Lack of Mutations in P450scc Gene (CYP11A) in Six Japanese Patients with Congenital Lipoid Adrenal Hyperplasia

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Abstract Congenital lipoid adrenal hyperplasia (CLAH) results from deficient cholesterol side chain cleavage activity in both adrenal and gonadal mitochondria. Previous studies, however, have failed to disclose a defect in the P450scc gene in six patients studied to date.

To investigate further a cause-and-effect relationship between a mutation in the P450scc gene and congenital lipoid adrenal hyperplasia phenotype, we studied the P450scc genes from five Japanese families, in which at least one member had the entity (six patients and 10 unaffected members), by direct sequencing of all the nine exons and by linkage analysis using restriction fragment length polymorphism and microsatellite polymorphism.

No major rearrangement or minor mutation was found in the entire exonic sequences of the gene in the six patients. The particular copy of the P450scc gene as defined by the microsatellite polymorphism did not cosegregate with the disease in one informative family.

We conclude that the primary lesion in congenital lipoid adrenal hyperplasia does not reside in the P450scc gene in most, if not all, of the patients.

Key words: congenital lipoid adrenal hyperplasia, P450scc gene, CYP11A, deoxyribonucleic acid sequencing, linkage analysis

Introduction

Congenital lipoid adrenal hyperplasia (CLAH), an autosomal recessive trait most commonly affecting Japanese, is a rare inborn error of metabolism caused by defective conversion of cholesterol into pregnenolone. Since the defect results in impaired biosynthesis of all three classes of adrenal steroids and of gonadal

sex steroids (1-3), the condition is characterized by profound adrenocortical and gonadal insufficiency (4-6). Although the cholesterol side chain cleavage system is mediated by the P450scc enzyme, previous studies in affected individuals have failed to detect a mutation in the P450scc gene (CYP11A). In 1986, Matterson *et al.* found no gross rearrangement in the P450scc gene in three unrelated patients by Southern blot analysis using cDNA probe (7). In 1991, Lin *et al.* reported that the amount of testicular messenger ribonucleic acid (mRNA) was normal in one Korean patient and that the

Received: February 28, 1995

Accepted: March 20, 1995

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cDNA sequence was normal in one Japanese and two Korean patients (8, 9). Further, in 1994, Sakai *et al.* reported normal testicular cDNA sequence in one Japanese patient (10).

To extend the above observations further, we studied the P450scc gene in six additional Japanese patients and their family members by direct sequencing of all the nine exons and by linkage analysis using Southern blot restriction fragment length polymorphism (RFLP) and single strand conformational polymorphism (SSCP).

Subjects

Six Japanese CLAH patients of five fami-

lies and ten unaffected family members were studied (Fig. 1). Each parent of the five families was unrelated. The clinical data of the patients are shown in Table 1. Three patients (patient A, B, C) had a 46,XY karyotype and the remaining three (patient D1, D2, E) had a 46,XX karyotype. All the patients were completely feminized and diffusely pigmented at birth.

Patient A

Signs and symptoms of acute adrenal failure started at two days of age: serum Na 128 mEq/L, K 6.1 mEq/L, plasma adrenocorticotropin (ACTH) 2,200 pg/mL, and plasma renin activity (PRA) >25 ng/mL/hour.

Fig. 1 Patient families. The individuals enclosed by the circles were analysed for the P450scc gene.

Table 1. Clinical features of the patients

Patient	Karyotype	External genitalia	Sexual ducts	Gonads	Enlarged adrenals of fat-tissue attenuation detected by CT scan
A	46,XY	Complete female	Wolffian derivatives	Testes	Yes
B	46,XY	Complete female	Wolffian derivatives	Testes	Yes
C	46,XY	Complete female	Wolffian derivatives	Testes	Not examined
D1	46,XX	Complete female	Uterus	Ovaries	Yes
D2	46,XX	Complete female	Uterus	Ovaries	Yes
E	46,XX	Complete female	Uterus	Ovaries	Not examined

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Patient B

At eight days of age, the patient was referred to our hospital because of diffuse pigmentation: serum Na 133 mEq/L, K 7.0 mEq/L, plasma ACTH 880 pg/mL, and PRA 60.0 ng/mL/hour (11).

Patient C

The patient began to vomit at eight hours of age: serum Na 126 mEq/L, K 6.1 mEq/L, and plasma ACTH 215 pg/mL. The eldest sibling died of adrenal insufficiency at 6 months of age.

Patient D1 and D2

Both patients had symptoms of adrenal insufficiency shortly after birth. Abdominal computed tomography revealed bilaterally enlarged adrenals of fat-tissue attenuation, excluding the congenital adrenal hypoplasia.

Patient E

The patient became dehydrated after repeated vomiting and diarrhea at two days of age. Her elder sibling died of adrenal insufficiency at 39 days of age. An autopsy showed bilaterally enlarged adrenal glands with fat deposition, confirming the diagnosis of CLAH.

All the patients were healthy on the standard doses of hydrocortisone and fludrocortisone at the time of the study.

Methods

Sequence analysis of the nine exons

The nine exons were amplified by polymerase chain reaction (PCR) under the condition programmed by Lin *et al.* (8). In this study, we designed the following two new primers:

5'-AGGGCTACCCAGTCCCCT-3' (antisense primer of exon 1); and 5'-TGTCTGCAATTCCAGCCAG-3' (antisense primer of exon 7). The sequences of the remaining primers were as described previously (8). The PCR products were directly determined for DNA sequences by dideoxyterminator method (Applied Biosystems). The sequence determination was done at least three times from independent amplifications.

Southern blot analysis

Genomic DNA of the patients, the family members, and ten Japanese controls was digested with *EcoR* I, *Hind* III, *Bam*H I, *Msp* I, *Pst* I, and *Pvu* II, and was hybridized with full-length human P450scc cDNA probe. Southern transfer, probe hybridization, and autoradiography were carried out by the standard methods.

SSCP analysis of the (CA)_n repeats

In family B and D, the DNA sequences encompassing the (CA)_n repeats, located at about 1,400 bp upstream of the exon 1 of CYP11A, was amplified by PCR and was subjected to SSCP analysis, to detect a possible microsatellite polymorphism (12). The primer sequences and conditions of the PCR analysis were as described previously (13). For SSCP analysis, PCR products were treated with formamide, displayed by electrophoresis (Pharmacia, Phast system) with native buffer strips through a homogenous gel 12.5 (Phast gel), and stained with silver.

Results

Sequence analysis of the nine exons

The sequences of the nine exons of the

Fig. 2 Southern blot analysis of family A. (F: father; P: patient; M: mother; ♂ : normal male; ♀ : normal female) Shown are *EcoR* I digests concomitantly hybridized with the cDNA probe for CYP11A and the genomic probe for TK. Both the size and the intensity of the three bands for CYP11A are comparable between the five individuals. The comparison of the band intensity for TK mapped to the long arm of chromosome 17 confirms that the amount of DNA loaded is very similar between the five lanes.

patients were identical with those of the reported human P450scc cDNA (14, 15). Exon-intron boundaries were also identical with the reported human P450scc gene sequences (16).

Southern blot analysis

No RFLP was detected for all the probe-enzyme combinations. The fragment pattern of the P450scc gene was identical in both length and signal intensity between the patients and the normal individuals (Fig. 2), excluding gross

Fig. 3 SSCP analysis of family D. The band pattern is different in two sibs with CLAHL, providing compelling evidence that CLAHL in the two sibs is not caused by the impairment of the P450scc gene (CYP11A).

rearrangements in the P450scc gene of the patients.

SSCP analysis of (CA)_n repeats

In family D, the two patients showed different SSCP patterns, indicating lack of the linkage between defective CYP11A and CLAHL (Fig. 3). In family B, the SSCP patterns were not informative.

Discussion

A minor mutation or gross rearrangement was not found in the entire nine exons of P450scc gene in all of our patients. In addition, the linkage between defective P450scc gene and CLAHL was precluded in one family, although the possibility of intragenic recombination can not be excluded. These results provide further credence to the belief that the primary lesion of CLAHL does not reside in the

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Fig. 4 The cholesterol side chain cleavage system. A schematic representation showing various factors involved in the cholesterol side chain cleavage system.

P450scc gene.

Provided there is an intact P450scc gene, there are several possible explanations for the

development of CLAH. As depicted in Fig. 4, two major steps are required for a successful conversion of cholesterol into pregnenolone: (1) transport of free cholesterol into inner mitochondrial membrane; and (2) enzymatic reaction within the mitochondria. The details of factors involved in the two steps are summarized in Table 2. Of the first step factors, steroid carrier protein 2, steroidogenesis activator peptide, endozepine, and endozepine receptor have been shown to be normal in the amount and size of mRNA (8, 9, 32) (The biological role of phosphoprotein 30, cytoskeleton, and 5-hydroperoxyeicosatetraenoic acid remains unclear). Similarly, of the second step components, adrenodoxin and adrenodoxin reductase have been reported to be normal in the amount and size of mRNA (8, 9). However, since detailed studies have not been performed

Table 2. Factors involved in the cholesterol side chain cleavage system

	Locus	Expression site	Known biological functions	Reference
			other than steroidogenesis	
P450scc	15q21-23	Adrenal, Gonad, Placenta	None	8,9,14-18
Adrenodoxin	11q22	Ubiquitous	Vitamin D metabolism	8,9,17-20
Adrenodoxin reductase	17q24-25	Ubiquitous	Vitamin D metabolism	8,9,17,20-22
SCP2	1p21-pter	Various	Non specific lipid transfer	23,24
Cytoskeleton	?	Ubiquitous	?	25,26
pp30	?	Adrenal, Gonad	None ?	27,28
SAP	? (Cloned)	Ubiquitous	None ?	8,9,29
Endozepine	? (Cloned)	Ubiquitous	Fat metabolism, Insulin secretion	8,30-32
5-HPETE	?	Ubiquitous	?	33

SCP2: Steroid carrier protein 2, pp30: Phosphoprotein 30, SAP: Steroidogenesis activator peptide

5-HPETE: 5-hydroperoxyeicosatetraenoic acid

for the corresponding genes, the possibility that a mutation in every one of the genes results in the development of CLAH remains tenable.

The lack of mutation in the P450scc gene is in sharp contrast to the presence of various mutations in other forms of congenital adrenal hyperplasia (2, 3). In this regard, it is noteworthy that the phenotype of CLAH is remarkably uniform (34), whereas that of other forms of congenital adrenal hyperplasia is highly variable (2, 3). Thus, the absence of the P450scc gene mutations may explain in part the difference in phenotypic spectrum between CLAH and other forms of congenital adrenal hyperplasia. Although the reason responsible for the lack of mutation in the P450scc gene remains to be clarified, one possibility would be that the loss of P450scc function results in fetal death while loss of other P450 enzyme function permits fetal survival. In this context, one may argue that the rabbits missing both alleles of the P450scc gene can survive the fetal life (35). However, this could be due to a species difference in the viability in terms of P450scc function.

In summary, the molecular basis of CLAH remains unknown. Our results, however, provide compelling evidence that CLAH is not caused by a defective P450scc gene.

Acknowledgements

We would like to thank Dr. Walter L. Miller, University of California, for kindly donating P450scc cDNA probe, Dr. Yutaka Igarashi, Tohoku University, for providing us with DNA samples of family D, and Dr. Fumiko Mitani, for fruitful discussion.

References

1. Degenhart HJ. Prader's syndrome (congenital lipoid adrenal hyperplasia). In: Pediatric and adolescent endocrinology 13. Adrenal diseases in childhood, Basel Karger, 1984: 125-44.
2. New MI, Levine LS. Congenital adrenal hyperplasia. Berlin/Heidelberg/New York /Tokyo Springer-Verlag 1984: 1-88.
3. New MI, White PC, Pang S, Dupont B, Speiser PW. The adrenal hyperplasias. In: Scriver CR, Beaudet AL, Sly WS, editors. The metabolic basis of inherited disease, New York: McGraw-Hill Book Co., 1989: 1296-305.
4. Prader A, Siebenmann RE. Nebenniereninsuffizienz bei Kongenitaler Lipoidhyperplasie der Nebennieren. *Helv Paediatr Acta* 1957; 12: 569-95.
5. Camacho AM, Kowarski A, Migeon CJ, Brough AJ. Congenital adrenal hyperplasia due to a deficiency of one of the enzymes involved in the biosynthesis of pregnenolone. *J Clin Endocrinol Metab* 1968; 28: 153-61.
6. O'Doherty NJ. Lipoid adrenal hyperplasia. *Guy's Hosp Rep* 1964; 113: 368-79.
7. Matterson KJ, Chung B, Urdea MS, Miller WL. Study of cholesterol side-chain cleavage (20, 22 desmolase) deficiency causing congenital lipoid adrenal hyperplasia using bovine sequence P450scc oligodeoxyribonucleotide probes. *Endocrinology* 1986; 118: 1296-305.
8. Lin D, Gitelman SE, Saenger P, Miller WL. Normal genes for the cholesterol side chain cleavage enzyme, P450scc, in congenital lipoid adrenal hyperplasia. *J Clin Invest*

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- 1991; 88: 1955-62.
9. Saenger P, Lin D, Gitelman SE, Miller WL. Congenital lipid adrenal hyperplasia-Genes for P450scc, side chain cleavage enzyme, are normal. *J Steroid Biochem Mol Biol* 1993; 45: 87-97.
10. Sakai Y, Yanase T, Okabe Y, Hara T, Waterman MR, Takayanagi R, *et al.* No mutation in cytochrome P450 side chain cleavage in a patient with congenital lipid adrenal hyperplasia. *J Clin Endocrinol Metab* 1994; 79: 1198-201.
11. Ogata T, Matsuo N, Saito M, Prader A. The testicular lesion and sexual differentiation in congenital lipid adrenal hyperplasia. *Helv Paediatr Acta* 1988; 43: 531-8.
12. Moore CCD, Brentano ST, Miller WL. Human P450scc gene transcription is induced by cyclic AMP and repressed by 12-O-tetradecanoylphorbol-13-acetate and A23187 through independent cis-elements. *Mol Cell Biol* 1990; 10: 6013-23.
13. Beckmann JS, Tomfohrde J, Barnes RI, Williams M, Broux O, Richard I, *et al.* A linkage map of human chromosome 15 with an average resolution of 2cM and containing 55 polymorphic microsatellites. *Hum Mol Genet* 1993; 2: 2019-30.
14. Chung B, Matterson kJ, Voutilainen R, Mohandas TK, Miller WL. Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in placenta. *Proc Natl Acad Sci USA* 1986; 83: 8962-6.
15. John ME, Jhon MC, Ashley P, MacDonald RJ, Simpson ER, Waterman MR. Identification and characterization of cDNA clones specific for cholesterol side-chain cleavage cytochrome p-450. *Proc Natl Acad. Sci USA* 1984; 81: 5628-32.
16. Morohashi k, Sogawa k, Omura T, Fujii-kuriyama Y. Gene structure of human cytochrome P-450 (SCC), cholesterol desmolase. *J Biochem (Tokyo)* 1987; 101: 879-87.
17. Sparkes RS, Klisak I, Miller WL. Regional mapping of genes encoding human steroidogenic enzymes: P450scc to 15q23-q24; adrenodoxin to 11q22; adrenodoxin reductase to 17q24-q25; and P450c17 to 10q24-q25. *DNA Cell Biol* 1991; 10: 359-65.
18. Chang C, Wu D, Lai C, Miller WL, Chung B. Cloning and structure of the human adrenodoxin gene. *DNA* 1988; 7: 609-15.
19. Picado-Leonard J, Voutilainen R, Kao L, Chung B, Straus JF III, Miller WL. Human adrenodoxin: Cloning of three cDNAs and cycloheximide enhancement in JEG-3 cells. *J Biol Chem* 1988; 263: 3240-4.
20. Hara T, Kimura T. Purification and catalytic properties of a cross-linked complex between adrenodoxin reductase and adrenodoxin. *J Biochem (Tokyo)* 1989; 105: 594-600.
21. Solish SB, Picardo-Lepnard J, Morel Y, Kuhn RW, Mohandas TK, Hanukoglu I, *et al.* Human adrenodoxin reductase: Two mRNAs encoded by a single gene on chromosome 17cen-q25 are expressed in steroidogenic tissues. *Proc Natl Acad Sci USA* 1988; 85: 7104-8.
22. Lin D, Shi Y, Miller WL. Cloning and sequence of the human adrenodoxin reductase gene. *Proc Natl Acad Sci USA* 1990; 87: 8516-20.
23. Yamamoto R, Kallen CB, Babalola GO, Rennert H, Billheimer JT, Strauss JF III. Cloning and expression of a cDNA encoding

- human sterol carrier protein 2. *Proc Natl Acad Sci USA* 1991; 88: 463-7.
24. He Z, Yamamoto R, Furth EE, Schantz LJ, Naylor SL, George H, *et al.* cDNAs encoding members of a family of proteins related to human sterol carrier protein 2 and assignment of the gene to human chromosome 1p21-pter. *DNA Cell Biol* 1991; 10: 559-69.
 25. Almahbobi G, Williams LJ, Hall PF. Attachment of mitochondria to intermediate filaments in adrenal cells: Relevance to the regulation of steroid synthesis. *Exp Cell Res* 1992; 200: 361-9.
 26. Hall PF. On the mechanism of action of ACTH; the role of actin. *Endocr Res* 1984-1985; 10: 431-61.
 27. Yanagibashi K. Short term stimulatory effect of ACTH: Mechanism of substrate cholesterol supply to P450_{scc} (in Japanese). *Tanpakusitu Kakusan Kouso* 1992; 37: 3024-33.
 28. Epstein LF, Orme-Johnson NR. Regulation of steroid hormone biosynthesis: Identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells. *J Biol Chem* 1991; 266: 19739-45.
 29. Li X, Warren DW, Gregorie J, Pedersen RC, Lee AS. The rat 78,000 dalton glucose-regulated protein (GRP78) as a precursor for the rat steroidogenesis-activator polypeptide (SAP): the SAP coding sequence is homologous with the terminal end of GRP78. *Mol Endocrinol* 1989; 3: 1944-52.
 30. Webb NR, Rose T, Marik N, Maquardt H, Shoyab M, Tadaro GJ, *et al.* Bovine and human cDNA sequences encoding a putative benzodiazepine receptor ligand. *DNA* (NY) 1987; 6: 71-9.
 31. Chang YJ, McCabe RT, Rennart H, Budarf ML, Sayegh R, Emanuel BS, *et al.* The human "peripheral-type" benzodiazepine receptor; regional mapping of the gene and characterization of the receptor expressed from cDNA. *DNA Cell Biol* 1992; 11: 471-80.
 32. Lin D, Chang YJ, Strauss JF III, Miller WL. The human peripheral benzodiazepine receptor gene: cloning and characterization of alternative splicing in normal tissues and in a patient with congenital lipid adrenal hyperplasia. *Genomics* 1993; 18: 643-50.
 33. Mikami K, Omura M, Tamura Y, Yosida S. Possible site of action of 5-hydroperoxyeicosatetraenoic acid derived from arachidonic acid in ACTH-stimulated steroidogenesis in rat adrenal glands. *J Endocrinol* 1990; 125: 89-96.
 34. Matsuo N, Tsuzaki S, Anzo M, Ogata T, Sato S. The phenotypic definition of congenital lipid adrenal hyperplasia: Analysis of the 67 Japanese patients. *Horm Res suppl* 1994; 41: 106.
 35. Pang S, Yang X, Wang M, Tissot R, Nino M, Maligod J, *et al.* Inherited congenital adrenal hyperplasia in the rabbit; absent cholesterol side chain cleavage cytochrome P450 gene expression. *Endocrinology* 1992; 131: 181-6.