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Identification of gene variants in 130 Han Chinese patients with hypospadias by targeted next-generation sequencing

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Abstract

Background: Hypospadias is a common congenital malformation of male external genitalia, which mainly manifests as an abnormal urethral opening on the ventral side of the penis. The etiology and clinical phenotype of hypospadias is highly heterogeneous, and its clinical diagnosis is challenging. Currently, over 70% of patients have an unknown etiology. Here, we performed a targeted analysis of gene mutations in 130 patients with hypospadias of unknown etiology to find the precise genetic cause.

Methods: We developed a targeted next-generation sequencing (NGS) panel, encompassing the exon coding regions of 105 genes involved in external genitalia and urogenital tract development and performed sequencing analysis on 130 children with hypospadias of unknown etiology.

Results: In total, 25 patients with hypospadias (19.2%) were found to have 20 mutations among the nine genes involved in external genitalia and urogenital tract development, including 16 reported and four novel mutation sites. Twenty-two patients (16.9%) had diagnostic variants. Multiple genetic mutations were identified in three of the 25 patients. Hypospadias combined with micropenis was the most common phenotype (68%) in 25 patients.

Conclusions: Higher frequency mutations were identified in *SRD5A2* (52%) and *AR* (24%) in our patient cohort. Middle or posterior hypospadias with micropenis may be significant indicators of genetic variations. Polygenic inheritance may be a rare genetic cause of hypospadias.

KEYWORDS
candidate gene, hypospadias, next-generation sequencing, pathogenic genes

Wanyu Zhang and Jinxiu Shi contributed equally to this work and share the first authorship.

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1 | INTRODUCTION

Hypospadias is a condition in which the opening of the urethra is found on the ventral surface of the penis, and is one of the most common congenital malformations. The prevalence of hypospadias varies greatly, ranging from 1 in 125 to 250 male live births in different countries (Singh et al., 2018). It may occur as an isolated defect or may be associated with other genital malformations (e.g., micropenis, cryptorchidism in one or both sides, and bifid scrotum) or with other organ malformations. According to the severity and the location of the urethral opening, hypospadias is often classified as anterior, middle, or posterior (van der Zanden et al., 2012). External genital malformations, as physical indicators of maleness, can have different physiological and psychological effects on children. Early surgical treatment is needed, and the different causes of hypospadias have different surgical requirements. Therefore, it is very necessary to identify the precise cause of hypospadias.

The etiology of hypospadias is complicated, and is thought to be related to genetic, endocrine, and environmental factors (Shih & Graham, 2014). To date, monogenic and chromosomal causes of hypospadias account for about 30% of all cases, indicating the significant role of genetics in the development of hypospadias (Sagodi, Kiss, Kiss-Toth, & Barkai, 2014). In addition, hypospadias has been described in over 200 syndromes (Blaschko, Cunha, & Baskin, 2012). There are three main molecular features of male external genitalia formation: (a) gonadal differentiation, (b) gonadal development, and (c) dependence on endocrine and environmental factors. In the early stages of genital tubercle development, a gonadal differentiation pathway is among the activated pathways, and relies on HOX signaling, WNT/β-catenin signaling, Hedgehog signaling, and bone morphogenetic protein (*BMP*) signaling (Y. Chen et al., 2018; Kojima, Kohri, & Hayashi, 2010). Other genes have been reported to be associated with hypospadias including sex-determining region Y (*SRY*) (OMIM 480,000), homeobox D13 (*HOXD13*) (OMIM 142,989), friend of *GATA2* (*FOG2*) (OMIM 603,693), and GATA-type zinc finger protein 4 (*GATA4*) (OMIM 600,576) (Brauner et al., 2016; Carmichael et al., 2013). Moreover, steroid-5-α-reductase (*SRD5A*) (OMIM 607,306) converts testosterone into dihydrotestosterone (DHT), which binds to the androgen receptor (*AR*) (OMIM 313,700) and thereby stimulates the formation of the external genitalia.

The etiology of hypospadias in most patients remains unknown, indicating that there are new candidate genes that have not yet been discovered. The aim of this study was to identify the pathogenic genes responsible for hypospadias in a cohort of 130 patients using next-generation sequencing (NGS) of a targeted gene panel. The gene panel contained 105 hypospadias-associated genes, some of which were not previously reported in patients but were implicated due to their known roles in gonadal and urethral development in male mice.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was ratified by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Informed consent from the parents or guardians of the subjects was obtained for all examinations.

2.2 | Patients

From May 2017 to March 2018, a total of 130 Chinese patients of age 6 months to 18 years old with variable degrees of hypospadias were enrolled in our cohort to screen for pathogenic genes. All patients were raised as males and had a 46, XY karyotype and SRY gene. Certain patients had hypospadias accompanied by additional genitourinary abnormalities including micropenis, inguinal hernia, unilateral or bilateral cryptorchidism, or bifid scrotum. All patients were being examined at Ruijin Hospital and Renjin Hospital affiliated with Shanghai Jiaotong University School of Medicine.

The clinical evaluation of the enrolled patients included chief complaints, history of present illness, birth history, personal history, family history, maternal pregnancy history, previous operation history, hormone therapy history, living environment, and history of contact with environmental pollutants. Physical examination involved measurement of height, weight, extended penis length, testicular volume size, and male genital evaluation.

Laboratory and imaging examinations included chromosome karyotype analysis and detection of *SRY* fragments, and measurement of blood biochemical and endocrine-related hormone levels (luteinizing hormone, follicle-stimulating hormone, testosterone, DHT, inhibin B, etc.). Gonadotropin-releasing hormone and human chorionic gonadotropin stimulation tests and gonadal ultrasound were performed in some of the patients.

Informed consent was obtained from each participating individual or their parents. This study was approved by the Ethics Committee of Ruijin Hospital.

2.3 | Targeted gene panel

Our hypospadias panel included 105 genes (Table 1) involved in gonadal development, gonadal differentiation, and/or specific syndromes. The targeted genes were obtained by referencing the GeneCards database and by entering the keywords "hypospadias," "disorders of sex development," or "gonad genesis" in PubMed.

TABLE 1 List of 105 genes in gene panel

Gonadal differentiation	HOX signaling	HOXA4, HOXA13, HOXB6, HOXD13
	Hedgehog signaling	DHH, GLI3, SHH
	FGF signaling	FGF8, FGF10, FGFR1, FGFR2, FGFR3
	SOX signaling	SOX2, SOX3, SOX9, SOX10
	BMP signaling	BMP4, BMP7
	Other	CBX2, CHD7, DGKK, DMRT1, GATA4, GATA5, MAP3K1, NR0B1, NR5A1, RSPO1, SRY, Wnt5a, WT1, ZFPM2
Gonadal development	Androgen-related	AR, FKBP4, FOXA1, FLNA, HSD17B3, HSD3B2, SRD5A2
	Estrogen-related	ATF3, CTGF, ESR1, ESR2, ZEB2
	Adrenal insufficiency	CYP11A1
	Other	AKR1C2, AKR1C4, AMH, ARX, ATRX, CYB5A, LHCGR, MAMLD1, POR, STAR
Central causes of hypogonadism	GNRHR, KAL1, LHX3, PROKR2, PROP1, TAC3, WDR11	
Toxin metabolism	CYP1A1, GSTM1, GSTT1	
Syndrome	KANSL1, MID1, MID2, SPECC1L	
Other	BBOX1, BNC2, CUL4B, FOXC1, FRAS1, GPC3, HBQ1, HYSP3, HYSP4, IGF2, INSL3, IRF6, KIF7, LHFPL5, LHX9, LIG4, LMNA, MECP2, MECP2, NLGN4X, PITX2, PCNT, PAX2, PAX6, RAD21, RPL35A, SRCAP, SNAP29, SETD5, SALL1, TRIM17, TP63, TMLHE, TDRD7, TBX22, WWOX	

2.4 | Next-generation sequencing

Genomic DNA was extracted from the patients' peripheral blood lymphocytes using a Qiagen kit according to the manufacturer's protocol. Total genomic DNA was sequenced using our targeted panel covering 105 relevant genes. Eight hundred nanograms of genomic DNA was randomly fragmented into 150–250 bp fragments using a Biorupter, and DNA libraries were prepared using the KAPA LTP Library Preparation kit and PCR amplification of the fragmented DNA. iGeneTech was used to synthesize the oligo pool containing 105 gene coding regions, which were then PCR amplified and transcribed to synthesize RNA probes. The sample DNA library was then hybridized with the RNA probes for 24 hr at 65°C and the target fragment was captured by binding to the specific probe after PCR amplification. The final products were sequenced using the Illumina-MiSeq sequencing platform.

2.5 | Data analysis and assessment of variants

Variants were checked for quality and depth, then filtered to include those with <1% minor allele frequency according to the GnomAD (East Asian). The NGS results were compared with the human genome reference sequence for single nucleotide polymorphism (SNP) and InDel annotation analysis. Mutations predicted to disrupt protein structure were identified using in silico tools, such as Mutation Taster, Polyphen-2, and SIFT. The ACMG standards and reference to the NCBI, ClinVar, and HGMD databases were used to classify the mutations into five categories: pathogenic, likely pathogenic (LP), variants of uncertain significance (VUS), likely benign, or benign (Amendola et al., 2016). Mutations were verified by Sanger sequencing. Finally, we analyzed the correlations between clinical phenotypes and genotypes.

3 | RESULTS

3.1 | Genetic variants identified by NGS

The average coverage of samples was 55.31X-2800X. Each variant labeled PASS had a quality value, ranging from 30.77 to 211,299. In total, 25 of 130 participants (19.2%, 25/130) carried genetic variants in the targeted genes. We identified 20 nonsynonymous single-nucleotide variants (SNVs) in nine different genes, including 16 reported and four novel mutational sites. The most commonly mutated gene in our study was *SRD5A2* (52%, 13/25), followed by *AR* (24%, 6/25) and *PROKR2* (12%, 3/25) (OMIM 607,123). Three patients in our hypospadias cohort had variants in multiple targeted genes (*PROKR2/SRD5A2*, *PROKR2/AR*, *AR/TRIM17*). These observations contribute to the hypothesis that polygenic inheritance is a rare genetic cause of hypospadias. Moreover, a variant in *TRIM17* (OMIM 606,123) was identified for the first time in hypospadias patients. The genetic variants of all patients are shown in Table 2.

TABLE 2 Genetic variants identified by targeted NGS

ID	Gene	Transcript	cDNA	Protein	Exon	GnomAD freq	Genotype	Origin	ACMG
P1	SRD5A2	NM_000348.3	c.G680A	p.R227Q	E4	0.006427	Compound	Mother	P/PS3 PM1 PM2 PM3 PP4
			c.C16T	p.Q6X	E1	0.000175	Het	Father	P/PVS1 PM1 PM2 PP3 PP4
P2	SRD5A2	NM_000348.3	c.G680A	p.R227Q	E4	0.006	Compound	Mother	P/PS3 PM1 PM2 PM3 PP4
			c.C16T	p.Q6X	E1	0.000175	Het	Father	P/PVS1 PM1 PM2 PP3 PP4
P3	SRD5A2	NM_000348.3	c.G607A	p.G203S	E4	0.00087	Compound	Father	P/PS3 PM1 PM2 PM3 PP4
			c.C16T	p.Q6X	E1	0.000175	Het	De novo	P/PVS1 PM1 PM2 PM6 PP3 PP4
P4	SRD5A2	NM_000348.3	c.G680A	p.R227Q	E4	0.006	Compound	Mother	P/PS3 PM1 PM2 PP4 PP5
			c.T59C	p.L20P	E1	0.00005851	Het	Father	LP/PM2 PM3 PP3 PP4 PP5
P5	SRD5A2	NM_000348.3	c.G680A	p.R227Q	E4	0.006	Compound	Father	P/PS3 PM1 PM2 PM3 PP4
			c.G607A	p.G203S	E4	0.00087	Het	Mother	P/PS3 PM1 PM2 PP4 PP5
P6	SRD5A2	NM_000348.3	c.G680A	p.R227Q	E4	0.006	Compound	Mother	P/PS3 PM1 PM2 PM3 PP4
			c.G607A	p.G203S	E4	0.00087	Het	Father	P/PS3 PM1 PM2 PP4 PP5
P7	SRD5A2	NM_000348.3	c.G281T	p.R94M	E1	0.000	Compound	Father	VUS/PM2 PP3 PP4
			c.G196A	p.G66R	E1	0.003	Het	Mother	VUS/PM2 PP3 PP4
P8	SRD5A2	NM_000348.3	c.C268T	p.H90Y	E1	0.000	Compound	Mother	VUS/PM2 PP3 PP4
			c.G196A	p.G66R	E1	0.003	Het	Father	VUS/PM2 PP3 PP4
P9	SRD5A2	NM_000348.3	c.T59C	p.L20P	E1	0.0000585	Compound	Father	LP/PM2 PM3 PP3 PP4 PP5
			c.C16T	p.Q6X	E1	0.000175	Het	Mother	P/PVS1 PM1 PM2 PP3 PP4
P10	SRD5A2	NM_000348.3	c.G680A	p.R227Q	E4	0.006	Hom	Mother/father	P/PS3 PM1 PM2 PM3 PP4
P11	SRD5A2	NM_000348.3	c.C736T	p.R246W	E5	0.000	Compound	Father	P/PS3 PM1 PM2 PM3 PP4
			c.G680A	p.R227Q	E4	0.006	Het	Mother	P/PS3 PM1 PM2 PP3 PP4
P12	SRD5A2	NM_000348.3	c.C751T	p.P251S	E5	0.000	Compound	Mother	LP/PM2 PM3 PP3 PP4
			c.G680A	p.R227Q	E4	0.006	Het	Father	P/PS3 PM1 PM2 PP4 PP5
	PROKR2	NM_144773.3	c.G533C	p.W178S	E2	0.002597	Het	Mother	LP/PS3 PM1 PP5
P13	SRD5A2	NM_000348.3	c.G607A	p.G203S	E4	0.00087	Compound	Mother	P/PS3 PM1 PM2 PM3 PP4
			c.C16T	p.Q6X	E1	0.000175	Het	Father	P/PVS1 PM1 PM2 PP3 PP4
P14	AR	NM_000044.4	c.C2612T	p.A871V	E8	0.000	Hem	Mother	P/PS3 PM1 PM2 PP4 PP5
P15	AR	NM_000044.4	c.C2612T	p.A871V	E8	0.000	Hem	Mother	P/PS3 PM1 PM2 PP4 PP5
P16	AR	NM_000044.4	c.G1823A	p.R608Q	E3	0.000	Hem	De novo	LP/PM1 PM2 PM6 PP3 PP4 PP5
P17	AR	NM_000044.4	c.G1823A	p.R608Q	E3	0.000	Hem	Mother	LP/PM1 PM2 PP3 PP4 PP5
	PROKR2	NM_144773.3	c.G533C	p.W178S	E2	0.002597	Het	Mother	LP/PS3 PM1 PP5

(Continues)

TABLE 2 (Continued)

ID	Gene	Transcript	cDNA	Protein	Exon	GnomAD freq	Genotype	Origin	ACMG
P18	AR	NM_000044.4	c.G1789A	p.A597T	E3	0.000	Hem	Na	P/PS3 PM1 PM2 PP4 PP5
P19	AR	NM_000044.4	c.A173T	p.Q58L	E1	0.00015	Hem	Mother	LP/PM1 PM2 PP3 PP4
	TRIM17	NM_001024940	c.C109T	p.R37X	E2	0.000	Het	Father	VUS/PM2 PP3
P20	ZFPM2	NM_012082.3	c.A2107C	p.M703L	E8	0.000448	Het	Mother	LP/PS3 PM2 PP4 PP5
P21	PROKR2	NM_144773.3	c.G533C	p.W178S	E2	0.002597	Het	Father	LP/PS3 PM1 PP5
P22	BMP4	NM_001202.5	c.C751T	p.H251Y	E4	0.0001	Het	Na	LP/PM1 PM2 PP3 PP5
P23	CHD7	NM_017780.3	c.C2189T	p.T730I	E4	0.001	Het	Na	VUS/PM2 PP3
P24	HOXD13	NM_000523.3	c.G32C	p.G11A	E1	0.0008	Het	Mother	LP/PS3 PM2 PP4 PP5
P25	GLI3	NM_000168.5	c.C2110T	p.Q704X	E14	0.000	Het	De novo	P/PVS1 PM1 PM2 PM6 PP3

Abbreviations: P, pathogenic; LP, likely pathogenic; VUS, variants of uncertain significance; NA, no related data; NGS, next-generation sequencing.

3.2 | Diagnostic gene mutations

In total, we identified diagnostic gene mutations in 22 patients. The overall diagnostic yield of 16.9% (22/130) was generated by adding the pathogenic and LP variants according the ACMG guidelines (Figure 1).

Among these 21 diagnosed patients, nine showed compound heterozygous mutations, four had a heterozygous mutation, four had a hemizygous mutation, and only one had a homozygous mutation. Additionally, three patients had polygenic mutations. Eleven patients carried *SRD5A2* gene mutations and six patients harbored *AR* gene mutations. Five patients carried rare gene mutations in *ZFPM2*, *PROKR2* (OMIM 607,123), *BMP4* (OMIM 112,262), *HOXD13*, and *GLI3* (OMIM 165,240).

3.3 | Clinical features of 130 patients

From the cohort, 130 patients presented hypospadias with varying degrees of severity, including 25 patients who manifested isolated hypospadias and 105 patients who had multiple hypospadias (with hypospadias accompanied by micropenis, cryptorchidism, and penoscrotal transposition).

3.4 | Clinical features of patients with genetic variant

Three types of external genital malformations were found in 25 patients, including hypospadias combined with micropenis (68%), hypospadias, micropenis combined with cryptorchidism (28%), and hypospadias combined with cryptorchidism (4%) (Figure 2a). In addition, posterior hypospadias accounted for 52% of patients, middle hypospadias accounted for 36%, and anterior hypospadias accounted for 12% of patients (Figure 2b). Hypospadias combined with micropenis was the most common malformation. However, all forms of posterior hypospadias, including the combined form, were the most common feature in patients with genetic mutations. Here, seven of 25 patients (28%) also showed thoracic spinal deformity, hydrocele, tricuspid regurgitation, inguinal hernia, tetralogy of Fallot, or finger deformities. Of note, none of the 25 patients had isolated hypospadias. The clinical phenotypes of the 25 patients with variants identified in the targeted genes are shown in Table 3.

Serum testosterone (T) and DHT after human chorionic gonadotrophin (HCG) stimulation were measured in 17 patients. The T/DHT ratio (after HCG stimulation) of 10 patients with *SRD5A2* gene variants was above the normal threshold of 10, indicating that this ratio has positive predictive value in clinical diagnosis (Maimoun et al., 2011). However, the T/DHT ratio was also higher than normal in three of five patients who were diagnosed with partial androgen insensitivity syndrome

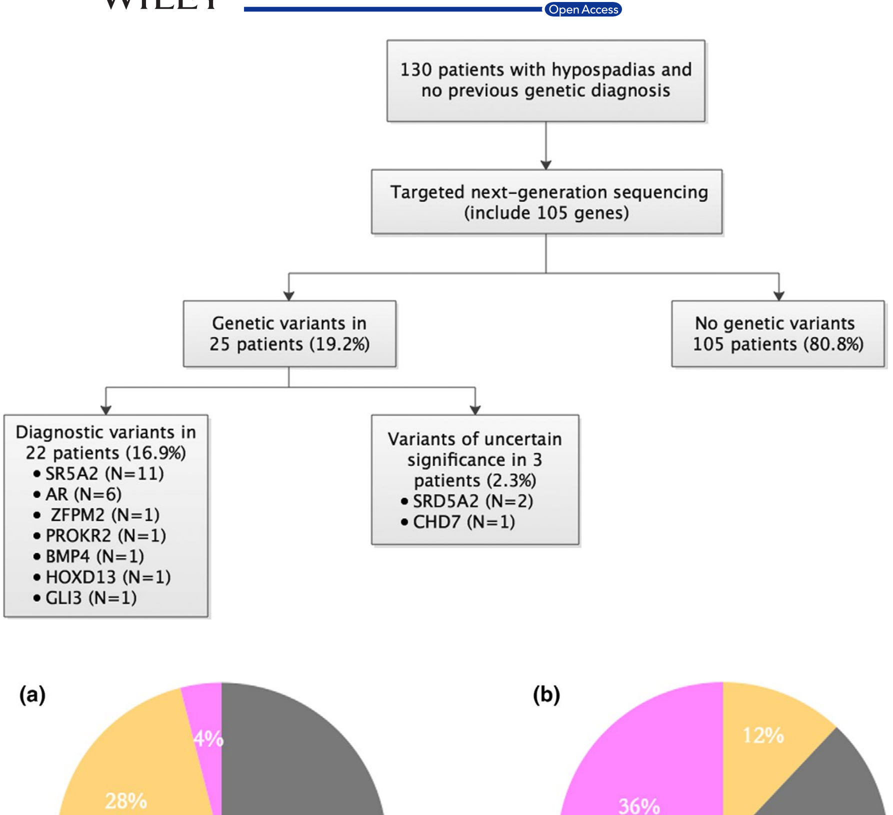


FIGURE 1 Genetic variants identified by targeted next-generation sequencing. Twenty-five patients with genetic variants were identified, 22 of whom carried diagnostic variants

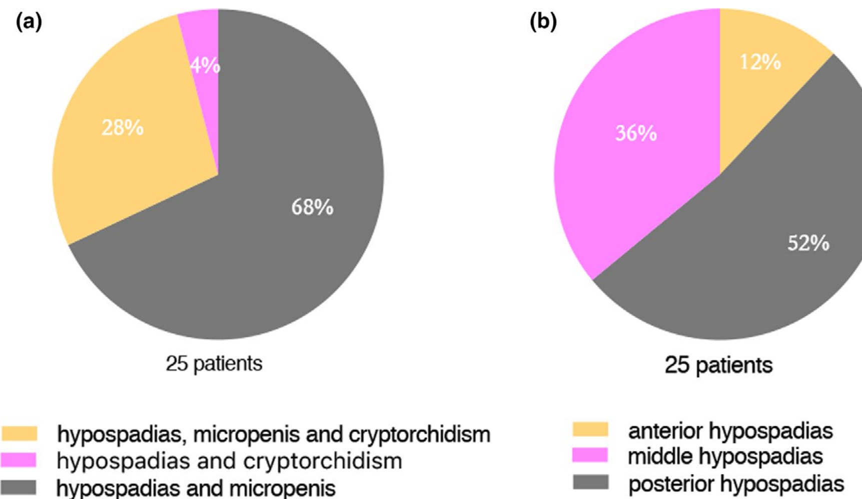


FIGURE 2 The external genital phenotypes of patients with genetic variations. (a) Proportion of 25 patients in the phenotypic categories. (b) Proportion of hypospadias features among the 25 patients

(PAIS). An elevated T/DHT ratio was observed in PAIS and among normal individuals. 5α-reductase deficiency, therefore, cannot be diagnosed even if the T/DHT ratio is higher than normal. Instead, it is necessary to diagnose 5α-reductase deficiency-associated hypospadias by genetic sequencing.

4 | DISCUSSION

Hypospadias is a common feature related to undervirilization of the male external genitalia. The development of external genitalia and the urogenital tract is complex, involving multiple factors, including coordinated genetics, cellular differentiation, tissue interactions, enzyme activities, and hormonal mediation (Kalfa, Philibert, & Sultan, 2009). The proper diagnosis of hypospadias is often challenging, given the significant heterogeneity of clinical presentations among patients with hypospadias. However, the diagnosis of hypospadias is important, and can facilitate sex assignment, selection of treatment strategies, and a more accurate evaluation of gonadal function. Here, we used a targeted NGS approach to identify 16 reported and four novel mutations in nine genes among 25 patients with various degrees of hypospadias.

SRD5A2 encodes the steroid 5α-reductase type 2 enzyme, which converts testosterone into DHT. In males, DHT induces the differentiation and growth of male external genitalia and development of secondary sexual characteristics during puberty (Yuan et al., 2017). To date, over 130 different mutations throughout the five exons of *SRD5A2* have been reported (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/validate.php). In our study, mutations in *SRD5A2* were the most common among those of the genes that were screened (52%, 13/25); this finding was consistent with that of previous study conducted in China (R. Wang et al., 2013). These observations suggest that the *SRD5A2* is a

hotspot for gene mutations in Han Chinese individuals with hypospadias. We identified nine different variants in 13 patients, including six previously reported variants (p.R246W, p.R227Q, p.G203S, p.G66R, p.L20P, p.Q6X) and three novel variants (p.P251S, p.R94M, p.H90Y). These mutations were clustered mainly in exons 1 and 4 (Figure 3a). Three novel variants were predicted to have a damaging effect on enzyme function by two in silico prediction tools and were located in highly conserved regions (Figure 3b). In addition, compound heterozygous mutations in the *SRD5A2* were identified in 12 of these patients (92.3%, 12/13). The p.R227Q variant was the most frequent variant (34.6%), followed by p.Q6X (19.2%) and p.G203S (15.4%), which is consistent with previous studies suggesting that these three variant positions are mutational hot spots in the Han Chinese population (Cheng et al., 2015; Song, Fan, Zhao, & Gong, 2019; Yuan et al., 2017). The p.Q6X variant is a nonsense mutation occurring in exon 1, which presumably results in the formation of a drastically truncated protein and lack of several important functional regions. Specifically, five patients (P1, P2, P3, P9, P13) who carried a p.Q6X variant displayed relatively severe clinical manifestations, which may be related to the complete loss of enzyme activity. Therefore, residual enzyme activity may be associated with phenotypic severity.

Hypospadias is often caused by PAIS. This is an X-linked recessive disorder caused by AR gene mutations (Su et al., 2017). Testosterone and DHT both bind the AR to activate a transcriptional response within the developing male external genitalia (Matsushita et al., 2018). In our cohort of patients, *AR* had the second highest number of all variants (24%, 6/25), with four different variants (p.Q58L, p.A597T, p.R608Q, and p.A871V) identified. These variants have all been previously reported and are distributed among multiple *AR* functional regions: two in the ligand binding domain (LBD), three in the DNA binding domain (DBD), and one in the amino-terminal transactivation domain (NTD). The p.A871V variant located in the LBD could lead to PAIS or mild AIS according to previous studies (Gottlieb, Beitel, Nadarajah, Paliouras, & Trifiro, 2012). Two patients (P14 and P15) who carried the p.A871V variant were diagnosed with PAIS; however, the clinical phenotype of the latter is more severe. Patients in our study with identical AR mutations were observed to display different phenotypes, which may have resulted from somatic mosaicism or other factors, such as genetic background or co-regulatory factors involved in the pathogenesis or phenotype of PAIS. In addition, three patients (P16, P17, and P18) carried *AR* mutations in the DBD and presented with PAIS, which is consistent with a previous report suggesting that *AR* mutations in the DBD region may directly underlie PAIS (Giwercman, Ivarsson, Richthoff, Lundin, & Giwercman, 2004). The p.Q58L variant located in the NTD was previously reported to be associated with male infertility and various degrees of hypospadias (Akcay et al., 2014; Kalfa et al., 2013). In addition to a p.Q58L variant in the *AR* gene, P19 also carried a heterozygous p.R37X variant in *TRIM17*. P19 presented with posterior hypospadias, microphallus, and testicular dysgenesis; long-term follow-up is needed to determine whether fertility will be affected in this patient. *TRIM17*, a member of the RING finger family, is expressed in the testis during embryonic development (Reymond et al., 2001). No *TRIM17* gene mutation has been previously found in hypospadias patients. We cannot determine conclusively whether the clinical phenotype of P19 is related to *TRIM17* gene mutation; thus, further studies are needed to clarify this case.

PROKR2 is related to isolated hypogonadotropic hypogonadism (IHH) and Kallmann syndrome (KS), which is an autosomal dominant trait. The p.W178S variant of *PROKR2* was identified in three patients, two of them (P12 and P17) with mutations in multiple genes, carrying *SRD5A2* and *AR* gene mutations, respectively. The p.W178S variant has been previously reported in Chinese patients, and according to a functional assay, it is associated with markedly reduced expression and protein instability (Monnier et al., 2009; Y. Wang, Gong, Qin, Liu, & Tian, 2017). It is interesting to identify IHH/KS-related pathogenic gene mutations in patients with hypospadias in our study, but these patients are too young to be diagnosed with IHH. It is worth noting that patients P12 and P17 are characterized by posterior hypospadias, especially P17, who showed penoscrotal transposition. It is possible that there are variants in genes controlling penile development in addition to *PROKR2* gene variations. Patient P21 displayed coronal hypospadias which could presumably involve additional undetected variants in genes controlling penile development or gene variations resulting from environmental factors.

ZFPM2 (also known as *FOG2*) is co-expressed with *GATA4* in the heart, brain, and gonads. *ZFPM2* has been demonstrated to regulate, with *SF-1* and *GATA4*, the expression of other key genes for sex determination or differentiation, including *SOX9* and *AMH* (Bashamboo et al., 2014). Mutations in *ZFPM2* have commonly been shown to be associated with congenital heart disease; however, they have only recently been detected in individuals with hypospadias (Eggers et al., 2016). A heterozygous missense variant p.M703L in *ZFPM2* was detected in patient P20, who presented with Tetralogy of Fallot, posterior hypospadias, and micropenis. The p.M703L variant found in our study has been previously reported as a pathogenic variant in congenital heart disease, although it has not been reported to be associated with hypospadias (Tan, Huang, Xu, Yang, & Yang, 2012; Zhang et al., 2014). The p.M703L variant is located in the sixth zinc finger structure. The first and sixth zinc finger structures of *ZFPM2* interact with conserved sequences in the N-terminal zinc finger of mammalian *GATA4*. Therefore, the p.M703L mutation may impair the gonadal development pathway initiated by the

TABLE 3 Clinical data of patients with genetic variants by gene-targeted NGS

ID	Gene	Age (years)	Classification	Complication		Inhibin B (pg/ml)	LH/FSH (mIU/ml)	T (ng/ml) (HCG stimulation test before /after)	DHT (pg/ml) (HCG stimulation test before /after)	T/DHT (HCG stimulation test after)	ther
				Micropenis	Cryptorchidism						
P1	SRD5A2	3.25	Perineal	Yes	No	107.14	0.1/0.98	<0.08/5.61	5.68/78.96	71.0	No
P2	SRD5A2	11.83	Proximal penile	Yes	No	107.99	2.04/3.14	0.25/28	30.93/48.22	58.1	No
P3	SRD5A2	1.08	Proximal penile	Yes	No	112.69	0.18/0.47	<0.08/4.23	44.39/107.08	39.5	No
P4	SRD5A2	4.58	Proximal penile	Yes	No	NA	NA	<0.08/NA	12.99/NA	NA	No
P5	SRD5A2	4.25	Perineal	Yes	No	NA	NA	NA	NA	NA	No
P6	SRD5A2	2.17	Proximal penile	Yes	Bilateral	157	NA	<0.08/NA	14.24/NA	NA	No
P7	SRD5A2	7.92	Perineal	Yes	No	NA	<0.07/0.85	<0.08/4.63	14.32/95.13	48.7	No
P8	SRD5A2	1.25	Proximal penile	Yes	No	83.81	<0.07/0.41	<0.08/4.08	11.41/89.74	45.5	No
P9	SRD5A2	2.83	Scrotal	Yes	Bilateral	NA	<0.07/0.58	<0.08/2.31	5.85/37.72	61.2	No
P10	SRD5A2	2.1	Proximal penile	Yes	No	70.17	<0.07/0.46	0.12/1.29	19.11/17.76	72.6	No
P11	SRD5A2	13.58	Scrotal	Yes	No	85.58	1.46/1.85	1.2/11.23	38.38/327.73	34.3	No
P12	SRD5A2 PROKR2	8.42	Penoscrotal	Yes	No	49.65	<0.07/1.16	<0.08/1.69	18.80/57.55	29.3	Thoracic spinal deformity Left hydrocele
P13	SRD5A2	1.42	Perineal	Yes	Bilateral	NA	6.5/5.4	1.21/15.88	22.54/247.46	64.2	Mild tricuspid regurgitation
P14	AR	0.3	Coronal	Yes	no	300.15	1.91/2.29	NA /1.25	NA /226.98	5.5	No
P15	AR	4.16	Scrotal	Yes	no	72.78	<0.07/1.18	<0.08/1.69	12.25/290.42	5.8	Right hydrocele penoscrotal transposition
P16	AR	6.25	Proximal penile	Yes	Bilateral	37.3	0.13/1.47	<0.08/1.68	18.10/152	11.0	No
P17	AR PROKR2	9	Scrotal	Yes	No	82.77	8.07/34	1.25/2.89	94.34/157.53	18.3	Bilateral hydrocele Penoscrotal transposition
P18	AR	4.25	Perineal	Yes	Bilateral	NA	NA	NA	NA	NA	Left inguinal hernia
P19	AR TRIM17	5	Scrotal	Yes	No	28.67	<0.07/0.49	<0.08/2.02	8.3/76.66	26.3	No
P20	ZFPM2	13.42	Scrotal	Yes	No	74.46	0.6/3.64	0.55/NA	NA	NA	Tetralogy of Fallot
P21	PROKR2	2.67	Coronal	Yes	Left	93.27	<0.07/0.49	<0.08/4.10	12.72/244.7	16.7	No

(Continues)

TABLE 3 (Continued)

ID	Gene	Age (years)	Classification	Complication		Inhibin B (pg/ml)	LH/FSH (mIU/ml)	T (ng/ml) (HCG stimulation test before /after)	DHT (pg/ml) (HCG stimulation test before /after)	T/DHT (HCG stimulation test after)	ther
				Micropenis	Cryptorchidism						
P22	BMP4	3.42	Midshaft	Yes	No	NA	NA	NA	NA	NA	No
P23	CHD7	4.5	Midshaft	No	Right	NA	NA	NA	NA	NA	No
P24	HOXD13	11	Scrotal	Yes	No	71.79	5.57/8.23	6.72/NA	394.22/NA	NA	Penoscrotal transposition Breast development
P25	GLI3	5.83	Coronal	Yes	Bilateral	106.5	0.14/3.2 bilateral	<0.08/4.35	16.34/181.71	23.94	Digital deformity

Note: Microphallus: length of penis is more than 2.5 *SD* from the average penile length in males of matched age.

Abbreviations: NA, no related data; NGS, next-generation sequencing; DHT, dihydrotestosterone.

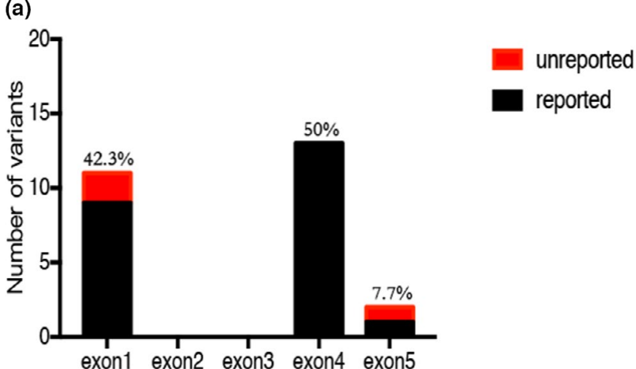
GATA4-ZFPM2 complex by affecting the binding of *ZFPM2* to *GATA4*(Brennan & Capel, 2004).

BMP4 is a member of the BMP family, which plays a vital role in embryonic development, including the regulation of the signaling cascades involved in urethral development. A heterozygous missense variant p.H251Y in *BMP4* was detected in patient P22, who presented with middle hypospadias and microphallus. The p.H251Y variant is located in the precursor peptide region of *BMP4*, which may affect the maturation and function of the whole protein. This missense variant of *BMP4* has been reported in Chinese patients with hypospadias in previous studies (T. Chen et al., 2007; H. Wang et al., 2018).

CHD7 is related to CHARGE syndrome or KS, which is of autosomal dominant inheritance. Several studies have found that not all patients carrying SNVs in *CHD7* develop the classical CHARGE syndrome phenotype (Van Nostrand et al., 2014). In our study, one *CHD7* heterozygous missense p.T730I variant was detected in patient P23, who presented with middle hypospadias and right cryptorchidism. However, no other malformations were consistent with the clinical diagnosis of CHARGE syndrome. Thus, according to ACGM guidelines, this is a VUS. However, this site is located in the SANT domain, which is highly conserved (Boyer, Latek, & Peterson, 2004). This region may be a histone tail binding module, which can bind to DNA or modified histones. In this case, we cannot establish whether the phenotype is related to the *CHD7* variants or an atypical phenotype associated with these gene variants.

The *HOXD13* is related to hypoplastic synpolydactyly and hypospadias. The *HOXD13* is expressed in the mesenchyme of the GT, and plays a major role in the development of the external genitalia and limbs (Tüzel et al., 2007). The p.G11A heterozygous mutation in *HOXD13* was found in patient P24, who presented posterior hypospadias and penoscrotal transposition. In vitro studies have shown that the p.G11A mutation causes a significant reduction in protein half-life (Brison et al., 2012). In combination with previous studies, it was found that males with *HOXD13* gene heterozygous mutations generally had milder limb deformities, some of whom also had mild hypospadias. However, patient P24 presented scrotal hypospadias, penoscrotal transposition, and breast development during puberty. The phenotypic diversity of *HOXD13* heterozygous variants could be attributed to incomplete penetrance and variable expressivity of autosomal dominant inheritance.

GLI3 is a transcription factor and is a downstream targets of the sonic hedgehog pathway during limb and craniofacial development. It may also be related to urethral and genital development. Several SNPs in the *GLI3* in Californian patients with hypospadias have been associated with increased risk of hypospadias (Carmichael et al., 2013). We identified a p.Q704X heterozygous mutation



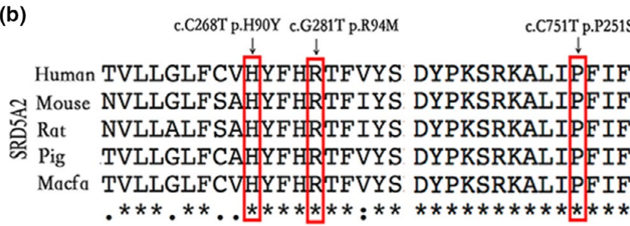


FIGURE 3 (a) Proportion of mutant alleles according to exon location. (b) Three novel variants in *steroid-5-α-reductase 2* were identified among our patients, all of which affect a highly conserved residue

in *GLI3* in patient P25, who presented with coronal hypospadias, micropenis, bilateral cryptorchidism, and digital deformity. To our knowledge, the current study is the first to identify a *GLI3* gene mutation in a patient with hypospadias, which provides a possible association between *GLI3* and urethral development.

5 | CONCLUSIONS

In summary, we performed targeted NGS of 105 genes associated with the molecular regulation of external genitalia and urogenital tract development in 130 patients with hypospadias of unknown etiology. A total of 25 patients (19.2%) were found to carry genetic variants in one or more of the nine targeted genes. Twenty-two (16.9%) had diagnostic variants.

The diagnostic rate observed in our study is lower than that observed in other studies (Eggers et al., 2016; H. Wang et al., 2018). Possible reasons include: (a) the panel of this study contains 105 genes, and may have discounted some variant genes or mutations in regulatory regions; (b) the criteria for selecting patients are different; in order to study the genetic factors affecting hypospadias development, the phenotype of hypospadias patients was not strictly screened in this study. Among 130 patients, 25 patients had hypospadias without genetic variation. In addition, the clinical phenotype of hypospadias in some patients is mild, and the probability of gene mutation in these patients is low; (c) the positive rate of gene mutations may be different for patients of different races; (d) this study did not detect DNA copy number variations.

In conclusion, we identified the precise molecular etiology for several patients in our cohort. The most frequently mutated gene in our study was *SRD5A2* (52%, 13/25), followed by *AR* (24%, 6/25). Although the contribution of variants in the other seven candidate genes is not high, it cannot be ignored. Hypospadias combined with micropenis accounted for 68% of all patients, which may represent a significant clinical sign for genetic detection. Specifically, combined with previous studies, we speculate that oligogenicity may be a rare genetic cause of hypospadias. Genetic diagnosis is useful for patients and clinicians alike. It contributes to the clinical understanding of hypospadias, and is invaluable for the genetic counseling of couples contemplating future pregnancies.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

ZD and WY conceived the study, designed the experiments, analyzed the data, and helped to draft the manuscript. WZ designed and performed the experiments, analyzed the results, and drafted the manuscript. WW, DW, JN and XJ participated in the experimental studies and helped in managing the patients. JS and CZ helped to conduct genetic testing and analyze data. JW was responsible for collecting samples and extracting DNA. LC, YX, and WL performed the literature search and conducted data synthesis.

CONSENT FOR PUBLICATION

We confirm that all of the family members involved in the case report gave their consent for their medical data to be published.

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