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Clinical, Hormonal, Behavioral, and Genetic Characteristics of Androgen Insensitivity Syndrome in a Brazilian Cohort: Five Novel Mutations in the Androgen Receptor Gene

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Androgen insensitivity syndrome (AIS) is caused by mutations in the androgen receptor gene and is associated with a variety of phenotypes in 46,XY individuals, ranging from phenotypic women [complete form (CAIS)] to men with minor degrees of undervirilization or infertility [partial form (PAIS)]. We studied 32 subjects with male pseudohermaphroditism from 20 families (9 CAIS, 11 PAIS) with the following criteria for AIS: 46,XY karyotype, normal male basal and human chorionic gonadotropin-stimulated levels of serum testosterone and steroid precursors, gynecomastia at puberty, and, in prepubertal patients, a family history suggestive of X-linked inheritance.

The entire coding region of the androgen receptor gene was analyzed, and mutations were found in all families with CAIS and in eight of 11 families with PAIS. Fifteen different mutations were identified, including five (S119X, T602P, L768V, I898F, and P904V) that have not been described previously.

Detailed clinical and hormonal features were compared with genotype in 25 subjects with AIS and confirmed by mutational analysis. LH hormone levels and the LH × testosterone product were high in all postpubertal subjects with AIS. All subjects with PAIS maintained at postpubertal age the gender identity and social sex that was assigned to them in infancy, in contrast to other forms of pseudohermaphroditism. (*J Clin Endocrinol Metab* 88: 3241–3250, 2003)

ANDROGENS HAVE A fundamental role in male sexual development and act by binding to the androgen receptor (AR), which is encoded by a gene located in the X chromosome (1). Androgen insensitivity syndrome (AIS) is a rare X-linked disorder in which 46,XY subjects have complete or partial impairment of androgen action throughout life due to abnormalities of the AR (2, 3). Subjects with the complete form of AIS (CAIS) have a female phenotype, including female breast development that begins at the age of expected puberty, and a paucity or absence of axillary and pubic hair. Partial AIS (PAIS) causes a spectrum of phenotypes, ranging from women with clitoromegaly to men with minor degrees of undervirilization; gynecomastia is common at puberty. In both CAIS and PAIS, androgen production is in the normal male range (4, 5).

The AR is encoded by a single gene that contains eight exons and is located on chromosome Xq11-12. The AR contains four functional domains: an N-terminal domain, encoded by exon 1; a DNA-binding domain, encoded by exons 2 and 3; a so-called hinge region, encoded by the 5′ portion of exon 4; and an androgen-binding domain, encoded by the 3′ portion of exon 4 and exons 5–8 (5).

More than 300 AR gene mutations have been described in individuals with AIS (http://www.mcgill.ca/androgendb/) (6). Most reports of mutations in the AR describe phenotypic characteristics in one or a few families (6); some reports of clinical and hormonal features of large groups do not include an analysis of the underlying mutations in all subjects and, therefore, may include other types of male pseudohermaphroditism. Long-term follow-up studies in this disorder are rare. Genotype and phenotype in families with AIS from The Netherlands were recently analyzed, but hormonal values were not reported (7). We describe the clinical, hormonal, molecular, and behavioral features of 25 Brazilian subjects with AIS confirmed by identification of mutations in the AR gene, including nine families with CAIS and eight with PAIS.

Subjects and Methods

Subjects

Informed parental consent, patient consent, and approval by the Hospital Ethics Committee were obtained before initiating the studies. Thirty-two individuals exhibited the following criteria for AIS: 46,XY karyotype and male levels of serum testosterone, testosterone precursors, and dihydrotestosterone (DHT) that excluded defects in testosterone synthesis or 5α-reductase 2 deficiency. Prepubertal subjects had family histories compatible with X-linkage, and subjects of postpubertal

Abbreviations: AIS, Androgen insensitivity syndrome; AR, androgen receptor; CAIS, complete AIS; DGGE, denaturing gradient gel electrophoresis; dGTP, deoxy-GTP; DHT, dihydrotestosterone; hCG, human chorionic gonadotropin; PAIS, partial AIS; T, testosterone.

age had gynecomastia. Women with CAIS had normal female external genitalia and primary amenorrhea. Subjects with PAIS had ambiguous external genitalia. Subject 11 was the maternal aunt of subject 2. Subjects 13, 14, and 16 were sisters; and subject 15 was their cousin. Subjects 5 and 7, 12 and 19, 20 and 23, and 21 and 24 were siblings. Subjects 3, 5–7, 21, 24, and 25 were previously reported (8, 9).

Phallus length was compared with the normal data of Schonfeld and Beebe (10). During puberty, penis length correlates better with pubertal status than with chronological age; therefore, phallus length was not expressed in SD scores in this age group. Development of breasts and pubic hair was classified according to Tanner stages for females (11), and axillary hair was quantified from 1+ to 4+. Subjects were submitted to pelvic ultrasonography and genitography. In two individuals with a urogenital sinus, vaginal length was measured by genitography from the bifurcation of the urogenital sinus to the end of the vagina. Eight sisters or mothers of AIS subjects were studied for purposes of genetic counseling by sequencing the exon that contained a mutation in the index case in each family.

Psychological evaluation

All patients were evaluated by the same psychologist (M.I.) to define gender identity and to help them understand their problems. The evaluation consisted of several interviews and projective tests, such as Szondi's test, free drawings of family, and the house, tree, person test. These are psychological tests in which the subject's response to the test material is indicative of personality traits and unconscious motivations. After psychological and hormonal evaluations, each subject was discussed in detail by the psychologist, the endocrinologists, and a surgeon to establish the appropriate management strategy.

Hormonal analysis

Serum LH and FSH were determined by commercial ^{125}I double-antibody RIA (Diagnostic Products Corp., Los Angeles, CA) or by immunofluorometric assays (AutoDelfia, Wallac, Inc., Turku, Finland). After extraction of serum with ethyl acetate and hexane, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, and estradiol levels were determined without previous chromatography after demonstration of specificity of antibodies used (12). 5α-DHT was measured by RIA after purification on Celite columns (Química Especializada Erich Ltda., São Paulo, Brasil) (12). Absolute values for serum testosterone (T) were multiplied by those of LH (T $\times$ LH product) to estimate androgen sensitivity. The normal range for T $\times$ LH product reported by Hiort *et al.* (13) in 53 fertile Caucasian men, ranging from 1,930–39,970 IU $\times$ ng/liter^2 (mean, 15,590 ng/liter^2), was used for comparison.

To assess human chorionic gonadotropin (hCG) responsiveness, prepubertal subjects were given 50–100 IU of hCG/kg body weight by im injection every 4 d for four doses, and blood was sampled before the first dose and 48 and 72 h after the last dose. These results were compared with those of boys with cryptorchidism (inguinal testis) and normal male external genitalia, submitted to the same protocol to treat cryptorchidism.

Molecular studies

Genomic DNA was obtained from peripheral blood leukocytes by salting out procedures. Exons 1–8 of the AR gene were amplified individually according to Lubahn *et al.* (1), except for the fragment containing GGN repeats in exon 1, which was amplified using primers A5 and A10, substituting 200 μM deoxy-GTP (dGTP) for 100 μM deaza-deoxy-dGTP and 100 μM dGTP. Exons 2–8 and the remaining fragments of exon 1 of the AR gene were amplified using 200 ng genomic DNA, 200 μM of each deoxynucleotide, 20 pmol of each primer, 2.5 U *Taq* polymerase, and the buffer given by the supplier (Pharmacia, Uppsala, Sweden) in a final volume of 100 μl. The polymerase chain reaction (PCR) assay was performed in the GeneAmp PCR System 9600 (PerkinElmer, Norwalk, CT). Amplification conditions consisted of an initial denaturing step of 98 C for 5 min, 30 cycles of 98 C for 1 min; 60 C for 1 min; 72 C for 2 min; followed by a final extension step at 72 C for 10 min. The fragment containing CAG repeats was amplified by 35 cycles with an annealing temperature of 60 C, and the fragment containing GGN repeats was amplified by 35 cycles with an annealing temperature of 55 C.

Initially, exons 5 and 7, which contain hot spots for mutations in the AR gene, were screened by denaturing gradient gel electrophoresis (DGGE) (8). Forward and reverse primers of exons 5 and 7 contained a 40-bp G-C clamp at the 5′ end, and PCRs were performed using one of the primers with a G-C clamp. Amplified DNA of affected subjects and normal controls was electrophoresed individually or after mixing normal and patient DNA that had been denatured at 98 C (10 min) and allowed to reanneal at room temperature (8) (modified by Russell, A., Glasgow University, Glasgow, Scotland, UK, personal communication). The fragments corresponding to exons 5 and 7 were electrophoresed in an 8% acrylamide gel, using denaturing concentrations of 40–80% and 35–70%, respectively (8). Fragments with abnormal migration were sequenced to identify the mutation. Fragments with normal migration were also sequenced to verify the sensitivity of DGGE. If a previously described mutation was identified in exons 5 or 7, the remaining exons were not sequenced. If a novel mutation in exons 5 or 7 was identified as well as in all subjects without mutations in exons 5 and 7, the remaining exons (1–4, 6, and 8) were sequenced without previous DGGE. For sequencing, 20 ng of the amplified DNA was submitted to a previous enzymatic treatment, using 10 U shrimp alkaline phosphatase and 2 U exonuclease I, following the supplier's instructions, and directly sequenced with the ABI PRISM Genetic Analyser 310 automatic DNA sequencer (PE Applied Biosystems, Foster City, CA).

Statistical analysis

Clinical and hormonal data of CAIS patients were compared with those of PAIS patients using the Mann-Whitney *U* test. Hormonal values of patients with PAIS were compared with those of subjects with 17-β hydroxysteroid dehydrogenase 3 deficiency (14) and 5-α reductase 2 deficiency (15) studied in the same laboratory, by the Kruskal-Wallis one-way ANOVA test, using the SigmaStat for Windows version 2.03 software (SPSS, Inc., Chicago, IL). Statistical significance was attributed to $P < 0.05$.

Results

Identification of mutations in the AR gene confirmed the diagnosis of AIS in 25 individuals with male pseudohermaphroditism. Seven subjects from three families fulfilled the diagnostic criteria for PAIS, but no mutation was identified in the coding region of the AR gene. In the present study, only data from the individuals with mutations are reported: clinical data are shown in Table 1, hormonal data in Table 2, and the results of molecular analysis in Table 3. Subjects were followed for 2–15.8 yr (median, 9.1 yr).

Clinical features (Table 1)

CAIS. Eleven subjects (two prepubertal and nine postpubertal) between 5.8 and 43 yr of age (median, 16 yr) from nine different families were diagnosed with CAIS. All were raised as girls, and family history was compatible with X-linked inheritance in six subjects. The most common postpubertal presentation was primary amenorrhea. Two postpubertal and the two prepubertal subjects were ascertained because of bilateral inguinal hernia.

Seven postpubertal women with CAIS with intact testes began breast development at the age of 11–15 yr (median, 13 yr) and developed pubic hair between the ages of 11 and 17 yr (median, 14 yr). Five of the seven women experienced breast development before pubic hair development. Subject 4 had persistent breast asymmetry.

Axillary hair was absent in six postpubertal subjects with CAIS and was sparse in the remaining three. All postpubertal

TABLE 1. Clinical data of Brazilian patients with androgen insensitivity due to mutations in the AR gene

Patient no./family	Mutation	Social sex	Age (yr)	Family history	Chief complaint	Onset of breast/PH (yr)	Breast development (Tanner)	Axillary hair	Pubic hair (Tanner)	Clitoris/ phallus (cm)	Vaginal and urethral openings	Vagina (cm)	Testes location
CAIS													
1/I[a]	P904V	F	5.8	N	Inguinal hernia	n.a./n.a.	I	Absent	I	<2	Separated	Present	Inguinal region
2/II[a]	S119X	F	9.5	P	Inguinal hernia	n.a./n.a.	I	Absent	I	<2	Separated	Present	Inguinal region
3/III	R779W	F	14	N	Amenorrhea + inguinal hernia	13/13.5	III	1+	II	<2	Separated	Present (3.0)	Inguinal region
4/IV	R752X	F	14.5	P	Amenorrhea	13/14	III	2+	II	<2	Separated	Present	Abdomen
5/V	R855C	F	16	P	Amenorrhea	12/14	IV	Absent	III (sparse)	<2	Separated	Present (4.0)	Abdomen
6/VI	M807V	F	16	N	Inguinal hernia	12/11.5	V	Absent	IV (sparse)	<2	Separated	Present (2.5)	Inguinal region
7/V[b]	R855C	F	17	P	Amenorrhea	13/16	V	Absent	III (sparse)	<2	Separated	Present (3.0)	Abdomen
8/VII	N705S	F	19	N	Amenorrhea	n.a./n.a.	IV	Absent	III (sparse)	<2	Separated	Present	Abdomen
9/VIII	L768V	F	19.8	U	Amenorrhea	15/17	V	Absent	IV (sparse)	<2	Separated	Present (5.0)	Labia majora
10/IX	N705S	F	34	P	Amenorrhea	11/11	V	Absent	IV (sparse)	<2	Separated	Present	Inguinal region
11/II[a]	S119X	F	43	P	Inguinal hernia	n.a./n.a.	IV	1+	II	<2	Separated	Present	Inguinal region
PAIS													
12/X	R855H	M→F	1	P	Ambiguous genitalia	n.a./n.a.	I	Absent	I	1.5	Single	Present	Inguinal region
13/XI	I898F	F	14	P	Ambiguous genitalia	11.5/13	IV	2+	IV	4.0	Separated	Present (3.0)	Labia majora
14/XI	I898F	F	18	P	Amenorrhea + inguinal hernia	14/14	V	Absent	IV (sparse)	4.5	Separated	Present (1.5)	Inguinal region
15/XI[c]	I898F	F	19	P	Ambiguous genitalia	>12/n.a.	V	3+	V	n.a.	Single	Present	Inguinal region
16/XI	I898F	F	20	P	Inguinal hernia	12.5/14	IV	3+	V	4.0	Separated	Present (4.0)	Inguinal region
17/XII	M742V	F	30	P	Ambiguous genitalia	13/13	IV	n.a.	IV	3.2	Single	Present	Inguinal region
18/XIII	W741C	M	2.5	P	Ambiguous genitalia	12.5/12.5	I	Absent	I	3.8	Single	n.a.	Scrotum
19/X	R855H	M	2.6	P	Ambiguous genitalia	n.a./n.a.	I	Absent	I	3.0	Single	Present	Inguinal region
20/XIV	T602P	M	7.3	P	Ambiguous genitalia	n.a./n.a.	I	Absent	I	2.5	Single	n.a.	Inguinal region
21/XV	R840S	M	7.8	P	Ambiguous genitalia	12/13.8	I	Absent	I	2.8	Single	n.a.	Inguinal region
22/XVI	R855H	M	13.8	N	Ambiguous genitalia	14.3/14.6	I	Absent	I	4.0	Single	Present	Inguinal region
23/XIV	T602P	M	16	P	Ambiguous genitalia	n.a./n.a.	IV	Absent	III	3.2	Single	Present	Right scrotum[d]
24/XV[c]	R840S	M	16.5	P	Ambiguous genitalia	12/12	Mastectomy	Absent	III	5.0	n.a.	n.a.	n.a.
25/XVII	Y763C	M	25	N	Ambiguous genitalia	n.a./n.a.	Gynecomastia	n.a.	n.a.	6.0	Single	Present (3.0)	Scrotum

Amenorrhea, Primary amenorrhea; PH, pubic hair; n.a., data not available; P, positive; N, negative; U, unknown.
[a] Patient submitted previously to bilateral gonadectomy.
[b] Patient submitted previously to unilateral gonadectomy.
[c] Patient submitted previously to genitoplasty.
[d] Surgery for bilateral cryptorchidism at age 4 yr resulted in atrophy of left testis and reduced size of right testis.

TABLE 2. Hormonal data of Brazilian patients with AIS due to mutations in the AR gene

Patient no./ family	Mutation	Age (yr)	LH (IU/liter)	FSH (IU/liter)	T (ng/dl)	DHT (ng/dl)	T:DHT Ratio	E2 (pg/ml)	T × LH (ng × U/liter^2)
CAIS/BAIS									
1/I[a]	P904V	5.8	<0.6[a]	11[a]	<14[a]	n.a.	n.a.	n.a.	n.a.
2/II[a]	S119X	9.5	<0.6[a]	5.5[a]	<14[a]	n.a.	n.a.	<13[a]	n.a.
3/III	R779W	14	22[c]	4.1[c]	790	9	88	<20	n.a.
4/IV	R752X	14.5	30	8	186	21	9	n.a.	55,800
5/V	R855C	16	43	13	1033	58	18	<10	444,190
6/VI	M807V	16	23[c]	11[c]	317	5	63	27	72,910
7/V[b]	R855C	17	32[b]	16[b]	286[b]	22[b]	13[b]	22[b]	91,520[b]
8/VII	N705S	19	14	3.5	365	n.a.	n.a.	36	51,100
9/VIII	L768V	19.8	27	5	275	51	5	30	74,250
10/IX	N705S	34	24	6.9	815	30	27	40	195,600
11/II[a]	S119X	43	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PAIS									
12/X	R855H	0.16	8	4.2	43 (600[d])	110[d]	5[d]	n.a.	n.a.
13/XI	I898F	14	15[c]	10[c]	1355	n.a.	n.a.	n.a.	203,250
14/XI	I898F	18	13	1.1	1592	71	22	49	206,960
15/XI	I898F	19	30[c]	7.5[c]	1042	n.a.	n.a.	56	312,600
16/XI	I898F	20	22[c]	11[c]	243	n.a.	n.a.	n.a.	53,460
17/XII	M742V	30	30[c]	9.2[c]	798	81	10	109	239,400
18/XIII	W741C	2.5	n.a.	4.6	355[d]	47[d]	8[d]	<10	n.a.
19/X	R855H	2.6	<0.6	<1	1140[d]	211[d]	5[d]	n.a.	n.a.
20/XIV	T602P	7.25	<0.1	2.5	<10	n.a.	n.a.	n.a.	n.a.
21/XV	R840S	7.8	<0.6	<1	386[d]	n.a.	n.a.	n.a.	n.a.
22/XVI	R855H	13.8	6	5.1	175 (413[d])	12 (29[d])	15 (14[d])	<10	n.a.
23/XIV	T602P	16	32[e]	34[e]	157[e]	26[e]	6[e]	<20[e]	50,240[e]
24/XV	R840S	16.5	9.3	11	1022	303	3	20	95,050
25/XVII	Y763C	25	49	11	1100	53	21	n.a.	539,000
Normal males			1.4–9.2	1.0–10.5	200–948	35–55	9–19	10–40	<39,970
Prepubertal boys after hCG[f]					133–648	20–38	11–31		

The T × LH products were obtained only in the patients with LH measurement by immunofluorometric assay. To convert to SI units, multiply testosterone by 0.0347 (nmol/liter), DHT by 0.0344 (nmol/liter), and E2 by 3.67 (pmol/liter). n.a. = Not available.

[a] Patient submitted previously to bilateral gonadectomy.

[b] Patient submitted previously to unilateral gonadectomy.

[c] Measured by RIA; normal adult male LH = 1.0 − 10 U/liter; FSH = 1.0 − 12 U/liter.

[d] After hCG stimulation 50–100 U/kg/dose, every 4 d, four times.

[e] Surgery for bilateral cryptorchidism at age 4 yr resulted in atrophy of left testis and reduced size of right testis.

[f] Boys with cryptorchidism (inguinal testis) and normal male external genitalia with same protocol.[d]

women with CAIS had fine, short pubic hair with light pigmentation, and the distribution pattern was Tanner II in three patients, Tanner III and sparse in three, and Tanner IV and sparse in another three.

The CAIS women had a normal clitoris (length, <2.0 cm). In five postpubertal women, the vaginal pouch varied from 2.5–5.0 cm in depth (median, 3.0 cm); the testes were localized in the inguinal region in six subjects, in the abdomen in four subjects, and in the labia majora in one subject.

PAIS. Mutations in the AR gene were identified in six prepubertal and eight postpubertal subjects from eight unrelated PAIS families, and their age at presentation ranged from 1–30 yr (median, 15 yr). In one (subject 12), because of poor virilization and lack of response to androgen therapy (testosterone esters, two 50-mg doses given 1 month apart), the social sex was changed from male to female at 1 yr of age, after extensive discussion by the medical team and the psychologist and acceptance by the family. Now, at age 9 yr she has a good adaptation as a girl, according to psychological evaluation. Five individuals were raised as females and eight as males, without any treatment with exogenous androgens before sex assignment, and all maintained their assigned social sex. Family history suggested X-linkage in seven of eight postpubertal subjects and was positive in all prepubertal subjects except subject 22, who was included because of the development of gynecomastia at puberty. These subjects were ascertained because of ambiguous genitalia (12 subjects), inguinal hernia in one, and primary amenorrhea and inguinal hernia in another.

Nine subjects with PAIS and intact testes had breast development at age 11.5–14.3 yr (median, 12.5 yr) and pubic hair development at 12–14.6 yr (median, 13.4 yr). Half of these subjects had breast enlargement before pubic hair development. Breast development corresponded to Tanner stage IV in four and Tanner V in two. The ages at which breast development occurred in subjects with CAIS and PAIS were not statistically different.

In postpubertal subjects with PAIS and intact testes, axillary hair was absent in three subjects, 2+ in one, and 3+ in two; and pubic hair was Tanner III in two, Tanner IV in three (sparse in one), and Tanner V in two subjects. The pubic hair was darker, longer, and coarser than in women with CAIS.

At the time of diagnosis, the length of the phallus of prepubertal subjects, all with male social sex, ranged between 2.5 cm (−3.7 SD) and 4.0 cm (−2.2 SD), with a median of 3.0 cm (−2.3 SD). The subject in whom social sex had been changed from male to female at age 1 yr had a 1.5 cm (−3.8 SD) phallus. Among subjects examined during puberty, the phallus mea-

TABLE 3. Mutations identified in the AR gene of Brazilian patients with AIS

Patient no./ family	Phenotype	Nucleotide	Exon/ domain	Mutation	Substitution	Amino acid[a]	CAG repeats	Binding data[b]	
								Bmax	Kd
2, 11/II	CAIS	TCG433TAG	1/TAD	S119X	Nonsense	—	25		
20, 23/XIII	PAIS	ACT2307CCT	3/DBD	T602P	Nonconservative	—	24		
8/VII	CAIS	AAT2469AGT	4/LBD	N705S	Conservative	Conserved	n.a.	0	
10/IX	CAIS	AAT2469AGT	4/LBD	N705S	Conservative	Conserved	22	0	
18/XIII	PAIS	TGG2585TGC	5/LBD	W741C	Nonconservative	Conserved	27	Low[c]	High
17/XII	PAIS	ATG2586GTG	5/LBD	M742V	Conservative	Conserved	21	0 (Ref. 39)	
4/IV	CAIS	CGA2616TGA	5/LBD	R752X	Nonsense	Conserved	16	0	
25/XVII	PAIS	TAC2650TGC	5/LBD	Y763C	Conservative	Nonconserved	22	Low (Ref. 8)	
9/VIII	CAIS	CTG2664GTG	5/LBD	L768V	Conservative	Conserved	20		
3/III	CAIS	CGG2697TGG	6/LBD	R779W	Nonconservative	Nonconserved	24		
6/VI	CAIS	ATG2781GTG	6/LBD	M807V	Conservative	Conserved	30	Low (Ref. 8)	
21, 24/XV	PAIS	CGT2880AGT	7/LBD	R840S	Nonconservative	Nonconserved	25		
5, 7/V	CAIS	CGC2925TGC	7/LBD	R855C	Nonconservative	Conserved	19	0	
12, 19/X	PAIS	CGC2926CAC	7/LBD	R855H	Conservative	Conserved	28	0 to nl	High
22/XVI	PAIS	CGC2926CAC	7/LBD	R855H	Conservative	Conserved	19	0 to nl	High
13–16/XI	PAIS	ATC3055TTC	8/LBD	I898F	Conservative	Conserved	19		
1/I	CAIS	CCC3073CGC	8/LBD	P904V	Conservative	Conserved	21		

nl, Normal; n.a., data not available; TAD, transcription activation domain; DBD, DNA-binding domain; LBD, ligand-binding domain.
[a] Homology between androgen, mineralocorticoid, progesterone, and glucocorticoid receptors.
[b] Binding studies, as published in http://www.mcgill.ca/androgendb, unless specified.
[c] The patient had two mutations: L547F in addition to W741C.

sured 4.0 cm in the subject with female social sex and 3.2 and 5.0 cm, respectively, in the two subjects with male social sex. Among the postpubertal subjects at diagnosis, phallus length was 6.0 cm (−4.6 SD) in the subject with male social sex and ranged between 3.2 cm (−6.3 SD) and 4.5 cm (−5.5 SD) in the three subjects with female social sex.

Ten subjects with PAIS had a single perineal opening (urogenital sinus), three had separate urethral and vaginal openings, and in one subject this information was not available. In seven subjects with a single orifice, a vagina was visualized by genitography. The vaginal length in four subjects ranged from 1.5–4 cm. These data were not available in four subjects who had been submitted to surgery previously. The testes were present in the inguinal region in nine subjects and in the labia majora or scrotum in three subjects.

Management, gender role, and sexual activity

CAIS and PAIS with female social sex. Subjects were submitted to gonadectomy and, at pubertal age, to estrogen replacement therapy. Subjects 10 and 11 with CAIS had sexual activity without vaginal dilation. Subjects 3, 5, 6, and 9 with CAIS and 14, 15, 16, and 17 with PAIS were submitted to vaginal dilation with acrylic molds of increasing width (16). Six women with CAIS and four females with PAIS were sexually active. Five women with CAIS (subjects 5, 6, 9, 10, and 11) expressed satisfaction with their overall sexual function, whereas one (subject 3) had persistent dyspareunia. Two subjects with PAIS and female social sex and gender identity (subjects 14 and 17) had satisfactory sexual function, and two sisters (subjects 13 and 16) had homosexual activity. Subjects 5, 6, 9, and 14 experienced transient dyspareunia at the beginning of sexual activity.

PAIS and male social sex. Subject 18 was treated with testosterone esters (two doses of 50 mg/month im) at age 4 yr, and his penis had a good response from 3.8–5.0 cm. At puberty, with endogenous testosterone therapy, his penis increased further to a normal length of 10 cm at age 16 yr. Subject 21 was treated at the age of 14.9 yr with testosterone esters (250 mg/wk im), and his penis length increased from 5.0–6.7 cm after 3 yr of irregular drug use. Now, he has sexual activity that he rates as somewhat unsatisfactory because of difficulty in maintaining erections and lack of ejaculation. His brother, subject 24, had a penis length of 5.6 cm at the age of 22.5 yr and was treated with testosterone esters (250–500 mg/wk); his penis length increased to 7.4 cm at the age of 24.2 yr. He now has regular sexual activity that he rates as satisfactory, with orgasm without ejaculation. Subject 22 was treated with testosterone esters (250 mg/wk im) at age 14 yr for approximately 1 yr, and his penis length increased from 4.0–5.0 cm. He is now 24 yr old with a penile length of 5.5 cm and refers to satisfactory sexual function.

Hormonal data (Table 2)

Testosterone levels in eight postpubertal women with CAIS ranged from 186-1033 ng/dl, with a median of 342 ng/dl (6.4–35.8 nmol/liter; median, 11.9 nmol/liter), and were in the normal range for males in six (75%) subjects, low in one subject with abdominal testes, and high in one subject.

In postpubertal subjects with PAIS, basal testosterone levels ranged from 157-1592 ng/dl, with a median of 1032 ng/dl (5.4–55.2 nmol/liter; median, 35.8 nmol/liter); levels were elevated in five (62%) subjects, in the normal male range in two subjects, and low in subject 23, who had complete atrophy of the left testis and reduced size of the right testis after surgery for cryptorchidism. Subject 22 was shown to be sexually immature by examination at age 13.8 yr (Tanner I) and had a basal serum testosterone level of 175 ng/dl (6.1 nmol/liter) that rose to 413 ng/dl (14.3 nmol/liter) after hCG stimulation. In five prepubertal subjects with PAIS, testosterone levels after hCG stimulation ranged between 355 and 1140 ng/dl, with a median of 413 ng/dl (12.3 and 39.5 nmol/liter; median, 14.3 nmol/liter), and were normal in four subjects

and high in one subject. Concentrations of testosterone precursors were normal, excluding defects in testosterone synthesis.

Serum LH was elevated in all postpubertal women with CAIS, ranging from 14–43 IU/liter (median, 26 IU/liter) and in all postpubertal subjects with PAIS, ranging from 9.3–32 IU/liter (median, 26 IU/liter).

The product of serum testosterone × serum LH (T × LH) was increased in all postpubertal women with CAIS (ranging from 51,100–444,190 ng × IU/liter^2; median, 74,250 ng × IU/liter^2) and in all postpubertal subjects with PAIS (ranging from 50,240–539,000 ng × IU/liter^2; median, 205,105 ng × IU/liter^2).

In postpubertal women with CAIS, FSH levels ranged from 3.5–16 IU/liter (median, 7.4 IU/liter) and were normal in six subjects and slightly elevated in two women with abdominal testes. In postpubertal subjects with PAIS, FSH levels were normal in five subjects, just above the upper limit in two subjects, and high in subject 23, who had signs of testicular atrophy after surgery for cryptorchidism.

In postpubertal women with CAIS, the testosterone to DHT ratio (T:DHT) ranged between 5 and 88 (median, 18) and was normal or decreased in four subjects and increased in three women. In the postpubertal subjects with PAIS, the basal T:DHT ratio ranged from 3–22 (median, 10) and was normal or decreased in three and elevated in two subjects. The T:DHT ratio after hCG stimulation, obtained from five subjects with PAIS, was normal or decreased, ranging from 5–14 (median, 8).

Estradiol levels in postpubertal women with CAIS were not elevated, compared with normal men, and ranged between less than 10 and 40 pg/ml (less than 36 and 147 pmol/liter; median, 27 pg/ml). In five postpubertal subjects with PAIS, the estradiol levels ranged from less than 20 to 109 pg/ml, with a median of 49 pg/ml (73–400 pmol/liter; median, 180 pmol/liter).

Serum LH, FSH, estradiol, DHT, the T × LH product, and T:DHT ratio were not different in subjects with CAIS and PAIS.

Postpubertal subjects with PAIS had a significantly higher testosterone and higher T × LH product when compared with male pseudohermaphroditism, owing to 17-β hydroxysteroid dehydrogenase 3 (14) and 5-α reductase 2 deficiencies (15). The T:DHT ratio was lower in postpubertal subjects with PAIS than in individuals with 5-α reductase 2 deficiency (15).

Mutational analysis (Table 3)

Initially, nine index subjects with CAIS and 11 index subjects with PAIS were studied by DGGE. The DNA-amplified fragment correspondent to exon 5 of subjects 4, 9, 17, 18, and 25 and to exon 7 of subjects 5, 7, 12, 19, 21, 22, and 24 showed altered migration on DGGE when compared with the normal control. The electrophoretic migration on DGGE of subject 9 resembled that of the normal control, but after mixing equal quantities of the PCR products of this subject and a normal control, we observed heteroduplex bands, indicating the presence of a mutation in this fragment.

Sequencing of exons 5 or 7 in samples with altered mobility on DGGE revealed a mutation in each subject (Table 3). Exons 5 and 7 of subjects with normal mobility on DGGE were also sequenced to verify the sensitivity of DGGE, and no mutations were identified.

After sequencing the remaining exons in the other index cases, 15 different mutations were identified in all families with CAIS and in eight families with PAIS of the 11 families originally studied (Table 3). All mutations involved single nucleotide substitutions. Five mutations (S119X, T602P, L768V, I898F, and P904R) have not been reported in the literature. The R840S mutation was previously described by us (9), and mutations Y763C, R779W, M807V, and R855C were previously reported in a collaborative study (8). Five mutations (N705S, W741C, M742V, R752X, and R855H) had been reported in the literature (6).

Twelve mutations occurred in conserved amino acids, whereas only three were seen in nonconserved amino acids. The proportion of mutations in conserved amino acids in families with CAIS (7 of 8) was similar to that in families with PAIS (5 of 7; $P = 0.57$) (Table 3).

Of the eight women who sought genetic counseling, six were heterozygous for mutations in the AR gene: a sister of subject 3 (R779W), the sister and mother of subject 1 (P904V), the mother of subject 8 (N705S), one sister of subjects 21 and 24 (R840S), and the mother of subjects 12 and 19 (R855H). Three of these carriers decided to have a child; the carrier of mutation R779W had one child with CAIS, the carrier of mutation R840S had one child with PAIS, and the carrier of P904V had a female infant who is also a carrier for the mutation in the AR gene.

Discussion

Since the original clinical description of AIS by Morris (17), when analysis of the AR gene was not yet available, few studies reported clinical and hormonal findings in large cohorts of subjects in whom the diagnosis of AIS was confirmed by identification of mutations in the AR gene. Ahmed *et al.* (18) reported the largest group of suspected cases of AIS (278 subjects), but the clinical and hormonal data refer to the complete series, whereas the diagnosis of AIS was confirmed in 83% of the women with CAIS and only 28% of subjects with PAIS.

The present study reports clinical and hormonal data of 25 Brazilian individuals with AIS that were confirmed by the identification of mutations in the AR gene. Most individuals were evaluated in a single center, and the median follow up was 9.1 yr. In 19 subjects, the mutation also was demonstrated in other affected family members or in maternal relatives, thus excluding postzygotic mutations and an influence of somatic mosaicism on phenotype.

Clinical and hormonal evaluations are sufficient for the diagnosis of CAIS, but the diagnosis of PAIS is more likely with a family history consistent with X-linked inheritance (19). Of the 173 subjects with clinical diagnosis of PAIS reported by Ahmed *et al.* (18), mutations in the AR gene were identified in 28%, and family history was negative in 67%. In the present study, the selection criteria, which included family history suggestive of X-linked inheritance in prepubertal subjects and the presence of gynecomastia in postpubertal

subjects, resulted in the identification of mutations in 100% of subjects with CAIS and 73% of families with PAIS. Only two postpubertal subjects with PAIS with mutations had negative family history. All prepubertal subjects with PAIS and positive family history had mutations in the AR gene.

Clinical presentation. The median age at presentation was 16 yr in subjects with CAIS (range, 5.8–43 yr) and 15 yr in PAIS (range, 1–30 yr). The diagnosis was made later than in the subjects described by Ahmed *et al.* (18) but similar to those studied by Sinnecker *et al.* (20). The most common presentation in women with CAIS is primary amenorrhea, and the most common presentation in prepubertal girls is inguinal hernia in this cohort as well as in the literature (5). Paucity in body hair was a common complaint in women with CAIS and in some with PAIS. The late diagnosis of our PAIS subjects with ambiguous genitalia at birth, many originating from distant villages, reflects cultural and financial problems.

The age of onset of breast and pubic hair development in subjects with AR gene mutations has not been described. In reports of subjects with clinical diagnosis of AIS, this age is more consistent with the age of pubertal onset of males (21, 22). In the present study, some women with AIS developed breasts and pubic hair later than in normal males and females (11, 23), but there was an overlap in the time of onset of pubertal signs between PAIS and CAIS. Quigley *et al.* (5) suggested that the delayed onset of pubertal development in 46,XY girls with CAIS (and some 46,XX carriers) would argue for a direct role of androgens in induction of puberty. If androgens have a major role in the induction of puberty, women with CAIS should have a later age of pubertal onset than subjects with PAIS, which was not observed in our cohort. The possibility of a direct role of Y-encoded genes on pubertal onset, not mediated by sex steroids, remains to be clarified. The occurrence of breast development before appearance of pubic hair probably is due to the impaired action of androgens and unopposed effect of estrogens secreted by the testes or formed from testosterone in extraglandular tissues.

Mature (longer, darker, and coarser) pubic and axillary hair is due to androgen action on the hair follicle (24). Absent or sparse axillary hair is characteristic of AIS, and one third of subjects with CAIS do not have axillary hair (25). In the present study, axillary hair was absent in two thirds of women with CAIS, and all had sparse pubic hair with Tanner stage III–IV distribution. Minimal pubic hair development occurs even in patients with complete defective AR (7). Therefore, absence of axillary hair is a stronger sign of AIS. In the subjects with PAIS, partial androgen action allowed development of pubic and axillary hair.

A relation of testicular position with phenotype, as reported by Barthold *et al.* (26), was found in our subjects with CAIS and PAIS as follows: location of the testes was abdominal in 36% and 0%, inguinal in 55% and 75%, and in the scrotum or labia majora in 9% and 25% of subjects, respectively (Table 1). This indicates an important, but not absolute, role of testosterone in testicular descent.

A vagina was present in all subjects with CAIS and in 10 of 11 individuals with PAIS (including those with a urogenital sinus). The average vaginal length was longer in CAIS, but there was an overlap between CAIS and PAIS. Women who used acrylic molds for vaginal dilation obtained sufficient vaginal length to allow successful sexual intercourse. Wisniewski *et al.* (27) studied 14 women with CAIS and reported that 11 were satisfied with their sexual functioning and three were dissatisfied. One of the latter subjects had become a lesbian, and the authors assumed that the two other women had dissatisfaction related to other variables. In the present study, seven women with AIS reported satisfactory sexual functioning. Four women experienced transitory dyspareunia in the beginning of sexual activity, which might have been due to small vaginal size or to psychological attitudes toward sex that dissipated with sexual activity. In family XI with PAIS associated with the I898F mutation, one woman functioned as heterosexual, and two others functioned as homosexuals. Therefore, the relation between genotype and sexual preference was inconsistent in this family. Two postpubertal males with PAIS had satisfactory sexual activity despite a penis length between 5.5 and 7.4 cm (−3.7 to −4.9 SD).

Gender role. All subjects with CAIS were raised as females and maintained female sex. The five individuals with PAIS who were raised as females maintained a female social sex after postpubertal age, despite clitoral growth and partial virilization. This is in distinct contrast to some other forms of male pseudohermaphroditism. In steroid 17β-hydroxysteroid dehydrogenase 3 deficiency and 5α-reductase 2 deficiency, several affected 46,XY individuals raised as females undergo a change to male social sex at the time of expected puberty (14, 15). The impairment of androgen action in subjects with PAIS is probably similar during embryogenesis and puberty, whereas the action of androgens is stronger at puberty in subjects with 17β-hydroxysteroid dehydrogenase 3 and 5α-reductase 2 deficiencies because of alternate pathways and maturation of isoenzymes. There is an overlap in phallus length at the time of diagnosis in our postpubertal PAIS subjects with female and male social sex, suggesting that sex assignment at birth and sex of rearing were more important than phallus size at puberty for development of gender identity. The sex of rearing was homogeneous within all families, except in the child in whom social sex was changed from male to female at 1 yr of age.

Hormonal findings. Serum gonadotropin and testosterone concentrations are often measured when AIS is suspected. Most published measurements have been obtained from subjects in whom mutations in the AR gene are not reported. The diagnosis of AIS requires careful exclusion of defects in testosterone biosynthesis and in the metabolism of testosterone to DHT. Subnormal levels of baseline or hCG-stimulated testosterone do not necessarily exclude AIS (28). Ahmed *et al.* (28) reported that in 42 subjects with confirmed AIS, basal LH levels, but not those of FSH, were often above the normal reference range.

In the present study, testosterone levels of most subjects were normal or elevated. In subjects with the I898F mutation, all from the same family, testosterone levels ranged from 243-1592 ng/dl, suggesting a lack of correlation of testoster-

one with genotype. Serum LH levels were increased in all subjects with CAIS and PAIS. Although the difference between groups was not significant, the median LH level was higher in CAIS (30 IU/liter) than in PAIS (18.5 IU/liter), suggesting a relationship with the severity of the mutation in the AR gene. Levels of FSH were normal in most subjects with AIS. Testosterone levels were low in one women with CAIS and abdominal testes, as the consequence of cryptorchidism, as has been reported (28). FSH was elevated in two women with CAIS with abdominal testes. One subject with PAIS who had testicular atrophy after surgery for bilateral cryptorchidism had low testosterone and high FSH levels. High FSH levels probably reflect impaired feedback by nonsteroidal factors, presumably inhibin B, resulting from the damage of seminiferous tubules. All of our postpubertal subjects with intact testes and mutations in the AR gene had a high T $\times$ LH product, compared with normal males (13). Therefore, in postpubertal male pseudohermaphrodites with normal testosterone precursors and T:DHT ratio, an elevated T $\times$ LH product is useful in the selection of subjects for the analysis of the AR gene.

In the present study, the T:DHT ratio was elevated in three subjects with CAIS, probably resulting from secondary 5α-reductase 2 deficiency, as previously reported by Imperato-McGinley *et al.* (29). In women with CAIS, the increased ratio of T:DHT is not a problem for the diagnosis because subjects with 5α-reductase type 2 deficiency have some degree of virilization. Two of 8 postpubertal subjects with PAIS had a slightly elevated T:DHT ratio but also had gynecomastia, which is not present in 5α-reductase type 2 deficiency (15). The T:DHT ratio was not different in subjects with CAIS and PAIS, but very high T:DHT ratios ($>$27), compatible with the diagnosis of 5α-reductase 2 deficiency, were only found in two women with CAIS.

In the present study, only three of 12 postpubertal subjects with AIS had increased levels of serum estradiol compared with normal men, and three had levels below the detection limit of the method, in contradistinction to the classical concept that estrogen production is increased in subjects with AIS (30). This suggests that breast development in these subjects occurred by the unopposed action of low levels of estrogens in the presence of androgen resistance (2).

Molecular analysis. Our experience confirms that DGGE is a method of high sensitivity for screening point mutations in exons 5 and 7 of the AR gene, especially with the mixture of equal quantities of the PCR products of a normal control and a patient (8).

We identified 15 different mutations in the AR gene in 17 families; 13 (87%) were located in the ligand binding domain and eight (53%) in exons 5 and 7, confirming these hotspot regions for mutations in the AR gene (6, 31). Mutations S119X, T602P, L768V, I898F, and P904R had not been reported before.

Mutation S119X in family II with CAIS creates a stop codon in exon 1 of the AR gene, probably resulting in a truncated protein. Nonsense mutations of the AR gene, are associated with CAIS, except in a subject with PAIS with the L172X mutation, in whom the blunted phenotype was thought to result from the expression of a wild-type allele due to mosaicism (32).

We identified the nonconservative mutation T602P in two brothers with PAIS (subjects 20 and 23). Threonine 602 is located in the second zinc finger of the DNA binding domain, adjacent to one of the four cysteines that anchor the zinc ion, and substitution by proline probably disrupts the organization of the backbone of the polypeptide, causing a transition in the direction of the chain and altering the structure of the AR (33).

The conservative mutation L768V was identified in subject 9 with CAIS. Leucine 768 is conserved between the receptors of the subfamily of AR. Another conservative substitution in this residue, L768P, is known to cause CAIS (18).

The conservative mutation I898F was identified in family XI with PAIS. Isoleucine 898 is conserved among the androgen, glucocorticoid, and mineralocorticoid receptors. The nonconservative substitution I898T has also been reported to cause CAIS (34).

Mutation P904R was identified in subject 1 with CAIS. This residue is conserved among the androgen, glucocorticoid, mineralocorticoid, and progesterone receptors (5). Two other mutations in this same residue, P904S (35) and P904H (31), also result in CAIS. The nitrogen in the proline ring is believed to have an important effect in the orientation of the polypeptide chain (33).

The mutations N705S, R752X, R779W, M807V, and R855C identified in our subjects with CAIS had been reported previously, also in women with CAIS (5, 6, 8), and phenotypes are homogenous with respect to normal female genitalia and female sex of rearing. Binding studies demonstrated the absence of binding between N705S mutant AR and androgens (5, 35, 36). N705 in the AR is one of hydrogen bond partners to the ligand R1881 (37). If this residue is replaced by serine, two effects are expected: loss of a hydrogen bond partner for the ligand, because serine is too small for the H-bond partner to ligand 17β-hydroxyl group, and destabilization of the structure (37).

We compared the phenotypes of our subjects with PAIS and the mutations W741C, M742V, Y763C, R840S, and R855H with the phenotypes of previously reported patients with the same mutation and found no major discrepancies.

Mutation W741C was reported previously in a patient born with perineal hypospadias, hypoplastic phallus and testes in nonfused labial folds, who also had a second mutation, L547F in exon 2 of the AR gene; the contribution of each mutation to the phenotype is not clear (38). Subject 18, with the W741C mutation, had perineal hypospadias, a phallus of 3.8 cm, and descended testes. He responded to testosterone therapy in childhood and achieved normal penile length, suggesting that the W741C mutation alone causes a mild disturbance of AR function.

Mutation M742V (subject 17) had been identified in one patient also reared as female and also with predominantly female ambiguous genitalia (39). Mutation Y763C (subject 25) had been identified in two patients also with ambiguous genitalia and male sex of rearing (40, 41). Mutation R840S, identified in two brothers with PAIS, was previously reported only by us (9).

There are arginine residues in the ligand-binding pocket

that carry a preponderance of mutations (6). Six of 15 (40%) of our families had mutations in these residues (R752X, R779W, R840S, R855C, and R855H). Replacement of basic arginine 855 by neutral and polar cysteine resulted in CAIS phenotype, whereas its replacement by also basic histidine resulted in PAIS with ambiguous genitalia, both in the literature (6) and in our patients (Table 3). Of eight patients with R855H mutation in the literature (6), five were females and three were males, whereas in our study, two subjects were males and the other one was changed from male to female sex.

The region in exon 1 that contains the CAG repeats was sequenced in all families, except in family VII. The number of CAG repeats ranged from 16–30, and the most frequent number of repeats was 19, found in four families. Families X and XVI, with PAIS caused by mutation R855H, had 28 and 19 CAG repeats, respectively, excluding a common origin for the mutation. Subjects 19 and 22, who had the same mutation (R855H) and a different number of CAG repeats, had similar degrees of virilization (penis length, −2.5 SD), suggesting that, in these cases, the number of CAG repeats did not influence the AR activity, or if they did, that other factors may have had compensatory effects on phenotype.

The identification of carriers of mutations in the AR gene is of clinical importance for genetic counseling. We studied eight women (mother, sister, or maternal relatives of subjects with mutations identified in the AR), and six of them were identified as carriers (heterozygotes) for mutations in the AR gene. The carrier of the mutation R840S had the diagnosis of infertility due to endometriosis and became pregnant by *in vitro* fertilization. It is not clear whether impaired androgen opposition to estrogen action in XX carriers for mutations in the AR might play a role in the etiology of endometriosis.

In conclusion, we identified mutations in the AR gene in all families with CAIS and 73% of families that fulfilled criteria for PAIS, including five mutations that have not been described previously. In 25 subjects with AIS confirmed by the presence of mutations in the AR, we describe detailed clinical, hormonal, and behavioral features and compare genotype to phenotype. The most novel finding is that all subjects with PAIS who were assigned a female sex rearing maintained a female social sex at postpubertal age, in distinct contrast to the outcomes in some forms of male pseudohermaphroditism, and clearly indicates that social or psychological factors interact with androgen action to influence the development of gender identity and gender role behavior.

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