of exome sequencing in individuals with a 46,XY karyotype and a range of DSD phenotypes. This data set contains all samples of 46,XY DSD submitted to our research lab that did not have a genetic diagnosis after all other testing methods had been exhausted and the first 13 sequential samples submitted to the UCLA Clinical Genomics Center for testing of 46,XY DSD. Material of X and Y origin was confirmed by exome sequencing. When

Table 2. Continued

Sample ID	Additional Clinical Findings	Other Diagnostic Tests	Gene Identified
CDSD033	No records	SRY present, AR, normal Sertoli cell function (AMH, InhibinB)	*HSD17B3*
CDSD034	No records	SRY present, aCGH	——
CDSD035	Neuropathy, hypotonia	No records	*MAMLD1*
CDSD036	No records	No records	——
CDSD037	No other malformations found	SRY present	*CHD7*
CDSD038	Congenital hypothyroidism	SRY present	*BNC2, FGFR1*
CDSD039	No records	SRY present	——
CDSD040	Premature birth, IUGR	SRY present, normal InhB, AMH, T, dihydrotestosterone	*MAP3K1*

known, phenotypic characteristics and results of previous genetic testing history are described in Table 2. The range of presenting phenotypes was wide, as is typical of DSD, with external genitalia classified as typical female with or without clitoromegaly (21 cases), ambiguous (12 cases), or typical male with or without micropenis (7 cases). Seven patients had associated nongenital malformations, not representing an easily recognizable syndrome.

Variants in MAP3K1

One of the most striking findings in our study was the identification of *MAP3K1* variants in a total of four cases. Variants in this gene have recently been associated with complete gonadal dysgenesis (16), and two of our cases had the same previously reported variant p.Gly616Arg. Patient RDSD014 was a female who presented in adolescence with complete gonadal dysgenesis. In contrast, patient RDSD023 was a male with ovotesticular DSD, ascertained at birth due to the presence of ambiguous genitalia, a finding not previously associated with this variant. We also identified novel, likely pathogenic variants in two additional patients. Patient RDSD017 had complete gonadal dysgenesis and a de novo p.Arg339Gln missense variant. Patient CDSD040 had a p.Pro257Leu missense variant predicted damaging by two of the three in silico algorithms (SIFT and PolyPhen) and presented as a male with complex ambiguous genitalia but no gonadal dysgenesis (Table 2). Pearlman et al (16) examined only patients with complete gonadal dysgenesis, whereas our study included a wider range of 46,XY DSD phenotypes. Most *MAP3K1* variants so far identified cluster in exons 2–4, and the p.Gly616Arg is in exon 10; thus, there is no obvious genotype-phenotype correlation.

Variants in WT1

We found two variants in *WT1*, a gene associated with 46,XY gonadal dysgenesis in several conditions including Denys-Drash syndrome (17). Patient RDSD024 presented with end-stage renal failure and Denys-Drash syndrome in the differential diagnosis. We identified a novel likely pathogenic p.His469Gln missense variant located in exon 9 of *WT1*, the location and type of variants most often associated with Denys-Drash syndrome (18). RDSD019, a patient with similar genital features, also had a novel missense variant (p.Arg458Gln) in exon 9 of *WT1*. Subsequent testing of parental samples showed that the variant was inherited from the unaffected father, making it less likely to be pathogenic. However, a new publication reported a familial case of Denys-Drash syndrome with the well-established exon 9 p.Arg394Trp variant identified in both the proband and his unaffected father (19), suggesting incomplete penetrance. With this report of incomplete penetrance in a case of an established *WT1* disease-causing variant, we decided that the p.Arg458Gln was in fact

Table 3. Details and Classification of Genetic Findings

Gene	Patient No.	Genomic Position	Zygosity	Transcript ID
Sex determination genes				
NR5A1	RDSD016	chr9:127255362	het	NM_004959.4
MAP3K1	RDSD014	chr5:56171018	het	NM_005921.1
MAP3K1	RDSD017	chr5:56160742	het	NM_005921.1
MAP3K1	RDSD023	chr5:56171018	het	NM_005921.1
MAP3K1	CDSD040	chr5:56155678	het	NM_005921.1
DHH	RDSD005	chr12:49483790	het	NM_021044.2
WT1	RDSD019	chr11:32413577	het	NM_024426.4
WT1	RDSD024	chr11:32413543	het	NM_024426.4
Sex differentiation genes				
AMHR2	RDSD026	chr12:53823984	het	NM_020547.2
		chr12:53823970	het	NM_020547.2
AMHR2	RDSD027	chr12:53819596	het	NM_020547.2
		chr12:53823970	het	NM_020547.2
HSD17B3	CDSD028	chr 9:99064233–388	hom	NM_000197.1
HSD17B3	CDSD033	chr9:99017188	het	NM_000197.1
		chr9:99060705	het	NM_000197.1
STAR	RDSD008	chr8:38008272	hom	NM_000349.2
AR	RDSD009	chrX:66766183	hemi	NM_000044.3
LHCGR	RDSD015	chr2:48941168	hom	NM_000233.3
MAMLD1	CDSD035	chrX:149639324	hemi	NM_005491.3
Central causes of hypogonadism				
CHD7	RDSD001	chr8:61655619	het	NM_017780.3
CHD7	CDSD037	chr8:61765478	het	NM_017780.3
Other				
NRP1	CDSD032	chr10:33491864	het	NM_001024628.2
BNC2	CDSD038	chr9:16435821	het	NM_017637.5
FGFR1	CDSD038	chr8:38287238	het	NM_023110.2

Abbreviations: hemi, hemizygous; het, heterozygous; hom, homozygous.

likely causative of the proband's phenotype and exhibits reduced penetrance in the apparently unaffected father. In consequence, both of the novel *WT1* variants identified in our study are likely the cause of the observed phenotype.

STAR variant and adrenal insufficiency

In a phenotypically female patient with suspected adrenal insufficiency and absent uterus (RDSD008), we found a homozygous variant at c.64+1G>A in the *STAR* gene. Splice site variants are considered mutations by definition because they generally result in a truncated protein (12). Homozygous mutations in *STAR* are associated with 46,XY sex reversal as part of lipoid congenital adrenal hyperplasia (20). This patient had four large regions of homozygosity greater than 10 Kb in size, equivalent to 1.97% of the genome being homozygous. The *STAR* variant was located within the largest homozygous region, spanning more than 20.6 Kb on chromosome 8. This sample had been subjected to microarray analysis for detection of large deletions and duplications, and none were detected (Table 2). The finding of this splice site variant in a homozygous interval of the patient's genome is probably a true homozygous finding, and the match with the reported phenotype of adrenal insufficiency makes this a likely genetic diagnosis.

Leydig cell hypoplasia

Patient RDSDO15 has typical female external genitalia and no response to T treatment, but no variant in the AR. Exome sequencing identified a homozygous c.562G>T nonsense variant in the *LHCGR* gene, predicted to lead to a truncated protein p.Glu188*, a likely null allele (12). We also sequenced the parents, who were known to be related, and several short homozygous interval(s) (5–10 Mb) were observed in the patient, encompassing 5.95% of the genome. The *LHCGR* variant identified here occurs in a region of homozygosity on chromosome 2. Inactivating variants of the *LHCGR* gene result in failure of Leydig cells to develop in the testis (21), leading to an extremely rare condition known as Leydig cell hypoplasia. Although these genetic findings are likely diagnostic for the Leydig cell hypoplasia, unfortunately they do not explain the lack of response to exogenous T in this patient.

Variants in the anti-Müllerian hormone (AMH) receptor/persistent Müllerian duct syndrome (PMDS)

AMH causes regression of the paramesonephric ducts through the AMH receptor, AMHR2 (22). In PMDS, Müllerian-derived structures remain in 46,XY individuals who are otherwise normal males (5). PMDS is a recessive

Table 3. Continued

Gene	cDNA Change	Protein Change	Effect of Variant	Variant Call
Sex determination genes				
NR5A1	c.937C>T	p.Arg313Cys	Reported mutation	Likely Pathogenic
MAP3K1	c.1846G>A	p.Gly616Arg	Reported mutation	Pathogenic
MAP3K1	c.1016G>A	p.Arg339Gln	Predicted damaging	Likely Pathogenic
MAP3K1	c.1846G>A	p.Gly616Arg	Reported mutation	Pathogenic
MAP3K1	c.770C>T	p.Pro257Leu	Predicted damaging	Likely Pathogenic
DHH	c.1043A>T	p.Glu348Val	Predicted damaging	VUS
WT1	c.1373G>A	p.Arg458Gln	Predicted damaging	Likely Pathogenic
WT1	c.1407C>A	p.His469Gln	Predicted damaging	Pathogenic
Sex differentiation genes				
AMHR2	c.1343C>G	p.Pro448Arg	Predicted damaging	Pathogenic
	c.1330_1356del	p.Leu444_Glu452delinsdel	Reported mutation	
AMHR2	c.745C>T	p.Leu249Phe	Predicted damaging	Pathogenic
	c.1330_1356del	p.Leu444_Glu452delinsdel	Reported mutation	
HSD17B3	Exon 1 deletion	likely null	Predicted damaging	Pathogenic
HSD17B3	c.239G>A	p.Arg80Gln	Reported mutation	Pathogenic
	c.194C>T	p.Ser65Leu	Reported mutation	
STAR	c.64 + 1G>A	p.Gly22*	Splice donor variant	Pathogenic
AR	c.1195T>C	p.Trp1195Arg	Predicted damaging	Likely Pathogenic
LHCGR	c.562G>T	p.Glu188*	Premature stop codon	Pathogenic
MAMLD1	c.1479_1480dupCAG	p.Gln502dup	Predicted damaging	VUS
Central causes of hypogonadism				
CHD7	c.1628C>T	p.Ser543Leu	Predicted damaging	VUS
CHD7	c.6194G>A	p.Arg2065His	Predicted damaging	VUS
Other				
NRP1	c.1819C>G	p.Glu607Glu	Likely benign	VUS
BNC2	c.2371T>C	p.Tyr791His	Likely damaging	VUS
FGFR1	c.320C>T	p.Ser107Leu	Predicted tolerated	VUS

condition generally caused by variants in the *AMH* or *AMHR2* genes. Unfortunately, testing for these genes is not available on a clinical basis in the United States. In two unrelated patients clinically diagnosed with PMDS, we identified a known 27-nucleotide deletion (23) and an additional variant in the *AMHR2* gene. In case RDSD026, the second variant was within the deleted region and therefore must be present on the other allele. In case RDSD027, the second variant was located in a different region of the gene, and without parental samples, we could not ascertain phase. However, given the strong association of *AMHR2* variants with the diagnosis of PMDS, we believe a genetic diagnosis has been achieved in both cases.

Likely pathogenic variants in partial androgen insensitivity syndrome (AIS)

Variants in the AR are well known causes of AIS (4, 24). We found a previously unreported missense variant in *AR* in RDSD009 that is likely causative of the DSD features in this patient. This patient had previously undergone deletion analysis for *AR* with no findings (25). To the best of our knowledge, there are no additional reports of such a constellation of clinical features as seen in this patient, and it seems unlikely that this single missense variant in *AR* would be responsible for them (Table 2).

NR5A1/SF1 is associated with 46,XY gonadal dysgenesis and adrenal insufficiency (3). In patient RDSD016, we identified an *NR5A1* variant previously reported in a patient with isolated distal hypospadias, generally considered a mild form of DSD (26). Because *NR5A1* variants are associated with a range of phenotypes, from severe gonadal dysgenesis to isolated hypospadias or even male infertility (27, 28), this variant is likely causative of the phenotype.

Variants in the *HSD17B3* gene were identified in two patients. In CDSD028, exon 1 of the gene was deleted in a region of homozygosity. Subsequent deletion/duplication analysis of the gene confirmed a 461-bp homozygous deletion of the gene. The deletion includes the initiating ATG and thus likely results in a complete lack of protein. In CDSD033, two different missense variants previously reported as damaging (29–31) were identified. HSD17B3 deficiency is a classic differential diagnosis for AIS (4). Our results show that it might be less rare than previously thought.

CHD7 variants in atypical CHARGE syndrome presentations

Mutations in the *CHD7* gene can cause CHARGE syndrome, a complex multiorgan disorder including genital abnormality (32), but not all variants in *CHD7* lead to the

full CHARGE syndrome phenotype (33). In two patients with very different presentations, we identified novel missense *CHD7* variants. Patient CDSD037 presented with a fairly typical genital presentation in CHARGE syndrome; however, no other anomalies were found that would warrant a clinical diagnosis of CHARGE even after follow-up by the clinician after the exome report. In contrast, the genital presentation of patient RDSD001 was less typical of CHARGE syndrome, but she had associated anomalies in organs typically affected in CHARGE syndrome (Table 2). However, follow-up by the referring physician showed that they were not typical of CHARGE syndrome; thus, this variant also remains a VUS. In both of these cases, we cannot determine whether the variants are benign and unrelated to the patient's phenotype or instead add to the growing body of evidence expanding the spectrum of phenotypes associated with *CHD7* variants (33).

Other VUS

VUS are potentially clinically actionable, and further clinical tests in patients in whom they are identified may assist in refining their categorization. We identified several VUS in our study. When these were in clinical samples, they were included on the report. Loss-of-function alleles of Desert Hedgehog (*DHH*) cause recessive 46,XY gonadal dysgenesis (OMIM no. 233420). Patient RDSD005 with complete gonadal dysgenesis had a heterozygous missense p.Glu348Val variant in *DHH*. The only previously reported heterozygous variant is a frameshift mutation on a 46XY, 45X mosaic background. The genital phenotype of this patient is an excellent fit with previous reports, and we feel the variant is potentially causative of this patient's phenotype; but, with the current evidence, it remains a VUS.

Hypospadias is a common malformation associated with DSDs, but the genetic etiology remains unclear. In two patients, we identified VUS in genes previously associated with hypospadias. A hemizygous variant in *MAMLD1* (OMIM no. 300758) was identified in a patient raised as male with undescended testes and "abnormal genitalia," associated with neuropathy and hypotonia (CDSD035). The variant cannot explain the patient's nongenital clinical symptoms but may be involved in the DSD part of the phenotype. CDSD038, a patient with penoscrotal hypospadias and a complex genital phenotype, harbored a missense *BNC2* variant, another gene associated with hypospadias (34, 35), and a variant in *FGFR1*, a gene associated with hypogonadotropic hypogonadism, especially in association with mutations *FGF8* or *GNRHR*, a case of oligogenic etiology in DSD (36). The *BNC2* variant, or the combination of the *FGFR1* and *BNC2* variants, may cause the genital phenotype in this patient.

When parental samples are available, we further mine the data outside of the primary gene list for de novo heterozygous variants and inherited compound heterozygous variants. In CDSD032, a de novo variant was found in the *Neuropilin 1* (*NRP1*) gene. Neuropilin 1 interacts with Sema3A, and variants in *SEMA3A* are associated with hypogonadotropic hypogonadism (MIM no. 614897). A mouse model preventing the interaction of Nrp1 and Sema3a proteins results in a Kallmann-like phenotype (37). This variant was reported as a VUS; however, endocrine testing in the child at age 12 did not identify hypogonadotropic hypogonadism. There are reports of spontaneous reversal of hypogonadotropic hypogonadism (38), but in this case endocrine testing did not support the diagnosis, and the genital phenotype of this patient remains unexplained genetically.

Discussion

Exome analysis of 46,XY DSD cases generated a genetic diagnosis in a total of 35% (14 of 40) of cases, with an additional six VUS that may be reclassified as literature evolves. Exome sequencing allowed an unprecedented level of genetic diagnostic success in this cohort, especially considering that, for most patients, other endocrine and genetic testing had been exhausted.

Historically, DSD patients have been diagnosed through a combination of endocrinology and phenotypic examination, with a genetic diagnosis being secondary. However, an early genetic diagnosis can guide future endocrine and imaging tests and help limit potentially unnecessary invasive testing and costs. For example, the variants in *HSD17B3* uncover a risk for virilization at puberty. Because 46,XY DSD can be associated with multiple genetic findings and variable clinical features, exome sequencing is also useful to identify a genetic cause without preconceived phenotype ideas. Patients with variants in the same gene can present very differently, as exemplified in our study by the *MAP3K1* variants we found in four patients with highly variable phenotypes. Conversely, patients with a clinical diagnosis of AIS were found to have likely pathogenic variants in the *NR5A1* or *HSD17B3* genes.

Exome sequencing identified genetic diagnoses of extremely rare conditions and identified variants in genes not currently available for clinical testing. Although *MAP3K1* and *AMHR2* mutations are known causes of DSD, clinical testing for these genes is not yet available in the United States, and the limited clinical testing of *LHCGR* available would not have detected the variant we identified here. Most samples here had already been tested for variants in *SRY* and *NR5A1*; thus we cannot directly address whether

the proportion of DSD cases accounted for by them will change as new genes such as *MAP3K1* are identified. However, from our study, we anticipate that in an unbiased cohort of 46,XY DSD individuals, these three genes will each account for 10–15% of cases.

Genetic diagnoses are useful for patients and clinicians, contribute to clinical knowledge of DSD, and are invaluable for genetic counseling of couples contemplating future pregnancies. A genetic diagnosis can also bring reassurance to patients and their families. The patient with the *NR5A1* variant was raised female but did not feel comfortable in that role. The diagnosis of AIS meant that she would be unlikely to respond to T treatment, but having self-administered T, she felt she had responded to it. The finding of an *NR5A1* variant previously reported in a male with isolated hypospadias was very reassuring for this patient. It supported her feeling that she should be male, validated her suspicion that she responded to T, and ultimately supported her transition to a male body habitus. Anecdotally, we have found that many families are relieved to receive a genetic diagnosis, even when prognosis and treatment options are not impacted.

In this study, we identified a number of VUS such as in *CHD7* or *DHH* (Table 3). Parental samples would be instructive in determining the pathogenicity of these variants, but were not available. For most of the 20 individuals for whom no interpretable variant was found, we found at least one variant in the DSD gene list, but they did not reach the level of clinical significance. Reasons included: a single variant in a gene associated with a recessive condition (in cases where copy-number variations had been ruled out by microarray analysis); published reports of a phenotypic spectrum that did not extend to the patient's findings; a potentially dominant variant present in the ESP with a minor allele frequency greater than 0.1%; and the variant was predicted to be benign or not affecting the canonical transcript. These variants may be reclassified as our understanding of DSD genetics evolves, but they currently cannot be interpreted or reported clinically. Ultimately, the combination of genetics with endocrine and imaging will validate the functionality of variants, thus advancing our understanding of DSD and treatment options for future patients. Additional reasons for not identifying a genetic cause include mechanisms that clinical exome sequencing cannot identify such as: nonexonic mutations; mutations in yet undiscovered DSD genes; oligogenic etiologies of DSD, as demonstrated in the case of *FGFR1* mutations (36); and epigenetic/environmental influences.

In summary, our data show that exome sequencing is an effective test for genetic diagnosis in DSDs. For a comparable cost of full sequencing of a single gene such as the *AR* or a limited-capture panel of genes, exome sequencing can examine all genes with known or suspected involvement in DSD. Exome sequencing should therefore be considered a good first-tier diagnostic or rule-out test by clinicians (39, 40). Recent advances in the sequencing technologies are leading to substantial decreases in turnaround time, and soon it will be possible to obtain results in under 2 weeks. This will allow the test to be useful even in urgent cases.

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