RESEARCH ARTICLE

A Potential Pathogenic *SRD5A2* Mutation and rs632148, rs523349 and rs522638 Polymorphisms in Increasing the Risk of Syndromic Hypospadias in Indonesian Population

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Abstract

BACKGROUND: Hypospadias, a congenital birth defect in male, is the opening of the urethra located on the ventral side of the penis. Several mutations in *SRD5A2* encoding steroid 5 alpha-reductase type 2 protein have been identified in hypospadias and polymorphisms in this gene have been known to be associated with an increased risk of hypospadias. In this study, several crucial molecular analyses of the *SRD5A2* gene and the association of the identified variants to the risk of syndromic hypospadias in Indonesian population were conducted.

METHODS: Thirty-two isolated and 29 syndromic hypospadias patients were enrolled in this study. DNA was isolated from whole blood for the amplification of all exons and exon-intron boundaries of *SRD5A2* by polymerase chain reaction (PCR), followed by Sanger sequencing. *In silico* analysis was performed using PolyPhen-2, Sorting Intolerant from Tolerant (SIFT) and Align GVGD. Statistical analysis was performed using Chi-squared test.

RESULTS: A novel missense mutation c.32T>C/p.Leu11Pro was identified in one isolated hypospadias patient and the *in silico* analysis predicted the mutation to be pathogenic. Three polymorphisms were identified, two in the non-coding region (c.-62G>C/rs632148 and c.281+15T>C/rs522638) and one in exon-1 (c.265C>G/p.Val89Leu/rs523349). Mutant alleles of these polymorphisms were significantly associated with syndromic hypospadias with odds ratios (OR) of 3.4, 3.13 and 2.54 respectively.

CONCLUSION: This study suggests that *SRD5A2* mutation is one of the causes of hypospadias in Indonesian population and rs632148, rs523349 and rs522638 polymorphisms are significantly associated with an increased risk of syndromic hypospadias.

KEYWORDS: mutation, polymorphism, *SRD5A2*, syndromic hypospadias

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Introduction

Hypospadias is one of the most common congenital anomalies in males characterized by abnormal location of urethral meatus.(1,2) The prevalence of hypospadias varies in different countries. The mean of hypospadias prevalence per 10,000 births was 19.9 in Europe, 34.2 in North America, 5.2 in South America, 0.6–62 in Asia and 17.1–34.8 in Australia.(1) Hypospadias presents mostly as isolated case while 15% of the cases are in syndromic forms. Syndromic hypospadias refers to hypospadias that

is accompanied by other phenotypes such as micropenis, anorectal malformation or other manifestations.(2-7) Hypospadias can occur following any disruption during the development of the penis, and previous studies showed that genetic and environmental factors were involved in the etiology of the disease.(5,6,8,9) Most hypospadias present as sporadic cases while few present as familial cases. Based on the anatomical displacement of the urethral meatus, the severity of hypospadias is classified into three groups namely mild, moderate and severe.(5,10) The more proximally the location of the urethral meatus (penoscrotal, scrotal, perineal) is, the more severe the hypospadias will be.(11)

The development of male genitalia occurs in several steps during weeks 8–16 of gestation. The initial phase of male genitalia development is a hormone-dependent phase, whereas the second phase is hormone-independent. Androgen-related genes such as *Androgen Receptor* (*AR*) and *Steroid 5 Alpha-reductase Type 2* (*SRD5A2*) play major roles in the hormone-dependent phase.(3,5,9,12)

The *SRD5A2* located on chromosome 2p23.1 encodes steroid 5α-reductase type 2 enzyme, which is required for the conversion of testosterone to dihydroxytestosterone (DHT). DHT binds to *AR* and initiate the signaling for the differentiation of male external genitalia and the development of male characteristics.(3,13-15) Any abnormality in the *SRD5A2* (mutations and/or polymorphisms) is linked with an increased risk of hypospadias.(9,11,16-19)

Previous study in the Chinese population showed that *SRD5A2* mutations were identified in 15 out of 33 hypospadias patients (~45%). Most of those mutations were identified in patients with posterior hypospadias.(17) Study in pediatrics with 46, XY disorder of sex development (46, XY DSD) in China showed that *SRD5A2* mutations were identified in 22 out of 76 patients (~29%). Seventy-two percent of those patients with *SRD5A2* mutations had hypospadias.(16) In Indonesia, similar study in pediatric with 46, XY DSD and 5-alpha-reductase type 2 deficiency have been done and showed that mutations were identified in 20 out of 37 patients (54%).(20) Hypospadias is a complex disorder caused by genetic alone or in combination with environmental factors. In complex disorders, polymorphisms are often associated with disease development. Polymorphisms in sex hormones biosynthesis including *SRD5A2* increased the risk of hypospadias. Among 10 single nucleotide polymorphisms (SNPs) they analyzed, rs7562326 gave the highest hypospadias risk.(19) The rs523349 (p.Val89Leu) variant had been reported to increase the risk of hypospadias in Indian population, and the homozygous genotype of this variant further increased the risk. Interestingly, when they combined the genetic factor (homozygous of rs523349 variant) with the environmental factor (having parents working in agricultural background), it increased the risk to a greater extent. This shows that combination of genetic and environmental factors indeed increases the risk of hypospadias.(21) However, from all of those studies, it is still unclear whether the prevalence of mutations and/or polymorphisms of this gene are different in each type of hypospadias, namely syndromic and isolated cases. Furthermore, little is known about the disruption of this gene in hypospadias among the Indonesian population. This study involved the molecular analysis of the *SRD5A2* gene and analysis of the association between the identified polymorphisms and hypospadias type.

Methods

Subjects Recruitment

Sixty-one hypospadias patients were enrolled in this study by consecutive sampling, with an inclusion criterion of boy under 18 years of age with hypospadias and an exclusion criterion of hypospadias with undervirilization phenotype. Twenty-nine patients presented with syndromic hypospadias (hypospadias accompanied by other phenotypes such as micropenis, penoscrotal transposition, undescended testis, anorectal malformation), while the other 32 had isolated hypospadias (hypospadias without any additional phenotype). The diagnosis was made based on physical examination performed by pediatric surgeon team in the Division of Pediatric Surgery, Dr. Hasan Sadikin General Hospital, Bandung, Indonesia. The subjects' ages ranged from 14 days to 15 years old and most of the hypospadias were identified as penoscrotal followed by mid-shaft (Table 1). Blood samples were collected consecutively following written informed consent from the parents. This study was approved by the Ethical Review Board of Faculty of Medicine Universitas Padjadjaran (No. 063/UNC6.C1.3.2/KEPK/PN/2015).

DNA Isolation and Genotyping

One to three mL whole blood samples were collected from each patient for genomic DNA extraction using DNA Isolation Kit (Roche Life Science, Boston, MA, USA). The primers were designed using Primer3 v.04 tools (*http://bioinfo.ut.ee/primer3-0.4.0/*), and the DNA sequences used for the template in primer design were downloaded from UCSC genome browser (*https://genome.ucsc.edu*)

Table 1. Subjects' characteristics.

Subject Characteristics	n (%)
Age	14 days – 15 years
Hypospadias type (n=61)	
Glandular	2 (3.3)
Coronal	9 (14.8)
Mid-shaft	20 (32.8)
Penoscrotal	25 (41)
Scrotal	3 (4.9)
Perineal	2 (3.3)
Complication in syndromic cases (n=29)	
Micropenis	16 (26.2)
Penoscrotal transposition	6 (9.8)
Undescended testis	4 (6.6)
Micropenis and penoscrotal transposition	1 (1.6)
Penoscrotal transposition and undescended testis	1 (1.6)
Undescended testis and anorectal malformation	1 (1.6)
Family history of hypospadias	2 (2.7)

(Supplementary 1). Each PCR reaction comprised of DNA template, dNTP, KCl, TRIS-HCl, MgCl2 and Taq DNA polymerase (AmpliTaq, Perkin Elmer, NJ, USA) in a total volume of 50 µL. DNA was amplified by a touch-down PCR method, with an initial denaturation of 94°C for 4 minutes, followed by 10 cycles of denaturation at 94°C for 1 minute, annealing at 70°C to 61°C (the temperature was reduced 1°C in each cycle) for 1 minute, and elongation at 72°C for 1 minute, then 25 cycles of annealing at 60°C for 1 minute. The PCR products were verified by DNA gel electrophoresis and sequenced using the forward primer with Big Dye Terminator v3.1 Sequencing Kit (Applied Biosystem, Foster City, CA, USA) on a Sanger Sequencer ABI 3730XL (Applied Biosystem).

In silico Analysis

The in silico analysis to predict pathogenicity of the identified mutation was conducted using the Polymorphism Phenotyping-2 (PolyPhen-2) (*http://genetics.bwh.harvard.edu/pph2/*) (22), Sorting Intolerant from Tolerant (SIFT) (*https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html*) (23), and Align GVG (*http://agvgd.hci.utah.edu/agvgd_input.php*) (24). To analyze p.Leu11Pro mutation in the *SRD5A2* using PolyPhen-2, protein accession number of SRD5A2 (NP_000339.2) was input to 'Protein or SNP identifier' box, followed by inputting amino acid position 11 into 'Position' box. The wild-type amino acid Leucine was put into the 'Substitution' box 'AA1' as 'L' while the alternate amino acid Proline was put into the 'AA2' box as 'P'. This was then finalized by submitting the query. SIFT sequence single protein tool analysis was conducted, starting with inputting Fasta sequence of SRD5A2 protein (NP_000339.2) into the 'protein query sequence' box, followed by submitting the amino acid substitution of interest as 'L11P'. The author's email address was informed then the query was submitted. The third mutation prediction analysis conducted was using Align GVGD, which was started by putting the Fasta sequence of SRD5A2 protein (NP_000339.2) into 'Paste your alignment' box, followed by submitting 'L11P' into the 'Paste your list of substitutions' box. The author email address was written then the query was submitted.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 10, and two-sided $p<0.05$ were considered statistically significant. The association between the identified polymorphism and syndromic or isolated hypospadias was assessed by odds ratio (OR) and 95% confidence of intervals (CIs) for alleles using Chi-square test.

Results

Novel Mutation in *SRD5A2*

PCR analysis of the *SRD5A2* gene revealed one heterozygous mutation in a subject with isolated mid-shaft hypospadias. The mutation was a missense mutation in exon-1, a single base substitution of thymine by cytosine at nucleotide position 32 (c.32T>C), resulting in a change of leucine to proline at codon 11 (p.Leu11Pro) (Figure 1). This variant has not been reported before and was not registered in the single nucleotide polymorphism (SNP) database (novel mutation).

Polymorphisms in *SRD5A2*

PCR followed by Sanger sequencing were conducted in all 29 individuals with syndromic hypospadias and 32 individuals with isolated hypospadias (Table 2). Sequencing of the *SRD5A2* showed one common variant (polymorphism) 62 bp upstream of the gene (c.-62G>C/rs632148), another one in the coding region of exon-1 (c.265C>G/p.Val89Leu/rs523349) and one in intron-1 (c.281+15T>C/rs522638) (Figure 2). The frequencies of the genotypes and alleles for all three polymorphisms were presented in Table 2 and Table 3 respectively. Interestingly, the homozygous mutant forms of all three polymorphisms more frequently occurred in the syndromic cases than the isolated cases (Table 2). Data

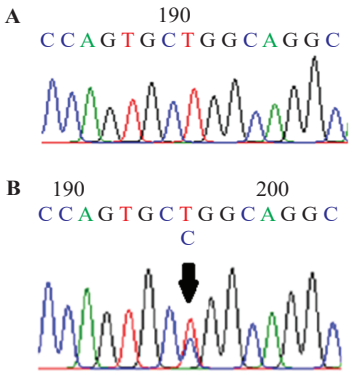

Figure 1. Electropherogram of SRD5A2 Mutation. A: Control population. B: Missense mutation in exon-1 of the *SRD5A2* gene (c.32T>C) with a change in amino acid from leucine to proline (p.Leu11Pro) (black arrow) identified in one patient with hypospadias.

analysis using Chi-square test showed that the mutant allele (C) c.-62G>C/rs632148 was significantly more common in syndromic hypospadias than in isolated hypospadias with OR 3.40 (Table 3). The mutant allele (G) of the c.265C>G/p.Val89Leu/rs523349 variant was also more common in syndromic hypospadias with OR 2.54 (Table 3). The third polymorphism identified was a non-coding variant located 15 bp downstream of exon 1 (c.281+15T>C/rs522638) (Figure 5). The minor allele frequency (MAF) was 0.27 (27%) in patients with syndromic hypospadias and 0.155 (15.5%) in patients with isolated hypospadias. An analysis using Chi-Square test revealed that the mutant allele (C) was associated with syndromic hypospadias with OR 3.13 (Table 3).

Pathogenicity Prediction of the Novel *SRD5A2* Mutation

Using PolyPhen-2 analysis, the prediction score for p.Leu11Pro mutation in *SRD5A2* was 0.999 for HumDiv (prediction for rare variant) and 0.980 for HumVar (prediction for rare variant in Mendelian disorder), both indicating that the mutation was probably damaging. Multiple sequence alignment of the amino acids from different species in PolyPhen-2 showed that the position of leucine was in the conserved region (Figure 3), therefore most likely to play an important role in protein function or folding. Using SIFT sequence single protein tool analysis, the substitution of *SRD5A2* at position 11 from leucine to proline was predicted to affect protein function, with a score of 0.02. SIFT score of ≤0.05 indicates that the mutation was predicted to be damaging. Align GVGD prediction tool identified the Leu11Pro substitution in *SRD5A2* as class 65, which means that the mutant amino protein was predicted to be deleterious. Those three tools gave aligned conclusion that the *SRD5A2* p.Leu11Pro mutation was a pathogenic variant.

Discussion

This study involved *SRD5A2* mutation and polymorphism analysis in syndromic and isolated hypospadias cases. To date, more than 100 mutations have been reported with most identified in the very wide range of external genitalia ambiguity and hypospadias.(25,26) Out of 61 hypospadias patients, a novel mutation was identified in an isolated case, hence the frequency of the *SRD5A2* mutation in

Table 2. Genotype of SRD5A2 polymorphisms identified in hypospadias.

Variant	Genotype	Hypospadias		Total n (%)
		Syndromic n (%)	Isolated n (%)	
rs632148	G/G	6 (9.8)	18 (29.5)	24 (39.3)
(c.-62G>C)	G/C	14 (23)	11 (18)	25 (41)
	C/C	9 (14.8)	3 (4.9)	12 (19.7)
		29 (47.5)	**32 (52.5)**	**61 (100)**
rs523349	C/C	7 (11.5)	17 (27.9)	24 (39.3)
(c.265C>G/p.Leu89Val)	C/G	14 (23)	11 (18)	25 (41)
	G/G	8 (13)	4 (6.6)	12 (19.7)
		29 (47.5)	**32 (52.5)**	**61 (100)**
rs522638	T/T	5 (8.2)	17 (27.9)	22 (36.1)
(c.281+15T>C)	T/C	15 (24.6)	11 (18)	26 (42.6)
	C/C	9 (14.8)	4 (7)	13 (21.3)
		29 (47.5)	**32 (52.5)**	**61 (100)**

C: cytosine; G: guanine; SRD5A2: steroid 5 alpha-reductase type 2; T: thymine.

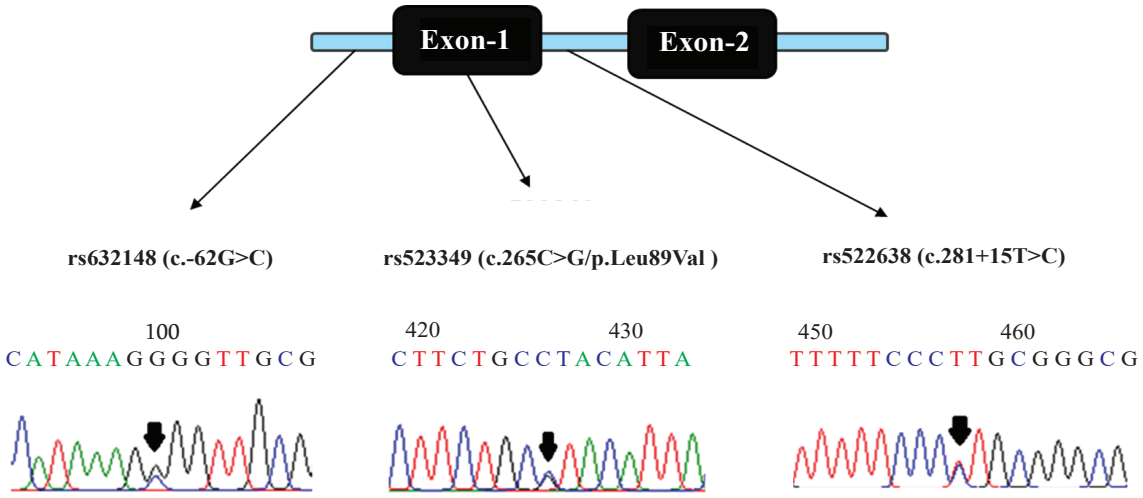

Figure 2. Location of the *SRD5A2* polymorphisms and the Sanger sequencing results. The rs632148 (c.-62G>C) was identified 62 bp upstream of SRD5A2 while the rs523349 (c.265C>G) was identified in exon 1 and rs522638 (c.281+15T>C) was identified in intron-1 of the gene.

Indonesian population with hypospadias but without *SRD5A2* deficiency is 1.6%. The identified mutation is a missense mutation at codon 11 which changes leucine to proline (c.32T>C/p. Leu11Pro) and is located in the conserved region within different species. Amino acids located in such a location usually has important protein function. The patient identified with this mutation had moderate (mid-shaft) hypospadias. Mutation analysis of the same gene in hypospadias in Swedish, Iranian, and Chinese populations has reported mutation frequencies of 0.6%, 1.8%, and 13.5% respectively.(11,15,27) From this study, the frequency of *SRD5A2* mutation in the Indonesian population is similar to that in the Iranian population, with *SRD5A2* mutations are more common in hypospadias in the Chinese population and less common in the Swedish population. However, the frequency of the *SRD5A2* mutation increased to 54% (harmful mutations were identified in 20 out of 37) in hypospadias patients with an 5-alpha-reductase type 2 deficiency.(20) In current study, the level of enzyme encoded by *SRD5A2* was not measured. Considering that phenotypically all of the patients in our cohort did not show undervirilization, if not all, we assume that most of them have adequate 5-alpha-reductase type 2. This might be the reason why we did not identify *SRD5A2* mutations as high as previous reported study.

Previous studies showed that polymorphisms in *SRD5A2*, particularly rs523349 (p.Leu89Val), are associated with hypospadias in Swedish, Turkish and Middle Eastern populations. It is known that the MAF of such polymorphisms is not significantly different between familial and sporadic cases (15), however, no study has reported whether the MAF of those polymorphisms is

Table 3. The allele of SRD5A2 polymorphisms identified in hypospadias.

Variant	Allele	Hypospadias		Total n (%)	RR	95% CI	OR	95% CI
		Syndromic n (%)	Isolated n (%)					
rs632148	G (Wild type)	26 (21.3)	47 (38.5)	73 (60)	1.83		3.40	
(c.-62G>C)	C (Mutant)	32 (26.2)	17 (14)	49 (40)	*p*=0.0013	1.27 - 2.67	*p*=0.0013	1.55 - 6.99
		58 (47.5)	**64 (52.5)**	**122 (100)**				
rs523349	C (Wild type)	28 (23)	45 (37)	73 (60)	1.60		2.54	
(c.265C>G/p.Leu89Val)	G (Mutant)	30 (24.5)	19 (15.5)	49 (40)	*p*=0.0132	1.11 - 2.31	*p*=0.0132	1.18 - 5.38
		58 (47.5)	**64 (52.5)**	**122 (100)**				
rs522638	T (Wild type)	25 (20.5)	45 (37)	66 (60)	1.78		3.13	
(c.281+15T>C)	C (Mutant)	33 (27)	19 (15.5)	48 (40)	*p*=0.0033	1.23 - 2.61	*p*=0.0033	1.45 - 6.71
		58 (47.5)	**64 (52.5)**	**122 (100)**				

C: cytosine; CI: confidence interval; G: guanine; OR: odds ratio; RR: relative risk; SRD5A2: steroid 5 alpha-reductase type 2; T: thymine.

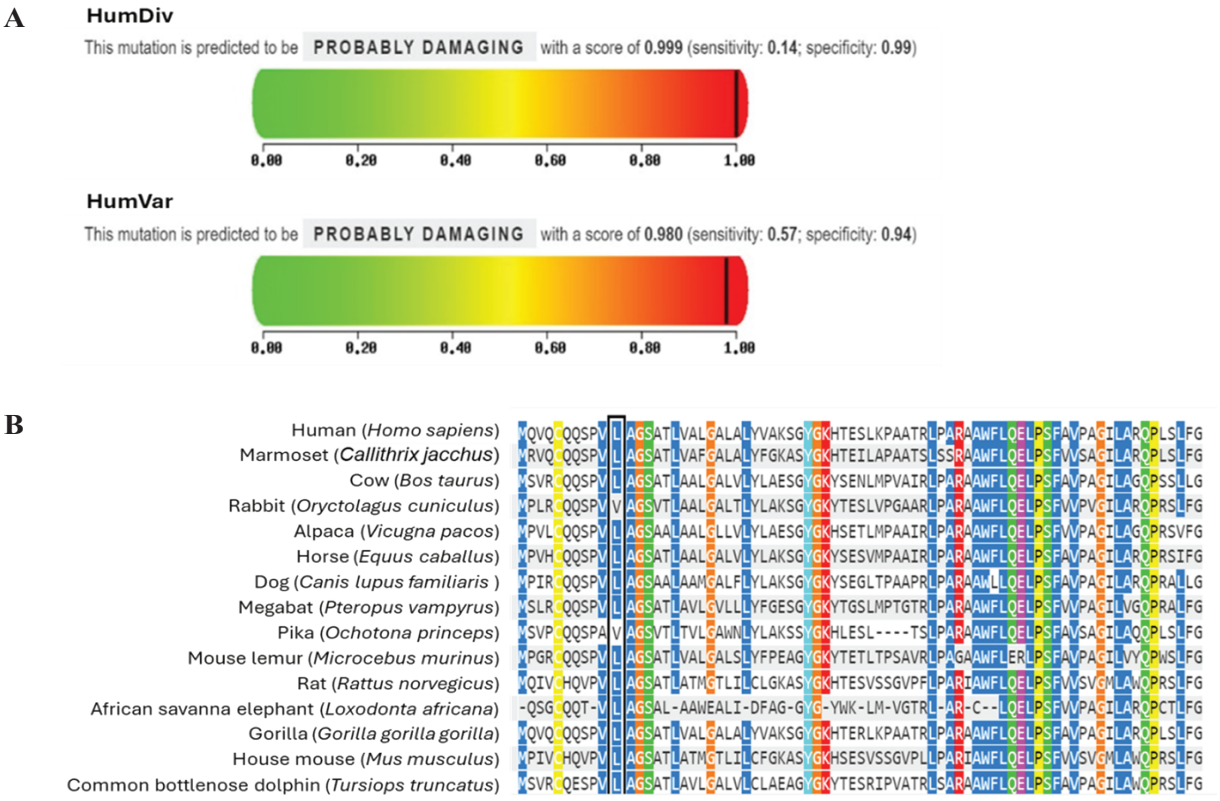

Figure 3. *In silico* analysis of the *SRD5A2* p.Leu11Pro mutation using PolyPhen-2. A: The mutation was predicted to be probably damaging with the score of 0.999 in HumDiv and 0.980 in HumVar. B: Multiple sequencing alignment of the p.Leu11Pro mutation showed that the amino acid was located in a conserved region across different species.

different between the isolated and syndromic hypospadias. We performed a cross-sectional association analysis of the three identified polymorphisms (rs632148, rs523349, rs522638) and the type of hypospadias (syndromic versus isolated), showing that the identified polymorphisms were associated with syndromic hypospadias with OR 3.4, 2.5 and 3.13 respectively. Other phenotypes besides hypospadias common in syndromic cases in our study population include micropenis (26%), followed by penoscrotal transposition (9.8%). These two comorbidities, as well as hypospadias, are anomalies of male external genitalia which typically indicate an androgen deficiency.(5) Unfortunately, the level of androgen hormones such as testosterone or DHT was not measured in these subjects. However, as *SRD5A2* encodes an enzyme important in the activation of the androgen signaling pathway, and identified polymorphisms are overrepresented in syndromic hypospadias with external genitalia-associated anomalies (micropenis, penoscrotal transposition, etc.), one could assume that the identified polymorphisms in this study are functional polymorphisms. Among these polymorphisms, the mutant allele of p.Val89Leu/rs523349 results in a 30% reduction of enzyme activity, thus reduces the level of testosterone metabolites.(28,29) Another polymorphism (c.-62G>C/rs632148) is located in the promoter region (5' UTR), which might disturb the binding of trans-activating factors like transcription factors or RNA polymerase, therefore reduces the activation of transcription. The polymorphism c.281+15T>C/rs522638 is located 15 bp downstream of exon-1, which might disturb the splicing process. Further studies involving more subjects were needed to confirm the findings from this study, and the measurement of the enzyme encoded by *SRD5A2* will aid us to better understand the role of *SRD5A2* in the etiology of hypospadias. *In silico* analysis was conducted to predict the pathogenicity of the variants, however functional studies are still required to clarify the effects of the variants associated with hypospadias.

Conclusion

A potential pathogenic mutation in *SRD5A2* (c.32T>C) resulting in a change of leucine to proline at the amino acid sequence 11 in the SRD5A2 protein (p.Leu11Pro) was

identified in one individual with isolated hypospadias. This study also reveals that the rs632148, rs523349 and rs522638 polymorphisms significantly associated with syndromic hypospadias, therefore, might be potential risk factors for the development of syndromic hypospadias.

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Authors Contribution

YS, RD and HH were involved in research conception and design. RD was involved in data acquisition and statistical analysis. RD, YP and YS analyzed and interpreted the data. RD, YS and YP drafted the original manuscript. HH and YS were involved in critical revision of the manuscript. RD, HH, and YS obtained funding. YS managed the administrative, technical, and material support. HH and YS supervised the study. All authors approved the final manuscript.

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