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Unique Dominant Negative Mutation in the N-Terminal Mitochondrial Targeting Sequence of StAR, Causing a Variant Form of Congenital Lipoid Adrenal Hyperplasia

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Context: Steroid acute regulatory (StAR) protein is a mitochondria-targeted protein that is part of the transduceosome complex crucial for transport of cholesterol to mitochondria. Recessive mutations cause classic and nonclassic congenital lipoid adrenal hyperplasia.

Objective: The aim of this study was to report the clinical, hormonal, genetic, and functional data of a novel heterozygous mutation in the *StAR* gene found in a 46,XY patient with ambiguous genitalia and neonatal severe steroidogenic deficiency.

Patient: Undetectable serum steroids with high ACTH and plasma renin activity but normal acute GnRH response were found in infancy. After gonadectomy (at 3 yr of age), serum LH and testosterone were undetectable, whereas FSH was normal but increased slowly afterward. Estrogen replacement therapy, started at 10.2 yr of age, suppressed gonadotropins (for 2 yr). However, after 1 month off estrogens, the patient showed castrated levels. At 11.9 yr old, after fludrocortisone withdrawal because of hypertension, plasma renin activity and aldosterone remained normal, suggesting mineralocorticoid recovery by a StAR-independent mechanism.

Results: We found a *de novo* heterozygous IVS-2A>G *StAR* mutation and the reported heterozygous p.G146A SF1 polymorphism with normal *CYP11A1*, *FDXR*, *FDX1*, *VDAC1*, and *TSPO* genes. The mutant StAR transcript lacked exon 2, resulting in the in-frame loss of amino acids 22 to 59 in the N-terminal mitochondrial targeting signal. *In vitro*, the mutant protein exhibited reduced StAR activity in a dominant-negative manner and almost no mitochondria localization.

Conclusions: A misfolded p.G22_L59del StAR might interfere with wild-type StAR activity by blocking the transduceosome complex, causing an autosomal dominant form of StAR deficiency, explaining the clinical phenotype. We speculated that estrogen might have modulated mineralocorticoid function and pubertal maturation in a human natural model lacking endogenous steroid production. (***J Clin Endocrinol Metab* 98: 0000–0000, 2013**)

Steroid acute regulatory (StAR) protein is required for adrenal and gonad steroidogenesis and for male sexual differentiation. It facilitates the movement of cholesterol from the outer to the inner mitochondrial membrane, thus providing the substrate for steroid hormone biosynthesis (1). In mitochondria of adrenal cortex and gonads, the conversion of cholesterol to pregnenolone is catalyzed by the cholesterol side-chain

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Abbreviations: CLAH, Congenital lipoid adrenal hyperplasia; DHEAS, dehydroepiandrosterone sulfate; DSD, disordered sexual development; ERT, estrogen replacement therapy; hCG, human chorionic gonadotropin; PRA, plasma renin activity; SF1, steroidogenic factor-1; StAR, steroid acute regulatory; T, testosterone; TSPO, translocator protein; VDAC1, voltage-dependent anion channel 1; WT, wild-type.

cleavage enzyme (*CYP11A1*) supported by its electron transport system consisting of reduced nicotinamide adenine dinucleotide phosphate, adrenodoxin reductase (*FDXR*) and adrenodoxin (*FDX1*) (2).

StAR is synthesized as a 37-kD 285-amino acid preprotein including a mitochondrial-targeting sequence (N-terminal 62-amino acid) that is cleaved during import into the mitochondria to yield the mature protein (30 kD) (3, 4). The mechanism of StAR translocation and action remains unclear. The evidence shows that StAR is part of a multiprotein complex, termed transduceosome (5), where the StAR pre-protein is the active molecule, and its phosphorylation is critical for the steroidogenesis-enhancing activity (6–10).

Recessive StAR mutations cause classic and nonclassic congenital lipoid adrenal hyperplasia (CLAH). Patients with classic CLAH usually present with neonatal adrenal insufficiency and have female external genitalia irrespective of genetic sex (11). Affected individuals with nonclassical CLAH present late-onset adrenal insufficiency with mildly disordered sexual development (DSD) (12–15).

We are reporting the clinical, hormonal, genetic, and functional data of a novel heterozygous mutation in the *StAR* gene found in a 46,XY patient with ambiguous genitalia and severe impairment of adrenal and gonadal steroidogenesis, who was raised as a female. Functional studies argued for the pathogenicity of the mutation. The molecular consequences of a novel heterozygous splice-junction *StAR* mutation disrupting mitochondrial targeting signal have been analyzed. During the long-term clinical follow-up after castration and during estrogen replacement therapy (ERT), we showed for the first time spontaneous recovery of mineralocorticoid function but not glucocorticoid and adrenal androgen function. In addition, a stimulatory effect of peripubertal ERT on pubertal maturation of LH secretion was observed in this human natural model of complete absence of endogenous sex steroids.

Subject and Methods

Case report

A 46,XY DSD Caucasian baby presenting with ambiguous genitalia was the first child born to nonconsanguineous healthy parents at term after an uneventful pregnancy (birth weight, 3960 g). The physical exam at 7 d of life revealed hyperpigmented skin and jaundice, a 0.5-cm width × 0.5-cm length phallus with penoscrotal hypospadias and poorly developed corporal tissue, complete labial fusion, and bilateral inguinal gonads approximately 1–1.5 cm in length (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). In the second week of life, the baby presented with severe adrenal insufficiency. In the neonatal period, laboratory tests revealed undetectable serum dehydroepiandrosterone sulfate (DHEAS), androstenedione, 17-hydroxyprogesterone, testosterone (T), cortisol, and aldosterone, high levels of ACTH and renin/plasma renin activity (PRA), as well as a lack of response to human chorionic gonadotropin (hCG), consistent with severe impairment of adrenal and testicular steroidogenesis (Table 1). The baby was started on hydrocortisone and fludrocortisone therapy with good clinical response. Adrenal glands were normally visible by abdominal ultrasound and computed

TABLE 1. Fourteen-year follow-up of blood hormone determinations and treatments in the 46,XY patient

Chronological age	7 days	3 months	2.8 yr	6.2 yr	10 yr	11.4 yr	12.1 yr	12.5 yr	13.2 yr	14.1 yr
Basal LH (mIU/ml)	<0.10	0.57	<0.10	0.19	0.27	<0.10	**29.6**	<0.10	5.45	11.2
Peak[a]		12.9	0.42		1.34					
Basal FSH (mIU/ml)	<0.10	3.8	1.54	**9.86**	**10.7**	<0.10	**66.7**	0.15	**13.8**	**15.7**
Peak[a]		15.2	6.5		**30.2**					
Basal T, ng/ml (nmol/liter)	**<0.05 (<0.17)**	**<0.05**	<0.05	<0.05	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**
Peak[b]	**0.29 (<1)**									
Basal progesterone, ng/ml (nmol/liter)	2.1 (6.68)		0.5	<0.10			0.21		<0.2	<0.2
Basal androstenedione, ng/ml (nmol/liter)	**<0.10 (<0.35)**		0.12	<0.10	**<0.10**	**<0.10**	**<0.10**	**<0.10**	**<0.10**	**<0.10**
17-Hydroxyprogesterone, ng/ml (nmol/liter)	**<0.10 (<0.3)**		<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
DHEAS, ng/ml (μmol/liter)	**<10 (<0.03)**		**<10**	**<10**	**<10**	**<10**	**<10**	**<10**	**<10**	**<10**
Cortisol, μg/ml (nmol/liter)	**<1 (<28)**									
ACTH, pg/ml (pmol/liter)	**143 (31.5)**		15.3	49	<10	12.9		31	124	19.6
Glucose, mg/dl (mmol/liter)	**15 (0.83)**									
Aldosterone (pmol/liter)	**<25**						124	193	92	164
PRA, ng/ml · h (pmol/liter · h)	**28 (22)**		0.27	7.5			<7		2.17	0.65
Serum renin (pg/ml)					15.7	19.5		15		
Sodium/potassium (mEq/liter)	**126/8.3**						145/3.6	142/3.8	139/4.2	136/3.8
Hydrocortisone dose (mg/m^2 · d)		30	13	10.8	11	11	10.2	13	9	11.4
Fludrocortisone dose (μg/d)		100	100	100	100	100	—	—	—	—
ERT conjugated estrogens (mg/d)						0.625	—	0.625	0.625	0.625

Data within *parentheses* are expressed in International System of Units. Abnormal values are shown in *boldface*. Conversion factors to International System of Units: T, ng/ml × 3.467 to nmol/liter; progesterone, ng/ml × 3.18 to nmol/liter; androstenedione, ng/ml × 3.49 to nmol/liter; 17-hydroxyprogesterone, ng/ml × 3.03 to nmol/liter; cortisol, μg/dl × 27.6 to nmol/liter; ACTH, pg/ml × 0.22 to pmol/liter; aldosterone, ng/dl × 27.7 to pmol/liter; PRA, ng/ml · h × 0.77 to pmol/ml · h; renin, pg/ml × 0.0237 to pmol/liter.

[a] Peak, acute GnRH test.

[b] Peak in the hCG test; bilateral castration at the age of 3 yr.

tomography scan. Genitography revealed the presence of a wide urogenital sinus. After careful evaluation, female sex assignment was recommended by the Hospital DSD Multidisciplinary Team, in agreement with the parents.

In early postnatal life, serum basal LH, FSH, and T levels were almost undetectable, but at 3 months of age, the response to GnRH was compatible with normal early postnatal activation of the gonadotropin-gonadal axis (Table 1). The gonads were removed at the age of 3 yr, and histological analysis showed normal bilateral immature testes (Supplemental Fig. 2).

During childhood, serum basal LH and T levels remained undetectable, whereas serum basal FSH levels were within the normal range for girls, but high for boys, up to 6 yr of age. Thereafter, FSH increased above the normal reference range for both sexes. At 10 yr of age, an exaggerated serum FSH but very low serum LH response to a GnRH stimulation test was observed (Table 1). The patient grew and developed normally; however, a clear delay in bone age was observed. ERT, started at 10.2 yr of age, suppressed gonadotropins to undetectable levels. At 12 yr of age, after ERT discontinuation for 1 month, serum LH and FSH levels increased above normal pubertal reference values for both sexes but returned to almost undetectable levels when ERT was restarted. Subsequently, however, a slight and persistent increment of both gonadotropins was observed (Supplemental Fig. 3).

During follow-up, renin/PRA levels remained within the lower normal range. Hypertension was recorded on a routine clinical exam at 11.9 yr of age. Discontinuation of fludrocortisone therapy and introduction of a low-sodium diet resulted in normalization of blood pressure. After fludrocortisone withdrawal, PRA remained within the lower normal range between 0.13 and 3.22 ng/ml · h (0.1–2.5 pmol/ml · h), whereas serum aldosterone levels were detected within the normal range between 92 and 193 pg/ml (255–535 pmol/liter) [normal range, 27–218 pg/ml (75–605 pmol/liter)], suggesting adrenal mineralocorticoid production recovery (Table 1).

The study was approved by the Ethical Review Board of the Garrahan Pediatric Hospital. Written informed consent was obtained from all participants.

Hormonal assays

Serum LH and FSH levels were determined by the Imx systems (Abbott Laboratories, Abbott Park, IL); assay sensitivity was 0.3 IU/liter for LH and 0.2 IU/liter for FSH; interassay coefficient of variation ranged from 3.1- 8.7% for LH and from 3.8–12% for FSH. Serum T was determined by a DPC Immulite Assay System (Diagnostic Products, Los Angeles, CA); assay sensitivity was 0.17 nmol/liter; interassay coefficient of variation ranged from 7.4 to 13%. Serum cortisol was determined by a DPC Immulite Assay System; assay sensitivity was 0.028 nmol/liter; interassay coefficient of variation ranged from 5.2 to 5.9%. Serum DHEAS was determined by a DPC Immulite Assay System; assay sensitivity was 0.052 nmol/liter; interassay coefficient of variation ranged from 6.3 to 8.8%. Serum androstenedione was determined by RIA [Diagnostic Systems Laboratories (DSL), Inc., Webster, TX]; assay sensitivity was 0.35 nmol/liter; interassay coefficient of variation ranged from 7 to 9.8%. Serum 17-hydroxyprogesterone was determined by RIA (DSL); assay sensitivity was 0.30 nmol/liter; interassay coefficient of variation ranged from 8.5 to 11%. ACTH was determined by the DPC Immulite Assay System; assay sensitivity was 5 pg/ml (16.6 pmol/liter); interassay coefficient of variation ranged from 6.1 to 10%. Serum aldosterone was determined by RIA (DSL); assay sensitivity was 7.64 pg/ml (21.2 pmol/liter); interassay coefficient of variation ranged from 5.9 to 9%. Serum renin was determined by immunoradiometric assay; assay sensitivity was 0.7 pg/ml; interassay coefficient of variation ranged from 1.78 to 2.64%. PRA was determined by RIAZEN; assay sensitivity was 0.033 ng/ml · h; interassay coefficient of variation ranged from 3.82 to 5.15%. Hormonal parameters were determined periodically.

At 7 d of age, a hCG stimulation test was performed as follows: after drawing a basal sample for T assay, two 2500-IU doses of hCG were administered im on d 0 and 3, and the response was evaluated on d 7. A GnRH test was performed at the ages of 3 months, 2.8 yr, and 10 yr. LH and FSH levels were measured at 0, 20, and 60 min after a GnRH (100 μg) iv bolus.

Genetic analysis

Genomic DNA from the patient, her relatives, and 50 healthy controls was isolated from peripheral blood lymphocytes by conventional methods (16). The coding and flanking intronic regions, including the 5′-flanking noncoding regulatory region, of *StAR*, *CYP11A1*, steroidogenic factor-1 (*SF1*), *FDXR*, *FDX1*, voltage-dependent anion channel 1 (*VDAC1*) and translocator protein (*TSPO*) genes were amplified by PCR using specific primers (primer sequences are listed in Supplemental Table 1) (17–19). PCR products were purified (QIAquick Gel Extraction Kit; QIAGEN, Buenos Aires, Argentina) and sequenced with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Buenos Aires, Argentina) using the Big Dye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems). The primers used for sequencing were the same as those used for PCR. The sequences were compared with the NCBI entries of *StAR* (NG_021399.1), *CYP11A1* (NG_007973.1), *SF1* (NG_008176.1), *FDXR* (NM_024417.2), *FDX1* (NM_004109.4), *VDAC1* (NG_027817.1), and *TSPO* (NM_000714.5).

RNA isolation and RT-PCR from gonad tissue

Total RNA was extracted from the patient's testicular tissue by homogenization in TRIzol reagent (Invitrogen, Buenos Aires, Argentina) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Amersham Biosciences, Buenos Aires, Argentina) and oligo dT following the manufacturer's instructions as previously described (20). The cDNA was then amplified by PCR with specific oligonucleotide primers for exons 1 and 5 of *StAR* gene (21) and with primers that span the entire coding region of *StAR* (1) and *FDXR* genes (Supplemental Table 1). A cDNA pool prepared from human prepubertal testicular tissues analyzed in a previous report (20) was used as positive control. Negative controls lacking cDNA were included in all PCRs. PCR products were analyzed on 2% agarose gels containing ethidium bromide and visualized using a UV transilluminator. The identities of RT-PCR products were verified by sequencing analysis.

Functional studies

Plasmid construction, cell transfection, steroid measurement, and Western blotting

StAR activity was measured as pregnenolone production in COS-1 cells cotransfected with either wild-type (WT) or mutant StAR and a vector expressing the F2 fusion of the cholesterol side-chain cleavage system (22). Vectors expressing cDNA of

WT StAR (23) and the fusion protein P450 side-chain cleavage/adrenoxin/adrenoxin reductase (F2) (22) were kindly provided by Dr. Walter L. Miller (University of California, San Francisco, CA). Mutant full-length StAR cDNA expression vector was generated by RT-PCR from the patient gonad tissue. The patient's StAR cDNA was PCR amplified using primers S1 and AS1 (1) (Supplemental Table 1) carrying *Bam*H1 and *Eco*RI restriction enzyme sites, respectively. The 860-bp PCR product corresponding to mutant StAR cDNA was 2% agarosa gel-purified using the QIAquick Gel Extraction Kit (QIAGEN). The fragment was digested with *Bam*H1/*Eco*RI and cloned into the *Bam*H1 and *Eco*RI sites of the WT StAR pCMV-Flag vector (23) after removing the 974-bp WT StAR cDNA from it. The accuracy of the construct was confirmed by sequencing.

Nonsteroidogenic COS-7 cells (fibroblast-like cell line derived from monkey kidney tissue, ATCC CRL-1651) were grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37 C in a humidified 5% CO_2 incubator. Cells were divided into 12-well plates and cotransfected using lipofectamine 2000 (Invitrogen) with empty vector, WT StAR, or mutant StAR constructs together with the F2 vector at approximately 80% confluence. To monitor transfection efficiency, cells were also cotransfected with 60 ng firefly luciferase reporter plasmid (pGL3-Promoter Vector) (Promega, Buenos Aires, Argentina) per well. An empty vector served as negative control to measure StAR-independent steroidogenesis (1, 11). 20α-Hydroxycholesterol (5 μg/ml) (Sigma-Aldrich, Buenos Aires, Argentina), which readily diffuses into mitochondria and thereby bypasses the action of StAR and provides an index of maximal mitochondrial steroidogenic capacity, was added as a positive control (1). Culture media were collected 48 h later, and pregnenolone production from cholesterol (endogenous or from the serum in the culture medium) was measured by enzyme immunoassay (ALPCO Diagnostics, Salem, NH). Sensitivity of this assay is 0.054 ng/ml (0.17 nmol/liter); interassay coefficient of variation ranged from 8.4 to 11.7%. Data are presented as the mean ± SD for three independent experiments, each performed in triplicate.

Cells were lysed in passive lysis buffer (Promega, Buenos Aires, Argentina). Protein content was determined using the Bradford method. Luciferase assay was performed following the standard protocol of the manufacturer (Luciferase assay System E1500; Promega, Madison, WI).

Western blot analysis using a rabbit polyclonal antihuman StAR antibody (kindly provided by Dr. Jerome F. Strauss, III, University of Pennsylvania, Philadelphia, PA) was used in a standard protocol to ensure the expression and translation of the intact WT StAR and mutant protein (3). Steroidogenic human adrenocortical H295R cell lysate was used as StAR positive control.

Immunofluorescence analysis

COS-7 cells transfected with the WT or mutant StAR vector were incubated with Mito tracker Red CMXRos reagent (Molecular Probes, Eugene, OR) 100 mM for 15 min to identify mitochondria (red emission). After washing, cells were harvested by trypsinization and fixed using PBS containing 4% formaldehyde for 15 min. Fixed cells were then cytocentrifugated in a Cytospin 3 (Shandon; Thermo Electron Corporation, Waltham, MA) centrifuge for 5 min at 750 rpm. After blocking with 1.5% normal goat serum in PBS for 1 h, slides were incubated with rabbit polyclonal antihuman StAR antibody diluted 1/200 for 1 h at room temperature, followed by the secondary Alexa Fluor 488 goat antirabbit antibody (5 μg/ml) (Molecular Probes) for 1 h. After the last wash, slides were mounted and examined in an Olympus FV300 Confocal laser scanning microscope.

To control for nonspecific immunofluorescent staining, cells were also incubated in solutions in which the primary antibody was omitted.

Results

Genetic analysis

The patient was found to be heterozygous for a novel A to G nucleotide change at the first base of the 3′ acceptor splice site of intron 1 in *StAR* gene (IVS1–2A>G) (Fig. 1A). Her parents did not contain this mutation. The sequence variation has not been detected in 100 independent *StAR* alleles.

Moreover, sequencing of the patient *SF1* gene revealed a G to C heterozygous substitution in exon 4 (Fig. 1B). This single nucleotide shift resulted in the previously reported G146A nonsynonymous polymorphism in 8.2% of the normal Japanese population (24). Interestingly, WuQiang *et al.* (24) have reported that G146A polymorphism slightly reduces the SF1 transactivation function. The father and the

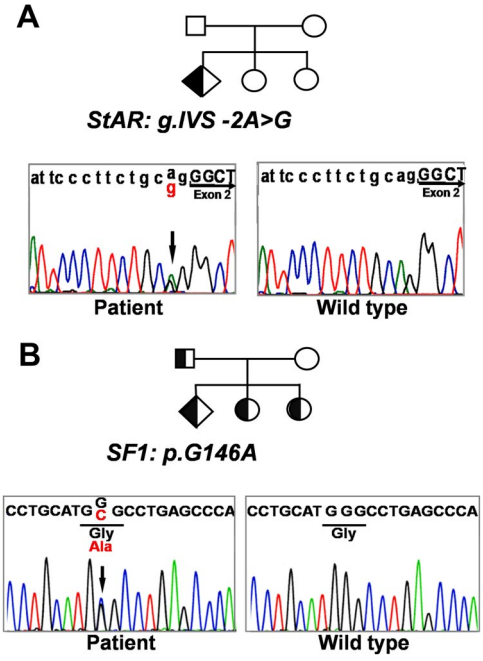

FIG. 1. Pedigree of affected family and mutation analysis by DNA sequencing. A, The proband is heterozygous for a novel *de novo* IVS1–2A>G substitution in *StAR* gene. B, The proband is heterozygous for a G to C substitution in exon 4 of *SF1* gene, resulting in the G146A nonsynonymous polymorphism. The father and the two unaffected sisters were also heterozygous for the SF1 polymorphism. The patient is marked with a *diamond* because of the congenital DSD. The *vertical arrows* indicate the position of the mutations. The exonic sequence is indicated in *capital letters*, whereas the intronic sequence is in *small letters*.

two unaffected sisters were also heterozygous for the SF1 polymorphism. Allele frequencies have varied distributions among ethnic groups ranging from 0.795 for allele G in individuals of African ancestry to 0.011 in individuals of European ancestry (1000 Genomes Project). No mutations were found in *CYP11A1*, *FDXR*, *FDX1*, *VDAC1*, and *TSPO* genes. We also found the following heterozygous single nucleotide polymorphisms in the patient's FDXR gene: rs35832918 C/T, rs1688149 A/G, rs2070920 C/T, rs35102176 C/G, rs2070921 C/T, rs552432 C/T, rs34495658 C/T, rs33991203 C/T, and rs690371 C/T. All these genetic variants were validated by the 1000 Genomes Project and also cluster and frequency (Supplemental Table 2).

mRNA expression analysis in the patient's testicular tissue

The A to G substitution in *StAR* gene disrupted the 3′ consensus sequence critical for splicing (the original acceptor sequence ag/G was changed to gg/G in mutant gene) (25). Therefore, the possibility that an IVS1–2A>G substitution at the 3′ splice acceptor site of intron 1 might reduce the efficiency of the acceptor site in splicing events was considered.

Because fresh gonad tissue of the patient was available, we could perform *in vivo* analysis of StAR mRNA to verify the hypothesis. As shown in Fig. 2A, amplification of cDNA from normal human prepubertal testes (20) showed the expected WT transcript. However, a smaller fragment was also detected in the patient's testicular tissue. Sequence analysis revealed that the smaller fragment lacked exon 2 (114 bp). Sequence analysis of the mutant cDNA suggested that an aberrant mRNA lacking exon 2 would result in the in-frame loss of amino acids 22 to 59 in the N-terminal mitochondrial targeting signal (p.G22_L59del StAR) (Fig. 2B).

Multiple alternatively spliced transcript variants have been found for the *FDXR* gene. The variant 1 represents the predominant transcript (26). It encodes isoform 1. To

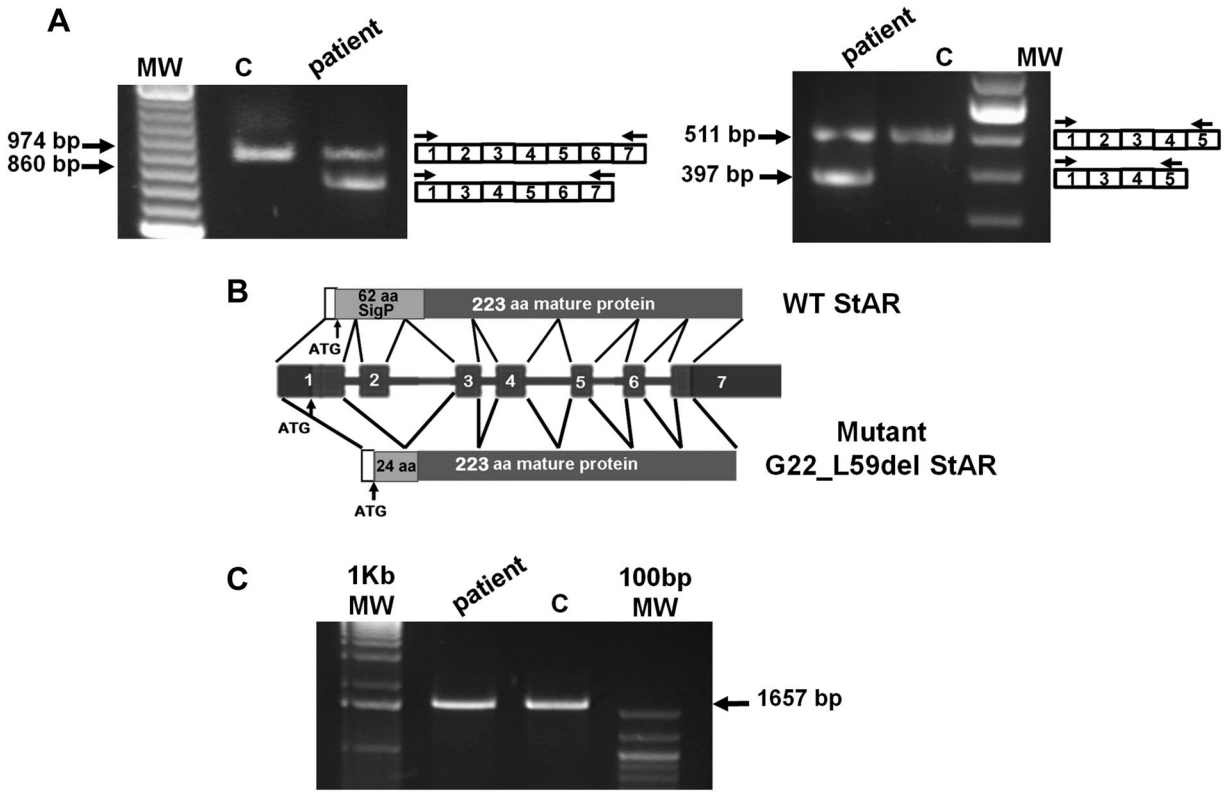

FIG. 2. RT-PCR analysis in the patient's testicular tissue. A, RT-PCR analysis of the StAR mRNA. mRNA from the patient's testicular tissue and from normal human testes were reverse-transcribed and amplified with primers S1 and AS1 (1) (*right*) and with primers StAR1→18 and StAR511→494 (23) (*left*). cDNA from the patient exhibited two spliced forms: the normally spliced coding region and a shorter product corresponding by sequencing to the complete exon 2 (114 bp) skipping. The primers used are indicated by *arrows*. C, cDNA pool of normal human testes; MW, 100-bp molecular weight marker (Invitrogen). B, Diagrammatic representation of *StAR* gene organization and the transcription products of normal and mutant genes. A mutation at position −2 in the acceptor splice site of intron 1 results in the skipping of exon 2. Normally spliced product (*top*) results in a transcript that encodes for the 285-amino acid StAR protein. The mutant transcript (*bottom*) lacks exon 2, resulting in the in-frame loss of amino acids 22 to 59 in the N-terminal mitochondrial targeting signal (G22_L59del StAR). C, RT-PCR analysis of the FDXR mRNA. RNA from the patient's testicular tissue and from normal human testes was used to perform end-point RT-PCR of FDXR full-length cDNA. Patient FDXR was normally spliced. C, cDNA pool of normal human testes; MW, molecular weight marker.

determine whether there was an effect of the *FDXR* genotype on its RNA expression, the patient's testicular RNA for FDXR was reverse-transcribed, amplified, sequenced, and compared with normal testicular tissues. No differences in transcript variant expression were found (Fig. 2C). Sequence analysis of full-length FDXR cDNA showed that normal human testicular tissue and the patient's testicular tissue only expressed FDXR isoform 1.

In vitro functional analysis

Western blot analysis confirmed that both the mutant and the WT StAR protein could be translated in COS-7 cells. Both the 37-kDa precursor and the 30-kDa mature forms were readily detected (Fig. 3A). However, the mutant p.G22_L59del StAR was processed to the mature form to a relatively lesser extent than the WT protein (Fig. 3A), suggesting an impairment of processing and entry of the mutant protein into the mitochondria. Therefore, we

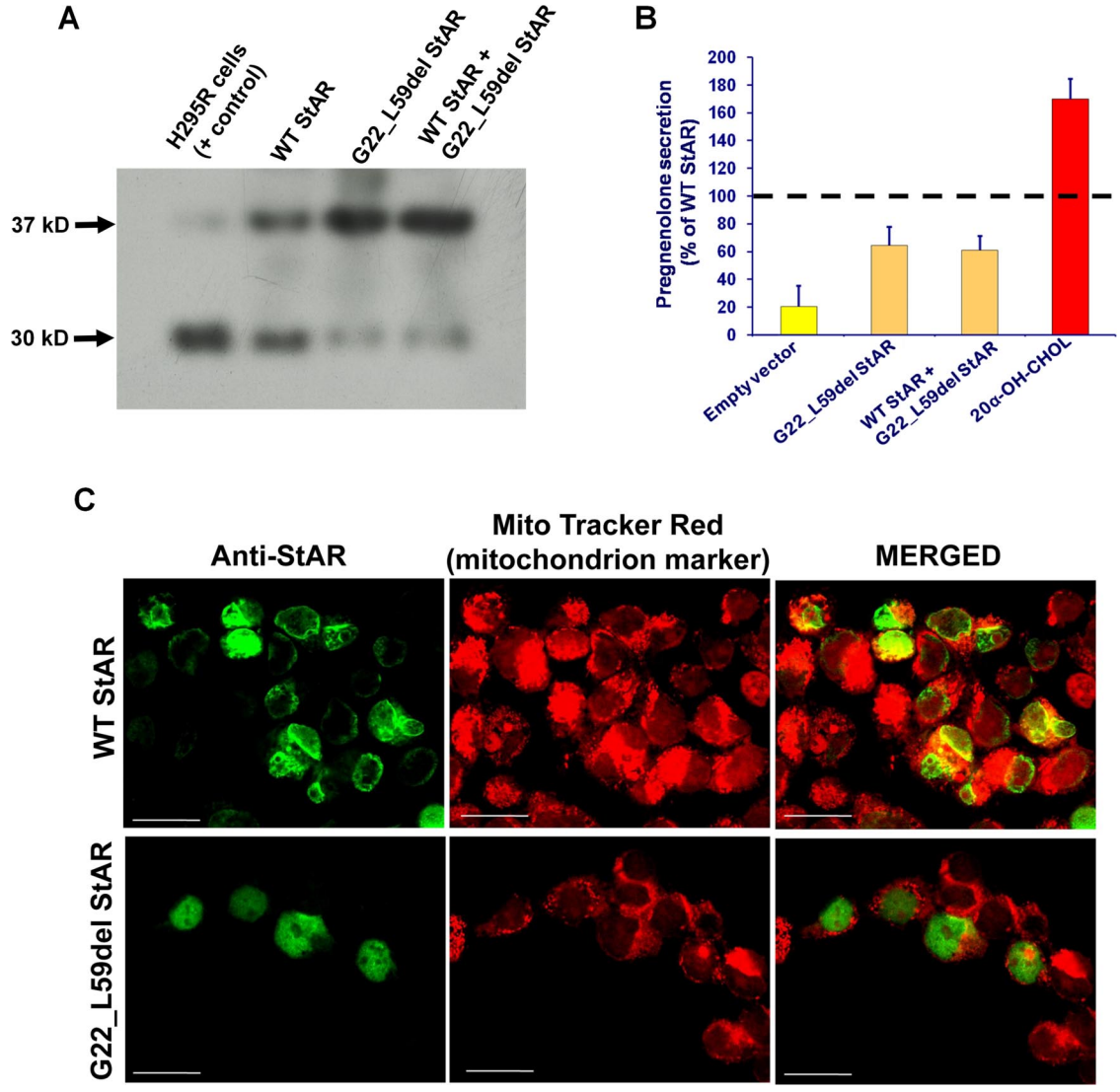

FIG. 3. *In vitro* studies of mutant StAR. A, Western blot analysis. COS-7 cells were transiently transfected with the indicated expression vectors. The *arrows* indicate the precursor and mature StAR-specific bands. StAR-specific bands for the WT StAR and the mutant p.G22_L59del StAR were detectable. H295R cell lysate was used as StAR positive control. B, Functional testing of the identified StAR mutation *in vitro*. COS-7 cells were transiently cotransfected with expression vectors for the side chain cleavage system (F2) and the indicated vectors. After 48 h, pregnenolone secreted into the culture medium was measured by a commercially available ELISA assay. 20α-OH-CHOL, steroidogenesis from control cells incubated with 20α-hydroxycholesterol (5 μg/ml); all other data show steroidogenesis from endogenous cellular cholesterol. The value for the WT StAR is set as 100% (*dashed line*). Results are given as mean ± SD for three independent experiments, each performed in triplicate. Pregnenolone secretion from endogenous cholesterol by COS-7 cells transfected with the F2 vector and the empty control vector was 5.2 ± 1.3 ng/ml (16.4 ± 4.1 nmol/liter). In the presence of WT StAR, pregnenolone secretion averaged 30.9 ± 8.1 ng/ml (97.6 ± 25.6 nmol/liter). C, Laser scanning confocal microscopy of transfected COS-7 cells. Cells were grown and incubated with MitoTracker Red CMXRos (*red*, *middle column*) and antihuman StAR polyclonal antiserum as primary and the antirabbit Alexa Fluor488 antibody as secondary antibody (*green*, *left column*). Image overlay (*right column*) shows colocalization of mitochondria and StAR in COS-7 cells expressing WT StAR. A very low colocalization of mutant p.G22_L59del StAR and mitochondria was observed. *Scale bar*, 20.0 μm.

performed immunofluorescence studies with Mito tracker Red (a mitochondrion marker) to assess the subcellular localization of the mutant protein. As shown in Fig. 3C, almost no colocalization of the mitochondria and the mutant p.G22_L59del StAR was observed. In addition, functional StAR activity studies revealed that the mutant p.G22_L59del StAR had 65% (±13%) of WT StAR activity (Fig. 3B).

Because IVS1–2A>G mutation was found in the heterozygous state, we tested the possibility that it might act as a dominant negative mutant. Cotransfection with WT and p.G22_L59del StAR vectors also reduced WT activity by 62% (±13.9%) (Fig. 3B), suggesting a dominant-negative effect.

Discussion

The present data describe the characterization of the novel heterozygous splice-junction IVS-2A>G StAR mutation found in a 46,XY patient with ambiguous genitalia, adrenal insufficiency, and severe impairment of adrenal and gonadal steroidogenesis characteristic of classic CLAH.

Surprisingly, after almost 2 yr of ERT, adrenal mineralocorticoid function was recovered at 11.9 yr of age. Although the mechanism is unknown, it can be speculated that estrogens might induce aldosterone production by a StAR-independent mechanism. In this regard, a recent report from Nishimoto *et al.* (27) showed that estrogens increased the number of cell clusters, located beneath the adrenal capsule, which produce aldosterone constitutively without renin-angiotensin system regulation.

After gonadectomy, a selective FSH increase with persistence of undetectable LH levels was observed. However, 2-yr ERT allowed for an increase of serum LH levels, detected after transient ERT withdrawal. This finding suggests that estrogen might have modulated pubertal maturation in a human model with complete postnatal absence of endogenous sex steroids. In this line, it has been reported in rodents (28) that the rise in the number of Kiss1 neurons at the anteroventral periventricular area critically depends on some degree of estrogenic input from the ovary. Furthermore, recent studies in gonadectomized animals showed that estradiol replacement induced LH secretion through neurokinin B signaling (29).

Sequence analysis of the mutant testicular cDNA showed that it produced an aberrant mRNA lacking exon 2, resulting in the in-frame loss of 38 of the 62 original residues in the N-terminal mitochondrial targeting signal (30). Splice mutations in *StAR* gene causing CLAH have already been described (21). However, most of them lead to premature translational termination resulting in a defective StAR protein. The impact that the mitochondrial targeting and processing of StAR has on steroidogenesis remains poorly defined. Intramitochondrial cleavage of StAR appears linked to StAR activity in rat adrenal glands (6). In addition, the loss of the mitochondrial leader sequence, while increasing the stability of StAR, strongly diminishes its ability to promote gonad and adrenal steroidogenesis in mice (31, 32). Moreover, studies in nonsteroidogenic COS-7 cells showed that cytoplasmic StAR protein retains full activity in the absence of its mitochondrial import sequence (4, 23, 33). In the present study, *in vitro* analysis of the mutant recombinant enzyme after its transient expression in COS-7 cells showed that the in-frame loss of N-terminal residues 22 to 59 in the StAR protein impaired enzyme activity. Furthermore, it seemed apparent that p.G22_L59del StAR lacked appropriate processing, which prevented its normal translocation into the mitochondria. This finding was substantiated by immunofluorescent analysis in COS-7 cells where almost no colocalization of mitochondria and mutant protein was observed.

To date, *StAR* gene defects appeared to follow an autosomal recessive mode of inheritance. By contrast, our patient was severely ill in a heterozygous state, indicating a dominant disease. StAR 479-bp promoter region (34) and the complete coding region of the *StAR* gene were sequenced. The presence of both normal and mutated cDNA in the patient's testicular tissue ruled out a partial gene deletion or a putative second mutation in regulatory regions further than 479 bp upstream of initiation codon 1, or in intron sequences affecting the normal *StAR* gene expression. Indeed, when tested *in vitro*, we found that mutant p.G22_L59del StAR pregnenolone production defect could not be rescued by coexpression with WT StAR, suggesting a dominant-negative effect. It is known that StAR participates in a large macromolecular complex to facilitate the movement of cholesterol into the mitochondria (5, 35) Considering the fact that many autosomal dominant diseases result from a "dominant-negative" mechanism, in which a small number of defective protein monomers disrupt a larger functional complex (36, 37), we suggest that an aberrant folding of p.G22_L59del StAR might occur. The p.G22_L59del StAR might interfere with WT StAR biological activity by blocking the transduceosome complex, causing an autosomal dominant form of StAR deficiency, explaining thereby the clinical phenotype in the heterozygous patient. In this regard, biophysical studies suggested that subtle changes in the folding of the StAR protein could have substantial effects on StAR activity (38). Moreover, we were unable to find mutations in other genes potentially causing the patient's phenotype (39, 40), *CYP11A1*, *FDXR*, *FDX1*, and *SF1*.

The existence of a synergism between the *StAR* mutation and the *SF1* hypomorphic polymorphism G146A seems improbable because the *SF1* G146A variant showed minimally decreased transactivation activity (24).

Previous reports showed that *StAR* mutations with more than 10–20% of *in vitro* WT activity may rather manifest as mild forms of CLAH (12–15). However, some cases without correlation between StAR activity *in vitro* and the clinical phenotypes have also been described (11, 41). Although the mutant p.G22_L59del StAR retained more than 60% of normal *in vitro* enzymatic activity, the affected patient presented neonatal disease onset and severe 46,XY DSD at birth. The reason for this dissociation between *in vitro* studies and the clinical phenotype remains poorly understood. Associated mutations in some other transduceosome members could contribute to the clinical phenotype. However, molecular analysis of the transduceosome components, VDAC1 and TSPO, known to directly interact with StAR, regulating the import and processing of StAR (5, 35) was completely normal in this affected patient. In addition, as previously suggested, it is possible that the artificial expression of the mutant p.G22_L59del StAR in nonsteroidogenic COS-7 cells inaccurately models the mechanism of this mutant StAR at the mitochondria (23, 32, 33).

In summary, we are reporting a case of CLAH associated to an as yet undescribed *de novo* heterozygous splice-junction mutation of the *StAR* gene that disrupted the N-terminal targeting domain. *In vitro* experiments argued for its functional impact and dominant-negative properties and also provided insight into the structure-function relationship of the StAR protein.

Long-term follow-up of our patient provided some unexpected clinical information regarding recovery of mineralocorticoid function and a change in the regulation of hypothalmo-pituitary FSH secretion after ERT.

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