図 1. ナチュラル 3-ヒドロキシ酪酸を負荷した in vitro probe assay の結果
（培養条件）37℃、96 時間培養
（培地）ブドウ糖フリー、脂肪酸フリー、カルニチン過剰、3-ﾋﾄﾞﾛｷｼ酪酸　25mM を負荷
（略字）3KTd＝βケトチオラーゼ欠損症；SCOTd＝サクシニル-CoA：CoA ケト酸トランスフェラーゼ欠損症；C2、C8＝それぞれアセチル、オクタノイルカルニチン；C4-OH＝ヒドロキシブチリルカルニチン。

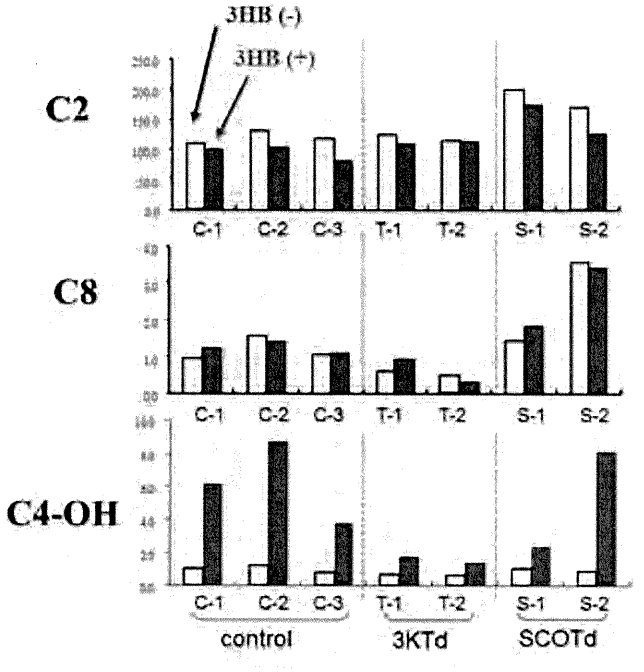
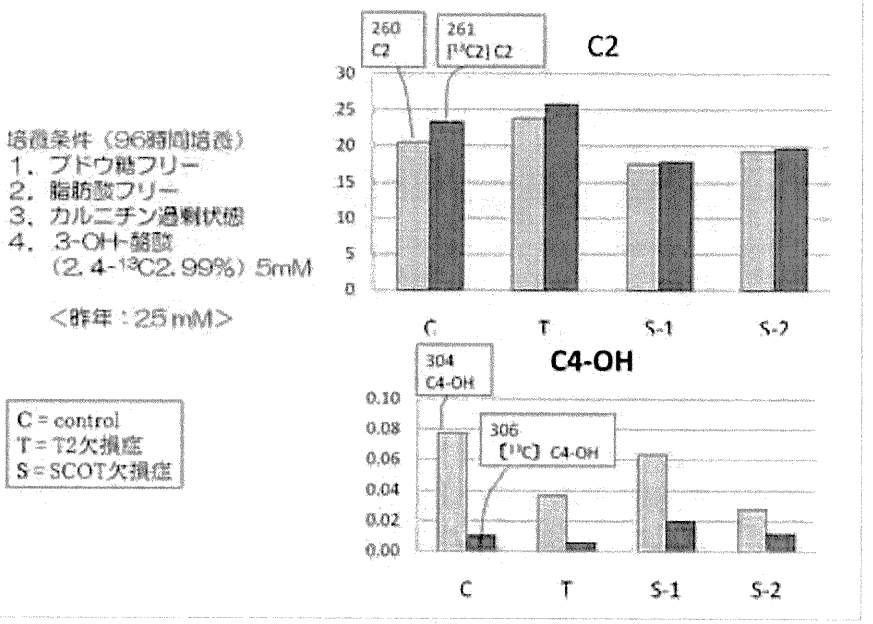


図２．　安定同位体ラベルの 3-ヒドロキシ酪酸を負荷した in vitro probe assay の結果



厚生労働科学研究費補助金（難治性疾患克服研究事業）

総合研究報告書

先天性ケトン体代謝異常症（HMG-CoA 合成酵素欠損症、HMG-CoA リアーゼ欠損症、β-ケトチオラーゼ欠損症、SCOT 欠損症）の発症形態と患者数の把握、診断治療指針に関する研究

分担研究課題　先天性ケトン体代謝異常症における治療法の検討

研究分担者　高柳正樹　　所属　千葉県こども病院

研究要旨

私どもはこれまで先天性ケトン体代謝異常症として、β-ケトチオラーゼ（T２）欠損症１例と HMG-CoA リアーゼ欠損症１例を経験した。これら先天性ケトン体代謝異常症は症例数も少なく、その治療法においても検討すべき点が多いものと考えられる。今回上記の２症例についての治療を通じて、先天性ケトン体異常症の治療法についての検討を行ったので報告する。

先天性ケトン体代謝異常症の治療原則について①ケトン合成の抑制、②低血糖時の対応、③アシドーシスに対する対応④血液浄化法、⑤カルニチンの補充などその他について検討した。

今後先天性ケトン体代謝異常症の新生児科医および小児科医における認知度を上げるために、本研究班を中心とした啓蒙活動が必要であると考えられる。

A. 研究目的

私どもはこれまで先天性ケトン体代謝異常症として、β-ケトチオラーゼ（T２）欠損症１例と HMG-CoA リアーゼ欠損症１例を経験した。これら先天性ケトン体代謝異常症は症例数も少なく、その治療法においても検討すべき点が多いものと考えられる。今回上記の２症例についての治療を通じて、先天性ケトン体異常症の治療法についての検討を行ったので報告する。

B. 研究方法

１）β-ケトチオラーゼ（T２）欠損症

症　例　S.Y. 2005年１１月２５日出生の女児

＜主訴＞痙攣、意識障害

＜家族歴＞血族婚なし。神経疾患なし。突然死なし。

＜既往歴＞在胎 38 週、3452g で出生。母が発熱、嘔吐、新生児仮死を認めたため、緊急帝切となる。仮死なし。黄疸のため光線療法受ける。発達は正常。発育も正常。

＜現病歴＞　生後６ヶ月半で突発性発疹。その後２週間咳が続き近医で与薬を受けていた。呼吸が荒かったと母親は表現している。６月３０日の明け方，患児が痙攣していることに気づき、救急病院へ搬送となった。

＜身体所見＞　体重 8287g。

ICU 入室時　体温35．8度。脈拍164．血圧　104/64

胸部　右肺野で粗な crackles,エア入りやや不良。心雑音なし。

腹部　肝は右鎖骨中線にて3cm 触れ、脾臓は触れない

皮膚　膨疹認めず。肛門部周囲の糜爛発赤あり。

意識障害を認める。

＜検査成績＞

BS	8mg/dl	
NH3	363 μg/dl	
Lactic	6.3mg/dl	
β hydroxybutyrate	21.68	mmol/l
acetoacetate	11.8	mmol/l
F-Carnitine	19.7	μmol/l
A-Carnitine	74.5	μmol/l
pH	6.796	
pCO2	19.4	Torr
HCO3-	2.8	mmol/l
BE	－28.2	
Anion gap	29.2	

＜治療法＞

急性期治療

高濃度の糖質を含む輸液をおこなった。

メイロンにてアシドーシスの補正をを図った。

安定期の治療

- 低タンパク食　2.0g/kg/day 以下
- カロリーは　80Kcal／kg／day以上
- カルニチン　300ｍｇ/day

２）HMG-CoAリアーゼ欠損症

Ｍ．Ｏ．（平成１１年１１月５日生まれ、女児）

＜家族歴＞血族婚なし。新生児、幼少時の死亡症例なし。痙攣などの中枢神経系の障害なし。

＜既往歴＞＜現病歴＞

在胎37週０日　2896gで出生。周産期に仮死などの問題点はなかった。

①　低ケトン性低血糖

日令２、生後６カ月、８か月時、１歳１カ月、２歳５か月時に低血糖発作を起こした。

②　症候性てんかん

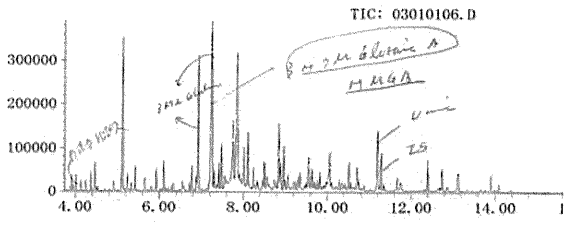
１歳１か月時の低血糖発作以後に意識消失発作が認められるようになった。この時から抗けいれん剤と投与始まる。１歳４カ月ころから全身性硬直性痙攣が認められている。以後脱力発作などを中心とする多様な発作型を示している。てんかん発作はコントロール困難で、つき最大５回程度おこり、抗けいれん剤の変更を余儀なくされている。２歳２カ月の脳波では左頭頂部を中心に棘波が認められ、時に左大脳半球全体に棘波は波及する所見が得られている。発達に関しては１歳７か月時の新版Ｋ式でDQ=67であった。

＜検査成績＞

尿中有機酸（非発作時）：

3-hydroxy-3-methylglutaric acid,

3-methylglutaconic acid の異常排泄が認められる。



患児有機酸分析（非発作時）

3-hydroxy-3-methylglutaric -CoA-lyase　活性（末梢リンパ球）　広島大学　佐倉伸夫先生測定

症例　32.69 pmol/min/10^6cell

対照　507.79(498.78+-192.85) pmol/min/10^6cell

と活性の低下を認めた。

＜治療法＞

急性期治療

高濃度の糖質を含む輸液をおこなった。

メイロンにてアシドーシスの補正をを図った。

安定期の治療

①低たんぱく食

タンパク摂取量　2g/kg/day

③　低脂肪食

日常的な制限は行わない。ファストフードなどによる過大な負荷は避けるように指導。

④　カルニチン内服

バルブロン酸ナトリウム服用中に著名な低フリーカルニチン血症を認めたため、補充療法を行っている。

⑤　抗けいれん剤

痙攣のコントロールが困難なため、抗けいれん剤の調整が必要とされた。

⑥　sick dayの対応

糖の補充に心がけることや、各種の低血糖の症状（傾眠などの意識障害等）が起きたら、補液のできる医療機関へ至急受診することなどを、十分に教育した。

C. 研究結果

先天性ケトン体代謝異常症の治療原則

①ケトン合成の抑制

HMG-CoA合成酵素欠損症、HMG-CoAリアーゼ欠損症、β-ケトチオラーゼ欠損症、SCOT欠損症の４つの疾患で空腹を避けることは重要である。これら４疾患では経口または輸液での糖質の補充は脂肪分解亢進を抑える。またHMG-CoAリアーゼ欠損症、β-ケトチオラーゼ欠損症、SCOT欠損症では糖質の補充はタンパク分解亢進も抑制する

②低血糖時の対応

低血糖時には10%ブドウ糖を2ml/kg（1.1mmol/kg）投与して低血糖を是正する。その

後患者の維持輸液量を 10%ブドウ糖濃度と適切な電解質濃度の輸液として持続投与する。10%ブドウ糖濃度の輸液は高浸透圧なので、血管外への漏れに十分注意する。

経過を観察して血糖は正常上限値を目標に管理する。

③アシドーシスに対する対応

HMG-CoA リアーゼ欠損症、β-ケトチオラーゼ欠損症、SCOT 欠損症ではアシドーシス発作が合併する。β-ケトチオラーゼ欠損症、SCOT 欠損症ではケトアシドーシスが臨床的問題の中心になる。一番重要な治療法は糖質を投与してケトン体合成を抑えることであり、これが達成できれば数時間後にアシドーシスは改善する。

代謝性アシドーシスに対する治療法は現在いろいろな意見がある。アシドーシスにおける最大の問題は、心機能の低下である。pH7.0 では負のイノトロピック効果とカテコールアミンに対する不応性の出現が実験的に確かめられている。極端なアシドーシスでは多呼吸が起き、呼吸性疲労を引き起こし衰弱していくことになる。低炭酸ガス血症においては大脳の血管収縮が生じる。PCO2 が 20 torr以下であるとこの血管収縮が起きることが脳波的に確かめられている。

神経系は極端なアシドーシスに対してもあまり障害を受けない。小児においては pH7.0 以上では臨床的に重大な心機能への悪影響はほとんど起きない。それゆえ多くの症例ではアシドーシスを即座に是正する必要はない。強力なアルカリ化は患者の状態を悪化させる。また高ナトリウム血症や高浸透圧血症、さらには中枢神経系のパラドキシカルなアシドーシスを引き起こす。

最小限のガイドラインとしては、pH が 7.1 以下で、循環器不全や呼吸器不全に陥っておらず、意識も清明のときには、重炭酸水素ナトリウム（メイロン 84®は 1mmol/ml）を 1mmol/kg ゆっくりと１０分以上かけて静注する。その後持続的に重炭酸ナトリウムを投与する。その目標値は pH>7.1, Pco2>20, Hco3->10 である。もちろん生化学的なデータの改善が見られたら、速やかにテーパリングしていかなくてはならない。

④血液浄化法など

さらなる侵襲的な治療法、例えば透析などの血液浄化法は、アシドーシスと代謝物のコントロールに有用ではあるが、これを必要とすることはほとんどない

これまでも HMG-CoA リアーゼ欠損症、β-ケトチオラーゼ欠損症、SCOT 欠損症に腹膜透析、血液透析が使用されたという報告はある。

持続透析の準備などで、糖質投与や他のケトン産生抑制の治療が遅れてしまわないように注意すべきである。

⑤その他

カルニチンの補充:特に血中カルニチン濃度が低い患者において考慮されるべきである。血中フリーカルニチンを 20 μ mol/l 以上に維持すべきである。

フォローアップ:神経学的機能と肝機能の定期的検査。

遺伝相談：無症状の兄弟の評価を含めた家族への遺伝相談は必須である。

D．考案

小児期にはケトーシスによく遭遇する．一方重篤なケトアシドーシスの中には死亡したり，後遺症を残すような先天性ケトン体代謝異常症が含まれている．

これらの疾患は診断が難しい疾患であり，日常診療の中ではいわゆる周期性嘔吐症（自家中毒）と区別がつきにくく診断が適切になされないために重篤なケトアシドーシスを繰り返し，中には精神発達遅滞をきたしたり死亡することもある疾患である．しかし診断さえつけば，高価な治療法を必要とせず，発作を未然に防ぎ正常発達ができる疾患である．そこにこれらの疾患の診断の重要性がある．

一方ケトン体産生障害を来す 3-hydroxy-3-methylglutaric -CoA-lyase 欠損症はロイシン代謝経路の先天代謝異常症で、その発生頻度は極めて低い。そのおもなる症状は低ケトン性低血糖であり、約半数の症例は生後７日以内の発症である。

新生児期において低血糖は多くの症例におこるこ

とから、本症例の様に新生児期に低血糖が認められた場合でも、正しい診断に至らない症例が多く存在するのでは考えられる。

本症例は繰り返して起きた低血糖によると思われる、中枢神経障害により症候性てんかんと軽度の発達障害を認めている。

新生児期における低血糖の原因は数多く知られているが、本疾患をふくめた系統的な鑑別疾患を確実に行うことが必要であると考えられる。

今後本疾患の新生児科医および小児科医における認知度を上げるために、本研究班を中心とした啓蒙活動が必要であると考えられる。

E. 結論

平成 22−23年度難治性疾患克服研究事業,, 研究奨励分野の「先天性ケトン体代謝異常症（HMG-CoA 合成酵素欠損症、HMG-CoA リアーゼ欠損症、β-ケトチオラーゼ欠損症、SCOT 欠損症）の発症形態と患者数の把握、診断治療指針に関する研究」班にて，４疾患の診断臨床指針を作成し広く広報していき、本疾患による障害を少しでも軽減して患者のＱＯＬの向上につながるように企画する。

F．研究発表

１．論文発表

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G．知的財産権の出願・登録状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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			GC/MSデータ解釈のノウハウ				
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Ⅳ. 研究成果の刊行物・別冊

Molecular Genetics and Metabolism 100 (2010) 339–344

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

A novel mutation (c.951C>T) in an exonic splicing enhancer results in exon 10 skipping in the human mitochondrial acetoacetyl-CoA thiolase gene

Toshiyuki Fukao [a,b,*], Reiko Horikawa [c], Yasuhiro Naiki [c], Toju Tanaka [d], Masaki Takayanagi [e], Seiji Yamaguchi [f], Naomi Kondo [a]

[a] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu 501-1194, Japan
[b] Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1196, Japan
[c] Division of Endocrinology and Metabolism, National Center for Child Health and Development, Tokyo 157-8535, Japan
[d] Division of Clinical Genetics and Molecular Medicine, National Center for Child Health and Development, Tokyo 157-8535, Japan
[e] Chiba Children's Hospital, Chiba 266-0007, Japan
[f] Department of Pediatrics, Faculty of Medicine, Shimane University, Izumo, Shimane 693-8501, Japan

ARTICLE INFO

Article history:
Received 9 February 2010
Received in revised form 16 March 2010
Accepted 16 March 2010
Available online 19 March 2010

Keywords:
Aberrant splicing
Exonic mutation
Splice site selection
Mitochondrial acetoacetyl-CoA thiolase
Inborn error of metabolism
SF2/ASF

ABSTRACT

Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency is an inherited disorder affecting isoleucine catabolism and ketone body metabolism. A Japanese female developed a severe ketoacidotic attack at the age of 7 months. Urinary organic acid analysis showed elevated excretion of 2-methyl-3-hydroxybutyrate but not tiglylglycine. She was diagnosed as having T2 deficiency by enzyme assay using fibroblasts. Mutation analysis revealed a compound heterozygote of c.556G>T(D186Y) and c.951C>T(D317D). Since c.951C>T does not cause amino acid change, we performed cDNA analysis and found that exon 10 skipping had occurred in the c.951C>T allele. A computer search using an ESE finder showed that an exonic splicing enhancer sequence, SF2/ASF, was located in CTGA^{951}CGC. We hypothesized that the exonic splicing enhancer is necessary for accurate splicing since the first nucleotide of exon 10 is C, which weakens the splice acceptor site of intron 9. We made a mini gene construct including exon 9-truncated intron 9-exon 10-truncated intron 10-exon 11 for a splicing experiment. We also made three mutant constructs which alter the SF2/ASF site (947C>T, 951C>T, 952G>A). An min-gene splicing experiment clearly showed that exon 10 skipping was induced in all three mutant constructs. Moreover, additional substitution of G for C at the first nucleotide of exon 10 resulted in normal splicing in these three mutants. These results confirmed that c.951C>T diminished the effect of the exonic splicing enhancer and caused exon 10 skipping.

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2[1]) (EC 2.3.1.9, gene symbol *ACAT1*) deficiency (OMIM 203750, 607809) is an autosomal recessive disorder, commonly known as β-ketothiolase deficiency. Since 1971 [1], more than 70 patients with it have been identified (including personal communications) [2]. This disorder affects the catabolism of isoleucine and the metabolism of ketone bodies, and is clinically characterized by intermittent ketoacidotic episodes with no clinical symptoms between episodes. T2-deficient patients usually have urinary excretion of 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate and tiglylglycine, derived from intermediates in isoleucine catabolism. The severity of the clinical features varies from patient to patient but follow-up studies reveal that, in general, T2 deficiency has a favorable outcome [3].

The T2 gene (gene symbol: ACAT1) spans approximately 27 kb, contains 12 exons [4], and is located at 11q22.3–q23.1 [5]. Human T2 cDNA is about 1.5 kb long and encodes a precursor protein of 427 amino acids, including a 33-amino-acid leader polypeptide [6]. We have identified more than 70 gene mutations [7–25], 15% of which cause aberrant splicing. Most were located at the highly conserved ag at the splice acceptor site and gt at the splice donor site. We also identified some exonic mutations which cause aberrant splicing by activating cryptic splice sites within their exons [15,24].

We herein report a novel exonic mutation—c.951C>T (the 11th nucleotide in exon 10). It was first regarded to be a silent mutation, D317D, but was associated with exon 10 skipping in cDNA analysis. The c.951C nucleotide is located in a possible exonic splicing enhancer (ESE) sequence, SF2/ASF, and C>T substitution results in a deviation from its consensus sequence. We showed by a

* Corresponding author at: Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan. Fax: +81 58 230 6387.
E-mail address: toshi-gif@umin.net (T. Fukao).

[1] *Abbreviation used:* T2, mitochondrial acetoacetyl-CoA thiolase.

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doi:10.1016/j.ymgme.2010.03.012

minigene splicing experiment that the substitutions in this ESE caused exon 10 skipping.

Materials and methods

Case report

The patient (GK64), a female, was born to non-consanguineous Japanese parents. She was well until 7 months of age when she presented with a one-week history of coughing and appetite loss. She developed convulsions and was admitted to a hospital. Laboratory findings showed blood pH 6.769, bicarbonate 2.8 mmol/L, base excess −28.2 mmol/L, ammonia 213 μmol/L, and blood glucose 0.45 mmol/L. She was referred to the National Center for Child Health and Development on a mechanical ventilation support. Urinary organic acid analysis at the time of crisis showed huge amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic acids; 2-methyl-3-hydroxybutyrate and tiglylglycine were not evident at that time. She was successfully treated by intravenous glucose infusion. Later, she had an upper respiratory infection and her urinary ketone was 2+ at the age of 8 months. At that time, urinary organic acid analysis showed the presence of 2-methyl-3-hydroxybutyrate. However, tiglylglycine, another characteristic urinary organic acid in T2 deficiency, was not detected. Skin biopsy and a fibroblast culture were performed and finally she was diagnosed as having T2 deficiency by enzyme assay.

Cell culture, enzyme assay and immunoblot analysis

The fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described in [26]. Immunoblot analysis was done, as described in [27].

Mutation detection

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 12 fragments including an exon and its intron boundaries [13]. RNA was prepared from the fibroblasts using an ISOGEN kit (Nippon Gene, Tokyo, Japan). RT-PCR and sequencing after subcloning into a pGEM-T Easy vector (Promega, Madison, USA) were performed as described previously [7], except for the following point. A full-coding sequence of human T2 cDNA was amplified as a single fragment using a sense primer (5′-$^{-40}$AGTCTACGCCTGTGGAGCCGA^{-20}-3′) and an antisense primer (5′-^{1326}TTCTGGTCACATAGGGTT^{1309}-3′).

Transient expression analyses

Transient expression analysis of T2 cDNAs was done using a pCAGGS eukaryote expression vector [28], as described in [19]. After transfection, the cells were further cultured at 37 °C for 72 h, and then they were harvested and kept at −80 °C until use. The cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0), 0.1% Triton X-100. After centrifugation at 10,000*g* for 10 min, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity and for immunoblot analysis.

Splicing experiment

A fragment (about 4 kb long) from the middle part of exon 9 to the middle part of exon 11 was amplified by Phusion DNA polymerase (New England BioLabs, Ipswich, USA) using control genomic DNA. The primers used in this amplification included the EcoR I linker sequence, as follows:

Ex 9 (EcoR I) primer (exon 9, sense) 5′-cagctgcgaatt^{842}CCAGTAC ACTGAATGATGGAGCAGCT^{873}-3′.

Ex 11 (EcoR I) primer (exon 11, antisense) 5′-cctccattggaatt^{1122}C ACTTTTTGGGGATCAATCT^{1103}-3′.

The amplified fragment, after digestion with EcoR I, was subcloned into an EcoR I site of the pCAGGS expression vector from which the Hind III and Pst I sites were deleted. The subcloned PCR fragment did not contain PCR errors, at least in the sequence of exon 9, the 3′ and 5′ splice sites of intron 9, exon 10, the 3′ and 5′ splice sites of intron 10, and exon 11. We deleted about a 0.5-kb Hind III-Pst I inner fragment in intron 9 and a 1.1-kb Hind III-Pst I inner fragment in intron 10 to reduce the minigene construct length. In order to make an mutant construct, in vitro mutagenesis was done on the wild-type fragment in the pUC118 vector, and then the mutant fragment was subcloned into the pCAGGS expression vector as a cassette of an about 870-bp Pst I-Hind III fragment including exon 10. We made three mutant constructs which altered the SF2/ASF site (c.947C>T, c.951C>T, and c.952G>A). Moreover, we also made three further mutant constructs with additional substitution of c.941G for C at the first nucleotide of exon 10.

Two μg of these expression vectors were transfected into 5×10^5 cells of SV40-transformed fibroblasts using Lipofectamine 2000. At 48 h after transfection, RNA was extracted from the cells. The first-strand cDNA was transcribed with a rabbit β-globin-specific antisense primer (β-glo2) (5′-^{461}AGCCACCACCTTCTGATA-3′) and then amplified with the Ex10 (EcoRI) primer on T2 exon 10, and another rabbit-specific antisense primer (β-glo3) (5′-^{443}GGCAGCCTGCACCTGAGGAGT-3′) to amplify the chimera cDNA of human T2 and rabbit β-globin.

Allele-specific RT-PCR

We performed allele-specific RT-PCR using mismatched primers:

c.556G allele (D186)-specific sense primer, 5′-^{530}TTTGATTGTA AAA GACGGGCTATCT**G**^{556}-3′.

c.556T allele (Y186)-specific sense primer, 5′-^{530}TTTGATTGTA AAA GACGGGCTATCT**T**^{556}-3′

The bold G or T represents the D186Y mutation site of c.556G>A. The underlined T indicates a mismatch introduced to the 4th nucleotide to assist allele-specific-RT-PCR.

Antisense primer 5′-^{1065}GGCTTCTTTACTTCCCACATTGCA^{1041}-3′.

cDNA with exon 10 gave a 535-bp fragment and cDNA with exon 10 skipping gave a 470-bp fragment.

Results and discussion

Enzyme assay and immunoblot analysis

Potassium-ion-activated acetoacetyl-CoA thiolase activity was absent in GK64's fibroblasts ($-K^+$ 3.8, $+K^+$ 3.9 nmol/min/mg of protein; Control fibroblasts $-K^+$ 4.7, $+K^+$ 7.8 nmol/min/mg of protein), confirming the diagnosis of T2 deficiency. Succinyl-CoA:3-ketoacid CoA transferase activity was 6.3 nmol/min/mg of protein (control fibroblasts 5.6 nmol/min/mg of protein). In immunoblot analysis, GK64's fibroblasts had a reduced but significant amount of T2 protein (Fig. 1). We then performed immunoblot analysis using two-fold serially diluted samples of two controls and GK64's fibroblasts from 30 to 3.75 μg. The relative amount of T2 protein in GK64 fibroblasts were estimated to be 25% of controls (data not shown).

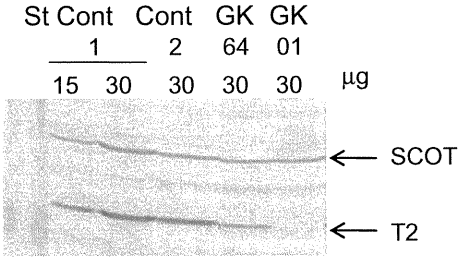

Fig. 1. Immunoblot analysis. The amount of fibroblast protein extract applied was indicated in each lane. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by arrows. Cont 1 and Cont 2 were healthy controls and GK01 was a disease control being cross reactive material-negative.

Mutation screening at the genomic level and cDNA level

Routine genomic PCR and sequencing of exons 1–12 identified two nucleotide substitutions, c.556G>T(D186Y) in exon 6 and c.951C>T(D317D) in exon 10. Both c.556G>T and c.951C>T were novel nucleotide substitutions in the T2 gene. No further mutations were identified by genomic mutation screening. Since the latter substitution does not alter amino acid, we performed RT-PCR analysis. A full-coding region was amplified using a pair of primers on a 5′-noncoding region and a 3′-non-coding region, allowing one to show the segregation of these two substitutions. After subcloning, 8 clones had c.556G>T(D186Y) but not c.951C>T(D317D). Two clones had exon 10 skipping without c.556G>T(D186Y). The exon 10 skipping causes a frame shift and premature termination at c.1011TAA. We re-sequenced the genomic region around exon 10 (IVS8 – 88–IVS9 + 44) again, but only c.951C>T(D317D) was detected. We regarded c.951C>T(D317D), the 11th nucleotide of exon 10, as the cause of exon 10 skipping which was detected in GK64's cDNA. Since the splice acceptor site of intron 9 might be weak because of the first nucleotide of exon 10 being C, we hypothesized that ESE sequences would be necessary for accurate exon recognition of exon 10 and that c.951C>T might disrupt the ESE and result in exon 10 skipping.

Transient expression analysis of D186Y mutant cDNA

Transfection of wild-type T2 cDNA gave a high acetoacetyl-CoA thiolase activity in the presence of potassium ion. Transfection of D186Y mutant cDNA gave no significant thiolase activity compared with mock cDNA transfection (Fig. 2A) Immunoblot analysis showed that mutant D186Y protein was detected with 1/3-fold amount of wild-type protein (Fig. 2B). These results indicate that the D186Y mutant protein is a stable protein but retains no residual activity. Even when incubation was done at a lower temperature (30 °C) after transfection, no residual T2 activity was detected (data not shown). This result confirmed that the D186Y mutation is a causative mutation in one allele, and is consistent with the fact that GK64's fibroblasts had T2 protein with about a 1/4-fold amount of controls'.

Searches for ESE sequence

We searched the possible ESE sequences which can be affected by c.951C>T, using ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) [30–31] and found that this mutated site, c.951C>T, was located in a possible SF2/ASF site, c.947CTGA951CGC (7th–13th nucleotides in exon 10). The substitution made a deviation from the consensus sequence of SF2/ASF, as shown in Fig. 3A.

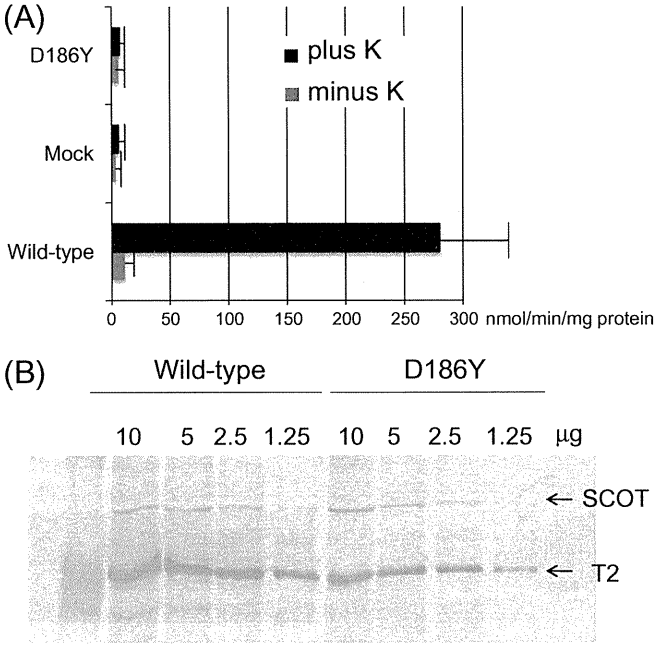

Fig. 2. Transient expression analysis of D186Y mutant cDNA. (A) Potassium-ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence and presence of the potassium ion are shown together with the standard deviation of three independent experiments. (B) Immunoblot analysis. The protein amounts applied are shown above the lanes. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody.

Minigene splicing constructs

We previously successfully performed minigene splicing experiments using a pCAGGS expression vector [8,24,29]. Since our minigene construct produces human T2-rabbit β-globin fusion mRNA, we could amplify this specific mRNA by RT-PCR using a combination of a human T2 sense primer and a rabbit β-globin antisense primer. We made a minigene construct including exon 9-truncated intron 9-exon 10-truncated intron 10-exon 11 for a splicing experiment, as shown in Fig. 3B. We made the c.951C>T mutant constructs and two additional mutant constructs (c.947C>T or c.952G>A) which also altered the SF2/ASF site, as shown in Fig. 3A. We hypothesized that the ESE is necessary for accurate splicing since the first nucleotide of exon 10 is C, which weakens the splice acceptor site of intron 9. Hence, we made three constructs with an additional substitution of 941G for C at the first nucleotide of exon 10 to strengthen the splice acceptor site of intron 9.

Splicing experiment

We performed a minigene splicing experiment. As shown in Fig. 3C, exon 10 skipping was induced in all three mutant constructs. Normally spliced transcripts with the inclusion of exon 10 were also produced in these mutant transcripts. The ratio of signal intensity of transcripts with exon 10 skipping to that of normally spliced transcripts in three independent experiments was highest in c.951C>T, followed by c.952G>A among these three mutants.

Moreover, additional substitution of G for C at the first nucleotide of exon 10 resulted in normal splicing in these three mutants. Hence, the ESE (SF2/ASF) was only necessary in the case of C at the first nucleotide of exon 10 in the experiment. This clearly showed that c.941C, the first nucleotide of exon 10, makes the recognition

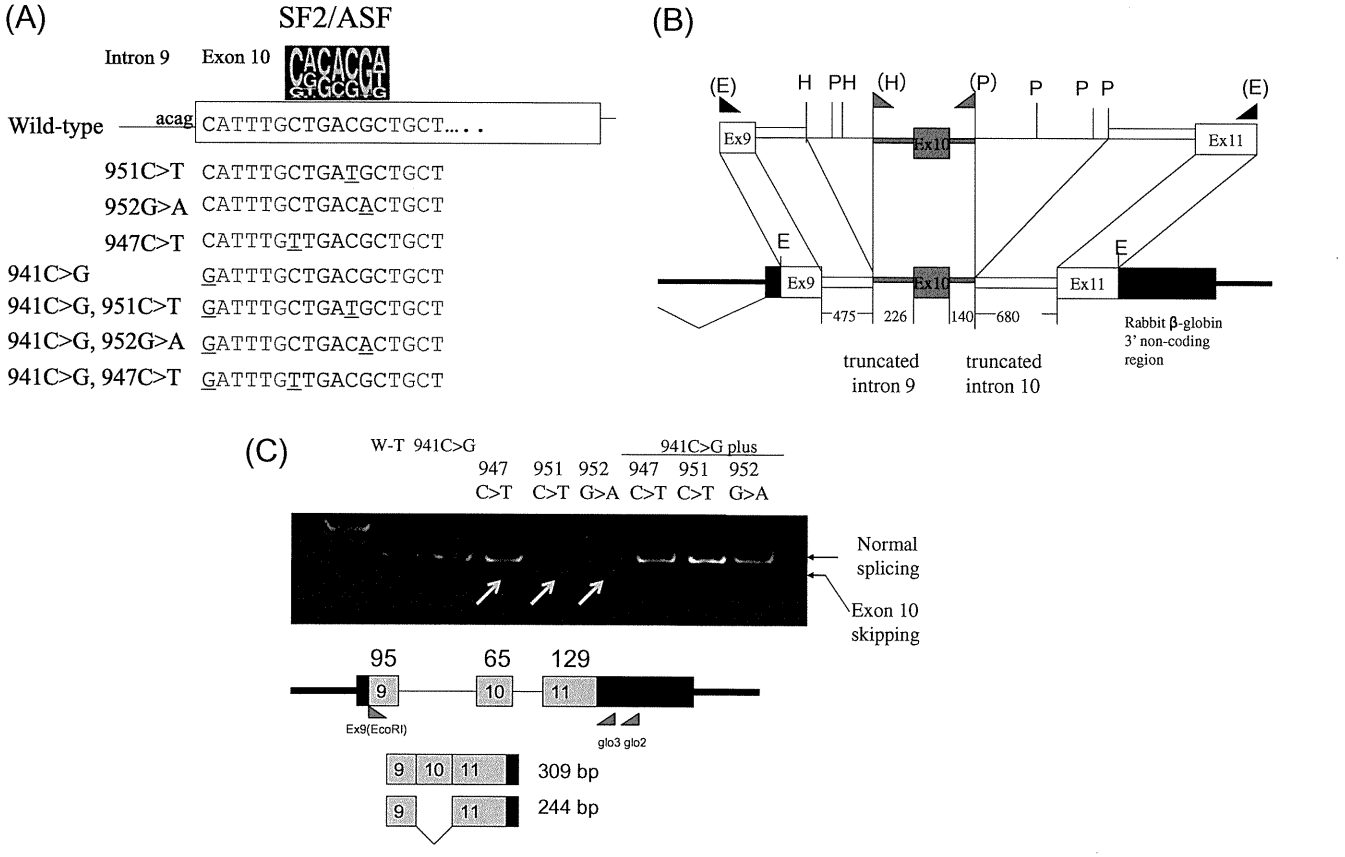

Fig. 3. Minigene splicing experiment. (A) Minigene splicing constructs. Sequence differences among 8 minigene splicing constructs. Mutations introduced are underlined. (B) Schematic presentation of minigene splicing construct. The minigene construct has a T2 gene fragment from c.842 of exon 9 and intron 9 (from +1 to a Hind III site, 475-bp open box) and intron 10 (from a Pst I site to −1, 680-bp open box) and exon 11 (to c. 1122). In the cases of mutant constructs, the region around exon 10, highlighted in gray, was replaced as a cassette. Thick lines and black boxes indicate pCAGGS vector sequences. (C) Detection of chimeric cDNAs derived from transfected minigenes. First-strand cDNA was reverse-transcribed using the glo2 primer. cDNA amplification was done using Ex9(EcoRI) and glo3 primers. Normal splicing and aberrant splicing produced 309-bp and 244-bp PCR fragments, respectively. The PCR fragments were electrophoresed on 5% polyacrylamide gel. Fragments with exon 10 skipping are shown by arrows.

of exon 10 or the splice acceptor site of intron 9 and requires an ESE for the accurate splicing of exon 10. These results confirmed that c.951C>T diminished the effect of the ESE and caused exon 10 skipping.

Effects of c.951C>T mutation on splicing

In the minigene splicing, normally spliced transcripts were detected in the construct with c.951C>T. This may mean that not only exon-10-skipped transcripts but also normally splicied transcripts can be produced in the c.951C>T mutant allele. However, when we analyzed 10 clones of full-length cDNA, 8 clones were from the allele with c.556G>T(D186Y). Two clones had exon 10 skipping but no cDNA clones with c.951C>T were found. In direct sequencing of full-length cDNA fragments, we found a possible faint signal for c.951T in the major signal for c.951C (Fig. 4B). Hence, the presence of normally spliced transcripts from c.951C>T was further confirmed by allele-specific RT-PCR. As shown in Fig. 4A, both c.556T(Y186) allele- and c.556G(D186) allele-specific RT-PCR gave a fragment with the expected size in the case of GK64, and only the latter gave a fragment in the case of a control. In direct sequencing of GK64's fragment of the c.556G(D186) allele, c.951 was T (normally spliced transcripts in the c.951C>T mutant allele) (Fig. 4B). An additional faint fragment with exon 10 skipping was also seen in GK64's c.556G(D186) allele-specific PCR. Exon 10 skipping causes frame shift and should result in nonsense-mediated mRNA decay; hence, the amount of cDNA with exon 10 skipping in the D186 allele was smaller than that of normally spliced cDNA. Based

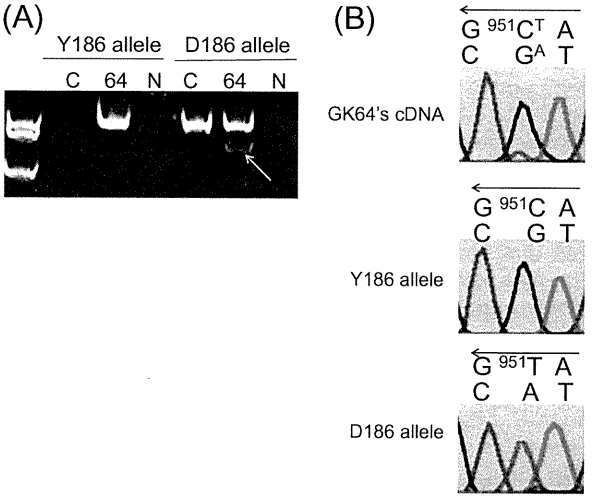

Fig. 4. Allele-specific cDNA amplification. (A) Allele-specific PCR fragments were electrophoresed on 5% polyacrylamide gel. C, control cDNA; 64, GK64's cDNA, N, negative control. An arrow indicates cDNA with exon 10 skipping. (B) Direct sequencing of the antisense strand at the c.951c>T (D186Y) site. Y186 allele, Y186 allele-specific PCR fragment; D186 allele, D186 allele-specific PCR fragment.

on cDNA analysis, a small amount of normally spliced mRNA with c.951C>T(D317D) was also produced and hence GK64 retained some residual T2 activity from this mutant allele. This finding is in accord with GK64's urinary organic acid profiles. We previously

showed that urinary organic acid analysis shows no elevated tiglylglycine and relatively small amount of 2-methyl-3-hydroxybutyrate even during ketoacidotic crisis and subtle elevation of 2-methyl-3-hydroxybutyrate under stable conditions in patients with mutations which retain some residual T2 activity [3,18,19].

The importance of the exonic splicing enhancer

The accurate removal of introns from pre-mRNA is essential for correct gene expression. However, the information contained in splice sites, including the splice donor site, branch site and splice acceptor site, is insufficient for a precise definition of exons [32–35]. Recently, it was established that exon sequence has elements which contribute to exonic recognition. Additional regulatory elements exist in the form of ESEs [32,33]. Exonic variants may inactivate an ESE, resulting in insufficient exon inclusion.

ESEs are known to play a particularly important role in exons with weak splice sites. Although the splice acceptor site of intron 10 has a relatively high Shapiro and Senapathy score [35] of 90.5, the site deviates from the consensus sequence at position +1, by the replacement of the G nucleotide with C. In computer analysis using ESE finder, the mutation c.951C>T was located on an ESE, the SF2/ASF site. SF2/ASF is a prototypical serine- and arginine-rich protein (SR family) with important roles in splicing and other aspects of mRNA metabolism. One classical function of SR proteins bound to exonic sequences is to stimulate recognition of the flanking splice sites [36]. Using the minigene approach, we have demonstrated that not only the c.951C>T substitution but also c.947C>T and c.952G>A, all of which affected the SF2/ASF site, resulted in insufficient exon 10 inclusion. This phenomenon was completely corrected by a substitution of G for C at the first nucleotide of exon 10. We therefore suggest that the weak splice acceptor site of intron 10 is normally compensated for by an ESE (SF2/ASF).

There are several precedent reports on ESE mutations in other genes [37–39]. For example, two synonymous mutations in exon 5 identified in pyruvate dehydrogenase-deficient patients (the c.483C>T and c.498C>T variants) disrupt a putative ESE, the SRp55 binding site [37]. These synonymous mutations result in the incomplete inclusion of PDHA1 exon 5 in the minigene splicing experiment and this effect is corrected following the restoration of a perfect consensus sequence for the 5′ splice site by site-directed mutagenesis. The mutation in the SRp55 binding site is affected in the case of the weak 5′ splice site selection in this case and the mutation in SF2/ASF site was affected in the case of the weak 3′ splice site selection in our case. c.1918C>G (pR640G) in exon 14 in the APC gene, which was found in a familial adenomatous polyposis (FAP) patient, was revealed to be sufficient to cause exon 14 skipping [38]. Minigene splicing experiments showed a mechanism involving disruption of an ASF/SF2 element. Systemic analysis of 24 mutations in PAH exon 9 showed that three of them affected ESE motifs and resulted in exon 9 skipping [39]. These facts indicate that we should consider that any mutations in an exon may affect splicing of the exon.

Importance of cDNA analysis

If mutation analysis were done only at the genomic level, this c.951C>T(D317D) mutation would be regarded as a silent mutation. However, the main character of this mutation was an ESE mutation which causes exon 10 skipping. Any nucleotide substitutions have the possibility to affect splicing efficiency. This indicates the importance of cDNA analysis to understand the character of mutations properly.

Acknowledgments

We thank N. Sakaguchi and K. Murase for technical assistance.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and by Health and Labor Science Research Grants for Research on Intractable Diseases and For Research on Children and Families from The Ministry of Health, Labor and Welfare of Japan.

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J Inherit Metab Dis
DOI 10.1007/s10545-010-9168-5

RESEARCH REPORT

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

Toshiyuki Fukao · Tomohiro Ishii · Naoko Amano · Petri Kursula · Masaki Takayanagi · Keiko Murase · Naomi Sakaguchi · Naomi Kondo · Tomonobu Hasegawa

Received: 30 March 2010 / Revised: 30 June 2010 / Accepted: 2 July 2010

Abstract Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency causes episodic ketoacidotic crises and no apparent symptoms between them. Here, we report a Japanese case of neonatal-onset SCOT deficiency. The male patient presented a severe ketoacidotic crisis, with blood pH of 7.072 and bicarbonate of 5.8 mmol/L at the age of 2 days and was successfully treated with intravenous infusion of glucose and sodium bicarbonate. He was diagnosed as SCOT deficient by enzymatic assay and mutation analysis. At the age of 7 months, he developed a second ketoacidotic crisis, with blood pH of 7.059, bicarbonate of 5.4 mmol/L, and total ketone bodies of 29.1 mmol/L. He experienced two milder ketoacidotic crises at the ages of 1 year and 7 months and 3 years and 7 months. His urinary ketone bodies usually range from negative to 1+ but sometimes show 3+ (ketostix) without any symptoms. Hence, this patient does not show permanent ketonuria, which is characteristic of typical SCOT-deficient patients. He is a compound heterozygote of c.1304C > A (T435N) and c.658-666dupAACGTGATT p.N220_I222dup. mutations in the *OXCT1* gene. The T435N mutation was previously reported as one which retained significant residual activity. The latter novel mutation was revealed to retain no residual activity by

Communicated by: K. Michael Gibson

References to electronic databases: Succinyl-CoA:3-ketoacid CoA transferase deficiency (OMIM 245050 601424): Succinyl-CoA:3-ketoacid CoA transferase (EC 2.8.3.5): *OXCT1* gene (gene ID 5019 NM_000436)

Competing interests: None declared.

T. Fukao · K. Murase · N. Sakaguchi · N. Kondo
Department of Pediatrics, Graduate School of Medicine,
Gifu University,
1-1 Yanagido,
Gifu, Gifu 501-1194, Japan

T. Fukao (✉)
Medical Information Sciences Division,
United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University,
1-1 Yanagido,
Gifu, Gifu 501-1194, Japan
e-mail: toshi-gif@umin.net

T. Fukao
Clinical Research Division, Nagara Medical Center,
Gifu, Gifu 502-8558, Japan

T. Ishii · N. Amano · T. Hasegawa
Department of Pediatrics, Keio University School of Medicine,
Shinjuku, Tokyo 160-8582, Japan

P. Kursula
Department of Biochemistry, University of Oulu,
P.O. Box 3000, FIN-90014 Oulu, Finland

M. Takayanagi
Chiba Children's Hospital,
Chiba, Chiba 266-0007, Japan

Published online: 21 July 2010

transient expression analysis. Both T435N and N220_I222 lie close to the SCOT dimerization interface and are not directly connected to the active site in the tertiary structure of a human SCOT dimer. In transient expression analysis, no apparent interallelic complementation or dominant negative effects were observed. Significant residual activity from the T435N mutant allele may prevent the patient from developing permanent ketonuria.

Abbreviations

SCOT succinyl-CoA:3-ketoacid CoA transferase
TKB total ketone bodies
FFA free fatty acids

Introduction

Ketone bodies, produced mainly in the liver, are an important source of energy for extrahepatic tissues (Mitchell and Fukao 2001). Succinyl-CoA: 3-ketoacid CoA transferase (SCOT; EC 2.8.3.5) is a mitochondrial homodimer essential for ketone body utilization. SCOT deficiency (OMIM 245050) causes episodic ketoacidosis and is part of the differential diagnosis of childhood ketoacidosis, a frequently occurring condition. In contrast with most organic acidemias, no diagnostic metabolites are observed in the blood and urine samples from SCOT-deficient patients, although the ketone bodies, acetoacetate, and 3-hydroxybutyrate are elevated (Mitchell and Fukao 2001). Since the first description of SCOT deficiency (Cornblath et al. 1971; Tildon and Cornblath 1972), fewer than 30 affected probands have been reported, including personal communication (Saudubray et al. 1987; Perez-Cerda et al. 1992; Sakazaki et al. 1995; Fukao et al. 1996, 2000, 2004, 2006; Pretorius et al. 1996; Niezen-Koning et al. 1997; Rolland et al. 1998; Snyderman et al. 1998; Berry et al. 2001; Longo et al. 2004; Yamada et al. 2007; Merron and Akhtar 2009; and seven other unpublished cases sent to TF). Because of the nonspecific metabolite profile of SCOT-deficient patients, in vitro methods of diagnosis are particularly important. Enzyme assays of SCOT activity are sufficient for clinical diagnosis, but current whole-cell assays can yield a spuriously high apparent residual activity (Perez-Cerda et al. 1992; Sakazaki et al. 1995; Kassovska-Bratinova et al. 1996). To assist clinical diagnosis, we cloned the human SCOT complementary DNA (cDNA) (Kassovska-Bratinova et al. 1996) and SCOT gene (*OXCT1*, Fukao et al. 2000), developed an anti-(human SCOT) antibody (Song et al. 1997), and described ten *OXCT1* gene mutations in SCOT-deficient patients (Kassovska-Bratinova et al. 1996; Song et al. 1998; Fukao et al. 2000, 2004, 2006, 2007; Longo et al. 2004; Yamada et al. 2007).

SCOT deficiency is one of the most important differential diagnosis of neonatal ketoacidotic crisis, since about half of the reported SCOT-deficient patients developed their first ketoacidotic crises in the neonatal period (Mitchell and Fukao 2001). Persistent ketosis and ketonuria are pathognomonic features of SCOT deficiency; however, these are not present in all SCOT-deficient patients. We previously pointed out that patients with mutation T435N, which retained some residual SCOT activity, do not show permanent ketosis (Fukao et al. 2004). In this study, we describe a Japanese SCOT-deficient patient with neonatal onset. One of his mutations was revealed to be T435N.

Materials and methods

Case presentation

The proband (GS21) is a Japanese boy born from non-consanguineous parents at 38 weeks of gestation. The pregnancy and delivery were uneventful. His birth length was 49.1 cm (50th–90th percentile), weight 2.59 kg (3rd–10th percentile), and head circumference 34.3 cm (50th–90th percentile). At the age of 2 days, he presented tachypnea and poor drinking ability. Physical examination revealed grunting and sternal retraction with a respiration rate of 60/min. Blood gas analysis showed severe metabolic acidosis [pH 7.072, partial pressure of carbon dioxide (PCO_2) 20.5 mmHg, bicarbonate 5.8 mmol/L]. The blood glucose level was 3 mmol/L, ammonia 95 μmol/L, sodium (Na) 151 mEq/L, potassium (K) 4.19 mEq/L, chlorine (Cl) 113.1 mEq/L, and urinary ketone bodies 3+ (ketostix, Siemens Healthcare Diagnostics, USA). GS21 was treated by intravenous infusion of glucose and sodium bicarbonate. Urinary organic-acid analysis by gas chromatography-mass spectrometry showed massive amounts of 3-hydroxybutyrate and acetoacetate with dicarboxylic acids. He was transferred to Keio University Hospital for further evaluation at 4 days of age. On admission, his general condition and blood gas data (pH 7.453, PCO_2 24.4 mmHg, bicarbonate 20.4 mmol/L) improved with intravenous infusion of glucose at 7 mg/kg/min. GS21 was suspected of having SCOT deficiency, and this was confirmed by enzyme assay using peripheral blood mononuclear cells and mutation analysis (see "Results and Discussion"). At the age of 3 weeks, serum free fatty acids (FFA) and total ketone bodies (TKB) were measured at 1, 3, and 6 h after feeding, as shown in Table 1. At the age of 1 month, the boy was discharged from the hospital. The feeding interval was kept at <6 h to avoid severe ketoacidosis.

At the ages of 3 months and 4 months, FFA and TKB (3 h after feeding) were measured, as shown in Table 1. TKB levels became higher than those at the age of 3 weeks.

Table 1 Serum free fatty acids (FFA) and total keytone bodies (TKB)

Age		FFA (mM)	TKB (mM)	FFA/TKB
3 weeks	1 h after feeding	0.20	0.24	0.83
	3 h after feeding	0.17	0.15	1.13
	6 h after feeding	0.46	0.81	0.57
3 months	3 h after feeding	0.53	2.54	0.21
4 months	3 h after feeding	0.39	1.49	0.26
7 months	Ketoacidotic crisis	1.84	29.1	0.06
	2 days after crisis	0.97	0.54	1.80
1.5 years	Mild ketoacidotic crisis	ND	10.3	NA
3 years 7 months	Mild ketoacidotic crisis	1.61	11.2	0.14
Reference values*	Fed state		0.10-0.30	
	15-h fast	0.5-1.6	0.10-0.70	0.6-5.2

ND not determined, *NA* not applicable

*Reference values are from Bonnefont et al. 1990

At the age of 7 months, he developed his second ketoacidotic crisis for no clear reason and was again hospitalized. The laboratory findings were blood pH 7.059, PCO_2 20.2 mmHg, bicarbonate 5.4 mmol/L, glucose 2.2 mmol/L, and TKB 29.1 mmol/L. Treatment was begun with intravenous infusion of glucose at 5.5–7.1 mg/kg/min. He had a bolus injection of 1 mEq/kg of sodium bicarbonate followed by continuous infusion of sodium bicarbonate at 1 mEq/kg/h for 8 h. Thirteen hours after admission, continuous infusion of insulin was also initiated at a glucose/insulin ratio of 6.2–8.3 g/U, since his blood glucose levels were rather high (11.0 mmol/L). The urinary ketone bodies turned negative 2 days after admission. At the ages of 1 year 7 months and 3 years 7 months, he exhibited his third and fourth episodes of ketoacidotic crisis due to acute gastroenteritis. Blood pH was 7.280, PCO_2 20.3 mmHg, bicarbonate 9.2 mmol/L, and TKB 10.3 mmol/L in the third episode, and blood pH was 7.192, PCO_2 17.3 mmHg, bicarbonate 6.4 mmol/L in the fourth episode. FFA and TKB during the episodes are shown in Table 1. GS21 recovered by continuous infusion of glucose only during both episodes. At the age of 3 years and 5 months, he was 93.4 cm in height (50th percentile), 11.95 kg in weight (10th percentile), and had a head circumference of 50.4 cm (50th–75th percentile). The patient is now 3 years and 9 months old, and his motor and mental development are within normal range. The feeding interval has been prolonged up to 12 h. His urinary ketone bodies usually range from negative to 1+ but sometimes show 3+ without any symptoms.

Enzyme assay

Informed consent for enzymatic diagnosis and molecular analysis was obtained from the parents of GS21. This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Assays for acetoacetyl-CoA thiolase and for SCOT were as previously described (Fukao et al. 1997; Song et al. 1997) using acetoacetyl-CoA as a substrate and measuring its disappearance spectrophotometrically.

Mutation analysis

Total RNA was purified from peripheral blood mononuclear cells with an ISOGEN kit (Nippon Gene, Tokyo, Japan). Real-time polymerase chain reaction (RT-PCR) was as previously described (Kassovska-Bratinova et al. 1996). Mutations were detected by amplifying cDNA spanning the full-length coding sequence and by sequencing ten clones. Genomic DNA was purified with a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). Mutation analysis at the genomic level was done by PCR for each exon and its intron boundaries (at least 50 bases from the exon/intron boundaries for both directions), followed by direct sequencing (Fukao et al. 2000).

Transient expression analysis

Mutant expression vectors were made using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and were confirmed by sequencing. Wild-type and mutant SCOT expression vectors (4 μg) were first transfected using Lipofectamine 2000 (GIBCO BRL Invitrogen Inc., Carlsbad, CA, USA) in ~10^5 SV40-transformed SCOT-deficient fibroblasts of GS01 (Kassovska-Bratinova et al. 1996; Fukao et al. 2004). One microgram of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct (Song et al. 1994), was cotransfected to monitor transfection efficiency. Transfection was done at 37°C for 24 h, then a further 48-h incubation was done at 37°C. The cells were harvested and stored at –80°C until SCOT and CT activities were assayed. Immunoblotting was done using a mixture of an anti-(human SCOT) antibody

(Song et al. 1997) and anti-(human CT) antibody (Song et al. 1994) as the first antibody (Fukao et al. 1997). The quantity of the mutant protein was estimated densitometrically, comparing it to the signal intensities of serially diluted samples of the wild-type SCOT protein.

Structural analysis

For analyzing putative structural effects of mutations on the SCOT protein, the recently determined crystal structure from the Structural Genomics Consortium [Protein Data Bank (PDB) entry 3DLX] of human SCOT was taken as a starting point. The structure was subjected to further crystallographic refinement using PHENIX (Adams et al. 2010) and COOT (Emsley and Cowtan 2004), including the addition of missing side chains and rebuilding the solvent network.

Results and discussion

Molecular diagnosis and characterization of mutations

SCOT activity in GS21's peripheral blood mononuclear cells was apparently much lower than two controls (GS21:0.25, control 1: 2.4, control 2: 3.9 nmol/min/mg of protein). Hence, we tentatively diagnosed him as having SCOT deficiency. Since we could not draw blood for a repeat of the enzyme assay at the age of 1 month and skin biopsy was not acceptable to the parents, we performed mutation analysis to confirm the diagnosis. The full coding sequence of SCOT cDNA from GS21 was sequenced after subcloning. Mutations c.658-666dupAACGTGATT p.N220_I222dup. and c.1304C > A (T435N) were separately identified in six and four clones, respectively. No other mutations were found at the cDNA level. We

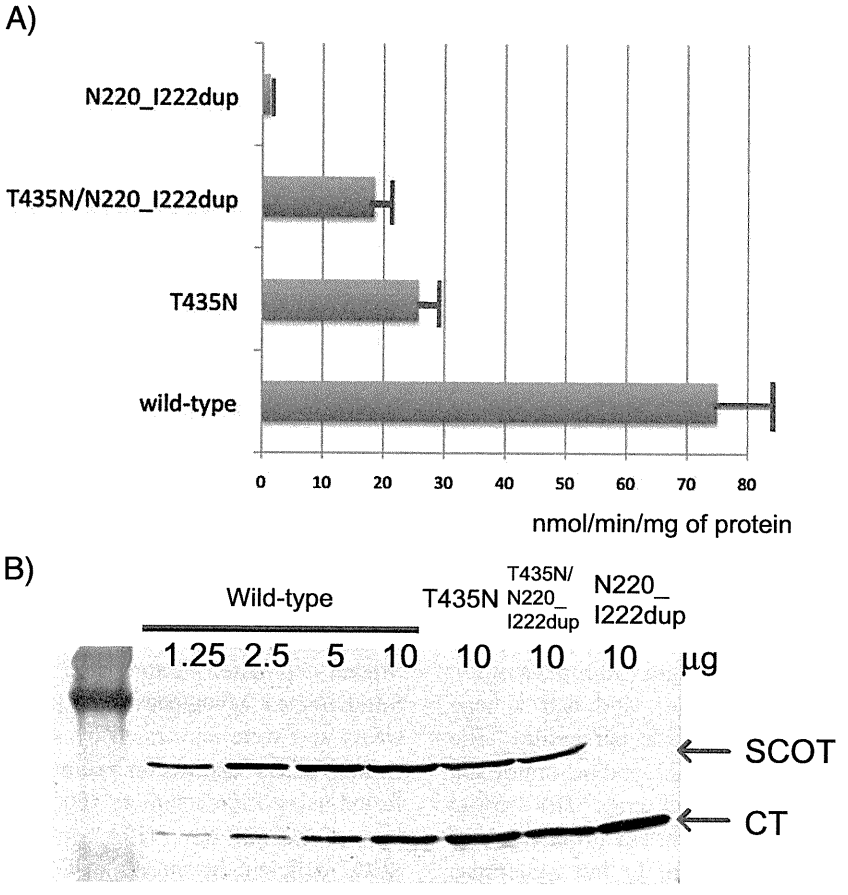

Fig. 1 Transient expression analysis of T435N and N220_I222dup mutant complementary DNAs (cDNAs). Transient expression analysis was performed at 37°C. Expression vectors (4 µg) were transfected. *T435N/ N220_I222dup* indicates a cotransfection of 4 µg each of two mutant expression vectors for T435N and N220_I222dup. **a** Succinyl-CoA:3-ketoacid CoA transferase (SCOT) enzyme assay. SCOT activity in the supernatant of the cell extract was measured. The mean values are shown together with the standard deviation (SD) of three independent experiments. **b** Immunoblot analysis. The protein amounts applied are shown *above the lanes*. We used previously described rabbit polyclonal antibodies, which we made (Song et al. 1994, 1997), and ProtoBlot Western blot AP system (Promega, Madison, WI, USA). The first antibody was a mixture of an anti-human cytosolic thiolase (CT) antibody and anti-human SCOT antibody. The positions of the bands for CT and SCOT are indicated by *arrows*. Immunoblotting was done using a mixture of an anti-(human SCOT) antibody (Song et al. 1997) and anti-(human CT) antibody (Song et al. 1994) as the first antibody (Fukao et al. 1997)