

**Table 2**

The expression of markers for skeletal muscle contractility, skeletal myogenesis, and skeletal muscle autocrine signaling in the myocytes differentiated from CPT II-deficient iPSCs. Gene expression was evaluated using quantitative real time RT-PCR as described in Materials and Methods; GAPDH was the internal control. Results are shown as fold change relative to control samples of undifferentiated CPT II-deficient iPSCs.

	Symbol	Fold change
Skeletal muscle contractility	ATP2A1	4.6
	CAV3	22.0
	DES	33.5
	DMPK	2.1
	DYSF	2.2
	LMNA	3.9
	MB	8.3
	MYH1	86.5
	MYOT	4.0
	NEB	4.3
	SGCA	4.1
	TNNC1	4.1
	TNNI2	8.4
	TNNT1	2.2
	TNNT3	35.7
	TTN	6.4
	Skeletal myogenesis	ACTA1
CAPN2		3.1
CAV1		3.8
IGF1		5.7
IGFBP3		5.9
IGFBP5		15.1
MEF2C		43.3
MSTN		74.8
MUSK		11.0
MYOG		477.3
PAX3		2.7
Skeletal muscle autocrine signaling	IGF1	5.7
	IGF2	59.7
	IL6	3.2

Many somatic cell types have been generated from iPSCs, but there have been limited reports describing directed differentiation into myocyte lineages [13]. Our protocol was used to generate CPT II-deficient myocytes from CPTIID-iPSCs [13]. These processes were validated by the detection of genes involved in skeletal muscle

development and function in CPT II-deficient myocytes. Culture in low glucose caused the death of undifferentiated iPSCs, which require large quantities of glucose, while the differentiated cells require limited glucose and produce lactic and pyruvic acids to more effectively obtain energy by mitochondrial oxidative phosphorylation. Thus, by using low-glucose medium, we increased the differentiation efficiency of iPSC-derived myocytes.

Yamaguchi et al. showed that an *in vitro* AC profiling assay could reliably detect various FAO disorders, consistent with reports from other groups [16–18]. In particular, C16 accumulation was found to be a reliable biomarker that could be used to diagnose CPT II deficiency. Consistent with these findings, our hiPSC-derived myocytes mimicked the metabolic characteristics of the disease.

The effects of PPAR agonists on mitochondrial FAO have been extensively examined [19]. Bezafibrate has proven efficacy in the treatment of long-chain FAO disorder [20–24]. We found that bezafibrate reduced long-chain ACs more effectively in myocytes from patient-derived iPSCs than in those from control hiPSCs. These findings indicate that bezafibrate could be a therapeutic drug for CPT II deficiency and other mitochondrial FAO disorders.

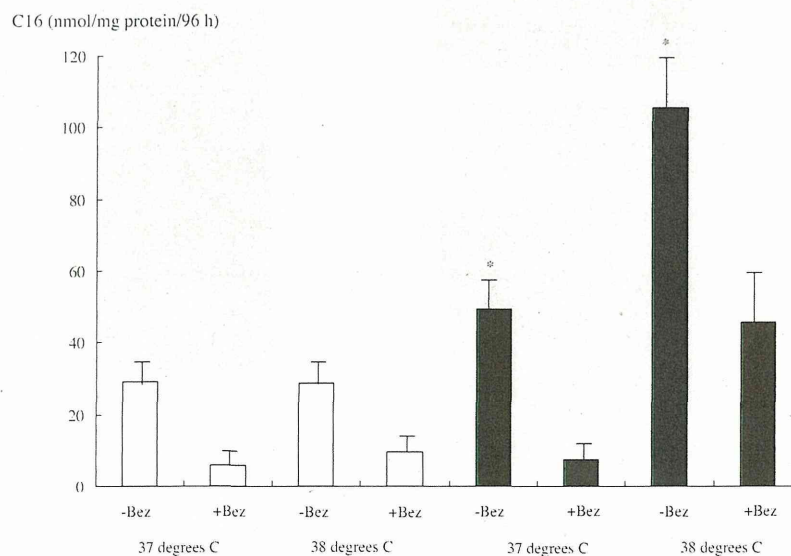
In conclusion, we successfully derived disease-specific iPSCs from a patient with CPT II deficiency and differentiated them into myocytes. Our results suggest that cellular models using patient-derived iPSCs will be of significant benefit for research groups studying CPT II deficiency-related diseases, and that these iPSC disease models may be a valuable resource for testing novel therapeutic strategies for these disorders.

#### Conflict of interest

The authors declare that they have no conflict of interest.

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**Fig. 4.** Acylcarnitine (AC) profiles in culture medium of iPSC-derived myocytes loaded with palmitic acid after bezafibrate (Bez) treatment. Data are shown as mean  $\pm$  SD (nmol/mg protein/96 h) ( $n = 3$ ) White bars: Myocytes from control iPSCs; Black bars: Myocytes from CPT II-deficient iPSCs. Statistically significant differences between 37 °C and 38 °C of control and 37 °C or 38 °C Bez (-) of patients shown as \* $p < 0.05$ .

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## Original article

## Carnitine–acylcarnitine translocase deficiency: Two neonatal cases with common splicing mutation and *in vitro* bezafibrate response

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### Abstract

**Background:** Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of acute encephalopathy- or myopathy-like illness. Carnitine–acylcarnitine translocase (CACT) deficiency is a rare FAO disorder, which represent an energy production insufficiency during prolonged fasting, febrile illness, or increased muscular activity. CACT deficiency is caused by mutations of the *SLC25A20* gene. Most patients developed severe metabolic decompensation in the neonatal period and died in infancy despite aggressive treatment.

**Patients and methods:** We herein report the clinical findings of two unrelated cases of CACT deficiency with mutation confirmation, and *in vitro* bezafibrate responses using *in vitro* probe acylcarnitine (IVP) assay. Patients 1 and 2 are products of nonconsanguineous parents. Both patients developed cardiac arrest at day 3 of life but survived the initial events. Their blood chemistry revealed hypoglycemia and metabolic acidosis. The acylcarnitine profiles in both patients demonstrated increased long-chain acylcarnitines, suggesting CACT or carnitine palmitoyltransferase-2 (CPT2) deficiency.

**Results:** The mutation analysis identified homozygous IVS2-10T>G in the *SLC25A20* gene in both patients, confirming the diagnosis of CACT deficiency. The IVP assay revealed increased C16, C16:1, but decreased C2 with improvement by bezafibrate in the cultured fibroblasts. The short-term clinical trial of bezafibrate in Patient 1 did not show clinical improvement, and died after starting the trial for 6 months.

**Conclusion:** This splicing mutation has been identified in other Asian populations indicating a possible founder effect. IVP assay of cultured fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial of more enrolled patients is required for evaluation of this therapy.

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**Keywords:** CACT deficiency; *SLC25A20* mutation; IVP assay; Bezafibrate

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## 1. Introduction

Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of neuromuscular symptoms as well as acute encephalopathy or even sudden death. In particular, the carnitine cycle is important in energy-producing pathway for cardiac and skeletal muscle and for preventing from hypoglycemia especially during prolonged fasting or increased muscular exercise. Carnitine–acylcarnitine translocase (CACT, EC 2.3.1.21) is one of the enzymes in the carnitine cycle, which catalyzes the transfer of the long-chain fatty acylcarnitines across the inner mitochondrial membrane in exchange of free carnitine. CACT deficiency (OMIM 212138) was first described in 1992 [1]. It is an autosomal-recessive disease caused by mutations of the *SLC25A20* gene located in chromosome 3p21.31 [2]. The gene consists of 9 exons and encodes protein comprising 301 amino acids [3]. CACT deficiency is a very rare disorder with so far as approximately 30 patients have been described, and accounted for 10% of patients with FAO disorders in French population [4]. However, it might be a common FAO disorder in some East Asian countries such as Hong Kong with the estimated incidence of 1 in 60,000 live births, and accounted for 33% of patients with FAO disorders [5]. Most patients develop neonatal-onset encephalopathy with nonketotic hypoglycemia, hyperammonemia, and hypothermia, or sudden death from cardiac arrhythmias. Cardiomyopathy and hepatic dysfunction may be the associated complications. CACT deficiency could be detected by elevations of C16 and C18 acylcarnitines, and low free carnitine in acylcarnitine profiles. However, the same profile could be found in neonatal carnitine palmitoyltransferase-2 (CPT2) deficiency. Therefore, confirmation of diagnosis requires CACT enzyme assay or molecular analysis of the *SLC25A20* gene [6]. Treatment includes intravenous glucose for acute decompensation, and avoidance of long fasting with frequent meals. Long-chain fatty acids may be restricted in diet, but medium-chain triglyceride (MCT) oil is supplemented instead. Carnitine therapy is still controversial. Despite aggressive treatment, most patients still died in infancy [7]. However, there have been some patients who received early treatment with good outcomes [8,9]. Novel therapy for FAOD using bezafibrate, which is a hypolipemic drug acting as a peroxisome proliferator-activated receptor (PPAR) agonist has been reported. The clinical trials of bezafibrate showed clinical improvement in adult patients with CPT2 deficiency [10], and a child with glutaric acidemia type 2 (GA2) [11]. *In vitro* probe acylcarnitine (IVP) assay can be used to evaluate FAO disorders [12], and determine the effect of bezafibrate [13]. We herein report the clinical findings of two unrelated cases with neonatal-onset CACT deficiency, and *in vitro* bezafibrate response using the IVP assay.

## 2. Patients and methods

### 2.1. Patients

#### 2.1.1. Case 1

This patient was the first child of possibly consanguineous parents from the southern province of Thailand. He was born at 37 weeks of gestation with birth weight of 2460 g (25th percentile), length 48 cm (3rd percentile), and head circumference 30 cm (<3rd percentile). He developed hypothermia at 10 h of age. Sepsis was suspected, but the patient rapidly responded to rewarming treatment. However, after rooming-in with the mother, he developed hypothermia again. At 60 h after birth, he had cardiac arrest. On physical examination, no abnormalities were found. Serum glucose was 1.2 mmol/L and acetoacetate was 0 mmol/L. Venous blood pH was 7.24 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 471  $\mu$ mol/L (normal, <110  $\mu$ mol/L). There were mildly elevated liver enzymes aspartate aminotransferase (AST) (97 U/L; normal, 0–32) and alanine aminotransferase (ALT) (78 U/L; normal, 0–33). Serum creatine kinase was 4439 U/L (normal, <190). He had a good response to treatment with intravenous glucose administration. Urine organic acids were unremarkable. A dried blood spot acylcarnitine profile by tandem mass spectrometry (MS/MS) showed free carnitine (C0), 5.26  $\mu$ M (10–60); C16-acylcarnitine, 14.14  $\mu$ M (0.6–7); C18-acylcarnitine, 2.71  $\mu$ M (0.15–2.1); C18:1-acylcarnitine, 4.3  $\mu$ M (0.3–3.2); and a (C16 + C18)/C0 ratio, 3.21 (0.007–0.5). The profile was consistent with CPT2 or CACT deficiency. The patient has been treated with a modular medical formula, which has been composed of modified fats (long-chain fatty acid restriction along with supplementation of 83% of fat as medium-chain triglyceride oil), protein, maltodextrins, minerals, and fat-, and water-soluble vitamins. L-Carnitine at a daily dosage of 100–150 mg/kg has been supplemented. Thereafter, he has had several episodes of hypoglycemia, hyperammonemia, and metabolic acidosis following infections. At 8 months of age, he developed cholestasis and hepatomegaly. At 9 months of age, an echocardiogram revealed hypertrophic cardiomyopathy. At the age of 15 months, he had mild developmental delay and generalized hypotonia. He could stand with support, put block in cup, and say one word. Then he had a metabolic crisis, and developed generalized weakness. After he recovered from encephalopathy, neurologic examination revealed normal cranial nerves, muscle weakness (grade 3/5), and decreased muscle tone and deep tendon reflexes (1+) in all extremities. A brain computed tomography scan was normal. Serum creatine kinase was elevated (1419 U/L). A nerve conduction study showed no evidence of demyelination. He had been ventilator-dependent since then. At 2½ years of



age, he had several complications including chronic liver disease, upper gastrointestinal bleeding, and osteoporosis. He died at the age of 2 years and 8 months from upper gastrointestinal bleeding and metabolic decompensation.

### 2.1.2. Case 2

The patient was the first child of nonconsanguineous parents. She was born at 35 weeks of gestation with a birth weight of 2.3 kg (50th percentile), length 44 cm (25th percentile), and head circumference 30 cm (10th percentile). At 2 days after birth, she developed lethargy, poor feeding, and cardiac arrest. Blood glucose was 0.56 mmol/L. She responded to cardiac resuscitation and intravenous glucose infusion. Serum acetoacetate was 0 mmol/L. Venous blood pH was 7.39 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 157  $\mu$ mol/L (normal, <110  $\mu$ mol/L). There were elevated liver enzymes AST (638 U/L; normal, 0–32) and ALT (83 U/L; normal, 0–33). Plasma lactate dehydrogenase (LDH) was 522 U/L (normal, 240–480). An echocardiogram revealed no cardiomyopathy. A dried blood spot acylcarnitine profile by MS/MS analysis showed C0, 13.8  $\mu$ M (10–60); C16-acylcarnitine, 15  $\mu$ M (0.6–7); C18-acylcarnitine, 4.3  $\mu$ M (0.15–2.1); C18:1-acylcarnitine, 5.9  $\mu$ M (0.3–3.2); and a (C16 + C18)/C0 ratio, 1.4 (0.007–0.5). The profile was consistent with either CPT2 or CACT deficiency. The patient had been treated with a high-MCT formula (Portagen<sup>®</sup>, Mead Johnson Nutritionals), and 100 mg/kg/day of L-carnitine. At 1 month of age, she developed anemia from Hb AE Bart's disease – a thalassemia intermedia resulting from the interaction between  $\alpha$ -thalassemia and heterozygous Hb E, which required monthly blood transfusion. At the age of 4 months, she had poor feeding and cardiac arrest. Blood glucose was 0.5 mmol/L. The patient died without any response to resuscitation. An autopsy revealed left ventricular hypertrophy, micro/macrovacuolar steatosis of the liver with focal areas of bridging fibrosis, and abnormal lipid accumulation in skeletal muscles and the proximal renal tubules.

### 2.2. Materials and methods

This study was approved by the Siriraj Institutional Review Board. The written informed consents for the mutation analysis, IVP assay, and bezafibrate trial were obtained from the parents. Genomic DNA was extracted from leukocytes. Mutation analyses of the *CPT2* and *SLC25A20* genes were performed in case 1, and only *SLC25A20* gene in case 2. All coding exons and their flanking intron sequences (up to 20 bases for both sides) of the *CPT2* and *SLC25A20* genes were PCR-amplified and directly sequenced according to the previously described method [14]. The IVP assay was performed using the skin fibroblasts in the absence

and presence of bezafibrate according to the previously described method [11].

## 3. Results

### 3.1. Mutation analysis and IVP assay

Mutation analysis of the *SLC25A20* gene identified homozygous c.199-10T>G (IVS2-10T>G) mutation in both patients, and heterozygous mutation in their parents (Fig. 1). Mutation analysis of the *CPT2* gene revealed no pathogenic mutation in Case 1. The IVP assay profiles revealed increased C16, C16:1 acylcarnitines, and decreased C2 (acetylcarnitine) indicating a typical pattern of CPT2 or CACT deficiency, with substantial reduction of long-chain acylcarnitines by the presence of bezafibrate in the cultured fibroblasts from both patients (Fig. 2). However, C2 acylcarnitine did not increase as expected.

### 3.2. Clinical trial of bezafibrate

We started a clinical trial of bezafibrate in case 1 at age of 2 years and 2 months, after the IVP assay which

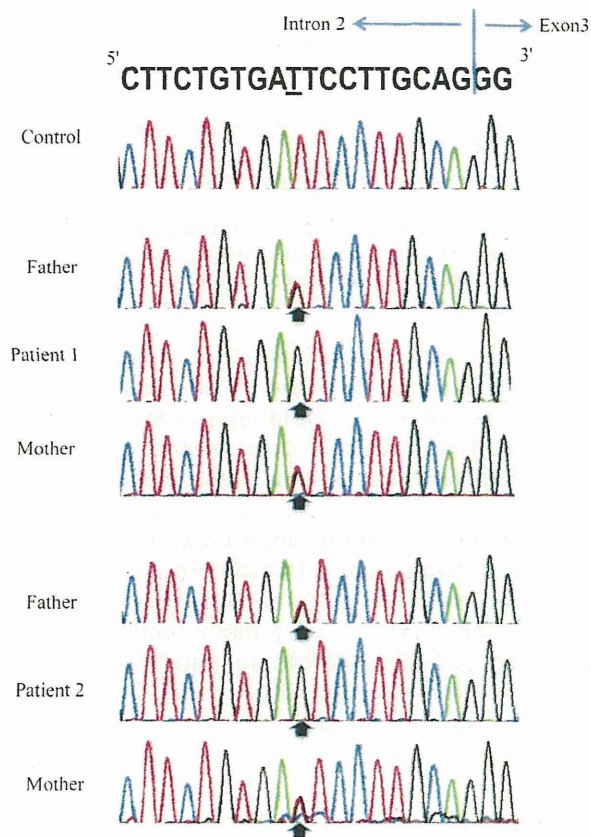


Fig. 1. The reference DNA sequence of an intron 2/exon 3 boundary of the *SLC25A20* gene, and the IVS2-10T>G mutation identified in both patients and their parents denoted by black arrows and the underlined letter.



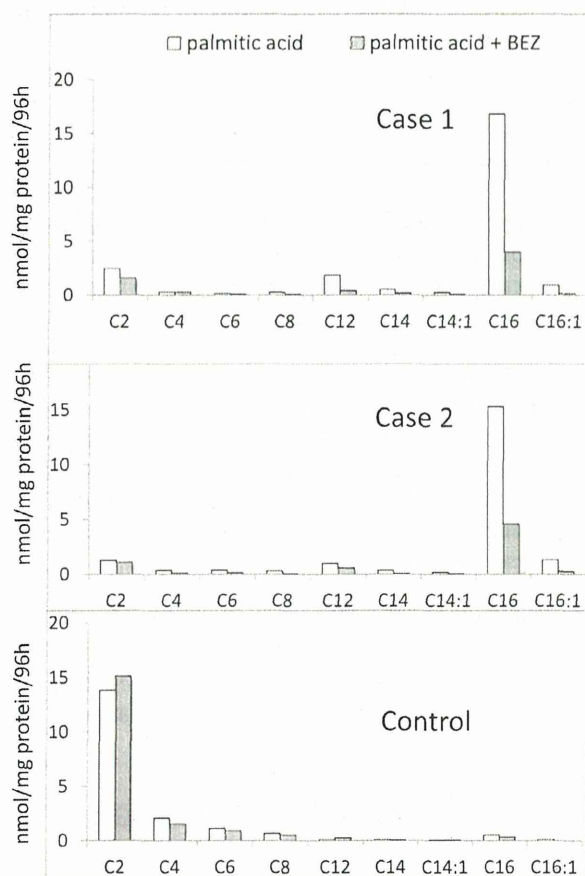


Fig. 2. Acylcarnitine profiles of IVP assay in the presence and absence of bezafibrate (BEZ) of cases 1, 2, and normal control respectively. Unit of vertical lines, nmol/mg protein of acylcarnitines (ACs); the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C12, C14, C14:1, C16, and C16:1. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars.

showed some improvement in acylcarnitine profiles with bezafibrate. We used a dosage of 17–25 mg/kg/day as previously described [11]. Monitoring of liver functions, lactate dehydrogenase (LDH), creatine kinase (CK), and lipid profiles showed no adverse effects of bezafibrate. A short-term evaluation, after 6 months of the trial, did not show clinical improvement except for slightly increased back muscle strength noted by the mother. An echocardiography showed stable but no improvement in a left ventricular mass index. Acylcarnitine profiles in dried blood spots and other biochemical parameters did not show improvement (data not shown). Case 2 died before a clinical trial was considered.

#### 4. Discussion

We report 2 unrelated cases of CACT deficiency with molecular confirmation first identified in Thailand. The c.199-10T>G (IVS2-10T>G) nucleotide change was the most prevalent mutation and identified in 14 out of 76 mutant alleles [15]. This mutation was homozygously

identified in three Vietnamese and three Chinese patients. In the present study, in spite that two families had no consanguineous history, both patients were also a homozygotes of the c.199-10T>G mutation. In Japan, three CACT deficient patients have been described. Among them the same mutation was identified heterozygously in only one patient [14]. We propose that this mutation is a founder mutation in Asian populations. Clinical history of the three Chinese patients with homozygous c.199-10T>G mutation were reported [16]. All of them developed cardiac arrest within two days of age, as well as our two patients. Hence the phenotype of homozygotes of c.199-10T>G mutation is severe. This mutation was suggested to reside at a consensus lariat branch point sequence resulting in skipping of exons 3 and 4 or exon 3 alone, which leads to truncation of the protein [17].

Although our cases 1 and 2 were homozygotes of the same mutation, Case 1 survived until 2 years and 8 months and Case 2 died at 4 months of age. Several factors might attribute to their different clinical outcomes: (1) Thalassemia disease in case 2 which required repeated blood transfusions might affect cardiac functions by chronic hypoxia, iron overload, or decreased carnitine [18]; (2) differences in possible modifier genes such as *SLC25A29* gene (CACT-like, *CACL*) which has palmitoyl-carnitine transporting activity [19]; and (3) different formulas using in our cases, one is a synthetic modular formula and the other is a commercial formula. However, the rationale of both special formulas for diet therapy is a reduction in long-chain fatty acids together with supplementation of medium-chain triglyceride oil to be a caloric source shunting an obstruction of long-chain fatty acid  $\beta$ -oxidation.

Although increased FAO flux induced by bezafibrate was clearly shown in fibroblasts only from patients with mild phenotypes of FAO disorders, increased mRNA expression after bezafibrate exposure also occurred in cell lines from patients with severe phenotypes [20]. This could explain *in vitro* response to bezafibrate observed in fibroblasts of patient 1 and 2. Despite the severe genotype leading to barely detectable enzyme activity [21], we believe that there should be some FAO flux which could be enhanced by bezafibrate in these patients. Our hypothesis is if there is entirely absent FAO flux in these patients, they should have anomalies like those found in a lethal neonatal form of CPT2 deficiency or GA2 [22], even though there has been no report of such findings in CACT deficiency. To our knowledge, patient 1 is the first case of neonatal-onset CACT deficiency who underwent a clinical trial of bezafibrate after showing an *in vitro* response by IVP assay. However, no beneficial short-term effect was shown. This might indicate the irreversible damage of the affected organs esp. the cardiac and skeletal muscles, and liver. Moreover, the difference between the *in vitro* and *in vivo* responses is



probably due to the difference of bezafibrate concentration used in the IVP assay (400  $\mu\text{mol/L}$ ) and typical concentrations obtained in patients on bezafibrate therapy (50–200  $\mu\text{mol/L}$ ) [23]. Another possible reason is inadequate acetyl-CoA production despite bezafibrate treatment. This hypothesis is supported by persistently low C2 acylcarnitines in IVP assays of our cases and a previous case with CACT deficiency [11]. Moreover, C16 acylcarnitine did not decrease to the control level after bezafibrate treatment. Overall, although some improvement of acylcarnitine profile was shown in the patient 1 and 2's fibroblasts in IVP assay with bezafibrate, the effect of bezafibrate was less than those in fibroblasts from patients with mild forms of FAO disorders [11,24]. Hence clinical improvement in this patient was thought to be limited. Since CACT-deficient patients who developed metabolic decompensation in early neonatal period had poor prognosis with routine management [7], we decided to use bezafibrate treatment in patient 1. He survived until two years of age with bezafibrate treatment. However, it is uncertain whether this longer survival owed to the effect of bezafibrate treatment or not, since no apparent improvement of clinical laboratory data was obtained.

In conclusion, CACT deficiency may be a common FAO disorder in East Asian populations probably from a founder effect. IVP assay of fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial and more enrolled patients are required for evaluation of this therapy.

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