Table 1 Clinical features of amino acidemia, urea cycle disorder, organic acidemia, and fatty acid oxidation disorder, and biochemical diagnosis

Clinical features	Tools for biochemical diagnosis
Amino acidemia	
・Neurological impairment ・Convulsion, unconsciousness ・Liver dysfunction ・Renal stone	Amino acid analysis (Organic acid analysis)
Urea cycle disorder	
・Convulsion, unconsciousness ・Mental retardation ・Hyperammonemia	Amino acid analysis Blood ammonia Organic acid analysis
Organic acidemia	
・Acute onset with hypotonia, unconsciousness from early infancy ・Intermittent episodes of ketosis, hypoglycemia ・Neurological retardation ・Other (ex. intractable eczema)	Organic acid analysis Acylcarnitine analysis
Fatty acid oxidation disorder	
・Lethargy, hypotonia, myalgia ・Acute encephalopathy, sudden death ・Cardiomyopathy ・Non-ketotic hypoglycemia ・Liver dysfunction, CK elevation	Acylcarnitine analysis Organic acid analysis

II. アミノ酸・有機酸・脂肪酸代謝異常症の臨床的特徴

Table 1 に示すように，アミノ酸代謝異常症は血液(または尿)のアミノ酸分析，尿素回路異常症では血中アンモニア値とアミノ酸分析などによって診断される。有機酸代謝異常症と脂肪酸代謝異常症は，GC/MS による尿中有機酸分析，タンデムマスによる血中アシルカルニチン分析によって生化学診断される。両者ともカルボン酸の増加する点で類似しているが臨床的特徴に違いがみられる。前者は有機酸の臓器毒性の所見がみられることが多く，後者はエネルギー産生不全による症状が前面に出る。

タンデムマス法ではアミノ酸とアシルカルニチン分析が可能であり，これら上記4種類の疾患群のスクリーニングに用いられる。GC/MS による尿中有機酸分析では，有機酸代謝異常症の生化学診断に最も威力を発揮するが，一部のアミノ酸血症でも補助的診断に用いられる。例えば，有機酸分析によって，フェニルケトン尿症でフェニルピルビン酸やフェニル乳酸，メープルシロップ尿症で分枝鎖 αケト酸の増加が観察される。尿素回路異常症では，一部の疾患でウラシル，オロット酸の増加がみられ診断に有用である。さらに脂肪酸代謝異常症の家長鎖脂肪酸代謝異常症では非ケトン性ジカルボン酸尿症がみられ，中鎖アシル-CoA 脱水素酵素欠損症ではヘキサノイルグリシンやスベリルグリシンのような診断的に有用な有機酸が検出される。

III. タンデムマスと GC/MS について

A. タンデムマス

MS が直列に並んだ構造(MS1 と MS2)構造を持ち，MS1 と MS2 で測定された粒子の質量数を比較して分子量が推定される。そしてそれぞれの分子のイオン強度によって定量される。非常に高感度な分析が可能であり[4]，新生児マススクリーニングでは，血液ろ紙の 3mm 大のディスクでよく，血清 10μL で分析可能である[5]。また分析試薬等がキット化され前処理も非常に簡単になっている。1検体の分析時間は2分程度であり，ランニングコストも安価なた

め，マススクリーニングに向いている。

タンデムマスでは，アミノ酸とアシルカルニチンが同時分析される。アミノ酸に関しては，アミノ酸分析計に比べると精度が低く限られたアミノ酸のみが測定可能である。このため代謝異常スクリーニングに用いられる。一方アシルカルニチン分析では，遊離カルニチン(C0)とアシルカルニチンが測定され，有機酸代謝異常症や，脂肪酸β酸化異常症(脂肪酸代謝異常症)の診断，スクリーニングに応用される。定性のための情報はイオンの質量数のみであるため，異性体の分離測定が困難である。しかしアシルカルニチンプロフィールが得られるため，後述のGC/MSに比べて，脂肪酸代謝異常症の診断には非常に有用である。

B. GC/MS

ガスクロマトグラフ(GC)と質量分析計(MS)が連結した構造を持つ機器である。GC分析するためには揮発性の物質に変える必要があるので，生体試料から抽出した物質を誘導体化してGC分析する[6]。GCから出てきた粒子は電子衝撃イオン化法で断片化されMSに導入される。マススペクトルは，分子構造の情報となる断片イオンが比較的多いため，異性体の同定も可能である。分析項目は尿中有機酸分析である。有機酸は弱酸であり体内に増加すると直ちに尿中に排出されるため尿の分析が用いられる。MSで一斉分析し，有機酸全体のプロフィールから代謝障害部位が特定される。

タンデムマスに比べGC/MSでは，機器のメンテナンスには比較的熟練を要し，また前処理も手間がかかり，分析時間も30～60分程度かかることが多い。しかし，詳細な代謝プロフィールが得られ，代謝異常の同定のみならず，乳酸ピルビン酸の増加，ケトーシス，ジカルボン酸尿症など患者の一過性の状態を把握することができ，病態評価，治療評価などに向いている。

IV. 質量分析による代謝異常症の生化学診断

体内に代謝障害があると，障害部位の上流物質とそれに由来する異常代謝産物がMSによる一斉分析で検出される。タンデムマスとGC/MSの分析プロフィールから，代謝障害部位が推定される。主な疾患とタンデムマスとGC/MS分析における診断マーカーを**Table 2**にあげた。

A. GC/MSによる尿中有機酸分析による生化学診断

ロイシンの代謝過程と有機酸代謝異常症を**Fig. 2**に例示している。最下段のメチルマロン酸血症では，メチルマロニル-CoAムターゼ欠損によって，methylmalonateとともにその上流のプロピオニル-CoAの代謝産物(methylcitrate，3-OH-propionate，propionylglycine等)が増加する。このプロフィールから生化学診断する。

その上流のプロピオン酸血症では，propionyl-CoA由来の状代謝産物との上流の2-methyl-3-OH-butyrate，tiglylglycine等が増加し，この特徴的なプロフィールから生化学診断される。

さらに，プロピオン酸血症の上流のβケトチオラーゼ欠損症では，上流の2-methyl-3-OH-butyrateやtiglylglycineが増加し，一方下流のpropionyl-CoA由来の代謝産物の増加はみられない。

B. タンデムマスによるアシルカルニチン分析

Fig. 2に示す3疾患のアシルカルニチン分析所見は，メチルマロン酸血症とプロピオン酸血症ではC3(propionylcarnitine)の増加がみられる。βケトチオラーゼ欠損症ではC5:1(tiglylcarnitine)とC5-OH(2-methyl-3-OH-butyrylcarnitine)の増加がみられる。メチルマロン酸血症とプロピオン酸血症は両疾患ともにC3の上昇しか見られないため，GC/MSによる尿中有機酸分析によって鑑別しなければならない。

V. GC/MSデータ自動解析・自動診断プログラム

GC/MS分析では，ガスクロマトグラムに相当するTIC(total ion current)が得られるが，この段階では，ピークの情報はピーク保持時間のみである。そのあと質量分析されると，各ピークのマススペクトルが得られる。**Fig. 3**にプロピオン酸血症の尿中有機酸分析所見とマススペクトルを例示している。しかし尿中に含まれる有機酸は数百以上あるので，すべてのピークをマススペクトルで同定してピーク強度などで定量することは，時間がかかるうえに，極めて困難な作業である。さらに代謝産物とその量がわかっても，代謝異常症に関する知識を必要とし，GC/MSデータをマニュアルで解析して生化学診断をすることは，専門知識のみならず，時間と労力を要する。

そこで我々は，独自に「GC/MSデータ自動解析・自動診断プログラム」を開発した[7]。これを使えば，GC/MS分析後に数分以内に，異常代謝産物を検出

Table 2 Main target diseases detectable by MS/MS and diagnostic markers

Disease	Diagnostic marker	
	MS/MS*	GC/MS**
Amino acidemia		
Phenylketonuria	Phe	PPA, PLA
Maple syrup urine disease	Ileu, Leu, Val	2KIV, 2M3VA, 2KIC
Homocystinuria	Met	−
Urea cycle disorder		
Citrullinemia (type I)	Cit	Orot, Uracil
Argininosuccinic aciduria	Cit (ASA)	Orot, Uracil
Organic acidemia		
Methylmalonic acidemia	C3, C3/C2	MMA, MC, 3HPA, PG
Propionic acidemia	C3, C3/C2	MC, 3HPA, PG
Isovaleric acidemia	C5	IVG
Methylcrotonylglycinuria	C5-OH	MCG, 3HiVA
HMG-CoA lyase deficiency	C5-OH	HMGA, MGA, MGCA
Multiple carboxylase deficiency	C5-OH	MC, MCG, 3HPA
Glutaric acidemia type I	C5-DC	GA, 3HGA
β-ketothiolase deficiency	C5-OH, C5:1	2M3HBA, TG
Fatty acid oxidation disorder		
MCAD deficiency	C8	HG, SG
VLCAD deficiency	C14:1	DIC
TFP deficiency	C16-OH, C18-OH	DIC, 3HDIC
CPT-1 deficiency	C0/[16+C18]	DIC
CPT-2 deficiency	(C16+C18:1)/C2, C16	DIC
Primary carnitine deficiency	C0 (reduced)	DIC
Glutaric acidemia type II	C8, C10, C12, etc	DIC, EMA, IVG, GA, etc

*MS/MS, amino acid and acylcarnitine in blood; GC/MS, urinary organic acid.
Abbreviations: MCAD and VLCAD, medium-chain- and very-long-chain-acyl-CoA dehydrogenases, respectively; TFP, mitochondrial trifunctional protein; CPT-1 and CPT-2, carnitine palmitoyltransferase-I and -II, respectively; PPA, phenylpyruvate; PLA, phenyllactate; 2KIV, α-ketoisovalerate; 2M3VA, α-keto-3-methylvalerate; 2KIC, α-ketoisocaproate; Orot, orotate; MMA, methylmalonate; MC, methylcitrate; 3HPA, 3-OH-pyruvate; PG, propionylglycine; IVG, isovalerylglycine; MCG, methylcrotonylglycine; 3HiVA, 3-OH-isovalerate; HMGA, 3-OH-3-methylglutarate, MGA, methylglutarate; MGCA, methylglutaconate; GA, glutarate; 3HGA, 3-OH-glutarate; 2M3HBA, 2-methyl-3-OH-butyrate; TG, tiglylglycine; HG, hexanoylglycine; SG, suberylglycine; DIC, dicarboxylate; 3HDIC-3-OH-dicarboxylate; EMA, ethylmalonate.

し，考えられる診断名をアウトプットできる。このため，代謝異常症やGC/MSデータに知識のない人でも短期間のうちに使えるようになる。**Fig. 4**に自動解析結果を例示している。これによると異常として検出した代謝産物に3-OH-propionate，propionylglycinem，およびmethylcitrateがあるので，下の段に「疑わしい疾患」としてプロピオン酸血症をあげている。患者の臨床経過やタンデムマスデータなどを総合すれば，プロピオン酸血症という最終診断に比較的容易に到達することができる。

VI. タンデムマスによるアシルカルニチン分析

血液ろ紙や血清10μL程度の微量検体で，約2分間の分析時間のあいだに，アミノ酸とアシルカルニチンを分析し，そして**Fig. 5**に示すような，アシルカルニチンとアミノ酸のデータを得ることができる。アシルカルニチンはアシル基の炭素数に対応して，C2，C3，C4.....と表示がされる。またアシル基に水酸基がついていればC5-OHのように表示され，不飽和結合をもつアシル基ならばC5:1，ジカルボキシル基であればC5-DCのように表現される。一方，

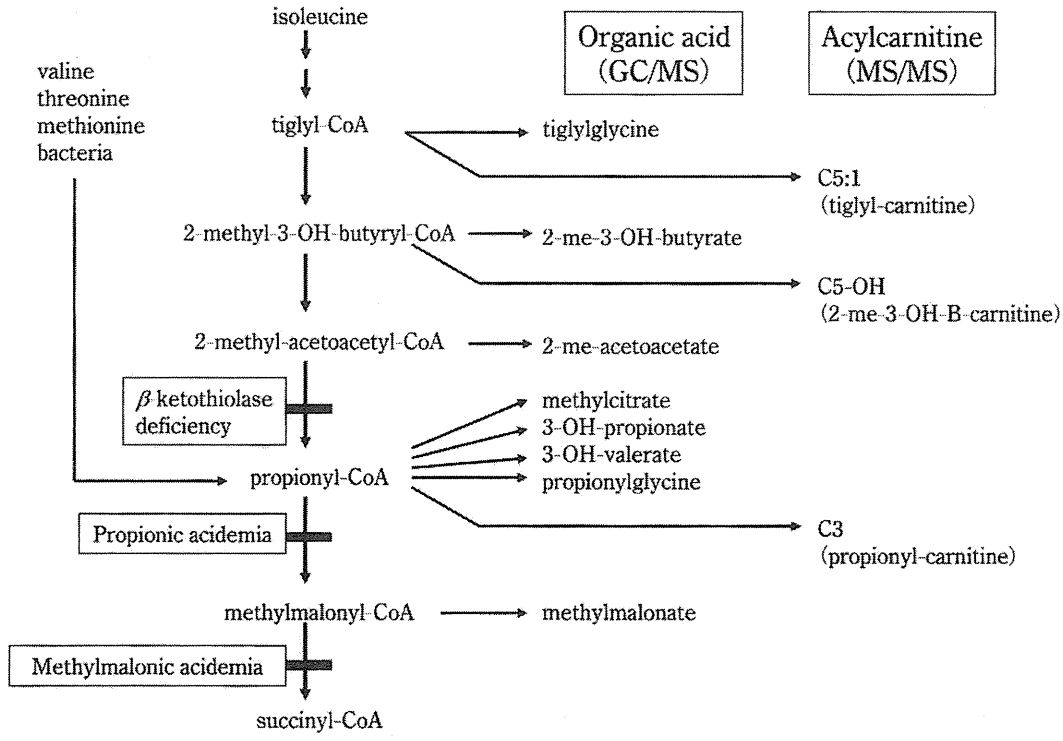

Figure 2 Metabolic pathways of isoleucine and related organic acidemia.

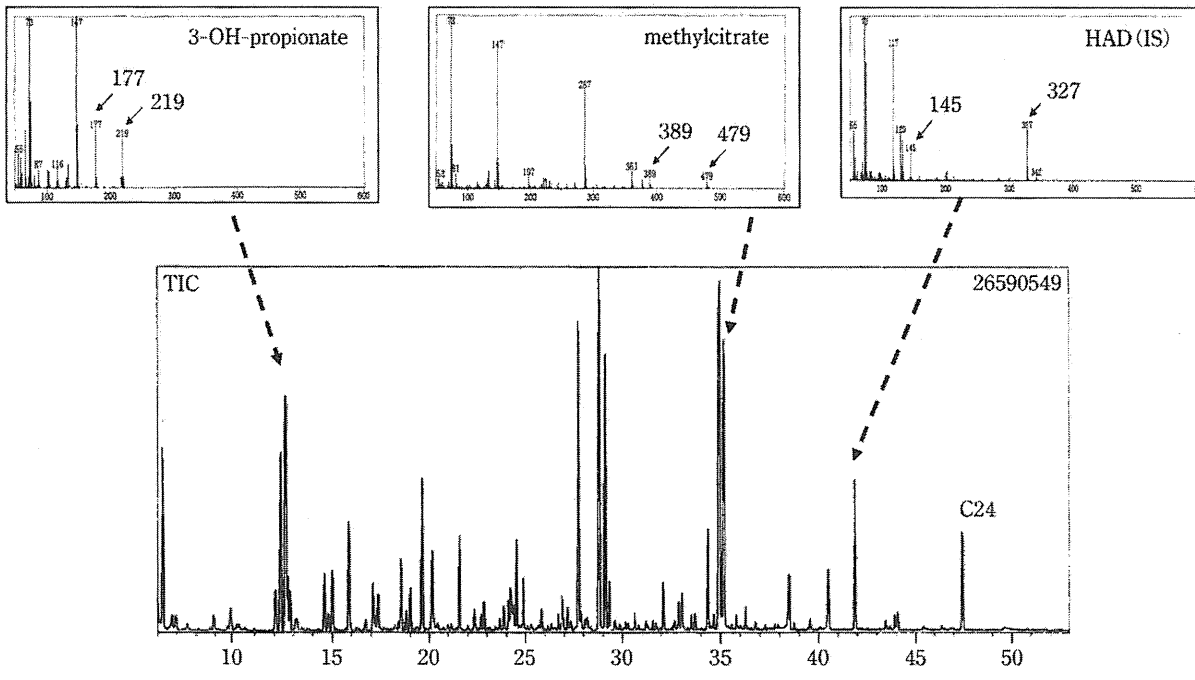

Figure 3 Gas chromatograms of urinary organic acids and mass spectrum of some peaks in propionic acidemia.
HDA, and C24 are heptadecanoate and tetracosane added internal standard.

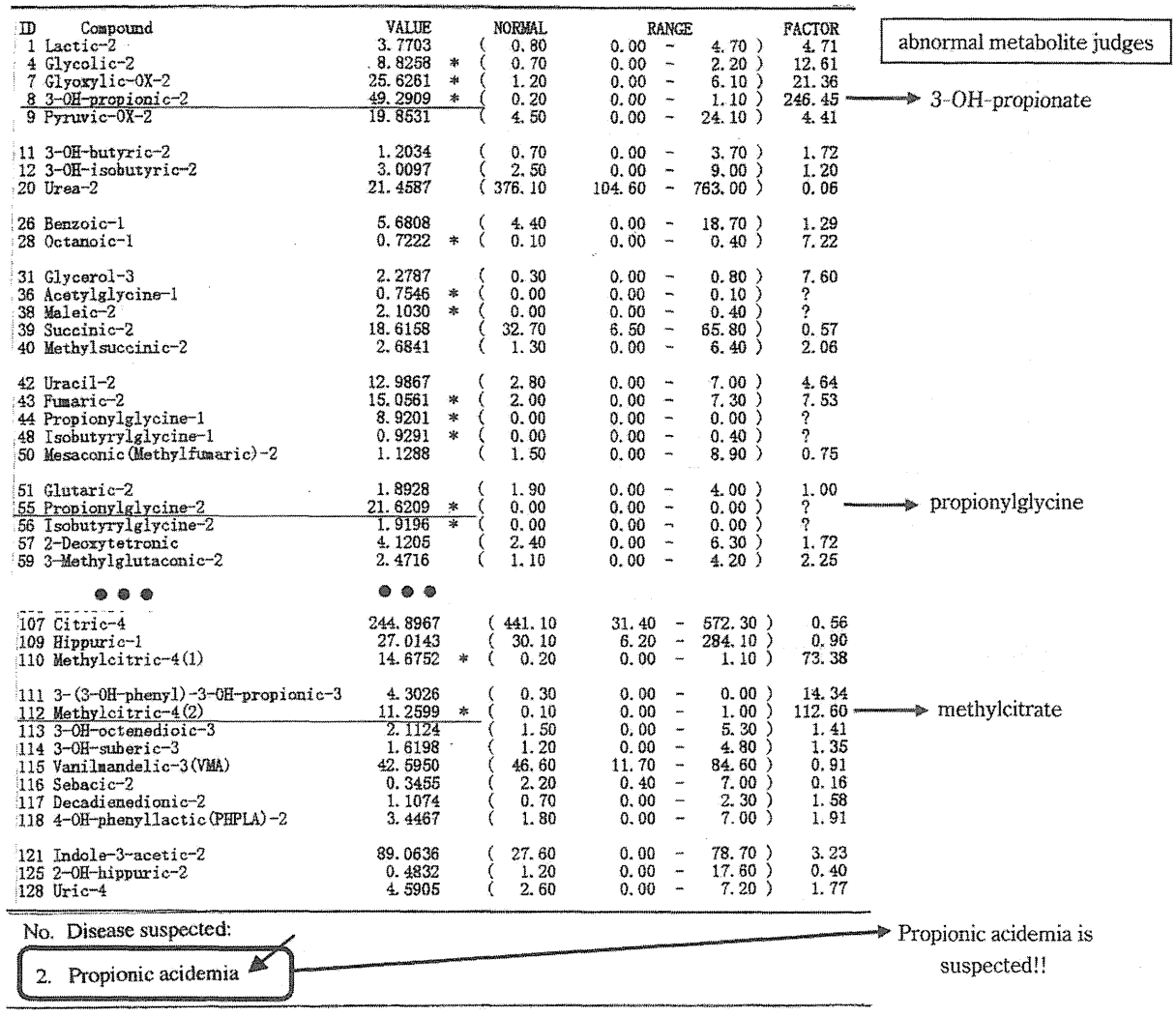

Figure 4 Results from the automated system of GC/MS data processing and autodiagnosis of propionic acidemia
*: abnormal metabolites flagged.

アミノ酸は，Phe，Tyr，Met，Leu，Val などのように表示される。

アシルカルニチンやアミノ酸の全体のプロフィールを見る方法を「スキャン法」というが，最初から測定する目標化合物を決めて，その化合物に対応するイオン質量数を設定して分析する方法を MRM 法という。MRM 法はスキャン法に比べて測定感度が数十倍に上がるが，全体のプロフィールを見ることはできなくなる。血液ろ紙を使用する新生児マススクリーニングでは，感度を上げるため MRM 分析の方がポピュラーになっている[2]。

VII. 後天性代謝異常症の診断

小児救急の場で急性脳症などに遭遇すると背景疾患のスクリーニングのために，タンデムマス分析や GC/MS 分析がポピュラーになりつつある。先天代謝異常の診断のみならず，後天性代謝障害を評価することもできる。例えば，ケトン性ジカルボン酸尿症は非常に強いケトーシスによって β 酸化が抑制されていることを表わし，非ケトン性ジカルボン酸尿症では，何らかの要因で β 酸化が抑制されていることが推測される。この他，食事性のビオチン欠乏やビタミン B12 欠乏，あるいは，チアミン欠乏による高乳酸血症，あるいは肝障害に伴うチロシン代謝の

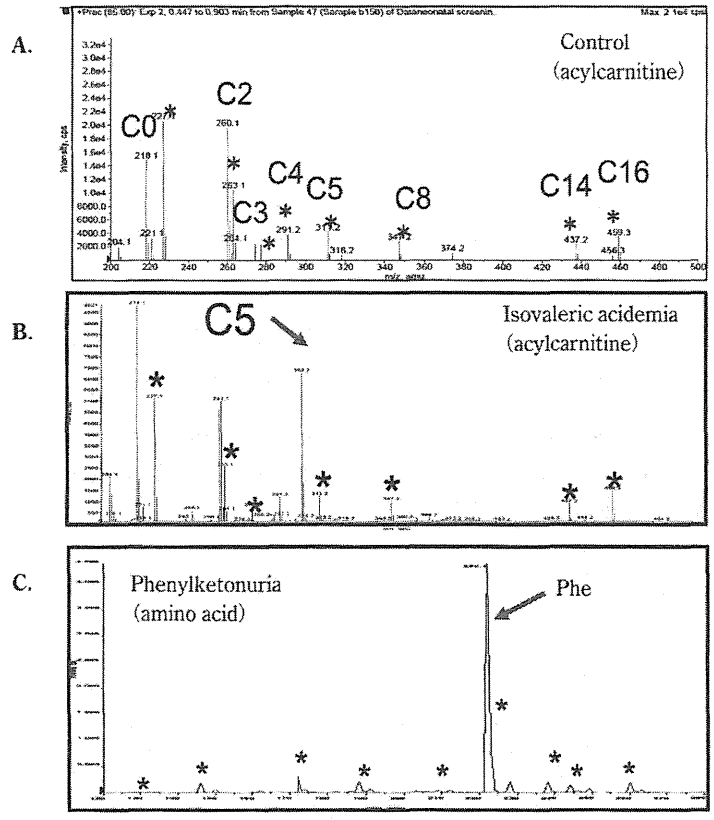

Figure 5　Mass spectrum of acylcarnitine and amino acid in MS/MS analysis (scan mode).
A, B: blood acylcarnitine analysis using MS/MS of control and isovaleric acidemia, respectively.　**C**: blood amino acid analysis using MS/MS of phenylketonuria.

障害なども生化学診断できるようになった[8]。小児救急の場にも必須の検査項目になりつつある。

VIII. おわりに

タンデムマスの新生児マススクリーニングへの導入に伴って，GC/MS による有機酸代謝異常症の診断も必須となりつつある。内科領域でも質量分析による網羅的代謝病態の評価を通じて，治療向上にも貢献することが期待される。質量分析は研究室レベルから臨床検査室へ応用が拡大しつつある。

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Bezafibrate can be a new treatment option for mitochondrial fatty acid oxidation disorders: Evaluation by in vitro probe acylcarnitine assay

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ABSTRACT

Background: The number of patients with mitochondrial fatty acid oxidation (FAO) disorders is recently becoming larger with the spread of newborn mass screening. Despite the advances in metabolic and molecular characterization of FAO disorders, the therapeutic studies are still limited. It was reported recently that bezafibrate (BEZ), an agonist of peroxisome proliferating activator receptor (PPAR), can restore FAO activity in cells from carnitine palmitoyltransferase-2 (CPT2) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiencies as well as clinical symptoms in the adult patients.
Methods: In this study, the therapeutic effect of BEZ was determined by in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry on various FAO disorders. The clinical trial of BEZ treatment for a boy with the intermediate form of glutaric acidemia type 2 (GA2) was also performed.
Results: The effect of BEZ was proven in cells from various FAO disorders including GA2, deficiencies of VLCAD, medium-chain acyl-CoA dehydrogenase, CPT2, carnitine acylcarnitine translocase and trifunctional protein, by the IVP assay. The aberrantly elevated long- or medium-chain acylcarnitines that are characteristic for each FAO disorder were clearly corrected by the presence of BEZ (0.4 mmol/L) in culture medium. Moreover, daily administration of BEZ in a 2-year-old boy with GA2 dramatically improved his motor and cognitive skills, accompanied by sustained reduction of C4, C8, C10 and C12 acylcarnitines in blood, and normalized the urinary organic acid profile. No major adverse effects have been observed.
Conclusion: These results indicate that BEZ could be a new treatment option for FAO disorders.

1. Introduction

Mitochondrial β-oxidation (FAO) is an essential energy producing pathway, particularly during the reduced energy supply from carbohydrate due to prolonged starvation or low caloric intake during infection, diarrhea or febrile illness. A number of FAO disorders have been recognized with the spread of tandem mass spectrometry (MS/MS) in the field of study of inborn metabolic disease as well as neonatal mass screening [1,2]. Many of them show episodic attacks like lethargy, acute encephalopathy or even sudden death due to energy production insufficiency.

It is considered that the FAO system consists of the following four groups : 1) carnitine cycle, which activates long-chain fatty acids for undergoing β-oxidation, including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 or -2 (CPT1 or CPT2, respectively, EC 2.3.1.21), or carnitine acylcarnitine translocase (CACT, EC 2.3.1.21); 2) long-chain FAO, whose enzymes are connected to the mitochondrial inner membrane, including very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) deficiency, and trifunctional protein (TFP, EC 1.1.1.211 and EC 2.3.1.16); 3) medium-chain FAO, whose enzymes are located in the mitochondrial matrix, including medium- and short-chain acyl-CoA dehydrogenases (MCAD, EC 1.3.99.3 and SCAD, EC 1.3.8.1) respectively), enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, or medium- and short-chain 3-ketothiolase (MCKAT and SCKAT, respectively); and 4) electron transfer system, from the dehydrogenases to respiratory chain, including electron transferring flavoprotein (ETF, EC 1.5.8.2) and ETF dehydrogenase (ETFDH, EC 1.5.5.1) [3–5].

Clinical features of FAO disorders can be roughly divided into the following three types: 1) severe form (neonatal form): patients present life-threatening illness with profound hypoglycemia, liver failure or hyperammonemia, and are often fatal in early infancy; 2) intermediate

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form (juvenile form): patients have intermittent episodic attacks like lethargy, encephalopathy, or even sudden death often onset in infancy or young childhood; 3) mild form (myopathic form): the patients may often show late onset after school ages or adulthood with episodes of hypotonia, myalgia, lethargy, myopathy-like symptoms, or liver dysfunction [6].

In vitro probe acylcarnitine profiling (IVP) assay was developed to evaluate FAO disorders recently [7,8]. Acylcarnitine (AC) profiles in the special culture medium as below after incubating with fatty acids as substrates are determined by MS/MS. Bezafibrate (BEZ) is a hypolipidemic drug, which is an agonist of peroxisome proliferating activator receptor (PPAR), and is claimed to act for induction of several FAO enzymes [9–11].

In this study, the effect of BEZ on various FAO disorders was evaluated using the IVP assay. Furthermore, we report an in vivo trial of BEZ on a boy with the intermediate form of GA2, presenting dramatic improvement with BEZ.

2. Materials and methods

2.1. Subjects and skin fibroblasts

Fibroblasts from 10 Japanese children with FAO disorders, one each of severe and intermediate forms of GA2, 2 each of severe and myopathic (mild) forms of VLCAD deficiency, one each of deficiencies of MCAD, CPT2, CACT, and TFP as well as 6 controls (healthy volunteers, passages 3 to 16) were used. The clinical types and genotypes are shown in Table 1. The child with MCAD deficiency was detected in a newborn mass screening and non-symptomatic, while one with the intermediate form of CPT2 deficiency had liver dysfunction in infancy. The child with the intermediate form of CACT deficiency had two life-threatening episodes in infancy, and after that no episodes were noted with normal development [12]. The child with TFP deficiency had an episode of liver failure in infancy, and then intermittent episodes of myalgia or hypotonia particularly following infection.

The clinical types and genotypes are shown in Table 1. In all cases, at least one allele has missense mutation, although the other alleles had missense or truncated mutations. In CACT deficiency (case 9), a missense mutation in an initiation codon (c.3G>A) in SLC25A29 was detected, but this could harbor a residual activity (Fukao et al., unpublished data).

Table 1
Clinical types and genotypes of patients with mitochondrial fatty acid oxidation disorders investigated.

Disease & case No.	Phenotype	Gene	Genotype, nucleotides (amino acids)	
			Allele 1	Allele 2
GA2				
1 (B)	Severe	*ETFA*	c.799G>A (G267R)	c.7C>T (R3X)
2 (C)	Intermediate	*ETFDH*	c.1217G>A (S406N)	c.1675C>T (R559X)
VLCAD deficiency				
3 (D)	Severe	*ACADV*	c.553G>A (G185S)	IVS9+1g>c
4 (E)	Severe	*ACADV*	c.454G>A (G152S)	c.997insT (A333fsX358)
5 (F)	Myopathic	*ACADV*	c.790A>G (K264E)	c.997insT (A333fsX358)
6 (G)	Myopathic	*ACADV*	c.1144A>C (K382Q)	c.1339G>A (G447R)
MCAD deficiency				
7 (H)	Non-symptomatic	*ACADM*	c.134A>G (Q45R)	c.449delCTGA (T150fsX153)
CPT2 deficiency				
8 (I)	Intermediate	*CPT2*	c.151A>G (R51G)	c.520G>A (E174K)
CACT deficiency				
9 (J)	Intermediate	*SLC25A29*	c.3G>A (M1I)	IVS4+1g>t
TFP deficiency				
10 (K)	Intermediate	*HADHB*	c.739C>T (R247C)	c.817delG (D273fsX292)

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; GA2, glutaric acidemia type 2; VLCAD, very-long-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase-2; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase. Case 2 (C) is a boy with GA2 who underwent the clinical trial of BEZ. Non-symptomatic case 7 (H) was detected in the newborn mass screening. Severe, intermediate, and myopathic forms are mentioned in the text. (B) to (K) correspond to those of Fig. 1.

2.2. In vitro probe assay with BEZ

Fibroblasts were cultured in 75 cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2/95% air incubator [13].

Fibroblasts harvested by trypsinization were seeded onto 6-well microplates (35 mm i.d., Iwaki, Japan) with the fresh above medium (2 mL/per well) until they reached confluence. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate (experimental medium). The experimental medium is MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabeled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, in the presence or absence of BEZ (0.4 mmol/L; Sigma). AC profiles in the culture medium were analyzed after 96 h. The experiments for each case were performed in triplicate.

2.3. Quantitative acylcarnitine analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) as described previously [13]. Briefly, methanol (200 μL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of the supernatant from culture medium. The portions were placed on ice for 30 min, and centrifuged at 1000×g for 10 min. Then, 150 μL of the supernatant was dried under a nitrogen stream, and butyl-derivatized with 50 μL of 3N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1 v/v). The ACs in 10 μL of the resultant aliquots were analyzed using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. The AC concentrations are expressed as nmol/mg protein.

2.4. Organic acid analysis using GC/MS

Urinary organic acids were analyzed according to the previous method [14]. Briefly, 40 μg of tropate (IS-2) and 20 μg each of heptadecanoate (IS-1) and tetracosane (C24) as internal standards were added to a urine specimen containing 0.2 mg creatinine. The samples were oxime-derivatized, and solvent extracted with ethylacetate, and trimethylsilylated (TMS-derivatization). The resultant aliquots were subjected to GC/MS (Shimadzu GC/MS QP2010 Plus, Kyoto, Japan), with a DB-5 column of 0.25 mm I.D × 30 m, 1 μm film thickness (J&W, Folsom, CA). The temperature program was from 100 °C to 290 °C at a rate of 4 °C/min).

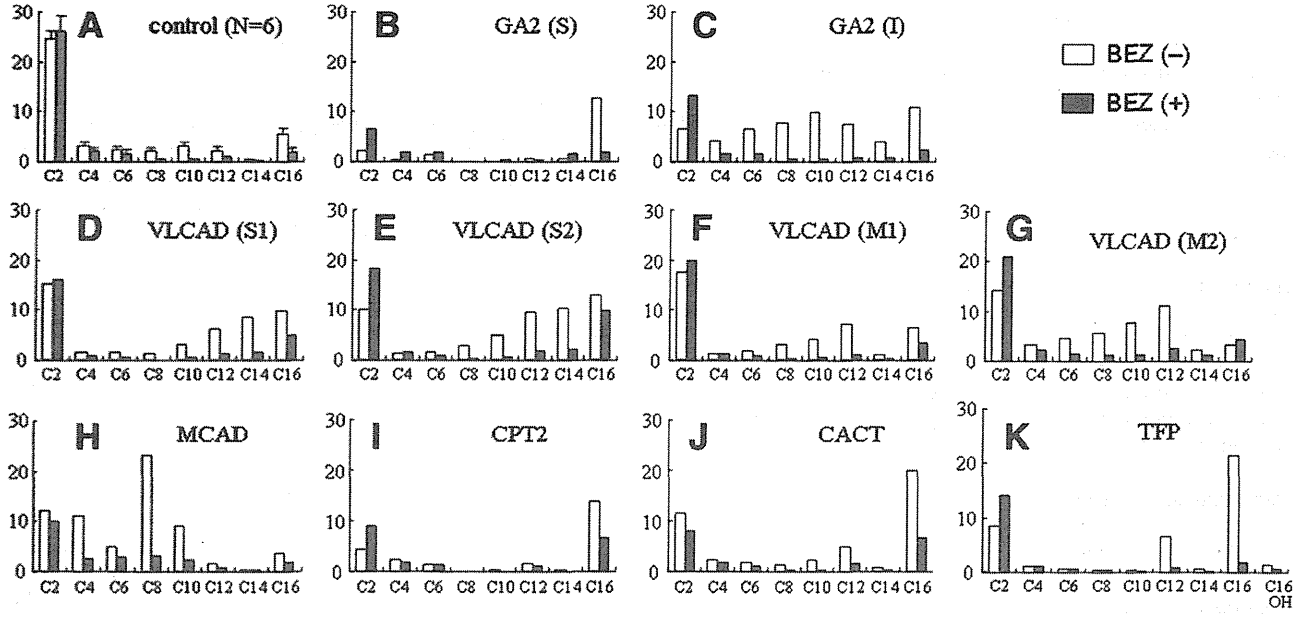

Fig. 1. Acylcarnitine profiles of in vitro probe assay in the presence and absence of bezafibrate. A, normal control; B, severe form of GA2; C, intermediate form of GA2 (the boy who underwent the clinical trial) (S and I, the clinically severe and intermittent form, respectively); D and E, severe form of VLCAD deficiency (S1 and S2, two cases, respectively); F and G: myopathic (mild) form of VLCAD deficiency (M1 and M2, two cases, respectively); H, I, J, and K: deficiencies of MCAD, CPT, CACT, and TFP, respectively. Unit of vertical lines, nmol /mg protein of acylcarnitines; the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C10, C12, C14, C16, and C16-OH. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars. In control (A), the mean plus SD values of 6 controls are shown.

2.5. BEZ trial on a child with the intermittent form of GA2

A Japanese boy with GA2 was detected in the newborn mass screening using MS/MS, and had no special symptoms in infancy with therapies of special formula and carnitine (approximately100 mg/kg/day, div. 3). After 1 year of age, however, he sometimes experienced episodes of hypotonia or lethargy following infection, and muscle weakness, often falling. At the age 2 years and 1 month, he was hospitalized for 2 and a half months, because of infection and lethargy, receiving treatments including artificial respiration to repeated aspiration pneumonia and unconsciousness in intensive care unit (ICU). At discharge, he could not walk alone, and could speak only a few words. So, his family consulted us, and strongly expressed a desire for any new therapies that might help their son.

Thereafter, under the approval by the ethical committee of Shimane University, we started a clinical trial of BEZ, continuing the dietary and carnitine therapies as before, since 2 years and 9 months of his age. His body weight ranged from 12 to 14 kg during the treatment, and 200 to 300 mg/day (approximately 17 to 25 mg/kg/day, div. 3) of BEZ was used in the trial. BEZ was purchased from Kissei Co Ltd, Tokyo, Japan. The study had no potential conflicts of interest (COI) to the authors.

3. Results

3.1. Effects of BEZ on FAO disorders by IVP assay

The AC profiles in the culture medium of fibroblasts from various FAO disorders in the presence and absence of BEZ are illustrated in Fig. 1. In control cells, C2 (acetylcarnitine) is the only prominent peak, and many of ACs further decreased in the presence of BEZ (Fig. 1A).

In the severe form of GA2 (Fig. 1B/S), C16 was apparently decreased, and C2 increased in the presence of BEZ, while C16 was extremely high before BEZ addition. The increase of C2 may indicate the acceleration of FAO, namely an increase of acetyl-CoA production. In the intermediate form of GA2 (Fig. 1C/I), all elevated ACs clearly decreased and normalized in the presence of BEZ, although broad ranges of ACs from C4 to C16 were extremely high before adding BEZ. This patient is the case 3 in Table 1, who underwent the clinical trial of BEZ treatment as illustrated in Fig. 2.

In 2 cases of the severe form of VLCAD deficiency (Figs. 1D/S1, and 1E/S2), elevation of C14 and C16 was larger, compared with that in 2 cases of the mild form (Figs. 1F/M1, and 1G/M2). The elevated ACs

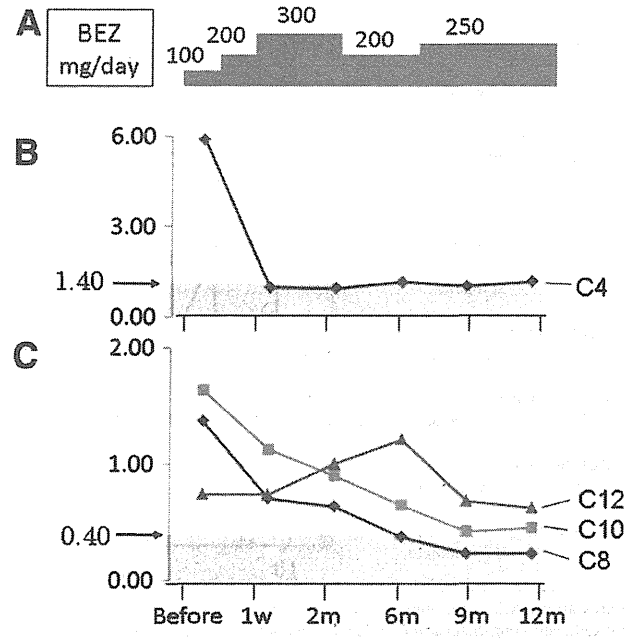

Fig. 2. Bezafibrate administration and changes in blood acylcarnitines. A, dose of bezafibrate, mg/day (approximately 17 to 25 mg/kg/day, div. 3); B, change of C4 acylcarnitine; C, changes in C8, C10, and C12. Arrows with the 1.40 and 0.40 indicate the cutoff values of blood acylcarnitines. Unit of acylcarnitine is nmol/mg protein.

Table 2
Time course of biochemical findings after initiation of bezafibrate administration.

	(Unit)	Before	After the start of BEZ treatment					Reference value*
			1w	2 m	6 m	9 m	12 m	
AST	(IU/L)	47	35	44	43	26	42	10–38
ALT	(IU/L)	27	17	22	24	20	21	5–40
LDH	(IU/L)	448	426	392	384	341	371	100–215
CK	(IU/L)	496	185	187	324	174	207	36–216
TChol	(mg/dL)	161	127	117	141	127	140	150–219

* : used in Shimane University Hospital. Abbreviations: AST, aspartate amino transferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; and TChol, total cholesterol.

such as C10, C12, C14, or C16 in both the severe and mild forms apparently decreased in the presence of BEZ.

In MCAD deficiency (Fig. 1H), the AC peaks of C4 to C10 were significant, but in the presence of BEZ, these AC peaks were almost normalized. In cases of CPT2 deficiency (Fig. 1I), CACT deficiency (Fig. 1J) and TFP deficiency (Fig. 1K), the extremely high AC peaks of C16 and/or C12 apparently decreased to an almost normal level, in the presence of BEZ.

3.2. Clinical trial of BEZ to a GA2 patient

Since the start of BEZ treatment, his motor and social development, and languages remarkably improved, and no metabolic episodes were noted. He became able to walk alone, showed improved muscle strength, and could speak markedly more words in a few weeks. Furthermore, several months later, he could ride a kid's tricycle by himself, although his intellectual ability was on the borderline for entrance into a kindergarten. For at least 1 year of the administration, no adverse effects of BEZ such as hypolipidemia or rhabdomyolysis have been observed.

The routine laboratory data such as blood AST, ALT, LDH or CK were in normal or subnormal ranges as shown in Table 2, showing stable levels of each test, although these laboratory data had sometimes fluctuated, in particular, when his condition was unstable before the initiation of BEZ. For example, during the stay in the ICU at the age of 2 years, the maximum levels of AST, ALT, LDH or CK were 1450 IU/L, 825 IU/L, 5200 IU/L, or 10,750 IU/L, respectively. The maximum level of blood ammonia at the ICU was 126 μg/dL, while no significant elevation was observed after that. Hypoglycemic attacks have not been noted.

BEZ is a hypolipidemic drug, and we have paid attention to the blood level of Cholesterol (TChol), because of the potential adverse effects. The dose of BEZ was 100 mg/day for the first 3 days, 200 mg/day for 4 days, and 300 mg/day for 2 months, respectively, as shown in Fig. 2A. At 2 months after starting BEZ of 300 mg/day, TChol level was a bit low, 117 mg/dL. Since then the dose has been lowered to 200 or 250 mg/day, and the TChol level has ranged between around 130 to 150 mg/mL, as shown in Table 2.

The changes in the AC levels of C4, C8, C10, and C12 are illustrated in Figs. 2B and C, respectively. All the increased ACs returned to approximately normal levels with the administration of BEZ after several months. In particular, C4 decreased to the normal range within a few weeks. Urinary organic acid analysis showed remarkable increases of ethylmalonate, methylsuccinate, adipate, 2-hydroxyglutarate, hexanoylglycine, suberate, and suberylglycine, before the BEZ treatment as shown in Fig. 3. The abnormalities in urinary organic acids were markedly corrected as early as 2 weeks after the initiation of BEZ therapy. The profile was almost normal but for a slight increase of ethylmalonate, and/or hexanoylglycine as illustrated in Fig. 3B.

4. Discussion

The treatments for FAO disorders have generally been described as follows: 1) avoiding a "long fasting": it prevents the increased requirement of fuel from FAO; 2) early infusion of glucose: it should be performed during the metabolic stress resulting from infection, diarrhea or overexercise, to prevent hypercatabolism; 3) carnitine therapy: it may be effective in many cases, although controversy

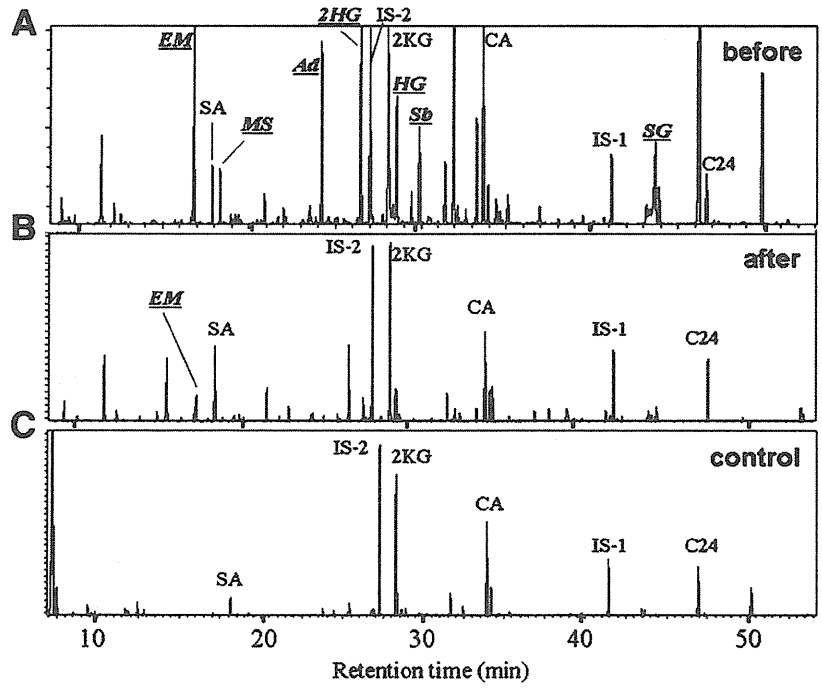

Fig. 3. Urinary organic acid profiles before and after bezafibrate administration. A, The total ion chromatogram (GC/MS) of urinary organic acids just before the start of BEZ; B, One year after the treatment; C, Normal control. Abbreviations: IS-2, IS-1 and C24 are tropate, heptadecanoate, and tetracosane, respectively, as internal standards; EM, ethylmalonate; SA, succinate; MS, methylsuccinate; Ad, adipate; 2HG, 2-hydroxyglutarate; 2KG, 2-ketoglutarate; HG, hexanoylglycine; Sb, suberate; CA, citrate; SG, suberylglycine. Metabolites judged as abnormal are shown in bold letters underlined.

remains in some cases; and 4) dietary therapy, including high carbohydrate/low lipid diet: Dietary restriction in FAO disorders may be less strict [15–18].

In this study, we demonstrated the effect of BEZ on various FAO disorders at both in vitro and in vivo levels. It was indicated by the IVP assay that FAO capacity was corrected by BEZ in various FAO disorders, and a clinical trial of BEZ in a boy with the intermediate form of GA2 showed a favorable consequence. Bastin, Djourdi and their colleagues reported the potential effect of BEZ for FAO disorders showing the increase of enzyme activity and mRNA production in several FAO enzymes from normal individuals, or reduced ACs in cells from VLCAD deficiency by the IVP assay using stable isotope-labeled palmitate [19]. Furthermore, they are performing a clinical trial on adult cases of mild form of CPT2 deficiency [20,21]. We should continue to pay attention to potential adverse effects of BEZ, including hypolipidemia or rhabdomyolysis, although such signs have never seen up to now.

We used the IVP assay to investigate the effect of BEZ in the other FAO disorders including GA2, deficiencies of MCAD, CACT, and TFP as well as CPT2 or VLCAD deficiencies. The beneficial effect of BEZ was clearly demonstrated in all these cases tested in this study, which included the clinically intermediate or severe forms as well as the mild form, having missense mutation of at least one allele. However, it is not yet clear whether the effect of BEZ is due to induction of mutant enzyme itself, or due to stimulation of the other FAO enzymes. If the effect is due to the latter mechanism, BEZ could potentially induce a "high pressure" on the FAO pathway, even resulting in devastating outcomes. We should further investigate the effect on the other severe forms of FAO disorders, the relation with the genotypes, or the dose dependency.

BEZ is an agonist of PPAR, which facilitates transcription of genes encoding FAO enzymes, and subsequently induces FAO enzyme production. Eventually, it can be considered to correct the FAO capacity in FAO disorders. Recently, it was reported that resveratrol which is a natural polyphenol and an activator of Sirtuin 1, is also expected to be a novel treatment option for FAO disorders [22]. The effect of resveratrol on FAO capacity can also be evaluated by the IVP assay like this study.

In conclusion, BEZ could be a new promising treatment option for FAO disorders. Many of patients with FAO disorders, particularly children with the milder form or adult cases, are intellectually normal, and their life prognosis is favorable if they can be prevented from severe episodes like encephalopathy. Symptoms or severity of FAO disorders are very heterogeneous depending on the disease, genetic background or lifestyle. Additional clinical studies of BEZ treatment will be essential for confirmation of its safety and practical utility.

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Brief Communication

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

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ABSTRACT

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

1. Introduction

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

2. Subjects and methods

2.1. Subjects

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

2.2. Mass spectrometric analysis

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

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

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

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

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

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

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

3. Results

3.1. Clinical features of patients

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

3.2. Biochemical results of patients

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

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

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

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

4. Discussion

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

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

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

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

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総　説

脂肪酸代謝異常症，ケトン体代謝異常症の最近の進歩

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キーワード：脂肪酸β酸化系異常症，ケトン体代謝異常症，新生児マススクリーニング，タンデムマススクリーニング，カルニチン，ケトン体

はじめに

脂肪酸のβ酸化系は遊離脂肪酸から心筋や骨格筋のエネルギーの多くを産生しているとともに，肝臓においてケトン体を産生することで，血糖コントロールにおいても重要な役割を果たしている[1,2]．

1980年代から1990年代前半にかけて脂肪酸β酸化系異常症が次々と報告され，とくに中鎖アシルCoA脱水素酵素（MCAD）欠損症が乳幼児突然死症候群，Reye様症候群の原因疾患として注目された[3]．1990年代前半からタンデムマスによるアシルカルニチン分析が開始され，脂肪酸代謝異常症のスクリーニングに有用であることから，1990年代後半からオーストラリア，米国の一部で公的事業としてタンデムマスが新生児マススクリーニングに導入され，2000年以降ドイツ，台湾などでも公的事業としてタンデムマススクリーニングが始まった．日本においては，1997年に福井大学の重松陽介先生らによるパイロット研究が始まり，2005年からは福井大学を含む全国5か所にパイロット研究が広がった[4]．

現在タンデムマスを用いた新生児マススクリーニングが，日本において多くの自治体で開始もしくは開始に向けて準備が進められている[4,5]．これまでの6疾患のスクリーニングから，新たなスクリーニングでは1次対象疾患だけでも19疾患が対象疾患となる．乳幼児期に突然の強い低血糖発作，Reye様症候群での発症や乳幼児突然死症候群と密接に関連する脂肪酸代謝異常症をはじめ，著しい代謝性アシドーシス，高アンモニア血症などで発症する有機酸代謝異常症などがその新たな対象疾患となっている．これらの疾患が初回発作を来す前に診断に至り，重篤な初回発作を防ぐことができれば，患者さんの予後を大きく変えることができると期待されている．

本稿では，脂肪酸代謝異常症，そして関連するケトン体代謝異常症について，新生児マススクリーニングの視点を入れながら概説したい．

脂肪酸酸化とケトン体代謝

1）意義

肝臓における脂肪酸β酸化系とそれに続くケトン体代謝系は，炭水化物からのエネルギー供給が低下したときに血糖を維持するために重要な代謝系である．特に骨格筋量が不十分で，アラニンからのグルコース供給が十分でない乳幼児期においては重要である．それは長鎖脂肪酸酸化系異常症やケトン体産生障害が，乳幼児期に著しい低血糖発作をきたすことからも明らかである．グルコースが不足した時に，以下で述べるようなホルモン系の作用により脂肪酸β酸化が亢進し，ケトン体が産生され，これが肝外組織でグルコースの代替えエネルギーとして用いられることで，低血糖になることを防いでいる．一方学童期以降は，骨格筋量が増してアラニンからのグルコース供給が十分となり，血糖維持に対するケトン体への依存度が低下してくるため，脂肪酸代謝異常症やケトン体産生異常症でも低血糖発作を来しにくくなる．同様にケトン体産生が亢進しにくくなることから，ケトン体利用障害におけるケトアシドーシスの危険性も少なくなる．

また脂肪酸の酸化は効率的なエネルギー産生系として重要である．心筋ではほとんどすべての栄養源を利用することができるが，なかでも脂肪酸β酸化系が主要なエネルギー供給源である．ケトン体を利用するた

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深尾　敏幸

めに必要なサクシニル-CoA：3-ケト酸CoAトランスフェラーゼ(SCOT)も心臓で最も強く発現しており[6]，ケトン体も重要なエネルギー源と考えられる．脂肪酸β酸化系が強く障害される場合，心筋はこの重要な心筋自体の脂肪酸β酸化系と肝臓から供給されるケトン体という両方のエネルギー源を失うことになる．カルニチン回路異常症，長鎖β酸化系異常症の重症型では，新生児期に心筋症，不整脈で死亡することからも心筋における脂肪酸β酸化系の重要性が理解できる．骨格筋では安静時のほとんどのエネルギーは脂肪酸β酸化でまかなわれており，激しい運動時にグリコーゲン代謝にスイッチされる．しかしさらに運動が持続すると再び脂肪酸代謝にスイッチされる．これは筋型糖原病においてセコンドウインド現象として知られている．比較的残存活性のあるカルニチン回路異常症，長鎖β酸化系異常症では，心筋障害や低血糖をきたさず，幼児期以降の筋痛，横紋筋融解が主症状となる．一方ケトン体産生障害やケトン体利用障害では，骨格筋や心筋自身のβ酸化系は正常であり，骨格筋や心筋における代謝障害は一般には認められない．

一般的に脳には脂肪酸のβ酸化系はなく，もっぱらグルコースと肝臓で産生されたケトン体などの直接のエネルギー源の供給を受ける必要があると考えられていたが，最近の研究によると，星状細胞にはβ酸化系やケトン体産生系酵素が存在し，肝臓と同様の調節機構を受けてケトン体を産生し，乳酸とともに神経細胞にケトンをエネルギーとして供給しているようである[7,8]．脳における脂肪酸β酸化，ケトン体産生系に関する報告は少なく，今後のさらに検討が必要である．神経系におけるケトン体の代謝は，ケトン食が難治性てんかんに効果があること[9]，さらに神経変性疾患などにも有効であるという報告もあり[10]，もっと注目すべき分野と考えられる．

2）代謝経路

脂肪酸β酸化系は，肝臓においてケトン体産生系とつながった代謝系で，4つのステップが存在する（図1）．1)脂肪組織から切り出された長鎖遊離脂肪酸が，カルニチン回路を利用して肝細胞のミトコンドリア内にはいるステップ，2)内膜結合型の極長鎖アシル-CoA脱水素酵素（VLCAD）と三頭酵素（TFP）からなる長鎖のβ酸化系というステップ，3)ミトコンドリアマトリックス内の中鎖～短鎖のβ酸化系のステップ，4）β酸化系から生成される大量のアセチル-CoAからHMG-CoAを介してアセト酢酸が産生されるケトン体産生系というステップである．

産生され血中に放出されたケトン体は肝外組織において取り込まれ，3-ヒドロキシ酪酸は再びアセト酢酸に変換された後，SCOTによってアセトアセチル-CoAと活性化され，アセチル-CoAとなってTCAサイクルを介してエネルギーとなる．これがケトン体利用系である．

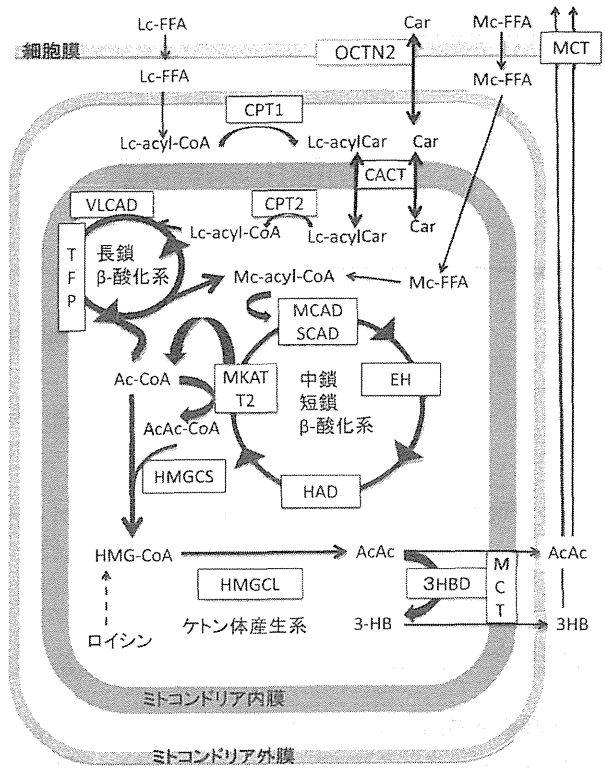

図1　肝細胞におけるミトコンドリア脂肪酸β-酸化系とケトン体産生系．酵素の略語については本文参照．β-酸化系は長鎖であれ中鎖，短鎖系であれ，1回転でアセチル-CoA（Ac-CoA）が1つ産生される．MCTはモノカルボン酸トランスポーターでミトコンドリア膜にも細胞膜にも存在する．Lc-FFA，長鎖遊離脂肪酸；Lc-acylCar，長鎖アシルカルニチン；Lc-acyl-CoA長鎖アシル-CoA；Mc-FFA，中鎖遊離脂肪酸；Mc-acyl-CoA中鎖アシル-CoA；Car，カルニチン；AcAc-CoA，アセトアセチル-CoA；HMG-CoA，3-ヒドロキシメチル-3-グルタリル-CoA；AcAc，アセト酢酸；3HB，3-ヒドロキシ酪酸

それぞれのステップについてもう少し詳細に記載する．

(a) カルニチン回路

上述のように長鎖脂肪酸はそのままでミトコンドリア内膜を通過できない．そのためにカルニチン回路が存在し，3つの酵素から成り立っている．すなわち長鎖脂肪酸は，ミトコンドリア膜間腔(外膜と内膜の間)でいったんアシル-CoAに活性化され，次いでカルニチンパルミトイルトランスフェラーゼ1（CPT1）によってアシルカルニチンとなり，ミトコンドリア内膜を貫通するカルニチンアシルカルニチントランスロカーゼ(CACT)によってミトコンドリア内に運び込まれ，そこで内膜に結合したカルニチンパルミトイルトランス

フェラーゼ2（CPT2）によって再びアシル-CoAに変換される．そして初めてミトコンドリア膜結合型の長鎖β酸化系に接続される．CPT1には肝臓で主に発現するCPT1A，筋肉で発現するCPT1B，脳で発現するCPT1Cが知られているが，これまで欠損症の報告はCPT1Aのみである．カルニチン代謝にはそのほか細胞膜でカルニチンの取り込みにカルニチントランスポーター（OCTN2）が必要である．

（b）長鎖β-酸化系

β-酸化系はアシル-CoA脱水素酵素，エノイル-CoAヒドラターゼ，3-ヒドロキシアシル-CoA脱水素酵素，3-ケトアシル-CoAチオラーゼという4ステップからなる．ミトコンドリア内膜に結合した長鎖のβ-酸化系はVLCADと，そのほかの3つの酵素活性をもつTFPからなる系である．この系の発見には日本人研究者が大きく貢献している[11][12]．

（c）中鎖，短鎖脂肪酸β-酸化系

ミトコンドリアマトリックスに存在し，中鎖アシル-CoA脱水素酵素（MCAD），短鎖アシル-CoA脱水素酵素（SCAD），エノイル-CoAヒドラターゼ（EH），3-ヒドロキシアシル-CoA脱水素酵素（HAD），中鎖3-ケトアシル-CoAチオラーゼ（MKAT），アセトアセチル-CoAチオラーゼ（T2）から成る．アセトアセチル-CoAチオラーゼ（T2）はケトン体利用のチオラーゼと同じチオラーゼである．

（d）ケトン体産生系

脂肪酸β酸化系は大量のアセチル-CoAを産生し，これがチオラーゼ反応でアセトアセチル-CoAとなり，このアセチル-CoAとアセトアセチル-CoAがHMG-CoA合成酵素の働きで重合してHMG-CoAが産生される．このHMG-CoA合成酵素（HMGCS）は細胞質の酵素とは全く異なったミトコンドリアの酵素である．HMG-CoAからHMG-CoAリアーゼ（HMGCL）によってアセト酢酸が産生され，一部はβヒドロキシ酪酸脱水素酵素（3HBD）反応によって3-ヒドロキシ酪酸に変換されて血中に放出される．

（e）ケトン体利用系

肝臓外組織はこの3-ヒドロキシ酪酸，アセト酢酸というケトン体を取り込んで，ミトコンドリアで3-ヒドロキシ酪酸は3HBD反応でふたたびアセト酢酸に変換されたのち，SCOTによってアセトアセチル-CoAに活性化され，そしてT2によりアセチル-CoAとなり，TCAサイクルを経てエネルギーとなる．

（f）モノカルボン酸トランスポーター（MCT）

上記ケトン体産生系と利用系において，アセト酢酸，3-ヒドロキシ酪酸ともにマイナス荷電があり，細胞膜やミトコンドリア膜を通過するためにモノカルボン酸トランスポーター（MCT）を必要とする[13]．MCTには水素イオン共役のSLC16Aファミリーに属するMCTs（MCT1～MCT4など）とNaイオン共役のSLC5Aに属するSMCT1，SMCT2が存在する[14]．ともに乳酸，ピルビン酸，分枝鎖αケト酸などと同じトランスポーターをケトン体も用いる．腎臓で近位尿細管の管腔面でSMCT1, SMCT2が乳酸，ケトン体などの再吸収系を形成している．上述の星状細胞と神経細胞の関連においては，星状細胞がMCT2とSMCT2が発現しているのに対し神経細胞はMCT1とSMCT1を発現することが知られており，星状細胞から神経細胞への乳酸，ケトン体の供給に関連していると考えられる[13]～[15]．ケトン体の放出，取込みにおいては，これらの乳酸，分枝鎖ケトン酸などの他のモノカルボン酸との競合となるため，これら他のモノカルボン酸の存在はケトン体代謝に影響を与えうる．

3）調節機構

脂肪酸β酸化系およびケトン体産生は，血糖維持機構の一部としてホルモンにより産生の調節を受けている[16]．主に脂肪組織からの遊離脂肪酸動員時のホルモン感受性リパーゼレベル，肝細胞への脂肪酸の取り込みレベルである．さらにケトン体産生においては，肝細胞でのミトコンドリアHMGCSレベルでも調節されている．インスリンは脂肪酸β酸化およびケトン体産生に抑制的に働き，グルカゴン，カテコールアミンは促進的に働く．

インスリンが十分分泌されている状態では，食後のパターンがそうであるように，低遊離脂肪酸，低ケトン状態となる．また病的状態として高インスリン血性低血糖症がある．一方グルカゴン，カテコールアミンの産生亢進状態（飢餓，発熱，精神的および肉体的ストレス状態）は，血中遊離脂肪酸，ケトン体ともに増加する．病的状態として脂肪酸β酸化およびケトン体産生系の異常があれば，遊離脂肪酸は高値にも拘らず，ケトン体が産生されず，高遊離脂肪酸，低ケトン性低血糖をきたす．この血糖，遊離脂肪酸，ケトン体の関係を理解することは低血糖の病態理解に重要である．

長鎖脂肪酸β酸化異常症の一般的事項

長鎖脂肪酸β酸化系疾患群にはカルニチン回路異常，長鎖β酸化系異常症を含む．疾患にかかわらず発症形態によって大きく3つの病型に分けられる[17]．また治療法も共通点が多い[18]．そこで3つの病型と基本治療について述べ，その後個々の疾患について概説する．

1）基本3病型

（a）新生児発症：新生児期から乳児期早期に発症し，しばしば心筋障害を伴い高い致死率を示す．出生

後の適応ストレスによって，痙攣，心筋肥大，不整脈，無呼吸で発症し，非ケトン性低血糖症，高アンモニア血症，肝逸脱酵素およびCKなどの上昇がみられる．疾患によっては，脳，腎臓の奇形を伴い胎児期からの異常が示唆される症例もある．このタイプは新生児マススクリーニングで結果が判明したとき既に発症している可能性が高い．どの3病型でも遊離脂肪酸は動員されているのに対してケトン体の産生が障害されるため遊離脂肪酸/総ケトン体比は高くなる．

(b) 乳幼児型：新生児期には症状がみられないかもしくは嘔吐，哺乳低下など非特異的な症状が一過性にみられ回復し，その後しばらくは症状に気づかれないことが多い．生後数か月から2歳ごろまでに感染，下痢などに伴って，筋力低下，急性脳症様発作，あるいは突然死のような形態で発症する．急性期には非ケトン性低血糖症，高アンモニア血症，トランスアミナーゼ，CKの高値を示す．いわゆる肝臓型とも呼ばれ肝腫大（脂肪肝）を示すことが多い，発作時に肥大型心筋症を示すこともある．この初回発作を防ぐことが新生児マススクリーニングで診断する大きな目的となる．

(c) 遅発型(骨格筋型)：発症時期は，幼児期，学童期あるいは成人期になって初めて運動やストレス時に筋痛，横紋筋融解症で発症する．ミオグロビン尿，高CK血症(トランスアミナーゼ高値を伴う)を示す．腎不全を来さなければ生命予後は良好と考えられている．CKは数千から数万IU/Lにまで上昇する．乳児型の患者も治療によって低血糖発作をきたさなくなると，その後は骨格筋型の所見を示すようになる．

2) 基本的な治療法

長鎖脂肪酸酸化異常症における治療法の概略は以下の様である[18][19]．

(a) 食事間隔の指導：低血糖発作を防ぐためには，頻回の食事などによって食事間隔に注意する必要がある．目安として新生児期は3時間以内，6か月まで4時間以内，1歳まで6時間以内，3歳まで8時間以内，4歳以上で10時間が推奨されている．

(b) MCTミルク，オイルの使用：長鎖脂肪酸代謝異常症では，中鎖～短鎖脂肪酸の代謝系が正常であるので中鎖脂肪酸の代謝は可能で，MCTミルク，オイルの使用は理にかなっている．症状，一般検査で異常のない症例では必ずしもMCTミルクを用いる必要はないと考えられるが．安全面を考えれば，必須脂肪酸強化MCTフォーミュラ(明治721)を用いて，母乳もしくは調整粉乳とMCTミルクを1：1の混合で投与する．低血糖時はMCTミルクのみにする．生後5か月以降はMCTミルクの割合を20%程度にする．離乳後はMCTオイルが利用できる．臨床症状がある場合はより強い食事介入が必要となる[18]．

(c) コーンスターチ：糖原病で用いられるように，消化管からの吸収が緩除な糖質であり，離乳後食事間隔が延びる場合，寝る前に2歳で20g(80kcal)程度を各種経腸栄養用のフレーバーで味付けして飲ませることは有効である．

(d) ストレス時の対応：発熱，下痢嘔吐など代謝ストレスがかかるような時にはできる限り脂肪酸代謝系が活性化されないようにするべきであり，糖分を十分にとるよう指導し，必要に応じて早期にグルコース輸液が望ましい．異化状態をさけて同化の方向に向けるために，中途半端な輸液にならないように注意する．グルコース投与量を6～8mg/kg/minとし，必要ならばインスリン併用(GI療法)も考慮する．発作時も原則的に同様である．

(e) L-カルニチン投与について：長鎖脂肪酸代謝異常症に対するL-カルニチンの投与については議論がある．L-カルニチン投与がβ酸化を促進させ，酵素異常のある患者にストレスになるという考え方である．一般原則は遊離カルニチンが低下していれば，補うのは理にかなっているということである．遊離カルニチンが15mmol/L以下にならないようにする．OCTN2欠損症では大量のL-カルニチン投与が必須である．

(f) その他：過剰な運動は横紋筋融解を引き起こすので避けることが望ましい．運動20分前に，MCT 0.5 g/kgをとると運動後の代謝も改善し，通常の運動による筋痛，横紋筋融解が抑えられるという報告もある[18]．

疾患各論

表に各疾患についてまとめてある．順に日本人症例の検討を含めて概説する．

1) カルニチン回路の異常症

(a) OCTN2欠損症（原発性全身性カルニチン欠乏症)：OCTN2はカルニチンの細胞膜での取り込みに重要なトランスポーターで，腎臓におけるカルニチンの再吸収の主要なトランスポーターである[20]．OCTN2欠損は原発性全身性カルニチン欠乏症をきたす．長鎖脂肪酸酸化系異常症の乳幼児期発症型となるが，進行性心筋症，筋力低下で発症することも多い．日本の研究者が病態解明に貢献した疾患の1つである[20][21]．日本において兄2名が8歳，2歳で死亡した家族歴をもつ5歳と6歳の兄弟例で，肥大型心筋症とミオパチーを呈し，カルニチン3g/日投与で血清フリーカルニチンレベルは低いものの30年間フォローされ健在であるという報告がある[22]．このことから早期診断，早期L-カルニチン投与が重要な疾患である．

(b) CPT1欠損症：CPT1A欠損症が通常CPT1欠

損症と呼ばれる．肝臓において長鎖脂肪酸はミトコンドリア内に取り込めず，ミトコンドリア膜間腔でのアシルカルニチンが産生できないため，遊離カルニチンの欠乏も生じない．このため血中の遊離カルニチンは高く，長鎖アシルカルニチンは低値となり，CACT欠損症やCPT2欠損症とは異なったパターンと成る[23]．CPT1欠損症では，原則肝臓における長鎖脂肪酸の酸化が障害されるが，心筋や骨格筋の長鎖脂肪酸酸化は障害されないため，一般には乳幼児期発症型（肝臓型）となるが，中には発症時期が早く新生児期の場合もある．タンデムマスによる新生児マススクリーニングでは1次疾患に分類され，初回発作以前に診断し，基本的な治療方針により治療すれば一般に予後はよいと考えられる[24]．

(c) CACT欠損症：次のCPT2欠損症とともに，長鎖アシルカルニチンが形成されるにもかかわらず，ミトコンドリア内で長鎖アシル-CoAに変換できない疾患であり，臨床的，代謝的にこの2つの疾患は区別できない．また骨格筋，心筋も同様に障害されることからCPT1Aとは異なった病態をとる[23]．すなわち遊離カルニチンが欠乏し，長鎖アシルカルニチンが蓄積する．CACT欠損症は全般的に新生児期発症型が多いようである[27]．

(d) CPT2欠損症：上述の3つの病型をとりうるが，日本ではCPT2欠損症11例の報告で乳幼児発症型が7例，遅発型4例と遅めの発症が多い[25][26]．CPT2欠損症，CACT欠損症では，タンデムマスによる新生児マススクリーニングでは，新生児期に見逃し例があることから2次疾患に分類されているが，C16+C18：1/C2比をとることで多くの症例でスクリーニングが可能と思われる[28]．初回発作以前に診断し，基本的な治療方針により治療すれば，重篤な低血糖発作を防ぎ，予後はよいと考えられる．

2) 長鎖β酸化系異常症

(a) VLCAD欠損症

日本人のVLCAD欠損症では，上記の3つの病型のうち成人発症の骨格筋型が多い．マススクリーニング結果によると頻度は13万人に1人である．新生児マススクリーニングでは1次疾患であり，日本人における変異について残存活性の程度に対する検討が比較的進んでおり，遺伝子変異解析が病型の推定に役立つ可能性がある[29]~[31]．

欧米においては[17]，新生児マススクリーニングで診断された症例は，10年までのフォローアップを通して無症状のままで経過している症例が多い．一部の症例で，強い運動後の筋症状を示す症例も報告されている．VLCAD欠損症患者数は，マススクリーニング以後に増加傾向にあるので，発症しない軽症型も多く診断されている可能性がある．

日本においても症例が蓄積され，遺伝子型と臨床病型の関係がさらに明らかになれば，一部の症例では乳幼児期の食事制限が軽減できると思われる．

(b) TFP欠損症

日本においてはタンデムマスのパイロットスタディでは症例はみつかっておらず，まだ日本人における頻度は不明である．これまでに日本で5例の報告があり，そのうち2例が新生児発症型，2例が乳幼児期発症型，1例が骨格筋型であり[32]，比較的早期発症例が多い可能性がある．

欧米においても[17]，半数が新生児スクリーニング時に臨床症状がみられ致死的な経過をとる症例が多い．それでも新生児スクリーニングはTFP欠損症の発症率や死亡率を低下させており[33]，新生児期発症以外の症例ではスクリーニングは有効である．本症の特徴的な事項として，罹患胎児の母（母は保因者）の約5人に1人がAcute fatty liver of pregnancy（AFLP）やHypertension, Elevated Liver Enzymes, and Low Platelet (HELLP) 症候群をきたすといわれており，注意が必要である[17]．

本症では長期経過のなかで不可逆性進行性末梢神経障害，網膜障害が問題となり，これらは新生児スクリーニング，早期治療によっても防げないとされている[17]．この合併症はTFP欠損症に特徴的である．

3) 中鎖～短鎖脂肪酸β酸化系異常症群

中鎖以降のβ酸化系異常症では，MCAD欠損症が代表的であり，エノイル-CoAヒドラターゼ欠損症の報告はなく，中鎖3-ケトアシル-CoAチオラーゼの欠損症は1例が報告されているが，遺伝子レベルでの確認がなされていない．ここでは以下の3疾患について述べる．

(a) MCAD欠損症

脂肪酸β-酸化系異常症の代表的疾患で，前述のように乳幼児突然死症候群をきたすことから注目された疾患である[3]．欧米では新生児スクリーニングでの頻度は1万人に1人の高頻度であり，実際マススクリーニング以前の発症頻度が約3万人に1名程度であることから，本症患者の半数以上は生涯無症状で過ごすと考えられている[34]．稀に新生児期に発症するが，おおくは3歳以前（中央値で1~1.5歳）の乳幼児期に，感染や長時間飢餓を契機に，嘔吐，意識障害，痙攣など急性脳症様発作をきたし，突然死したり神経学的後遺症を残す．マススクリーニング導入以前は，診断症例の約1/4が発作時死亡し，1/3まではいかないが多くの症例で発達遅滞，行動異常など神経学的後遺症を認めていた[34]．マススクリーニング導入以後，新生児期早期発症による死亡を除くと，発症例は著しく低下している[34]．