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

Bing-Qing Yu, Zhao-Xiang Liu, Yin-Jie Gao, Xi Wang, Jiang-Feng Mao, Min Nie, Xue-Yan Wu

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.

Asian Journal of Andrology (2021) 23, 69–73; doi: 10.4103/aja.aja_36_20; published online: 25 September 2020

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.\(^6\) 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 (ZFP2), chromobox 2 (CBX2), sex-determining region Y (SRY), SRY-box 9 (SOX9), mitogen-activated protein 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 oxido-reductase (POR), cytochrome b5 type A (CYB5A), 3β-hydroxysteroid dehydrogenase 2 (HSD3B2), anti-Müllerian hormone (AMH), anti-Müllerian hormone type II
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 in specific pubertal stages. During the prepubertal 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 deeper12 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 (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 | |
| Prepuberty, n/total (%) | 18/58 (31.0) |
| Puberty, n/total (%) | 40/58 (69.0) |

* One patient with missing clinical data of age; ** 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; MAP2K1: 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 (0.0–0.7) | 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

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%. 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. 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% (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. Previous studies have demonstrated that
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 Chinese21 and a Korean study4 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.12,21 This may be related to the higher consanguineous rates in other countries compared to China.21 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. ZXH, 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.

ACKNOWLEDGMENTS
This work was supported by the National Natural Science Foundation of China (No. 81971375 and No. 81771576), the National Key Research and Development Program of China (2016YFC0905100), the CAMS Innovation Fund for Medical Sciences (2016-I2M-1-002), and the Nonprofit Central Research Institute Fund of the Chinese Academy of Medical Sciences (No. 2017PT32020 and No. 2018PT32001).

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

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

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| 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 (MAML1)                         | Xq28     | 300120   |
| Mitogen-activated protein 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)                                  | 17q24.3-25.1 | 608160 |
| **Disorders of hormone synthesis or action**                        |          |          |
| Anti- Müllerian hormone (AMH)                                       | Yp11.2   | 480000   |
| Anti-Müllerian hormone type II receptor (AMHR2)                     | 6q22.1   | 604714   |
| Androgen receptor (AR)                                              | 1p36.12  | 603490   |
| Cytochrome b5, type A (CYB5A)                                       | 11p13    | 607102   |
| Cytochrome P450, subfamily A, polypeptide 1 (CYP11A1)               | 8q24.3   | 604809   |
| Cytochrome P450, subfamily B, polypeptide 1 (CYP11B1)               | 15q24.1  | 118485   |
| Cytochrome P450, subfamily B, polypeptide 2 (CYP11B2)               | 8q24.3   | 610613   |
| 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  | 608845   |
| 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     |
|---------------------------------------------------|-----------|----------|
| Spermatogenesis                                   |           |          |
| 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    | 300836   |
| 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   |
| Prokinecin 2 (PROK2)                              | 3p13      | 607002   |
| Prokinecin 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   | 162332   |
| Tachykinin receptor 3 (TACR3)                    | 4q24      | 162332   |
| Tripartite motif-containing protein 32 (TRIM32)   | 9q33.1    | 602290   |
| Tetrapecptide repeat domain-containing protein 8 (TTC8) | 14q31.3   | 608132   |
| WD repeat-containing protein 11 (WDR11)          | 10q26.12  | 606417   |
| Wingless-type mmjv 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. |
|----------|----------------------|------------------------|--------|-------------------------------|---------------------|----------------------|-----------------------|------------------------|
| 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    | 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   | 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   | 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±9.7            | 100.0                  |        | Ovotestes 50.0 (2/2)           | 21.4±8.5            | 45.5±22.7            | 0.4±0.2               | 24.1±7.1               |

Clit: clitoromegaly; Amb: ambiguous; Hyp: hypoplasia; s.d.: standard deviation; SRD5A2: steroid 5α-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 | p.Ala421ala | Heterozygote | Reported | Pathogenic |
| 2013-003 | 14 | Female | AR | c.2696T>C | p.Ile899Thr | Heterozygous | Reported | Likely pathogenic |
| 2013-004 | 2.5 | Female | SRD5A2 | c.16C>T | p.Gln6 | Heterozygous | Reported | Pathogenic |
| 2013-005 | 11 | Female | NR5A1 | c.1075_1089delCTTG | p.Leu363_Asp364insLeuAlaValGlu | Heterozygous | Reported | VUS |
| 2013-007 | 29 | Female | NR5A1 | c.763_764insCACCAAG | p.Arg255Profs*44 | Heterozygous | Novel | Likely pathogenic |
| 2013-009 | 25 | Female | AR | c.2328G>T | p.Met776Ile | Hemizygous | Reported | Pathogenic |
| 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.Leu363_Asp364insLeuAla | Heterozygous | Reported | VUS |
| 2013-014 | 1 | Male | SRY | c.2740C>T | p.Gln362Serfs | Hemizygous | Reported | Likely pathogenic |
| 2013-016 | 25 | Female | AR | c.226C>T | p.Arg76G | 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 | Heterozygous | 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 | Heterozygous | Reported | Pathogenic |
| 2013-021 | 15 | Female | SRD5A2 | c.607G>A | p.Gly203Ser | Heterozygous | Reported | Pathogenic |
| 2013-022 | 11 | Male | AR | c.2344T>A | p.Thr782Asn | Hemizygous | Novel | VUS |
| 2013-023 | 8 | Male | AR | c.2344T>A | p.Thr782Asn | Hemizygous | Novel | VUS |
| 2013-024 | 10 | Female | SRD5A2 | c.607G>A | p.Gly203Ser | Heterozygous | Reported | Likely 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 | Heterozygous | Novel | Pathogenic |
| 2013-029 | 2.5 | Male | SRD5A2 | c.100G>C | p.Gly34Arg | Heterozygous | Reported | Pathogenic |
| 2013-030 | 3 | Female | AR | c.2087A>T | p.Gln6 | Heterozygous | Reported | Pathogenic |
| 2013-031 | 12 | Female | NR5A1 | c.267G>T | p.Arg93Ser | Heterozygous | Novel | VUS |
| 2013-032 | 0.8 | Female | NR5A1 | c.62C>T | p.Ile21Ilefs*131 | Heterozygous | Novel | VUS |
| 2013-033 | 2 | Female | AR | c.1035_1038delCTTT | p.Leu347Thrfs*131 | Hemizygous | Reported | Likely pathogenic |
| 2013-034 | 4 | Female | HSD17B3 | c.74_75delTG | p.Val229Leufs*54 | Heterozygous | Novel | Likely pathogenic |
| 2013-036 | 28 | Male | HSD17B3 | c.179T>C | p.Ile60Thr | Heterozygous | Novel | VUS |
| 2013-037 | 12 | Male | HSD17B3 | c.179T>C | p.Ile60Thr | Homozygous | Novel | VUS |
| 2015-001 | 14 | Female | NR5A1 | c.250C>T | p.Arg84Cys | Heterozygous | Reported | Pathogenic |
| 2015-002 | 25 | Female | NR5A1 | c.104G>A | p.Gly35Asp | Heterozygous | Reported | Pathogenic |
| 2015-003 | 2.5 | Male | AR | c.2522G>A | p.Arg841His | Hemizygous | Reported | Pathogenic |
| 2015-005 | 9 | Female | NR5A1 | c.950G>A | p.Ser32Asn | Heterozygous | Reported | Pathogenic |
| 2015-010 | 26 | Male | SRY | c.392C>A | p.Pro131His | Hemizygous | Novel | VUS |
| 2015-011 | 8 | Male | NR5A1 | c.272G>A | p.Gly91Asp | Heterozygous | Reported | Pathogenic |
| 2015-012 | 19 | Female | MAP3K1 | c.1985T>C | p.Leu662Pro | Heterozygous | Novel | VUS |
| 2015-013 | 25 | Male | CYP17A1 | c.985delTACinsAA | p.Ala421ala | Heterozygous | Reported | Pathogenic |
| 2015-014 | 46 | Male | AR | c.1823G>A | p.Ala608Gln | Hemizygous | Reported | Pathogenic |
| 2015-015 | 56 | Male | AR | c.1823G>A | p.Ala608Gln | 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 | Heterozygous | Reported | Pathogenic |
| 2015-019 | 13 | Female | SRD5A2 | c.16C>T | p.Gln6 | Heterozygous | Reported | Pathogenic |
| 2015-020 | 22 | Male | SRD5A2 | c.16C>T | p.Gln6 | Heterozygous | Reported | Pathogenic |
| 2015-021 | 1 | Male | SRD5A2 | c.737G>A | p.Ala246Glu | Hemizygous | Reported | Pathogenic |
| 2015-022 | 22 | Female | AR | c.2522G>A | p.Arg841His | Hemizygous | Reported | Pathogenic |
| 2015-023 | 1.3 | Female | AR | c.1213C>T | p.Glu405 | Hemizygous | Reported | Pathogenic |

Contd...
### Supplementary Table 3: Contd...

| Patient  | Age  | Sex  | Gene                | Variants               | AA change         | Genotype      | ACMG     |
|----------|------|------|---------------------|------------------------|-------------------|---------------|----------|
| 2015-025 | 15   | Male | LHCGR               | c.437C>G               | p.Leu153Pro       | Heterozygote  | Novel    |
| 2015-028 | 25   | Female | NR5A1              | c.699_700insCTGCAGCTG | p.Leu233_Glu234insLeuGlnLeu | Heterozygote  | Reported  |
| 2015-031 | 1.2  | Female | SRD5A2             | c.737G>A               | p.Arg246Gln       | Hemizygous    | Reported  |
| 2015-032 | 23   | Female | AR                 | c.2248A>G              | p.Met750Val       | Homozygous    | Reported  |
| 2018-001 | 12.1 | Female | AR                | c.2608A>T              | p.Ile870Phe       | Hemizygous    | Novel    |
| 2018-004 | 13.5 | Female | SRD5A2             | c.16C>T                | p.Gln6'           | Homozygous    | Reported  |
| 2018-005 | 18   | Female | MAP3K1             | c.629C>T               | p.Pro210Leu       | Heterozygote  | Novel    |
| 2018-007 | 12.3 | Female | AR                | c.2069_2071del         | 690_691del        | Hemizygous    | Reported  |
| 2018-008 | 15.3 | Female | SRD5A2             | c.16C>T                | p.Gln6'           | Homozygous    | Reported  |
| 2018-009 | 28   | Female | AR                 | c.1847G>A              | p.Arg616His       | Hemizygous    | Reported  |
| 2018-015 | 12.5 | Female | NR5A1             | c.133A>G               | p.Lys45Glu        | Heterozygote  | Novel    |
| 2018-017 | 15.5 | Male  | CYP17A1            | c.1263G>A              | p.Ala421Ala       | Heterozygote  | Reported  |
| 2018-018 | 2    | Female | SRD5A2             | c.16C>T                | p.Gln6'           | Heterozygote  | Novel    |
| 2018-020 | 38   | Female | LHCGR             | c.988G>A               | p.Asp330Asn       | Heterozygote  | Novel    |

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 a-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: 17j-Hydroxysteroid dehydrogenase III

### Supplementary Table 4: The detailed 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   | Male | Female             | Testes (inguinal)   | Absent               | Clitoris further enlarged    | B1P1         |
| 2013-004 | 2.5  | Female | Ambiguous         | Testes (inguinal)  | -                    | -                           | -            |
| 2013-005 | 11   | Male  | 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)| -                    | -                           | -            |
| 2013-015 | 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 (inguinal)  | Absent               | Female                      | B5P1         |
| 2013-020 | 2.2  | Female | Female           | Testes (labioscrotal)| -                    | -                           | -            |
| 2013-021 | 15   | Female | Female           | 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 | Female           | Testes (labioscrotal)| Absent               | Clitoris further enlarged    | B1P1         |
| 2013-025 | 22   | Female | Female           | Ovaries (abdominal) | Uterus               | Clitoris further enlarged    | B3P5         |
| 2013-027 | 16   | Female | Female           | 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 | Female           | Ambiguous (left/right)| 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                       | B2P3         |
| 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                       | B2P4         |
| 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 (%) | AR (%) | SRD5A2 (%) | NR5A1 (%) |
|------|---------|-----------------|-------------------------------|-----------------|--------------------------------|---------------------|--------|------------|-----------|
| 2013 | America | Arboleda et al. | 5                             | 35              | 40.0                           | 0.0                 | 0.0    | 50.0       | 0.0       |
| 2015 | America | Baxter et al.   | 40                            | 64              | 50.0                           | 35.0                | 7.1    | 0.0        | 7.1       |
| 2016 | China   | Dong et al.     | 13                            | 219             | 69.2                           | 46.2                | 55.6   | 0.0        | 0.0       |
| 2016 | Australia| Eggers et al.  | 278                           | 1031            | 57.2                           | 42.4                | 23.9   | 12.8       | 13.7      |
| 2017 | Korea   | Kim et al.      | 37                            | 67              | 35.1                           | 24.3                | 33.3   | 0.0        | 11.1      |
| 2017 | Turkey  | Ozen et al.     | 20                            | 2761            | 45.0                           | 45.0                | 0.0    | 0.0        | 0.0       |
| 2018 | China   | Wang et al.     | 80                            | 70              | 74.3                           | 42.9                | 26.7   | 23.3       | 33.3      |
| 2019 | UK      | Hughes et al.   | 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      |

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