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 were clinical samples submitted 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, and an additional 12.5% with likely pathogenic findings, and 15% with variants of unknown clinical significance (VUS).

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* 99: 0000–0000, 2014)**

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Abbreviations: ●●●.

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). While 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 DSD 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.

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 out of five previously undiagnosed patients. Here we have expanded this approach by using exome sequencing to capture most 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 forty 46,XY DSD patients sequenced at the 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 (#11–001 775-AM-00 007; Principal Investigator: E. Vilain) or to the UCLA Clinical Genomics Center. Exomes were captured using Agilent SureSelect All Exon 50Mb capture kit and sequenced on an Illumina HiSeq2000 or HiSeq2500 as 50bp or 100bp 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/index.html). The output BAM file was sorted, merged, and PCR duplicates were removed using Picard (http://picard.sourceforge.net/). INDEL (insertions and deletions) realignment and recalibration were performed using Genome Analysis Took Kit (GATK) (http://www.broadinstitute.org/gatk/). Mean coverage was over 80x for each sample and approximately 93% of the RefSeq gene coding regions +/-2bp was covered at 10x or greater (individual gene coverage is indicated in Table 1). Single-nucleotide variants (SNVs) 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 $>$ 1Mb of homozygosity using linkdatagen (8) and Plink software (http://pngu.mgh.harvard.edu/~purcell/plink/) (9). All high-quality variants were annotated using Variant Annotator X (VAX), a custom-designed variant effect predictor (10).

Variants with a minor allele frequency (MAF) 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 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 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 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 SNVs with high exome sequencing quality score (11). All INDELs are validated by Sanger sequencing.

Using ACMG 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 are classified likely pathogenic. To assess 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, ABMG board-certified geneticists, genetic counselors, and clinicians in-

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 (*e.g.* steroid synthesis/receptors)			
AKR1C4	3-a-HSD, C11/CDR/DD4/HAKRA	100%	46,XY DSD
AMHR2	MISR2	100%	Persistent Müllerian Duct Syndrome (PMDS)
ATRX	RAD54	100%	α-thalassemia X-linked intellectual disability syndrome
CYP11A1	P450SCC	100%	Congenital adrenal hyperplasia (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%	Androgen Insensitivity Syndrome (CAIS/PAIS)
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%	Persistent Müllerian Duct Syndrome (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
BBS10	FLJ23560	100%	Bardet Biedl syndrome
BBS12	FLJ35630/ FLJ41559	100%	Bardet Biedl syndrome
CHD7	FLJ20357/FLJ20361/KIAA1416	100%	Kallman syndrome; normosmic isolated GnRH deficiency (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 (Kallman 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%	Kallman syndrome, normosmic IGD, and Pfeiffer Syndrome
PCSK1	PC1/PC3/SPC3	98%	Morbid obesity
KAL1	anosmin-1/KALIG-1	95%	IGD with anosmia (Kallman syndrome)
LEPR	CD295/ OBR	95%	Morbid obesity
LHX3		87%	Combined pituitary hormone deficiency
FGF8	AIGF	79%	IGD with anosmia (Kallman syndrome) and normosmic IGD
PROK2	BV8/KAL4/MIT1/PK2	76%	IGD with anosmia (Kallman syndrome) and normosmic IGD
KISS1R	AXOR12/HOT7T175	54%	Isolated abnormality in GnRH secretion or response

cluding, 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 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 (10 cases) or typical male with or without micropenis (9 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. RDSD014 was a female who presented in adolescence with complete gonadal dysgenesis. In contrast, 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. 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 out 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, while 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). 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 likely causative of the probands' phenotype and exhibits reduced penetrance in the apparently unaffected father. In consequence, both 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 since 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 10Kb in size equivalent to 1.97% of the genome being homozygous. The *STAR* variant was located within the largest homozygous region spanning more than 20.6Kb 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 make this a likely genetic diagnosis.

Leydig cell hypoplasia

RDSDO15 has typical female external genitalia and no response to testosterone treatment, but no variant in the androgen receptor (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. While these genetic findings are likely diagnostic for the Leydig cell hypoplasia, unfortunately they

Table 2. Phenotypic descriptions, previous clinical findings, and genetic findings from current study

Sample ID	External Genitalia	Anatomy	Gonads	Additional Clinical Findings	Other Diagnostic Tests	Gene Identified
RDSD001	female	no uterus; blind vagina	none found by ultrasound	ASD, VSD, BAV, hydrocephalus, cerebellar hypoplasia, optic nerve hypoplasia, ear pits, blindness	aCGH; SLO*	*CHD7*
RDSD002	female	no records	complete gonadal dysgenesis	no records	no records	——
RDSD003	female	no uterus; blind vagina; hypoplastic labia; Fallopian tubes	no records	Tall stature, breast Tanner 3	Familial; AR; SRY present	——
RDSD004	female	no records	gonadal dysgenesis	no records	aCGH; TESCO**	——
RDSD005	female	no uterus; bilat. Fallopian tubes	streak, with rete testis & seminiferous tubules	no records	Familial; aCGH; SRY present	*DHH*
RDSD006	female	no records	no records	amelia	SRY; SOX9; WNT4; aCGH	——
RDSD007	female	no records	complete gonadal dysgenesis	adrenal rests	SRY; SF1	——
RDSD008	female	no uterus	no records	adrenal insufficiency; small adrenals	Consanguinity; aCGH; TESCO	*STAR*
RDSD009	female	no uterus;blind vagina; typical labia	Immature testis with Fallopian tubes on Left; Immature testis with vas on Right	jejunal atresia (corrected) and microcephaly	aCGH; TESCO, SRY promoter	*AR*
RDSD010	female, enlarged clitoris	no records	no records	no records	aCGH	——
RDSD011	female	uterus & 1/3 vagina present	no records	short stature; dysmorphic features, failure to thrive	SRY present; aCGH	——
RDSD012	female	no records	no records	kidney disease	WT1	——
RDSD013	female	uterus & Fallopian tubes present	Bilat. Inguinal streak	gonadal dysgenesis, normal uterus	Sry present; SRY	——
RDSD014	female	no records	complete gonadal dysgenesis	presentation at age 15	no records	*MAP3K1*
RDSD015	female	no clitoromegaly, non rugated labia, vagina	small bilat. Inguinal gonads; fibrous tissue with Sertoli cells	raised as male	AR; RHOA; SRY; DHH; SF1; MAP14; CXORF6; MAP3K1	*LHCGR*
RDSD016	female, enlarged clitoris	no uterus	two testes	clinical diagnosis of androgen insensitivity syndrome	no variants in AR	*NR5A1*
RDSD017	female	hypoplastic partial bicornuate uterus with cervix, Fallopian tubes	Bilat. streak gonads	Tall stature, primary amenorrhea	no records	*MAP3K1*
RDSD018	ambiguous (raised male)	partial labioscrotal fusion, micropenis, penoscrotal hypospadias; urogenital sinus; Müllerian remnants	no palpable gonads	no records	CGH 311kb dup on chr 11 of unknown clinical significance	——
RDSD019	ambiguous (raised female)	uterus, cervix, Fallopian tube on L. long urethra	disorganized testicular tissue on L; normal testis on R	no records	SRY present	*WT1*
RDSD020	ambiguous (raised female)	vas & Müllerian structures	no records	developmental delay, agenesis of corpus callosum, Dandy Walker malformation	no Y chromosome abnormality, aCGH	——
RDSD021	ambiguous (raised male)	microphallus, posterior labioscrotal fusion	no records	hypotonia, congenital adrenal hypoplasia, dysmorphic features, cardiac defect	Sry present; aCGH, no 22q del	——
RDSD022	ambiguous	no records	no records	microcephaly, intestinal dymotility, optic nerve hypoplasia	no records	——
RDSD023	ambiguous (raised male)	Microphallus, chordee, hypospadias; incompletely fused scrotum	ovotestis with Fallopian tube & partial uterus; contralateral normal testis	no records	Familial; normal male hormonal profile	*MAP3K1*
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.	Denys Drash syndrome; end-stage renal disease; bilateral nephrectomy before age 2; no Wilms tumor.	SRY present	*WT1*
RDSD025	male, micropenis	no records	cryptorchidy	hypoplastic adrenal gland; dysmorphic features, hypotonia, brain malformations	Consanguinity; aCGH	——
RDSD026	male	*** see detail in legend	no gonad on Right; Left: inguinal fibrotic & atrophic testis, no Sertoli; exuberant Leydig cell proliferation, in complex specimen	persistent Müllerian duct syndrome (PMDS)	no records	AMHR2
RDSD027	male	Fallopian tubes and small uterus	two abdominal testes, normal testicular tissue	persistent Müllerian duct syndrome (PMDS)	elevated AMH; normal LH, FSH, Testosterone	AMHR2
CDSD028	female	no uterus, no adnexa (U/S)	inguinal(No other info)	no records	SRY present	*HSD17B3*
CDSD029	male	bifid scrotum, penoscrotal transposition, penoscrotal hypospadias. No micropenis.	bilat. descended gonads, likely testes (normal male hormones)	normal renal U/S, normal EKG	no records	*CHD7*
CDSD030	female, clitoromegaly	no posterior fusion, vaginal opening, no uterus (U/S)	palpable in inguinal hernia; testes with no malignancies	Primary amenorrhea, elevated T, DHT and T/DHT ratio	SRY present; AR	——
CDSD031	ambiguous (raised female)	posterior labial fusion, no rugation, no clitoromegaly, UG sinus	Complete gonadal dysgenesis; abdominal; no oocytes, no seminiferous tubules	bilateral gonadectomy	SRY present	——
CDSD032	female	Vagina, UG sinus, no uterus, no Fallopian tubes	Inguinal testes with calcifications and immature seminiferous tubules lacking spermatogonal development	deafness, impaired cognition	no records	*NRP1*
CDSD033	female	no records	bilateral hernias with palpable gonads, normal appearing testes descended into labial folds after orchiopexy	no records	SRY present; AR; normal Sertoli cell function (AMH, InhB)	*HSD17B3*
CDSD034	ambiguous (raised male)	penoscrotal transposition, penoscrotal hypospadias, bifid scrotum	Likely testes (normal hormonal function & U/S) in upper scrotum requiring orchiopexy	no records	SRY present, aCGH	——
CDSD035	male	"abnormal genitalia"	undescended testes	neuropathy, hypotonia	no records	*MAMLD1*
CDSD036	ambiguous (raised male)	midshaft hypospadias, chordee	bilateral descended testes	no records	no records	——
CDSD037	male, micropenis		vanishing testes	no other malformations found	SRY present	*CHD7*
CDSD038	ambiguous (raised male)	2 cm phallus, penoscrotal hypospadias, penoscrotal transposition, micropenis, chordee	bilateral descended testes	congenital hypothyroidism	SRY present	*BNC2 FGFR1*
CDSD039	male, micropenis	no ovaries or uterus (U/S), 1 cm phallus, small scrotum	vanishing testes; nubbin with no testicular tissue (laparoscopy)	no records	SRY present	——
CDSD040	ambiguous (raised male)	Perineal hypospadias, 2 cm phallus, bifid scrotum, penoscrotal transposition, no Müllerian structures	bilateral descended testes; no gonadal dysgenesis	premature birth, IUGR	SRY present; Normal InhB, AMH, Testosterone, diOHT	*MAP3K1*

aCGH: array CGH 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.

*SLO: endocrine test for Smith-Lemli Opitz

**TESCO: testis-specific enhancer of Sox9 was analyzed and did not detect any variants

***Hypoplastic vas, epididymis, rete testis, microcalcifications, portion of seminal vesicle & prostatic tissue, primitive Fallopian tube, vagina, endocervix, uterine structure.

do not explain the lack of response to exogenous testosterone in this patient.

Variants in the AMH receptor/PMDS

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

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 RDSD016 we identified an *NR5A1* variant previously reported in a patient with isolated distal hypospadias, generally considered a mild form of DSD (26). As *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 result in 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, patient RDSD001's genital presentation 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 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

VUSs 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 #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 VUSs in genes previously associated with hypospadias. A hemizygous variant in *MAMLD1* [OMIM #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. Neuropilin1 interacts with Sema3A, and variants in *SEMA3A* are associated with hypogonadotropic hypogonadism [MIM #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 out of 40) of cases, with an additional six variants of uncertain clinical significance 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 a 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. While *MAP3K1* and *AMHR2* mutations are known causes of DSD, clinical testing for these genes is not yet available in the USA, 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 testosterone treatment, but having self-administered testosterone, 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 testosterone 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 VUSs 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 These reasons included: a single variant in a gene associated with a recessive condition (in cases where CNVs had been ruled out by microarray analysis); published reports of a phenotypic spectrum that did not extend to the patients' findings; a potentially dominant variant present in the ESP with a minor allele frequency greater than 0.1%; 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

Table 3. Details and classification of genetic findings

Gene	Patient	Genomic Position	Zygosity	Transcript ID	cDNA Change	Protein Change	Effect of Variant	Variant Call
Sex Determination Genes								
NR5A1	RDSD016	chr9: 127 255 362	het	NM 004959.4	c.937C>T	p.Arg313Cys	reported mutation	Likely Pathogenic
MAP3K1	RDSD014	chr5: 56 171 018	het	NM 005921.1	c.1846G>A	p.Gly616Arg	reported mutation	Pathogenic
MAP3K1	RDSD017	chr5: 56 160 742	het	NM 005921.1	c.1016G>A	p.Arg339Gln	predicted damaging	Likely Pathogenic
MAP3K1	RDSD023	chr5: 56 171 018	het	NM 005921.1	c.1846G>A	p.Gly616Arg	reported mutation	Pathogenic
MAP3K1	CDSD040	chr5:56 155 678	het	NM 005921.1	c.770C>T	p.Pro257Leu	predicted damaging	Likely Pathogenic
DHH	RDSD005	chr12: 49 483 790	het	NM 021044.2	c.1043A>T	p.Glu348Val	predicted damaging	VUS
WT1	RDSD019	chr11: 32 413 577	het	NM 024426.4	c.1373G>A	p.Arg458Gln	predicted damaging	Likely Pathogenic
WT1	RDSD024	chr11: 32 413 543	het	NM 024426.4	c.1407C>A	p.His469Gln	predicted damaging	Pathogenic
Sex Differentiation Genes								
AMHR2	RDSD026	chr12: 53 823 984	het	NM 020547.2	c.1343C>G	p.Pro448Arg	predicted damaging	Pathogenic
		chr12: 53 823 970	het	NM 020547.2	c.1330 1356del	p.Leu444 Glu452delinsdel	reported mutation	
AMHR2	RDSD027	chr12: 53 819 596	het	NM 020547.2	c.745C>T	p.Leu249Phe	predicted damaging	Pathogenic
		chr12: 53 823 970	het	NM 020547.2	c.1330 1356del	p.Leu444 Glu452delinsdel	reported mutation	
HSD17B3	CDSD028	chr 9:99 064 233–388	hom	NM 000197.1	exon 1 deletion	likely null	predicted damaging	Pathogenic
HSD17B3	CDSD033	chr9:99 017 188	het	NM 000197.1	c.239G>A	p.Arg80Gln	reported mutation	Pathogenic
		chr9:99 060 705	het	NM 000197.1	c.194C>T	p.Ser65Leu	reported mutation	
STAR	RDSD008	chr8: 38 008 272	hom	NM 000349.2	c.64 + 1G>A	p.Gly22*	splice donor variant	Pathogenic
AR	RDSD009	chrX: 66 766 183	hemi	NM 000044.3	c.1195T>C	p.Trp1195Arg	predicted damaging	Likely Pathogenic
LHCGR	RDSD015	chr2: 48 941 168	hom	NM 000233.3	c.562G>T	p.Glu188*	premature stop codon	Pathogenic
MAMLD1	CDSD035	chrX:149 639 324	hemi	NM 005491.3	c.1479 1480dupCAG	p.Gln502dup	predicted damaging	VUS
Central Causes of Hypogonadism								
CHD7	RDSD001	chr8: 61 655 619	het	NM 017780.3	c.1628C>T	p.Ser543Leu	predicted damaging	VUS
CHD7	CDSD037	chr8:61 765 478	het	NM 017780.3	c.6194G>A	p.Arg2065His	predicted damaging	VUS
Other								
NRP1	CDSD032	chr10:33 491 864	het	NM 001024628.2	c.1819C>G	p.Glu607Glu	likely benign	VUS
BNC2	CDSD038	chr9:16 435 821	het	NM 017637.5	c.2371T>C	p.Tyr791His	likely damaging	VUS
FGFR1		chr8:38 287 238	het	NM 023110.2	c.320C>T	p.Ser107Leu	predicted tolerated	VUS

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 two weeks. This will allow the test to be useful even in urgent cases.

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