Identification of new mutations in sterol 27-hydroxylase gene in Japanese patients with cerebrotendinous xanthomatosis (CTX)

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Abstract Cerebrotendinous xanthomatosis (CTX) is a hereditary sterol storage disease associated with accumulation of cholesterol and cholestanol in various tissues, especially tendons and neural tissues. The biochemical defect that causes CTX is a deficiency of the mitochondrial sterol 27-hydroxylase which oxidizes the side chain of cholesterol in connection with formation of bile acids. Japan has a relatively high prevalence of CTX and more cases of the disease are found here than in any other country. In the present study two new different point mutations are described in the heme-ligand binding domain of the sterol 27-hydroxylase gene in three Japanese CTX patients and one CTX heterozygote. Two of the homozygotes as well as the heterozygote subject have a single base substitution of A for G at codon 441 [CGG (Arg) to CAG (Gln)]. Another homozygote has a transition of C to T at codon 441 [CGG (Arg) to TGG (Trp)]. These two different mutations result in two restriction fragment length polymorphisms (RFLPs) for the enzymes *Stu*I or *Hpa*II. We also assayed sterol 27-hydroxylase activity using skin fibroblasts derived from three CTX patients, one CTX heterozygote, and normal subjects. While two of the homozygous subjects have undetectable levels of the enzyme activity, one homozygous subject and one heterozygous subject have decreased levels of the enzyme activity, about 1.4% and 10% of normal, respectively. The results suggest that the newly identified point mutations in the sterol 27-hydroxylase gene could account for the sterol 27-hydroxylase deficiency in the Japanese CTX patients.—**Kim, K-S., S. Kubota, M. Kuriyama, J. Fujiyama, I. Björkhem, G. Eggertsen, and Y. Seyama.** Identification of new mutations in sterol 27-hydroxylase gene in Japanese patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1994. **35:** 1031–1039.

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Cerebrotendinous xanthomatosis (CTX) is an autosomal recessive sterol storage disease characterized by tendon xanthomas, dementia, cerebellar ataxia, premature atherosclerosis, and cataracts (1–4). Biochemically, CTX patients have a defect in bile acid biosynthesis associated with incomplete oxidation of the cholesterol side chain, leading to excretion of great amounts of C27-bile alcohols in bile, feces, and urine (5) as well as accumulation of cholestanol. There was a controversy in the past concerning the location of this defect; Salen et al. (6) suggested that a reduced activity of the microsomal 5β-cholestane-3α,7α,12α,25-tetrol-24S-hydroxylase in CTX patients leads to the subnormal production of bile acid, whereas Oftebro et al. (7) suggested that the primary metabolic defect in CTX is a deficiency of mitochondrial sterol 27-hydroxylase which catalyzes the first step in the oxidation of side chain of sterol intermediates in the bile acid synthesis pathway. The latter hypothesis was supported by Skrede et al. (8) who demonstrated a deficiency of the sterol 27-hydroxylase activity in skin fibroblasts from CTX patients. It was also shown that the above microsomal 5β-cholestane-3α,7α,12α,25-tetrol-24S-hydroxylase is of little or no importance in the biosynthesis of cholic acid in humans under normal conditions (for a review see ref. 1). Recent molecular cloning of the human sterol 27-hydroxylase cDNA and characterization of cDNAs in two CTX patients confirmed a deficiency of sterol 27-hydroxylase (9, 10). Two different point mutations (in position 362, Arg to Cys, and in position 446, Arg to Cys) were identified in an African-American and a Canadian, respectively (10). These two mutations were present in functional domains of the sterol 27-hydroxylase gene, the adrenodoxin and heme-ligand binding sites,

Abbreviations: CTX, cerebrotendinous xanthomatosis; RT-PCR, reverse transcription-polymerase chain reaction; EDTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; RFLP, restriction fragment length polymorphism; HPLC, high performance liquid chromatography; DTT, dithiothreitol; CPS, counts per second.

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respectively. Very recently Leitersdorf et al. (11) also described mutations in the sterol 27-hydroxylase gene in Jews of Moroccan origin with CTX. In these cases the frameshift and splice-junction mutations result in a lack of expression of mRNA. Considering the relatively high prevalence of CTX in Japan and the fact that there are more known cases here than in any other country (4, 12), it was considered important to elucidate the molecular basis for CTX in Japanese patients and to establish a method to diagnose the homozygous or heterozygous states.

METHODS

Patients

Three homozygous subjects (K.O., H.M., and Y.S.) and one heterozygous subject (N.M.), diagnosed by clinical and laboratory findings were studied. These patients have been described in detail previously (4, 12). Briefly, CTX-1 (K.O.) and CTX-2 (H.M.) had typical manifestations of CTX, and their parents were first cousins. CTX-1 showed severe neurological symptoms including mental deficiency and intractable convulsions. CTX-2 had a gait disturbance and visual impairment in both eyes. Bilateral cataracts were diagnosed at age 34. CTX-3 (Y.S.) had mild neurological symptoms, but experienced attacks of severe chest pain at age 35 and 37. Coronary angiography revealed obstruction and marked stenosis of the coronary arteries. His parents were not consanguineous. Serum cholestanol levels were markedly increased in CTX-1 (54 μg/ml), CTX-2 (42 μg/ml), and CTX-3 (22 μg/ml). The serum cholestanol level of heterozygote (CTX-4, N.M.) was 3.0 μg/ml, which was within the normal range (2.7 $\pm$ 0.8 μg/ml, n = 17). CTX-4, the father of CTX-2 had a past history of gallstone and pancreatitis, but had no manifestations of CTX. Informed consent was obtained from all patients and healthy controls. This study was approved by the Third Department of Internal Medicine, Kagoshima University School of Medicine.

Cell culture

The fibroblasts were biopsied from skin in the forearm of the patients, heterozygote and normal subjects (n = 17). The cells were grown and maintained as a monolayer in Dulbecco's Modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (1.5 mmol/ml). The cells were harvested with trypsin (200 U/ml) after 5–15 passages, washed twice with phosphate-buffered saline, and stored as pellets at −80°C for RNA preparation and sterol 27-hydroxylase assay.

Preparation of RNA from fibroblasts

Total RNA was isolated from cultured skin fibroblasts (10^7 cells) by the guanidium cesium chloride method as described previously (13), and kept at −80°C until used. Approximately 1 μg of total RNA was used for reverse transcription-polymerase chain reaction (RT-PCR).

Preparation of oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystems Inc. 318A DNA synthesizer. The primers used for RT-PCR were designed according to the published sterol 27-hydroxylase cDNA sequence (9) and shown as follows. The primers (SP3 and P2) were used to amplify the upstream region of the cDNA encompassing nucleotides 555 to 1274. The sequence of SP3 represented as "upstream primer" was 5'-CAATGAGGTGATTGATGACT-3' (nucleotides 555–574) and P2 represented as "downstream primer" was 5'-AAGAGGAAGCCATCAACTTCAATTT-3' (nucleotides 1250–1274, antisense). The primers (P3 and P4) were used for amplification of the target region encoding two functional domains. The sequence of P3 represented as "upstream primer" was 5'-TACCACCTCTCAAAGGACCCTGAGA-3' (nucleotides 1066–1090). The P4 represented as "downstream primer" was 5'-GCAAGGAGTTCCTCCCACCTCTCGA-3' (nucleotides 1728–1752, antisense). The primers used for direct sequencing were as follows; SP3, PA, 5'-TGGCATCCAGGTG TCTGGCT-3' (nucleotides 930–949), λP1, 5'-ATCCAGGAGG CCTTGCACGA-3' (nucleotides 1090–1109), PC, 5'-CCTTCTCTGAGCCTGAAAGCTTCC-3' (nucleotides 1331–1354), and SP7, 5'-GCTGGAGATGCAGCTACTCC-3' (nucleotides 1470–1489). The primers used at RT-PCR for *Stu*I digestion were PA and PB, 5'-TCTCCCTCCTCATCTGA GAC-3' (nucleotides 1691–1710, antisense). The primers used at RT-PCR for *Hpa*II digestion were PC and PD, 5'-ACTTCTGGATCAGCCTTGCG-3' (nucleotides 1491–1510, antisense).

5'-Phosphorylation of oligonucleotide primer

For generating single-stranded DNA for direct sequencing from PCR products, 5' terminus of upstream primers (SP3 and P3) were phosphorylated prior to PCR reaction by the kination reaction (14) as follows; the reaction mixture (50 μl) containing 200 pmol primer, 100 U T4 polynucleotide kinase, 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 10 mM DTT, 0.1 mM EDTA, and 2 mM ATP was incubated at 37°C for 1 h followed by heat treatment at 70°C for 10 min.

RT-PCR amplification

We used the polymerase chain reaction (PCR) coupled to reverse transcription (RT-PCR) (15). RT-PCR was performed using the Gene Amp RNA Amplification Reagent

Kit (Perkin-Elmer Cetus, Norwalk, CT). The amounts of RT-PCR products were almost the same in patients and normal controls. The amplification of the target region (nucleotides 1066–1752; 687 bp) comprising two catalytic domains (adrenodoxin and heme-ligand binding) was carried out in a final volume of 100 μl in two steps (e.g., reverse transcription and cDNA PCR amplification). For PCR analysis, total RNA from cultured fibroblasts was converted to cDNA using reverse transcriptase (RT). The RT mixture (20 μl) contained 5 mM $MgCl_2$, 1 × PCR buffer II, 1 mM dNTPs, 1 U RNase inhibitor, 2.5 U reverse transcriptase, 1 μg total RNA, and 0.15 mM oligonucleotide primer P4. One hundred μl of mineral oil was overlayered to prevent evaporation during the high temperature incubations. The reaction tubes were incubated at 42°C for 15 min (annealing and extension), heated at 99°C for 5 min (inactivation of reverse transcriptase and denaturation of RNA-cDNA hybrids) in the DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT), and then soaked at 5°C for 5 min. PCR amplification was immediately performed after the RT reaction by adding 80 μl of a PCR Master Mix containing 2 mM $MgCl_2$, 1 × PCR buffer II, 66.5 μl sterile distilled water, 2.5 U *Taq* DNA polymerase, and 0.15 mM oligonucleotide primer P3 (kinationed primer). The amplification reaction was performed for 30 cycles in a thermal cycler using the following cycle conditions: 1 min for denaturation at 95°C and 4 min for annealing and extension at 68°C. Similarly, amplification of another region was performed as control using primers SP3 and P2 spanning 555–1274 nucleotides region. Ten μl of amplification product was electrophoresed on 2% agarose gel to confirm successful amplification. A single band of expected size, 720 bp (in case of SP3-P2 amplification) or 687 bp (in case of P3-P4 amplification), was identified by ethidiumbromide staining of the gel. Molecular size marker (ΦX 174 *Hae*III marker, Wako Inc., Japan; 1357, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp) was used to calculate the size of the amplified fragments.

Direct nucleotide sequencing

The PCR products were purified using Magic PCR Preps (Promega, Marison, WI) and then digested with lambda exonuclease (GIBCO BRL, Gaithersburg, MD) (16) which is a 5' to 3' nuclease that attacks double-stranded DNA only if there is a 5' terminal phosphate. The single-stranded DNA produced by exonuclease digestion was directly sequenced with T7 DNA polymerase (Sequenase; U.S. Biochemicals, Cleveland, OH) and the oligonucleotide primers (SP3, PA, λP1, PC or SP7; 0.5 pmol/μl, respectively), using the dideoxynucleotide chain termination method (17). Primers (SP3 and PA), primer (λP1), primer (PC), and primer (SP7) were used for direct sequencing of the 555–1274 nucleotides region, adrenodoxin binding domain, heme-ligand binding domain, and 3' region of sterol 27-hydroxylase cDNA (1490–1617 nucleotides region), respectively.

Restriction fragment length polymorphism (RFLP) analysis

The substitution (G→A) in amino acid residue 441 creates a new *Stu*I cleavage site and another nucleotide transition (C→T) eliminates a cleavage site for restriction enzyme *Hpa*II. Amplified PCR products spanning the catalytic domains were digested with 10 units of *Stu*I or *Hpa*II for 1 h at 37°C. All of the digested PCR products were electrophoresed using 4% Nusieve:agarose (3:1) or 3.5% agarose gels, and the resulting fragments were identified by ethidiumbromide staining of the gels.

Assay of enzyme activity and protein concentration

Assay of sterol 27-hydroxylase activity in fibroblasts was performed essentially according to the previously described method (8), using mitochondrial fractions prepared from fibroblasts. The mitochondrial fraction of fibroblasts was prepared with slight modification of the procedures described previously (18). Protein concentrations were determined according to the Bradford's method (19) using bovine serum albumin as a standard. One–1.2 mg/ml of protein was used in the assay. Labeled substrate (10 μmol/l), 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (sp act 150 μCi/mg) was prepared as described previously (20). The converted 27-hydroxylated products were detected by combined HPLC (LC-10A series, Shimadzu, Kyoto, Japan) using an LC-18 column (250 × 4.6 mm, Supelco, USA), and the radioactivity in the converted products was measured by a radiodetector (RLC-700, Aloka, Tokyo, Japan).

RESULTS

Amplification and sequencing of functional domains of sterol 27-hydroxylase gene

One μg of total RNA isolated from the fibroblasts derived from three CTX patients, one CTX heterozygote, and seventeen normal subjects was used for the amplification of target region (nucleotides 1066–1752) containing functional domains (adrenodoxin and heme-ligand binding) and another region (nucleotides 555–1274) of sterol 27-hydroxylase gene. The nucleotide sequence of the region (nucleotides 555–1274) in the patients and normal subjects was completely identical with the cDNA sequence reported by Cali and Russell (9) (data not shown). PCR amplification of the target region using P3 and P4 as primers (see Methods) generated a DNA fragment of 687 nucleotides which was an expected size. The sequences obtained from purified PCR product derived from normal subjects were identical with those reported for sequence of normal human sterol 27-hydroxylase cDNA by Cali and Russell (9). On the contrary, we

identified two different single base substitutions in the heme-ligand binding region of three CTX patients and one CTX heterozygote. *i)* In two homozygous patients (CTX-1 and CTX-2) and one heterozygous subject (CTX-4) we identified a G→A transition at codon 441, replacing arginine (CGG) by glutamine (CAG) (**Fig. 1A**). *ii)* In one homozygous patient (CTX-3) we identified a C→T transition at codon 441, replacing arginine (CGG) by tryptophan (TGG) (Fig. 1B). These two mutations were present in a functional domain of the sterol 27-hydroxylase and would be expected to disrupt enzyme activity.

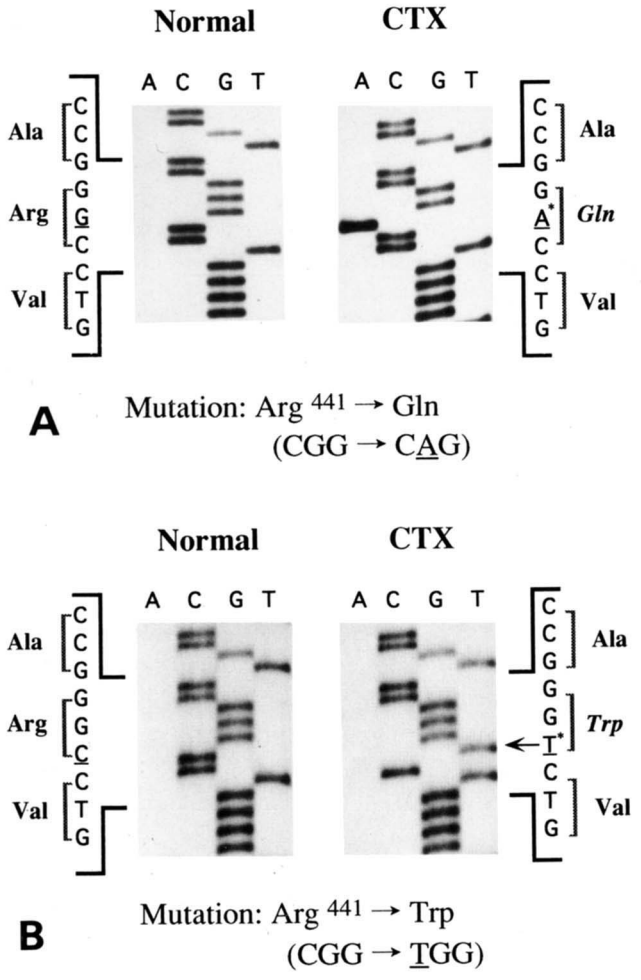

Fig. 1. Identification of two mutations of sterol 27-hydroxylase gene in CTX patients. Amplified PCR products from a normal subject and CTX patients were sequenced as described in Methods. Arrows denote the positions of each substitution showing (A) the presence of glutamine (CAG) in place of arginine (CGG) at the position of amino acid 441 in the patient of CTX-1, and (B) a replacement of arginine (CGG) with tryptophan (TGG) at the same position (codon 441) in CTX-3. Nucleotide sequence and amino acid numbers were taken from the data of human sterol 27-hydroxylase cDNA (9).

Detection of point mutation by RFLP

Two different mutations resulted in two RFLPs for the enzymes *Stu*I or *Hpa*II. As shown in **Fig. 2A,** RT-PCR amplification of the target region using primers PA and PB was performed. Both PCR products derived from normal subjects and patients resulted in 781 nucleotides which was an expected size. As shown in Fig. 2B, there is one *Stu*I restriction site in normal subjects and two sites in patients with (Arg^{441}→Gln). To detect a G→A transition at codon 441, the PCR product of 781 nucleotides was cut by *Stu*I enzyme (Fig. 2C). This treatment generated two bands of 611 and 170 nucleotides in case of normal subject (Fig. 2C, lane 3). In case of the homozygous patients (CTX-1 and CTX-2) this treatment generated three bands of 345, 266, and 170 nucleotides due to a creation of *Stu*I restriction site (Fig. 2C, lanes 4 and 5). In case of the homozygote CTX-1 (Fig. 2C, lane 4) a very faint unexplained band was repeatedly observed corresponding to 611 nucleotides. The pattern was, however, definitely different from the heterozygote pattern. In case of the heterozygous subject (CTX-4) this treatment thus generated four clear bands of 611, 345, 266, and 170 nucleotides (Fig. 2C, lane 6). The band of 611 nucleotides remained uncut (as compared to the intensity of the band on lane 3). The results suggest the presence of one normal allele. In case of the homozygous patient (CTX-3), the *Stu*I digestion generated two bands of 611 and 170 nucleotides as likely was observed in the normal subject (Fig. 2C, lane 7). RT-PCR amplification of the target region using primers PC and PD was performed (**Fig. 3A**). As shown in Fig. 3B, the PCR product contains one *Hpa*II restriction site in the normal subject, and this restriction site is eliminated by the mutation (Arg^{441}→Trp). Both PCR products derived from the normal subjects and patient (CTX-3) gave 180 nucleotides which was an expected size. To detect a C→T transition at codon 441, the PCR product of 180 nucleotides was cut by *Hpa*II (Fig. 3C). This treatment generated two bands of 110 and 70 nucleotides (Fig. 3C, lane 3) in the case of normal subjects. On the other hand, the band of 180 nucleotides remained uncut in the case of homozygous patient (CTX-3) (Fig. 3C, lane 5). The result suggests that *Hpa*II restriction site was eliminated due to the C→T transition at codon 441.

Sterol 27-hydroxylase activity in skin fibroblasts

We next measured sterol 27-hydroxylase activities in skin fibroblasts derived from five normal subjects, three CTX patients, and one CTX heterozygote to see whether there was any correlation between RFLP pattern (normal, homozygote, and heterozygote) and enzyme activity. The assay was performed using mitochondrial fractions from fibroblasts as described in Methods. We used 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol as a substrate, which

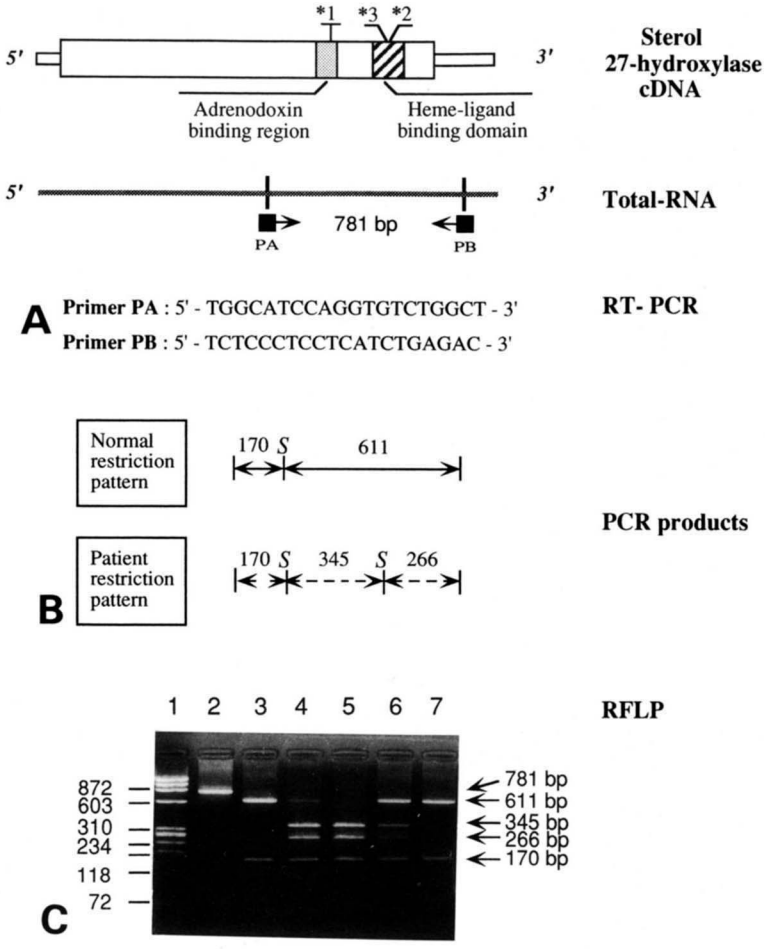

Fig. 2. PCR-detection of Gln mutation with *Stu*I digestion. (A) Schematic representation of the amplified portion (781 bp) of the sterol 27-hydroxylase cDNA by RT-PCR using oligonucleotide primers PA (nucleotides 930–949) and PB (nucleotides 1691–1710). Two point mutations, *1 (Arg^{362}→Cys) and *2 (Arg^{446}→Cys), were previously described by Cali et al. (10), and new different mutations, *3 (Arg^{441}→Gln, and Arg^{441}→Trp) were identified in the present study. (B) There is one *Stu*I restriction site designated as letter *S* in the normal subject, and there are two sites in patients with a mutation (Arg^{441}→Gln). The horizontal solid and dotted arrows indicate the size of the restriction fragments generated by *Stu*I digestion of the amplified DNAs. (C) Analysis of the PCR products amplified from a normal subject and the patients was performed by electrophoresis using a 4% Nusieve:agarose (3:1) gel after digestion with *Stu*I. Lane 1, standard size marker (ΦX174/*Hae*III); lane 2, uncut amplified DNA of normal subject; lane 3, normal subject after *Stu*I digestion; lane 4, patient CTX-1 after *Stu*I digestion; lane 5, patient CTX-2 after *Stu*I digestion; lane 6, CTX-4 (heterozygote) after *Stu*I digestion; and lane 7, patient CTX-3 after *Stu*I digestion. The sizes of restriction fragments were estimated by comparison with the standard molecular size marker (ΦX174/*Hae*III).

is converted to 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol by the action of sterol 27-hydroxylase. The converted 27-hydroxylated products were detected by HPLC and the radioactivity was measured by a radiodetector. The HPLC procedure separated very efficiently the product (5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol, retention time 9 min) from the substrate (5β-[7β-^{3}H]cholestane-3α,7α, 12α-triol, retention time 34 min). Incubation of the 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol with a mitochondrial fraction of the normal fibroblasts significantly converted the substrate to 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol (**Fig. 4A**). The degree of conversion in five normal subjects was 4–12% and the enzyme activities were calculated to be 493 ± 86.3 nmol/mg per h (**Table 1**). The radio scanning data of three homozygous subjects and one heterozygous subject are shown in Fig. 4 B–E. Two homozygous subjects (CTX-1 and CTX-2) showed undetectable levels of the enzyme activities. Another homozygous subject (CTX-3) and one heterozygous subject (CTX-4) had decreased levels of the enzyme activities about 1.4% (7

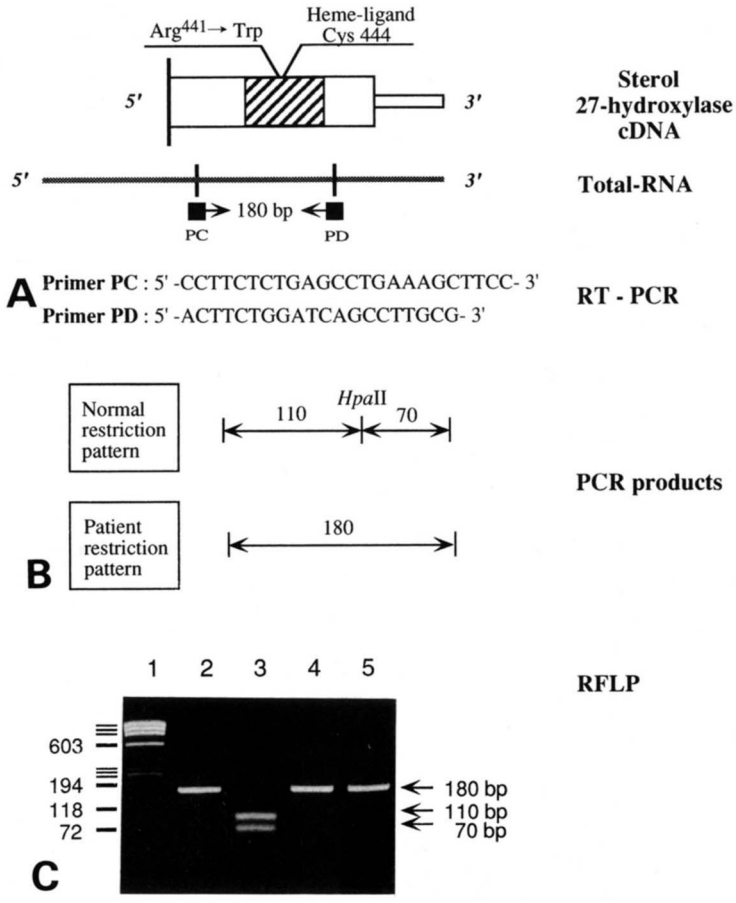

Fig. 3. PCR-detection of Trp mutation with *Hpa*II digestion. (A) Schematic representation of the portion of the sterol 27-hydroxylase cDNA amplified by the RT-PCR. Oligonucleotide primers PC (nucleotides 1331–1354) and PD (nucleotides 1491–1510) were used for amplification of target region (180 bp) including the mutation. (B) In normal subjects there is one *Hpa*II restriction site, and this site is eliminated by the Trp mutation. The horizontal arrows indicate the size of the fragments generated by the digestion. (C) Analysis of amplified DNAs from normal subject and the patient (CTX-3) was performed by electrophoresis using a 3.5% agarose gel after digestion with *Hpa*II. Lane 1, size marker (ΦX174/*Hae*III); lane 2, uncut amplified DNA of normal subject; lane 3, normal subject after *Hpa*II digestion; lane 4, uncut amplified DNA of the CTX-3; lane 5, the patient CTX-3 after *Hpa*II digestion. The sizes of restriction fragments were estimated by comparison with the standard molecular size marker (ΦX174/*Hae*III).

nmol/mg per h) and 10% (49 nmol/mg per h), respectively, as compared to those of normal subjects (Table 1). Thus there is a correlation between RFLP patterns (homozygote, heterozygote, or normal) and sterol 27-hydroxylase activities. Taken together, the newly identified point mutations in the functional domain of the sterol 27-hydroxylase could account for sterol 27-hydroxylase deficiency in the Japanese CTX patients who were studied.

DISCUSSION

CTX is a rare autosomal recessive disease characterized by tendon xanthomas, neurological manifestations, early onset of atherosclerosis, and cataracts (1). CTX patients can be diagnosed by these clinical findings and by biochemical findings, such as increased levels of serum cholestanol, increased cholestanol/cholesterol ratio, and the presence of abnormal bile alcohols (5). In view of the fact that CTX can be effectively treated by chenodeoxycholic acid (21) it is important to diagnose it at an early presymptomatic stage. Recently, Cali et al. (10) reported two different point mutations in the sterol 27-hydroxylase gene in two CTX patients. They identified a missense mutation at codon 362 (arginine to cysteine) in an African-American. The affected codon is located in a highly conserved region in mitochondrial cytochrome P450s which is thought to be the adrenodoxin binding site (22). In another Canadian, a missense mutation was found at codon 446 (arginine to cysteine) (10). This codon

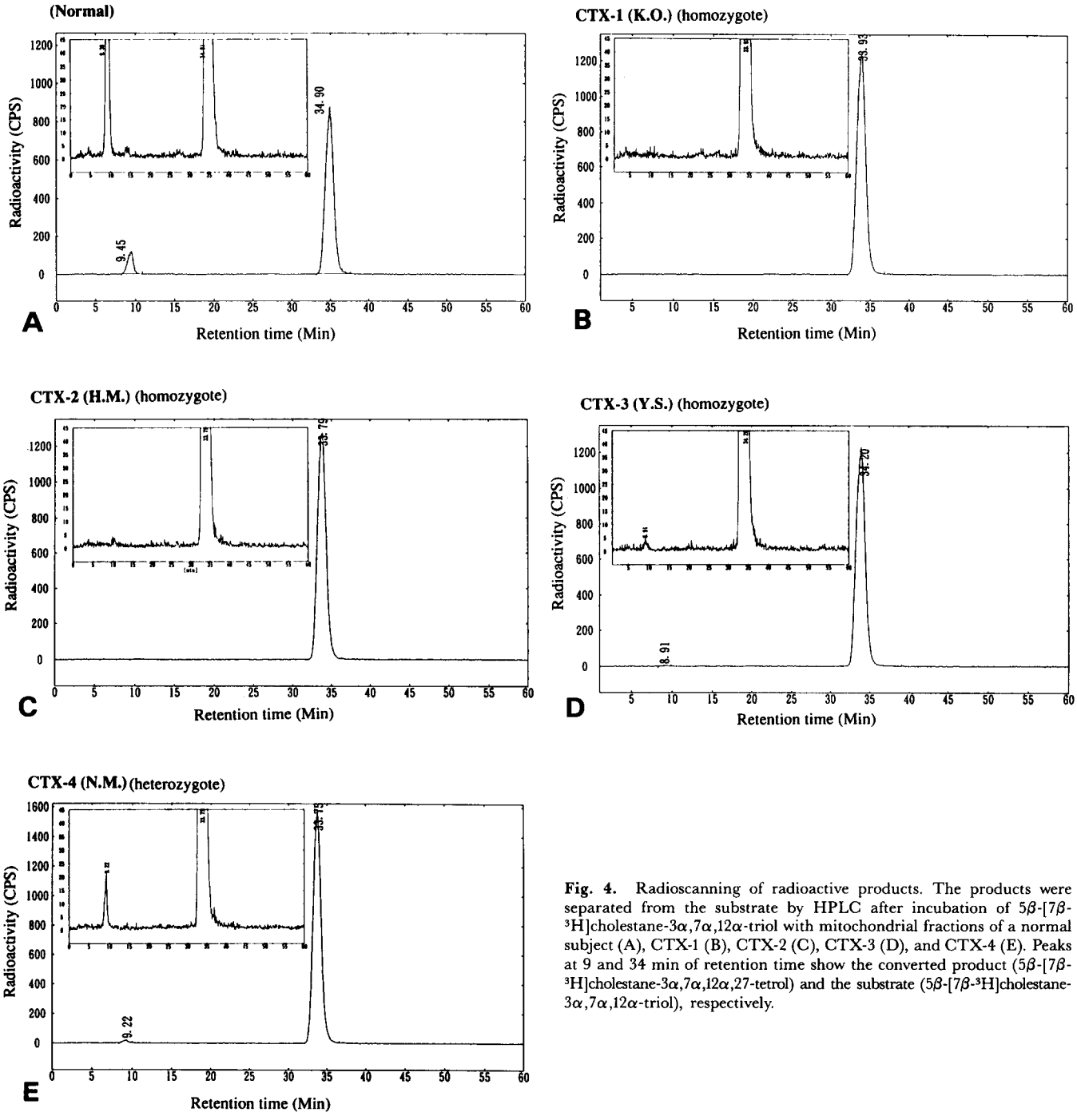

Fig. 4. Radioscanning of radioactive products. The products were separated from the substrate by HPLC after incubation of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol with mitochondrial fractions of a normal subject (A), CTX-1 (B), CTX-2 (C), CTX-3 (D), and CTX-4 (E). Peaks at 9 and 34 min of retention time show the converted product (5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol) and the substrate (5β-[7β-^{3}H]cholestane-3α,7α,12α-triol), respectively.

is located in the region corresponding to the heme-ligand binding site (23). In both cases the mutations occurred in the functional domains of sterol 27-hydroxylase gene and would thus be expected to disrupt enzyme activity. This expectation was confirmed by transfecting both mutant cDNAs in COS cells, which resulted in low or undetectable levels of enzyme activity (10). Leitersdorf et al. (11) have recently described a completely different type of mutations in Moroccan Jews with CTX, resulting in a lack of expression of mRNA. In similarity to the patients studied by Cali et al. (10) and in contrast to the patients studied by Leitersdorf et al. (11), the Japanese CTX patients expressed mRNA in normal amounts and showed a normal pattern in Northern blotting experiments (results not shown).

By direct sequencing of RT-PCR products from skin

TABLE 1. Detection of mutations and determination of sterol 27-hydroxylase activity in normal subjects, CTX patients, and a CTX heterozygote

Subject	Age (at onset)	Phenotype	Sex	Mutation		Sterol 27-Hydroxylase Activity
				Amino Acid	Nucleotides	
	yr					*nmol/mg/h*
CTX-1 (K.O.)	35 (10)	homozygote	M	$Arg^{441} \rightarrow Gln$	CGC→CAG	ND
CTX-2 (H.M.)	36 (20)	homozygote	M	$Arg^{441} \rightarrow Gln$	CGG→CAG	ND
CTX-3 (Y.S.)	37 (30)	homozygote	M	$Arg^{441} \rightarrow Trp$	CGG→TGG	7
CTX-4 (N.M.)	66	heterozygote	M	$Arg^{441} \rightarrow Gln$	CGG→CAG	49
Normal controls	30–50		M			493 ± 86[a] (n = 5)

[a] Mean ± SD; ND: not detectable.

fibroblasts, two different new point mutations were found in a functional domain of the sterol 27-hydroxylase gene in three patients and one heterozygote. In two homozygotes (CTX-1 and CTX-2) and one heterozygote (CTX-4, who is the father of CTX-2), a single substitution of A for G at codon 441 was found. Another homozygous patient (CTX-3) had a single base substitution of T for C at codon 441. Both mutations are different from two mutations reported by Cali et al. (10) but are present in the same functional domain. Interestingly, two new mutations in the present study as well as two mutations in the study by Cali et al. (10) occurred in CG dinucleotides, which is thought to be hypermutable in the human genome (24).

The newly identified mutations resulted in RFLPs for the restriction enzymes, *Stu*I or *Hpa*II. The clinically diagnosed homozygous and heterozygous states could thus be diagnosed by RFLP patterns for the *Stu*I or *Hpa*II enzymes.

Although the CTX heterozygote (CTX-4) was found to have decreased levels of the residual enzyme activity (about 10% of normal subject), he had normal levels of serum cholestanol and was asymptomatic. As the sterol 27-hydroxylase activity is not rate-limiting in the overall biosynthesis of bile acids, a very marked reduction in enzyme activity may be required to reduce bile acid biosynthesis and increase cholestanol biosynthesis. More information is needed, however, before we can evaluate how much reduction in sterol 27-hydroxylase activity is required to seriously affect the biosynthesis of bile acids and cholestanol. The results of the present work suggest that a reduction of the enzyme activity by 98% or more leads to increased formation of cholestanol (CTX-1, CTX-2, and CTX-3), whereas a reduction by 90% (the heterozygote, CTX-4) has no significant consequences for formation of cholestanol. Reduced enzyme activity in heterozygotes without clinical symptoms has previously reported also in other genetic diseases such as Tay-Sachs disease (25–27) and metachromatic leukodystrophy (MLD) (28, 29). The cerebroside sulfate sulfatase activity of leukocytes derived from MLD heterozygote was about 10% of the control level (29).

Although we have not yet performed mutant cDNAs transfection experiments, it is reasonably assumed that two mutations shown in the present study account for the sterol 27-hydroxylase deficiency in the Japanese CTX patients by the following criterias. *1)* Two newly identified mutations are present in the heme-ligand binding site of the sterol 27-hydroxylase and in CG dinucleotides which are hypermutable in the human genome as described above. *2)* There is a correlation between clinically diagnosed homozygous or heterozygous states, RFLP patterns for the *Stu*I enzyme, and sterol 27-hydroxylase activity in fibroblasts.

In summary, we have identified two new mutations in Japanese CTX patients. The new mutations resulted in two RFLPs. The RFLP technique used here may be useful for future presymptomatic diagnosis of CTX homozygotes and for genetic counseling in the specific families studied here. In order to use RFLP as a general diagnostic technique, however, more knowledge must be accumulated about possible mutations in the sterol 27-hydroxylase gene.

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