Novel (60%) and Recurrent (40%) Androgen Receptor Gene Mutations in a Series of 59 Patients with a 46,XY Disorder of Sex Development

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Background: Androgen receptor (AR) gene mutations are the most frequent cause of 46,XY disorders of sex development (DSD) and are associated with a variety of phenotypes, ranging from phenotypic women [complete androgen insensitivity syndrome (CAIS)] to milder degrees of undervirilization (partial form or PAIS) or men with only infertility (mild form or MAIS).

Objective: The aim of the study was to characterize the contribution of the AR gene to the molecular cause of 46,XY DSD in a series of Spanish patients.

Setting: We studied a series of 133 index patients with 46,XY DSD in whom gonads were differentiated as testes, with phenotypes including varying degrees of undervirilization, and in whom the AR gene was the first candidate for a molecular analysis.

Methods: The AR gene was sequenced (exons 1 to 8 with intronic flanking regions) in all patients and in family members of 61% of AR-mutated gene patients.

Results: AR gene mutations were found in 59 individuals (44.4% of index patients), of whom 46 (78%) were CAIS and 13 (22%) PAIS. Fifty-seven different mutations were found: 21.0% located in exon 1, 15.8% in exons 2 and 3, 57.9% in exons 4–8, and 5.3% intronic. Twenty-three mutations (40.4%) had been previously described and 34 (59.6%) were novel.

Conclusions: AR gene mutation is the most frequent cause of 46,XY DSD, with a clearly higher frequency in the complete phenotype. Mutations spread along the whole coding sequence, including exon 1. This series shows that 60% of mutations detected during the period 2002–2009 were novel. **(*J Clin Endocrinol Metab* 95: 1876–1888, 2010)**

Androgen action is mediated by binding of testosterone (T) and dihydrotestosterone (DHT) to the intracellular receptor (AR; OMIM no. 313700).

AR-mediated androgen action is essential for normal primary male sexual development before birth and for normal secondary male sexual development around puberty, whereas in females, androgens also participate in sexual development around puberty and in adult female sexual function.

46,XY individuals have a single copy of the *AR* gene because of its location on the X chromosome (1, 2). Inac-

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Abbreviations: AIS, Androgen insensitivity syndrome; AR, androgen receptor; CAIS, complete AIS; DHT, dihydrotestosterone; DSD, disorder of sex development; GSF, genital skin fibroblast(s); hCG, human chorionic gonadotropin; K_d, dissociation constant; MAIS, mild AIS; PAIS, partial AIS; T, testosterone.

tivating mutations in the *AR* gene in 46,XY genetic males can therefore have marked effects upon masculinization because of the critical role of the AR in male differentiation. AR dysfunction in XY individuals results in androgen insensitivity syndromes (AIS; OMIM no. 300068), which are estimated to be present in 1:20,000 to 1:64,000 male births, and variable phenotypic expression has permitted the classification of AIS into complete (CAIS) and partial (PAIS) forms, as well as a rare group of phenotypically normal men with azoospermia (2–4). Subjects with CAIS have a female phenotype, including female breast development that begins at the expected pubertal age, and a paucity or absence of axillary and pubic air. PAIS causes a spectrum of phenotypes, ranging from women with clitoromegaly to men with minor degrees of undervirilization; gynecomastia is common at puberty, and in both cases, androgen production is in the normal male range (3). AIS has been reported to be the most frequent cause of 46,XY disorder of sex development (DSD), although frequencies vary depending on the series. Moreover, its molecular characterization is important when sex assignment and therapy outcomes are discussed and genetic counseling is requested.

Cloning of human *AR* complementary DNA has permitted characterization of the molecular defects causing AIS. Different strategies have revealed over 500 entries of mutations, representing over 300 different *AR* gene mutations, from more than 600 patients with AIS (http://www.mcgill.ca/androgendb/) (5).

We report the clinical, biochemical, and molecular features of 59 patients with 46,XY DSD in whom the clinical diagnosis of AIS was confirmed by a combination of biochemical [in peripheral blood and in genital skin fibroblasts (GSF)] and molecular studies that led to identification of 57 different *AR* gene mutations.

Patients and Methods

Patients and hormonal study

A series of 133 index patients with 46,XY DSD in whom gonads were confirmed as testes was consecutively studied for a molecular diagnosis during the period 2002–2009 (they originated from our center and 22 other public and private centers in Spain); external genitalia were completely feminized in 54 (41%) and partially virilized to varying degrees in the remaining 79 (59%). *AR* gene was analyzed in all as the first candidate gene.

Informed consent for the genetic study was obtained from patients and/or parents at each center.

Mutations in *AR* gene were found in 59 individuals (44.4% of index patients) from whom family member studies were performed in 36 families (61% of affected patients).

Serum hormone determinations had been performed at each center with commercialized RIA assays.

AR gene analysis

Genomic DNA from patients and siblings was isolated from blood leukocytes using standard procedures. *AR* gene exons 1–8 were amplified by PCR. Primers used for amplification are listed in Table 1. All molecular analyses were performed at a central laboratory.

PCR amplification of exons 1–8, except segment AR1G of exon 1, was carried out in 12.5 μl containing 50 ng genomic DNA, 0.3 mM of each primer, 0.05 mM of each dNTP, 1 mM $MgCl_2$, and 0.25 U Taq polymerase (ECOGEN). PCR amplification of segment AR1G was carried out in 12.5 μl containing 50 ng genomic DNA, 0.5 mM of each primer, 0.375 U FailSafe PCR Enzyme Mix, and Premix G (EPICENTRE). Reactions were denatured at 94 C for 5 min and then subjected to 40 cycles of amplification at 94 C for 1 min, annealing at 60 C for 30 sec and elongation at 72 C for 1 min, followed by a final extension at 72 C for 7 min.

After PCR, the products were analyzed in ethidium bromide-stained agarose gels and showed a single band with expected size. Products were sequenced in an automated sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the specifications provided by the manufacturer. The primers used in sequencing were the same as those used for PCR.

GenBank accession numbers used were GI:178897 to 178904 and M27423 to M27430 and GI:178627 and M20132. Nucleotide changes were reconfirmed in each DNA by antisense sequence and resequencing after a new PCR from original DNA.

Androgen binding and 5α-reductase activities in GSF

When genital skin was obtained at surgery, androgen binding activity was determined twice in GSF (at cell passages 5 and 7) in

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TABLE 1. Primers used for *AR* gene amplification and sequencing

Reaction	Primer name	Sequence (5′→3′)	Location	Product size
AR1A	Sense	GCCTGTTGAACTCTTCTGAGC	Exon 1	366 bp
	Antisense	GCCTGTTGAACTCTTCTGAGC	Exon 1	
AR1C	Sense	AGCAAGAGACTAGCCCCAGGCAGC	Exon 1	300 bp
	Antisense	CGGAGCAGCTGCTTAAGCCGGGG	Exon 1	
AR1D	Sense	CTGCCCCATCCACGTTGTCCCTGCT	Exon 1	300 bp
	Antisense	GACTCAGATGCTCCAACGCCTCCAC	Exon 1	
AR1E	Sense	CAAGGAGTTGTGTAAGGCAG	Exon 1	283 bp
	Antisense	TCTCGCCTTCTAGCCCTTTG	Exon 1	
AR1F	Sense	CAGGCAAGAGCACTGAAGATACTGC	Exon 1	279 bp
	Antisense	GGTTCTCCAGCTTGATGCGAGCGTG	Exon 1	
AR1G	Sense	CGCGACTACTACAACTTTCCACTGG	Exon 1	438 bp
	Antisense	GCCAGGGTACCACACATCAGGT	Exon 1	
AR1I	Sense	TAGCCCCCTACGGCTACACTCGG	Exon 1	245 bp
	Antisense	CAGAACACAGAGTGACTCTGC	Exon 1	
AR2	Sense	GCCTGCAGGTTAATGCTGAA[a]	Intron 1	374 bp
	Antisense	GTTATTTGATAGGGCCTTGC[a]	Intron 2	
AR3	Sense	GTTTGGTGCCATACTCTGTC[a]	Intron 2	410 bp
	Antisense	ATGGCCACGTTGCCTATGAA[a]	Intron 3	
AR4	Sense	GAGTTTAGAGTCTGTGACCA[a]	Intron 3	455 bp
	Antisense	GATCCCCCTTATCTCATGCT[a]	Intron 4	
AR5	Sense	AACCCGTCAGTACCCAGACT[a]	Intron 4	283 bp
	Antisense	GCTTCACTGTCACCCCATCA[a]	Intron 5	
AR6	Sense	GGGCTTATTGGTAAACTTCC[a]	Intron 5	290 bp
	Antisense	GTCCAGGAGCTGGCTTTTCC[a]	Intron 6	
AR7	Sense	TCAGATCGGATCCAGCTATC[a]	Intron 6	412 bp
	Antisense	TCTATCAGGCTGTTCTCCCT[a]	Intron 7	
AR8	Sense	GAGGCCACCTCCTTGTCAAC[a]	Intron 7	302 bp
	Antisense	AAGGCACTGCAGAGGAGTAG[a]	Intron 8	

[a] According to Imai *et al.* (51).

10 cases of CAIS and in five cases of PAIS according to the technique published by Carrascosa *et al.* (6). Parameters measured included DHT binding capacity (B_{max}) that reflects the receptor concentration and receptor-binding dissociation constant (K_d).

5α-Reductase activity (determined as the total amount of DHT, androstanediol, and androstandione generated from ^{3}H-T at 5×10^{-8} M in 1 h by 1 mg of cell protein) was determined in GSF at cell passage 5 in 10 cases of CAIS and five cases of PAIS according to the technique published by Audí *et al.* (7). The rate of androstendione formation from tritiated testosterone (17β-hydroxysteroid dehydrogenase activity) was also determined in the same incubations.

All of the *in vitro* studies were performed at the same central laboratory as the molecular studies.

Results

AR gene analysis resulted in the characterization of an abnormal sequence in 59 patients (44.4% of the total number of 46,XY DSD patients), which represented an 85% (46 patients) incidence of *AR* mutations in the complete female external genitalia (CAIS) and 16% (13 patients) in the partially virilized phenotype (PAIS). Phenotype, mutational, and family data are shown in Table 2, and hormonal and GSF data are shown in Table 3.

A molecular diagnosis was reached in 22 of the remaining 74 patients after analysis of other candidate genes, according to clinical and biochemical phenotypes (*SRD5A2*, *HSD17B3*, *SF1*, *WT1*, *CYP17A1*, and *LHCGR* gene mutations). Among the remaining 52 patients (39.1% of the whole series) without mutations in the analyzed genes, only one with completely female external genitalia presented a clearly X-linked family history, whereas three with ambiguous genitalia presented a family history. In 37 patients, biochemistry was consistent with androgen insensitivity, and 11 with ambiguous genitalia were premature infants with or without intrauterine growth retardation.

Phenotypes and hormonal data

Among the 46 CAIS index cases (Table 2), two were aborted fetuses diagnosed owing to discordant geno/phenotype (C11 and C17), one was prenatally suspected because of fetal sex discordance (C37), 22 (47.8%) were diagnosed during infancy owing to the presence of an inguinal hernia, and 21 (45.6%) at puberty because of amenorrhea. All had female sex assignment. Gonadectomy had been performed at diagnosis before pubertal age in eight patients (18.2%) or after pubertal development in 20 (45.5%), whereas testes had not been removed in 13 (29.5%) who remained prepubertal or in three (6.8%) after puberty.

The 13 patients with PAIS (Table 2) presented ambiguous external genitalia prenatally (patient P1) and at birth.

TABLE 2. Phenotype, genotype, and family data in index patients carrying AR gene mutations

Patient phenotype	Age at diagnosis/ gonadectomy	Reason for consultation	Social sex	Mutation[c] GI:178897-178904, GI:178627	Nucleotides GI:178897-178904	Location	Previously described	CAG repeats	GGC repeats	Family investigations		
										Heterozygote carriers	Only wild-type sequence	Other members affected
CAIS												
C1	5 m/8 m	Inguinal hernia	F	E2K	GAA2AAA	Exon 1	Yes	27	13	Mother		
C2	16 yr/22 yr	Amenorrhea	F	Q76X	CAG76TAG	Exon 1	No	Truncated	18		1 cousin	
C3	10 m	Inguinal hernia	F	InsA,R79fsX81		Exon 1	No	21	18	Mother, 2 sisters	1 aunt	
C4	2 m	Inguinal hernia	F	InsGCCG,A15fsX82		Exon 1	No	20	9			
C5	16 yr/20 yr	Amenorrhea	F	Q84X	CAG84TAG	Exon 1	No	20		Mother		
C6	24 yr/24 yr	Amenorrhea	F	Del 10bp,S82fsX169		Exon 1	No	20	18			1 sister
C7	15 yr/40 yr	Amenorrhea	F	DelC,P219fsX224		Exon 1	No	18	17			
C8	16 yr/20 yr	Amenorrhea	F	InsT, K239X		Exon 1	No	21	18		2 sisters	
C9	16 yr/26 yr	Amenorrhea	F	DelCT,Q346fsX499		Exon 1	No	21	17		Sister	
C10[a]	15 yr/15 yr	Amenorrhea	F	G453S	GGT453AGT	Exon 1	No	22	17			1 sister, 2 aunts
				Y571C	TAT571TGT	Exon 2	Yes					
C11	22 wk (GA)	Fetal sex discordance	Aborted fetus	Del 17bp,H543fsX544		Exon 2	No	23	17	Mother	2 aunts, great-aunt, grandmother	
C12	3.5 yr	Inguinal hernia	F	G568E	GGG568GAG	Exon 2	No	26	18			
C13	1 yr	Inguinal hernia	F	A573P	GCT573CCT	Exon 2	No	24	18			1 sister
C14	3 m	Inguinal hernia	F	C579W	TGC579TGG	Exon 2	No	23	17	Mother, 1 aunt		1 aunt
C15	13.5 yr	Amenorrhea, gender dysphoria	F	F583L	TTC583TTG	Exon 2	No	29	17	Mother, 2 sisters		1 sister, 1 aunt
C16[b]	1 yr	Inguinal hernia	F	IVS2–2 A>C + K590E		Intron 2	No	13	18	Mother, 1 aunt, 1 cousin	2 cousins	1 cousin
					AAA590GAA	Exon 3	No					
C17	Prenatal	Fetal sex discordance	Aborted fetus	IVS2–3 C>G		Intron 2	No	26	18	Mother		
C18	1.5 yr	Inguinal hernia	F	Y593X	TAC593TAA	Exon 3	No	18	18			
C19	36 yr	Amenorrhea	F	C619R	TGT619CGT	Exon 3	No	20	18	Mother		1 sister
C20	16 yr/16 yr	Amenorrhea	F	D695Y	GAC695TAC	Exon 4	No	19	18	Mother		
C21	16 yr/20 yr	Amenorrhea	F	D695N	GAC695AAC	Exon 4	Yes	22	17	Mother, 1 aunt	Sister, 1 cousin	1 cousin, 1 aunt
C22	3.7 yr	Inguinal hernia	F	S703C	AGC703TGC	Exon 4	No	19	18	Mother, sister	1 aunt	
C23	16 yr/25 and 40 yr	Amenorrhea	F	N705S	AAT705AGT	Exon 4	Yes	18	18			
C24	3 yr/3 yr	Inguinal hernia	F	N705S	AAT705AGT	Exon 4	Yes	22	18	1 sister	2 sisters	1 sister
C25	6 yr/14 yr	Inguinal hernia	F	W741R	TGG741CGG	Exon 5	Yes	23	17			
C26	16 yr/18 yr	Amenorrhea	F	G743V	GGG743GTG	Exon 5	Yes	24	17			
C27	17 yr/17 yr	Amenorrhea	F	L744F	CTC744TTC	Exon 5	Yes	21	17	1 sister	1 sister	
C28	16 yr/17 yr	Amenorrhea	F	F747C	TTT747TGT	Exon 5	No	23	17		Sister	
C29	4 yr/4 yr	Inguinal hernia	F	R774C	CGC774TGC	Exon 6	Yes	27	17			
C30	16 yr/18 yr	Amenorrhea	F	R774H	CGC774CAC	Exon 6	Yes	20	17			
C31	2 m/2 yr	Inguinal hernia	F	Y781D	TAC781GAC	Exon 6	No	20	18	Mother, sister	2 aunts	
C32	15 yr/17 yr	Amenorrhea	F	DelGT,P785fsX827		Exon 6	Yes	19	18			
C33	18 yr	Amenorrhea	F	M787I	ATG787ATA	Exon 6	No	23	18			
C34	11 m	Inguinal hernia	F	M787I	ATG787ATA	Exon 6	No	23	17			1 sister
C35	17 yr/17 yr	Amenorrhea	F	M787I	ATG787ATT	Exon 6	No	26	17			
C36	17 yr/20 yr	Amenorrhea	F	DelAA,R792fsX827		Exon 6	No	20	17	Sister		

(*Continued*)

TABLE 2. Continued

Patient phenotype	Age at diagnosis/ gonadectomy	Reason for consultation	Social sex	Mutation[c] GI:178897-178904, GI:178627	Nucleotides GI:178897-178904	Location	Previously described	CAG repeats	GGC repeats	Family investigations: Heterozygote carriers	Family investigations: Only wild-type sequence	Family investigations: Other members affected
C37	Prenatal	Fetal sex discordance	F	IVS6-44 G>A		Intron 6	No	28	17			
C38	4 m	Inguinal hernia	F	R831Q	CGA831CAA	Exon 7	Yes	23	17	Mother	Grandmother	
C39	6 m	Inguinal hernia	F	R831X	CGA831TGA	Exon 7	Yes	19	18			
C40	18 yr/20 yr	Amenorrhea	F	N833del		Exon 7	No	19	18			
C41	8 m/10 m	Inguinal hernia	F	L838V	CTC838GTC	Exon 7	No	8	17			
C42	3 m/5 m	Inguinal hernia	F	R855H	CGC855CAC	Exon 7	Yes	20	14	Mother, 2 aunts	1 aunt	
C43	10 m/10 m	Inguinal hernia	F	V889M	GTG889ATG	Exon 8	Yes	21	17	Mother	2 aunts	1 sister
C44	15 yr/17 yr	Inguinal hernia	F	M895T	ATG895ACG	Exon 8	Yes	26	17			
C45	10 m/10 m	Inguinal hernia	F	V903L	GTG903TTG	Exon 8	No	23	18	Mother		
C46	1 yr	Inguinal hernia	F	DelC,R905fsX942		Exon 8	No	22	17	Mother	2 aunts	
PAIS												
P1	Prenatal/5 yr	Fetal sex discordance, hypospadias	M	P378R	CCT378CGT	Exon 1	No	17	18			
P2	2 yr/no	Hypospadias	M	P390S	CCG390TCG	Exon 1	Yes	22	19			
P3	3 m/1 yr	Inguinal hernia, hypospadias	F	I680N	ATT680AAT	Exon 4	No	21	4	Mother, 3 aunts, grandmother, 1 great-aunt, great-grandmother		1 sister, 1 cousin, 2 great-aunts
P4	15 yr/no	Hypospadias, pubertal gynecomastia	M	M742I	ATG742ATA	Exon 5	Yes	18	18	Mother		
P5	8 m/no	Hypospadias	M	M745L	ATG745CTG	Exon 5	No	23	17	Mother, grandmother		
P6	15 yr/16 yr	Ambiguous genitalia	F	R840H	CGT840CAT	Exon 7	Yes	23	11	1 niece		
P7	3 yr/4 yr	Ambiguous genitalia	F	R855H	CGC855CAC	Exon 7	Yes	18	17	Mother, 2 aunts	1 aunt	1 sister
P8	1 yr/no	Hypospadias, pubertal gynecomastia	M	A870V	GCG870GTG	Exon 8	Yes	25	11			
P9	8.5 m/15 m	Ambiguous genitalia	F	S888S	AGC888AGT	Exon 8	Yes	23	17	Mother		
P10	4 m/5.5 m	Ambiguous genitalia	F	S888S	AGC888AGT	Exon 8	Yes	25	18			
P11	27 yr	Ambiguous genitalia	M	V889L	GTG889CTG	Exon 8	No	18	14			1 sister
P12	4 m/7 m	Ambiguous genitalia	F	Q902K	CAA902AAA	Exon 8	Yes	21	17	Mother		
P13	14 yr/14 yr	Amenorrhea	F	P913S	CCC913TCC	Exon 8	Yes					

M, Male; F, female; m, months; GA, gestational age.

[a] Patient C10 presented two mutations (G453S and Y571C).

[b] Patient C16 presented two mutations (IVS2 −2 A>C and K590E).

[c] Missense mutations are indicated by the amino acid number preceded by the normal and followed by the substituted amino acid. Ins indicates an insertion followed by the nucleotide/s; Del indicates a deletion followed by the nucleotide/s; fs indicates a frameshift (an abnormal amino acid sequence beginning after the previously indicated amino acid); X indicates a stop codon at the indicated amino acid number. Intronic nucleotide point mutations are indicated by the intron number (IVS) followed by the nucleotide number preceding the following exon and the nucleotide change.

TABLE 3. Biochemical studies in index patients carrying AR mutations

						Before hCG stimulation				After hCG stimulation			GSF studies			
													DHT binding		5-α-R	δ-4
Patient	Age	LH (IU/liter)	FSH (IU/liter)	Estradiol (ng/dl)	SHBG (nmol/liter)	T (ng/dl)	DHT (ng/dl)	T/DHT ratio	hCG test protocol	T (ng/dl)	DHT (ng/dl)	T/DHT ratio	Bmax 10^{-15} M/mg	K_d 10^{-9} M	(pM/mg/h)	(pM/mg/h)
CAIS																
C1	5 m												4.3	0.95	70.7	60.4
C4	2 m					49			1500 IU/48 h × 3	589	46	12.8				
C5	16 yr	7.7	2.1	4.0	22	398			1500 IU/48 h × 7	470			2.8	0.63	12.1	7.4
C6	24 yr	10.5	0.6	2.7		831							0	0	69.1	95.8
C7	40 yr	10.0	4.5	3.3	113	590							0	0	1.7	1.3
C14	3 m												11	0.18	48.4	121.0
C15	13.5 yr	14.8	0.6			144	23	6.2	2500 IU/48 h × 3	303	32	9.5				
C18	1.8 yr	1.3	2.3	0.5		23										
C19	36 yr	27.3	4.7	7.3	42	645										
C22	3.7 yr	<0.1	1.2			10	4	2.5	1000 IU/48 h × 6	310	32	9.7	4.4	0.32	222.3	37.8
C23	40 yr	16.7	23.4	2.2	102	365							0	0	22.9	5.8
C25	14 yr					490										
C31	2 yr	0.1	0.6	5.9		26			1500 IU/48 h × 7	1120			18.5	1.7	29.7	110.0
C32	15 yr	23.0	6.0	2.7	51.8	570							0	0	2.3	59.6
C33	24 yr	23.8	21.8	3.6		1350	77	17.5								
C34	11 m	3.5	0.9			134	13	10.3								
C35	17 yr	7.6	4.1	2.0		1000	38	26.3								
C37	4 yr												13.3	0.14	98.5	8.5
C39	6 m	0.4	0.4		128	26	24	1.1	500 IU/48 h × 7	688						
C40	20 yr	35.0	16.0	6.5	43.4	606										
C41	8 m					58	8	7.2	1500 IU/48 h × 7	812	144	5.6				
C42	3 m					109	28	3.9	1500 IU/48 h × 7	827	46	17.9				
C45	10 m					30	10	3	1500 IU/48 h × 7	464	31	14.9				
PAIS																
P1	5 d	4	5.1			267										
P2	2 yr												30.0	0.28	102.3	4.4
P3	6 m					557			1500 IU/48 h × 7	1790			28.0	1.0	151.8	25.0
P4	15 yr	4.9	2.4			92			3000 IU/m^2 × 3	1502			14.3	0.73	29.4	19.4
P8	3 m					109	28	3.9	1500 IU/48 h × 7	827	46	17.9				
P9	8.5 m	1.2	1.6	0.9		117			1000 IU/48 h × 3	544			0	0	79.8	31.8
P10	4 m	1.6	4.1	1.1		158										
P11	27 yr	18.4	5.6	3.0	30	752	99	7.6								
P13	14 yr	30.4	27.3	3.6		913							0	0	25.4	6.0
Normal GSF													27.2 ± 7.3 (n = 26)	0.35 ± 0.35 (n = 26)	174 ± 85 (n = 9)	8 ± 8 (n = 9)
Normal NGSF													4.9 ± 2.5 (n = 11)	0.25 ± 0.25 (n = 11)	15 ± 12 (n = 4)	3 ± 4 (n = 4)

m, Months; NGSF, non-GSF; 5-α-R, 5a-reductase activity (amount of DHT + androstanediol + androstandione formed from tritiated testosterone at 5 × 10^{-8} M in 1 h by 1 mg of cell protein); δ-4, amount of androstendione formed from ^{3}H-T at 5 × 10^{-8} M in 1 h by 1 mg of cell protein); Bmax, maximal binding.

Female sex was assigned in seven (53.8%) who had been gonadectomized at prepubertal age (in five) or at puberty (in two), male sex was assigned in six (46.2%) in whom phalloplasty was performed during childhood (in five), and gynecomastia developed in the three who reached pubertal development (patients P4, P8, and P11).

Two patients, one CAIS (patient C5) and one PAIS (patient P1), presented Müllerian duct remnants consisting of uterus and Fallopian tubes to which the intraabdominal testes were adhered; this led to Müllerian duct and testes removal even in patient P1 who had male sex assignment.

No cases of malignancy or carcinoma *in situ* were reported in the 13 cases in which testicular histology results were available.

Hormonal data were available in 28 cases (20 CAIS and eight PAIS) (Table 3) showing normal to high T concentrations, either at baseline or after human chorionic gonadotropin (hCG) stimulation, except in three prepubertal cases (patients C18, C34, and P10) in whom no stimulation was performed. DHT was measured in 11 cases, resulting in a T/DHT ratio, either at baseline or under hCG stimulation, ranging from 5.6 to 26.3. SHBG levels were highly variable at postpubertal age (from 22 to 102 nmol/liter). Baseline LH and/or FSH were raised in most postpubertal patients.

Molecular data

Mutational analysis revealed 57 different mutations in 59 index patients (Table 2): two patients presented two different mutations in the same allele (patients C10 and C16) and each of four different mutations was present in two unrelated patients (patients C23 and C24, C33 and C34, C42 and P7, and P9 and P10). Twelve mutations were located in exon 1 (21.0%), six in exon 2 (10.5%), three in exon 3 (5.3%), five in exon 4 (8.8%), six in exon 5 (10.5%), seven in exon 6 (12.3%), six in exon 7 (10.5%), nine in exon 8 (15.8%), two in intron 2 (3.5%), and one in intron 6.

The 46 *AR* gene mutations identified in CAIS patients comprised 32 single nucleotide substitutions, eight partial deletions (from 1 to 10 bp), three insertions (from 1 to 4 bp), and three splice site defects; they predicted a single amino acid change in 28 substitutions (patients C1, C10 bearing two different substitutions, C12-C16, C19-C31, C33-C35, C38, and C41-C45), a stop codon in four substitutions (patients C2, C5, C18, and C39), a single amino acid deletion in one codon deletion (patient C40), a frameshift sequence with a shortened protein in six partial deletions (patients C6, C7, C9, C11, C32, and C36) and in all insertions (patients C3, C4, and C8), and a frameshift sequence with a longer protein in one single nucleotide deletion (patient C46).

The 12 *AR* gene mutations identified in PAIS patients were all single nucleotide substitutions predicting an amino acid change in 11, but one was a silent mutation (patients P9 and P10).

Thirty-four of these mutations were novel (59.6%) because they were not present in the *AR* database (5) or in more recent reports: InsA,R79fsX81; InsGCCG,A15fsX82; Q84X; Del10bp,S82fsX169; DelC,P219fsX224; InsT, K239X; DelCT,Q346fsX499; G453S; Del17bp, H543fsX544; G568E; A573P; C579W; F583L; IVS2 –2 A>C; K590E; IVS2 −3 C>G; Y593X; C619R; D695Y; S703C; F747C; Y781D; M787I x 2; DelAA,R792fsX827; IVS6 –44 G>A; N833del; L838V; V903L and DelC, R905fsX942 in CAIS patients; and P378R, I680N, M745L, and V889L in PAIS.

The remaining 23 mutations (40.4%) had been previously described: E2K in one PAIS male (8); Q76X in one CAIS (9); P390S in three mild AIS (MAIS) with oligospermia and male infertility, and in one male with a seminoma (10–12); Y571C in one CAIS (13); D695N in three CAIS, one PAIS female, and one MAIS with infertility (12, 14–17); N705S in five CAIS and one PAIS (3, 18–22); W741R in one CAIS (23); M742I in one PAIS female (24); G743V in one PAIS female, one PAIS male, and one CAIS (25–27); L744F in one CAIS (28) and as a somatic mutation in a male with quiescent prostate cancer (29); R774C in 10 CAIS (13, 15, 30–35); R774H in eight CAIS and one PAIS (3, 19, 20, 24, 30, 36, 37); DelGT,P785fsX827 in one CAIS (38); R831Q in eight CAIS (20, 33, 39–43); R831X in five CAIS (16, 19, 40, 44, 45); R840H in one CAIS, six PAIS males, and six PAIS females (23, 38, 39, 46–55); R855H in four CAIS, six PAIS males, six PAIS females, and one male with infertility (12, 16, 21–24, 28, 36, 39, 52, 53, 56–58); A870V in one PAIS male (59); S888S in three PAIS males (60, 61); V889M in four CAIS and two PAIS females (18, 20, 37, 50, 62, 63); M895T in two CAIS (38, 44); Q902K in one PAIS male (64); and P913S in one PAIS (65).

The regions in exon 1 that contain the CAG and GGC repeats were sequenced in all patients. The number of CAG repeats ranged from 8 to 29, whereas the number of GGC repeats ranged from 4 to 19, with the most frequent number of repeats being 23 and 17, respectively (Table 2). Patients C23 and C24, C33 and C34, C42 and P7, and P9 and P10 had the same mutations (N705S, M787I, R855H, and S888S, respectively) and a different number of CAG or GGC repeats, suggesting that the number of CAG or GGC repeats did not influence AR activity in these cases because they had a similar phenotype, except for patients C42 and P7. The family of patient P3 is an extended one, with three generations of affected patients in which all 46,XX were carriers of an *AR* allele with only four GGC

(polyglycines); in addition, they presented a high rate of spontaneous abortion.

Family studies were performed in 36 families (Table 2). All of the 24 mothers analyzed were heterozygous carriers, whereas two grandmothers were carriers and two were not (in the last two cases, exon 1 CAG repeat length confirmed that the mutated *AR* allele originated in the mother from the maternal grandfather's allele). One great-grandmother was also a carrier. Among sisters of index patients, 11 in 11 families were 46,XY-affected, nine in seven families were heterozygous carriers, and eight in six families were noncarriers. Among 28 aunts, 10 in six families were heterozygous carriers, 13 in eight families were noncarriers, and five in four families were 46,XY-affected. Among great-aunts, one was a heterozygous carrier, one a noncarrier, and two in one family were 46,XY-affected.

GSF studies

DHT binding assay was performed in 15 GSF from 10 CAIS and five PAIS (Table 3). Binding was undetectable in the following mutations: Del10bp,S82fsX169 (patient C6), DelC,P219fsX224 (patient C7), N705S (patient C23), DelGT,P785fsX827 (patient C32), S888S (patient P9), and P913S (patient P13). Binding capacity was diminished in E2K (patient C1), Q84X (patient C5), C579W (patient C14), S703C (patient C22), IVS6–44G>A (patient C37), and M742I (patient P4). Affinity was slightly decreased (elevated K_d) in Y781D (patient C31) and I680N (patient P3). Binding assay yielded normal parameters in P390S (patient P2).

Five-α-reductase activity was determined in the same 15 GSF cultures (Table 3). Activity was markedly reduced, yielding similar results as in control non-GSF in seven mutations (46.7%): Q84X (patient C5), DelC,P219fsX224 (patient C7), N705S (patient C23), Y781D (patient C31), DelGT,P785fsX827 (patient C32), M742I (patient P4), and P913S (patient P13). 17-β-Hydroxysteroid dehydrogenase activity was markedly increased in five cases [E2K (patient C1), Del10bp,S82fsX169 (patient C6), C579W (patient C14), Y781D (patient C31), and DelGT,P785fsX827 (patient C32)] and slightly increased in four others [S703C (patient C22), I680N (patient P3), M742I (patient P4), and S888S (patient P9)]. In summary, both 5α-reductase and 17β-hydroxysteroid dehydrogenase activities were normal for a GSF culture in only two cases [IVS6–44G>A (patient C37) and P390S (patient P2)].

Discussion

The present study reports the frequency, variety, location, and phenotypes of 57 *AR* gene mutations detected in 59 Spanish patients successively analyzed during the period 2002–2009 in a series of 133 46,XY DSD index patients (one patient per family). Selection criteria were karyotype, presence of testicular gonads and normal T secretion (although data on this latter criterion were unavailable in some cases). *AR* gene was considered the first candidate, and its analysis yielded some abnormality in the sequence in 44.4% of index patients, with the percentage being 5-fold higher in the female than in the partially virilized phenotype (85 *vs.* 16%). Patients without mutations in *AR* or other subsequently analyzed genes (39.1% of the whole series) might present clinical and biochemical phenotypes consistent with androgen insensitivity or even an X-linked family history; such patients constitute a challenge for the diagnosis of 46,XY DSD. As also pointed out in other series, prematurity accompanied or not by intrauterine growth retardation was present in 21.1% of those with ambiguous genitalia, suggesting that this high incidence may be related to a developmental immaturity.

This series shows that, in the complete phenotype (CAIS patients), the first diagnosis in a family may now be prenatal (4.3%) but is almost equally distributed between infancy owing to an inguinal hernia (47.8%) and puberty because of amenorrhea (45.7%). Gonadectomy was consequently performed after puberty in the highest percentage (45.5%), whereas a tendency was observed to preserve the gonads during infancy and puberty (36.3%) *vs.* those gonadectomized before puberty (18.2%).

The incidence of *AR* gene mutations in the ambiguous genitalia group was low (16%), and sex assignment was almost equally distributed (53.8% for female and 46.2% for male sex). Gonadectomy was performed early in females when diagnosed, and males undergoing spontaneous puberty developed gynecomastia.

It is unclear whether testicular tumors are more common in AIS patients compared with those with simple cryptorchidism in whom the prevalence of the premalignant state of carcinoma *in situ* has been reported to be as high as 3% (66). Histological study of the testes was not available in a number of cases in this study, but no signs of malignant degeneration were detected in any of the ip gonads removed after puberty (data not shown). A rare association with bilateral complete Müllerian duct remnants was present in one CAIS patient and one PAIS patient, and although the mechanism for such a presence has not been explained, the association has been noticed in a few patients in several series (9, 67–77). In some patients, mostly the earlier, the diagnosis was not molecular; however, in more recent patients, the *AR* gene mutations had been characterized, as were those in our patients, and in one report (69), *AMH* and *AMHR* genes were sequenced to rule out any AMH protein dysfunction. Because AMH is

negatively regulated by T and AMH protein levels were elevated in patients with CAIS during the first year of life and from puberty development (78), the mechanism(s) for such Müllerian duct persistence in a very small proportion of AIS patients cannot yet be established.

The molecular results underline the diversity of mutations present in any studied population and show that they are usually family-based, although several have been described in unrelated families.

Fifty-seven different mutations, 34 of which had not been described previously in the literature, were identified in the *AR* gene in 59 patients.

In our series, exon 1 presented the highest number of mutations (21%) whereas, to date, except in the recent report by Philibert *et al.* (9), exon 1 has been considered to bear the lowest rate of mutations despite encoding more than half of the AR protein (5). This may be due to the fact that this exon may not have been analyzed in all series. Interestingly, except for the first two exon 1 mutations reported (E2K in patient C1 and Q76X in patient C2) (8, 9), all the other exon 1 mutations found in CAIS patients were novel, being stop codons, insertions, or deletions, except for the single amino acid change G453S that was combined in the same allele with another amino acid change in exon 2 (Y571C, patient C10). Only two mutations in exon 1 were found in PAIS patients: both were a single amino acid change (P378R and P390S), and the latter had previously been reported (10–12). In summary, single amino acid changes were less frequent in exon 1 than stop codons, insertions, or deletions, probably because the unstructured N-terminal domain of AR can more often tolerate single amino acid changes with no alterations in AR function.

Interestingly, the 11 mutations present in exons 2 and 3 (encoding the AR protein DNA binding domain) and in intron 2 were only detected in CAIS patients.

Mutations InsA,R79fsX81; InsGCCG,A15fsX82; Q84X; Del10bp,S82fsX169; DelC,P219fsX224; InsT,K239X; DelCT,Q346fsX499; P378R; G453S; Del17bp,H543fsX544; G568E; A573P; C579W; F583L; IVS2 –2 A>C; K590E; IVS2 −3 C>G; Y593X; C619R; I680N; D695Y; S703C; M745L; F747C; Y781D; M787I; DelGT,P785fsX827; DelAA, R792fsX827; IVS6 –44 G>A; N833del; L838V; V889L; V903L and DelC,R905fsX942 had not been reported previously.

Insertions, deletions, and nonsense mutations of the *AR* gene [Q76X (9); InsA,R79fsX81; InsGCCG,A15fsX82; Q84X; Del10bp,S82fsX169; DelC,P219fsX224; InsT,K239X; DelCT,Q346fsX499; Del17bp,H543fsX544; Y593X; DelGT,P785fsX827 (38); DelAA,R792fsX827; R831X; N833del, and DelC,R905fsX942] found in the present series are all associated with CAIS phenotype, probably due to the fact that these mutations produce a truncated protein. Thus, androgen binding in GSF, which was determined in four of those patients, was undetectable in three (patients C6, C7, and C32) and diminished in one (patient C5). Patient C5 carried a novel premature stop codon (Q84X), and similarly reduced androgen binding has been explained in other patients by the possible expression of a protein with some DNA and ligand binding domains from a downstream translation initiating codon (79).

We found splice site mutations in three CAIS patients: IVS2 –2 A>C, IVS2 −3 C>G, and IVS6 –44 G>A, which raised the possibility that these mutations can cause anomalies at mRNA level. The IVS2 –2 A>C mutation is accompanied by the K590E mutation in the same *AR* allele, and diminished DHT binding in GSF was observed in the intron 6 anomaly.

Interestingly, all nonsense and splice site mutations in which 5α-reductase activity was determined resulted in a clearly diminished enzyme activity, which may add to aggravating the phenotype. Diminished 5α-reductase activity in AIS patients has been described as a possible explanation for phenotype variation (80).

Point mutations predicting a single amino acid change were the most frequent (n = 39; 68.4% of all mutations), similar to all published series (http://www.mcgill.ca/androgendb/) (5).

Single amino acid changes found in the present series and also previously described were E2K in a CAIS patient and described in a PAIS patient (8); P390S in a PAIS patient and described in three MAIS with oligospermia and male infertility and in one male with a seminoma (10–12); Y571C in a CAIS described in one CAIS (13); D695N in a CAIS described in three CAIS, one PAIS female, and one MAIS with infertility (12, 14–17); N705S in two unrelated CAIS and described in five CAIS and one PAIS (3, 18–22); W741R in a CAIS and described in one CAIS (23); M742I in one PAIS and described in one PAIS female (24); G743V in a CAIS and described in one PAIS female, one PAIS male, and in one CAIS (25–27); L744F in a CAIS and described in one CAIS and in a male with prostate cancer (28, 29); R774C in a CAIS and described in 10 CAIS (13, 15, 30–35); R774H in a CAIS and described in eight CAIS and one PAIS (3, 19, 20, 24, 30, 36, 37); R831Q in a CAIS and described in eight CAIS (20, 33, 39–43); R840H in a PAIS and described in one CAIS, six PAIS males, and six PAIS females (23, 38, 39, 46–55); R855H in a PAIS and described in four CAIS, six PAIS males, six PAIS females, and one male with infertility (12, 16, 21–24, 28, 36, 39, 52, 53, 56–58); A870V in a PAIS and described in one PAIS male (59); S888S in two unrelated PAIS and described in three PAIS males (60, 61); V889M in a CAIS and described in four CAIS and two PAIS females (18, 20, 37,

50, 62, 63); M895T in a CAIS and described in two CAIS (38, 44); Q902K in a PAIS and described in one PAIS male (64); and P913S in a PAIS and described in one PAIS (65).

The P390S mutation, present in a PAIS patient (scrotal hypospadias) with male sex assignment, confirmed that this single amino acid change previously only reported in three MAIS patients presenting infertility (10, 12) has a partial effect. In our study, *in vitro* GSF studies showed completely normal DHT binding parameters as well as T metabolism, and mutant *in vitro* androgen-stimulated transcriptional activity was found to be normal (10). If polyglutamine $(CAG)_n$ and/or polyglycine $(GGN)_n$ tract variation may explain different phenotypes, then our patient presented a normal $(CAG)_{22}$ but the longest $(GGC)_{19}$ repeat leading to a $(GGN)_{25}$, and diminished transcriptional activity and protein synthesis have been found for shorter or longer $(GGN)_n$ tracts, with $(GGN)_{24}$ and $(GGN)_{27}$ presenting 35 and 58% lower transactivating activities, respectively, than the most frequent $(GGN)_{23}$ (81).

Novel single amino acid changes were G453S, G568E, A573P, C579W, F583L, K590E, C619R, D695Y, S703C, F747C, Y781D, M787I x 2, L838V, and V903L in CAIS patients and P378R, I680N, M745L, and V889L in PAIS.

Six of the novel single amino acid changes in CAIS index patients were present in the *AR* DNA-binding domain encoded by exons 2 and 3: G568E, A573P, C579W, F583L, K590E (this combined with IVS2 –2 A>C), and C619R. The complete phenotypes in these patients, with other affected family members in five (except G568E), raised the hypothesis that all these amino acid changes may produce complete loss of DNA-binding activity. GSF study in C579W showed diminished DHT binding capacity, diminished 5α-reductase, and increased 17β-hydroxysteroid dehydrogenase activities.

Finally, the mutation I680N was present in an extended family with several PAIS patients (index patient, P3). GSF in one patient showed diminished DHT affinity. In addition, the allele presented, in all affected and carrier members, a short polyglycine exon 1 repeat: $(GGC)_4$ repeats following the initial six polyglycine codon $(GGT)_3(GGG)(GGT)_2$ tract, which was constant in all patients of the present series. $(GGN)_n$ exon 1 repeat length has been shown to modulate *AR* transactivation and translation activity with the shortest $(GGC)_4$ showing the lowest activity (81, 82); this could, thus, increase the pathogenic effect of the I680N mutation.

In summary, the present study conducted in a series of 133 index patients with 46,XY DSD, in whom *AR* was the first candidate gene, showed *AR* to be abnormal in 44.4%, with a clearly higher frequency in the complete phenotype (78%). The 57 different *AR* mutations spread along the whole coding sequence, including exon 1 (21%). This series shows that 60% of mutations detected during the period 2002–2009 were novel. Functional studies are required to confirm the mechanism and the pathogenicity of novel mutations, mainly those in the partial phenotypes. Variation in exon 1 $(GGC)_n$ tract length may influence some *AR* mutation pathogenicities and also partly explain phenotypic differences among different families bearing the same mutation.

Acknowledgments

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