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Genetic Spectrum of hypospadias in ethnic minority children from Yunnan

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Abstract

Background: Hypospadias is the second most common congenital anomaly in male infants, characterized by an abnormal urethral opening on the ventral surface of the penis. It is typically classified as anterior, middle penile, or posterior based on the location of the urethral meatus. However, the high phenotypic heterogeneity of hypospadias poses considerable challenges for clinical diagnosis. Currently, the aetiology remains unknown in more than 70% of patients. Few genetic studies on hypospadias have been conducted in ethnic minority populations in Yunnan Province.

Methods: Whole-exome sequencing was performed in 23 children with hypospadias of unknown aetiology. Based on 105 hypospadias-associated genes reported in humans and mice, we identified candidate variants and confirmed mutations in family members by Sanger sequencing.

Results: Among the 23 patients, the most common phenotype was middle hypospadias (16/23, 69.6%), and 22 of 23 patients presented with additional genital anomalies, most frequently micropenis. Seventeen patients harboured 17 mutations in 11 genes, including 10 reported and 7 novel mutations. Of these 11 genes, 4 were previously reported in humans with hypospadias and

7 in mice. No higher frequency of AR gene mutations was observed in our cohort. Although SRD5A2 is commonly implicated, it was not a hotspot gene in this study. Five novel mutations and one known mutation were identified in hypospadias-associated genes (BMP4, NR5A1, SRD5A2, and FLNA).

Conclusions: To the best of our knowledge, this is the first study to investigate genetic mutations in ethnic minority children with hypospadias in Yunnan. Our findings may provide genetic and reproductive counselling for these families.

Keywords: hypospadias, whole-exome sequencing , genetic mutation, ethnic minority, Yunnan

Introduction

Hypospadias is the second most common congenital anomaly in males [1]. This malformation of the external genitalia can have profound physiological and psychological consequences. Affected children may need to sit while urinating, and adults with untreated hypospadias often experience sexual inhibition, erectile and ejaculatory dysfunction, and negative genital self-appraisal [2, 3][. Even patients who undergo surgery within the first two years of life may still face social, sexual, and medical complications later in life [4].

Based on urethral orifice location, hypospadias is classified into three types: anterior, middle, and posterior [4]. The urethral opening is located within the glans in most cases, at the midshaft or penile base less frequently, and rarely within or below the scrotum [5].. Anterior hypospadias accounts for approximately 70% of cases, while posterior forms (scrotal or perineal) comprise about 30% [6]. Clinical severity varies according to anatomical type [7].

The prevalence of hypospadias varies substantially across Western countries, ranging from 2 to 8 per 1,000 live male births[8]. Ethnic disparities are evident: incidence is highest among Caucasians, intermediate among Black individuals, and lowest among Asians and Hispanics [4]. Within the United States, Arkansas reports the highest prevalence at 3.91 per 1,000 male births, whereas Argentina shows the lowest at 2.1 per 1,000 births [9]. Latin American countries including Argentina, Chile, Colombia, Mexico, and Costa Rica demonstrate lower overall prevalence. European rates are highly variable, ranging from 1.06 per 1,000 births in France to 3.74 in Lombardy, Italy[9].. Notably, prevalence data from China remain limited, particularly for ethnically diverse regions.

The heritability of hypospadias ranges from 57% to 77% [10]. The condition can be transmitted through both paternal and maternal lineages [10]. Approximately 7% of patients have affected relatives [11], and brothers of affected individuals carry a 9-17% risk of hypospadias [10]. Seven percent of cases with hypospadias have relatives with hypospadias If one family has a male patient with hypospadias, and the brother of the patient has a 9-17% risk of hypospadias [10].

Both genetic and environmental factors contribute to hypospadias pathogenesis. Genetic evidence includes mutations associated with syndromic forms, increased heritability, and ethnic variation [6].To date, 18 genomic regions and 30 candidate genes have been implicated, includingMAMLD1,AR,HOXA4,SRD5A2,WT1,SF1,HOXB6,HSD3B2,SHH,GLI1-3,FGF8,FGF10,FGFR2,BMP7,DGKK,ESR1,ESR2,MAP3K1,CHD7,NR5A1,CYP11A1,AKR1C3,ATF3,BNC2,SP1, andSP7[12,13, 14]. These aberrations may occur on sex chromosomes or autosomes [15].

Embryologically, the urethral meatus can terminate anywhere along the penile shaft, within the scrotum, or in the perineum, depending on the timing of urethral developmental arrest [12]. However, the etiology remains unknown in most patients.

Despite substantial progress in identifying candidate genes, critical gaps persist. First, the vast majority of genetic studies have been conducted in Caucasian populations, with limited representation from Asian cohorts. Second, the genetic architecture of hypospadias in Chinese populations, particularly in ethnically diverse regions, remains virtually unexplored. Third, no systematic genetic screening using comprehensive approaches has been performed in these understudied populations, precluding precise genotype-phenotype correlations and personalized clinical management.

Yunnan Province, southwestern China, presents a unique opportunity to address these gaps. With a permanent population of 47.4 million-comprising 66.9% Han Chinese and 33.1% ethnic minorities-Yunnan offers exceptional genetic diversity for investigating population-specific risk variants. However, genetic testing for hypospadias has not been systematically implemented in this region.

Whole-exome sequencing (WES) enables comprehensive detection of coding variants with established pathogenicity, facilitating simultaneous analysis of known hypospadias genes and discovery of novel candidates. The aim of this study was to identify pathogenic genetic variants

in boys with hypospadias from Yunnan Province using WES, thereby establishing a foundation for regional genetic counseling and precision surgical planning.

Materials and Methods

Patient selection

This study was approved by the Ethics Committee of Kunming Children's Hospital of Kunming Medical University. Written informed consent was obtained from the parents or legal guardians of all participants under 16 years of age for the use of medical records and blood samples in research publications.

The study population comprised 23 nonconsanguineous families with hypospadias probands admitted to Kunming Children's Hospital in 2017. Probands were classified by hypospadias severity based on the anatomical location of the urethral opening documented prior to surgery. Families with incomplete anatomical descriptions or chromosomal abnormalities were excluded. The control group consisted of 20 healthy boys (aged 4 to 8 years; mean ± SD: 5.62 ± 3.22 years) who underwent elective circumcision at the day surgery unit. All controls had normal genital examination findings and no personal or family history of urogenital anomalies or endocrine disorders.

Clinical evaluations

Clinical assessments were conducted at Kunming Children's Hospital, Kunming Medical University. Evaluations included birth history, present illness history, anthropometric measurements (weight, height), and male genital examination (testicular volume and stretched penile length). Chromosomal karyotype analysis was performed for all probands.

DNA extraction and library preparation

Genomic DNA was extracted from 2 ml venous blood (EDTA anticoagulated) using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Three micrograms of genomic DNA were fragmented using Tagment DNA Enzyme 1 (TruSight One Sequencing Panel, Illumina Inc., San Diego, CA, USA). Following fragmentation, DNA was purified with magnetic beads and amplified by ligation-mediated PCR. Adapter sequences "TAAGGCGA" and "CTCTCTAT"

were incorporated during a 10-cycle PCR program: initial denaturation at 98° C for 30 sec; 10 cycles of 98° C for 10 sec, 60° C for 30 sec, 72° C for 30 sec; final extension at 72° C for 5 min. Target enrichment was performed using the TruSight One Sequencing Panel (Illumina China, Shanghai, China) according to the manufacturer's protocol. Post-capture PCR amplification was conducted with 5 μl PCR Primer Cocktail and 20 μl Enrichment Amp Mix per reaction (total volume 50 μl), followed by purification to generate the final captured library.

High-throughput sequencing and quality control

Paired-end sequencing (2 × 150 bp) was performed on the Illumina NextSeq 500 platform. Raw sequencing data were processed to remove adapter sequences and low-quality reads. Quality control metrics for all samples were as follows: mean sequencing depth >100×; target region coverage at 20× depth >95%; Q30 score >85%; mapping rate to the human reference genome (GRCh37/hg19) >95%; PCR duplication rate <20%. Only samples meeting these criteria were included in downstream analysis.

Variant calling and annotation

Clean reads were aligned to the human reference genome (UCSC hg19) using Burrows-Wheeler Aligner (BWA-MEM). Variant calling was performed using Genome Analysis Toolkit (GATK) best practices. Identified variants were annotated and filtered against dbSNP v142, the Human Gene Mutation Database (HGMD), ClinVar, the 1000 Genomes Project, and the NHLBI Exome Sequencing Project (ESP6500). Functional impact was predicted using SIFT, PolyPhen-2, MutationTaster, Mutation Assessor, FATHMM, GERP++, PhyloP, and SiPhy. Variants were annotated using ANNOVAR.

Candidate gene selection

A panel of 105 candidate genes associated with hypospadias was compiled from published literature on male genital development in humans and mouse models (reference 19). These genes were enriched using the TruSight One Sequencing Panel.

Protein function prediction

The pathogenicity of novel nonsynonymous variants was evaluated using PolyPhen-2, PANTHER, SIFT, and PMut.

Co-segregation analysis

Candidate variants were validated by Sanger sequencing in probands and family members. Primers were designed using NCBI Primer-BLAST (primer sequences provided in Supplementary Table 1). Co-segregation patterns were determined to assess variant inheritance within families.

Results

Clinical findings

Twenty-three children with hypospadias, aged 9 months to 8 years (mean ± SD: 3.62 ± 2.22 years), were recruited for mutation analysis. Seven patients were from ethnic minority groups (Miao, Yi, Bai, and Zhuang). Phenotypic severity varied: 4 (17.4%) had anterior hypospadias, 16 (69.6%) middle, and 3 (13.0%) posterior (Fig. 1). One patient had isolated hypospadias; the remaining 22 had additional genital anomalies including micropenis, cryptorchidism, penoscrotal transposition, or inguinal hernia. Karyotype analysis confirmed 46,XY in all patients.

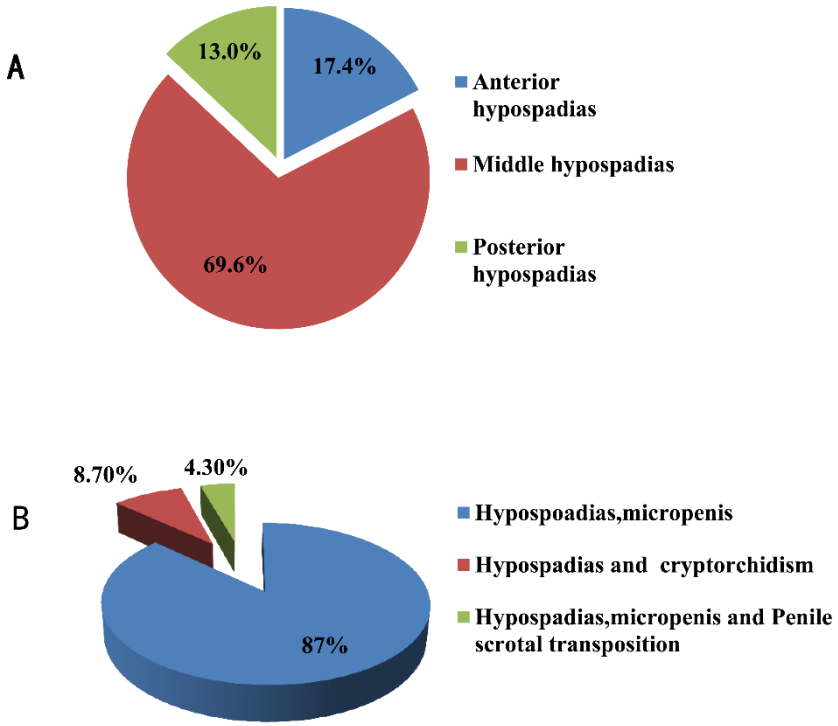


Figure 1. The external genital phenotypes of patients. A: Proportion of 23 patients in the phenotypic categories. B: Proportion of hypospadias features among the 23 patients.

Whole-exome sequencing analysis

Genetic variants were identified in 17 of 23 probands (73.9%) across 11 candidate genes (Table 1). These included 17 nonsynonymous SNVs: 10 previously reported and 7 novel. Functional categorization was as follows: BMP4 and NR5A1 (gonadal differentiation); FLNA, SRD5A2, and AMH (gonadal development); PROP1 FRAS1, KIF7, LIG4, SRCAP, and PCNT (other developmental pathways).

PCNT was the most frequently mutated gene (4 patients), followed by FLNA (3 patients). All variants are listed in Table 2.

Among the 17 mutation-positive patients, 13 had heterozygous variants, 3 had hemizygous variants (X-linked genes), and 1 had a homozygous variant.

Table 1. 17 of 23 probands carried genetic variants in 11 genes

Gonadal differentiation	**BMP signaling**	***BMP4***
	Other	***NR5A1***
Gonadal development	**Androgen−related**	***FLNA, SRD5A2***
	Other	***AMH***
Central causes of hypogonadism	***PROP1***	
Other	***FRAS1, KIF7, LIG4, PCNT, SRCAP***	

Table 2 Gene mutations of family with hypospadias

Family		**Gene**	***Exon***	**Transcript**	**Amino Acid Changes**	**Nucleotide Changes**	**Gene Type**	**Inheritance mode**	**GnomAD freq**	
1	**Chr2-31754395**	***SRD5A2***	**5**	**NM-000348**	**pR227Q**	**c.680C>T**	**Hom**	**AR**	**0.00560**	**Novel**

2	Chr14-54417493	*BMP4*	4	NM-001202	p.R162W	c.484C>T	Het	AD	0.0000008347	Known
3	chrX-153589891	*FLNA*	21	NM_001110556	p.A998T	c.2992G>A	Hem	XR	0. 00000	Novel
4	Chr21-47863848	*PCNT*	45	NM_006031	c.9826C>T	p.H3276Y	Het	AR	0.0022	Known
5	ChrX-153588550	*FLNA*	22	NM_001110556	c.3613G>A	p.A1205T	Hem	XR	–	Novel
6	Chr19-2251835	*AMH*	5	NM_000479	c.1562C>T	p.A521V	Het	AR	0.0030	Known
7	Chr9-127253449	*NR5A1*	6	NM_004959	c.1049G>C	P.R350P	Het	AD	-	Novel
8	Chr4-79285133	*FRAS1*	22	NM_025074	c.2647G>A	p.V883M	Het	AR	0.0030	Known
9	Chr21-47754588	*PCNT*	3	NM_006031	c.545G>A	p.R182H	Het	AR	0.0025	Known
10	Chr21-47847674	*PCNT*	42	NM_006031	c.7459C>G	p.L2487V	Het	AR	0. 0109	Known
11	Chr21-153581444	*FLNA*	38	NM_001110556	c.6151C>T	p.R2051W	Hem	XR	0.0003	Novel
12	Chr15-90172022	*KIF7*	19	NM_198525	c.3665-5G>A	splicing	Het	AR	0.0140	Novel
13	chr13-108861645	*LIG4*	2	NM_002312	c.1972A>G	p.I658V	Het	AR	0.0184	Known
14	Chr16-30749693	*SRCAP*	34	NM_006662	c.8332G>C	p.V2778L	Het	AD	0. 006	Novel
15	Chr5-177421270	*PROP1*	2	NM_006261	*c.179G>A*	p.G60E	Het	AR	0.0031	Known
16	Chr21-47817314	*PCNT*	22	NM_006031	*c.4352G>A*	p.R1451H	Het	AR	0.00570	Known
17	chr15-90196002	*KIF7*	2	NM_198525	*c.160C>T*	p.H54Y	Het	AR	0.0000	Known

Identification of pathogenic mutations

Sanger sequencing confirmed variants in 17 patients (73.9%, 17/23), comprising 10 previously reported and 7 novel mutations (Fig. 2, Table 2). All 8 novel mutations were absent in 20 healthy controls.

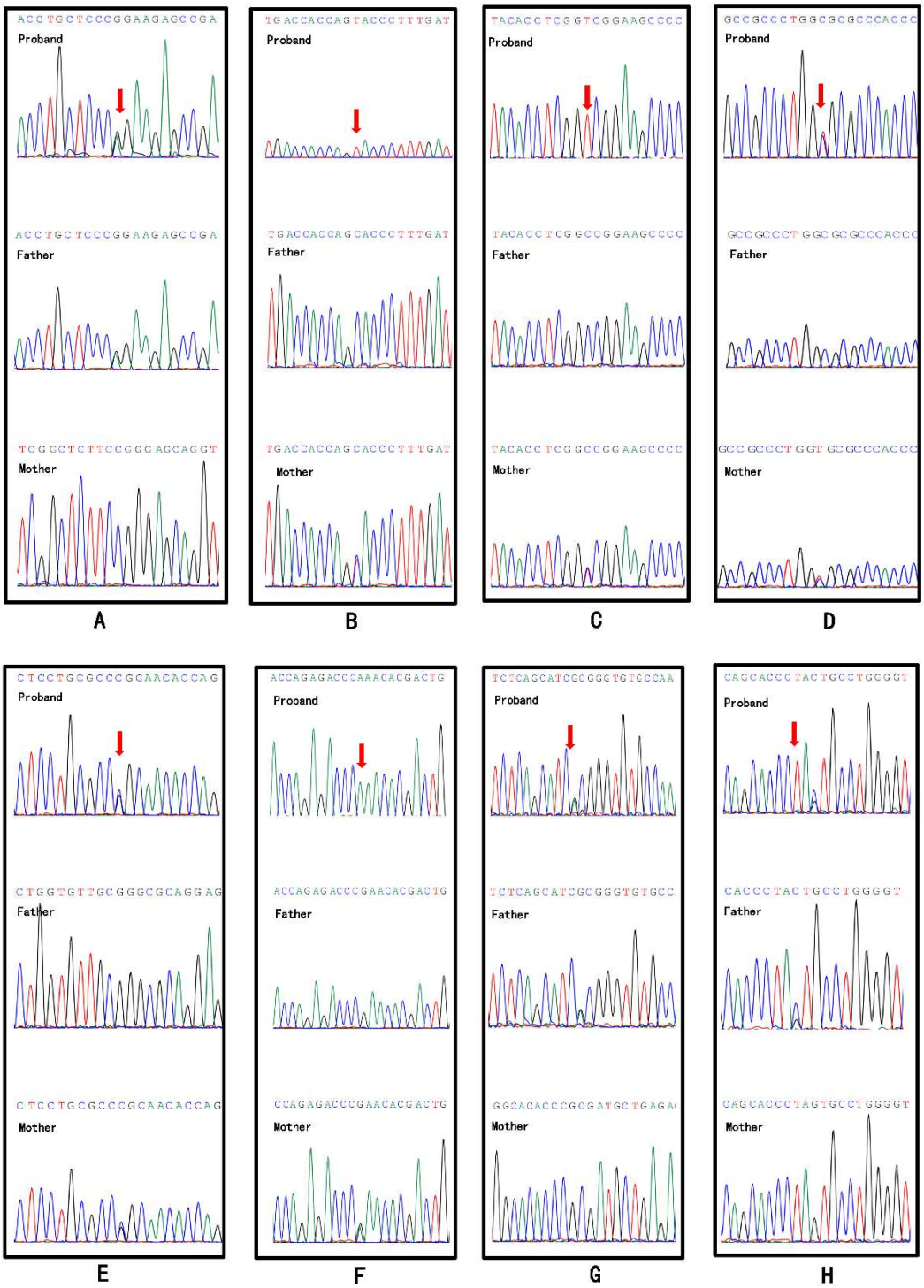


Figure 2. The variants of the probands. Arrows denote the mutations (the proband). Mutations in the "genes of hypospadias" identified by Sanger sequencing in families 2, 3, 5, 6, 7, 11, 14, and 16. A: The mutation in family 2; B: The mutation in family 3; C: The mutation in family 5; D:

The mutation in family 6; E: The mutation in family 7; F: The mutation in family 11; G: The mutation in family 14; H: The mutation in family 16.

Among the 17 mutation-positive patients, phenotypic distribution was: 3 (17.6%) anterior, 11 (64.7%) middle, and 3 (17.6%) posterior hypospadias. Six patients (35.3% of mutation-positive group) were from ethnic minority groups (Yi, Bai, and Zhuang).

Restricting analysis to genes with established pathogenicity in human hypospadias (BMP4, NR5A1, SRD5A2, and FLNA), 6 patients (26.1%, 6/23) carried pathogenic variants, including five novel and one known mutation.We identified 17 mutations among these 23 probands with hypospadias. The overall rate of diagnosis was73.9% (17/23) according to the "candidate genes": 3 patients (13.0%) had anterior hypospadias, 11 patients (47.9%) had middle hypospadias, and 3 patients (13.0%) had posterior hypospadias. The minority nationalities of Yi, Bai and Zhuang accounted for 26.1% (6/23).

According to the reported genes in humans with hypospadias [14] [20], the overall diagnostic rate was 26.1% (6/23). The mutations include five novel mutations and one known mutation of these genes (*BMP4*, *NR5A1*, *SRD5A2*, and *FLNA*).

Discussion

Hypospadias is among the most common congenital genital malformations in male newborns, arising from complex interactions between genetic, endocrine, and environmental factors [21]. In this study, we used whole exome sequencing to investigate the genetic basis of hypospadias in 23 children from Yunnan Province, China. We identified nonsynonymous variants in 11 candidate genes in 17 patients (73.9% variant detection rate). However, only four of these genes (BMP4, NR5A1, SRD5A2, and FLNA) have been previously implicated in human hypospadias; the remaining seven genes (PROP1, FRAS1, KIF7, LIG4, SRCAP, PCNT, AMH) were selected based on mouse models [19] and require further validation to establish their role in human disease. When restricting analysis to genes with established human disease association, the diagnostic yield was 26.1% (6/23 patients), consistent with prior reports [19,30].

Genotype-phenotype correlations and comparison with prior studies

Our findings diverge from prior studies in Han Chinese populations that identified SRD5A2 and AR as mutation "hotspots" [19, 22-24]. We detected only one SRD5A2 mutation (p.R227Q, previously reported) and no AR mutations. Several factors may explain this discrepancy. First, our sample size (n=23) is relatively small, limiting our ability to capture rare variants. Second, Yunnan Province is home to 26 ethnic minorities with distinct genetic backgrounds; population stratification may influence variant distribution. Third, phenotypic heterogeneity in our cohort-22 of 23 patients had hypospadias with additional anomalies (micropenis, cryptorchidism, penoscrotal transposition, or inguinal hernia)-may reflect different underlying genetic architectures compared to isolated hypospadias.

Among the four established genes, we identified five novel and one known mutation. The SRD5A2 p.R227Q mutation in Family 1 is a well-characterized variant causing 5 α -reductase type 2 deficiency [23]. We also identified three novel FLNA variants. FLNA encodes filamin A, which crosslinks actin cytoskeleton and regulates cell migration and signal transduction [27,28]. Mutations in FLNA have been associated with hypospadias and gonadal dysgenesis [20], supporting the potential pathogenicity of our findings. The BMP4 c.484C>T variant was identified in a patient with middle hypospadias; BMP4 mutations have been reported in Chinese patients with hypospadias [19, 25, 26]. Finally, one patient carried a novel NR5A1 variant; NR5A1 mutations account for 8-15% of 46,XY disorders of sex development [29,30].

Novel variants and limitations

We identified eight novel variants in seven genes (PROP1, FRAS1, KIF7, LIG4, SRCAP, PCNT, AMH) that have not been previously reported in human hypospadias. These variants were absent from 20 population controls, and all were predicted to be damaging by multiple in silico tools. However, we acknowledge several critical limitations. First, functional validation is lacking: we did not perform in vitro splicing assays, protein stability studies, or cellular rescue experiments to confirm pathogenicity. Second, familial segregation data were incomplete; co-segregation analysis was performed but not all family members were available for testing. Third, our control group (circumcision patients) was not perfectly matched for age or ethnicity, though

all had normal genital examination. Fourth, the sample size is insufficient to draw conclusions about genotype-phenotype correlations or population-specific variant frequencies.

Conclusion

In summary, this study identified potentially pathogenic variants in 17 of 23 children with hypospadias using a targeted candidate gene approach. While the overall variant detection rate was high (73.9%), the diagnostic yield for established human disease genes was 26.1%, consistent with existing literature. We report five novel mutations in four established genes (BMP4, NR5A1, SRD5A2, FLNA) and eight additional variants in genes requiring functional validation. Our findings expand the mutation spectrum of hypospadias-associated genes and highlight the need for larger, multi-center studies with functional follow-up to establish the clinical significance of variants in genes currently supported only by animal models.

Discussion

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Conclusion

In summary, this study identified potentially pathogenic variants in 17 of 23 children with hypospadias using a targeted candidate gene approach. While the overall variant detection rate was high (73.9%), the diagnostic yield for established human disease genes was 26.1%,

consistent with existing literature. Seventeen patients harboured 17 mutations in 11 genes, including 10 previously reported and 7 novel mutations/variantstotal. Of these,5 novel mutationswere in four genes with established pathogenicity in human hypospadias (NR5A1, SRD5A2, and FLNA), and 2 novel variantswere in seven genes previously implicated only in mouse models (PROP1, FRAS1, KIF7, LIG4, SRCAP, PCNT, AMH).

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Abbreviations

SIFT: Sorting Intolerant from Tolerant

WES: Whole-exome sequencing

PCR: Polymerase chain reaction

SNVs: Single-nucleotide variants

AR: Androgen receptor

BMP4: Bone morphogenetic protein 4

NR5A1: Steroidogenic Factor 1

FLNA: Filamin A

SRD5A2: Steroid 5α-reductase type 2

PROP1: PROP1 Paired-Like Homeobox 1

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Kunming Children's Hospital, Kunming Medical University. Written informed consent was obtained from all participants. For participants under 16 years of age, written informed consent was obtained from their parents or legal guardians. Our team was authorized by the Ethics Committee to access the data used in this research.

Consent to publish

All participants provided written informed consent for the publication of identifying images, personal details, and clinical details in an anonymous manner. For participants under 18 years of age, written informed consent for publication was obtained from their parents or legal guardians.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author (B Y) or first author (WJH) upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Z Z, X Z, C C W, C K Y and W J H conceived and designed the experiments; Z Z, B Y, W J H, X Z and C C W performed the experiments; Z Z, X Z, C C W, C K Y and W J H analyzed the data; W J H and X Z recruited patients and collected clinical information; Z Z, C C W, X Z and W J H contributed to accumulation and interpretation of clinical data; W J H, B Y, C C W and Z Z coordinated the project. All authors read and approved the final manuscript.

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Figure legends

Figure 1. External genital phenotypes of the patients. (A) Distribution of phenotypic categories among the 23 patients. (B) Distribution of hypospadias features among the 23 patients.

Figure 2. Genetic variants identified in the probands. Arrows indicate the mutation sites. Sanger sequencing confirmed mutations in "hypospadias-associated genes" in families 2, 3, 5, 6, 7, 11, 14, and 16. Pedigrees 2 – 16 show the inheritance patterns and mutation carriers in each family.

Supplementary Files

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- SupplementaryTable1.docx