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Advances in genomic diagnosis of a large cohort of Egyptian patients with disorders of sex development

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Abstract

Disorders/differences of sex development (DSD) comprise a group of congenital disorders that affect the genitourinary tract and usually involve the endocrine and reproductive system. The aim of this work was to identify genetic variants responsible for disorders of human urogenital development in a cohort of Egyptian patients. This three-year study included 225 patients with various DSD forms, referred to the genetic DSD and endocrinology clinic, National Research Centre, Egypt. The patients underwent thorough clinical examination, hormonal and imaging studies, detailed cytogenetic and fluorescence in situ hybridization analysis, and molecular sequencing of genes known to commonly cause DSD including *AR*, *SRD5A2*, *17BHSD3*, *NR5A1*, *SRY*, and *WT1*. Whole exome sequencing (WES) was carried out for 18 selected patients. The study revealed a high rate of sex chromosomal DSD (33%) with a wide array of cytogenetic abnormalities. Sanger sequencing identified pathogenic variants in 33.7% of 46,XY patients, while the detection rate of WES reached 66.7%. Our patients showed a different mutational profile compared with that reported in other populations with a predominance of heritable DSD causes. WES identified rare and novel pathogenic variants in *NR5A1*, *WT1*, *HHAT*, *CYP19A1*, *AMH*, *AMHR2*, and *FANCA* and in the X-linked genes *ARX* and *KDM6A*. In addition, digenic inheritance was observed in two of our patients and was suggested to be a cause of the phenotypic variability observed in DSD.

KEYWORDS
46,XY DSD, disorders of sex development, sex chromosomal abnormalities, syndromic DSD, whole exome sequencing

1 | INTRODUCTION

The term disorders of sex development (DSD) encompasses a spectrum of phenotypes that include three main groups: sex chromosome DSD, 46,XX DSD, and 46,XY DSD (Hughes, 2008). The overall incidence of DSD is not precisely detected, owing to the difficulties of achieving accurate clinical diagnosis and the lack of diagnosis of many patients until late childhood. Many of DSD phenotypes are rare (e.g., 46,XY complete gonadal dysgenesis; 1:100,000 births), whereas others are relatively common (e.g., cryptorchidism; 1:50 births). The incidence of atypical genitalia in European countries is about 1:4500–1:5500. However, it seems to be increased in populations with a higher consanguinity rate. The incidence was estimated to be

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higher reaching 1:2500 and 1:3000 livebirths in Saudi Arabia and in Egypt, respectively (Abdullah et al., 1991; Mazen et al., 2008). An increased rate of DSD in other societies with a high consanguinity rate was also observed in other studies, supporting this hypothesis (Thyen et al., 2006). The rate of parental consanguinity among patients with genetic diseases in Egypt was reported to be as high as 55%, with a resultant increase in the rate of autosomal recessive disorders including DSD recessive forms (Afifi et al., 2010). There is also evidence that the incidence of DSD-related phenotypes may be increasing in some countries (Virtanen et al., 2007). DSD represent a complex emotional and social problem for the patients and their families, especially in Middle Eastern/African countries. It also constitutes a great burden on public health services due to its sophisticated management options, lack of professional counselors, and long-term surgical decisions. Various forms of fetal genital abnormalities and syndromes with sex chromosomal aneuploidy could be prenatally diagnosed (Forrester & Merz, 2003). The prognosis and pregnancy outcome are highly variable depending on the type of aneuploidy and the association of somatic malformations. Early and precise prenatal diagnosis can pose a great impact on prenatal counseling and subsequent elective termination (Cheikhelard et al., 2000).

Although DSD-related disorders exhibit a broad range of phenotypes and vary in incidence, there is evidence that they may have a common genetic etiology (Lourenço et al., 2009). The genetic mechanisms that control sex determination and differentiation are overly complex and highly dependent on gene dosage and gene expression threshold levels that are crucial for driving the genetic cascade required for gonadal development. Although the understanding of the genetic basis of human gonadal development has been greatly progressed, since the sex-determining region Y (*SRY*) was identified in 1990, molecular network mechanisms are still largely obscure (Ahmed et al., 2014). Overall, a definite molecular diagnosis is usually identified in approximately 30% of DSD cases (Baxter et al., 2015; Hughes, 2008). Recently, the expeditious development in exome sequencing has resulted in the identification of several new genes associated with DSD (Hughes et al., 2019).

The aim of this work was to use a stepwise combination of approaches to improve the overall diagnostic yield in a large cohort study of DSD patients from Egypt.

2 | PATIENTS AND METHODS

2.1 | Editorial policies and ethical considerations

This study was approved by the Ethical Scientific Committee of the National Research Centre (NRC), Cairo, Egypt, and conducted in accordance with the declaration of Helsinki ethical principles for medical research involving human subjects. An informed consent was obtained from the patients or their guardians.

A total of 225 DSD patients referred to the genetic DSD and endocrinology clinic, NRC, Cairo, Egypt, were studied over a period of 3 years. The patients exhibited different presenting features and their ages ranged from 2 months to 32 years. All patients were subjected to detailed clinical and genital examinations, pedigree analysis, family history of similar or other conditions, and the presence of associated abnormalities. The patients underwent anthropometric measurements of height, weight, and arm span, and secondary sexual characters were classified according to Tanner et al. (1969). Sexual ambiguity was assessed following the classification of Quigley et al. (1995). Hormonal evaluation of congenital adrenal hyperplasia (CAH) was carried out for suspected patients. Hormonal assay of basal and post human chorionic gonadotropin (HCG) of serum testosterone and its precursors and dihydrotestosterone (DHT) was done for classification of defects in hormonal biosynthesis. Serum FSH and LH, anti-Müllerian hormone (AMH), inhibin B, and serum estradiol were also evaluated according to each case. Pelvic ultrasonography and genitography were executed for all the patients. Laparotomy, pelvic laparoscopy, and gonadal biopsy were performed whenever indicated.

2.1.1 | Cytogenetic studies

Chromosomal analysis of peripheral blood lymphocytes, using Giemsa-Trypsin-G (GTG)-banding technique, was carried out for the patients and suspected family members through standard procedures. Karyotype description followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN, 2016). Fluorescence in situ hybridization (FISH) analysis was performed, using commercial probes, on metaphases and interphase cells, when indicated. FISH was also done on gonadal tissue cells in selected patients. FISH procedure was performed according to the manufacturer's instructions (Cytocell Inc., Cambridge, United Kingdom).

2.1.2 | DNA extraction and Sanger sequencing of common DSD genes

DNA was extracted from peripheral blood lymphocytes of the patients and available family members using the PAXgene Blood DNA Kit (Qiagen, Germany). Amplification of *AR*, *17BHSD3*, *SRD5A2*, *NR5A1*, *WT1*, and *SRY* genes was performed using intron-specific primers designed by Primers3 software. Purification of PCR products was done using the Exo-SAP PCR Clean-up kit (Thermo, Germany), and it was then sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, United States). Analysis was performed using the ABI 3500 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

2.1.3 | Whole exome sequencing

Whole exome sequencing (WES) was performed according to Bashamboo et al. (2014). In brief, Agilent SureSelect Human All Exon

V4 was used for exon enrichment. Then, paired-end sequencing was done using TruSeq v3 chemistry on the Illumina HiSeq2000 platform. Through the manufacturer's proprietary software, read files (Fastq) were generated from the sequencing platform and were mapped using the Burrows-Wheeler Aligner. Local realignment of the mapped reads at potential insertion/deletion (indel) sites was performed using GATK, version 1.6. Picard, version 1.62, was used to mark duplicate reads, and additional manipulations of the BAM file were carried out with Samtools (0.1.18). GATK Unified Genotyper was used for calling SNP and indel variants for each sample. Analysis of variants was performed through a range of web-based bioinformatics tools, using EnsEMBL SNP Effect Predictor (http://www.ensembl.org/homosapiens/userdata/uploadvariations). Manual screening of variants against the Human Gene Mutation Database Professional (Biobase) was also performed (http://www.biobase-international.com/product/hgmd). In silico analysis was carried out using SIFT and PolyPhen2 to identify the variants' potential pathogenicity. Candidate phenotype causal variants were verified using Sanger sequencing. Potentially pathogenic variants identified by exome sequencing were screened against a number of databases including the Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/), dbSNP138 databases (http://www. ncbi.nlm.nih.gov/snp/), the ExAC database (http://exac.broadinstitute.org/), and the 1000 Genomes Project database (http://browser.1000genomes.org/index.html), in addition to our internal database (700 exomes).

3 | RESULTS

The most common presenting feature was atypical genitalia (75 patients) followed by male infertility (58 patients) and then primary amenorrhea (50 patients). A total of 23 patients presented with genital anomalies associated with other malformations, 10 patients presented with undescended testis, and nine presented with delayed puberty (Table 1). The parental consanguinity rate was about 53% with a particular rise in the 46,XY DSD group, reaching 70%.

TABLE 1 Presentations of different categories of studied DSD patients

Classification	Presentation	No.
Sex chromosome DSD (No = 75)	Primary male infertility/small testes	51
	Primary amenorrhea	13
	Atypical genitalia	7
	DSD with other congenital anomalies	4
46,XX DSD (No = 46)	Atypical genitalia, of them 11 were diagnosed with CAH (21-hydroxylase deficiency)	15
	Male infertility	7
	Undescended testis	1
	Primary amenorrhea	17
	DSD with other somatic anomalies	6
46,XY DSD (No = 100)	Atypical genitalia	53
	Primary amenorrhea	20
	Undescended testis	9
	Delayed puberty	9
	DSD with other somatic anomalies	9
DSD associated with somatic anomalies and autosomal abnormalities		4
Total number	225	

Abbreviations: CAH, congenital adrenal hyperplasia; DSD, disorders of sex development.

3.1 | Cytogenetic results

Sex chromosomal aberrations were detected in 75/225 patients (33.3%). Autosomal abnormalities were detected in four patients with DSD associated with multiple congenital anomalies (all the cytogenetic results are presented in Table 2). 46,XX DSD was present in 46/225 patients (20.4%). A total of 11 46,XX patients were diagnosed with 21-hydroxylase deficiency CAH, as evidenced by increased blood levels of 17-hydroxyprogesterone, DHEA, and androstenedione and low cortisol levels. Eight of them were of the salt-losing type and revealed hyponatremia, hyperkalemia, and high plasma renin, while the remaining had the simple virilizing type. A total of 10 patients were diagnosed as 46,XX testicular DSD, seven presented with primary infertility and azoospermia, two with atypical genitalia, and one with undescended testis. Five of those patients with infertility were proved by FISH analysis to have the *SRY* gene translocated to the distal part of the short arm of one X chromosome. The remainders were all negative for *SRY* gene. Two patients had 46,XX ovotesticular (OT) DSD, and one of them had a gonadal 46,XX/45,X/46,XY cell line mosaicism. Other two OT DSD patients revealed 46,XX/46,XY chimerism by karyotype and FISH analysis.

A total of 100/225 patients (44.4%) were diagnosed as 46,XY DSD. One of the patients presenting with short stature, low serum testosterone, Mullerian derivatives, and gonadal dysgenesis was found to have a 45,X gonadal mosaicism by FISH analysis.

3.2 | Molecular results

3.2.1 | Sanger sequencing studies

46,XY patients were classified according to the good response of androgenic hormones into either androgen insensitivity syndrome or 5-alpha reductase deficiency syndromes, while those with bad response and low T/A (testosterone/androstenedione) ratio were provisionally diagnosed as 17 beta hydroxysteroid dehydrogenase deficiency. Sequencing analysis of *AR* gene (exons 2,3 encoding the

TABLE 2 Cytogenetic results of studied DSD patients in relation to presentations

Classification	Presentation	Karyotype and FISH results	No.
Sex chromosome DSD (No = 75)	Primary male infertility/small testes (no = 51)	47,XXY	45
		45,X/46,XY	2
		45,X; FISH: SRY+	1
		48,XXXY	1
		47,XYY	1
		47,XXY/48,XXXY/49,XXXY/46,XY	1
	Primary amenorrhea (no = 13)	46,X,i(Xq); 45,X/46,X,i(Xq)	6
		45,X/46,XY	2
		47,XXX	2
		46,X,add(Xq)	2
		46,X,del(Xq)	1
	Atypical genitalia (no = 7)	45,X/46,XY	2
		45,X/46,X,idic(Yp) FISH: idicY:SRY++, CEP Y++	3
		46,XX /46,XY (OT DSD)	2
	DSD with other congenital anomalies (no = 4)	47,XXX	1
		47,XXY	1
		49,XXXXY	2
46,XX testicular DSD (No = 10)	Male infertility	FISH: SRY+ (no = 5)	7
		FISH: SRY– (no = 2)	
	Atypical genitalia	FISH: SRY–	2
	Undescended testis	FISH: SRY–	1
46,XX OT DSD (No = 2)	Atypical genitalia	FISH: SRY–	1
		FISH on gonadal cells: mos.46,XX/45,X/46,XY	1
DSD associated with somatic anomalies and autosomal abnormalities (No = 4)		47,XY,+mar. FISH: Marker is derived from chromosome 15	1
		47,XX,+mar. FISH: t(11q;22q) (wcp 11+; wcp 22+).	1
		46,XY,add(18p)	1
		46,XY,add(5p). FISH: added material was derived from chr. 14.	1

Abbreviations: DSD, disorders of sex development; FISH, fluorescence in situ hybridization; OT, ovotesticular.

DNA-binging domain and exons 4–8 encoding the ligand-binding domain) was done for 28 46,XY patients and revealed pathogenic variants in five patients (18%); one of them revealed a novel variant in exon 8 c.2731delTC that led to a frameshift and premature stop codon resulting in a truncated protein. Sequencing of both *HSD17B3* gene in 20 patients and *SRD5A2* gene in 26 patients showed pathogenic variants in 50%. Eight novel variants were detected in *HSD17B3* gene in seven patients; one of them was a compound heterozygous variant, while three patients showed previously reported gene variants (Table 3). Sequencing analysis of *SRD5A2* gene revealed a characteristic pattern including homozygous variants in nine patients: p.Gly34Arg (c.100G > A) in exon 1 in three patients, p.Gly196Ser (c.586G > A) in three patients, p.Tyr91His (c.271 T > C) in two patients, and p.Glu56Arg (c.167C > G) in one patient, while four patients showed compound heterozygous variants: p.Ala207Asp (c.620C > A) + p.Leu89Val (c.265C > G) in three patients and p.Gly196Ser + p.Leu89Val in one patient. Pathogenic variants in the *WT1* gene were detected in two patients, the first was a boy with gonadal dysgenesis and gonadoblastoma, who carried a de novo heterozygous p.Arg462Try (c.1384C > T) variant. A novel heterozygous variant p.Lys459Arg (c.689A > G) in *WT1* gene was detected in the other patient who presented with short stature, atypical genitalia with mixed gonadal dysgenesis, and a mosaic karyotype: 45,X[90]/46,X,idic(Y)(q11.2)[10] (in both blood and gonadal cells). All the identified variants are presented in Table 3. Wild-type sequences of *NR5A1* and *SRY* genes were detected in 10 male patients presenting with hypospadias or gonadal dysgenesis and in three 46,XY female patients with gonadal dysgenesis, respectively. Moreover, sequencing of *NR5A1* and *SRY* genes was carried out for twelve 46,XX patients with primary amenorrhea and for five patients with 46,XX testicular DSD, respectively, and revealed positive amplification of *SRY* and wild-type sequence of both genes.

TABLE 3 Distribution of gene variants identified by Sanger sequencing in known DSD genes

Gene	Variants	No. of patients with the variant	ACMG novel variants classification	No. of sequenced patients	Reference
AR No = 5	NM_000044.6:c.2731delTC[a]	1	Pathogenic (PVS1 + PS2)	28	Akcay et al., 2014 Mazen et al., 2014
	NM_000044.6:c.2317G > T; p. Glu773X	1	Previously reported		
	NM_000044.6:c.2343G > T; p. Met781Ile	1	Previously reported		
	NM_000044.6:c.2567G > A; p. Arg856His	1	Previously reported		
	NM_000044.6:c.2137C > T p. Leu713Phe	1	Previously reported		
HSD17B3 No = 10	NM_000197.1:c.198G > A; p. Trp50X[a]	1	Pathogenic (PVS1 + PM2+ PM3)	20	Rosler, 2006 Hassan et al., 2016
	NM_000197.1:c.575A > C; p. Gln176Pro	2	Previously reported		
	NM_000197.1: c.777-783delGATAACC	1	Previously reported		
	NM_000197.1:c.539 T > C; p. Met164Thr[a]	1	Pathogenic (PS3 + PM2 + PM3)		
	NM_000197.1:c.565delG[a]	1	Pathogenic (PVS1 + PM2 + PM3)		
	NM_000197.1:c.200C > T; p. Ala51Val * + c.575A > C; p. Gln176Pro	1	Pathogenic (PS3 + PM2 + PM3)		
	NM_000197.1:c.629 T > C; p. Leu194Pro * + c.588C > T; p. Ile180 = [a]	1	Pathogenic (PS3 + PM2 + PM3)		
	NM_000197.1:c.208A > G; p. Thr54Ala[a]	2	Pathogenic (PS3 + PM1 + PM3)		
SRD5A2 No = 13	NM_000348.4:c.100G > A; p. Gly34Arg	3	Previously reported	26	Mazen et al., 2003. Maimoun et al., 2011. Akcay et al., 2014. Soliman et al., 2015. Nagaraja et al., 2019.
	NM_000348.4:c.586G > A; p. Gly196Ser	3	Previously reported		
	NM_000348.4:c.620C > A; p. Ala207Asp + c.265C > G; p. Leu89Val	3	Previously reported		
	NM_000348.3:c.167C > G; p. Glu56Arg	1	Previously reported		
	NM_000348.4:c.586G > A; p. Gly196Ser + c.265C > G; p. Leu89Val	1	Previously reported		
	NM_000348.4:c.271 T > C; p. Tyr91His	2	Previously reported		
WT1 No = 2	NM_024426.4:c.1384C > T; p. Arg462Try	1	Previously reported	2	Lehnhardt et al., 2015 Mazen, Hassan, et al., 2017
	NM_024426.4:c.689A > G; p. Lys459Arg[a]	1	Likely pathogenic (PS2 + PM2)		

Note: N.B: Total sequenced 46,XY patients = 89.

Abbreviations: ACMG, American College of Medical Genetics and Genomics; DSD, disorders of sex development.

[a]Indicates novel mutations.

3.2.2 | Whole exome sequencing

WES was performed for 18 patients and for family members, when indicated. Pathogenic variants were detected in 12 families (66.7%). The patients were selected after exclusion of common monogenic causes according to specific selection criteria:

1. Patients with positive consanguinity and family history of similar conditions.
2. Patients with atypical phenotypes or with multiple congenital anomalies after excluding the presence of pathogenic copy number variants by chromosomal microarray analysis.
3. Patients with 46,XY gonadal dysgenesis showing negative results by Sanger sequencing.
4. Patients with XX testicular and OT DSD after excluding chromosomal abnormalities and obtaining negative FISH signal for the *SRY* gene.

Additional two pathogenic variants were identified in *WT1* gene by WES: a de novo p.Arg462Try (c.1384C > T) variant in a 46,XY gonadal dysgenesis patient and a de novo p:Arg495Gly (c.1483C > G) variant in a patient with atypical genitalia and 46,XX testicular DSD. Digenic variants were suggested in two unrelated patients with 46,XY DSD and atypical genitalia, associated with gonadal dysgenesis in the first one and with congenital heart defect in the second. They showed pathogenic variants in *NR5A1/MAP3K* genes and *FGFR1/STARD3* genes, respectively. A novel *NR5A1* gene variant, p.Glu206Thrfs*20 (c.614_615insG), was also detected in a 46,XY DSD patient with primary amenorrhea and gonadal dysgenesis. Another male patient with 46,XX DSD was found to have a splice site variant in *CYP19A1* gene. Novel pathogenic variants in *AMH*, *AMHR2*, *ARX*, and *KDM6A* genes were identified in 46,XY DSD-unrelated patients, in addition to rare *HHAT* and *FANCA* variants. Interpretation of novel sequence variants was performed according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology standards and guidelines (Richards et al., 2015). All the detected novel variants were found to be either pathogenic or likely pathogenic. The details of gene variants identified by WES are depicted in Table 4.

4 | DISCUSSION

The clinical manifestations of DSD are heterogenous, and patients may present at any stage of life from the neonatal stage to adolescence or adulthood. Our routine strategy for genetic diagnosis of DSD patients, after the initial clinical, hormonal, and imaging evaluation, depends on chromosomal and FISH analysis followed by sequencing of known DSD genes and chromosomal microarray analysis of patients with multiple congenital anomalies. In an attempt to increase our diagnostic yield, we have introduced WES technique to solve out some undiagnosed patients or patients with atypical phenotypes.

In this cohort, the patients were categorized into four groups: sex chromosome DSD, 46,XX DSD, 46,XY DSD, and DSD-associated with autosomal abnormalities (Table 1). The frequency of sex chromosomal DSD was 33.3%, which is higher than that reported in other studies (≈ 20%) (Juniarto et al., 2016). This may be due to a high referral rate, as our specialized center is the main referral center for cytogenetic studies in Egypt. A wide range of sex chromosome and some autosomal anomalies were detected (Table 2). Sex chromosome dosage change may lead to abnormal expression of dosage-sensitive sex-chromosome genes that regulate coexpression networks of autosomal genes (Raznahan et al., 2018). DSD may also occur as a part of several malformation syndromes or present with different congenital anomalies due to imbalances affecting autosomal or X-linked genes involved in sex development machinery (Kim et al., 2015). Among our 46,XX DSD patients, 11 patients had the 21-hydroxylase deficiency CAH; of them, eight had the salt-losing type and three showed the simple virilizing type. The majority of virilized 46,XX infants were diagnosed as 21-hydroxylase deficiency, the most prevalent type of CAH (Baronio et al., 2019). Ten of our patients had 46,XX testicular DSD (4.4%), seven presented with infertility, two with atypical genitalia, and one with undescended testis. 46,XX OT DSD was detected in two patients as identified by gonadal histopathology, one of them revealed a gonadal 46,XX/45,X/46,XY cell line mosaicism, "while other two OT DSD patients had 46,XX/46,XY chimerism detected by FISH analysis on blood lymphocytes" (Table 2). Unfortunately, examination of other tissues was not feasible in the latter two patients, thus it still be a possibility of sex-chromosomal mosaicism. Most of our 46,XX males presenting with infertility were positive for *SRY* gene (five out of seven), while the remainders were all *SRY*-negative. The *SRY* gene is present in about 80% of classic XX testicular DSD as a result of abnormal X/Y interchange during paternal meiosis and could be detected by FISH analysis on the X short arm, while most patients with atypical genitalia and OT DSD patients are *SRY*-negative (McElreavey & Cortes, 2001; Mekkawy et al., 2020). In patients with no *SRY* gene, the presence of gonadal 46,XY cell line may explain testicular development, as evidenced in one of our 46,XX OT DSD patients. In other cases, dysregulation of the expression of other *SOX* family members (Sry-related HMG-box) may lead to testis formation (Kim et al., 2015; Sutton et al., 2011). Loss-of-function variants in WNT signaling factors including *WNT4* and *RSPO1* could be also responsible for the phenotype (Mandel et al., 2008; Parma et al., 2006). Recently, loss-of-function variants in the nuclear receptor NR2F2, as well as specific changes involving the Arginine 92 residue of *NR5A1*, have been described in association with testis formation in 46,XX children (Bashamboo et al., 2016; Bashamboo et al., 2018). However, none of our 46,XX DSD patients examined by WES revealed abnormalities in those candidate genes. On the other hand, WES identified a de novo *WT1* variant, p.Arg495Gly (c.1483C > G) in a female-reared child with 46,XX testicular DSD who presented with atypical genitalia and had a male gender identity. The variant was located in the highly conserved fourth zinc finger DNA-binding domain of *WT1* required for testis formation (Eozenou et al., 2020) (Table 4). Similarly, Gomes et al. (2019) reported a patient with 46,XX testicular DSD who had a novel heterozygous p.Arg485Glyfs*14 pathogenic variant of *WT1*, located in the fourth zinc finger of the protein. *WT1* (OMIM # 194070) encodes a

TABLE 4 Variants identified by WES

Patient phenotype	Karyotype	Sex of rearing	Gene	Variants	ACMG novel variants classification	Ref.
Ambiguous genitalia, gonadal dysgenesis	46,XY	Male	*WT1*	NM_024426.4: c.1384C > T (p.Arg462Try) de novo missense variant	Previously reported	Mazen et al., 2018
Ambiguous genitalia, bilateral testicular dysgenesis	46,XX SRY− (both in blood and gonadal tissue)	Female; Changed to male	*WT1*	NM_024426.4: c.1483C > G (p.Arg495Gly) Novel de novo missense variant	Pathogenic (PS2 + PS3)	Eozenou et al., 2020
Primary amenorrhea, Gonadal dysgenesis	46, XY	Female	*NR5A1*	NM_004959.5: c.614_615insG (p.Glu206Thrfs*20) Novel de novo frameshift variant	Pathogenic (PVS1 + PS2)	Mazen et al., 2016
Ambiguous genitalia gonadal dysgenesis	46, XY	Female	Digenic *NR5A1* *MAP3K1*	NM_004959.5:c.937C > T (p.Arg313Cys); de novo missense variant NM_005921.2:c.710A > G (p.Glu237Arg) Heterozygous missense variant	Previously reported Likely pathogenic (PM2 + PP1+ PP2 + PP4 + PP5).	Mazen et al., 2016
Ambiguous genitalia, congenital heart defect	46,XY	Male	Digenic *FGFR1* *STARD3*	NM_001174063.2: c.1418G > A (p.Arg473Glu) Novel homozygous variant NM_001165937.2: c.879C > T (p.Ala247Val) Homozygous variant	Likely pathogenic (PM1 + PM2 + PM3) Likely pathogenic (PM2 + PM3 + PP3 + PP5)	Mazen et al., 2016
Microcephaly, delayed puberty, gonadal dysgenesis, mild Fanconi anemia	46,XY	Female	*FANCA*	NM_000135.4: c.4232C > T (p.Pro1411Leu) Novel homozygous variant	Likely pathogenic (PM2 + PM3 + PP3 + PP4 + PP5)	Mazen et al., 2018
Ambiguous genitalia, bilateral inguinal testes, no Müllerian derivatives, microcephaly, multiple café au lait patches	46,XY	Female with strong male gender identity	*HHAT*	NM_001170580: c.1329C > A (p.Asp443Lys), Novel homozygous variant	Likely pathogenic (PM2 + PM3 + PP3 + PP4 + PP5)	
Bilateral cryptorchidism, uterus and fallopian tubes	46, XY	Male	*AMH*	NM_000479.5:c.208delC (p.Leu70Cysfs*7) Novel homozygous frame shift variant	Pathogenic (PVS1 + PM2 + PM3)	Mazen, El-Gammal, et al., 2017.
Bilateral cryptorchidism, uterus and fallopian tubes	46, XY	Male	*AMHR2*	NM_001164691.2: c.847A > C (p.His256Pro) Novel homozygous missense variant	Likely pathogenic (PM2 + PM3 + PP3 + PP4 + PP5)	Mazen, El-Gammal, et al., 2017.
Impalpable testes, hypogenitalism, and penoscrotal hypospadias	46,XX	Male	*CYP19A1*	NM_001205254.1:c.1263 + 1G > T Novel homozygous splice site variant in the donor splice site	Pathogenic (PVS1+ PM2 + PM3)	Mazen, McElreavey, et al., 2017.

TABLE 4 (Continued)

Ambiguous genitalia, cardiac defects, lissencephaly, agenesis of the corpus callosum, and colpocephaly	46,XY	Male	*ARX*	NM_139058:c.196 + 1G > T Novel variant in the donor splice site of the first exon	Pathogenic (PVS1 + PS2)
Kabuki syndrome dysmorphic features, undescended testes, and severe intellectual disability.	46,XY	Male	*KDM6A*	NM_021140:c.1760G > A (p.Ser587Asp) Novel missense variant	Likely pathogenic (PS2 + PM2)

Abbreviations: ACMG, American College of Medical Genetics and Genomics; WES, Whole exome sequencing.

zinc finger DNA-binding protein that has a role in transcriptional activation or repression depending on chromosomal context. It is essential for the normal development of mesothelial tissues and genitourinary system (Lehnhardt et al., 2015). The −KTS protein isoform acts as a direct activator of the *NR5A1* gene and can bind the *SRY* promoter region leading to transactivation (Hossain & Saunders, 2001). WES also identified a novel homozygous splice site variant in the donor splice site in aromatase (*CYP19A1)* gene (c.1263 + 1G > T). The variant occurred in a 46,XX male patient of consanguineous parents who presented at the age of 21 years with impalpable testes and atypical genitalia (Mazen, McElreavey, et al., 2017). Aromatase is a member of the cytochrome P450 superfamily. It catalyzes the conversion of androgen to estrogen. This was the first report of this rare disorder in an Egyptian patient. It was recommended to consider aromatase deficiency in patients with 46,XX DSD after excluding CAH.

In our cohort, 46,XY DSD was the largest category, constituting 44.4% of the total number. Similarly, Juniarto et al. (2016) and Mazen et al. (2008) reported 46,XY DSD as the most common category among DSD patients constituting 68.2% and 65.9%, respectively. 46,XY DSD is largely heterogeneous and often challenging in molecular diagnosis. Patients usually present with atypical genitalia or female genitalia with amenorrhea or delayed puberty. While single gene analysis of six genes in our 46,XY DSD group gave a 33.7% diagnostic yield, WES in 18 selected patients lacking a molecular diagnosis reached a 66.7% diagnostic yield (including two 46,XX DSD patients). The most commonly detected pathogenic variants by Sanger sequencing were in the *HSD17B3* and *SRD5A2* genes (50%, each), followed by the *AR* gene (18%) (Table 3). The most frequently detected pathogenic variants in *SRD5A2* were the p.Gly34Arg and p.Gly196Ser amino acid changes (Soliman et al., 2015). p.Gly34Arg represents a mutational hotspot in Egyptians and was previously reported in other Egyptian patients, suggesting a founder effect (Mazen et al., 2003). Similarly, p.Gly196Ser is one of the mutational hotspots in the *SRD5A2* gene, which was reported in several populations (Akcay et al., 2014; Nagaraja et al., 2019), while p.Tyr91His variant, which has a deleterious effect on protein function, was reported in Turkish and Palestinian populations (Maimoun et al., 2011). Similar to our findings, *SRD5A2* variants were the second most frequently reported cause of 46,XY DSD in Indian populations (Nagaraja et al., 2019). We have found that 17β-Hydroxysteroid dehydrogenases III (HSD17B3) deficiency is relatively common in our cohort with a different mutational profile compared with that observed in other populations (Hassan et al., 2016) (Table 3). In contrast, only five pathogenic variants were detected in the *AR* gene out of 28 patients with suspected androgen insensitivity. The higher prevalence of the autosomal recessive disorders, HSD17B3 deficiency and 5α-Reductase deficiency (5α-RD2) deficiency, may be due to the high rate of parental consanguinity (nearly 70%). Similarly, a high rate of HSD17B3 deficiency (1:100–300) was identified in the Gaza strip, indicating the relevant role of consanguinity in increasing the prevalence of HSD17B3 deficiency (Rosler, 2006). A rise in the prevalence of heritable DSD causes was also reported in India due to the high frequency of consanguineous marriages (Nagaraja et al., 2019). The lower rate of *AR* pathogenic variants detected here may be ascribed to the sequencing limited to exons 2–8 and/or the relative increase in the mutations of genes with autosomal recessive inheritance. *WT1* gene variants were detected in three of our 46,XY DSD patients with atypical genitalia and variable degrees of gonadal dysgenesis. Two patients carried a de novo heterozygous p.Arg462Try (c.1384C > T) variant in exon 9, identified by direct sequencing in one patient who manifested an early developing gonadoblastoma (Mazen, Hassan, et al., 2017), while the other was diagnosed by WES. A novel heterozygous variant in exon 9, p.Lys459Arg (c.689A > G), was also detected in a patient carrying a mosaic karyotype: 45,X[90]/46,X,idic(Y)(q11.2)[10], who had shown pelvicalyceal dilatation of the left kidney. (Table 3). *WT1* gene variants can result in a range of phenotypes characterized by glomerulopathy associated with varying degrees of genital anomalies in 46,XY patients. p.Arg462Try is a mutational hotspot in exon 9 that has been described in patients with Denys–Drash syndrome and patients with steroid-resistant nephrotic syndrome (Lehnhardt et al., 2015). However, none of our patients presented with signs of nephrotic syndrome. Patients carrying *WT1* pathogenic variants are at an increased risk of Wilms tumor or gonadoblastoma, and it is recommended to perform prophylactic nephrectomy or gonadectomy when expected (Ahn et al., 2017). Further analysis by WES in the selected undiagnosed patients revealed more interesting findings: Two pathogenic de novo *NR5A1* variants were detected in two unrelated patients with 46,XY gonadal dysgenesis (Mazen, Abdel-Hamid, et al., 2016). The first patient presented with amenorrhea and primary

infertility and carried a novel heterozygous frameshift variant in exon 4, p.Glu206Thrfs*20 (c.614_615insG), while the other patient presented at the age of 4 years as a female with atypical genitalia and gonadal dysgenesis and carried the variant p.Arg313Cys (c.937C > T). This variant was previously reported by Allali et al. (2011) in a patient presenting with mild isolated hypospadias. The more severe phenotype in our patient may be due to the association of a rare *MAP3K1* gene variant inherited from her mother (Table 4). *MAP3K1* (OMIM # 600982) is a protein coding gene that has been associated with an autosomal dominant form of 46,XY gonadal dysgenesis (Le Caignec et al., 2003). Loke et al. (2014) reported rare missense *MAP3K1* variants in 46,XY DSD patients and postulated that it could alter the cofactor-binding and/or MAP kinase activity above a required threshold, thereby disrupting the normal testicular development. Different pathogenic *MAP3K1* variants were also identified by Baxter et al. (2015) in 46,XY DSD patients with variable phenotypes. Digenic inheritance was suggested in another patient in our cohort with atypical genitalia, 46,XY DSD, and congenital heart disease, in whom WES detected a novel homozygous variant, p.R473Q in *FGFR1* gene (OMIM#136350), affecting exon 10. In addition, a homozygous (p.A247V) variant in the START domain of the STARD3 protein was detected and predicted to be deleterious on the protein function (Mazen, Hassan, et al., 2016). These gene mutations were confirmed by Sanger sequencing performed for the patient and his normal brother. It revealed that the normal brother was heterozygous for the same variant affecting *FGFR1* gene and carried a wild-type sequence of the *STARD3* gene, raising the possibility of the contribution of these two mutations to the phenotype. *STARD3* (OMIM# 607048) encodes a cholesterol-binding protein that acts in the endosomal cholesterol transport and is expressed in many adult human tissues at high levels, including testis and adrenal gland (Watari et al., 1997). It is worth mentioning that digenic inheritance should be formulated with caution in consanguineous couples as multiple homozygous variants could be frequently encountered in their offspring. Thus, studying of the functional consequences of these variants as well as the analysis of normal sibs will be of help. Two of our 46,XY DSD boys with bilateral cryptorchidism were shown by laparoscopy to have a uterus and fallopian tubes. WES identified a homozygous novel frameshift variant (p.L70Cfs*7) in the *AMH* gene in the first child, while the second child had a novel homozygous *AMHR2* (p.H256P) missense variant (Mazen, El-Gammal, et al., 2017). Persistent Müllerian duct syndrome is a very rare form of 46,XY DSD that results from inactivating mutations affecting the *AMH* gene or AMH receptor type II (*AMHR2*) gene. It is mostly transmitted in an autosomal recessive pattern, and most cases were detected among families with a high consanguinity rate (Picard & Josso, 2019). The majority of *AMH* gene variants were identified in the Mediterranean region and Saudi Arabia, while most of *AMHR2* variants were identified in Northern Europe (Ren et al., 2017).

WES had identified other rare variants in patients presenting with complex phenotypes. A homozygous rare variant, p.Pro1411Leu (c.4232C > T), involving *FANCA* gene was detected in a 46,XY gonadal dysgenesis female patient presenting with atypical external genitalia and microcephaly. This variant was found to be inherited from the heterozygous parents (Mazen et al., 2018). The phenotype of Fanconi anemia patients is highly variable and may be associated with varying degrees of skeletal, genital, renal, and central nervous system anomalies. Another 46,XY female-reared patient had presented with atypical genitalia, bilateral inguinal testes with no Müllerian derivatives, microcephaly (−3 SD), and multiple café-au-lait patches and had a strong male gender identity. This patient was found to harbor a homozygous missense variant in exon 11 of the *HHAT* gene p.Asp443Lys (c.1329C > A) inherited from her heterozygous parents. This novel variant is predicted to be deleterious by various bioinformatics tools. *HHAT* (Hedgehog Acyltransferase; OMIM# 605743) encodes an enzyme that catalyzes amino-terminal palmitoylation of Hedgehog gene family and is expressed in XY and XX gonads at the sex determination critical time (Callier et al., 2014; Chamoun et al., 2001). A single loss-of-function variant of *HHAT* has been reported to disrupt testicular organogenesis and skeletal and embryonic growth development in humans (Callier et al., 2014).

Interestingly, two different X-linked variants were detected by WES in two of our patients with multiple congenital anomalies. The first was a novel *ARX* gene variant (c.196 + 1G > T) detected in an eight-day-old 46,XY DSD infant with cardiac defects, lissencephaly, agenesis of the corpus callosum, and colpocephaly. Similar to this patient, 46,XY DSD with lissencephaly, agenesis of the corpus callosum, intractable neonatal seizures, congenital heart anomalies, and atypical genitalia with small dysgenetic testes was separately reported by Ogata et al. (2000) and Spinosa et al. (2006). Kato et al. (2004) also identified two recurrent and 13 novel variants in the *ARX* gene in 20 patients with brain and genital malformations; most of the patients had X-linked lissencephaly with atypical genitalia (XLAG). XLAG is a relatively recently recognized syndrome resulting from mutation in the *ARX* gene located in Xp22.13. *ARX* is an X-linked recessive gene (OMIM# 300382) that encodes the Aristaless-related homeobox protein, belonging to Aristaless-related subset of the paired (Prd) class of homeodomain proteins (Lee, 2017), which have a crucial role in cerebral development and patterning (Bienvenu et al., 2002). A novel missense variant p.Ser587Asp (c.1760G > A) in the X-linked gene *KDM6A* was also identified in a patient with dysmorphic facies characteristic of Kabuki syndrome (KS), with undescended testes and severe intellectual disability. Most KS patients (OMIM#147920 and 300867) had variants in *KMT2D* gene, whereas about 3% to 5% are due to *KDM6A* gene variants with X-linked dominant inheritance (OMIM # 300128) (Bögershausen et al., 2016; Lederer et al., 2012). Genitourinary anomalies including hypospadias and cryptorchidism may occur in males with KS, while severe intellectual disability is very rare as the majority exhibit mild to moderate forms (Bögershausen & Wollnik, 2013).

The early prenatal identification of fetal anomalies allows for proper parental counseling regarding the expected prognosis and management options and give the opportunity to proceed to in utero MR imaging or genetic testing. Nevertheless, some abnormalities including lissencephaly could not be detected until late pregnancy (20 and 24 weeks) (Williams & Griffiths, 2017). In most Middle Eastern countries, elective pregnancy termination is forbidden unless the life of mother is in imminent danger. However, the diagnosis of a

serious fetal anomaly with a poor outcome by an authorized doctor may allow termination in specific cases.

In conclusion, we report a large number of patients with DSD from the same ethnic group. The study enlarges the scope of both unusual cytogenetic and monogenic pathogenic variants and suggests digenic inheritance as a cause of phenotypic variability observed in DSD patients. The role of exome sequencing was emphasized for more accurate diagnosis and management of DSD patients and for better genetic counseling.

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CONFLICT OF INTEREST

The authors declare that there are no competing financial interests in relation to the work described.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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