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ORIGINAL ARTICLE

Male Endocrinology

Identification of three novel *SRD5A2* mutations in Chinese patients with 5α-reductase 2 deficiency

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In this study, we investigated the genetics, clinical features, and therapeutic approach of 14 patients with 5α-reductase deficiency in China. Genotyping analysis was performed by direct sequencing of PCR products of the steroid 5α-reductase type 2 gene (*SRD5A2*). The 5α-reductase activities of three novel mutations were investigated by mutagenesis and an *in vitro* transfection assay. Most patients presented with a microphallus, variable degrees of hypospadias, and cryptorchidism. Eight of 14 patients (57.1%) were initially reared as females and changed their social gender from female to male after puberty. Nine mutations were identified in the 14 patients. p.G203S, p.Q6X, and p.R227Q were the most prevalent mutations. Three mutations (p.K35N, p.H162P, and p.Y136X) have not been reported previously. The nonsense mutation p.Y136X abolished enzymatic activity, whereas p.K35N and p.H162P retained partial enzymatic activity. Topical administration of dihydrotestosterone during infancy or early childhood combined with hypospadia repair surgery had good therapeutic results. In conclusion, we expand the mutation profile of *SRD5A2* in the Chinese population. A rational clinical approach to this disorder requires early and accurate diagnosis, especially genetic diagnosis.

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Keywords: 5α-reductase type 2 deficiency; dihydrotestosterone; mutation; *SRD5A2*

INTRODUCTION

In male sexual differentiation, steroid 5α-reductase type 2 (5α-RD2), the enzyme that catalyzes the irreversible conversion of testosterone (T) to dihydrotestosterone (DHT), plays a crucial role in the formation of the external genitalia, urethra, and prostate.[1] Deficiency of 5α-RD2 is an autosomal recessive disorder first described in 1974.[2,3] At birth, affected 46,XY individuals often have disordered sexual development (DSD) characterized by perineoscrotal hypospadias, microphallus, and undescended testes with normal Wolffian duct derivatives.[4] The disorder presents a spectrum of phenotypes, ranging from female external genitalia to hypospadias with microphallus to apparently normal male external genitalia.[5–8]

There are two isoenzymes of 5α-reductase, both of which are hydrophobic and membrane bound. They share approximately 60% of amino acid sequence identity.[9] Steroid 5α-reductase type 2 gene (*SRD5A2*), the gene encoding 5α-RD2, is located on chromosome 2p23, whereas the gene encoding 5α-reductase-1 is located on chromosome 5. Both genes contain five exons and four introns.[4] It was suggested that 5α-reductase-1 is the major enzyme in the ovary, testis, nongenital skin, and liver, while 5α-RD2 predominantly exists in the male urogenital tract and female genital skin.[10] To date, more than 100 mutations of *SRD5A2* have been identified in individuals with different geographic and ethnic backgrounds.[11–13] Most mutations are detrimental to the enzymatic activity, leading to various degrees of undervirilization in 46,XY individuals.[6,13–16]

The initial diagnosis of 5α-RD2 deficiency was usually based on characteristic clinical signs, including microphallus, hypospadias, and gonads in the labial folds or inguinal region. Abnormal hormonal profiles, especially the elevated T/DHT ratio, are a widely used diagnostic tool,[17–19] but prepubertal patients require the measurement of T and DHT after human chorionic gonadotropin (hCG) stimulation. It has been found that DHT measurement by liquid chromatography-mass spectrometry (LC-MS) was superior to equivocal T/DHT ratios. As LC-MS assays are not available in most clinical centers in the mainland of China, genotyping analysis appears to be the most reliable way to diagnose the deficiency of 5α-RD2.

We investigated 14 Chinese patients with 5α-RD2 deficiency with variable clinical findings. Nine *SRD5A2* mutations, including three novel mutations, were identified in these patients. A functional study was performed to analyze the enzymatic activity by site-directed mutagenesis assays.

PATIENTS AND METHODS

Patients

Fourteen patients aged from 5 years to 34 years (diagnosis age) with 46,XY DSD were included in this study. Clinical diagnosis of 5α-reductase-2 deficiency was based on undermasculinization after

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birth (clitoromegaly, hypospadias with various degrees of microphallus, undescended testes, *etc.*), obvious virilization after puberty, normal T levels, and elevated ratios of plasma T to DHT (**Table 1**). The definitive diagnosis of steroid 5α-reductase-2 deficiency was confirmed by the combination of a clinical diagnosis and sequencing of the *SRD5A2* gene. The experimental protocols were approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China). Written informed consent was obtained from all of the adult patients themselves or from the child patients' parents, and the methods were carried out in accordance with the approved guidelines.

Hormonal studies

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and T were measured by chemiluminescent microparticle immunoassay (CMIA) on Abbott Architect plus system (Abbott Diagnostics, Il, USA). Moreover, serum DHT concentrations were assayed using a radioimmunoassay kit (Beckman Coulter, Texas, USA). The antibody used in the immunoassay is highly specific for DHT. The analytical sensitivity, or minimum detection limit, was 9.14 pg ml^{-1}. Extremely low cross-reactivities were obtained with several related molecules. The cross-reactivities with T, estradiol, androstenedione, and androstanediol were 0.02, 1.41, 1.90, and 0.25, respectively. Moreover, the cross-reactivities with cortisol, progesterone, and 11-deoxycortisol were nondetectable (≤0.01%). The reference range of DHT was 33.7–756 pg ml^{-1} in normal males and 17.7–246 pg ml^{-1} in healthy females. For hCG stimulation test, prepubertal patients received an injection of 1000–2000 U hCG (I-44020673, Lizhu, Shanghai, China) on days 1, 3, and 5, and serum samples were obtained on day 1 before injection and poststimulation on day 6.

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using a kit (TIANGEN Biotech, Beijing, China). Exons 1–5 of *SRD5A2* gene were amplified by PCR using five pairs of primers that had been described previously,[20] and direct sequencing was performed. PCRs were performed in a volume of 20 μl containing 10 μl of 2× Taq PCR Master Mix (TIANGEN Biotech), 100 ng of genomic DNA, and 5 pmol of each primer. DNA was first denatured at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C–60°C for 30 s, and 72°C for 40 s. A final DNA extension was performed at 72°C for 10 min.

Construction of mutant expression vector

The vectors pCDNA3.1-*SRD5A2*-WT and p.P212R mutant (used as a positive control) have been described previously.[20] The pCDNA3.1-*SRD5A2*-WT plasmid was used to generate mutants such as p.K35N, p.Y136X, and p.H162P by site-directed mutagenesis using the following primers:

K35N FOR: 5'-CTCCGGCTACGGGAATCACACGGAGA-3'; K35NREV:5'-ATTCCCTAGCCGGAGGGCTTCGCGA-3'; Y136X FOR: 5'-TTACTGTGCTGAATAACCTGATGGGT-3'; Y136X REV: 5'-TTATTCAGCACAGTAAATCAGATAG-3'; H162P FOR: 5'-TGGGAATAAACATTCCTAGTGACTAT-3'; H162P REV: 5'-GGAATGTTTATTCCCATTCCCAAAA-3'. Plasmids were isolated

Table 1: Clinical and genetic characteristics of the patients

Patient number	*Age of diagnosis (year)*	*Sex of rearing at birth*	*Phenotype*	*LH (U l^{-1})*	*FSH (IU l^{-1})*	*T (nmol l^{-1})*	*DHT (nmol l^{-1})*	*Basal T/DHT*	*SRD5A2 mutation*
1	22	Female to male	FEG+CM+G bilateral in labia majora No breast development; virilization at 13 years; phalloplasty at 32 years	4.2	2.5	32.9	0.3	99.6	p.Q6X/p.K35N/p.F234L
2	23	Male	MP (4 cm in length), G bilateral in scrotum, perineoscrotal hyp	7.1	3.1	25.5	0.5	50.9	p.G203S/p.R227Q/p.G34R
3	20	Male	MP (2 cm in length), G bilateral in inguinal position, perineoscrotal hyp	5.5	4.7	35.0	0.6	63.6	p.L20P/p.R246Q
4	19	Male	MP (2 cm in length), G bilateral in scrotum, perineoscrotal hyp; virilization	5.4	8.5	22.6	0.3	80.6	p.G203S/p.G203S
5	23	Female to male	FEG, CM, virilization, G bilateral in inguinal position, no breast development	18.4	32.9	15.5	0.2	91.1	p.A228V/---
6	24	Female	FEG, CM, G bilateral in inguinal position, single orifice	18.8	32.1	9.5	UD	UD	p.Q6X/---
7	30	Male	FEG, MP, G bilateral in inguinal position	3.6	11.8	11.0	UD	UD	p.Y136X/p.Y136X
8	18	Female to male	FEG, CM, G bilateral in inguinal position, virilization	12.3	23.5	29.6	0.2	123.2	p.Q6X/p.H162P
9	18	Female to male	FEG, CM, G bilateral in inguinal position, virilization	7.5	24.0	32.9	0.5	71.5	p.Q6X/p.H162P
10	5	Female to male	FEG, CM, G bilateral in inguinal position, perineoscrotal hyp	0.09	0.5	0.5-21.9	1.1-0.2	0.4-114.9	p.G203S/p.G203S
11	11	Female to male	FEG, CM, G bilateral in inguinal position, perineoscrotal hyp	1.4	2.0	1.0-8.4	0.05-0.6	20-14.7	p.Q6X/p.R227Q
12	23	Female to male	MP (2 cm in length), G bilateral in inguinal position, perineoscrotal hyp; virilization at 15 years	13.1	24.5	18.7	0.5	40.7	p.Q6X/p.N193S
13	18	Male	MP, G bilateral in inguinal position, perineoscrotal hyp	3.6	11.3	30.0	0.5	63.7	p.R171S/G196V
14	34	Female to male	MP (3.5 cm in length); perineoscrotal hyp; virilization at 15 year	5.7	17.4	24.4	0.6	43.5	p.L20P/p.R227X

FEG: female external genitalia; MP: micropenis; hyp: hypospadias; MPH: microphallus; CM: clitoromegaly; PA: primary amenorrhea; LH: luteinizing hormone; FSH: follicle-stimulating hormone; T: testosterone; DHT: dihydrotestosterone; UD: undefined. G: gonads. Reference ranges: LH 1.3–10.1 IU l^{-1}; FSH 1.4–13.6 IU l^{-1}; T 6.24–29.12 nmol l^{-1}; DHT 0.06–1.99 nmol l^{-1}

and purified with a NucleoBond® Xtra Midi EF kit (MACHEREY-NAGEL, Duren, Germany).

Transfection of 293T cells and 5α-reductase activity assays

Human embryonic kidney 293T cells were incubated in 12-well plates and transfected with 1.6 μg of purified plasmid using Lipofectamine 2000 (Life Technologies, Waltham, USA). After 48 h, the medium was replaced with 500 μl of fresh medium containing 100 000 cpm of ^{14}C-testosterone (PerkinElmer, Massachusetts, USA) and 500 nmol l^{-1} unlabeled testosterone. After incubation for 30 min, the medium was collected and the steroids were extracted twice using cyclohexane/ethylacetate (7:3). The steroids were harvested from the organic phase by speed-vac lyophilization. The steroids were dissolved in 20 μl of chloroform, spotted onto a thin-layer chromatography (TLC) plate (MACHEREY-NAGEL), and developed in toluene/acetone (80:20) for 1 h. The TLC plate was then exposed to hyperfilm (General Electric Company, Connecticut, USA) for 72 h. All experiments were replicated three times.

RESULTS

Clinical characteristics

The clinical features, hormonal data, and molecular analysis results of 14 patients with 5α-RD2 deficiency are summarized in **Table 1**. All patients had 46,XY karyotypes and various degrees of genital ambiguity. Nine of the 14 patients (64.3%) were initially raised as females. Among these, eight patients (57.1%) had changed their social gender from female to male (patient 6 had not). Two patients were prepubertal (patients 10 and 11). Twelve patients were postpubertal, and patient 4, who came from a consanguineous family, had a brother with a similar genital phenotype. Patients 8 and 9 were twin brothers who had similar phenotypes and were reared as girls until virilization occurred at puberty. Patient 1 was born with a predominantly female phenotype, including clitoromegaly and perineoscrotal hypospadias. Partial virilization (deepening of voice, laryngeal prominence) occurred from the age of 13 years, and the patient was diagnosed at 22 years old. Patient 1 changed social gender and accepted phalloplasty at 32 years old. Microphallus with various degrees of hypospadias and virilization after puberty were the most frequent phenotypes. None of the patients had gynecomastia. Topical DHT gel was used by patients 3, 4, 5, and 10 from different diagnostic ages. It was suggested that DHT therapy would be helpful to increase the phallic length and facilitate hypospadia repair in patient 10 (**Figure 1**). However, for patients 3, 4, and 5, DHT administration did not show obvious results of phallic enlargement.

Serum hormones

Baseline plasma T was determined in all cases, and the DHT concentrations were available in 12 patients. The mean (s.d.) values for T, DHT, and the baseline T/DHT ratio were 23.95 ± 8.68 nmol l^{-1}, 0.40 ± 0.14 nmol l^{-1}, and 72.85 ± 26.22, respectively. All of the basal T/DHT ratios were above 8.5 except in patient 10. HCG stimulation tests were conducted in patients 10 and 11.

DNA sequencing

All the five exons of *SRD5A2* were sequenced in all patients, and nine different mutations were identified in these 14 patients from 13 unrelated families (**Table 1**). Three mutations, p.K35N, p.Y136X, and p.H162P (**Figure 2a–2c**), have not been reported previously and were not found in 100 control individuals (**Figure 2d**). Compound heterozygous mutations were found in nine patients and homozygous mutations in three patients. Protein alignment showed that Y136 and H162 were conserved in different species. However, K35 was not conserved (**Figure 3**). In addition, patients 5 and 6 carried single heterozygous mutations, along with the p.V89L polymorphism. The most frequent mutation in our study was the nonsense mutation p.Q6X, found in one allele of six patients (patients 1, 6, 8, 9, 11, and 12). Interestingly, two affected individuals carried three different mutations: patient 1 with p.Q6X, p.F234L, and p.K35N and patient 2 with p.G203S, p.R227Q, and p.G34R. The polymorphism p.V89L was identified in 11 patients.

Steroid 5α-reductase enzymatic activity

The three novel mutations were functionally studied by expressing the mutant enzymes *in vitro* and assaying their steroid 5α-reductase activities with ^{14}C-labeled testosterone (the substrate). In 293T cells transfected with wild-type *SRD5A2* cDNA, a majority of ^{14}C-labeled testosterone was converted to dihydrotestosterone. By contrast, cells transfected with constructs expressing the novel mutant p.Y136X or the previously described mutant p.P212R[21] produced nearly undetectable DHT, whereas cells transfected with the p.K35N or p.H162P mutant

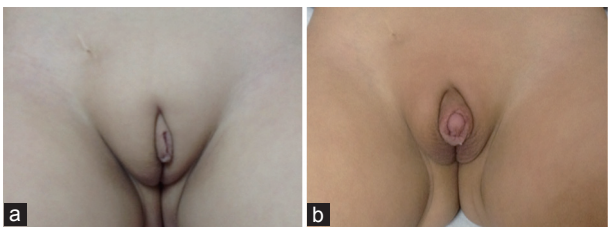

Figure 1: Clinical manifestation of patient 10. (**a**) Patient 10 accepted the first stage of hypospadias repair surgery when he was 4 years old. (**b**) Patient 10 after 2 months of DHT gel application. DHT: dihydrotesterone.

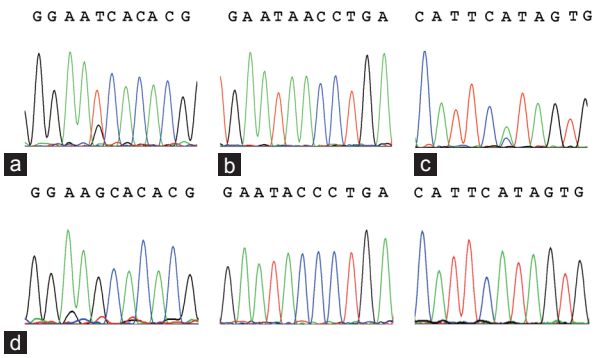

Figure 2: Sequencing result of three novel mutations in *SRD5A2* gene. (**a**) c.105 G>T (p.K35N) was identified in exon 1 of *SRD5A2* gene in patient 1. (**b**) c.408 C>A (p.Y136X) was detected in exon 2 of *SRD5A2* gene in patient 7. (**c**) c.485 A>C (p. H162P) was found in exon 3 of *SRD5A2* gene in patient 8. (**d**) Normal sequencing results, respectively.

	K35N ↓	Y136X ↓	H162P ↓
Homo sapens	VAKPSGYGKHTES-L	YLIYCAEYPDGWYTD	LGMGINIHSDYILRQ
Canis familiaris	LAKPSGYGKYSEG-L	YLIYCAEYPAEWYMD	LGMGINIHSDYILRQ
Pan troglodytes	VAKPSGYGKHTES-L	YLIYCAEYPDGWYTD	LGMGINIHSDYILRQ
Mus musculus	FGKPASYGKHSES-V	YLVYCAEYPEEWYTD	LGMGINIHSDCMLRQ
Rattus norvegicus	LGKPASYGKHTES-V	HMLHCTQYHSGWHRD	TGMAINIHSDYILRN
Cyprinus carpio	ITSHIPYGRYVNT--	CLVYVAEYPKDWCMD	LGMGINIHSDHILRR
Xenopus gallus	FTYPAAYGKHVATGK	YLIYCAEYPKDWCMD	LGMGINIHSDLLLRQ
Ornithorhynchus anatinus	MRRDINEGCHTPH-I	YLTYCAEYPDDWYTD	LGMGVNIHSDHLLRQ

Figure 3: Protein alignment among different species. Protein alignment showed that Y136 and H162 were conserved in different species. However, the conservative did not exist in K35 among different species.

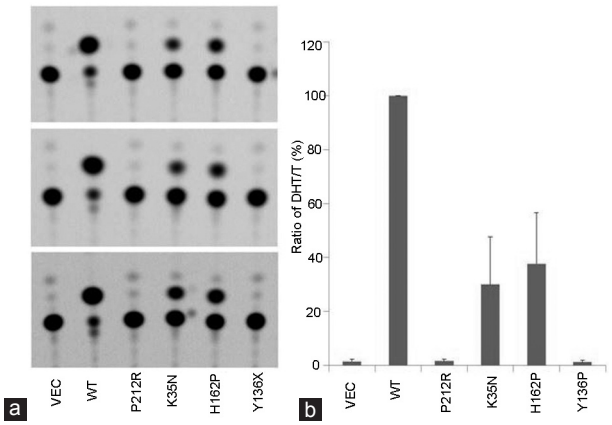

Figure 4: Enzymatic activity of wild-type and mutant steroid 5α-reductase. (**a**) 5α-Reductase activity assay in transfected HEK-293 cells. The conversion from T to DHT was obviously impaired in cells transfected with p.Y136X and p.P212R. Cells transfected with p.K35N and p.H162P mutant still could catalyze the conversion to some extent. (**b**) Figures were scanned, and conversion rate was calculated by the ratio of DHT/T. DHT: dihydrotestosterone; WT: wild type; VEC: empty vector; p.P212R: negative control.

catalyzed the conversion to some extent (**Figure 4a**). Figures were scanned, and the conversion rate was calculated by the ratio of DHT/T. The enzyme activities of SRD5A2-P212R, SRD5A2-K35N, SRD5A2-H162P, and SRD5A2-Y136X were 1.6%, 30.0%, 37.7%, and 1.2%, respectively (**Figure 4b**). Michaelis constant (Km) and maximum velocity of enzyme-catalyzed reaction (Vm) were calculated in WT (0.488 and 434.78), H162P (0.071 and 41.49), and K35N (0.392 and 5000.00) mutants by TLC (**Supplementary Figure 1**).

DISCUSSION

Andersson *et al.*[22] first reported that deletion of the *SRD5A2* gene is responsible for 46,XY DSD in a tribe in the New Guinea Highlands in 1991. Since then, molecular analysis of this disorder has revealed more than 100 *SRD5A2* mutations, including missense and nonsense mutations, splice-junction alterations, deletions, and insertions.[20] In our study, nine mutations were identified, among which p.G203S, p.Q6X, and p.R227Q were known mutations, whereas p.Y136X, p.H162P, and p.K35N have not been reported previously. By *in vitro* enzymatic activity assay, p.Y136X was found to abolish the enzymatic activity completely, whereas p.K35N and p.H162P reduced enzymatic activity moderately.

Consistent with a previous report,[20] one heterozygous mutation together with polymorphism p.V89L was identified in three patients. Nevertheless, we cannot exclude the possibility that these three patients had additional mutations of other DSD-causative genes. According to the GWAS data, the minor allele frequency (MAF) of V89L in the Chinese population is 31.7% (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=523349, last accessed on: May 30, 2018). The V89 allele has substantially higher enzymatic activity than L89, and heterozygotes for V89L have intermediate enzymatic activity.[23] Makridakis *et al.*[24] reported that the homozygous V89L variation of the *SRD5A2* gene decreased 5α-RD2 activity by approximately 30% and that heterozygotes for V89L had intermediate enzymatic activity. Although substitution of p.V89L in 81 Japanese patients showed no discernible effect on the development of micropenis, this relatively common nucleotide variation is considered a factor for the development of androgen-related disorders.[23]

A functional study of the three novel mutations in terms of enzymatic activity was performed. p.Y136X, found in patient 7 in a homozygous form and predicted to encode a truncated 135-amino-acid protein with a large portion of the C-terminus deleted, caused a complete loss of enzymatic activity. Meanwhile, mutagenesis and the *in vitro* enzymatic activity assay indicated that the p.H162P mutation led to synthesis of an enzyme with partial catalytic activity. p.K35N, one of the three mutations detected in patient 1, had a moderate impact on enzymatic activity. Interestingly, two patients carried three mutations in the *SRD5A2* gene, including patient 1 with p.Q6X, p.F234L, and p.K35N and patient 2 with p.G203S, p.R227Q, and p.G34R. Cloning and sequencing results suggested that p.Q6X and p.K35N in patient 1 did not reside in the same allele, and p.G203S and p.R227Q did not reside in the same allele in patient 2. Therefore, p.K35N appears not to have been the causative mutation in patient 1.

Since first reported by Nie *et al.*[25] the p.A228V substitution has only been identified in three Chinese patients and has not been detected in other countries. Although the impact of p.A228V on enzymatic activity has not been assessed before, the p.A228T mutation impairs the affinity of the enzyme to T and shortens the half-life of the protein.[10] In addition, Wilson *et al.*[26] demonstrated that nearly all mutations located between codons 197 and 230 caused the complete inactivation of the enzyme. Similarly, the change of arginine to glutamine at codon 227 reduced enzymatic activity to 3.2% of controls.[27] This mutation, which we found in two patients, has been reported in China, Japan, Vietnam, Mongolia, and Laos, indicating its prevalence among Asians.[5,28–31] In addition, p.R227X, detected in patient 14, has been reported previously in one Pakistani and one Mexican-American.[4]

Patient 10, a homozygote for p.G203S, underwent gender reassignment at 9 months of age after an operation for "hernias." He responded well after first-stage surgery and 2 months of DHT gel treatment. However, the use of DHT gel in patients 3 and 4, who were diagnosed after puberty, exerted little effect on external genital virilization, although they had more obvious masculine phenotypes. Topical application of DHT gel above the pubic area (25–50 mg daily) was recommended for prepubertal patients, in combination with hypospadia repair surgery. However, the effects were limited in the adult patients 3, 4, and 5, indicating the importance of early diagnosis and early treatment. By contrast, even after puberty, patients with hypogonadotropic hypogonadism always achieved good improvement with the administration of androgens, as a sexually functional penis is an important concern. Topical DHT gel shows effectiveness in the enlargement of penis size in prepubertal patients, which is also helpful for urethral reconstruction. The high frequency of social gender changes may partly reflect inappropriate sex assignment during infancy. The therapeutic window of DHT administration of 5α-RD2 remains to be addressed. Apparently, accurate diagnosis by mutational analysis is crucial to the early prognosis of DSD.

In conclusion, nine mutations were identified in 14 Chinese patients within 5α-RD2, including three novel mutations. They exerted various degrees of effects on enzymatic activity. Mutational analysis of *SRD5A2* is crucial to the early diagnosis of DSD. Application of DHT gel treatment from an early age, along with corrective surgery for cryptorchidism and hypospadias, has proved effective in improving the virilization of external genitalia when the patient is considering assuming the male gender.

AUTHOR CONTRIBUTIONS

JQ conceived and designed the study; YL contributed to patient recruitment and data collection; TC and HW contributed to plasmid construction and manuscript writing; BH performed enzymatic

activity analysis and contributed to the discussion; HZ performed DNA extraction and sequencing; HLZ participated in enzymatic activity analysis; WJZ supervised this investigation; HLZ contributed to the discussion; and FGC, HDS, HJY and KXC contributed to patient recruitment and follow-up. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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

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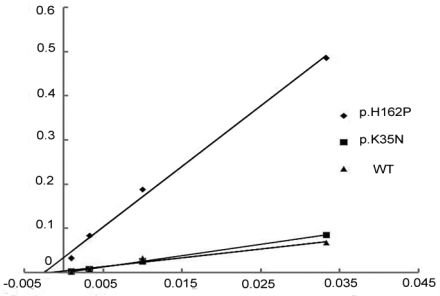

Supplementary Figure 1: Km and Vm values of wild-type and mutant steroid 5α-reductase.