A Novel Homozygous Mutation in *CYP11A1* Gene Is Associated with Late-Onset Adrenal Insufficiency and Hypospadias in a 46,XY Patient

Petr Rubtsov, Maksim Karmanov, Polina Sverdlova, Pavel Spirin, and Anatoly Tiulpakov

Department of Endocrine Genetics (A.T.), Endocrinology Research Center, Moscow 117036, Russian Federation; Engelhardt Institute of Molecular Biology (P.R., P.Sv., P.Sp.), Moscow 119991, Russian Federation; and Republican Children's Hospital (M.K.), Moscow 117513, Russian Federation

Context: The first and the rate-limiting step in the biosynthesis of hormones in all steroidogenic tissues, conversion of cholesterol to pregnenolone, is catalyzed by the cholesterol side-change cleavage cytochrome P450 (P450scc) encoded by a single gene, *CYP11A1*. To date, mutations in *CYP11A1* gene have been reported in six patients, all of whom presented with adrenal insufficiency within the first 4 yr of life and severely underandrogenized external genitalia (Prader stages 1–2).

Objective: Our aim was to characterize *in vitro* and *in vivo* effects of a novel homozygous *CYP11A1* gene mutation identified in a patient with an unusual presentation of P450scc deficiency.

Methods and Patients: A 46,XY patient presented with mid-shaft hypospadias and cryptorchidism at birth and signs of adrenal failure at 9 yr. Mutational analysis of *CYP11A1* gene was performed by PCR, followed by direct sequencing. P450scc activity was determined by measuring concentration of pregnenolone synthesized from cholesterol in the medium after a transient transfection of HEK293 cells with P450scc, adrenodoxin, adrenodoxin reductase, and steroidogenic acute regulatory protein expression plasmids.

Results: The sequencing of *CYP11A1* gene in the proband revealed a novel homozygous L222P mutation, whereas both parents were heterozygous carriers for this mutation. *In vitro* P450scc activity of L222P mutant was approximately 7% compared with the wild type.

Conclusions: This case represents the mildest phenotype of P450scc deficiency to be described. The phenotypic presentation was consistent with the partial reduction of P450scc activity of L222P mutant. **(*J Clin Endocrinol Metab* 94: 936–939, 2009)**

The first and the rate-limiting step in the biosynthesis of hormones in all steroidogenic tissues is conversion of cholesterol to pregnenolone (1). This involves three consecutive chemical reactions, 20α-hydroxylation, 22α-hydroxylation, and cleavage of the cholesterol side-chain, all of which are catalyzed by cytochrome p450 cholesterol side-chain cleavage enzyme (P450scc), encoded by a single *CYP11A1* gene on chromosome 15q23-24 (2). Defects of *CYP11A1* gene are associated with a rare form of congenital adrenal hyperplasia (CAH). Up to now, *CYP11A1* mutations have been identified in six patients (one female and five males), all of whom presented with salt-losing adrenal crisis early in life, whereas all genetic males were severely undervirilized and assigned as females.

Here we describe an unusual case of P450scc deficiency associated with a novel homozygous L222P mutation in *CYP11A1* gene. The 46,XY proband presented with ambiguous genitalia at birth, was assigned as male, and showed no overt signs of adrenal insufficiency until the age of 9 yr.

The case, to our knowledge, represents the mildest phenotype of P450scc deficiency described so far. Functional expression studies showed significantly reduced, but detectable activity of L222P mutant.

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Abbreviations: ADX, Adrenodoxin; ADXR, ADX reductase; CAH, congenital adrenal hyperplasia; P450scc, cytochrome p450 cholesterol side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein.

Patients and Methods

Case reports

The proband was the first of twins, apparently dizygotic (as reported by the local pediatrician), born to a consanguineous Russian couple. The second of the twins (phenotypically normal female) died at the age of 2.5 yr after an unknown illness presenting with weakness and cyanosis. No autopsy was performed. The proband's birth weight was 2.6 kg, the length 51 cm. At birth he presented with bilateral cryptorchidism with inguinal location of testes, mid-shaft hypospadias, and bilateral talipes equino-varus. He underwent procedures for talipes correction at the ages of 1 yr and 1 yr 2 months. At the age of 2 yr 7 months, surgery for hypospadias and cryptorchidism was performed. All operations were performed under general anesthesia and were uneventful. At the age of 9 yr, the boy started to experience episodes of weakness, dizziness, and vomiting. At the age of 10 yr 4 months, he was admitted to the emergency department of the local hospital with a history of severe weakness, vomiting, and seizures; serum sodium was 112 mmol/liter and potassium was 6.5 mmol/liter. Treatment with prednisolone and fludrocortisone was started, resulting in dramatic improvement.

At presentation to our endocrinology department at the age of 10.7 yr, his height was 125 cm (−2.3 SD) (3), his weight was 25 kg (body mass index SD score, −0.4) (4), his puberty staging was Tanner G1P1 with 2 ml testes; and bone age was 9.5 yr (5). The plasma ACTH level (while on treatment with prednisolone 3.75 mg/d and fludrocortisone 0.025 mg/d) was elevated (325.7 pg/ml; normal range, 0.8–58), whereas dehydroepiandrosterone sulfate and 17-hydroxyprogesterone were below detection limits. The karyotype result was 46,XY. Ultrasound and magnetic resonance imaging showed normal-sized adrenals.

At later referral at the age of 14.2 yr, the patient was on treatment with hydrocortisone 15 mg/d and fludrocortisone 0.05 mg/d. His height was 144.5 cm (−2.1 SD), his weight was 46 kg (body mass index SD score, 1.2), pubertal stage was Tanner G1P1 with 6 ml testes, and penile length was 5 cm (−1.3 SD) (6). The LH level was 37.2 mU/liter (normal range, 0.5–2.0), FSH was 25.7 mU/liter (normal range, 0.4–1.7), whereas testosterone was 0.26 ng/ml (normal range, 1.6–4.0).

The association of 46,XY disorder of sexual development and primary adrenal failure pointed to a congenital disorder affecting both adrenal and gonadal steroidogenesis. Low dehydroepiandrosterone sulfate in the presence of elevated ACTH ruled out 3β-hydroxysteroid dehydrogenase deficiency. A defect of either steroidogenic acute regulatory protein (StAR) or P450scc deficiency was considered in the differential diagnosis.

DNA analysis

The study was approved by the institutional review board, and the proband and the parents gave informed consent for DNA analysis. Genomic DNA was extracted from peripheral leukocytes by standard procedure. *STAR* and *CYP11A1* genes were amplified by PCR using pairs of intronic primers and conditions published elsewhere (7–9). Amplification products were purified and directly sequenced using automated DNA sequencer (model 310, Applied Biosystems, Foster City, CA). GenBank cDNA entries with accession numbers U17280 and M14565 were used as reference sequences for analyses of mutations and numbering of nucleotides for *STAR* and *CYP11A1* genes, respectively.

Construction of mammalian expression plasmids

Wild-type human P450scc and StAR cDNAs cloned in pRK5 vector (10) were provided by Dr. Noriyki Katsumata (National Research Institute for the Child Health and Development, Tokyo, Japan). The L222P amino acid substitution was introduced in the wild-type P450scc cDNA by recombinant PCR method (11). Human adrenodoxin reductase (ADXR) and adrenodoxin (ADX) cDNA were amplified from total adrenal cDNA by PCR and cloned in mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The same vector was used to create StAR, wild-type P450scc, and mutant (L222P) P450scc expression plasmids.

Functional expression of the replacement mutant P450scc

Functional expression analysis was performed using the procedure described by Katsumata *et al.* (10, 12) with minor changes. HEK293 cells were transfected with 100 ng each of human ADX, ADXR, and StAR expression plasmids and 100 ng of either wild-type or mutant P450scc expression plasmids or empty pcDNA3.1(+) vector by lipofectamine method. In addition, 100 ng of Renilla luciferase expression plasmid pRL-TK (Promega, Promega, WI) was included in the transfection mixture and used as internal control for transfection efficiency. Renilla luciferase activity was determined using Renilla Luciferase Assay System (Promega, Madison, WI). P450scc activity was determined by measuring concentration of pregnenolone in the culture medium after 48 h of incubation by ELISA using CAN-PRE-450 kit (Diagnostics Biochem Canada Inc., London, Ontario, Canada). The results were presented as mean ± SD from four independent experiments, each performed in triplicate.

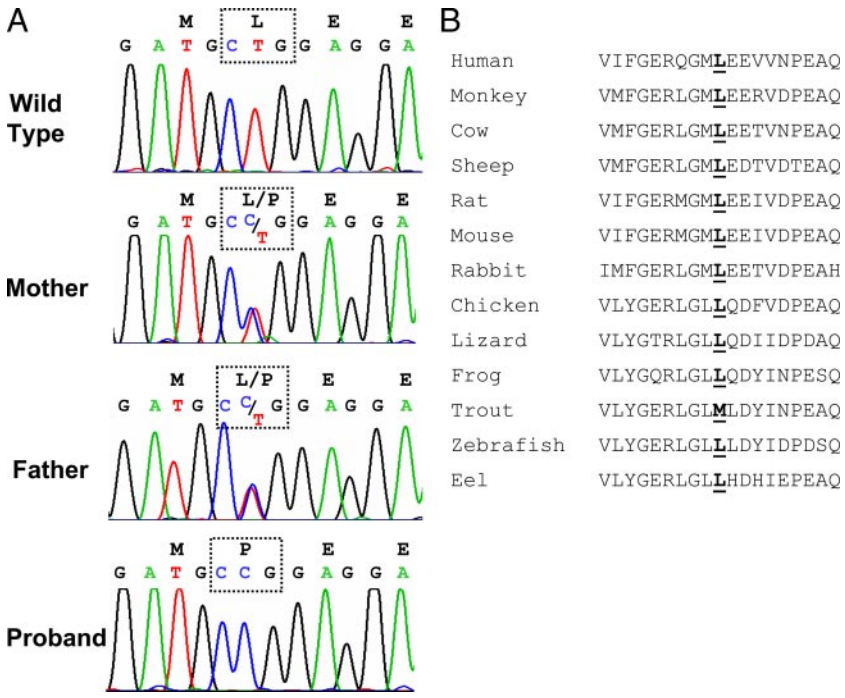

FIG. 1. A, Electrophoreograms of DNA sequences. *Top to bottom*, Wild-type control, mother, father, proband; residues 222 are marked by *dotted boxes*. B, Alignment of the sequences of human (residues 213–231), monkey, cow, sheep, rat, mouse, rabbit, chicken, lizard, frog, trout, zebrafish, and eel P450scc proteins; residue 222 in the human protein and corresponding residues in the other species are *bold* and *underlined*.

Results

Sequencing of *STAR* and *CYP11A1* genes

Sequencing of coding regions and splice sites of the *STAR* gene showed no mutations. By sequencing *CYP11A1* gene in the proband, we detected a homozygous c.666T>C mutation in exon 3, which resulted in p.L222P substitution (Fig. 1). Both parents were heterozygous for this single nucleotide change (Fig. 1).

P450scc activity of L222P mutant

To evaluate the functional consequence of L222P substitution, we transiently expressed the mutant along with ADX, ADXR, and StAR in HEK293 cells (Fig. 2). The HEK293 cells expressing the wild-type P450scc successfully converted cholesterol to pregnenolone (concentration in the medium, 60 ± 5.6 ng/ml), whereas those transfected with the empty pcDNA3.1(+) plasmid produced virtually no pregnenolone (2.4 ± 0.2 ng/ml). The L222P replacement resulted in markedly reduced, but significantly higher than baseline (empty vector) pregnenolone production (6.4 ± 1.6 ng/ml).

Discussion

We have described a case of 46,XY disorder of sexual development and adrenal insufficiency associated with a novel L222P mutation in the *CYP11A1* gene.

The residue L222 is highly conserved in P450scc across species, with the exception of the rainbow trout in which it is replaced by a close hydrophobic amino acid, methionine; P450scc entries for all other species in the GenBank (http://www.ncbi.nlm.nih.gov) contain leucine in the corresponding position (Fig. 1). L222 resides between α-helixes E and F (13), and replacement to proline at this position may change the angle of the polypeptide chain turn at the junction between the helixes.

The functional expression analyses demonstrated that the mutant L222P P450scc protein had significantly reduced but detectable activity, which was estimated to be 6.9% of normal (Fig. 1). Comparable reduction of enzymatic activity in other forms of CAH is usually associated with partial loss of function phenotypes, *e.g.* non-salt-wasting forms of 21-hydroxylase deficiency (14) or 3β-hydroxysteroid dehydrogenase deficiency (15). The initial presentation of the proband was compatible with partial P450scc deficiency. The patient presented with midshaft hypospadias, which was indicative of a substantial level of androgen production at least until the 14th week gestation. As to the adrenal function, approximately 1 yr before the proband's admission with the salt-losing crisis he might have already had signs of adrenal insufficiency accounting for his episodes of weakness, dizziness, and vomiting. Although the etiology of short stature in the proband is not completely understood, we speculate that it could be the consequence of chronic mineralocorticoid deficiency, similar to cases of poorly controlled salt-losing CAH or aldosterone synthase deficiency. Nevertheless, the fact that the boy underwent three uneventful surgeries under general anesthesia suggests that, at least during early childhood, his adrenal function was not critically impaired.

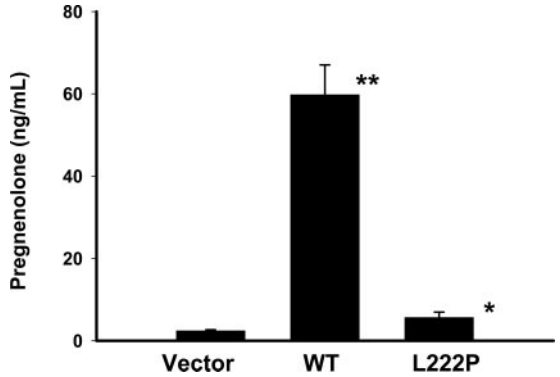

FIG. 2. Activity of L222P mutant. HEK293 cells were transfected with human ADX, ADXR, and StAR expression plasmids and either wild-type or mutant P450scc expression plasmids. Empty pcDNA3.1(+) vector was used as a negative control. Pregnenolone secreted into the culture medium was determined by ELISA. Renilla luciferase expression plasmid was included in each of the transfection reactions and used as internal control for transfection efficiency. Values are the mean ± SD from four independent transfection experiments, each performed in triplicate. Vector, Empty pcDNA3.1(+) plasmid; WT, wild-type P450scc; L222P, L222P replacement P450scc. *, $P < 0.01$ *vs.* vector; **, $P < 0.001$ *vs.* L222P.

To date, five cases of *CYP11A* mutation in 46,XY individuals have been reported (8, 10, 12, 16, 17). Although partially preserved L222P mutant activity could explain the mild undervirilization and late-onset adrenal insufficiency in our patient, the overall genotype-phenotype correlation among previously described cases of P450scc deficiency has been poor. Thus, patients with R353W (10) and A359V (12) mutations resulting in reductions of *in vitro* activity (8.1 and 11.7%, respectively) that were comparable to our case presented with delayed but still significantly earlier manifestations of adrenal insufficiency (7–9 months and 21 months, respectively). On the other hand, a replacement of nonconserved leucine to triptophan at position 141, showing *in vitro* 38.5% of the wild-type enzyme activity, presented clinically with an adrenal crisis in the first days of life (17), similar to the two cases associated with nil function mutations (16, 17). With respect to gonadal function, four of the five previously described 46,XY patients with P450scc deficiency had a completely female phenotype (12, 16, 17), whereas the other male, who had late-onset adrenal insufficiency, showed only a minor degree of virilization described as "female external genitalia with clitoromegaly, no labial fusion, and separate vaginal and urethral opening" (8).

It is not clear whether the partial preservation of P450scc function in our case contributed to completion of the pregnancy to term. This mechanism was proposed in the first documented case of P450scc deficiency, in which clinical phenotype was also suggestive of the partial activity of the enzyme (8). However, later observations showed that even nil function defects (16, 17) were compatible with term pregnancies.

The mechanism of deterioration of residual P450scc activity is not completely understood. In our case, disease progression could be traced from the stage of partial loss of steroidogenic function in the first years of life to the complete adrenal and gonadal failure, evident from the hormone profiles at the ages of 10 and 14 yr, respectively. In the first reported case of P450scc deficiency (8, 12), the loss of residual enzyme activity was explained by the same two-hit model that was proposed for CAH due to StAR deficiency (9). According to this paradigm, initial impairment of steroidogenesis as a result of genetic defect (first hit) is followed by cell damage due to accumulation of cholesterol esters (the second hit). The latter is believed to be the cause of massive enlargement of adrenal glands, one of the frequent findings in StAR deficiency (18, 19). It is noteworthy that in our case as well as in all other reported patients with P450scc deficiency, for whom imaging data were available (8, 12, 16, 17), no adrenal

enlargement was detected. This strengthens the hypothesis of al Kandari *et al.* (12), who pointed to a possible difference between the mechanisms of the second hit in StAR and P450scc deficiency, suggesting that in the latter the intramitochondrial accumulation of cholesterol might lead to cell apoptosis before accumulation of lipids in the cytoplasm.

Baker *et al.* (20) have recently described two male patients with congenital lipoid adrenal hyperplasia presented with adrenal insufficiency at 2–4 yr, normal male genitalia, and absence of adrenal enlargement. These two cases were associated with missense *STAR* gene mutations that retained *in vitro* approximately 20% of the wild-type activity and were designated as nonclassical lipoid CAH (20). The case reported in the current paper represents a nonclassical variant of another disorder, deficiency of cholesterol side-chain cleavage enzyme. This broadens our view of the clinical spectrum of P450scc deficiency, showing that it may present in males with almost complete androgenization of external genitalia and absence of overt signs of adrenal failure during the first decade of life.

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Address all correspondence and requests for reprints to: Anatoly Tiulpakov, M.D., D.Sc., Department of Endocrine Genetics, Endocrinology Research Center, 11 Ulitsa Dmitriya Ulianova, Moscow 117036, Russian Federation. E-mail: ant@endocrincentr.ru.

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