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Delayed-onset adrenal hypoplasia congenita and hypogonadotropic hypogonadism caused by a novel mutation in *DAX1*

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Abstract

In this study, we described a male who presented with delayed-onset adrenal hypoplasia congenita (AHC) and mild hypogonadotropic hypogonadism (HHG) without a relevant family history. A novel mutation in the *DAX1* (dosage-sensitive sex reversal, congenital adrenal hypoplasia critical region on the X chromosome, gene 1) gene was shown to cause X-linked AHC and HHG. Genetic analysis revealed a novel nonsense mutation, c.154G > T (p.Glu52Term), in the *DAX1* gene. Molecular testing demonstrated that the milder phenotype caused by this mutation was due to expression of a partially functional, amino-truncated *DAX1* protein generated from an alternate in-frame translation start site (methionine at codon 83). This unusual case revealed a potential mechanism for a novel mutation that resulted in an unusual delayed-onset mild clinical phenotype. It expands the spectrum of adrenal hypoplasia congenita and hypogonadotropic hypogonadism.

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Introduction

Adrenal hypoplasia congenita (AHC) is a rare developmental disorder of the adrenal gland. Congenital adrenal deficiency can derive from a number of disorders, including X-linked adrenal hypoplasia congenita (AHC).[1] In affected patients, the adrenal glands lack the permanent adult zone of the adrenal cortex, thus they usually show adrenal insufficiency in infancy and childhood and hypogonadotropic hypogonadism (HHG) in adolescence, resulting in delayed puberty and, sometimes, cryptorchidism.[2] AHC is severe unless appropriate steroid replacement therapy is provided; therefore, the diagnosis has important implications for patients and their families.[3–7]

The gene responsible for AHC is *DAX1* (dosage-sensitive sex reversal, congenital adrenal hypoplasia critical region on the X chromosome, gene 1; also known as *NROB1*),[8–21] which encodes repressor of steroidogenic factor-1 (SDF1), reduces early growth response-1 (Egr1) and SDF1 synergy, and diminishes gonadotropin-releasing hormone (GnRH) stimulation of the luteinizing hormone beta (LHβ) promoter.[22]

To date, a wide variety of mutations in *DAX1* have been described. Most nonsense mutations cause lethal phenotypes unless appropriate steroid replacement therapy is provided. However, a few cases with nonsense mutations have milder clinical manifestations.[23–25] The underlying mechanism of these nonsense mutations with mild phenotypes has not been completely elucidated.

Here, we describe a case of mild AHC and HHG caused by a novel nonsense mutation in *DAX1*. Laboratory tests revealed a potential mechanism to explain the mild clinical phenotype associated with this mutation.

Case report

This study was approved by the Research Ethics Committee of Tongji Hospital (Wuhan, Hubei, China), and written informed consent was obtained from the adult patient and his parents. The propositus was the only child of non-consanguineous parents of Chinese origin. The pregnancy was normal with a normal delivery at 40 weeks of a boy of normal birth weight (3400 g). The child was admitted to the Tongji Hospital at the age of 9 years due to failure to thrive, darkening of the skin, and decreased appetite for the previous 4 months. The physical exam showed generalized pigmentation. Laboratory tests were consistent with a diagnosis of primary adrenal insufficiency. Serum cortisol was 1.3 μg/dL (normal range: 5.0–25.0 μg/dL) at 8 a.m. with a high adrenocorticotropic hormone (ACTH) level of 388 pmol/L (normal range: 1.6–13.9 pmol/L). The patient was treated with hydrocortisone and dexamethasone and the symptoms of hyperpigmentation and poor appetite were controlled.

The patient's medical history remained uneventful until age 28, when he presented for infertility after 3 years of unprotected

intercourse. According to the patient's memory, he went through a normal puberty. Formal testing revealed a normal sense of smell and normal male genital appearance. Biochemical tests showed low endogenous gonadotropin concentrations: luteinizing hormone (LH) was 1.93 mIU/mL (normal adult range: 1.24–8.62 mIU/mL) and follicle-stimulating hormone (FSH) was 5.79 mIU/mL (normal adult range: 1.27–19.26 mIU/mL), but the patient had a low endogenous serum testosterone level of 0.53 ng/mL (normal adult range: 1.75–7.81 ng/mL). LH and FSH did not increase significantly after an injection of 100 μg of gonadotropin-releasing hormone (GnRH). Concentrations of serum testosterone, dehydroepiandrosterone, and androstenedione increased significantly in response to human chorionic gonadotropin (hCG). The relative increases of these hormone levels were $>500\%$ after stimulation by hCG. The subnormal LH response to GnRH stimulation and the normal testosterone response to hCG confirmed the diagnosis of hypogonadotropic hypogonadism (Tables 1 and 2). Anatomy of the hypothalamus and pituitary by magnetic resonance imaging was normal. Combined with the gonadotropin deficiency and primary adrenal insufficiency that occurred in childhood, these results indicated a diagnosis of AHC and HHG.

After obtaining informed consent for blood collection and genomic DNA analysis, genomic DNA was isolated from peripheral blood leukocytes of the patient and his parents using the TIANamp Blood DNA kit (Tiangen Biotech, Beijing, China). Sequencing reactions were performed in the forward and reverse directions using BigDye Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA), and direct sequencing of PCR products was performed for both exons of the *DAX1* gene from both strands using an ABI-Prism 3130xlgenetic analyzer (PE Applied Biosystems, Edison, NJ).

pCDAN3.1(+) expression vectors containing the DAX1 wild-type (WT), c.107C > T (Q37X), c.154G > T (E52X), and c.1197C > G (Y399X) mutations were created by overlapping PCR.[25–27] Expression vectors containing cDNA for WT DAX1 and artificial c.1197C > G (Y399X) mutants were used as positive and negative controls for DAX1 function and in vitro translation. Mutations were verified by direct DNA sequencing.

Total RNA of tissues and cells was extracted with Magzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of mRNA was done using the FastQuant RT Kit (with gDNase; Tiangen Biotech). Quantitative reverse transcription (RT)-PCR analysis was performed with the Fast SYBR Green Master Mix (Invitrogen, Shanghai, China) using synthesized primers from GeneCopoeia (Guangzhou, China).

Human *DAX1* cDNAs for WT, E52X, and Y399X mutants were cloned into the pSicoR/Flag plasmid expression vector

Table 2. hCG testicular stimulation test.

	−15 min	24 h
Testosterone (ng/mL)	0.52	4.92
Dehydroepiandrosterone (ng/mL)	59.36	440
Androstenedione (ng/mL)	0.01	2.82

Table 1. GnRH stimulation gonadotropin secretion test.

	−15 min	0 min	15 min	30 min	45 min	60 min	90 min	120 min
LH (IU/L)	4.2	4.71	5.28	5.12	4.75	3.6	2.25	2.33
FSH (IU/L)	7.1	6.8	7.11	6.88	6.21	6.18	4.99	5.93

and then transfected into human embryonic kidney 293T cells. Protein extraction and immunoblot analysis was performed as previously described.[28] Recombinant *DAX1* was probed with a 1:2000 dilution of the primary antibody toward the Flag epitope and a 1:10,000 dilution of the secondary anti-mouse antibody. Reactive bands were detected using a Millipore kit (Billerica, MA, USA) with Kodak MS X-ray film (ProteinSimple, San Jose, CA, USA).

DAX1-luciferase fusion cDNA sequences were produced by overlapping PCR; the 3′ end of the DAX1 CDS after c.247_249ATG was replaced by the luciferase CDS from the pGL3 basic vector (Promega Corp., Madison, WI, USA) for WT, Q37X, and E52X mutant reporter, and the stop codon of c.1411_1413 TAA was replaced by the luciferase CDS for DAX1 Y399X reporter. All fusion cDNA sequences were then cloned into pCDNA3.1(+) for the mammalian luciferase assay. The 293T cells were transfected with the pCDNA3.1 (+)-DAX1-luciferase reporter vector, and luciferase assays were performed 48 hours after transfection. The results of triplicate transfections are expressed as the mean ± standard error of the mean (SEM).

Mammalian expressed vector containing full-length human *DAX1* WT, Q37X, E52X, and Y399X were constructed in pCDNA3.1(+). A luciferase reporter was added to the pGL3 basic vector (Promega Corp.) containing the native rat *LHβ* promoter (−154 to 5). The reporter vector was co-transfected with expression vectors containing full-length human steroidogenic factor 1 (SDF1; 20 ng) and full-length rat early growth response-1 (Egr1; 20 ng), and then co-transfected with full-length human *DAX1* WT, Q37X, E52X, and Y399X expression vectors separately (50 ng each) into GH-3 cells. Luciferase assays were performed 48 hours after transfection. The results of triplicate transfections are expressed as the mean ± SEM.

Continuous data were compared using one-way analysis of variance. Data are represented as mean ± SEM. In all cases, $P < 0.05$ was considered statistically significant.

Exon sequencing revealed a novel nonsense mutation from GAG to TAG, corresponding to codon 52 (Figure 1a), leading to a translation termination. We did not find other mutations in the *DAX1* gene. Gene analysis of the patient's mother indicated that the mutation was inherited from her allelic variant. A search for the mutation in the patient's father yielded normal results.

Western blotting showed that compared with WT *DAX1* (51 kDa), proteins translated in vitro from cDNA constructs containing the E52X mutation generated a shorter protein product of 43 kDa; and protein was not translated from cDNA constructs containing the Y399X mutation (Figure 1b). Levels of the mutant protein were lower than levels of WT DAX1 protein. This observation appears to reflect a lower translation efficiency from the alternate start site, as mRNA levels were not different for the three constructs (Figure 1c). These results confirmed an amino-truncated form of the protein.

The *DAX1* WT, Q37X, E52X, and Y399X coding sequences, including the initiation codon, were cloned into the pcDNA3.1 vector and the initiation codon of luciferase protein was removed. The recombinant vectors were transfected into 293T cells and incubated for 48 hours. We observed an obvious decrease in luciferase activity in the Y399X group compared with *DAX1* WT, Q37X, and E52X groups, although luciferase activity in the E52X and WT groups were slightly lower than that of Q37X (Figure 1d). This result demonstrated that the initiation codon at position 83 could initiate a truncated DAX1 protein.

DAX1 encodes a repressor of SDF1 activity, reduces Egr1 and SDF1 synergy, and diminishes GnRH stimulation of the

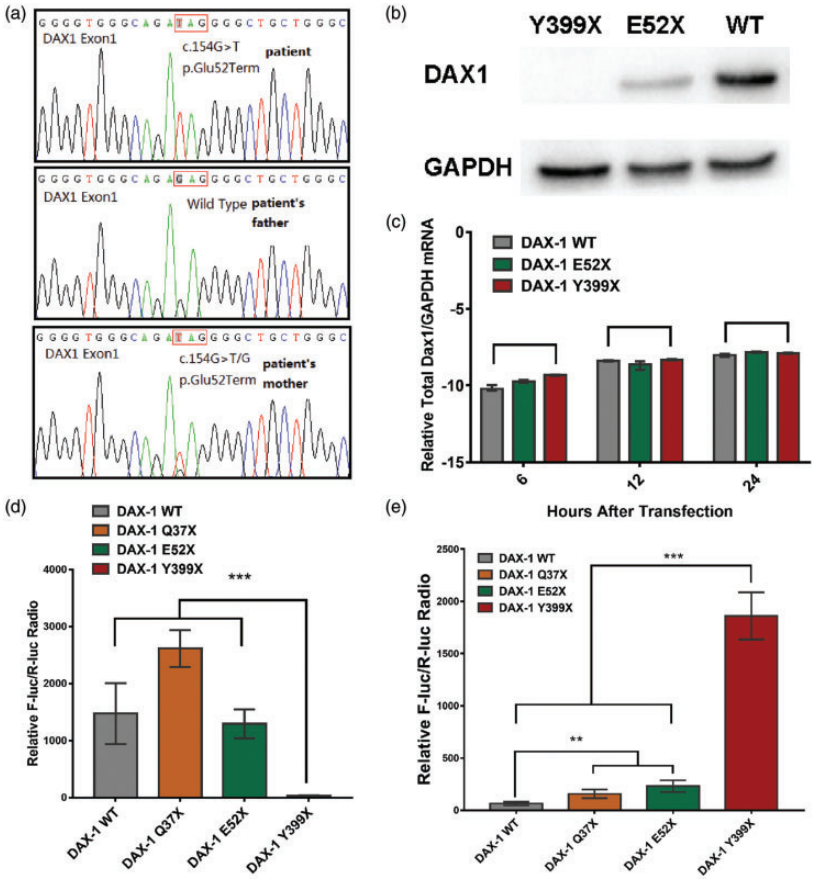

Figure 1. (a) Exon sequencing revealed a novel nonsense mutation from GAG to TAG at codon 52, leading to a translation termination; no other mutations were found in the *DAX1* gene. Gene analysis of the mother indicated that the patient's nonsense mutation was inherited from her allelic variant. A search for the mutation in the father yielded normal results. (b) Detection of the recombinant DAX1 proteins containing the carboxyl terminal Flag epitope. Whole-cell extracts were probed with the anti-Flag antibody by western blotting. Products of approximately 45 and 53 kDa were represented in the reactive bands for the E52X mutation and wild-type (WT), respectively (the tag and epitope add approximately 2 kDa to the molecular mass of the relative proteins). (c) Transcript expression levels of WT, E52X, and Y399X *DAX1* mutants were measured by quantitative PCR after 6, 12, and 24 hours of transfection. The bars represent the copy number of mRNA (the results of triplicate transfections are expressed as the mean ± SEM). (d) The *DAX1* WT, Q37X, E52X, and Y399X coding sequences including the initiation codon were cloned into pcDNA3.1 vectors and the initiation codon of luciferase was removed. The recombinant vectors were transfected into 293T cells and incubated for 48 hours. An obvious decrease in luciferase activity was observed in Y399X compared with the WT, Q37X, and E52X groups, and the luciferase activity of the E52X and WT groups was slightly less than that of the Q37X group. The results of triplicate relative firefly/Renilla luciferase ratio (F-luc/R-luc) are expressed as the mean ± SEM, $^{***}P < 0.001$. (e) The *LHβ* promoter was cloned into a pGL3 vector and co-transfected with *DAX1* WT, Q37X, E52X, and Y399X vectors in GH-3 cells. After 48 hours of co-incubation, the luciferase activity of *DAX1* WT group was obviously decreased compared with the Y399X group and lower levels of luciferase activity were observed in the Q37X and E52X groups compared with the Y399X group. The results of triplicate relative firefly-luciferase/Renilla-luciferase ratio are expressed as the mean ± SEM, $^{**}P < 0.01$; $^{***}P < 0.001$.

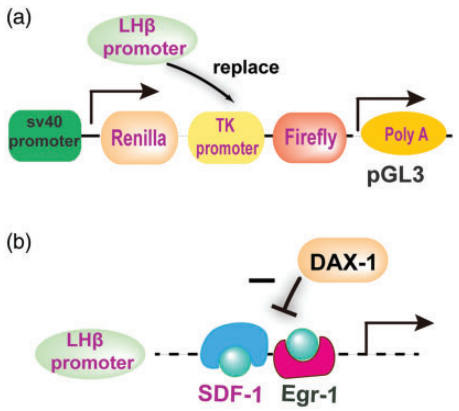

Figure 2. (a) A luciferase reporter was cloned into a pGL3 basic vector containing the native rat *LHβ* promoter (−154 to 5). (b) DAX1 represses SDF1 activity, reduces Egr1-SDF1 synergy, and diminishes GnRH stimulation of the *LHβ* promoter.

LHβ promoter.[22] To further support this conclusion and prove the relationship between DAX1 and LHβ, the *LHβ* promoter was co-transfected with *DAX1* WT, Q37X, E52X, and Y399X vectors (Figure 2). We detected an obvious decrease in luciferase activity of *DAX1* WT compared with Y399X. We also observed lower activities of luciferase reporter in Q37X and E52X groups compared with the Y399X group (Figure 1e). These data validated the partial function of the truncated DAX1 protein induced by these nonsense mutations.

Discussion

In this study, we present a male with AHC and HHG caused by a novel mutation, c.154G > T (p. Glu52Term) in exon 1 of the *DAX1* gene. The clinical phenotype of this patient was unusual due to the delayed onset and normal testes and development of male external genitalia. This mutation resulted in a premature stop codon at the 52nd amino acid. Simultaneously, the codon at 83 could serve as a secondary start codon, which could lead to a truncated yet functional DAX1 fragment. This is the first report of this nonsense mutation occurring in *DAX1*.

Although this novel nonsense mutation should have exerted a great impact on the DAX1 protein, the patient presented only mild symptoms. The partially preserved function of this mutant protein was surprising and led us to consider the connection between genotype and phenotype, as well as the importance of investigating this disease that manifested with only mild symptoms.

This nonsense mutation may result in partly functional truncated proteins, and these truncated proteins may reduce the severity of the clinical phenotype. Clearly, the impact of these different protein-truncating mutations depends on the stability and function of the important protein domains. During translation, a protein is gradually extended from the N-terminal to the C-terminal; in this case, the mutant stop codon at position 52 truncated the N-terminal domain of the DAX1 protein. The function of the amino-terminal part of DAX1 remains unclear, but DAX-1 protein consists of 470 amino acids, and its N-terminal portion includes four incomplete repeats of a new structural motif encoding a DNA binding function. The C-terminal half of the protein demonstrates high homology, and it includes a ligand-binding area of nuclear hormone receptor superfamily.[29] DAX1 is characterized by its repeating motif structure in the N-terminus with assumed functional redundancy among the motifs. In contrast, the loss of the C-terminus of DAX1 normally triggers a severe clinical phenotype.[30]

The presumed functional redundancy of DAX1 is not enough to explain the unusual clinical phenotype of our patient. We assumed that translation could initiate again at an alternate site downstream of the start codon, resulting in a truncated,

yet partially functional protein. There are a few rare cases in which a downstream initiator produces a truncated protein after extreme premature termination in the N-terminal region.[23–25] The Kozak consensus sequences flanking methionine at codon 83 have been identified as a putative internal in-frame translation initiation site of the *DAX1* sequence.[25,31] In our study, methionine 83 was shown to be an alternative internal translation initiation codon by western blotting and the dual luciferase assay, and the resultant shorter DAX1 protein was shown to partially rescue the clinical phenotype of this patient, consistent with the delayed onset of adrenal insufficiency. Here, we showed that translation from an internal in-frame start site downstream of a nonsense mutation could reduce the clinical severity of AHC. Translation efficiency initiating from the backup start site was normal, because mRNA levels did not differ for the three constructs.

Furthermore, *DAX1* gene dosage seems to play a key role in protein function.[32–34] Thus, it is possible that the clinical features reflect reduced expression from a hypomorphic allele in addition to effects on the protein itself. Our findings also showed that the residual DAX1 function was sufficient to delay the onset of overt adrenal failure but was not capable of supporting normal testis development and function.

No similar phenotypes were found in the patient's family. Taking the Chinese one-child policy into account, the diagnosis of X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism should not be eliminated without similar family history. *DAX1* gene analysis is recommended when the clinical picture is suggestive of AHC and HHG.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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