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ORIGINAL ARTICLE

Male Health

Prevalence of gene mutations in a Chinese 46,XY disorders of sex development cohort detected by targeted next-generation sequencing

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46,XY disorders of sex development (DSD) is characterized by incomplete masculinization genitalia, with gonadal dysplasia and with/without the presence of Müllerian structures. At least 30 genes related to 46,XY DSD have been found. However, the clinical phenotypes of patients with different gene mutations overlap, and accurate diagnosis relies on gene sequencing technology. Therefore, this study aims to determine the prevalence of pathogenic mutations in a Chinese cohort with 46,XY DSD by the targeted next-generation sequencing (NGS) technology. Eighty-seven 46,XY DSD patients were enrolled from the Peking Union Medical College Hospital (Beijing, China). A total of fifty-four rare variants were identified in 60 patients with 46,XY DSD. The incidence of these rare variants was approximately 69.0% (60/87). Twenty-five novel variants and 29 reported variants were identified. Based on the American College of Medical Genetics and Genomics (ACMG) guidelines, thirty-three variants were classified as pathogenic or likely pathogenic variants and 21 variants were assessed as variants of uncertain significance. The overall diagnostic rate was about 42.5% based on the pathogenic and likely pathogenic variants. Androgen receptor (*AR*), steroid 5-alpha-reductase 2 (*SRD5A2*) and nuclear receptor subfamily 5 Group A member 1 (*NR5A1*) gene variants were identified in 21, 13 and 13 patients, respectively. The incidence of these three gene variants was about 78.3% (47/60) in patients with rare variants. It is concluded that targeted NGS is an effective method to detect pathogenic mutations in 46,XY DSD patients and *AR*, *SRD5A2*, and *NR5A1* genes were the most common pathogenic genes in our cohort.

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Keywords: 46,XY disorders of sex development; mutations; targeted next-generation sequencing

INTRODUCTION

Gonadal differentiation originates from the bipotential primordium during embryonic development and is determined by sex chromosomes to either differentiate into testes or ovaries. This process is termed sex determination.[1] Hormones are synthesized and secreted by the developing testes or ovaries to promote the differentiation of the genitalia. This process is termed sex differentiation.[2] Sex differentiation and sex determination are complicated processes controlled by several genetic factors. They induce sex development in a tissue-specific and time-dependent manner.[3,4] Any genetic defects affecting the process of sex determination and sex differentiation could lead to disorders of sex development (DSD), where the development of chromosomal, gonadal, or anatomical gender is atypical.[5]

46,XY DSD is the most complicated type of DSD. It is characterized by incomplete masculinization genitalia, with gonadal dysplasia, and with/without the presence of Müllerian structures.[6] The incidence of 46,XY DSD is about 1/6000.[5] At present, more than 30 genes have been identified associated with 46,XY DSD; these include (1) genes related to testicular development: Wilms' tumor 1 (*WT1*), nuclear receptor subfamily 5 Group A member 1 (*NR5A1*), GATA-binding protein 4 (*GATA4*), zinc finger protein, FOG family member 2 (*ZFPM2*), chromobox 2 (*CBX2*), sex-determining region Y (*SRY*), SRY-box 9 (*SOX9*), mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*), doublesex- and mab-3-related transcription factor 1 (*DMRT1*), TSPY like 1 (*TSPYL1*), desert hedgehog signaling molecule (*DHH*), alpha thalassemia/mental retardation syndrome X-linked (*ATRX*), mastermind-like domain-containing 1 (*MAMLD1*), nuclear receptor subfamily 0 Group B member 1 (*NR0B1*), and Wnt family member 4 (*WNT4*); and (2) genes related to hormone synthesis and action: androgen receptor (*AR*), steroid 5-alpha-reductase 2 (*SRD5A2*), 7-dehydrocholesterol reductase (*DHCR7*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), steroidogenic acute regulatory protein (*STAR*), cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), hydroxysteroid 17-beta dehydrogenase 3 (*HSD17B3*), cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*), cytochrome p450 oxidoreductase (*POR*), cytochrome b5 type A (*CYB5A*), 3β-hydroxysteroid dehydrogenase 2 (*HSD3B2*), anti-Müllerian hormone (*AMH*), anti-Müllerian hormone type II

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receptor (*AMHR2*), and aldo-keto reductase family 1 member C2 (*AKR1C2*).[7,8]

46,XY DSD patients with different gene mutations have similar clinical manifestations, *i.e.*, patients with androgen synthesis or action related gene mutations are difficult to distinguish. During the prepuberty stage, the clinical phenotype of patients with 5α-reductase deficiency induced by *SRD5A2* gene mutations and androgen insensitive syndrome (AIS) induced by *AR* gene mutations is often indistinguishable.[9] Hence, it is difficult to accurately diagnose patients solely based on clinical manifestations. Gene sequencing may offer accurate etiological diagnosis for 46,XY DSD.

Whole exome sequencing (WES) and targeted next-generation sequencing (NGS) are the most commonly used methods to detect multiple pathogenic mutations in a variety of genetic diseases.[10] WES captures nucleotide sequences in protein-coding regions of the genome, while targeted NGS captures nucleotide sequences in specific genomic regions, which may constitute introns, exons, and regulatory sequences of a particular gene.[11] Although WES has the capability of comprehensively sequencing all the genes within the genome and could be used for discovery purposes, the coverage of targeted NGS is much deeper[12] and less expensive.[13] Clinical interpretation of WES is difficult due to the large amounts of data generated and the limitation of current bioinformatic analysis capabilities.[14] Numerous studies have demonstrated that targeted NGS could achieve a diagnostic rate similar to WES for Mendelian diseases. The purpose of this study was to identify gene mutations in a Chinese 46,XY DSD cohort using targeted NGS technology.

PATIENTS AND METHODS

Patients

Eighty-seven patients with 46,XY DSD were enrolled in this study from the Endocrinology Department of Peking Union Medical College Hospital (Beijing, China) between January 2013 and April 2018. Clinical characteristics and gene mutations of patients harboring *HSD17B3* or *NR5A1* have been published previously.[15,16]

The patient inclusion criteria were as follows: (1) patients with 46,XY karyotype and (2) patients with external genital malformation, including female external genitalia, clitoromegaly, ambiguous external genitalia, and perineal hypospadias. Informed written consent was obtained from all participants and the study protocol was reviewed and approved by the Peking Union Medical College Hospital Ethics Committee (No. JS-2111).

Targeted gene panel

Thirty-two reported 46,XY DSD pathogenic genes and 51 genes related to gonadal development or differentiation were selected using PubMed, OMIM, and Genetic testing registry database (**Supplementary Table 1**).

Targeted next-generation sequencing

Genomic DNA was extracted from peripheral blood leukocytes using the Qiagen DNA Blood kit (Qiagen, Dusseldorf, Germany). The gene panel (NimblegenSeqCap EZ system, Roche, Basel, Switzerland) was designed to capture all exons and 50 bp flanking intron sequences of the 83 DSD-related genes. The DNA samples were analyzed using massive parallel sequencing (100-bp paired-end reads) on an Illumina HiSeq2500 sequencing system (Illumina, Inc., San Diego, CA, USA) after hybridization to the capture array. Bioinformatic analysis including quality control, read alignment, and variant calling (including single-nucleotide variants [SNVs] and small indels) were performed using bioinformatic pipelines previously described.[17] The variants identified by NGS were validated using Sanger sequencing.

Assessment of variants

A variant was recognized as an underlying disease-causing variant if it was not found in the following databases: dbSNP (http://www.ncbi.nlm.nih.gov/snp/), exome variant server (http://evs.gs.washington.edu/EVS/), ensemble database or in 500 Chinese healthy controls, or the allele frequency was found to be <0.001 in the database. Based on the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) published in 2015, variants were classified into five categories: pathogenic, likely pathogenic, variants of uncertain clinical significance (VUS), likely benign, and benign.[18]

RESULTS

Clinical features

Sixty unrelated 46,XY DSD Chinese patients were identified harboring 54 rare mutations. The median age of these patients at the initial visit was 14.0 years old, and 75.0% of the patients were assigned as females and 25.0% assigned as males. Genital examination revealed that 51.7% of the patients had female external genitalia, 11.7% had female external genitalia with clitoromegaly, 15.0% had ambiguous external genitalia, and 21.7% had hypospadias (**Table 1**). The distribution of gene mutations in 46,XY DSD patients with different external genitalia is shown in **Figure 1**.

Table 1: Clinical characteristics of patients with 46,XY disorders of sex development harboring mutations

Clinical characteristics	*Value*
Age[a] (year, *n*=59), median (range)	14.0 (7.0–22.5)
Sex	
Male, *n*/total (%)	15/60 (25.0)
Female, *n*/total (%)	45/60 (75.0)
External genitalia	
Female, *n*/total (%)	31/60 (51.7)
Clitoromegaly, *n*/total (%)	7/60 (11.7)
Ambiguous, *n*/total (%)	9/60 (15.0)
Hypospadias, *n*/total (%)	13/60 (21.7)
Developmental stage[b]	
Prepuberty, *n*/total (%)	18/58 (31.0)
Puberty, *n*/total (%)	40/58 (69.0)

[a]One patient with missing clinical data of age; [b]Two patients with missing laboratory results.

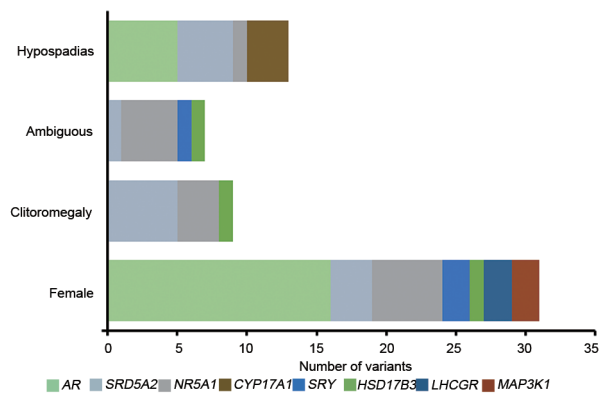

Figure 1: Distribution of gene mutations in 46,XY DSD patients based on different external genitalia. DSD: disorders of sex development; *AR*: androgen receptor; *SRD5A2*: steroid 5-alpha-reductase 2; *NR5A1*: nuclear receptor subfamily 5 Group A member 1; *SRY*: sex-determining region Y; *CYP17A1*: cytochrome P450 family 17 subfamily A member 1; *HSD17B3*: hydroxysteroid 17-beta dehydrogenase 3; *MAP3K1*: mitogen-activated protein kinase kinase kinase 1; *LHCGR*: luteinizing hormone/choriogonadotropin receptor.

Among these patients, except two patients with missing laboratory results, 18 patients were in prepuberty stage (prepuberty group) and 40 patients reached the age of puberty (puberty group). Laboratory tests indicated that serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone (T), and estradiol (E2) levels in patients from the puberty group were higher compared to patients in the prepuberty group ($P < 0.001$; **Table 2**). Based on the external genitalia of patients in the puberty group, patients were classified as "female," "clitoromegaly," "ambiguous," and "hypospadias." The serum LH, FSH, T, and E2 levels in these four groups were not statistically different ($P > 0.05$; **Table 3**).

Mutational analysis

Targeted next-generation sequencing demonstrated that 69.0% (60/87) of the patients had detectable mutations (**Figure 2a**), 53.7% (29/54) of these mutations had been reported previously and 46.3% (25/54) were novel mutations (**Figure 2b**). Of these mutations, missense mutations were the most common and accounted for 66.7% (36/54), followed by indel mutations accounting for 22.2% (12/54). Nonsense mutations, splicing mutations and gross deletion mutations each accounted for 3.7% (2/54; **Figure 2c**). Based on the ACMG guidelines, 40.7% (22/54) of these mutations were assessed as pathogenic, 20.4% (11/54) were likely pathogenic mutations and 38.9% (21/54) were assessed as VUS (**Figure 2d**).

AR gene mutations, including 14 reported mutations and 3 novel mutations, were detected in 21 patients. *SRD5A2* gene mutations, which included 7 reported mutations and 2 novel mutations, were identified in 13 patients. Thirteen patients had *NR5A1* gene mutations and included 7 reported mutations and 6 novel mutations. *SRY*, *CYP17A1,* and *HSD17B3* gene mutations were detected in three patients, and *MAP3K1* and *LHCGR* gene mutations were detected in two patients (**Figure 3**).

Table 2: The laboratory test results of 46,XY disorders of sex development patients in different age stage

Clinical characteristics	*Prepuberty (n=18)*	*Puberty (n=40)*	*Reference values*
Age (year), median (range)	2.5 (1.3–6)	17.0 (13.6–25.0)	NA
LH (IU l^{-1}), median (range)	0.1 (0–0.7)	18.1 (12.5–27.4)	1.24–8.62
FSH (IU l^{-1}), median (range)	1.7 (0.9–3.1)	7.8 (10.9–71.8)	1.27–19.26
T (ng ml^{-1}), median (range)	0 (0–0.1)	1.2 (0.2–4.2)	1.75–7.81
E2 (pg ml^{-1}), median (range)	3.6 (1.2–11.7)	23.0 (15.6–31.9)	<47

DSD: disorders of sex development; LH: luteinizing hormone; FSH: follicle-stimulating hormone; T: testosterone; E2: estradiol; NA: not applicable

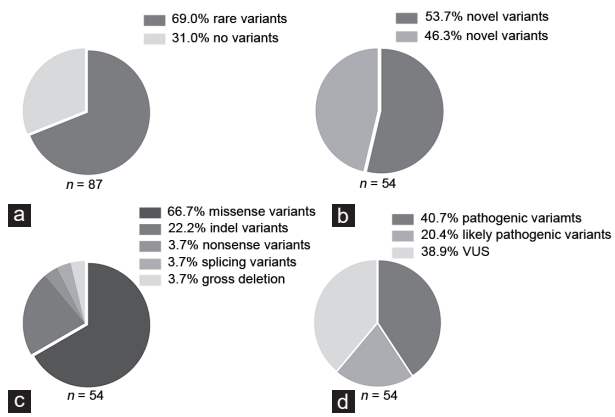

Figure 2: Genetic diagnosis of the 46,XY DSD cohort. (**a**) Proportion of 46,XY DSD patients with identified variants in DSD genes. (**b**) Proportion of novel variants. (**c**) Proportion of the different variant types. (**d**) Clinical significance of variants and their proportions. DSD: disorders of sex development; VUS: variants of uncertain clinical significance.

The clinical information of the 46,XY DSD patients with different gene mutations is shown in **Supplementary Table 2**. Detailed clinical and mutation information is shown in **Supplementary Table 3** and **4**.

DISCUSSION

In this study, targeted NGS was used to identify pathogenic gene mutations in a Chinese 46,XY DSD cohort. Sixty out of eighty-seven unrelated patients were identified with 54 rare variants. The incidence of these rare variants was approximately 69.0% (60/87). Based on the ACMG guidelines, the overall diagnostic rate was about 42.5% and was based on the ratio of pathogenic and likely pathogenic mutations.

Gene mutations in 46,XY DSD patients have been previously identified using traditional PCR combined with Sanger sequencing. Gene sequencing performed on a gene-by-gene basis is time-consuming and expensive. Previous studies have demonstrated that only 13% of DSD patients undergo molecular diagnosis, of which the diagnostic rate for identifying pathogenic genes is only about 20%.[19,20] Next-generation sequencing technology has gradually become the leading method to detect pathogenic genes due to its high throughput to detect variants.

In 2013, Arboleda *et al*.[20] were the first to use NGS technology to identify gene mutations in 46,XY DSD patients. A total of 10 patients were included in that study, five of whom were known to have pathogenic mutations. This was performed to determine the accuracy of NGS. Their study demonstrated that NGS was able to consistently identify known mutations in patients, in addition to pathogenic mutations in two of the remaining five patients. Since then, NGS has been widely used for the molecular diagnosis of 46,XY DSD patients. Numerous studies have demonstrated that the diagnostic rate of NGS to identify pathogenic mutations in 46,XY DSD patients was about 40%–66%[7,8,20–25] (**Supplementary Table 5**). In our study, we screened 87 patients using a targeted gene panel designed to include genes involved in sex development. Sixty patients were identified with rare variants with a diagnostic rate of about 42.5%. Our results were consistent with previous studies and suggested that targeted technology is an effective method to improve the molecular diagnostic rate in 46,XY DSD patients.

AR, *NR5A1,* and *SRD5A2* gene mutations were the most common and accounted for 35.0%, 21.7%, and 21.7% of the variants in our cohort, respectively. This was consistent with a previous study performed in Shanghai.[21] Previous studies have demonstrated that

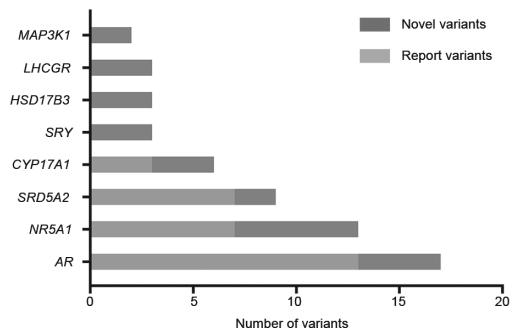

Figure 3: Variants identified in eight genes. *AR*: androgen receptor; *SRD5A2*: steroid 5-alpha-reductase 2; *NR5A1*: nuclear receptor subfamily 5 Group A member 1; *SRY*: sex-determining region Y; *CYP17A1*: cytochrome P450 family 17 subfamily A member 1; *HSD17B3*: hydroxysteroid 17-beta dehydrogenase 3; *MAP3K1*: mitogen-activated protein kinase kinase kinase 1; *LHCGR*: luteinizing hormone/choriogonadotropin receptor.

Table 3: The laboratory test results of different phenotype in puberty group

Hormones	*Female (n=21)*	*Clitoromegaly (n=7)*	*Ambiguous (n=4)*	*Hypospadias (n=8)*	*P*
LH (IU l^{-1}), mean±s.d.	21.1±9.9	21.6±18.3	16.6±9.9	17.2±7.9	0.677
FSH (IU l^{-1}), mean±s.d.	51.4±33.8	51.6±18.3	33.8±37.1	18.7±11.7	0.179
T (ng ml^{-1}), mean±s.d.	1.9±3.2	3.1±2.2	2.9±2.4	3.5±2.8	0.070
E2 (pg ml^{-1}), mean±s.d.	27.1±27.3	22.2±8.6	22.3±5.1	34.1±19.2	0.477

s.d.: standard deviation; LH: luteinizing hormone; FSH: follicle-stimulating hormone; T: testosterone; E2: estradiol

the incidence of *MAP3K1* gene mutations in 46,XY DSD is about 13%–18%.[26–28] However, in our cohort, only 3.3% of the patients were identified with *MAP3K1* gene mutations. A Chinese[21] and a Korean study[8] showed that the incidence of *MAP3K1* gene mutations in DSD patients was 4% and 7.7%, respectively. This suggested that *MAP3K1* gene mutations may have different roles in different ethnic groups. In addition, the incidence of *HSD17B3* gene mutations in our cohort was 4.9% and was lower compared to previous studies.[7,22,25] This may be related to the higher consanguineous rates in other countries compared to China.[22] These studies suggest that the incidence of different gene mutations in 46,XY DSD patients may be associated with patient race.

46,XY DSD patients with comorbidities of hypertension and hypokalemia may have mutations in the *CYP17A1* gene,[29] and hence were excluded from our study. However, we found three patients with *CYP17A1* gene mutations. Previous studies have shown that 10%–15% of patients with *CYP17A1* gene mutations do not manifest hypertension and hypokalemia.[30] In addition, these patients are difficult to distinguish from other types of 46,XY DSD. This suggests that gene sequencing could accurately diagnose 46,XY DSD patients.

There is a limitation of this study that should be addressed. Genomic rearrangements were not analyzed in this study, which has been identified accounting for a significant proportion of 46,XY DSD cases.[31,32]

CONCLUSION

We performed targeted NGS using a gene panel that included 83 genes related to sex development. Sixty out of the eighty-seven unrelated patients were identified using targeted NGS. The overall diagnostic rate was about 42.5% and was based on pathogenic and likely pathogenic variants according to the ACMG criteria. Our study demonstrated that targeted NGS was an effective method to detect pathogenic genes in 46,XY DSD patients.

AUTHOR CONTRIBUTIONS

XYW and MN conceived of the study and participated in its design. XW and JFM collected the clinical data and the blood sample. ZXL, BQY, and YJG carried out the genetic studies. BQY wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: Genes included in the targeted gene panel

Gene	*Location*	*OMIM*
Disorders of testicular development		
Aldo-Keto Reductase Family 1, member C2 (AKR1C2)	10p15.1	600450
Aldo-Keto Reductase Family 1, member C2 (AKR1C4)	10p15.1	600451
Aristaless-related homeobox (ARX)	Xp21.3	300382
α-Thalassemia/mental retardation syndrome X-linked (ATRX)	Xq21.1	300032
Chromobox homolog 2, Drosophila polycomb class (CBX2)	17q25.3	602770
Desert hedgehog (DHH)	12q13.12	605423
Doublesex- and MAB3-related transcription factor 1 (DMRT1)	9p24.3	602424
GATA-binding protein 4 (GATA4)	8p23.1	600576
Mastermind-like domain-containing 1 (MAMLD1)	Xq28	300120
Mitogen-activated protein kinase kinase kinase 1 (MAP3K1)	5q11.2	600982
Nuclear receptor subfamily 0 Group B member 1 (NR0B1)	Xp21.2	300473
Nuclear receptor subfamily 5, Group A, member 1 (NR5A1)	9q33.3	184757
R-spondin family, member 1 (RSPO1)	1p34.3	609595
SRY-BOX 9 (SOX9)	17q24.3-25.1	608160
Sex-determining region Y (SRY)	Yp11.2	480000
Testis-specific Y-encoded-like protein 1 (TSPYL1)	6q22.1	604714
Wingless-type mmtv integration site family, member 4 (WNT4)	1p36.12	603490
Wilms' tumor gene 1 (WT1)	11p13	607102
Zinc finger protein, multitype 2 (ZFPM2)	8q23.1	603693
Disorders of hormone synthesis or action		
Anti- Müllerian hormone (AMH)	19p13.3	600957
Anti-Müllerian hormone type II receptor (AMHR2)	12q13.13	600956
Androgen receptor (AR)	Xq12	313700
Cytochrome b5, type A (CYB5A)	18q22.3	613218
Cytochrome P450, subfamily XIA, polypeptide 1 (CYP11A1)	15q24.1	118485
Cytochrome P450, subfamily XIB, polypeptide 1 (CYP11B1)	8q24.3	610613
Cytochrome P450, subfamily XIB, polypeptide 2 (CYP11B2)	8q24.3	124080
Cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1)	10q24.32	609300
7-Dehydrocholesterol reductase (DHCR7)	11q13.4	602858
17β-Hydroxysteroid dehydrogenase III (HSD17B3)	9q22.32	605573
3β-Hydroxysteroid dehydrogenase 2 (HSD3B2)	1p12	613890
Luteinizing hormone/choriogonadotropin receptor (LHCGR)	2p16.3	152790
Cytochrome P450 oxidoreductase (POR)	7q11.23	124015
Steroid 5 α-reductase 2 (SRD5A2)	2p23.1	607306
Steroidogenic acute regulatory protein (StAR)	8p11.23	600617
Other syndromes		
Adp-Ribosylation factor-like 6 (ARL6)	3q11.2	608845
BBS1	11q13.2	209901
BBS10	12q21.2	610148
BBS12	4q27	610683
BBS2	16q13	606151
BBS4	15q24.1	600374
BBS5	2q31.1	603650
BBS7	4q27	607590
BBS9	7p14.3	607968
Chromodomain helicase DNA-binding protein 7 (CHD7)	8q12.2	608892
Fras1-related extracellular matrix protein 2 (FREM2)	13q13.3	608945
Homeobox A13 (HOXA13)	7p15.2	142959
17β-Hydroxysteroid dehydrogenase IV (HSD17B4)	5q23.1	601860
Interferon regulatory factor 6 (IRF6)	1q32.2	607199
Lysine-specific methyltransferase 2D (KMT2D)	12q13.12	602113
Midline 1 (MID1)	Xp22.2	300552
MKKS	20p12.2	604896

Contd...

Supplementary Table 1: Contd...

Gene	*Location*	*OMIM*
Spermatogenisis		
Aurora kinase C (AURKC)	19q13.43	603495
Solute carrier family 26 member 8 (SLC26A8)	6p21.31	608480
Spermatogenesis-associated protein 16 (SPATA16)	3q26.31	609856
Zinc finger mynd-containing protein 15 (ZMYND15)	17p13.2	614312
Other related genes		
Cystic fibrosis transmembrane conductance regulator (CFTR)	7q31.2	602421
Cytochrome P450 family 19 subfamily A member 1 (CYP19A1)	15q21.2	107910
Doublesex- and MAB3-related transcription factor 2 (DMRT2)	9p24.3	604935
Fibroblast growth factor 8 (FGF8)	10q24.32	600483
Fibroblast growth factor receptor 1 (FGFR1)	8p11.23	136350
Fibroblast growth factor receptor 2 (FGFR2)	10q26.13	176943
Follicle-stimulating hormone, beta polypeptide (FSHB)	11p14.1	136530
Gonadotropin-releasing hormone 1 (GNRH1)	8p21.2	152760
Gonadotropin-releasing hormone receptor (GNRHR)	4q13.2	138850
HFE	6p22.2	613609
Heparan sulfate 6-o-sulfotransferase 1 (HS6ST1)	2q14.3	604846
Kallmann syndrome interval gene 1 (KAL1)	Xp22.31	300836
KiSS-1 metastasis-suppressor (KISS1)	1q32.1	603286
KISS1 receptor (KISS1R)	19p13.3	604161
Luteinizing hormone, beta polypeptide (LHB)	19q13.33	152780
NMDA receptor synaptonuclear signaling and neuronal migration factor (NSMF)	9q34.3	608137
Prokinecitin 2 (PROK2)	3p13	607002
Prokinecitin receptor 2 (PROKR2)	20p12.3	607123
Semaphorin 3A (SEMA3A)	7q21.11	603961
Semaphorin 3E (SEMA3E)	7q21.11	608166
SRY-BOX 3 (SOX3)	Xq27.1	313430
Tachykinin 3 (TAC3)	12q13.3	162330
Tachykinin receptor 3 (TACR3)	4q24	162332
Tripartite motif-containing protein 32 (TRIM32)	9q33.1	602290
Tetratricopeptide repeat domain-containing protein 8 (TTC8)	14q31.3	608132
WD repeat-containing protein 11 (WDR11)	10q26.12	606417
Wingless-type mmtv integration site family, member 5A (WNT5A)	3p14.3	164975
WW domain-containing oxidoreductase (WWOX)	16q23.1-q23.2	605131

Supplementary Table 2: The clinical information of 46,XY disorders of sex development patients with different gene mutations

Gene	*Age (year) mean±s.d.*	*External genitalia*				*Gonads*	*Müllerian structures (%) (n/N)*	*LH (IU/L) mean±s.d.*	*FSH (IU/L) mean±s.d.*	*T (ng ml^{-1}) mean±s.d.*	*E2 (pg ml^{-1}), mean±s.d.*
		Female (%)	*Cli (%)*	*Amb (%)*	*Hyp (%)*						
AR (*n*=21)	17.1±14.9	76.2			23.8	Testes	11.1 (1/9)	13.7±11.3	30.1±30.9	2.4±3.5	22.8±30.3
SRD5A2 (*n*=13)	8.9±7.2	23.1	7.7	38.5	30.8	Testes	16.6 (1/6)	5.4±6.1	6.5±7.9	2.3±2.8	18.7±15.0
NR5A1 (*n*=13)	14.2±8.1	38.5	30.8	23.1	7.7	Testes	37.5 (3/8)	14.2±14.6	48.0±41.9	0.8±1.1	15.9±9.3
CYP17A1 (*n*=3)	18.5±5.6				100.0	Testes	0.0 (0/1)	20.8±8.1	12.9±3.6	1.1±0.2	32.3±29.3
HSD17B3 (*n*=3)	14.6±12.2	33.3	33.3	33.3		Testes	100.0 (1/1)	10.9±9.7	15.6±15.9	2.9±2.6	20.2±18.0
SRY (*n*=3)	24.3±2.1	66.7	33.3			Ovaries	100.0 (3/3)	38.4±15.6	82.9±21.6	0.6±0.6	24.2±6.3
LHCGR (*n*=2)	38.0	100.0				Testes	0.0 (0/1)	27.2	41.9	0.5	30.5
MAP3K1 (*n*=2)	18.5±0.7	100.0				Ovotestes	50.0 (1/2)	21.4±8.5	45.5±22.7	0.4±0.2	24.1±7.1

Cli: clitoromegaly; Amb: ambiguous; Hyp: hypospadias; s.d.: standard deviation; *SRD5A2*: steroid 5 α-reductase 2; *NR5A1*: nuclear receptor subfamily 5, Group A, member 1; *CYP17A1*: cytochrome P450, family 17, subfamily A, polypeptide 1; *HSD17B3*: 17β-Hydroxysteroid dehydrogenase III; *SRY*: Sex-determining region Y; *LHCGR*: Luteinizing hormone/choriogonadotropin receptor; *MAP3K1*: mitogen-activated protein kinase kinase kinase 1; AR: androgen receptor; LH: luteinizing hormone; FSH: follicle-stimulating hormone

Supplementary Table 3: The detail information of variants detected in 46,XY disorders of sex development patients

Patient	*Age*	*Sex*	*Gene*	*Variants*	*AA change*	*Genotype*		*ACMG*
2013-002	15	Male	*CYP17A1*	c.985delTACinsAA		Heterozygote	Reported	Pathogenic
				c.1263G>A	p.Ala421Ala	Heterozygote	Reported	Pathogenic
2013-003	14	Female	*AR*	c.2696T>C	p.Ile899Thr	Hemizygous	Reported	Likely pathogenic
2013-004	2.5	Female	*SRD5A2*	c.16C>T	p.Gln6*	Homozygous	Reported	Pathogenic
2013-005	11	Female	*NR5A1*	c.1075_1089dupCTTGCGCTGCAGCTG	p.Leu363_Asp364insLeuAlaLeuGlnLeu	Heterozygote	Reported	VUS
2013-007	29	Female	*NR5A1*	c.763_764insCACCAAAG	p.Arg255Profs*44	Heterozygote	Novel	Likely pathogenic
2013-009	25	Female	*AR*	c.2328G>T	p.Met776Ile	Hemizygous	Novel	VUS
2013-010	11	Female	*AR*	c.2567G>A	p.Arg856His	Hemizygous	Reported	Pathogenic
2013-011	6	Female	*AR*	c.2522G>A	p.Arg841His	Hemizygous	Reported	Pathogenic
2013-012	16	Female	*NR5A1*	c.1083delG	p.Gln362Serfs*20	Heterozygote	Novel	VUS
2013-014	1	Female	*AR*	c.2740C>T	p.Pro914Ser	Hemizygous	Reported	Likely pathogenic
2013-016	25	Female	*SRY*	c.226C>T	p.Arg76Cys	Hemizygous	Novel	VUS
2013-017	16	Female	*AR*	c.2168T>C	p.Leu723Ser	Hemizygous	Novel	VUS
2013-018	15	Female	*SRD5A2*	c.211C>T	p.Gln71*	Homozygous	Novel	VUS
2013-019	35	Female	*AR*	c.2301delT	p.Asp768Ilefs*21	Hemizygous	Reported	Pathogenic
2013-020	2.2	Female	*SRD5A2*	c.16C>T	p.Gln6*	Homozygous	Reported	Pathogenic
2013-021	15	Female	*SRD5A2*	c.607G>A	p.Gly203Ser	Heterozygote	Reported	Pathogenic
				c.239_240insT	p.Thr81Aspfs*55	Heterozygote	Novel	Likely pathogenic
2013-022	11	Male	*AR*	c.2344T>A	p.Tyr782Asn	Hemizygous	Novel	VUS
2013-023	8	Male	*AR*	c.2344T>A	p.Tyr782Asn	Hemizygous	Novel	VUS
2013-024	10	Female	*SRD5A2*	c.607G>A	p.Gly203Ser	Heterozygote	Reported	Likely pathogenic
				c.16C>T	p.Gln6*	Heterozygote	Reported	Pathogenic
2013-025	22	Female	*SRY*	c.103_106delCTTT	p.Leu35Alafs*25	Hemizygous	Novel	Likely pathogenic
2013-027	16	Female	*NR5A1*	c.244G>T	p.Ala82Ser	Heterozygote	Novel	Pathogenic
2013-029	2.5	Male	*SRD5A2*	c.100G>C	p.Gly34Arg	Heterozygote	Reported	Pathogenic
				c.16C>T	p.Gln6*	Heterozygote	Reported	Pathogenic
2013-030	3	Female	*AR*	c.2087A>T	p.Asp696Val	Hemizygous	Reported	VUS
2013-031	12	Female	*NR5A1*	c.267G>T	p.Arg89Ser	Heterozygote	Novel	VUS
2013-032	0.8	Female	*NR5A1*	c.62C>T	p.Ser21Phe	Heterozygote	Novel	VUS
2013-033	2	Female	*AR*	c.1035_1038delGTCT	p.Leu347Thrfs*131	Hemizygous	Reported	Likely pathogenic
2013-034	4	Female	*HSD17B3*	c.74_75delTG	p.Val25Glufs*54	Heterozygote	Novel	Likely pathogenic
				del exon 1		Heterozygote	Novel	Likely pathogenic
2013-036	28	Male	*HSD17B3*	c.179T>C	p.Ile60Thr	Heterozygote	Novel	VUS
				del exon 1		Heterozygote	Novel	Likely pathogenic
2013-037	12	Male	*HSD17B3*	c.179T>C	p.Ile60Thr	Homozygous	Novel	VUS
2015-001	14	Female	*NR5A1*	c.250C>T	p.Arg84Cys	Heterozygote	Reported	Pathogenic
2015-002	25	Female	*NR5A1*	c.104G>A	p.Gly35Asp	Heterozygote	Reported	Pathogenic
2015-003	2.5	Male	*AR*	c.2522G>A	p.Arg841His	Hemizygous	Reported	Pathogenic
2015-005	9	Female	*NR5A1*	c.95G>A	p.Ser32Asn	Heterozygote	Reported	Pathogenic
2015-010	26	Female	*SRY*	c.392C>A	p.Pro131His	Hemizygous	Novel	VUS
2015-011	8	Male	*NR5A1*	c.272G>A	p.Gly91Asp	Heterozygote	Reported	Pathogenic
2015-012	19	Female	*MAP3K1*	c.1985T>C	p.Leu662Pro	Heterozygote	Novel	VUS
2015-013	25	Male	*CYP17A1*	c.985delTACinsAA		Heterozygote	Reported	Pathogenic
				c.1343C>T	p.Ala448Val	Heterozygote	Novel	VUS
2015-014	46	Male	*AR*	c.1823G>A	p.Arg608Gln	Hemizygous	Reported	Pathogenic
2015-015	56	Male	*AR*	c.1823G>A	p.Arg608Gln	Hemizygous	Reported	Pathogenic
2015-016	24	Male	*AR*	c.528C>A	p.Ser176Arg	Hemizygous	Reported	VUS
2015-018	6	Female	*NR5A1*	c.132_134delCAA	p.Asn44del	Heterozygote	Reported	Pathogenic
2015-019	13	Female	*SRD5A2*	c.16C>T	p.Gln6*	Heterozygote	Reported	Pathogenic
				del exon 2		Heterozygote	Reported	Likely pathogenic
2015-020	22	Male	*SRD5A2*	c.16C>T	p.Gln6*	Heterozygote	Reported	Pathogenic
				c.737G>A	p.Arg246Gln	Heterozygote	Reported	Pathogenic
2015-021	1	Male	*SRD5A2*	c.680G>A	p.Arg227Gln	Heterozygote	Reported	Pathogenic
				c.211C>T	p.Gln71*	Heterozygote	Novel	VUS
2015-022	22	Female	*AR*	c.2522G>A	p.Arg841His	Hemizygous	Reported	Pathogenic
2015-023	1.3	Female	*AR*	c.1213C>T	p.Gln405*	Hemizygous	Reported	Pathogenic

Contd...

Supplementary Table 3: Contd...

Patient	*Age*	*Sex*	*Gene*	*Variants*	*AA change*	*Genotype*		*ACMG*
2015-025		Female	*LHCGR*	c.458T>C	p.Leu153Pro	Heterozygote	Novel	VUS
				c.437C>G	p.Ser146Cys	Heterozygote	Novel	VUS
2015-028	25	Female	*NR5A1*	c.699_700insCTGCAGCTG	p.Leu233_Glu234insLeuGlnLeu	Heterozygote	Reported	Pathogenic
2015-031	1.2	Female	*SRD5A2*	c.737G>A	p.Arg246Gln	Homozygous	Reported	Pathogenic
2015-032	23	Female	*AR*	c.2248A>G	p.Met750Val	Hemizygous	Reported	Likely pathogenic
2018-001	12.1	Female	*AR*	c.2608A>T	p.Ile870Phe	Hemizygous	Novel	VUS
2018-004	13.5	Female	*SRD5A2*	c.16C>T	p.Gln6*	Homozygous	Reported	Pathogenic
2018-005	18	Female	*MAP3K1*	c.629C>T	p.Pro210Leu	Heterozygote	Novel	VUS
2018-007	12.3	Female	*AR*	c.2069_2071del	p. 690_691del	Hemizygous	Reported	Pathogenic
2018-008	15.3	Female	*SRD5A2*	c.16C>T	p.Gln6*	Homozygous	Reported	Pathogenic
2018-009	28	Female	*AR*	c.1847G>A	p.Arg616His	Hemizygous	Reported	Pathogenic
2018-015	12.5	Female	*NR5A1*	c.133A>G	p.Lys45Glu	Heterozygote	Novel	VUS
2018-017	15.5	Male	*CYP17A1*	c.1263G>A	p.Ala421Ala	Heterozygote	Reported	Pathogenic
				c.437-1G>C		Heterozygote	Novel	Pathogenic
2018-018	2	Female	*SRD5A2*	c.16C>T	p.Gln6*	Heterozygote	Reported	Pathogenic
				c.548-1G>A		Heterozygote	Reported	Pathogenic
2018-020	38	Female	*LHCGR*	c.988G>A	p.Asp330Asn	Homozygous	Novel	VUS

ACMG: American College of Medical Genetics and Genomics; VUS: variants of uncertain clinical significance; *LHCGR*: luteinizing hormone/choriogonadotropin receptor; AR: androgen receptor; *SRD5A2*: steroid 5 α-reductase 2; *NR5A1*: nuclear receptor subfamily 5, Group A, member 1; *CYP17A1*: cytochrome P450, family 17, subfamily A, polypeptide 1; *MAP3K1*: mitogen-activated protein kinase kinase kinase 1; *HSD17B3*: 17β-Hydroxysteroid dehydrogenase III

Supplementary Table 4: The detail clinical information detected in 46,XY disorders of sex development patients

Patient	*Age*	*Sex*	*External genitalia*	*Gonads (left/right)*	*Müllerian structures*	*External Genitalia (puberty)*	*Tanner stage*
2013-002	15	Male	Hypospadias	Testes (labioscrotal)	Absent	Penis, testes enlarged	B3P2
2013-003	14	Female	Female	Testes (inguinal)	Absent	Clitoris further enlarged	B1P1
2013-004	2.5	Female	Ambiguous	Testes (inguinal)	-		
2013-005	11	Female	Clitoromegaly	-	-	Clitoris further enlarged	-
2013-007	29	Female	Female	Testes (abdominal)	Uterus	Female	B3P4
2013-009	25	Female	Female	Testes (inguinal)	Absent	Female	B5P2
2013-010	11	Female	Female	Testes (labioscrotal)	Absent	Clitoris further enlarged	B1P1
2013-011	6	Female	Female	Testes (labioscrotal)	-		
2013-012	16	Female	Female	Testes (abdominal)	Uterus	Female	B3P4
2013-014	1	Female	Female	Testes (labioscrotal)	-		
2013-016	25	Female	Female	-	Uterus	Female	B1P1
2013-017	16	Female	Female	Testes (inguinal/abdominal)	Absent	Female	B5P1
2013-018	15	Female	Female	Testes (inguinal/labioscrotal)	Absent	Testes enlarged	B1P1
2013-019	35	Female	Female	Testes-	Absent	Female	B5P1
2013-020	2.2	Female	Female	Testes (labioscrotal)	-		
2013-021	15	Female	Hypospadias	Testes (labioscrotal)	Absent	Penis enlarged	B1P6
2013-022	11	Male	Hypospadias	Testes (labioscrotal)	Absent	Penis enlarged	B5P1
2013-023	8	Male	Hypospadias	-			
2013-024	10	Female	Ambiguous	Testes (labioscrotal)	Absent	Clitoris further enlarged	B1P1
2013-025	22	Female	Clitoromegaly	Ovaries (abdominal)	Uterus	Clitoris further enlarged	B3P5
2013-027	16	Female	Clitoromegaly	Testes (abdominal)	Absent	Clitoris further enlarged	B1P5
2013-029	2.5	Male	Hypospadias	Testes (labioscrotal)	-		
2013-030	3	Female	Female	Testes (inguinal)	-		
2013-031	12	Female	Ambiguous	Testes (labioscrotal)	Absent	Clitoris further enlarged	B2P3
2013-032	0.8	Female	Female	-	-		
2013-033	2	Female	Female	Testes (inguinal)	-		
2013-034	4	Female	Female	Testes (inguinal)	-		
2013-036	28	Male	Ambiguous	Testes (labioscrotal/inguinal)	-	Penis, testes enlarged	B1P5
2013-037	12	Male	Clitoromegaly	Testes (inguinal/labioscrotal)	Primordial uterus	Clitoromegaly	B1P3
2015-001	14	Female	Female	Testes (inguinal/abdominal)	Absent	Female	B1P2
2015-002	25	Female	Female	Testes (abdominal)	Uterus	Female	B1P1
2015-003	2.5	Male	Hypospadias	Testes (labioscrotal/inguinal)	-		

Contd...

Supplementary Table 4: Contd...

Patient	*Age*	*Sex*	*External genitalia*	*Gonads (left/right)*	*Müllerian structures*	*External Genitalia (puberty)*	*Tanner stage*
2015-005	9	Female	Ambiguous	Testes (inguinal/labioscrotal)	-		
2015-010	26	Female	Female	Ovaries (abdominal)	Uterus	Female	B2P1
2015-011	8	Male	Hypospadias	Testes (inguinal/labioscrotal)	-		
2015-012	19	Female	Female	-	Uterus	Female	B3P3
2015-013	25	Male	Hypospadias	-	-	-	-
2015-014	46	Male	Hypospadias	-	-	-	-
2015-015	56	Male	Hypospadias	-	-	-	-
2015-016	24	Male	Female	-	-	-	-
2015-018	6	Female	Ambiguous	Testes (labioscrotal)	-		
2015-019	13	Female	Ambiguous	Testes (inguinal)	-	Penis enlarged	B2P3
2015-020	22	Male	Hypospadias	Testes (labioscrotal)	Absent	Penis, testes enlarged	B1P4
2015-021	1	Male	Ambiguous	Testes (inguinal)	-		
2015-022	22	Female	Female	Testes (inguinal)	Absent	Female	B5P5
2015-023	1.3	Female	Female	Testes (inguinal)	-		
2015-025		Female	Female	-			
2015-028	25	Female	Clitoromegaly	Testes (inguinal)	Absent	Clitoris further enlarged	-
2015-031	1.2	Female	Hypospadias	Testes (inguinal)			
2015-032	23	Female	Female	Testes (inguinal)	-	Female	B2P3
2018-001	12.1	Female	Female	Testes (inguinal)	Absent	Female	B1P1
2018-004	13.5	Female	Ambiguous	Testes (labioscrotal)	Primordial uterus	-	B2P4
2018-005	18	Female	Female	ovotestis (abdominal)	Absent	Female	B2P2
2018-007	12.3	Female	Female	Testes (inguinal)	Primordial uterus	Female	B1P1
2018-008	15.3	Female	Clitoromegaly	Testes (inguinal)	Absent	Clitoris further enlarged	B1P4
2018-009	28	Female	Female	Testes (inguinal)	Absent	Female	B5P1
2018-015	12.5	Female	Clitoromegaly	Testes (inguinal)	Absent	Clitoris further enlarged	B1P5
2018-017	15.5	Male	Hypospadias	Testes (labioscrotal)	-	Clitoris further enlarged	B4P4
2018-018	2	Female	Female	Testes (inguinal)	-		
2018-020	38	Female	Female	Testes (inguinal)	Absent	Female	B3P1

Supplementary Table 5: Summary results of previous studies using Targeted next-generation sequencing analysis for 46,XY disorders of sex development

Year	*Country*	*Author*	*Number of 46, XY DSD patients*	*Number of genes*	*Patients with rare variants (%)*	*Diagnostic Rate[a] (%)*	*AR (%)*	*SRD5A2 (%)*	*NR5A1 (%)*
2013	America	Arboleda *et al.*[20]	5	35	40.0	0.0	0.0	50.0	0.0
2015	America	Baxter *et al.*[25]	40	64	50.0	35.0	7.1	0.0	7.1
2016	China	Dong *et al.*[24]	13	219	69.2	46.2	55.6	0.0	0.0
2016	Australia	Eggers *et al.*[23]	278	1031	57.2	42.4	23.9	12.8	13.7
2017	Korea	Kim *et al.*[8]	37	67	35.1	24.3	33.3	0.0	11.1
2017	Turkey	Ozen *et al.*[22]	20	2761	45.0	45.0	0.0	0.0	0.0
2018	China	Wang *et al.*[21]	80	70	74.3	42.9	26.7	23.3	33.3
2019	UK	Hughes *et al.*[7]	73	30	45.2	34.2	28.0	16.0	8.0
2019	This study		87	83	69.0	42.5	35.0	21.7	21.7

[a]Based on the pathogenic and the likely pathogenic variants. DSD: disorders of sex development; AR: androgen receptor; SRD5A2: steroid 5 α-reductase 2; *NR5A1*: nuclear receptor subfamily 5, Group A, member 1