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 (Wu et al., 2010). 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).

**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

### Table I The primer sequences, the annealing temperatures and the product sizes for SRD5A2 and AR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
<th>Annealing temperature (°C)</th>
<th>Amplified product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRD5A2_1</td>
<td>CGGCGAGTGTGGAGGAGGCTGTGAG</td>
<td>TGGGGGAGTGAAGGCGGCCTCGTG</td>
<td>70</td>
<td>650</td>
</tr>
<tr>
<td>SRD5A2_2</td>
<td>GATAATTTGATTTGGGTGTAAG</td>
<td>TGGCGATGTAAGGTTG</td>
<td>52</td>
<td>369</td>
</tr>
<tr>
<td>SRD5A2_3</td>
<td>CCCACCTGTCCACCGCTCTAGGA</td>
<td>TGTATCATCTGGTCCACACTGCCTG</td>
<td>55</td>
<td>350</td>
</tr>
<tr>
<td>SRD5A2_4</td>
<td>TTGGCATGATTGACCTTCCGATTC</td>
<td>CAATACAGGCGAGCAAGTCGAG</td>
<td>55</td>
<td>352</td>
</tr>
<tr>
<td>SRD5A2_5</td>
<td>CCAGAGAAACACTATGAAAGCTCAC</td>
<td>GGCCAGGAGGACACTG</td>
<td>52</td>
<td>340</td>
</tr>
<tr>
<td>AR_1a</td>
<td>CGGATCTACAGGCTGCTGTAAC</td>
<td>GCGGTTCGAGAAATGTCG</td>
<td>52</td>
<td>796</td>
</tr>
<tr>
<td>AR_1b</td>
<td>GGCAGGAGTACGAGTCAACT</td>
<td>ACCACACCGTTGATCATACAC</td>
<td>62</td>
<td>796</td>
</tr>
<tr>
<td>AR_1c</td>
<td>CATCCCACTGCGTCGTAGCAG</td>
<td>TCGACCAAGGGGTCGACC</td>
<td>52</td>
<td>683</td>
</tr>
<tr>
<td>AR_2</td>
<td>AATGCCAGGAGACTCTGCTTAAC</td>
<td>TAAATTTTCTTTTCTTAGG</td>
<td>55</td>
<td>653</td>
</tr>
<tr>
<td>AR_3</td>
<td>GAAATGCAAAATATAGTGGCACA</td>
<td>CATCAGGGCAGTATCAGTAAATG</td>
<td>55</td>
<td>640</td>
</tr>
<tr>
<td>AR_4</td>
<td>TATGAAAAATGGGTTATAGCAG</td>
<td>CACATAAGACACCCGATAAT</td>
<td>55</td>
<td>716</td>
</tr>
<tr>
<td>AR_5</td>
<td>GTCCCTTTTCCCCACACTTAAATG</td>
<td>GGGGCTATCCGCTGCGAATC</td>
<td>62</td>
<td>432</td>
</tr>
<tr>
<td>AR_6</td>
<td>TGGACAGGAGAGATGAGTATGA</td>
<td>TCTAGCAATAAAAAATTGCTC</td>
<td>55</td>
<td>577</td>
</tr>
<tr>
<td>AR_7</td>
<td>AACTACAGGAGCGCAAGTAGCTAG</td>
<td>TCCCAAGAGATCTTATACATT</td>
<td>55</td>
<td>571</td>
</tr>
<tr>
<td>AR_8</td>
<td>GGGAGGAGCAAGAAGTAGCAG</td>
<td>GCCATGGGAGGTTGATAGG</td>
<td>55</td>
<td>511</td>
</tr>
</tbody>
</table>
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, no vaginal pouch, bilateral testes palpable in the labio-scrotum and left testis palpable in the inguinal canal as further confirmed by ultrasound examinations.

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).
c.607 G>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.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 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 tgttctagtgttt+ins AC, p.L185Ts,fs,192X: a new mutation in heterozygous state was found in Patient 6 in exon 4.

Deletion of 16 nucleotides and insertion of 2 nucleotides between positions 553 and 568 at cDNA caused a frameshift and yielded a premature stop codon at 192, which was not found in 50 ethnic-matched control samples.
c.578 A>G, p.N193S: Patient 8 was heterozygous for this known substitution 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 a C > T transversion at nucleotide position 683 in exon 4. At the same codon, another mutation (GCT → ACT substitution, p.A228T) was reported and found in a boy presented with hypospadias and micropenis (Nordenskjöld et al., 1998). The A228T mutant product has been found to be associated with impairment of T binding capacity and the half life of the protein shortening (Wigley et al., 1994). In addition, other evidence explains this variant as a causative mutation. First, the amino acid A228 is highly conserved (Fig. 2). This position contains an identical amino acid in seven organisms displayed in the National Center for Biotechnology Information database. Second, the ‘SIFT’ software was used to predict protein function affected by the mutant p.A228V. 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 (‘polyphe’) which also predicted a probable pathogenic nature for this alteration. Last, Wilson and Vilchis et al. found that almost all mutations located between codons 197 and 230 can render the enzyme completely inactive (Wilson et al., 1993; Vilchis et al., 2008, 2009).

Two novel nonsense mutations, p.Q71X and p.W140X, were located at exon 1 and exon 2, respectively. Therefore, the mutant products of p.Q71X and p.W140X were expected to be drastically truncated proteins only containing 70 and 139 amino acids, respectively. These two mutant products lost several important regions, including two or three transmembrane domains (the second to the fourth transmembrane domains were encoded by codons 72-92, 146-166 and 206-226, respectively) (http://www.uniprot.org/uniprot/P31213), the C-terminal end and the important functional region between codons 197 and 230. Thus, it is conceivable that 5α-reductase activity was abolished.

The p.C222Ffs232X and L185Tfs192X mutations were two novel deletion mutations which caused frameshifts and then yielded the premature stop codon at 232 and 192, respectively with a large deletion of the C-terminal end. In addition, the L185Tfs192X mutation entirely lost the important functional region between codons 197 and 230 and the p.C222Ffs232X mutation severely changed the normal sequence of this region. These two new mutations were also not found in 50 ethnic-matched control samples. We therefore predict these two variants are disease-causing mutations.

The remaining mutations found in our study have been previously reported by other groups. The p.N193S, p.R227Q and p.R246Q variants impair 5α-reductase activity as a consequence of a reduced NADPH binding (Thigpen et al., 1992; Wigley et al., 1994; Makridakis et al., 2000) and p.R246Q variant is common among patients with SRD5A2 gene defect from the Northern states of India (Nagaraja et al., 2010). Functional analysis of the p.L20P, p.G203S, p.Q6X and p.F219Sfs278X mutations, however, has not been reported. To our knowledge, the L20P mutation has firstly reported in a compound heterozygous state in a person of Thai origin (p.L20P/p.G183S) who exhibited a female external genitalia and palpable gonads in the labio-scrotum (Sahakitrungruang et al., 2008). Similarly, 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 mutation 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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