Five novel mutations of SRD5A2 found in eight Chinese patients with 46,XY disorders of sex development

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ABSTRACT: Individuals with male karyotype (46,XY) affected by 5α-reductase type 2 deficiency, a rare autosomal recessive inherited disorder, can have an almost female phenotype or partially virilized external genitalia. Mutations in the steroid-5α-reductase (SRD5A2) gene, leading to functional impairment of 5α-reductase type 2, are responsible for this disorder. Our present study analyzed SRD5A2 gene mutations in eight unrelated 46,XY Chinese patients with disorders of sex development. Direct sequencing of genomic DNA for SRD5A2 gene revealed the presence of one homozygous (p.Q6X) and seven compound heterozygous mutations (p.G203S/R227Q, p.L20P/R227Q, p.Q6X/p.A228V, p.C222Ffs232X/p.R246Q, p.W140X/F219Sfs278X, p.Q71X/L185Tfs192X and p.Q6X/p.N193S) in our patients. Among them, p.C222Ffs232X, p.A228V, p.Q71X, L185Tfs192X and p.W140X mutations have not been previously reported. These novel mutations may provide us new insights into the molecular mechanism of 5α-reductase type 2 deficiency. Seven out of eight patients had at least one variant in exon 4, and 8 of 12 (66.7%) mutations were located in exon 4. The expanded mutation database of the SRD5A2 gene should benefit patients in the diagnosis and treatment of this disease.

Key words: 5α-reductase type 2 deficiency / 46,XY disorders of sex development / SRD5A2 / gene mutations / ambiguous genitalia

Introduction

5α-Reductase type 2 deficiency (OMIM number #264600) is a rare autosomal recessive inherited disorder. Affected 46,XY individuals who at birth usually present with ambiguous genitalia characterized by pseudovagina, microphallus, cryptorchidism and perineoscrotal hypospadias, and are often raised as females (Wilson et al., 1993; Imperato-McGinley and Zhu, 2002). During puberty, however, 56–63% of patients present spontaneous virilization without evidence of gynecomastia, frequently leading to a switch from female to male gender (Cohen-Kettenis, 2005). These changes may strongly affect patients’ mental and physical health.

In peripheral androgen target tissues, the conversion of testosterone (T) to more biologically active dihydrotestosterone (DHT), required for the normal masculinization of external genitalia, prostate and urethra in male embryo, is catalyzed by the 5α-reductase type 2 (Wilson et al., 1993; Imperato-McGinley and Zhu, 2002). The abnormal 5α-reductase type 2 as a result of mutations in the SRD5A2 gene could lead to 46,XY disorders of sex development (46,XY DSD). This gene is located at chromosome 2p23 with 5 exons, encoding a 254 amino acid protein containing at least four putative transmembrane regions and an androgen-binding domain at its N-terminal end (Wilson et al., 1993; Griffin et al., 2001; Vilchis et al., 2008). To date, more than 50 different mutations (http://www.hgmd.cf.ac.uk/, 20 January 2010 date last accessed) scattering throughout the gene have been reported worldwide, most are missense mutations, although premature stop codons and small deletions have also been described (Griffin et al., 2001).

Differential diagnosis of 5α-reductase type 2 deficiency with androgen insensitive syndrome (AIS) caused by mutations in the androgen receptor (AR) gene is quite difficult since the clinical features and hormonal tests are very similar (Griffin et al., 2001). The test of a post-hCG stimulation T/DHT ratio, which may help to distinguish 5α-reductase type 2 deficiency from AIS to some extent, however, is not available in most laboratories in China. In addition, the post-hCG stimulation T/DHT ratio in 5α-reductase type 2 deficiency...
may overlap with the values expected in AIS (Hiort et al., 1996; Mazen et al., 2003). Thus, genetic testing is a useful tool for a definitive diagnosis of 5α-reductase type 2 deficiency.

In this study, we performed mutational analysis of the SRD5A2 gene and the AR gene in 12 unrelated Chinese individuals with 46,XY DSD referred to our hospital for a better diagnosis and treatment. We found eight patients had defects in the SRD5A2 gene including five novel mutations and four patients affected by AIS confirmed by mutations in the AR gene, which had been described in our previous study (Wu et al., 2010).

### Patients and methods

#### Patients

Eight unrelated patients with incomplete virilization (mean of age: 10.5 years) were referred to our laboratory for genetic testing. In all cases, there was no history of consanguineous marriage for three generations. Each patient had a 46,XY karyotype. The clinical diagnosis of AIS or 5α-reductase type 2 deficiency was raised on the basis of medical history, the clinical presentation of hypospadias, microphallus, cryptorchidism, pseudovagina, lack of Mullerian derivatives and measurements of related hormones (serum FSH, LH and T levels). Informed written consent was obtained from all patients, and the study was approved by our institutional review board.

#### Hormonal testing

FSH, LH and T were measured using chemiluminescent immunoassays (Bayor Diagnostics Corporation, USA). HCG stimulation test was performed as below: T levels were measured before and after three alternate-day intramuscular injections of 3000 units of HCG (Zhuhai Pharmaceuticals, China).

#### Genetic analysis

Coding sequence abnormalities in the SRD5A2 or AR genes were assessed by PCR and then direct sequencing analysis. The complete coding region including intron–exon boundary, was amplified from genomic DNA obtained from peripheral blood leukocytes using standard methods. The primers used for PCR were designed by primer 3.0 (available at: http://frodo.wi.mit.edu/, the sequence listed in Table I). All PCRs included 20 pmol of each primer, 100 ng of DNA template, 25 μl of 2x easytaq mix (Tiangen Biotech, Beijing, China) and double-distilled water to obtain a final volume of 50 μl in a thermocycler (MPCR System PTC-200). Direct sequencing of PCR products was performed using a Taq big dye terminator sequencing kit and an ABI3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences generated from patients were compared with the published SRD5A2 sequence (accession no. DNA: NG_008365.1, cDNA: NM_000348, protein: NP_000339) and the AR sequence (accession no. DNA: NG_009014, cDNA: NM_000044, protein: NP_000035). When a mutation was detected, both sense and antisense strands were sequenced to confirm the change. To confirm the frameshift mutation, subclone sequencing was performed for those exons that were identified with heterozygous frameshift mutations by direct PCR sequencing. The purified PCR products were ligated to pGEM-T Easy vectors (Promega, Madison, WI, USA) and transformed into DH5α-competent cells. Positive clones were picked and sequenced by ABI PRISM 3700 genetic analyzer.

#### Predicted effects of SRD5A2 mutations

In order to predict the protein function affected by an amino acid substitution, we performed in silico analysis using two different softwares—‘SIFT’ (http://sift.jcvi.org) and ‘polyphen’ (http://genetics.bwh.harvard.edu/pph/). ‘SIFT’ predicts protein function based on sequence homology and the physical properties of amino acids. Whereas ‘polyphen’ predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

### Results

#### Clinical features

All patients with 5α-reductase type 2 deficiency had karyotypes of 46,XY and presented at different ages with various degrees of...
ambiguous genitalia as described in Table II. They had no family history of any related condition except Patient 8 whose brother had similar genital phenotype. Unfortunately, most of patients we studied were referred to our laboratory mainly for genetic analysis; therefore, the data of hormonal evaluation and medical history were incomplete.

Four patients were prepubertal at first examination (Table II, Cases 1–4). Cases 1–3, reared as females, were brought to our clinic due to ambiguous genitalia in the form of perineo-scrotal hypospadias, microphallus and palpable testes in the labia-scrotum. Pelvic ultrasonography revealed no Mullerian derivatives. Case 4, raised as a male, showed microphallus, penoscrotal hypospadias and palpable testes in the right inguinal canal and left testis palpable in the labio-scrotum.

Four patients were postpubertal (Table II, Cases 5–8). Case 5 presented at age 13 with female phenotype but with mild clitoromegaly and a blind ending vaginal pouch first came to medical attention mainly due to bilateral palpable masses in inguinal regions. Sex of rearing was maintained as female since she did not present overt virilization at puberty. Cases 6 and 7 presented with similar external genital phenotypes and showed prominent signs of virilization in the form of deepened voice, male pubic hair and temporal hairline recession during puberty. However, sex of rearing was maintained as females. Case 8, reared as female, was born with severe hypospadias. He had a simple hypospadias repair at the age of 21 at his local hospital due to presenting virilization and then changed his gender from female to male, but he had not acquired a definitive diagnosis and received any related drug treatment.

**Hormonal profile**

Among all patients, normal or increased basal T levels were measured (ranged from 5.8 to 788.5 ng/dl) according to age-related reference values in our laboratory. Three prepubertal patients were tested for hCG stimulation test, whose T-value increased to 689.7, 335 and 569.7 ng/dl, respectively and these were a marked rise compared with normal T-value of post-hCG. Other detailed hormone levels for each patient were listed in Table II.

**Mutational analysis**

After the entire SRD5A2 gene and AR gene had been sequenced in these 8 patients, we did not find any genomic mutation in the AR gene and identified 12 different mutations with 5 being novel in the SRD5A2 gene (Table II and Fig. 1).
A, p.G203S: this known causative mutation located in exon 4 was heterozygous in patient 1.
c.680 G>A, p.R227Q: this known mutation located in exon 4 was found in a heterozygous state in Patients 1 and 2, respectively. The father of Patient 2 was the carrier for the mutation.
c.59 T>C, p.L20P: a reported mutation located in exon 1 was heterozygous in Patient 2.
c.16 C>T, p.Q6X: this common nonsense mutation was found in heterozygous state in Patients 3 and 8, and in homozygous state in Patient 7.
c.683 C>T, p.A228V: a novel mutation in exon 4 was found in Patient 3, which was not found in 50 ethnic-matched control samples using direct sequencing analysis of exon 4.
c.663_664 del TT, p.C222F,fs,232X: in Patient 4, deletion of TT located in exon 4 was discovered in a heterozygous state. This new mutation caused a frameshift and yielded a premature stop codon at 232 which was not found in 50 ethnic-matched control samples.
c.737 G>A, p.R246Q: Patient 4 was heterozygous for this known substitution in exon 5.
c.419 G>A, p.W140X: this unreported nonsense mutation located in exon 2 was heterozygous in Patient 5.
c.655 del T, p.F219S,fs,278X: this known causative mutation located in exon 4 was found in heterozygous state in Patient 5, and the mother’s DNA analysis demonstrated its origin.
c.211 C>T, p.Q71X: a novel nonsense mutation in exon 1 was found in Patient 6 in a heterozygous state.
c.553_568 del tgctagtctgtt+ins AC, p.L185T,fs,192X: a new mutation in heterozygous state was found in Patient 6 in exon 4.

Results of in silico analysis
To predict the functional impairment of the mutant A228V protein, the ‘SIFT’ software was used. The score obtained for this new substitution was zero, which placed the mutation in the ‘Affect protein function’ class. This result was confirmed by another software application (‘polyphen’), with which this mutation was predicted to be probably damaging with a score of 0.997 (sensitivity: 0.33; specificity: 0.98).

Discussion
In our present study, we examined eight unrelated patients affected by 5α-reductase type 2 deficiency. Based on the observation of ambiguous genital phenotype, male karyotype and hormonal levels, and combined with prominent virilization at puberty in three postpubertal patients (Cases 6–8), the clinically tentative diagnosis of 5α-reductase type 2 deficiency was made. However, in other patients including four prepubertal (Cases 1–4) and one postpubertal (Case 5) with slight virilization during puberty, the clinical diagnosis of 5α-reductase type 2 deficiency was somewhat difficult because of clinical manifestations similar to those of other conditions, such as AIS, Leydig cell
hypoplasia, 17α-hydroxylase/17,20-lyase deficiency and 17β-hydroxysteroid dehydrogenase deficiency (Faienza et al., 2008; Kossack et al., 2008; Rosa et al., 2010; Wu et al., 2010). In light of normal levels of serum basal T, hydroxyproline (17-OHP), adrenocorticotropic hormone, plasma renin activity (data not shown) and elevated serum T levels after hCG stimulation, 17α-hydroxylase/17,20-lyase deficiency, 17β-hydroxysteroid dehydrogenase deficiency and Leydig cell hypoplasia could be ruled out. The exclusion of AIS, however, was quite difficult because the test of a post-hCG stimulation T/DHT ratio, which may help to distinguish 5α-reductase type 2 deficiency from AIS to some extent, was not available in our laboratories. Moreover, the post-hCG stimulation T/DHT ratios are variable according to age and the severity of the enzyme defects (Hiort et al., 1996; Mazen et al., 2003). Therefore, precise diagnosis may be achieved by mutational analysis of the genes encoding 5α-reductase type 2.

In our patients, 12 different mutations with 5 being novel were identified in the SRD5A2 gene and 7 of 8 affected individuals were compound heterozygotes. Moreover, we have observed that 7 of our 8 (87.5%) patients had at least one variant in exon 4, and 8 of 12 (66.7%) mutations were located in exon 4. This is consistent with previous reports (Vilchis et al., 2008, 2009), suggesting that exon 4 may be the mutational hotspots in the SRD5A2 gene.

p.A228V, p.W140X, p.Q71X, p.C222Ffs232X and L185Tfs192X are novel mutations we identified in this study. Although no studies were performed to assess the functional consequences of these mutations such as enzymatic activity, there is some evidence indicating them as pathogenic mutations. p.A228V missense mutation is caused by an ACT substitution, p.A228T) was predicted a probable pathogenic nature for this alteration. The result was confirmed by another software application ('polyphen') which placed the mutation in the ‘Affect protein function’ class. The score obtained for this new substitution was zero. However, was quite difficult to confirm the p.A228T mutation.

A homozygote of two Korean sisters with mild clitoromegaly (Kim et al., 1997; Sasaki et al., 2008, 2009) and have been described in several different populations (Canto et al., 2004; Sahakitrungruang et al., 2008). Similar, the p.F219Sfs278X mutation introduces a frameshift at codon 219, leading to an aberrant long protein product due to an extended termination signal at codon 278 which was also described only once in a homozygote of two Korean sisters with mild clitoromegaly (Kim et al., 2006). In contrast, the p.G203S and p.Q6X mutations are recurrent and have been described in several different populations (Canto et al., 1997; Sasaki et al., 2003; Wang et al., 2004; Sahakitrungruang et al., 2008; Vilchis et al., 2009).

In conclusion, we have identified 12 different mutations with 5 being novel in the SRD5A2 gene from 8 patients with 46,XY DSD. These novel mutations may provide new insights into the molecular mechanism of 5α-reductase type 2 deficiency. The expanded mutation database of the SRD5A2 gene should benefit patients in the diagnosis and treatment of this disease.

**Authors’ roles**

M.N. was involved in every aspect of this project, produced a large part of the results and helped to prepare the manuscript. Q.Z. did
a part of experiment and prepared the manuscript. S.L. measured the levels of hormone. J.M. collected the blood samples and involved in research consent and ethics. X.W. designed the study and provided mentorship.

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