CASE REPORT

Identification of mutations in the *SRD5A2* gene in Thai patients with male pseudohermaphroditism

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Objective: To describe two unrelated Thai patients with suspected 5α-reductase type 2 deficiency and perform mutation analysis of the *SRD5A2* gene.
Design: Case report.
Setting: A pediatric endocrinology clinic at a university hospital.
Patient(s): Two unrelated patients with 46,XY karyotype, born with ambiguous genitalia, were studied. One was reared as a boy and the other was reared as a girl.
Intervention(s): The entire coding regions of the *SRD5A2* gene were assessed by polymerase chain reaction (PCR) and sequencing analysis.
Main Outcome Measure(s): Molecular characterization of the *SRD5A2* gene.
Result(s): Four different pathogenic mutations (three missense and one nonsense) were identified. These were located at exon 1 (p.Q6X and p.L20P), exon 3 (p.G183S), and exon 4 (p.G203S). The T>C transition (c.59T>C) resulting in a leucine-to-proline substitution at codon 20 (p.L20P) has not been previously described and was not detected in 100 unaffected, ethnic-matched control chromosomes. In addition, p.G183S, previously identified only among patients from mixed African–European ancestry and in the Dominican Republic, was also detected in a Thai patient.
Conclusion(s): This study demonstrates that the *SRD5A2* gene is responsible for 5α-reductase type 2 deficiency across different populations and emphasizes the important role of genetic testing for the definite diagnosis and genetic counseling before gender assignment or any surgical intervention. (Fertil Steril® 2008;■:■–■. ©2008 by American Society for Reproductive Medicine.)

Key Words: *SRD5A2*, 5α-reductase type 2 deficiency, ambiguous genitalia, novel mutation

The 5α-reductase type 2 deficiency (OMIM 607306), an autosomal recessive disorder, is characterized by a phenotype at birth that can range from normal female structures to a male phenotype with ambiguous genitalia, or isolated infertility (1, 2). This enzymatic deficiency leads to impairment in the conversion of T into more biologically active dihydrotestosterone (DHT), the androgen essential for normal development of external genitalia in the male fetus. Dihydrotestosterone also mediates most events of male puberty, including temporal hairline recession, growth of the facial and body hair, and development of the prostate (1).

The *5 alpha-reductase type 2* (*SRD5A2*), which encodes the 5α-reductase type 2 enzyme, is found to be mutated in individuals with 46,XY with various clinical and biochemical phenotypes (1–5). Affected neonates are usually assigned female gender at birth and raised as girls, although they have 46,XY with bilateral testes and male Wolffian duct structures. During puberty, however, they can exhibit virilization without breast development, which is often accompanied by gender identity change from female to male (6). There are also other forms of 46,XY disorders of sex differentiation (46,XY DSD) mimicking the phenotype caused by 5α-reductase type 2 deficiency, especially before puberty. Although the diagnosis of 5α-reductase type 2 deficiency can be made on the basis of an elevated T/DHT serum ratio after hCG administration, the disease cannot be ruled out by a lack of an elevated T/DHT ratio, especially in patients with partial enzyme deficiency and prepubertal subjects (1, 4, 7). The use of molecular studies, therefore, becomes necessary for a definite diagnosis of this disorder.

At least 54 different disease-causing mutations scattered throughout the gene have been described (http://www.hgmd.cf.ac.uk, accessed October 2007). Of these, 42 are missense/nonsense mutations. The splice-junction

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alterations and nucleotide or whole gene deletions have also been reported. Some of the mutations are recurrent and reported in various ethnic groups, whereas others seemingly reflect a founder effect.

In this study we performed mutation analysis of all the coding regions of the *SRD5A2* gene in two Thai unrelated individuals with suspected 5α-reductase type 2 deficiency. Four different mutations, with one being novel, were identified.

MATERIALS AND METHODS

Patients

The two unrelated patients were referred for diagnosis and treatment of ambiguous genitalia at the Pediatric Endocrinology Unit, Department of Pediatrics, Chulalongkorn University. There was no history of consanguinity in both families. All patients had 46,XY karyotype.

Patient 1 was born with ambiguous genitalia and subsequently reared as a girl. She was referred to us at 11 months old. Physical examination revealed perineoscrotal hypospadias with a single perineal opening, a clitoral-like phallus, and a bifid scrotum/labia majora with palpable gonads (Fig. 1, left panel). Pelvic ultrasonography was unable to identify Mullerian remnants. Genitogram showed a blind-ending pseudovagina. The basal levels of gonadotropins were normal (LH, 0.1 mIU/mL [normal, <0.5 mIU/mL] and FSH, 0.4 mIU/mL [normal, 0.4–1.6 mIU/mL] in prepubertal male). The T and DHT basal levels were 2.8 ng/dL and 3 ng/dL, respectively. After hCG stimulation test, T and DHT levels increased to 585 ng/dL and 8.49 ng/dL, respectively, yielding a T/DHT ratio of 68.9. After a discussion with the parents, reassignment to male gender was made.

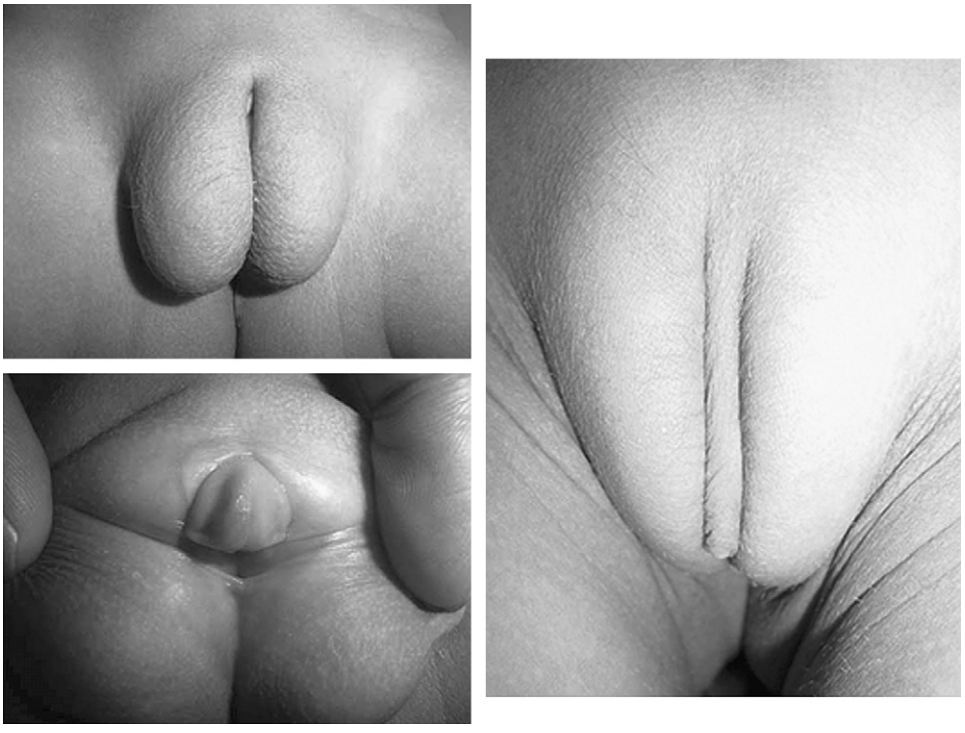
Patient 2 was born with apparent female external genitalia and palpable gonads in the labia majora. Pelvic ultrasonography showed an absence of Mullerian structures. An hCG stimulation test performed at the age of 24 months reported no increment in plasma T level. The DHT level was not measured because the assay was unavailable in our center at that time. The initial presumptive diagnosis was androgen insensitivity. At the age of 30 months, bilateral gonadectomy was performed. The pathological results revealed normal structures of testes without spermatogenesis. She had been raised as a female. On her last visit at 12 years old, the clitoromegaly was still present ((Fig. 1, right panel). She also developed male attitude and gender dysphoria.

Hormonal Studies

The hCG stimulation test was performed to assess testicular T biosynthesis and its peripheral 5α-reduction. The patient received three daily IM injections of 1,500 IU of hCG. The

FIGURE 1

Photographs showing apparent female external genitalia in Thai patients with 5α-reductase type 2 deficiency. (*Left*) patient 1 at 11 months old. (*Right*) patient 2 at 12 years old.



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basal blood samples were drawn on day 1 before administration of the first hCG dose and the poststimulation samples were drawn on day 4. Measurement of T and DHT was carried out using commercial RIA kits and ELISA kits, respectively (IBL, Hamburg, Germany).

Molecular Methods

Written informed consent for testing was obtained from the patients or their parents after receiving counseling. The institutional review board (IRB) approval was not obtained as the investigation was considered a part of clinical care.

Genomic DNA was extracted from peripheral leukocytes according to standard protocols. All five exons of the *SRD5A2* gene were amplified by polymerase chain reaction (PCR) using sets of primers and reaction conditions (Table 1). We used 100 ng of genomic DNA, 1 × PCR buffer (Promega, Madison, WI), 1.875 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM of each primer, and 0.5 U *Taq* DNA polymerase (Promega) in a total volume of 20 μL. The PCR products were verified for correct size on ethidium bromide-stained 1.5% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI). For a novel missense mutation, restriction enzyme digestion was used to confirm its presence in the patients and to screen in 100 control chromosomes from unaffected, ethnic-matched individuals. In addition, the parents of the patient 2 whose DNA samples were available were screened by restriction fragment length polymorphism for the same variant.

Protein Sequence Comparisons

SRD5A2 orthologues were first identified through a BLAST search of the nonredundant database using *Homo sapiens* SRD5A2, accession NP_000339, as the reference sequence. All known and complete SRD5A2 sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by ClustalX 1.81 program. The human SRD5A2 was aligned with rhesus monkey (*Macaca mulatta*; XP_001105329), horse (*Equus caballus*; XP_001501572), cow (*Bos taurus*; XP_605410), wild boar (*Sus scrofa*; NP_999153), mouse (*Mus musculus*; NP_444418.1), and worm (*Caenorhabditis elegans*; NP_510077). The program classified amino acids by the variation in polarity, assessing both amino acid class conservation and evolutionary conservation at any given site.

RESULTS

Analysis of the *SRD5A2* gene by PCR sequencing revealed four different sequence variants, one of which was previously undescribed. Both patients were compound heterozygotes. Patient 1 harbored two different mutations, located in exons 1 and 4. In exon 1, a single base substitution of cytosine by thymine at nucleotide position 16 (c.16C>T) leading to an amber stop codon TAG (p.Q6X) was detected. In exon 4, a G>A transversion at nucleotide position 607 (c.607G>A) resulted in a glycine-to-serine substitution at codon 203 (p.G203S). Parental DNA was not available for analysis. Patient 2 was also found to carry two heterozygous mutations located in exons 1 and 3. A T>C transition was identified at nucleotide position 59 (c.59T>C) in exon 1. This was expected to result in a leucine-to-proline substitution at codon 20 (p.L20P). The mutation was confirmed by digestion of the PCR products with the restriction enzyme *Sty*I. This mutation has never been previously described and was not detected in 100 ethnic-matched unaffected control chromosomes (data not shown). In exon 3, a G>A transversion was detected at nucleotide position 547 (c.547G>A) leading to a glycine-to-serine substitution (p.G183S). Sequence analysis of exons 1 and 3 of parental genomic DNA confirmed that the mother was heterozygous for the c.59T>C mutation and the father, heterozygous for the c.547G>A mutation (data not shown).

DISCUSSION

We described two unrelated Thai patients suspected of 5α-reductase type 2 deficiency with mutations in the *SRD5A2* gene.

TABLE 1

Oligonucleotides and PCR conditions for *SRD5A2* mutation analysis.

Exon	Primer sequences for PCR 5′ to 3′		Annealing temperature (°C)
	Forward	Reverse	
1	GCAGCGGCCACCGGCGAGG	CCGGGAGCAGGGCAGTGC	68
2	AGCTTAAGAAAGAGGTGGGG	GATGGGATCATTACGAGGTC	60
3	GCCACGTCTTAGGACCATTC	GGTACTGTGATGGGACTGGG	60
4	TGTTTCCCCTTCTCCCCAAG	CTCTCATCCAGCTAACTTCC	60
5	CAGCCATCACCACTACCCTC	GCAGACACCACTCAGAATCC	60

Note: PCR = polymerase chain reaction.

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One novel mutation was identified. In addition, this study demonstrated the recurrence of already known mutations.

Both patients were compound heterozygotes for the mutations. Patient 1 presented two different mutations (p.Q6X/p.G203S). Both have been identified previously. The p.Q6X mutation has been reported in an adult Japanese phenotypic female patient in whom an inguinal testis presented a giant seminoma (8). This patient was homozygous for the mutation. The nonsense mutation (p.Q6X) presumably results in the formation of drastically truncated protein. The p.G203S mutation has been observed in a compound heterozygous Mexican patient (p.G115D/p.G203S) who was reared as a male and exhibited a more masculine phenotype (9). Although no in vitro assay has been performed to confirm the causative role of the p.G203S substitution, its position at the highly conserved residue argues for its functional significance (Fig. 2). Our patient had perineoscrotal hypospadias with a single perineal opening, a clitoral-like phallus, and a bifid scrotum/labia majora with palpable gonads. To our knowledge, the combination of these two point mutations has not been described to date.

Patient 2 with apparent female external genitalia and palpable gonads also harbored two different mutations (p.L20P /p.G183S). The p.G183S substitution has been initially reported in a homozygous form in a black Brazilian patient who was raised as a female but changed gender role behavior to male at age 30 years. The patient had microphallus, perineoscrotal hypospadias, a vaginal pouch, and right-sided cryptorchidism (3). The 5α-reductase activity in cultured genital skin fibroblasts from this patient was undetectable. The causative role of the p.G183S substitution was also demonstrated by site-directed mutagenesis and in vitro assays (10). This mutation was subsequently detected among a subset of African–Brazilian patients (11–13). Because it was reported only in Brazilian patients from mixed African–European ancestry and in the Dominican Republic, it was considered a founder effect allele (13, 14). However, the presence of this mutation in a Thai patient in our study suggests a possible recurrent mutation. This report provided evidence supporting that the 183 position could be a hot spot of the *SRD5A2* gene, as suggested by Cai et al. (14).

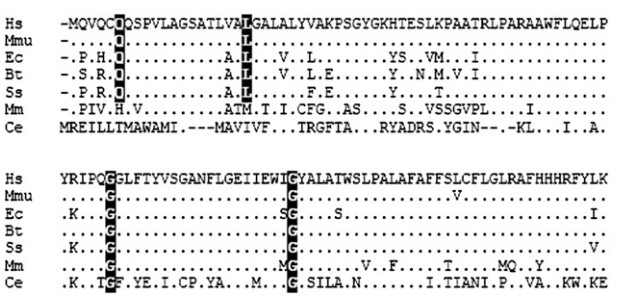
Another substitution, the p.L20P, found in patient 2, has never been described. Although no in vitro study was performed to investigate the functional consequence of this mutation on 5α-reductase activity, there are several lines of evidence supporting it as a disease-causing mutation. First, the amino acid, L20, is extremely well conserved (Fig. 2). This position contains an identical or similar amino acid in every organism represented in the National Center for Biotechnology Information (NCBI) database. Second, PolyPhen (http://coot.embl.de/PolyPhen/) predicts it to be possibly damaging. Third, this variant has not been reported to be a polymorphism in NCBI SNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), Ensembl (http://www.ensembl.org/index.html), and PupaSUITE/PupaSNP (http://pupasuite.bioinfo.cipf.es/) databases. And lastly, it was not detected in 100 ethnic-matched control chromosomes.

The degree of external genitalia virilization at birth in *SRD5A2* mutations has been described as extremely variable, ranging from a simple micropenis to an enlarged clitoris, labioscrotal fusion, single urethral meatus with palpable gonads in the more highly affected cases. Both of our patients had male pseudohermaphroditism with predominantly female external genitalia at birth. The newborn phenotypes of male pseudohermaphrodites with 5α-reductase type 2 deficiency, partial androgen insensitivity, or 17β-hydroxysteroid dehydrogenase (11βHSD) enzyme deficiency may be indistinguishable. Although diagnosis can be made on the basis of an elevated T/DHT ratio after administration of hCG, the disease cannot be ruled out by a lack of an elevated T/DHT ratio (1, 4, 7). Therefore, molecular analysis of the *SRD5A2* gene for the correct diagnosis of 5α-reductase type 2 deficiency and for genetic counseling is crucial. The clinical history of patient 2 emphasizes the necessity of early diagnosis before gender assignment is decided. Although the female gender was assigned at birth in this patient, and bilateral gonadectomy was performed, the patient had predominantly a male psychosexual orientation and identity particularly at puberty. These observations should lead physicians to consider male gender assignment in individuals with 5α-reductase type 2 deficiency, even if the external genitalia are predominantly female at birth. In fact, it has been suggested that male assignment of neonatal diagnosed patients would be the recommendation of choice considering the natural history of this disease (15).

Testicular morphology has rarely been studied in patients with 5α-reductase type 2 deficiency. The testicular pathology of patient 2 was studied at 30 months of age, revealing a normal testicular structure without spermatogenesis.

FIGURE 2

Sequence alignment of the SRD5A2. The sites of all amino acid variants found in this study are shaded in all conserved species. Sites that are 100% conserved across all sequences are indicated by dots. Hs = *Homo sapiens*; Mmu = *Macaca mulatta*; Ec = *Equus caballus*; Bt = *Bos taurus*; Ss = *Sus scrofa*; Mm = *Mus musculus*; Ce = *Caenorhabditis elegans.*



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Previous reports have also demonstrated normal testicular morphology, spermatogonial index, and Sertoli cell number in 4-month and 20-month-old individuals with compound heterozygous mutations in the *SRD5A2* gene (16, 17). There were some reports describing testis morphology in patients at pubertal and postpubertal ages. Absent or incomplete spermatogenesis and abnormal Sertoli cell maturation were found (18, 19). However, fertility in two patients from a Swedish family having two mutations in exon 4 has also been demonstrated (20). Whether abnormal spermatogenesis is a direct effect of the mutation or the secondary consequence of incomplete testicular descent is still uncertain.

Risk of gonadal tumors in 5α-reductase type 2 deficiency is quite low (21). However, there was one study reporting a case of a giant seminoma originating from an inguinal testis in an adult Japanese phenotypic female who was homozygous for the p.Q6X substitution (8). This finding suggests that early testis descent is advisable when the male gender is officially assigned or gonadectomy is to be performed when the female gender is chosen. Our patient 1 with descended testes also harbored this mutation in one of the two alleles (p.Q6X/p.G203S). This patient was eventually reared as a boy.

In summary, we reported two unrelated Thai patients with male pseudohermaphroditism caused by 5α-reductase type 2 deficiency. A novel pathogenic mutation of the *SRD5A2* gene, p.L20P, was identified. In addition, p.G183S and p.G203S substitutions, previously identified only in patients from mixed African–European ancestry and Mexican individuals, respectively, were also detected in Thai patients. This study demonstrates that the *SRD5A2* gene is responsible for 5α-reductase type 2 deficiency across different populations and emphasizes the important role of genetic testing for a definite diagnosis as well as genetic counseling.

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