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# Functional effects of *DAX-1* mutations identified in patients with X-linked adrenal hypoplasia congenita

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ABSTRACT

X-linked adrenal hypoplasia congenita with hypogonadotropic hypogonadism and adrenal insufficiency is a rare disorder caused by mutations of *DAX-1*. In this study, we investigated the functional defects of DAX-1 caused by mutations identified in 3 unrelated Korean patients with adrenal hypoplasia congenita. The *DAX-1* gene was directly sequenced using genomic DNA isolated from peripheral blood leukocytes. The functional defects of DAX-1 caused by mutations were evaluated using an in vitro promoter assay. After mutagenesis of *DAX-1* complementary DNA in the pcDNA3.1 vector, steroidogenic factor 1 and the promoter region of steroidogenic acute regulatory protein (*StAR*) genes in pGL4.10[*luc2*] were transiently cotransfected into human embryonic kidney 293 cells, followed by luminometry measurements of the luciferase activity of StAR. Mutation analysis of 3 patients revealed p.L386delfsX2, p.W105X, and p.Q252X mutations of the *DAX-1* gene. The mutant DAX-1 proteins showed lower repressive activity on the *StAR* gene promoter when compared with normal DAX-1. Nonsense and frameshift mutations of the *DAX-1* gene partially eliminated the ability of DAX-1 to repress the transcription of StAR in an in vitro assay.



## 1. Introduction

X-linked adrenal hypoplasia congenita (AHC) with hypogonadotropic hypogonadism and adrenal insufficiency is a rare disorder caused by mutations of the *DAX-1* (Dosage-sensitive sex reversal-Adrenal hypoplasia congenita critical region on the X chromosome gene-1) gene [1]. DAX-1 is an orphan nuclear receptor that encodes a 470–amino acid protein and

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consists of 2 exons separated by a 3.4-kilobase intron [2]. DAX-1 serves to activate target genes involved in hypothalamic-pituitary-adrenal-gonadal (HPAG) axis development and function. *DAX-1* is a general repressor of steroid biosynthesis that acts by repression of steroidogenic factor-1 (SF-1)–mediated transactivation of many genes in the steroid biosynthetic pathway, such as steroidogenic acute regulatory protein (StAR), 3$\beta$-hydroxysteroid dehydrogenase, and P450scc genes. It also inhibits its own transcription and LH$\beta$ transcriptional activities and reduces *GnRH* expression [3-6].

*DAX-1* mutations found in AHC patients result in reduced transcriptional silencing activity, which could contribute to AHC pathogenesis [7,8]. Some studies have suggested that this repressor function may involve specific transcriptional silencing domains within the C-terminal region of DAX-1 [7,8], whereas others have proposed that repression by DAX-1 may involve direct binding of DAX-1 to DNA hairpin loop structures in the promoters of certain target genes [5] or an interaction between DAX-1 and SF-1. This protein-protein interaction may involve specific silencing domains within the C-terminal region of DAX-1 [8] or the recruitment by DAX-1 of repressors [9]. The majority of functional data reported suggest that DAX-1 represses the transcription of several genes expressed in the adrenal and reproductive axes, either directly or through an interaction with the related orphan nuclear receptor SF-1 [7]. Transfection assays demonstrate that AHC-associated *DAX-1* mutations abrogate its ability to act as a transcriptional repressor of SF-1. Functional studies showed that mutant DAX-1 was impaired in its abilities for nuclear localization, for transrepression of *StAR* and *LH*$\beta$ transcriptional activities, and for reduction of *GnRH* expression [6].

We previously reported clinical features and molecular analysis of the *DAX-1* gene in 2 patients with X-linked AHC that were due to mutations of the *DAX-1* gene and 1 patient with Xp21 contiguous gene deletion syndrome [10]. This report describes the clinical features, molecular analysis, and analysis of functional effects of these mutations of *DAX-1* in 3 unrelated Korean patients with X-linked AHC. Transient gene expression assays were undertaken to assess the effects of the mutant DAX-1 protein on transcriptional repression.

## 2. Materials and methods

### 2.1. Patients

With approval from the Institutional Review Board at Asan Medical Center, 3 unrelated Korean patients were included in the study after obtaining informed consent. Patients 1 and 2 presented with adrenal insufficiency in the neonatal period and have been treated with hydrocortisone and 9$\alpha$-fludrocortisone. We previously reported the clinical features and gene abnormalities of patients 1 and 2 [10]. They have developed normally, and hyperpigmentation has resolved. The current age of patient 1 is 12 years, and he did not show any secondary sexual characteristics. In patient 2, leutenizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone levels were not measured because he is in prepubertal stage. Patient 3 was brought to the hospital because of skin hyperpigmentation and fatigue at the age of 6 years. The patient did not have family history of adrenal insufficiency. Laboratory findings indicated elevated serum corticotropin level and plasma renin activity, suggesting adrenal insufficiency (Table 1). He has been treated with hydrocortisone and 9$\alpha$-fludrocortisone. The patient's current age is 19 years, and testosterone enanthate was added because of hypogonadotropic hypogonadism. The clinical and endocrinological characteristics of the 3 patients are summarized in Table 1. In a corticotropin stimulation test, patient 1 did not show an increase in serum cortisol level. The corticotropin stimulation test was not performed in patients 2 and 3. The diagnosis of X-linked AHC was established by DNA analysis of the *DAX-1* gene.

### 2.2. Mutation analysis of the DAX-1 gene

Genomic DNA was isolated from peripheral blood leukocytes after informed consent was obtained from the patients. The *DAX-1* gene was amplified by polymerase chain reaction (PCR) using primer pairs for the *DAX-1* gene, according to the previous report [10]. The amplified PCR products were directly sequenced on both strands using a BigDye Terminator V3.0 Cycle Sequencing Ready reaction kit (Applied

**Table 1 – Clinical characteristics of the 3 patients with X-linked AHC at presentation**

| | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| Age at presentation | 3 d | 24 d | 6 y |
| Current age, y | 12 | 9 | 19 |
| Family history | None | None | None |
| Pubic hair Tanner stage | I | I | IV |
| Testicular volume, mL | 2 | 1 | 2 |
| Na/K/Cl, mEq/L | 129/5.6/92 | 122/6.4/91 | 136/4.0/101 |
| Plasma corticotropin, pg/mL | 1,180 | 24,300 | 3,470 |
| Plasma cortisol, $\mu$g/dL | 1.4 | 2.8 | Not available |
| Plasma renin activity, ng/(mL h) | 76 | >80 | 27 |
| Aldosterone, pg/mL | 3.4 | 13.2 | Not available |
| 17-Hydroxyprogesterone, ng/mL | 2.5 | 1.2 | Not available |
| LH, mIU/mL | 0.87 | Not done | Not done |
| FSH, mIU/mL | 0.52 | Not done | Not done |
| Testosterone, ng/mL | 0.04 | Not done | 4.16 |
| Corticotropin stimulation test | No response | Not done | Not done |

LH indicates luteinizing hormone; FSH, follicle-stimulating hormone.

Biosystems, Foster City, CA) and an ABI3100 Genetic analyzer (Applied Biosystems).

### 2.3. *Plasmid construction and mutagenesis*

Human DAX-1 and SF-1 complementary DNA (cDNA) clones were purchased from OriGene (Rockville, MD). Their coding regions, generated by PCR amplification, were subcloned into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA). *StAR* promoter sequences (+3 to –1222) from human genomic DNA were cloned into pGL4.10 (Promega, Madison, WI), encoding the luciferase reporter gene *luc2* using *pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The mutations of *DAX-1* were created using a PCR-based *Dpn*I treatment method [11]. Mutagenic primers were designed to create p.L386delfsX2, p.W105X, and p.Q252X, corresponding to the mutation in the amino acid sequence of *DAX-1* from each patient. Mutation sites and cloned plasmids were confirmed via sequence analysis with a BigDye Terminator V3.0 Cycle Sequencing Ready reaction kit (Applied Biosystems).

### 2.4. *Transient transfection and promoter assays*

Human embryonic kidney 293 cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum and 1% streptomycin/penicillin in 5% $CO_2$ atmosphere at 37°C. A luciferase reporter construct containing the human StAR promoter region (+3 to –1222) was cotransfected with expression constructs containing human full-length cDNA of SF-1 and DAX-1 (wild type [WT] or mutants) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Luciferase activity was assayed from cell lysates 24 hours after transfection with the Luciferase Assay system (Promega) according to the manufacturer's instruction. The cells were cotransfected with $\beta$-gal plasmid (Promega) to normalize luciferase activity. The luciferase activity of StAR was determined by luminometry (Microlumat Plus; Berthold Technologies, Wildbad, Germany). Results are expressed as mean ± SD from at least 3 independent experiments. The 2-tailed, paired Student t test was used to determine the statistical significance of repressive activity of mutants when compared with WT activity.

## 3. Results

### 3.1. *Mutation analysis of the DAX-1 gene*

Direct sequencing of *DAX-1* in patient 1 revealed deletions of cytosine and thymine at nucleotide positions 1156 and 1157 (c.1156_1157delCT), which led to a frameshift at amino acid codon 386 (p.L386delfsX2) (Fig. 1). This patient's mother was a heterozygous carrier of this 2-nucleotide deletion. Mutation analysis of *DAX-1* in patient 2 showed that guanine was substituted with adenine at nucleotide position 314, leading to tryptophan replacement, with stop codon at codon position 105 (p.W105X). This mutation disrupts the DAX-1 function because of premature truncation of the protein. This patient's mother did not carry a *DAX-1* mutation, indicating that the mutation in this patient had arisen de novo or germline; this also indicates a risk of recurrence. Molecular data for patient 3 disclosed that cytosine was substituted with thymine at base 754 (c.754C>T), leading to a glutamine replacement, with a stop codon at codon 252 (p.Q252X).

### 3.2. *Transient gene expression assays*

*DAX-1* has been shown to inhibit the transcription of reporter genes that are driven by *SF-1* [7]. Therefore, we tested whether the mutations in these patients altered this property of DAX-1. The mutant DAX-1 proteins exhibited lower repressive activity than normal DAX-1 on the promoter of the *StAR* gene. Transient cotransfection assays showed that all 3 mutations abolished the repression activity of both *StAR* and *SF-1* gene promoter activation (Fig. 2). This inhibition was reduced with each of the DAX-1 mutant vectors examined. Coexpression of WT DAX-1 greatly repressed SF-1 activity (relative luciferase activity: 52% in p.L386delfsX2, 41% in p.W105X, and 48% in p.Q252X).

## 4. Discussion

In this study, 2 patients presented with adrenal insufficiency in the neonatal period. We previously reported these 2 mutations of *DAX-1*, p.L386delfsX2 and p.W105X, in patients 1 and 2, respectively [10]. These mutations disrupt DAX-1 function because of premature truncation of the protein. The *DAX-1* mutations described in this study resulted in abnormal DAX-1 proteins that either completely lacked or had truncated ligand-binding domains. In vitro promoter assays showed that mutations of the *DAX-1* gene could partially (3- to 4-fold) eliminate the ability of DAX-1 to repress transcriptional activity of the *StAR* gene promoter when compared with normal DAX-1. The p.Q252X mutation identified in patient 3 was also reported previously [12]. Patient 3 revealed unusual delayed-onset adrenal insufficiency. Nonsense mutations of the distal N-terminal region were reported to have a milder phenotype due to the expression of a partially functional, amino-truncated DAX-1 protein [13].

To date, more than 140 different *DAX-1* mutations have been identified (http://www.hgmd.org/). Most *DAX-1* mutations reported so far have arisen from gene deletions or premature stop codons that cause a loss of the C-terminal region, resulting in premature truncation of the DAX-1 protein and loss of the DAX-1 repressor function [14]. Relatively few missense mutations have been reported in *DAX-1*. Thirty missense mutations have been reported (http://www.hgmd.org/), all of which are located in the C-terminal ligand-binding domain of *DAX-1* [15]. These may provide insight into potentially important domains for DAX-1 function [12]. Inversions located in a conserved noncoding region upstream of *DAX-1* relocate regulatory sites crucial in DAX-1 expression and result in X-linked AHC [16]. Rare cases of AHC have been reported in female carriers of *DAX-1* mutation or deletion, which are caused by the skewed inactivation of one of the X chromosomes [17].

Most boys affected with X-linked AHC present with adrenal insufficiency in early infancy, although a significant fraction presents in later childhood or even as young adults. The degree of gonadotropin deficiency is also variable. Current data suggest that little correlation exists between the type or

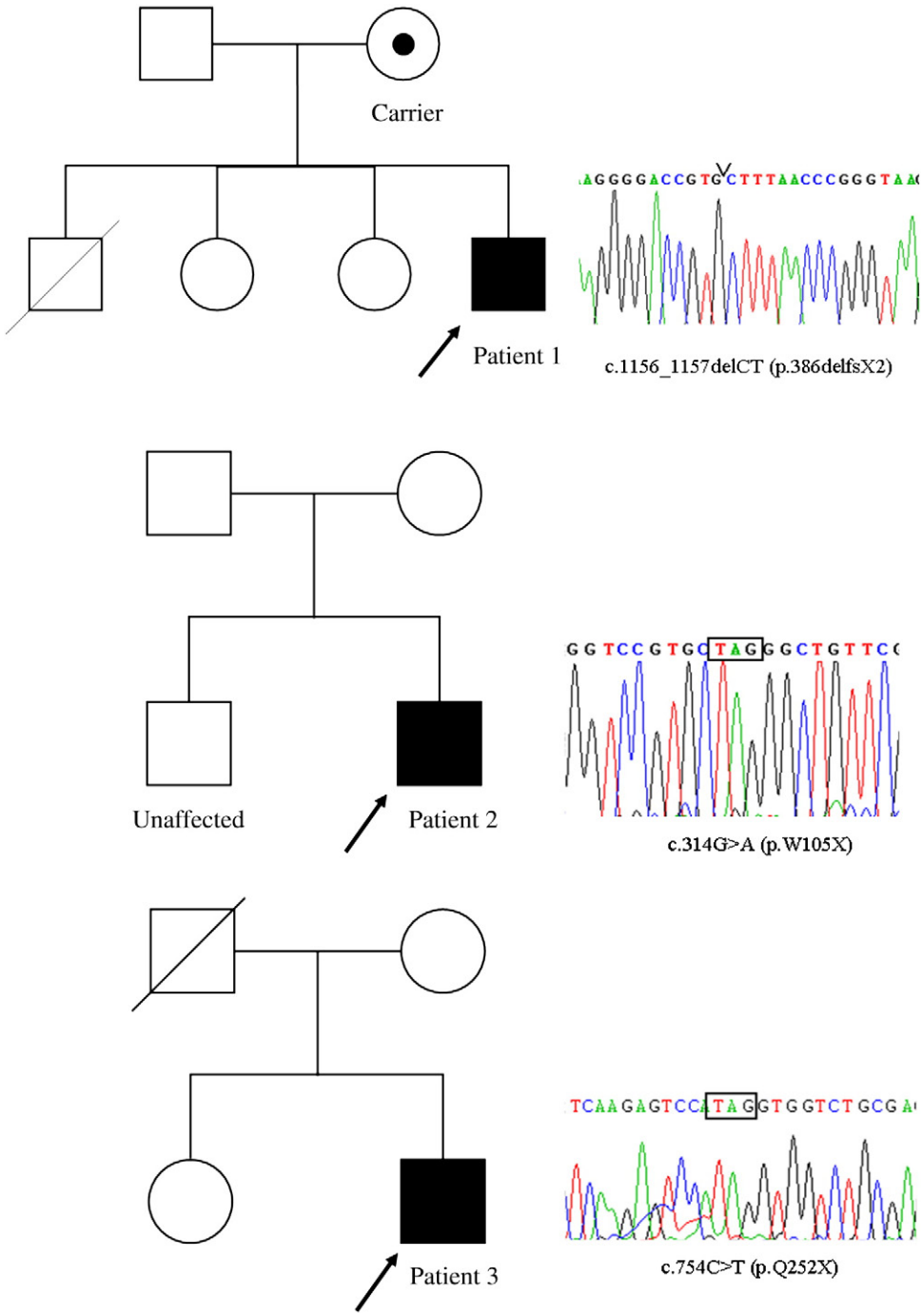


**Fig. 1 – Family pedigrees and genotypes of patients and their families.**

position of the DAX-1 mutation and the age at presentation or diagnosis [14]. Little correlation also is evident between the activity of DAX-1 mutants in functional assays and the age at presentation, other than the case of a patient who first presented in adulthood [18]. Variability of phenotype can occur with the same mutation within a family [19]. With the exception of one mild missense mutation, modifier genes and epigenetic factors are important in determining the ultimate genotype-phenotype correlations of X-linked AHC [9,14].

Although loss of DAX-1 function is associated with adrenal failure and hypogonadotropic hypogonadism in humans, the majority of functional data suggest that DAX-1 is a transcriptional repressor of other nuclear receptors that are expressed in the HPAG axis. The function of DAX-1 as a negative regulator of steroidogenesis may have a more significant role in adult adrenal glands. DAX-1 is likely involved in the control of adrenal steroid production, possibly through repression of steroidogenic genes (this would keep steroid production in control when synthesis is required) as well as through regulation of the transcription of specific biosynthetic genes required for the steroid being secreted [20]. SF-1 functions as a transcriptional activator of many genes

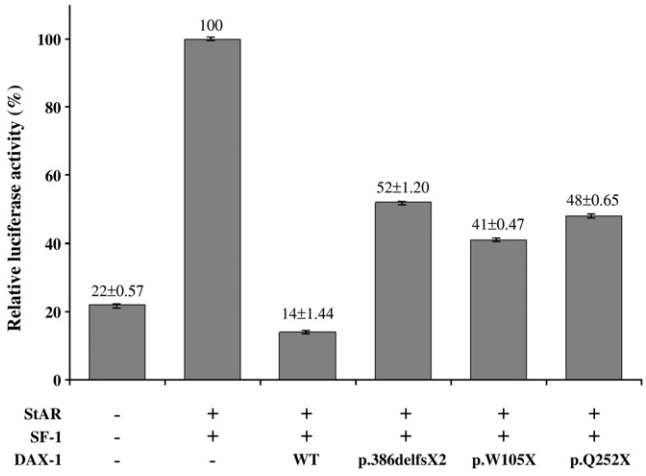


**Fig. 2 – The effect of DAX-1 and its mutants on SF-1–mediated transactivation. Repression by WT DAX-1 was lost when the mutations were introduced. Wild-type DAX-1 suppresses basal transcription activity of the pcDNA3.1 vector down to 14%, whereas this activity is only suppressed down to 52% for p.L386delfsX2, 41% for p.W105X, and 48% for p.Q252X mutants (mutants vs WT, $P < .05$). These results confirm that these mutants display only partial activity.**

involved in steroid hormone biosynthesis in the HPAG axis; and DAX-1 appears to act by complexing with, and inhibiting, the activator function of SF-1 [4]. SF-1 has been characterized as a transcription factor required for the expression of a variety of steroidogenic enzymes [21]. How disruption of DAX-1 leads to AHC and hypogonadotropic hypogonadism appears to be more complex than previously considered and likely involves molecular functions beyond its role as a repressor of SF-1 action [20]. DAX-1 increased GnRH expression in the presence of SF-1, whereas the DAX-1 mutants reduce the regulation of GnRH expression in the hypothalamus [6].

Wild-type DAX-1 was shown recently to inhibit the transcriptional effects of SF-1 [7]. DAX-1 has also been shown to suppress expression of the SF-1–regulated StAR promoter [8]. Deletion of the C-terminal region of DAX-1 reduces its ability to silence gene expression [7]. Therefore, X-linked AHC appears to be correlated with loss of DAX-1 transcriptional repression through the disruption of its silencing domain function. We used these features of DAX-1 to test whether the p.L386delfsX2, p.W105X, and p.Q252X mutations reported here altered DAX-1 function. Each one of these mutations was found to eliminate the ability of DAX-1 to inhibit SF-1–mediated transcription. These types of reporter gene assays may also prove useful for assessing the functional effects of *DAX-1* mutations.

In conclusion, we identified 3 loss-of-function mutations of the *DAX-1* gene (ie, p.L386delfsX2, p.W105X, and p.Q252X) in 3 unrelated Korean patients with X-linked AHC. This study has demonstrated that nonsense and frameshift mutations of the *DAX-1* gene resulted in partial elimination of the ability of DAX-1 to repress the transcription of StAR in an in vitro assay.

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## REFERENCES

[1] Lehmann SG, Wurtz JM, Renaud JP, et al. Structure-function analysis reveals the molecular determinants of the impaired biological function of DAX-1 mutants in AHC patients. Hum Mol Genet 2003;12:1063-72.
[2] Muscatelli F, Strom TM, Walker AP, et al. Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. Nature 1994;372:672-6.
[3] Jimenez P, Saner K, Mayhew B, et al. GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. Endocrinology 2003;144:4285-8.
[4] Clipsham R, McCabe ER. DAX1 and its network partners: exploring complexity in development. Mol Genet Metab 2003;80:81-120.
[5] Zazopoulos E, Lalli E, Stocco DM, et al. DNA binding and transcriptional repression by DAX-1 blocks steroidogenesis. Nature 1997;390:311-5.
[6] Li N, Liu R, Zhang H, et al. Seven novel DAX1 mutations with loss of function identified in Chinese patients with congenital adrenal hypoplasia. J Clin Endocrinol Metab 2010;95: E104-11.
[7] Ito M, Yu R, Jameson JL. DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. Mol Cell Biol 1997;17: 1476-83.
[8] Lalli E, Bardoni B, Zazopoulos E, et al. A transcriptional silencing domain in DAX-1 whose mutation causes adrenal hypoplasia congenita. Mol Endocrinol 1997;11:1950-60.
[9] Achermann JC, Meeks JJ, Jameson JL. Phenotypic spectrum of mutations in DAX-1 and SF-1. Mol Cell Endocrinol 2001;185: 17-25.
[10] Choi JH, Shin YL, Kim GH, et al. Identification of novel mutations of the DAX-1 gene in patients with X-linked adrenal hypoplasia congenita. Horm Res 2005;63:200-5.
[11] Li S, Wilkinson MF. Site-directed mutagenesis: a two-step method using PCR and DpnI. Biotechniques 1997;23:588-90.
[12] Zhang YH, Guo W, Wagner RL, et al. *DAX1* mutations map to putative structural domains in a deduced three-dimensional model. Am J Hum Genet 1998;62:855-64.
[13] Ozisik G, Mantovani G, Achermann JC, et al. An alternate translation initiation site circumvents an amino-terminal DAX1 nonsense mutation leading to a mild form of X-linked adrenal hypoplasia congenita. J Clin Endocrinol Metab 2003;88:417-23.
[14] Reutens AT, Achermann JC, Ito M, et al. Clinical and functional effects of mutations in the DAX-1 gene in patients with adrenal hypoplasia congenita. J Clin Endocrinol Metab 1999;84:504-11.
[15] Achermann JC, Ito M, Silverman BL, et al. Missense mutations cluster within the carboxyl-terminal region of DAX-1 and impair transcriptional repression. J Clin Endocrinol Metab 2001;86:3171-5.
[16] Skinningsrud B, Husebye ES, Gilfillan GD, et al. X-linked congenital adrenal hypoplasia with hypogonadotropic hypogonadism caused by an inversion disrupting a conserved

noncoding element upstream of the NR0B1 (DAX1) gene. J Clin Endocrinol Metab 2009;94:4086-93.
[17] Shaikh MG, Boyes L, Kingston H, et al. Skewed X inactivation is associated with phenotype in a female with adrenal hypoplasia congenita. J Med Genet 2008;45:e1.
[18] Tabarin A, Achermann JC, Recan D, et al. A novel mutation in DAX1 causes delayed-onset adrenal insufficiency and incomplete hypogonadotropic hypogonadism. J Clin Invest 2000;105:321-8.
[19] Bernard P, Ludbrook L, Queipo G, et al. A familial missense mutation in the hinge region of DAX1 associated with late-onset AHC in a prepubertal female. Mol Genet Metab 2006;88:272-9.
[20] Iyer AK, McCabe ER. Molecular mechanisms of DAX1 action. Mol Genet Metab 2004;83:60-73.
[21] Parker KL, Schimmer BP. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 1997;18:361-77.