Table 3 Mutations reported in this study

	Subject	*Final diagnosis*	*Mutation*	*Location*	*Genetic defect*
1	Case 1	CACT deficiency	c.199-10t>g	Intron 2	Skipping of exon 3 or exons 3 and 4
	Cases 1 and 2		c.576G>A[a]	Exon 6	Premature stop codon W192ter
2	Case 2	CACT deficiency	c.106-2a>t[a]	Intron 1/exon 2 junction	Splicing acceptor mutation
3	Case 3	Non-traumatic rhabdomyolysis (a possible carrier of CACT deficiency)	c.804delG	Exon 8	Frameshift from codon 269
4	Case 4	Healthy	c.516T>C[a]	Exon 5	Synonymous mutation T172T

Abbreviation: CACT, carnitine–acylcarnitine translocase.
[a]Newly identified mutations in this study.

worldwide in the literature, and among them, the partial genotype of one Japanese patient was briefly described by a researcher from our group (TF).[6,7,22,23] In many cases, CACT deficiency was seen during the neonatal period, with a few cases seen in their infantile period. The rarity of the disease may be explained in part by miscarriage and stillbirth.

Because of the resemblance of clinical features, including the acylcarnitine profiles determined by tandem mass spectrometry, between CACT deficiency and the neonatal form of CPT2 deficiency, genotyping is expected to be a pivotal tool for differentially diagnosing these two disorders. Here, we report the genotypes of two cases with CACT deficiency. Two *CACT* mutations, c.576G>A and c.106-2a>t, have never been reported in the literature; the former mutation was detected in both the affected babies and could be Japanese patient-specific. The c.576G>A mutation changes the tryptophan at the amino-acid residue 192 to a stop signal. The three-dimensional structure of rat CACT indicates that such a mutation might result in an incomplete binding-site structure for cytosomal fatty acids and therefore lead to impaired enzymatic functions.[24] The importance of the splicing mutation c.106-2a>t has been emphasized by Korman *et al.*[25] Another identified mutation, c.199-10t>g, was the commonest one among the patients reported, especially the Chinese population.[7]

Among the patients with non-traumatic rhabdomyolysis, we found the c.804delG mutation in the *CACT* gene, which has been reported to be pathogenetic in a Cape Indian individual and a Caucasian individual.[7] Although heterozygous CPT2 deficiency has also been reported to cause rhabdomyolysis,[26] we propose that our patient bearing this mutation was a heterozygous carrier of CACT deficiency, because the CACT enzymatic activity of his fibroblasts was maintained within the normal range. Acylcarnitine analysis was not performed for these patients; hence, we could not completely exclude the possibility that they were affected by other β-oxidation defects, such as very long chain acyl-CoA dehydrogenase (VLCAD) deficiency. However, the incidence of VLCAD deficiency is believed to be less than that of CPT2 deficiency in the Japanese population.[27] c.516T>C was found to be a synonymous variant in the case of the healthy volunteers. Table 3 summarizes all the genetic variations found in this study.

CACT deficiency needs to be diagnosed genetically as early as possible because of the following reasons: (1) patients with the disorder die during the neonatal period, (2) tests for measurement of the enzyme activity are not necessarily available worldwide, (3) differential diagnosis between CPT2 and CACT deficiency can be achieved only by genetic methods, (4) immediate induction of adequate therapies is required, and (5) genetic analysis can distinguish patients with CACT deficiency-like diseases but without genetic mutations.[28,29] However, due to the rarity of the disease, the structure and characteristics of the *CACT* gene are still not well understood. Recently, DHPLC has been developed to screen mutations in these types of rare diseases, as it is particularly useful for detecting unknown polymorphisms and mutations. In this study, we demonstrate that all the PCR products producing heteroduplexes during DHPLC indeed contained mutated sequences, whereas normal sequences did not cause heteroduplex formation. These findings were further confirmed by family studies of each CACT-deficiency patient.

In conclusion, we identified five mutations in the *CACT* gene, three of which were responsible for CACT deficiency. We have also demonstrated the successful screening of unknown *CACT* gene mutations with DHPLC.

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Case report

Metabolic encephalopathy in beta-ketothiolase deficiency: The first report from India

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Abstract

Beta-ketothiolase deficiency, or mitochondrial acetoacetyl-CoA thiolase (T2) deficiency, is a rare autosomal recessive disorder affecting isoleucine catabolism and ketone body metabolism. A patient from South India presented with acute ketoacidosis at 11 months of age. During the acute crisis the C5OH (2-methyl-3-hydroxybutyryl) carnitine and C5:1 (tiglyl) carnitine were elevated and large amounts of 2-methyl-3-hydroxybutyrate, tiglylglycine, and 2-methylacetoacetate were excreted. Brain CT showed bilateral basal ganglia lesions. Potassium ion-activated acetoacetyl-CoA thiolase activity was deficient in the patient's fibroblasts. The patient is a homozygote for a novel c.578T>G (M193R) mutation. This is the first report of T2 deficiency confirmed by enzyme and molecular analysis from India.

Keywords: T2 deficiency; 2-Methyl-3-hydroxybutyrate; Tiglylglycine; C5OH; *ACAT1*; Ketoacidosis

1. Introduction

Beta-ketothiolase deficiency (OMIM 203750), also known as mitochondrial acetoacetyl-coenzyme A (CoA) thiolase (T2, gene symbol *ACAT1*) deficiency, is a rare autosomal recessive disorder that affects the metabolism of isoleucine and ketones. T2 deficiency is clinically characterized by severe ketoacidosis triggered by ketogenic stresses such as infections and fasting [1]. The disorder is usually suspected when increased excretion of 2-methyl-3-hydroxybutyrate, tiglylglycine, and 2-methylacetoacetate is detected by urinary organic acid analysis and/or elevated levels of 2-methyl-3-hydroxybutyrylcarnitine (C5OH) and tiglylcarnitine (C5:1) are detected in blood plasma using tandem mass spectrometry [1–4]. However, some patients do not show such typical profiles in these analyses [2–4].

Here we provide the first report of a T2-deficient patient from India, with typical urinary organic acid and blood acylcarnitine profiles, who presented with severe metabolic acidosis and metabolic encephalopathy.

2. Case report

An 11-month-old male child (GK95, GK number is an internal identifier for T2 deficient patients) was admitted in the pediatric intensive care unit with a history of fever, cough, and rapid breathing. The child

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was born to parents who are first cousins and he is the first child to the parents. The development at 11 months was appropriate. Ten days prior to the admission he had diarrhea and dehydration and was treated with intravenous fluids. Following 2 days of febrile episodes, he developed tachypnea, poor perfusion and tachycardia, and unconsciousness. Initial laboratory tests indicated metabolic acidosis with an arterial pH of 6.9, Pco_2 of 10 mmHg, and bicarbonate level of 4.4 mM. The blood glucose was low at 1.8 mmol/L. Urine ketones were strongly positive (180 mg/dL). Serum lactate was normal. On the second hospital day, he had generalized tonic–clonic seizures and was treated with levetriacetam 20 m/kg and then started on maintenance dose of 10 mg/kg/dose. Brain CT showed hypodensities in the bilateral lentiform nucleus and caudate head, suggestive of metabolic encephalopathy (Fig. 1). The child was intubated and kept on a ventilator on the second hospital day. There was no improvement with sodium bicarbonate correction and the child was put on dialysis for 2 days. Fluid and electrolyte balance was maintained and the child received a glucose infusion stepwise in 2 mg/kg/min increments up to 12–15 mg/kg/min with monitoring of blood glucose levels. Following dialysis, the biochemical parameters improved. The child was extubated on the fourth hospital day. Acylcarnitine analysis showed a C5OH concentration of 3.08 μM (cut-off value 1.0) and a C5:1 concentration of 1.69 μM (cut-off value 0.3). Urinary organic acid analysis showed elevated levels of 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate, and tiglylglycine. A tentative diagnosis of T2 deficiency was made. The child regressed, with loss of social smile, recognition, and the ability to sit or crawl. Management following the acute stage included a low-protein (1.5 g/kg), high-carbohydrate diet supplemented with 50 mg/kg carnitine. The child was discharged on the 15th hospital day on the same diet with the antiepileptic drug and baclofen for dystonia.

One week after discharge, dystonia of all four limbs, predominant in lower limbs and mild irritability were noted. A month later, irritability subsided and the child could follow objects and started recognizing the parents. Physiotherapy was started. At 15 months of age, social smile with partial head control was attained but central hypotonia persisted. Dystonia of the trunk with intermittent arching was also noted by 15–16 months of age. Trihexyphenydyl was used at dose of 4 mg twice a day. At 18 months, good head control was achieved and the child could sit and stand with support. At 24 months, he could walk with support; social interac-

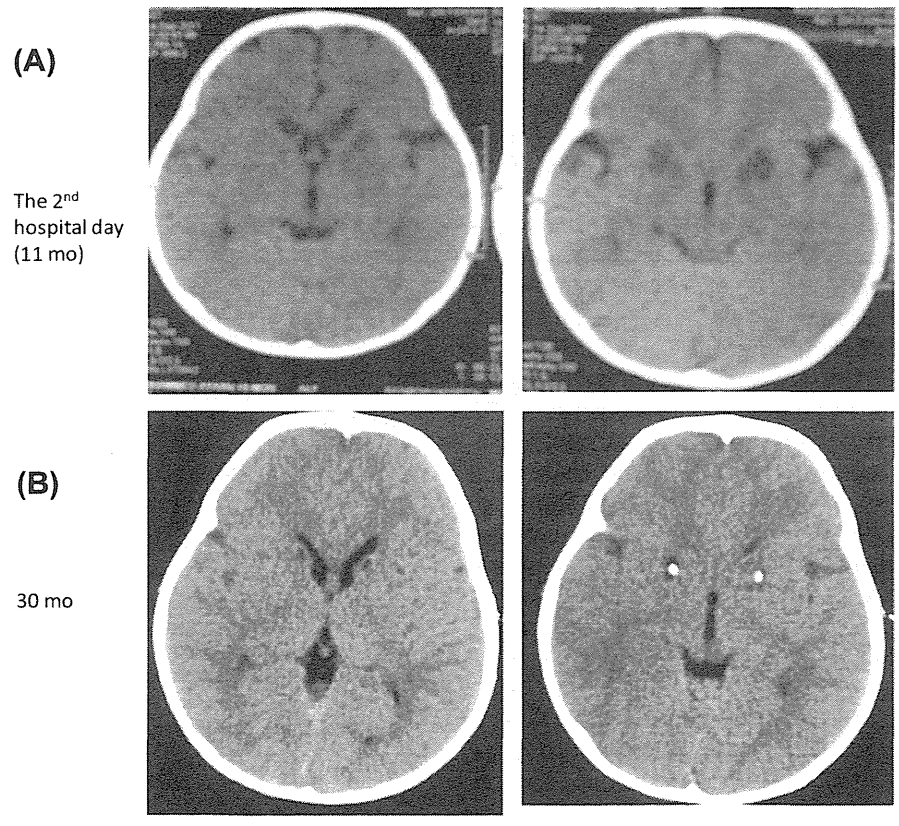

Fig. 1. Brain CT findings in patient GK95. (A) Plain axial images show symmetrical hypodensities involving bilateral basal ganglia, suggestive of metabolic encephalopathy. (B) Plain axial images show bilateral symmetrical calcification involving the anterior part of the lentiform nucleus with surrounding low-density areas.

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tion was good and he was able to say 4–6 two-syllable words. On a recent follow-up, at 30 months of age, speech had improved to 24–30 two-syllable words and the child was toilet trained. Although the child had truncal hypotonia with mild bilateral lower limb dystonia, he was able to walk with support. Social quotient was 96 and developmental quotient was 74 with predominant motor delay. Height was 84 cm (85th centile) and body weight was 13 kg (85th centile). Head circumference was 48 cm (50th centile). Follow-up brain CT showed bilateral calcification in the basal ganglia (Fig. 1).

An acetoacetyl-CoA thiolase assay was done in the absence and presence of potassium ion using cultured fibroblasts. Potassium ion-activated acetoacetyl-CoA thiolase activity was absent in GK95's fibroblasts ($-K^+$ 6.3, $+K^+$ 5.5 nmol/min/mg protein; control fibroblasts $-K^+$ 5.6, $+K^+$ 8.9 nmol/min/mg protein), confirming the diagnosis of T2 deficiency. Mutation analysis was then performed at the genomic level. We identified a homozygous c.578T>G (M193R) mutation. We confirmed that both parents were heterozygous carriers of the mutation. Transient expression of mutant T2 cDNA showed that the M193R mutant retained no residual T2 activity.

3. Discussion

Beta-ketothiolase deficiency, or T2 deficiency, was first described in 1971 [5], and more than 100 patients have been identified worldwide (including unpublished patients). Although most reports have come from Western countries, the Middle East, and Japan [1], T2 deficiency has recently been reported in other countries including China and Vietnam [6]. Furthermore, a possible founder mutation, R208X, was identified in the Vietnamese population [6]. The incidence of T2 deficiency is not yet defined in most populations. Newborn screening by tandem mass spectrometry is now ongoing in several countries and regions. The incidence of T2 deficiency was reported to be 1 in 232 000 over the period January 2001 to November 2010 in one study from Minnesota, USA [4]. A Japanese pilot study found no T2-deficient patients among roughly 2 million newborns screened by tandem mass spectrometry (Yamaguchi et al., unpublished data). However, newborn screening can yield false negative results, especially in individuals with mutations that allow some residual T2 activity [2–4]. Six of seven T2-deficient probands had such "mild" mutations in the Japanese population [2,3]. This is characteristic for the Japanese population. In India, a pilot screening of 5000 newborns from the state of Andhra Pradesh by tandem mass spectrometry detected several disorders but not T2 deficiency [7]. Because most T2-deficient patients can be identified by urinary organic acid analysis or acylcarnitine analysis during acute metabolic decompensation, popularization of these analyses in India may increase the detection of T2 deficiency.

Unconsciousness during severe ketoacidosis is one of the common clinical symptoms of T2 deficiency [1]. "Metabolic stroke-like episodes" comprises acute focal neurological deficits in connection with acute metabolic decompensation and associated focal lesions on brain imaging. Metabolic encephalopathy involving basal ganglia has been reported in other types of organic acidemia such as propionic acidemia and methylmalonic acidemia [8]. The basal ganglia have high energy requirements in childhood and this may make them particularly vulnerable to damage by impaired energy metabolism. Bilateral basal ganglia lesions in T2 deficiency have been reported only in patients GK06 [1,9] and GK70 [6] among our records of about 100 T2-deficient patients; both of these patients presented a severe ketoacidotic crisis. One T2-deficient patient (GK85) showed basal ganglia lesions without apparent severe ketoacidosis, perhaps because of chronic metabolic insufficiency, and presented with non-progressive choreiform movements [10]. The present patient, GK95, is the fourth patient to have basal ganglia lesions among our records. Because this patient showed no neurological problems before the severe ketoacidotic episode, it is likely that the metabolic encephalopathy was a sequela of the severe ketoacidotic episode.

In conclusion, we report a case of T2 deficiency with a novel mutation. This case with T2 deficiency had the uncommon presentation of metabolic encephalopathy with neurological sequela.

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CASE REPORT

Three Japanese Patients with Beta-Ketothiolase Deficiency Who Share a Mutation, c.431A>C (H144P) in *ACAT1*: Subtle Abnormality in Urinary Organic Acid Analysis and Blood Acylcarnitine Analysis Using Tandem Mass Spectrometry

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Abstract Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency affects both isoleucine catabolism and ketone body metabolism. The disorder is characterized by intermittent ketoacidotic episodes. We report three Japanese patients. One patient (GK69) experienced two ketoacidotic episodes at the age of 9 months and 3 years, and no further episodes until the age of 25 years. She had two uncomplicated pregnancies. GK69 was a compound heterozygote of the c.431A>C (H144P) and c.1168T>C (S390P) mutations in T2 (*ACAT1*) gene. She was not suspected of having T2 deficiency during her childhood, but she was diagnosed as T2 deficient at the age of 25 years by enzyme assay using fibroblasts. The other two patients were identical twin siblings who presented their first ketoacidotic crisis simultaneously at the age of 3 years 4 months. One of them (GK77b) died during the first crisis and the other (GK77) survived. Even during severe crises, C5-OH and C5:1 were within normal ranges in their blood acylcarnitine profiles and trace amounts of tiglylglycine and small amounts of 2-methyl-3-hydroxybutyrate were detected in their urinary organic acid profiles. They were H144P homozygotes. This H144P mutation has retained the highest residual T2 activity in the transient expression analysis of mutant cDNA thus far, while the S390P mutation did not retain any residual T2 activity. The "mild" H144P mutation may result in subtle profiles in blood acylcarnitine and urinary organic acid analyses. T2-deficient patients with "mild" mutations have severe ketoacidotic crises but their chemical phenotypes may be subtle even during acute crises.

Abbreviations

SCOT Succinyl-CoA:3-ketoacid CoA transferase
T2 Mitochondrial acetoacetyl-CoA thiolase

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2, gene symbol ACAT1) deficiency (OMIM 203750) is an autosomal recessive inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. This disorder, first described by Daum et al. (1971), is characterized by intermittent episodes of metabolic ketoacidosis associated with vomiting and unconsciousness often triggered by infections (Fukao et al. 2001). There are no clinical symptoms between episodes. Typical T2 deficiency is easily diagnosed by urinary organic acid analysis, characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate both during ketoacidotic episodes and between episodes (Fukao et al. 2001, 2003). Diagnosis is confirmed by measurement of T2 activity on cultured skin fibroblasts (Robinson et al. 1979; Zhang et al. 2004). T2 deficiency is caused by mutations in the *ACAT1 (T2)* gene located on chromosome 11q22.3-q23.1 (Fukao et al. 1990; Kano et al. 1991). T2 deficiency is very heterogeneous at the genotype level, with at least 50 different mutations described (Fukao et al. 1995, 1997, 1998, 2001, 2002, 2003, 2007, 2008, 2010a, b; Wakazono et al. 1995; Nakamura et al. 2001; Zhang et al. 2004, 2006; Sakurai et al. 2007).

Some T2-deficient patients with mutations which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003). We herein describe three Japanese patients with T2 deficiency whose H144P mutation retains significant residual activity. Their urinary organic acid and blood acylcarnitine profiles were atypical and subtle even during severe ketoacidotic crises.

Materials and Methods

Case Reports

GK69

This Japanese woman (GK69), born in 1984, developed severe metabolic acidosis at the age of 9 months. On admission to a third-level hospital, she was semicomatose, polypneic (48/min), and hypotonic. Laboratory values were: blood glucose 6.8 mmol/L, NH_3 92 μmol/L, blood pH 7.225, pCO_2 7.2 mmHg, bicarbonate 3 mmol/L, base excess −21.3, Na 153 mEq/L (normal range: 139–146), BUN 28.5 mg/dL (normal range: 10–18), and creatinine 1.1 mg/dL (normal range: 0.18–0.46). Metabolic acidosis was refractory to sodium bicarbonate therapy. Peritoneal dialysis was performed for 2 days. On the second hospital day, polypnea and unconsciousness disappeared and the blood gas data improved. Urinary organic acid analysis showed massive amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic aciduria. No increases in 2-methyl-3-hydroxybutyrate or tiglylglycine were noted, although this analysis was performed in an outside laboratory and no urine samples were available for reanalysis. At that time, T2 deficiency was excluded from differential diagnosis based on this organic acid data and the tentative diagnosis was succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. However, an enzyme assay for SCOT was not performed. At the age of 3 years, the patient had a similar but milder episode. Subsequently, she had no further ketoacidotic episodes. Growth and development were normal. She had two uncomplicated pregnancies.

Twin Siblings (GK77b and GK77)

GK77b is a twin Japanese boy. He was born at 36 weeks gestation weighing 2,400 g. His parents had no known consanguinity but both were from a small island in Amami islands in Japan. He experienced several febrile illnesses without ketoacidosis. However, at 3 years 4 months of age, after a 3-day history of fever, cough, and vomiting, he developed anorexia, lethargy, and polypnea. He was admitted to a local hospital. His blood glucose level was 2.3 mmol/L. Blood gas analysis was not performed. Hypoglycemia was corrected with intravenous glucose injection of 20 ml of 20% glucose solution followed by continuous infusion of a 2.6% glucose solution. About 30 h after admission, his condition worsened. Blood gas analysis revealed severe metabolic acidosis showed pH 6.88, pCO_2 6.1 mmHg, and bicarbonate 1.1 mmol/L. He was transferred to a regional hospital. On arrival at the hospital, he was unconscious with a heart rate of 168/min and respiratory rate of 39/min. Blood laboratory data were: WBC 19,050/μL, CRP 0.2 mg/dL (normal values: <0.15), BUN 36.2 mg/dL (normal range: 10–18) creatinine 0.5 mg/dL (normal range: 0.25–0.49), NH3 33.5 μmol/L, glucose 3.8 mmol/L, pH 7.17, pCO_2 20 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 16.3 mmol/L. He received continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.4–0.47 mEq/kg/h. However, unconsciousness and metabolic acidosis did not improve. On the fifth hospital day, he died before being transferred to a third-level hospital.

GK77 is the twin brother of GK77b. Pyloric stenosis was diagnosed at the age of 1 month and corrected surgically;

thereafter, he was well until 3 years 4 months of age. Two days after the onset of his twin brother, he developed frequent repeated vomiting after cough and nasal discharge. Therefore, he was admitted to the regional hospital at the same time as his twin. On admission, he was lethargic. Laboratory findings were: WBC 7,760/μL, CRP 0.5 mg/dL (normal values: <0.15), BUN 20.2 mg/dL (normal range: 10–18), creatinine 0.4 mg/dL (normal range: 0.25–0.49), glucose 3.7 mmol/L, NH3 25 μmol/L, blood pH 7.135, pCO_2 19.5 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 10.1 mmol/L. He received a continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.3 mEq/kg/h. On the third hospital day, his condition worsened and he was transferred to a third-level hospital. On admission, the blood gasses were pH 7.372, pCO_2 21.6 mmHg, bicarbonate 12.2 mmol/L, and base excess −11.2 mmol/L. A glucose infusion rate was further increased to 6.5 mg/kg/min with 10% glucose solution. Acidosis normalized with 9 h (pH 7.399, bicarbonate 21.7 mmol/L, base excess −2.6 mmol/L). Two days later, the urinary ketones became negative and he started eating.

GK77 is now 4 years 8 months and has experienced no further ketoacidotic episodes. The family has been advised to avoid fasting and to come to the local hospital if he has a high fever or appetite loss. His growth and development are within normal ranges.

Urinary Organic Acid Analysis and Acylcarnitine Analysis

Urine samples containing 0.2 mg of creatinine were used for our high risk screening of organic acids. As internal standards, 20 mg each of tropate (TA, C9), margarate (MGA, C17), and tetracosane (C24) were added to these samples. Trimethylsilylated samples were analyzed using capillary gas chromatography-mass spectrometry (QP 5050A, Shimadzu Co. Ltd., Kyoto, Japan), as described earlier (Kimura et al. 1999). The values of organic acids were expressed as the peak area (%) relative to IS-1 (margarate) on the mass chromatogram. Quantification of 2-methyl-3-hydroxybutyrate and tiglylglycine in urine samples from GK77b and GK77 was kindly done by Dr. Sass (Freiburg University) (Lehnert 1994). For comparison, quantification was also done in urine samples from T2-deficient patients whose urinary screening profiles had typical T2 deficient ones. We used urine sample in stable condition from GK01 who is a compound heterozygote of A333P and c.149delC (Fukao et al. 1998) and samples in acute and stable conditions from T2-deficient patients from India (GK(Ind)) in our high-risk screening. Blood spot and serum acylcarnitine analysis using tandem mass spectrometry was also done, as described (Kobayashi et al. 2007), and blood spot samples from GK75 and GK79, who are R208X homozygotes (Fukao et al. 2010b) were used as positive controls.

Enzyme Assay and Immunoblot Analysis Using Fibroblasts

Control and patients' fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described (Robinson et al. 1979; Zhang et al. 2004). We assayed acetoacetyl-CoA thiolase activity in the presence and absence of potassium-ion, since T2 is the only thiolase which is activated by the ion. Immunoblot analysis was done, as described (Fukao et al. 1997). In the cases of the controls, twofold serial dilution samples from 30 to 3.75 μg were electrophoresed together with samples (30 μg) of GK68 and GK77 to determine the amount of T2 protein in the patients' fibroblasts relative to that in the control fibroblasts.

Mutation Analysis

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Genomic DNA was extracted from fibroblasts using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed by PCR and direct sequencing of genomic fragments that included each exon and its surrounding intron sequences (Fukao et al. 1998). For GK77b and the parents, exon 5 was amplified from a dried blood spot 1.25 mm in diameter, which was used for tandem mass spectrometry, using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

Restriction Enzyme Assay to Detect c.431A>C (H144P)

The c.431A>C (H144P) mutation creates a new BmgT120 I site (GGACC). DNAs from 110 Japanese controls were examined using a restriction enzyme assay, as follows.

A fragment (314 bp), including exon 5 and its surrounding introns, was amplified using the following primers:

In4 as (in intron, −69 to −48)5′-CATGCTCTATTAAG-TTCTGCAG-3′

In5 as (in intron, +137 to +119) 5′-ATCCAGACACTCT-TGAGCA-3′

An aliquot of the resulting amplicon was digested with BmgT120 I, then resolved on a 5% polyacrylamide gel. The c.431A fragment (wild-type) is 314-bp long and the c.421C fragment is cut into 162-bp and 152-bp fragments.

Transient Expression Analysis of Mutant cDNAs

Transient expression of T2 cDNAs was performed using a pCAGGS eukaryote expression vector (Niwa et al. 1991), as described (Sakurai et al. 2007). After transfection, cells were cultured at 37°C or 40°C for 48 h, then harvested and kept at −80°C until use. Cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100. After centrifugation at 10,000 × g for 10 min, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity and for immunoblot analysis.

Results and Discussion

Confirmation of the Diagnosis

GK69's fibroblasts were assayed for SCOT activity to confirm the diagnosis in 2008, when GK69 was 24 years old. As shown in Table 1, she was diagnosed as having T2 deficiency but not as having SCOT deficiency.

SCOT deficiency was first suspected in GK77 and GK77b, based on the following facts (1) Two of the four SCOT deficient Japanese families were from the Amami islands, the population of which is about 120,000. They were T435N homozygotes (Fukao et al. 2004). (2) The acylcarnitine profiles and urinary organic acid analysis during acute ketoacidotic crisis in both patients had no typical profile for T2 deficiency, as discussed below. As shown in Table 1, GK69's and GK77's fibroblasts had normal SCOT activity and a higher ratio (1.3) of acetoacetyl-CoA thiolase activity in the presence to the absence of potassium ions than typical T2-deficient fibroblasts (the ratio was around 1.0). Immunoblot analysis also showed a clearly detectable amount of T2 protein in GK77's fibroblasts, and a lower amount in GK69's fibroblasts. Densitometric analysis showed that the amounts of T2 protein in GK77 and GK69 were estimated to be 50% and 25% of control, respectively (Fig. 1).

Table 1 Acetoacetyl-CoA thiolase activities in the absence and presence of potassium ions

Fibroblasts	Acetoacetyl-CoA thiolase activity			SCOT activity
	$-K^+$	$+K^+$	$+K^+/-K^+$	
Controls ($n = 5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1
GK69	3.6 ± 0.5	4.1 ± 0.9	1.2 ± 0.1	4.7 ± 1.4
GK77	4.2 ± 0.3	5.8 ± 1.5	1.4 ± 0.3	3.9 ± 0.5
T2D	4.5 ± 1.4	4.7 ± 1.6	1.0 ± 0.1	5.6 ± 0.5

Enzyme activity is expressed as nmol/min/mg of protein. In cases of patients, enzyme assay was done three times and shows average ± SD. T2D, A disease control

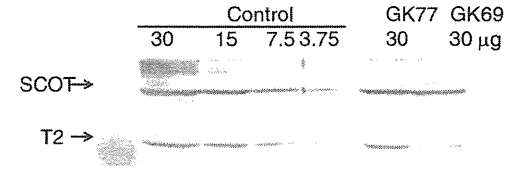

Fig. 1 Immunoblot analysis. In the cases of the controls, serial twofold dilutions from 30 to 3.75 μg were studied together with samples (30 μg) from GK68 and GK77. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

Mutations and Their Effects on T2 Protein

Mutation screening revealed that GK69 was a compound heterozygote of c.431A>C (H144P) and c.1168T>C (S390P). Her mother had S390P heterozygously but did not have H144P. The father's DNA was not available for analysis. GK77 had an H144P mutation homozygously, shown by mutation screening at the genomic level. Their parents and GK77b were heterozygous carriers and a homozygote of H144P, respectively. The c.431A>C (H144P) mutation creates a BmgT120I site (GGACA to GGACC). We could not find c.431A>C (H144P) in the 110 Japanese controls using the restriction enzyme assay with BmgT120I.

We performed transient expression analysis of wild-type and mutant cDNAs in T2-deficient SV40-transformed fibroblasts. Following expression of T2 cDNAs for 48 h at 37°C, an enzyme assay and immunoblots were performed (Fig. 2a,b). The transfection of wild-type T2 cDNA produced high potassium ion-activated acetoacetyl-CoA thiolase activity (T2 activity), whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. The H144P mutant retained a residual T2 activity of ~25% of the wild-type value (Fig. 2a). The S390P mutant did not retain any residual T2 activity. In immunoblot analysis (Fig. 2b), the H144P mutant protein was detected, whereas no S390P protein was detected. The relative amount of the H144P mutant protein, as compared to the wild-type, was estimated to be 50%. Hence, the specific activity (unit/mg of T2 protein) of the H144P mutant protein was estimated to be about 50% of the wild type. Protein-folding and post-folding stability is predicted to vary with the incubation temperature. Hence, we also performed transient expression at 40°C for 48 h. The H144P mutant in expression at 40°C had a similar level of residual activity to that at 37°C.

We reported the tertiary structure of the human T2 tetramer (Haapalainen et al. 2007). Figure 3a shows the positions of the H144P and S390P mutations on the dimer.

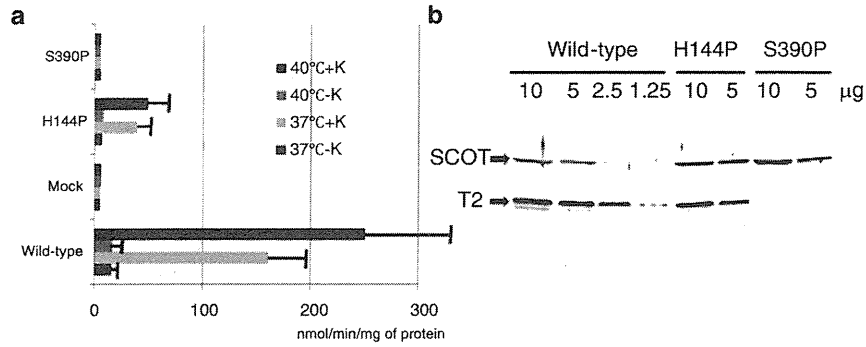

Fig. 2 Transient expression analysis of H144P and S390P mutant cDNAs. Transient expression analysis was performed at 40°C and 37°C. (**a**) Potassium ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence (−K) and presence (+K) of potassium ions are shown together with the SD of three independent experiments. (**b**) Immunoblot analysis. The protein amounts applied are indicated above the lanes. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

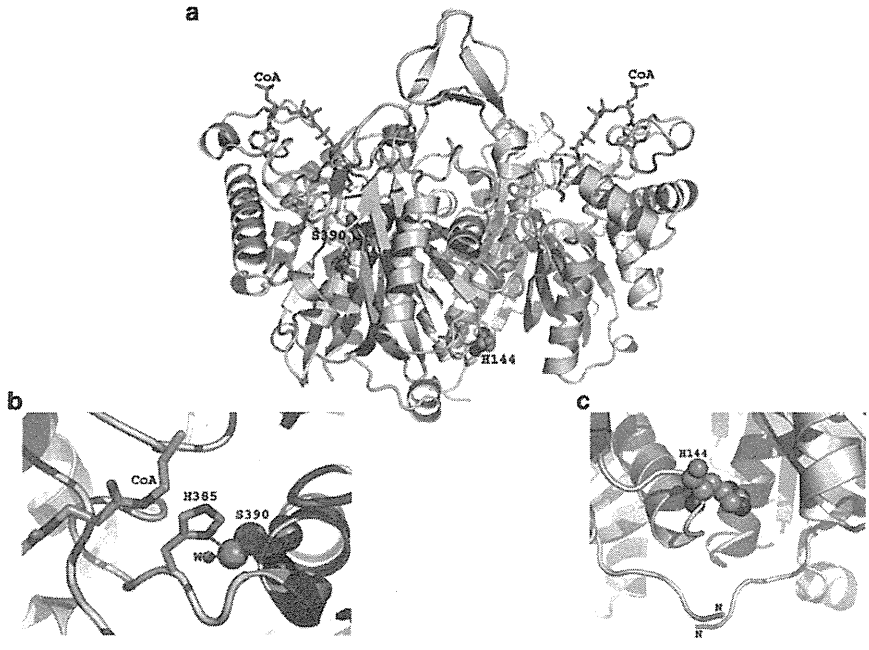

Fig. 3 The positions of H144P and S390P on the tertiary structure of human T2 dimers with substrates of coenzyme A

As seen in the figure, S390 is close to the active site and H144 is at the dimer interface close to the surface of the protein. Figure 3b shows a zoomed-in view around S390. This mutant is located at the active site. S390 is hydrogen-bonded to catalytic histidine, H385; it could be that this serine is needed to orient histidine in a way that the histidine can stabilize the transient negative charge of the substrate optimally. S390 is also hydrogen-bonded to a water molecule that is needed in stabilizing parts of the enzyme. So, if S390 is mutated into proline, these two hydrogen bonds do not exist. Hence, this S390P is expected to bring about a serious change in T2 catalytic cavity. In our expression analysis, this S390P was also too unstable to detect mutant protein. Figure 3c shows a zoomed-in view at the dimer interface. H144 is interacting with the residues of the neighboring subunit. If this residue is mutated into Pro, there is less dimeric interaction, which in turn might destabilize the overall structure. Since this residue is far from the active site and substrate binding site, it is difficult to explain why this H144P mutant had reduced specific activity in transient expression analysis from the viewpoint of structural analysis.

Urinary Organic Acid Analysis

GK69 was first suspected to having T2 deficiency as a probable diagnosis; however, urinary organic acid analysis at the first ketoacidotic crisis indicated no characteristic profile for T2 deficiency such as elevated 2-methyl-3-hydroxybutyrate and tiglylglycine in 1985 (no data was available). The results of the urinary organic acid analysis of our patients are shown in comparison with those of typical T2-deficient patients, GK01 and GK(Ind) (Table 2, Fig. 4). At the age of 24 years when her condition was stable, GK69's urinary organic acid analysis showed that there were only trace amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine (Table 2). In our screening, this low level of tiglylglycine was difficult to detect. Urinary organic acid analysis during the acute crises of GK77 and GK77b showed huge amounts of 3-hydroxybutyrate and acetoacetate with elevated 2-methyl-3-hydroxybutyrate but only trace amounts of tiglylglycine. The levels of 2-methyl-3-hydroxybutyrate and tiglylglycine during a stable condition in GK77 are similar with those in GK69.

In cases of typical T2-deficient patients, it is easy to suspect T2 deficiency based on large amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine as shown in Fig. 4. However, even in cases of trace amounts of tiglylglycine (possibly under the detection limit), T2 deficiency cannot be excluded. An H144P mutation, which retained high residual activity, may contribute to atypical profiles in the presented cases. These findings strengthen our previous observations that some T2-deficient patients with mutations, which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003).

Table 2 Quantitative analysis of urinary organic acid analysis during acute crises and stable conditions

Patients	Acute crises		Stable conditions	
	2M3HB	Tiglylglycine	2M3HB	Tiglylglycine
GK69	NA	NA	14.0	13.3
GK77b	405.7	45.8	NA	NA
GK77	160.2	6.7	27.3	14.8
GK01	NA	NA	399.1	732.1
GK(Ind)	484.6	503.9	195.1	797.6
Controls ($n = 42$)			10.7 ± 7.6	24.6 ± 14.6

Values are expressed as mmol/mol creatinine

NA means that samples were not available for the analysis. GK01 is a compound heterozygote of c.149delC and A333P, which retained no residual activity (Fukao et al. 1998). GK(Ind) indicates a patient with typical T2-deficient profiles of urinary organic acids in our screening

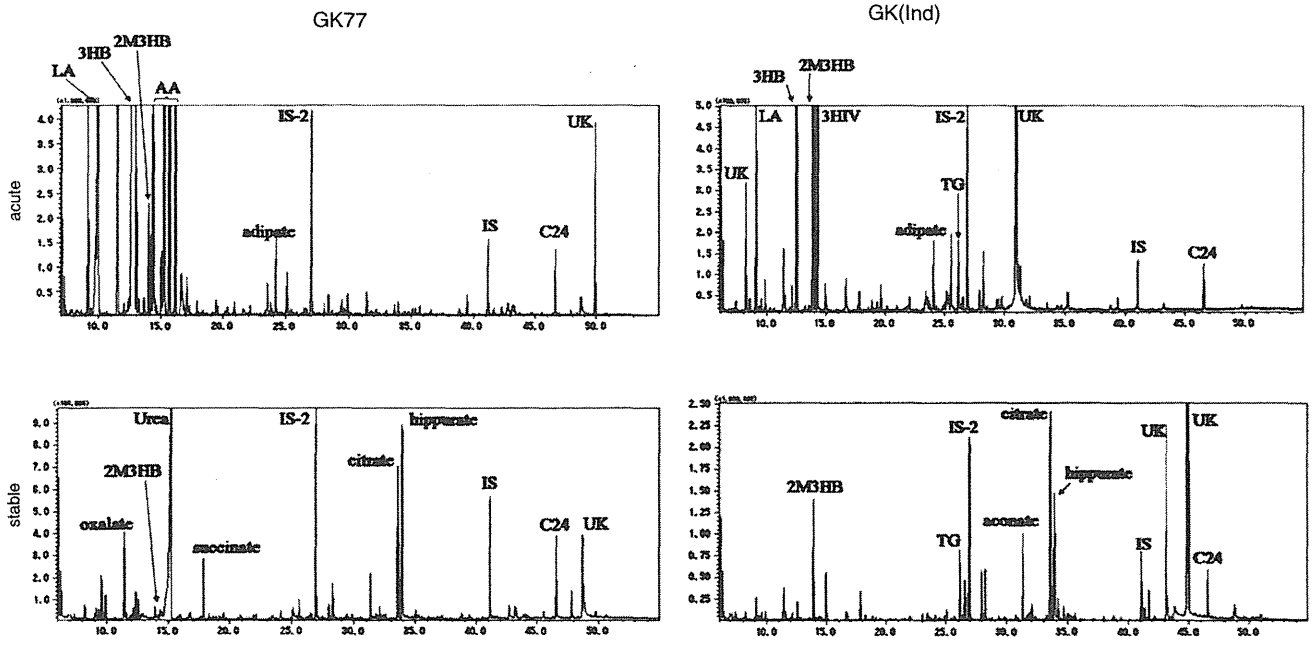

Fig. 4 Urinary organic acid profiles of GK77 during the acute episode and an asymptomatic period in comparison with those of a typical T2-deficient patient (GK(Ind)). *LA* Lactate, *3HB* 3-OH-butyrate, *3HIV* 3-OH-isovalerate, *AA* Acetoacetate, *2M3HB* 2-Methyl-3-OH-butyrate, *TG* Tiglylglycine, *IS-2* and *IS* Internal standards, *UK* Unknown. Since acetoacetate is unstable and samples from GK(Ind) were shipped on filter papers after thoroughly drying, the levels of acetoacetate are likely underestimated

Table 3 C5-OH and C5:1 carnitines in blood filters and serum samples from GK77 and GK77b during acute crises

Patients	Dried blood spots		Serum	
	C5:1	C5-OH	C5:1	C5-OH
GK77b	0.027	0.11	ND	0.12
GK77	0.012	0.11	0.044	0.10
R208X homozygotes				
GK75 (acute)	0.89	2.89	NA	NA
GK79 (stable)	1.20	2.35	NA	NA
Controls ($n = 30$)				
Average ± SD	0.015 ± 0.016	0.26 ± 0.15	0.015 ± 0.013	0.059 ± 0.024

ND not detected, *NA* not applicable

The values are expressed as μmol/L

GK75 and GK79 are positive controls for T2 deficient patients who are R208X homozygotes (Fukao et al. 2010b)

Blood and Serum Acylcarnitine Analyses

Acylcarnitine analysis was done using samples during the acute crises of GK77 and GK77b. Table 3 shows the results in comparison with those of typical T2-deficient patients (R208X homozygotes) (Fukao et al. 2010b). C5:1 and C5OH elevation in blood spots, characteristic for T2 deficiency, was clearly detected in the samples from the typical T2-deficient patients but was absent in samples from GK77 and GK77b. We previously reported that the abnormality of the acylcarnitine profiles in T2-deficient patients with mutations which retain some residual activity is subtle during nonepisodic conditions (Fukao et al. 2003), but the present study clearly showed that it could be also subtle even during severe ketoacidotic episodes. This means that acylcarnitine analysis using blood spots cannot detect some T2-deficient patients like GK77 and GK77b. Serum acylcarnitine analysis might detect elevation of these compounds to some extent, but we need to analyze more cases to clarify the usefulness of serum acylcarnitine analysis in such T2-deficient patients with mutations which retain some residual activity.

T2 deficiency cannot be excluded even if acylcarnitine profiles during acute episodes are within normal ranges. Careful evaluation of urinary organic acids, especially for the presence of 2-methyl-3-hydroxybutyrate, is necessary not to overlook T2 deficiency.

Clinical Issues

Since they were confirmed as identical twins by DNA analysis (data not shown), their genetic backgrounds were identical and most environmental factors were also very similar between them. One died during the ketoacidotic crisis and the other survived.

In Japan, intravenous infusion therapy for vomiting, appetite loss, and dehydration is commonly performed with commercially available initial infusion solution, such as Solita T1 (2.6% glucose) followed by maintenance solution, such as Solita T2 and T3 (4.3% glucose). These solutions are effective for physiological ketosis. However, in the case of T2 deficiency, a higher concentration of glucose may be necessary. Accordingly, we had the impression that GK77 became much better after the glucose concentration was changed from 5% to 10%. In the case of prolonged ketoacidosis, consideration should be given to increasing the infusion rate of glucose to ensure high normal blood glucose level to suppress ketone body synthesis and isoleucine catabolism via insulin secretion.

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Concise One-Sentence Take-Home Message

Patients with beta-ketothiolase deficiency having a mutation which retains some residual activity showed subtle abnormality in urinary organic acid analysis and blood acylcarnitine analysis even during acute ketoacidotic episodes.

Details of the Contributions of Individual Authors

Toshiyuki Fukao and Naomi Kondo performed the enzyme assays, immunoblot/mutation analysis, and expression analysis of cDNAs. Toshiyuki Fukao mainly wrote this manuscript. Shinsuke Maruyama, Toshihiro Ohura, Mitsuo Toyoshima, Naomi Kuwada, and Mari Imamura are the physicians responsible for the patients. Yuki Hasegawa and Seiji Yamaguchi performed gas chromatography-mass spectrometry and tandem mass spectrometry analyses and first suspected the disorder. Isao Yuasa confirmed GK77 and 77b as identical twins by DNA analyses. Antti M Haapalainen and Rik K Wierenga analyzed the tertiary structural effects of the mutations.

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Alpha-methylacetoacetic acidura, mitochondrial acetoacetyl-CoA thiolase deficiency (OMIM 203750, 607809)

Mitochondrial acetoacetyl-CoA thiolase, acetyl-CoA acetyltransferase 1 (EC 2.3.1.9)

ACAT1 gene (gene ID 38, NM_000019.3)

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Details of Ethics Approval

This study has been approved by the Ethical Committee of the Graduate School of Medicine, Gifu University.

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Brief Communication

Clinical and molecular aspects of Japanese children with medium chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

We report the outcome of 16 Japanese patients with medium chain acyl-CoA dehydrogenase deficiency. Of them, 7 patients were diagnosed after metabolic crisis, while 9 were detected in the asymptomatic condition. Of the 7 symptomatic cases, 1 died suddenly, and 4 cases had delayed development. All 9 patients identified by neonatal or sibling screening remained healthy. Of 14 mutations identified, 10 were unique for Japanese, and 4 were previously reported in other nationalities. Presymptomatic detection including neonatal screening obviously improves quality of life of Japanese patients, probably regardless of the genotypes.

1. Introduction

Medium chain acyl-CoA dehydrogenase deficiency (MCADD) (MIM #201450) is an autosomal recessive inherited metabolic disorder of mitochondrial fatty acid oxidation. The number of MCADD patients has recently become larger in Japan with the spread of acylcarnitine analysis using tandem mass spectrometry (MS/MS). The disease frequency was estimated to be approximately 1:100,000 in Japan according to a newborn screening pilot study of 1.57 millions babies (unpublished report). Clinical symptoms of MCADD are heterogeneous, ranging from asymptomatic to severe handicaps followed by metabolic crisis or sudden unexpected death (SUD) [1,2]. Approximately 20% of previously undiagnosed patients die during their first metabolic decompensation [3–7]. Blood acylcarnitine, urinary organic acid analyses, MCAD activity and mutation analyses are major tools for diagnosis of MCADD. A common c.985A>G mutation has been reported in 80–90% of Caucasian patients [8–16] while c.449–452delCTGA mutation was identified in 45% of mutant alleles in Japanese patients with MCADD [17]. In recent years, the detection incidence of the presymptomatic patients with MCADD has increased since the neonatal mass screening was expanded in Japan. However, there are few reports of the outcomes of the Japanese patients. Herein, we report the relation of clinical onsets, genotypes and outcomes of 16 Japanese children with MCADD, and 4 heterozygote carriers, which were analyzed in Shimane University.

2. Subjects and methods

2.1. Subjects

Sixteen Japanese patients with MCADD from 15 unrelated families, including previously reported 9 cases [17], and 4 carriers were studied (Table 1). The patients were analyzed for confirmation of diagnosis in Shimane University from 2001 to 2011. Of them, 8 (cases 8 to 16) were identified by neonatal mass screening, 7 (cases 1 to 7) were diagnosed after metabolic crisis, and 1 was detected by sibling screening. Cases 2 and 8 were siblings, and cases 19 and 20 were parents of case 16. Diagnosis of the patients was confirmed by urinary organic acid, blood acylcarnitine and mutation analyses.

2.2. Mass spectrometric analysis

Acylcarnitines in blood spots on filter paper were analyzed by a method standardized for neonatal mass screening using MS/MS, an API 3000 instrument (Applied Biosystems, Foster City, CA, USA) [8,18]. Urinary organic acids were analyzed using the solvent extraction method by the QP 2010 capillary GC/MS system (Shimadzu Co., Ltd., Kyoto, Japan) [19]. The determination of test values was assessed using reference values set at the Shimane University.

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Table 1
Clinical and genetic characteristics of Japanese patients with MCAD deficiency.

Patient	Sex	Age at onset	Age at diagnosis	Neonatal screening	Primary clinical symptoms	Hypoglycemia	Hyperammonemia	Tandem MS		GC/MS (RPA%)		Genotype		Outcome
								C8 <0.35 μM	C8/C10 (<3)	HG	SG	Allele 1	Allele 2	
Symptomatic group														
1	F	1 y	1 y	–	Cardiopulmonary arrest, dyspnea, poor feeding	(+)	(−)	4.52	8.69	n.a	n.a	IVS4+1G≥A	c.422A≥T (Q116L)	Sudden death
2 [a] *	M	1 y 4 m	1 y 4 m	–	Gastroenteritis, seizures	(+)	(−)	3.33	17.53	9.9	15.3	c.449–452delCTGA	c.449–452delCTGA	Severe handicapped
3 [a]	M	8 m	8 m	–	Cardiopulmonary arrest	(n.a)	(+)	5.97	3.49	11.1	44.5	c.449–452delCTGA	c.157C>T (R28C)	Developmental delay
4	F	1 y 1 m	1 y 1 m	–	Developmental regression	(+)	(+)	7.00	21.00	14.7	112.2	del. ex 11–12	del. ex 11–12	Developmental delay
5 [a]	F	2 y 2 m	2 y 2 m	–	Cold, gastroenteritis	(+)	(−)	1.71	15.55	n.a	n.a	c.449–452delCTGA	c.449–452delCTGA	Developmental delay
6 [a]	F	1 y 3 m	1 y 3 m	–	Unconsciousness, apnea, vomiting	(n.a)	(−)	n.a	n.a	n.a	n.a	del. ex 11–12	del. ex 11–12	Normal
7 [a]	F	1 y 7 m	1 y 7 m	–	Unconsciousness, fever	(+)	(+)	4.12	10.05	6.1	6.4	c.275C>T (P67L)	c.157C>T (R28C)	Normal
Asymptomatic group														
8 [a] *	M	–	5 y 5 m	–	Normal	(−)	(−)	1.37	39.14	n.a	n.a	c.449–452delCTGA	c.449–452delCTGA	Normal
9 [a]	F	–	5 d	+	Normal	(−)	(−)	5.92	11.38	12.9	14.8	c.1085G>A (G337E)	c.843A>T (R256S)	Normal
10	F	–	5 d	+	Normal	(−)	(−)	5.37	12.49	6.33	39.88	c.449–452delCTGA	c.157C>A (R28H)	Normal
11	M	–	5 d	+	Normal	(−)	(−)	4.82	13.03	15.3	3.8	IVS3+2T≥C	c.843A>T (R256S)	Normal
12	F	–	5 d	+	Normal	(−)	(−)	4.04	14.96	n.a	n.a	c.449–452delCTGA	c.212G≥A (G46D)	Normal
13 [a]	F	–	5 d	+	Normal	(−)	(−)	2.78	15.44	11.5	5.9	c.449–452delCTGA	c.134A>G (Q20R)	Normal
14	F	–	5 d	+	Normal	(−)	(−)	2.59	10.00	3.08	3.20	c.1085G≥A (G337E)	c.1184A≥G (K370R)	Normal
15	M	–	5 d	+	Normal	(−)	(−)	2.58	8.32	(−)	1.50	c.449–452delCTGA	IVS3+5G≥A	Normal
16 [a]	M	–	5 d	+	Normal	(−)	(−)	0.49	3.77	9.7	(−)	c.449–452delCTGA	c.820A>C (M249V)	Normal
Carrier group														
17	M	–	5 d	+	Normal	(−)	(−)	0.44	1.02	(−)	(−)	c.845C>T (P257L)	n.d	Normal
18	F	–	4 m	–	Eczema	(−)	(−)	0.51	0.88	(−)	(−)	c.843A>T (R256S)	n.d	Normal
19	M	–		–	Normal	(−)	(−)	0.37	1.00	n.a	n.a	c.449–452delCTGA	n.d	Normal
20	F	–		–	Normal	(−)	(−)	0.20	0.95	n.a	n.a	c.820A>C (M249V)	n.d	Normal

[a]: Purevsuren et al. [17] reported; *: siblings; sex: M, male; F, female; age: y, year; m, month; d, day; +, involved to neonatal mass screening; (−), not detected; n.a, not available; RPA%, relative peak area percentage; HG, hexanoylglycine; SG, suberylglycine; novel mutations are underlined.

2.3. DNA sequencing of gene, acyl-CoA dehydrogenase, medium chain (ACADM)

Genomic DNA was purified from the patients' fibroblasts or blood filter papers using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Mutation analysis on genomic DNA was performed by PCR for each exon and its intron boundaries followed by direct sequencing [17].

Informed consent to perform DNA analysis was obtained from the parents of the patients. This study was approved by the Ethical Committee of the Shimane University Faculty of Medicine.

3. Results

3.1. Clinical features of patients

The clinical features of 16 Japanese patients with MCADD and 4 carriers (9 males and 11 females) are summarized in Table 1, including previously reported cases [17]. All 7 patients that were diagnosed after metabolic crisis were born before the initiation of newborn screening in their local area. The mean age at onset of the symptomatic cases was 1 y 3 m (range: 8 m to 2 y 2 m). The symptomatic patients were all in good general health with normal development until metabolic crisis. Metabolic crises were triggered by common cold or gastroenteritis in 5 cases. One of them died of SUD. Four cases had mild to severe handicaps, and 2 cases developed normally. The patients who were identified by neonatal screening remain healthy at this time.

3.2. Biochemical results of patients

The results of mass spectrometric analysis are shown in Table 1. Blood acylcarnitine analysis was available in 15 of the 16 patients. Octanoylcarnitine (C8) and octanoyl:decanoylcarnitine (C8/C10) ratio were assessed for detection of MCADD. Marked elevation of C8 and C8/C10 was observed in 14 cases (1.37–7 μmol/L), and slight elevation of C8 and C8/C10 (0.49 μmol/L and 3.77) was found in one case (case 16). The level of C8 was also mildly elevated in 3 (0.44, 0.51 and 0.37 μmol/L, respectively) of the 4 carriers while C8/C10 value was under cut-off (1.02, 0.88 and 1.00). Case 20, who is a mother of case 16, showed no abnormal findings.

Urinary organic acids were analyzed in 11 cases with MCADD and 4 carriers. Both hexanoylglycine and suberylglycine were elevated in 9 patients, and hexanoylglycine or suberylglycine was increased in one case each. However, neither hexanoylglycine nor suberylglycine was identified in the carriers.

3.3. Mutations in acyl-CoA dehydrogenase, medium chain (ACADM) gene

Fourteen types of mutations were identified in 30 independent alleles, 7 of which were novel. These included three types of splice site alterations (IVS3+2T>C, IVS3+5G>A and IVS4+1G>A), and four missense mutations (G46D, Q116L, G337E and K395R). These novel mutations were not detected in 120 alleles from unaffected Japanese individuals. All mutations are summarized in Table 1, together with previously reported cases (cases 2, 3, 5–9, 13 and 16) [17]. A c.449–452delCTGA [20,21] was detected in 10 (33.3%) of 30 independent alleles (2 cases with homozygous and 6 cases with compound heterozygous). A homozygous large deletion including exons 11 and 12 [22] was identified in 4 (13.3%) alleles. R28C (2/30 alleles), R256S (2/30 alleles), P67L (1/30 alleles), M249V (1/30 alleles) and G337E (1/30 alleles) were also observed (Table 1) [9,17,22].

4. Discussion

We investigated the relationship between clinical and molecular spectrums of 16 Japanese patients with MCADD. While symptomatic patients remained undiagnosed until metabolic crisis, asymptomatic patients were identified by neonatal mass screening (8 cases), or by sibling screening (1 case). Most of the symptomatic cases developed metabolic crisis associated with hypoglycemia triggered by common infection and prolonged fasting [3,4]. Those patients had poor outcomes such as mild to severe impairments or SUD. However, expansion of blood acylcarnitine analysis using MS/MS for neonatal mass screening in Japan allowed earlier detection of MCADD in the asymptomatic/presymptomatic stage. Subsequent prophylactic management for those children was conducted in a more appropriate and timely manner during metabolic stress such as fever, viral infection and other medical procedures.

Fourteen mutations were identified in 30 independent alleles including seven novel mutations. The amino acids affected by the novel missense mutations (G46D, Q116L, G337E and K395R) are highly conserved among different species (*Pan Troglodytes, Rattus norvegicus, Xenopus laevis* and *Danio rerio*), suggesting that these amino acids play an important role in medium acyl-CoA dehydrogenase activity. There are also splice site alterations such as IVS3+2T>C, IVS3+5G>A and IVS4+1G>A positioned at a 5′ donor splice site. Shapiro and Senapathy 5′ splice site scores [23] of altered sites changed from 76.4 to 58.6 for IVS3+2T>C, from 76.4 to 62.4 for IVS3+5G>A, and from 86.3 to 68.1 for IVS4+1G>A, respectively, suggesting that these changes are likely responsible for aberrant mRNA splicing. It is reported that point mutations in donor splice site produced exon skipping or aberrant 5′ donor splice site activation [24]. Since these changes likely resulted in aberrant splicing and premature truncation, non-sense mediated mRNA decay [25] or translation into shorter proteins with unlikely residual activity would result.

Most of the mutations detected in Japanese patients were unique, but Q20R, R28C, R256S and c.449–452delCTGA were previously reported in other nationalities [9,22,26,27]. The Japanese patient with compound heterozygous of R28C was one quarter of Caucasian. In contrast, a common missense mutation c.985A>G (80–90%) of Caucasian [8,15,28–30] was not detected in any Japanese patients in this study.

Our study demonstrates that detection in the asymptomatic/presymptomatic stage is essential to achieve favorable outcomes of patients with MCADD. Neonatal mass screening is absolutely a beneficial system to improve the quality of life of patients with MCADD. Genetic background of Japanese patients with MCADD is different from those in Caucasians. It is likely that there is no correlation between genotype and phenotype in Japanese patients with MCADD, and a specific genotype does not predict the clinical outcome.

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Effects of idursulfase enzyme replacement therapy for Mucopolysaccharidosis type II when started in early infancy: Comparison in two siblings

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Enzyme replacement therapy

ABSTRACT

Mucopolysaccharidosis type II (MPS II) is a lysosomal storage disorder that is progressive and involves multiple organs and tissues. While enzyme replacement therapy (ERT) with idursulfase has been shown to improve many somatic features of the disease, some such as dysostosis multiplex and cardiac valve disease appear irreversible once established, and little is known about the preventative effects of ERT in pre-symptomatic patients. We report on two siblings with severe MPS II caused by an inversion mutation with recombination breakpoints located within the *IDS* gene and its adjacent pseudogene, *IDS-2*. The siblings initiated treatment with idursulfase at 3.0 years (older brother) and 4 months (younger brother) of age, and we compared their outcomes following 2 years of treatment. At the start of treatment, the older brother showed typical features of MPS II, including intellectual disability. After 34 months of ERT, his somatic disease was stable or improved, but he continued to decline cognitively. By comparison, after 32 months of ERT his younger brother remained free from most of the somatic features that had already appeared in his brother at the same age, manifesting only exudative otitis media. Skeletal X-rays revealed characteristic signs of dysostosis multiplex in the older brother at the initiation of treatment that were unchanged two years later, whereas the younger brother showed only slight findings of dysostosis multiplex throughout the treatment period. The younger brother's developmental quotient trended downward over time to just below the normal range. These findings suggest that pre-symptomatic initiation of ERT may prevent or attenuate progression of the somatic features of MPS II. Follow-up in a larger number of patients is required to confirm the additive long-term benefits of ERT in pre-symptomatic patients.

1. Introduction

Mucopolysaccharidosis (MPS) type II (Hunter syndrome, OMIM #309900), is an inborn error of glycosaminoglycan (GAG) metabolism caused by deficient activity of lysosomal iduronate 2-sulfatase (IDS, EC 3.1.6.13). The responsible gene, IDS, is located on chromosome Xq28, and the disease shows classic X-linked recessive inheritance. Rarely, females may be affected as a result of biallelic mutations, skewed X-inactivation, uniparental isodisomy, or X-autosome translocations [1,2]. Dermatan sulfate and heparan sulfate, the substrates for IDS, accumulate in the lysosomes of various tissues and organs of affected patients, leading to the development of characteristic signs and symptoms of MPS II after the first year of life. (HOS reference). Somatic features include coarse facies, straw-like hair, rough and thickened skin, macrocephaly, disproportionate short stature due to dysostosis multiplex, decreased joint mobility, cardiac valve disease and left ventricular hypertrophy, hepatosplenomegaly, obstructive sleep apnea, and restrictive lung disease. Frequent otitis media and hernias (inguinal and umbilical) may be the earliest presenting signs, but are non-specific. Patients with little to no IDS activity (severe form) exhibit progressive somatic disease, cognitive decline, and death during adolescence (HOS). Patients with some residual IDS activity (mild form) have largely somatic disease with normal intellectual development [3].

In recent years, enzyme replacement therapy (ERT) with recombinant human iduronate-2sulfatase (idursulfase, Elaprase®, Genzyme, a Sanofi Company and Shire Human Genetic Therapies, Cambridge, MA) has been available for the treatment of MPS II. Weekly infusions of idursulfase have been shown to improve walking capacity, hepatosplenomegaly, and urinary GAG levels [4]. However, ERT appears to be less effective in correcting disease manifestations once developed in the skeletal system and heart valves [5,6]. Intravenously administered ERT has not been shown to slow or prevent the deterioration of the central nervous system in patients with the severe phenotype, most likely because it does not cross blood–brain barrier at the labeled dose [7]. Although idursulfase is approved for use only in patients who are at least 5 years of age, a recent report from the Hunter Outcome Survey (HOS) suggests that it can safely reduce urinary GAG levels and hepatomegaly in young children, some

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