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New mutations, hotspots, and founder effects in Brazilian patients with steroid 5α-reductase deficiency type 2

Abstract Mutations of the steroid 5α-reductase type 2 (*SRD5A2*) gene in 46,XY subjects cause masculinization defects of varying degrees, due to reduced or impaired enzymatic activity. In this study, sequence abnormalities of the *SRD5A2* gene were assessed by polymerase chain reaction with specific primers and automated sequencing analysis in DNA samples from 20 patients with suspected steroid 5α-reductase type 2 deficiency from 18 Brazilian families. Eleven subjects presented *SRD5A2* homozygous single-base mutations (two first cousins and four unrelated patients with G183S, two with R246W, one with del642T, one with G196S, and one with 217_218insC plus the A49T variant in heterozygosis), whereas four were compound heterozygotes (one with Q126R/IVS3+1G>A, one with Q126R/del418T, and two brothers with Q126R/G158R). Three pa-

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tients were heterozygous for A207D, G196S, and R266W substitutions. The V89L polymorphism was found in heterozygosis in one of them (with A207D) and in one case with an otherwise normal gene sequence. The A49T variant was also detected in heterozygosis in the second case without other sequencing abnormalities. Four patients harbor yet non-described *SRD5A2* gene mutations: a single nu-

cleotide deletion (del642T), a G158R amino acid substitution, a splice junction mutation (IVS3+1G>A), and the insertion of a cytosine (217_218insC) occurring at a CCCC motif. This is the first report of a single-nucleotide insertion in the coding sequence of the *SRD5A2* gene. In addition to these new mutations, this investigation reveals the prevalence of G183S substitution among a subset of African–Brazilian patients and presents evidences of the recurrence of already known mutations.

Introduction

The steroid 5α-reductase type 2 enzyme is encoded by the *SRD5A2* gene on chromosome 2 and is predominantly expressed in external genital tissues and prostate. Steroid 5α-reductase type 2 deficiency, an autosomal recessive disorder (OMIM 607306), leads to the impairment of the conversion of testosterone (T) to dihydrotestosterone (DHT). Both hormones bind to the same androgen-receptor protein in cell nuclei, but perform different physiological roles: T plays a major role in the stimulation of Wolffian ducts during sexual differentiation and probably in the control of spermatogenesis, whereas DHT is considered the essential androgen for normal development of external genitalia in the male fetus [1]. DHT also mediates most events of male puberty, including maturation of facial and body hair, genital, and prostate growth.

The diagnosis of 5α-reductase type 2 deficiency can be difficult, mainly before the age of puberty. It should be suspected in all prepubertal male pseudohermaphrodites, especially those with perineal hypospadias and in those who virilize at puberty without evidence of gynecomastia. In the majority of cases, this diagnosis can be confirmed by demonstration of an abnormally high T/DHT serum ratio before and/or after hCG administration [2–4]. Early diagnosis of 5α-reductase type 2 deficiency is important because of its bearing on the assignment of sex in the affected infant. If the gonads are not removed, virilization occurs during puberty; as a consequence, individuals who were raised as females frequently experience a postpubertal change in gender identity and role from female to male [5]. Considering the natural history of this disease, male assignment of neonatal diagnosed patients would be the recommendation of choice [2], but differentiation of a female gender identity also occurs in a minority of patients [6].

Mutations of the *SRD5A2* gene in 46,XY subjects result in a spectrum of external genital phenotypes ranging from complete female to nearly complete male with mild symptoms of undermasculinization [5, 7, 8]. To date, over 40 different mutations scattered throughout the gene have been described. Most of them are missense or nonsense mutations, although total deletion of the gene and nucleotide deletions as well as splice-junctions alterations have also been reported [9–11]. Some of them are recurrent mutations reported among various ethnic groups, whereas others pre-sumably reflect a founder effect. In addition, polymorphic variations at codons 49 (A49T) and 89 (V89L) have been correlated with masculinization defects and may represent genetic risk factors for the occurrence of hypospadias [11, 12].

In this paper, we report the molecular data of *SRD5A2* gene from 18 families and 20 patients with suspected steroid 5α-reductase type 2 deficiency from three different services in Brazil, in order to provide evidence of the mutational spectrum of this disease and its relevance for correct diagnosis and genetic counseling.

Patients and methods

Twenty patients with clinical signs of 5α-reductase type 2 deficiency from 18 unrelated families were included in this study. Informed consent was obtained from all patients or parents according to the approved by the Ethics Committee of Faculty of Medical Sciences from State University of Campinas (UNICAMP), Sao Paulo, Brazil. The clinical diagnosis was based on ambiguous genitalia in patients with 46,XY karyotype and elevated testosterone/DHT ratio. Some patients, in pubertal age, had also virilization without gynecomastia.

Eleven patients from ten families were followed in an interdisciplinary service for diagnosis and treatment of intersex cases in Campinas, Brazil (UNICAMP, patient numbers 5–9, 12, 14, 15, 18, 19, and 20 in Table 1). The other eight patients from seven families were followed in a genetics service in Salvador, BA, Brazil (State University of Bahia, patient numbers 1 to 4, 10, 11, 13, 17 in Table 1) and one patient in a pediatrics service in Sao Paulo, SP, Brazil (State University of Sao Paulo, patient number 16 in Table 1). Patients 1 and 2 are cousins, and patients 14 and 15 are brothers.

The clinical data, as age at diagnosis, sex at first consultation, definitive sex assignment, family history of sex ambiguity, consanguinity, classification of the external genitalia according to Sinnecker et al. [7] criteria and pubertal development were obtained retrospectively. Total T and DHT serum levels were also obtained retrospectively and confirmed with new evaluation at UNICAMP. The total T measurement was performed with commercial kit by electrochemiluminescence (Roche Elecsys 2010) and the DHT measurement by solid phase radioimmunoassay, with the *n*-hexane technical extraction procedure. In the prepubertal patients who were older than 4 months, the samples for total T and DHT determinations were collected following hCG stimulation (Profasi-Serono, 2000 IU, IM, for 3 consecutive days and collected at the 4th day).

Genomic DNA was isolated from blood leukocytes of the patients according to routine protocols. Polymerase chain reaction (PCR) products generated by exon flanking primers [13] were purified using the Wizard PCR Preps kit (Promega, Madison, Wis., USA) and were sequenced using the ABI377 Automated DNA Sequencer (PerkinElmer, Applied Biosystems) according to the manufacturers' procedure. All sequencing reactions were performed with PCR

Table 1 Clinical features, ethnic background, geographical origin, and molecular data of 20 subjects with 5α-reductase deficiency from 18 Brazilian families. *T* Testosterone, *DHT* dihydrotestosterone

Patient	Consanguinity/ family history	Age at diagnosis (years)	External genitalia[a]	Gonadal position R/L	T/ DHT ratio	Social sex	Ethnic background/ geographical origin	Molecular lesion			Other molecular variation
								Type	Location	Mutation	
								Class 1: homozygotes			
1[b]	+/+	5	4b	I/I	86[c]	F	AE/BA	Missense	Exon 3	G→A, G183S	
2[b]	+/+	2	4b	I/I	50[c]	F	AE/BA	Missense	Exon 3	G→A, G183S	
3	−/−	16	3b	S/S	87[d]	M	AE/BA	Missense	Exon 3	G→A, G183S	
4	−/−	27	3b	I/I	95[d]	F	AE/BA	Missense	Exon 3	G→A, G183S	
5	+/−	21 days	3b	S/S	70[d]	F	AE/BA	Missense	Exon 3	G→A, G183S	
6	−/−	20	3b	S/S	96[d]	F→M	AE/SP	Missense	Exon 3	G→A, G183S	
7	+/+	16	3b	I/S	82[d]	F[e]	E/MG	Missense	Exon 4	G→A, G196S	
8	+/+	3 months	3b	S/S	70[d]	?→F	AE/BA	Missense	Exon 5	C→T, R246W	
9	+/−	14	3b	I/I	65[d]	F→M	AE/SP	Missense	Exon 5	C→T, R246W	
10	−/−	19	3b	S/S	87[d]	M	AE/BA	Frameshift	Exon 1	217_218 insC	A49T (heterozygous)
11	−/−	19	3b	I/S	30[f]	F	AE/BA	Frameshift	Exon 4	del642T	
								Class 2: compound heterozygotes			
12	−/−	18	3b	S/S	15[d]	F→M[g]	E/SP	a) Missense	Exon 2	A→G, Q126R	
								b) Frameshift	Exon 2	418delT	
13	−/−	15	3b	S/S	96[f]	F	AE/BA	a) Missense	Exon 2	A→G, Q126R	
								b) Splice junction	Exon 3/ intron3	IVS +1G>A	
14[h]	−/+	2	3b	S/S	51[c]	?→M	E/SP	a) Missense	Exon 2	A→G, Q126R	
								b) Missense	Exon 3	G→C, G158R	
15[h]	−/+	4	3b	S/S	38[f]	F→M	E/SP	a) Missense	Exon 2	A→G, Q126R	
								b) Missense	Exon 3	G→A, G158R	
								Class 3: heterozygotes			
16	−/−	2	3a	S/S	91[c]	M	E/SP	Missense	Exon 5	C→T, R246W	
17	−/−	8	3b	I/I	21[c]	M	AE/BA	Missense	Exon 4	C→A, A207D	V89L (heterozygous)
18	−/−	1 month	3b	S/S	25[d]	M	E/SP	Missense	Exon 4	G→A, G196S	
								Class 4: no abnormality identified			
19	−/+	23	3a	S/S	87[d]	M	E/SP	−	−	−	V89L (heterozygous)

Table 1 (continued)

Patient	Consanguinity/ family history	Age at diagnosis (years)	External genitalia[a]	Gonadal position R/L	T/ DHT ratio	Social sex	Ethnic background/ geographical origin	Molecular lesion			Other molecular variation
								Type	Location	Mutation	
20	−/−	4	3b	S/S	25[c]	F→M	E/SP	−	−	−	A49T (heterozygous)

I Inguinal, *S* scrotal, *F* female, *M* male, *?* undefined, *AE* African–European-derived, *E* European-derived, *BA* State of Bahia, *MG* State of Minas Gerais, *SP* State of Sao Paulo, *R* right, *L* left
[a]According to the classification of Sinnecker et al. [7]
[b]First cousins
[c]After hCG stimulation
[d]Basal
[e]Ferraz et al. [14]
[f]Data before gonadectomy
[g]Ferraz et al. [15]
[h]Brothers

Fig. 1 Partial sequences of the *SRD5A2* gene showing the new mutations detected in the present study. **a** In patient no. 10, the insertion of a C between nucleotides 217 and 218 of the first exon leads to a premature stop at codon 135; **b** In patient no. 11, the deletion of a T in exon 4 leads to an ORF alteration. **c** In patient no. 13, a G→A mutation in the donor splice site of the third intron and **d** In patient no. 14, a G→A mutation in the third exon replaces a glycine (G) to an arginine (R) at position 158; The arrows point to the mutations. *N* = G/A
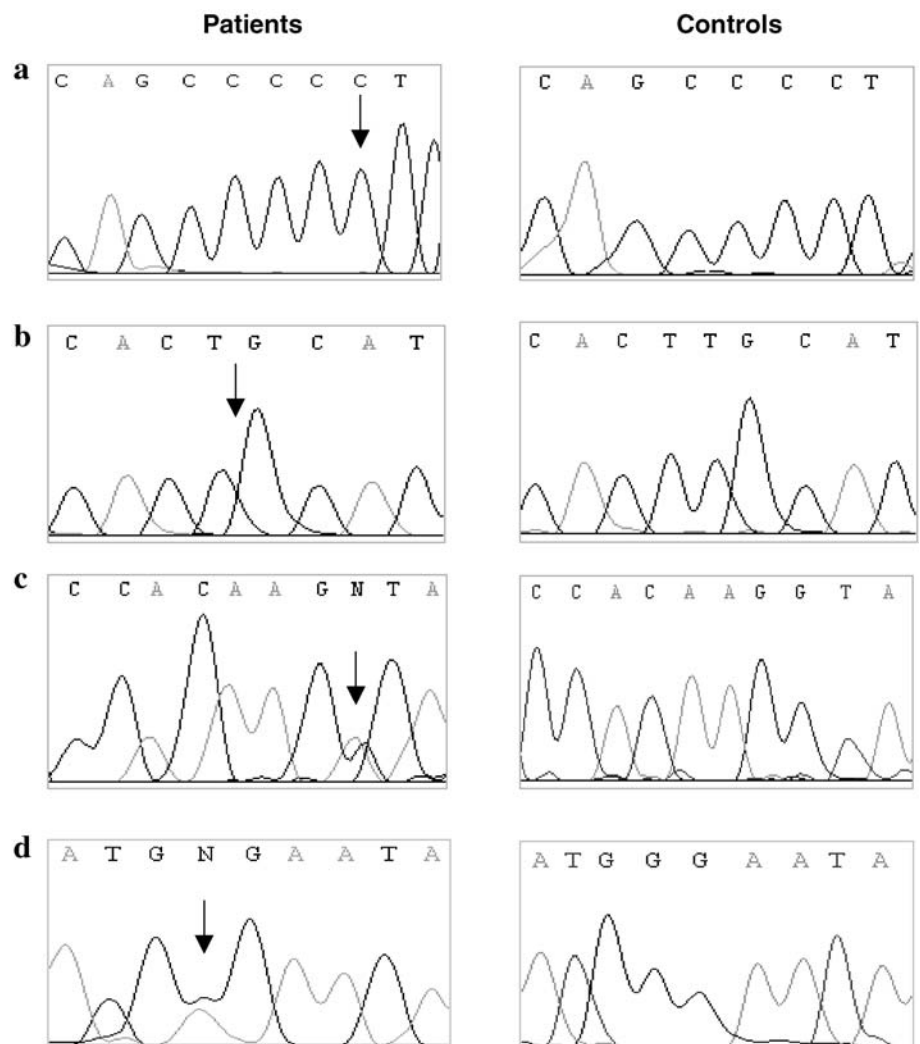
products from two separate reactions, using sense and antisense primers. The A49T and the V89L genotypes were determined for the heterozygous patients, as well as for the two without sequencing abnormalities. In one case (patient no. 11), the exon 1 PCR product was cloned in the vector pGEM^T, using the Easy Vector Cloning kit (Promega), followed by sequencing analysis with the exon-specific primers.

Results

The clinical features of the subjects are summarized in Table 1. Patients' ages varied from 21 days to 27 years; ten were more than 14 years old and only one has not already reached puberty (case 13), whereas the others were below 8 years of age and in prepubertal stage. Female was the initial sex assignment in 12 patients, male in six, and undefined in two. The definitive sex of rearing was female in seven cases and male in 13 cases. Consanguinity was registered in five families, and five others had similar cases (cases 1 and 2 are cousins, and cases 14 and 15 are brothers). The genital ambiguity according to Sinnecker et al. [7] criteria was grade 4b in two patients (cases 1 and 2), grade 3a in other two (cases 16 and 19), and grade 3b in the remainder cases, with palpable gonads in all cases. The total T levels varied from normal to elevated levels for age and pubertal stage, whereas the majority of patients had DHT values close to the lower limit of the method, varying from 0.03 ng/ml to 0.06 ng/ml. Three patients had been submitted to gonadectomy before present evaluation, and T/DHT ratio was not repeated.

The patients' ethnical and geographical origins and mutations are also summarized in Table 1. Twelve patients belonged to families with reported mixed ancestries (African- and European-derived) and, with exception of patient no. 9, were born in Bahia, a northeastern Brazilian state with a highly miscigenated population. Present-day African-Brazilians are considered the product of an original African gene pool that received European and Amerindian genes for about 12 generations [16]. The other eight patients were reported as European-derived only and were born either in Minas Gerais state (one case) or in Sao Paulo state (seven cases). Both states are located in a southeastern region, which received a greater contingent of European immigration in the last two centuries, composed mainly by Portuguese, Italian, Spanish, and German individuals.

Four new mutations were identified: the insertion of a cytosine between nucleotides 217 and 218 in the first exon (217_218insC), the deletion of a thymine at position 642 of exon 4, a point mutation at the donor-splicing site of the third intron (IVS3+1G>A), and a C→G transition at codon 158 leading to amino acid substitution (G158R) (Fig. 1). Eleven subjects presented SRD5A2 homozygous single-base mutations (six with G183S, two with R246W, one with del642T, one with G196S, and one with 217_218insC), whereas other four were compound heterozygotes (one with Q126R/IVS3+1G>A, one with Q126R/del418T and two brothers with Q126R/G158R). In addition, three were het-

erozygous for A207D, G196S, and R266W substitutions, and in one case, no sequencing abnormalities were detected. Two of them were heterozygous for the V89L polymorphism: patient no. 17 with the A207D substitution, and patient no. 19 with no sequencing abnormalities. Patient no. 20 was heterozygous for the A49T variant, without other molecular alterations. The finding of both homozygous 217_218insC and heterozygous A49T mutations in the first exon of patient no. 10 on direct sequencing of the PCR product was confirmed by the cloning procedure.

Discussion

Because the diagnosis of steroid 5α-reductase type 2 deficiency is sometimes difficult due its clinical variability and cannot be ruled out by a lack of an elevated T/DHT ratio, the molecular characterization of the mutations is a relevant tool for the correct ascertainment of this disease. The investigation of this series of Brazilian patients' suspected steroid 5α-reductase type 2 deficiency leads to the findings of four new mutations, reveals the prevalence of G183S substitution, and presents evidences of the recurrence of already known mutations.

The six missense mutations detected in the present series (Q126R, G158R, G183S, G196S, A207D, and R246W) are mutations that lead to amino acid substitutions occurring in conserved regions of the type 2 enzyme of humans, rats, and monkeys [17], suggesting that these positions are important for enzymatic activity. Indeed, the causative role or the Q126R, G183S, A207D, and R246W substitutions was confirmed by site-directed mutagenesis and in vitro assays [18]. In addition, with the exception of G158R, these mutations have already been found in patients with steroid 5α-reductase type 2 deficiency and are included in the SRD5A2 Human Gene Mutation Database [9].

The prevalence of G183S substitution can probably been attributed to a founder gene effect, because it has been observed in five individuals from Bahia State (two were first-degree cousins) and in one patient from Sao Paulo State, all of them with mixed African–European descent. This substitution has been reported before in two Brazilian patients [19] and in four affected members of a family from the Dominican Republic [20]. Although the 183 code position might be a hot spot of the SRD5A2 gene as suggested by Cai et al. [20], it is noteworthy to mention that, until now, this mutation was reported only among Brazilian patients from mixed African–European ancestry and in the Dominican Republic. A founder effect can be considered when we take into account that both countries have a strong component of African descendent in their nowadays populations in consequence from the slave trade that occurred between the sixteenth and seventeenth centuries, in the Dominican Republic, and during the Colonial Period in Brazil [16, 21].

A second founder gene effect may be illustrated by the Q126R substitution, which was found in four compound heterozygotes of this series, one of them previously described by Ferraz et al. [15]. This mutation has been detected in subjects from Brazil, Belgium, Louisiana, and

New York, all with presumed Portuguese ancestry [22]; in a French sibship [23]; and in a German individual [7]. These observations may indicate that the Q126R mutation is relatively common among Caucasians and could have been spread through European migration in the last centuries, although the possibility of a hotspot cannot be excluded. In contrast, the R246W substitution seen in three unrelated patients of this series seems to be a good example of recurrent mutation due to a hotspot in the exon 5 of the gene. Indeed, two mutant alleles (*R246W* and *R246Q*) arise from mutation at either position of a CG dinucleotide and have been found in seven different ethnic groups: African-American, White-European, Pakistanis, Dominican Republicans, Brazilians, Egyptians, [22], and Chinese [11].

Other hotspots of the *SRD5A2* gene may be represented by the G196S and A207D substitutions. The first one has been described in Greek, Turkish, Swedish, and Brazilian patients (7, 8, 14, 24, and this series), whereas the second was initially identified in a compound heterozygous Austrian individual [24], followed by the description of two siblings of Mexican origin, with this specific alteration in homozygous state [25]. To our knowledge, this is the third report of A207D substitution, occurring in a Brazilian male of African–European descent in heterozygous state (patient no. 17). This patient is also heterozygous for the V89L polymorphism, but the methodology employed in the present work does not allow us to establish if the A207D substitution is in *trans* or in *cis* with the 89L allele and parents' DNA, which could help us to determine the inheritance of these alleles, was not available. If we presume that they are in different alleles, it would be possible to suggest that the reduction of enzymatic activity of the 89L variant by approximately 30% [26] could significantly contribute to clinical manifestation of genital ambiguity, because the 207D mutation leads to complete inactivation of the enzyme [22]. Because neither functional determination of 5α-reductase activity in genital skin fibroblasts or in vitro experiments with *SRD5A2* expression vectors containing the targeted mutations have been performed, this question remains widely open.

One of the new mutations described in the present study was detected in a compound heterozygote (Q126R/IVS3 +1G>A, patient no. 13). It has been demonstrated that the Q126R substitution inactivates the enzymatic function in vitro and is associated with a drastically decreased half-life of the mutant protein [18]. The second mutation takes place at the first nucleotide of the donor-splicing site of the third intron (IVS3+1G>A). Thus, it can be predicted that altered RNA messenger processing would lead to an anomalous protein, either by intron retention or by the use of an alternative splicing site. Moreover, the high T/DTH ratio (96) observed in this patient is a good indication that this new mutation also leads to an inactive enzyme. Thus, the partial virilization of external genitalia observed in this patient can hardly be attributed to the residual function of the proteins coded by these alleles. This mechanism of virilization can also not be true for patients homozygous for *SRD5A2* gene deletions or for mutations introducing premature stop codons, not allowing any translation of a functional enzyme [27, 28]. Together, these observations are in agreement with the hypotheses proposed by Hiort et al. [28] that [1] significant levels of T or its precursors have enough androgenic potential to induce marked virilization of the external genitalia in early fetal life, and [2] variable fetal T levels could be a major determinant for the variable genotype–phenotype correlation in this disorder.

In the present series, three other patients (nos. 12, 14, and 15) were compound heterozygotes where one of the alleles carried the Q126R codon alteration. In the other allele, patient no. 12 exhibited a del418T on exon 2, which leads to a frameshift at codon 140 and introduced a premature stop codon at amino acid 159 [15]. Patient nos. 14 and 15 were brothers, and a new missense mutation was found (**G**GA→**A**GA, G158R). Although no in vitro study had been done to evaluate the effect of this mutation on 5α-reductase activity, its function may be affected by the substitution of a negatively charged amino acid (glycine) by a positively charged one (arginine) in a conserved protein domain. It is interesting to note that the four compound heterozygous patients in which one of the alleles leads to the Q126R amino acid change exhibited the same external genitalia phenotype (3b, according to Sinnecker et al. [7]).

A yet non-described, one-nucleotide deletion was detected in homozygosis in patient no. 11 (del642T). This thymine deletion on exon 4 predicts a frameshift at amino acid position 215, resulting in the addition of 22 amino acids at the C-terminal of this 254-amino acid isozyme. It is likely that this alteration would compromise enzymatic activity but, in this case, the possible effect of postpubertal T production must be taken into account, because gonadectomy occurred at age of 18 years, 1 year before being evaluated by us. At age of 19 years, this patient remains at the female-rearing sex.

The finding of the insertion of a cytosine between the 217 and 218 bases of the first exon of the gene on both alleles of the *SRD5A2* gene of patient no. 10 is very intriguing, because parental consanguinity is denied, and heterozygosis for the A49T rare variant is also present. The single nucleotide insertion at codon 73 results in a disruptive alteration, because a premature stop codon is predicted to occur at amino acid position 135. This is the first report of an insertion in the *SRD5A2* gene, arising in a C-rich sequence. Insertions of less than ten nucleotides are assumed to arise by non-random mechanisms [29], and it has been suggested that even short repetitive motifs such as CCCC have ~10- to 15-fold susceptibility to insertions and deletions than nonrepetitive sequences, due to slippage-mediated process [30]. In the present case, parents' DNA was not available, and we might consider either the possibility of independent origin of this rare mutation in two different alleles or a single origin in an unknown common ancestor, with the A49T substitution arising as a secondary event.

The presence of one 49T allele was the sole change detected in patient no. 20 from the present series, which exhibited a perineoscrotal hypospadias and a T/DHT ratio of 25. The 49T enzyme has been correlated with an increase of 5α-reductase activity in vitro [31], but it was further

demonstrated that the 49T allele, both in homozygous or heterozygous condition, leads to lower serum androgen concentration, indicating that this rare variant may be less efficient that initially thought [32]. Interestingly, the A49T codon substitution was found in one or both alleles of the *SRD5A2* gene in five out of seven patients with isolated hypospadias [12]. Thus, Silver and Russel [12] suggested that partial deficiency of 5α-reductase activity and inadequate levels of DHT in the fetal urethra may be sufficient to cause the phenotype of hypospadias without other clinical features of 5α-reductase deficiency. This hypothesis is reinforced by the observations that the expression of 5α-reductase type 2 is localized to the stroma surrounding the urethra, especially along the urethral seam area in the ventral portion of the remodeling urethra in male fetuses between 16 and 22 weeks of gestation [33].

We did not find a second mutation or polymorphic variants that could negatively affect the 5α-reductase type 2 activity in two patients (nos. 16 and 18) and only the polymorphic V89L was found in heterozygosis in patient no. 19. It is possible that undetected mutations could map outside of the exons and the immediate flanking introns analyzed here or could be present in the promoter region of SRD5A2, which was not explored in our study.

In the present series, all patients had severe external genitalia defects (from frankly ambiguous to male genitalia with severe undervirilization) not allowing genotype–phenotype correlations. Moreover, as discussed above, two distinct phenomena may counteract in the degree of virilization exhibited by 5α-reductase deficiency patients: the absence or partial 5α-reductase type 2 enzymatic activity and the individually variable testosterone levels during the development of male external genitalia. Probably, genotype–phenotype correlation in these patients would be more reliable through the analysis of the grade of virilization at puberty (such as the penile length and the distribution of genital and facial hair), or the sexual orientation (sexual attraction and relationship). Unfortunately, this analysis has not been done, because 11 patients were still at the prepubertal stage.

In conclusion, the investigation of this series of Brazilian patients leads to the findings of four new mutations, reveals the prevalence of G183S substitution, presents evidences of the recurrence of already known mutations, and reinforces the importance of molecular analysis for the correct diagnosis of 5α-reductase type 2 deficiency and for genetic counseling.

References

1. Wilson JD, Griffin JE, Russell DW (1993) Steroid 5 alpha-reductase 2 deficiency. Endocr Rev 14:577–593
2. Odame I, Donaldson MD, Wallace AM, Cochran W, Smith PJ (1992) Early diagnosis and management of 5 alpha-reductase deficiency. Arch Dis Child 67:720–723
3. Saenger P, Goldman AS, Levine LS, Korth-Schutz S, Muecke EC, Katsumata M, Doberne Y, New MI (1978) Prepubertal diagnosis of steroid 5 alpha-reductase deficiency. J Clin Endocrinol Metab 46:627–634
4. Greene S, Zachmann M, Manella B, Hesse V, Hoepffner W, Willgerodt H, Prader A (1987) Comparison of two tests to recognize or exclude 5 alpha-reductase deficiency in prepubertal children. Acta Endocrinol (Copenh) 114:113–117
5. Imperato-McGinley J, Zhu YS (2002) Androgens and male physiology the syndrome of 5 alpha-reductase-2 deficiency. Mol Cell Endocrinol 198:51–59
6. Zucker KJ (1999) Intersexuality and gender identity differentiation. Annu Rev Sex Res 10:1–69
7. Sinnecker GH, Hiort O, Dibbelt L, Albers N, Dorr HG, Hauss H, Heinrich U, Hemminghaus M, Hoepffner W, Holder M, Schnabel D, Kruse K (1996) Phenotypic classification of male pseudohermaphroditism due to steroid 5 alpha-reductase 2 deficiency. Am J Med Genet 63:223–230
8. Nordenskjold A, Ivarsson S-A (1998) Molecular characterization of 5 alpha-reductase type 2 deficiency and fertility in a Swedish family. J Clin Endocrinol Metabol 83:3236–3238
9. Human Gene Mutation Database *SRD5A2* gene (http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/127343.html). Accessed September 28, 2004
10. Fernandez-Cancio M, Nistal M, Gracia R, Molina MA, Tovar JA, Esteban C, Carrascosa A, Audi L (2004) Compound heterozygous mutations in the *SRD5A2* gene exon 4 in a male pseudohermaphrodite patient of Chinese origin. J Androl 25:412–416
11. Wang Y, Li Q, Xu J, Liu Q, Wang W, Lin Y, Ma F, Chen T, Li S, Shen Y (2004) Mutation analysis of five candidate genes in Chinese patients with hypospadias. Eur J Hum Genet 12:706–712
12. Silver RI, Russell DW (1999) 5 alpha-reductase type 2 mutations are present in some boys with hypospadias. J Urol 162:1142–1145
13. Katz MD, Cai LQ, Zhu Y, Herrera C, DeFillo-Ricart M, Shackleton HL, Imperato-McGinley J (1995) The biochemical and phenotypic characterization of females homozygous for 5 alpha-reductase deficiency. J Clin Endocrinol Metab 80:3160–3167
14. Ferraz LFC, Guerra Jr G, Baptista MTM, Maciel-Guerra AT, Hackel C (1998) Detection of Gly-196-Ser mutation in 5 alpha-reductase type II gene in a Brazilian patient with female assignment and behavior. J Pediatr Endocrinol Metab 11:465–466
15. Ferraz LFC, Baptista MTM, Maciel-Guerra AT, Guerra Jr G, Hackel C (1999) New frameshift mutation in the 5 alpha-reductase type 2 gene in a Brazilian patient with 5 alpha-reductase deficiency. Am J Med Genet 87:221–225
16. Bortolini MC, Salzano FM, Zago MA, Da Silva WA Jr, Weimer TA (1997) Genetic variability in two Brazilian ethnic groups: a comparison of mitochondrial and protein data. Am J Phys Anthropol 103:147–156
17. Levy MA, Brandt M, Sheedy KM, Holt DA, Heaslip JI, Trill JJ, Ryan PJ, Morris RA, Garrison LM, Bergsma DJ (1995) Cloning, expression and functional characterization of type 1 and type 2 steroid 5 alpha-reductases from Cynomolgus monkey: comparisons with human and rat isoenzymes. J Steroid Biochem Mol Biol 52:307–319
18. Wigley WC, Prihoda JS, Mowszowicz I, Mendonca BB, New MI, Wilson JD, Russell DW (1994) Natural mutagenesis study of the human steroid 5 alpha-reductase 2 isozyme. Biochemistry 33:1265–1270

19. Mendonça BB, Inácio M, Costa EM, Arnhold IJ, Silva FA, Nicolau W, Bloise W, Russell DW, Wilson JD (1996) Male pseudohermaphroditism due to steroid 5 alpha-reductase 2 deficiency. Diagnosis, psychological evaluation, and management. Medicine (Baltimore) 75:64–76

20. Cai LQ, Zhu YS, Katz MD, Herrera C, Baez J, DeFillo-Ricart M, Shackleton CH, Imperato-McGinley J (1996) 5 alpha-reductase-2 gene mutations in the Dominican Republic. J Clin Endocrinol Metab 81:1730–1735

21. Weil TE, Black JK, Blutstein HI, Johnston KT, McMorris DS Munson FP (1973) Social System. In: Area handbook for the Dominican Republic. US Government Printing Office, Superintendent of Documents, 2nd edn, pp 49–55

22. Russell DW, Wilson JD (1994) Steroids 5 alpha-reductases: two genes/two enzymes. Annu Rev Biochem 63:25–61

23. Boudon C, Lumbroso S, Lobaccaro JM, Szarras-Czapnik M, Romer TE, Garandeau P, Montoya P, Sultan C (1995) Molecular study of the 5 alpha-reductase type 2 gene in three European families with 5α-reductase deficiency. J Clin Endocrinol Metab 80:2149–2153

24. Thigpen AE, Davis DI, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, Francke U, Wilson JD, Russell DW (1992) Molecular genetics of 5 alpha-reductase deficiency. J Clin Invest 90:799–809

25. Canto P, Vilchis F, Chavez B, Mutchinick O, Imperato-McGinley J, Perez-Palacios G, Ulloa-Aguirre A, Mendez JP (1997) Mutations of the 5 alpha-reductase type 2 gene in eight Mexican patients from six different pedigrees with 5α-reductase-2 deficiency. Clin Endocrinol 46:155–160

26. Makridakis NM, Ross RK, Pike MC, Chang L, Stanczik FZ, Kolonel LN, Shi C-Y, Yu MC, Henderson BE, Reichardt JKV (1997) A prevalent missense substitution that modulates activity of prostatic steroid 5 alpha-reductase. Cancer Res 57: 1020–1022

27. Andersson S, Berman DH, Jenkins EP, Russell DW (1991) Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism. Nature 354:159–161

28. Hiort O, Schütt MS, Bals-Pratsch MT, Holterhus P-M, Marschke C, Struve D (2002) A novel homozygous disruptive mutation in the *SRD5A2* gene in a partially virilized patient with 5 alpha-reductase deficiency. Int J Androl 25:55–58

29. Cooper DN (1990) Structure and function in the human genome. In: Cooper DN (ed) Human gene evolution. BIOS Scientific, Guilford, UK, pp 3–54

30. Nishizawa M, Nishizawa K (2002) A DNA sequence evolution analysis generalized by simulation and the Markov chain Monte Carlo method implicates strand slippage in a majority of insertions and deletions. J Mol Evol 55:706–717

31. Makridakis NM, Ross RK, Pike MC, Crocitto LE, Kolonel LN, Pearce CL, Henderson BE, Reichardt JKV (1999) A missense substitution in the SRD5A2 gene is associated with prostate cancer in African–American and Latino men in Los Angeles. Lancet 354:957–958

32. Allen NE, Reichardt JKV, Nguyen H, Key TJ (2003) Association between two polymorphisms in the SRD5A2 gene and serum androgen concentrations in British men. Cancer Epidemiol Biomark Prev 12:578–581

33. Kim KS, Liu W, Cunha GR, Russell DW, Huang H, Shapiro E, Baskin LS (2002) Expression of the androgen receptor and 5 alpha-reductase type 2 in the developing human fetal penis and urethra. Cell Tissue Res 307:145–153