0013-7227/01/$03.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 86(8):3820–3825

Heterozygous Mutation in the Cholesterol Side Chain Cleavage Enzyme (P450scc) Gene in a Patient with 46,XY Sex Reversal and Adrenal Insufficiency

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Cytochrome P450scc, the mitochondrial cholesterol side chain cleavage enzyme, is the only enzyme that catalyzes the conversion of cholesterol to pregnenolone and, thus, is required for the biosynthesis of all steroid hormones. Congenital lipoid adrenal hyperplasia is a severe disorder of steroidogenesis in which cholesterol accumulates within steroidogenic cells and the synthesis of all adrenal and gonadal steroids is impaired, hormonally suggesting a disorder in P450scc. However, congenital lipoid adrenal hyperplasia is caused by mutations in the steroidogenic acute regulatory protein StAR; it has been thought that P450scc mutations are incompatible with human term gestation, because P450scc is needed for placental biosynthesis of progesterone, which is required to maintain pregnancy. In studying patients with congenital lipoid adrenal hyperplasia, we identified an individual with normal StAR and SF-1 genes and a heterozygous mutation in P450scc. The mutation was found in multiple cell types, but neither parent carried the mutation, suggesting it arose *de novo* during meiosis, before fertilization. The patient was atypical for congenital lipoid adrenal hyperplasia, having survived for 4 yr without hormonal replacement before experiencing life-threatening adrenal insufficiency. The P450scc mutation, an in-frame insertion of Gly and Asp between Asp271 and Val272, was inserted into a catalytically active fusion protein of the P450scc system (H2N-P450scc-Adrenodoxin Reductase-Adrenodoxin-COOH), completely inactivating enzymatic activity. Cotransfection of wild-type and mutant vectors showed that the mutation did not exert a dominant negative effect. Because P450scc is normally a slow and inefficient enzyme, we propose that P450scc haploinsufficiency results in subnormal responses to ACTH, so that recurrent ACTH stimulation leads to a slow accumulation of adrenal cholesterol, eventually causing cellular damage. Thus, although homozygous absence of P450scc should be incompatible with term gestation, haploinsufficiency of P450scc causes a late-onset form of congenital lipoid adrenal hyperplasia that can be explained by the same two-hit model that has been validated for congenital lipoid adrenal hyperplasia caused by StAR deficiency. (*J Clin Endocrinol Metab* 86: 3820–3825, 2001)

THE MITOCHONDRIAL CHOLESTEROL side chain cleavage enzyme P450scc converts cholesterol to pregnenolone, thus catalyzing the first and rate-limiting step in steroidogenesis (1, 2). Congenital lipoid adrenal hyperplasia (lipoid CAH) is a severe inborn error of steroid hormone synthesis that disrupts the synthesis of all adrenal and gonadal steroids and leads to the accumulation of cholesterol esters (3–5). Mitochondria isolated from affected tissues fail to convert cholesterol to pregnenolone (6–8); hence, it was long thought that lipoid CAH was caused by P450scc mutations. However, genetic analysis of P450scc was normal in several patients (9–11). Mutations causing lipoid CAH were then found in the gene for the steroidogenic acute regulatory (StAR) protein (12–15). StAR facilitates the transport of cholesterol into mitochondria (12–17), where the P450scc system resides (2). StAR is expressed in the adrenals and gonads but not in the placenta (18), and placental biosynthesis of progesterone, which requires P450scc, is unaffected in lipoid CAH (19). Placentally produced progesterone is essential for the maintenance of human pregnancy. In some animals, such as rabbits and goats, the maternal corpus luteum secretes progesterone throughout pregnancy, but the human corpus luteum of pregnancy secretes progesterone only during the first trimester, during which there is a luteoplacental shift to the placental production of progesterone (20, 21). Therefore, we have suggested that mutations in P450scc would be incompatible with term gestation (22). However, in studying patients with the clinical findings of CAH (15, 23), we encountered a patient with normal StAR genes and a heterozygous mutation in P450scc. We now present clinical, hormonal, genetic, and functional data showing that heterozygosity for P450scc mutation causes a clinical syndrome resembling delayed-onset lipoid CAH, even though homozygous P450scc deficiency should cause early spontaneous abortion.

Abbreviations: lipoid CAH, Congenital adrenal hyperplasia; StAR, steroidogenic acute regulatory; SF-1, steroidogenic factor 1.

Materials and Methods

DNA analysis

The study was approved by the institutional review board, and the parents gave informed consent for DNA analysis. DNA was prepared from blood leukocytes as described (15, 23). The genes for StAR and for steroidogenic factor 1 (SF-1) were amplified by PCR and sequenced using oligonucleotides and conditions previously reported (15, 24). To analyze the P450scc gene from this family, PCR primers were designed for each exon within introns near the intron/exon borders, according to the published human P450scc sequence (25, 26). The sequences of these oligonucleotides are given in Table 1. The PCR conditions consisted of 5 min at 94 C, followed by 30 cycles of 40 sec at 94 C, 40 sec at 57 C, and 60 sec at 72 C. If nonspecific bands were present, the expected PCR product was purified by gel electrophoresis through 2% NuSieve (FMC

TABLE 1. Oligonucleotides using PCR for P450scc gene and site-directed mutagenesis

Exon		Location	Sequence
Exon 1	Forward	5′-flanking	5′-TGGTAGTTATAATCTTGGCCCTG-3′
	Reverse	Intron 1	5′-TACAGCAGGGCTACCCAGTCCCCT-3′
Exon 2	Forward	Intron 1	5′-CTTCCATCAGCCCCTCTCCCCCA-3′
	Reverse	Intron 2	5′-TCCCTCCAGTCCCTGGGGAGA-3′
Exon 3	Forward	Intron 2	5′-TGTTCACCAGGCCTGGGGTCT-3′
	Reverse	Intron 3	5′-AACACTGAGTCCTCCCACCCCCAT-3′
Exon 4	Forward	Intron 3	5′-AGTTGGGCTCTCAGATGGCCCCG-3′
	Reverse	Intron 4	5′-AGGAGCCGGCTGAGGCCTGGGGCT-3′
Exon 5	Forward	Intron 4	5′-TTCCCTCCCGCCTGCAGGGAACCT-3′
	Reverse	Intron 5	5′-GGGCCTGGTGGGGAAGGGGCACGT-3′
Exon 6	Forward	Intron 5	5′-GATAATACCCTACTCCCCACC-3′
	Reverse	Intron 6	5′-GGCCCTGCCCAGGGATTGGA-3′
Exon 7	Forward	Intron 6	5′-ATCAGCTTCTGAGGTCCCCT-3′
	Reverse	Intron 7	5′-ACGGTCAGTCTCTCCCGACCCCG-3′
Exon 8	Forward	Intron 7	5′-TGTGGGGAAGTGGGGCTTCACT-3′
	Reverse	Intron 8	5′-AAGATTGGTGCCTTCATTAGGG-3′
Exon 9	Forward	Intron 8	5′-TAGGGAGACAGATCCTCCCCTG-3′
	Reverse	3′-flanking	5′-TGAAGATGCAGAGACCCCATGG-3′
Primer 1	Sense	Exon 4 and exon 5	5′-*GGG GAC GTG ATT TTC AGT AAA GCT GAC ATA*-3′
Primer 2	Antisense	Exon 4	5′-*GTC CCC GTC CCA TGC AGC CAC ATG GTC* CTT-3′

These primers were used for PCR amplification and direct sequencing for the P450scc gene. PCR primers were designated to correspond to sequences of introns of the human P450scc gene (26). Italic primers were used for site-directed mutagenesis. *Underline* indicates the six bases insertion mutation.

Bioproducts, Rockland, ME). Direct sequencing of the PCR products was done with an ABI PRISM Dye Terminator Cycle Sequencing Kit and an ABI 373A automated fluorescent sequencer (PE Applied Biosystems, Foster City, CA), as described (15, 23).

In vitro analysis of mutant P450scc activity

The six-base insertion in the P450scc sequence was inserted by oligonucleotide-mediated site-direct mutagenesis into the P450scc segment of a vector termed F2, which encodes a fusion of the cholesterol side-chain cleavage enzyme and its electron-donating cofactors: H2N-P450scc-adrenodoxin reductase-adrenodoxin-COOH (27). The sequences of the oligonucleotides used for site-directed mutagenesis are given in Table 1. Primer 1 was the sense strand, and primer 2 was the antisense strand containing the six inserted nucleotides (*underlined*). The accuracy of the construct was confirmed by sequencing. Nonsteroidogenic COS-1 cells were transfected with 1 μg of either the wild-type or mutant F2 plasmid, using 10 μl lipofectramine, as described (12). The soluble hydroxysterol, 20α-hydroxycholesterol, which is converted to pregnenolone by P450scc but bypasses the action of StAR (12), was added as substrate at a concentration of 12.5 μM, which is well above the 2.8 μM Km (Michaelis constant) of the F2 fusion protein (27). Culture media were harvested 48 h after transfection and assayed for pregnenolone by high performance liquid chromatography, as described (15, 23).

Case Report and Results

The proband, the first child of her parents, had a birth weight of 3160 g and a length of 51 cm following an uneventful pregnancy and birth. Inguinal hernias were diagnosed at 2 yr of age, and inguinal masses were resected at another clinic, but the details of that surgery are unknown. When first seen in our clinic at age 4 yr, she was lethargic and had hyperpigmentation. Her weight was 17.4 kg, and her height was 109.4 cm. She had clitoromegaly, no labial fusion, and separate vaginal and urethral openings. Gonads were not palpable. Her serum Na was 142 mEq/liter, and serum K was 4.2 mEq/liter (both normal). However, ACTH was extremely elevated (>880 pmol/liter; normal range, 2.33–6.04), cortisol was 59.4 nmol/liter (normal range, 110–496.6), PRA was 11.73 μg/liter·h (normal range, 0.87–2.76), and plasma aldosterone was 88.7 nmol/liter (normal range, 15.8–49.7) (Table 2). ACTH administration did not increase serum cortisol (30.4–34.2 nmol/L), and a salt-restricted diet did not stimulate urinary aldosterone excretion. Administration of GnRH increased LH from 6.7 mIU/ml to 75.3 mIU/ml and increased FSH from 23.3 mIU/ml to 75.2 mIU/ml. Human CG stimulation did not increase serum testosterone levels (1.03 to 0.85 nmol/liter). Antibodies to P450scc, P450c21, and P450c17 (Cosmic, Tokyo, Japan) were not detected in her serum, ruling out the common form of autoimmune Addison's disease (28, 29). Her karyotype was 46,XY. Computed tomographic and ultrasonographic examinations revealed no hypertrophy of the adrenal glands, and no uterus. Vaginography demonstrated a blind vaginal pouch. She was treated with hydrocortisone and 9α-fluorocortisone; she became more active, and her pigmentation disappeared.

Identification of the P450scc mutation

StAR missense mutations that retain partial activity and cause a later onset of the clinical symptoms of lipoid CAH have been described (13–15); hence, we first sequenced this patient's StAR gene, but this was homozygously normal. Recently, Achermann *et al.* (24) described a patient with physical and hormonal findings similar to lipoid CAH in whom a heterozygous mutation was found in the gene for SF-1, a transcription factor required for adrenal and gonadal expression of all steroidogenic enzymes. However, the SF-1 heterozygote had normal müllarian structures, whereas our patient lacked müllarian structures, and the SF-1 gene was homozygously normal in our patient. We then sequenced the exons of her gene for P450scc and identified heterozygosity for a six-base insertion in exon 4 (Fig. 1A). This mutation (TGGGACGTGATT→TGGGAC*GGGGAC*GTGATT), which may have occurred by slipped strand mispairing (30), inserts codons for Gly and Asp between Asp 271 (GAC) and Val 272 (GTG) without altering the P450scc reading frame (Fig. 1B)

TABLE 2. Endocrinological data

		Normal range
ACTH	>880 pmol/liter	2.33–6.04 pmol/liter (2–4 yr)
Cortisol	59.4 nmol/liter	110–496.6 nmol/liter
Aldosterone	88.7 nmol/liter	15.8–49.7 nmol/liter (3–6 yr)
PRA	11.73 μg/liter·h	0.87–2.76 μg/liter·h (3–6 yr)
17-hydroxyprogesterone	<0.3 nmol/liter	0.39–1.26 nmol/liter (3–6 yr)
11-DOC	0.056 nmol/liter	0.03–0.28 nmol/liter (3–5 yr)
Corticosterone	0.358 nmol/liter	6–23 nmol/liter (1–14 yr)
DHEA-S	<0.54 μmol/liter	<0.54 μmol/liter

ACTH stimulation[a]	Before	After	Na restriction test[b]	Before	After
Cortisol	30.4	34.2 nmol/liter	Urinary aldosterone (day)	3.2	3.8 nmol/d

Measurements were obtained in the patient at the time of diagnosis at the age of 4 yr.
[a] $^{1-24}$ACTH (0.25 mg) administered iv.
[b] Intake of salt restricted to 1.0 g/d for 4 d. Urinary aldosterone: normal range, 61–120 μg/d.

TABLE 3. Effect of the two amino acid insertion mutation on P450scc activity

Vector	Pregnenolone (ng/dish)
Control	ND
WT	320 ± 42
Mutant	ND
WT+Mutant	275 ± 18

The mutant P450scc cDNA with the six-base insertion was cloned into the F2 construct, which expresses the functional protein H2N-P450scc-adrenodoxin reductase-adrenodoxin-COOH (27). Nonsteroidogenic COS-1 cells were cultured and transfected with 1 μg of the wild-type and mutant F2, using 10 μl lipofectamine, as described previously (12). The substrate added was 20α-hydroxycholesterol (5 μg/ml), which bypasses the action of StAR. Forty-eight hours after transfection, the culture medium was collected for assays of pregnenolone by high-performance liquid chromatography. Control, pECE vector; WT, wild type; F2 Mutant, mutant F2; Wt+Mutant, both of 1.0 μg of the wild-type and mutant vector were transfected simultaneously; ND, not detected.

(25, 26). Analysis of the patient's genomic DNA from hair follicles and urinary sediment confirmed that this mutation was heterozygous and was inconsistent with mosaicism. Leukocyte genomic DNA from her mother and father did not contain this mutation, suggesting that the patient's mutations occurred *de novo* (Fig. 1C). Direct sequencing of all other exons, splice sites, and 620 bases of 5′ flanking DNA did not reveal any other mutations.

The three-dimensional structure of P450scc is not known. We have previously aligned the sequences of the various human mitochondrial (Type I) P450 enzymes (31), and we have used computational methods to align one of these with the secondary structures of three bacterial Type Ip450 enzymes (P450cam, P450terp, P450eryf), whose complete structures are known by crystallography (32). These comparisons indicate that residues 271 and 272 of P450scc lie in the G-helix. The presence of the very highly conserved Trp 270 residue nearby in this helix confirms this localization. The insertion of Gly and Asp between residues 271 and 272 would probably not disrupt the helix, but would lengthen it and change the orientation downstream from 272 by half a turn, meaning the next segment would begin in the wrong location. Thus, we propose that the insertion of Gly and Asp between residues 271 and 271 results in a conformational mutant.

Activity of the P450scc mutant

To determine whether this in-frame insertion altered P450scc enzymatic activity, we created the Gly-Asp insertion mutant by site-directed mutagenesis. To catalyze conversion of cholesterol to pregnenolone, P450scc must receive electrons from NADPH through the intermediacy of a flavoprotein, termed "adrenodoxin reductase," and an iron-sulfur protein, termed "adrenodoxin" (for review see Ref. 2). Because the level of P450scc activity in transfected cells is crucially dependent on the molar ratio of adrenodoxin to P450scc (27, 33), we sought to eliminate this variable by using a vector (termed F2) that expresses the P450scc system as a single, catalytically active fusion protein; H2N-P450scc-adrenodoxin reductase-adrenodoxin-COOH (27). Similarly, P450scc activity as measured by production of pregnenolone normally depends on the StAR protein to deliver the cholesterol substrate into the mitochondria (17). However, we circumvented the action of StAR by providing the cells with 20α-hydroxycholesterol, which readily diffuses into mitochondria without StAR's action (12, 34). The wild-type, but not the mutant, efficiently converted 20α-hydroxycholesterol to pregnenolone (Table 3). Because the mutation was found in a heterozygous state, we tested the possibility that it might act as a dominant negative mutant; however, cotransfecting COS-1 cells with both the wild-type and mutant vectors had no significant affect on the wild-type's enzymatic activity (Table 3).

Discussion

The differential diagnosis of male pseudohermaphroditism (46 XY sex reversal) includes: 1) errors in testosterone biosynthesis and action (*e.g.* defects in testosterone biosynthetic enzymes, 5α-reductase deficiency, androgen receptor defects); 2) dysgenetic male pseudohermaphroditism (*e.g.* XY gonadal dysgenesis, XO/XY mosaicism, mutations in SOX9, WT1, SF-1 or other factors involved in testicular development); 3) testicular unresponsiveness to LH/hCG; and iv) defects in the synthesis or action of müllarian-inhibiting substance (35). Our patient's hyperresponsiveness of LH and FSH to GnRH stimulation and the unmeasurable testosterone response to hCG would normaly implicate an error in testosterone biosynthesis, but because the testes were probably removed at age 2, these are the expected responses. The minimal cortisol concentrations in the face of grossly ele-

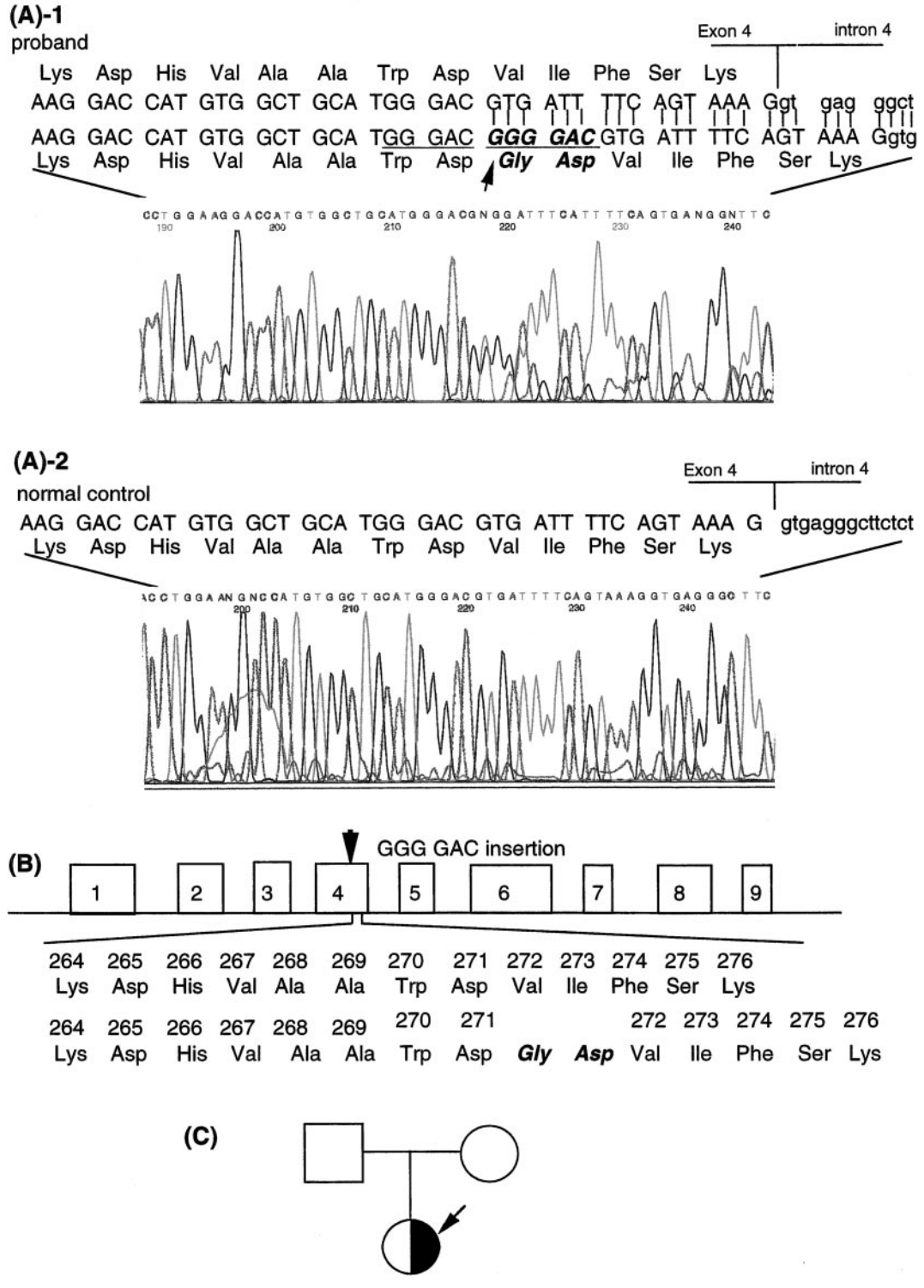

FIG. 1. Mutation of P450scc gene. A-1, Chromatograms of DNA sequences in the proband demonstrating the heterozygous six-base insertion (*italic bases*) in exon 4. Note the double peaks after the mutation site (denoted by an *arrow*). *Underlines* indicate that this mutation is presumably caused by slipped strand mispairing. A-2, Direct sequence of wild type. B, Schematic representation of the mutation sites and part of the amino acid sequence of P450scc. The 6-bp insertion adds two amino acids (Gly and Asp) in frame between Asp271 and Val272. Amino acids numbers follow previous reports (26, 27). C, Family tree. The *half-solid circle* in the proband designates a heterozygous mutation of P450scc.

vated ACTH concentrations indicate an error in an enzyme or factor affecting both adrenal and gonadal steroidogenesis. Dysgenetic male pseudohermaphroditism cannot be excluded totally because the testes were not available for inspection. However, the karyotype was 46, XY, an SF-1 mutation was inconsistent with the absence of a uterus and was ruled out by sequencing, and defects in WT1 and SOX9, as well as unresponsiveness to LH/hCG and disorders of

müllarian-inhibiting substance synthesis and action, would all be inconsistent with adrenal insufficiency. Similarly, mutations in DAX-1 would cause adrenal insufficiency but not cause male pseudohermaphroditism (36). Thus, it is clear that our patient had a combined error in adrenal and gonadal steroidogenesis.

Among the errors in the combined testosterone and cortisol biosynthesis, one might consider defects in 3βHSD, P450c17, and classical lipoid CAH due to StAR mutations. All described defects in 3βHSD are in the 3βHSD II gene and can be either salt-wasting or nonsalt-wasting (37). However, such patients tend to have normal or elevated plasma concentrations of 17-hydroxyprogesterone due to peripheral conversion of 17-hydroxypregnenolone to 17-hydroxyprogesterone by extraglandular 3βHSDI (37, 38), and 17-hydroxyprogesterone was unmeasurable in our patient. A severe defect in P450c17 would cause cortisol and testosterone deficiency with male pseudohermaphroditism, but would also result in elevated concentrations of DOC and corticosterone (39), which were not seen in our patient. StAR mutations causing lipoid CAH are usually, but not always associated with massively enlarged adrenals (40) but our patient's StAR gene sequence was normal. Thus, it appeared that our patient had a wholly novel defect in steroidogenesis.

This patient had clinical features similar to patients with lipoid CAH (8, 14, 15, 23) but also differed from the usual phenotype of lipoid CAH in two respects. First, the age of onset of clinical symptoms was at age 4 yr, whereas most patients with lipoid CAH have symptoms of adrenal insufficiency within the first month of life (14, 15). A compound heterozygous patient carrying the StAR mutations A218V and L275P, each of which retains 20–24% of StAR activity, survived 4 months without hormonal replacement (14), and another compound heterozygous patient carrying M225T, which retains 43% of activity, and the wholly inactive Q258$\times$ mutation survived 10 months without therapy (15). Thus, our patient clearly had a slower, more insidious onset of adrenal insufficiency. Second, our patient did not have enlarged adrenals detected by computed tomography or ultrasonography. Massive adrenal enlargement is a classical feature of lipoid CAH described in virtually all of these patients (3–15), however, a lack of radiologically demonstrable adrenal hyperplasia has been described in one patient with lipoid CAH caused by StAR mutations (40). Thus, adrenal hyperplasia remains a useful diagnostic feature of classic lipoid CAH.

The heterozygous mutation in our patient's P450scc gene was found in her peripheral leukocytes and in cells from her urinary sediment and hair follicles, but was not found in leukocyte DNA from either parents. Thus, our patient exhibited a spontaneous mutation that was found in all three tissues examined, as well as in her adrenals and testes. This indicates that the mutation occurred very early, quite possibly during meiosis of a gamate before fertilization, rather than being a somatic cell mutation causing a patchy distribution of abnormal cells as seen in the McCune-Albight syndrome. The sequence of our patient's mutation, which suggests slipped strand mispairing, also strongly favors a mutation occurring during meiosis rather than a somatic cell mutation occurring during mitosis. The severe phenotype associated with heterozygosity for a P450scc mutation indicates that it is unlikely that persons carrying such mutations could reproduce and thus transmit the mutation. Thus, any individual with lipoid CAH phenotype carrying a P450scc mutation is likely to carry a *de novo* mutation.

Heterozygotes for other forms of CAH are clinically normal and have minimal, if any hormonal manifestations; hence, we did not anticipate that heterozygotes for P450scc mutations would have an obvious phenotype. By contrast, our patient was severely ill in a heterozygous state, indicating a dominant disease. Many autosomal dominant diseases result from a "dominant negative" mechanism, in which a small number of defective protein monomers disrupt a larger functional complex. However, expression of the P450scc mutant in the same cells transfected with the wild-type P450scc construct did not impair the activity of the wild type. Thus, we sought another explanation for the dominant nature of the mutation in the gene for this enzyme.

The reason why our patient developed incomplete virilization and mild adrenal insufficienty despite retaining 50% of normal enzymatic activity can probably be explained by the same "two-hit" mechanism that accounts for the pathophysiology of lipoid CAH (14) and has been confirmed by studies of human ovarian physiology (23) and by examination of StAR-knockout mice (41, 42). P450scc is the rate-limiting enzyme in steroidogenesis. This is a very slow enzyme, turning over only 1 mole of cholesterol per mole of P450scc per second (43); thus, haploinsufficiency of P450scc may prevent the elaboration of an appropriate steroidogenic response. The insufficient steroidogenesis will lead to increased tropic stimulation of the adrenals by ACTH and of the gonads by gonadotropins, stimulating the uptake of low-density lipoproteins and cholesterol; this cholesterol accumulates, eventually causing cell death (14). The principal difference with lipoid CAH is that haploinsufficiency of P450scc permits more steroidogenesis than the low levels of StAR-independent steroidogenesis seen in StAR deficiency, so the progression from the mutation-induced impairment in steroidogenesis (the first hit) to the loss of steroidogenesis from cell death (the second hit) is slower. Consistent with this view, rabbits with homozygous deletion of P450scc gene have a phenotype that very closely resembles lipoid CAH although, unlike our patient, the heterozygotes are healthy and can reproduce (44), but, unlike a hypothetical human homozygous deletion for P450scc, these rabbits are born normally because the maternal corpus luteum rather than the placenta synthesizes progesterone (19–21). Thus, while our patient shows that haploinsufficiency of P450scc causes late-onset lipoid CAH, it remains true that complete P450scc deficiency should cause spontaneous abortion at the time of the luteo-placental shift (21).

Lipoid CAH has been studied in at least 57 patients (40), and only one of the initially described patients lacked a StAR mutation (14). However, haploinsufficiency of SF-1 with normal StAR has been reported in one patient with a clinical syndrome resembling lipoid CAH (24), and we now show that haploinsufficiency of P450scc can also produce this phenotype. Thus, lipoid CAH is the common phenotypic manifestation of several genetic disorders in the early steps in steroidogenesis.

Acknowledgments

Received January 1, 2001. Accepted April 20, 2001.

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This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Science, Education, and Culture of Japan and by NIH Grants DK37922 and HD34449 (to W.L.M.).

T.T., K.F., and N.K. contributed equally to this work.

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