論　文　目　録

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主　論　文

A nonsense mutation (TGG [^{116}Trp] →TAG [Stop]) in *CYP11B1* causes steroid 11β-hydroxylase deficiency　1冊

平成５年12月発行　J. Clin. Endocrinol. Metab. 77 : 1677～1682

参考論文

1）Congenitally defective aldosterone biosynthesis in humans : the involvement of point mutations of the P-450$_{C18}$ gene (*CYP11B2*) in CMO Ⅱ deficient patients　1冊

平成４年１月発行　Biochem Biophys Res Commun. 182 : 974～979

2）Congenitally defective aldosterone biosynthesis in humans : inactivation of the P-450$_{C18}$ gene (*CYP11B2*) due to nucleotide deletion in CMO Ⅰ deficient patients　1冊

平成５年２月発行　Biochem Biophys Res Commun. 190(3) : 864～869

0021-972X/93/7706-1677$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 77, No. 6
Printed in U.S.A.

A Nonsense Mutation (TGG [Trp^{116}] → TAG [Stop]) in *CYP11B1* Causes Steroid 11β-Hydroxylase Deficiency*

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ABSTRACT

Steroid 11β-hydroxylase deficiency (11βOHD), an autosomal recessive hereditary disease, accounts for 5–8% of cases of congenital adrenal hyperplasia. In this study, we carried out a molecular genetic analysis of *CYP11B1* encoding steroid 11β-hydroxylase (P450c11) from a Japanese patient affected with this disease. Nucleotide sequence analysis of polymerase chain reaction-amplified products of the patient's genome revealed the occurrence of a stop codon in exon 2 due to a point mutation, TGG → TAG (Trp^{116} → Stop). To further analyze the role of *CYP11B2* encoding steroid 18-hydroxylase (P450c18) in the 11βOHD patient, *CYP11B2* of the patient was also amplified and sequenced. In contrast to *CYP11B1*, there was no mutation in *CYP11B2*. Restriction fragment length polymorphism analysis indicated that the 11βOHD patient is homozygous and his unaffected parents are heterozygous for the mutation. When a cDNA corresponding to *CYP11B1* of the 11βOHD patient was transfected into COS-7 cells, steroid 11β-hydroxylase activity was not detectable in mitochondria of the cells. These results demonstrate that intact P450c11 was not produced at all due to the nonsense mutation in *CYP11B1* of the patient without any mutation in *CYP11B2*. (*J Clin Endocrinol Metab* **77:** 1677–1682, 1993)

CONGENITAL adrenal hyperplasia (CAH) is a disease caused by an inherited defect in cortisol biosynthesis (1a, 1b). Cortisol is normally synthesized from cholesterol in the zona fasciculata of the adrenal cortex by five enzymatic steps: the cholesterol side-chain is cleaved by a reaction catalyzed by cholesterol desmolase to form pregnenolone, which is then dehydrogenated at the 3β position to yield progesterone, and two successive hydroxylation reactions at the 17α and 21 positions of progesterone yield 11-deoxycortisol, which is then converted to cortisol by the catalytic action of steroid 11β-hydroxylase (P450c11).

Five to 8% of reported cases of CAH are due to steroid 11β-hydroxylase deficiency (11βOHD), which is characterized by virilization and hypertension. Decreased cortisol production stimulates ACTH secretion, which results in the excessive production of adrenal androgens and non-11β-hydroxylated steroid precursors. The excessive androgens cause fetal virilization in females and precocious virilization in males. Elevated levels of 11-deoxycorticosterone or other metabolites with mineralocorticoid activity induce hypokalemia and sodium retention. These events cause hypertension accompanied by renin suppression (2, 3). Glucocorticoid administration to the patient suppresses ACTH secretion and causes a reduction of 11-deoxycorticosterone secretion as well as normalization of plasma volume, resulting in the normalization of hypertension, hypokalemia, and renin suppresion. Thus, the recovery of renin secretion induces an increase in angiotensin-II production, bringing the level of aldosterone within the normal range (4–8).

Recently, Mornet *et al.* (9) reported the isolation and nucleotide sequences of the two genes, *CYP11B1* and *CYP11B2*. The former was ascribed to the gene encoding P450c11, and the latter was postulated to be a pseudogene closely related to *CYP11B1* (9). Kawamoto *et al.* (10–12) and Shizuta *et al.* (13, 14) isolated full-length cDNAs corresponding to transcripts of *CYP11B1* and *CYP11B2*, each encoding P450c11 and P450c18, respectively. They demonstrated that both P450c11 and P450c18, as expressed in COS-7 cells, exhibit 11β-hydroxylase activity, but only P450c18 retains 18-hydroxylase/oxidase activity. The nucleotide sequences of both cDNAs for P450c11 and P450c18 are 93% identical (10–14). These observations indicate that corticosterone methyloxidase type I and II (CMO I and CMO II), previously designated as the enzymes catalyzing the penultimate and ultimate steps in the synthesis of aldosterone, are identical to P450c18 (11–14). These findings are further confirmed by other investigators using polymerase chain reaction (PCR) (15) and established by a protein chemical approach (16).

More recently, two independent studies describing genetic defects in 11βOHD have been reported. One defect is derived from a missense mutation in codon 448 (Arg → His) in

Received June 23, 1993. Accepted August 19, 1993.
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* This article is dedicated to Dr. Osamu Hayaishi (President of Osaka Bioscience Institute), who has been awarded the highest Japanese decoration, Kun-Itto-Zuihou-Sho. This work was supported in part by grants from Naito Memorial Foundation, the Takeda Science Foundation, Sankyo Co. Ltd., Shionogi Pharmaceutical Co., Kobayashi Magobei Foundation, Special Research Fund of Kochi Medical School, and a Grant-in-Aid for Science from the Ministry of Education, Science, and Culture of Japan.

CYP11B1 of Jewish patients of Moroccan origin (17). The other defect is derived from a 2-basepair (bp) insertion in codon 394 in *CYP11B1* of a patient of Turkish origin (18). The latter mutation results in a frame shift to form a stop codon at position 469. In these works, however, expression experiments to demonstrate a lack of 11β-hydroxylase activity of the mutant P450c11s have not been performed, so it remains to be elucidated whether these two types of mutations actually cause 11βOHD.

In an effort to investigate the pathogenesis of 11βOHD and the relation between clinical phenotypes and deficiencies of *CYP11B1* and/or *CYP11B2*, we attempted to perform a molecular genetic analysis of a Japanese patient who has been diagnosed as 11βOHD on the basis of clinical and biochemical data. In this paper, we present evidence to show that a nonsense mutation occurs in exon 2 of *CYP11B1* without any mutation in *CYP11B2*, and that the mutation causes a complete lack of 11β-hydroxylase activity in the patient.

Subjects and Methods

Subjects

A 27-yr-old Japanese man with a 46,XY karyotype, 145.6 cm in height and 53.2 kg in weight, is the offspring of a consanguineous marriage. He was admitted to a hospital at 1 yr of age because of excessive growth, but without complaints of poor feeding, nausea, or vomiting. Clinical symptoms and laboratory data, including serum electrolytes and evaluation of urinary excretion of steroids, suggested CAH due to 11βOHD as the most likely diagnosis. Since then, he had been treated with 30 mg cortisol daily. At 23 yr of age, he was readmitted to confirm the diagnosis, and laboratory tests were performed under the condition of cessation of cortisol replacement for 2 weeks. Blood pressure was 140/90 mm Hg. Using commercially available kits, levels of PRA (Renin RIA beads, Dainabot Co., Tokyo, Japan), ACTH (ACTH IRMA kit SRL II, Euro-Diagnostic BV, Apeldoorn, Netherlands), aldosterone (Aldosterone RIA kit, Shionogi Pharmaceutical Co., Osaka, Japan), cortisol (Gamma Coat Cortisol kit, INCSTAR Co., Stillwater, MN), and progesterone, and 17α-hydroxyprogesterone (progesterone and 17α-hydroxyprogesterone ^{125}I RIA kits, Diagnostic Products Co., Los Angeles, CA) were measured. Plasma levels of 11-deoxycorticosterone, corticosterone, and 11-deoxycortisol were measured by RIA, using specific antibodies (Teikoku-Hormone Mfg. Co., Tokyo, Japan) after separation of extracted plasma with Sephadex LH-20 column (Pharmacia Fine Chemical, Uppsala, Sweden). Table 1 summarizes plasma levels of various steroids, PRA, and ACTH in the patient, confirming the diagnosis of 11βOHD. Since the age of 23 yr, he has been treated with 0.75 mg dexamethasone daily, resulting in normalization of increased levels of plasma steroids (Table 1). There are no other members of his family or relatives that have had similar abnormalities.

TABLE 1. Levels of plasma steroids, ACTH, and PRA before and after treatment with dexamethasone (0.75 mg daily for 6 months)

Steroids	Before	After	Normal value
17α-Hydroxyprogesterone (nmol/L)	13.1	2.1	<4.8
11-Deoxycortisol (nmol/L)	49.35	0.63	0.13–3.35
Cortisol (nmol/L)	33	ND	160–580
Progesterone (nmol/L)	3.18	0.95	0.51–1.34
11-Deoxycorticosterone (nmol/L)	3.76	1.23	0.02–0.80
Corticosterone (nmol/L)	2.4	ND	1.1–24.3
Aldosterone (pmol/L)	BD	140	42–352
ACTH (pmol/L)	85.3	16.3	2.2–12.1
PRA (ng/L·s)	BD	1.39	0.08–0.56

ND, Not determined; BD, borderline detectability.

DNA extraction

Peripheral blood samples were obtained from the patient, his family members, and unrelated healthy individuals. Blood samples of CMO I- and CMO II-deficient patients were kindly supplied by Dr. A. Rösler at Hadassah Medical Center (Jerusalem, Israel) and by Dr. S. Ulick at V.A. Hospital (New York, NY), respectively. Total DNA was isolated from the blood samples as previously described (19).

Amplification by PCR

PCR was performed by two steps for selective amplification of each exon of *CYP11B1* and *CYP11B2*. In the first PCR amplification, two sets of oligonucleotides (Table 2) were used to amplify exons 1–4 together with their surrounding introns and to amplify exons 5–9 together with their surrounding introns. Subsequently, the second PCR amplification of each exon with its surrounding introns was performed as previously described (19).

DNA sequencing

Each of the PCR products was blunt ended with T4 DNA polymerase and subcloned into the *Sma*I site of pUC118. Nucleotide sequence was determined by the dideoxy chain termination method (20), using *Bca* BEST DNA polymerase (Takara Shuzo Co., Kyoto, Japan).

Southern blot analysis

Genomic DNA (10 μg) was digested with *Bln*I (recognition sequence: CCTAGG), then electrophoresed on a 0.7% agarose gel and transferred to a Hybond-N membrane (Amersham, Aylesbury, Buckinghamshire, United Kingdom) (21). The membrane was subjected to hybridization with a ^{32}P-labeled probe of the 319-bp fragment containing exon 2 in *CYP11B1*. The filter was finally soaked in 0.1 × SSC (150 mmol/L NaCl/15 mmol/L sodium citrate) containing 1% sodium dodecyl sulfate at 50 C. The probe hybridizes to both *CYP11B1* and *CYP11B2*.

Restriction fragment length polymorphism (RFLP) analysis of PCR products

The PCR products amplified from exon 2 with its surrounding introns in *CYP11B1* were digested with *Bln*I and electrophoresed on a 5% polyacrylamide gel.

Construction of expression plasmids and measurement of steroid hydroxylase activity

We constructed cDNA corresponding to *CYP11B1* of the 11βOHD patient (see Table 3) for expression experiments, as follows. The *Sma*I-*Bam*HI fragment of the full-length human cDNA for P450c11 was cloned into M13mp18. Single stranded DNAs of M13mp18 containing the cDNA for P450c11 were prepared. Complementary strands were synthesized by T7 DNA polymerase simultaneously using three primers that were designed to replace Gln^{43} with Arg, Trp^{116} with stop codon, and Val^{386} with Ala (see Table 3). The double stranded DNA was ligated by T4 DNA ligase and transformed into JM109. Each mutant cDNA construct was totally sequenced to validate its structure. Finally, the *Sma*I-*Bam*HI fragment containing the full-length mutant cDNA was inserted into the pSVL expression vector using *Xba*I and *Bam*HI sites (pSV11βOHD). The wild-type human cDNA for P450c11 constructed in pSVL (pSV11β) and another mutant or polymorphic cDNA construct (pSVCMO) corresponding to amino acid substitutions, Gln^{43} → Arg and Trp^{116} → Ala, in pSVL, were also used. Steroid hydroxylase activity in the mitochondrial fraction of COS-7 cells transfected with each expression plasmid was determined as previously described (12).

TABLE 2. Sequences of oligonucleotide primers used for PCR amplification and RFLP analysis

Sense primer	Location	Anti-sense primer	Location	Purpose for amplification
For first amplification				
1) TCATGCACCCCCAATGAGTCCCTG	5′-flanking	GCCAGCTAGCCCAGCACCAAGT	Intron 4	Exon 1–Exon 4 (*CYP11B1*)
2) TCCTTCATCTACCTTTGGCTGGGG	5′-flanking	CCGAGACTGCCCCGACACCCAAAT	Intron 4	Exon 1–Exon 4 (*CYP11B2*)
3) AGACTTGGTGCTGGGCTAGCTGGC	Intron 4	GACAAGGTCAGCAAGATCTTCCCC	3′-UT	Exon 5–Exon 9 (*CYP11B1*)
4) ATTTGGGTGTCGGGGCAGTCTCGG	Intron 4	TTGCTATTTGACAAGCCTGGCAAG	3′-UT	Exon 5–Exon 9 (*CYP11B2*)
For second amplification				
5) CGAAGGCAAGGCACCAGGCAAGAT	5′-flanking	TGGCAGTGCTGAGTGCC	Intron 1	Exon 1 (*CYP11B1*)
6) GTTTCCAGAGCAGGTTCCTGGGTG	5′-flanking	TGGCAGTGCTGAGTGCC	Intron 1	Exon 1 (*CYP11B2*)
7) CTGTGAAGCCGCTAAT	Intron 1	CAGGCTGCCCACCCAGC	Intron 2	Exon 2 (Both genes)
8) TTGCTGGGGCGGCCTCA	Intron 2	CCACTCCAGGGTCTCTG	Intron 3	Exon 3 (Both genes)
9) CCTTGTGCTCAGCAGTG	Intron 3	CCTTCCCCATAGCACTG	Intron 4	Exon 4 (Both genes)
10) GAGGACACTGAAGGATG	Intron 4	GCTTGGCATCACCCTCT	Intron 5	Exon 5 (Both genes)
11) TCCTCCTGTGCAAGGTC	Intron 5	CCAGGGCCACAGGGAGG	Intron 6	Exon 6 (Both genes)
12) GGTGCAGAGAGCACAGG	Intron 6	GGATCAGGGAATGACTG	Intron 7	Exon 7 (Both genes)
13) CAGTCATTCCCTGATCC	Intron 7	TGCTGCTTAGCCTGGCAAACCCTG	3′-UT	Exon 8–Exon 9 (*CYP11B1*)
14) CAGTCATTCCCTGATCC	Intron 7	GGTCAGGCAGAGGGAAGCTGGTGG	3′-UT	Exon 8–Exon 9 (*CYP11B2*)

TABLE 3. Mutation and polymorphism of *CYP11B1* or corresponding cDNA

Patient number	exon 1	exon 2		exon 6	exon 7	Subject	Reference
	43	82	codon 116	362	386		
1	Gln (CAG)	Asp (GAC)	Trp (TGG)	Leu (CTC)	Val (GTG)	Normal healthy control	This study
2	*Arg* (C*GC*)	Asp (GAT)	*Stop* (TAG)	Leu (CTG)	*Ala* (G*C*G)	11βOHD	This study
3	*Arg* (C*GC*)	Asp (GAT)	Trp (TGG)	Leu (CTG)	*Ala* (G*C*G)	CMO I deficiency	(27)
4	*Arg* (C*GC*)	Asp (GAT)	Trp (TGG)	Leu (CTG)	*Ala* (G*C*G)	CMO II deficiency	(19)
5	Gln (CAG)	Asp (GAC)	Trp (TGG)	Leu (CTC)	Val (GTG)	Primary aldosteronism	(10, 11)

Codons and deduced amino acid residues are from *CYP11B1* of the normal healthy individual (No. 1), the 11βOHD patient (No. 2), the corticosterone methyloxidase (CMO) type I deficient patient (No. 3), and the CMO type II deficient patient (No. 4). Codons and deduced amino acid residues displayed for patient 5 are from cDNA for P450c11 derived from surgically excised adenoma of the patient suffering from primary aldosteronism. Note that nucleotides or amino acid residues different from those of the normal healthy control are underlined. Complete sequence data of *CYP11B1* including those of eight introns and nine exons are deposited in the GenBank/EMBL/DDBJ Data Bank under the accession nos. D16153 and D16154. The sequence of *CYP11B1* is somewhat different from that reported by Mornet *et al.* (9).

Results

Sequence analyses of CYP11B1 and CYP11B2

By PCR, we amplified a total of nine exons with their surrounding introns of both *CYP11B1* and *CYP11B2* from the patient affected with 11βOHD and then cloned the PCR products into pUC118 for nucleotide sequencing analysis. The analysis of each PCR product revealed that a point mutation TGG → TAG (Trp^{116} → Stop) occurs in exon 2 of *CYP11B1* of the patient, as shown in Fig. 1. This mutation is detectable in all 11 clones examined. No other mutation was observed in the other 8 exons of *CYP11B1* and the promoter region up to −1100 relative to the transcriptional initiation site. Also, no mutation was detectable in any exon of *CYP11B2*. As control experiments, we performed sequencing of *CYP11B1* derived from normal healthy individuals, CMO I-deficient patients, and CMO II-deficient patients, compared with the sequence of cDNA for P450c11 derived from a patient suffering from primary aldosteronism (11). There were some polymorphisms in *CYP11B1* of these individuals, as shown in Tables 3 and 4, but the nonsense mutation observed in the 11βOHD patient was not detectable.

RFLP analyses of genomic DNA and PCR products

The nonsense mutation in exon 2 in *CYP11B1* from the 11βOHD patient resulted in the formation of a recognition site of the restriction enzyme *Bln*I. This allowed us to investigate by genomic Southern blot analysis whether the 11βOHD patient was homozygous or heterozygous for the mutation. The pedigree of the family and results of Southern blot hybridization are shown in Fig. 2, A and B, respectively. When genomic DNA was digested with *Bln*I and hybridized with the ^{32}P-labeled exon 2 of *CYP11B1*, three bands of 7.7, 5.6, and 0.7 kilobases (kb) were detectable in the normal healthy individual. The band of 7.7 kb corresponded to *CYP11B1*, and the band of 5.6 kb corresponded to *CYP11B2*. The band of 0.7 kb mapped to the region from the 5′-flanking region to intron 2 of *CYP11B2*, where a polymorphic site for *Bln*I existed in the normal healthy individual, the

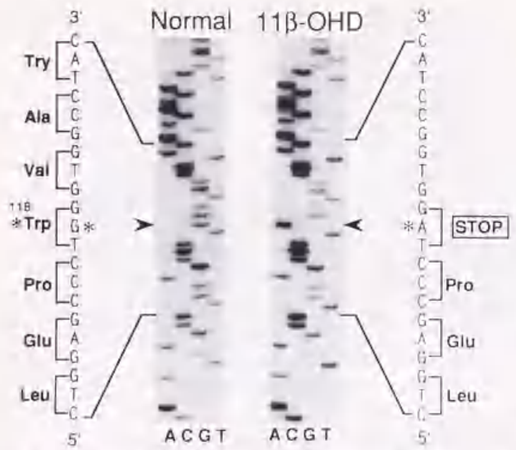

FIG. 1. Nucleotide sequence analysis of a portion of exon 2 in *CYP11B1*. A portion of nucleotide sequence ladders of exon 2 in *CYP11B1* from a normal healthy individual (Normal) and a 11βOHD patient is shown. The *left and the right sequences* represent the nucleotide sequences corresponding to the autoradiograms together with their deduced amino acid residues. *Star symbols along the sequences* denote the point mutation. The *arrowhead* represents the site of mutation. Note that the point mutation results in the formation of a stop codon in the gene of the patient.

patient's mother, and his sibling, but was lacking in the patient and his father. In the 11βOHD patient, the band of 7.7 kb was not detectable, but two smaller bands with sizes of 4.3 and 3.4 kb were found (lane 3). RFLP analysis of DNA samples from the clinically unaffected father and mother of the patient showed a heterozygous pattern; the bands corresponding to *CYP11B1* consisted of the 7.7-, 4.3-, and 3.4-kb fragments (lanes 1 and 2). His unaffected sibling showed the same pattern as the normal healthy individual (lane 4). These results establish that disappearance of the 7.7-kb band with concomitant appearance of the 4.3- and 3.4-kb bands is caused by the point mutation in exon 2 of *CYP11B1* of the patient.

To further confirm the above results, we amplified exon 2 with its surrounding introns of *CYP11B1* from the patient, his family members, and the normal healthy individual, respectively. The strategy for screening the nucleotide substitution is shown in Fig. 3B. The *Bln*I restriction site newly produced by the G → A substitution at codon 116 was easily detectable by the digestion of amplified exon 2 with *Bln*I. The electrophoretic patterns of the *Bln*I-digested PCR products are shown in Fig. 3A. The size of a single band (319 bp) of his unaffected sibling (lane 4) was identical to that of the normal control (lane N). The PCR product from the patient (lane 3) was cleaved to form two fragments of 228 and 91 bp. On the other hand, PCR products from his father and mother were cleaved with *Bln*I to yield three fragments that correspond to the two fragments (228 and 91 bp) seen in the patient and the uncleaved fragment (319 bp) seen in his unaffected sibling and the normal healthy individual (lanes 1 and 2). These results indicate that the 11βOHD patient is homozygous for the mutation, whereas his unaffected parents are heterozygous (as expected on the basis of their consanguinity), and his sibling's *CYP11B1* is as intact as that of the normally healthy individual.

Expression of P450c11 in COS-7 cells transfected with expression plasmids

We constructed expression plasmids containing cDNAs corresponding to *CYP11B1* of the 11βOHD patient, the CMO I- or CMO II-deficient patients, and the normal healthy individual and transfected them into COS-7 cells, as described in *Materials and Methods*. As shown in Table 4, steroid 11β-hydroxylase activity was detectable in the mitochondria of the cells transfected with pSVCMO containing the mutated or polymorphic cDNA for P450c11. Nevertheless, the activity was not detectable in cells transfected with pSV11βOHD containing the mutant cDNA for P450c11. These results indicate that 11βOHD in this study is exclusively caused by the nonsense mutation in exon 2 of *CYP11B1*.

Discussion

In the present study, we found a nonsense mutation at codon 116 in *CYP11B1* from a Japanese patient affected with 11βOHD. A stop codon present in place of a tryptophan codon at amino acid position 116 causes the production of a truncated inactive protein. RFLP analyses of genomic DNA and PCR products of exon 2 of *CYP11B1* demonstrated that the patient in the consanguineous family is homozygous and the unaffected parents are heterozygous for the mutation. His sibling is healthy and normal for *CYP11B1*. Furthermore, expression studies using COS-7 cells transfected with an expression plasmid carrying the mutant cDNA revealed that this mutation causes a lack of 11β-hydroxylase activity in the patient, but these compounds appear to be formed by the catalytic action of P450c18 (12, 14), as his gene, *CYP11B2*, is intact. The simultaneous analysis of *CYP11B2* and *CYP11B1* will be useful for diagnosis of biochemical and clinical variants affected by this disease.

Methods for prenatal diagnosis of CAH have been developed in recent years for the purpose of genetic counseling for parents in regard to early prenatal and/or postnatal therapy (22–26). Recent reports (17, 18) have indicated that individuals affected with 11βOHD have no identifiable gene deletions or rearrangements in *CYP11B1*, and both mutated genes have not yet been associated with specific RFLP. In this study, we identified a unique type of mutation in *CYP11B1* that produces an extra restriction site, *Bln*I. This is the first report showing that 11βOHD can be detected by RFLP analysis. Therefore, the restriction enzyme, *Bln*I, may be a useful tool for DNA analysis of 11βOHD in future studies. It should be emphasized, however, that the current case is different from those of 11βOHD found previously by other investigators, as mentioned above. It is, therefore, important to determine whether these differences may reflect racial or ethnic variation. Further analysis of mutated *CYP11B1* of patients affected with 11βOHD is required for elucidation of the above point.

TABLE 4. Steroid hydroxylase activity of various P450c11s expressed in COS-7 cells

Patient number	Products					Plasmid
	Corticosterone	18-OH-Corticosterone	Aldosterone	18-OH-DOC	19-OH-DOC	
			(pmol/mg)			
1 and 5	395.8 ± 4.9	1.0 ± 0.1	<0.02[a]	18.1 ± 1.3	12.8 ± 0.7	pSV11β
2	<0.05[a]	<0.05	<0.02	<0.05	<0.05	pSV11βOHD
3 and 4	371.0 ± 5.8	1.0 ± 0.1	<0.02	16.1 ± 1.0	11.0 ± 1.1	pSVCMO

Hydroxylase activities of various P450c11s expressed in COS-7 cells were measured as described under *Materials and Methods*. Values represent mean ± SD of triplicate experiments for determination of the amount of each product formed in a 20-min incubation with [^{3}H]DOC as a substrate. Each indicated number (1–5) at the left side of this table corresponds to that shown in Table 3.

[a] Values below the limit of detection (12).

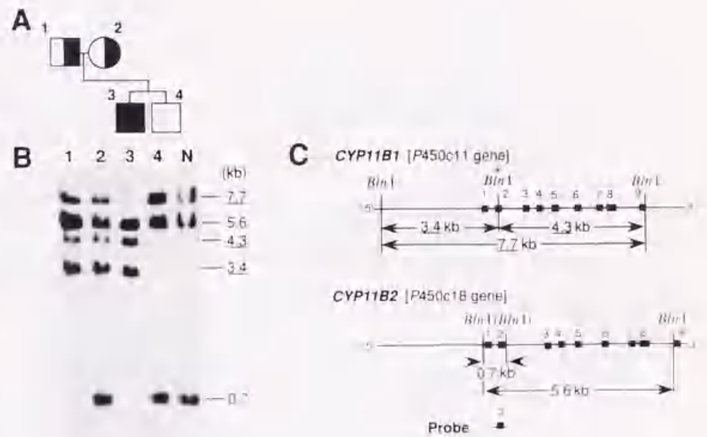

FIG. 2. Southern blot analysis of *CYP11B1*. A, The pedigree of a Japanese family carrying an allele for 11βOHD. *Squares and circles* represent males and females, respectively. A *solid symbol* shows existence of mutant *CYP11B1* in the homozygous state, and *half-solid symbols* show the existence of mutant *CYP11B1* in the heterozygous state. B, Autoradiogram of Southern blot analysis of the genomes after digestion with *Bln*I. Lane N corresponds to the normal healthy individual; lanes 1, 2, and 4 correspond to the unaffected family members; and lane 3 corresponds to the 11βOHD patient. Kilobase sizes of the bands are shown on the *right*. The sizes of bands originated from *CYP11B1* are indicated by *underlines*. C, Schematic illustrations of *CYP11B1* and *CYP11B2* along with that of the probe used in this study. *Bln*I sites and sizes of predicted restriction fragments (in kilobases) are marked. Each exon is numbered starting from 1–9. Coding regions are represented by *thick lines*, and 5′- and 3′-flanking regions by *thin lines*. *Bln* I denotes restriction sites for *Bln*I. A *star symbol* indicates the recognition site produced by the G → A substitution. *Bln* I with *parentheses* shows a polymorphic recognition site.

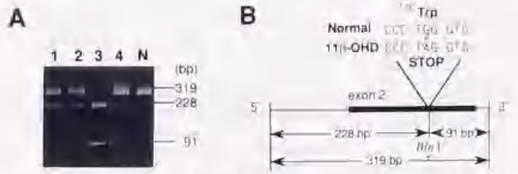

FIG. 3. RFLP analysis of PCR products from exon 2 in *CYP11B1*. A, Gel electrophoretic patterns of RFLP analysis of the PCR products of exon 2 of *CYP11B1* after digestion with *Bln*I. Lane N corresponds to the normal healthy individual; lanes 1, 2, and 4 correspond to the unaffected family members; and lane 3 corresponds to the 11βOHD patient. The G → A substitution at codon 116 results in the formation of a *Bln*I site. B, A schematic illustration of exon 2 and its flanking regions with the sizes of *Bln*I restriction fragments. *Thin and thick solid lines* represent the amplified region (319 bp) by PCR. *Bln* I shows the recognition site of this restriction enzyme. A *star symbol* indicates the exact locus of the recognition site newly produced by the G → A substitution.

patient.

11βOHD occurs in a wide spectrum of clinical variants, including a form with a concurrent defect in 18-hydroxylation and a form with apparent aldosterone biosynthesis after appropriate glucocorticoid administration (4–8). Two types of 11βOHD may exist: one accompanied by a simultaneous mutation(s) in *CYP11B2*, and the other without any mutation in *CYP11B2*. In fact, it is notable that very low amounts of cortisol and corticosterone are detected in the

Acknowledgments

We are grateful to Drs. K. Miura, K. Yasuda, N. Yamakita, H. Tanaka, T. Nakamura, and K. Ohashi at Gifu University, and to Drs. T. Kurashige, K. Araki, and Y. Yamamoto at Kochi Medical School for their

contribution to the data presented in Table 1. Thanks are also due to I. Kuribayashi and Y. Tsuno at Kochi Medical School for technical and secretarial assistance.

Note Added in Proof

Just after submitting this paper, we noticed the report by Curnow *et al.* (28) dealing with congenital adrenal hyperplasia caused by mutations in *CYP11B1*. In their report, they described mutations on eight different loci in *CYP11B1* causing 11βOHD.

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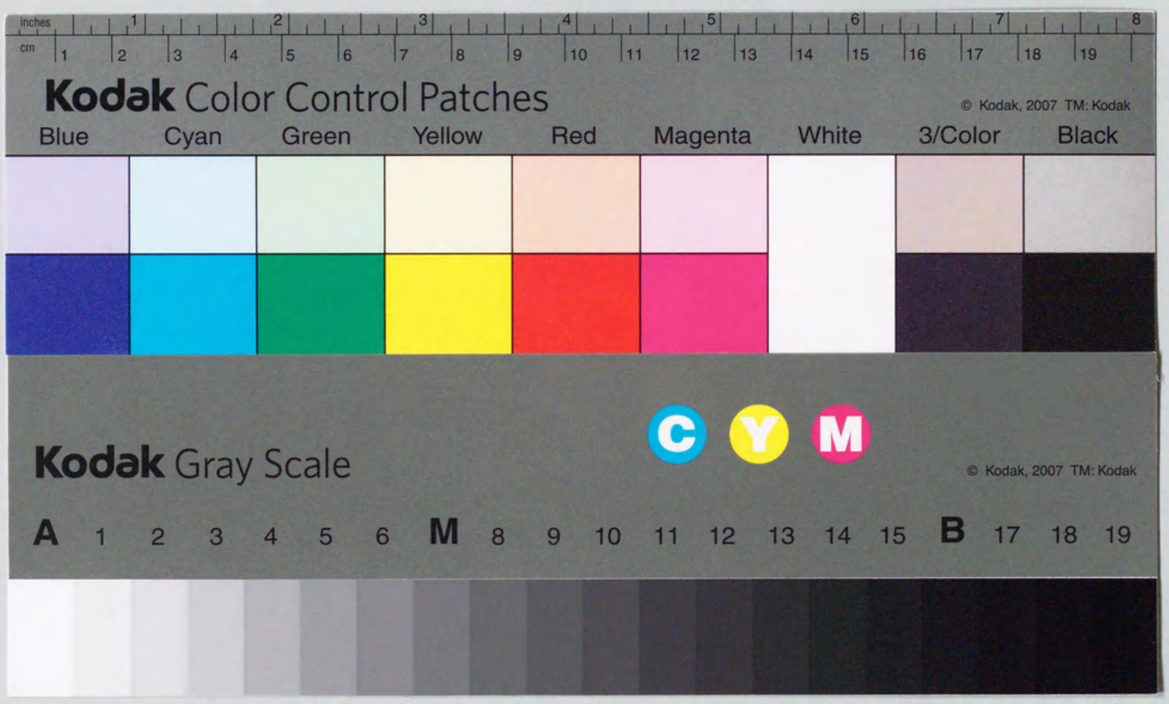
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