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ORIGINAL ARTICLE

A structural mapping of mutations causing succinyl-CoA: 3-ketoacid CoA transferase (SCOT) deficiency

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Abstract Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency is a rare inherited metabolic disorder of ketone metabolism, characterized by ketoacidotic episodes and often permanent ketosis. To date there are ~20 disease-associated alleles on the *OXCT1* gene that encodes the mitochondrial enzyme SCOT. SCOT catalyzes the first, rate-limiting step of ketone body utilization in peripheral tissues, by transferring a CoA moiety from succinyl-CoA to form acetoacetyl-CoA, for entry into the tricarboxylic acid cycle for energy production. We have determined the crystal structure of human SCOT, providing a molecular understanding of the reported mutations based on their potential structural effects. An interactive version of this manuscript (which may contain additional mutations appended after acceptance of this manuscript) may be found on the web address: http://www.thesgc.org/jimd/SCOT.

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Abbreviations

SCOT	Succinyl-CoA:3-ketoacid CoA transferase
OXCT1	3-oxoacid CoA transferase 1

Introduction

Ketone bodies (acetoacetate, 3-hydroxybutyrate, acetone), predominantly produced in the liver, provide extrahepatic organs such as heart and brain with energy when glucose supply is limited (Sass 2012). Any enzyme malfunction in ketone body utilization (ketolysis) could lead to a buildup of unused ketone bodies and result in ketoacidosis. Succinyl-CoA:3-ketoacid CoA transferase (SCOT; gene name *OXCT1*; EC 2.8.3.5) catalyzes the first and rate-determining

step of ketolysis (Fukao et al 2000; Kassovska-Bratinova et al 1996). SCOT is a member of the CoA transferase family I that activates acetoacetate by transferring a CoA moiety from succinyl-CoA to form acetoacetyl-CoA. Acetoacetyl-CoA is further metabolized by acetoacetyl-CoA thiolase into two acetyl-CoA molecules which enter the citric acid cycle for energy production (Williamson et al 1971). SCOT is a mitochondrial enzyme expressed in all extrahepatic tissues, but abundant in the heart, brain and kidney (Fukao et al 1997). There exists also a testis-specific isoform SCOT-t (gene name *OXCT2*), sharing 74 % amino acid identity with SCOT (Tanaka et al 2002).

Mutations in the human *OXCT1* gene on chromosome location 5p13 result in the rare autosomal recessive deficiency of SCOT (OMIM 245050) (Mitchell and Fukao 2001). SCOT-deficient patients, often with neonatal onset, present with recurrent ketoacidosis episodes that could be life-threatening, but with no symptoms between episodes (Niezen-Koning et al 1997). The number of reported cases is few, and symptoms, which may vary among individuals, include vomiting, lethargy and tachypnea, as well as unconsciousness caused by severe ketoacidosis (Berry et al 2001; Sakazaki et al 1995; Snyderman et al 1998). Permanent ketosis or ketonuria is a characteristic symptom but may be absent in patients retaining residual SCOT activity (Fukao et al 2010; Fukao et al 2011; Fukao et al 2004). Approximately 30 affected probands were reported to date and 24 mutations identified (Table 1). Here we report the crystal structure of human SCOT, and present an interactive mapping of missense mutations to understand the molecular basis of SCOT deficiency.

Table 1 Mutations reported for the human OXCT1 gene causing SCOT deficiency. Additional phenotype information can be found in the supplementary text

#	DNA change	Exon	Mutation site	Mutated residue	Protein change	Conservation	Reference
1[a,c,d]	c.112C>T[b]	2	Arg38	Cys	p.R38C		(Alkén 2008)
2[c]	c.335T>A[b]	4	Val112	Asp	p.V112D	Semi-Conserved	(Alkén 2008)
3	c.398T>A	4	Val133	Glu	p.V133E	Semi-Conserved	(Song et al 1998)
4	c.644C>T	6	Ala215	Val	p.A215V	Conserved	(Fukao et al 2011)
5	c.656G>A	6	Gly219	Glu	p.G219E	Conserved	(Fukao et al 2000)
6	c.661G>A	6	Val221	Met	p.V221M	Semi-Conserved	(Fukao et al 2000)
7	c.677G>A	7	Ser226	Asn	p.S226N	Semi-Conserved	(Fukao et al 2011)
8[c]	c.785C>G	8	Pro262	Arg	p.P262R	Conserved	Sass et al (unpublished)
9[c]	c.802C>T	8	Arg268	Cys	p.R268C	Conserved	Sass et al (unpublished)
10	c.803G>A	8	Arg268	His	p.R268H	Conserved	(Fukao et al 2007)
11	c.971G>A	10	Gly324	Glu	p.G324E	Conserved	(Fukao et al 2000)
12	c.980T>C	10	Leu327	Pro	p.L327P	Conserved	(Fukao et al 2011)
13[†]	c.1162A>G[b]	12	Met388	Val	p.M388V	Conserved	(Alkén 2008)
14	c.1210G>T	13	Val404	Phe	p.V404F	Conserved	(Fukao et al 2011)
15	c.1213T>C	13	Ser405	Pro	p.S405P	Conserved	(Fukao et al 2011)
16[†‡]	[b]	14	Leu429	Phe	p.L429F	Conserved	(Alkén 2008)
17	c.1304C>A	14	Thr435	Asn	p.T435N	Conserved	(Fukao et al 2010; Fukao et al 2004)
18	c.1367G>T	15	Cys456	Phe	p.C456F	Conserved	(Song et al 1998)
19	c.1402C>T	15	Arg468	Cys	p.R468C	Low-conserved	(Fukao et al 2011)
Insertion, deletion, frameshift mutations							
20	c.649C>T	6	Arg217	X	p.R217X	Semi-Conserved	(Longo et al 2004)
21	c.817G>T	8	Glu273	X	p.E273X	Variable	(Fukao et al 2011)
22	c.848C>G	9	Ser283	X	p.S283X	Conserved	(Yamada et al 2007)
23	c.658-666dup	6	Asn220-Ile222		p.N220-I222 dup	Conserved (Asn220), Semi-conserved (Val221,Ile222)	(Fukao et al 2010)
24	c.1561T>C[b]	6	X521R	Arg	Adding 20 AAs		(Alkén 2008)

[a] The R38C mutation resides in the N-terminus of the protein that is disordered and not modelled in the crystal structure

[b] DNA change in the R38C, V122D, M388V and X521R mutations are deduced from amino acid substitution. The DNA change in the L429F mutation is not reported and cannot be deduced from amino acid substitution

[c] These mutations have not been confirmed as pathogenic by expression analysis

[d] R38C and L429F mutations coexisted in one mutant allele

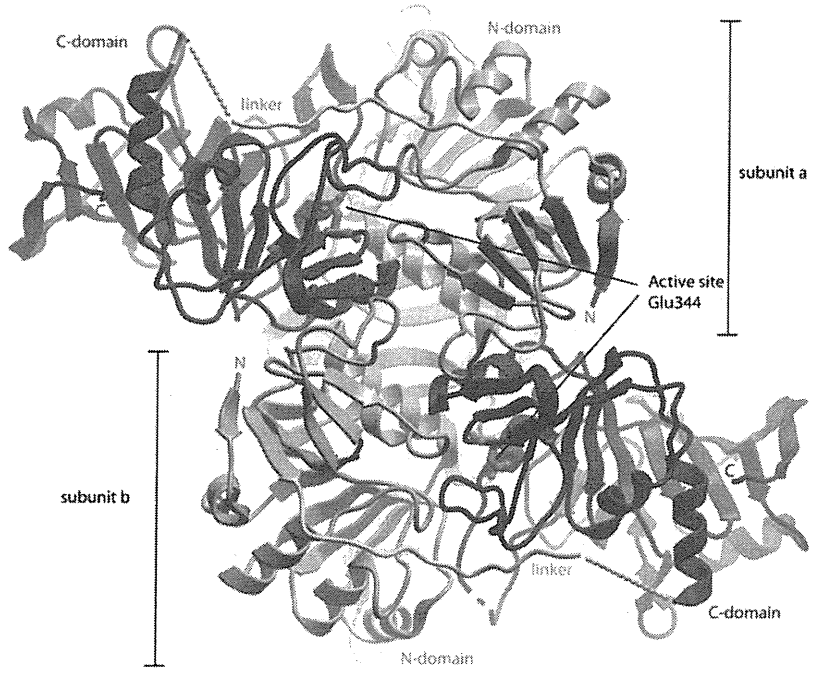

Fig. 1 Ribbon diagram of human SCOT structure illustrating the homodimeric assembly. The two monomeric subunits **a** and **b** are coloured distinctively (*blue* and *magenta*). The active site in each subunit is indicated by the Glu344 residue shown in *orange sticks*

Experimental procedures

Expression, purification & crystallization

DNA fragment encoding the catalytic domain of human SCOT (aa 40–520; GenBank entry 4557817) was subcloned into pNIC-CTHF vector incorporating a C-terminal His_6-tag. The plasmid was transformed into BL21(DE3)-R3-pRARE2, cultured in Terrific Broth at 37 °C, and induced with 0.5 mM IPTG. Cells were homogenized in lysis buffer (50 mM HEPES pH7.5, 500 mM NaCl, 5 % glycerol, 5 mM imidazole, 1 mM PMSF, 0.5 mM TCEP), centrifuged to remove cell debris, and the supernatant was purified by Nickel affinity (HisTrap 1 ml GE/Amersham) and size exclusion (HiLoad 16/60 Superdex S200) chromatography. Purified protein was concentrated to 21 mg/ml and stored in 10 mM HEPES pH7.5, 500 mM NaCl, 5 % (w/v) glycerol and 0.5 mM TCEP at −80 °C. Crystals were grown by vapour diffusion at 20 °C, in sitting drops mixing 100 nl protein pre-incubated with 2 mM acetyl-CoA and 300 nl reservoir solution containing 0.20 M sodium chloride, 0.1 M Tris pH9.0 and 25 % (w/v) polyethylene glycol 3,350. Crystals were cryo-protected in mother liquor containing 20 % (w/v) glycerol and flash-frozen in liquid nitrogen.

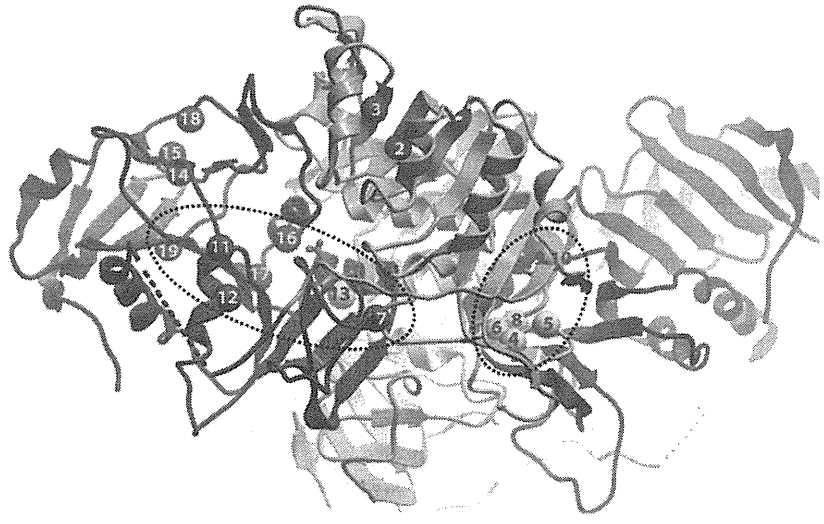

Fig. 2 Clustering of human SCOT missense mutations, displayed in the same colour scheme as Fig. 1. The positions of amino acid mutations are indicated by *small spheres* and *numbered* according to Table 1. Mutations that affect the monomeric fold or dimerization interaction are coloured *red* and *yellow* respectively

Data collection & structure determination

Diffraction data to maximum resolution of 2.20 Å were collected on beamline X10A at the Swiss Light Source, and processed using the CCP4 Program suite (CCP4 1994). SCOT crystallized in the $P2_1$ space group with four molecules in the asymmetric unit (Supplementary Table 1). The structure of human SCOT was solved by molecular replacement with PHASER (McCoy et al 2005), using the pig heart structure as search model (PDB code 1M3E)(Bateman et al 2002). Initial automated model building was performed with ARP/wARP (Perrakis et al 2001), followed by cycles of iterative manual model building with COOT (Emsley and Cowtan 2004) and REFMAC5 refinement (Murshudov et al 1997). The refined model consists of protein residues 40–285 and 297–519. No electron density was observed for part of the inter-domain linker (residues 286–296). No ligand density for acetyl-CoA was found in the active site though it was added during crystallization. Structure factors and coordinates were deposited in the Protein Data Bank under the accession code 3DLX.

Results and discussion

We have determined the crystal structure of human SCOT which exhibits a homodimer architecture containing two active sites (Fig. 1). Each monomer consists of the amino-terminal (N-; aa 40–272) and carboxy-terminal (C-; aa 298–510) domains, connected by a linker region (aa 273–297). The N- and C-domains share a common α/β structural fold for CoA transferase family I members (Heider 2001), as previously seen in the pig SCOT structure (89 % sequence identity)(Bateman et al 2002). The active site of each monomer is situated at the interface of the two domains, where a strictly conserved residue Glu344 (Fig. 1, orange sticks) attacks the incoming succinyl-CoA substrate and forms an enzyme-CoA thioester intermediate, as an integral part of the catalytic mechanism (Solomon and Jencks 1969). Residues in the active site of human SCOT are also conserved in the testis-specific isoform SCOT-t, suggesting it may have enzymatic activity.

To date, three nonsense, two insertion and 19 missense mutations leading to SCOT deficiency are known from literature or newly reported here (Table 1 and Supplementary text), although six missense mutations have not been confirmed as pathogenic mutations by expression analysis. There is a polymorphism c.173C>T (T58M) which retains full enzyme activity (Song et al 1998). The three nonsense mutations (R217X, E273X, S283X) are expected to cause premature translation termination, resulting in truncated SCOT proteins that lack completely the C-domain and hence abolish part of the active site. Another mutation c.1561T>C at the termination codon results in X521R and adds 20 amino acids in the C terminus of SCOT peptide (Alkén 2008). The missense mutations are broadly distributed between the N- and C-domains of the protein, although two clusters of 'mutational hotspots' can be observed (Fig. 2). One cluster is close to the interface between two SCOT subunits in a dimer. A duplication mutation (N220-I222dup) is also present in this region (Fukao et al 2010). The other cluster is located in secondary structure elements that make up the active site and CoA-binding site of the enzyme.

The mapping of missense mutations onto the human SCOT structure allows us to classify their potential structural consequences broadly into three types. In the first type, amino acids tightly packed in the enzyme are substituted to bulkier and/or charged residues (Fig. 2 and Table 1, *#2–7,11,14,16,17*). This likely results in severe steric and electrostatic clashes in the local environment which in turn can compromise severely the folding, architecture and stability of the enzyme. These mutants often result in the more severe phenotype (permanent acidosis), consistent with their much diminished enzyme activity (Fukao et al 2000; Song et al 1998). The second type disrupts the integrity of a secondary structure element, either by introducing a conformationally-restrained residue (e.g. Pro, Gly) into an α-helix/β-strand (Fig. 2 and Table 1, *#12,15*), or by removing such residues from their critical involvement in a loop/turn segment (Fig. 2 and Table 1, *#8*). The third type involves the substitution of arginine residues where their guanidinium side-chains are involved in salt bridge formation. These charged interactions contributed to stabilizing two neighbouring regions in 3D space that are distant apart in the polypeptide sequence. Substitution of arginine to a weakly positive-charged (Fig. 2 and Table 1, *#10*) or uncharged amino acid (Fig. 2 and Table 1, *#9,19*) will abolish these salt bridges. Though retaining partial enzyme activities, these mutant proteins are thermally less stable compared to wild-type (Fukao et al 2007; Fukao et al 2011).

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Conflict of interest None

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A Treatable New Cause of Chorea: Beta-Ketothiolase Deficiency

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Inherited metabolic diseases are increasingly identified in patients with movement disorders. Chorea can occur in Lesch-Nyhan disease, glucose transporter type 1 (*GLUT-1*) deficiency, respiratory chain disorders, and some organic acidurias (propionic, methylmalonic, and type 1 glutaric acidurias).[1] We describe a patient with deficiency of mitochondrial acetoacetyl-coenzyme A (CoA) thiolase (beta-ketothiolase [T2]), a classic organic aciduria, who presented with nonprogressive chorea since infancy and stable basal ganglia abnormalities on imaging.

Clinical Details

A 17-year-old male was evaluated for nonprogressive choreiform movements, dysarthria, myoclonic jerks, and ataxia since infancy. He was the third child of a nonconsanguineous French-Canadian couple. Pregnancy, delivery, and perinatal period were normal. At age 4.5 months, he presented with hypotonia. Nerve conduction studies and electromyography were normal. At age 10 months, he had good eye contact but severe axial hypotonia. Continuous involuntary upper extremity movements were noted. Blood lactate, pyruvate, and glucose levels, a karyotype, a cerebral computerized tomography study and a muscle biopsy were all normal.

His development was delayed. He sat at 13 months and walked at 3.5 years. His first words and sentences were at ages 2 years and 5 years, respectively. No developmental regression occurred.

At age 4 years, daily activities (dressing, eating) were difficult because of involuntary movements. His gait was unstable. Brain magnetic resonance imaging (MRI) revealed small, bilateral, T2 hyperintensities in both the putamen and cerebral peduncles. His audiogram and cardiac ultrasound were normal. Pyruvate dehydrogenase, cytochrome oxidase, succinate cytochrome C reductase assays, and the lactate/pyruvate ratio in fibroblasts were normal, as were a deletion analysis of leukocyte mitochondrial DNA and plasma amino acid levels. Urinary organic acids and acylcarnitine profile were not obtained.

At age 5 years, he had acute gastroenteritis with dehydration, ketotic odor, a blood pH of 7.17 (normal, 7.36–7.44), and a bicarbonate level of 7.7 mmol/L (normal, 21–25 mmol/L). During a 3-day hospitalization, he responded well to rehydration, with no observed sequelae. He had other childhood infections but did not develop systemic metabolic changes.

He started a normal class at age 6 years. His constant movements restricted his school performance. Neurological examination showed persistent, nonprogressive gait disturbance truncal ataxia; chorea; dysmetria (greatest in the upper limbs); and slight hypotonia but normal strength.

At age 7 years, brain MRI revealed stability of the previously described abnormalities. Neuropsychological evaluation showed an IQ of 105 with normal verbal function and high nonverbal function but with limitations because of constant involuntary movements.

Additional Supporting Information may be found in the online version of this article.

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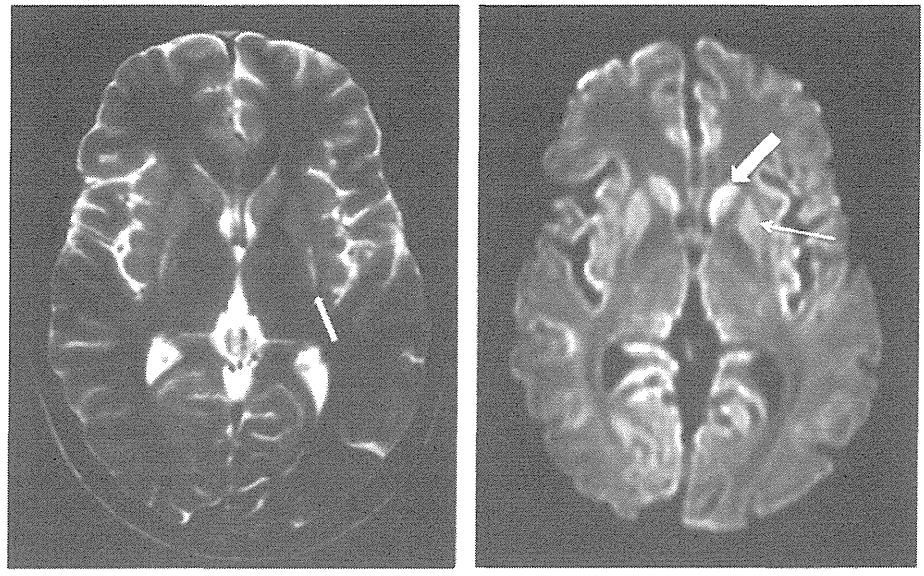

A. B.

FIG. 1. (**A**) This T2-weighted axial image shows high signal and atrophy of the posterolateral putamen (white arrow). (**B**) This diffusion weighted image shows atrophy of the putamen with high signal intensity in the putamen (small white arrow) and caudate (large white arrow) nuclei.

He was lost to follow-up until age 17 years. In the interval, he had enjoyed good health, completed secondary school, and planned to train in wood manufacturing.

Since he was diagnosed with T2 deficiency (T2D) (see Metabolic Investigations, below), the patient has remained clinically stable with mild protein restriction (approximately 60 g protein daily) and avoidance of fasting longer than 15 hours. He was advised to seek medical care in case of repeated vomiting or other stress and to wear a bracelet identifying his diagnosis.

Metabolic Investigations

At age 17 years, urinary organic acid analysis revealed elevated levels of 2-methyl-3-hydroxybutyrate (410 μmol/mmol creatinine; normal, ≤12 μmol/mmol) and 2-methylacetoacetate (1566 μmol/mmol creatinine; normal, ≤ 6 μmol/mmol) and high levels of plasma C5:1 acylcarnitine (0.29 μmol/L; normal, <0.05μmol/L), with normal levels of free carnitine, plasma amino acids, blood lactate, and pyruvate. T2D was confirmed enzymatically in fibroblasts: potassium-dependent acetoacetyl-CoA thiolase activity was reduced (activity ratio with/without K^+: 0.9 vs 2.1 in control cells). *T2* gene analysis revealed 2 previously described mutations: G152A and N158D.[2]

Neurological Findings

The patient had mild dysarthria, generalized choreic movements, and myoclonic jerks (see video). Cranial nerve examination revealed abnormal saccade initiation. Tandem gait was difficult. The remainder of the neurological examination was unremarkable.

Brain MRI

Brain MRI at age 18 years showed marked atrophy of the putamen and hyperintensities of the putamen and caudate nuclei (diffusion-weighted image) (Fig. 1). T2-weighted axial imaging revealed hyperintensity of the posterolateral putamen (see Fig. 1.).

Discussion

To our knowledge, this is the first description of chorea as the main feature of T2D. Previously reported neurological findings in T2D include ataxia,[3,4] dystonia,[4] and myoclonus.[3] Hypotonia and developmental delay in the absence of identifiable ketoacidotic crises were reported in 4 T2D patients,[5] including 3 who had bilateral defects in the posterolateral putamen.

Intriguingly, our patient had no recorded severe ketoacidosis. The episode of gastroenteritis and acidosis at age 5 years was less severe than most reported metabolic decompensations in undiagnosed T2D,[6] and the abnormal movements were present long before this episode.

Under normal circumstances, degradation of the amino acid isoleucine (see Supplementary Fig. 1), which is obstructed in T2D, can occur in the brain[7] and in the liver, where most isoleucine is metabolized. This patient's clinical course suggests that neurological complications in T2D, which usually are attributed to severe ketoacidotic episodes, may result from a brain-specific mechanism, such as toxic accumulation of isoleucine-derived acyl-CoA esters in brain mitochondria. Such a cell-autonomous, tissue-specific mechanism could occur even in the absence of systemic decompensations.[8]

Evidence supports a direct neurotoxic mechanism in T2D. A metabolite that is specific to T2D, 2-methylacetoacetate, can induce oxidative stress in rat cerebral cortex.[9] Also, hereditary deficiency of the preceding enzyme of isoleucine degradation, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, has been associated with choreoathetoid movements[10] and abnormal basal ganglia imaging. Such brain-specific events with the characteristics of ischemia on imaging studies, but with a nonvascular distribution ("metabolic strokes") are increasingly recognized in diseases of the mitochondrial respiratory chain and organic acidurias.

T2D should be considered in patients with unexplained chorea. History should include careful questioning about previous ketoacidotic episodes; however, as reported here, neurological complications of T2D may occur even without such decompensations. In patients with T2D, urinary organic acid analysis usually strongly suggests the diagnosis, which can be confirmed by enzymatic and molecular testing. Because of the broad clinical spectrum of T2D, and because preventive treatment can reduce or eliminate the occurrence of metabolic crises, siblings of an affected individual should be tested, even if they are neurologically asymptomatic and have no history of ketoacidotic crises. ■

Legend to the Video

Video 1. The patient presents choreic movements combined with some myoclonic jerks.

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Commentary

There is an increasing body of evidence implicating mitochondrial dysfunction in the pathogenesis of Huntington's disease.[1] It is known that patients who have defects of the mitochondrial respiratory chain occasionally present with chorea and have bilateral symmetric lesions of the pallidum on MRI.[2] There is also a well established relation between organic acidurias and chorea. For instance, in a review of 25 patients who had acidurias of several etiologies, Gaston and colleagues identified chorea in 7 of their patients.[3]

β-Ketothiolase (also known as 3-oxothiolase, or 2-methylacetoacetyl-coenzyme A [CoA] thiolase, or mitochondrial acetoacetyl-CoA thiolase [T2]) deficiency (T2D) is an autosomal recessive aciduria that has only rarely been reported in association with movement disorders. In the current issue of *Movement* Disorders, Buhas and colleagues describe a patient with generalized chorea, myoclonus, ataxia, and delayed motor development but with preserved cognition.[4] Their report is interesting not only because this seems to be the first case of chorea in relation to T2D but also because the movement disorder seems to be unrelated to episodes of acidosis, the usual cause of neurological deterioration in most individuals with T2D. Finally, a word of caution before considering T2D as a treatable cause of chorea: The dichotomy between effective treatment of the metabolic acidosis in such cases and failure of improvement of the movement disorder is also well exemplified by another

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Brief Communication

Development of MLPA for human *ACAT1* gene and identification of a heterozygous Alu-mediated deletion of exons 3 and 4 in a patient with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency

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ABSTRACT

Mitochondrial acetoacetyl-CoA thiolase deficiency is an autosomal recessive disorder, characterized by intermittent ketoacidosis. We developed a multiplex ligation-dependent probe amplification method for mutation detection in the *ACAT1* gene, which encodes this enzyme, and validated it using DNAs from two previously reported patients having partial deletion and duplication in this gene. Using this method, we identified a heterozygous deletion including exons 3–4 in a third patient, likely due to Alu-mediated non-equal homologous recombination between Alu sequences.

1. Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9) deficiency is an autosomal recessive disorder, affecting isoleucine and ketone body metabolism. The disorder is clinically characterized by intermittent ketoacidotic crises with no symptoms in between episodes [1,2]. More than 100 patients have been reported (including personal communications). However, the incidence of T2 deficiency has not yet been defined in most populations. An incidence of 1 in 232 000 has been reported in Minnesota, USA [3]. The T2 gene (*ACAT1*), located on chromosome 11q22.3–23.1, comprises 12 exons spreading over approximately 27 kb [4]. So far, at least 50 different mutations have been identified in T2-deficient patients ([5–10] and unpublished data). Most of the mutations reported in *ACAT1* are point mutations, small insertions and deletions. Only one large deletion including exons 2–4 [9] and a homozygous tandem duplication of exons 8 and 9 [8], which were caused by Alu element-mediated non-equal homologous recombination, have been identified.

Alu sequences are the most abundant repeats in the human genome, which contains more than 1 million copies of Alu sequences distributed throughout the genome with an average spacing of 4 kb. Alu-mediated rearrangement events have long been recognized as a common cause of local deletion and duplication events associated with human genetic disease [11–14].

Homozygous large deletions can be suspected by absence of PCR amplification of deleted exons [9]. However, detection of heterozygous deletions is difficult using routine genomic PCR amplification. Multiplex ligation-dependent probe amplification (MLPA) has been proven to be an efficient and reliable technique for copy number analysis for each exon [15–19].

In the present study, we describe the establishment of MLPA for *ACAT1* and the identification of a deletion including exons 3–4. We show this deletion to be caused by Alu-mediated non-equal homologous recombination between Alu sequences in introns 2 and 4 in the *ACAT1* in a T2-deficient patient.

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2. Materials and methods

2.1. Patient

GK44 is a Caucasian female born to non-consanguineous parents. She presented with a severe ketoacidotic crisis at 9 months of age. Laboratory testing showed blood gas pH < 7.05, base excess −23 mmol/L, blood glucose 3.4 mmol/L, ammonia 61 mol/L (normal < 50), 3-hydroxybutyrate 7.9 mmol/L (normal 0–0.3), and urinary ketones 3+. Urinary organic acid analysis showed massive excretion of 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate (with total butanone > 500 mol/mmol creatinine; normal < 5). Enzyme assay confirmed the diagnosis of T2 deficiency (see Supplementary data 1). Fasting avoidance was recommended and 'sick day regime' was implemented. She had no further ketoacidotic crises following the confirmation of the diagnosis. She is now 18 years old, healthy and of normal psychomotor development.

2.2. DNA and RNA extraction and mutation analysis

Genomic DNA was purified from the fibroblasts with Sepa gene kits (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed at the genomic level, by PCR and direct sequencing, using a primer set for fragments including exons and their intron boundaries [6]. RNA was prepared from the fibroblasts using the ISOGEN kit (Nippon Gene, Tokyo, Japan). RT-PCR and sequencing was performed as described previously [5,6,10].

2.3. Structural analysis

The T2 crystal structure 2IBW [20] has been used for the structural analysis. The program COOT [21] has been used for analyzing the structure and CCP4MG [22] has been used for making the figures.

2.4. MLPA probe set development and analysis

The target sequence of each synthetic half probe was designed using H-MAPD program [23]. One probe pair for each exon of the *ACAT1* gene was designed (see Supplemental data 2). MLPA reactions were performed according to the manufacturer's instructions (MRC Holland, The Netherlands) with 100 ng genomic DNA and EK1 MLPA reagent kit and the P200-A1 human DNA Reference kit that includes reference probes and MLPA control fragments (MRC Holland, The Netherlands). The PCR products were separated by capillary electrophoresis on an ABI 3130XL genetic analyzer (Applied Biosystems, Warrington, UK). The Gene Mapper v 4.0 software (Applied Biosystems) was used to analyze the runs and to retrieve peak intensities corresponding to each probe in the different samples, and integrated peak areas were exported to an Excel 2003 spreadsheet. Data generated by the combination of *ACAT1* synthetic probe mix and P200-A1 probe mix were intra-normalized by dividing the peak area of amplification product of each probe by the total area of only the reference probes in P200-A1. Secondary normalization was achieved by dividing this intra-normalized probe ratio in a sample by the average intra-normalized probe ratio of all reference samples.

2.5. Deletion breakpoint characterization.

The region surrounding the deletion from intron 2 to intron 5 in the T2-deficient patient was amplified with a sense primer (Ps: 5′-AAGGAG GGCCATTACAGCATCTCCTAGGAC-3′) located at positions g.15781–15810 in intron 2 and an antisense primer (Pa: 5′-GGACTTAGCAAA TCCAGACACTCTTGAGCA-3′) located at positions g.18868–18838 in intron 5 (Gen-Bank accession: NG_009888.1).

The PCR was carried out for 40 cycles at 94 °C for 1 min, 54 °C for 2 min, 72 °C for 2 min, followed by a 5-min extension at 72 °C, using the Takara Ex-Taq (Takara Shuzo, Japan) and Takara PCR thermal cycler (Takara Shuzo, Japan). After subcloning into the pGEM-T Easy vector system (Promega, USA), the fragment was sequenced.

3. Results and discussion

Enzyme assay and immunoblot analysis confirmed that GK44 was T2 deficient (see Supplemental data 1). However, mutation screening by direct sequencing of all 12 exons in a genomic DNA preparation, including exon/intron boundaries, only detected a heterozygous mutation c. 602C > T(A201V) in exon 7. This A201 is a highly conserved residue among thiolases [20] and structural analysis indicated A201V pathogenicity (Supplemental data 3). cDNA analysis showed that this mutation was identified in all 8 clones analyzed. Hence we considered that the other mutation might be a large deletion, potentially of some exons. Since an MLPA probe kit for *ACAT1* was not available commercially, we established an MLPA method for *ACAT1* gene.

We validated our MLPA method by re-analyzing the DNA of a patient in whom we previously identified a homozygous large deletion including exons 2–4 [9] and a patient in whom we identified a homozygous duplication including exons 8 and 9 [8]. As shown in Fig. 1A, these abnormalities could be detected by our MLPA method. Then a mixture of DNAs from these patients was analyzed to mimic compound heterozygocity of the deletion including exons 2–4 and the duplication including exons 8–9. This abnormality could also be detected by our MLPA method. We then applied our MLPA method to GK44's DNA and a heterozygous deletion including exons 3 and 4 was found. Since skipping of exons 3 and 4 results in frame shift in T2 mRNA, such truncated mRNA is subject to nonsense-mediated mRNA decay and is therefore hardly detected in cDNA analysis. Clearly, without an MLPA analysis, it would have been very difficult to identify this mutation.

The molecular basis of exons 3 and 4 deletion was then investigated. *ACAT1* has a high density of Alu elements and contains 33 partial and complete Alu sequences, comprising 32.7% of the whole T2 gene. As shown in Fig. 1B, intron 2 has three Alu sequences and intron 4 has two Alu sequences. We hypothesized that a non-equal homologous recombination between Alu sequences in intron 2 and intron 4 has occurred, generating a deletion including exons 3 and 4. Hence a long-range PCR was carried out on genomic DNA with a pair of primers in intron 2 (5′ from three Alu sequences) and intron 5 (Fig. 1B). This PCR successfully amplified a 3.1-kb fragment in controls and 1.2 kb fragment in GK44 (Fig. 1C). Direct sequencing this junction fragment confirmed the breaking point in GK44. The recombined sequence was compared with the control sequences of introns 2 and 4. An Alu–Y sequence in intron 2 had recombined with another Alu–Sc sequence in intron 4, resulting in the formation of a new full-length Alu sequence (see Supplemental data 4). The breakpoints occurred within the region of a completely homologous sequence of 35 bp in between two Alu sequences. We previously identified a similar homologous recombination between Alu sequences in intron 1 and intron 4 [9].

In summary, we have successfully developed MLPA analysis for the *ACAT1* gene in which defects are responsible for T2 deficiency. We identified a heterozygous deletion of exons 3 and 4 in a patient with T2 deficiency. This is the third patient with *ACAT1* gene rearrangement caused by Alu-mediated unequal homologous recombination. We now have a useful tool for mutation detection in the *ACAT1* gene.

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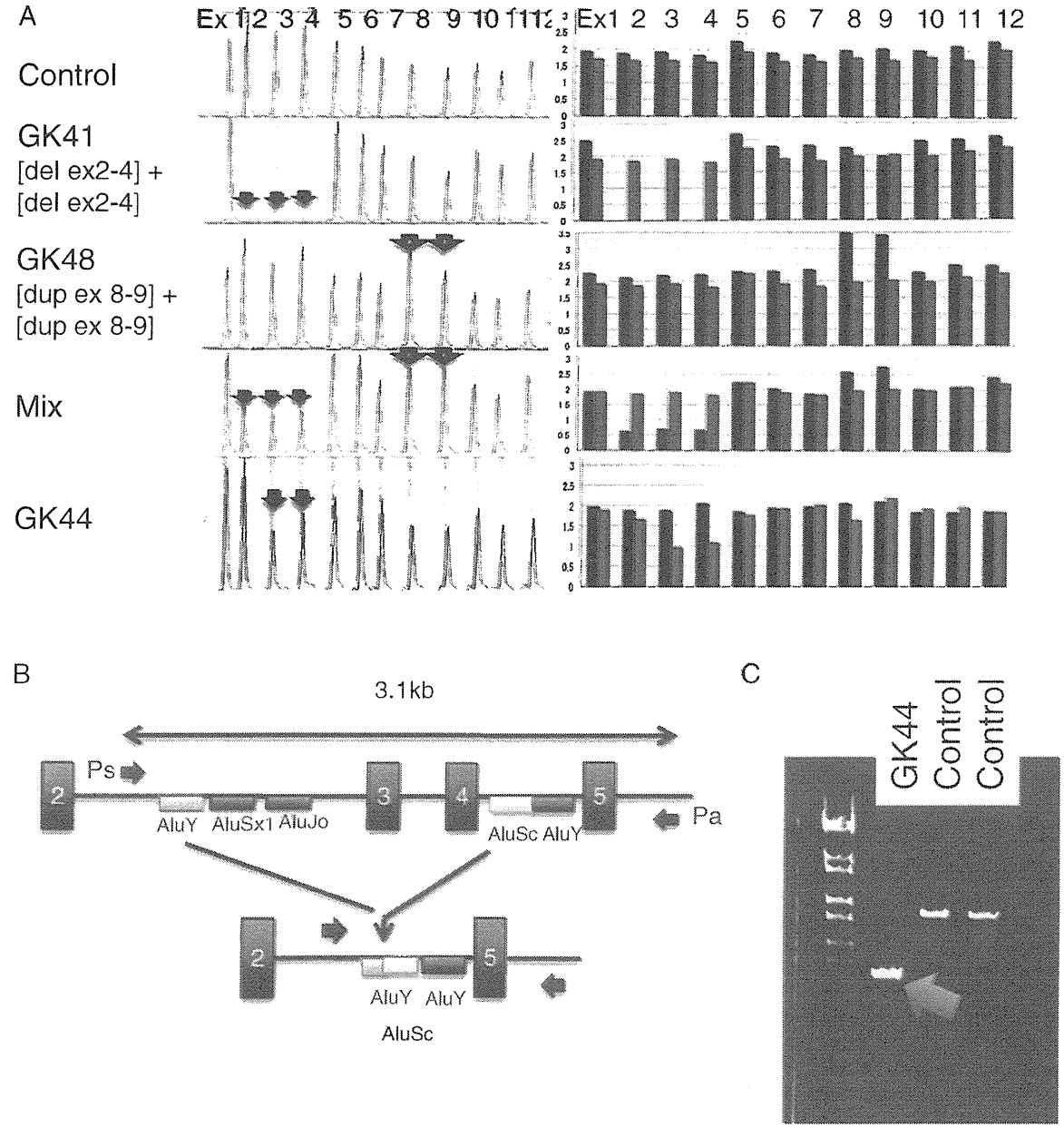

Fig. 1. A) MLPA analysis of *ACAT1* gene. The MLPA profiles of each *ACAT1* exon are shown. The peaks derived from P200-A1 reference probes followed by the peak of exon 12 are not shown. The histogram of a calculated exonic dosage normalized as described in Materials and methods is shown. Blue bars and red bars indicate patient's and one control's dosages, respectively. Closed arrows and open arrows indicate deletions and duplications, respectively. 'Mix' indicates a mixture of equal micrograms of GK41 and GK48's DNA samples. B) Schematic presentation of PCR amplification using genomic DNA. The positions of PCR primers (Ps and Pa) are shown. Intron 2 and intron 4 have three and two Alu sequences, respectively. An arrow indicates a non-equal homologous recombination site. GK44 had breakpoints within Alu-Y and Alu Sc elements in introns 2 and 4, respectively. (C) Long-range PCR analysis. A 3.1-kb (approx.) fragment was amplified in controls but only a 1.2 kb fragment was amplified in GK44.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgme.2013.07.004.

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ORIGINAL ARTICLE

Three novel mutations in the carnitine–acylcarnitine translocase (*CACT*) gene in patients with CACT deficiency and in healthy individuals

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Carnitine–acylcarnitine translocase (CACT) and carnitine palmitoyltransferase II (CPT2) are key enzymes for transporting long-chain fatty acids into mitochondria. Deficiencies of these enzymes, which are clinically characterized by life-threatening non-ketotic hypoglycemia and rhabdomyolysis, cannot be distinguished by acylcarnitine analysis performed using tandem mass spectrometry. We had previously reported the *CPT2* genetic structure and its role in CPT2 deficiency. Here, we analyzed the *CACT* gene in 2 patients diagnosed clinically with CACT deficiency, 18 patients with non-traumatic rhabdomyolysis and 58 healthy individuals, all of whom were confirmed to have normal *CPT2* genotypes. To facilitate *CACT* genotyping, we used heat-denaturing high-performance liquid chromatography (DHPLC), which helped identify five distinct patterns. The abnormal heteroduplex fragments were subjected to *CACT*-specific DNA sequencing. We found that one patient with CACT deficiency, Case 1, carried c.576G>A and c.199-10t>g mutations, whereas Case 2 was heterozygous for c.106-2a>t and c.576G>A. We also found that one patient with non-traumatic rhabdomyolysis and one healthy individual were heterozygous for c.804delG and the synonymous mutation c.516T>C, respectively. In summary, c.576G>A, c.106-2a>t and c.516T>C are novel *CACT* gene mutations. Among the five mutations identified, three were responsible for CACT deficiency. We have also demonstrated the successful screening of *CACT* mutations by DHPLC.
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Keywords: β-oxidation; CACT deficiency; carnitine-acylcarnitine translocase (CACT); CPT2 deficiency; denaturing high-performance liquid chromatography (DHPLC); rhabdomyolysis

INTRODUCTION

Carnitine–acylcarnitine translocase (CACT), as well as carnitine palmitoyltransferase II (CPT2), has pivotal roles in the carnitine cycle, which is the β-oxidation pathway of long-chain fatty acids.[1] The *CACT* gene is known to be associated with the *SLC25* gene family, *SLC25A20*. In 1997, the human *CACT* cDNA was cloned and sequenced by Huizing *et al.*[2] The entire gene, spanning about 42 kb on chromosome 3p21.31, contains 9 exons and codes a 301-amino-acid protein.[3,4] Cases of CACT deficiency are rare, and only 40 cases at most have been reported worldwide.[5–7] Although defects in CACT induce lethal neonatal episodes of coma due to hypoketotic hypoglycemia, cardiomyopathy, cardiac arrhythmia and rhabdomyolysis, quite a few patients exhibit milder phenotypes compatible with longer life spans.[8,9]

CACT deficiency (OMIM No. 212138) cannot be easily differentiated from severe cases with CPT2 deficiency (OMIM No. 255110) on the basis of clinical manifestations and blood acylcarnitine profiles analyzed using tandem mass spectrometry;[10–13] however, genetic analysis could be utilized to distinguish between the two conditions. CACT deficiency is so rare that the characteristics of the *CACT* gene are not yet fully understood. In this study, we analyzed the *CACT* gene in 2 patients diagnosed clinically with CACT deficiency, 18 Japanese patients with non-traumatic rhabdomyolysis and 58 healthy Japanese individuals. As little information is available regarding *CACT* polymorphisms and mutations, we introduced heat-denaturing high-performance liquid chromatography (DHPLC) to screen for the *CACT* gene.[14] To confirm that DHPLC would be suitable for screening these gene mutations and variants, the following strategy was carried out. First, we sequenced all fragments showing heteroduplex patterns; second, we sequenced all the exons of the *CACT* gene in randomly selected individuals; third, we included data for two patients who had died of CACT deficiency; and fourth, the data were confirmed by family studies.

In this study, we identified mutations responsible for CACT deficiency and screened *CACT* mutations with DHPLC. To the best

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of our knowledge, the two infant cases are only the second and third cases wherein the genotypes of Japanese patients with CACT deficiency have been fully confirmed.

SUBJECTS AND METHODS

Patients diagnosed clinically with CACT deficiency

Case 1. The patient was the second child of non-consanguineous Japanese parents. His sister was in good health. His mother had pregnancy-induced hypertension. He was born at 36 weeks and 5 days of gestation by cesarean section. He had decreased respiratory functions and experienced asphyxia. At 2 days of age, repeated apnea, muscular hypotonia, irritability, oliguria, hypoglycemia, liver dysfunction and cryptogenic rhabdomyolysis were noted. Although CPT2 or CACT deficiency was suspected on the basis of acylcarnitine profiles of plantar blood spots (Table 1), the subsequent DNA analysis performed at our laboratory showed that his *CPT2* gene was normal (data not shown).[15] The patient was clinically diagnosed with CACT deficiency. At the age of 2 years and 9 months, he died of symptoms resembling those of Reye syndrome. (Case 1 is going to be reported in Japanese from a clinical point of view in an upcoming issue of the Journal of the Japan Pediatric Society by Sugiyama, *et al.*)

Case 2. The patient was the second child of non-consanguineous Japanese parents. His elder sister was healthy. The pregnancy was uneventful, and at 37 weeks of gestation, he was born by normal vaginal delivery. At 2 days of age, he suddenly became cyanotic and flaccid and then went into cardiac arrest. He died at 3 days of age. Tandem mass spectrometry analysis of acylcarnitines in a postmortem blood spot card showed increases in C14, C16 and C18 acylcarnitines (Table 1), which led us to consider a defect in either CACT or CPT2. After the patient died, the physician in charge brought the blood disc of the patient and whole blood from his parents to our laboratory for genetic analysis in order to reach a final diagnosis. Direct DNA sequencing performed on genomic DNA from the parents confirmed the absence of mutations in the *CPT2* gene (data not shown).[15] We highly suspected that the patient had died of CACT deficiency.

Patients with non-traumatic rhabdomyolysis

Eighteen patients with non-traumatic rhabdomyolysis (12 men and 6 women; mean age ± s.d., 51.1 ± 19.6 years) were retrospectively recruited from the outpatient clinic of Fukuoka University Hospital and Clinic, Fukuoka, Japan. The mean ± s.d. value of their peak serum creatine kinase levels was 22 522.7 ± 7 224.4 IU l^{-1}. The patients had no history of statin therapy. DNA sequencing carried out at our laboratory indicated that all patients had normal *CPT2* genotypes (data not shown).[15]

Healthy volunteers

The 58 individuals in our healthy panel included 47 men and 11 women (mean ± s.d. age, 28.3 ± 7.1 years). They had no recent history of either heavy sport activities or specific drug usage. The *CPT2* genotypes of these individuals were determined by DNA sequencing and were found to be normal without exception (data not shown).[15]

Table 1 Acylcarnitine levels in dried blood spots from Cases 1 and 2

Acylcarnitine	*Case 1 (μM)*	*Case 2 (μM)*	*Upper 95% limit (μM)*
C0	9.37	11.85	60.0
C14	0.66	1.35	0.4
C14:1	0.17	0.56	0.1
C14-OH	0.064	0.24	0.050
C16	4.77	12.07	1.9
C16:1	0.78	1.84	0.3
C18:1	ND	3.07	2.1

Abbreviation: ND, not determined.

Amplification of genomic DNA

Genomic DNA purified from peripheral blood cells was PCR amplified using nine pairs of *CACT*-specific primers (Table 2), as previously described.[4,16] PCR conditions were as follows: 95 °C for 1 min; 30 cycles each of 95 °C for 1 min, specific annealing temperature for 1 min, and 72 °C for 1 min, followed by a final extension step at 60 °C for 10 min.

DNA sequence analysis

CACT-specific direct DNA sequencing was performed on all PCR products using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the results were analyzed on the automated ABI Prism 310 Genetic Analyzer (Applied Biosystems), as described by the manufacturer. Cumulative sequences were compared with the *CACT* sequence published in the OMIM database (http://www.ncbi.nlm.nih.gov/nuccore/319918843).

Homoduplex or heteroduplex formation

CACT-specific PCR products were purified using Microcon (Millipore Corp., Billerica, MA, USA). PCR product of each test was mixed with the comparable PCR product from a normal donor. The mixtures were denatured at 95 °C for 10 min and then slowly cooled down to 65 °C at a rate of 1 °C min^{-1}. Following 5-min incubation at 65 °C, the samples were cooled to 4 °C at a rate of 1 °C every 5 s.

DHPLC analysis

PCR-amplified products were analyzed using the Nucleic Acid Fragment Analysis System (Transgenomic, Inc., San Jose, CA, USA). The results have been presented in the form of chromatographic peaks by using the Navigator software. Complete sequence-matched hybridization produced a single homoduplex peak, whereas hybridization with sequences containing mutation(s) resulted in heteroduplexes and aberrant peaks with different retention times. PCR products that showed heteroduplex patterns by DHPLC were subjected to *CACT*-specific direct sequencing using PCR primers as described above.

Ethical consideration

Written informed consent for genetic investigations was obtained from all patients and family members, as well as from volunteers. The Fukuoka University Ethics Committee investigated and approved this research project (12-12 [08-77]).

Table 2 Primers for amplifying and sequencing the *CACT* gene

PCR product	*Exon*	*Primer*	*Sequence (5′–3′)*	*Size (bp)*	*Tm (°C)*
CACT 1	1	1F	GGTCGAGAACTGCAGACGGAG	247	70
		1R	ACACATGCCCTCTTCTGCCCAG		
CACT 2	2	2F	CAGGCAGTTCTGATTCTGGT	194	60
		2R	AACCCCGTGAATGTGTTCTG		
CACT 3	3	3F	AAAGGTGGTGGTGTCTGTAAAC	228	64
		3R	GTCACGCTACCAGGCACAAC		
CACT 4	4	4F	CTCGGTGGTTAGTCACAGG	251	66
		4R	GCCACTGCACCCAGTCCTGA		
CACT 5	5	5F	CCTGCTGGGTCTGTGACTCTGA	224	70
		5R	CCACTTCAGGTGACCTTCCCCA		
CACT 6	6	6F	TGGCGAAGAGTTTATGTACTTT	233	60
		6R	CAGACATGGAGCCAAGAACA		
CACT 7	7	7F	GCCACTCTCACAGCCTTTGTCTA	237	70
		7R	TATGAGCTTTGCACCCCAGGATTA		
CACT 8	8	8F	GACTTAACTCGTAGTTTCTCCT	255	62
		8R	GGAACAAGCAAAAGTCAAACCA		
CACT 9	9	9F	AATAGCCTATGAATAGTTATTCC	187	66
		9R	TTACTACTCCTTCTCCTCAACGA		

Abbreviation: CACT, carnitine–acylcarnitine translocase.

RESULTS

DHPLC analysis and subsequent DNA sequencing of the *CACT* gene

We used DHPLC to facilitate the screening of unknown mutations in the *CACT* gene. PCR-amplified fragments of all nine *CACT* exons from a randomly chosen healthy volunteer were directly sequenced and were found to have no mutations. On using autologous DHPLC analysis, no heteroduplex pattern was found in any exon (Figure 1); therefore, this DNA fragment was used as the reference for further DHPLC studies.

We performed DHPLC analysis for nine exons of the *CACT* gene amplified from Case 1, Case 2, and their parents. As expected, we found heteroduplex patterns at exons 3 and 6 (namely, CACT 3 and CACT 6, respectively) in Case 1, as well as exon 2 (CACT 2) and CACT 6 in Case 2 (Figure 2). The segregations of CACT heteroduplexes were subsequently confirmed by family studies.

Our results from direct DNA sequencing of the *CACT* gene showed that Case 1 had the c.576G>A and c.199-10t>g mutations. The former mutation, which has never been described elsewhere, introduces a premature stop codon at the amino-acid residue 192, tryptophan, whereas the latter is a previously reported splicing acceptor mutation at the intron 1/exon 2 junction and results in the skipping of both exons 3 and 4 or only exon 3.[17] The genetic segregation was subsequently confirmed by family studies using genomic DNA from his parents (data not shown). Based on these genetic analyses, we confirmed that Case 1 had CACT deficiency.

For Case 2, at first only genomic DNA samples from the parents were sequenced, because the only samples that were available for Case 2 were a few residual blood spots obtained for neonatal screening. We found that the father had c.106-2a>t at the splice acceptor site of intron 1, which was another novel mutation identified in this study, whereas the mother had the c.576G>A mutation in exon 6, the same novel mutation shared by Case 1. As Case 2 was found to have a normal *CPT2* gene, we highly suspected that he had CACT deficiency. Next, exons 2 and 6 of Case 2 were sequenced to determine the presence of these mutations. The DHPLC and partial sequencing results described above, together with full-length exon sequencing and DHPLC of the *CACT* gene of his parents, confirmed the presence of the c.106-2a>t mutation at the junction of intron 1 and exon 2, as well as c.576G>A in exon 6. Based on these genetic analyses, Case 2 was finally diagnosed with CACT deficiency. To the best of our knowledge, the c.576G>A and c.106-2a>t genotypes have never been reported in the literature. It is also worth noting that c.576G>A was seen in both Cases 1 and 2.

In parallel, we investigated *CACT* mutations in 18 patients with non-traumatic rhabdomyolysis and 58 healthy volunteers by using DHPLC. Among the 18 patients, only 1 heteroduplex was found in exon 8 (CACT 8; Figure 2). The mutation, identified as c.804delG by DNA sequencing, resulted in a frameshift at codon 269. This patient, Case 3, was a 74-year-old Japanese man with hypertension and previously undetected rhabdomyolysis (peak serum creatine kinase level, 4832 IU ml^{-1}) with renal injury (serum creatinine level, 2.2 mg dl^{-1}). The CACT enzymatic activity in the fibroblasts was determined on a later day and found to be within the normal range (101 pmol · min^{-1} · mg^{-1} vs the normal range, 59–308 pmol · min^{-1} · mg^{-1}; data from Dr Ronald J. A. Wanders).[18]

Among the 58 healthy individuals, 1 heteroduplex was found at exon 5 of the *CACT* gene (CACT 5). This individual was a 33-year-old man, namely, Case 4. This exon 5 mutation was subsequently confirmed by DNA sequencing as c.516T>C, which resulted in the synonymous mutation T172T (Figure 2). This genetic alteration is the third novel *CACT* mutation identified in this report.

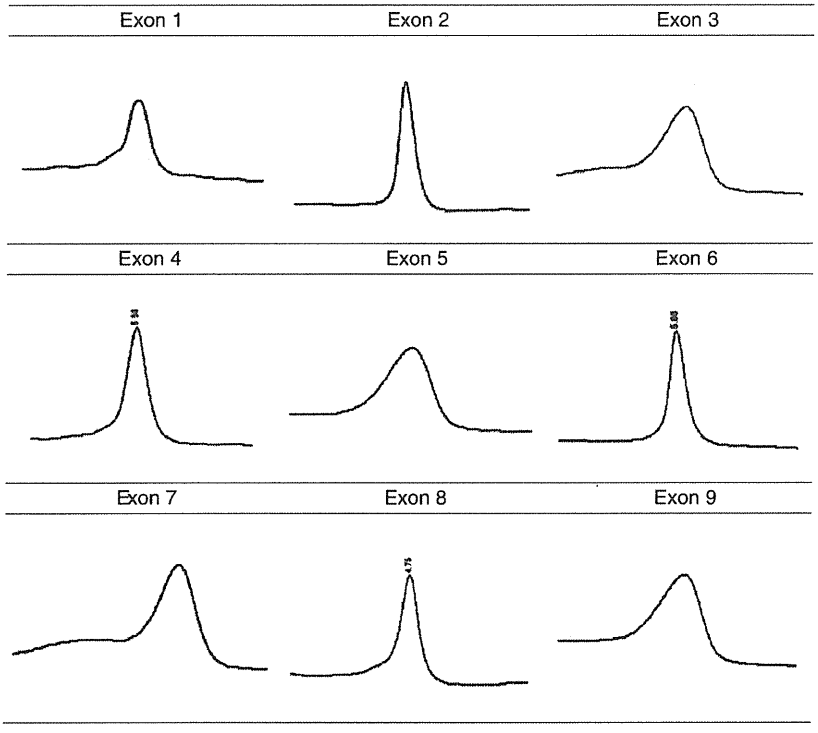

Figure 1 DHPLC patterns of homoduplexes obtained with normal *CACT* exons. Complete sequence-matched hybridization between two normal or autologous sequences produced a single peak for each exon.

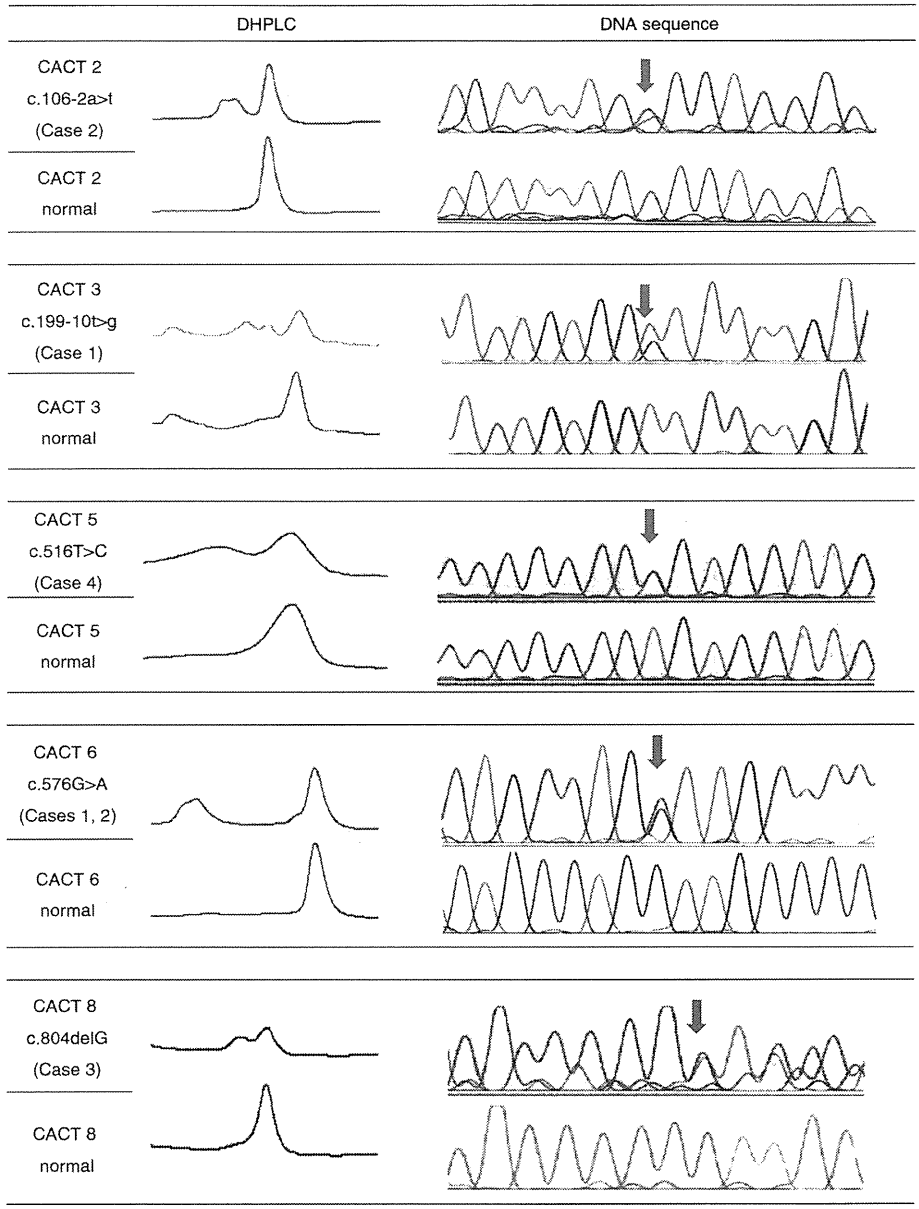

Figure 2 Aberrant DHPLC patterns of heteroduplexes and corresponding DNA sequences. Hybridization of unmatched normal and mutated sequences generated heteroduplexes, thereby resulting in aberrant peaks with different retention times. Red arrows indicate positions of mutations identified by direct DNA sequencing.

To confirm the reliability of DHPLC, 13 healthy individuals without any DHPLC heteroduplex were randomly selected, and their DNA was subjected to full-length DNA sequencing. No mutations were found in any exon of their *CACT* gene (data not shown).

DISCUSSION

CACT, as well as CPT2, is one of the key enzymes that participate in the intake of long-chain fatty acids into the mitochondrial matrix; therefore, defective activities of these enzymes result in impaired β-oxidation of fatty acids and lack of indispensable energy. CPT2 deficiency and CACT deficiency are known to induce a wide range of clinical manifestations, from lethal neonatal episodes of coma due to hypoketotic hypoglycemia, cardiomyopathy and cardiac arrhythmia to the adult-type of non-traumatic rhabdomyolysis.[5,8,19] CPT2 deficiency consists of a lethal neonatal form, an infantile (hepatic) form and an adult-onset (muscular) form. The genetic characteristics of CPT2 deficiency have been well analyzed. We have previously described gene mutations in seven patients with definitive CPT2 deficiency, and by determining their genotypes, we found Japanese patient-specific mutations; however, we failed to find any relationship between their genotypes and clinical manifestations.[15,20,21] On the other hand, the genetic characteristics of CACT deficiency have not been well described, due to the scarcity of the disease. To the best of our knowledge, only 40 patients at most have been reported

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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