以外にサクシニル-CoA：３ケト酸トランスフェラーゼ(SCOT)欠損症が知られているが、SCOT欠損症の代謝プロフィールはケトン体増加のみで非特異的である。このためケトン体代謝異常を生化学診断することは容易ではない。そこで、培養細胞とタンデムマスを用いるin vitro probe assay法によるケトン体代謝異常の酵素診断測定系を確立するために、基礎実験を行った。

B. 研究方法

１） βケトチオラーゼ欠損症の代謝プロフィールの検討

2000年から2009年までに島根大学（有機酸分析およびアシルカルニチン分析）、福井大学（主にアシルカルニチン分析）、および岐阜大学（酵素、遺伝子解析）で診断した症例の尿中有機酸所見と血中アシルカルニチン所見を、日本人患者７名とアジア諸国28名（中国６例、インド11例、ベトナム10例、およびタイ１例）で比較検討した。

２） in vitro probe assayによるケトン体代謝異常診断の基礎的検討

培養皮膚線維芽細胞とタンデムマスを用いるin vitro probe assayに3-OH-酪酸（ナチュラル）25 mMを負荷して96時間培養し、培養液中のアシルカルニチン分析を行った。細胞は、正常対照３例、βケトチオラーゼ（T2）欠損症２例、サクシニル-CoA：CoAトランスフェラーゼ（SCOT）欠損症２例を検討した。

（倫理面への配慮）

分析結果を診断後の治療に役立てるため、患者の尿、血液検体は連結匿名化して分析を行った。その他「臨床研究に関する倫理指針」に基づき、患者の個人情報について管理を行った。

C. 研究結果と考察

１） βケトチオラーゼ欠損症の代謝プロフィールの検討

(a) 代謝プロフィールの異常の頻度：表１に示すように、日本人患者のうち尿中有機酸で異常（2-メチル-3-ヒドロキシ酪酸の上昇）が検出されたのは７例中６例であった。しかしチグリルグリシンの明らかな上昇は７例中１例のみであった。残る１例は代謝プロフィールで明らかな異常なく、臨床症状から疑い酵素活性測定・遺伝子解析によって診断された症例であった。血中アシルカルニチンでは、７例中３例で異常（C5:1またはC5-OHの上昇）が観察され、両方とも高値を示したのは７例中１例のみであった。

一方アジア諸国の患者では、28例中全例が尿中有機酸分析で異常がみられた。2-メチル-3-ヒドロキシ酪酸の上昇は28例全例、チグリルグリシンの上昇は28例中27例にみられた。血中アシルカルニチン所見では、C5:1の上昇は28例中23例、C5-OHの上昇は28例中20例であった。両方とも上昇が見られたのは、28例中18例であった。

(b) 2-メチル-3-ヒドロキシ酪酸の排泄量（表１）：日本人ではマスクロマトグラムでのピーク面積比による定量値は4.0～33.9（正常＜1.3）であった。アジア諸国の患者では、21.1～950.1以上と日本人患者に比べて明らかに高値を示した。

(c) アシルカルニチン所見：日本人患者のC5:1濃度は７例中２例に異常がみられ、それぞれ0.48と0.85 μmol/L（正常＜0.2）であった。C5-OH濃度は、異常の判明している患者１例でありその値は3.61μmol/L（正常＜１）であった。一方アジアの患者で異常値は、C5:1濃度は0.22～1.21 μmol/L（正常＜0.2）、C5-OH濃度は1.03～2.93 μmol/L（正常＜1.0）であった。

（考察）

日本人患者では尿中有機酸分析でもアシルカルニチン分析でも、異常検出頻度は他のアジア諸国の患者に比べ明らかに低い傾向がみられた。日本人患者では、酵素欠損の証明される患者であっても代謝産物の濃度は低い傾向があり、生化学診断の難しい症例が多い可能性がある。わが国で行われているタンデムマスによる新生児マススクリーニングのパイロットスタディーにおいて、約127万人検査している段階でまだ発見例がないこと、乳幼児期に発症して初めて診断された小児で後方視的に新生児血液ろ紙のアシルカルニチン分析をしても異常を示さなかったことを考えると、日本人患者ではGC/MSでもタンデムマスでも異常が検出しにくいかもしれない。現時点では、新生児期に見逃す可能性がある疾患として、わが国のタンデムマス•スクリーニング一次対象疾患から外されている。

２） in vitro probe assayによるケトン体代謝異常診断の基礎的検討

培養皮膚線維芽細胞とタンデムマスを用いるin vitro probe assayは、脂肪酸β酸化能を評価する方法である。グルコースフリー、遊離脂肪酸フリーのメディウムにパルミチン酸、またはオクタン酸を培養液に添加して、96時間後の培

養液中に分泌されたアシルカルニチンを測定する方法である。図１に示すように、中鎖脂肪酸代謝異常（MCAD欠損症）ではC8, C6, C4などの中鎖～短鎖のアシルカルニチンの上昇がみられ、長鎖脂肪酸代謝異常（VLCAD欠損症）ではC16, C14, C12などの長鎖アシルカルニチンの上昇がみられる。

この原理を応用して、図２に示すような代謝マップで、ケトン体である3-ヒドロキシ酪酸を負荷してケトン体利用能を評価できるかどうか基礎実験を行った。ケトン体が利用されてアセチル-CoAが生成されると仮定して、アセチル-CoAを反映するマーカーとしてアセチルカルニチンの量を測定した。また参考としてC8およびC4-OH（3-ヒドロキシブチリルカルニチン）を測定した。その結果、図３に示すように、3-ヒドロキシ酪酸負荷前後で、正常、T2欠損症、SCOT欠損症ともにアシルカルニチンの変化に明らかな差はみられなかった。また３群ともに、3-ヒドロキシ酪酸の負荷後にC4-OHの著明は増加がみられた。

（考察）

今回の方法では、ケトン体負荷によるアセチル-CoAの生成量を評価できなかった。今後の課題として、今回用いたケトン体濃度は25 mMであったがこの量は正常人のケトン体血中濃度の100倍以上にあたる（正常者の血中ケトン体濃度は＜0.15mM）。負荷するケトン体の量について検討する必要がある。また今回負荷したケトン体はナチュラル型であり、安定同位体ラベルしたケトン体を負荷してC2をトレースすることも検討してみる必要がある。ケトン体でなく通常のように脂肪酸を負荷した時に生成されるケトン体、あるいはC2をトレースするとケトン体代謝異常を評価できるかもしれない。

D. 結論

代表的なケトン体代謝異常であるβケトチオラーゼ欠損症は、日本人で診断された患者数はこれまで10例前後と少ない。これに対し、日本以外のアジア諸国（特に南アジア）では、βケトチオラーゼ欠損症の頻度が高くまた生化学的異常も目立つ。日本人の患者ではβケトチオラーゼ欠損症は生化学的には異常が検出されにくいのかもしれない。またケトン体代謝異常を酵素学的に評価する目的で、in vitro probe assayの応用を試みたが、ケトン体代謝異常に特異的所見は得られなかった。今後安定同位体ラベルの化合物などで検討する価値はある。

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F. 知的財産権の出願・登録状況

特になし

表1. βケトチオラーゼ欠損症の有機酸とアシルカルニチンプロフィール：アジア諸国との比較

A＝急性期
S＝安定期

2M3HB＝2-me-3-OH-butyrate;
TG＝tiglylglycine

×＝検査せず
（すでに他施設で診断）
OR＝振り切れ(over range)
nd ＝検出感度以下
？ ＝詳細不明
＋ ＝異常値しかし量は不明

国	Case No.	診断時年齢	状態	GC/MS		MS/MS	
				2M3HB < 1.3	TG 0.0	C5:1 < 0.2	C5-OH < 1.0
日本	1	8m	A	31.5	nd	nd	nd
	2	3y4m	A	23.6	nd	trace	nd
	3	3y4m	A	21.8	nd	nd	nd
	4	3y8m	A	4.0	nd	×	×
	5	?	A	＋	nd	0.06	0.54
	6	>20y	S	33.9*	140.6*	0.85	3.61
	7	?	?	?	?	0.48	0.54
中国	1	5m	A	129.2	80.0	0.24	nd
	2	1y1m	S	67.0	30.0	0.29	1.30
	3	1y1m	A	125.9	29.4	0.70	1.68
	4	2y6m	?	×	×	trace	0.19
	5	14y10	?	×	×	0.60	0.35
	6	?	S	72.9	384.6	×	×
インド	1	6m	A	950.4	853.5	0.66	2.98
	2	7m	A	83.5	OR	0.01	0.16
	3	7m	A	31.4	128.8	0.29	1.03
	4	1y1m	S	76.7	trace	0.22	0.91
	5	2y5m	A	32.0	nd	0.02	0.57
	6	11y5m	S	43.5	185.1	×	×
	7	?	S	21.1	42.8	×	×
	8	?	S	160.5	64.0	0.32	2.69
	9	?	S	＋	＋	×	×
	10	1y0m	A	23.93	15.7	0.11	0.54
	11	10m	A	27.04	155.0	0.44	2.93
ベトナム	1	3m	A	203.1	228.0	0.42	1.10
	2	3m	S	53.7	OR	0.19	1.09
	3	6m	A	120.1	207.8	0.60	2.73
	4	7m	A	132.7	101.7	0.78	1.92
	5	11m	A	419.1	192.7	0.89	2.46
	6	2y9m	A	73.9	472.1	0.53	1.52
	7	3y1m	S	34.1	168.2	1.20	2.35
	8	?	A	118.7	212.7	0.44	1.38
	9	1y0m	A	80.8	129.54	0.23	0.94
	10	10m	A	27.0	62.79	0.19	1.90
タイ	1	1y	?	×	×	1.21	2.83

図1. in vitro probe assay の結果の例

MCAD= 中鎖アシル-CoA 脱水素酵素
VLCAD= 極長鎖アシル-CoA 脱水素酵素

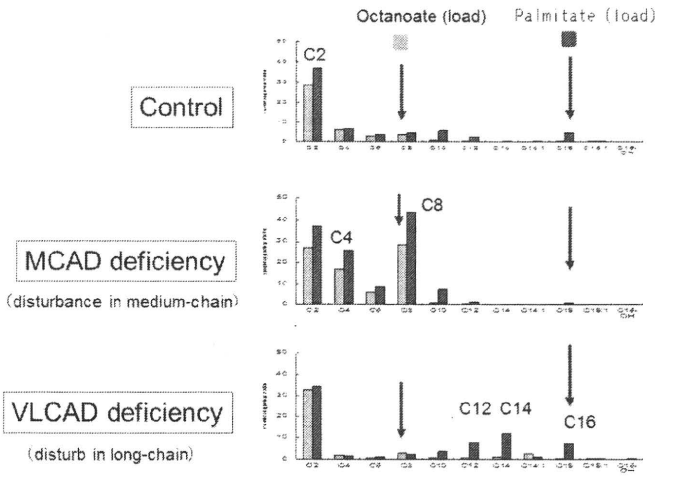


図2. ケトン体代謝の代謝マップ

3-OH-butyrate を負荷する in vitro probe assay

★:3-OH-酪酸を負荷する。(**略字**)T2 = ミトコンドリアアセトアセチル-CoA チオラーゼ(短鎖型 β ケトチオラーゼ)、;HMGS = ヒドロキシメチルグルタリル-CoA(HMG-CoA)合成酵素;HMGLHMG-CoA リアーゼ = ;SCOT = サクシニル-CoA:3-ケト酸 CoA トランスフェラーゼ。

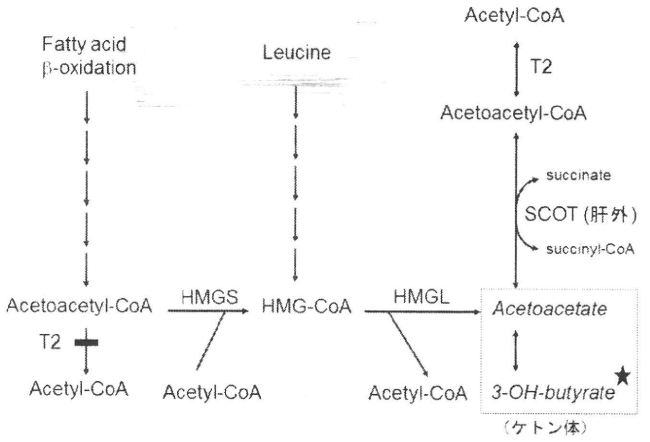
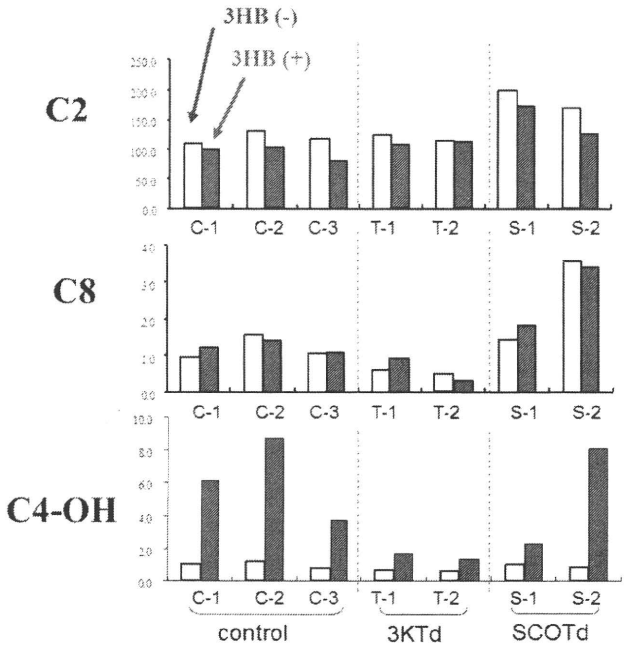


図3. In vitro probe assay の結果

(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
カルニチン過剰状態
3-OH-酪酸(25 mM)を負荷

(略字)
C = control
T, 3KTd = 3KT deficiency
S, SCOTd = SCOT deficiency
3HB = 3-OH-butyrate



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：ケトン体代謝異常症の急性期治療に関する研究

研究分担者　高柳正樹　千葉こども病院

研究要旨

「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斑」ので次年度診断治療指針を作成するために、特に急性期治療に関してケトン体合成の抑制、低血糖、アシドーシスの治療について検討し記載した。

A．研究目的

「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斑」で次年度診断治療指針を作成するために、治療に関する検討を行った。

B．研究方法

これまでの症例報告、総説、教科書等から急性期治療に関する情報を得て、それから妥当と考えられる治療法を選択する。

略語

HL: HMG-CoA lyase

SCOT:succinyl-CoA: 3-ketoacid CoA transferase

T2: mitochondrial AcAc-CoA thiolase

mHS: mitochondrial HMG-CoA synthase

C．研究結果

ケトン合成の抑制

HL, SCOT, T2, mHS 欠損症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 HL, SCOT, T2 欠損症では糖質の補充はタンパク分解亢進も抑制する

低血糖

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。

アシドーシス

HL, SCOT, T2 欠損症ではアシドーシス発作が合併する。SCOT, T2 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。

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 HL, SCOT, T2 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。

D. 考察

適切な急性期治療が、ケトン体代謝異常症の症例において重要なことは言うまでもない。ケトン体産生障害では低血糖が、ケトン体利用障害ではケトアシドーシスが臨床的に問題となるが、重要なことはともにケトン体産生の刺激をおさえることであり、十分な糖分の早期補給ということになる。ともすればアシドーシス治療を生理食塩水で開始するということがされがちであるが、糖分の十分な補給が重要であることを強調したい。

なお今回のこの急性期治療の方針は、Mitchell GA, Fukao T: Chapter102 Inborn errors of ketone body metabolism. (Scriver CR, Beaudet AL, Sly WS, Valle D eds: Metabolic and Molecular Bases of Inherited Disease (8th edition), McGraw-Hill, Inc, NewYork) 2001, vol 2, pp2327-2356 を多く参考にした。

G. 研究発表

1. 論文発表

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2. 学会発表

１）深尾敏幸、山口清次、重松陽介、高柳正樹、新宅治夫、堀川玲子　「先天性ケトン体代謝異常症の発症形態と患者数の把握、診断指針に関する研究」班

先天性ケトン体代謝異常症(T2 欠損症、SCOT 欠損症)の診断指針―日本での診断された症例の検討から　第５２回日本先天代謝異常学会総会　１０月２１～２３日、大阪、２０１０

H. 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

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：T２欠損症の遺伝子診断に関する研究　MLPA 法の確立

研究分担者　深尾敏幸　　所属　岐阜大学大学院医学系研究科

研究要旨

T2 欠損症の遺伝子診断において、遺伝子内の１つ以上のエクソンを含む挿入、欠失変異は。その代表が遺伝子内の１つ以上のエクソンを含む欠失や挿入変異は、ゲノム PCR—ダイレクトシークエンス法では同定できないため問題である。MLPA 法はエクソンのコピー数の異常を検出する感度のいい方法であり、多くの疾患では既製の MPLA プローブキットが販売されている。しかし稀な疾患においては独自で作成する必要があり、今回 T２欠損症について MLPA 法を独自で作成して、このような遺伝子内欠失、挿入変異を検出することが可能とすることができた。

A．研究目的

β-ケトチオラーゼ欠損症はミトコンドリアアセトアセチル-CoA チオラーゼ（T２）の欠損症である。演者らはこれまで世界各地からの約 85 症例の遺伝子変異を同定してきた．今回本遺伝子の Multiplex ligation-dependent probe amplification assays (MLPA)を独自に確立し、それによるスクリーニングで遺伝子内欠失を同定した

。

B．研究方法

MLPA probe design というソフトを用いてT２遺伝子のプローブを設計し、独自の T2 遺伝子 MLPA 法を確立した。すでに報告したエクソン２−４を含む欠失およびエクソン８−９のタンデム重複のホモ接合体および人工的に作成したそれらのヘテロ接合体が同定可能であるかどうかを調べた。

まだ遺伝子変異が１アレルしか同定されていない症例 GK06,, GK44, GK51, GK83 について本法を用いて、各エクソンの欠失、重複の確認を行った。

これら疾患の遺伝子解析は岐阜大学の医学研究等倫理委員会の承認を得て、個人情報の取り扱いに注意して行っている。

C．研究結果

1) これまで同定されたエクソン重複例、欠失例の検出

コントロール（図１A）エクソン２−４の欠失ホモ接合体例（図１B）、エクソン８−９の重複例（図１C）、人工的に作成したエクソン２−４欠失とエクソン８−９に重複のヘテロ接合体（図１D）の MLPA 増幅パターンを示した。十分に異常を検出でき、相対コピー数を示したプロット（図２A~D）においても異常を検出できた。

2)一方のアレルの変異の同定できていない４名について本法を用いたところ、GK44 でエクソン３−４を含む欠失をヘテロで同定できた(図３)。

D．考察

遺伝子変異の同定率は 86 症例中で最終的に９６％（７アレル変異不明）である。β-ケトチオラーゼ欠損症では遺伝子内のエクソンを含む欠失挿入例として、エクソン２−４の欠失、エクソン８−９の重複、エクソン３−４の欠失がこれで同定されたが、すべてイントロン内の Alu 配列での不均等組み換えによるものであった。遺伝子によってこのような遺伝

子内の比較的大きな欠失、挿入変異の頻度は異なっているが、常にこのような変異を考慮する必要がある。

MLPA 法は各エクソンの欠失、重複を同定するのに非常に有用な方法で、ジストロフィン遺伝子など既に臨床応用されているが、T２欠損症のような稀な疾患ではコマーシャルベースでの開発はされず、独自に作成することが必要であった。

E. 結論

本研究によりβ-ケトチオラーゼ欠損症の MLPA 法を確立することができた。これによりこれまで変異が同定できなかった症例においても異常を検出できる可能性が高まり、遺伝子診断の向上が得られた。

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2) Fukao T, Sass, JO, Thimm E, Wendel U, Ficicioglu C, Monastiri K, Guffon N, Baric I, Zabot M-T, Kondo N. Clinical and molecular studies of five patients with succinyl-CoA:3-ketoacid CoA transferase deficiency. 2010 Annual symposium of Society for the Study of Inborn Errors of Metabolism. August 31-September 3, 2010 (Istanbul, Turkey)

3) 深尾敏幸、山口清次、重松陽介、高柳正樹、新宅治夫、堀川玲子 「先天性ケトン体代謝異常症の発症形態と患者数の把握、診断指針に関する研究」班 先天性ケトン体代謝異常症(T2 欠損症、SCOT 欠損症)の診断指針—日本での診断された症例の検討から 第５２回日本先天代謝異常学会総会 １０月２１～２３日、大阪、２０１０

4) 深尾敏幸、堀友博、近藤直実: サクシニル-CoA:3-ケト酸 CoA トランスフェラーゼ(SCOT)欠損症５症例の検討。 第５２回日本先天代謝異常学会総会 １０月２１～２３日、大阪、２０１０

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H. 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

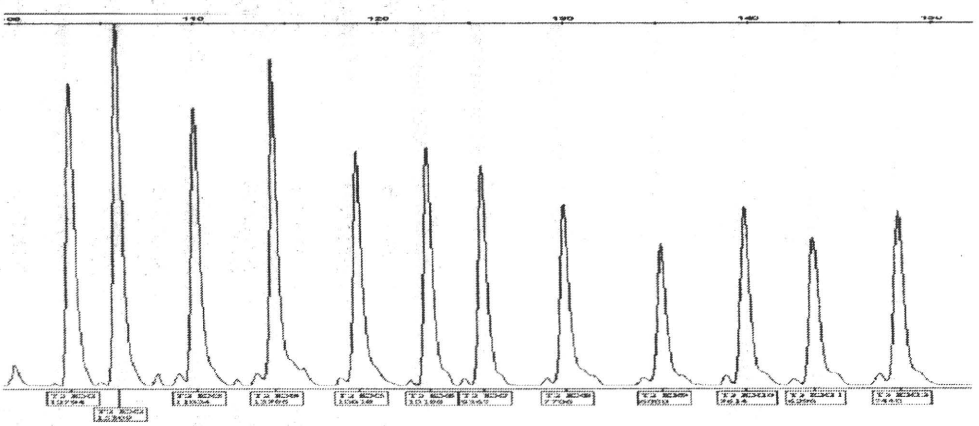
3. その他

なし

図1

A)

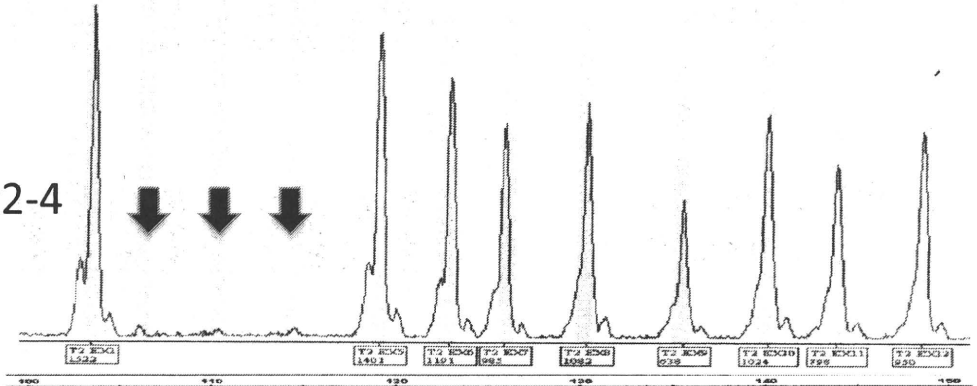
Control



B)

GK41

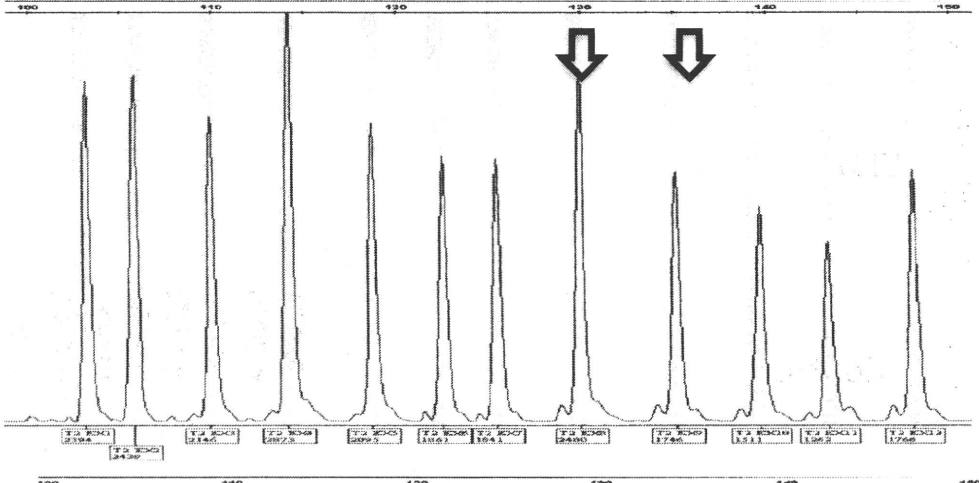
del ex2-4/del ex2-4



C)

GK48

dup8-9/dup8-9



D)

del ex2-4/

dup ex8-9

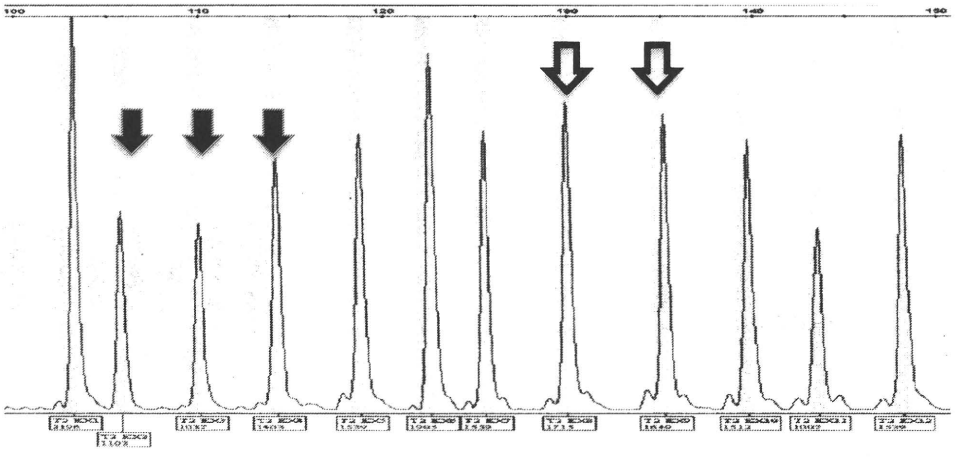


図2）

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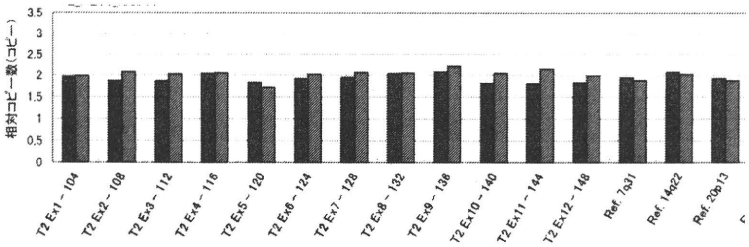
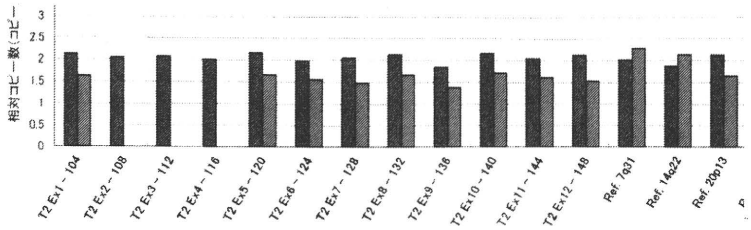
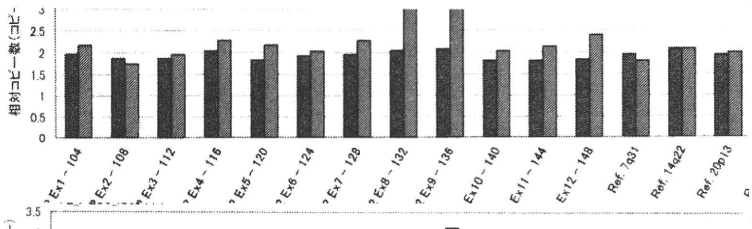


B) GK41
del ex2-4/del ex2-4



C) GK48
dup8-9/dup8-9



D) del ex2-4/
dup ex8-9

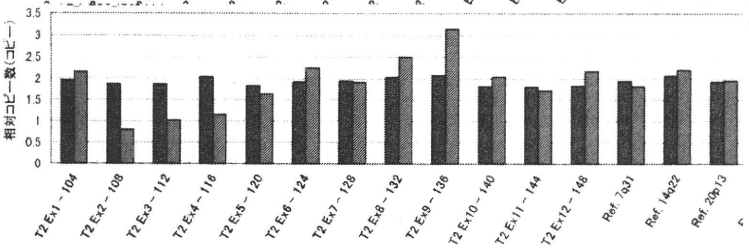
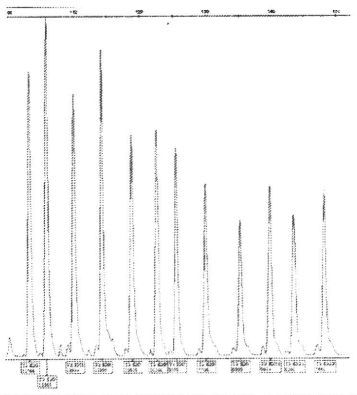
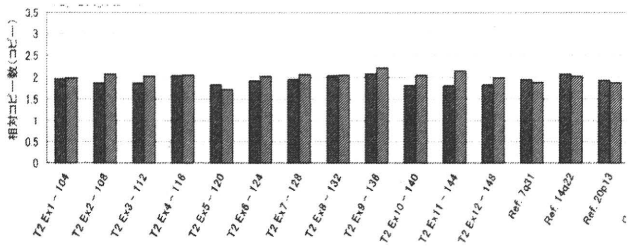


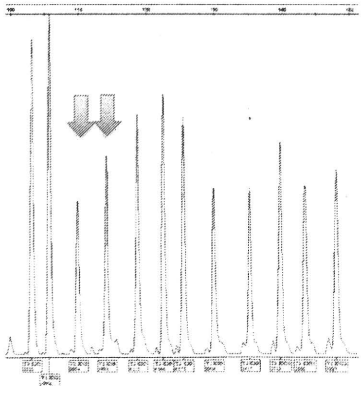
図3）

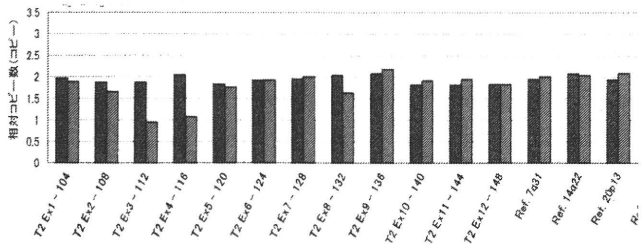
Control





GK44





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

書籍

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
深尾敏幸	ケトン体	高柳正樹	小児科臨床ピクシス 見逃せない先天代謝異常	中山書店	東京	2010	93-95
深尾敏幸	ケトーシス	高柳正樹	小児科臨床ピクシス 見逃せない先天代謝異常	中山書店	東京	2010	14-17
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雑誌

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Fukao T, Sass JO, Kursula P, Thimm E, Wendel U, Ficicioglu C, Monastiri K, Guffon N, Varic I, Zabot M-T, Kondo N.	Clinical and molecular characterization of five patients with Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency	Biochimica Biophysica Acta Molecular Basis of Disease	In press		
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A novel mutation (c.951C>T) in an exonic splicing enhancer results in exon 10 skipping in the human mitochondrial acetoacetyl-CoA thiolase gene

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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

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

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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 10,000g 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).

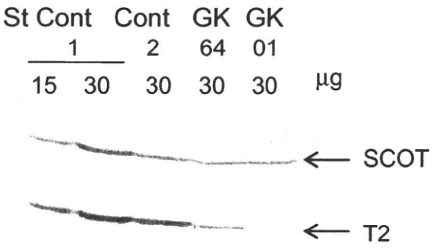

Fig. 1. Immunoblot analysis. The amount of fibroblast protein extract applied was indicated in each lane. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by arrows. Cont 1 and Cont 2 were healthy controls and 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. 3**A.

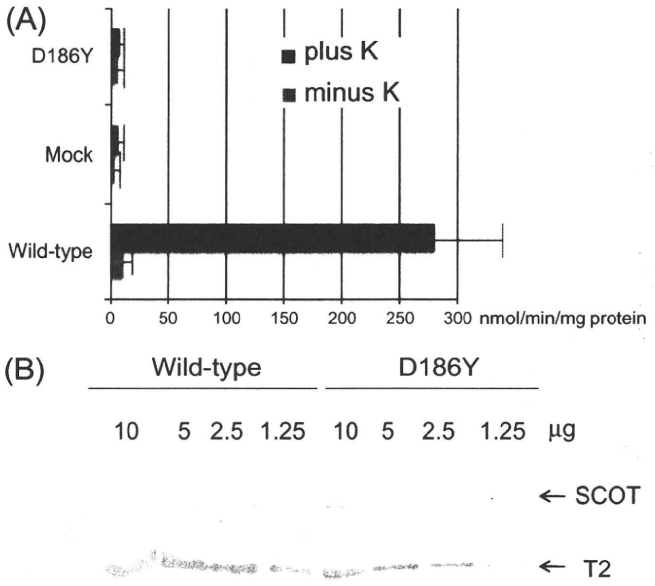

Fig. 2. Transient expression analysis of D186Y mutant cDNA. (A) Potassium-ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence and presence of the potassium 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. 3**A. 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

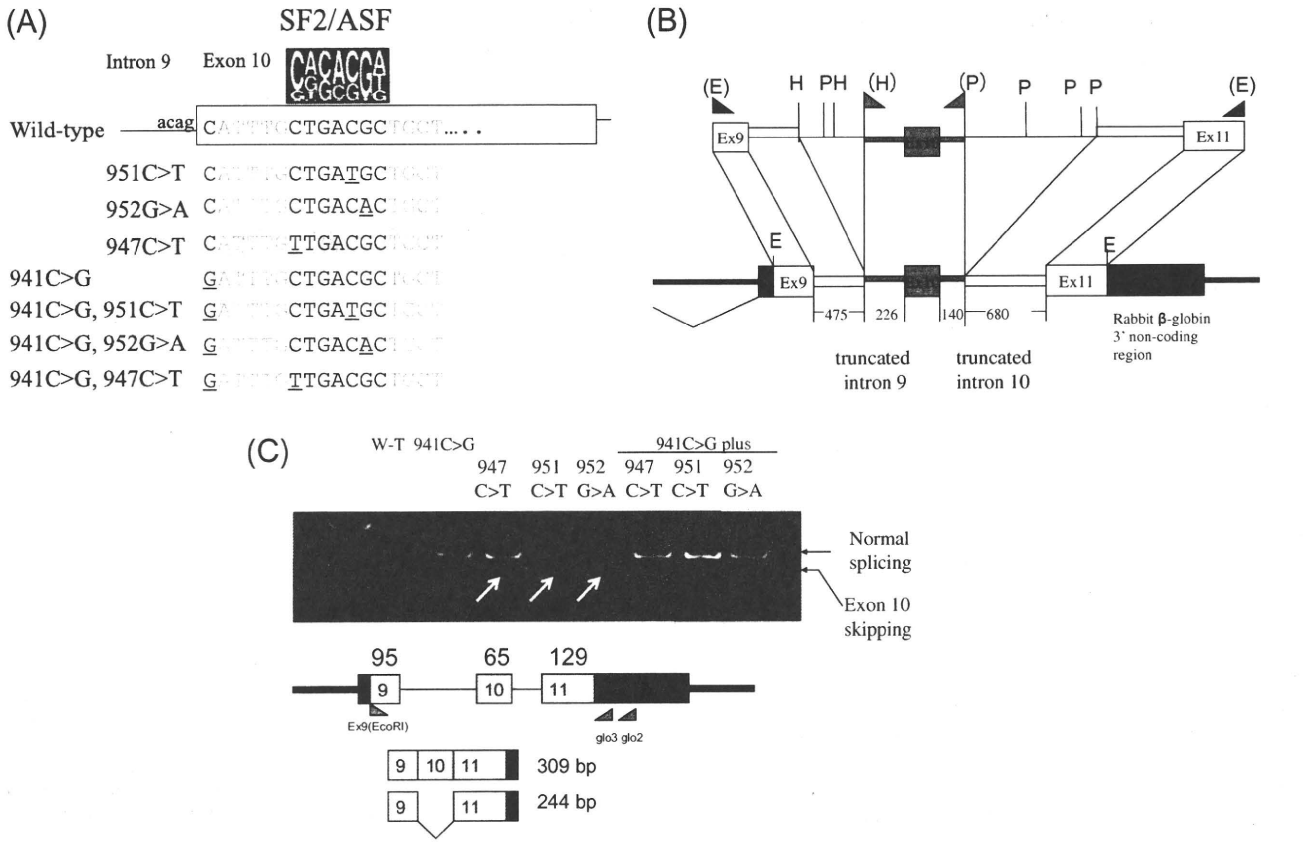

Fig. 3. Minigene splicing experiment. (A) Minigene splicing constructs. Sequence differences among 8 minigene splicing constructs. Mutations introduced are underlined. (B) Schematic presentation of minigene splicing construct. The minigene construct has a T2 gene fragment from c.842 of exon 9 and intron 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

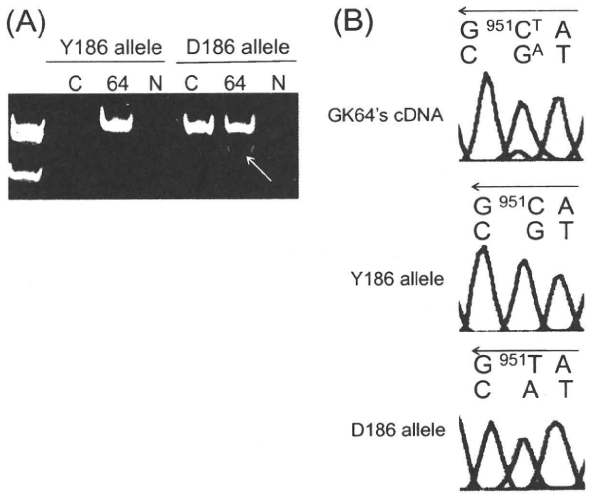

Fig. 4. Allele-specific cDNA amplification. (A) Allele-specific PCR fragments were electrophoresed on 5% polyacrylamide gel. C, control cDNA; 64, GK64's cDNA, N, negative control. An arrow indicates cDNA with exon 10 skipping. (B) Direct sequencing of the antisense strand at the c.951c>T (D186Y) site.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

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