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The Identification of 5α-Reductase-2 and 17β-Hydroxysteroid Dehydrogenase-3 Gene Defects in Male Pseudohermaphrodites from a Turkish Kindred*

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ABSTRACT

Male pseudohermaphroditism (MPH) is characterized by incomplete differentiation of male genitalia in the presence of testicular tissue. Enzymatic defects involving androgen synthesis or action are causes of MPH. We studied the molecular genetics of a large isolated inbred Turkish kindred with MPH due to either 5α-reductase-2 (*SRD5A2*) or 17β-hydroxysteroid dehydrogenase-3 (*17βHSD3*) gene defects. Using single strand DNA conformational polymorphism analysis and DNA sequencing, a new mutation in exon 5 of *SRD5A2* gene was detected in certain male pseudohermaphrodites from this kindred. This single base deletion (adenine) resulted in a frame shift at amino acid position 251 resulting in the addition of 23 amino acids at the carboxyl-terminal of this 254-amino acid isozyme. Transfection expression of the mutant isozyme in CV1 cells showed a complete loss of enzymatic activity in the conversion of [^{14}C]testosterone to dihydrotestosterone, without a change in the messenger ribonucleic acid level compared to that of the wild-type isozyme. Analysis of the *17βHSD3* gene in other male pseudohermaphrodites from this kindred revealed a single point mutation (G→A) at the boundary between intron 8 and exon 9, disrupting the splice acceptor site of exon 9.

In this kindred, in addition to the identification of male pseudohermaphrodites with either a homozygous *SRD5A2* or *17βHSD3* gene defect, other male pseudohermaphrodites were found to be genetically more complex: *e.g.* homozygous for the *SRD5A2* defect and heterozygous for the *17βHSD3* defect, or homozygous for the *17βHSD3* defect and heterozygous for the *SRD5A2* defect. Also, phenotypically normal carriers were identified with either one or both gene defects.

Homozygous male pseudohermaphrodites with *SRD5A2* or *17βHSD3* gene defects were phenotypically distinguishable by the presence of mild gynecomastia in the latter. Hormone data were consistent with the particular homozygous gene defect. In summary, we show 1) the novel existence of two gene defects, *SRD5A2* and *17βHSD3*, each causing MPH within a large isolated Turkish kindred; 2) that the two defects segregate independently and may be inherited from two different progenitors; and 3) analysis of a new mutation in exon 5 of *SRD5A2* gene, supporting the functional importance of the carboxyl-terminal of 5α-reductase-2 isozyme. (*J Clin Endocrinol Metab* **83:** 560–569, 1998)

A MALE pseudohermaphrodite is an incompletely masculinized individual with a 46,XY karyotype and testes. Genetic defects in enzymes necessary for the synthesis or action of androgens may result in incomplete development of the Wolffian ducts and male genitalia (1, 2). An isolated Turkish kindred of male pseudohermaphrodites with biochemical evidence of two distinct enzyme defects was previously reported by this laboratory in 1987 (3). This kindred is from a remote village located in the Taurus mountains of southern Turkey. Pedigree analysis demonstrated consanguinity within the kindred (Figs. 1 and 2). Some of the affected males had 5α-reductase-2 (5α-RD-2; gene *SRD5A2*) deficiency, whereas others had 17β-hydroxysteroid dehydrogenase 3 (17βHSD-3; gene *17βHSD3*) deficiency.

The diagnosis of 5α-RD-2 deficiency in several male pseudohermaphrodites was previously established biochemically by an elevated plasma testosterone (T) to dihydrotestosterone (DHT) ratio coupled with increased urinary C_{19} and C_{21} 5β/5α-steroid metabolite ratios (3–5). Intermediate elevations of urinary C_{19} and C_{21} 5β/5α-steroid metabolite ratios identified heterozygous individuals. The biochemical diagnosis of 17βHSD deficiency in other affected male pseudohermaphrodites was based on elevated ratios of plasma androstenedione (Δ^4) to T (3, 6). Heterozygosity for 17βHSD-3 deficiency, however, could not be demonstrated with this methodology.

This paper identifies gene defects for both *SRD5A2* and *17βHSD3* within this kindred. Homozygous and genetically complex heterozygous subjects are also described. In addition to molecular genetic studies of this large kindred (3, 5), affected subjects and families not yet connected to the master pedigree are described.

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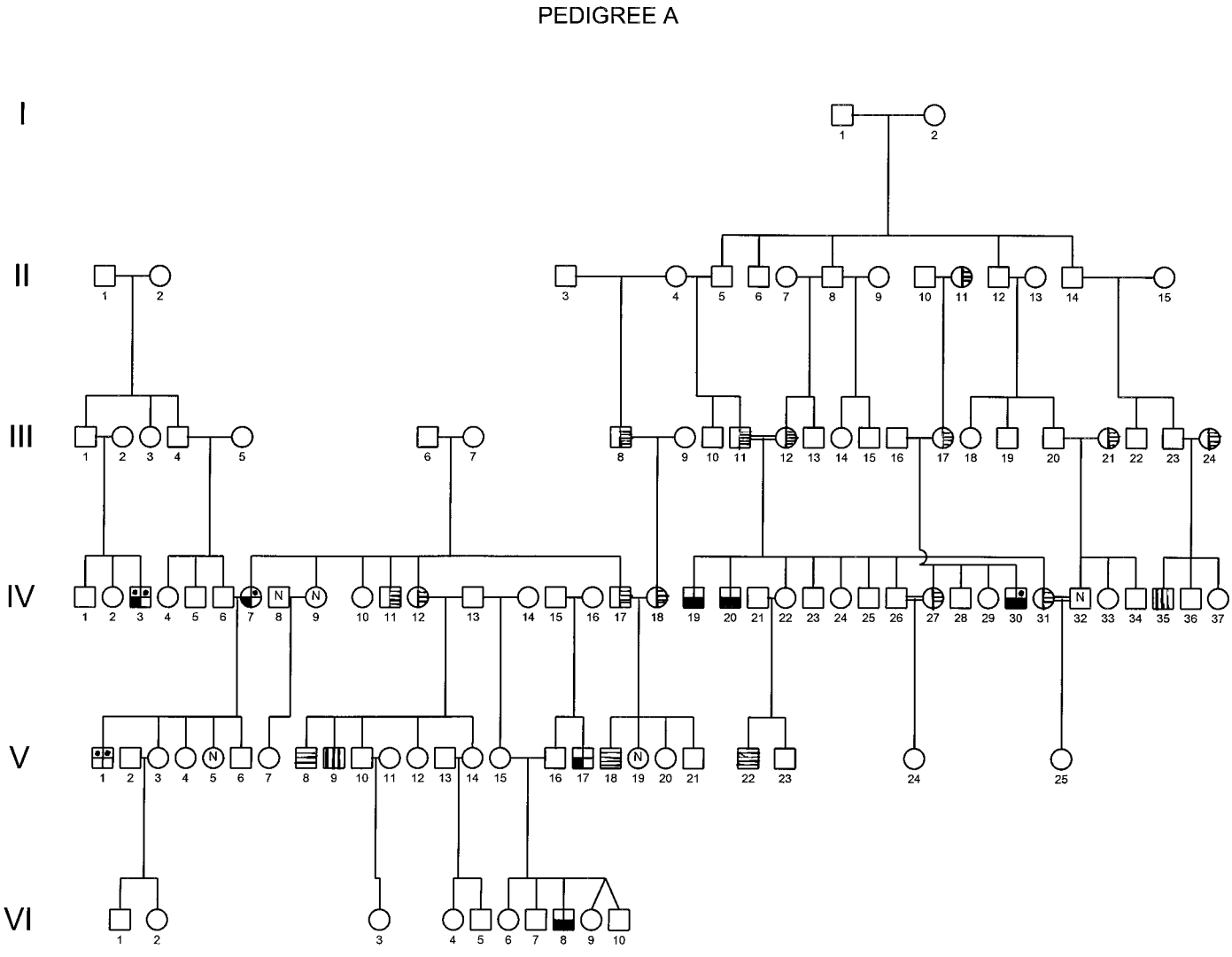

FIG. 1. Pedigree A of a large Turkish kindred with 5α-RD-2 and 17βHSD-3 deficiency. *Circles* and *squares* indicate females and males, respectively. The Roman numerals indicate the generation. ◪, Homozygous *SRD5A2* gene defect; ◧, heterozygous *SRD5A2* gene defect; ▣, homozygous *17βHSD3* gene defect; ◪-, both homozygous *SRD5A2* and heterozygous *17βHSD3* gene defects; ◪, both homozygous *17βHSD3* and heterozygous *SRD5A2* gene defects; ◔ , both heterozygous *SRD5A2* and heterozygous *17βHSD3* gene defects; ▤, 5α-RD-2-deficient patients identified by clinical and biochemical studies; ▥, 17βHSD-3-deficient patients identified by clinical and biochemical studies; ◨, 5α-RD-2-deficient carriers identified by biochemical studies.

Subjects and Methods

Subjects

Twenty-one subjects (nine male pseudohermaphrodites, five phenotypically normal males, and seven phenotypically normal females), as shown in Table 1 and Figs. 1 and 2, were studied. All participants provided written informed consent. This study was approved by the institutional review board of the New York Hospital-Cornell Medical Center.

Subjects A-IV-20, A-IV-19, A-IV-30 (Fig. 1), and D-II-5 (Fig. 2) have clinical and biochemical evidence of steroid 5α-RD-2 deficiency and were evaluated previously as subjects adult-1, adult-2, child-7, and child-8, respectively (5). Subject A-V-1 (Fig. 1) is subject IV-7 in the previously published study and has clinical and biochemical evidence of 17βHSD-3 deficiency (3) (Table 1). His mother (subject A-IV-7) is subject III-1 from the previous study (3). The clinical features of these male pseudohermaphrodites were described previously (3, 5).

A-IV-3 is a 45-yr-old subject, born with ambiguous genitalia and raised as a female, although known in the community as an affected child. The subject did not change to a male gender role at puberty and remains the only known affected person not to do so in the community. A moderate amount of facial hair was present. Blood and urine samples were provided, but a physical examination was declined.

A-VI-8 is a 46-yr-old male pseudohermaphrodite who was raised as a female, but changed to a male gender role at puberty. He supposedly underwent unsuccessful corrective genital surgery 20 years ago. On physical examination he had penoscrotal hypospadias and a phallus with a stretched length of 3 cm. His left testis was in the inguinal canal, and the right testis was in the scrotum (10 and 15 mL, respectively). He had a deep voice, but no acne or temporal hair line recession.

Subject B-III-7 is a 6-month-old child, who was recognized to be a male pseudohermaphrodite at birth by his family and is being raised as a male. He had perineoscrotal hypospadias, a bifid scrotum and a right scrotal testis. The left testis could not be palpated. Phlebotomy was not possible due to poor venous access.

C-II-8 is a 23-yr-old male pseudohermaphrodite, who was raised as a female. At puberty there was no breast development or menstrual periods. There was clitoral enlargement, a muscular body habitus, and deepening of the voice. A gender role change to male occurred during this time. On physical examination there was moderate facial and body hair, a female escutcheon, and no evidence of acne or temporal hair line recession. There was perineoscrotal hypospadias as well as a bifid scro-

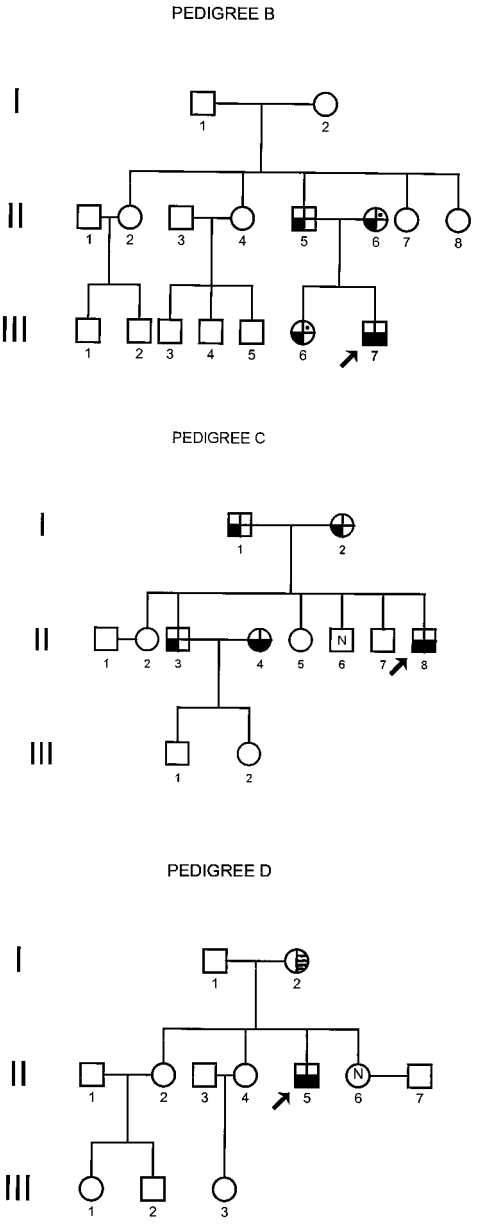

FIG. 2. Pedigrees of families B–D from a Turkish community, who are not yet linked to the large pedigree A (see Fig. 1). See Fig. 1 for the details of symbol labeling.

tum, a pseudovagina, and a right inguinal and a left scrotal testis (10 and 15 mL, respectively). His phallus measured 2 cm in length when stretched, and a chordee was present.

Subjects A-V-17, B-II-5, C-I-1, C-II-3, and C-II-6 are healthy adult males (aged 23–60 yr) from the community with normal secondary sexual characteristics and normal sexual function. Subjects B-II-6 and N1 are healthy adult females (aged 24 and 45 yr old, respectively) from the community with normal secondary sexual characteristics, reproductive function, and physical examinations. C-II-4 is a 36-yr-old woman who underwent menarche at age 14 yr and has two children. Her menstrual periods were regular, at 25-day intervals, with scanty bleeding for 3 days. Her physical examination was within normal limits. The Ferriman-Galloway hirsutism score was 3 of 11. There was no history of acne, infertility, menstrual irregularity, or hirsutism in these females. Two subjects (C-I-2 and N-2) were postmenopausal, one subject (B-III-6) was a healthy 4-yr-old girl with a normal physical examination.

Methods

Blood samples from representative members of the master pedigree and three smaller pedigrees (see *Subjects* section above; Figs. 1 & 2) were collected for molecular genetic analysis and plasma hormone assays. Urine samples were also collected for analysis of steroid $5\beta/5\alpha$ metabolites.

Plasma androgens and urinary steroid metabolites. Plasma Δ^4, T, and DHT were measured by RIA after separation by paper chromatography using a previously described method (7, 8). Urinary C_{19} and C_{21} $5\beta/5\alpha$-steroid metabolite ratios (etiocholanolone to androsterone, 11β-hydroxyetiocholanolone to 11β-hydroxyandrosterone, tetrahydrocortisol to 5α-tetrahydrocortisol, and tetrahydrocorticosterone to 5α-tetrahydrocorticosterone) were analyzed as methyloxime trimethylsilyl ether derivatives by capillary gas chromatography, as described previously (8, 9).

PCR amplification and DNA sequencing. Blood was drawn into ethylenediamine tetraacetate (EDTA)-containing tubes, and genomic DNA from white blood cells was isolated by the Qiagen genomic DNA isolation kit (Qiagen, Chatsworth, CA). The concentrations of DNA were determined by UV absorbance. Exons 1–5 of the *SRD5A2* gene (10) were amplified and sequenced by PCR as previously described with minor modifications (11). Briefly, PCR amplification was carried out in 25 μL containing 0.12 μg genomic DNA, 1 μmol/L of each oligonucleotide primer, 200 μmol/L of each of four deoxyribonucleotide triphosphates, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.1% Triton X-100, 1.5 mmol/L $MgCl_2$, and 2.5 U thermostable DNA polymerase (Promega, Madison, WI). For hot PCR, 10 μCi [α-^{32}P]deoxy-ATP were added to the reaction. The samples were heated at 94 C for 2 min, and then at 94 C for 1 min, 65 C for 1 min, and 72 C for 1 min for a total of 35 cycles; a final cycle consisted of 72 C for 10 min.

PCR amplification and labeling of exons 1–11 of the *17βHSD3* gene were carried out using primers and annealing temperatures summarized in Table 2. The reaction mixture contained 0.12 or 0.36 μg genomic DNA, 200 μmol/L of each of four deoxyribonucleotide triphosphates, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.1% Triton X-100, and 2.5 U thermostable DNA polymerase. Ten microcuries of [α-^{32}P]deoxy-ATP were added for hot PCR. The samples were denatured at 94 C for 2 min, and then sequentially heated at 94 C for 30 s, at the annealing temperature shown in Table 2 for 15 s, and at 72 C for 30 s for a total of 30 or 35 cycles. A final extension cycle was performed at 72 C for 10 min. The *17βHSD3* gene was sequenced using the fmol DNA sequencing kit (Promega, Madison, MI) with ^{32}P-labeled primer (11).

Single strand DNA conformational polymorphism (SSCP) analysis. SSCP analysis was performed as described previously (11) with certain modifications. Exon DNA was amplified and radiolabeled as described above. One microliter of the PCR product was added to 9 μL formamide denaturing dye (98% formamide, 20 mmol/L EDTA, 10 mmol/L NaOH, and 0.05% each of xylene cyanol and bromophenol blue), denatured at 100 C for 6 min, and immediately cooled on ice. Three microliters of this solution were loaded onto a 0.5 X Hydrolink MDE gel (J. T. Baker, Phillipsburg, NJ) containing 10% glycerol and electrophoresed at 350 V at room temperature overnight in 0.6 $\times$ TBE buffer (54 mmol/L Tris-borate, pH 8.3, and 2.4 mmol/L EDTA). An aliquot of hot PCR sample was diluted in loading buffer (60% sucrose, 50 mmol/L EDTA, and 0.05% each of xylene cyanol and bromophenol blue) and loaded in an adjacent lane without denaturation to determine the migration position of the double-stranded DNA fragment. After electrophoresis, the gel was dried and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at room temperature.

Site-directed mutagenesis and expression of 5α-RD-2 isoenzymes. A plasmid (pCMV-5αRD2) containing the entire encoding region of human 5α-RD-2 complementary DNA (cDNA) (12) was obtained from Drs. Luu-The and Labrie (CHUL Research Center, Quebec, Canada). A new subclone (pCMV-5αRD2w) was constructed by insertion of a PCR-amplified fragment of 3′-untranslated region [from 2335–2394 in the GenBank sequence (10)] of human *SRD5A2* gene to *Sac*I-*Hin*dIII sites of pCMV-5αRD2. This new construct, pCMV-5αRD2w, was used for *in vitro* mutagenesis to generate a single base deletion using Altered Sites II *in vitro* Mutagenesis Systems from Promega (Madison, WI). The sequences of all constructs were confirmed by DNA sequencing.

TABLE 1. Summary of genetic and biochemical data of the subjects

Subject no.	Age (yr)	Sex	5αRD-2 mutation[a]	17βHSD-3 mutation[a]	Δ^4 (ng/dL)	T (ng/dL)	DHT (ng/dL)	T/DHT	E/A	11OHE/11OHA	THB/5αTHB	THF/5αTHF
Homozygous for 17βHSD-3 deficiency												
A-V-1	26	MPH	−/−	+/+	1860	259	35	7	1.12	0.36	0.48	2.37
Homozygous for 17βHSD-3 deficiency and heterozygous for 5αRD-2 deficiency												
A-IV-3	45	MPH	−/+	+/+	869	286	22	13	2.5	1.22	2.41	15.7
Homozygous for 5αRD-2 deficiency												
A-IV-19	48	MPH	+/+	−/−	88	682	12	57	4.41	2.00	13.7	12.7
A-IV-20	42	MPH	+/+	−/−	95	795	26	31	4.98	1.31	NM	>20
A-VI-8	46	MPH	+/+	−/−	123	877	17	52	4.99	1.12	5.71	25.1
B-III-7	6m	MPH	+/+	NA	NA	NA	NA	NA	1.06	0.09	2.2	>20
C-II-4	36	F	+/+	−/−	82	23	3	8	8.03	1.64	4.55	NM
C-II-8	23	MPH	+/+	−/−	108	517	17	30	5.33	0.68	4.41	54.5
D-II-5	24	MPH	+/+	−/−	134	724	21	34	4.7	2.14	12.04	37.14
Homozygous for 5αRD-2 deficiency and heterozygous for 17βHSD-3 deficiency												
A-IV-30	26	MPH	+/+	−/+	NA	NA	NA	NA	1.40	1.50	1.00	6.75
Heterozygous for 5αRD-2 deficiency												
A-V-17	49	M	−/+	−/−	52	529	47	11	2.13	1.41	0.83	3.12
B-II-5	26	M	−/+	−/−	64	492	33	15	3.9	0.67	1.84	6.9
C-I-1	60	M	−/+	−/−	102	1120	57	20	1.27	0.47	1.38	6.11
C-I-2	59	F	−/+	−/−	61	8	2	4	2.81	0.62	1.68	7.32
C-II-3	39	M	−/+	−/−	132	774	83	9	1.54	0.74	0.83	3.16
Heterozygous for 5αRD-2 deficiency and heterozygous for 17β-HSD-3 deficiency												
A-IV-7	55	F	−/+	−/+	67	8	1	8	3.19	1.8	1.25	4.52
B-II-6	24	F	−/+	−/+	147	109	6	18	2.14	0.66	0.89	3.4
B-III-6	4	F	−/+	−/+	27	NM	NM	NA	0.97	0.80	0.61	1.55
Normal												
C-II-6	23	M	−/−	−/−	125	889	72	12	1.17	0.29	0.94	3.89
N1	45	F	−/−	−/−	78	11	5	2	1.92	1.11	0.66	2.18
N2	51	F	−/−	−/−	67	15	8	2	1.42	0.81	0.24	1.46
Ref. values												
Female					50–200	14–55	3.8–25.3	2.6–5.4	0.9 ± 0.4	0.4 ± 0.2	0.5 ± 0.2	1.66 ± 0.62
Male					50–150	300–950	30–70	8–17	0.9 ± 0.4	0.4 ± 0.2	0.5 ± 0.2	1.11 ± 0.4
Prepubertal child					10–30	5–10	1–5		1.08 ± 0.14	0.6 ± 0.24	0.43 ± 0.42	1.21 ± 1.63

5αRD-2, 5α-Reductase-2 gene; 17βHSD-3, 17β-hydroxysteroid dehydrogenase-3; Δ^4, androstenedione; T, testosterone; DHT, dihydrotestosterone; E/A, etiocholanolone to androsterone; 11OHE/11OHA, 11β-hydroxyetiocholanolone to 11β-hydroxyandrosterone; THB/5αTHB, tetrahydrocorticosterone to 5α-tetrahydrocorticosterone; THF/5αTHF, tetrahydrocortisol to 5α-tetrahydrocortisol; m, months; MPH, male pseudohermaphrodite; F, female; M, male; NA, not applicable; NM, nonmeasurable (below the detection limit of the assay); −/−, wild type (normal); −/+, heterozygote (carrier); +/+, homozygote (patient).

[a] The 5αRD-2 gene mutation is a single base (adenine) deletion; the 17βHSD-3 gene mutation is 655-1, G→A at intron 8.

TABLE 2. Summary of primer sequences and PCR conditions for amplification of 17βHSD-3 gene

Primer	Location	Amplification target	Sequence 5′→3′	Primer conc. (μmol/L)	$MgCl_2$ conc. (mmol/L)	Anneal temperature (°C)
1N	5′-Untranslated,	Exon 1	ACGGCCAGGGCTGAAACAGTCTGTT	1	1.5	65
1C	Intron 1		AGTAACAAGCAGGAACAACAGCAG			
2N	Intron 1	Exon 2	TGAATTACTGTCTTTTAAAAGCA	0.5	1.5	54
2C	Intron 2		AATACAAGGGAGGAGAAAGTCCCCA			
3N	Intron 2	Exon 3	GCTCATCATCCTTGTCTCTTGGTTT	1	1.5	65
3C	Intron 3		GAGGGCTCCACACACATCTCCCTTA			
4N	Intron 3	Exon 4	TGGATCCCTGTTCATTAAAAAAACT	1	1.5	65
4C	Intron 4		GATGTATGACAACAAGCTTTGCATC			
5N	Intron 4	Exon 5	CTGATCTTCTGACACATTTTTGTTT	0.5	2	62
5C	Intron 5		AGCCAGGGGACCCAGAACCTGGG			
6N	Intron 5	Exon 6	GAGAATTTCTCTAATCATCCGGCTG	0.5	1	60
6C	Intron 6		ACATGTTAATGCATTTCGCACA			
7N	Intron 6	Exon 7	AGTTCCTTGTCGGGCTTACCTTTGG	1	1.5	65
7C	Intron 7		AGGGCAGGGAGGCCATGTTGCTCCA			
8N	Intron 7	Exon 8	CAACAAAGCCATGGGAAC	1	1.5	62
8C	Intron 8		AAGGAAGAGACTTGGAAGTCATGAC			
9N	Intron 8	Exon 9	AGCTCACTCTGGGGCCTCAGGTGTC	1	1.5	65
9C	Intron 9		GATGACAAGGACTCCACAGTCG			
10N	Intron 9	Exon 10	GATTGCTTCTGTGCCATGGTCTTTG	0.5	2	54
10C	Intron 10		TTCAAGAAAAGGAGAAGTT			
11N	Intron 10	Exon 11	GATGAACTGAGGTACTTGTTATTCC	1	1.5	65
11C	3′-Untranslated		GAGGAAAAGGTTGTGCTGGACTCCT			

Oligonucleotide sequences were reported by Andersson and co-workers (20).

CV1 cells (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% FBS, 20 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells were plated on 60-mm dishes with a density of 0.4×10^6 cells/dish and transfected using the calcium phosphate precipitation method (ProFection, Promega) with 5–10 μg expression vector, 2 μg RSV-β-galactosidase plasmid, and pBluescript-SK plasmid to total 15 μg DNA/dish as previously described (13). After 12–16 h of transfection, the cells were washed and continued to grow in fresh medium for 48 h before harvesting. The transfection efficiencies were monitored by measuring β-galactosidase activity in the cell homogenates.

Assay of 5α-RD-2 enzymatic activity. 5α-RD-2 enzymatic activity was assessed as previously described with modifications (14, 15). Briefly, the transfected cells were harvested by scraping with a rubber policeman, pelleted by centrifugation, washed three times with phosphate-buffered saline, resuspended in 50 mmol/L sodium phosphate buffer (pH 7.4) containing 20% glycerol, homogenized, frozen, and stored at −80 C until analysis. For the enzymatic assay, cell homogenate proteins (20 μg) were incubated at 37 C for 1 h in 0.1 mol/L Tris-citrate buffer (pH 5.0), 2 mmol/L NADPH, and 5 μmol/L [^{14}C]T. Immediately after incubation, the steroids were extracted in ethyl ether, dried, and resuspended in chloroform-methanol (2:1) solution. The steroid products were separated by TLC on silica gel A/chloroform-ethyl-acetate (5:1, vol/vol) developing system, exposed to Kodak X-Omat film, and identified by comparison with known standards. The conversion of T to DHT was determined by liquid scintillation counting of radioactive steroid products.

RT-PCR analysis of messenger ribonucleic acid (mRNA) levels. Total cellular RNA from transfected CV1 cells was extracted using TRIzol reagents (Life Technologies, Grand Island, NY), and the concentrations were determined by UV absorbance (13). One microgram of total cellular RNA or pCMV-5αRD2w plasmid DNA was treated with 5 U ribonuclease (RNase)-free deoxyribonuclease I (DNase I; Promega, Madison, WI) in 50 mmol/L Tris-HCl (pH 7.5) and 10 mmol/L $MgCl_2$ at 37 C for 30 min. Ten micrograms of transfer RNA were added to the reaction as a carrier. The samples were purified by a High Pure PCR Product Purification Kit (Boehringer Mannheim, Indianapolis, IN), treated with 10 U RNase-free DNase I (Boehringer Mannheim), and repurified. One tenth of each sample was subjected to RT-PCR amplification using the Titan One Tube RT-PCR System (Boehringer Mannheim) to quantitate the levels of 5α-RD-2 mRNA in transfected cells according to the manufacturer's instruction with some modifications. A pair of primers, 5′-GACATTTGTG TACTCACTGCTC-3′ and 5′-CGAAGCTTCATTGACA GTTTTCAT-CAGCATTGTGG-3′, located at the beginning of exon 2 and the untranslated region of exon 5 of 5α-RD-2 mRNA, respectively (11), were used in amplification. The conditions of RT-PCR amplification were: incubation of the samples at 50 C for 30 min, then one cycle at 94 C for 2 min to denature the template, 35 cycles each of 94 C for 30 s, 60 C for 30 s, and 68 C for 45 s, followed by a final cycle of 68 C for 5 min. One fifth of each sample was analyzed by electrophoresis in a 1.5% agarose gel and visualized with ethidium bromide staining.

Statistics. The 5α-RD-2 enzymatic activity is presented as the mean ± SEM percent conversion of T to DHT. One-way ANOVA followed by *post-hoc* Student-Newman-Keuls test was used to determine the difference among multiple groups. $P < 0.05$ was accepted as the level for statistic significance.

Results

The interrelationship of the male pseudohermaphrodites is apparent in the master pedigree A (Fig. 1). Pedigrees B–D (Fig. 2) are not yet linked to pedigree A. Table 1 summarizes the genetic, plasma, and urinary data.

SRD5A2 gene mutation

Analysis of the *SRD5A2* gene by SSCP revealed a mutation in exon 5 in subjects A-IV-20 and C-II-8, as shown in Fig. 3A. The migration pattern of radiolabeled single stranded PCR products of affected subjects was compared with that of normal controls (Fig. 3A, A-IV-20 and C-II-8 *vs.* normal). This mutation was confirmed by DNA sequencing. As shown in Fig. 3B, a single base, adenine, at exon 5 of the *SRD5A2* gene was deleted in affected subjects, A-VI-8 and C-II-8. The remaining four exons of the *SRD5A2* gene were normal in the affected subjects by both SSCP and DNA sequencing (data not shown). Heterozygosity of the mutation was detected by both SSCP analysis (see lanes C-I-1 and A-IV-7 of Fig. 3A) and DNA sequencing (see lane C-I-2 of Fig. 3B). In addition, heteroduplex formulation of double-stranded DNA was de-

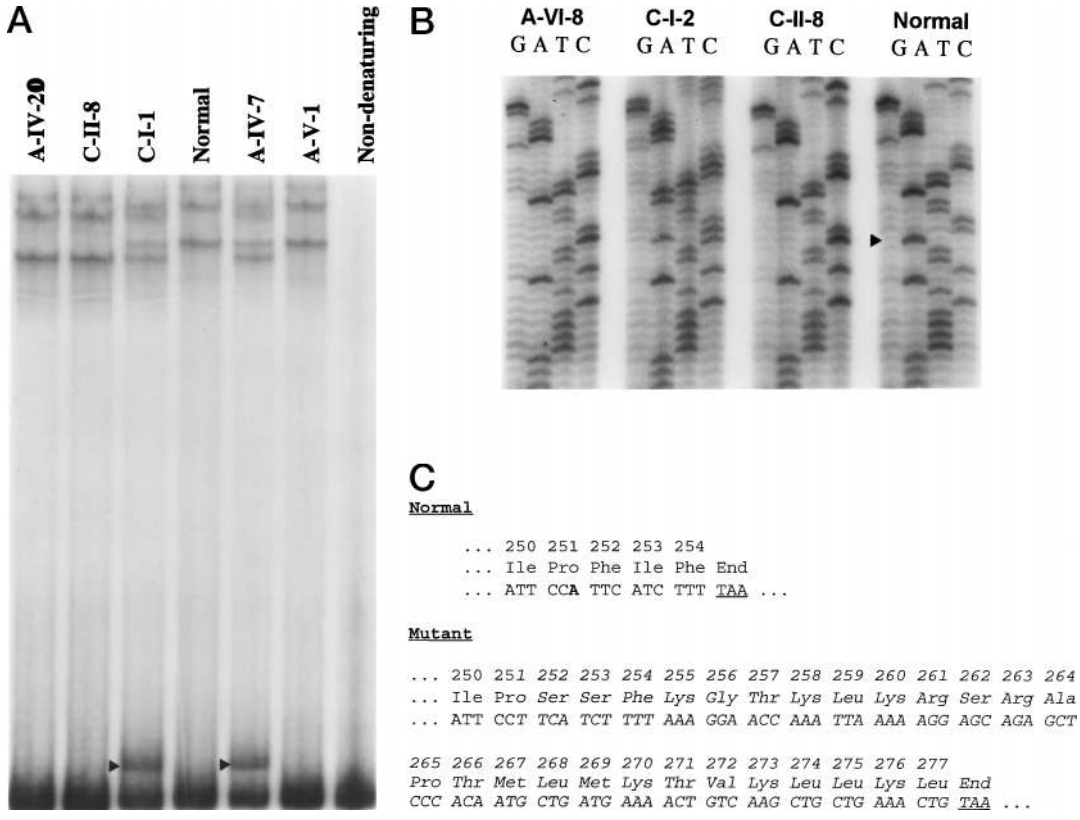

FIG. 3. Exon 5 mutation of the 5α-RD-2 gene (*SRD5A2*) in male pseudohermaphrodites from a Turkish community. A, Representative SSCP analysis of exon 5 of the *SRD5A2* gene in male pseudohermaphrodites with 5α-RD-2 deficiency (A-IV-20 and C-II-8), two carriers (A-IV-7 and C-I-1), a 17βHSD-3-deficient subject (A-V-1), and a normal subject. The bands on the *top* represent the various mobilities of the single strand DNA conformations, and the band on the *bottom* is the double-stranded DNA as indicated in the nondenaturing lane. The *solid arrowheads* mark the heteroduplex DNA in heterozygous subjects. B, Representative DNA sequencing of exon 5 of the *SRD5A2* gene in male pseudohermaphrodites with 5α-RD-2 deficiency (A-VI-8 and C-II-8), a carrier (C-I-2), and a normal subject. The *solid arrowhead* indicates that an adenine (A) in the normal sequence is deleted in affected subjects. C, Partial sequences of exon 5 of human *SRD5A2* gene to illustrate the single base (adenine) deletion in the exon 5 of *SRD5A2* gene in male pseudohermaphrodites from the Turkish community resulted in a frame shift at amino acid position of 251 and an additional 23 amino acids in the carboxyl-terminal of 5α-RD-2 isozyme. The number in the first row shows the amino acid position of 5α-RD-2 isozyme. The boldface A in the third row of the normal sequence indicates the deleted adenine in the mutated gene. The sequences after the mutation are indicated as italic letters.

tected in heterozygous subjects by SSCP analysis (Fig. 3A, *arrowheads* C-I-1 and A-IV-7).

The single base deletion in exon 5 of the *SRD5A2* gene caused a frame-shift mutation at amino acid position 251, with the addition of 23 amino acids to the carboxyl-terminal of this normally 254-amino acid isozyme (Fig. 3C). The functional significance of this mutation was assessed by *in vitro* mutagenesis-transfection expression in CV1 cells. The mutation was generated in an expressible cDNA by site-directed mutagenesis. Wild-type and mutated cDNAs in expression vector were transfected to CV1 cells, and the enzymatic activity was determined in the cell homogenates. As shown in Fig. 4, A and B, the conversion of [^{14}C]T to DHT was completely lost in the mutant isoenzyme (only 0.5% of the wild-type isoenzyme) and was the same as that in the nontransfected or mock-transfected controls. However, the level of mutant *SRD5A2* mRNA was comparable to that in the wild-type isoenzyme using RT-PCR analysis (Fig. 4C).

Plasma androgen levels and urinary steroid metabolite ratios in subjects homozygous for the mutation in the *SRD5A2* gene (Table 1) were consistent with 5α-RD-2 deficiency (2, 4, 16–18). As shown in Table 1, the genetically confirmed homozygotes had high normal plasma T levels with low plasma DHT levels, an increased T to DHT ratio, and elevated 5β/5α-steroid metabolite ratios. The heterozygous subjects had intermediate C_{19} and C_{21} urinary 5β/5α-steroid metabolite ratios, consistent with their carrier status, as previously described (3).

17βHSD3 gene mutation

Using SSCP analysis and DNA sequencing, a single point mutation at the boundary between intron 8 and exon 9 of *17βHSD3* gene was detected in subjects A-V-1 and A-IV-3. Figure 5A shows a representative SSCP, demonstrating the distinctly abnormal migration pattern of radiolabeled single-stranded PCR products of exon 9 of the *17βHSD3* gene. DNA sequencing (Fig. 5B) revealed a substitution of an adenine (A) for a guanine (G) preceding the first base of exon 9. This mutation disrupted the splice acceptor site of exon 9 (19, 20). Heterozygosity of this mutation was detectable by SSCP (Fig. 5A, A-IV-7) and was confirmed by DNA sequencing (Fig. 5B, A-IV-30 and B-II-6).

Analysis of plasma steroids in this homozygote with

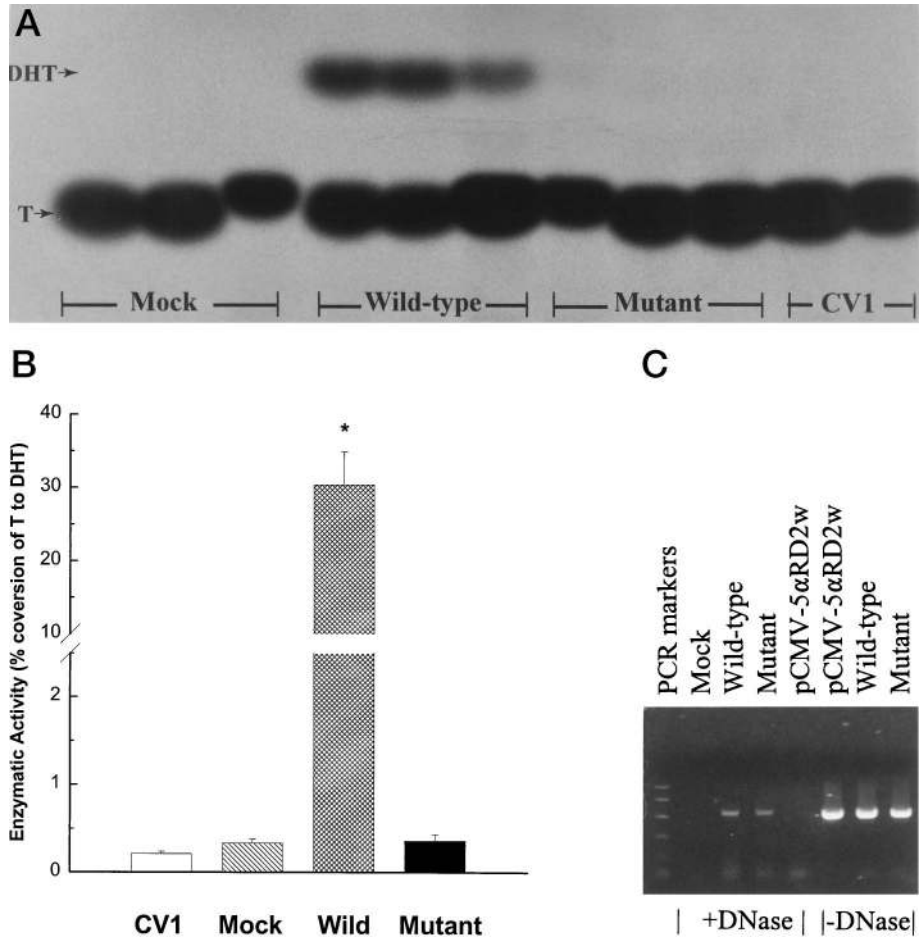

FIG. 4. Transfection analyses of mutant enzyme activity and gene expression levels of *SRD5A2* gene in CV1 cells. A, Representative assay of 5α-RD enzyme activity. Cell homogenate from pCMV vector (mock)-transfected, wild-type or mutant *SRD5A2* gene-transfected, or untransfected (CV1) CV1 cells were assayed for 5α-RD activity in the presence of [^{14}C]T as described in *Materials and Methods*. The positions of T and DHT are indicated. B, The levels of 5α-RD activity (percent conversion of T to DHT) by transfection analysis in CV1 cells. The values are the mean ± SEM of six individual samples. *, $P < 0.05$ compared to all other groups (by Student-Newman-Keuls test). C, RT-PCR analysis of *SRD5A2* gene expression in transfected cells. Total cellular RNA was isolated from pCMV vector (mock)-transfected or wild-type or mutant *SRD5A2* gene-transfected CV1 cells, treated with (+DNase) or without (−DNase) RNase-free DNase I. The transcript levels were examined by RT-PCR as described in *Materials and Methods*. pCMV-5αRD2w containing the wild-type human 5α-RD-2 cDNA was used as a control for the assay. The PCR size markers on the *left* are 1000, 750, 500, 300, 150, and 50 bases from *top* to *bottom*, respectively. The expected size of the specific product is 545 bases.

17βHSD-3 deficiency (subject A-V-1) revealed a markedly elevated plasma Δ^4 level (1860 ng/dL; normal male range, 50–150 ng/dL), a decreased T level (259 ng/dL; normal male range, 300–950 ng/dL), and an increased Δ^4 to T ratio (7.2; normal, <0.5; Table 1), consistent with 17βHSD-3 deficiency (3, 19, 20). The plasma DHT level, plasma T to DHT ratio, and ratios of urinary C_{19} and C_{21} 5β/5α-steroid metabolites were normal.

A male pseudohermaphrodite homozygous for a SRD5A2 gene defect and heterozygous for the 17βHSD3 gene defect

A novel feature of this kindred is the coexistence of two gene mutations in the same family as well as in the same individual. Subject A-IV-30 (Fig. 1 and Table 1) was homozygous for the *SRD5A2* exon 5 mutation and heterozygous for the *17βHSD3* gene mutation, as determined by both SSCP and DNA sequencing. Phenotypically, this subject did not differ from those homozygous for the *SRD5A2* gene defect. Plasma androgen levels are not available, but urinary C_{19} and C_{21} 5β/5α-steroid metabolite ratios were consistent with 5α-RD-2 deficiency (Table 1).

A male pseudohermaphrodite homozygous for the 17βHSD3 gene defect and heterozygous for a SRD5A2 gene defect

Subject A-IV-3 (Fig. 1, pedigree A) was both homozygous for the *17βHSD3* gene defect and heterozygous for the *SRD5A2* gene mutation (Table 1). Plasma androgen levels (Table 1) were similar to those of subject A-V-1, a homozygote for the *17βHSD3* defect, with the exception of a slightly lower DHT level and a slightly higher T to DHT ratio. The urinary 5β/5α-steroid metabolite ratios are compatible with heterozygosity for 5α-RD-2 deficiency (5, 18). This subject was reluctant to have a physical examination. Thus, we cannot comment about the phenotypic expression of this genetically complex subject.

Heterozygosity for both SRD5A2 and 17βHSD3 gene mutations in the same individuals

Heterozygosity for both *SRD5A2* and *17βHSD3* gene defects was detected in subject A-IV-7 (the mother of subject A-V-1, homozygous for the *17βHSD3* gene defect), subject B-II-6, and her daughter B-III-6 (see *Subjects and Methods*, pedigrees A and B, and Table 1). These females were fertile and had normal secondary sexual characteristics. As shown in Table 1, their plasma Δ^4 concentrations were within the normal range. Their absolute plasma levels of T and DHT were variable. However, the T to DHT ratios were elevated, and the urinary 5β/5α-steroid metabolite ratios were in the heterozygous range for 5α-RD-2 deficiency.

Discussion

We have identified defects in the *17βHSD3* gene and/or the *SRD5A2* gene in male pseudohermaphrodites from a

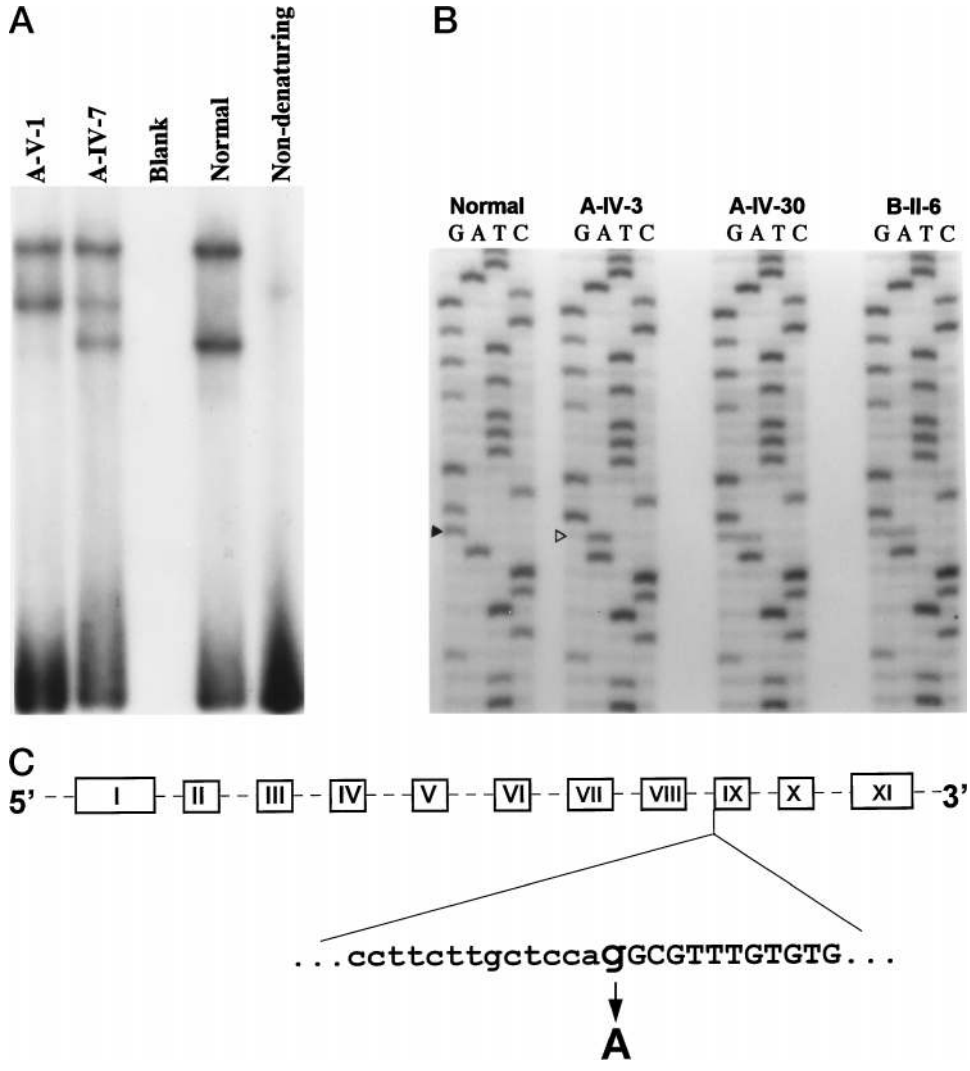

FIG. 5. A, Point mutation in the *17βHSD3* gene in male pseudohermaphrodites from the Turkish community detected by SSCP analysis (A) and DNA sequencing (B). A, Representative SSCP analysis of exon 9 of the *17βHSD3* gene in a male pseudohermaphrodite with 17βHSD deficiency (A-V-1), a carrier (A-IV-7), and a normal subject. The bands on the *top* represent the single strand DNA, and the band on the *bottom* is the double-stranded DNA. B, Representative DNA sequencing of exon 9 of the *17βHSD3* gene in a male pseudohermaphrodite (A-IV-3), two carriers (A-IV-30 and B-II-6), and a normal subject. The *solid arrowhead* indicates that base G in the normal control sequence is substituted by base A in the affected subject, as indicated by an *open arrowhead*. C, The genomic structure of the human *17βHSD3* gene and the mutation detected in male pseudohermaphrodites from a Turkish community. This mutation causes a disruption of splicing acceptor of exon 9.

large isolated kindred with known consanguinity. This is the only known kindred in the world with two rare gene defects causing male pseudohermaphroditism (MPH). The particular mutation responsible for 5α-RD-2 deficiency is newly described. A single base (adenine) deletion at exon 5 of the *SRD5A2* gene caused a frame shift at amino acid position 251, adding 23 amino acids to the carboxyl-terminal of the 254-amino acid isozyme. This carboxyl-terminal mutation resulted in complete loss of enzymatic activity without altering the level of gene expression in transfection analysis. Other studies indicate that mutations of the carboxyl-terminal of this isozyme decrease both cofactor (NADPH) binding and substrate binding (21). However, decreases in cofactor and substrate binding alone cannot entirely account for the overwhelming loss of enzymatic activity of this mutant enzyme, because high concentrations of NADPH and T were used in the assay, and further increases in cofactor and substrate concentrations (5-fold higher) did not improve the enzymatic activity of the mutant enzyme (data not shown). Additional mutations of the *SRD5A2* gene in Turkish and Mediterranean subjects are summarized in Table 3.

The *17βHSD3* gene mutation in MPH from this kindred is a single point mutation (G→A, 655-1) in the boundary between intron 8 and exon 9 that disrupts the splice acceptor site for exon 9 and alters the sequence of the enzyme (19, 20). This mutation has previously been reported in two subjects of Mediterranean descent: a Greek American and a Syrian (19, 20). Other mutations of the *17βHSD3* gene identified in 17βHSD-deficient subjects from the eastern Mediterranean and Middle East include a missense mutation in exon 2 (S65L) and a missense mutation in exon 3 (R80Q) (19, 20).

As the two genes are located on different chromosomes (*SRD5A2* on chromosome 2 and *17βHSD3* on chromosome 9), they segregate independently during meiosis (22). Therefore, combinations of homozygosity and/or heterozygosity for the two gene defects are possible in the same individual within this kindred. Not surprisingly, we have detected genetically complex male pseudohermaphrodites: one who is homozygous for a *SRD5A2* gene mutation and heterozygous for the *17βHSD3* gene defect, one who is homozygous for the *17βHSD3* gene defect and heterozygous for a *SRD5A2* gene mutation, and three females who are compound heterozygotes for the two enzyme defects. To date, a male pseudohermaphrodite who is homozygous for the two gene defects has not been detected.

The phenotypes of the male pseudohermaphrodites with

TABLE 3. Summary of 5αRD-2 gene mutations in eastern Mediterranean and Middle East

Ethnic group/subject	Consanguinity/family history	Type	Location	Mutation	Ref. no.
Algerian	No/no	Missense	Exon 1	P59R	27
Egyptian	Yes/no	Missense	Exon 5	R246W	27
Greek/American	No/no	Missense	Exon 4	G→A, G196S	28
Jordanian	No/no	Missense	Exon 1	L55Q	27, 28
		Missense	Exon 1	Q56R	
		Missense	Exon 2	G115D	
Lebanese	Yes/yes	Missense	Exon 1	T→A, L55Q	29
Maltese	No/no	Deletion	Exon 2	TC deletion at nucleotide 359	27, 28
		Missense	Exon 3	G→C, R171S	
Turkish	Yes/no	Deletion (no frame shift)	Exon 3	ATT deletion, ΔMet^{157}	30, 31
Turkish	Yes/no	Missense	Exon 1	T→A, L55Q	31
Turkish	No/yes	Missense	Exon 4	G196S	31
Turkish	Yes/yes	Deletion	Exon 5	A deletion, frame shift	This report

either of the two enzyme defects from this community are similar. Wolffian-derived structures (epididymis, vasa deferentia, seminal vesicles, and ejaculatory ducts) are present, with a clitorus-like phallus and a blind vaginal pouch within a urogenital sinus (2, 23). The testes are either descended or located in the inguinal canal. At puberty, virilization of the external genitalia with deepening of the voice and increased musculature occurs, frequently followed by a gender change from female to male. The subjects with the *17βHSD3* gene defect develop mild gynecomastia at puberty and have slightly increased facial and body hair (3, 19).

There are at least five isozymes of 17βHSD in the human (23, 24). The 17βHSD-3 isozyme is expressed in the testes and is responsible for MPH due to 17βHSD deficiency (19, 20). The type 1 17βHSD isozyme mainly catalyzes the conversion of estrone (E_1) to estradiol (E_2). Types 3 and 5 are the rate-limiting enzymes in T biosynthesis. Types 2 and 4 predominantly affect T and E_2 inactivation. The ontogeny of these five isozymes in human tissues has not been studied. However, the fact that adults homozygous for a *17βHSD3* gene defect have ambiguous genitalia suggests that 17βHSD-3 is the primary isozyme expressed early *in utero* and is responsible for T biosynthesis from Δ^4 during the critical period of sexual differentiation. At puberty, the expression of the other 17βHSD isozymes in the peripheral tissues compensates to some extent for testicular 17βHSD-3 deficiency, resulting in significant pubertal masculinization.

MPH in this community has been known to occur for at least seven generations spanning over 100 yr (5). The elders state that their ancestors came from Konya, in the central Anatolian plateau of present day Turkey. The village is 1800 meters above sea level and has a population of less than 3000. It was an isolated area until 25 yr ago, when dirt roads were built, and electric and telephone lines were established. The inhabitants are shepherds and farmers who work seasonally along the Mediterranean coast. Affected children are currently recognized at birth, given male names, and raised as boys. In the past, affected babies were raised as girls, and after a pubertal gender change, the community would call them kiz-oğlan (girl-boy) and regard them as incomplete men.

Pedigree analysis demonstrating intermarriage suggests that a founder effect is responsible for the dissemination of genetic abnormalities within this community. The geographic isolation of this kindred is analogous to that of the large Dominican kindred with an *SRD5A2* gene defect (2, 4). The coexistence of both *SRD5A2* and *17βHSD3* gene defects in this kindred may be derived from either one progenitor carrying both defects on different alleles or from two progenitors. Thus, it is possible that one gene defect was initially present in this area, and at some point later in time another progenitor introduced the second mutant gene into this population. Studies to date reveal an asymmetric distribution of the two gene defects in this kindred, *i.e.* there are more individuals with homozygous or heterozygous *SRD5A2* gene defects than individuals with homozygous or heterozygous *17βHSD3* gene defects identified by molecular genetic analysis. This finding supports the concept that the *SRD5A2* gene defect may have been introduced first.

Our present findings support the previous demonstration that females homozygous or heterozygous for the *SRD5A2* gene defect or the *17βHSD3* gene defect are fertile (4, 25) (Fig. 2, pedigree C). It has been shown that the *17βHSD3* gene is expressed in the testes (19, 23), but not in the ovary (26). The consanguineous marriages together with the lack of adverse expression of either of these defects in women contributes greatly to the dissemination of both gene defects in this community.

In summary, we have identified defects in both the *17βHSD3* and *SRD5A2* genes that are responsible for MPH in a geographically isolated large Turkish kindred. The coexistence of the two gene defects in the same individual has been identified, although homozygosity for the two gene defects in the same individual has yet to be identified. The phenotypic expression of subjects homozygous for the *SRD5A2* gene defect or homozygous for the *17βHSD3* gene defect is similar to those previously reported (2–5, 19, 20). Two progenitors, each carrying one of the defects, or one progenitor with both defects could be responsible for the coexistence of the two gene defects in this kindred. Identification of unique haplotypes for the two genes may aid in the resolution of this mystery.

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References

1. **Imperato-McGinley J, Peterson RE.** 1976 Male pseudohermaphroditism: complexities of male sexual development. Am J Med. 61:251–272.
2. **Imperato-McGinley J.** 1996 Male pseudohermaphroditism. In: Adashi EY, Rock JA, Rosenwaks Z, eds. Reproductive endocrinology, surgery, and technology. Philadephia: Lippincott-Raven; 936–955.
3. **Imperato-McGinley J, Akgun S, Ertel NH, Sayli B, Shackleton CHL.** 1987 The coexistence of male pseudohermaphrodites with 17-ketosteroid reductase deficiency and 5α-reductase deficiency within a Turkish kindred. Clin Endocrinol (Oxf). 27:135–143.
4. **Imperato-McGinley J, Guerrero L, Gautier T, Peterson RE.** 1974 Steroid 5α-reductase deficiency in man: an inherited form of male pseudohermaphroditism. Science. 186:1213–1216.
5. **Akgun S, Ertel N, Imperato-McGinley J, Sayli B, Shackleton CHL.** 1986 Familial male pseudohermaphroditism in a Turkish village due to 5α-reductase deficiency. Am J Med. 81:267–274.
6. **Imperato-McGinley J, Peterson RE, Stoller R, Goodwin WE.** 1979 Male pseudohermaphroditism secondary to 17β-hydroxysteroid dehydrogenase deficiency: gender role change with puberty. J Clin Endocrinol Metab. 49:391–395.
7. **Peterson RE, Imperato-McGinley J, Gautier T, Sturla E.** 1977 Male pseudohermaphroditism due to steroid 5α-reductase deficiency. Am J Med. 62:170–191.
8. **Shackleton CHL.** 1993 Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. J Steroid Biochem. 45:127–140.
9. **Peterson RE, Imperato-McGinley J, Gautier T, Shackleton CHL.** 1985 Urinary steroid metabolites in subjects with male pseudohermaphroditism due to 5α-reductase deficiency. Clin Endocrinol (Oxf). 23:43–53.
10. **Labrie F, Sugimoto Y, Luu-The V, et al.** 1992 Structure of human type II 5α-reductase gene. Endocrinology. 131:1571–1573.
11. **Cai L-Q, Zhu Y-S, Katz MD, et al.** 1996 5α-Reductase-2 gene mutation in the Dominican Republic. J Clin Endocrinol Metab. 81:1730–1735.
12. **Luu-The V, Sugimoto Y, Puy L, et al.** 1994 Characterization, expression, and immunohistochemical localization of 5α-reductase in human skin. J Invest Dermatol. 102:221–226.
13. **Zhu YS, Yen PM, Chin WW, Pfaff DW.** 1996 Estrogen and thyroid hormone interaction on regulation of gene expression. Proc Natl Acad Sci USA. 93:12587–12592.
14. **Normington K, Russell DW.** 1992 Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. J Biol Chem. 267:19548–19554.
15. **Sugimoto Y, Lopez-Solache I, Labrie F, Luu-The V.** 1995 Cations inhibit specifically type I 5 alpha-reductase found in human skin. J Invest Dermatol. 104:775–778.
16. **Imperato-McGinley J, Peterson RE, Gautier T, Sturla E.** 1979 Male pseudohermaphroditism secondary to 5α-reductase deficiency: a model for the role of androgens in both the development of the male phenotype and the evolution of a male gender identity. J Steroid Biochem Mol Biol. 11:637–645.
17. **Imperato-McGinley J, Gautier T.** 1986 Inherited 5α-reductase deficiency in man. Trends Genet. 2:130–133.
18. **Imperato-McGinley J, Peterson RE, Gautier T, Shackleton CHL, Arthur A.** 1985 Decreased urinary C19 and C21 steroid 5α-metabolites in parents of male pseudohermaphrodites with 5α-reductase deficiency: detection of carriers. J Clin Endocrinol Metab. 60:553–558.
19. **Geissler WM, Davis DL, Wu L, et al.** 1994 Male pseudohermaphroditism caused by mutations of testicular 17 beta-hydroxysteroid dehydrogenase 3 [see comments]. Nat Genet. 7:34–39.
20. **Andersson S, Geissler WM, Wu L, et al.** 1996 Molecular genetics and pathophysiology of 17 beta-hydroxysteroid dehydrogenase 3 deficiency. J Clin Endocrinol Metab. 81:130–136.
21. **Wigley WC, Prihoda JS, Mowszowicz I, et al.** 1994 Natural mutagenesis study of the human steroid 5α-reductase 2 isozyme. Biochemistry. 33:1265–1270.
22. **Embury SH, Steinberg MH.** 1994 Genetic modulators of disease. In: Embury SH, Hebbel RP, Mohandas N, Steinberg MH, eds. Sickle cell disease: basic principles and clinical practice. New York: Raven Press; 279–294.
23. **Andersson S, Moghrabi N.** 1997 Physiology and molecular genetics of 17β-hydroxysteroid dehydrogenases. Steroids. 62:143–147.
24. **Labrie F, Luu-The V, Lin SX, et al.** 1997 The key role of 17β-hydroxysteroid dehydrogenases in sex steroid biology. Steroids. 62:148–158.
25. **Katz MD, Cai L-Q, Zhu Y-S, et al.** 1995 The biochemical and phenotypic characterization of females homozygous for 5α-reductase-2 deficiency. J Clin Endocrinol Metab. 80:3160–3167.
26. **Zhang Y, Word RA, Fesmire S, Carr BR, Rainey WE.** 1996 Human ovarian expression of 17β-hydroxysteroid dehydrogenase types 1, 2, and 3. J Clin Endocrinol Metab. 81:3594–3598.
27. **Wilson JD, Griffin JE, Russell DW.** 1993 Steroid 5α-reductase 2 deficiency. Endocr Rev. 14:577–593.
28. **Thigpen AE, Davis DL, Milatovich A, et al.** 1992 The molecular genetics of steroid 5α-reductase 2 deficiency. J Clin Invest. 90:799–809.
29. **Hochberg Z, Chayen R, Reiss N, et al.** 1996 Clinical, biochemical, and genetic findings in a large pedigree of male and female patients with 5α-reductase 2 deficiency. J Clin Endocrinol Metab. 81:2821–2827.
30. **Boudon C, Lobaccaro JM, Lumbroso S, et al.** 1995 A new deletion of the 5 alpha-reductase type 2 gene in a Turkish family with 5α-reductase deficiency. Clin Endocrinol (Oxf). 43:183–188.
31. **Hiort O, Sinnecker GH, Willenbring H, Lehners A, Zollner A, Struve D.** 1996 Nonisotopic single strand conformation analysis of the 5α-reductase type 2 gene for the diagnosis of 5α-reductase deficiency. J Clin Endocrinol Metab. 81:3415–3418.