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Genetic analysis of a Japanese cerebrotendinous xanthomatosis family: identification of a novel mutation in the adrenodoxin binding region of the *CYP* 27 gene

Wengen Chen [a], Shunichiro Kubota [a], Yukiko Nishimura [a], Shuichi Nozaki [b], Shizuya Yamashita [b], Tsutomu Nakagawa [b], Kaoru Kameda-Takemura [b], Masakazu Menju [b], Yuji Matsuzawa [b], Ingemar Björkhem [c], Gösta Eggertsen [c], Yousuke Seyama [a,*]

[a] *Department of Physiological Chemistry and Nutrition, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*
[b] *Second Department of Internal Medicine, Osaka University School of Medicine, Osaka, Japan*
[c] *Division of Clinical Chemistry, Karolinska Institutet, Huddinge University Hospital, Huddinge, Sweden*

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Abstract

Cerebrotendinous xanthomatosis (CTX), an autosomal recessive lipid-storage hereditary disorder, is caused by mutations in the sterol 27-hydroxylase gene (*CYP* 27). A 24-year-old female Japanese CTX patient and her parents were studied for a *CYP* 27 mutation. Multiple xanthomas were the main complaint of the patient and plasma cholestanol level was markedly elevated. Sterol analysis of a xanthoma biopsy confirmed cholesterol and cholestanol deposition, and the cholestanol accounted for 8.1% of the total sterols. Sterol 27-hydroxylase activity in fibroblasts derived from the patient was undetectable, while the activities in fibroblasts from her mother and father were 54% and 41% of the normal level, respectively. Direct sequence analysis showed a missense mutation of A for G substitution in the *CYP* 27 gene at codon 362 (CGT ^{362}Arg to CAT ^{362}His) with a homozygous pattern in the patient, and a heterozygous pattern in the parents. The mutation, which eliminates a normal *Hga*I endonuclease site at position 1195 of the cDNA and is located at the adrenodoxin binding region of the gene, is most probably responsible for the decreased sterol 27-hydroxylase activity in this Japanese CTX family. The combined data strongly support that the primary enzymatic defect in CTX is the disruption of sterol 27-hydroxylase and that the disease is inherited in an autosomal recessive trait.

Keywords: Cerebrotendinous xanthomatosis (CTX); Cholestanol; Serum 27-hydroxycholesterol; Sterol 27-hydroxylase; Point mutation

1. Introduction

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive sterol storage disease clinically characterized by xanthomas, progressive neurological dysfunctions, cataracts [1], osteoporosis [2,3] and premature atherosclerosis [4]. The underlying biochemical defect of CTX is a lack of sterol 27-hydroxylase [5–7]. The enzyme is of importance for bile acid biosynthesis [1], and the possibility has also been discussed that the product of the enzyme, 27-hydroxycholesterol, is involved in the regulation of cholesterol homeostasis [8]. The metabolic defect in the bile acid synthesis leads to reduced levels of bile acids, especially, chenodeoxycholic acid level. In addition, CTX patients excrete large amounts of C_{27}-bile alcohols in bile, feces, and urine [9], and there is an accumulation of cholestanol in different tissues [10–12]. The defect of the sterol 27-hydroxylase in extrahepatic organs may be partly related to the premature atherosclerosis in CTX patients, as the enzyme seems to be involved in a defense mechanism for macrophages exposed to excess cholesterol [13].

Following the cloning of human sterol 27-hydroxylase gene by Cali et al. [14] and determination of its structure by Leitersdorf et al. [15], several mutations of this gene have been identified in clinically diagnosed CTX patients, including single base substitution [15–20], deletion [15,21]

Abbreviations: CTX, cerebrotendinous xanthomatosis; RT-PCR, reverse transcription polymerase chain reaction; CPS, count per second; HPLC, high performance liquid chromatography

* Corresponding author. Fax: 81-3-5689-2704. e-mail: yousuke@m.u-tokyo.ac.jp

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and insertion [22]. However, most of these mutations were identified in sporadic cases or incompletely characterized CTX family members.

A relatively high prevalence of this disease has been noticed in some distinct populations like Jews of Moroccan origin, Dutch and especially Japanese [1,23]. The latter account for nearly one third of all the cases reported till now [24]. Treatment with chenodeoxycholic acid can reduce plasma cholestanol level and may prevent the disease progression [25] or even reverse some of the neurological disabilities [26]. If untreated, CTX may develop serious neurological defects insidiously and may even lead to death [27]. In view of this, it is important to search for new mutations causing CTX and to set up appropriate genetic analysis methods to screen and diagnose presymptomatic CTX patients or heterozygotes in distinct populations at risk.

In the present study, we report a novel point mutation at the adrenodoxin binding region of the *CYP* 27 gene in a Japanese CTX family. The mutation eliminates a normal *Hga*I restriction endonuclease site at position 1195 of the cDNA which allows us to establish a simple method to screen and diagnose this type of mutation in populations.

2. Materials and methods

2.1. Subjects

A clinically diagnosed 24-year-old female CTX patient and her parents were studied. The patient complained of gradually enlarging bilateral Achilles tendons (left: 25 mm, right: 24 mm, normal: 5–9 mm) as well as similar subcutaneous swellings over her triceps, knees and dextral 2nd finger joint when we examined her at the age of 17. Mild mental retardation with an IQ of 67 was noticed. No other remarkable neurological defects and visual impairment could be observed at the time of the diagnosis. Cardiovascular investigations were also normal. Although electroencephalograph examination showed predominant slow α waves, magnetic resonance imaging didn't show any organic change in cerebellum. Biochemical analysis showed a markedly elevated plasma concentration of cholestanol.

No abnormal biochemical findings and CTX manifestations were observed in the parents except that the mother had slightly enlarged Achilles tendons (left: 13 mm, right: 13 mm). Informed consent was obtained from all the subjects and the study was approved by the Second Department of Internal Medicine, Osaka University School of Medicine.

2.2. Biochemical analysis

Cholesterol and cholestanol levels were determined on fasting blood samples by high performance liquid chromatography (HPLC) as described previously [28]. Triglyceride [29] and HDL-cholesterol [30] were measured by enzymatic methods. Protein concentration was determined according to the Bradford's method [31] using a kit from Japan Bio Rad (Tokyo, Japan).

2.3. Cell culture

Fibroblasts derived from the patient, her parents and 4 healthy control subjects were grown and maintained as monolayer in culture dishes (10 cm) in Dulbecco's Modified Eagle's Medium (Life Technologies, Tokyo, Japan) supplemented with 10% fetal calf serum (Life Technologies, Tokyo, Japan), penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (1.5 mM) in a 95% air and 5% CO_2 atmosphere at 37°C in a humidified incubator. The cells were harvested with trypsin (200 U/ml), washed three times in phosphate buffered saline, and then frozen at −80°C for a short time to measure the sterol 27-hydroxylase activity.

2.4. Assay of sterol 27-hydroxylase activity

Assay of sterol 27-hydroxylase activity was performed by a procedure described by Skrede et al. [32]. Labeled substrate, 5β-[7β-^{3}H] cholestane- 3α,7α,12α-triol with specific activity 150 cpm/pmol was synthesized as described previously and purified by HPLC [33]. The substrate (480,000 cpm, 3.2 nmol) was dissolved in 10 μl acetone (which was evaporated under a nitrogen stream), and then was solubilized in 250 μl of 5% bovine serum albumin followed by the addition of the following incubation mixture: 33 mM Hepes (pH 7.4); 5 mM ATP; 5 mM potassium malate; 1 mM glucose 6-phosphate; 0.5 IU glucose-6-phosphate dehydrogenase; 1.2 mM NADPH; and 15 mM $MgCl_2$. The fibroblasts from 2 dishes were suspended in 250 μl of 0.25 M sucrose and added to the incubation mixture to start the reaction, giving a final volume of 608 μl. The final protein concentration was 0.53 to 1.13 mg/ml. After incubation at 37°C for 2 h, the reaction was terminated by adding 0.1 ml 1 M HCl. Extraction with 5 ml ethylacetate was performed twice and the converted 27-hydroxylated product was detected by HPLC (LC-10A Shimadzu, Kyoto, Japan) using a LC-18 column (250 × 4.6 mm, Supelco, USA). The radioactivity of the product was measured by a radiodetector (RLC-700, Aloka, Tokyo, Japan). The enzyme activity was expressed as pmol/mg protein per h.

2.5. PCR and RT-PCR amplification

All the 9 exons of sterol 27-hydroxylase gene were amplified. Primers used to amplify exon 1 to exon 9 were listed in Table 1. Genomic DNA (500 ng) extracted from the cultured fibroblasts was used for amplifying each of the exons. The PCR reaction mixtures (100 μl) contained 1 × PCR buffer, 0.2 mM of each dNTP, 0.2 μM upstream

Table 1
Sequence and location of primers used for amplifying the exons of *CYP* 27 gene [a]

Primer	Location	Amplification	Sequence 5′ to 3′	Position
E1up	5′-flanking	exon 1	ACTCAGCACTCGACCCAAAGGTGCA	−42 to −17 [b]
E1d	intron 1	exon 1	CCACTCCCATCCCCAGGACGCGATG	14 [c]
E2up	intron 1	exon 2	TGGCCCAGTTATTCAGTTTTGATTG	10 [c]
E2d	intron 2	exon 2	GGGCCCTGTTCCAGTCCCTTCAGGC	10 [c]
E3up	intron 2	exon 3	GCTTATCTTTGTGCTGTTCCTCTGC	9 [c]
E3d	intron 3	exon 3	GAGCACAACCTCTCCCTGACCCATT	33 [c]
E4up	intron 3	exon 4	TCTGCCTCCTGTGATGGCCTCTGTG	10 [c]
E4d	intron 4	exon 4	GCTGATGCACAGACCTGGAGTCACC	39 [c]
E5up	intron 4	exon 5	GCTCTTGGTCCTTGGAGATCATGAC	40 [c]
E5d	intron 5	exon 5	ACTGGTTACGGTTGGGAGCTGGGGG	30 [c]
E6up	intron 5	exon 6	TTCCTAGAATCGCCTCACCTGATCT	17 [c]
E6d	intron 6	exon 6	TTCCCTCCCCACAAAGAGATCCTGT	27 [c]
E7up	intron 6	exon 7	GCAGACTCCAGACATTCTTTTCCCT	4 [c]
E7d	exon 8	exon 7–8	TGGAAGCTTTCAGGCTCAGAGAAG	1355–1332 [b]
E8up	exon 8	exon 8	CCTTCTCTGAGCCTGAAAGCTTCC	1331–1354 [b]
E8d	intron 8	exon 8	GTGGATTGTGTGTTTGCCATCCACT	28 [c]
E9up	intron 8	exon 9	AGTGGATGGCAAACACACAATCCAC	28 [c]
E9d	3′-untranslated	exon 9	CCCAGCAAGGCGGAGACTCA	1620–1639 [b]

[a] Those for amplifying exon 1 to exon 5 were the same as in Ref. [15].
[b] The A of the ATG initiation codon is number 1.
[c] Minimal distance from exon.

and downstream primers listed in Tables 1 and 2. 5 U Taq DNA polymerase. The PCR amplification reaction was performed for 30 cycles in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) using the following conditions: 1 min at 95°C for denaturation and 4 min at 68°C for annealing and extension. After DNA amplification, 8 μl of the products were electrophoresed on a 2% agarose gel and subjected to ethidium bromide staining to confirm the successful amplification. For RT-PCR, upstream primer (P359) 5′TTCGTCAGATCCATCGGGTT3′ (nucleotide 739–758) and downstream primer (dP367) 5′GCAAGGAGTTCCTCCCACCTCTCG 3′ (nucleotide 1729–1752) were designed. Total RNA was extracted from fibroblasts by acid guanidine-phenol-chloroform [34]. Total RNA (1 μg) was first converted to cDNA in a 20 μl reaction mixture containing 5 mM $MgCl_2$, 1 × PCR buffer II, 1 mM of each dNTP, 1 U RNase inhibitor, 1 μM downstream primer (dP367) and 2.5 U reverse transcriptase. The reaction tube was incubated at 42°C for 30 min (annealing and extension), heated at 95°C for 5 min (inactivation of reverse transcriptase and denaturation of RNA-cDNA hybrids) 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 1.25 mM $MgCl_2$, 1 × PCR buffer II, 0.25 μM upstream primer (P359), and 2.5 U Taq DNA polymerase. The amplification reaction was performed for 30 cycles using the following conditions: 1 min at 95°C for denaturation and 4 min at 68°C for annealing and extension. The 1014 bp RT-PCR product was used for *Hga* I restriction enzyme analysis.

Table 2
Plasma lipid concentrations (mg/dl) in the patient and her parents

	Patient	Mother	Father
Triglyceride	155	174	145
HDL-cholesterol	62	60	ND
Cholesterol [a]	207	216	218
Cholestanol [b]	4.06	0.17	0.11

[a] Normal level: 175 ± 26 mg/dl (mean ± S.D., $n = 17$).
[b] Normal level: 0.27 ± 0.08 mg/dl (mean ± S.D., $n = 17$).
ND, not determined.

2.6. Direct sequence analysis

The non-RI-SSCP method [35] was first tried to screen for mutations of the *CYP* 27 gene. The method, which was successful in identifying the 2 point mutations at codon 441 of *CYP* 27 (data not shown), failed in offering any information in this study: all the exons amplified from the patient, her parents and a normal subject migrated in the same patterns, although several conditions of electrophoresis were tried. It may be possible to detect point mutation by PCR-SSCP, but the sensitivity of the method is also dependent on several factors [36]. To this end, all the exons of the *CYP* 27 gene from the patient were sequenced, since the sterol 27-hydroxylase activity assay strongly indicated that there should exist a mutation in the *CYP* 27 gene in this family.

In order to generate single-strand DNA for direct sequence analysis, 5′ terminus of primers were phosphorylated prior to the PCR reaction by the kination reaction as follows: the reaction mixture (50 μl) containing 200 pmol primer, 20 U T4 polynucleotide kinase (Life Technologies, Tokyo, Japan), 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. The PCR products amplified from the phosphorylated primers were first purified using SUPREC™ column (TaKaRa, Kyoto, Japan) and then

digested with 2 U λ exonuclease (Life Technologies, Tokyo, Japan) to produce single-strand DNA for direct sequence analysis [37]. Sequence was conducted by the dideoxynucleotide chain termination method using T7 DNA polymerase (Sequenase, US Biochemicals, Cleveland, OH, USA). All the exons including the splice junctions were sequenced using the primers listed in Table 1.

2.7. Restriction endonuclease analysis with HgaI

The mutation detected by sequence analysis abolished a cleavage site for restriction enzyme *Hga*I at cDNA position 1195. 16 μl of amplified RT-PCR product was digested with 2 U of *Hga*I (New England Biolabs, Beverly, MA, USA) for 2 h at 37°C. The digested products were electrophoresed on a 2% agarose gel and the fragments were confirmed by ethidium bromide staining of the gel.

3. Results

3.1. Biochemical analysis

The patient had a slightly elevated plasma cholesterol concentration of 207 mg/dl but a markedly elevated cholestanol level of 4.06 mg/dl. Total triglyceride and

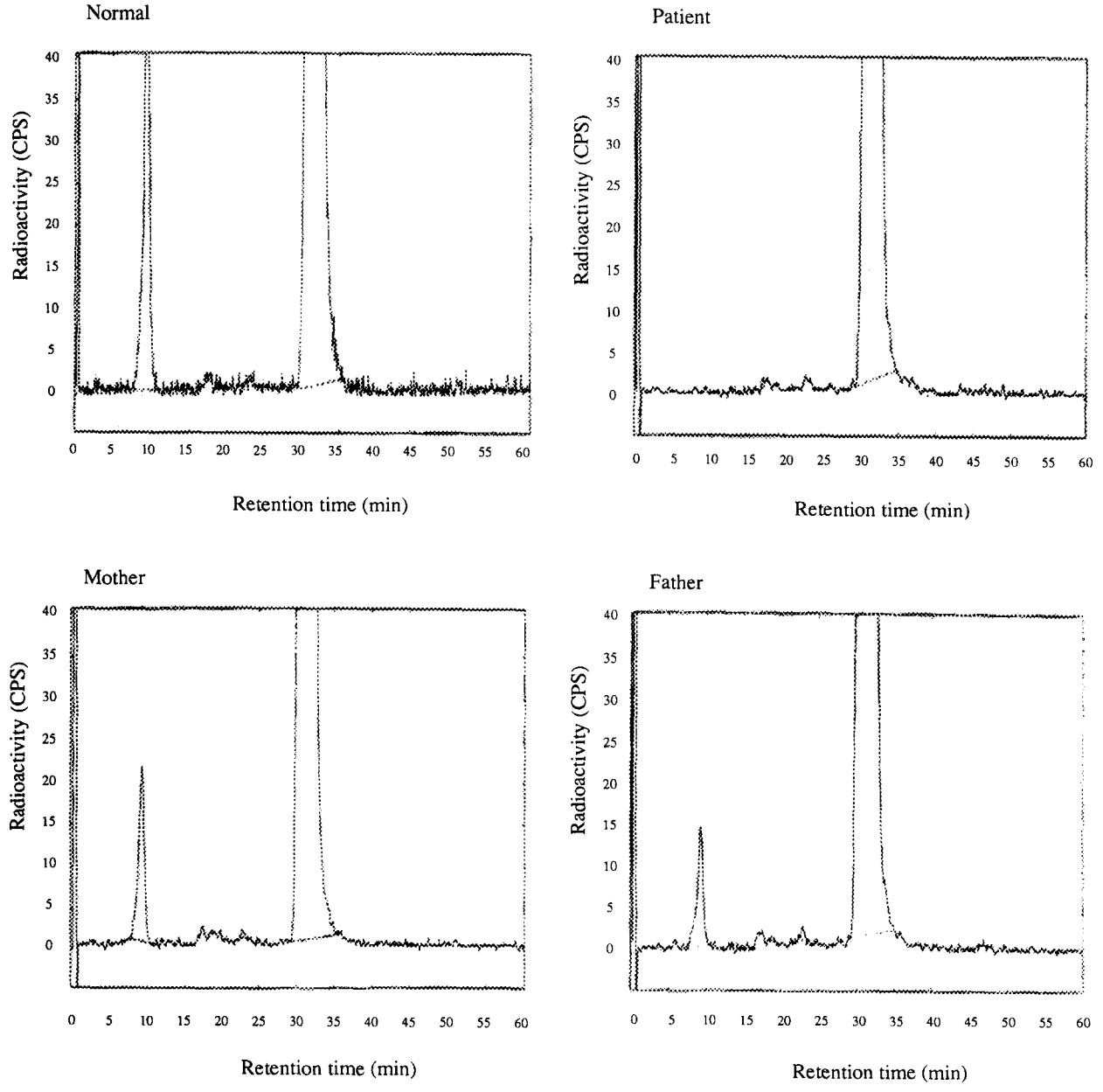

Fig. 1. Detection of radioactive product and substrate. The product was separated from the substrate by HPLC after incubation of 5β-[7β-^{3}H] cholestane-3α,7α,12α-triol with fibroblasts derived from a normal subject (A), the patient (B), mother (C) and father (D). Peaks at 9 and 34 min of retention time showed 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.

HDL-cholesterol were within normal range (Table 2). Sterol assay on a biopsied tuberous xanthoma showed that the amount of cholesterol, cholestanol and sitosterol was 192 mg %, 17 mg % and 0.2 mg %, respectively. Cholestanol thus accounted for 8.1% of the total sterols measured in the xanthoma. Both the mother and father had normal plasma concentration of cholestanol.

3.2. Sterol 27-hydroxylase activity in fibroblasts

Sterol 27-hydroxylase activity was assayed in fibroblasts derived from the patient, her parents and 4 normal subjects. Fibroblasts from the normal subject significantly converted the substrate 5β-[7β-^{3}H] cholestane-3α,7α,12α-triol into 5β-[7β-^{3}H] cholestane-3α,7α,12α,27-tetrol. The 27-hydroxylated product was efficiently separated from the substrate by HPLC and the radioactivity was monitored by a radiodetector (Fig. 1). The mean enzyme activity in normal fibroblasts was 110 20 pmol/mg protein per h (mean ± SD, $n = 4$). Fibroblasts from the patient showed undetectable enzyme activity while corresponding cells from her mother and father had 54% (60 pmol/mg protein per h) and 41% (45 pmol/mg protein per h) of the normal level, respectively (Table 3). The results indicated presence of a homozygous mutation in *CYP* 27 gene in the patient and a heterozygous mutation in the parents. Therefore, direct sequence analysis was conducted.

Table 3
Fibroblast sterol 27-hydroxylase activity in the patient and her parents

	Enzyme activity (pmol/mg per h)	Percentage of normal (%)
Patient	UD	–
Mother	60	54
Father	45	41
Normal ($n = 4$)	110 ± 20	100

UD, undetectable.

3.3. Sequence analysis of genomic DNA

By direct sequence of all the exons and splice junctions of the *CYP* 27 gene, a single base substitution of A for G at codon 362 (CGT ^{362}Arg to CAT ^{362}His) was identified (Fig. 2): homozygous pattern in the patient and heterozygous pattern in her parents. The mutation was confirmed on both strands of duplicately amplified PCR products (exon 6). No other mutation in all exons and splice junctions was found in the *CYP* 27 gene of the patient (data not shown).

3.4. Restriction endonuclease analysis

The mutation eliminated a normal *Hga*I site at position 1195 of cDNA. In normal subjects, digestion of the 1014 bp RT-PCR product amplified using the primer P359 and dP367, with *Hga*I generated 2 fragments of 557 bp and

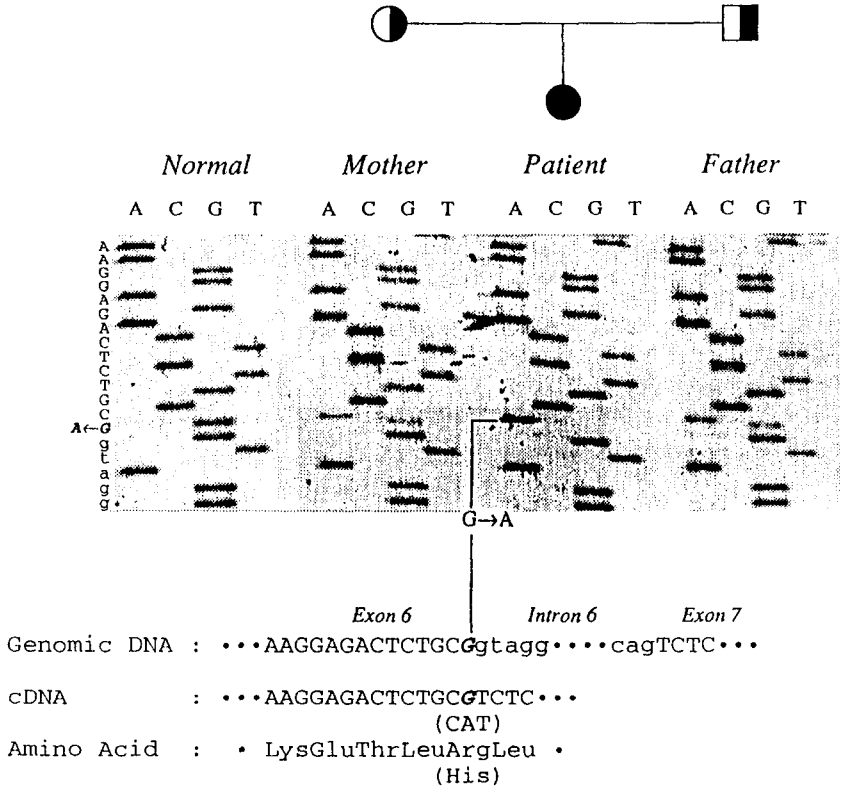

Fig. 2. Direct sequence analysis of sterol 27-hydroxylase gene in the CTX family and a normal subject. All the exons of the gene were PCR-amplified using genomic DNA and sequenced. The normal sequences of the region across the mutation and their corresponding amino acids were shown. The A for G substitution at nucleotide 1205 was indicated by an arrow.

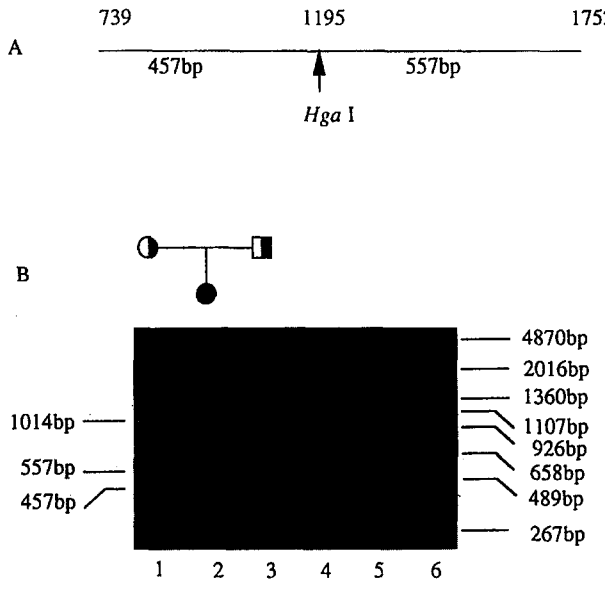

Fig. 3. Identification of the mutation by *Hga*I endonuclease digestion. (A) Part of the normal restriction map of enzyme *Hga*I in the cDNA. (B) *Hga*I restriction analysis of RT-PCR products from the patient, parents and a control subject. Lane 1, mother; lane 2, patient; lane 3, father; lane 4, normal subject; lane 5, normal sample without *Hga*I digestion; lane 6, standard size marker (*pHY*).

457 bp (Fig. 3). On the contrary, digestion of the RT-PCR product from the patient did not result in any change of the fragment length. The parents showed both the original band (1014 bp) and the two new bands (557 and 457 bp). The results indicated that the patient is homozygous for the mutation and that her parents are heterozygous.

4. Discussion

By PCR direct sequence analysis a homozygous missense mutation of A for G substitution at codon 362 (CGT ^{362}Arg to CAT ^{362}His) was identified in a Japanese CTX patient, and a corresponding heterozygous mutation in her parents (Fig. 2). The results support that CTX is inherited in an autosomal recessive pattern. Interestingly, the single base pair substitution occurred only one base pair away from the mutation (CGT ^{362}Arg to TGT ^{362}Cys) reported by Cali et al. [16]. Both the mutations disrupt the normal Arg at codon 362 but result in different mutant amino acids. The normal ^{362}Arg is highly conserved among mitochondrial cytochrome *P*-450s and serves as a binding region for the adrenodoxin cofactor of the sterol 27-hydroxylase [38]. No other nucleotide change was found in any of the exons of the *CYP* 27 gene, including splice junctions. Therefore, although we have not conducted expression study of the mutant *CYP* 27 cDNA into COS cells, the mutation is most probably responsible for the decreased sterol 27-hydroxylase activity in this family. CTX is inherited in an autosomal recessive pattern and the disease is often seen in consanguinity family. However, the parents of our case were not consanguineous. A genetic screening using the *Hga*I restriction analysis described here would be useful for testing relatives or other populations at risk.

Bilateral xanthoma of the Achilles tendon was the most important clinical finding in CTX patients and occurred in 95% of the 144 patients reviewed by Kuriyama et al. [24]. Most tendon xanthomas seemed to develop in the third or fourth decade but may occur as early as at the age of 15 [1,24]. The main complaint of the patient in the present study was the multiple enlarging xanthomas, which were large enough to be noticed by the patient at the age of 15. Sterol analysis on a biopsied tuberous xanthoma showed cholesterol and cholestanol deposition and cholestanol accounted for 8.1% of the total sterols. The result was consistent with the 7.3% reported by Salen et al. [12]. Other manifestations of CTX such as cataracts, and neurological dysfunctions were not observed at the time of diagnosis except for mild mental retardation with an IQ of 67. The development of the symptoms in CTX patients is extremely variable. Some of the patients are already mentally retarded in the early period of life, while others have normal intelligence even in the sixth decade of life [1]. It is intriguing to notice that her mother who is a heterozygote also has enlarged Achilles tendons (left 13 mm, right 13 mm). As the grandfather who is healthy with normal plasma cholesterol and cholestanol levels also has slightly enlarged Achilles tendons (left 11 mm, right 11 mm), the small xanthomas in the mother may have been caused by other genetic and/or environmental factors in her family rather than by her heterozygous mutation in the *CYP* 27 gene. In addition, the father who is also a heterozygote for the same mutation has normal Achilles tendon thickness (left 9 mm, right 7 mm).

The fibroblast sterol 27-hydroxylase activities in the parents were about half of the normal level as can be assumed in heterozygous state. The plasma cholestanol levels in heterozygous parents were normal. According to the results, a 50% activity reduction of the enzyme had no significant effect on plasma cholestanol level. Leitersdorf et al. reported that the age-adjusted mean plasma cholestanol concentration in 28 heterozygotes with cytosine deletion at nucleotide 376 of the cDNA was identical to that found in non-carrier members of their families [21]. Although mild hypercholestanolemia was reported in the asymptomatic parents of 5 CTX patients [28] and in 2 heterozygous individuals with C to T substitution at nucleotide 430 of the cDNA [19], cholestanol measurement seemed less significant for diagnosing heterozygote. Other genetic and/or environmental factors may play a role in the regulation of cholestanol levels, as the plasma concentrations of cholestanol varied remarkably among CTX patients [39] and heterozygotes [21]. Two non-carriers in a CTX family were reported to have moderately elevated cholestanol levels [21]. The severity of CTX symptoms was unrelated to plasma concentration of cholestanol [39].

Although useful in CTX diagnosis, plasma cholestanol concentrations should thus be evaluated cautiously unless they are markedly elevated. On the other hand, sterol 27-hydroxylase assay may be adopted for diagnosing CTX, in particular, for heterozygote diagnosis, as our data showed that the heterozygotes had about half of the normal activity and that there was no overlap between the levels of heterozygote and normal control. Since skin biopsy is not an easily acceptable method and fibroblast culture is time-consuming, measuring the enzyme activity in the mitochondrial fraction of peripheral leukocytes may be a suitable alternative (unpublished data). Serum 27-hydroxycholesterol measurement may also be considered for CTX diagnosis as it is the direct product of sterol 27-hydroxylase. This was partly supported by our data of the serum 27-hydroxycholesterol concentrations in a homozygous CTX patient and the heterozygous daughter from another CTX family (14 ng/ml and 80 ng/ml, respectively, unpublished data). The heterozygote had about half of the serum 27-hydroxycholesterol concentration of normal subjects as reported by Dzeletovic et al. using the same method (mean $\pm$ SD: 154 ± 43 ng/ml, $n = 31$) [40]. More data are needed, however, before reference ranges of serum 27-hydroxycholesterol concentration in heterozygotes and homozygotes can be defined.

In conclusion, a novel missense mutation of A for G substitution at codon ^{362}Arg (CGT ^{362}Arg to CAT ^{362}His) was detected in a Japanese CTX family. The patient had undetectable level of sterol 27-hydroxylase activity. On the contrary, the heterozygous parents had about 50% of normal activity level. The results strongly support that the primary enzymatic defect in CTX is located at the sterol 27-hydroxylase and that the disease is inherited in an autosomal recessive pattern.

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