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Mutations of the 5α-reductase Type 2 gene in eight Mexican patients from six different pedigrees with 5α-reductase-2 deficiency

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Summary

BACKGROUND AND OBJECTIVE Male pseudohermaphroditism due to 5α-reductase deficiency was originally described in 1974. Recently, 5α-reductase Type 2 gene defects have been found generally to be due to point mutations within the 5 exons of the 5α-reductase-2 gene. In this report, we describe the molecular study of patients with 5α-reductase deficiency.

DESIGN Previously diagnosed patients with 5α-reductase deficiency were sampled in order to perform molecular studies.

PATIENTS Eight 5α-reductase deficient individuals from 6 unrelated families.

MEASUREMENTS Single-strand conformational polymorphism and DNA sequencing were performed after polymerase chain reaction amplification of each of the 5 exons of the gene.

RESULTS Five different missense mutations were found. In 4 patients a cytosine to guanine substitution was observed at codon 212 in exon 4. Two siblings presented a cytosine to adenine substitution at codon 207 in exon 4. Another patient exhibited a guanine to adenine substitution at codon 34 in exon 1, whilst one individual presented 2 mutations: a guanine to adenine substitution at codon 115 in exon 2 and a guanine to adenine substitution at codon 203 in exon 4 (previously undescribed mutation).

CONCLUSIONS The presence of the same mutation in 4 patients from 3 families indicates the increased prevalence of this mutation in a particular ethnic group, suggesting a common ancestry for the gene defect in these patients. The existence of hot spots is supported by the mutations in codons 34 and 207 which have also been found in other ethnic groups. Interestingly, the patient who presented 2 different mutations, one of them previously undescribed, was reared as a male and exhibited a more masculine phenotype. Further studies in patients with this and other mutations will be needed to verify genotype–phenotype correlation.

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5α-Reductase-2 deficiency is a specific form of hereditary male pseudohermaphroditism originally described in humans in 1974 (Imperato-McGinley *et al.*, 1974; Walsh *et al.*, 1974). This deficiency impairs the conversion of testosterone (T) to dihydrotestosterone (DHT), the androgen essential for normal development of external genitalia in the male fetus (Griffin & Wilson., 1989). Affected individuals are 46,XY males who generally present with pseudovaginal perineo-scrotal hypospadias at birth (Imperato-McGinley *et al.*, 1974; 1991;Walsh *et al.*, 1974; Saenger *et al.*, 1978; Price *et al.*, 1984; Griffin & Wilson, 1989) and often with bilaterally cryptorchid testes, characteristics that almost invariably lead to a female gender assignment. During puberty, significant virilization occurs and without any therapeutic intervention masculinization is often accompanied by a gender identity change from female to male (Imperato-McGinley *et al.*, 1979; 1991; Méndez *et al.*, 1995).

The cloning of two genes (Types 1 and 2) that encode two steroid 5α-reductase isoenzymes has been described (Andersson *et al.*, 1991; Jenkins *et al.*, 1992) and specific gene mutations have been defined (Andersson *et al.*, 1991; Thigpen *et al.*, 1992a). Molecular genetic evidence, such as gene deletions and point mutations, have demonstrated that the gene designated as Type 2, located in band p23 on the short arm of chromosome 2,

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is the specific locus for 5α-reductase deficiency (Wilson *et al.*, 1993; Wigley *et al.*, 1994; Boudon *et al.*, 1995a,b). Several different mutations in patients bearing this defect have been identified; only one complete deletion of the gene has been detected (Andersson *et al.*, 1991). Except for a dinucleotide and a trinucleotide deletion, all other molecular abnormalities detected have been point mutations, which occur throughout the 5 exons that constitute the gene and are mainly responsible for amino acid substitutions (Thigpen *et al.*, 1992a, b; Wilson *et al.*, 1993; Wigley *et al.*, 1994; Boudon *et al.*, 1995a, b; Forti *et al.*, 1996).

Here we present the molecular study of 8 patients with 5α-reductase deficiency from 6 unrelated families. Five different missense mutations are described, including a new mutation detected in exon 4.

Patients and methods

The clinical, endocrinological and biochemical features of the first five patients have been previously described; patients 1–5 of this study, correspond to patients 3–7 of our previous report (Méndez *et al.*, 1995).

The diagnosis of 5α-reductase deficiency was established in all cases by physical examination, measurements of plasma T and DHT, as well as the T/DHT ratio, urinary 5β/5α C_{19} and C_{21} metabolite ratios, karyotype and studies of 5α-reductase in cultured skin fibroblasts.

Subject 6 is a 24-year-old patient with a female gender identity. There was no history of consanguinity. The phallus was 4·5 cm in length. Both gonads were in the scrotum (11·0 and 6·3 ml), perineo-scrotal hypospadias and a vaginal pouch were present. The basal T/DHT ratio was 28 and all urinary 5β/5α C_{19} and C_{21} metabolite ratios measured were elevated.

Subject 7 is a 22-year-old individual with a male gender identity who was raised as a female but changed his gender identity spontaneously 4 years before admission. There was no history of consanguinity in his family. The phallus measured 4·5 cm and the gonads, located in the scrotum, had volumes of 20 and 22 ml. Perineo-scrotal hypospadias and a vaginal pouch were present. The basal T/DHT ratio was 29 and all urinary 5β/5α C_{19} and C_{21} metabolite ratios measured were elevated.

Subject 8 is 17 years old. He was raised as a male, presenting penoscrotal hypospadias and descended testes at birth. There was no history of consanguinity. The phallus was 4·3 cm in length and the gonads presented volumes of 11·8 and 19·9 ml and a penoscrotal hypospadias was observed. The basal T/DHT ratio was 44.

Polymerase chain reaction

Genomic DNA for the polymerase chain reaction (PCR) was isolated from blood leucocytes of the patients and controls (unrelated normal individuals), by standard methods (John *et al.*, 1991). Five sets of oligonucleotide primers (see Table 1) were used in the PCR amplifications of exons 1–5 of the 5α-reductase 2 gene (Labrie *et al.*, 1992). Reactions were carried out in a total volume of 50 μl containing 0·5–1·0 μl genomic DNA, 1·5 μM of each oligonucleotide primer, 25 μM mixed dNTPs, 1·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl pH 8·3, 4% DMSO and 2·0 U of thermostable polymerase (Ampli-*Taq*, Perkin Elmer Corp. NJ, USA). Twenty-five cycles of PCR amplification were performed in a thermal cycler with

Table 1 Oligonucleotides used for amplification of each exon of the 5α-SR2 gene

Amplification target	Primer name	Sequence (5′ → 3′)	PCR Fragments length (bp)	Annealing temperature (°C)
Exon 1	501	GCAGCGGCCACCGGCGAGG	358	65
	502	AGCAGGGCAGTGCGCTGCACT		
Exon 2	503	TGAATCCTAACCTTTCCTCCC	235	58
	504	AGCTGGGAAGTAGGTGAGAA		
Exon 3	505	TGTGAAAAAAGCACCACAATCT	208	58
	506	CAGGGAAGAGTGAGAGTCTGG		
Exon 4	507	TGATTGACCTTCCGATTCTT	232	54
	508	TGGAGAAGAAGAAAGCTACGT		
Exon 5	509	TCAGCCACTGCTCCATTATAT	166	58
	510	CAGTTTTCATCAGCATTGTGG		

Designed from the 5α-SR2 sequence reported by Labrie *et al.* (1992).

denaturation at 94°C for 1 minute, annealing at 54–65°C for 1 minute (Table 1) and extension at 72°C for 1 minute. After amplification, PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide in order to verify the correct size of the expected fragments.

Analysis of single-strand conformation polymorphism (SSCP)

To screen variant sequences, SSCP analysis was performed on amplified genomic DNA from patients and control subjects according to the method described by Orita *et al.* (1989) with minor modifications previously described (Thigpen *et al.*, 1992a). Briefly, each 50 μl reaction contained 20–30 ng purified exon DNA (Centricon-100 columns; Amicon Inc., Beverly, MA, USA), 25 μM dNTPs, 2·5 U Taq DNA polymerase, 1·25 μM each of sense and antisense primers, and 10 μCi of α-^{32}P-dCTP (sp.act. 3000 Ci/mmol; NEN-DuPont, Boston, MA, USA), in 10 mM Tris (pH 8·8), 50 mM KCl and 1·5 mM $MgCl_2$. All exons were amplified using the same conditions described above. After PCR, 2·0 μl of each reaction mixture were transferred into 18 μl of loading buffer (95% formamide, 40 mM EDTA, 0·05% bromophenol blue); the samples were heated at 94°C for 5 minutes and then cooled on ice. Two microlitres of each mixture were loaded onto neutral polyacrylamide gels (5·4% for exons 1, 2, 3, 5 and 8% for exon 4) containing or not containing 10% glycerol. Electrophoresis was carried out with a sequencing apparatus at 200 V for 14–16 hours at room temperature. After electrophoresis, gels were dried and exposed to Kodak XAR-5 X-ray films for 4–14 hours.

Sequencing of mutation-containing exons

DNA samples showing altered electrophoretic patterns on SSCP were sequenced by the dideoxynucleotide-termination method (Sanger *et al.*, 1977). Direct sequencing of the PCR products was done in both directions using an AmpliCycle Sequencing Kit (Perkin Elmer-Roche, Branchburg, NJ, USA), following the protocol supplied by the manufacturer. Sequencing reactions were run on a 5·0% polyacrylamide–7·5 M urea gel. After electrophoresis, gels were dried and exposed to Kodak XAR-5 X-ray films.

Results

No deletions of the 5α-reductase 2 gene were detected by PCR analysis. Mutations were observed, in all cases, by the SSCP analyses (Fig. 1). Abnormalities in migration were detected by SSCP analysis in two exons in one case (subject 8) and in one exon in the remaining patients. However, in subjects 4 and 5 (siblings) it was necessary to modify the SSCP technique by increasing the acrylamide concentration from 5·4 to 8% in order to observe differences in migration. In all cases it was possible to determine the presence of homozygosity or compound heterozygosity (subject 8) by this methodology.

The DNA sequences of each mutation detected by SSCP analysis were determined by PCR sequencing. Five different point mutations were found in the 8 patients studied (Table 2). In patients 1, 2 and 3 (siblings), as well as 6, a cytosine to guanine substitution was observed at codon 212 in exon 4. This point mutation was responsible for a proline to arginine substitution (codon CGA instead of CCA). Siblings 4 and 5 presented a cytosine to adenine substitution at codon 207 in exon 4, which was responsible for an alanine to aspartate substitution (codon GAC instead of GCC). In subject 7, a guanine to adenine substitution was detected at codon 34 in exon 1. This mutation was responsible for a glycine to arginine substitution (codon GGA instead of GGG). Patient 8 was a compound heterozygote who presented two mutations. First, a guanine to adenine substitution at codon 115 in exon 2, was responsible for a glycine to aspartate substitution at the codon mentioned (codon GAC instead of GGC). This patient also presented a guanine to adenine substitution at codon 203 in exon 4 which was responsible for a glycine to serine substitution (codon AGC instead of GGC) (Fig. 2).

Discussion

In the male, external genital and prostate differentiation is regulated by the reduction of T to DHT. Impairment of this reduction causes male pseudohermaphroditism. A wide clinical, and biochemical spectrum of 5α-reductase-2 deficiency has been reported. Several different mutations have been identified in patients with 5α-reductase-2 deficiency. Only one complete deletion of the gene has been detected (Andersson *et al.*, 1991). In almost all the other cases, point mutations have been identified throughout the 5 exons that constitute the gene and are responsible for amino acid substitutions (Thigpen *et al.*, 1992a, b; Wilson *et al.*, 1993; Wigley *et al.*, 1994; Boudon *et al.*, 1995a, b).

Identical mutations have been found in individuals with widely divergent geographic and ethnic backgrounds, suggesting the existance of mutational hot spots in the gene. However, in other cases, similar mutations within the same ethnic group have been described and are almost certainly derived from common ancestral mutations, due to a founder-gene effect in some populations (Thigpen *et al.*, 1992a, b; Wilson *et al.*, 1993; Wigley *et al.*, 1994; Boudon *et al.*, 1995a, b; Forti *et al.*, 1996).

In our eight patients missense mutations were identified. The six families were from different geographic locations in Mexico

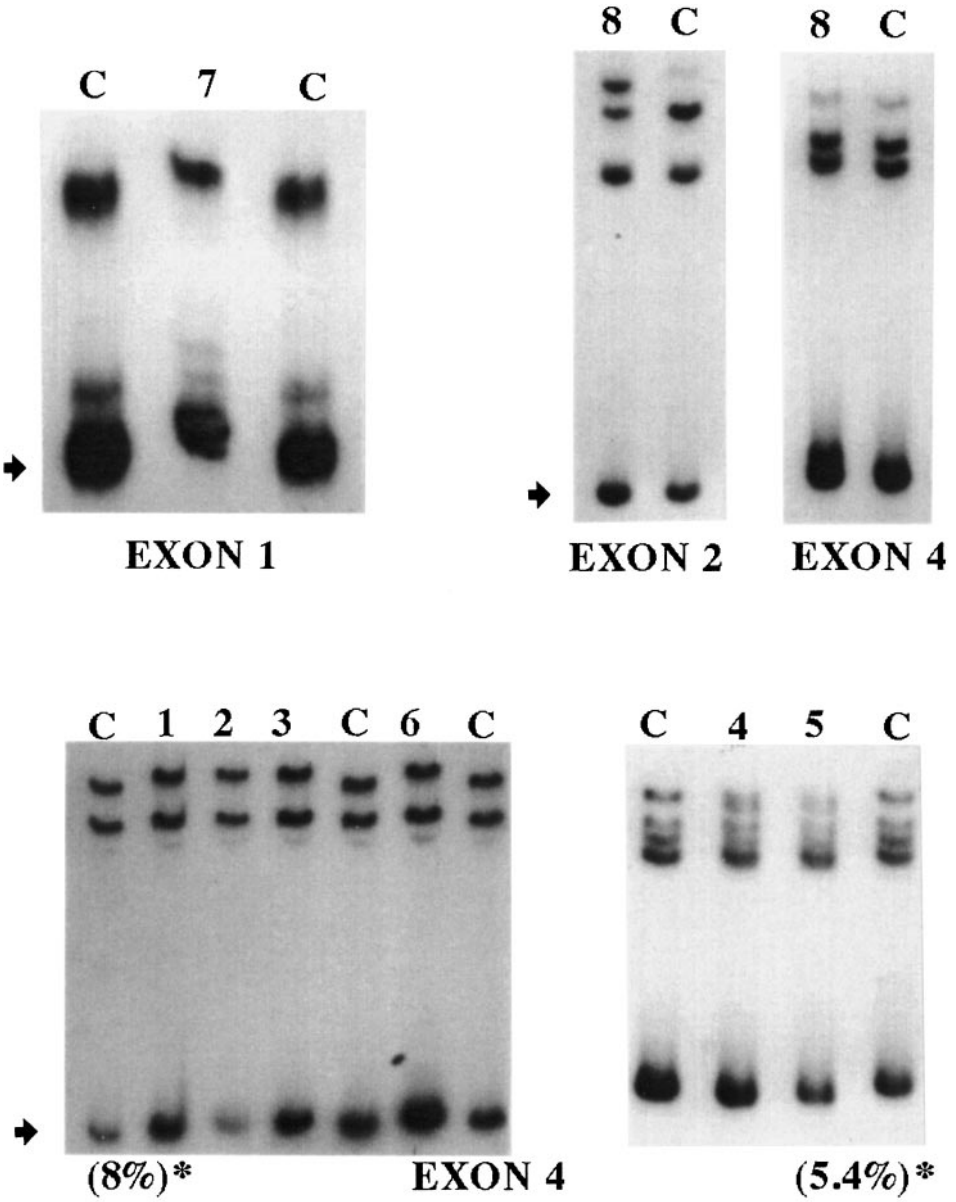

Fig. 1 Detection of mutations in the 5α-reductase Type 2 gene by SSCP analysis in the 8 patients studied. Individual exons were amplified from genomic DNA extracted from the indicated patients, as well as from the controls (C), and screened for SSCPs. In exon 4 the polyacrylamide concentration was 8 or 5·4%. Subject 8 has one mutation in exon 2 and one in exon 4; all other subjects exhibited a single mutation.

and the families were not related one to another. All patients, except one, were homozygotes. Four patients from three families presented the same, P212R, mutation in exon 4 which has been previously described in a Mexican–American subject (Wigley *et al.*, 1994), thus indicating the increased prevalence of this mutation in this particular ethnic group. Another family with two affected siblings had a A207D mutation in exon 4. This has been previously identified only in an Austrian individual (Thigpen *et al.*, 1992a). Subject 7 had a G34R mutation in exon 1 that had previously been reported in Mexican–American, a Sicilian and Vietnamese subjects (Thigpen *et al.*, 1992a). The latter two mutations confirm and extend previous information about the existence of mutational hot spots in the 5-α reductase-2 gene. Finally, in one affected subject, 2 different mutations were identified; one of them, located in codon 115, exon 2, had been previously reported in a Mexican–American (Thigpen *et al.*, 1992a); however, the mutation in codon 203 of exon 4 has not been described

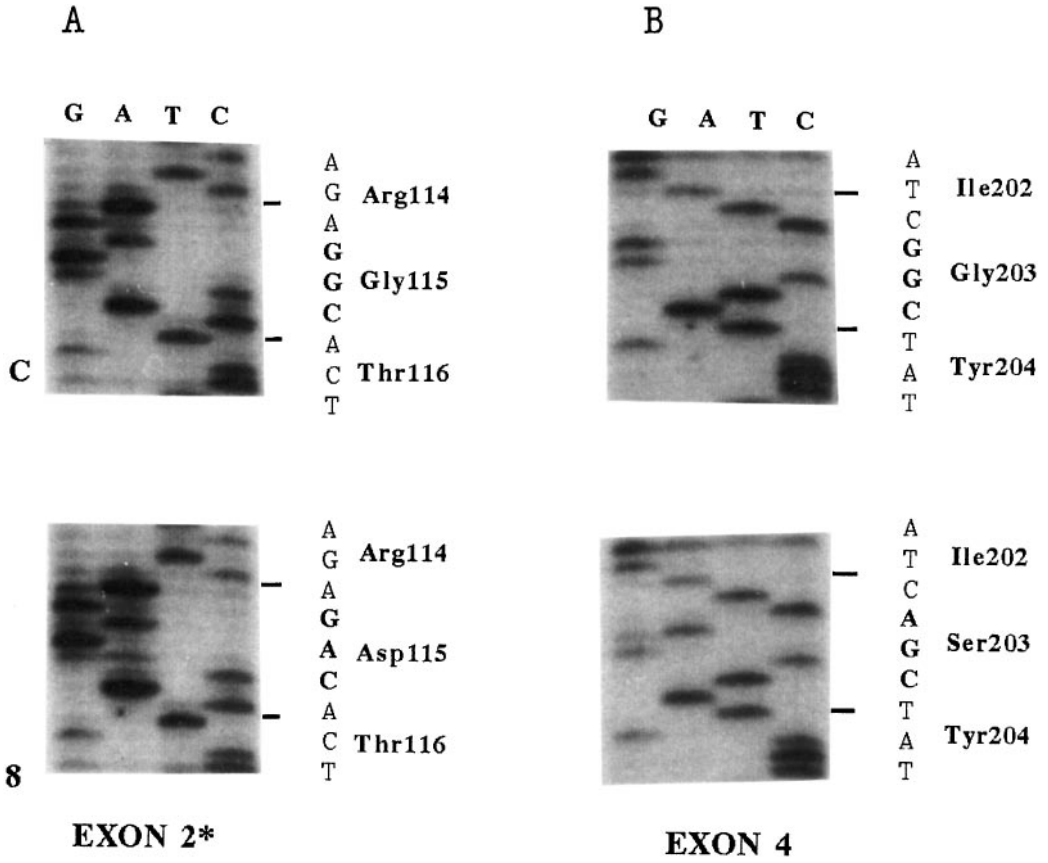

Fig. 2 a, Partial sequence of exon 2 of the 5αR2 gene of patient 8 and a control (C), showing the G to A substitution which changes the sense of codon 115 from glycine to asparagine. b, Partial sequence of exon 4 of the 5αR2 gene of patient 8 and a control (C), showing the G to A substitution which changes the sense of codon 203 from glycine to serine.

Table 2 Missense mutations of the 5α-reductase 2 gene in 8 patients with 5α-reductase deficiency

Patient no.	Exon	Mutation
1	4	C → G P212R*
2[a]	4	C → G P212R*
3[a]	4	C → G P212R*
4[b]	4	C → A A207D*
5[b]	4	C → A A207D*
6	4	C → G P212R*
7	1	G → A G34R*
8	2	G → A G115D†
	4	G → A‡ G203S

* Homozygotes. † Compound heterozygote.
[a,b] Siblings. ‡ Novel mutation.

previously. Interestingly, this individual was reared as a male and did not present the usual phenotype observed. He had descended gonads, and penoscrotal hypospadias. Although it has not been possible to clearly correlate the phenotypes with the existing mutations or with the enzymatic characteristics (Thigpen *et al.*, 1992a; Wigley *et al.*, 1994), Thigpen *et al.* (1992a) suggested a correlation between clinical expression and severity of the impairment of enzyme function. The mutation present in exon 4 in our last patient could have been responsible for the more male genitalia. However, 5α-reductase activity determined in cultured genital skin fibroblasts revealed diminished or absent DHT synthesis depending on the experimental conditions. At pH 7·4 (whole cells), this patient synthesized $< 2{\cdot}0$ nmol/g protein/h *vs* $6{\cdot}4 + 1{\cdot}9$ (SD) nmol/g protein/h in control subjects. At pH 5·5 (sonicates) in the presence of increasing concentrations of NADPH, there was no enzymatic activity in the patient, while the controls synthesized > 160 nmol/g protein/h. However, we should take into consideration that the instability of the enzyme could make it difficult to assay by this methodology (Wilson *et al.*, 1993).

Further studies, including the re-creation of the G203S mutation in patients bearing this specific defect, will be needed to verify genotype–phenotype correlation.

In summary, we describe 8 patients from 6 unrelated families who exhibit missense mutations of the 5α-reductase 2 gene. Of the 5 mutations detected, 3 were found in exon 4 and one was a previously undescribed mutation. Of the 4 mutations previously reported, all but one had been identified in Mexican–American subjects reinforcing the founder effect. Regarding the A207D mutation existing in two siblings, only an Austrian individual has been reported with this specific alteration, supporting the existance of hot spots in the gene (Thigpen *et al.*, 1992a, b; Wilson *et al.*, 1993; Wigley *et al.*, 1994; Boudon *et al.*, 1995a, b).

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