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ARTICLE

Mutation analysis of five candidate genes in Chinese patients with hypospadias

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Hypospadias is the displacement of the urethral meatus from the tip of the glans to the ventral side of the phallus. During fetal development, *SRY*, *SOX9*, *WT1*, *SRD5A2* and *AR* are important at different stages in the differentiation and development of the male genital system. Mutations in these genes impair masculinization and may be associated with hypospadias. In order to explore these possibilities, we employed polymerase chain reaction and direct sequencing to analyze the coding regions of these five genes in 90 Chinese hypospadias patients. We found a total of 16 different mutations in *SRD5A2*, *AR* and *WT1* in 24 of these 90 patients. Seven mutations are novel. No mutation was found in *SRY* or *SOX9*. SNP V89L found in *SRD5A2* was statistically significant between patients and controls. Our results indicated that mutations in *SRD5A2*, *AR* and *WT1* were associated with hypospadias. In conclusion, mutations are frequently found in genes that control androgen action and metabolism, but are seldom found in genes active in the early phase of sex determination and differentiation. Mutations in *AR*, *SRD5A2* or *WT1* seem to be associated not only with hypospadias but also with micropenis.
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Introduction

Hypospadias (OMIM 146450) is a disorder of male external genital development in which the urethral orifice is found on the ventral surface of the penis rather than at the tip of the glans. It is a common congenital abnormality with an incidence of 0.03–0.4%.[1] It may be classified as simple (glandular or penile) or severe (penoscrotal, scrotal or perineal) on the basis of the anatomical location of the urethral meatus. The cause of hypospadias is complex and poorly understood. In most cases it occurs sporadically, but families exist in which hypospadias segregates as a dominant trait, showing that a genetic cause is undoubtedly involved in at least a subset of cases.[2] Multifactorial models have yielded heritability indices ranging from 57 to 74% from pedigree data, indicating a multifactorial model of inheritance.[3]

Normal male genital development involves a coordinated interplay between several gene products (Figure 1), all of which are important for normal male reproductive tract development. The sex-determining region Y gene (*SRY*) is the initial switch to male sexual differentiation by triggering development of Sertoli cells. SRY box 9 (*SOX9*) may be the direct downstream target of SRY in the sex-determination cascade,[4] and is involved in the regulation of Sertoli cell-specific expression of anti-Müllerian hormone and the synthesis of testosterone in fetal testes.[5] The Wilms' tumor 1 gene (*WT1*) is a long-term expression gene during embryonic sex development and has a male-specific role in earlier and later sex determination.[4,6] Heterozygous

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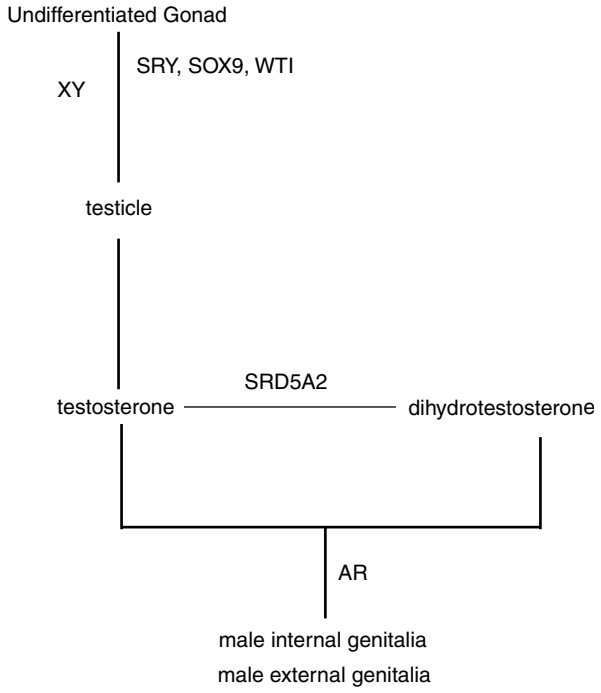

Figure 1 Simplified scheme of male sex differentiation and sex organ formation.

loss-of-function germ-line mutations in *WT1* are associated with mild effects on sexual differentiation, including hypospadias and cryptorchidism.[7] Development of the urethral and external genital system in the male fetus is an androgen-dependent process, requiring dihydrotestosterone (DHT) formed from testosterone by the action of steroid 5α-reductase type 2 (*SRD5A2*). Mutations of *SRD5A2* that reduce or inhibit enzyme activity result in diminished DHT formation, and cause masculinization defects of varying degrees, including hypospadias.[8] Both DHT and its precursor, testosterone, have their biological activities via binding to the androgen receptor (*AR*), which subsequently binds to the androgen-responsive element on DNA and regulates gene transcription.[9] It has been demonstrated that mutations of *AR* are definitely associated with hypospadias.[10–12]

SRY, *SOX9*, *WT1*, *SRD5A2* and *AR* are important in the development of the male genital system and act at different stages, with *SRY* and *SOX9* acting at early stages, *SRD5A2* and *AR* at late stages, and *WT1* at all stages. Mutations in these genes impair masculinization and may be associated with hypospadias. In order to explore these possibilities and to investigate the mutation distribution, the five genes were selected for analysis in Chinese hypospadias patients and normal controls.

Materials and methods

Experimental subjects

We identified 90 Chinese patients with hypospadias from the Hypospadias Treating Center of the Plastic Surgery Hospital affiliated with the Peking Union Medical College. Clinical phenotypes, family histories and complications of the 90 patients are listed in Table 1. All patients included were 46,XY. Informed consent was obtained from all patients or their parents.

A total of 276 unrelated, normal Chinese males were included as the comparison group. Control group 1 consisted of 96 subjects whose DNA was sequenced for all loci where mutations were found in hypospadias cases; control group 2 consisted of 180 subjects whose DNA was examined to determine the occurrence of novel mutations first identified in cases. The mean age of both control groups was 27 years. Routine medical examinations were performed on all controls before they were enrolled in the study.

Table 1 Clinical data of 90 patients with hypospadias

Type of hypospadias	*Glandular*	*Penile*	*Penoscrotal*	*Scrotal*	*Perineal*
No. of cases	7 (7.8%)	22 (24.4%)	33 (36.7%)	17 (18.9%)	11 (12.2%)
Average age (years)	9.0	10.3	9.2	14.6	10.9
Family history	5	6	19	14	7
Hypospadias	2	0	3	4	3
Sterile	3	2	3	0	2
Other deformities[a]	0	3	7	6	2
Complications	5	4	16	17	11
Micropenis[b]	3	3	9	10	5
Cryptorchidism	0	1	3	3	2
Gynecomastia	2	0	2	1	3
Hernia	0	0	1	1	0

[a]Other deformities include: cleft lip, cleft palate, deformities of hand and heart, etc.
[b]Micropenis: length of penis is more than −2.5SD from the average penile length in males of matched age.

Polymerase chain reaction (PCR) amplification and sequencing

Genomic DNA was extracted from the peripheral blood leukocytes of patients using standard procedures. PCR was performed to amplify all the exons and the exon–intron boundaries of *SRD5A2*, *AR*, *WT1*, *SOX9* and the single exon of *SRY* with primers that we designed using Premier 5.0 software (Table 2). Exon 1 of *AR*, exons 1 and 5 of *SRD5A2*, exon 1 of *WT1*, single exon of *SRY* and exons 1 and 3 of *SOX9* were divided into seven, two, five, two, two, two and three segments, respectively, during their amplification in order to facilitate their subsequent sequencing. PCR was performed in a 50 μl volume and thermal cycling was performed in a GeneAmp PCR System 2700 (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) (detailed PCR conditions are available upon request).

PCR products were purified using MultiScreen-PCR plate (Millipore). Using sequencing primers (Table 2), the purified PCR products were sequenced directly with the ABI 3700 DNA sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). Sequencing results were analyzed using the Phred/Phrap/Consed software. When differences from the normal were found, purified PCR products from these patients were sequenced again to

Table 2 Primers sets for PCR

Gene	*Exons*	*Forward primers*	*Reverse primers*
SRD5A2	1A	tgagaaaggggtattgctgcg[a]	aggtggcccgaagagggag
	1B	tgcaggttcagtgccagcagagc	ttggcgttcctcggtgcgcg[a]
	2	gaggtggggatgagaccatgttc[a]	gttttgcgatgggaaatggc
	3	ccctcctttcattttagcttagtttg[a]	ttcgtgccctcactgtccc
	4	ttcttgctgcctttgtgtattttg[a]	ttcggtttctcaatcttcctctgc
	5A	taactgtaggttgacttgtaacaaag[a]	catgtacttggattgcccgg
	5B	agtttactcctacccttccag[a]	aatagccataccagttttccg
	5C	agaccttgaatacaggagccc[a]	ttttctgcccagacgtgcc
	5D	tacagcagaagccccaagcaac[a]	gcattccgcaaacataggcc[a]
	5E	atacagagcccacatttccacacc	tctttatttccagcacacagccc[a]
AR	1A	gggaggcggggtaagggaag[a]	ccgactgcggctgtgaagg[a]
	1B	ctccccaagcccatcgtag[a]	tgtcagaaatggtcgaagtgc
	1C	agcagcagcagcagcaagag[a]	ttaagccggggaaagtggg
	1D	acgaggatgactcagctgccccatc[a]	gtagtccagcgggttctccagcttg[a]
	1E	caagggaggttacaccaaag	atacaactggccttcttcgg[a]
	1F	gcttcctcatcctggcacac	cgaaaggcgacatttctgg[a]
	1G	tgccgctatggggacctgg[a]	cggtgaagtcgctttcctg
	2	aatgctgaagacctgagacttcac[a]	taagttatttgatagggccttgcc
	3	tgtccacttttttcatgtggtagg[a]	cacgttgcctatgaaagggtcag
	4	tctgtgaccagggagaatg[a]	cttatctcatgctcccacttc
	5	tcagtacccagactgaccactgc	ccatcaccaccaaccaggtctg[a]
	6	ctgggcttattggtaaacttcccc	tttaatggcaaaagtggtcctctc[a]
	7	tatcctttcccctgagatctc[a]	gctgttctccctgataaagcacc
	8	ctccttgtcaaccctgtttttctccc	gaacatgttcatgacagactgtac[a]
WT1	1A	gccagagcagcagggagtc	ccggaaaagtggacagtgaagg[a]
	1B	ttcggcttacgggtcgttg	cgcttccgctatcctcacg[a]
	2	caccgctgacactgtgcttc	ccttttagatgtcctcctttgc[a]
	3	ccaggctcaggatctcgtg	tccaagacccagcatgcc[a]
	4	tttgtggttatgtgttctaactc	aggaggaaagcgttctaatgtcac[a]
	5	gggacttagttcagcactcttg[a]	aatgctaccctgattacccac
	6	aaaaccatcattccctcctg[a]	gggccaaagagtccatcag
	7	taaagcctcccttcctcttac	aaagcagtgcttactttccatc[a]
	8	atccccttttccagtatcattttc	gggtgtttccttttctttctttttc[a]
	9	ggggactggggaaatctaag	agccacgcactattccttc[a]
	10	tcagggacagaatgatggg	taaaagtaggcagggcagag[a]
SRY	1A	tcagcagggcaagtagtc[a]	gtggctttcgtacagtcatc[a]
	1B	tagagtgaagcgacccatg[a]	tgtccgtttgtatcaagttag[a]
SOX9	1A	tgagccacagttacaccccattc	gcgagcccgatctgaagaag[a]
	1B	cttgacgtgcggcttgttc	cgccttcctaagtgctcgc[a]
	2	gtgttgtttggatgcctcttag[a]	ggaagccgagtggtctgg
	3A	tttgggtacgagttgcctttag[a]	gcactcgccccaacagatc
	3B	ggtgaaggtggagtagaggc[a]	tacacgggcagctacgg
	3C	ctcagcgtggtcagcgtg	gcactcgccccaacagatc[a]

[a]Also used for sequencing.

confirm the original results. In addition, purified PCR products from some patient parents and normal controls were sequenced for analysis.

Statistical analysis

The software Epi 5.0. was used for statistical analysis of genotypic and allele distribution of detected SNPs. Test for statistical differences was performed using the χ^2 test or Fisher's exact test, as appropriate. Odds ratios (OR) and 95% confidence intervals (95% CIs) were also calculated for SNPs found to be significant by pairwise comparisons.

Results

We found a total of 16 different mutations in *SRD5A2*, *AR* and *WT1* genes in 24 of our 90 hypospadias patients and seven parents (Table 3). No mutation was found in *SRY* or *SOX9*. Eight different mutations were found in *SRD5A2* in 14 of our cases. Three are novel (Q6X, nt656 delT, H232H). Three different novel mutations were found in *WT1* in four of our patients. Five different mutations were found in *AR* in six of our patients. One is novel (L859L). The *AR* contains a polymorphic CAG repeat sequence for the polyglutamine tract in exon 1. CAG repeat numbers were obtained only in 61 patients because repeat sequences were often unreadable with the direct sequencing methods used. The repeat lengths were from 22 to 25 and within the normal range. No mutations in *SRY*, *SOX9*, *WT1* and *AR* were found in control group 1. G203S and F234L in *SRD5A2* were found in four and one controls, respectively, in control group 1, and H232H in *SRD5A2* was found in one control in control group 2.

In all, 10 SNPs were found in *SRD5A2*, *WT1* and *SOX9* genes. Three SNPs in *SRD5A2* were detected. One is novel: G/A in 3′UTR of exon 5 (dbSNP accession number ss# 12675709). One was statistically significant ($P=0.007$) (Table 4): nucleotide 265 G/C in exon 1, which leads to amino acid V89L. Six SNPs in *WT1* and one SNP in *SOX9*

Table 3 Genotype and phenotype of patients carrying mutations

Gene	Patient no.	Nt. change	Amino-acid change	Exon	Allele[a]	Phenotype				Others
						Hypospadias type	Micropenis[b]	Bifid scrotal	Other abnormal	
AR	4	1991C>T	I664T	4	Hem	Glanular	No	No	Gynecomastia	
	86	2519G>A	R840H	7	Hem	Perineal	Yes	Yes	Not found	Mother was Het[a] R840H
	84	2525T>C	I842T	7	Hem	Scrotal	Yes	Yes	Not found	Mother and maternal-grandmother were Het[a] I842T
	8	2564G>A	R855H		Hem	Perineal	Yes	Yes	Not found	Mother was Het[a] R855H
	9	2564G>A	R855H	7	Hem	Perineal	Yes	Yes	Not found	
	1	2577C>A	L859L	7	Hem	Penile	No	No	Not found	
SRD5A2	83	16C>T	Q6X	1	Hom	Scrotal	Yes	Yes	Cryptorchidism	
	44	607G>A	G203S	4	Hom	Perineal	Yes	Yes	Not found	
	39	680G>A	R227Q	4	Hom	Scrotal	Yes	Yes	Not found	
	73	680G>A	R227Q	4	Hom	Penile	No	Yes	Not found	
	81	737G>A	R246Q	5	Hom	Scrotal	No	Yes	Cryptorchidism	
	16	16C>T	Q6X	1	Het	Scrotal	Yes	Yes	Cryptorchidism	Father was Het[a] Q6X
		607G>A	G203S	4	Het					Mother was Het[a] G203S
	87	607G>A	G203S	4	Het	Scrotal	Yes	Yes	Not found	
		671T>A	L224H	4	Het					
	88	607G>A	G203S	4	Het	Scrotal	Yes	Yes	Not found	Father was Het[a] L224H
		671T>A	L224H	4	Het					
	89	607G>A	G203S	4	Het	Scrotal	Yes	Yes	Not found	
		671T>A	L224H	4	Het					
	64	607G>A	G203S	4	Het	Penile	No	Yes	Not found	
	20	656delT		4	Het	Perineal	Yes	Yes	Cryptorchidism	
	56	680G>A	R227Q	4	Het	Glanular	No	No	Not found	
	60	696T>C	H232H	4	Het	Scrotal	No	Yes	Not found	
	40	702C>G	F234L	5	Het	Penoscrotal	Yes	Yes	Not found	
WT1	74	390C>T	N130N	1	Het	Penoscrotal	Yes	No	Not found	
	79	390C>T	N130N	1	Het	Penile	No	No	Not found	
	85	391G>A	A131T	1	Het	Penile	No	No	Not found	
	65	477C>T	S159S	2	Het	Glanular	No	No	Not found	

[a]Hem: hemizygous; Het: heterozygous; Hom: homozygous.
[b]Micropenis: length of penis is more than −2.5SD from the average penile length in males of matched age.

Table 4 Genotype distribution of V89L in patients and controls (exon 1 of *SRD5A2*; nucleotide 265)

		Patients	*Controls*
Genotype	C/C	22	12
	G/C	52	44
	G/G	16	31
	Total	90	87
Allele frequencing	C[a]	0.53	0.39
	G	0.47	0.61

[a]Significantly elevated in patients, $P = 0.007$ (χ^2 test).

were found, respectively, and all of them were not statistically significant.

Discussion

In our study, four homozygous mutations of *SRD5A2* were identified in five patients. Patients 83 and 81 had scrotal hypospadias with cryptorchidism and bifid scrotum. Q6X was found in patient 83, and the mutant product is expected to contain only six amino acids, resulting in loss of enzyme activity. R246Q was found in patient 81. Thigpen *et al*[13] reported two patients who carried homozygous R246Q with perineoscrotal hypospadias, microphallus and cryptorchidism. This mutation reduces enzyme activity and affects the nicotinamide adenine dinucleotide phosphate (NADPH) Km in cultured genital skin fibroblasts. G203S was found in patient 44 with perineal hypospadias, bifid scrotum and micropenis. G203 is a conserved amino acid between human and mice, and it is possible that this variant contributed to severe phenotype of patient 44. Patients 39 and 73 carried the same mutation, R227Q, which is one of the most common mutations in Chinese patients.[14] It is known to inhibit NADPH binding, to reduce the binding of testosterone to the enzyme and the half-life of the protein.[8,13] Patient 39 had scrotal hypospadias with micropenis and bifid scrotum, while patient 73 had penile hypospadias with bifid scrotum. Thus, patients carrying the same mutation may present with different phenotypes. A similar phenomenon was also described by Sinnecker *et al*:[15] two brothers who carried homozygous R227Q mutations presented with different phenotypes: one had scrotal hypospadias with bifid scrotum and micropeins, and the other had only micropeins.

We identified two kinds of compound heterozygous mutations in *SRD5A2* in four patients who all had scrotal hypospadias with bifid scrotum and micropenis. Patient 16, with Q6X and G203S, also had cryptorchism. His father, the carrier of Q6X, and his mother, the carrier of G203S, were all healthy. Patients 87, 88 and 89 were brothers. They carried the same mutations (G203S and L224H) and presented with similar phenotypes. Their father was healthy and was the carrier of L224H. Canto *et al*[16] reported a patient with penoscrotal hypospadias, who carried G203S and G115D, and studies indicated that the activity of SRD5A2 and the synthesis of DHT decreased in cultured genital skin fibroblasts carrying these mutations.

In this study, some heterozygous mutations in *SRD5A2* were also found in five patients, healthy parents (Table 3) and controls. The role of heterozygous variants in disease occurrence is uncertain. H232H, which is novel and was found in one control out of 276, is considered a rare SNP. It has been reported that over half of the affected individuals with steroid 5α-reductase 2 deficiency were homozygotes, whereas 40% were either compound heterozygotes or inferred compound heterozygotes, suggesting that the carrier frequency of *SRD5A2* mutations in the populations studied may be quite high.[13] For these five patients, there might also be mutations that mapped outside of the exons and promoter region of *SRD5A2*, which were not explored in our study.

For the V89L SNP in *SRD5A2* (Table 4), 18% of cases and 36% of controls had the wild-type G/G genotype ($P = 0.007$, OR = 2.56 and 95% CI = 1.21–5.46). The frequency of the variant C allele is significantly elevated in cases compared to controls ($P = 0.007$, OR = 1.78 and 95% CI = 1.14–2.78). This substitution results in approximately a 30% reduction in enzyme activity.[17] These results suggest that the presence of the C allele is a genetic risk factor for hypospadias occurrence.

Homozygous and compound heterozygous mutations were found in nine out of 90 cases (10%) in the present study, suggesting that *SRD5A2* is a strong candidate gene for hypospadias. Our results indicated that genetic variants of *SRD5A2* contributed to hypospadias, although there were no apparent correlations between the genotype and severity of phenotype.

Five mutations in the *AR* gene were identified in six patients (46, XY), who are the hemizygotes for the *AR* gene. R840H was found in patient 86 with perineal hypospadias, bifid scrotum and micropenis. De Bellis A *et al*[18] reported a subject with the same mutation, who had an ambiguous external genital phenotype. Androgen-binding affinity in genital skin fibroblasts of this subject was seven-fold lower than control, and the mutant receptor had reduced transcriptional activity. R855H was found in patient 8 and his maternal uncle, patient 9. These cases had perineal hypospadias, bifid scrotum and micropenis. R855H has also been reported in two brothers with severe perineal hypospadias, bilateral cryptorchidism and micropenis. This amino-acid change is consistent with the more severe binding defect and clinical phenotype.[19] R840H and R855H are located in 'hot spot' regions, important for the formation and function of hormone–receptor complex

and within regions that are involved in AR dimerization, and could lead to the functional defect of receptors.[20,21] L859L, a synonymous mutation, was found in patient 1 with penile hypospadias, and it is unlikely to have a major impact on the patient phenotype.

All of the healthy fathers of patients 86, 84 and 8 had normal genotypes, while all their healthy mothers were heterozygous for the mutations found in their sons. The healthy maternal grandmother of patient 84 was also heterozygous for the mutation found in her grandson. Patient 8 and his maternal-uncle, patient 9, had the same mutation. These findings indicate that mutations in *AR* are transmitted according to the pattern of X-linked recessive disorders and play an important role in disease occurrence.

As the blood samples of normal children were difficult to collect, the mean age of controls was a little older than that of our patients. This is not likely to influence the findings from the present study as we focused on genomic variants that do not change with age, and we can be confident that the genitals of controls are normal as they were fully developed in adults.

A total of 24 patients out of the 90 studied (27%) had some mutations in the *SRD5A2*, *AR* and *WT1*. Of these 24, 14 had micropenis (accounting for 58%), whereas patients with micropenis accounted for 33% of all patients. This condition in patients 9, 86 and 87 was so serious that they had to undergo penile reconstruction. Our findings of an association between mutations in *AR*, *SRD5A2* or *WT1* and micropenis are in contrast to prior reports showing no associations between mutations in *AR* and *SRD5A2* and micropenis.[22] This could be due to different racial compositions in patients in each study.

Mutations in the candidate genes studied were found in 27% of patients, indicating that mutations in *SRD5A2*, *AR* and *WT1* are associated with hypospadias. Only five patients had hypospadias without any associated anomalies, which is consistent with previous reports.[23] Our results indicate that mutations are frequently found in genes that control androgen action and metabolism (*SRD5A2*, *AR*, *WT1*), but are seldom found in genes active in the early phase of sex determination and differentiation (*SRY*, *SOX9*).

Mutations in candidate genes were not found in 73% of our patients. Further studies should include evaluation of noncoding regions and the promoter areas of the candidate genes, other genes that mediate the process of male sex differentiation, abnormal expression of the genes, more detailed correlation between genotype and phenotype, and effects of certain environmental toxins.

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