A point mutation in the bile acid biosynthetic enzyme sterol 27-hydroxylase in a family with cerebrotendinous xanthomatosis

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Abstract Cerebrotendinous xanthomatosis (CTX) is a rare familial disorder characterized by progressive neurological dysfunction, atherosclerosis, and xanthomas with sterol storage in the nervous system, vessels, and tendons. Increased serum cholestanol, derived from intermediates of cholesterol catabolism, may possibly be a major cause of the disease. An examination was made of the cDNA encoding cytochrome P450 sterol 27-hydroxylase (CYP27) in hepatic mitochondria, considered a defective enzyme inducing CTX, in a Japanese housewife afflicted with CTX and her family. The proposita and one of her brothers, who also had CTX symptoms and hypercholestanolemia, were found to be homozygotic, carrying a point mutation in the CYP27 gene at Arg^{104} (CGG) to Trp^{104} (TGG). The mutant position has a 100% conserved positive charge in all known vertebrate cytochrome P450s and even in bacterial cytochrome P450cam. The mother of the proposita and another brother were both free of CTX symptoms and were heterozygotic for the mutation, although their plasma cholestanol increased moderately. An increase in plasma cholestanol alone would, thus, not appear to be a direct cause of sterol storage in CTX, while CTX is strongly suggested to be caused by defects in both alleles of the CYP27 gene.—**Nakashima, N., Y. Sakai, H. Sakai, T. Yanase, M. Haji, F. Umeda, S. Koga, T. Hoshita, and H. Nawata.** A point mutation in the bile acid biosynthetic enzyme sterol 27-hydroxylase in a family with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1994. **35:** 663–668.

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Cerebrotendinous xanthomatosis (CTX), a rare disorder of cholesterol metabolism, exhibits an inherited autosomal recessive pattern first described in 1937 (1). The most prominent symptoms are tendinous xanthomas, neurological dysfunction with dementia, pyramidal-tract signs and cerebellar ataxia, and premature coronary heart disease due to sterol accumulation (2).

In CTX, excess cholestanol (5α-cholestan-3β-ol) is present in the brain, xanthomas, plasma, and bile (3–5). The capacity to convert cholesterol to bile acids has been shown to be impaired (5), and the incomplete oxidation of the cholesterol side chain leads to an accumulation of cholestanol and abnormal tetra- and penta-hydroxylated bile alcohols (6), accompanied by enhanced activity of cholesterol 7α-hydroxylase in the liver through feedback control of decreased bile acids synthesis (2). Increased plasma cholestanol may lead to sterol storage in general tissues (4), although the mechanism for this has yet to be determined.

Oftebro et al. (7) found evidence that the primary enzymatic defect in CTX may be a deficiency of mitochondrial sterol 27-hydroxylase (CYP27) which initially catalyzes the oxidation of side chains of sterol intermediates. Recently, Cali and Russell (8) uncovered human CYP27 cDNA and Cali et al. (9) pointed out point mutations in two CTX patients.

The purpose of the present report is to describe a point mutation found in the CYP27 gene of a Japanese family afflicted with CTX, and to discuss the relationship between the genotype of the mutation and clinical features, especially plasma cholestanol concentrations and CTX symptoms in the family.

MATERIALS AND METHODS

Assay of plasma cholesterol, cholestanol, bile acids and bile alcohols

Plasma cholesterol and cholestanol levels were measured as free sterols by the gas chromatographic methods

Abbreviations: CTX, cerebrotendinous xanthomatosis; CYP27, sterol 27-hydroxylase; PCR, polymerase chain reaction; bp, base pair; LDL, low density lipoprotein; HDL, high density lipoprotein; CDCA, chenodeoxycholic acid.

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of Ishikawa et al. (10). Bile alcohols were assayed by the methods as described previously (11). Plasma 7α-hydroxycholesterol was assayed as a kind favor by Dr. H. Oda and Dr. S. Kuroki, Kyushu University, using the methods as described (12).

Fibroblast culture

The fibroblasts were biopsied from forearm skin of the proposita as well as from three normal subjects, and cultured as previously described (13). Genomic DNA and total RNA were isolated from the cells as described (14).

Synthesis of CYP27 cDNA by polymerase chain reactions (PCR)

Single-stranded cDNA was synthesized from total RNA by specifically primed reverse transcription reaction using Rous-associated virus 2-reverse transcriptase (Takara Shuzo, Co., Kyoto, Japan). Ten μg of total RNA was reverse-transcribed in the presence of random primer (hexadeoxynucleotide mixture; $dp(N)_6$) (Takara Shuzo, Co., Kyoto, Japan), 2.5 mM of each dNTP and reverse transcriptase buffer, and 7 U of the enzyme at 42°C for 1 h. An aliquot of the reaction mixture was heated to 95°C for 7 min. The coding region of CYP27, except for exon 1, was then amplified using primers established by Cali et al. (9). Exon 1 of the CYP27 gene was amplified from genomic DNA as described by Cali et al. (9).

DNA sequence analysis

We determined the DNA sequence of the amplified fragments from two separate aliquots of cells of the proposita by direct sequencing (15), using bacteriophage T7 DNA polymerase (Sequenase, U.S. Biochemicals).

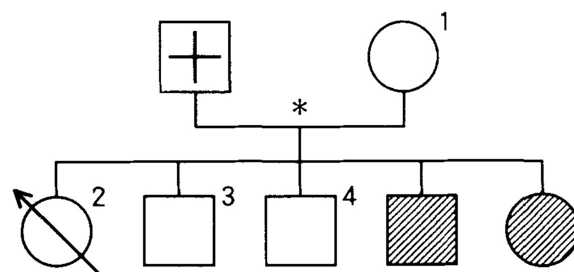
Restriction genotyping of amplified CYP27 gene with Msp I and gel analysis in the family members

The genomic DNA of family members and 50 normal subjects were prepared from their peripheral leukocytes. To detect single base substitution in the nucleotide 430 of CYP27 cDNA, restriction genotyping analysis was conducted after PCR amplification using intra exon primers (nucleotides number 390–410 (8) 5' GTACCCAGTACGG AACGACA3' and 452–472 5'TTCCGTGGTGAACGGC CCAT3'). Msp I (Takara Shuzo Co., Kyoto, Japan) was added directly to each reaction mixture (5 units Msp I /300 ng DNA). After 3 h at 37°C, each reaction mixture was loaded onto a 2% agarose gel and electrophoresed. The gel was then treated with ethidium bromide (0.2 mg/l) for 20 min and DNA fragments were visualized under UV illumination.

RESULTS

Case report

The proposita was a Japanese housewife, aged 62 (**Fig. 1**). She had grown up in good health. She married at 28, but gave birth to no children. She had a history of tendon xanthomas that started in her thirties. The xanthomas gradually enlarged and came to involve numerous extensor tendons on her feet, bilaterally on her Achilles tendons, and bilaterally on her tibial tuberosities, complicated by bilateral xanthelasma palpebrarums. She was admitted to our hospital due to pain in the Achilles tendon xanthomas. On examination, coronary heart disease was detected by an electrocardiogram. No cataracts could be



Subject No.	age (years)	CTX	cholestanol (μg/ml)	cholesterol (μg/ml)	TG (mg/dl)	HDL-C (mg/dl)
1	92	–	7.5	1780	114	49.7
2	62	+	15.3	1900	140	36.0
3	58	+	10.4	2220	85	52.6
4	56	–	5.4	1960	74	50.6

Fig. 1. Pedigree of the family and their plasma lipid profiles. The arrow indicates the proposita and the cross indicates a deceased subject. Subjects No. 1–4 were evaluated in the study but not those with shadows; * represents consanguineous marriage; CTX, cerebrotendinous xanthomatosis; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol. Normal range of serum cholestanol is 2.4 ± 0.7 μg/ml, mean ± SD, n = 17.

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detected. Neurological examination indicated pyramidal-tract signs including hyperactive deep-tendon and pathological reflexes, mild mental retardation, and muscular atrophy in the lower extremities. There was no cerebellar ataxia. An electroencephalogram showed predominant α waves with considerably diffuse δ waves. She had a normal cholesterol level (1900 μg/ml) with increased plasma cholestanol concentration (15.3 μg/ml, normal: 2.4 ± 0.7 μg/ml, mean ± SD, n = 17). There was an increased cholestanol/cholesterol ratio in bile (7.3%, mean value of normal controls is 0.71%) and biopsied xanthoma (2.1%, mean value of xanthoma from subjects with hypercholesterolemia is 0.3%). There was an increase of bile alcohols in the plasma (**Table 1**), an increased ratio of cholic acid/chenodeoxy cholic acid in plasma (6.5%, mean value of normal controls was 0.6%) and bile (25.3%, mean value of normal controls was 1.0%). An increased plasma 7α-hydroxycholesterol concentration (1,858 pmol/ml as total, and 222 pmol/ml as free fraction; normal: 240 ± 116 pmol/ml as total, and 56 pmol/ml as free fraction, n = 8) was also observed. Phytosterol and sitosterol were not detected in the plasma. Thyroidal function was within the normal range. The patient was thus diagnosed as CTX. She was the offspring of a consanguineous marriage. Her father, having no obvious symptoms of CTX, died of an unknown cause at 70 years of age. Her mother, aged 92, and one brother, aged 56, were healthy, but with moderate hypercholestanolemia (7.5 μg/ml and 5.4 μg/ml, respectively). Another brother, aged 58, had general tendon xanthomas and mild mental retardation with increased plasma cholestanol (10.4 μg/ml).

DNA sequence analysis

We used PCR to amplify CYP27 gene coding sequences from genomic DNA and cDNAs prepared from total RNA of fibroblasts using reverse transcriptase. A homozygous mutation of a single base substitution from arginine (CGG) to tryptophan (TGG) at codon 104 was identified by direct sequencing (**Fig. 2**). A silent polymorphism was found in codon 89 (GGA:Gly (8)→GGC:Gly) in both alleles in CTX cells and three normal Japanese subjects' cells.

Restriction genotyping of the mutation in the family members

To determine whether the mutation at codon 104 was present in the proposita genome and the genotype of the mutation in the family members, PCR amplification of the region containing the codon 104 from genomic DNAs of the family and 10 healthy subjects was conducted. In 50 normal subjects, Msp I digestion of PCR-amplified products of 82 base pairs (bp) generated new 41bp fragments. The digestion of amplified DNA from the proposita (subject No. 2 in Fig. 1, lanes 3 and 4) and one brother (No. 3, lanes 5 and 6) caused no change in the fragment length with the disappearance of half-size fragments due to the loss of Msp I sites (**Fig. 3**). Similar digestion of PCR fragments from the mother (No. 1, lanes 1 and 2) and another brother (No. 4, lanes 7 and 8) showed both 82 bp fragments and half-size fragments. Thus, as shown in Fig. 3, the proposita and one brother (No. 3) were homozygotes of the mutation of the CYP27 gene. The mother and another brother (No. 4) had the heterozygous mutation.

DISCUSSION

In CTX, excess cholestanol accumulation is observed in the nervous system, xanthoma, bile, and plasma (4, 5). Bile acid concentrations in the bile are subnormal and high levels of abnormal bile alcohols are found, as shown by Setoguchi et al. (6). Based on these and previous findings in vitro (7) and in vivo (16), Skrede et al. (17) concluded that the primary enzymatic defect in this disease was present in CYP27. Cali et al. (9) reported two cases of CTX caused by separated homozygous point mutations in the CYP27 gene. One had a point mutation at po-

TABLE 1. Bile alcohol glucuronide profiles in plasma and bile of the propositus

Compound	Plasma	Bile
	ng/ml	*% of bile alcohols*
5β-Cholestane-3α,7α,12α,23-tetrol	ND (ND)[a]	1.8
5β-Cholestane-3α,7α,12α,24-tetrol	ND (ND)	26.7
5β-Cholestane-3α,7α,12α,25-tetrol	1304 (5.1 ± 0.5)[b]	49.1
5β-Cholestane-3α,7α,12α,23,25-pentol	434 (ND)	17.2
5β-Cholestane-3α,7α,12α,24,25-pentol	386 (ND)	5.2
5β-Cholestane-3α,7α,12α,25,26-pentol	118 (10.0 ± 7.7)	ND
27-nor-5β-Cholestane-3α,7α,12α,24,25-pentol	42 (32.2 ± 12.6)	ND
Bile acids/bile alcohols	6.9	18.6 (455-833)

[a] ND, not detected.
[b] Control values are shown as mean ± SD (n = 7).

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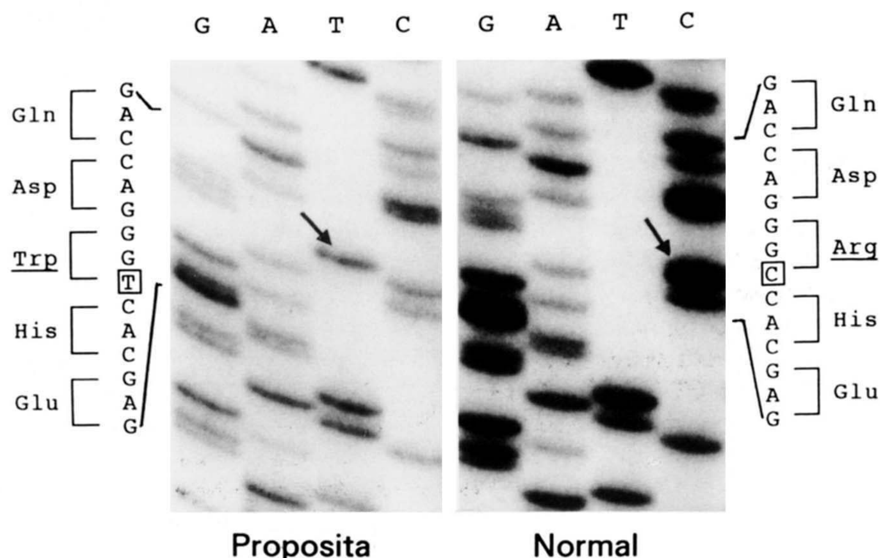

Fig. 2. Sequence analysis of cDNA of sterol 27-hydroxylase from a normal subject and the proposita. Sequences were determined on both strands by direct sequencing of amplified DNA. The regions across a mutation were sequenced on separately amplified DNAs. Direction of 5'-3' is from the bottom to top. A portion of the autoradiogram from a 6% polyacrylamide/8 M urea gel is shown. A single base substitution (C→T) is indicated by arrows.

sition 446, which is two amino acids away from a ubiquitously conserved cysteine of cytochrome P450 proteins that serve as a ligand for the heme cofactor (18). The other had a point mutation at position 362 in a sequence highly conserved among mitochondrial cytochrome P450s and thought to bind to the adrenodoxin cofactor (19). In our case, a homozygous point mutation occurred at amino acid position 104, in which arginine was replaced by tryptophan. This position has a 100% conserved position charge in 33 aligned vertebral cytochrome P450s, as demonstrated by Nelson and Strobe (20), in cytochrome P450 vitamin D_3 25-hydroxylase (21), and even in bacterial cytochrome P450cam (22). This arginine interacts with a heme propionate group (22), and position 100 tryptophan, four residues away, possibly may be involved in electron transfer between NADPH-cytochrome P450 reductase and P450 heme in microsomal P450s (20). The observed mutation in our case was present in the functional domain of CYP27 and possibly disrupted enzyme activity, perhaps owing to disturbance of the electron transfer system, although CYP27 is one of mitochondrial P450s. In consideration of the high degree of conservation in cytochrome P450s at the site of the mutation in the family, further study, including actual measurement of CYP27 activity in the family and site-directed mutagenesis of this region of CYP27, should be conducted to prove that the CYP27 obtained from the family is, in fact, defective, and to provide a greater understanding of the functional domain of CYP27 and other cytochrome P450s.

Both CTX patients reported by Cali et al. (9) had homozygotic mutations in the CYP27 gene. In the present study, the proposita and a brother, both with typical symptoms of CTX and hypercholestanolemia, had the homozygous missense mutation in codon 104 of the CYP27 gene. The mother and another brother are heterozygotes of the mutation without the typical symptoms of

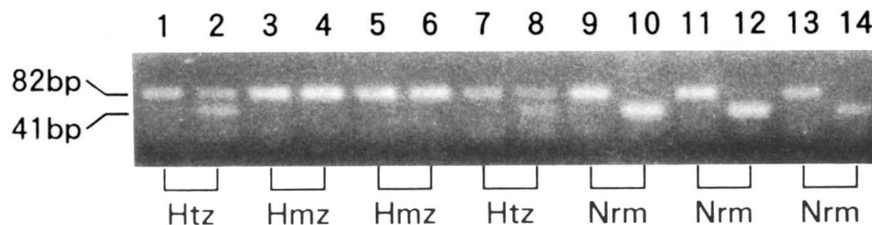

Fig. 3. Msp I digestion of PCR fragments around the mutation. Odd numbers are PCR fragments, and even numbers are Msp I-digested fragments. Msp I divides the 82 bp products (lanes 9, 11, 13) into half-size fragments of 41 bp (lanes 10, 12, 14) in normal subjects. Lanes 1 and 2 represent subject No. 1; lanes 3 and 4, No. 2; lanes 5 and 6, No. 3; and lanes 7 and 8, No. 4.

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CTX. Plasma cholestanol concentrations, however, had moderately increased (Fig. 1). This mutation was not observed in 100 alleles from 50 normal subjects. CTX would thus appear to be caused by defects of both alleles of the CYP27 gene. This is consistent with the fact that CTX is inherited in an autosomal recessive way (2). Interestingly, all three CTX mutations (at codons 104, 362 and 446) occurred in CG dinucleotides, thought to be hypermutable in the human genome (23, 24).

The direct mechanism for cholestanol accumulation in general tissues in CTX is unknown, while the primary defect of this disease has been clarified. Menkes, Schimschock, and Swanson (3) found cholestanol to be deposited in the nervous system, and considered the possibility that cholestanol transport system from the tissues was impaired in CTX. Philippart and van Bogaert (4) considered that CTX is likely generalized cholestanolosis, in that cholestanol accumulation was detected in tendon xanthomas and plasma from CTX subjects as well as in the nervous system. As for the mechanisms of cholestanol accumulation, Lussier-Cacan et al. (25) considered that increased plasma cholestanol, like sitosterol, possibly interferes with the normal uptake and degradation of LDL by peripheral cells. An LDL turnover study demonstrated that both production and catabolic rates of LDL increases in CTX (26). Shore et al. (27) found an abnormality of apoA-I isoform with decreased serum HDL-cholesterol that possibly enhances cholestanol accumulation in CTX. However, not all CTX patients, including our case, have an abnormality in the apoA-I isoform with decreased HDL-cholesterol, and other mechanisms remain to be clarified. The accumulated cholestanol/cholesterol ratio differed remarkably in tissues, particularly that of the nervous system compared with other tissues (5), and accordingly, the mechanism of cholestanol deposition could differ among tissues. The presence of bile alcohols in the plasma may also have pathological significance in the presence of hypercholestanolemia. Batta et al. (28) found increased amounts of albumin and apoB in the cerebrospinal fluid of CTX patients, and concluded that large plasma bile alcohol glucuronide pools were importantly involved in abnormal blood-brain barrier permeability in these patients. This would, consequently, lead to increased transport of cholestanol and cholesterol in the brain.

It is of interest in this study that the complete absence of CTX symptoms in subjects carrying the heterozygotic mutation with moderate hypercholestanolemia (Fig. 1) suggests that increased plasma cholestanol alone would not induce CTX. Beppu et al. (29) found that the plasma cholestanol concentrations of ten CTX patients ranged from 6.4 μg/ml to 32.3 μg/ml, and the severity of CTX symptoms was unrelated to plasma cholestanol concentration. Kuriyama et al. (30) noted that parents of five CTX patients appeared to have mild hypercholestanolemia, but no CTX symptoms, as we also observed in this study. These findings may indicate that an increase in plasma cholestanol concentration alone does not lead to the presence of CTX symptoms, and CTX may be due to defects in both alleles of the CYP27 gene.

The procedure shown in this study may be useful to screen even early stage CTX exhibiting no typical CTX symptoms, and medication in the early stages by CDCA and/or HMG-CoA reductase inhibitors may prevent the progress of CTX (2, 31).

We conclude that the point mutation at codon 104 in both alleles of the CYP27 gene causes CTX in the family, and that an increase in plasma cholestanol concentration alone would not give rise to the present CTX symptoms.

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