# A novel mutation in the cytochrome $P450_{27}$ (*CYP27*) gene caused cerebrotendinous xanthomatosis in a Japanese family

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**Abstract** Cerebrotendinous xanthomatosis (CTX) is an autosomal recessive lipid storage disease caused by mutations in the cytochrome $P450_{27}$ (*CYP27*) gene. This disease is characterized by the accumulation of a bile alcohol, cholestanol, in diverse tissues. Accumulation in the central nervous system leads to neurological dysfunction including dementia, spinal cord paresis, and cerebellar ataxia. Accumulation in other tissues causes tendon xanthomas, premature atherosclerosis, and cataracts. In a Japanese family with CTX, we identified two point mutations in the *CYP27* gene at different sites. One is a novel transversion, which substitutes G for C at Pro [368] (CCC) to Arg (CGC). The other is a transition, which substitutes A for G at Arg[441] (CGG) to Gln (CAG), this being the same mutation that Kim et al. reported (1994. *J. Lipid Res.* **35:** 1031–1039). Allele-specific polymerase chain reaction analysis indicated that the father and mother of this family, who themselves had no clinical manifestations of CTX, had the former and latter mutations heterozygously, respectively. On the other hand, the patients each had both mutations heterozygously. These results are highly suggestive, but not conclusive, that the newly identified transversion in the *CYP27* gene accounts for the sterol 27-hydroxylase (EC 1.14.13.15) deficiency in these patients.—**Okuyama, E., S. Tomita, H. Takeuchi, and Y. Ichikawa.** A novel mutation in the cytochrome $P450_{27}$ (*CYP27*) gene caused cerebrotendinous xanthomatosis in a Japanese family. *J. Lipid Res.* 1996. **37:** 631–639.

**Supplementary key words** cerebrotendinous xanthomatosis (CTX) • cytochrome $P450_{27}$ • cholestanol • sterol 27-monooxygenase • mitochondrial cytochrome P450 • single-strand conformation polymorphism

Cerebrotendinous xanthomatosis (CTX) is a rare, recessively inherited lipid storage disease that was first described by van Bogaert, Scherer, and Epstein (1). In CTX, excess cholestanol accumulation is observed in the nervous system, xanthoma, bile, and plasma (2, 3), and the patients exhibit clinical manifestations of tendon xanthomas, progressive neurological dysfunction, and juvenile cataracts (4). The increased biosynthesis of cholestanol may be due to increased utilization of bile acid intermediates as precursors for cholestanol (5). These symptoms often develop during the second and third decades of life (6), and become more severe with increasing age, leading to profound incapacitation. The slowly progressive nature of the disease and the non-uniformity of the clinical manifestations, even within the same CTX family, may preclude clinical diagnosis at an early stage (7).

The metabolic defect underlying the failure to synthesize bile acids is controversial and has been postulated to involve either a sterol 24-hydroxylase or a 27-hydroxylase. Based on the results of intermediary metabolic studies, Salen et al. (8) postulated that a defect in microsomal sterol 24S-hydroxylase causes CTX. Oftebro et al. (9) reported, however, that a defect in the mitochondrial sterol 26-hydroxylase (currently 27-hydroxylase) accounts for CTX. Several subsequent studies using fibroblasts from different patients with CTX have confirmed a lack of mitochondrial sterol 27-hydroxylase activity in this disease (10). Recent molecular cloning of human sterol 27-hydroxylase cDNA and characterization of cDNAs in two patients with CTX also confirmed a deficiency of sterol 27-hydroxylase (11, 12).

Japan has a relatively high prevalence of CTX as compared with other countries (4, 6). Therefore, it is important to investigate the distribution of and to establish a method for genetic diagnosis of this disease in Japan. We investigated a Japanese family with CTX, and detected two different non-synonymous substitutions at amino acid residues 368 and 441. The former was a novel transversion adjacent to the ferredoxin-binding region of mitochondrial cytochromes P450, and the latter was the same mutation as that reported by Kim et

Abbreviations: CTX, cerebrotendinous xanthomatosis; *CYP27*, cytochrome $P450_{27}$; SSCP, single-strand conformation polymorphism.

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al. (13). In this paper, we discuss the possibility of the newly identified transversion in the *CYP27* gene being the cause of CTX.

## MATERIALS AND METHODS

### Materials

All the enzymes used in this study were purchased from Takara Shuzo, Promega, or Perkin Elmer. The enzymes were used according to the manufacturers' recommendations and standard procedures. Oligonucleotides for polymerase chain reactions were from Nihon Seifun Co.

### Patients

Three Japanese patients diagnosed as having CTX according to the defined clinical and laboratory criteria were studied. Although CTX-2 and -3 (they are sisters) showed typical manifestations of CTX, mental retardation, intractable convulsions, gait disturbance, visual impairment in both eyes, tendon xanthomas, and cataracts, CTX-1 showed very weak symptoms. The levels of plasma cholestanol were markedly increased in these patients (see Table 2). Their parents had no manifestations of CTX and were not consanguineous.

Informed consent was obtained from all subjects. This study was approved by the Department of Internal Medicine (3rd Division), Kagawa Medical School.

### Assaying of plasma cholestanol

Measurement of the levels of plasma cholestanol in the patients with CTX and their parents was entrusted to SRL Co. and performed by gas chromatographic methods. It should be noted that the levels of plasma cholestanol in the family members were measured at different times.

### Extraction of genomic DNA

Genomic DNAs were extracted from leucocytes of the sisters with CTX, their parents, and healthy controls using a commercially available genomic DNA extraction kit, Sepa Gene (Sanko Jyunyaku Co.), according to the manufacturer's recommendations.

### PCR amplification

We amplified the 5′ flanking region and all exons of the cytochrome $P450_{27}$ (*CYP27*) gene by means of PCR amplification (14) to identify the mutation(s) causing CTX. For this amplification, oligonucleotides were basically synthesized according to Cali et al. (12), and Leitersdorf et al. (15). As our attempt to amplify the 5′ flanking region of the *CYP27* gene was unsuccessful, we substituted the RL14′ and RL15′ oligonucleotides for the RL14 and RL15 ones, respectively (see Table 1). Furthermore, in order to amplify exons 6 to 9, we originally synthesized six oligonucleotides (see Table 1).

The PCR protocol for the amplification of the 5′ flanking region included 5 min melting of the strands at 95°C, and then 1 min of denaturation at 95°C, and 5 min of annealing and extension at 68°C, using Taq DNA polymerase (Promega), for 35 cycles. With respect to the amplification of exon 1, a further one cycle, 1 min of denaturation at 95°C, and 10 min of annealing and extension at 68°C, followed the above protocol. The

TABLE 1. Sequences and locations of oligonucleotides in the *CYP27* gene that were used for amplification of exons and allele-specific PCR

| Oligonucleotide | Location | Target | Sequence 5′ → 3 | Position[a] |
|---|---|---|---|---|
| Oligonucleotides for amplification of exons[b] | | | | |
| RL14′ | 5′flanking | 5′-flanking | AGGGATCAGATGACTGGCCC | -498 to -479 |
| RL15′ | exon 1 | 5′-flanking | GCGCAGCCCAGCGCAGCCAT | 20 to 1 |
| 6b | exon 7 | exon 6 | GTTTGTGGGGACCACAGGGT | 1209 to 1190 |
| 7a | exon 6 | exon 7 | AAGCTGTGCTTAAGGAGACT | 1160 to 1179 |
| 7b | exon 8 | exon 7 | ACATAGTGGCAGAACACAAA | 1289 to 1270 |
| 8a | exon 7 | exon 8 | GATGGCTTCCTCTTCCCCAA | 1240 to 1259 |
| 8b | exon 9 | exon 8 | ACCACCTTGTACTTCTGGAT | 1499 to 1480 |
| 9a | exon 8 | exon 9 | CTGGAGATGCAGCTACTCCT | 1450 to 1469 |
| Oligonucleotides for allele-specific PCR | | | | |
| CTX-1202C | exon 7 | C or G(1202) | TCTCTACCCTGTGGTCCC | 1185 to 1202 |
| CTX-1202G | exon 7 | C or G(1202) | TCTCTACCCTGTGGTCCG | 1185 to 1202 |
| CTX-1421G | exon 8 | G or A(1421) | CCCTTTGGCTATGGGGTCCG | 1402 to 1421 |
| CTX-1421A | exon 8 | G or A(1421) | CCCTTTGGCTATGGGGTCCA | 1402 to 1421 |

[a]A of the ATG initiation codon is number +1.
[b]The following oligonucleotides were also used for amplification of other exons; 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 4c, 4b, 5a, 5b, 6a, and 9b (ref.15).

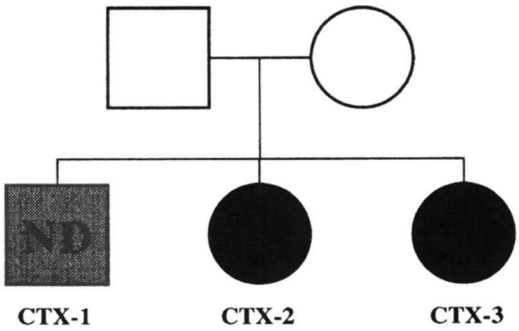


**Fig. 1.** Pedigree of the family in which three children were diagnosed as having cerebrotendinous xanthomatosis (CTX). The filled pedigree symbols indicate typical CTX cases and the shaded one indicates a mild CTX case. ND indicates a molecularly uncharacterized subject.

PCR protocol for amplification of exons 2 to 5 comprised 5 min melting of the strands at 94°C, and then 1 min of denaturation at 94°C, 2 min of annealing at 65°C and 2 min of extension at 72°C, using AmpliTaq DNA polymerase (Perkin Elmer), for 35 cycles. Final extension was then performed for 10 min at 72°C. All these reactions were performed with a Program Temp Control System PC-700 (ASTEC). The following ones were performed with a 1605 Air Thermo-Cycler (Idaho Technology). The PCR protocol for the amplification of exons 6 to 9 comprised 15 sec melting of the strands at 94°C, and then 15 sec of denaturation at 94°C, 15 sec of annealing at 55°C and 20 sec of extension at 72°C, using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) for 35 cycles. Final extension was then performed for 20 sec at 72°C.

### SSCP analysis

To identify the mutation(s) that caused CTX in our patients, we performed single-strand conformation polymorphism (SSCP) analysis (16) using Silver Stain Plus (Bio-Rad), according to the manufacturer's recommendations.

### Allele-specific PCR

In order to determine the genotypes of the sisters with CTX and their parents, allele-specific PCR was performed. In this analysis, the 7b and 8b oligonucleotides described above and four newly synthesized ones were used. These oligonucleotides are summarized in **Table 1.** The PCR protocol for this analysis was the same as that used for amplification of exons 2 to 5 except that we substituted AmpliTaq DNA polymerase of Promega for that of Perkin Elmer.

### Molecular cloning and sequencing

The PCR products were directly cloned into the pT7Blue T-Vector (Novagen). The inserted fragments were cut out with appropriate restriction enzymes, and then subcloned into the M13 mp18 and mp19 phage vectors using standard procedures. Single strand DNAs were prepared from these recombinant phages. The sequencing reactions were performed according to the modified dideoxy method (17), using a commercially available sequencing kit; *Bca*BEST (Takara Shuzo Co., Ltd.). The nucleotide sequences were determined for both strands of the subcloned DNA for the sake of precision.

### Analysis of DNA sequences

Analyses of the sequences were carried out using the packages, ODEN (18) and GENETYX.

## RESULTS

### Screening of mutation(s) in the cytochrome $P450_{27}$ (*CYP27*) gene by SSCP

A Japanese family with three CTX patients was investigated (**Fig. 1**). At first, we amplified the 5′-flanking region and nine exons of the *CYP27* gene of the sisters with CTX and eight normal subjects to detect mutation(s) in this gene. The oligonucleotides used for this experiment are listed in Table 1. Screening from the 5′-flanking region to exon 9 of the *CYP27* gene of the sisters by single-strand comformation polymorphism (SSCP) analysis revealed that only exons 7 and 8 of the sisters each exhibited a band shift (data not shown).

### DNA sequence

To specify the sites, we used the translation initiation site of the *CYP27* gene and the first amino acid residue (alanine) from the N-terminus of the mature form of *CYP27* as reference points (1), respectively. On sequencing analysis of exons 7 and 8 of the *CYP27* gene of the sisters, we identified two kinds of clones, respectively, namely, with respect to exon 7, some clones had guanine substituted at 1202 for cytosine, and with respect to exon 8, some clones had adenine substituted at 1421 for guanine (**Fig. 2**). Except for these mutations, the nucleotide sequences of both exons 7 and 8 of the sisters were completely identical with the cDNA sequence reported by Cali and Russell (11) (data not shown). The mutation in exon 7 is a novel transversion, which substitutes G for C at $Pro^{368}$ (CCC) to Arg (CGC), and that in exon 8 is a transition, which substitutes A for G at $Arg^{441}$ (CGG) to Gln (CAG). The latter was the same mutation that Kim et al. (13) reported. These mutations were located just downstream of the ferredoxin-binding region (19), and in heme-binding region of mitochondrial cytochrome P450 (20), respectively (Fig. 2 and **Fig. 3**). It should be noted that, according to previous reports, CTX mutations often occur in CG dinucleotides, which are thought to be hypermutable in the human genome (21).

However, this was not the case for the novel mutation we identified.

### Allele-specific PCR

Although the sisters in this family showed typical manifestations of CTX, there were no symptoms of CTX in the parents. On the basis of their phenotypes and the above nucleotide sequences, we supposed that the father of this family harbored one of the two mutations heterozygously and the mother harbored the other one heterozygously, and that the simultaneous inheritance of these mutations from the parents accounted for the CTX phenotype of the sisters. Therefore, we performed allele-specific PCR to determine the genotypes of the family members. The oligonucleotides used for this experiment are listed in Table 1. The PCR reactions were unsuccessful for the combinations of the mother's genomic DNA-1202G/7b primers and the father's genomic DNA-1421A/8b primers (**Fig. 4**). These results indicated that the father and mother had a substitution of $C^{1202}$ to G, and $G^{1421}$ to A heterozygously, respectively. On the other hand, the sisters each had both mutations heterozygously. Then we investigated the genotypes of both exons 7 and 8 in 19 normal subjects using allele-specific PCR and detected neither of these mutations in them (data not shown).

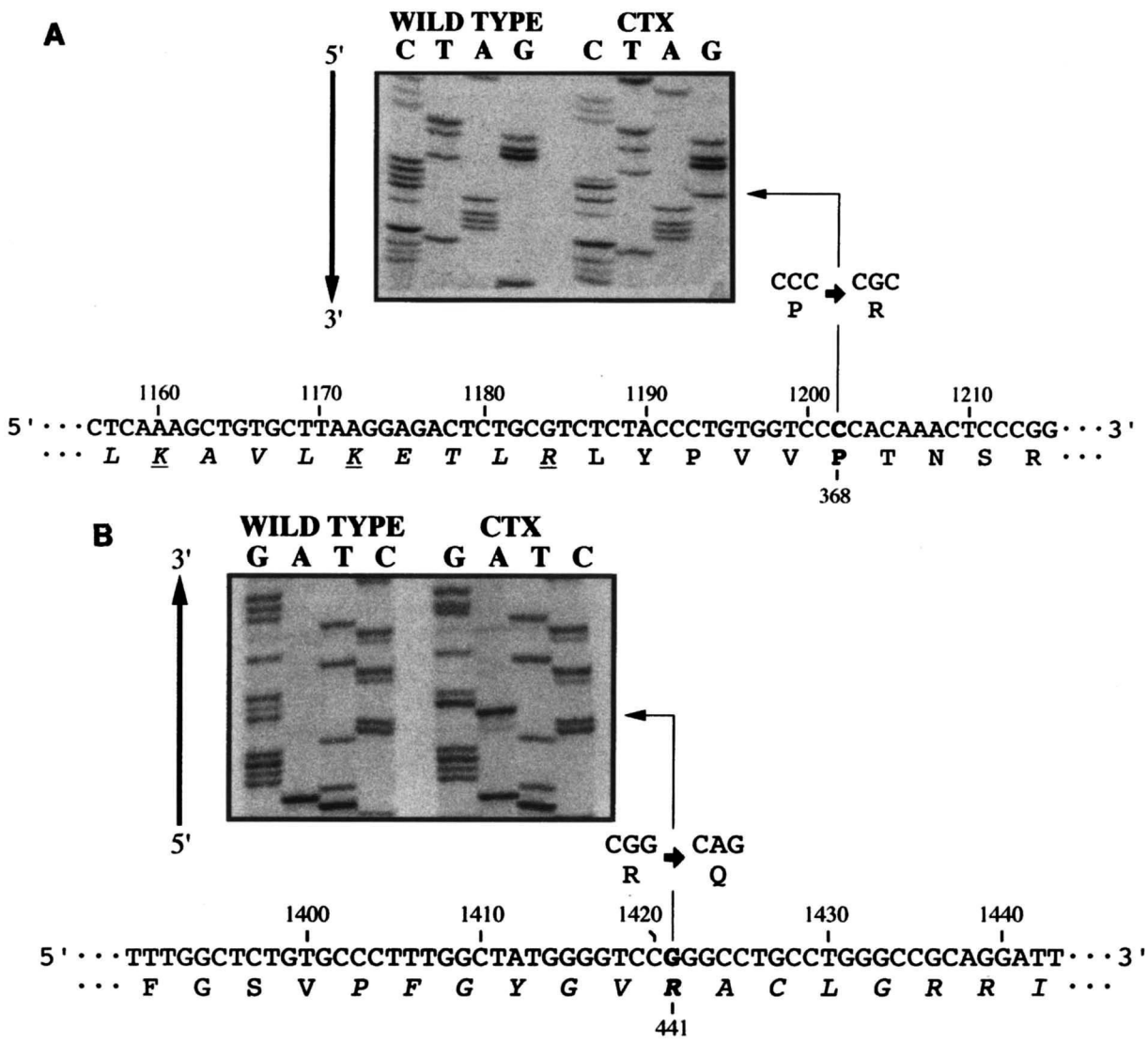


**Fig. 2.** DNA sequence analysis of the mutant allele of CTX. Amplified PCR products from a wild type subject and the CTX patients were sequenced as described under Materials and Methods. The nucleotide sequence of the coding strand of the wild type allele and the corresponding amino acid sequences are shown at the bottom. The translation initiation site of the *CYP27* gene and the first amino acid residue (alanine) from the N-terminus of the mature form of *CYP27* are numbered as reference points (1), respectively. The arrows at the left indicate the orientation of the DNA strand. Bold letters indicate the positions of the mutations. (A) Sequence around the mutation in exon 7. Italics and underlining indicate the putative ferredoxin-binding region and three adjacent basic amino acid residues, respectively (see the text). (B) Sequence around the mutation in exon 8. Italics indicate the heme-binding region.

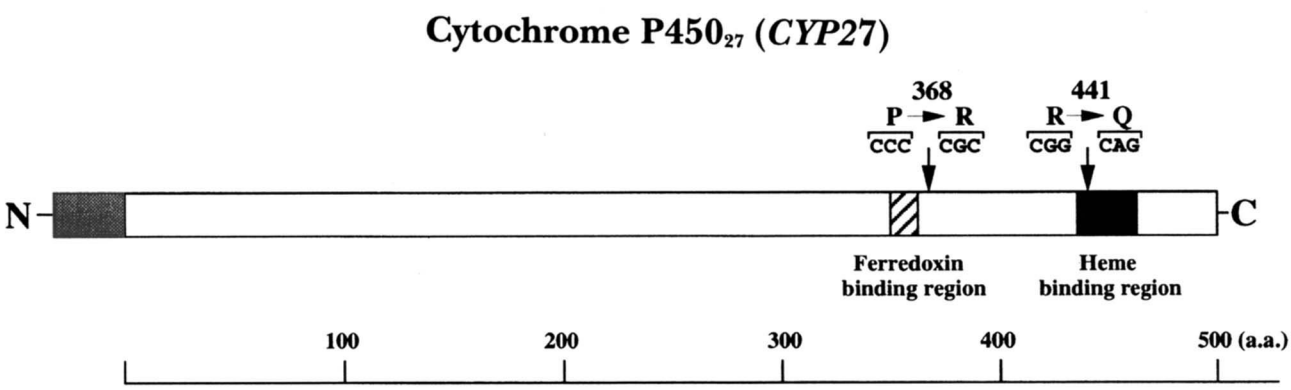


**Fig. 3.** Structure of *CYP27* and the positions of the mutations. Shaded, crosshatched, and filled regions indicate the 33-residue mitochondrial signal peptide, ferredoxin-binding region, and heme-binding region, respectively. The arrows indicate the positions of the mutations. The first amino acid residue (alanine) from the N-terminus of the mature form of *CYP27* is numbered as a reference point (1).

### Plasma cholestanol level

**Table 2** shows the levels of plasma cholestanol with the genotypes of the family members and normal subjects. The levels of plasma cholestanol were markedly increased in all the patients, but those in the parents seemed to be within normal range. Furthermore, the levels in the parents were equivalent to that of CTX-4 who harbored the same mutation heterozygously as that found in exon 8 (13).

## DISCUSSION

We report two point mutations in the gene for cytochrome P450$_{27}$ (*CYP27*) in Japanese sisters with CTX. In exon 8 of the *CYP27* gene, a mutation encoding Gln (CAG) in place of Arg (CGG) at position 441 was detected in the heme-binding region of cytochromes P450 (20) (Figs. 2 and 3). This mutation had already been identified by Kim et al. (13), who clarified that this mutation could account for the *CYP27* deficiency in patients with CTX. Contrary to that study (13), the sisters we investigated both harbored this mutation heterozygously. Another mutation that we isolated, in exon 7 of the *CYP27* gene, was a transversion that substitutes G for C at Pro$^{368}$ (CCC) to Arg (CGC), and was different from other mutations in this gene reported previously (7, 12, 13, 15, 22–25). These mutations are summarized in **Fig. 5.** This novel Arg was located five amino acids away from the ferredoxin-binding region of mitochondrial cytochromes P450 (19) (Figs. 2 and 3). Although not every mutation in the *CYP27* gene will produce deficient or inactive *CYP27* and we have not yet performed expression experiments on the mutant cDNAs, from the following results, it can be reasonably assumed that this novel mutation accounts for the CTX phenotype in the sisters. *1)* There were no mutations that were detectable on SSCP analysis in the *CYP27* gene other than the two mutations described here, and one of these mutations could cause the *CYP27* deficiency. *2)* There is a correlation between the clinical diagnosis and the genotype of the *CYP27* gene of the sisters. In other words, the mutation in exon 7 seemed to co-segregate with the CTX phenotype in the sisters. Although the mechanism by which this amino acid change accounts for the abnormal enzymatic activity is unclear, we consider two models to explain this mechanism.

The first model asserts that the substitution from Pro to Arg causes a serious conformational change of *CYP27,* because Pro residue mainly prefers the turn conformation. On the other hand, Arg residue prefers α-helix conformation in the second structure. However, the Pro residue at 368th is not so conservative among mitochondrial cytochromes P450 (data not shown). Therefore, the amino acid residue of this position in *CYP27* seems to be flexible in some degree.

In the second model, we suppose that this mutation, a positive charge of Arg residue, disturbs the binding action of ferredoxin or electron transfer to *CYP27* with the result that the enzymatic activity is disrupted. All mitochondrial cytochromes P450 require two electrons from ferredoxin as a common electron donor to exhibit their enzymatic activities. Tsubaki et al. (19) identified a putative ferredoxin-binding region of cytochrome P450$_{scc}$ on chemical modification with pyridoxal 5′-phosphate. The region corresponding to this putative ferredoxin-binding region among mitochondrial cytochromes P450 {cytochromes P450$_{scc}$ *(CYP11A1)*, P450$_{11\beta}$ *(CYP11B1)*, P450$_{ald}$ *(CYP11B2)*, P450$_{24}$ *(CYP24)*, and P450$_{27}$ *(CYP27)*} exhibited great similarity. In particular, the pattern of three adjacent basic amino acid residues (Lys at 338, Lys at 341, and Arg at 345; positions according to the amino acid sequence of sheep mature

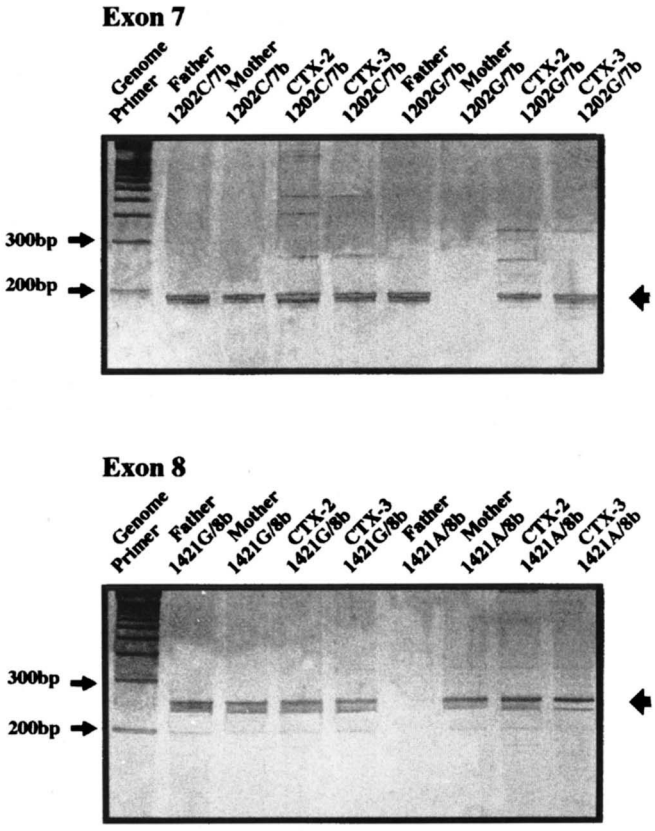


**Fig. 4.** Analysis of the allele-specific PCR products from family members with CTX. PCR products were electrophoresed using PAGEL (polyacrylamide gel, Atto Co.). Standard size markers are indicated at the left with their lengths. The origins of the template genomic DNA and primers used for the allele-specific PCR are indicated at the tops of the lanes. The arrows at the right indicate the products of the allele-specific PCR.

cytochrome $P450_{scc}$) was completely conserved, except that in some *CYP11B1* and *CYP11B2* the basic Lys residue was substituted for Arg at 338 (data not shown). In 1991, Coghlan and Vickery (26) clarified that there was a highly conserved acidic region (positions 68–86) in human ferredoxin, Asp-76 and Asp-79 in this region playing a critical roles in electron transfer partner recognition and complex formation. Furthermore, they presented schematic models for the interaction of ferredoxin with NADPH-ferredoxin reductase and cytochrome $P450_{scc}$. In these models, Asp-76 and Asp-79 interact with complementarily charged residues in NADPH-ferredoxin reductase and cytochrome $P450_{scc}$. Therefore, we believe that the patterns of three adjacent basic amino acid residues described above are very important for the binding action of ferredoxin and subsequent electron transfer. The novel mutation we identified in exon 7 produced the additional polar residue, Arg, just downstream of the putative ferredoxin-binding region. Thus, we consider the first model is less likely than the second model. In order to obtain definitive proof that the novel mutation described here is responsible for the CTX phenotype, in vitro expression studies are currently underway in our laboratory.

CTX is inherited in an autosomal recessive manner (27), and almost all the CTX patients reported so far harbored mutations in the *CYP27* gene homozygously. This is partly due to parental consanguinity. In 1991, Kuriyama et al. (6) reported that in 95 families, the parents of patients with CTX, who themselves had no clinical manifestations of CTX, were consanguineous in 41 families and non-consanguineous in 18 families (a family history was not mentioned for 36 families). In our case, the parents, who themselves also have no clinical manifestations of CTX, are not consanguineous and the sisters have the mutations heterozygously; all the same, the sisters have typical symptoms of CTX. Namely, a mutation in exon 7 in the sisters was derived from the father and the other one in exon 8 was derived from the mother (see Fig. 4). Consequently, both the alleles in the sisters were disrupted by these mutations, respectively. This is a very rare case and a first report that presented distinct genealogy of different mutations causing CTX.

It is of interest that the complete absence of CTX symptoms in subjects carrying the heterozygotic mutation with moderate hypercholestanolemia suggests that increased plasma cholestanol alone does not induce CTX (22). In 1982, Beppu et al. (28) found that the plasma cholestanol concentrations in ten CTX patients

TABLE 2. Genotypes and the levels of plasma cholestanol in the members of the CTX family

| Subject | Genotype | Sex | Mutation | Cholestanol |
|---|---|---|---|---|
| | | | | μg/ml |
| Father | C/g (1202); G/G (1421) | | $Pro^{368}$ to Arg | 3.5 |
| Mother | C/C (1202); G/a (1421) | | $Arg^{441}$ to Gln | 3.1 |
| CTX 1 | ND | F | ND | 31.2 |
| CTX 2 | C/g (1202); G/a (1421) | M | $Pro^{368}$ to Arg; $Arg^{441}$ to Gln | 30.8 |
| CTX 3 | C/g (1202); G/a (1421) | M | $Pro^{368}$ to Arg; $Arg^{441}$ to Gln | 32.3 |
| Control | | | | 2.4 ± 0.73 |

The lowercase letters (g and a) indicate mutations; ND, not done.

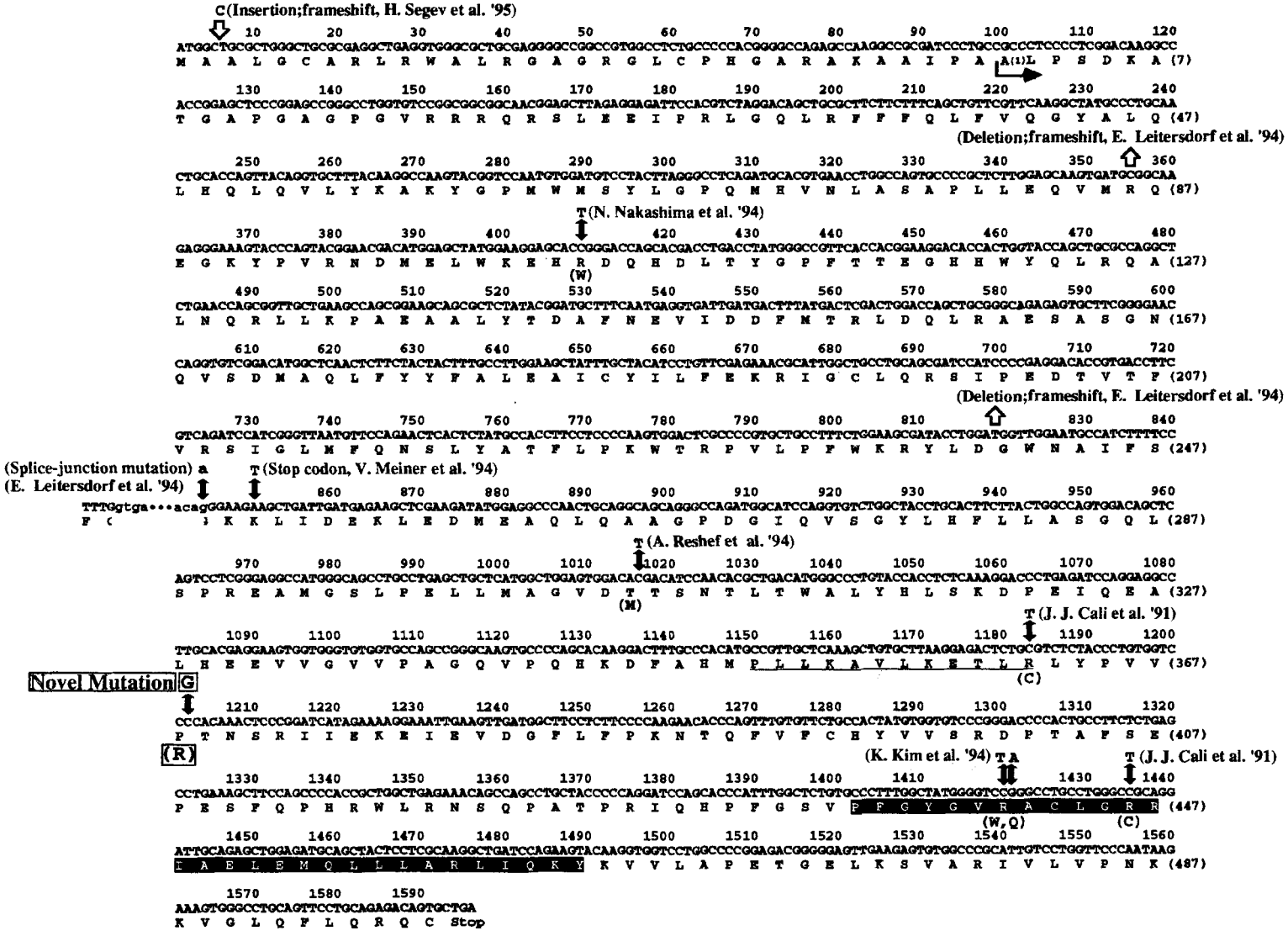


**Fig. 5.** The mutations in the *CYP27* gene causing CTX. The nucleotide sequence of the *CYP27* gene and the corresponding amino acid sequences are shown. Amino acid residues are numbered in the parentheses, with position 1 being assigned to the first amino acid residue (Ala) of the mature form of *CYP27*. Upper-case letters indicate exon sequences and lower-case letters indicate intron sequences. Underlining and shaded regions indicate the putative ferredoxin-binding region and the heme-binding region, respectively. Non-synonymous substitutions are indicated by double-headed arrows and resultant amino acid residues are indicated in the parentheses under those codons.

ranged from 6.4 μg/ml to 32.3 μg/ml, and the severity of the CTX symptoms was unrelated to the plasma cholestanol concentration. The same phenomenon was observed in our cases. Although the levels of plasma cholestanol in three CTX patients, including the sisters, were about 31 μg/ml, which is within the range described above, the symptoms of CTX-1 were much weaker than those of the sisters (CTX-2 and -3) (see Patients in Materials and Methods and Table 2). We have not yet investigated the genotype of CTX-1, however, from the present situation, we suppose that the genotype of CTX-1 is the same as that of the sisters. The clinical features of CTX are thus quite variable and they may depend considerably on the circumstances of the individual. In view of the fact that early treatment with bile acids could retard or even reverse the course of the disease (29) and that CTX could be effectively treated with chenodeoxycholic acid (30), it is important to diagnose it at an early presymptomatic stage. Our finding of a novel mutation in a Japanese family may be useful for future presymptomatic diagnosis of CTX and for genetic counseling in specific families. For these purposes, we need more knowledge about mutation in the *CYP27* gene.

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