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Hypogonadotropic Hypogonadism in Subjects with *DAX1* Mutations

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Abstract

DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; also known as *NROB1*, nuclear receptor subfamily 0, group B, member 1) encodes a nuclear receptor that is expressed in embryonic stem (ES) cells, steroidogenic tissues (gonads, adrenals), the ventromedial hypothalamus (VMH), and pituitary gonadotropes. Humans with *DAX1* mutations develop an X-linked syndrome referred to as adrenal hypoplasia congenita (AHC). These boys typically present in infancy with adrenal failure but later fail to undergo puberty because of hypogonadotropic hypogonadism (HHG). The adrenal failure reflects a developmental abnormality in the transition of the fetal to adult zone, resulting in glucocorticoid and mineralocorticoid deficiency. The etiology of HHG involves a combined and variable deficiency of hypothalamic GnRH secretion and/or pituitary responsiveness to GnRH resulting in low LH, FSH and testosterone. Treatment with exogenous gonadotropins generally does not induce spermatogenesis. Animal models indicate that DAX1 also plays a critical role in testis development and function. As a nuclear receptor, DAX1 has been shown to function as a transcriptional repressor, particularly of pathways regulated by other nuclear receptors, such as steroidogenic factor 1 (SF1). In addition to reproductive tissues, DAX1 is also expressed at high levels in ES cells and plays a role in the maintenance of pluripotentiality. Here we review the clinical manifestations associated with *DAX1* mutations as well as the evolving information about its function based on animal models and in vitro studies.

Human Studies

Clinical Features of AHC

X-linked AHC is a disorder that presents in infancy with adrenal insufficiency and occurs in fewer than 1:12, 500 live births (Sikl, 1948, Mitchell and Rhaney, 1959, Dacou and Di George, 1968, Uttley, 1968, Petersen, Bille, Jacobsen et al., 1982, Kelch, Virdis, Rapaport et al., 1984). The age at presentation has a bimodal distribution: the majority of patients present within the first 2 months of life; the remainder present insidiously later in childhood (Yu, Ito, Saunders et al., 1998, Reutens, Achermann, Ito et al., 1999, Lin, Gu, Ozisik et al., 2006). Histologically, the disorder is characterized by absence of the adrenal cortex

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permanent zone, with residual cytomegalic cells (Uttley, 1968, Kerenyi, 1961, Mamelle, David, Riou et al., 1975).

Clinical signs and symptoms include typical features of adrenal insufficiency: hyperpigmentation, vomiting, poor feeding, failure to thrive, convulsions, vascular collapse and sudden death. Biochemical findings include hyponatremia, hyperkalemia, hypoglycemia, reduced serum cortisol and aldosterone, and increased plasma ACTH (Kelch et al., 1984). The diagnosis of adrenal insufficiency can be made on the basis of baseline low cortisol and elevated ACTH levels or after ACTH stimulation testing (Achermann, Silverman, Habiby et al., 2000). Some patients have residual cortisol or mineralocorticoid production but their circulating levels are inadequate to prevent the clinical manifestations. In some pedigrees, unexplained deaths of male infants may be attributed to X-linked AHC before recognition of the disorder in the family.

Treatment with glucocorticoids and mineralocorticoids allows affected individuals to survive into adolescence at which point hypogonadotropic hypogonadism (HHG) is manifest and distinguishes AHC from other causes of adrenal insufficiency (Petersen et al., 1982, Prader, Zachmann and Illig, 1975, Golden, Lippe and Kaplan, 1977, Zachmann, Illig and Prader, 1980, Kelly, Joplin and Pearson, 1977).

Genetics of AHC

Prior to the identification of the *DAX1* gene, the underlying cause of AHC was unknown. The observation that some patients with X-linked AHC have a contiguous gene syndrome, and present with various combinations of glycerol kinase deficiency, Duchenne muscular dystrophy, ornithine transcarbamoyltransferase deficiency, and mental retardation allowed the locus to be narrowed to Xp21.3-p21.2 (Hammond, Howard, Brookwell et al., 1985, Bartley, Patil, Davenport et al., 1986, Francke, Harper, Darras et al., 1987, Mandel, Willard, Nussbaum et al., 1989, Goonewardena, Dahl, Ritzen et al., 1989, Worley, Ellison, Zhang et al., 1993). Subsequently, mutations were identified in *DAX1*, confirming that it is the causative gene for AHC (Muscatelli, Strom, Walker et al., 1994, Zanaria, Muscatelli, Bardoni et al., 1994).

Since the initial identification of *DAX1* as the gene responsible for AHC, numerous additional mutations have been discovered including deletions, alterations of splice-sites, missense mutations, nonsense mutations and frameshift mutations (Fig. 1) (Reutens et al., 1999, Lin et al., 2006, Achermann et al., 2000, Muscatelli et al., 1994, Zanaria et al., 1994, Guo, Mason, Stone et al., 1995, Takahashi, Shoji, Haraguchi et al., 1997, Meloni, Cao and Rosatelli, 1996, Nakae, Abe, Tajima et al., 1997, Schwartz, Blichfeldt and Muller, 1997, Peter, Viemann, Partsch et al., 1998, Seminara, Achermann, Genel et al., 1999, Bassett, O'Halloran, Williams et al., 1999, Caron, Imbeaud, Bennet et al., 1999, Merke, Tajima, Baron et al., 1999, Tabarin, Achermann, Recan et al., 2000, Domenice, Latronico, Brito et al., 2001, Wiltshire, Couper, Rodda et al., 2001, Sekiguchi, Hara, Matsuoka et al., 2007, Bernard, Ludbrook, Queipo et al., 2006, Verrijn Stuart, Ozisik, de Vroede et al., 2007, Landau, Hanukoglu, Sack et al., 2010, Li, Liu, Zhang et al., 2010, Sykiotis, Hoang, Avbelj et al., 2010, Abe, Nakae, Yasoshima et al., 1999, Achermann, Meeks and Jameson, 2001, Ahmad, Paterson, Lin et al., 2007, Argente, Ozisik, Pozo et al., 2003, Balsamo, Antelli, Baldazzi et al., 2005, Brown, Scobie, Townsend et al., 2003, Calliari, Longui, Rocha et al., 2007, Frapsauce, Ravel, Legendre et al., 2011, Garcia-Malpartida, Gomez-Balaguer, Sola-Izquierdo et al., 2009, Guo, Burris, Zhang et al., 1996, Habiby, Boepple, Nachtigall et al., 1996, Hamaguchi, Arikawa, Yasunaga et al., 1998, Kinoshita, Yoshimoto, Motomura et al., 1997, Krone, Riepe, Dorr et al., 2005, Lam, Cheng, Poon et al., 2006, Mantovani, Ozisik, Achermann et al., 2002, Mericq, Ciaccio, Marino et al., 2007, Nakae, Tajima, Kusuda et al., 1996, Ozisik, Mantovani, Achermann et al., 2003, Salvi, Gomez, Fiaux et al., 2002, Tsai and

Tung, 2005, Wang, Killinger and Hegele, 1999, Wu, Zhang, Zhou et al., 2011, Yanase, Takayanagi, Oba et al., 1996, Zhang, Guo, Wagner et al., 1998, Zhang, Huang, Anyane-Yeboa et al., 2001, Franzese, Brunetti-Pierri, Spagnuolo et al., 2005, Laissue, Copelli, Bergada et al., 2006, Okuhara, Abe, Kondo et al., 2008). After other known causes (i.e., congenital adrenal hyperplasia, CAH) have been excluded, it is estimated that as many as 50% of boys with idiopathic primary adrenal insufficiency may have mutations in *DAX1* (Lin et al., 2006). Because females are typically silent carriers of AHC, a careful family history is required to detect the X-linked pattern of transmission.

About one-third of AHC patients harbor *DAX1* deletions, which can range from microdeletions within the coding sequence or promoter, to very large deletions involving adjacent genes (Lin et al., 2006). Deletions are important to recognize because *DAX1* PCR reactions in an affected male will yield no product and the *DAX1* sequence of an unaffected carrier will be normal, reflecting amplification of the normal X-chromosomal allele.

The locations of naturally occurring *DAX1* mutations provide insights into the structure-function relationships in the protein (Fig. 1). Truncation of as little as 39 amino acids from the carboxy-terminus is sufficient to eliminate transcriptional repression by DAX1 (Ito, Yu and Jameson, 1997). Missense mutations account for about one-quarter of *DAX1* mutations and tend to cluster in the carboxy-terminal ligand binding-like domain (Lin et al., 2006). These frequently occur in conserved amino acids and structural models suggest that most alter protein folding, thereby impairing nuclear localization and cofactor recruitment (Lehmann, Wurtz, Renaud et al., 2003, Lehmann, Lalli and Sassone-Corsi, 2002). It is notable that relatively few missense mutations occur in the amino-terminal region of the protein, possibly because of redundant function of the repeated LXXLL protein interaction domains (Verrijn Stuart et al., 2007, Kawajiri, Ikuta, Suzuki et al., 2003, Suzuki, Kasahara, Yoshioka et al., 2003).

Phenotypic Expression

The phenotypes of patients with AHC are heterogeneous. The type or location of a *DAX1* mutation does not consistently predict the age of onset of adrenal insufficiency or disease severity (Muscatelli et al., 1994, Peter et al., 1998, Merke et al., 1999, Wiltshire et al., 2001, Sekiguchi et al., 2007, Bernard et al., 2006, Verrijn Stuart et al., 2007). Phenotypic heterogeneity occurs within a family with the same mutation, as well as with different *DAX1* mutations, suggesting an effect of modifier genes or environmental effects on the expression of clinical manifestations (see the Overview Section and Chapter 1).

Although AHC usually presents during infancy, some patients are not diagnosed until later in life. One individual was diagnosed at 28 years of age. He presented with isolated adrenal insufficiency and undiagnosed partial HHG, as there was sufficient testosterone to produce some masculinization at puberty (Tabarin et al., 2000). Of note, this mutation (I439S) resulted in incomplete transcriptional repression in cell-based assays. Another patient presented at 28 years of age with HHG and was shown to have compensated adrenal insufficiency (Mantovani et al., 2002). He had a Y380D mutation that also caused partial loss of function in transfection assays. A 20 year old male presented with adrenal insufficiency and was found to have hypogonadism with low testosterone and inappropriately normal LH, but elevated FSH. He had an unusual amino-terminal (Q37X) frameshift mutation predicted to cause a severe truncation of the DAX1 protein (Ozisik et al., 2003). However, utilization of a downstream alternate translation site allowed production of an amino-terminally truncated protein, presumably accounting for his milder phenotype. A patient with a *DAX1* missense mutation (W105C) had isolated mineralocorticoid deficiency, without evidence of glucocorticoid deficiency or HHG (Verrijn Stuart et al., 2007). This same amino acid substitution was present in 3 unaffected

male relatives. This mutation was located in the amino-terminal repeat region of the DAX1 protein and the mild phenotype was suggested to be caused by redundant function of the other repeat domains.

Although the majority of AHC patients are male, mutations with phenotypic expression in females have been reported. The first was a homozygous nonsense mutation (codon 172) in a woman with isolated HHG. This mutation was proposed to involve gene conversion (Merke et al., 1999). The second was a heterozygous missense mutation in a female with late-onset AHC; of note, her hemizygous father and heterozygous sister were asymptomatic (Bernard et al., 2006). In some pedigrees with affected males, delayed puberty has been reported in carrier females (Seminara et al., 1999). A female with a contiguous gene deletion including *DAX1* and *DMD* presented with adrenal insufficiency in infancy (Shaikh, Boyes, Kingston et al., 2008). The deletion was located on the maternal X-chromosome and she was found to have skewed X-inactivation, resulting in relatively low expression of paternal genes in some tissues. Thus, while most female carriers of *DAX1* mutations are asymptomatic, some have been reported with clinical features of AHC. Like other X-linked disorders, this may reflect variable gene expression or oligogenicity (Sykiotis et al., 2010).

The onset of puberty is also variable in AHC. Most commonly, boys fail to enter puberty and gonadotropin levels are low and unresponsive to GnRH. Rarely, there is delayed or incomplete puberty associated with partial gonadotropin deficiency. Surprisingly, some affected boys develop gonadotropin-independent precocious puberty, including pubic hair, penile growth, increased testicular volume and advanced bone age (Domenice et al., 2001, Landau et al., 2010). Although testosterone levels are elevated, gonadotropin levels are usually low. There is variable responsiveness to GnRH stimulation. When present, the features of precocious puberty wane over time. The mechanism of precocious puberty is unclear and may be multifactorial. Possible causes include Leydig cell autonomy, persistent androgen production by the fetal adrenal, ACTH-stimulation of steroidogenic cells in the testis, and impaired gonadal steroid negative feedback of the HPG axis (Peter et al., 1998, Domenice et al., 2001, Landau et al., 2010).

Unilateral or bilateral cryptorchidism may occur, likely because of low gonadotropin production in utero (Zachmann et al., 1980, Muscatelli et al., 1994, Peter et al., 1998, Kruse, Sippell and Schnakenburg, 1984). As adults, affected patients typically have oligospermia or azoospermia that is unresponsive to gonadotropin therapy, raising the possibility of Sertoli, peritubular myoid, or germ cell dysfunction. In a limited number of cases in which biopsies are available, there is evidence of Sertoli cell only syndrome (Seminara et al., 1999) or testicular dysgenesis (Ozisik et al., 2003). As noted below, mice with *Dax1* mutations exhibit gonadal dysgenesis, including abnormal testis cord development (Yu et al., 1998, Jeffs, Meeks, Ito et al., 2001, Meeks, Weiss and Jameson, 2003).

The phenotypic heterogeneity of AHC may be due to multiple factors including compensation from other genes (Dipple and McCabe, 2000, Scriver and Waters, 1999), epigenetic or non-genetic factors (Peter et al., 1998, Sekiguchi et al., 2007), and modulation of DAX1 localization, and therefore potentially function, in the fetal adrenal gland by components of the extracellular matrix and hormones such as ACTH (Battista, Otis, Cote et al., 2005) As noted above, some *DAX1* mutations cause incomplete loss of function. There is also evidence of skewed X-inactivation in some female carriers (Shaikh et al., 2008). In addition, the symptoms of adrenal insufficiency can be nonspecific and the diagnosis may be delayed until adrenal crisis is precipitated by other illnesses. A family history of AHC and access to medical care can also lead to earlier recognition of the disorder (Reutens et al., 1999).

The Hypothalamic-Pituitary Axis in AHC

Due to the heterogeneity of the phenotype in AHC, it has been challenging to pinpoint the site of deficiency within the hypothalamic-pituitary axis (Golden et al., 1977, Hay, Smail and Forsyth, 1981). Early studies suggested that HHG resulted from either a primary pituitary (Gordon, Cohen, Beastall et al., 1984, Kikuchi, Kaji, Momoi et al., 1987, Bovet, Reymond, Rey et al., 1988, Pitteloud, Thambundit, Dwyer et al., 2009) or a primary hypothalamic defect (Peter et al., 1998, Kruse et al., 1984, Kletter, Gorski and Kelch, 1991, Partsch and Sippell, 1989). The current theory is based largely on a detailed study of two kindreds with HHG, each with mutations that truncated the protein, albeit at different amino acids (Habiby et al., 1996). In the first kindred, the proband (MH) had low baseline LH levels but normal free alpha subunit (FAS) levels (Fig. 2). In the second kindred, the proband (JW) had normal LH and FSH but 24h profiles of pulsatile LH secretion were erratic, without a clear pulsatile pattern. After administration of exogenous pulsatile GnRH, MH failed to mount a gonadotropin response, consistent with a pituitary defect. In contrast, JW responded to the first pulse but did not respond to subsequent GnRH pulses. This response contrasts with GnRH deficient patients (e.g., Kallmann syndrome), who exhibit a progressive increase in GnRH-stimulated gonadotropin responses as the pituitary is primed over several days. The lack of a gonadotropin response to GnRH in AHC patients with HHG confirms a defect at the level of the pituitary (Seminara et al., 1999, Habiby et al., 1996). However, because the initial bolus of exogenous GnRH stimulated LH release in JW, it is postulated that he had a low level of pre-existing GnRH, sufficient for priming, but insufficient for maintaining normal gonadotropin secretion (Habiby et al., 1996). Thus, the hormonal responses in JW are consistent with a hypothalamic defect in GnRH production, as well as a pituitary defect in GnRH responsiveness and gonadotropin production. Unanticipated pituitary and gonadal defects have been identified in other subsets of patients with isolated GnRH deficiency receiving pulsatile GnRH (Sykiotis et al; JCEM, 2011), suggesting that, like DAX1, mutations in genes expressed in the hypothalamus, pituitary, and gonads may reveal phenotypes that are masked by abnormalities in other components of the reproductive axis.

Diagnosis and Treatment of AHC

Molecular genetic techniques now provide the opportunity for genetic testing, early diagnosis, and steroid replacement (Schwartz et al., 1997, Wiltshire et al., 2001). Sequencing of *DAX1* has demonstrated the value of presymptomatic diagnosis of AHC, allowing close clinical monitoring and early treatment before an Addisonian crisis as well as appropriate hormonal therapy at the time of puberty (Achermann et al., 2000). In families known to harbor *DAX1* mutations, it is reasonable to perform measurements of plasma cortisol and aldosterone before and after ACTH stimulation, as well as plasma ACTH and plasma renin activity, pending genetic testing (Peter et al., 1998). If the location of a *DAX1* mutation is known, and is absent in a potentially affected boy, the diagnosis has been excluded, although it may be reasonable to confirm the DNA sequence independently before eliminating all monitoring. If the location of a mutation is not known, a normal coding sequence does not necessarily exclude the disorder, as some mutations may alter promoter activity or *DAX1* mRNA splicing (Goto and Katsumata, 2009). Submicroscopic deletions upstream of *DAX1*, resulting in loss of regulatory sequences, has been reported to result in sex-reversal, reminiscent of duplication of the *DAX1* locus (Smyk, Berg, Pursley et al., 2007).

The treatment of adrenal insufficiency in AHC is similar to that of other causes. Most patients require replacement of both glucocorticoids and mineralocorticoids. The treatment of HHG includes several options. Testosterone replacement therapy has been used to directly stimulate virilization (Prader et al., 1975, Hay et al., 1981). Human chorionic

gonadotropin has also been used to stimulate testosterone production (Kelly et al., 1977, Tabarin et al., 2000, Hay et al., 1981). Gonadotropins have been administered in an attempt to induce spermatogenesis but without success, possibly pointing to an independent DAX1-mediated defect in the gonad (Seminara et al., 1999). As noted above, pulsatile GnRH is ineffective in these patients. It remains to be seen whether earlier interventions or ICSI might be used to achieve fertility.

Differentiating AHC from Other Causes of Congenital Adrenal Insufficiency

AHC must be differentiated from a variety of other adrenalopathies as the clinical manifestations, treatment, and prognosis vary considerably. Congenital adrenal hyperplasia (CAH) can be caused by mutations in several genes encoding steroidogenic enzymes involved in glucocorticoid synthesis (*CYP21A2, CYP17A1, HSD3B2, CYP11B1*) or in the co-factor enzyme P450 oxidoreductase (POR) that serves as an electron donor to CYP21A2 and CYP17A1. 21-hydroxylase (*CYP21A2)* deficiency is by far the most common cause of neonatal adrenal insufficiency. It is characterized by increased levels of 17-hydroxyprogesterone and other steroids upstream of the enzymatic block. Mutation of *CYP11A1* (side-chain cleavage enzyme) is a rare cause of adrenal insufficiency and undervirilization. XY patients with StAR mutations (Lipoid Congenital Adrenal Hyperplasia, Lipoid CAH) also present with adrenal insufficiency and severe undervirilization (female phenotype) because they lack the StAR cholesterol transport protein and the resulting lipid accumulation is ultimately toxic to steroidogenic cells (Tee, Lin, Sugawara et al., 1995). XY patients with heterozygous SF1 mutations exhibit a range of features that can include gonadal dysgenesis and adrenal insufficiency (Achermann et al., 2001). Adrenoleukodystrophy can be excluded by measurement of serum long chain fatty acids, which are elevated in adrenoleukodystrophy but normal in AHC (Reutens et al., 1999). Aldosterone deficiency precedes overt glucocorticoid deficiency in some AHC infants. (Wiltshire et al., 2001, Sills, Voorhess, MacGillivray et al., 1983). Thus, AHC should be considered in patients initially diagnosed with primary hypoaldosteronism who later develop cortisol deficiency. Not uncommonly, AHC is not recognized as the cause of adrenal insufficiency until patients fail to enter puberty.

Mouse Models of *Dax1* Function

Mapping and Structure of Dax1

The human and mouse *Dax1* genes are structurally similar with two exons and about 75% similarity in the coding region (Burris, Guo and McCabe, 1996). Exon 1 encodes the amino-terminal region with three and one-half repeated sequences containing hydrophobic LXXLL motifs, which are thought to be involved in protein-protein interactions (Suzuki et al., 2003, Iyer and McCabe, 2004) (Fig. 1). A region homologous to the ligand binding domain (LBD) of other nuclear receptors is encoded by portions of exons 1 and 2. Notably, the DAX1 protein lacks the zinc-finger DNA binding domain that characterizes other members of the nuclear receptor superfamily. The carboxy-terminus contains transcriptional regulatory sequences (Iyer and McCabe, 2004). To date, no ligand has been found for DAX1 (Iyer and McCabe, 2004).

Dax1 Expression

Dax1 is expressed in the adrenal and in all regions of the HPG axis during development and in the adult. *In situ* hybridization demonstrates *Dax1* expression at embryonic day 10.5 (E10.5) in the urogenital ridge (Swain, Zanaria, Hacker et al., 1996). *Dax1* expression is sexually dimorphic in the developing gonads. By E12.5, *Dax1* expression is extinguished in the testis although it persists in the ovaries. In the testis, both Sertoli cells and Leydig cells stain positive for DAX1; in the ovaries, the granulosa and theca cells show DAX1

expression. In the adrenal gland, *Dax1* is expressed predominantly in the zona glomerulosa (Ikeda, Takeda, Shikayama et al., 2001). Studies using RT-PCR reveal that *Dax1* is expressed in the adult ovary, testis, adrenal gland, hypothalamus, cerebral cortex, spinal cord, thymus, heart, lung, kidney, and spleen (Bae, Schaefer, Partan et al., 1996).

Dax1 Mouse Models

Multiple *Dax1* mouse models have been generated to characterize its biological function and its role in AHC. The dosage-sensitive sex-reversal (DSS) phenotype was first replicated in mice by overexpression of *Dax1* in the *Poschiavinus* mouse strain (Swain, Narvaez, Burgoyne et al., 1998, Eicher, Washburn, Schork et al., 1996). *Dax1* was overexpressed using multiple copies of an 11kb genital ridge specific region of the gene on the genetic background of a weak *Sry* allele in this carefully selected murine model. XY sex-reversal occurred in a subset of mice. Although this model confirmed the important role of *Dax1* in sex determination, the phenotype was not replicated on backgrounds other than the *Poschiavinus* mouse, presumably due to modifying effects of the genetic background.

When *Dax1* was deleted from ES cells in an effort to generate a *Dax1* knock-out mouse, rapid ES cell differentiation was observed (Yu et al., 1998), precluding reimplantation of the cells into blastocysts. Subsequent studies showed that *Dax1* is expressed in the blastocyst and is important for maintenance of ES cell pluripotency (Clipsham, Niakan and McCabe, 2004, Niakan, Davis, Clipsham et al., 2006). DAX1 is part of a protein network of molecules that includes OCT4 and other regulators of pluripotency (Kelly and Hammer, 2010, Kelly, Xu, Kuick et al., 2010, Kim, Chu, Shen et al., 2008) (Fig. 3). DAX1 is also highly expressed in certain poorly differentiated malignancies, such as Ewing's sarcoma (Garcia-Aragoncillo, Carrillo, Lalli et al., 2008).

A *Dax1* knock-out model was developed by Cre-mediated deletion of exon 2 (Yu et al., 1998). This approach allowed integration of the floxed version of *Dax1* during ES cell manipulation, followed by deletion of exon 2 in female carriers, and transmission of the mutant gene to XY offspring. Deletion of *Dax1* was hypothesized to impair ovarian development, largely because excess doses of *Dax1* (i.e., duplication) caused XY sex reversal, suggesting that *Dax1* might be an ovary-determination gene. Unexpectedly, ovarian development was normal, and instead, the mice had testis dysgenesis and a spermatogenesis defect. Gonadotropin and testosterone levels were normal, suggesting a non-hormonal testis defect. The dysgenetic testis was determined to be a result of disorganized testis cord formation, apparently the result of impaired differentiation of peritubular myoid and Sertoli cells (Jeffs et al., 2001, Meeks et al., 2003, Meeks, Crawford, Russell et al., 2003). Additional genetic crosses substantiated the role of *Dax1* in testis development. *Dax1*-deficient mice were crossed with the *Poschiavinus* strain. If *Dax1* was an ovary-determining gene, the absence of *Dax1* in this cross should have favored normal testis development. On the other hand, if *Dax1* played a role in testis development, a combined deficiency of *Dax1* with a weakened *Sry* allele could accentuate intersex phenotypes or result in sex reversal. All XY mice in this cross were phenotypic females with activation of the ovary pathway, confirming the importance of *Dax1* in testis development (Meeks et al., 2003). Levels of *Sry* expression were normal, indicating that *Dax1* acted downstream of *Sry* in testis development. Similarly, crosses of *Dax1*-deficient mice onto C57BL/6J and other genetic backgrounds resulted in varying degrees of sex reversal (Bouma, Albrecht, Washburn et al., 2005, Park, Lee, Emge et al., 2008).

These *Dax1*-deficient mice do not develop adrenal insufficiency, although they do have expanded X-zones, which correspond to the fetal zone in humans (Yu et al., 1998, Beuschlein, Keegan, Bavers et al., 2002). Thus, *Dax1*-deficient mice lack some of the classic features of AHC, such as adrenal insufficiency and gonadotropin deficiency.

However, they reveal new aspects of DAX1 function in the testis. It should also be noted that *Dax1* function is very sensitive to genetic background, with phenotypic expression being dramatically modified by mouse strain (Bouma et al., 2005, Park et al., 2008). It is also possible that the deletion of exon 2 does not eliminate all aspects of Dax1 function, although analogous mutations in exon 2 have been shown in humans to cause AHC.

Molecular Mechanisms of DAX1 Action

DAX1 is structurally related to another nuclear receptor, SF1 (NR5A1). SF1 is essential for the development of the HPA and HPG axes and the expression of various steroidogenic enzyme genes (Worley et al., 1993). Disruption of the *Sf1* gene in mice causes complete agenesis of the gonads and adrenals, as well as hypothalamic and pituitary defects (Luo, Ikeda and Parker, 1994). *Dax1* and *Sf1* are also known to have overlapping expression patterns during development and in several adult tissues(Ikeda et al., 2001, Ikeda, Swain, Weber et al., 1996). These observations suggested the possibility of a functional interaction between DAX1 and SF1. In addition, because many nuclear receptors interact as homodimers or heterodimers, their coexpression raised the possibility that Sf1 and Dax1 might interact directly as transcription factors. As described below, there is convincing evidence that DAX1 modulates the transcriptional activity of SF1 (Ito et al., 1997). However, this action appears to involve direct protein-protein interactions and intermediary factors rather than traditional binding of SF1-DAX1 heterodimers to DNA.

Nuclear receptors either repress or activate target genes, dependent in part on the recruitment of cofactor proteins (Lonard, Lanz and O'Malley, 2007). In many cases, receptors switch from a repressor to an activator in response to ligand-mediated conformational changes. DAX1 has been found to function largely as a transcriptional repressor. It possesses basal repressive activity, even when fused to other transcription factors (Ito et al., 1997). Moreover, DAX1 is a potent inhibitor of SF1-mediated transcriptional activation (Ito et al., 1997, Clipsham and McCabe, 2003). Corepressor recruitment, and the resulting transcriptional silencing by DAX1, occurs through a C-terminal LBD-like (LBD-L) domain (Ito et al., 1997). DAX1 recruits the nuclear receptor corepressor (N-CoR) to SF1 through two conserved regions within the LBD-L domain of DAX1 (Crawford, Dorn, Sadovsky et al., 1998). DAX1 is also known to interact with the corepressor Alien through its C-terminal domain (Altincicek, Tenbaum, Dressel et al., 2000). The ability of DAX1 to repress SF1-mediated transcription has been used to assess the function of naturally occurring human DAX1 mutations (Lin et al., 2006).

It remains somewhat puzzling how this inhibitory activity of DAX1 *in vitro* is related to its function *in vivo*. SF1 stimulates the DAX1 promoter (Yu, Ito and Jameson, 1998). Although DAX1 typically represses SF1-mediated transcription, low doses of DAX1 can activate certain SF1-regulated promoters, such as CYP11A1 and CYP11B1 (Verrijn Stuart et al., 2007). Perhaps more important from a physiologic perspective, both SF1 and DAX1 play important, and incompletely understood, roles in development. Current models suggest that DAX1 may favor a pluripotential state whereas SF1 directs cell differentiation (Kim, Barlaskar, Heaton et al., 2009).

In addition to SF1, DAX1 acts as a repressor of other nuclear receptors including the estrogen receptor (ER or NR3A1-2), the progesterone receptor (PR or NR3C3), the androgen receptor (AR or NR3C4), and liver receptor homologue-1 (LRH-1 or NR5A2) (Suzuki et al., 2003, Zhang, Thomsen, Johansson et al., 2000, Holter, Kotaja, Makela et al., 2002, Agoulnik, Krause, Bingman et al., 2003). DAX1 antagonizes cooperation between different transcription factors like SF1 and Wilms Tumor 1 (WT1) in the regulation of the Mullerian inhibiting substance (MIS) gene (Nachtigal, Hirokawa, Enyeart-VanHouten et al.,

1998). DAX1 also inhibits SF1/EGR1 stimulation of LH3 gene expression (Dorn, Ou, Svaren et al., 1999) and SF1/GATA6 regulation of androgen biosynthesis genes (Jimenez, Saner, Mayhew et al., 2003).

In cells coexpressing DAX1 and SF1, DAX1 interacts with SF1 through its LXXLL motifs and translocates to the nucleus (Kawajiri et al., 2003). DAX1 mutations in AHC result in aberrant localization to the cytoplasm (Lehmann et al., 2002). Subcellular localization of DAX1 also plays an important role in DAX1 function in various cell types, including ES cells (Clipsham et al., 2004). DAX1 has been proposed to play a novel role as an RNA binding protein in the cell cytoplasm (Lalli, Ohe, Hindelang et al., 2000).

DAX1 Function and AHC

AHC patients fail to develop the adult zone of the adrenal gland, emphasizing the role of DAX1 in development, as well as a regulator of steroidogenesis. DAX1 is expressed in the adrenal cortex throughout development, although its precise role in adrenal morphogenesis is not fully understood. In *Dax1*-deficient mice, the X-zone fails to regress in male mice at puberty (Yu et al., 1998), suggesting that *Dax1* may play an important role in fetal adrenal regression as opposed to directing zonation of the adult adrenal gland. The function of DAX1 in the development of the adrenal gland may be related to its role in maintaining pluripotency (Niakan et al., 2006). In the adult, expression of *Dax1* and *Sf1* is layer-specific (Kim et al., 2009). Neither *Dax1* nor *Sf1* are expressed in the outermost layer, the capsule. Both *Dax1* and *Sf1* are expressed in the subcapsular cortex whereas the mature steroidogenic layers of the cortex express only *Sf1*. It is plausible that DAX1 maintains a progenitor pool whereas SF1 directs various stages of cell differentiation (Kim et al., 2009). This layer-specific expression pattern suggests that *Sf1* and *Dax1* expression, along with paracrine and endocrine signals, control the differentiation and development of the adrenal cortex by maintaining a pool of stem or progenitor cells (Kim et al., 2009).

As noted above, the development of HHG in AHC is thought to reflect a combined defect of hypothalamic and pituitary function (Habiby et al., 1996). *Dax1* is expressed in the VMH, Rathke's pouch, and in the pituitary (Ikeda et al., 2001). These observations again raise the possibility that DAX1 plays a role in development of the pituitary and hypothalamus.

Conclusions

The majority of X-linked AHC patients present with adrenal insufficiency during the first few months of life. It is estimated that more than 50% of boys with idiopathic primary adrenal insufficiency have mutations in *DAX1* (Lin 2006). As our understanding of the disease has evolved, improved treatments have extended patient life expectancy. Patients with HHG secondary to AHC have low serum gonadotropins. It appears that both the hypothalamus and pituitary are affected by mutations in *DAX1*; however, the mechanism of DAX1 action in these tissues remains unclear.

Although the mouse models of *Dax1* have provided a wealth of information about the developmental expression pattern of *Dax1* and the molecular mechanism of Dax1 action, these models do not faithfully replicate the AHC and HHG phenotypes seen in humans. Mouse studies have shown that *Dax1* is expressed not solely in the adult steroidogenic tissues but also in the early embryo and in the developing gonad, adrenal, hypothalamus, and pituitary. Increasing evidence supports a model in which DAX1 functions in a network of other transcription factors to regulate the transition from pluripotency (DAX1 high) to differentiation (DAX1 low). Future questions will focus on the developmental roles of DAX1, its target genes, and its interplay with SF1 and other transcription factors. From a clinical perspective, the main challenges continue to be early diagnosis and treatment of

affected infants, distinguishing AHC from other causes of neonatal and childhood adrenal insufficiency, and the hormonal management of puberty and fertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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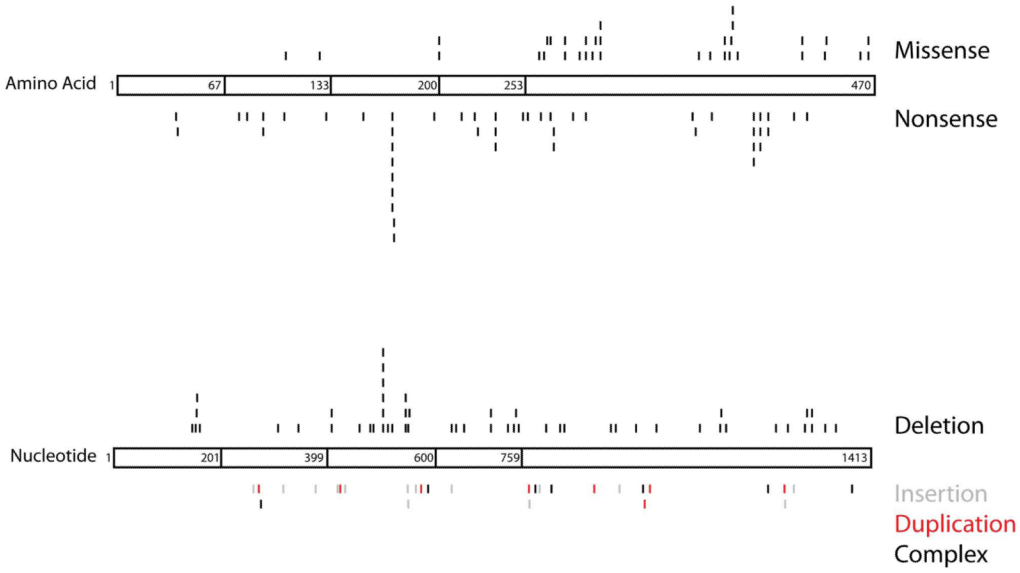

Figure 1.

Schematic of known DAX1 mutations. The DAX1 protein structure is divided into three and a half amino-terminal repeats (amino acids 1–67, 68–133, 134–200 and 201–253) and one nuclear receptor-like domain (amino acids 254–470). In the top diagram, missense (top) and nonsense (bottom) mutations are shown at specific amino acids along the length of DAX1. The bottom figure depicts deletions (black above protein), insertions (gray below protein), duplications (red below protein) and complex deletions/insertions (black below protein), which are assigned based on nucleotide number. Visualization of all naturally occurring mutations demonstrates clustering at specific points along the DAX1 protein. Numbering of mutations began at the A of the ATG translational initiation codon (Ensembl transcript ID ENST00000378970 and protein ID ENSP00000368253). The locations of the frameshift mutations (deletions, insertions, duplications and complex deletions/insertions) are designated by the first nucleotide altered. References are found above in the section "Genetics of AHC." This represents a comprehensive list of *DAX1* mutations to the best of our knowledge. Please refer to the original articles for details.

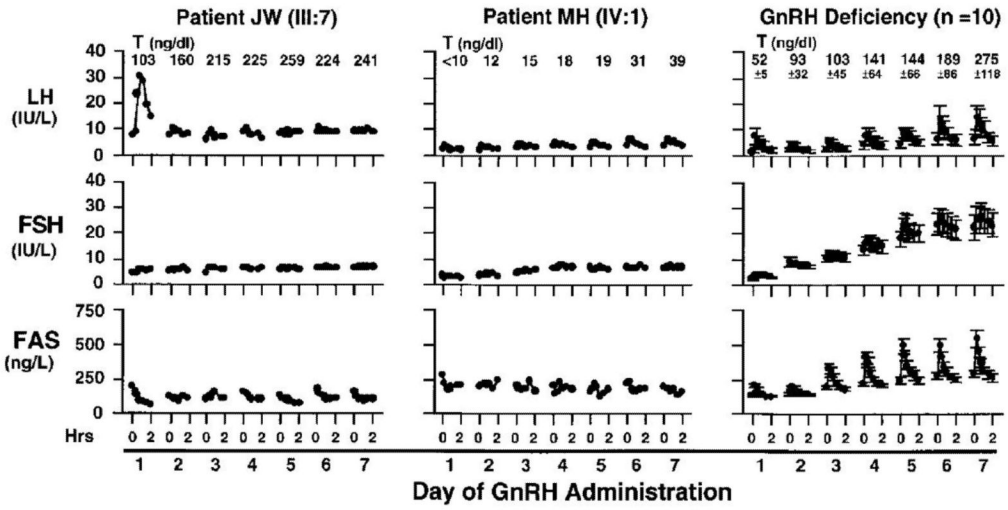

Figure 2.

Gonadotropin levels after 1 week of GnRH administration to two probands with HHG and ten patients with Kallmann Syndrome (GnRH deficiency). Intravenous pulsatile GnRH (25ng/kg) was administered every 2 hours after baseline secretory studies. Daily testosterone levels are shown above each graph.

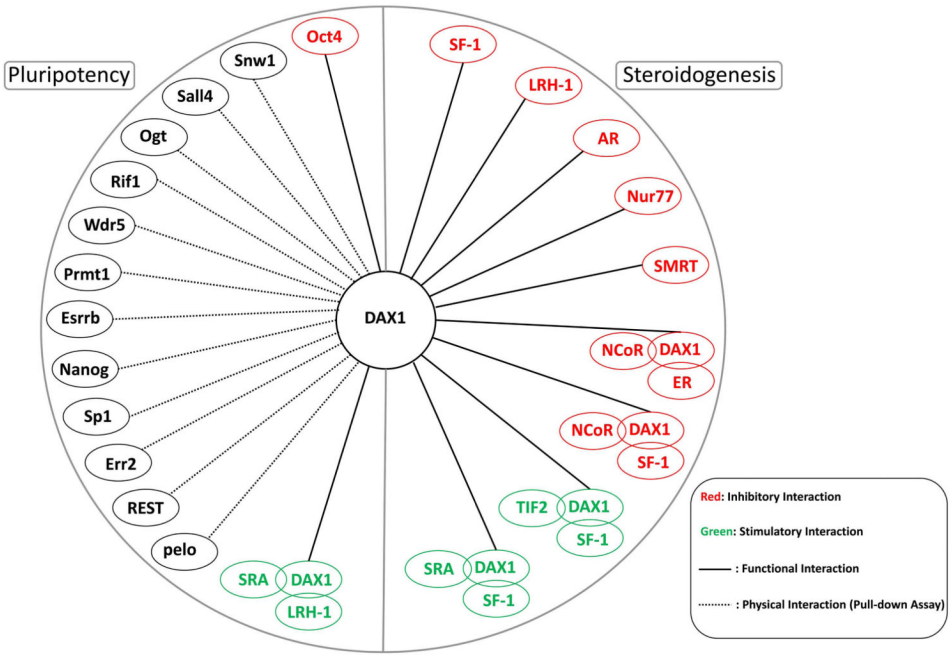

Figure 3.

DAX1 protein interactions. DAX1 acts in either an inhibitory (red) or a stimulatory (green) manner by interacting with proteins involved in pluripotency and steroidogenesis. These protein complexes may contain other transcription factors, coactivators, and corepressors. Physical interactions (dotted line) have been determined using mutagenesis studies and pull-down assays.