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Mutational Analysis of *DAX1* in Patients with Hypogonadotropic Hypogonadism or Pubertal Delay*

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ABSTRACT

Although delayed puberty is relatively common and often familial, its molecular and pathophysiologic basis is poorly understood. In contrast, the molecular mechanisms underlying some forms of hypogonadotropic hypogonadism (HH) are clearer, following the description of mutations in the genes *KAL*, *GNRHR*, and *PROP1*. Mutations in another gene, *DAX1* (*AHC*), cause X-linked adrenal hypoplasia congenita and HH. Affected boys usually present with primary adrenal failure in infancy or childhood and HH at the expected time of puberty.

DAX1 mutations have also been reported to occur with a wider spectrum of clinical presentations. These cases include female carriers of *DAX1* mutations with marked pubertal delay and a male with incomplete HH and mild adrenal insufficiency in adulthood. Given this emerging phenotypic spectrum of clinical presentation in men and women with *DAX1* mutations, we hypothesized that *DAX1* might be a candidate gene for mutation in patients with idiopathic sporadic or familial HH or constitutional delay of puberty. Direct sequencing of *DAX1* was performed in 106 patients, including 85 (80 men and 5 women) with sporadic HH or constitutional delay of puberty and patients from 21 kindreds with familial forms of these disorders. No *DAX1* mutations were found in these groups of patients, although silent single nucleotide polymorphisms were identified (T114C, G498A). This study suggests that mutations in *DAX1* are unlikely to be a common cause of HH or pubertal delay in the absence of a concomitant history of adrenal insufficiency. (*J Clin Endocrinol Metab* **84:** 4497–4500, 1999)

THE ASSOCIATION of *DAX1* (*AHC*) gene mutations with X-linked adrenal hypoplasia congenita (AHC) and hypogonadotropic hypogonadism (HH) is well established (OMIM: 300200) (1, 2). More than 50 different mutations in the gene encoding DAX-1 have been reported in this condition (3–6). Affected boys typically present with primary adrenal insufficiency in infancy or childhood. HH usually becomes evident later in life with failure of pubertal development (7, 8).

DAX-1 is an orphan nuclear hormone receptor that is expressed in the adrenal gland, gonads, hypothalamus, and pituitary gonadotropes (9). The HH caused by *DAX1* mutations seems to involve deficits at both hypothalamic and pituitary levels (10–13). DAX-1 is also expressed in Sertoli cells (14), and male *Ahch* (*Dax1*) knockout mice have disordered spermatogenesis and infertility (15). DAX-1 has a crucial role, therefore, in the development and function of the reproductive axis at multiple levels. Different approaches to counseling and treatment are needed for patients with *DAX1* mutations compared to those with hypothalamic forms of HH, such as Kallmann syndrome (3, 16).

Recently, *DAX1* gene mutations have been found in several men and women who have less typical reproductive phenotypes. These cases include: 1) partial HH in a man who presented later in life with mild adrenal failure (13); 2) HH, but normal adrenal function, in a woman who is homozygous for a *DAX1* mutation through gene conversion (17); and 3) extreme pubertal delay, but normal fertility, among heterozygous female carriers of *DAX1* mutations (12). Given the phenotypic spectrum of reproductive disorders now reported, we hypothesized that *DAX1* mutations might cause idiopathic familial or sporadic HH or constitutional delay of puberty (CD) among patients lacking a history of overt adrenal failure. DNA sequence analysis of over 100 such patients suggests, however, that coding sequence mutations in *DAX1* are unlikely to be a common cause of such conditions.

Subjects and Methods

Subjects

DAX1 was sequenced directly in 106 patients who had sporadic (nonfamilial) or familial HH (see *Patient Characteristics* for definitions) or

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CD (testicular volume <4 mL and delay of sexual maturation at 14 yr of age). Patients were not recruited if they had multiple pituitary hormone deficiencies or if a likely cause for their altered hypothalamic-pituitary-gonadal (HPG) function was evident (for example, a history of tumors, cranial irradiation, or syndromes associated with HH). Mutations in the genes encoding the GnRH receptor and anosmin-1 (*KAL*) had been excluded in 75% and 80% of the patients, respectively.

PCR and direct sequencing of DAX1

After obtaining Institutional Review Board approval, DNA was extracted from patients' blood leukocytes using standard methods. Both exons of *DAX1*, their splice sites, and a 240-bp 5′ region of the *DAX1* promoter region were PCR amplified using the following six primer pairs: DAX1.1 For: 5′-TGAGACAGGGAAAGGGGTAAT-3′; DAX1.1 Rev: 5′-CCGGGCTCATCGCCGCACGAA-3′; DAX1.2 For: 5′-TGGTGGATCAGTGTTGGGGC-3′; DAX1.2 Rev: 5′-CCGGGATCAGAGCCGCACGAA-3′; DAX1.3 For: 5′-AAGCAAACGTACGCGGCAC-3′; DAX1.3 Rev: 5′-CCTCTGCGCGAAGTAGGAGC-3′; DAX1.4 For: 5′-TAGCTCAAAGCAAACGCACGTG-3′; DAX1.4 Rev: 5′-GACGCCCAGCAGTTGCGCAC-3′; DAX1.5 For: 5′-GCCTCAGCGGGCCTGTTGAAG-3′; DAX1.5 Rev: 5′-CCCGATGCTTTTGTGAGCTGGGAA-3′; DAX2.1 For: 5′-GCTAGCAAAGGACTCTGTGGT-3′; DAX2.1 Rev: 5′-TGTGTGGCCCACATGACTTTA-3′.

PCR conditions were: 1-min predenaturation at 96 C; 35 cycles of 1 min at 94 C, annealing for 1 min at 55–58 C, and extension for 1 min at 72 C; and 15-min elongation at 72 C. Buffer conditions have been described previously (18). Direct sequencing was performed in forward and reverse using dRhodamine (PE Applied Biosystems, Foster City, CA) or Thermo Sequenase II (Amersham Pharmacia Biotech Pharmacia, Piscataway, NJ) dye terminator sequencing kits and automated sequencers (Models 373A and 377; PE Applied Biosystems, Foster City, CA).

Results

Patient Characteristics

Patient characteristics are shown in Fig. 1.

Sporadic HH/CD (n = 85). The majority of patients investigated had sporadic (nonfamilial) reproductive disorders (n = 85) (Fig. 1, *left*). Isolated sporadic HH was present in 83 patients (78 men and 5 women), and CD was present in 2 boys. Of those with HH, seven had an adult-onset form of HH in which normal pubertal development occurs but HH, apulsatile LH secretion, and low testosterone develops in adult life (19). An additional four men have the fertile eunuch syndrome. This condition is diagnosed when testicular development and spermatogenesis occurs, but systemic testosterone concentrations are insufficient for full virilization (20–22). In the cases of CD, pubertal development was particularly delayed, as no spontaneous testicular enlargement or sexual maturation was evident by 15 yr of age.

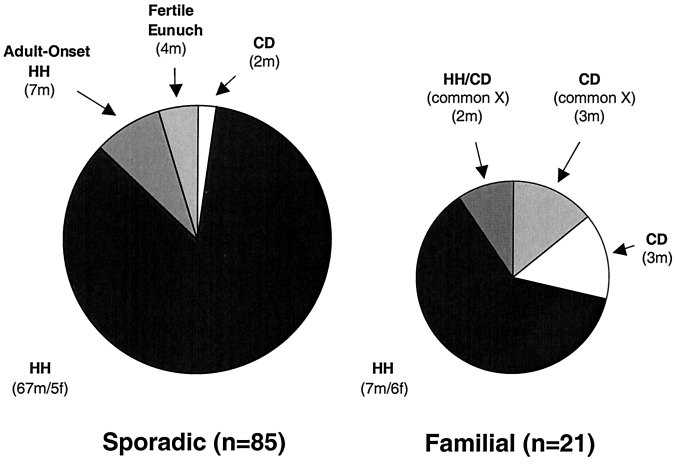

FIG. 1. Patient characteristics. The majority of patients studied (n = 85) had sporadic (nonfamilial) forms of HH or CD (*left*). The remaining patients (n = 21) had familial forms of HH or CD (*right*). A subset of patients who had an X-linked pattern of familial HH or CD were screened first using microsatellite markers in the region of the *DAX1* (*AHC*) locus. Sequencing analysis was only undertaken in those families where two affected brothers inherited a common X-allele from their mother, thus making *DAX1* a potential candidate gene for mutation. (m, male; f, female).

Familial HH/CD (n = 21). DAX1 was sequenced in a total of 21 kindreds with familial forms of HH, CD, or both (Fig. 1, *right*). Families were excluded if the phenotype appeared to be inherited from the proband's father or father's family, as this precluded an X-linked gene as the cause of their condition. A total of 13 of the 21 kindreds had familial HH affecting both male and female family members. Familial CD with a classic X-linked pattern of inheritance was present in three kindreds. An additional nine families had two affected brothers in the same generation. In these cases, polymorphic microsatellite markers in the region of the *DAX1* locus (DXS1202, DXS1214, DXS1226; PE Applied Biosystems) were used first to determine whether both affected sons inherited the same X-chromosome from their mother. Common descent of the same maternal X-allele (*DAX1* locus) to both sons occurred in five of the nine families (two HH/CD, three CD/CD). Because a common allele segregated with the phenotype in these cases, *DAX1* was considered a candidate gene, and direct sequencing was undertaken. In four families, however, affected brothers inherited different maternal X-alleles, making *DAX1* an unlikely candidate gene for the phenotype seen in these cases (four CD/CD). These families were excluded from further analysis.

Mutational Analysis

Direct sequencing of the coding region, splice sites, and promoter (−240 bp) of *DAX1* did not reveal any mutations in the 106 patients studied. Single nucleotide polymorphisms were detected at two sites [T114C and G498A, the A of the ATG translation initiation codon being designated +1 (23)] (Table 1). These nucleotide changes did not alter the amino-acid sequence of DAX-1 (C38C and R166R, respectively) and were detected at a similar frequency in a control population.

Discussion

Pubertal delay is a common clinical problem that is multifactorial in its origin. Environmental and nutritional factors can delay the onset and progression of puberty. A variety of hormonal disorders and acquired structural defects that affect the HPG axis can affect the process of sexual maturation (for reviews, see Refs. 24–27). Based on family histories and twin studies, genetic components also seem to contribute to the timing of puberty (24, 28). The genetic basis of pubertal development is poorly understood at present. However, the coexistence of pubertal delay with variable degrees of HH in some families suggests that a common factor may be responsible for these phenotypes in a subset of patients with pubertal disorders (29).

TABLE 1. Single nucleotide polymorphisms in the coding region of *DAX1*

Nucleotide polymorphism	Codon	Males (n = 95) (%)		Females (n = 11) (%)		
		T	C	TT	TC	CC
T114C	C38C	12 (13)	83 (87)	3 (27)	2 (18)	6 (55)
		G	A	GG	GA	AA
G498A	R166R	69 (73)	26 (27)	8 (73)	1 (9)	2 (18)

Several single gene disorders have now been shown to cause HH in humans (30). Affected patients may show a spectrum of mild to severe phenotypes, even within a kindred with the same mutation. Mutations in these genes may affect the HPG axis at various levels. For example, mutations in *KAL* (anosmin-1) cause the hypothalamic HH observed in patients with X-linked Kallmann syndrome (31, 32), whereas mutations in the gene encoding the GnRH receptor (33, 34) primarily affect gonadotrope function. Mutations in the pituitary transcription factors PROP-1 (35) and HESX-1 (36) can also cause HH, although in these cases additional anterior pituitary hormones are affected. Defining the molecular basis of these reproductive disorders is important because approaches to treatment and counseling are different. At present, however, the underlying pathogenesis of most forms of familial or sporadic HH/CD remains unclear (16, 29, 30, 37).

The association of HH with X-linked adrenal hypoplasia congenita and *DAX1* gene mutations is well established. Although HPG activity may be relatively preserved in infancy (38–40), the majority of affected patients show marked HH at the expected time of puberty. In rare cases, partial pubertal development has been observed (5). In this study, we hypothesized that mutations in *DAX1* might be found in a subset of patients with HH or delayed puberty alone. In addition to its functional characteristics, the location of *DAX1* on the X chromosome makes it an attractive candidate gene for a relatively common disorder because phenotypic effects are likely to be manifest in hemizygous males.

We included patients with sporadic as well as familial disorders in our cohort, as over one third of AHC patients reported to date have no other affected family members and they have *de novo DAX1* mutations (4, 41, 42). The recent report of a man who first presented at 28 yr of age with partial HH, but only mild adrenal failure, demonstrates that a reproductive phenotype may precede adrenal symptoms in certain individuals with *DAX1* mutations (13). In addition, several females with sporadic HH were included in this study following the report of a woman with a homozygous *DAX1* mutation who has HH and normal adrenal function (17). Finally, we included families in which both males and females have HH, as extreme pubertal delay has been reported in some female carriers of *DAX1* mutations (12). Such a phenotype in heterozygous women could result from skewed X-inactivation.

Identifying patients with *DAX1* mutations among those attending clinics for HH is important for a variety of reasons. First, different approaches to treatment might be needed for such patients, given their variable response to GnRH (10–13). Although data are limited at present, spermatogenesis may be affected by *DAX1* mutations in humans as it is in mice (15), and the response to gonadotropin treatment may be impaired (12, 13). Second, these patients may have subclinical adrenal failure that could become clinically significant if left undiagnosed, as highlighted by the patient who presented with increasing symptoms of adrenal insufficiency in his late twenties (13). Third, when the genetic basis for a disorder is identified in a proband, appropriate genetic counseling can be provided to additional family members. In the case of *DAX1* mutations, female carriers of the mutation can be advised regarding testing of male offspring for adrenal insufficiency. Boys with *DAX1* mutations can be given glucocorticoids and mineralocorticoids, as indicated, and hormonal replacement can be provided at the time of puberty. Finally, any mutations found to be associated with a varied reproductive phenotype could provide important insight into the structure and function of the DAX-1 protein. The majority of *DAX1* mutations reported to date are frameshift or nonsense mutations (3, 4). Missense mutations, which might cause relatively subtle alterations in protein function, have been rare among the early, classical cases of AHC and seem to be localized to the putative ligand-binding (carboxy-terminal) domain of DAX-1 (1, 5, 6, 38, 43–45). A direct sequencing approach was used, therefore, to optimize our sensitivity for detecting missense mutations. Although two previously reported polymorphisms were discovered in a significant number of patients (4), no *DAX1* mutations were found in the 106 patients studied. These findings indicate that *DAX1* mutations are unlikely to be a significant cause of HH or pubertal delay in the absence of a personal or family history of adrenal failure.

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