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Androgen Insensitivity Syndrome: Clinical Phenotype and Molecular Analysis in a Single Tertiary Center Cohort

Touzon MS et al. Molecular studies in Androgen Insensitivity Syndrome

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What is already known on this topic?

Androgen insensitivity syndromes the most frequent monogenic known cause of 46,XY DSD. Mutations of variable severity in androgen receptor gene are associated with a wide phenotypic spectrum, ranging from complete androgen insensitivity syndrome to a partial form or a mild form.

What this study adds?

Characterization of the clinical phenotype, long term follow up, in particular gender identity and the contribution of the *AR* gene to the molecular cause of 46,XY DSD in a single tertiary pediatric center of Buenos Aires, Argentina. Nine novel *AR* mutations are described.

Abstract

Objective: The aim of this study was the molecular characterization of the *AR* gene as the cause of 46,XY DSD in our population.

Methods: We studied 41 non related 46,XY DSD index cases with characteristics consistent with AIS. Genomic DNA was isolated from peripheral blood leukocytes of all patients and 25 family members from 17 non-related families.

Results: The *AR* gene analysis revealed an abnormal sequence in 58.5% of total index patients. All of the CAIS cases were genetically confirmed, while in PAIS only in 13 (43.3%) patients a mutation in *AR* was detected. Molecular studies revealed other affected/carrier relatives in 87% of the index cases. The *AR* mutations were found spread along the whole coding sequence, with a higher prevalence in LBD. Nine (39%) out of 23 *AR* mutations were novel. In 17% of *AR*-mutated gene patients, somatic mosaicism was detected in DNA derived from blood leukocytes. In our cohort long-term follow up gender dysphoria raised as male or female was not found. Finally, in suspected PAIS, the identification of *AR* mutation was clearly less than in CAIS patients.

Conclusion: The improvement of the knowledge of the components of the *AR* complex and signaling network might contribute to the long term outcome and genetic counseling in AIS patients.

Key words: 46,XY disorders of sex development; androgen insensitivity syndrome; Androgen Receptor gene mutations; mosaicism; clinical phenotype

INTRODUCTION

The endogenous androgens, testosterone (T) and dihydrotestosterone (DHT) exert their effects via a single intracellular receptor protein, the androgen receptor (AR) (1). 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 (2). The *AR* gene is located on the X-chromosome at Xq11–12 region and encodes a protein with a molecular mass of approximately 110 kDa. The gene consists of eight coding exons (I to VIII) (3). The AR is a transcription factor that belongs to the nuclear receptor subfamily 3, group C, member 4. The protein consists of 920 amino acids that, like other nuclear receptors, is composed of an N-terminal domain (NTD, located on exon 1), a DNA-binding domain (DBD, located on exons 2 and 3) containing two zinc fingers, a hinge region connecting the ligand-binding domain (LBD) to the DBD and a C-terminal LBD, located on exons 4–8) (4).

Androgen insensitivity syndrome (AIS; OMIM 300068) is the most frequent monogenic known cause of 46,XY disorders of sex development (DSD), an X-linked recessive condition. Mutations of variable severity in androgen receptor gene are associated with a wide phenotypic spectrum, ranging from complete androgen insensitivity syndrome (CAIS) to a partial form (PAIS) or a mild form (MAIS). Patients who present CAIS exhibit female external genitalia, testes located in the inguinal or abdominal area, and complete breast development with

sparse to absent axillary and pubic hair. Patients with PAIS present a predominantly male phenotype with hypospadias or a predominantly female phenotype with clitoromegaly and/or posterior labial fusion, ambiguous genitalia, and variable degrees of gynecomastia at puberty. Patients with MAIS present normal external male genitalia associated with infertility.(5) The aim of this study was to characterize the clinical phenotype and the contribution of the *AR* gene to the molecular cause of 46,XY DSD in our population.

SUBJECTS AND METHODS

Clinical Cases

We studied 41 non related 46,XY DSD patients, with clinical and hormonal characteristics consistent with androgen insensitivity syndrome. Eleven patients were suspected with CAIS and 30 with PAIS. **The patients included presented with female or ambiguous external genitalia, adequate testosterone production without evidence of steroidogenic blockade and no Müllerian structures by abdominal ultrasound. Patients with hormonal determinations previous to gonadal biopsy or gonadectomy presented no biochemical evidence of gonadal dysgenesis: normal male FSH levels. In this individuals, the *AR* gene was the first candidate to molecular analysis.** Informed consent for the genetic study was obtained from all adult patients or patient's parents or tutors. This study was approved by the Independent Ethics Committee "Prof. Dr J.P.Garrahan Pediatric Hospital" (reference number 971). Consent was obtained from each patient after full explanation of the purpose and nature of all procedures used.

Hormonal assays

Serum LH and FSH were measured by MEIA AxSYM (Abbott); testosterone by chemiluminescence assays (Immulite; Siemens), and AMH by ELISA (Diagnostic Systems).

***AR* gene mutation analysis**

Genomic DNA was isolated from peripheral blood leukocytes of all patients (41 index cases) and 25 family members from 17 families according to standard procedures. There were seven families in which family members were not available for molecular studies. The entire coding region (exons 1-8) and splice sites in flanking intronic regions of *AR* gene were PCR amplified and automated sequenced(6).

After PCR, the products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and showed a single band with expected size. The PCR products were purified (Qia Quick PCR purification kit, Qiagen, Buenos Aires, Argentina) and sequenced using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Buenos Aires, Argentina) on an ABI PRISM 3130 Genetic Analyzer capillary DNA Sequencer (Applied Biosystems,

Buenos Aires, Argentina). The primers used for sequencing were the same as those used for PCR. Previously reported intronic mutations were also analysed (Human Gene Mutation Database (HGMD), www.hgmd.cf.ac.uk/). The nucleotide sequences obtained were compared with those from Genebank accession number: NG_009014.2. Nucleotide changes were reconfirmed in each DNA by antisense sequence and resequencing after a new PCR product from original DNA.

***In Silico* Protein Analysis**

Nonsense and frameshift mutations which implicate a premature stop codon and a truncated protein were considered deleterious.

The sequence homology-based tool SIFT (Sorting Intolerant from Tolerant; <http://sift.jcvi.org/>), version 2.0.6, the structure-based tool PolyPhen-2 (Polymorphism Phenotyping v2; <http://genetics.bwh.harvard.edu/pph2/>) and Mutation Taster (<http://www.mutationtaster.org/>) were used to predict the pathogenicity of the missense variants previously not described using default settings. To evaluate the implication of a novel synonymous mutation, we used The Berkeley Drosophila Genome Project (BDGP) (<http://www.fruitfly.org/>) as a splice site prediction program.

The SIFT algorithm predicts the functional importance of the substitutions based on the alignment of orthologous and/or paralogous protein sequences. The PolyPhen-2 algorithm predicts the functional effects of amino acid changes by considering conservation, physicochemical differences and the proximity of the substitution to the predicted functional domains. Unlike SIFT or PolyPhen which handle only single amino acid substitutions, MutationTaster works on DNA level and allows insertions and deletions up to 12 base pairs.

The original sequence of the protein was obtained from the Ensembl and Uniprot/Swiss-Prot databases.

Statistical analysis

This study describes the genotype and clinical phenotype of patients with AIS. A statistical analysis was not necessary.

RESULTS

In our study the *AR* gene analysis revealed an abnormal sequence in 24 individuals (58.5% of total index patients). All of the CAIS cases (n=11) were genetically confirmed, while in PAIS (n=30) only in 13 patients a mutation in *AR* was detected.

Family studies were performed in 25 family members from 17 families. The molecular studies and affected family members are shown in Table 1. Molecular studies revealed other affected/carrier relatives in 87% of the index cases.

Denovo AR mutations in 3 (P3, P5 and P6) out of 13 mothers analyzed were found. In two non-related index cases (P12 A and P15), two 46,XY affected siblings raised as female were detected. Interestingly, even though in P12A PAIS was established, normal external female genitalia, in the affected sister, was observed (P12 B). As it is shown in Table 1, 23 *AR* mutations were detected. The

AR mutations were found spread along the whole coding sequence, with a higher prevalence in LBD: 8.3% were located in NTD; 16.6% in the DBD; 70.8% in the LBD and 4.3% were gross deletions(7).

Nine(39%) out of 23(P1,P6,P8,P13,P15, P16, P22, P23 and P24) AR mutations were novel. Two novel mutations were located in the NTD domain (P1 and P16). They were both out of frame deletions that ultimately created a nonsense stop codon and premature truncation of the protein. The others, located in the LBD, were as follows: four missense mutations, a nonsense mutation as well as a 2bp deletion and a duplication of 7bp that produce a frameshift with a premature stop codon. Three patients (P6, P8 and P16) harboured somatic mosaicism: a nonsense mutation, a 7bp duplication and a 20bp deletion which result in a truncating frameshift mutation. One missense mutation was located in the DBD. All novel mutations were predicted to be pernicious by *all in silico* tools. In four individuals (P5, P6, P8 and P16), 17% of AR-mutated gene patients, somatic mosaicism of mutant and wild type alleles was detected in DNA derived from blood leukocytes.

Of the 17 individuals without a defect in the AR, 2 patients were finally diagnosed (and genetically confirmed) with 5-alpha reductase deficiency. In the others, diagnosis remains unknown.

The clinical phenotype and follow-up of the genetically confirmed patients is shown in supplemental data Table 1. Interestingly, during follow-up, no gender dysphoria including those PAIS patients assigned male or female was observed. Unfortunately, in toddler patients, gender identity could not be evaluated.

According to previous reports, very low frequency of gonadal tumors was found – only in P17 a Sertoli cell tumor was detected(8).

DISCUSSION

In the present study, we describe a series of unrelated patients affected by different degrees of AIS. Androgen receptor gene mutations are the main cause of 46,XY DSD. To date, the AR gene mutations database (<http://www.mcgill.ca/androgendb/>) has reported more than 800 different AR mutations from patients with androgen insensitivity syndrome.

In all CAIS cases, AR mutations responsible for the phenotype were identified. However, similarly to other cohorts, in PAIS phenotype cases, AR mutations were identified in 38% only. On this line, in a series of 41 index patients AR gene showed to be abnormal in 58.5%, confirming the diagnosis. Similarly, Boehmer *et al*(9) and Audi *et al*(2) report a frequency of detection of 44-65% approximately. On the contrary, de Silva *et al* (10) and Akcay *et al* (11) describe cohorts with 15-18% of genetically confirmed AIS. In these studies, the significantly lower percentage of AR mutation detection could be due to the presence of overlaps in clinical presentation of the patients, such as 5- α reductase deficiency or the fact that patients with a testosterone biosynthetic defect were also included. Therefore, it has been proposed that even though AR is essential for virilization, other components of the AR complex and signaling

network are required for complete masculinization. It has been suggested in non-detected cases that the androgen resistance might be secondary to mutations in the 5'UTR, or other regulatory regions; moreover, necessary AR cofactor(s) should also be taken into consideration. In this respect, several cofactors, such as coactivators SRC1(**Steroid receptor coactivator 1**), TIF2(**Transcriptional mediators/intermediary factor 2**), SRC3(**Steroid receptor coactivator 3**) and corepressors NRIP1(**Nuclear receptor-interacting protein 1**), NR0B1 (**nuclear receptor subfamily 0 group B member 1**), are actively involved in the regulation of AR-mediated transcription, and might play an important role in AIS etiopathogenesis(**12-15**). Interestingly, in order to confirm androgen resistance, Horning *et al* study developed a DHT-dependent transcriptional induction of the androgen-regulated *APOD*(**Apolipoprotein D**) gene in cultured genital fibroblasts (APOD-assay). However, the usefulness of this APOD assay in a large cohort should be addressed(**16**).

Mutations in the *AR* are distributed throughout the gene with a preponderance (70.8%) located in the LBD(**17**). In our cohort nine novel *AR* mutations were found, expanding the mutational spectrum of 46, XY DSD. In 3 novel mutations, located in the LBD, a truncated significantly reduced or inactive protein was predicted due to a premature stop codon secondary to gene deletion (P15), gene duplication (P8) or nonsense mutation (P6). The p.Phe726Cys missense mutation located in the LBD was also detected. On this line, Quigley *et al* study demonstrated by functional assays that a missense mutation in the same position (p.Phe726Leu), caused the disruption of N/C terminal interaction of the mutated protein. Hence it could be suggested that the novel missense mutation found in our cohort might also affect the transactivation activity of the AR impairing the binding of the ligand to its LBD(**18**). The remaining novel mutations, two gene deletions (P1 and P16) located in the NTD domain, result in a truncated protein due to a premature stop codon.

According to other reports, lack of correlation between genotype and clinical phenotype was found(**19**). Interestingly, in siblings of family 12, harbouring p.Asp691del mutation, a clinical variability was evident. In one a CAIS while in the other a PAIS phenotype was observed. Petroliet *al* study showed in N/C terminal interaction assays, different profiles of the mutant AR protein in response to DHT stimulation, explaining the phenotypic diversity observed in PAIS cases(**20**).

Somatic mosaicism has been reported. Interestingly, even though the patients carried severe *AR* mutations, PAIS clinical phenotype was reported. In these affected patients the *de novo* mutation occurred after the zygote stage and probably very early during the first cell divisions. Thus, different proportions of cells containing mutant or wild-type protein are present in various tissues of the same individual explaining the mild phenotype. Similarly, in four patients of our cohort (P5, P6, P8 and P16) a severe mutation was detected presenting a PAIS phenotype. It is noteworthy that detection of somatic mosaicism in *AR* has a

great impact for patients with AIS because further virilization is possible after birth and is an important consideration for genetic counseling(21).

No gender dysphoria was observed in our cohort, even though no systematic assessment was available in all cases.

In contrast to previous reports, in this cohort AMH serum levels during the neonatal period were within the normal male reference range in the only 2 PAIS cases in whom it was assessed (22,23). AMH gene expression in Sertoli cells is inhibited via AR receptor pathway (24). The lack of AR expression in Sertoli cells during minipuberty, could explain our findings, suggesting that other unknown factors might be involved in the regulation of AMH synthesis (25).

In agreement with previous reports, normal gonadotropin levels were the most frequent finding (26).

Study limitations

Even though all *in silico* tools predicted the *novel* mutations to be damaging for protein structure and function, functional assays should be performed to confirm pathogenicity.

CONCLUSION

In summary, in this study we report a series of 41 46,XY DSD index patients in whom *AR* was the candidate gene. The molecular diagnosis is useful for genetic counseling of the families. However, similar to other series the percentage of *AR* mutations found is almost 60% of the suspected cases.

Emerging technological advances might contribute to increase in the accuracy of the etiology in the suspected AIS cases.

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Authorship contributions

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Supplemental table 1. Clinical Phenotype at diagnosis and long term follow-up

Patient	CA at diagnosis (years)	Clinical form	Phenotype	Gonadal position	EMS at diagnosis (PAIS)	Social sex	Fe pu dy
1	4	CAIS	Female genitalia, inguinal hernia	inguinal		F	Fe
2	5.7	CAIS	Female genitalia, inguinal hernia	inguinal		F	Lo
3		CAIS	Female genitalia, inguinal hernia	inguinal		F	
4	6.7	CAIS	Female genitalia, inguinal hernia	inguinal		F	Fe
5	1.7	PAIS	Penoscrotal hypospadias	scrotal	9	M	Pu gy ide
6	0.5 months	PAIS	micropenis, penoscrotal hypospadias	scrotal	6	M	Sp on de Ge
7	0.3	PAIS	micropenis,	inguinal /	5.5	F	Fe

	months		penoscrotalhypospadias	scrotal				Sp
8	0.8 months	PAIS	micropenis, penoscrotalhypospadias	scrotal	6	M	Lo	Fe
9	9.8	PAIS	Femalegenitalia, clitoris hypertrophy	NA	NA	F	hy	re
10	4 months	CAIS	Femalegenitalia, inguinal hernia	inguinal		F	Fe	re
11	9.8	CAIS	Femalegenitalia, inguinal hernia	inguinal		F	Fe	W
12	1.5 months	PAIS	Penoscrotalhypospadias	scrotal	9	M	sy	Pr
13	2 months	CAIS	Femalegenitalia, inguinal hernia	inguinal		F	de	ye
14	7.3	PAIS	female genitalia, complete fusion of the labia majora	inguinal	5	F	ma	Fe
15	1.8	PAIS	Femalegenitalia, posterior fusion of the labia majora, inguinal hernia	inguinal	2	F	Fe	Ob
16	14.6	CAIS	Femalegenitalia	abdominal		F	Sp	Pu

17	7	CAIS	Female genitalia, inguinal hernia	inguinal		F
18	1.3	PAIS	micropenis, penoscrotal hypospadias	scrotal	6	M
19	17	CAIS	Female genitalia, inguinal hernia	inguinal		F
20	0.7 months	PAIS	Penoscrotal hypospadias	scrotal	9	M
21	7 months	PAIS	micropenis, penoscrotal hypospadias	scrotal	6	M
22	0.5 months	PAIS	micropenis, penoscrotal hypospadias, inguinal hernia	inguinal	5	M
23	2 months	PAIS	micropenis, penoscrotal hypospadias	scrotal	6	M
24	0.3 months	PAIS	Penoscrotal hypospadias, unilateral cryptorchidism	inguinal / scrotal	8.5	M

CA: chronological age; EMS(39): External masculinization score; CAIS: Complete androgen insensitivity syndrome; PAIS: Partial androgen insensitivity syndrome; F: female; M: male; NA: not available; BMT: Bone marrow transplant. **T: testosterone.**

Male reference Gonadotropin levels (MU/ml): 0-3 months: FSH 2.43 ± 1.67 , LH 2.52 ± 1.74 ; 3-12 months: FSH 1.35 ± 0.81 , LH 1.21 ± 1.65 ; 12-24 months: FSH 0.90 ± 0.59 , LH 0.15 ± 0.17 ; >24 months: FSH 1.10 ± 0.82 , LH 0.13 ± 0.32 ; 9-12 years: FSH (MU/ml) 2.26 ± 0.96 , LH 0.78 ± 0.99 .

Male reference testosterone levels (ng/ml): 1-5 months < 0.05 – 1.77; 6-11 months \leq 0.07; 1-5 years \leq 0.25; 6-9 years \leq 0.30; 10-11 years 0.05 – 0.50; 12-14 years 0.10 – 5.72; 15-17 years 2.20 – 8.00.

Male reference AMH levels (pmol/l): 0-14 days: 250-1000; 15 days-3 years 400-2400; >3 years prepubertal Tanner 1 300-1400; >3 years pubertal Tanner 2 70-1000; >3 years pubertal Tanner 3 30-400; >3 years pubertal Tanner 4/5 30-180.

Uncorrected proof