A

B

Figure 2. Analysis of RNA intermediates encompassing exons 12 and 13. **A:** Positions of the SCOT-specific primers for analysis of RNA intermediates encompassing exons 12 and 13. **B:** RT-PCR. cDNA was synthesized with isolated hnRNA using a mixture of SCOT-specific antisense primers including 12R′, 13R′, and 14R′. PCR was done using the indicated primers. A fragment with arrow 1 was a SECRI in which intron 12 was spliced out. A fragment with arrow 2 had skipping of exons 12 and 13. Fragments with arrow 3 had a retention of intron 12.

(ex15R), a SECRI in which intron 14 was spliced out was faintly detected until 40 min in the control.

GS23 fibroblasts

Intermediates that retained intron 12 remained with similar amounts for the full duration of the chase and no SECRIs in which intron 12 was spliced out were detected, in either primer combinations of in11F/ex13R or ex12F/in13R. The SECRIs in which intron 13 was spliced out were also not amplified in either combination of in12F/ex14R or ex13F/in14R. The SECRI in which intron 14 was spliced out was amplified with in13F and ex15R primers.

Discussion

We identified an apparently homozygous mutation c.1248+5g>a in a SCOT-deficient patient. This mutation resulted in major aberrant mRNA with skipping of exons 12 and 13. It is well known that, among donor splice-site mutations, changes at the G residue at position 1 are most commonly described, followed by mutations at position 5. Mutations at these 2 positions are thought to significantly reduce the pairing of the donor splice site with the complementary site in the small nuclear ribonucleoprotein particle U1snRNP [Kramer, 1996]. Buratti et al. (2007) summarized 346 aberrant splice donor sites that were activated by mutations in 166 human diseases. Point mutations leading to cryptic splice donor site activation were most common in the first intron nucleotide, followed by the fifth nucleotide. Substitutions at position +5 were exclusively g>a transitions. In our case, the c.1248+5g>a mutation

accordingly resulted in a drastically reduced Shapiro and Senapathy score [Shapiro and Senapathy, 1987] at the authentic splice donor site of intron 13 from 79.0 (CT/gtaagt) to 64.6 (CT/gtaaat). Hence, it is predicted that c.1248+5g>a should cause aberrant splicing such as exon 13 skipping, but surprisingly, this mutation caused skipping of both exons 12 and 13.

In RT-PCR analysis using RNA from CHX-treated and CHX-untreated fibroblasts, we clearly showed that cDNA with skipping of exons 12 and 13 was the major transcript and was subjected to NMD. Both the cDNA with exon 13 skipping and the cDNA with exons 8–13 skipping should be minor and side transcripts because the former was not detected even in the CHX-treated condition and the latter is in-frame and had a relatively smaller amount than the major cDNA with skipping of exons 12 and 13 in the CHX-treated condition.

There are several reports of mutations at single-splice junctions that result in the skipping of two or more exons [Fang et al., 2001; Haire et al., 1997; Hayashida et al., 1994; Schneider et al., 1993; Takahara et al., 2002; Yamada et al., 2007]. The splicing order of introns can explain the events of aberrant splicing. Takahara et al. (2002) analyzed the molecular basis of skipping of exons 5 and 6 of COL5A1 due to a splice acceptor site of intron 5 mutation, by determination of the splicing order of introns 4, 5, and 6. They suggested that the acceptor-site mutation of intron 5 leads to the removal of the two downstream exons (exons 5 and 6), because those exons can be fused to a "single" exon, whereas the next downstream intron (intron 6) and the mutation-bearing intron (intron 4) remain in the transcript. Their finding could be applicable for a splice donor-site mutation, as the removal of the upstream intron creates a "single" exon that, if the next upstream intron is retained, allows
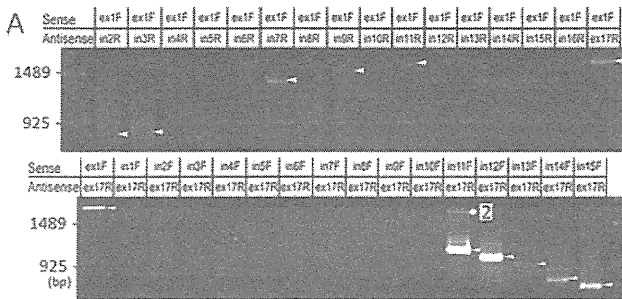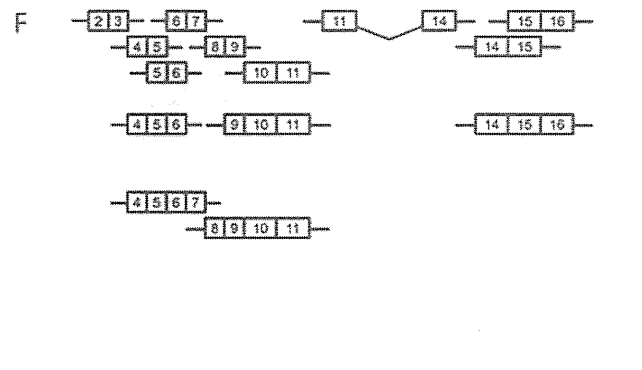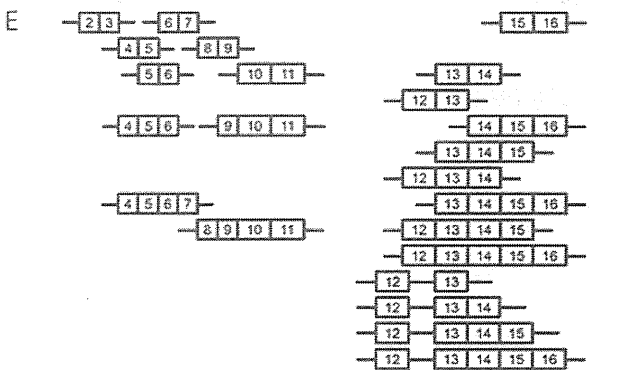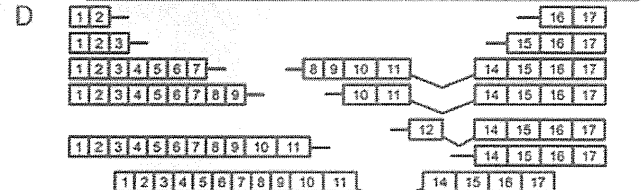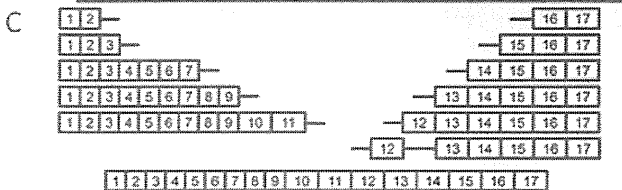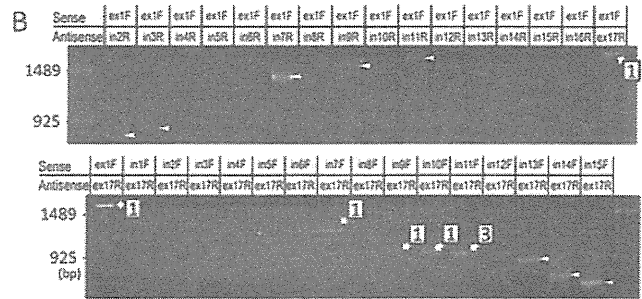
Figure 3. Detection of large SECRIs including exon 1 or exon 17. **A and B**: Detection of large SECRIs including exon 1 or exon 17. All fragments indicated by arrows and arrowheads were analyzed by direct sequencing. Fragments indicated by arrowhead without number had neither skipping of any exon nor retention of any intron. Fragments with arrow 1 had skipping of exons 12 and 13. A fragment with arrow 2 had a retention of intron 12. A fragment with arrow 3 had exon 13 skipping. **C and D**: Schematic presentations of SECRIs identified. **E and F**: All SECRIs flanked by introns which were detected (Supp. Fig. S1) are also schematically presented.

the skipping of the two exons upstream from the mutation site. We initially hypothesized that our case had the same molecular basis as described above. This outcome was actually reported in the case of a splice-donor site in the neurofibromatosis type 1 gene [Fang et al., 2001]. However, a SECRI comprising exons 12 and 13 was not detected in GS23 fibroblasts, suggesting that this pathway was not a major one.

Kessler et al. (1993) reported a method to determine the order of intron removal using RT-PCR in conjunction with appropriate combinations of intron and exon primers for any small pre-mRNA in vivo. Several reports determined the order of intron removal by this method [Attanasio et al., 2003; Schwarze et al., 1999]. Kessler's original method requires amplification spanning one intron; for example, amplification including whole intron 3 using 5′ primer in exon 3 and 3′ primer in intron 4. However, SCOT gene (gene symbol OXCT1) includes 17 exons and spans more than 150 kb. Because all introns except intron 12 were too long to amplify (1.2–33 kb), we could not determine a precise splicing order of OXCT1 gene by this original method.

Our strategy involved (1) detection of a possible last intron to be spliced by amplification of SECRIs using combinations of sense primer in exon 1 and antisense primer in each intron and using combinations of antisense primer in the last exon and sense primer in

each intron and (2) detection of any SECRIs comprising two, three, four, five, six sequential exons, and so on using intronic primers flanking adjacent exons or larger exon clusters (Fig. 3). This method is less definitive than the original method. Because the method relies on the analysis of steady-state RNA, we cannot rule out the possibility that some RT-PCR products may be side products or dead-end molecules in nuclear RNA. Moreover, the failure to identify a SECRI could be the consequence of at least two factors. The first would be retention of a larger upstream or downstream intron and the second would be the very rapid removal of the intron in which one primer was placed. Hence, even though we could construct several potential pathways of splicing, such pathways may not represent the major pathway. Even with the above limitations, our strategy may provide useful information on the molecular basis of splicing abnormality in a large gene such as SCOT gene.

We tried to assess order of removal of introns 11, 12, 13, and 14 in a dynamic fashion using actinomycin D treatment to halt transcription and with primer setting according to the Kessler's original method, as performed in several reports [Attanasio et al., 2003; Kessler et al., 1993; Schwarze et al., 1999]. As discussed above, because introns 11, 13, and 14 are too long to amplify intermediates including these introns, we could not determine the definite order of intron removal. However, these results could be best explained
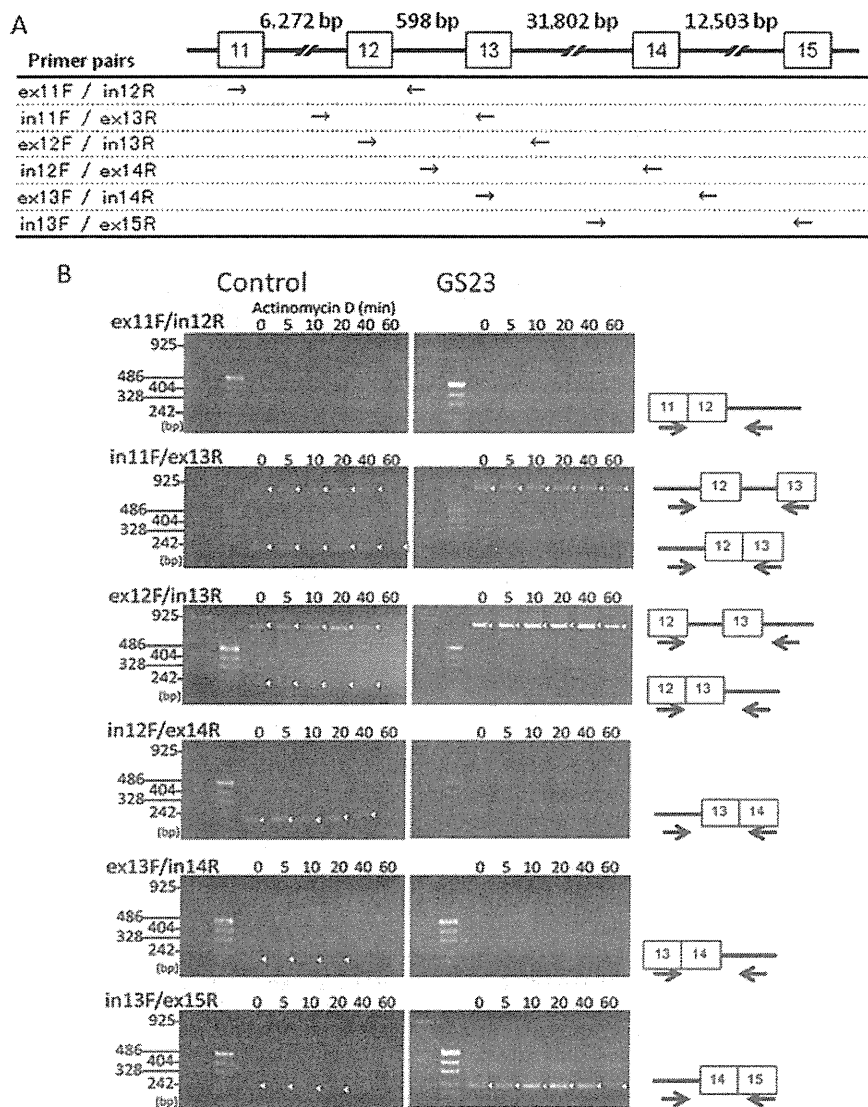
Figure 4. Analysis of intron removal around exons 11–14 using Actinomycin D. A: PCR primer combinations used for the analysis of intron removal. B: Total RNA was prepared from cultured fibroblasts after defined time intervals (0, 5, 10, 20, 40, and 60 min) of exposure to Actinomycin D, treated with DNase, reverse transcribed, amplified with indicated primer pairs, and electrophoresed on 2% agarose gels. Amplified fragments are indicated by arrowhead.

as follows: in control, intron 11 was the last intron to be spliced and intron 12 removal was also rather slow and occurred after intron 13 removal in the major pathway, although a minor pathway in which intron 12 splicing occurred before intron 13 was also present. In GS23, since intron 13 was mutated, retention of intron 13 occurred for a long time. This halt of intron13 splicing may result in intron 12 retention and intron 11 retention. This "splicing paralysis" [Schwarze et al. 1999] may be a molecular basis of two-exon skipping. This splicing paralysis could be solved by skipping of the whole intron 11–exon 12–intron 12–exon 13–mutated intron 13. The presence of all SECRIs detected, as shown in Figure 3, is consistent with the above explanation.

According to recent studies, splicing occurs during transcription (co-transcriptional splicing) and introns are removed in a general 5′–3′ order. However, as in our case, splicing does not always occur as a linear process starting at 5′ end of the primary transcript.

Many different factors can influence the order of splicing of SCOT transcripts, including sequences at splice junctions, the length and sequences of introns, the sequences of adjacent exons, and RNA secondary structures. Supp. Table S2 summarizes the lengths of introns and exons in the SCOT transcript, and Shapiro and Senapathy's scores [Shapiro and Senapathy, 1987] of splice-site junctions. This analysis did not reveal a definitive factor that would explain the order of intron removal.

In summary, our study showed that (1) a single nucleotide substitution at the 5′ splice site of intron 13 (c.1248+5g>a) in GS23's fibroblasts causes skipping of exons 12 and 13 predominantly, that (2) the formation of SECRI comprising exon 12 and 13 was not the cause of skipping of exons 12 and 13 in GS23's fibroblasts, that (3) the mutation resulted in retention of intron 13, thus causing the retention of introns 12 and 11, and this "splicing paralysis" was the molecular basis of two-exon skipping.

Acknowledgment

References

Attanasio C, David A, Neerman-Arbez M. 2003. Outcome of donor splice site mutations accounting for congenital afibrinogenemia reflects order of intron removal in the fibrinogen alpha gene (FGA). Blood 101:1851–1856.

Baric I, Sarnavka V, Fumic K, Maradin M, Begovic D, Ruiter JP, Wanders RJ. 2001. A new case of succinyl-CoA:acetoacetate transferase deficiency: favourable course despite very low residual activity. J Inherit Metab Dis 24:81–82.

Berget SM. 1995. Exon recognition in vertebrate splicing. J Biol Chem 270:2411–2414.

Berry GT, Fukao T, Mitchell GA, Mazur A, Ciafre M, Gibson J, Kondo N, Palmieri MJ. 2001. Neonatal hypoglycaemia in severe succinyl-CoA: 3-oxoacid CoA-transferase deficiency. J Inherit Metab Dis 24:587–595.

Buratti E, Chivers M, Kralovicova J, Romano M, Baralle M, Krainer AR, Vorechovsky I. 2007. Aberrant 5′ splice sites in human disease genes: mutation pattern, nucleotide structure and comparison of computational tools that predict their utilization. Nucleic Acids Res 35:4250–4263.

Cornblath M, Gingell RL, Fleming GA, Tildon JT, Leffler AT, Wapnir RA. 1971. A new syndrome of ketoacidosis in infancy. J Pediatr 79:413–418.

Fang LJ, Simard MJ, Vidaud D, Assouline B, Lemieux B, Vidaud M, Chabot B, Thirion JP. 2001. A novel mutation in the neurofibromatosis type 1 (NF1) gene promotes skipping of two exons by preventing exon definition. J Mol Biol 307:1261–1270.

Fukao T, Song XQ, Watanabe H, Hirayama K, Sakazaki H, Shintaku H, Imanaka M, Orii T, Kondo N. 1996. Prenatal diagnosis of succinyl-coenzyme A:3-ketoacid coenzyme A transferase deficiency. Prenat Diagn 16:471–474.

Fukao T, Song XQ, Mitchell GA, Yamaguchi S, Sukegawa K, Orii T, Kondo N. 1997. Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. Pediatr Res 42:498–502.

Fukao T, Mitchell GA, Song XQ, Nakamura H, Kassovska-Bratinova S, Orii KE, Wraith JE, Besley G, Wanders RJ, Niezen-Koning KE, Berry GT, Palmieri M, et al. 2000. Succinyl-CoA:3-ketoacid CoA transferase (SCOT): cloning of the human SCOT gene, tertiary structural modeling of the human SCOT monomer, and characterization of three pathogenic mutations. Genomics 68:144–151.

Fukao T, Shintaku H, Kusubae R, Zhang GX, Nakamura K, Kondo M, Kondo N. 2004. Patients homozygous for the T435N mutation of succinyl-CoA:3-ketoacid CoA Transferase (SCOT) do not show permanent ketosis. Pediatr Res 56:858–863.

Fukao T, Sakurai S, Rolland MO, Zabot MT, Schulze A, Yamada K, Kondo N. 2006. A 6-bp deletion at the splice donor site of the first intron resulted in aberrant splicing using a cryptic splice site within exon 1 in a patient with succinyl-CoA: 3-Ketoacid CoA transferase (SCOT) deficiency. Mol Genet Metab 89:280–282.

Fukao T, Kursula P, Owen EP, Kondo N. 2007. Identification and characterization of a temperature-sensitive R268H mutation in the human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene. Mol Genet Metab 92:216–221.

Fukao T, Ishii T, Amano N, Kursula P, Takayanagi M, Murase K, Sakaguchi N, Kondo N, Hasegawa T. 2010. A neonatal-onset succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patient with T435N and c.658-666dupAACGTGATT p.N220_I222dup mutations in the OXCT1 gene. J Inherit Metab Dis 33:636.

Fukao T, Sass JO, Kursula P, Thimm E, Wendel U, Ficicioglu C, Monastiri K, Guffon N, Baric I, Zabot MT, Kondo N. 2011. Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. Biochim Biophys Acta 1812:619–624.

Haire RN, Ohta Y, Strong SJ, Litman RT, Liu Y, Prchal JT, Cooper MD, Litman GW. 1997. Unusual patterns of exon skipping in Bruton tyrosine kinase are associated with mutations involving the intron 17 3′ splice site. Am J Hum Genet 60:798–807.

Hawkins JD. 1988. A survey on intron and exon lengths. Nucleic Acids Res 16:9893–9908.

Hayashida Y, Mitsubuchi H, Indo Y, Ohta K, Endo F, Wada Y, Matsuda I. 1994. Deficiency of the E1 beta subunit in the branched-chain alpha-keto acid dehydrogenase complex due to a single base substitution of the intron 5, resulting in two alternatively spliced mRNAs in a patient with maple syrup urine disease. Biochim Biophys Acta 1225:317–325.

Hernan I, Gamundi MJ, Planas E, Borras E, Maseras M, Carballo M. 2011. Cellular expression and siRNA-mediated interference of rhodopsin cis-acting splicing mutants associated with autosomal dominant retinitis pigmentosa. Invest Ophthalmol Vis Sci 52(6):3723–3729.

Kassovska-Bratinova S, Fukao T, Song XQ, Duncan AM, Chen HS, Robert MF, Perez-Cerda C, Ugarte M, Chartrand C, Vobecky S, Kondo N, Mitchell GA. 1996. Succinyl CoA: 3-oxoacid CoA transferase (SCOT): human cDNA cloning, human chromosomal mapping to 5p13, and mutation detection in a SCOT-deficient patient. Am J Hum Genet 59:519–528.

Kessler O, Jiang Y, Chasin LA. 1993. Order of intron removal during splicing of endogenous adenine phosphoribosyltransferase and dihydrofolate reductase pre-mRNA. Mol Cell Biol 13:6211–6222.

Kramer A. 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu Rev Biochem 65:367–409.

Longo N, Fukao T, Singh R, Pasquali M, Barrios RG, Kondo N, Gibson KM. 2004. Succinyl-CoA:3-ketoacid transferase (SCOT) deficiency in a new patient homozygous for an R217X mutation. J Inherit Metab Dis 27:691–692.

Maniatis T, Reed R. 2002. An extensive network of coupling among gene expression machines. Nature 416:499–506.

Maquat LE. 2005. Nonsense-mediated mRNA decay in mammals. J Cell Sci 118:1773–1776.

Merron S, Akhtar R. 2009. Management and communication problems in a patient with succinyl-CoA transferase deficiency in pregnancy and labour. Int J Obstet Anesth 18:280–283.

Mitchell GA, Fukao T. 2001. Chapter 102. Inborn errors of ketone body catabolism. In: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, editors. Metabolic and Molecular Bases of Inherited Disease (8th edition). New York: McGraw–Hill. p2327–2356.

Niezen-Koning KE, Wanders RJ, Ruiter JP, Ijlst L, Visser G, Reitsma-Bierens WC, Heymans HS, Reijngoud DJ, Smit GP. 1997. Succinyl-CoA:acetoacetate transferase deficiency: identification of a new patient with a neonatal onset and review of the literature. Eur J Pediatr 156:870–873.

Perez-Cerda C, Merinero B, Sanz P, Jimenez A, Hernandez C, Garcia MJ, Ugarte M. 1992. A new case of succinyl-CoA: acetoacetate transferase deficiency. J Inherit Metab Dis 15:371–373.

Pretorius CJ, Loy Son GG, Bonnici F, Harley EH. 1996. Two siblings with episodic ketoacidosis and decreased activity of succinyl-CoA:3-ketoacid CoA-transferase in cultured fibroblasts. J Inherit Metab Dis 19:296–300.

Rolland MO, Guffon N, Mandon G, Divry P. 1998. Succinyl-CoA:acetoacetate transferase deficiency. Identification of a new case; prenatal exclusion in three further pregnancies. J Inherit Metab Dis 21:687–688.

Sakazaki H, Hirayama K, Murakami S, Yonezawa S, Shintaku H, Sawada Y, Fukao T, Watanabe H, Orii T, Isshiki G. 1995. A new Japanese case of succinyl-CoA: 3-ketoacid CoA-transferase deficiency. J Inherit Metab Dis 18:323–325.

Schneider S, Wildhardt G, Ludwig R, Royer-Pokora B. 1993. Exon skipping due to a mutation in a donor splice site in the WT-1 gene is associated with Wilms' tumor and severe genital malformations. Hum Genet 91:599–604.

Schwarze U, Starman BJ, Byers PH. 1999. Redefinition of exon 7 in the COL1A1 gene of type I collagen by an intron 8 splice-donor-site mutation in a form of osteogenesis imperfecta: influence of intron splice order on outcome of splice-site mutation. Am J Hum Genet 65:336–344.

Shapiro MB, Senapathy P. 1987. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res 15:7155–7174.

Snyderman SE, Sansaricq C, Middleton B. 1998. Succinyl-CoA:3-ketoacid CoA-transferase deficiency. Pediatrics 101:709–711.

Song XQ, Fukao T, Mitchell GA, Kassovska-Bratinova S, Ugarte M, Wanders RJ, Hirayama K, Shintaku H, Churchill P, Watanabe H, Orii T, Kondo N. 1997. Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT): development of an antibody to human SCOT and diagnostic use in hereditary SCOT deficiency. Biochim Biophys Acta 1360:151–156.

Song XQ, Fukao T, Watanabe H, Shintaku H, Hirayama K, Kassovska-Bratinova S, Kondo N, Mitchell GA. 1998. Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency: two pathogenic mutations, V133E and C456F, in Japanese siblings. Hum Mutat 12:83–88.

Takahara K, Schwarze U, Imamura Y, Hoffman GG, Toriello H, Smith LT, Byers PH, Greenspan DS. 2002. Order of intron removal influences multiple splice outcomes, including a two-exon skip, in a COL5A1 acceptor-site mutation that results in abnormal pro-alpha1(V) N-propeptides and Ehlers–Danlos syndrome type I. Am J Hum Genet 71:451–465.

Tildon JT, Cornblath M. 1972. Succinyl-CoA: 3-ketoacid CoA-transferase deficiency. A cause for ketoacidosis in infancy. J Clin Invest 51:493–498.

Williamson DH, Bates MW, Page MA, Krebs HA. 1971. Activities of enzymes involved in acetoacetate utilization in adult mammalian tissues. Biochem J 121:41–47.

Yamada K, Fukao T, Zhang G, Sakurai S, Ruiter JP, Wanders RJ, Kondo N. 2007. Single-base substitution at the last nucleotide of exon 6 (c.671G>A), resulting in the skipping of exon 6, and exons 6 and 7 in human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene. Mol Genet Metab 90:291–297.

ORIGINAL ARTICLE

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

Naeem Shafqat · Kate L. Kavanagh · Jörn Oliver Sass ·
Ernst Christensen · Toshiyuki Fukao · Wen Hwa Lee ·
Udo Oppermann · Wyatt W. Yue

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.

N. Shafqat · K. L. Kavanagh · W. H. Lee · U. Oppermann ·
W. W. Yue (✉)
Structural Genomics Consortium, University of Oxford,
Oxford OX3 7DQ, UK
e-mail: wyatt.yue@sgc.ox.ac.uk

J. O. Sass
Zentrum für Kinder- und Jugendmedizin, Universitätsklinikum
Freiburg, 79106 Freiburg, Germany

J. O. Sass
Klinische Chemie & Biochemie, Universitäts-Kinderspital,
8032 Zürich, Switzerland

E. Christensen
Department of Clinical Genetics, Juliane Marie Centre,
2100 Copenhagen, Denmark

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

T. Fukao
Department of Pediatrics, Graduate School of Medicine,
Gifu University, Gifu 501-1194, Japan

T. Fukao
Medical information Sciences Division, United Graduate
School of Drug Discovery and Medical Information Sciences,
Gifu University, Gifu 501-1194, Japan

U. Oppermann
Botnar Research Centre, Oxford Biomedical Research Unit,
OX3 7LD Oxford, UK

🖄 Springer

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

🖄 Springer

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*
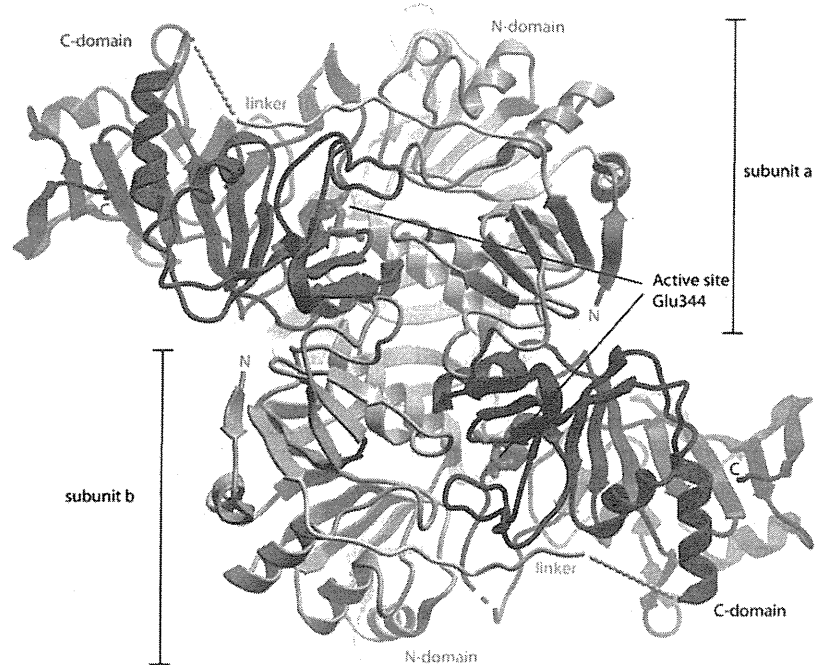

Experimental procedures

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
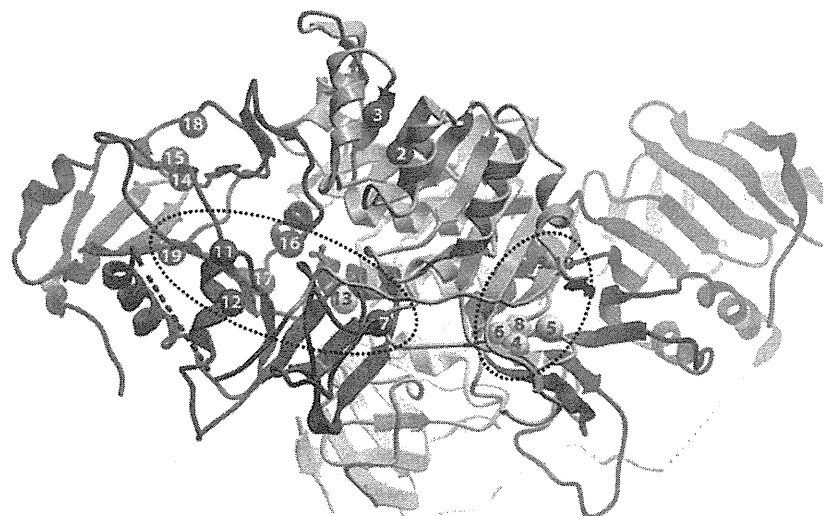
Data collection & structure determination

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).

References

Alkén J (2008) Glucose and ketone body metabolism—with emphasis on ketotic hypoglycemia. Ph.D. Thesis, Karolinska Institutet, Stockholm

Bateman KS, Brownie ER, Wolodko WT, Fraser ME (2002) Structure of the mammalian CoA transferase from pig heart. Biochemistry 41:14455–14462

Berry GT, Fukao T, Mitchell GA, Mazur A, Ciafre M, Gibson J, Kondo N, Palmieri MJ (2001) Neonatal hypoglycaemia in severe succinyl-CoA: 3-oxoacid CoA-transferase deficiency. J Inherit Metab Dis 24:587–595

CCP4 (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr D: Biol Crystallogr 50:760–763

Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr D: Biol Crystallogr 60:2126–2132

Fukao T, Song XQ, Mitchell GA, Yamaguchi S, Sukegawa K, Orii T, Kondo N (1997) Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. Pediatr Res 42:498–502

Fukao T, Mitchell GA, Song XQ, Nakamura H, Kassovska-Bratinova S, Orii KE, Wraith JE, Besley G, Wanders RJ, Niezen-Koning KE, Berry GT, Palmieri M, Kondo N (2000) Succinyl-CoA:3-ketoacid CoA transferase (SCOT): cloning of the human SCOT gene, tertiary structural modeling of the human SCOT monomer, and characterization of three pathogenic mutations. Genomics 68:144–151

Fukao T, Shintaku H, Kusubae R, Zhang GX, Nakamura K, Kondo M, Kondo N (2004) Patients homozygous for the T435N mutation of succinyl-CoA:3-ketoacid CoA Transferase (SCOT) do not show permanent ketosis. Pediatr Res 56:858–863

Fukao T, Kursula P, Owen EP, Kondo N (2007) Identification and characterization of a temperature-sensitive R268H mutation in the human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene. Mol Genet Metab 92:216–221

Fukao T, Ishii T, Amano N, Kursula P, Takayanagi M, Murase K, Sakaguchi N, Kondo N, Hasegawa T (2010) A neonatal-onset succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patient with T435N and c.658-666dupAACGTGATT p.N220_I222dup mutations in the OXCT1 gene. J Inherit Metab Dis doi:10.1007/s10545-010-9168-5

Fukao T, Sass JO, Kursula P, Thimm E, Wendel U, Ficicioglu C, Monastiri K, Guffon N, Baric I, Zabot MT, Kondo N (2011) Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. Biochim Biophys Acta 1812:619–624

Heider J (2001) A new family of CoA-transferases. FEBS Lett 509:345–349

Kassovska-Bratinova S, Fukao T, Song XQ, Duncan AM, Chen HS, Robert MF, Perez-Cerda C, Ugarte M, Chartrand C, Vobecky S, Kondo N, Mitchell GA (1996) Succinyl CoA: 3-oxoacid CoA transferase (SCOT): human cDNA cloning, human chromosomal mapping to 5p13, and mutation detection in a SCOT-deficient patient. Am J Hum Genet 59:519–528

Longo N, Fukao T, Singh R, Pasquali M, Barrios RG, Kondo N, Gibson KM (2004) Succinyl-CoA:3-ketoacid transferase (SCOT) deficiency in a new patient homozygous for an R217X mutation. J Inherit Metab Dis 27:691–692

McCoy AJ, Grosse-Kunstleve RW, Storoni LC, Read RJ (2005) Likelihood-enhanced fast translation functions. Acta Crystallogr D: Biol Crystallogr 61:458–464

Mitchell GA, Fukao T (2001) Inborn errors of ketone body catabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) Metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 2327–2356

Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr D: Biol Crystallogr 53:240–255

Niezen-Koning KE, Wanders RJ, Ruiter JP, Ijlst L, Visser G, Reitsma-Bierens WC, Heymans HS, Reijngoud DJ, Smit GP (1997) Succinyl-CoA:acetoacetate transferase deficiency: identification of a new patient with a neonatal onset and review of the literature. Eur J Pediatr 156:870–873

Perrakis A, Harkiolaki M, Wilson KS, Lamzin VS (2001) ARP/wARP and molecular replacement. Acta Crystallogr D: Biol Crystallogr 57:1445–1450

Sakazaki H, Hirayama K, Murakami S, Yonezawa S, Shintaku H, Sawada Y, Fukao T, Watanabe H, Orii T, Isshiki G (1995) A new Japanese case of succinyl-CoA: 3-ketoacid CoA-transferase deficiency. J Inherit Metab Dis 18:323–325

Sass JO (2012) Inborn errors of ketogenesis and ketone body utilization. J Inherit Metab Dis 35:23–28

Snyderman SE, Sansaricq C, Middleton B (1998) Succinyl-CoA:3-ketoacid CoA-transferase deficiency. Pediatrics 101:709–711

Solomon F, Jencks WP (1969) Identification of an enzyme-gamma-glutamyl coenzyme A intermediate from coenzyme A transferase. J Biol Chem 244:1079–1081

Song XQ, Fukao T, Watanabe H, Shintaku H, Hirayama K, Kassovska-Bratinova S, Kondo N, Mitchell GA (1998) Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency: two pathogenic mutations, V133E and C456F, in Japanese siblings. Hum Mutat 12:83–88

Tanaka H, Kohroki J, Iguchi N, Onishi M, Nishimune Y (2002) Cloning and characterization of a human orthologue of testis-specific succinyl CoA: 3-oxo acid CoA transferase (Scot-t) cDNA. Mol Hum Reprod 8:16–23

Williamson DH, Bates MW, Page MA, Krebs HA (1971) Activities of enzymes involved in acetoacetate utilization in adult mammalian tissues. Biochem J 121:41–47

Yamada K, Fukao T, Zhang G, Sakurai S, Ruiter JP, Wanders RJ, Kondo N (2007) Single-base substitution at the last nucleotide of exon 6 (c.671 G>A), resulting in the skipping of exon 6, and exons 6 and 7 in human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene. Mol Genet Metab 90:291–297

A Treatable New Cause of Chorea:
Beta-Ketothiolase Deficiency

Daniela Buhaş, MD,[1,3] Geneviève Bernard, MD,[2,3] Toshiyuki Fukao, MD, PhD,[4] Jean-Claude Décarie, MD,[5,8]
Sylvain Chouinard, MD,[6] and Grant A. Mitchell, MD[7,8]*

[1]Department of Medical Genetics, Montreal Children's Hospital, Montréal, Quebéc, Canada
[2]Department of Neurology and Neurosurgery, Montreal Children's Hospital, Montréal, Québec, Canada
[3]McGill University Health Centre, Montreal Children's Hospital, Montréal, Québec, Canada
[4]Department of Pediatrics, Gifu University Graduate School of Medicine, Gifu, Japan
[5]Department of Medical Imaging, CHUM, Université de Montréal, Montréal, Québec, Canada
[6]CHU Sainte-Justine, Université de Montréal, Montréal, Québec, Canada
[7]André Barbeau Movement Disorders Unit, CHUM, Université de Montréal, Montréal, Québec, Canada
[8]Division of Medical Genetics, Department of Pediatrics, Université de Montréal, Montréal, Québec, Canada

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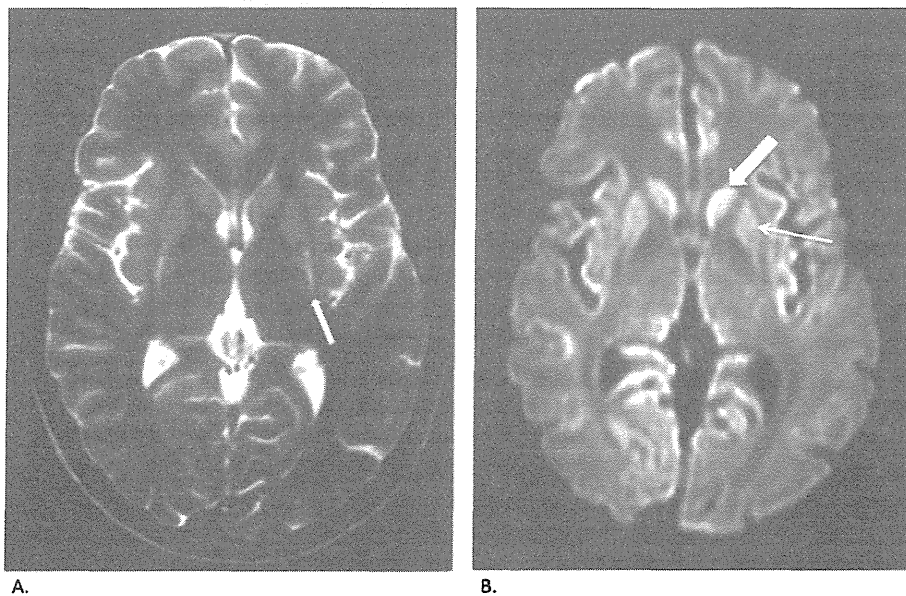
FIG. 1. (A) This T2-weighted axial image shows high signal and atrophy of the posterolateral putamen (white arrow). **(B)** This diffusion weighted image shows atrophy of the putamen with high signal intensity in the putamen (small white arrow) and caudate (large white arrow) nuclei.

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, $\leq$12 µmol/mmol) and 2-methylacetoacetate (1566 µmol/mmol creatinine; normal, $\leq$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.

References

1. Sedel F, Saudubray JM, Roze E, Agid Y, Vidailhet M. Movement disorders and inborn errors of metabolism in adults: a diagnostic approach. J Inherit Metab Dis 2008;31:308–318.

2. Zhang GX, Fukao T, Rolland Mo, et al. Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency: T2-deficient patients with "mild" mutation(s) were previously misinterpreted as normal by the coupled assay with tiglyl-CoA. Pediatr Res 2004;56:60–64.

3. Middleton B, Bartlett K, Romanos A, et al. 3-ketothiolase deficiency. Eur J Pediatr 1986;144:586–589.

4. Yalcinkaya C, Apaydin H, Ozekmekci S, Gibson KM. Delayed-onset dystonia associated with 3-oxothiolase deficiency. Mov Disord 2001;16:372–375.

5. Ozand PT, Rashed M, Gascon GG, et al. 3-Ketothiolase deficiency: a review and four new patients with neurologic symptoms. Brain Dev 1994;1(suppl):38–45.

6. Fukao T, Scriver CR, Kondo N; T2 Collaborative Working Group. The clinical phenotype and outcome of mitochondrial acetoacetyl-CoA thiolase deficiency (beta-ketothiolase or T2 deficiency) in 26 enzymatically proved and mutation-defined patients. Mol Genet Metab 2001;72:109–114.

7. Murin R, Mohammadi G, Leibfritz D, Hamprecht B. Glial metabolism of isoleucine. Neurochem Res 2009;34:194–204.

8. Mitchell GA, Gauthier N, Lesimple A, Wang SP, Mamer O, Qureshi I. Hereditary and acquired diseases of acyl-coenzyme A metabolism. Mol Genet Metab 2008;94:4–15.

9. Leipnitz G, Seminotti B, Amaral AU, Fernandes CG, Dutra-Filho CS, Wajner M. Evidence that 2-methylacetoacetate induces oxidative stress in rat brain. Metab Brain Dis 2010;25:261–267.

10. Zschocke J, Ruiter JP, Brand J, Lindner M, et al. Progressive infantile neurodegeneration caused by 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency: a novel inborn error of branched-chain fatty acid and isoleucine metabolism. Pediatr Res 2000;48:852–855.

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]

----

*Correspondence to: Dr. Francisco Cardoso, Professor of Neurology, Av Pasteur 89/1107, 30150-290 Belo Horizonte MG Brazil; cardosofe@terra.com.br

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
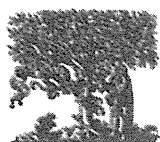
Brief Communication

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

Toshiyuki Fukao [a,b,*], Yuka Aoyama [b], Keiko Murase [a], Tomohiro Hori [a], Rajesh K. Harijan [c], Rikkert K. Wierenga [c], Avihu Boneh [d], Naomi Kondo [a]

[a] Department of Pediatrics, Graduate School of Medicine, Gifu University, Yanagido 1-1, Gifu 501-1194, Japan
[b] Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1194, Japan
[c] Department of Biochemistry, University of Oulu, Oulu, Finland
[d] Metabolic Genetics, Royal Children's Hospital, Murdoch Children's Research Institute, University of Melbourne, Victoria, Australia

ARTICLE INFO

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.

© 2013 Elsevier Inc. All rights reserved.

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.

* Corresponding author at: Department of Pediatrics, Graduate School of Medicine, Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1194, Japan. Fax: +81 58 230 6387.
E-mail address: toshi-gif@umin.net (T. Fukao).

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, anmonia 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.
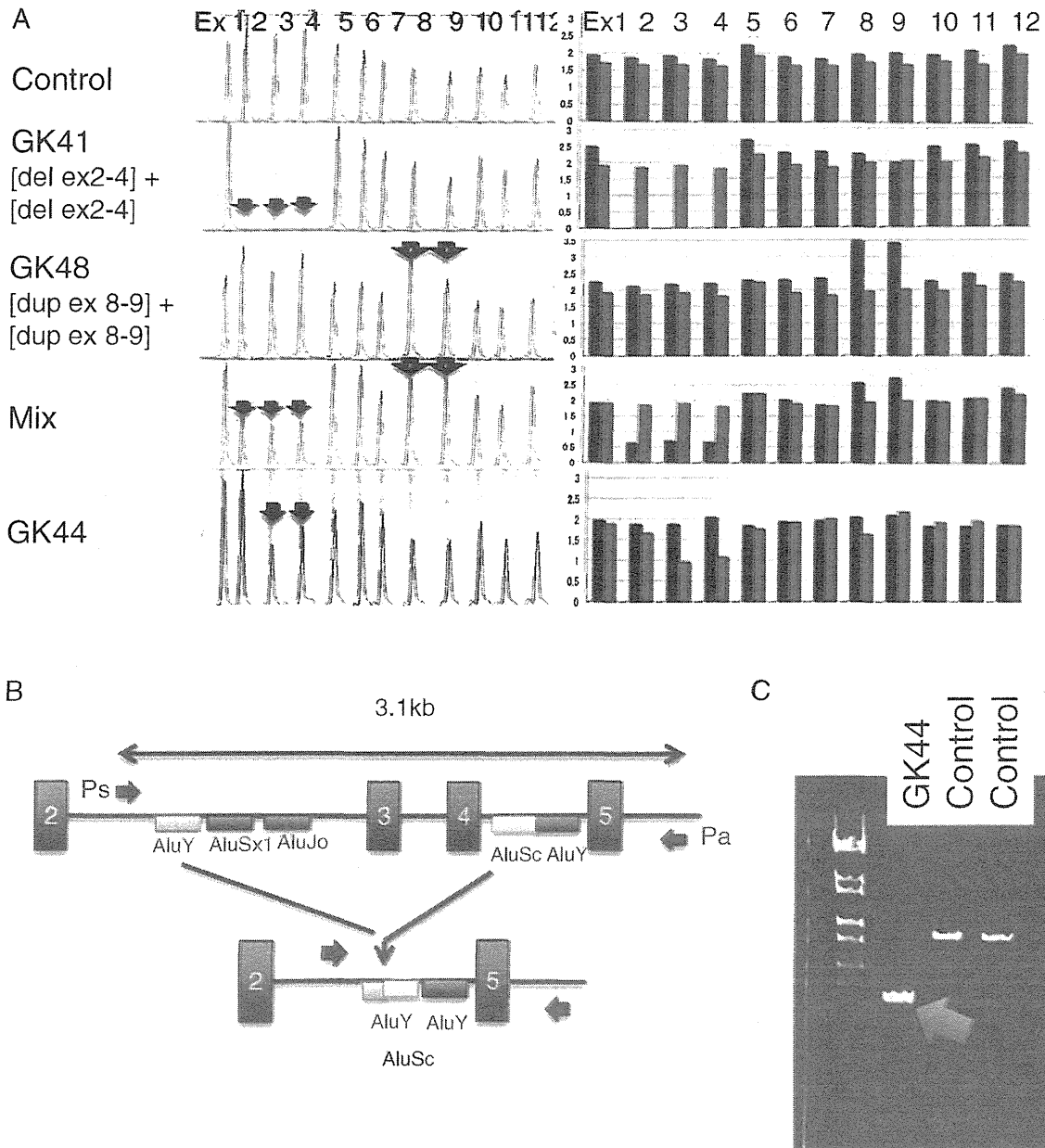
Acknowledgments

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.

References

[1] R.S. Daum, P.H. Lamm, O.A. Mamer, C.R. Scriver, A "new" disorder of isoleucine catabolism, Lancet 2 (1971) 1289–1290.

[2] G.A. Mitchell, T. Fukao, Inborn errors of ketone body metabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic & Molecular Basis of Inherited Disease, McGraw-Hill, New York, 2001, pp. 2327–2356.

[3] K. Sarafoglou, D. Matern, K. Redlinger-Grosse, K. Bentler, A. Gaviglio, C.O. Harding, P. Rinaldo, Siblings with mitochondrial acetoacetyl-CoA thiolase deficiency not identified by newborn screening, Pediatrics 128 (2011) 246–250.

[4] M. Kano, T. Fukao, S. Yamaguchi, T. Orii, T. Osumi, I. Hashimoto, Structure and expression of the human mitochondrial acetoacetyl-CoA thiolase-encoding gene, Genetics 109 (1991) 285–290.

[5] T. Fukao, H. Nakamura, K. Nakamura, C. Perez-Cerda, A. Baldellou, C.R. Barrionuevo, F.G. Castello, Y. Kohno, M. Ugarte, N. Kondo, Characterization of six mutations in five Spanish patients with mitochondrial acetoacetyl-CoA thiolase deficiency: effects of amino acid substitutions on tertiary structure, Mol. Genet. Metab. 75 (2002) 235–243.

[6] T. Fukao, H. Nakamura, X.Q. Song, K. Nakamura, K.E. Orii, Y. Kohno, M. Kano, S. Yamaguchi, T. Hashimoto, T. Orii, N. Kondo, Characterization of N93S, I312T, and A333P missense mutations in two Japanese families with mitochondrial acetoacetyl-CoA thiolase deficiency, Hum. Mutat. 12 (1998) 245–254.

[7] T. Fukao, C.R. Scriver, N. Kondo, The clinical phenotype and outcome of mitochondrial acetoacetyl-CoA thiolase deficiency (beta-ketothiolase or T2 deficiency) in 26 enzymatically proved and mutation-defined patients, Mol. Genet. Metab. 72 (2001) 109–114.

[8] T. Fukao, G. Zhang, M.O. Rolland, M.T. Zabot, N. Guffon, Y. Aoki, N. Kondo, Identification of an Alu-mediated tandem duplication of exons 8 and 9 in a patient with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency, Mol. Genet. Metab. 92 (2007) 375–378.

[9] G. Zhang, T. Fukao, S. Sakurai, K. Yamada, K. Michael Gibson, N. Kondo, Identification of Alu-mediated, large deletion-spanning exons 2–4 in a patient with mitochondrial acetoacetyl-CoA thiolase deficiency, Mol. Genet. Metab. 89 (2006) 222–226.

[10] G.X. Zhang, T. Fukao, M.O. Rolland, M.T. Zabot, G. Renom, E. Touma, M. Kondo, N. Matsuo, N. Kondo, Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency: T2-deficient patients with "mild" mutation(s) were previously misinterpreted as normal by the coupled assay with tiglyl-CoA, Pediatr. Res. 56 (2004) 60–64.

[11] M.A. Batzer, P.L. Deininger, Alu repeats and human genomic diversity Nature reviews, Genetics 3 (2002) 370–379.

[12] B. Calabretta, D.L. Robberson, H.A. Barrera-Saldana, T.P. Lambrou, G.F. Saunders, Genome instability in a region of human DNA enriched in Alu repeat sequences, Nature 296 (1982) 219–225.

[13] P.L. Deininger, M.A. Batzer, Alu repeats and human disease, Mol. Genet. Metab. 67 (1999) 183–193.

[14] S.K. Sen, K. Han, J. Wang, J. Lee, H. Wang, P.A. Callinan, M. Dyer, R. Cordaux, P. Liang, M.A. Batzer, Human genomic deletions mediated by recombination between Alu elements, Am. J. Hum. Genet. 79 (2006) 41–53.

[15] V. Gatta, O. Scarciolla, A.R. Gaspari, C. Palka, M.V. De Angelis, A. Di Muzio, P. Guanciali-Franchi, G. Calabrese, A. Uncini, L. Stuppia, Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA), Hum. Genet. 117 (2005) 92–98.

[16] K.K. Lai, I.F. Lo, T.M. Tong, L.Y. Cheng, S.T. Lam, Detecting exon deletions and duplications of the DMD gene using Multiplex Ligation-dependent Probe Amplification (MLPA), Clin. Biochem. 39 (2006) 367–372.

[17] T. Lalic, R.H. Vossen, J. Coffa, J.P. Schouten, M. Guc-Scekic, D. Radivojevic, M. Djurisic, M.H. Breuning, S.J. White, J.T. den Dunnen, Deletion and duplication screening in the DMD gene using MLPA, Eur. J. Hum. Genet. 13 (2005) 1231–1234.

[18] P. Quarello, E. Garelli, A. Brusco, A. Carando, C. Mancini, P. Pappi, L. Vinti, J. Svahn, I. Dianzani, U. Ramenghi, High frequency of ribosomal protein gene deletions in Italian Diamond-Blackfan anemia patients detected by multiplex ligation-dependent probe amplification assay, Haematologica 97 (2012) 1813–1817.

[19] M. Schwartz, M. Duno, Improved molecular diagnosis of dystrophin gene mutations using the multiplex ligation-dependent probe amplification method, Genet. Test. 8 (2004) 361–367.

[20] A.M. Haapalainen, G. Merilainen, P.L. Pirila, N. Kondo, T. Fukao, R.K. Wierenga, Crystallographic and kinetic studies of human mitochondrial acetoacetyl-CoA thiolase: the importance of potassium and chloride ions for its structure and function, Biochemistry 46 (2007) 4305–4321.

[21] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtan, Features and development of Coot, Acta Crystallogr. D: Biol. Crystallogr. 66 (2010) 486–501.

[22] S. McNicholas, E. Potterton, K.S. Wilson, M.E. Noble, Presenting your structures: the CCP4mg molecular-graphics software, Acta Crystallogr. D: Biol. Crystallogr. 67 (2011) 386–394.

[23] J. Zhi, E. Hatchwell, Human MLPA Probe Design (H-MAPD): a probe design tool for both electrophoresis-based and bead-coupled human multiplex ligation-dependent probe amplification assays, BMC Genomics 9 (2008) 407.

ORIGINAL ARTICLE

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

Takao Fukushima[1], Hidetoshi Kaneoka[1,2], Tetsuhiko Yasuno[1], Yukari Sasaguri[2], Tomoko Tokuyasu[2], Kuniko Tokoro[3], Toshiyuki Fukao[4] and Takao Saito[1,5]

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.
Journal of Human Genetics advance online publication, 3 October 2013; doi:10.1038/jhg.2013.103

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

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 ($101 \, pmol \cdot min^{-1} \cdot mg^{-1}$ vs the normal range, $59–308 \, pmol \cdot min^{-1} \cdot mg^{-1}$; 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.
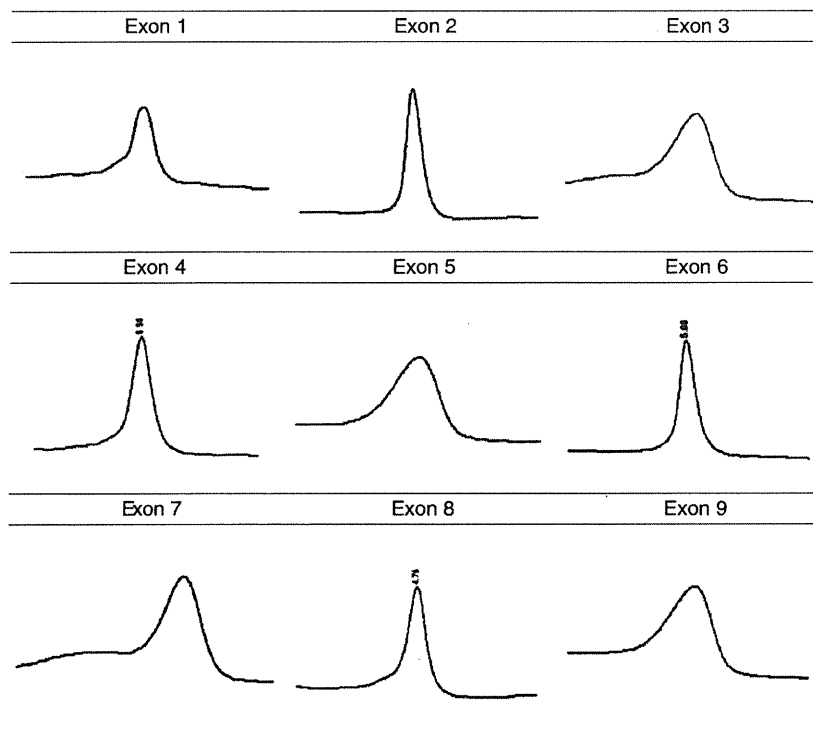

Exon 1	Exon 2	Exon 3
Exon 4	Exon 5	Exon 6
Exon 7	Exon 8	Exon 9

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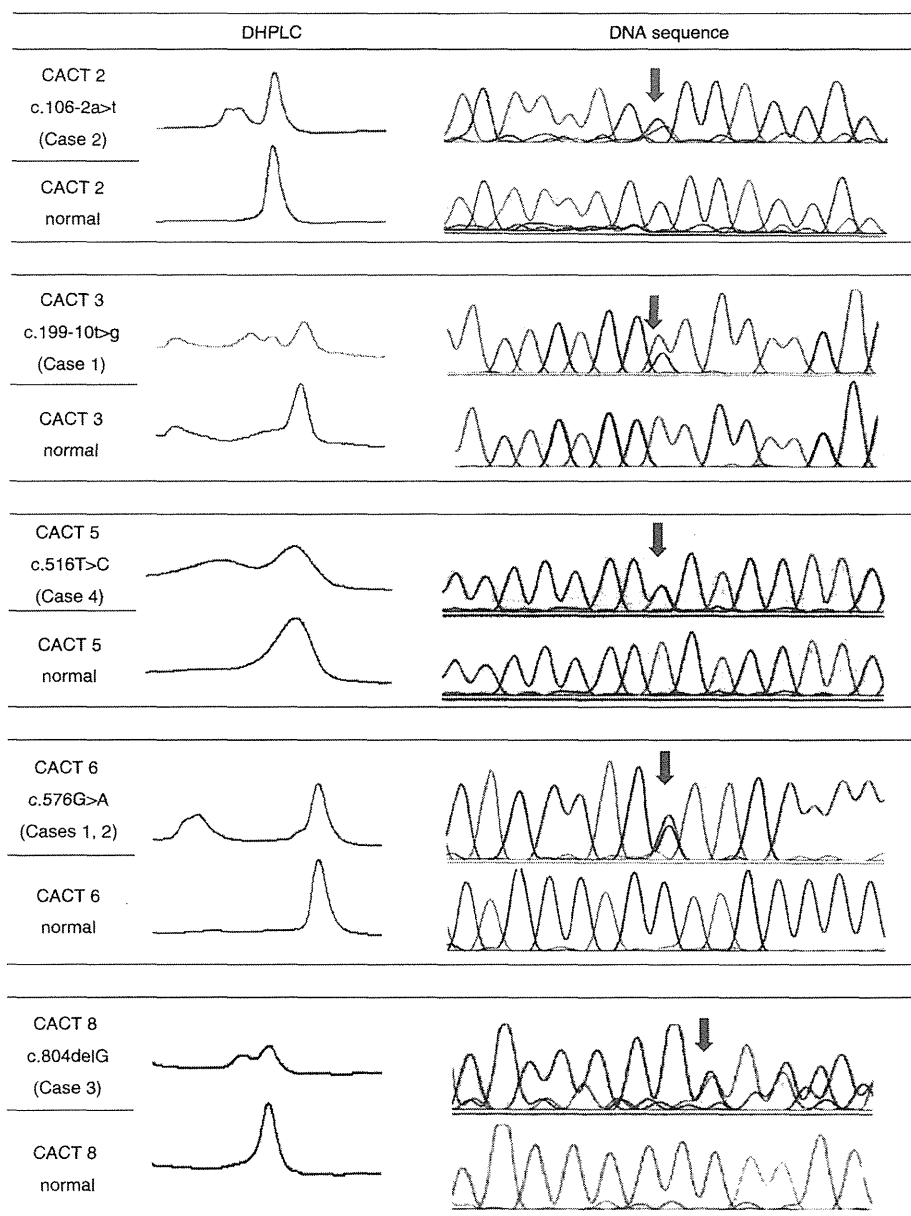
Figure 2 Aberrant DHPLC patterns of heteroduplexes and corresponding DNA sequences. Hybridization of unmatched normal and mutated sequences generated heteroduplexes, thereby resulting in aberrant peaks with different retention times. Red arrows indicate positions of mutations identified by direct DNA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