Clinical and molecular characterization of 5α-reductase type 2 deficiency due to mutations (p.Q6X, p.R246Q) in SRD5A2 gene

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Abstract. Early diagnosis and optimal management for steroid 5α-reductase type 2 deficiency (5α-RD2) patients are major challenges for clinicians and mutation analysis for the 5α-reductase type 2 (SRD5A2) gene is the golden standard for the diagnosis of the disease. In silico analysis of this enzyme has not been reported due to the lack of appropriate model. Moreover, the histological and pathological changes of the gonads are largely unknown. In the present study, a 5α-RD2 patient born with abnormal external genitalia was studied and mutation analysis for SRD5A2 gene was conducted. Moreover, we constructed the homology modeling of 5α-reductase using SWISS-MODEL, followed by the molecular docking study. Furthermore, immunohistochemical staining of Ki67 for the testes tissue was conducted to investigate the potential pathological characteristics. The patient had male (46, XY) chromosomes but presented female characteristics, and the mutation analysis identified a heterozygotes mutation (p.Q6X, p.R246Q) in SRD5A2 gene. In silico analysis elucidated the potential effect of the mutation on enzyme activity. Immunohistochemical staining for the excised testes showed that 30%–50% of the germ cells were Ki67 positive, which indicated the early neoplastic potential. In conclusion, we analyzed the genotype-phenotype correlations of 5α-RD2 caused by a heterozygotes mutation (p.Q6X, p.R246Q). Importantly, we conducted the homology modeling and molecular docking for the first time, which provided a homology model for further investigations. Immunohistochemical results suggested gonadectomy or testis descent should be performed early for 5α-RD2 patient, as delayed treatment would have maintained the testes in a tumorigenic condition.

Key words: Steroid 5α-reductase type 2 deficiency (5α-RD2), Phenotypic analysis, Genotypic analysis, In silico analysis, Pathological characteristic

DISORDERS OF SEX DEVELOPMENT (DSD) with ambiguous genitalia are medical conditions requiring prompt investigation and early gender assignment. Obviously, it is most important to diagnose DSDs correctly as soon as possible which will allow the patient to live as normal a life as possible. However, confirming a cause for DSD and devising a management plan is one of the most challenging clinical conditions for the clinicians.

One of the more unusual DSDs is 5α-reductase type 2 deficiency (5α-RD2). In this condition, although the affected individuals have male (46, XY) chromosomes, they usually present with ambiguous external genitalia at birth and are assigned female gender and raised up as girls [1, 2]. As is shown in Fig. 1, it is caused by a deficiency of 5α-reductase isoenzymes, an enzyme that
convert testosterone (T) into more biologically active dihydrotestosterone (DHT).

5α-RD2 is clinically and genetically heterogeneous. The clinical spectrum ranges from a total female appearance to a nearly complete male phenotype with mild symptoms of under-masculinization. Detailed genotype-phenotype analysis may provide information necessary to improve diagnosis and treatment of 5α-RD2. 5α-RD2 is related to mutations in the 5α-reductase type 2 (SRD5A2, NM_000348.3) gene, which is located in chromosome 2p23 and contains five exons. So far, more than 100 different mutations have been reported in the SRD5A2 gene. However, it is difficult to investigate how the structure changes affect the enzyme activity as no homological model of the enzyme is available. Moreover, due to the low morbidity and different awareness of the disease, the histological and pathological changes of the genital system of 5α-RD2 patient are largely unknown. And it remains to be discussed which sex is suitable for a certain patient as most of the patients will be faced with a choice to be a male or female.

In the present study, we identified a DSD patient and made a detailed analysis of his clinical features, laboratory data, and management. Mutation analysis of the SRD5A2 gene was performed to make a molecular diagnosis. For the first time, homology modeling and molecular docking of 5α-reductase were carried out to investigate the effect of structural changes on the enzyme activity. Moreover, immunohistochemical staining of the testes tissue was conducted to investigate the potential histological and pathological characteristics of the genital system of 5α-RD2 patient. These results provide information necessary to improve early diagnosis and treatment of DSD for clinicians and better understand the genotype-phenotype correlations for 5α-RD2.

**Patient and Methods**

The study was approved by the Institutional Review Board of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China). All methods were carried out in accordance with the approved ethical guidelines and written informed consent was obtained from the patient and his parents.
A patient aged 15 years old was referred to our hospital (Shandong Provincial Hospital Affiliated to Shandong University, China) for the diagnosis and treatment of ambiguous genitalia. The patient was born with ambiguous genitalia and has been reared as a girl. She presented typical symptoms such as clitoris-like phallus, perineoscrotal hypospadias, cryptorchidism and undeveloped breast. There was no history of consanguineous marriage for three generations in the family.

Surgical treatments were carried out after the diagnosis confirmed, including bilateral testectomy, clitoridectomy and labiaplasty. The surgery was successful and the patient discharged from the hospital three days after the surgery. The patient was followed-up for one year, and the social gender, appearance, living conditions were recorded in detail.

Serum hormone studies
In order to facilitate the diagnosis of the patient, human chorionic gonadotropin (hCG, Livzon, China) stimulation test was carried out. The patient received daily intra-muscular injections of 1,500 IU of hCG for 3 days. The basal blood samples were taken on day 1 prior to administration of the first hCG dose, and the post stimulation samples were taken on day 4 for the measurement of T and DHT. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), T and DHT levels were measured by chemiluminescence immunoassay (Roche, Switzerland).

DNA extraction and amplification
Genomic DNA of the patient was extracted from peripheral leucocytes using genomic DNA kit (TIANGEN BIOTECH, Beijing, China, DP304-03). All five exons of the SRD5A2 gene were amplified by polymerase chain reaction (PCR) in a 25 μL system, which includes 2.0 μL dNTP, 2.5 μL 10 × PCR buffer, 0.15 μL Taq Hot Start (Takara Bio, Ohtsu, Japan), 2.0 μL genomic DNA and 0.5 μL 10 μM forward and reverse primers (Table 1). The initial denaturation was 95°C for 3 min, subsequently followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 70°C/55°C/52°C for 45 s and elongation at 72°C for 60 s. The amplifications were performed in an Eppendorf Mastercycler 5333 PCR amplification (Eppendorf Scientific, Germany).

Mutation analysis
PCR products were purified and sequenced in both the sense and the antisense directions on an ABI 3700 DNA sequencer (Applied Biosystems, PerkinElmer, Foster City, CA, USA). Sequences were compared with the reference sequence (NM_000348) by using CHROMAS software (v.2.01; Techne1ysium Pty Ltd, Tewantin, Qld, Australia). The mutation was named using the recommendations of the Nomenclature Working Group, in which the cDNA and protein sequence positions were designed by the prefixes c. and p. respectively.

Homology modeling and molecular docking
The NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/) was used to search the amino acid sequence of the human 3-oxo-5-alpha-steroid 4-dehydrogenase 2

### Table 1: Primers for SRD5A2 amplification

| Exons | Primers          | Sequence (5'-3') | Annealing temperature (°C) |
|-------|------------------|------------------|---------------------------|
| Exon 1 | 1 Forward        | CGGCGAGGGTGGGAGGCGAGATGGAG | 70                        |
|       | 1 Reverse        | TGGGGGAGTGAAGGCGGCTCTGTG  |                           |
| Exon 2 | 2 Forward        | GATAATTTGTATTGGGTAAAG | 52                        |
|       | 2 Reverse        | TGGCGATAGATTGT      |                           |
| Exon 3 | 3 Forward        | CCCACTTTCTGCCACGTCTTAGGA | 55                        |
|       | 3 Reverse        | TGTATCATCCTGTCACACTGTC |                           |
| Exon 4 | 4 Forward        | TTGCAATGATTCACCTGCCATTTC | 55                        |
|       | 4 Reverse        | CAATACAAGGCCAGCAAGTCAGA |                           |
| Exon 5 | 5 Forward        | TGGGAAGGAAGGAAATAGAATCATAA | 52                        |
|       | 5 Reverse        | CGCCAGGAGACCTACTATTACATA |                           |
The employed protein sequence was NP_000339.2 reported by Hata. We applied methylomicrobium alcaliphilum delta(14)-sterol reductase (PDB ID: 4QUV) as the template, and the homology of amino acid sequence was aligned. Homology modeling of 5α-reductase was carried out using SWISS-MODEL, a fully automated protein structure homology-modelling server.

Molecular docking study was performed to investigate the binding mode between the NADPH, testosterone and the human 5α-reductase using Autodock vina 1.1.2. The 2D structures of the NADPH and testosterone were drawn by ChemBioDraw Ultra 14.0 and converted to 3D structure using ChemBio3D Ultra 14.0 software. The AutoDockTools 1.5.6 package was employed to generate the docking input files. The search grid of the 5α-reductase for NADPH was identified as center_x: –21.072, center_y: –9.114, and center_z: 25.63 with dimensions size_x: 15, size_y: 15, and size_z: 15, while for testosterone was identified as center_x: –29.454, center_y: –4.392, and center_z: 14.88 with dimensions size_x: 15, size_y: 15, and size_z: 15. The value of exhaustiveness was set to 20. For Vina docking, the default parameters were used if it was not mentioned. The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMoL 1.7.6 software (http://www.pymol.org/).

Structural evaluation
The effect of the mutation Arg246Gln on the structure of the human 5α-reductase was evaluated by studying the homology modeling structure using PyMoL 1.7.6.

Immunohistochemistry (IHC)
Testicular morphology was assessed on haematoxylin-eosin (HE) staining, followed by immunohistochemical analysis for the expression and localization of Ki67. IHC was performed using paraffin-embedded tissue sections. The sections were dewaxed and hydrated, followed by antigen retrieval (in 0.01 mol/L citrate buffer solution, pH 6.0, heated to boiling for 2–3 min in a stainless steel pressure cooker). Endogenous peroxidase was blocked using a 3% hydrogen peroxide solution. The section was incubated with the blocking goat serum for 15 min, then immunostained with rabbit antibody against Ki67 (dilution 1:100; Abcam) at 4°C overnight. Secondary staining was performed with HRP-conjugated anti-rabbit IgG using a MaxVision Kit and a 3,5-diaminobenzidine (DAB) peroxidase substrate kit (Maixin Co, Fuzhou, China). The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

Results
Clinical features and hormone studies
The patient came from a unconsanguineous family and was referred to our department due to genital dysplasia for 15 years. She was the only child of her family and her parents both presented normal phenotype. She had a female face and presented typical symptoms such as clitoris-like phallus, perineoscrotal hypospadias, cryptorchidism and undeveloped breast (Fig. 2A–D). No other positive physical signs were shown. Pelvic ultrasonography presented no signs of uterus and accessories, while bilateral testes were shown within the prominence of the perineum. She has been raised as a girl since born.

Results showed that after hCG stimulation, the serum T level was almost doubled compared to the previous serum T level, while the serum DHT level only increased a little. The T/DHT ratio increased to 1.5 times compared with the basal ratio. Other detailed hormone levels of the patient were listed in Table 2. Combined with the aforementioned results, the primary diagnosis of the patient was 5α-reductase type 2 deficiency.

Management and follow-up
After the diagnosis of 5α-RD2 was confirmed by SRD5A2 gene mutation analysis, the patient received bilateral testectomy, clitoridectomy and labiaplasty (Fig. 2E–F).

The patient was followed-up 1 month, 3 months, 6 month, 12 months after discharged from the hospital, respectively. Now the social gender of the patient is female, and the patient manifests slim shape and little body hair. She lives a normal female life by taking conjugated estrogens (0.3 mg/day, dosage adjusted according to the development of breast every month). She plans to undergo vaginoplasty in the near future.

Mutational analysis of SRD5A2 gene
In order to specify the diagnosis, the sequence analysis of the SRD5A2 gene was performed. The analysis revealed a heterozygotes mutation (Fig. 3A). A nonsense mutation (c.16 C > T, p.Q6X) was found in exon 1, and a missense mutation (c.737 G > A, p.R246Q) was found in exon 5. The former mutation derived from the mother of the patient, while the latter mutation came from her father. This result confirmed the primary diagnosis. As is shown in Fig. 3B, the missense mutation c.737 G > A
affected the strictly conserved domains among vertebrate orthologs, while the nonsense mutation c.16 C > T leaded to a truncated protein.

**In silico analysis of 5α-reductase**

Till now, no research have reported how the structure changes affect the enzyme activity as no homological model of the enzyme is available. To further investigate the potential influence of the mutation on the functions of 5α-reductase, here we conducted homology modeling and molecular docking for the first time. Homology modeling of 5α-reductase was carried out using SWISS-MODEL. It was shown that human 5α-reductase features 6 α-helices (Fig. 4A). The structural impact of the Arg246Gln mutation was further investigated by studying the human 5α-reductase structure using PyMol 1.7.6. Results showed that a change to glutamine at position 246 results in loss of electrostatic interactions with the NADPH (Fig. 4B–C).

**Immunohistochemical studies**

Gonadal tumors often occur in abnormal sexual development disorders and Ki67 is considered as a marker for the proliferative potential of tumor cells [3, 4]. Therefore, we explored the expression of Ki67 in the testicular sections by immunohistochemistry to investigate the malignant potential of the testes of this patient. As shown in Fig. 5, germ cells with hyperchromatic nucleus could be seen in the tubules and germ cell maturation was occasionally observed. Moderate Ki67 staining cells

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**Table 2** Detailed hormone levels of the 5α-RD2 patient

| Baseline | After hCG stimulation |
|----------|-----------------------|
| T (nmol/L) | DHT (nmol/L) | T/DHT | FTI (%) | FSH (mIU/mL) | LH (mIU/mL) | E2 (pg/mL) | PRL (ng/mL) | PRG (ng/mL) | T (nmol/L) | DHT (nmol/L) | T/DHT |
|----------|-------------------|-------|---------|-----------|------------|-----------|-----------|------------|-----------|-------------------|-------|
| 19.11 (4.9–32.1) | 0.25 (40.5–355) | 76.44 | 34.73% (0.29–5.62%) | 3.47 (1.5–12.4) | 7.49 (1.7–8.6) | 15.72 (<18.4–73.4) | 17.83 (4.1–15.2) | 0.67 (0.73–4.3) | 33.72 | 0.30 | 112.4 |

Abbreviations: T, testosterone; DHT, dihydrotestosterone; FTI, free testosterone index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; PRL, prolactin; PRG, progesterone.
accounts for more than 50% in the left testis tissues and 30% in the right testis of the patient. These results indicated the early neoplastic potential for the testes of this patient. No interstitial fibrosis was observed between the oval seminiferous outlines.

**Discussion**

In the present study, a 5α-RD2 patient born with abnormal external genitalia was recorded in detail. Mutational analysis of *SRD5A2* gene was conducted and etiological mutations (p.Q6X and p.R246Q) were found. Moreover, we conducted the homology modeling and molecular docking for the first time, which provide a homology model for further investigations. Furthermore, the histological changes of the genital system was investigated, which may contribute to direct and improve the treatment of the disease.

The clinical manifestations of the patient here is typical, including clitoris-like phallus, perineoscrotal hypospadias, cryptorchidism and undeveloped breast [1, 2, 5-7]. In order to identify the patient from diseases that with the similar clinical manifestations such as androgen insensitive syndrome (AIS) and Leydig cell hypoplasia, hCG stimulation test was performed. Results showed a significant elevation of serum T/DHT ratio, indicating that the testes of the patient were able to secret T and the conversion from T to DHT was suppressed. However, the post hCG stimulation T/DHT ratios varies according to age and the severity of the enzyme defects, and the false-negative results [8-11]. There are patients that diagnosed with 5α-RD2 by gene mutational analysis even they had normal T/DHT ratio after the hCG stimulation, which means the precise diagnosis was not accomplished for this patient [9, 12].

Importantly, we conducted the homology modeling of 5α-reductase using SWISS-MODEL, followed by the molecular docking study was performed to investigate the binding mode between the NADPH, testosterone and the human 5α-reductase. The detailed mechanisms of
how the mutations affect the enzyme activity of 5α-reductase has never been investigated due to the lack of homology model. Our in silico results here showed that the residue Arg-246 is located in the loop region near the C-terminal, and forms electrostatic interaction with the NADPH. The substitution of the polar amino acid arginine for neutral glutamine in the protein will lead to a severe loss of enzyme function. As far as we know, this is the first report on the homology modeling and molecular docking of human 5α-reductase, which provides a homology model for further investigations.

Moreover, we should notice that gonadal tumors often occur in abnormal sexual development disorders. Due to the increased level of gonadotropin and maldescent of testes, 5α-RD2 patients who are assigned male gender or received surgery during their elder age may have high risk to develop gonadal tumors. Ki67 is a nuclear protein that expressed by cells in G1, S, G2, and M phases of the cell cycle. It is rapidly metabolized after M phase and is not synthesized in G0 phase. Therefore, the expression of Ki67 reflects the proliferative potential of tumor cells [3, 4]. Here we performed the Ki67 immunohistochemical staining for the surgical specimens of the patient. Importantly, the immunohistochemical results showed that the Ki67 positive germ cells account for 30%–50% of all the germ cells in the testes tissues of the patients, significantly higher than that in the normal testis, which reveals a potential in developing gonadal tumors for this patient. It has been reported that the risk of gonadal tumors in 5α-RD2 patients is low, this may due to the limited data and cases [13]. Theoretically, the malignant potential of gonadal tissues in 5α-RD2 patients should be further investigated in detail. As the testes of these patients are usually hypofunctional, which subsequently results to an increased gonadotropin level. Gonadal tumors tend to occur in degenerative testes and augmented gonadotropin stimulation has been demonstrated to be important in gonadal tumors’ development [14, 15]. In addition, as testicular maldescent is common in 5α-RD2 patients, the increased temperature within the intra-abdominal environment also increased the susceptibility to the development of gonadal tumors [14, 16]. Accordingly, a giant

Fig. 4  The structural change caused by the mutation of Arg246Gln.
A: Total view of the mutation. B: 5α-reductase wt: The yellow dotted lines show the electrostatic interactions between the side chain of Arg-246 (green) and NADPH (salmon). C: 5α-reductase Arg246Gln: In the mutant protein, the electrostatic interaction between Gln-246 (green) and NADPH (salmon) is lost. The figures were generated from the human 5α-reductase using PyMoL 1.7.6 to visualize the structural effect of mutation.
seminoma in an adult Japanese phenotypic female who was homozygous for the p.Q6X substitution was reported, demonstrating that the potential of developing gonadal tumor in 5α-RD2 patients should be paid more attention [17].

It has been reported that most of the patients with 5α-RD2 are typically first assigned a female gender, and due to the significant masculinization, psychosexual orientation and cultural biases, a number of these patients may switch to male gender during puberty [18, 19]. Our aforementioned immunohistochemical results suggested that gonadectomy should be performed when the female gender is chosen, and early testis descent is necessary when the male gender is assigned, as delayed treatment would have maintained the testes in a tumorigenic condition.

Generally, genetic analysis is essential for the diagnosis and treatment of patients with gonadal abnormalities. Thanks to the genetic analysis, a number of etiologic mutational genes have been identified, such as SF1, StAR, HSD3B2 and CYP17 [20, 21]. As for SRD5A2, more than 100 different mutations scattered throughout the five exons have been reported in different ethnic groups so far. Over 90% of all the mutations are missense mutations, nonsense mutation, deletions and insertions. It has been reported that more than 60% of patients were homozygotes and others were heterozygotes [22-26]. Here we also performed mutational analysis of SRD5A2 gene, the results revealed two different mutations (p.Q6X and p.R246Q). Both of these mutations have been identified previously. The p.Q6X mutation was a common nonsense mutation that has been reported mainly in Asiatic patients from Thailand, Korean and Chinese origins [17, 27-29]. This mutation results in the formation of completely truncated protein missing the binding sites to T and its cofactor [17, 27, 29-32]. The p.R246Q is a missense mutation that impairs 5α-reductase activity due to a reduced NADPH binding.

![Fig. 5](image_url)
It has been reported that the p.R246Q mutation is common among patients with SRD5A2 gene defect from the Northern states of India. Ambiguous genitalia is the common clinical manifestation, including enlarged clitoris or clitoris-like underdeveloped glans, perineal hypospadias, microphallus and labial fusion. Spontaneous virilization usually emerges at puberty [27, 34]. The detailed data of related patients were summarized in Table 3 [19, 27-29, 35-37].

There are several issues need to be further investigated. We should pay more attention to the histological and pathological changes of the genital system of 5α-RD2 patients. A long time follow-up is needed to present the outcomes of 5α-RD2 patients that assigned different genders.

In conclusion, we analyzed the genotype-phenotype correlations of 5α-RD2 caused by a heterozygotes mutation (p.Q6X, p.R246Q) in detail. Our research emphasized the importance of mutation analysis of SRD5A2 gene in diagnosing the disease. Importantly, we conducted the homology modeling and molecular docking for the first time, which provided a homology model for further in silico investigations on 5α-reductase. Moreover, immunohistochemical results suggested gonadectomy or testis descent should be performed early for 5α-RD2 patient, as delayed treatment would have maintained the testes in a tumorigenic condition. All these results emphasized the importance of early diagnosis.

### Table 3  Detailed data of the 5α-RD2 patient with p.Q6X and/or p.R246Q mutation

| Mutation       | Origin | Social gender | Phenotype                                                                                                                                  | Changes of T/DHT after hCG stimulation | Ref  |
|----------------|--------|---------------|--------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|------|
| p.Q6X/p.R246Q  | Korea  | Female        | Microphallus, clitoris-like underdeveloped glans, bifid scrotum and palpable testes                                                        | From 1.4 to 8.7                        | [26] |
| p.Q6X/A228V    | China  | Female        | Microphallus, hypospadias, palpable testes                                                                                               | NA                                     | [25] |
| p.Q6X/p.Q6X    | China  | Female        | Mild clitoromegaly, hypospadias, palpable testes                                                                                          | NA                                     | [25] |
| p.Q6X/p.N193S  | China  | Female to male| Hypospadias and palpable testes                                                                                                         | NA                                     | [25] |
| p.Q6X/p.G203S  | China  | Female to male| Clitoromegaly, hypospadias, undeveloped breast and virilization at puberty                                                               | NA                                     | [19] |
| p.Q6X/p.G203S  | Thailand| Female to male| Perineoscrotal hypospadias, clitoral-like phallus, and labia majora with palpable gonads                                                  | From 0.93 to 68.9                      | [27] |
| p.R246Q/p.G203S| China  | Female        | Clitoromegaly, hypospadias and spontaneous virilization at puberty                                                                       | From 20.3 to 20.4                      | [33] |
| p.R246Q/p.G203S| China  | Female        | Clitoromegaly, hypospadias and spontaneous virilization at puberty                                                                       | From 18.5 to 20.1                      | [33] |
| p.R246Q/p.C222Fs232X | China  | Male          | Microphallus, hypospadias, palpable testes                                                                                               | NA                                     | [25] |
| p.R246Q/p.R246Q| Korea  | Female        | Clitoromegaly, labial fusion, palpable testes, blind-ending vagina                                                                       | From 6 to 1.2                          | [26] |
| p.R246Q/p.R246Q| Korea  | Female        | Slight clitoromegaly and bilateral inguinal hernia                                                                                       | NA                                     | [26] |
| p.R246Q/p.R246Q| China  | Female to male| Clitoromegaly, hypospadias and bilateral testes                                                                                          | NA                                     | [19] |
| p.R246Q/p.R246Q| India  | Female        | Clitoromegaly, hypospadias and inguinal testes                                                                                           | NA                                     | [34] |
| p.R246Q/p.R246Q| India  | Female        | Clitoromegaly, hypospadias, blind vaginal pouch and virilization at puberty                                                              | NA                                     | [34] |
| p.R246Q/p.R246Q| Mexico | Female        | Clitoromegaly, hypospadias                                                                                                               | NA                                     | [35] |
| p.R246Q/p.G203S| Korea  | Male          | Micropenis, severe hypospadias and a bifid scrotum                                                                                         | From 7.4 to 8.8                        | [26] |
| p.R246Q/p.N193S| China  | Female to male| Hypospadias and prostate dysplasia                                                                                                        | NA                                     | [19] |
| p.R246Q/p.G203S| China  | Female to male| Hypospadia                                                                                                                               | NA                                     | [19] |
and surgical treatment, which may benefit the final prognosis of the 5α-RD2 patients.

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**Disclosure**

There are no conflicts of interest to disclose.

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