Transient Expression Analysis of Mutant cDNAs

Transient expression of T2 cDNAs was performed using a pCAGGS eukaryote expression vector (Niwa et al. 1991), as described (Sakurai et al. 2007). After transfection, cells were cultured at 37°C or 40°C for 48 h, then harvested and kept at −80°C until use. Cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 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.

Results and Discussion

Confirmation of the Diagnosis

GK69's fibroblasts were assayed for SCOT activity to confirm the diagnosis in 2008, when GK69 was 24 years old. As shown in Table 1, she was diagnosed as having T2 deficiency but not as having SCOT deficiency.

SCOT deficiency was first suspected in GK77 and GK77b, based on the following facts (1) Two of the four SCOT deficient Japanese families were from the Amami islands, the population of which is about 120,000. They were T435N homozygotes (Fukao et al. 2004). (2) The acylcarnitine profiles and urinary organic acid analysis during acute ketoacidotic crisis in both patients had no typical profile for T2 deficiency, as discussed below. As shown in Table 1, GK69's and GK77's fibroblasts had normal SCOT activity and a higher ratio (1.3) of acetoacetyl-CoA thiolase activity in the presence to the absence of potassium ions than typical T2-deficient fibroblasts (the ratio was around 1.0). Immunoblot analysis also showed a clearly detectable amount of T2 protein in GK77's fibroblasts, and a lower amount in GK69's fibroblasts. Densitometric analysis showed that the amounts of T2 protein in GK77 and GK69 were estimated to be 50% and 25% of control, respectively (Fig. 1).

Table 1 Acetoacetyl-CoA thiolase activities in the absence and presence of potassium ions

Fibroblasts	Acetoacetyl-CoA thiolase activity			SCOT activity
	$-K^+$	$+K^+$	$+K^+/-K^+$	
Controls ($n = 5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1
GK69	3.6 ± 0.5	4.1 ± 0.9	1.2 ± 0.1	4.7 ± 1.4
GK77	4.2 ± 0.3	5.8 ± 1.5	1.4 ± 0.3	3.9 ± 0.5
T2D	4.5 ± 1.4	4.7 ± 1.6	1.0 ± 0.1	5.6 ± 0.5

Enzyme activity is expressed as nmol/min/mg of protein. In cases of patients, enzyme assay was done three times and shows average ± SD. T2D, A disease control

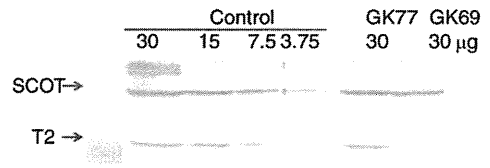

Fig. 1 Immunoblot analysis. In the cases of the controls, serial twofold dilutions from 30 to 3.75 μg were studied together with samples (30 μg) from GK68 and GK77. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

Mutations and Their Effects on T2 Protein

Mutation screening revealed that GK69 was a compound heterozygote of c.431A>C (H144P) and c.1168T>C (S390P). Her mother had S390P heterozygously but did not have H144P. The father's DNA was not available for analysis. GK77 had an H144P mutation homozygously, shown by mutation screening at the genomic level. Their parents and GK77b were heterozygous carriers and a homozygote of H144P, respectively. The c.431A>C (H144P) mutation creates a BmgT120I site (GGACA to GGACC). We could not find c.431A>C (H144P) in the 110 Japanese controls using the restriction enzyme assay with BmgT120I.

We performed transient expression analysis of wild-type and mutant cDNAs in T2-deficient SV40-transformed fibroblasts. Following expression of T2 cDNAs for 48 h at 37°C, an enzyme assay and immunoblots were performed (Fig. 2a,b). The transfection of wild-type T2 cDNA produced high potassium ion-activated acetoacetyl-CoA thiolase activity (T2 activity), whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. The H144P mutant retained a residual T2 activity of ~25% of the wild-type value (Fig. 2a). The S390P mutant did not retain any residual T2 activity. In immunoblot analysis (Fig. 2b), the H144P mutant protein was detected, whereas no S390P protein was detected. The relative amount of the H144P mutant protein, as compared to the wild-type, was estimated to be 50%. Hence, the specific activity (unit/mg of T2 protein) of the H144P mutant protein was estimated to be about 50% of the wild type. Protein-folding and post-folding stability is predicted to vary with the incubation temperature. Hence, we also performed transient expression at 40°C for 48 h. The H144P mutant in expression at 40°C had a similar level of residual activity to that at 37°C.

We reported the tertiary structure of the human T2 tetramer (Haapalainen et al. 2007). Figure 3a shows the positions of the H144P and S390P mutations on the dimer.

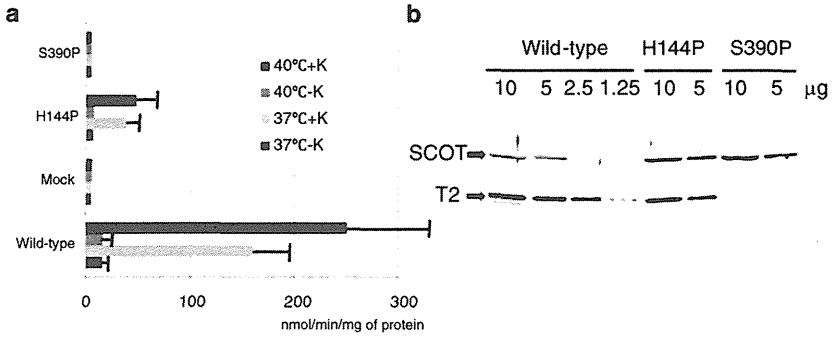

Fig. 2 Transient expression analysis of H144P and S390P mutant cDNAs. Transient expression analysis was performed at 40°C and 37°C. (**a**) Potassium ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence (−K) and presence (+K) of potassium ions are shown together with the SD of three independent experiments. (**b**) Immunoblot analysis. The protein amounts applied are indicated above the lanes. 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*

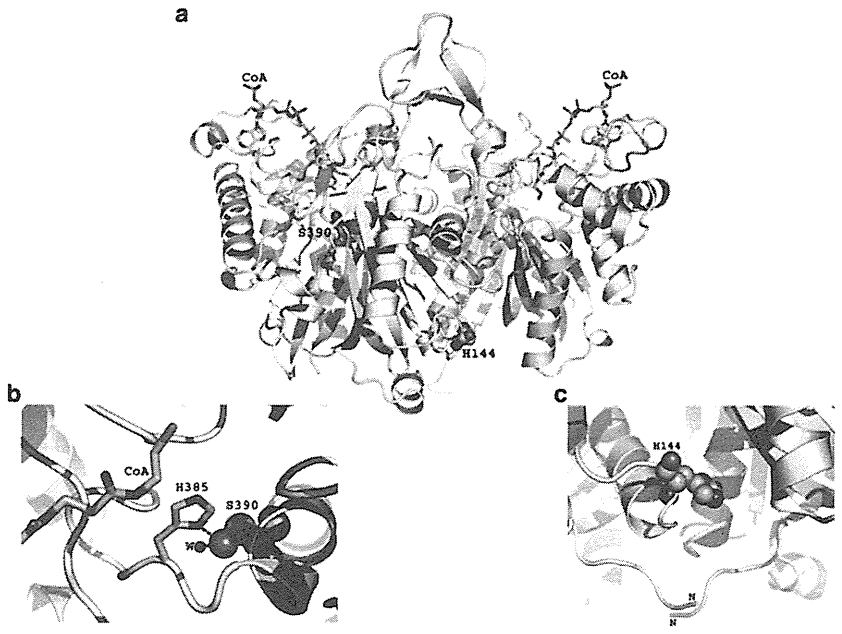

Fig. 3 The positions of H144P and S390P on the tertiary structure of human T2 dimers with substrates of coenzyme A

As seen in the figure, S390 is close to the active site and H144 is at the dimer interface close to the surface of the protein. Figure 3b shows a zoomed-in view around S390. This mutant is located at the active site. S390 is hydrogen-bonded to catalytic histidine, H385; it could be that this serine is needed to orient histidine in a way that the histidine can stabilize the transient negative charge of the substrate optimally. S390 is also hydrogen-bonded to a water molecule that is needed in stabilizing parts of the enzyme. So, if S390 is mutated into proline, these two hydrogen bonds do not exist. Hence, this S390P is expected to bring about a serious change in T2 catalytic cavity. In our expression analysis, this S390P was also too unstable to detect mutant protein. Figure 3c shows a zoomed-in view at the dimer interface. H144 is interacting with the residues of the neighboring subunit. If this residue is mutated into Pro, there is less dimeric interaction, which in turn might destabilize the overall structure. Since this residue is far from the active site and substrate binding site, it is difficult to explain why this H144P mutant had reduced specific activity in transient expression analysis from the viewpoint of structural analysis.

Urinary Organic Acid Analysis

GK69 was first suspected to having T2 deficiency as a probable diagnosis; however, urinary organic acid analysis at the first ketoacidotic crisis indicated no characteristic profile for T2 deficiency such as elevated 2-methyl-3-hydroxybutyrate and tiglylglycine in 1985 (no data was available). The results of the urinary organic acid analysis of our patients are shown in comparison with those of typical T2-deficient patients, GK01 and GK(Ind) (Table 2, Fig. 4). At the age of 24 years when her condition was stable, GK69's urinary organic acid analysis showed that there were only trace amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine (Table 2). In our screening, this low level of tiglylglycine was difficult to detect. Urinary organic acid analysis during the acute crises of GK77 and GK77b showed huge amounts of 3-hydroxybutyrate and acetoacetate with elevated 2-methyl-3-hydroxybutyrate but only trace amounts of tiglylglycine. The levels of 2-methyl-3-hydroxybutyrate and tiglylglycine during a stable condition in GK77 are similar with those in GK69.

In cases of typical T2-deficient patients, it is easy to suspect T2 deficiency based on large amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine as shown in Fig. 4. However, even in cases of trace amounts of tiglylglycine (possibly under the detection limit), T2 deficiency cannot be excluded. An H144P mutation, which retained high residual activity, may contribute to atypical profiles in the presented cases. These findings strengthen our previous observations that some T2-deficient patients with mutations, which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003).

Table 2 Quantitative analysis of urinary organic acid analysis during acute crises and stable conditions

Patients	Acute crises		Stable conditions	
	2M3HB	Tiglylglycine	2M3HB	Tiglylglycine
GK69	NA	NA	14.0	13.3
GK77b	405.7	45.8	NA	NA
GK77	160.2	6.7	27.3	14.8
GK01	NA	NA	399.1	732.1
GK(Ind)	484.6	503.9	195.1	797.6
Controls (n = 42)			10.7 ± 7.6	24.6 ± 14.6

Values are expressed as mmol/mol creatinine

NA means that samples were not available for the analysis. GK01 is a compound heterozygote of c.149delC and A333P, which retained no residual activity (Fukao et al. 1998). GK(Ind) indicates a patient with typical T2-deficient profiles of urinary organic acids in our screening

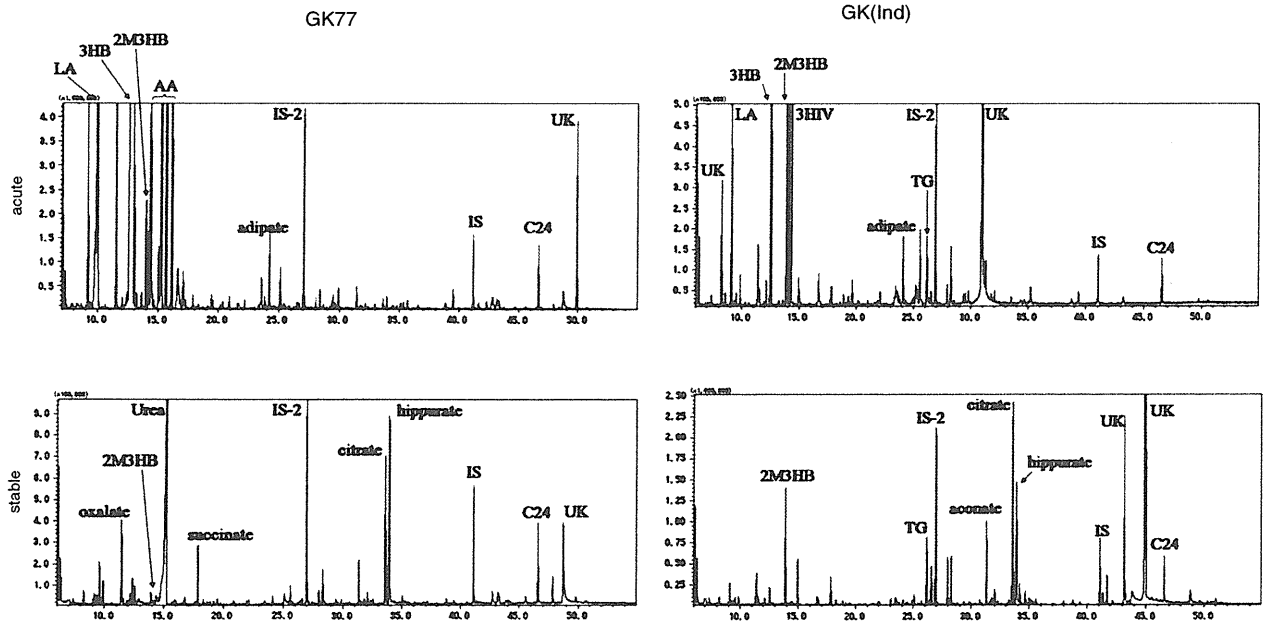

Fig. 4 Urinary organic acid profiles of GK77 during the acute episode and an asymptomatic period in comparison with those of a typical T2-deficient patient (GK(Ind)). *LA* Lactate, *3HB* 3-OH-butyrate, *3HIV* 3-OH-isovalerate, *AA* Acetoacetate, *2M3HB* 2-Methyl-3-OH-butyrate, *TG* Tiglylglycine, *IS-2* and *IS* Internal standards, *UK* Unknown. Since acetoacetate is unstable and samples from GK(Ind) were shipped on filter papers after thoroughly drying, the levels of acetoacetate are likely underestimated

Table 3 C5-OH and C5:1 carnitines in blood filters and serum samples from GK77 and GK77b during acute crises

Patients	Dried blood spots		Serum	
	C5:1	C5-OH	C5:1	C5-OH
GK77b	0.027	0.11	ND	0.12
GK77	0.012	0.11	0.044	0.10
R208X homozygotes				
GK75 (acute)	0.89	2.89	NA	NA
GK79 (stable)	1.20	2.35	NA	NA
Controls (n = 30)				
Average ± SD	0.015 ± 0.016	0.26 ± 0.15	0.015 ± 0.013	0.059 ± 0.024

ND not detected, *NA* not applicable

The values are expressed as μmol/L

GK75 and GK79 are positive controls for T2 deficient patients who are R208X homozygotes (Fukao et al. 2010b)

Blood and Serum Acylcarnitine Analyses

Acylcarnitine analysis was done using samples during the acute crises of GK77 and GK77b. Table 3 shows the results in comparison with those of typical T2-deficient patients (R208X homozygotes) (Fukao et al. 2010b). C5:1 and C5OH elevation in blood spots, characteristic for T2 deficiency, was clearly detected in the samples from the typical T2-deficient patients but was absent in samples from GK77 and GK77b. We previously reported that the abnormality of the acylcarnitine profiles in T2-deficient patients with mutations which retain some residual activity is subtle during nonepisodic conditions (Fukao et al. 2003), but the present study clearly showed that it could be also subtle even during severe ketoacidotic episodes. This means that acylcarnitine analysis using blood spots cannot detect some T2-deficient patients like GK77 and GK77b. Serum acylcarnitine analysis might detect elevation of these compounds to some extent, but we need to analyze more cases to clarify the usefulness of serum acylcarnitine analysis in such T2-deficient patients with mutations which retain some residual activity.

T2 deficiency cannot be excluded even if acylcarnitine profiles during acute episodes are within normal ranges. Careful evaluation of urinary organic acids, especially for the presence of 2-methyl-3-hydroxybutyrate, is necessary not to overlook T2 deficiency.

Clinical Issues

Since they were confirmed as identical twins by DNA analysis (data not shown), their genetic backgrounds were identical and most environmental factors were also very similar between them. One died during the ketoacidotic crisis and the other survived.

In Japan, intravenous infusion therapy for vomiting, appetite loss, and dehydration is commonly performed with commercially available initial infusion solution, such as Solita T1 (2.6% glucose) followed by maintenance solution, such as Solita T2 and T3 (4.3% glucose). These solutions are effective for physiological ketosis. However, in the case of T2 deficiency, a higher concentration of glucose may be necessary. Accordingly, we had the impression that GK77 became much better after the glucose concentration was changed from 5% to 10%. In the case of prolonged ketoacidosis, consideration should be given to increasing the infusion rate of glucose to ensure high normal blood glucose level to suppress ketone body synthesis and isoleucine catabolism via insulin secretion.

Acknowledgments We thank professor Jörn Oliver Sass (Freiburg Univ) for quantification of urinary 2-methyl-3-hydroxybutyrate and tiglylglycine, Drs Hironori Kobayashi and Yuichi Mushimoto (Shimane University) for urinary organic acid analysis and tandem mass analysis, Dr Tamayo Ishikawa (Kagoshima University) for patients' care, and Ms Keiko Murase and Ms Naomi Sakaguchi (Gifu University) for technical assistance. We also thank Paul Langman, PhD for his assistance with scientific English usage.

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Concise One-Sentence Take-Home Message

Patients with beta-ketothiolase deficiency having a mutation which retains some residual activity showed subtle abnormality in urinary organic acid analysis and blood acylcarnitine analysis even during acute ketoacidotic episodes.

Details of the Contributions of Individual Authors

Toshiyuki Fukao and Naomi Kondo performed the enzyme assays, immunoblot/mutation analysis, and expression analysis of cDNAs. Toshiyuki Fukao mainly wrote this manuscript. Shinsuke Maruyama, Toshihiro Ohura, Mitsuo Toyoshima, Naomi Kuwada, and Mari Imamura are the physicians responsible for the patients. Yuki Hasegawa and Seiji Yamaguchi performed gas chromatography-mass spectrometry and tandem mass spectrometry analyses and first suspected the disorder. Isao Yuasa confirmed GK77 and 77b as identical twins by DNA analyses. Antti M Haapalainen and Rik K Wierenga analyzed the tertiary structural effects of the mutations.

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Alpha-methylacetoacetic acidura, mitochondrial acetoacetyl-CoA thiolase deficiency (OMIM 203750, 607809)

Mitochondrial acetoacetyl-CoA thiolase, acetyl-CoA acetyltransferase 1 (EC 2.3.1.9)

ACAT1 gene (gene ID 38, NM_000019.3)

Details of Funding

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

Details of Ethics Approval

This study has been approved by the Ethical Committee of the Graduate School of Medicine, Gifu University.

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日本小児科学会雑誌　115巻10号　1573～1579（2011年）

論　策

新生児マススクリーニング対象疾患の保険契約の現状について

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東北大学大学院医学系研究科遺伝病学[4]，国立成育医療センター研究所成育政策科学研究部[5]，島根大学医学部小児科[6]

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要　旨

新生児マススクリーニング（MS）対象疾患患児の学資・生命などの保険加入について，①民間会社（38社）の動向調査，②フェニルケトン尿症（PKU）患者335家族への加入状況調査を行った．

①では学資保険について16社，生命保険について15社から回答を得た．生命保険では当該患児が加入するための明確な条件を示した会社は1社のみであった．②では，有効回答のべ124家族中30家族が加入時に病名告知し，うち5家族が入院保障の特約を付加できなかった．また，「成人後に保険加入できるか心配である」という回答を多く得た．

新生児マススクリーニングが始まり30年が経過し，対象疾患の患者が正常に発達し社会に貢献するようになっているにもかかわらず，ほんの数社の学資保険にのみ加入できるだけで特約や生命保険などへの加入はほとんど不可能である現状が明らかになった．

キーワード：フェニルケトン尿症，新生児マススクリーニング，学資保険，生命保険，QOL

緒　言

日本における新生児マススクリーニング（MS）対象疾患患児の保険加入の実態については，蒔田らが2002（平成14）年の第105回日本小児科学会学術集会において報告している[1]．その内容は，生命保険への加入時には遺伝子検査の義務付けがなく，従って遺伝情報による加入差別はないものと考えられてきたが，実情は存在するというものであった．

この報告の後，郵政事業庁（現かんぽ生命保険）は，2003（平成15）年3月25日付（同年4月1日より実施）で先天性甲状腺機能低下症，PKU，ガラクトース血症，先天性副腎過形成の4症に対する保険加入機会拡大を次のような条件付きで発表した[2]．「次の点を踏まえ，申込時の告知内容により，健康状態に問題がないことが確認できたときには，保険にご加入できることとします．①生後1歳を経過していること，②生後早期に疾患を発見・治療を開始し，申込みの時点も治療を継続していること，③合併症もなく症状が安定し，通常の生活を送っておられること．なお，ご加入できる保険種類は，当面，加入ニーズの高い学資保険，育英年金付学資保険及び22歳までに満期となる養老保険に限らせていただきます」（この発表内容は，郵政事業庁，郵政公社時代を通してホームページに報道発表資料として掲載されていたが，かんぽ生命保険会社への変更時に削除されている）．

なお，本邦以外の国における保険加入問題を扱う調査論文は今のところ見当たらない．

これらの経緯を踏まえ，我々は2004（平成16）年，2005（同17）年にPKUのこども達の保険加入状況調査[3,4]を行い，民間保険会社や日本郵政公社の保険加入の際には病名告知の上で円滑に加入できるような商品の設定や情報が必要であること，また，病名のみでの拒否がなく，審査が十分な医学的根拠のもとで適正に行われるように働きかける必要があることを明らかにした．これらの実現に向け，①民間保険会社の動向調査，②2006（平成18）年度PKU患者家族への保険加入状況調査を行ったので，内容と結果を報告する．

方　法

①民間保険会社の動向調査

調査は社団法人生命保険協会所属の38社を対象とし，3回（2006年1月，2007年11月，2008年3月）行った．1回目，2回目は保険会社の審査担当部署に記名方式でアンケート用紙を送付し，同封の返信用封筒にて回収した．1回目の内容は，既出の郵政事業庁の保険加入機会拡大の内容にもとづいて，学資・養老保険に相当する保険について先天性甲状腺機能低下症，PKU，ガラクトース血症，先天性副腎過形成の4症に対する

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「加入制限の有無」「制限の内容」「加入できる商品」などとした．2回目は，メープルシロップ尿症，ホモシスチン尿症を含めた6疾患患児を被保険者とする生命保険加入が可能かどうかについて尋ねた．3回目は，6疾患患児について，学資保険（メープルシロップ尿症とホモシスチン尿症を追加）および生命保険加入の可否について，確認を依頼する文書とした．1回目および2回目の調査用紙は付表1，2に示す．

②PKU患者家族への保険加入状況調査

PKU親の会（現PKU親の会連絡協議会）の協力を得て，2006年12月～2007年1月にかけて335家族に無記名方式のアンケート用紙を郵送し，同封の返信用封筒で回収した．内容は，「保険加入の可否」，「加入保険の種類」，「病名告知の有無」，「機会拡大後の日本郵政公社の保険加入」および「今後の保険加入の希望」を尋ねる項目とした．用紙には，調査の目的および不利益にならないよう匿名で行う旨の説明文を添付した．回答数は同じ家族に複数の患者がいる場合も1家族として扱った．対象となる患者家族の人権の擁護のため，調査は文科省・厚労省の疫学調査に関する倫理指針（平成14年6月17日）に基づいて行った．本研究の公表は大阪市立大学医学部倫理委員会の承認を受けている．

結　果

①民間保険会社の動向調査

【1】日本郵政公社の学資保険・養老保険に相当する保険への加入

3回の調査のいずれかで回答が得られたのは38社中16社であった．3回の調査のまとめを表1に示す．

日本郵政公社（当時）が設けた加入機会拡大時の条件は，前述の4疾患児について「生後1歳を経過していること」，「生後早期に疾患を発見・治療を開始し，申し込みの時点も治療を継続していること」，さらに「合併症もなく症状が安定し，通常の生活を送っていること」となっている．この条件が満たされた上で，商品は学資保険，育英年金付学資保険，および22歳までに満期となる養老保険に限られていた．

この内容に照らし合わせ，学資保険を想定し基本契約では「制限なし」と回答したのは16社中4疾患に対しては0社であり，同様にホモシスチン尿症，メープルシロップ尿症を含めた6疾患に対しては2社であった．学資保険の基本契約においては，患児ではなく，保険料を負担する保護者のみの健康状態を審査する．ただし，医療保険や特約である死亡保障の締結の場合には，患児本人の健康状態を審査する必要があるということであった．

一方，「審査あり」と回答した会社は16社中8社であった．この8社のうち，1社は日本郵政公社に準じて条件項目を設定しており，4疾患に対して2歳以降に加入可という年齢制限を設けているが学資保険については多数が加入でき，他の保険種類にも加入でき得ると回答した．8社中3社（1社は4疾患のみ）は，疾患名のみでなく各個人が希望する保障内容と告知内容，治療状況を総合的に判断すると回答した．8社中2社は，4疾患に対して年齢以外の条件で総合判断とした．また，8社中1社は，4疾患に対して年齢を含めた条件により判断するとした．残り1社は，6疾患に対して基本契約では保険料の支払い能力を確認するため保護者の健康状態を審査すると回答した．

16社中1社は「現時点では加入できない」が，今後「制限付きで加入できるよう検討している」と回答し，16社中4社は学資保険に相当する商品がないか，停止している状態であった．16社中1社は回答が明確でなかった．

養老保険については，16社中3社で相当する保険があると回答した．このうち2社は65歳，80歳を満期とした商品に加入できるとし，3社中1社は「奇形がないこと，治療へのコンプライアンスがよいこと，知的及び身体的発育が順調であることを確認する」と回答した．残り13社は回答がなかったか，あるいは商品がなかった．

【2】生命保険への加入

38社中13社から回答があり，そのうち生命保険商品があるのは11社であった．3回の調査のまとめを表1に示す．11社中1社は，がん保険，生命保険（死亡保障），医療保険に関して比較的明確に条件を示している．がん保険については6疾患とも基本契約及び特約（死亡・医療保障）の締結が可能であった．また生命保険，医療保障については，10歳以上であること，長期入院なく長期に亘って安定してコントロールされていること，主治医の意見書で病状が確認できることの3つを条件とし，クレチン症・古典的PKU患児については条件をすべて満たしていれば生命保険では標準体料率で，医療保障ではさらなる条件付加で締結可能であるとしている．他の4疾患については，生命保険，医療保障ともに上記3条件を満たした上で社内の医長による審査で加入可否を決定するとしている．11社中9社は，6疾患に対して総じて商品と申込者の告知内容，症状・合併症の状況を総合判断し，加入可否を決定するとし，リスクに応じて保険料を高く設定する場合や，リスクが高ければ引受けできない場合もあるとした．残り1社は，現時点では6疾患児とも加入ができないとした．

②PKU患者家族への保険加入状況調査

335家族のうち122家族より返送があった（有効回答数122，有効回答率36%）．患児の平均年齢は15歳で

付表1　社団法人生命保険協会所属38社に送付した1回目のアンケート用紙

フェニルケトン尿症（PKU）をもつこどもの保険加入制限に関する調査

以下の質問に対し、当てはまる数字を○で囲み、必要事項をご記入ください。

1．日本郵政公社は、「先天性甲状腺機能低下症」、「フェニルケトン症」、「ガラクトース血症」及び「先天性副腎過形成」について、各種データ、治療の効果等を検討した結果、早期に治療を開始したときには、症状の発現を相当程度抑制できること等を確認し、平成15年4月1日から上記疾患患者について保険加入の適応拡大を承認しました。
この措置をご存じですか。
（1）はい　　（2）いいえ

2．貴社における上記疾患患者の保険加入時の措置はどのようなものでしょうか
(1) 特に制限していない。（→5．へ）
(2) 制限を設け、審査のうえ加入可能かどうかを判定する（→3．へ）
(3) 現時点では加入できない。（→4．へ）

3．〔2．で（2）を選択された場合にお答えください〕
日本郵政公社では上記疾患患者の保険加入の適応拡大について「具体的には、次の点を踏まえ、申込時の告知内容により、健康状態に問題がないことが確認できたときには、保険にご加入できることとします。」というような制限を付けています。
(ア)生後1歳を経過していること
(イ)生後早期に疾患を発見・治療を開始し、申込みの時点も治療を継続していること
(ウ)合併症もなく症状が安定し、通常の生活を送っておられること
なお、ご加入できる保険種類は、当面、加入ニーズの高い学資保険、育英年金付学資保険及び22歳までに満期となる養老保険に限らせていただきます。

質問1）貴社における年齢制限について伺います。
（1）年齢制限を設けている　（　　歳から）（→質問2）へ）
（2）制限はない

質問2）質問1）で（1）と答えられた場合は
年齢制限を設けている理由を教えてください。
（　　）

質問3）貴社における（イ）の制限について伺います。
（1）日本郵政公社と同様の制限をしている
（2）内容は異なるが制限をしている
この場合どのような制限を設けておられますか
（　　）
（3）制限は設けていない。

質問4）貴社における（ウ）の制限について伺います。
（1）日本郵政公社と同様の制限をしている。
（2）内容は異なるが制限をしている
この場合どのような制限を設けておられますか。
（　　）
（3）制限はもうけていない

質問5）日本郵政公社において加入できる保険種類は、学資保険、育英年金付学学資保険及び22歳までに満期となる養老保険に限られていますが、貴社において上記疾患患児が加入できる保険の種類はどのようなものでしょうか。
（　　）

質問6）養老保険の満期については年齢制限がありますか。
(1) 年齢制限を設けている　（　　才まで）（→質問7）へ）
(2) 制限はない

質問7）質問6）で（1）と答えられた場合は
年齢制限を設けている理由を教えてください。
（　　）

4．〔2．で（3）を選択された場合にお答えください〕
当該疾患患者の保険加入について、貴社では将来加入できる方向で検討されていますでしょうか。
（1）制限付きで加入できるよう検討している
（2）制限なしで加入できるよう検討している
（3）その他
（　　）

5．特記すべきことがございましたら、下欄にご記入ください
（　　）

あった.

【1】保険加入状況については，延べ124家族の回答があった（回答率37.0%）.「加入を試みたことがない」という回答は25家族（20.2%）で平均年齢13歳であった.「円滑に加入できた」は59家族（47.6%）で，平均年齢17歳（加入時4歳）であった.「1度以上加入を拒否されたことがある」は40家族（32.3%）で，平均年齢13歳（加入拒否時3歳）であった．この割合は，

付表2　社団法人生命保険協会所属38社に送付した2回目のアンケート用紙

新旧マススクリーニング対象疾患患児の保険加入についての調査
調査記入用紙

（1）　現在、貴社商品の基本契約のうち、旧マススクリーニング6疾患患児〔フェニルケトン尿症、メープルシロップ尿症、ホモシスチン尿症、ガラクトース血症、クレチン症（先天性甲状腺機能低下症）、先天性副腎過形成〕は、どの契約も締結可能である。

【はい　・　いいえ　・　その他（　　）】

（2）　現在、貴社商品の基本契約に付加される特約保障のうち、旧マススクリーニング6疾患患児は、どの特約も付加可能である。
（養育者が加入する学資保険などに付加される特約も含みます。）

【はい　・　いいえ　・　その他（　　）】

（1）（2）ともに「はい」の場合、質問（4）、（5）へお進みください。

（3）　上記（1）（2）で対象6疾患患児の貴社商品の基本契約、または特約保障付加について、現在、加入・付加可否も含めて疾患ごとの条件（年齢や健康状態、特約保障の範囲）などが設けられているものをご記入ください。（個々の商品について「審査条件は社内秘のため公表できない」、または「加入希望者ごとに個別に対応する」という回答でも結構です。）

（4）新体制のマススクリーニング対象疾患患児の保険加入について、貴社の対応をお聞かせください。（「未定である」との回答でも結構です。）

（5）その他、特筆するべきことがございましたら、ご記入ください。

表1　社団法人生命保険協会所属38社に送付したアンケートの結果より会社ごとの保険商品と加入条件をまとめたもの
表中「○」は学資保険，生命保険については「商品あり」，公開については回答内容の「公開可」を示す．「？」は不明確，「—」は回答なし，「×」は「商品なし」または「公開不可」を示す．また，5の会社は，当該疾患にかかわらずどの疾患患者に対しても契約の公平性を保つため審査は必要と一般化した説明であった．8の会社は社内の引受け基準は公開していないため，情報公開には慎重を期すよう申し入れがあった．

	学資保険		公開	生命保険		公開
1	○ 4疾患	年齢などの条件により判断	○	○ 6疾患	総合判断	× 不公表
2	○ 6疾患	基本契約で保護者の健康状態	○	○ 6疾患	個別に条件あり	○
3	○ 加入できない		○	—	—	—
4	×	商品なし	×	—	—	—
5	○ 6疾患？		× 一般化	○ 6疾患	総合判断	× 一般化
6	—		—	○ 6疾患	総合判断	○
7	○ 6疾患	基本契約では制限なし	×	○ 6疾患	総合判断	×
8	○ 6疾患	基本契約では制限なし	× 留意	○ 6疾患	総合判断	× 留意
9	—		—	○ 6疾患	総合判断	○
10	○ 6疾患	総合判断	×	○ 6疾患	総合判断	×
11	—		—	○ 加入できない		？
12	×	商品なし	？	—		—
13	—		—	×	商品なし	？
14	×	商品なし	？	×	商品なし	？
15	○ 4疾患	総合判断	？	○ 6疾患	総合判断	？
16	○ 4疾患	年齢以外の条件で総合判断	？	—		？
17	○ 4疾患	2歳以上で多くが加入可	？	—		—
18	○ 4疾患	総合判断	？	○ 6疾患	総合判断	？
19	○ 4疾患	年齢以外の条件で総合判断	？	—		—
20	×	商品なし		—		—

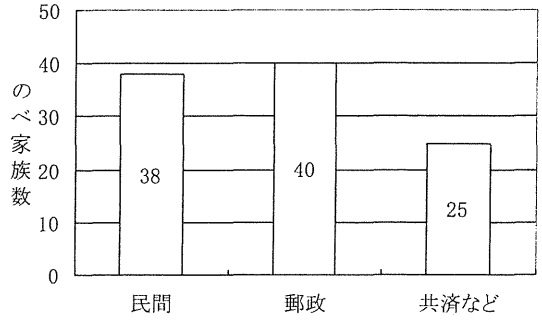

図1　PKU患者家族への保険加入状況調査にて回答のあったのべ124家族のうち，保険加入できた99家族について，民間，郵政，共済などの内訳

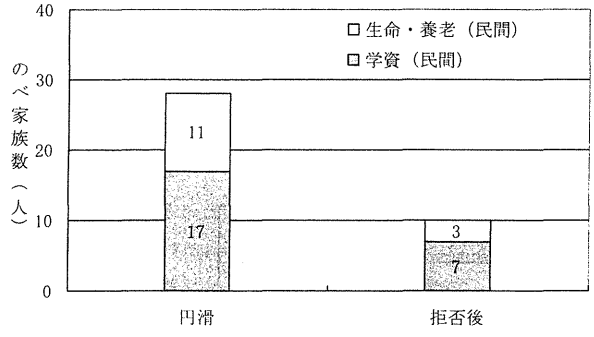

図1-1　図1のうち民間保険会社に加入した38家族について加入を希望した商品と内訳．学資，生命・養老の区分をさらに円滑に加入できたか，または他の保険加入を一度拒否されて加入したか（円滑または拒否）に分けて示す．

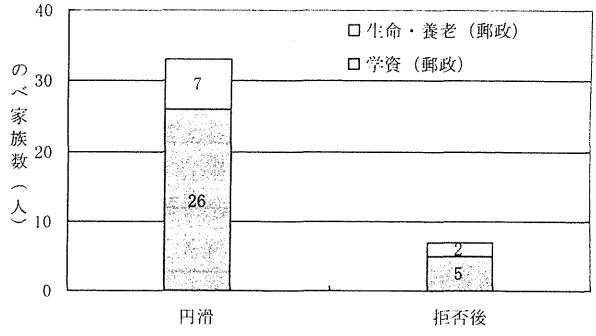

図1-2　図1のうち郵政公社の保険に加入した40家族について，加入を希望した商品と内訳．学資，生命・養老の区分をさらに円滑に加入できたか，または他の保険加入を一度拒否されて加入したか（円滑または拒否）に分けて示す．

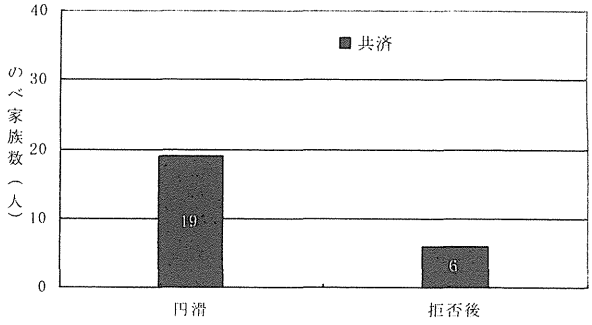

図1-3　図1のうち共済組合などの保険に加入した25家族についての内訳．学資，生命・養老の区分は回答されていないため不明であったが，円滑に加入できたか，または他の保険加入を一度拒否されて加入したか（円滑または拒否）に分けて示す．

過去2回の調査[3][4]においても同様であった．

「円滑に加入できた」家族と「1度以上加入を拒否されたことがある」家族について，加入を希望する会社・商品の内訳（複数回答）は，日本郵政公社（当時）の学資保険（31家族），ついで民間保険会社の学資保険（24家族）が多かった（図1）．

【2】「円滑に加入できた」57家族と，「1度以上加入を拒否されたことがある」うち後に他の保険に加入した24家族を合わせた合計81家族の病名告知の状況は，「判明前」の加入が8家族，内訳は国内5例，不明3例，さらに「判明後加入」のうち，病名告知後に加入できる商品のある外資系保険会社に4家族，国内保険会社に加入したうち，加入時に病名を「告知しなかった（非告知加入）」のは39家族，「告知した（告知後加入）」のは30家族であった（図2）．

【3】今後の保険加入の希望などについては，「今のところ新たな保険会社を探すつもりはない」が22家族，「今すぐにでも入れる保険を探したい」が12家族，「成人後の保険加入が円滑にできるか心配である」が45家族で最も多く，「特約付加を拡大してほしい」が11家族，「加入できる保険を知りたい」が3家族であった（図3）．

考　察

民間保険会社の保険商品への加入可否については，各社商品ごとに特長を持ち容易に比較検討できないため，加入機会拡大時に条件を明らかにした日本郵政公社の商品に照らし合わせ調査した．3度の調査を通して回答率が低かった（最終的に学資保険42.1%，生命保険34.2%）が，できるだけ広い範囲で保険加入できる可能性を調査したかったため保険協会に属する全ての会社に調査書を送付した．その結果，該当する商品がなかった会社からは回答がなかったと思われ，また，患者家族にとって厳しい現状であることが示唆された．

MS対象疾患児の加入に際しては，ほとんどの会社が個々の告知内容，健康状態（合併，奇形なし），治療へのコンプライアンスなどを総合的に判断するが，引き受け基準は開示できないとしている．学資保険は基

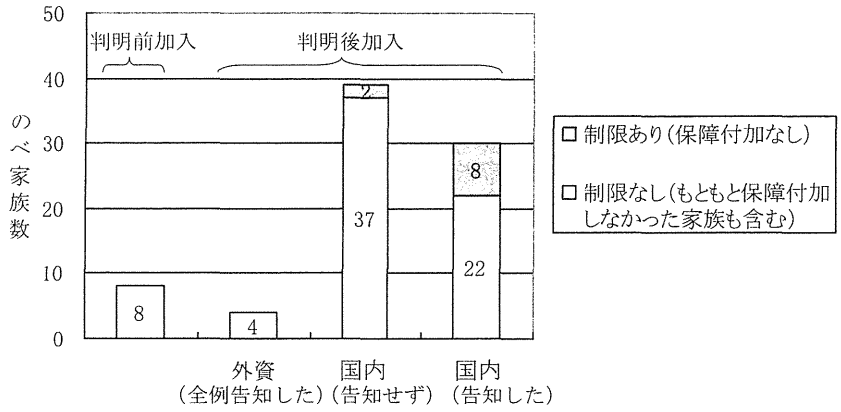

図2　PKU患者家族への保険加入状況調査にて回答のあったのべ124家族のうち，「円滑に加入できた」57家族と「1度以上加入を拒否されたことがある」うち後に他の保険に加入した24家族を合わせた，のべ81家族の病名告知の状況．「告知した」は，加入時に保険会社に病気について告知した患者家族数，「告知せず」は告知せずに加入した患者数を指す．「外資」は，病名告知後に加入できる商品のある外資系保険会社を指し，4家族全例告知後に加入している．「制限なし」には，加入手続き時に保障付加を希望しなかった家族と，希望し付加できた家族を含む．

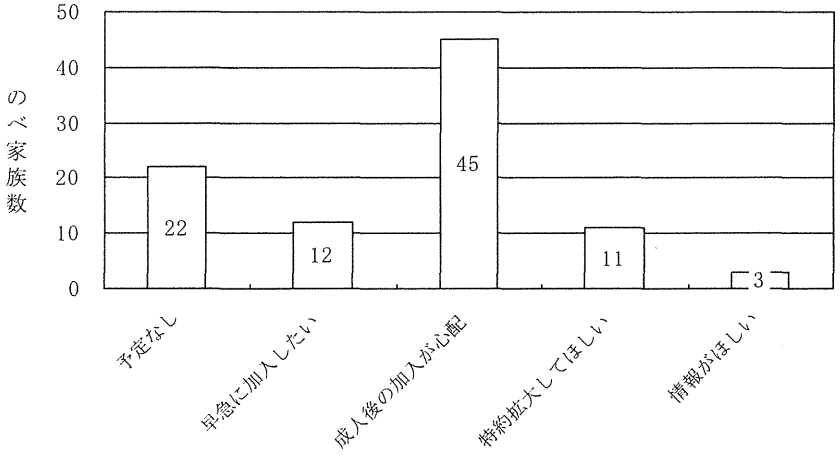

図3　PKU患者家族への保険加入状況調査にて回答のあったのべ124家族のうち，今後の保険加入について望むことなどの回答（複数回答）

本契約のみでは，保険料負担者である保護者の支払い能力が問われ，被保険者である患児の健康状態は問題にはならないようである．患児の加入状況調査において，「病名を告知して加入を拒否された」などの円滑に加入できなかったケースでは，疾病入院や障害疾病入院に対する保障特約が加入制限の理由になっていると思われる．また，図2に示すように，円滑に加入できたケースのうち，「病名を告知せずに加入」した理由については，加入前に上記のような手続き上の問題を認識していた家族と，疾患自体が告知するに値するものではないと認識していた家族に分けられる．

さらに，今回の生命保険会社からの回答内容について社名を含めて公開することへの同意を依頼したが，学資保険，生命保険とも同意を表明したのは3社のみであり，社内の引き受け基準を公にすることへの懸念が強く現れている．

社団法人日本アレルギー学会は，気管支喘息患者の生命保険加入が難しいことに関して，2007年に社団法人生命保険協会加盟41社に対して，我々のものと同様の調査および加入差別の撤廃の要望を行っている[5]．回答結果によると，多くの生命保険会社から，「引受け基準について適宜見直しを実施」など，本調査と同様に厳しい結果であったとしているが，1社のみ平成20年6月より「『適切な喘息管理を行っている』ことを前提に喘息患者さん向け生命保険が誕生」したと報告している[6]．要望に際し，喘息治療として吸入ステロイド剤導入以来，30年間有病率が上昇しているにもかかわらず喘息死者数が大幅に減少していることを示している．PKUにおいてもMS事業開始から30年以上経過し，患児は早期発見・早期治療の開始および治療継続

により知能発達遅滞を呈することなく成長できることが確認されている．このことを裏付けることができる長期予後のデータを示すことが必要である．

患者家族に対する加入状況調査は，出生前後に加入する学資保険の一般的な性格上，新しく発見された患児の最新の加入調査を行う目的で，3年連続で実施した．そのため，繰り返し調査書を送られた家族からの回答が減少し回答率が低くなった．しかし，新規発見の患児家族の加入状況を把握することにより保険会社の対応の変化があったかどうかを捉えることが可能であり，連続調査は有用であったと考える．3度目の調査において，新規保険加入は2歳で生命保険会社の学資保険，1歳で共済組合の保険（告知なし），2歳でかんぽ生命保険の学資保険（審査により入院保障の付帯なし）の3家族であった．

前年2005（平成17）年に行ったPKU患児の保険加入状況調査[1]では，加入機会拡大後の保険への加入拒否が3件報告され，その内訳は「窓口担当者が加入できるようになったことを知らなかったため円滑に加入手続きが取れなかった」が1件，「保険の担当者に病名を告げた時点で断られた」が2件であった．

この結果を踏まえ，2006年11月に日本郵政公社総裁ならびに簡易保険事業本部長宛に，窓口と審査担当各部署に改正内容を再度周知し，当該疾患児が加入する際の対応を統一するよう，また，特約について，特に疾病入院，疾病傷害特約には対象疾患の治療目的での通・入院を除くという条件を付けるなど，特約付加希望に対してひきつづき柔軟に対応するよう，書簡にて要請した．

これに対しては，2006年11月に日本郵政公社総裁の指示のもと簡易保険事業本部長から文書による回答を得た．回答書には窓口や審査担当部署における対応統一については，周知徹底を行う旨が記載されていた．

この要請後に，新規加入を希望する患児家族が円滑に手続きを行えたかについて，今後確認調査が必要である．

日本郵政公社は，2007（平成19）年10月1日より民営化され日本郵政グループとなり，従来の保険業務は株式会社かんぽ生命保険に引き継がれている．新会社の保険商品はこれまでの簡易保険と商品名は同じであるものの，まったく別のものとして扱われることにあるとしており，また，日本郵政公社時のMS対象疾患4症に対する加入機会拡大条件についてのホームページ上の報道発表は削除されている．しかし，問い合わせの結果，旧簡易保険商品では，日本郵政公社時の拡大条件に則って加入可否を判断するとしている．従って，かんぽ生命保険の新規商品の契約に際しては，家族が患児の同席のもと患児の状態についてよく説明し，特に特約付加については外傷などの当該疾患以外の理由による入院が保障されるように交渉することが必要であると考える．

また，民間の保険会社については，告知の上で加入できる保険商品を選択することが契約続行のために必要であると考えられるが，PKU患者家族への保険加入状況調査で，「成人後の保険加入が円滑にできるか心配である」が45家族で最も多かったことは，MS疾患患児の生命保険加入が依然厳しい状況であることを浮き彫りにしたものである．これから成人者が増加するにつれ，生命保険への加入希望がさらに高まるものと予測され，日本小児科学会を含め30年の経過と実績があるMS事業に関わる者の重要な検討課題であると考えられる．

謝辞　アンケートにご協力頂いたPKU親の会連絡協議会会員各位に深謝する．

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日本小児科学会の定める利益相反に関する開示事項はありません．

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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).

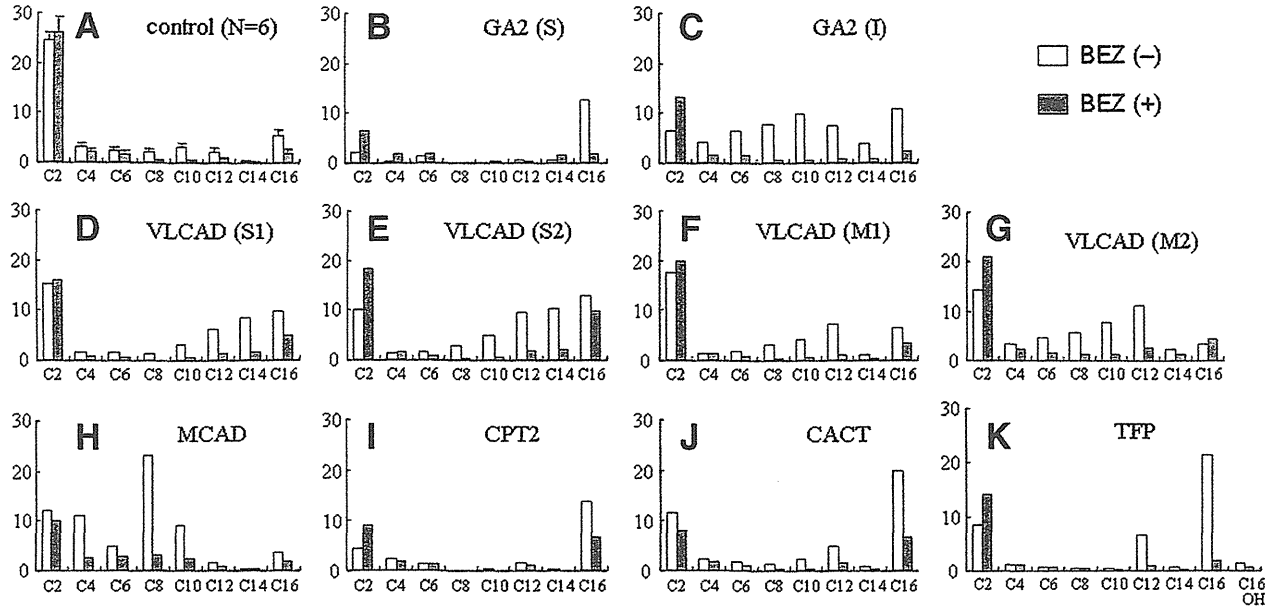

Fig. 1. Acylcarnitine profiles of in vitro probe assay in the presence and absence of bezafibrate. A, normal control; B, severe form of GA2; C, intermediate form of GA2 (the boy who underwent the clinical trial) (S and I, the clinically severe and intermittent form, respectively); D and E, severe form 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

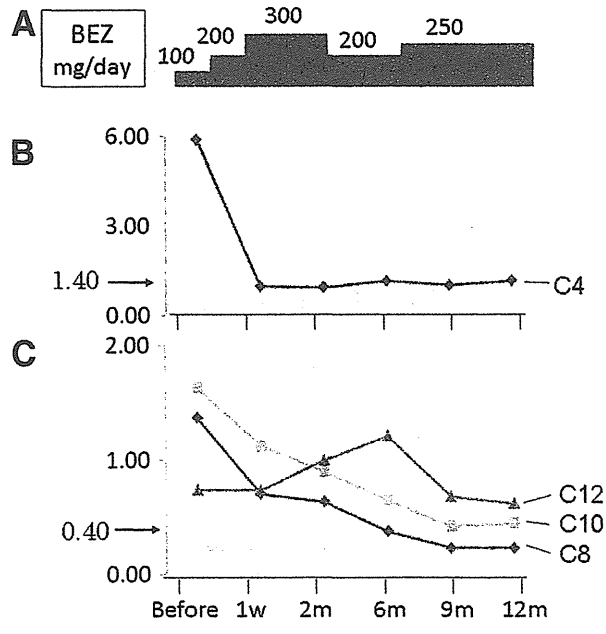

Fig. 2. Bezafibrate administration and changes in blood acylcarnitines. A, dose of bezafibrate, mg/day (approximately 17 to 25 mg/kg/day, div. 3); B, change of C4 acylcarnitine; C, changes in C8, C10, and C12. Arrows with the 1.40 and 0.40 indicate the cutoff values of blood acylcarnitines. Unit of acylcarnitine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

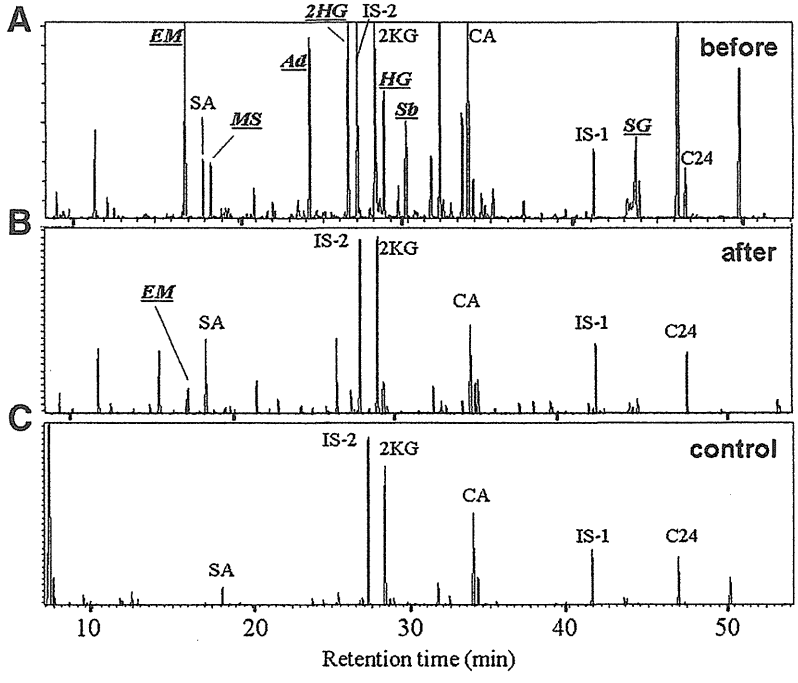

Fig. 3. Urinary organic acid profiles before and after bezafibrate administration. A, The total ion chromatogram (GC/MS) of urinary organic acids just before the start of BEZ; B, One year after the treatment; C, Normal control. Abbreviations: IS-2, IS-1 and C24 are tropate, heptadecanoate, and tetracosane, 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 <0.35 μM	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.