Molecular Study of the 3β-Hydroxysteroid Dehydrogenase Gene Type II in Patients with Hypospadias

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To determine whether some patients with idiopathic hypospadias have HSD3B2 mutations, we genotyped this locus in 90 patients with hypospadias (age, 6.0 ± 0.4 yr) and 101 healthy fertile male controls. We measured basal plasma renin activity and performed an ACTH test for determination of 17-OH-pregnenolone, 17-OH-progesterone, cortisol, dehydroepiandrosterone sulfate, and androstenedione and an human chorionic gonadotropin test for determination of androstenedione, testosterone, and dihydrotestosterone. We did not observe a clear steroidogenic pattern suggestive of 3β-HSD deficiency in any patient. DNA was extracted from peripheral lymphocytes; and exons 1, 2, 3, and 4 were amplified by PCR and analyzed by denaturing gradient gel electrophoresis. An abnormal electrophoretic migration pattern of exon 4 was observed in five patients. Two patients had missense heterozygous mutations (S213T and S284R). In another three patients, we observed heterozygous nucleotide variants in exon 4 that did not produce a change in amino acids (A238, T259, T260). In vitro enzymatic activity was diminished by 40% and 32% in the S213T and S284R heterozygous mutations, respectively. One control exhibited a heterozygous mutation in exon 3 (V78I), which did not alter in vitro enzyme activity. In addition, we observed possible polymorphisms in intron 1 in four patients and one control. We conclude that subtle molecular abnormalities in the HSD3B2 gene may be observed in some patients with apparent idiopathic hypospadias but that this finding is uncommon. (J Clin Endocrinol Metab 89: 957–964, 2004)

HYPOSPIADAS IS ONE of the most frequent congenital malformations with an incidence around one per 125 to one per 300 live male births (1–3), which has apparently increased during the last decades (4, 5). The significance of the hypospadias relates not only to its frequency but to important functional and cosmetic impairment. In this malformation, the urethral meatus is located on the ventral side of the penis proximal to the tip of the glans, from the balanopreputial sulcus to the perineal area. The urethra is formed by fusion of the urethral folds along the ventral surface of the penis, a process that occurs during the first trimester of gestation, and depends on the proper secretion and action of androgens (6).

One important enzyme involved in the synthesis of mineralocorticoids, glucocorticoids, and sex steroids is the 3β-hydroxysteroid dehydrogenase (3β-HSD), which belongs to aldo-keto reductase family and is a membrane-bound protein in the endoplasmic reticulum and mitochondria (7–10). This enzyme is necessary for the conversion of pregnenolone to progesterone, 17-OH-pregnenolone (17OH Preg) to 17OH progesterone (17OH Prog), and of dehydroepiandrostenedione (DHEA) to androstenedione. It catalyzes the oxidation and isomerization of 5-ene-3β-hydroxy pregnene and 5-ene-hydroxyandrostene steroid precursors into the corresponding 4-ene-ketosteroids.

In humans, there are two isoenzymes, encoded by two genes on chromosome 1p13.1 (11–13), which are designated type I and type II. Both genes consist in four exons and three introns, and both enzymes have high homology in their amino acid sequence (14, 15). The type I gene (HSD3B1) is expressed in placenta, mammary gland, prostate, liver, kidney, and skin. The type II gene (HSD3B2) is expressed in the adrenal gland, ovary, and testis (16).

The clinical presentation of HSD3B2 deficiency is heterogeneous and may range from salt-losing congenital hyperplasia (17) to premature puberty, and mild hyperandrogenism (18). However, the possibility that isolated abnormalities in male genital development, such as hypospadias, without other clinical manifestations, may be caused by a mutation in the HSD3B2 gene has not been documented up to now. We hypothesized that some cases of apparent idiopathic hypospadias may be consequence of HSD3B2 gene mutations, which may affect androgen production during fetal life. We conducted a prospective endocrine and molecular study in 90 patients with hypospadias with no evidence of salt loss during infancy, to determine the possible presence of molecular abnormalities in the HSD3B2 gene.

Subjects and Methods

Patients

Ninety patients (6.0 ± 0.4 yr old) with penile or perineoscrotal hypospadias were studied. Subjects with glandular hypospadias, genetic
syndromes, or major anatomical abnormalities of the genital urinary or gastrointestinal tract were excluded. Some patients had additional evidence of ambiguous genitalia, such as cryptorchidism or microopenis. A complete physical examination was performed in each patient. Each parent gave signed informed consent, and the patient gave verbal assent when pertinent. The study was approved by the Ethics Committee of the San Borja Arriaran Hospital.

A standard ACTH stimulation test (Cortrosyn, 0.25 mg iv; Alliance Pharmaceutical, Whitshire, UK) was performed, and we determined the serum levels of 17OHP, 17OHP, cortisol, DHEA, and androstenedione before and 60 min after ACTH administration. In addition, a standard human chorionic gonadotropin (HCG) stimulation test (Profasi, 100 IU/kg im; Serono, Randolph, MA) was performed, and serum androstenedione, testosterone, and dihydrotestosterone were determined before and 24 h after drug administration. Baseline renin plasma activity was determined in most subjects, provided that they were resting comfortably for at least 30 min. DNA was prepared from peripheral leukocytes. A karyotype was performed in all subjects with hypospadias associated with bilateral cryptorchidism.

Hormone assays

To measure 17OHP Preg, the sera were extracted with ethyl acetate: n-hexane (6:4, vol/vol) and purified in LC-18 columns by sequential elution with iso-octane/ethyl acetate (82:6, vol/vol), evaporated, and dissolved in PBS. The recovery was approximately 90%. A competitive binding RIA for 17OHP Preg using antibody, tracer, and standard was performed, and the assay was validated using positive control samples. This process enabled us to determine the normal electrophoretic migration pattern by DGGE.

**Site-directed mutagenesis**

We generated vectors carrying the identified mutations using site-directed mutagenesis. (QuikChange Site-Directed Mutagenesis Kit; Stratagene Cloning System, La Jolla, CA) following the manufacturer’s instructions. Briefly, the point mutations documented by sequencing were included in oligo sequences designed such that the desired nucleotide change was in the middle of the primer with at least 15 bases of correct sequence on each side. The point mutation was introduced into pcDNA3 human type II 3β-HSD (kindly donated by Drs. Anne-Marie Moisan and Jacques Simard, University of Laval, Quebec, Canada) using Pfu Turbo DNA Polymerase that replicates both plasmid DNA strands. The product was treated with Dpn1 endonuclease selecting the mutated synthesized DNA. The DNA sequence of the newly introduced mutation in pcDNA3 type II 3β-HSD was confirmed by double-strand DNA sequence analysis or by PCR amplification followed by DGGE of linearized mutated vector.

**Transfection of COS 7 cells and Western immunoblot analysis**

COS 7 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM/high glucose (GibcoBRL LifeTechnology, Bethesda, Maryland) containing 10% fetal calf serum and 1% penicillin-streptomycin. The cells were seeded on a 9 cm plate at 10°/cm² the day prior to transfection. The following day, the medium was changed to DMEM containing 1% penicillin-streptomycin. The gDNA, genomic DNA; us, upstream; ds, downstream; V/t, voltage (volts)/time (hours).

**Table 1.** Primers and condition used for PCR amplification of exon 1–4 of HSD2B2

<table>
<thead>
<tr>
<th>Primers</th>
<th>Exon</th>
<th>Size of amplified fragment (bp)</th>
<th>Annealing temperature (C)</th>
<th>DQE conditions gel (%)</th>
<th>Gradient (%)</th>
<th>V/t (h)</th>
<th>Position of primers on gDNA</th>
<th>Sequence of oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 a b</td>
<td>1 and 2</td>
<td>540</td>
<td>51</td>
<td>30–70</td>
<td>60/12</td>
<td>1219–1235</td>
<td>3′-AGAGCATAAAGGCTCCAG-3′</td>
<td>5′-CCACACCCCATGAGATG-3′</td>
</tr>
<tr>
<td>1b</td>
<td>a CG</td>
<td>Anti (intron 2)</td>
<td>264</td>
<td>48</td>
<td>30–70</td>
<td>70/20</td>
<td>5488–5504</td>
<td>3′-CCAAATGCTCGTACGCTG-3′</td>
</tr>
<tr>
<td>3 k</td>
<td>b</td>
<td>Sense (intron 2)</td>
<td>7865 us 5′-CCAGATCTCTTATCC-3′</td>
<td>10</td>
<td>30–70</td>
<td>5714–5698</td>
<td>3′-CCACGCTCTTGGTATC-3′</td>
<td>5′-CCAACTTGTCTTTATAC-3′</td>
</tr>
<tr>
<td>3</td>
<td>l</td>
<td>Anti (intron 3)</td>
<td>460</td>
<td>54</td>
<td>20–60</td>
<td>80/17</td>
<td>7849–7865</td>
<td>3′-TGTTGTCGATCTCCTT-3′</td>
</tr>
<tr>
<td>4 Ae</td>
<td>m</td>
<td>Sense (intron 3)</td>
<td>7820–8250</td>
<td>300</td>
<td>54</td>
<td>40–60</td>
<td>120/2.5</td>
<td>7849–7865</td>
</tr>
<tr>
<td>4 Ae</td>
<td>f</td>
<td>Anti (ex 4)</td>
<td>8111–8095</td>
<td>300</td>
<td>54</td>
<td>40–60</td>
<td>120/2.5</td>
<td>7849–7865</td>
</tr>
<tr>
<td>4 Af</td>
<td>d</td>
<td>Sense (ex 4)</td>
<td>8752–8741</td>
<td>600</td>
<td>54</td>
<td>40–60</td>
<td>120/2.5</td>
<td>8151–8197</td>
</tr>
<tr>
<td>4 Dg</td>
<td>h</td>
<td>Anti (3′ UTR)</td>
<td>8747–8758</td>
<td>473</td>
<td>54</td>
<td>30–70</td>
<td>80/20</td>
<td>8340–8356</td>
</tr>
<tr>
<td>4 Ei</td>
<td>g</td>
<td>Anti (3′ UTR)</td>
<td>8774–8758</td>
<td>473</td>
<td>54</td>
<td>30–70</td>
<td>80/20</td>
<td>8340–8356</td>
</tr>
</tbody>
</table>
the enzymatic activity of 3β-HSD was determined by the conversion of DHEA to androstenedione by RIA. Briefly, 43 h after transfection, intact transfected COS-7 cells were incubated with 10 nM DHEA (Sigma) for 30, 90, and 360 min in serum-free DMEM media. Subsequently, we determined the concentration of DHEA and androstenedione by RIA in the media as indicated above. Results were normalized by protein concentration determined by BCA protein Assay Kit (Pierce, Rockford, IL).

Statistical analysis
Data are reported as mean ± SEM of serum determinations performed in duplicate, or of culture media of at least two separate transfection experiments in duplicate.

Results
The 90 subjects studied were classified based on their genital abnormalities. Fifty-nine subjects had isolated hypospadias, six had hypospadias and bilateral cryptorchidism, four had hypospadias and unilateral cryptorchidism, eight had hypospadias and microopenis, and 13 had severe hypospadias with bifid scrotum and chordee. The hypospadias was classified as penile in 71 and perineoscrotal in 19 patients. The average age at the time of the study was 6.0 ± 0.4 yr, with a range of newborn to 15 yr. The patients’ weight was 0.1 ± 0.2 sd, and their height was −0.2 ± 0.2 sd for chronological age. Surgery for correction of hypospadias was performed in 69 patients, with a single operation in 43 patients and two or more surgeries in 26. Twenty-one patients had not been subject to surgery at the time of the study, mostly because of their young age. Karyotype was 46 XY in all patients studied. The results of the hormonal profile obtained during the ACTH and HCG tests were within the normal range. Specifically, we did not observe definite evidence of a steroidogenic profile suggestive of 3β-HSD, 17 ketoreductase, 5α-reductase deficiency, or androgen insensitivity in the 90 patients studied.

Assay of 3β-HSD enzymatic activity
The enzymatic activity of 3β-HSD was determined by the conversion of DHEA to androstenedione by RIA. Briefly, 43 h after transfection, intact transfected COS-7 cells were incubated with 10 nM DHEA (Sigma) for 30, 90, and 360 min in serum-free DMEM media. Subsequently, we determined the concentration of DHEA and androstenedione by RIA in
Identification of HSD3B2 gene mutations in patients and control samples

Exons 1–4 were amplified using the primers described in Table 1. The amplified PCR products were analyzed by DGGE, allowing rapid screening for mutations. With this method, we identified an altered electrophoretic mobility pattern by DGGE in four patients and one control for intron 1, and in five patients for exon 4 (Fig. 1). In addition, an abnormal electrophoretic migration pattern for exon 3 was observed in one control.

To identify possible specific mutations responsible for the abnormal patterns in DGGE, the HSD3B2 gene was determined by sequencing in both directions in these five patients. Sequencing studies revealed the following heterozygous mutations: in patient 1, the G→C transition predicts a modification of codon 213 (AGT) encoding Ser into Thr (ACT); whereas in patient 2, the nucleotide transition of C→G predicts a change in codon 284 (AGC) encoding Ser into Arg (AGG). These findings were confirmed by allele-specific PCR. In the other three patients, the nucleotide transition did not predict an amino acid change in the primary structure of the enzyme. In patient 3, the substitution of T→C at codon 238 (GCT) codes for the same amino acid Ala (GCC). Patient 4 showed a G→A substitution in codon 259 (ACG), which encodes for the same amino acid Thr (ACA) and, in patient 5, the nucleotide substitution A→C at codon 320 (ACA) codifies for the same amino acid Thr (ACC) (Fig. 2). We observed an abnormal DDGE pattern in patients 6, 7, and 8 consisting of an heterozygous T→G substitution in nucleotide 1362 of intron 1. In addition, patient 9 showed a heterozygous A→C substitution in nucleotide 1372 of intron 1 (Fig. 2).

In addition, we studied the four exons of the HSD3B2 gene by DGGE in DNA obtained from 101 healthy fertile men. Two control samples showed abnormal electrophoretic mobility by DGGE. One control sample (control 1) showed the same abnormal DGGE migration pattern in exons 1 and 2 as patients 6, 7, and 8 (Fig. 1); and the sequencing of the PCR-fragment confirmed the same heterozygous T→G substitution change in nucleotide 1362 of intron 1 (Fig. 2). The other control sample (control 2) showed an abnormal DGGE pattern in exon 3 (Fig. 1), and sequencing identified a G→A substitution at codon 78 (GTC) that encoded Val into Ile (ATC) in one allele (Fig. 2).

![Fig. 2. Direct sequence analysis of the amplified spanning exons 1 and 2, exon 3, and exon 4 of HSD3B2 from patients and controls with abnormal DGGE patterns. Direct sequencing of exon 1 and 2, and 3 (upstream primers) and patients 3 and 4 (downstream primers) showed heterozygous substitution (arrows); of exon 1 and 2 of patients 6, 7, 8, and 9 and in control 1 (upstream primer) and heterozygous substitution (arrows); of exon 3 of control 2 (upstream primer). Results represent at least four separate sequencing experiments in both directions with internal primers of purified PCR fragments for exon 4 and at least two separate sequencing experiments in both directions of purified PCR fragments for exon 1 and 2, and exon 3.](https://academic.oup.com/jcem/article-abstract/89/2/957/2840823)
The clinical, hormonal, and molecular characteristics of the nine patients with changes in HSDB2 are shown in Tables 2 and 3. We should note that patient 4 was diagnosed with bilateral Wilms’ tumor at the age of 2 yr 8 months, with no known family history for this condition. The genetic analysis of WT1, kindly performed by Dr. Vicky Huff (Department of Molecular Genetics/Cancer Genetics, M. D. Anderson Cancer Center, Houston, TX), did not detect any mutations of this gene.

**Family studies**

PCR amplification and DGGE for exons 1, 2, 3, and 4 of the HSD3B2 gene were performed in DNA obtained from the parents and siblings in eight of the nine patients (Fig. 3). DGGE analysis was performed in parallel with negative and positive controls, when the relatives were available for study. All samples with an abnormal DGGE pattern were sequenced. The family of patient 1 consists of both parents and a brother, and we documented that the mother and the brother, who had normal external genitalia, were also heterozygous for the S213T mutation, explained by possible phenotypic heterogeneity. The parents of patient 2 did not carry the S284R mutation, suggesting a de novo mutation. The mother of patient 3 did not carry the A238 mutation, and the father was not available for study. Both parents and a brother of patient 4 did not show any abnormality, indicating a de novo T259 mutation in this patient. The study of both parents and three siblings of patient 5 detected the T320 mutation only in the father, who showed a bifid prepuce and a wide urethral meatus. The family of patient 6 consists of both parents, a brother, and a sister, with the father showing the same n1362 T→G heterozygous mutation. The father of patient 7 was also heterozygous for this mutation, whereas the mother did not show the nucleotide change, and his sister was not available for study. The family of patient 8 was not available for study. Finally, the family of patient 9 consists of

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Type of hypospadias</th>
<th>Other abnormalities</th>
<th>Molecular findings</th>
<th>Clinical family study</th>
<th>Molecular family study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.9</td>
<td>Scrotal</td>
<td>Bilateral cryptorchidism, chordee, precocious pubarche</td>
<td>S213T</td>
<td>Normal</td>
<td>Mother and brother heterozygous for S213T</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>Midshaft</td>
<td>None</td>
<td>S284R</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>Midshaft</td>
<td>Premature and small for gestational age</td>
<td>A238</td>
<td>Father not available for study</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>2.2</td>
<td>Proximal third of the penis</td>
<td>Micropenis, chordee, Wilms’ tumor</td>
<td>T259</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>1.9</td>
<td>Subcoronal</td>
<td>None</td>
<td>T320</td>
<td>Father has a bifid prepuce and a wide urethral meatus</td>
<td>Father is heterozygous for T320</td>
</tr>
<tr>
<td>6</td>
<td>1.9</td>
<td>Perineoscrotal</td>
<td>Bilateral cryptorchidism, micropenis</td>
<td>n1362 T→G</td>
<td>Normal</td>
<td>Father is heterozygous for n1362 T→G</td>
</tr>
<tr>
<td>7</td>
<td>7.1</td>
<td>Midshaft</td>
<td>None</td>
<td>n1362 T→G</td>
<td>Normal</td>
<td>Father is heterozygous for n1362 T→G</td>
</tr>
<tr>
<td>8</td>
<td>2.4</td>
<td>Subcoronal</td>
<td>None</td>
<td>n1362 T→G</td>
<td>Not available for study</td>
<td>Not available</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>Scrotal</td>
<td>Bilateral cryptorchidism, micropenis, bifid scrotum</td>
<td>n1372A→C</td>
<td>Normal. Father not available for study</td>
<td>Sister carries n1372A→C</td>
</tr>
</tbody>
</table>

**TABLE 3.** Steroid levels before and 60 min after ACTH test in the patients with mutations of 3βHSD2.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>12.9</td>
<td>7.0</td>
<td>0.9</td>
<td>2.3</td>
<td>1.9</td>
<td>1.9</td>
<td>7.1</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>17OH Preg (ng/ml)</td>
<td>1.0</td>
<td>0.8</td>
<td>1.7</td>
<td>0.1</td>
<td>1.3</td>
<td>0.9</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>0 min</td>
<td>4.1</td>
<td>1.9</td>
<td>6.8</td>
<td>0.5</td>
<td>1.5</td>
<td>1.8</td>
<td>0.6</td>
<td>0.7</td>
<td>2.3</td>
</tr>
<tr>
<td>17OH Preg (ng/ml)</td>
<td>0.7</td>
<td>0.6</td>
<td>0.4</td>
<td>0.1</td>
<td>1.0</td>
<td>0.6</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>0 min</td>
<td>2.4</td>
<td>2.5</td>
<td>4.0</td>
<td>1.5</td>
<td>2.1</td>
<td>1.4</td>
<td>0.9</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>DHEA (ng/ml)</td>
<td>3.8</td>
<td>1.8</td>
<td>1.2</td>
<td>1.0</td>
<td>0.4</td>
<td>1.1</td>
<td>2.7</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>0 min</td>
<td>7.4</td>
<td>2.8</td>
<td>1.3</td>
<td>1.6</td>
<td>0.7</td>
<td>1.2</td>
<td>4.6</td>
<td>0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Androstenedione (ng/ml)</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0 min</td>
<td>0.6</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>17OH Preg/17OH Preg</td>
<td>1.7</td>
<td>0.9</td>
<td>1.7</td>
<td>2.9</td>
<td>0.6</td>
<td>1.3</td>
<td>0.6</td>
<td>1.2</td>
<td>6.4</td>
</tr>
<tr>
<td>DHEA/androstenedione</td>
<td>13.2</td>
<td>18.7</td>
<td>13.0</td>
<td>26.7</td>
<td>0.4</td>
<td>8.4</td>
<td>7.6</td>
<td>17.0</td>
<td>18.3</td>
</tr>
<tr>
<td>17OH Preg/cortisol</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

To covert ng/ml in nmol/liter, multiply by the following factors: 17OH Preg × 3.01, 17OH Preg × 3.00, DHEA × 3.47, and androstenedione × 3.49. * These ratios are obtained from the steroid values of 60 min after ACTH administration.
both parents and a sister; the patient and sister carried the
n1362 T→G substitution in intron 1, whereas the mother was
normal, and the father was not available for study.

Effect of mutations on type II 3β-HSD activity

To assess the influence of the mutations on type II 3β-HSD
eexpression and/or activity, COS-7 cells were transfected

Fig. 3. Mutational analysis of the patients and their families. The
schematic representation illustrates the pedigree of the families
of eight patients. The patients are indicated with arrows. ?, Subject
unavailable for the study.

Fig. 4. Western blot (A) and enzymatic activity (B) of transient transfections of
wild-type and mutated human type II 3β-HSD. A. Western blot was per-
formed using antiserum raised in rabbit
against purified human type II 3β-HSD in
COS 7 cell homogenates transfected
with wild-type and mutant pcDNA3 as
described in Subjects and Methods.
Lane 1, S213T-homozygous; lane 2,
S213T-heterozygous; lane 3, S284R-ho-
mozygous; lane 4, S284R-heterozygous;
lane 5, wild-type; lane 6, human corpus luteum homogenate; lane 7, molecular
weight marker. B. Comparison of the
time course of enzymatic conversion of
DHEA into androstenedione in trans-
fected COS-7 cells with homozygous
wild-type human type II 3β-HSD, or
heterozygous or homozygous -S213T,
-S284R, or -V78I mutants expressing
vector as indicated in Subjects and
Methods. The results are representa-
tive as the mean ± SEM (n = 2 in
duplicate) after normalization by protein
content.

Discussion

The first cases of HSD3B2 deficiency were described by
Bongiovanni (22). During the neonatal period, ambiguous
genitalia with or without salt loss may be seen in both sexes
(2, 23–26). Male newborn patients with mutations of the
HSD3B2 gene may manifest salt-losing congenital adrenal
hyperplasia, with undermasculinized genitalia resulting
from the lack of secretion of mineralocorticoids and glu-
corticoids by the adrenals, and of androgens by the testis.
Older boys may show precocious pubarche, and varying
degrees of hypogonadism may be observed during adoles-
cence (1–3). Some patients with less severe mutations in

with the pcDNA3 vector containing wild-type human type
II 3β-HSD or mutant type II 3β-HSD-S213T, -S284R, or -V78I
variants alone or in equimolar concentrations with the wild-
type human type II 3β-HSD. Because an equal amount of
each vector was transfected, we assumed that the enzyme
expression was 50% from the wild-type vector and the other
50% from the variant. This was not confirmed by other meth-
ods, however. In support of this assumption, transfected cells
expressed comparable amounts of either normal or mutated
type II 3β-HSD, and COS-7 cells did not express endogenous
enzyme, as determined by Western immunoblotting (Fig.
4A). The type II 3β-HSD activity, studied by the conversion of
DHEA to androstenedione, in transfected mammalian
nonsteroidogenic cells with the vector expressing heterozy-
gous S213T or S284R mutants, was reduced by 40 and 32%,
respectively, compared with wild-type human type II 3β-
HSD (Fig. 4B). The heterozygous V78I mutation detected in
exon 3 of the control subject did not affect enzyme activity,
as shown in Fig. 4B. In the cells transfected with the vector
expressing the homozygous S213T, S284R, or V78I mutants,
the enzyme activity was reduced by 96, 80, and 99%, respec-
tively, compared with transfected cells with the wild-type
human type II 3β-HSD vector (Fig. 4B).
33). Hypospadias has been observed in patients with Wilms' disease, and his hypospadias was caused by a WT1 gene mutation (31). After our study was performed opens the question of whether the patient's fathers of patients 6 and 7 harbored the same nucleotide substitution but had a normally located urethral meatus. Patient 9 showed heterozygous n1372A substitution but had a normally located urethral meatus. Direct confirmation of this hypothesis, however, cannot be concluded from this study.

We observed an S213T mutation in patient 1, and our in vitro studies demonstrated that this mutation reduced in vitro enzyme activity. The mother and the brother of this patient, however, harbored the same heterozygous mutation but did not exhibit any signs of 3β-HSD deficiency. Thus, it is unclear whether this mutation caused the hypospadias in our patient. Moisan et al. (18) reported a mutation S213G in this same codon, in a girl with premature pubarche. We should mention that serine in codon 213 is conserved in primates and porcines but not in other species; whereas in nonprimates, species a threonine is not observed in this codon (28, 29).

The S284R mutation harbored by patient 2 is located in a conserved hydrophobic domain localized between amino acids 283 and 310 in the COOH-terminal. The deletion of this region produces a mutant cytosolic enzyme that lacks a targeting sequence that directs the translated protein to specific organelles, so a mutation of this hydrophobic domain may be critical for subcellular localization (28–30).

Patients 3, 4, and 5 carried heterozygous silent mutations in exon 4, which might be polymorphic variants of the HSD3B2 gene. In our 101 healthy fertile control men, however, no polymorphic changes were observed in exon 4, although we documented a V78I heterozygous mutation in exon 3 in one control individual that displayed normal enzymatic activity. The number of control individuals we studied, though, is not sufficient to define with certainty the genotype distribution.

The fact that patient 4 developed a bilateral Wilms’ tumor after our study was performed opens the question of whether his hypospadias was caused by a WT1 gene mutation (31–33). Hypospadias has been observed in patients with Wilms’ syndrome attributable to WT1 gene abnormalities, even without Wilms’ tumor or overt nephropathy. The molecular study of the WT1 gene, however, did not show mutations of this gene in this patient.

An n1362T→G substitution was observed in intron 1 in patients 6, 7, and 8 and in one healthy fertile control. The fathers of patients 6 and 7 harbored the same nucleotide substitution but had a normally located urethral meatus. Patient 9 showed heterozygous n1372A→C mutation in intron 1, which was also carried by his father and sister, who did not exhibit genital anomalies. These substitutions in intron 1 are likely to represent polymorphisms. Both nucleotide changes in the intron 1 detected in this study are highly conserved through the human 3β-HSD gene family. Simard et al. (34) reported two sibs with nonsalt-losing form of classic 3β-HSD, who had a nucleotide mutation n6651G→A in intron 3 in one allele, and a missense mutation in exon 4 in the other allele, which reduced enzymatic function. Some intronic nucleotide mutations may affect mRNA processing by modification of splicing sites. Previously, it has been reported that a complex dinucleotide repeat in the HSD3B2 located in the intron 3 constitutes a highly polymorphic region with variable racial/ethnic distribution (35). The present study is the first report of nucleotide changes in intron 1 of this gene.

The hormonal abnormalities observed in patients with classical 3β-HSD deficiency show increased levels of 17OHPreg and DHEA and increased ratios of 17OHPreg/17OHPreg, 17OHPreg/cortisol, and DHEA/androstenedione (36). We did not observe increased 17OHPreg or DHEA concentrations either in the basal state or after stimulation with ACTH or HCG in our patients with mutations of the HSD3B2 gene, except possibly in patient 3. This is not totally unexpected, however, because there are several difficulties in establishing the criteria for the diagnosis of 3β-HSD deficiency based on hormonal levels: the levels of 17OHPreg may not be elevated due to normal activity of the 3β-HSD type I, which is present in other tissues such as skin and liver. In addition, normal hormonal levels vary with chronological age and have broad ranges at different ages (19). Furthermore, Lutfallah et al. (37) studied 12 heterozygous carriers of severe HSD3B2 mutations, and these patients had normal hormonal findings; however, subtle clinical findings or 3β-HSD in vitro activity were not reported in that study. Recently Lutfallah et al. (38) proposed new hormonal criteria for the diagnosis of 3β-HSD deficiency. One of the limitations of our study is the lack of normal hormonal values for the ACTH and HCG tests from our own laboratory, which complicates the interpretation of these results.

In summary, we have performed a complete hormonal and molecular study of the HSD3B2 gene in a large group of patients with apparent idiopathic hypospadias. Two patients showed heterozygous missense mutations in exon 4, leading to decreased in vitro enzymatic activity, suggesting a possible cause of reduction in androgen synthesis during the first trimester of gestation. We conclude that subtle molecular abnormalities of the HSD3B2 gene may be observed in some patients with apparent idiopathic hypospadias but that this finding is uncommon.

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