Severe Combined Adrenal and Gonadal Deficiency Caused by Novel Mutations in the Cholesterol Side Chain Cleavage Enzyme, P450scc

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Context: Mitochondrial cytochrome P450scc converts cholesterol to pregnenolone in all steroidogenic tissues. Although progesterone production from the fetally-derived placenta is necessary to maintain pregnancy to term, four patients with mutations in the gene encoding P450scc (CYP11A1), have been described, one in a 46,XX female and three in underandrogenized 46,XY individuals, all with primary adrenal failure.

Objective: Our aim was to determine whether P450scc mutations might be found in other children and to explore genotype/phenotype correlations.

Methods and Patients: We performed mutational analysis of CYP11A1 in individuals with 46,XY disorders of sex development and primary adrenal failure, followed by functional studies of P450scc activity and of P450scc RNA splicing.

Results: Among nine 46,XY infants with adrenal failure and disordered sexual differentiation, two infants had compound heterozygous mutations in CYP11A1. One patient harbored the novel P450scc missense mutations L141W and V415E, which retained 38 and 0% activity, respectively. The other carried a CYP11A1 frameshift mutation c835delA (0% activity) and a splice site mutation [IVS3+(2-3)insT] that prevented correct splicing of P450scc mRNA.

Conclusions: P450scc deficiency is a recently recognized disorder that may be more frequent than originally thought. The phenotypic spectrum ranges from severe loss-of-function mutations associated with prematurity, complete underandrogenization, and severe, early-onset adrenal failure, to partial deficiencies found in children born at term with clitoromegaly and later-onset adrenal failure. In contradistinction to congenital lipoid adrenal hyperplasia caused by steroidogenic acute regulatory protein mutations, adrenal hyperplasia has not been reported in any of the six patients with P450scc deficiency. (J Clin Endocrinol Metab 93: 696–702, 2008)
order was thought to represent an enzymatic defect originally termed “20,22 desmolase deficiency” (for review, see Ref. 8). However, studies of DNA from affected individuals demonstrated that the CYP11A1 gene encoding P450scc was normal in this disease (9–12), and in 1995 it was shown that this disease, more properly termed “congenital lipoid adrenal hyperplasia” (lipoid CAH) was caused by mutations in the gene encoding the steroidogenic acute regulatory protein (StAR) (13, 14).

Studies of lipoid CAH showed that only the acute stimulation of steroidogenesis requires StAR; in the absence of StAR there is still a low level of StAR-independent steroidogenesis (15), such as that seen in the placenta, which lacks StAR (16). The successful outcome of pregnancies harboring fetuses with severe defects of adrenal and gonadal steroidogenesis, including fetuses with adrenal hypoplasia congenita due to mutation of DAX-1 (17–20) or steroidogenic factor 1 (SF1) (21–23), indicates that fetal steroidogenesis is not required for human fetal development (24). By contrast, placental production of progesterone is essential to suppress uterine contractility, permitting pregnancy to progress. Thus, human pregnancy depends on progesterone from the mother’s corpus luteum of pregnancy during the first trimester, whereupon there is a “luteo-placental shift” to production of progesterone by the placental syncytiotrophoblasts, a fetal tissue (25). In the pregnancies of some animals, such as the rabbit and rodent, progesterone is produced by the corpus luteum throughout pregnancy, so that ablation of P450scc remains compatible with term gestation. Thus, it had been thought that interfering with progesterone synthesis by the human placenta would result in second trimester spontaneous abortion (26), but several cases of severe P450scc mutations have now been reported (27–30). Because such cases represent novel experiments of nature that probe the roles of fetal steroidogenesis in human pregnancy, there is substantial interest in the rare patients with genetic defects in P450scc. We now report the clinical, molecular genetic, and functional analysis of P450scc mutations in two additional patients and review the findings in all reported patients with P450scc defects.

**Subjects and Methods**

**Mutational analysis**

With Institutional Review Board approval and informed consent, genomic DNA was extracted by standard methods from peripheral blood leukocytes of nine infants with 46,XY disorders of sexual differentiation and primary adrenal failure. Among these nine infants, four were preterm (two with mild hypospadia, one with clitoromegaly, and one with normal female genitalia), and five were full term (one with ambiguous genitalia and four with female genitalia; two of these had P450scc mutations). Mutations in StAR and SF1 were excluded by DNA sequencing, as described (15, 22).

All nine exons and all splice sites of CYP11A1 were PCR-amplified using variations on conditions reported previously (27, 29). PCR products were purified using 1 U/μl Exonuclease I (New England Biolabs, Beverly, MA) and 0.1 U/μl shrimp alkaline phosphatase (USB Corp., Cleveland, OH) and then subjected to direct sequencing using BigDye terminator v1.1 (Applied Biosystems, Foster City, CA) and a MegaBACE1000 capillary DNA sequencer (Amersham Biosciences Inc., Little Chalfont, UK). Sequence Analyser v3.0 (Amersham Biosciences) and Sequencher v4.1 (Gene Codes Corp., Ann Arbor, MI) were used to analyze the data.

Compound heterozygous mutations were confirmed by studying parental DNA, as well as by subcloning PCR products into a TA-cloning vector (Invitrogen, Carlsbad, CA) and sequencing independent clones. Genscan splice prediction software (http://genes.mit.edu/GENSCAN.html) was used to assess the consequences of the splice site mutation detected.

**Functional studies of P450scc activity**

The P450scc moiety of the F2 plasmid expressing a fusion protein (H,N-P450scc-adrenodoxin reductase-adrenodoxin-COOH) of the human cholesterol side chain cleavage system (31) was mutagenized by PCR-based, site-directed mutagenesis as described (27) using the primers shown in Table 1. The PCR conditions were: 95°C for 1 min, 15 cycles of 95°C for 30 sec, 55°C for 1 min, and 68°C for 16 min. Mutagenized cDNA plasmids were sequenced in their entirety before functional studies.

Wild-type and mutant P450scc function was assessed using an in vitro system with pregnenolone production as a measure of enzyme activity. Nonsteroidogenic COS-1 cells were cultured to 90–95% confluency at 37°C in 5% CO2 in DMEM/Ham’s 21 medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin and transfected with 1 μg of either the wild-type or mutant F2 plasmid, using Lipofectamine 2000 (Invitrogen), as described (13). Culture media were treated with 5 μg/ml 22R-hydroxycholesterol, which bypasses the action of StAR and is converted to pregnenolone by P450scc (31). Media were collected 48 h later, and pregnenolone production was assayed by RIA.

**TABLE 1. Oligonucleotide sequences**

<table>
<thead>
<tr>
<th>Nucleotide change (codon sequence)</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>422T→G (L141W)</td>
<td>Sense: CCATAGGAGTCTCTGGAGAGAAGTGGCGGAGC</td>
<td>Cloning</td>
</tr>
<tr>
<td></td>
<td>Antisense: CGTGCAGCACTCTTCACAGGACTCTATGGC</td>
<td>Cloning</td>
</tr>
<tr>
<td>1244T→A (V415E)</td>
<td>Sense: CTCGCCAGACACTGAGCAAGTGCCACTCTA</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>c835delA</td>
<td>Antisense: TAGATGGCCACTTGCCTCAGTGTCCGAG</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>Name for minigene experiment</td>
<td>Sense: TCAGATAAAGCTGACTATACCCACAGAAGCTCTTACG</td>
<td>PCR</td>
</tr>
<tr>
<td>SCC-x3x4-F</td>
<td>Antisense: GAAACTCTGGGTGTATACGCTGCTTTACTGA</td>
<td>PCR</td>
</tr>
<tr>
<td>SCC-x3x4-R</td>
<td>Cloning</td>
<td></td>
</tr>
<tr>
<td>IVS3- S</td>
<td>Mutagenesis</td>
<td></td>
</tr>
<tr>
<td>IVS3-AS</td>
<td>Mutagenesis</td>
<td></td>
</tr>
<tr>
<td>GAPDH-sense</td>
<td>Cloning</td>
<td></td>
</tr>
<tr>
<td>GAPDH-antisense</td>
<td>PCR</td>
<td></td>
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</table>
using standard reagents (MP Biomedicals Corp., Irvine, CA) as described (32). Data are presented as the mean ± SEM for four independent experiments, each performed in triplicate.

**Minigene construction and splicing analysis**

A segment of the wild-type human CYP11A1 gene encoding P450scc, consisting of exon 3, intron 3, and exon 4, was amplified by PCR using oligonucleotides 5′-ctggactggcttctgctgc-3′ and 5′-cactggctccctctgctgc-3′ (Table 1). PCR was done with pfu polymerase (Stratagene, La Jolla, CA), starting at 95°C for 5 min, followed by 15 touchdown cycles starting at 95°C for 30 sec, followed by a gradient of 67 to 60°C with the temperature reduced by 0.5°C for each 30-sec cycle, concluding with 90 sec at 72°C. This was followed by 35 PCR cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, ending with a final extension at 72°C for 7 min. The PCR product was cleaved with BamHI and XhoI and cloned into the corresponding sites of pcDNA 3.1, placing the construct under the transcriptional regulation of the T7 promoter in the vector. The intron 3 mutant was created by PCR-based site-directed mutagenesis of this wild-type plasmid using the oligonucleotides listed in Table 1. The wild-type and mutant vectors were confirmed by sequencing using GenBank sequences NC_000015.8 (33) as the genomic reference and NM_000781 (34) as the mRNA reference.

HeLa cells were grown to 50–80% confluence at 37°C in 5% CO2 in DMEM/Ham’s 21 medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin. The wild-type and mutant minigene constructs (2 μg) were transfected into HeLa cells using 16 μl Enhancer and 60 μl Effectene transfection reagents (QIAGEN, Hilden, Germany). After transfection for 20 h, the cells were washed with PBS and incubated in fresh medium for an additional 24 h; then total cellular RNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH). The RNA was treated with DNaseI to degrade any contaminating genomic DNA; 1 μl 25 mM EDTA was added to stop the reaction, and the DNaseI was heat-inactivated at 65°C for 10 min; the RNA was then used as template for cDNA synthesis using reverse transcriptase (Invitrogen). The single-stranded cDNA was used as template for PCR amplification using vector-specific primers (Invitrogen) and primers for GAPDH as an internal control (Table 1). PCR was started at 95°C for 2 min, followed by 15 touchdown cycles beginning with 95°C for 30 sec, followed by a gradient of 57 to 50°C with the temperature reduced 0.5°C for each 30 sec cycle, and 72°C for 90 sec. This was followed by 40 cycles of PCR of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec, ending with a final extension at 72°C for 7 min.

**Case Reports**

Four previous patients have been reported with P450scc deficiency (27–30), hence our cases are termed patients 5 and 6.

**Patient 5**

Patient 5 is a phenotypically normal female without clitoromegaly born at term after an uneventful pregnancy; birth weight was 3.1 kg and her clinical and hormonal data have been reported (35). There was no maternal history of miscarriage, but an older sibling had died from pneumonia at 5 months of age. Patient 5 became lethargic and hypoglycemic (10 mg/dl) at 9 h of age and developed hyperkalemia (7.1 mmol/liter and hypernatremia (121 mmol/liter) during the first week of life. At 9 d of age her plasma ACTH was grossly elevated (>1000 pg/ml), cortisol was 8.5 μg/dl but did not respond to cosyntropin (peak, 9.2 μg/dl), and other measured steroids (in nanograms per deciliter) were low (compared with reference ranges): 17OH-pregnenolone 41 (70–850); aldosterone 4.6 (5–175); 17OH-progesterone 9.0 (60–150); and dehydroepiandrosterone (DHEA) 29 (50–760). She responded well to treatment with hydrocortisone, 9α-fludrocortisone, and salt.

At 4.5 yr, pelvic ultrasonography revealed bilateral intraabdominal gonads and no uterus. Karyotype was 46,XY. Basal gonadotropins were elevated (FSH 18 IU/liter, LH 27 IU/liter) and administration of 5000 U of human chorionic gonadotropin (hCG) daily for 3 d produced no detectable testosterone. Anti-Müllerian hormone (AMH) was 9.9 ng/ml (normal range 48–83 ng/ml), indicating Sertoli cell failure. The testes were removed at 8 yr of age, and estrogen replacement was initiated at 11 yr to induce feminization.

**Patient 6**

The mother of patient 6 had a prior miscarriage and after the birth of patient 6, had another miscarriage at 10–12 wk gestation of a 46,XY fetus with unfused labioscrotal folds. During the pregnancy with patient 6, the mother had low serum estradiol and high hCG; amniocentesis showed that the fetus was 46,XY. Labor was induced at 39 wk, and failure to progress necessitated a cesarean section. At birth, the weight was 2.98 kg and the external genitalia were normal female; no gonads, internal reproductive structures, or adrenals were identified by ultrasound or magnetic resonance imaging (MRI), and a genitogram revealed a blind vaginal pouch. At 6–7 d of age she had hypoglycemia (6 mg/dl), hypernatremia (125 mmol/liter), hyperkalemia (6.1 mmol/liter), and undetectable plasma cortisol (<1 μg/dl). All steroid values obtained between 4 and 9 d of age were low (compared with reference ranges in parentheses; all values in nanograms per deciliter at Endocrine Sciences, Tarzana, CA); pregnenolone 127 (150–2000); progesterone <10 (84–1360); 17OH-progesterone <20 (7–77); DHEA 121 (65–1250); androstenedione 25 (20–290); testosterone <3 (75–400); dihydrotestosterone <2 (5–60). AMH was low but detectable (11.0 ng/ml; reference range 16–49), suggesting the presence of some Sertoli cells. She responded well to treatment with hydrocortisone, 9α-fluorocortisone, and salt. Despite the absent gonadal steroids, gonadotropins were undetectable in the first 12 d of life; the reason for this is not clear. At 8 wk of age, after administration of luprolide (10 μg/kg), LH was only 0.87 IU/liter, free α-subunit was 1.0 ng/ml, and androstenedione, DHEA, 17OH-progesterone, and testosterone did not rise after administration of three doses of 1500 IU hCG given every other day. Adrenal failure combined with hypogonadotropic hypogonadism might suggest a defect in DAX-1, and combined adrenal and gonadal failure might suggest a lesion in SF1 (23), but the sequences of both genes were normal. Patient 6 also had a tethered spinal cord due to a lipoma, hyperlordosis, strabismus, central hypothyroidism, and subnormal GH secretion. At 9 yr, FSH was 73 IU/liter and LH was 4 IU/liter, suggesting primary gonadal failure rather than hypogonadotropic hypogonadism. Consistent with this, AMH was only 1.2 ng/ml (normal range 34–60 ng/ml at 7–8 yr). A repeat MRI scan detected no adrenal or gonadal tissue. However, because AMH remained detectable, laparoscopy was performed and bilateral atrophic testes were removed, one of which had a small Sertoli cell adenoma.
Mutational analysis

Patient 5 was a compound heterozygote for the novel CYP11A1 missense mutations L141W (c.422T > G), inherited from the mother, and V415E (c.1244T > A), inherited from the father. Patient 6 was a compound heterozygote for the frameshift mutation c.835delA inherited from the mother and for the splice site mutation IVS3/IVS11 (2–3)insT inherited from the father. Homozygosity for the c.835delA mutation was previously reported in patient 3 (29).

Studies of P450scc enzyme activity

The activity of P450scc requires the presence of the electron-transfer proteins, adrenodoxin, and adrenodoxin reductase (36). Because P450scc activity is crucially dependent on the molar ratio of each of these three components and, in different systems, both adrenodoxin (31, 37) and adrenodoxin reductase (38, 39) can be limiting, a single plasmid (F2) was used that expresses a fusion protein of the three components of the cholesterol side chain cleavage system (H2N–P450scc–adrenodoxin reductase–adrenodoxin–COOH) with a fixed molar ratio (1:1:1) (31). Using this system ensures that any variations in mutant P450scc activity seen in transfection assays is due to the amino acid change in the mutation rather than to variations in the level of cofactor expression.

COS-1 cells expressing wild-type F2 plasmid made 1350 ± 189 ng pregnenolone/ml culture medium (mean ± SEM), whereas cells transfected with the empty vector produced no detectable pregnenolone. The L141W mutant found in patient 5 produced 520 ± 78 ng pregnenolone/ml (38.5% of wild-type F2 protein), whereas the V415E mutation also found in patient 5 lacked any detectable ability to convert cholesterol to pregnenolone. Similarly, when the c.835delA mutation identified in patient 6 was introduced into the P450scc moiety of the F2 plasmid, no activity was detected (Fig. 1).

Minigene analysis of the splice site mutation

The IVS3/IVS11 (2–3)insT mutation found in patient 6 introduces an additional thymidine after the second base in the third intron so that TTG GAG Tgttaaggg becomes TTG GAG Tgttaaaa. This insertion is predicted to disrupt the tgaaggg splice donor site, so that the intronic sequence will be retained in the mRNA. The retention of the intron with the additional thymidine would create a TAA stop codon: TTG GAG TGT TAA ggg that would result in a truncated protein devoid of activity. Alternatively, it is possible that some transcripts might be spliced correctly. To distinguish between these possibilities, we constructed a “minigene” comprising exon 3, intron 3, and exon 4 and compared its splicing when the intron 3 sequence was either wild-type or carried the mutation found in patient 6.

PCR amplification of the RNA produced from the transcription of the wild-type minigene showed that intron 3 was correctly spliced, yielding a product of the predicted size (580 bp) (Fig. 2). By contrast, PCR amplification of the RNA produced by the mutant minigene produced a product of 1631 bp and no 580 bp product was seen, indicating that the sequences corresponding to intron 3 had been retained in the mRNA. Thus the intronic mutation found in patient 6 prevents correct splicing of the P450scc RNA, so that functional protein cannot be made.

Discussion

The maintenance of human pregnancy requires progesterone, which suppresses uterine contractility, permitting gestation to proceed to term (25, 40, 41), and interruption of the action of progesterone will induce abortion. Because P450scc is the only known enzyme that can convert cholesterol to pregnenolone (1–3), it has been thought that inactivating mutations of P450scc or of any other factor required for placental synthesis of progesterone would be incompatible with term human gestation (26). Consistent with this, no mutations have been reported in ferredoxin, ferredoxin reductase, or the placental isozyme of 3β-hydroxysteroid dehydrogenase. However, four previous pa-
patients with mutations in P450scc have been described, to which we now add two more. Review of these cases shows imprecise correlation between the clinical findings and the enzymology of the P450scc mutations identified in these individuals (Table 2). Patients 1 (27), 2, (28), and 4 (30) all came to medical attention well after the newborn period (9 months to 4 yr of age) and all had P450scc lesions that retained substantial activity: patient 1 had P450scc lesions that retained substantial activity: patient 1 was a manifesting heterozygote for a mutation devoid of activity and hence would be predicted to have about 50% of activity (from the normal allele). Patient 2 carried a missense mutation (R353W) with about 8% of activity on one allele, and the other allele carried a P450scc mRNA splicing defect that generated a small amount of correctly spliced RNA, but the proportion of correctly to incorrectly spliced RNA was not quantitated. Patient 4 had two missense mutations, A359V and R353W, reported to retain 11.7 and 2.8% of activity, respectively, although the same report also noted that R353W retained 8.1% of activity in their report of patient 2. By contrast, patient 3 was homozygous for the frameshift mutation c835delA, predicted to cause the pre-mature stop codon L288X; we have now tested this frameshift in vitro, because it was also found in patient 6, and have shown that it is devoid of detectable activity. Consistent with the lack of detectable enzymatic activity using an in vitro assay, patient 3 had the most severe clinical course, being born at 31 wk and having profound neonatal adrenal failure.

Patients 5 and 6, reported here, also had adrenal failure in the first week of life but had succeeded in reaching term gestation, suggesting that some placental progesterone was synthesized throughout pregnancy. Patient 6 had mutations that eliminated detectable activity, yet she reached term gestation. However, the functional data in patient 5 are more confusing. The L141W mutation retained 38.5% of activity so that in the heterozygous state one would expect about 19% of net activity. This is substantially greater activity than was seen in patients 2 and 4, yet the clinical presentation was substantially more severe. In sum, the data in the six reported patients with P450scc deficiency (Table 2) show a spectrum of clinical presentations from intractable early labor and prematurity to adrenal insufficiency in early childhood. The phenotype/genotype correlations are poor, but the precise reasons for this are unknown.

P450scc deficiency is a novel, rare disorder that can present as acute adrenal insufficiency at any time from infancy to early childhood. In all cases, ACTH and plasma renin activity are grossly elevated and adrenal steroids are inappropriately low or absent; the 46,XY patients have female external genitalia, sometimes with clitoromegaly. In contradistinction to the massive adrenal enlargement typically seen in congenital lipoid adrenal hyperplasia caused by mutations in StAR (7), none of the six patients with P450scc deficiency has been reported to have adrenal hyperplasia. Although a small number of patients with StAR mutations have normal-sized adrenals (42, 43), this may be useful in distinguishing these disorders. Affected tissue has not been available to determine whether P450scc-deficient adrenals can respond to ACTH in vitro. With the exception of patient 3 (29) and patient 6, described here, other reported patients have had at least one allele that conferred some activity. This would seem to explain the term gestation because P450scc is needed to make the progesterone required to maintain pregnancy. Patient 3 (29) remains enigmatic; the survival of that pregnancy to 31 wk may have been caused by an unusually long survival of the progesterone biosynthetic capacity of the maternal corpus luteum, which eventually involuted, causing premature labor. Several patients have had other clinical problems (absent corpus callosum in patient 4, tethered spinal cord in patient 6, short stature in patients 3 (O. Hiort, personal communication) and 6, but a link to P450scc deficiency is not established for any of these conditions. Additional cases, especially those studied hormonally during pregnancy, may provide further information about the hormonal control of parturition and illuminate the pathophysiology of P450scc deficiency.

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