Congenital Lipoid Adrenal Hyperplasia: Functional Characterization of Three Novel Mutations in the *STAR* Gene

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Context: The steroidogenic acute regulatory protein (StAR) has been shown to be essential for steroidogenesis by mediating cholesterol transfer into mitochondria. Inactivating StAR mutations cause the typical clinical picture of congenital lipoid adrenal hyperplasia.

Objective: The objective of the investigation was to study the functional and structural consequences of three novel StAR mutations (p.N148K in an Italian patient; p.P129fs and p.Q128R in a Turkish patient).

Methods and Results: Transient *in vitro* expression of the mutant proteins together with P450 side-chain cleavage enzyme, adrenodoxin, and adrenodoxin reductase yielded severely diminished cholesterol conversion of the p.N148K mutant, the combined p.P129fs and p.Q128R mutant, and the p.P129fs mutant by itself. The p.Q128R mutant led to a higher cholesterol conversion than the wild-type StAR protein. As derived from three-dimensional protein modeling, the residue N148 is lining the ligand cavity of StAR. A positively charged lysine residue at position 148 disturbs the hydrophobic cluster formed by the α4-helix and the sterol binding pocket. The frame shift mutation p.P129fs truncates the StAR protein. Residue p.Q128 is situated at the surface of the molecule and is not part of any functionally characterized region of the protein.

Conclusion: The mutations p.N148K and p.P129fs cause adrenal insufficiency in both cases and lead to a disorder of sex development with complete sex reversal in the 46, XY case. The mutation p.Q128R, which is not relevant for the patient's phenotype, is the first reported variant showing a gain of function. We speculate that the substitution of hydrophilic glutamine with basic arginine at the surface of the molecule may accelerate cholesterol transfer. **(*J Clin Endocrinol Metab* 95: 1301–1308, 2010)**

Lipoid congenital adrenal hyperplasia (LCAH) is a rare autosomal recessive disorder that is characterized by diminished or absent adrenal and gonadal steroid hormone biosynthesis. As a result, patients present with adrenal insufficiency and typically, but not always, with complete sex reversal in 46,XY males (1, 2). LCAH is caused by inactivating mutations of the steroid acute regulatory (*STAR*) gene (3–7). Several studies could confirm that the rate-limiting step in steroid biosynthesis is the StAR protein-mediated delivery of cholesterol from the outer to the inner mitochondrial membrane and to P450 side-chain cleavage enzyme (P450scc) for further processing (8). The exact details of the mechanism by which StAR shuttles cholesterol from the outer to the inner mitochondrial membrane remain unclear (9).

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Abbreviations: LCAH, Lipoid congenital adrenal hyperplasia; P450scc, P450 side-chain cleavage enzyme; STAR, steroid acute regulatory; STARD, StAR domain.

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Bose *et al.* (4) proposed a two-hit model as pathophysiological explanation for LCAH. In response to a stimulus (*e.g.* ACTH), the normal steroidogenic cell recruits cholesterol from endogenous synthesis, stored lipid droplets, or low-density lipoprotein-receptor mediated endocytosis. Subsequently StAR promotes the cholesterol transport from the outer to the inner mitochondrial membrane in which cholesterol is further processed to pregnenolone. In cells with mutant StAR (first hit), there is no rapid steroid synthesis, but still some StAR-independent cholesterol flows into the mitochondria, resulting in a low level of steroidogenesis. Due to increased steroidogenic stimuli in response to inadequately low steroid levels, additional cholesterol accumulates. Massive cholesterol storage and resulting biochemical reactions eventually destroy all steroidogenic capacity (second hit) (4). This two-hit model has been confirmed by clinical studies (10, 11) as well as StAR knockout mice studies (12, 13).

The human *STAR* gene is localized on chromosome 8p11.2 and consists of seven exons (14). It is translated as a 285-amino acid protein including a mitochondrial target sequence (N terminal 62 amino acids), which guides StAR to the outer mitochondrial membrane and a cholesterol binding site, which is located at the C-terminal region. *In vitro* studies revealed that StAR protein lacking the N-terminal targeting sequence (N-62 StAR) can still stimulate steroidogenesis in transfected COS-1 cells, whereas mutations in the C-terminal region lead to severely diminished or absent function (1, 15, 16). Most of the *STAR* gene mutations associated with LCAH are located in the C-terminal coding region between exon 5 and 7 [StAR-related lipid transfer (START) domain] (4).

Herein we describe three novel *STAR* gene mutations in exon 4 that lead to LCAH and in one case complete sex reversal. We studied the functional and structural consequences of these mutations *in vitro* to provide further insight in the pathophysiology of StAR mutations.

Patients and Methods

Patient 1

The term neonate (weight 3100 g, length 47.5 cm) was born as the first child by cesarean section to healthy consanguineous parents of Italian origin. At birth the child presented with normal female external genitalia. Neither neonatal hypoglycemia nor respiratory distress was detected. Newborn screening for congenital adrenal hyperplasia was not performed. Hyperpigmentation of the skin was noted since the first week of life as well as poor growth during the first 2 months of life. At the age of 3 months, the child presented with acute crises including vomiting, dehydration, hypotension (73/42 mm Hg), hyponatremia of 123 mmol/liter (normal range 133–145 mmol/liter), and hyperkalemia of 6.7 mmol/liter (normal range 3.6–5.5 mmol/liter) after a first dose of hexavalent vaccination. ACTH was extremely elevated with greater than 330 pmol/liter (normal range 2–11 pmol/liter) as well as plasma renin activity with 22.6 nmol/liter · h (normal range 0.2–2.2 nmol/liter · h). Subsequent laboratory investigations revealed 17-hydroxyprogesterone of 1.8 nmol/liter (normal range 0.9–6.6 nmol/liter), aldosterone of less than 14 pmol/liter (normal range 28–416 pmol/liter), and cortisol of 425 nmol/liter (normal range 166–662 nmol/liter). LH was 0.5 mIU/ml (normal range boys <0.3–2.5 mIU/ml, girls <0.3–0.5 mIU/ml). Further diagnostic workup showed a 46,XY karyotype. Magnetic resonance imaging scans demonstrated the absence of Müllerian structures. The adrenal glands were enlarged. Vaginoscopy demonstrated a blind pouch. Laparoscopy revealed small gonads in the inguinal canal that were removed at the age of 1.5 yr. Hydrocortisone and fludrocortisone replacement therapy was started in conjunction with sodium chloride supplementation during initial presentation.

Patient 2

The infant is the only child of a large consanguineous Turkish family. The child presented at the age of 4 months with hyponatremia of 120 mmol/liter (normal range 133–145 mmol/liter), hyperkalemia of 6.5 mmol/liter (normal range 3.6–5.5 mmol/liter), cortisol of 346 nmol/liter (normal range 83–690 nmol/liter), elevated ACTH of greater than 275 pmol/liter (normal range 2–11 pmol/liter), and plasma renin activity levels of 45 nmol/liter · h (normal range 1.9–2.9 nmol/liter · h). 17-hydroxyprogesterone was low with less than 0.1 nmol/liter (normal range 0.1–6.6 nmol/liter) as well as testosterone with 0.07 nmol/liter (normal range 0.5–2.6 nmol/liter). Aldosterone was measured with 194 pmol/liter (normal range 55–400 pmol/liter) and dehydroepiandrosterone-SO_4 was measured with 15 nmol/liter (normal range 89–1100 nmol/liter). Newborn screening for congenital adrenal hyperplasia was not performed in the neonatal period. Hypoglycemia with a lowest glucose level of 40 mg/dl was present during the first week of life. Infant respiratory distress was not reported. Clinical examination revealed normal female external genitalia with mild hyperpigmentation of the labia majora. Further investigation revealed a normal female karyotype 46,XX, bilateral adrenal hyperplasia, a normal uterus on abdominal ultrasound, and enlarged ovaries containing multiple cysts. Computed tomography scans confirmed large adrenal glands (right 3 × 2 cm; left 4 × 2.5 cm) of nearly exclusively lipoid content. Hydrocortisone and fludrocortisone replacement therapy was started. Of interest three siblings of the father and two siblings of the mother died at the age of 1–2 yr.

Mutational analysis

Blood samples were sent by the treating physicians for further molecular investigation after informed consent by the parents or the relatives. The study was approved by the Ethical Review Board of the Christian-Albrechts-University in Kiel. Preparation of genomic DNA from peripheral blood leukocytes followed a standard protocol. All seven exons of *STAR* were amplified by PCR as described previously (primer sequences available online in Supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) (4). The PCR products were verified for correct DNA size on an agarose gel and subsequently purified. The mutational analysis was performed by direct DNA sequencing of the complete *STAR* coding region. The samples were electrophoresed on an automated sequencer (ABI PRISM

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310) and analyzed with the ABI SeqScape 3.7 software (PerkinElmer, Wellesley, MA). Mutants were designated according to the recommendations of the Nomenclature Working group (17).

Site-directed mutagenesis

Human full-length *STAR* wild-type cDNA was reversed transcribed from human common reference RNA, cloned in the *Eco*RI restriction site of a pcDNA3 vector, and subjected to direct sequencing. The mutagenesis to introduce the c.383A>G, c.384-385insCT, and c.444C>A mutations in exon 4 was performed from the pcDNA wild-type *STAR* construct using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) (primer sequences available in Supplemental Table 1) (4). The introduction of the mutation between the *Eco*RI restriction sites was verified by direct sequencing of the insert. The integrity of the expression plasmid was ensured by recloning the mutated *StAR* cDNA insert into the *Eco*RI restriction sites of a newly restricted pcDNA3 expression vector. *StAR* cDNA was again verified by direct sequencing. The resulting pcDNA3 vector was used for *in vitro* expression.

In vitro expression and assay of StAR activity

COS-7 cells were transiently transfected using Lipofectamine (Invitrogen, Karlsruhe, Germany) with 1 μg of each pcDNA3-STAR construct, 1 μg P450scc enzyme [pE-scc (18)], 0.5 μg adrenodoxin [pE-adx (18)], 0.5 μg adrenodoxin reductase [pE-ar (19)], and 100 ng pRK-TK (Promega, Mannheim, Germany) coding for Renilla luciferase. Posttransfection treatment followed a standard protocol (Invitrogen) using DMEM supplemented with glutamine, antibiotics, and 10% fetal calf serum. Cells were incubated with 250 μl human plasma as physiological source of cholesterol for 24 h (cholesterol concentration in plasma was 5.0 mmol/liter, pregnenolone concentration in plasma was <0.03 nmol/liter). As positive control for StAR-independent cholesterol conversion, 22(R)-hydroxycholesterol (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used. For steroid extraction a volume of 150 μl cell supernatant was mixed with an 8-fold deuterium labeled internal standard (17-hydroxyprogesterone-D_8) and 3 ml benzene and extracted for 2 min at room temperature. The organic phase was separated and evaporated to dryness under nitrogen. The dry residue was reconstituted in 60 μl isopropanol followed by 100 μL of high purity grade water. Pregnenolone concentration in the resultant supernatant was measured by liquid chromatography-tandem mass spectroscopy in the multireaction monitoring mode with the transition m/z 299/159. The lower detection limit of the assay was 0.1 nmol/liter and the intra- and interassay variation was 8.2 and 12.7% at a concentration of 75 nmol/liter, respectively.

Cells were lysed in lysis buffer (Promega). Protein content was determined using the Bradford method. Renilla luciferase assay was performed following the standard protocol of the manufacturer (Promega Corp., Madison, WI). Western blot analysis using an antihuman StAR rabbit polyclonal antibody (Acrio Antibodies, Herford, Germany) was used in a standard protocol to

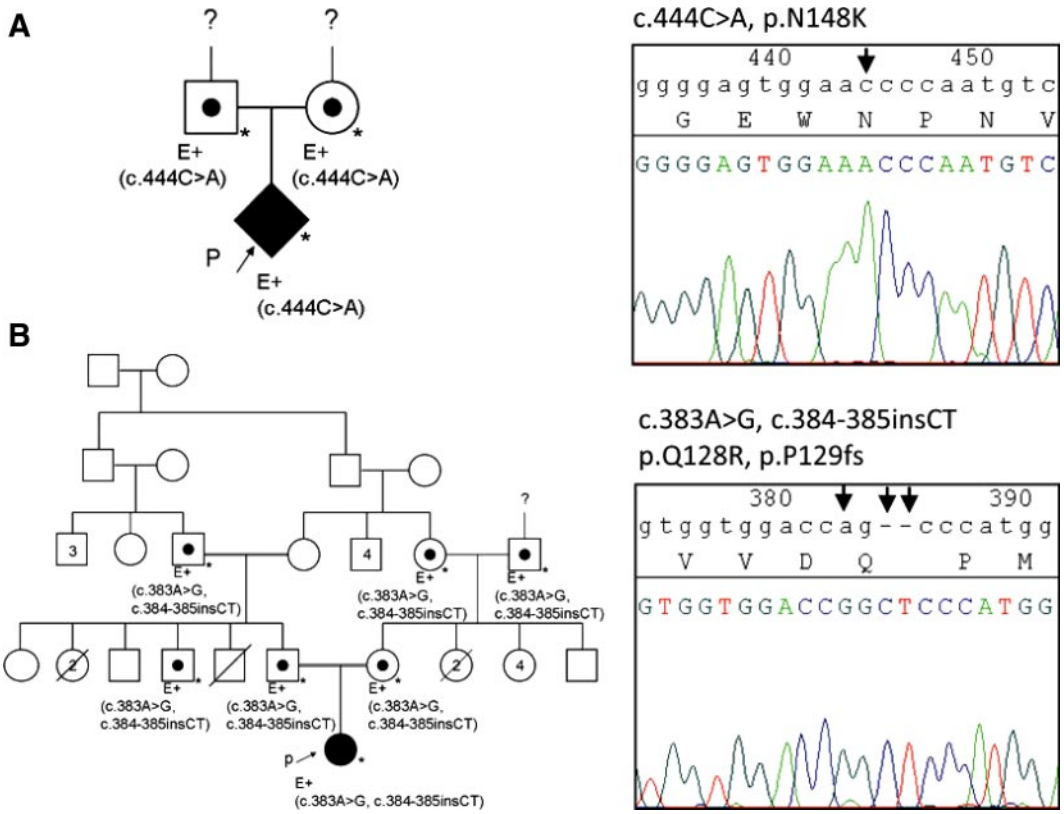


FIG. 1. Pedigrees of affected families and mutation analysis by direct DNA sequencing. A, The patient is marked with a *diamond* because of congenital disorder of sex development. The index is homozygous for the mutation c.444C>A predicting an amino acid exchange of asparagine at codon 148 with lysine (p.N148K). Both parents are heterozygous carriers of this mutation. B, The patient is homozygous for the mutation c.383A>G as well as a small insertion c.384-385insCT within exon 4 of the *StAR* gene. The parents, maternal grandparents, paternal grandfather, and paternal uncle are heterozygous carriers of both mutations. E+ represents a positive evaluation.

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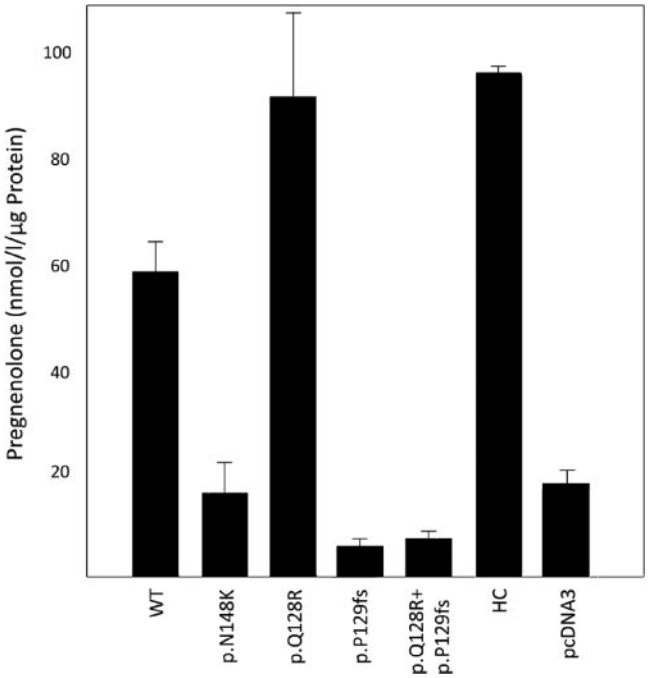


FIG. 2. COS-7 cells were transiently transfected with the respective pcDNA3-*StAR* construct and adrenodoxin, adrenodoxin reductase, P450scc, and pRK-TK coding for Renilla luciferase followed by incubation with cholesterol. Cholesterol conversion to pregnenolone was measured after 24 h incubation by liquid chromatography-tandem mass spectrometry. WT, wild-type StAR; HC, 22(R)-hydroxycholesterol.

ensure the expression and translation of the intact StAR wild-type and mutant proteins (8).

Immunofluorescence

Immunofluorescence followed a standard protocol (20). We used the antihuman polyclonal rabbit StAR antibody (Acrio Antibodies) and the mouse anti-Grp 75 antibody (Stressgen Bioreagents, Victoria, British Columbia, Canada) as primary antibodies in a 1:200 dilution. The Alexa Fluor 488 goat antirabbit and Alexa Fluor 594 goat antimouse antibodies (Molecular Probes, Leiden, The Netherlands) were used as secondary antibodies in a 1:500 dilution. The slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), allowing for nuclear 4, 6-diamidino-2-phenlyindole staining.

Molecular modeling

To study the impact of the new mutations on protein conformation, we generated a three-dimensional protein model of the StAR ligand binding domain. StAR shares similar biophysical and functional properties with MLN64, which also belongs to the cholesterol-specific START proteins (8, 21). *MLN64* was first identified as a gene being overexpressed in malignant compared with benign breast tumors (22). Because the crystal structure of the START domain of MLN-64/StAR domain (STARD)-3 (PDB code 1em2) has been solved (23), we used it as template for our three-dimensional StAR ligand binding domain protein model. According to the alignment obtained by a fold recognition procedure (ProHit package; ProCeryon Biosciences GmbH, Salzburg, Austria), amino acid residues were exchanged in the template (alignment available in Supplemental Fig. 1). Finally, the model structures were energy minimized, using the steepest descent algorithm implemented in the GROMOS program package (W. F. van Gunsteren, Laboratory of Physical Chemistry, University of Groningen, Groningen, The Netherlands; distributed by BIOMOS Biomolecular Software BV). To generate the StAR/cholesterol complex, the model structure of StAR has been superimposed onto the model structure of the MLN64/cholesterol model (PDB code 2i92) (24) and the coordinates of the cholesterol molecule were used. The structural representation was generated with the Ribbons software (25).

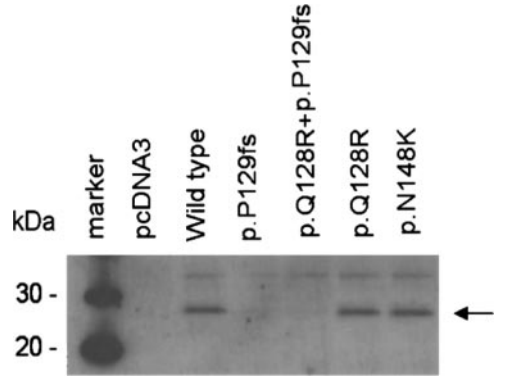


FIG. 3. Results of Western blot analysis from transiently transfected COS-7 cells with the indicated expression vectors. The *arrow* indicates the StAR specific band. StAR-specific bands for the wild-type StAR protein and the p.N148K and p.Q128R StAR mutants were detectable. No specific band was detectable for the premature truncated StAR mutants.

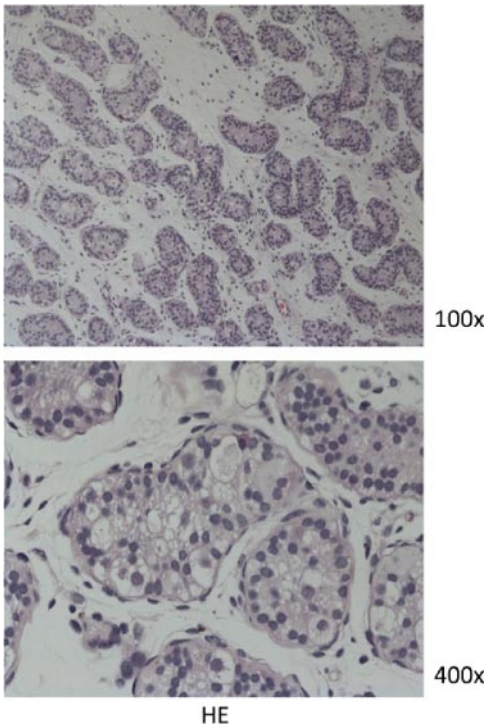


FIG. 4. Hematoxylin and eosin (HE) staining of the excised gonads of index patient 1 showed relatively small, oval seminiferous tubular outlines without basal lamina thickening and no interstitial fibrosis. Leydig cells were not visible. Staining with inhibin and StAR antibodies was negative (data not shown).

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Histological studies

Gonads were fixed in 10% buffered formaldehyde solution, embedded in paraffin, cut into 5 μm sections, and further deparaffinized and stained with hematoxylin and eosin, as well as periodic acid-Schiff stain. Consecutive sections were stained with polyclonal antibodies against human inhibin-α (AbD Serotec Morphosys, Düsseldorf, Germany) and human StAR (Acrio Antibodies).

Results

Mutational analysis

Amplification and sequencing of all seven *StAR* gene exons revealed a homozygous missense mutation c.444C>A within exon 4 in patient 1, predicting an amino acid exchange of asparagine at codon 148 with lysine (p.N148K). Both parents were heterozygous for this mutation (Fig. 1). Mutational analysis in patient 2 revealed a homozygous missense mutation c.383A>G as well as a small homozygous insertion c.384-385insCT within exon 4 of the *StAR* gene. Both parents, the paternal grandfather, and both the maternal grandmother and grandfather were heterozygous carriers of both mutations (Fig. 1). It can be assumed that the paternal grandmother is also carrier of the mutations because the couple gave birth to two children who died during infancy. However, she was not available for genetic analysis. The small insertion leads to a frameshift and a premature stop codon at residue 186 (p.P129L . . . p.S186X, called p.P129fs for reasons of length), leading to a truncation of the mature StAR protein. The missense mutation leads to an amino acid exchange of glutamine at codon 128 with arginine (p.Q128R). Neither sequence variation has been detected in 100 independent StAR alleles.

Functional analysis

Transient *in vitro* expression of all mutant proteins was performed in intact COS-7 cells. We used 22(R)-hydroxycholesterol as positive control to bypass StAR-mediated cholesterol transfer. The empty pcDNA3.1 vector served as negative control to measure StAR-independent steroidogenesis. Transient *in vitro* expression of the mutation p.N148K resulted in a low capability for cholesterol conversion (15.5 $\pm$ 6.8 nmol pregnenolone per liter per microgram protein) comparable with the cholesterol conversion of our negative control (17 $\pm$ 3.3 nmol pregnenolone per liter per microgram protein), reflecting complete inactivation of the StAR function. The two mutations detected in patient 2 were individually studied as well as in combination. The p.P129fs mutant leads as single mutation and in combination with p.Q128R to a severely reduced cholesterol conversion of 5.4 $\pm$ 1.2 nmol pregnenolone per liter per microgram protein and 6.7 $\pm$ 2.8 nmol pregnenolone per liter per microgram protein, respectively, comparable with the empty vector. The mutant p.Q128R showed no impaired cholesterol conversion (91.3 $\pm$ 16.2 nmol pregnenolone per liter per microgram protein). On the contrary, it led to an even significantly higher pregnenolone concentration than wild-type StAR (58.1 $\pm$ 5.5 nmol pregnenolone per liter per microgram protein, $P < 0.05$) (Fig. 2). Overall, the relative StAR activities seen with wild-type StAR, hydroxycholesterol, and the empty vector are well comparable with the existing data in literature (9, 26).

Western blotting of the mutant proteins p.N148K and p.Q128R derived from COS-7 cells showed a StAR-specific band of the expected size, whereas the mutant proteins p.P129fs and p.Q128R with p.P129fs derived from COS-7 cells showed no StAR-specific band (Fig. 3). This may be due to insufficient antibody binding to the truncated protein or nonsense decay of the StAR mRNA. Immunofluorescence studies on transiently transfected COS-7 cells showed that wild-type StAR and the mutant proteins p.N148K and p.Q128R colocalized with mouse Grp75, a marker of the mitochondrion (Supplemental Fig. 2). This demonstrates that the p.N148K and the p.Q128R mutations have no effect on the intracellular lo-

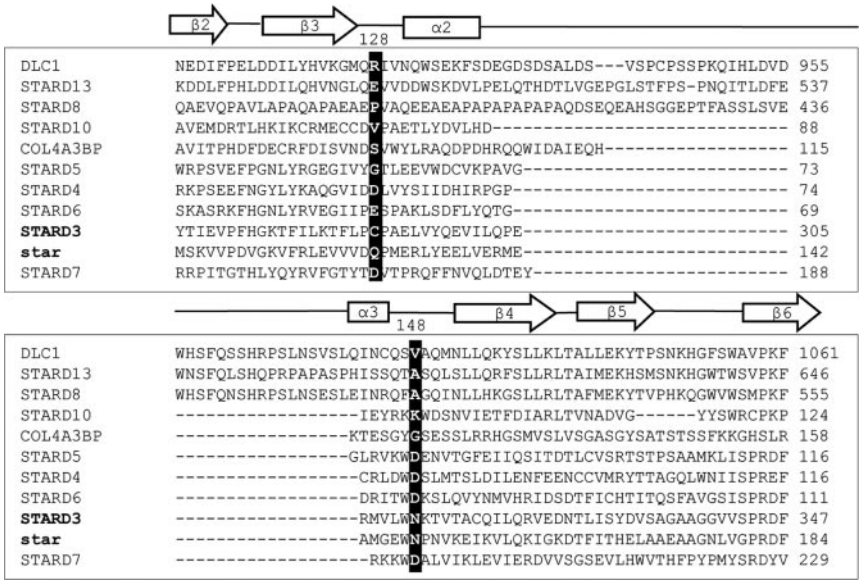


FIG. 5. Alignment of 11 human STARDs designed with ClustalW. *Black boxes* indicate the two mutant positions. The amino acid residue asparagine at position 148 is strictly conserved in the cholesterol binding STARD1/STAR subfamily (in *bold letters*) and is directly following the α3-helix.

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calization of the mutant proteins. In analogy to our findings in Western blotting, we detected no anti-StAR antibody binding for the mutant proteins p.P129fs as well as p.Q128R with p.P129fs.

Histological studies

Laparoscopy of patient 1 revealed small gonads in the inguinal canal, which were removed at the age of 1.5 yr. The excised gonads showed relatively small, oval seminiferous tubular outlines without basal lamina thickening and no interstitial fibrosis (Fig. 4). As expected at this age, Leydig cells were not visible. Consequently, staining for inhibin was not detectable. Staining with StAR antibody was also negative. Large and round germ cells were observed in the tubules. No apparent abnormality was noticed at this stage of gonadal maturation.

Discussion

We present two patients with adrenal insufficiency and adrenal hyperplasia combined in one of the cases with a 46,XY complete sex reversal. The association between LCAH and *StAR* mutations can be regarded as well established (3–5, 27). There are 15 different proteins that contain a START domain. Multiple sequence alignments of the 15 STARD domains (28) in humans allows the construction of a phylogenetic tree that divides the group into six subfamilies (28, 29). The cholesterol binding STARD1/StAR subgroup consists of the StAR and the MLN64 (STARD 3) protein. Recent studies, including three-dimensional protein modeling of the MLN64 START domain and StAR (23, 24, 30, 31), could demonstrate that the putative sterol-binding pocket is formed by nine twisted antiparallel β-sheets and four α-helices. The β-sheet forms a U-shaped unclosed barrel, building the floor and walls of a hydrophobic cavity accommodating the ligand. The roof is formed by helices-α3 and -α4 and an Ω-loop inserted between β5 and β6. A second Ω-loop, located between β7 and β8 buttresses the first. Although there is only approximately 30% sequence conservation within the STARD1/StAR subgroup, residue N148 is conserved (Fig. 5). This indicates a crucial role of asparagine at position 148 for cholesterol binding specificity.

To study the pathomechanisms of the reported StAR mutations, we generated a three-dimensional model structure of StAR using the crystal structure of MLN64 (STARD 3) as template (23). Several molecular models have been published for StAR (24, 30, 31). Although all were generated by different methodologies and approaches, they are almost identical with our model as judged from the calculated root-mean square deviation. The conserved asparagine at position 148 of the STARD1/StAR subgroup, mutated to lysine in our Italian patient, is directly following the α3 helix and is in proximity to a hydrophobic cluster formed by the α4-helix and the sterol binding pocket (Figs. 5 and 6). This hydrophobic interaction helps in maintaining the internal structure of the binding pocket. This was thoroughly studied and discussed in recent publications (32). Therefore, the introduction of the long positively charged side chain of lysine at this position would highly disrupt the hydrophobic cluster and perturb the binding of cholesterol, thereby reducing the activity of StAR.

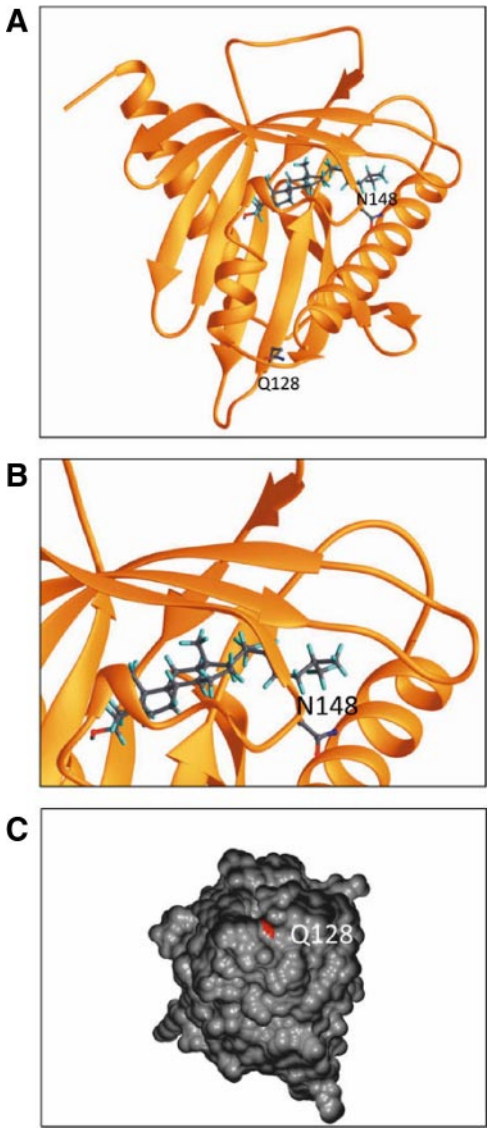


FIG. 6. Three-dimensional model structure of the StAR protein. A, Overview of StAR with bound cholesterol depicting the residue p.Q128 and p.N148. B, Close-up view, The residue N148 is lining the binding pocket accommodating the ligand cholesterol. The substitution with a lysine residue at this position disrupts the hydrophobic cluster formed by the α4-helix and the sterol binding pocket. C, Surface model, p.Q128 is located at the surface of the molecule. The substitution to a basic arginine residue may facilitate the contact to cofactors accelerating cholesterol transfer.

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The residue p.Q128 is situated on the surface of the protein without any contact to the ligand binding cavity (Fig. 6). Baker *et al.* (33) reported the clinical case of severe LCAH due to a p.R182L mutant StAR protein. *In vitro* studies revealed normal cholesterol binding and transfer between liposomes but impaired steroidogenic activity in a transfected cell system (33). Cholesterol binding therefore does not necessarily indicate normal StAR function. Protein modeling could show that p.R182L is also part of the surface of the molecule, indicating that *StAR* function also relies on intact protein-protein interactions (34). In agreement with the literature, we speculate that the exchange of polar glutamine to basic arginine at position 128 may facilitate interaction with cofactors on the outer mitochondrial membrane accelerating cholesterol conversion. To our knowledge we hereby report the first gain-of-function mutation of the StAR protein. Further studies may use protein import assays and measurements of cholesterol binding abilities of this mutant to learn more regarding StAR physiology and cholesterol shuttling.

The frame shift mutation detected in patient 2 leads to a truncated protein lacking the C-terminal end. Early functional studies on truncated StAR proteins could show that the C-terminal end is the functionally essential part for cholesterol binding, whereas the N-terminal end serves as guide to the mitochondria (16). Interestingly, the functional activity of the truncated protein in our study is significantly lower than the baseline activity of our empty vector control ($P < 0.05$). The truncated protein still contains the N-terminal mitochondrial guiding sequence. Whether this leads to a trapping of the mutant protein at the mitochondrial membrane and blockage of cholesterol diffusion into the organelle or whether the low ability to convert cholesterol is just a side effect of *in vitro* experimental conditions remains speculative.

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Disclosure Summary: The authors have nothing to declare.

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