CLINICAL STUDY

DAX1 and X-linked adrenal hypoplasia congenita: clinical and molecular analysis in five patients

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Abstract

Objective: Mutations in the gene coding for the orphan nuclear receptor DAX1 cause X-linked adrenal hypoplasia congenita (AHC). Affected boys usually present with primary adrenal failure in early infancy or childhood. Impaired sexual development due to hypogonadotropic hypogonadism becomes manifest at the time of puberty. Moreover, evidence from *Dax1* knockout mice and a limited number of patients with AHC, suggests that mutations in *DAX1* may directly cause abnormalities in spermatogenesis. The aim of this study was to characterize clinically and genetically five patients with AHC. **Design:** DNA sequencing analysis, endocrine testing, testicular ultrasound and semen analysis with 1-year follow-up after gonadotropin treatment.

Methods: We report on five men with classic AHC manifestations. Genomic DNA was extracted from patients' peripheral blood leukocytes and the coding region, splice sites, and promoter (–240 bp) region of *DAX1* were directly sequenced.

Results: Three known and two novel mutations were detected in the *DAX1* coding sequence in these patients. Semen analysis was performed in four of the five patients and showed azoospermia. Twelve-month treatment with gonadotropins did not restore fertility in these patients. All patients showed a normal testicular Doppler ultrasound, in contrast with that observed in *Dax1*-deficient mice, which display abnormalities in the rete testis.

Conclusions: These cases further expand the number of *DAX1* mutations reported in the literature, as well as our clinical knowledge of this rare disease.

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Introduction

The orphan nuclear receptor DAX1 plays a crucial role in the development and function of the adrenal gland and the hypothalamic–pituitary–gonadal axis (1–4). Mutations in the gene encoding DAX1 (*DAX1*, OMIM: 300473) cause X-linked adrenal hypoplasia congenita (AHC, OMIM: 300200) (1, 2). AHC is an inherited disorder of adrenal gland development, characterized by lack of the permanent zones of the adrenal cortex. Boys with this condition usually present with severe primary adrenal failure in infancy or childhood. Hypogonadotropic hypogonadism (HH) becomes apparent at puberty and infertility results from gonadotropin deficiency in combination with a primary defect in spermatogenesis (5, 6). Over 80 different mutations in *DAX1* have been described, most of which are nonsense

or frameshift mutations causing premature truncation of the protein (7–9).

During the last years, other variant phenotypes including an adult-onset form of AHC (10–12), isolated HH in a female homozygous for a *DAX1* mutation and extreme pubertal delay in heterozygous female carriers have been described (6, 13). Moreover, evidence from both *Dax1* knockout mice (14) and a limited number of patients with AHC (6, 10–12), suggests that mutations in *DAX1* may directly cause abnormalities in spermatogenesis. In particular, either Sertoli cell (15) or Leydig cell (16) 'rescue' of *Dax1* expression showed that these rescued animals have restored fertility, although abnormalities in testicular architecture persist. The way in which DAX1 affects fertility seems to be, at least in part, mediated by blockage of the rete testis, with subsequent dilatation of the proximal efferent ductules (17). Dilatation or ectasia of the rete

testis, that is the anastomosing spaces where the tubuli recti enter the mediastinum testis, is a rare and benign condition that may be visualized on scrotal sonography. Interestingly, among all patients described to date in the literature, only one has been reported to show abnormalities of the rete testis, and in particular ectasia of the rete testis at testicular ultrasound (12), although most of these patients are not usually investigated for such an alteration.

Here, we report clinical and molecular analysis of five young males with classic AHC manifestations and HH due to *DAX1* mutations, two of which have not previously been reported in the literature. This study further expands the present knowledge about the disease and reports two novel alterations in this gene.

Materials and methods

DNA sequencing and mutational analysis

After obtaining written consent, genomic DNA was extracted from peripheral blood leukocytes using standard procedures. Both exons of *DAX1* (GenBank accession no. NM_000475) were amplified by PCR using specific oligonucleotide primer pairs and conditions described previously (11, 12). Direct sequencing of PCR products was performed using a Taq big dye terminator sequencing kit and an ABI310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA).

Informed consent was obtained in all cases, after project approval by the local Ethics Committee.

Testicular ultrasound

Ultrasound of the scrotum has been reported to be highly sensitive in the detection of rete testis blockage (18) and it consisted of imaging the testes, epididymides, paratesticular structures and spermatic cords in a systematic fashion. The examination was performed with the patient supine and the scrotum elevated by a sheet wrapped around the patient's pelvis. A 7 to 15 MHz. transducer was used. Transverse and longitudinal images were obtained, and color and spectral Doppler ultrasound was performed.

Results

Patients

The five patients presented either at neonatal age (patient 2) or childhood (patients 1, 3, 4 and 5) with a history of fatigue, nausea, dehydration and weight loss. Patient 4 has been extensively described in a previous report (19). Clinical laboratory investigations revealed hypocortisolemia, hypoaldosteronism and elevated adrenocorticotropin (ACTH) in all boys, consistent with primary adrenal failure. All patients later presented with delayed puberty, with low testosterone

levels in the presence of low/normal follicle-stimulating hormone and luteinizing hormone (LH) levels, and both were unresponsive to gonadotropin releasing hormone (GnRH) stimulation test. All of them, with the exception of patient 2 who is still growing, reached a normal final height, consistent with their target height (Table 1). Androstenedione, progesterone and 17-hydroxyprogesterone were below the normal range. An abdominal computed tomography scan revealed small adrenal glands. Anti-adrenocortical antibodies were negative, and very-long-chain fatty acids were normal, thus excluding autoimmune Addison's disease and adrenoleukodystrophy respectively. Moreover, all patients underwent a testicular Doppler ultrasound. The typical appearance of the rete testis is an echogenic structure communicating from the mediastinum testis to the epididymal head. The rete is usually located at the posterolateral aspect of the testis. Occasionally, the rete may be anechoic with numerous tiny round or tubular structures, indicating tubular ectasia of the rete testis. Ultrasound of the five patients included in the study did not detect any abnormality of the rete testis.

Treatment with cortisone acetate and/or hydrocortisone and testosterone was started in all patients. Semen analysis was performed in four patients (1, 3, 4 and 5) and showed azoospermia. In these four patients, testosterone replacement was withdrawn and treatment with exogenous gonadotropins was started and maintained for at least one year (human chorionic gonadotropin 2000 IU and urofollitropin 75 IU, three times weekly). In all patients, this treatment failed to improve the sperm count and restore fertility. Consequently, intramuscular testosterone enanthate (250 mg every 4 weeks) replacement was restored. Of note, while 1-year treatment induced normalization of testosterone levels in patient 4, patients 1, 3 and 5 did not respond either in terms of hormonal testing, or in terms of testicular volume.

No patient had a family history of affected males. Clinical and endocrine details are given in Table 1.

Mutational analysis

Direct DNA sequencing analysis of *DAX1* in patients 1, 2 and 4 revealed three frameshift alterations, of which two are deletions, the first novel to the literature, and one an insertion (1011delC, codon 337; 543delA, codon 181; 259insAGCG, codon 86) (19, 20). All lead to premature stop codons at positions 371 (1011delC) and 263 (543delA and 259insAGCG), thus resulting in non-functional truncated proteins. Patient 3 revealed a missense mutation in the C-terminal region of *DAX1* (K382N), which has already been reported and shown to result in a protein devoid of repressor activity (21). In patient 5, we detected a C-terminal missense mutation (W291S), novel to the literature. Nevertheless, this codon, which is highly

Table 1 Clinical, biochemical and molecular data of AHC patients included in the study. The endocrine data refer to the first evaluation in an adult endocrine unit, after replacement therapies were withdrawn.

Patient no.	Age at diagnosis (years)	Present age (years)	Height/target (cm)	Cortisol (nmol/l) (nv: 140–700)	ACTH (ng/l) (nv: 10–65)	Testosterone (nmol/l) (nv: 13–42)	Testes vol. of the rete testis (ml)	Blockage	Treatment with Gn			
									Sperm count	↑ Testosterone	DAX1 Mutation	
1	13	19	176/176	49	1900	3.1	2	No	No response	No response	1011delC	
2	<0.1	18	167*/174	<10	1063	3.0	6	No	No response	No response	543delA	
3	1	34	174/177	<10	790	0.7	3	No	No response**	No response**	K382N	
4	3	39	178/175	<10	24980	0.6	3	No	No response	No response	259insAGCG	
5	5	39	178/176	<10	970	0.7	5	No	No response**	No response**	W291S	

nv, normal values; NP, not performed; Gn, gonadotropins.

*Patient #2 is still growing. **These patients were treated both with gonadotropins and, subsequently, with GnRH.

conserved among other related orphan nuclear receptor superfamily members, has already been demonstrated to be subject to substitution (W291C), resulting in a non-functional protein (21). A schematic representation of the human *DAX1* gene is shown in Fig. 1.

Four of the five mothers of the affected boys were screened and shown to be heterozygous for the same mutations. At the time of the screening, patient 3's mother had already died. No other family members were available for the study.

Discussion

The association of mutations in the orphan nuclear receptor *DAX1* with X-linked AHC and hypogonadotropic hypogonadism (HH) is well established. Affected boys often present with salt-wasting primary adrenal insufficiency in early infancy (5, 22, 23). Children who do not present symptoms early in life may undergo a period of relatively good health and tend to present with more insidious signs and symptoms of the disease throughout childhood or, rarely, in adulthood. At puberty, boys also display delayed sexual development due to a combined hypothalamic and pituitary form of HH (5). The patients described here all presented with the classical, early-onset clinical and endocrine features (Table 1).

Direct DNA sequencing analysis revealed two novel and three known mutations in *DAX1* (two deletions, one insertion and two missense mutations in the C-terminus) (19–21). Most mutations of *DAX1* are nonsense or frameshift mutations that cause premature truncation of the protein, and it has been demonstrated that deletion of as few as the last nine amino acids of *DAX1*, which constitute the putative activation function-2 domain, is associated with severe clinical phenotype (24). Therefore, the two deletions and the insertion described here are predicted to result in non-functional truncated proteins. On the other hand, the K382N missense mutation has already been reported and results in a protein devoid of repressor activity (21). In patient 5, we detected a missense mutation (W291S) novel to the literature. Nevertheless, this codon, which is highly conserved among other related orphan nuclear receptor superfamily

Figure 1 Schematic representation of the human *DAX1* gene. The amino-terminal repeat motif is shown. The positions of the 5 mutations described in this report are also shown. LBD, ligand binding domain; DBD, DNA binding domain.

members, has already been demonstrated to be subject to substitution (W291C), resulting in a non-functional protein (21). Our report of these two missense mutations further strengthens the hypothesis of a potential clustering of *DAX1* missense mutants in the putative ligand binding domain.

Evidence from overexpression of *DAX1/Dax1* in humans (25) and mice (26) has shown that this nuclear receptor plays a key dosage-sensitive role in gonadal development. Furthermore, targeted deletion of *Dax1* in the mouse causes impaired spermatogenesis and infertility (14). *Dax1* knockout mice have quite marked abnormalities of testicular structure, with dilated seminiferous tubules, blockage of the rete testis and of proximal/middle efferent tubules due to aberrantly located Sertoli cells, and ectopic and hyperplastic Leydig cells (17). Both Sertoli and Leydig cell 'rescue' of *Dax1* expression have been performed by crossing *Dax1* knockout mice with transgenic lines expressing *Dax1* from an anti-Müllerian hormone and an LH receptor promoter respectively (15, 16). These rescued animals have restored fertility, but the abnormalities in testicular architecture persist.

Limited data are available about the role of *DAX1* in human fertility. Azoospermia has been reported in several patients with classic X-linked AHC, and attempts to induce fertility using gonadotropins have been unsuccessful to date in a limited number of patients (6, 10–12, 27). Given the *Dax1* knockout mice phenotype, it could be expected that blockage of the rete testis should be a hallmark in AHC patients. Interestingly, of all patients described to date in the literature, only one has been reported to show blockage of the rete testis at testicular ultrasound (12), although most of them were not evaluated for such an alteration. Our report of a normal testicular Doppler ultrasound in all five patients described does not seem to support this hypothesis. On the contrary, the lack of effect on spermatogenesis of one-year treatment with gonadotropins in four of our patients provides further evidence that *DAX1* directly affects spermatogenesis in man and that these patients are relatively resistant to gonadotropin treatment. The observed increase in circulating testosterone values during treatment indicates that Leydig cell function can be, at least partially, rescued in some of these patients, suggesting that Leydig cell defects are mainly dependent on gonadotropin deficiency, at least in humans. Therefore, gonadotropin treatment may be able to rescue, partially, the Leydig cell function but not the exocrine function.

The lack of blockage of the rete testis in our *DAX1*-deficient men seems to indicate that human spermatogenesis also does not depend on factors connected with structural disorganization of the gonads. A testicular biopsy could provide additional information to reveal the mechanism underlying spermatogenic failure in these patients. To date, the only data on testicular histology in *DAX1*-deficient men have been reported

by Brown and colleagues (27) who described normal testicular architecture in an infant who died of AHC. Accordingly, the 'rescue' experiments in *DAX1* knockout mice seem to exclude the possibility that abnormalities of the testicular structure are mainly responsible for their infertility. Unfortunately, this hypothesis could not be confirmed by our study since none of our patients underwent a testicular biopsy, as there was no existing clinical and ethically correct indication for this invasive procedure. A testicular specimen could be obtained perhaps in the future if the patients opt for intracytoplasmic sperm injection, but, at the moment, none of the patients included in this study is seeking paternity.

This report further expands the number of *DAX1* mutations reported in the literature, and adds to our clinical knowledge of this rare disease. Further studies will seek to clarify the real impact of *DAX1* mutations on human spermatogenesis, in order to achieve the appropriate management of fertility in these patients.

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