と算定された。本疾患は早期診断、早期治療の介入により神経学的予後を改善しうるという観点からは、この地域においてのスクリーニングの有用性は十分に考慮されると考えられる。さらに、我が国で同定されているその他の遺伝子変異も、ほとんどが日本固有の変異である。表現型と遺伝子型との関連については海外の症例も含め、明らかな相関は見いだされていないが、治療方針決定や予後推定の一助となるよう、今後も症例の蓄積と詳細な検討が望まれる。

２成人期の管理について

乳幼児期から思春期にかけての成長発達の問題をクリアした成人期は神経学的後遺症や成長障害などのリスクが減少する一方で、骨粗鬆症や免疫異常、肺腎合併症、加齢に伴う諸変化が問題となる。

本研究における患者実態調査でも患者年齢の平均は 26.3 歳であり、主治医の大半が小児科医であるにも関わらず成人症例が６割程度を占めている。すなわち晩期合併症の認識と対策が今後の課題である。本症例では 52 歳から L-シトルリン内服を開始しており身長や脳萎縮の改善は見込めないも、歯牙の萌出や毛髪の色素増加、脱毛の減少、蛋白摂取量の増加（刺身等）、全身状態の安定などが確認されている。シトルリンの継続的な投与は小児期を過ぎても有用であることを示している。

E.　結論

北東北地域における患者集積の実態を把握し、遺伝学的検討を行った。本地域において高頻度変異が存在する点からは、マススクリーニングへの応用も含め本地域での疾患の啓蒙が重要であり、早期診断確立に寄与できることが望まれる。

また本邦におけるリジン尿性蛋白不耐症患者の長期経過について報告した。青壮年期以降に L-シトルリンを開始した症例においても、蛋白摂取量や全身状態のみならず毛髪や歯牙などの理学所見に関して改善が得られた。リジン尿性蛋白不耐症患者の長期生存例は今後増加することが予想され、晩期合併症の認識と対策、QOL の向上が今後の課題である。

F 健康危険情報

なし

G研究発表

Noguchi A et al. SLC7A7 genomic structure and novel variants in three Japanese lysinuric protein intolerance. HUMAN MUTATION 15:367.372 (2000)

Koizumi A et al. A cluster of lysinuric protein intolerance (LPI) patients in a northern part of Iwate, Japan due to a founder effect families. HUMAN MUTATION Mutation in Brief #356 (2000) Online

H知的財産権の出願・登録状況

１　特許取得　なし

２　実用新案登録　なし

３　その他　なし

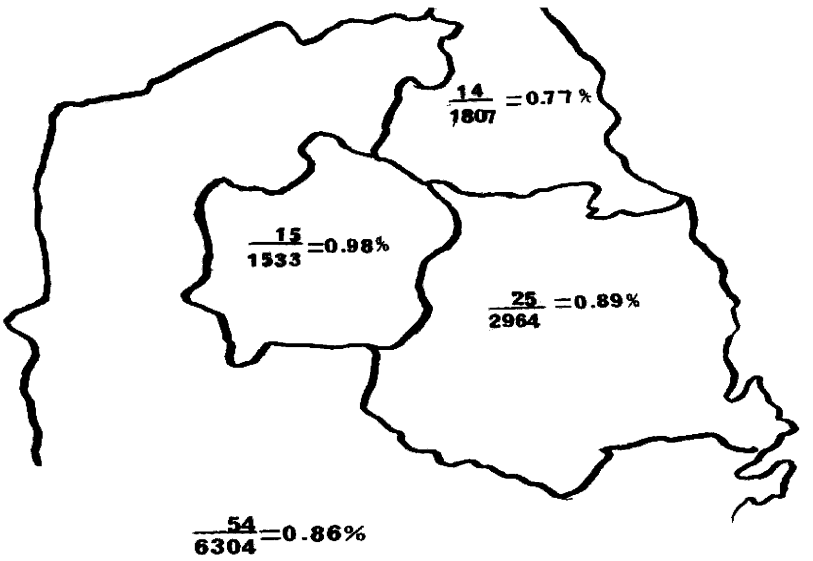


図1　新生児LPIのマススクリニング（R410）を用いた保因者の発見

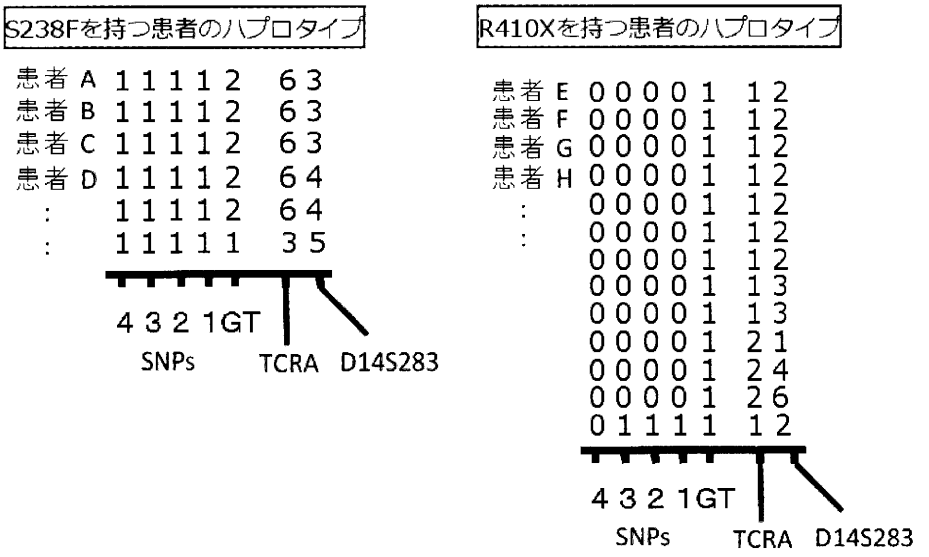


図2　同一遺伝子変異を持つLPI症例のハプロタイプ解析

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究年度終了報告書

当院におけるリジン尿性蛋白不耐症の４例の臨床的多様性

分担研究者：　高柳　正樹
千葉県こども病院医療局長

研究要旨
　今回は当院におけるリジン尿性蛋白不耐症の４例の臨床的多様性について報告する。
4 症例の発症年齢は、3 カ月から 13 歳と幅広く分布している。初発症状は症例により異なっており、しかも非特異的な症状である。
シトルリンを100-150mg/kg/dayの投与を行い、症状の増悪や急性転化を防げている。
治療上の問題はその臨床上の多様性から、症例毎に異なるものと思われる。
今後さらなる臨床研究が必要と考えられる。

研究協力者氏名：
千葉県こども病院　代謝科　　村山　圭

A．研究目的

　リジン尿性蛋白不耐症はその臨床像が非常に多彩であることが知られている。

　今回は当院におけるリジン尿性蛋白不耐症の４例の臨床的多様性について報告する。

B．研究方法

　症例：当院で経験したリジン尿性蛋白不耐症４症例をまとめて表に示した。1例は千葉大学病院小児科へ転院しているので最新の情報はない。

（倫理面への配慮）

個人が特定されないように配慮した。

C．研究結果

　4症例の発症年齢は3カ月から13歳と幅広く分布している。また診断年齢も8カ月から19歳となっている。発症から診断までの期間は5カ月から10年となっており、３症例では適切な診断に至るまでに5年以上かかっている。初発症状は症例により異なっており、しかも非特異的な症状である。さらに経過中の主なる症状としては、ＳＬＥ，抗リン脂質抗体症候群を示す症例、知能発達遅延を示す症例もおり、症例を通じて共通なものはないと言っていい。骨粗しょう症は1例の患者に著名に見られるのみであった。現在シトルリンを100-150mg/kg/dayの投与を行い、症状の増悪や急性転化を防げている。

D．考察

　リジン尿性蛋白不耐症の臨床像は非常に多様である。この疾患の可能性に気がつけばその後の診断は現在でも困難ではない。この疾患の一般小児科医、内科医への啓蒙教育活動が最も重要と思われる

。当院での症例でも発症から診断まで長期間を要していることから、このことが裏付けられる。

現在におけるリジン尿性蛋白不耐症の治療上の問題点は以下のように考えられる。

・シトルリン至適投与量は？
・適切な投与量設定のためのマーカーは？
・患者にシトルリンだけ投与していけば問題はないのか？
・リジン低下に対する治療法は？
・臨床的多様性の理由は？
・Genotype-phenotype relationship?
・osteoporosisの発症頻度は？
・ビスフォスフォネート製剤の適応は
・免疫学的異常との関連、機序は？

E．結論

リジン尿性蛋白不耐症はその臨床像が非常に多彩である。この疾患の存在の教育啓蒙活動が診断を早め、患者のＱＯＬの向上に寄与すると考える。

治療上の問題はその臨床上の多様性から、症例毎に異なるものと思われる。
今後さらなる臨床研究が必要と考えられる。

F．研究発表

1．論文発表

北澤克彦　他。Hemophagocytic lymphohistiocytosis(HLH)発症を契機に診断されたリジン尿性蛋白不耐症の１例。日小血会誌　14:pp125-129,2000.

永田正人　他。免疫異常を伴った Lysine Protein Intolerance の 1 例。日児誌 92:pp1799-1804,1988.

山本重則　他。Lysine Protein Intolerance の 2 症例－ε-N-Acetyl-L-lysine による低リジン血症の治療について。日児誌 90:pp198-204,1986.

G．知的財産権の出願・登録状況
（予定を含む。）

1．特許取得
なし
2．実用新案登録
なし
3.その他
なし

表１：当科で経験したリジン尿性蛋白不耐症４例の臨床概略

	現在年齢	性別	発症年齢	初発症状	診断年齢	主なる症状
症例1	27	男	3ヶ月	体重増多不良	8ヶ月	肝機能障害
症例２	30	男	13歳	血球呑食症候群	19歳	知能発達遅滞
症例3	33	女	2，3歳	低身長	8歳	高リン脂質症候群
症例4	41	女	3，4歳	嘔吐、頭痛	14歳	肝腫大

表２：当科で経験したリジン尿性蛋白不耐症４例の臨床概略（続）

	特記事項	シトルリン量	体重	遺伝子
症例1	SFD40W2160g	8g	60kg	
症例 2		6g	56kg	
症例3	SLE、脳梗塞、	6g	40kg	R410X,S238F 秋田小児科
症例4	osteoporosis			

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

リジン尿性蛋白不耐症のシトルリン治療

分担研究者　遠藤文夫
熊本大学医学薬学研究部
小児科学分野

研究要旨
リジン尿性蛋白不耐症は高アンモニア血症を伴う先天代謝異常症である。治療に用いられているシトルリンは研究試薬を内服薬として用いることが多く、その安全性は十分とはいえなかった。シトルリンがサプリメントとして利用できるようになり、食品としてのシトルリンを治療薬として利用することが可能となった。今回、シトルリンの効果と安全性についての検証を試みたので報告する。

研究協力者氏名
中村公俊　　熊本大学医学薬学研究部小児科学分野　講師

A．研究目的

リジン尿性蛋白不耐症、OTC（オルニチントランスカルバミラーゼ）欠損症、CPS（カルバミルリン酸合成酵素）欠損症、などは高アンモニア血症を伴う先天代謝異常症であり、適切な治療を行ったとしても死亡または重篤な障害をきたすことがある疾患である。尿素サイクル異常症においては、肝移植などの根治治療の適応は限られているため、アルギニン、安息香酸ナトリウム、シトルリンなどの投与が試みられ、効果をあげている。安息香酸ナトリウム、シトルリンは研究試薬を内服薬として用いることが多く、その安全性は十分とはいえなかった。シトルリンがサプリメントとして利用できるようになり、食品としてのシトルリンを治療薬として利用することが可能となった。今回、シトルリンの効果と安全性についての検証を試みたので報告する。

B．研究方法

リジン尿性蛋白不耐症においてシトルリン、アルギニン、安息香酸などの投与が試みられている患者を対象とする。これまでに試薬のシトルリンを用いている患者さんも対象とする。日本先天代謝異常学会のシトルリン使用に関する委員会事務局からリジン尿性蛋白不耐症患者の担当医に対して、協和発酵キリン株式会社から提供を受けた、食品であるシトルリンを供給する。その効果について体重変化、アンモニア、肝障害等を評価する。また、供給先の医療機関における診察、検査によって安全性を確認する。

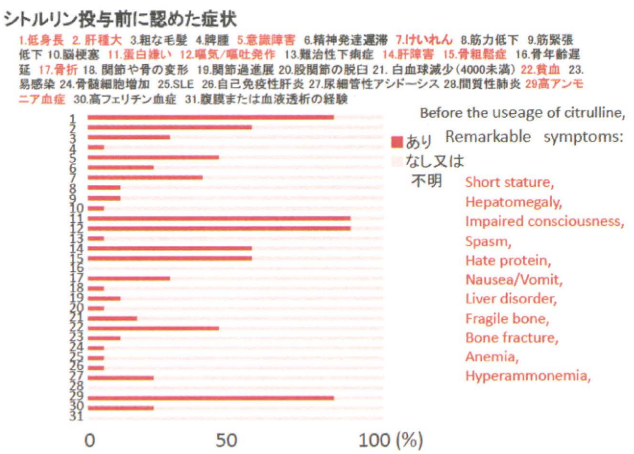
C．研究結果

調査対象となったリジン尿性蛋白不耐症患者のすべてがシトルリンによる治療を行っていた。一方、尿素サ

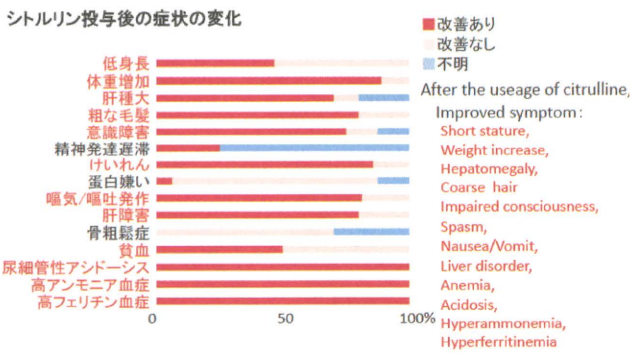
イクル異常症の患者のなかで、OTC 欠損症患者の約 20%のみがシトルリンを用いて治療されていた。本委員会からシトルリンを供給している施設は全国で 22 施設であり、リジン尿性蛋白不耐症 17 例、OCT 欠損症 17 例、CPS 欠損症 3 例であった。これまでに重篤な副作用は報告されていない。

調査対象となったリジン尿性蛋白不耐症患者では、シトルリンの投与前には、低身長、肝種大、意識障害、けいれん、蛋白嫌い、嘔気/嘔吐発作、肝障害、骨粗鬆症、骨折、貧血、高アンモニア血症などを主な症状として認めた（図 1）。その中で、シトルリン投与によって改善を認めた症状として、低身長、肝種大、意識障害、けいれん、嘔気/嘔吐発作、肝障害、貧血、高アンモニア血症があげられた（図 2）。蛋白摂取量が増やせるようになったことや、シトルリンを多く含むスイカを大量に食べなくても体調管理ができるようになったことも効果としてあげられていた。しかし骨折や骨粗しょう症に対する効果は乏しいと考えられた。

（図 1）シトルリン投与前に認めた症状

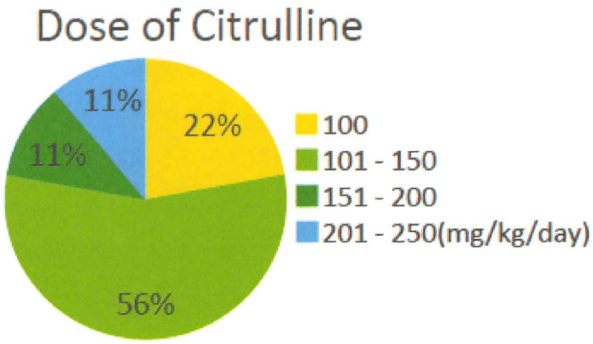


（図 2）シトルリン投与後の症状の変化



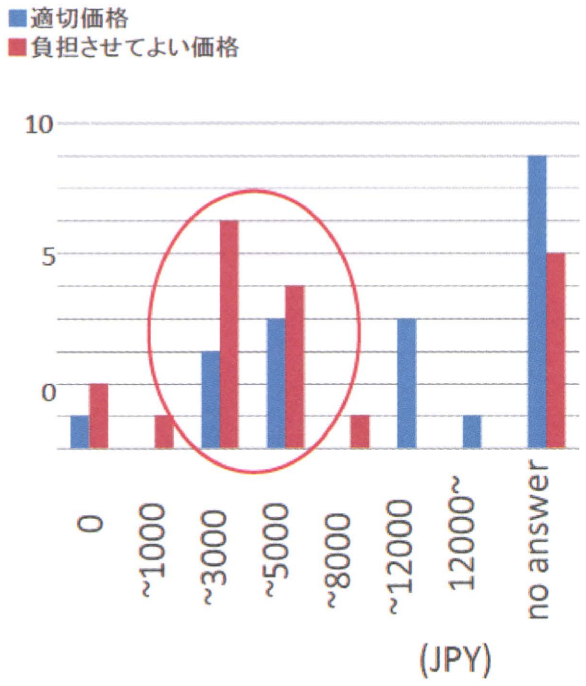
シトルリンの投与量を（図 3）に示す。ほとんどの症例で推奨されている投与量である 100－200mg/kg/日が用いられていた。約 1 割の患者ではそれよりやや多い寮の投与がされていた。

（図 3）リジン尿性蛋白不耐症患者における
シトルリンの投与量



また、担当医が考えるシトルリンの適正価格、患者が負担してよいと考えられる価格を（図 4）に示した。試薬のシトルリンを用いた場合には学童期の小児で 1 ヶ月あたり約 30 万円必要となる。食品としてのシトルリンであれば、3000 円から 5000 円程度が適当であると考えた担当医が多かった。

（図 4）担当医が考えるシトルリンの適切な価格



D．考察・結論

尿素サイクル異常症における治療薬として、アルギニン、安息香酸ナトリウム等を使用している施設が多く、シトルリンを使用している施設はまだ少ない。OTC 異常症 CPS 欠損症やリジン尿性蛋白不耐症などで、適応症例の拡大が可能であると考えられる。シトルリンの使用は、尿素サイクル異常症における有効な治療戦略のひとつになり得ると考えられる。

E．健康危険情報

なし

F．研究発表

1．論文発表

（発表誌名巻号・頁・発行年等も記入）

Nakamura K, Sekijima Y, Nakamura K, Hattori K, Nagamatsu K, Shimizu Y, Yasude T, Ushiyama M, Endo F, Fukushima Y, Ikeda S. Cerebral hemorrhage in Fabry's disease. J Hum Genet. 55, 259-61. (2010)

Nakamura K, Hattori K, Endo F. Newborn Screening for lysosomal disorders. Am J Med Genet. (2011 in press)

Shigeto S, Katafuchi T, Okada Y, Nakamura K, Endo F, Okuyama T, Takeuchi H, Kroos MA, Verheijen FW, Reuser AJJ, Okumiya T Improved assay for differential diagnosis between Pompe disease and acid alpha-glucosidase pseudodeficiency on dried blood spots. Mol. Genet. Metab. (2011 in press)

中村公俊　Fabry病の疫学と診断　神経内科73,(2010)

中村公俊　アミノ酸代謝異常症、尿素サイクル異常症、糖原病の新しい治療法　小児内科　42, 1191-1194 (2010)

中村公俊、遠藤文夫　先天性アミノ酸代謝異常症　高チロシン血症　日本臨床　別冊　肝･胆道系症候群 I　肝臓編（上）　486-489 (2010)

2．学会発表

城戸淳、中村公俊ほか

「尿素サイクル異常症におけるシトルリン治療」　日本先天代謝異常学会雑誌 26 巻 2 号 p116, 2010

G．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

III.　研究成果の刊行物に関する一覧表

研究成果の刊行に関する一覧

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
高柳正樹	代謝救急	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	2-4
山本重則	肝不全、Reye様症候群	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	48-50
高柳正樹	肝機能異常、黄疸、肝腫大	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	51-53
村山　圭	乳幼児突然死症候群	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	57-59
高柳正樹	特殊な代謝検査を行う必要のない病態	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	72-73
中村公俊	代謝のプロファイリング	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	124-126
村山　圭	ミトコンドリアレスキュー	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	186-187
野口篤子	カルニチントランスポーター欠損症	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	229-231
高橋　勉	ニーマンピック病	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	235-237
高柳正樹	治療用調整粉乳によるカルニチンビオチン欠乏症	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	244-245
高柳正樹	酵素診断	五十嵐隆 高柳正樹	小児科臨床ピクシス	中山書店	東京	2010	268-269

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
SShigeto S, Katafuchi T, Okada Y, Nakamura K, Endo F, Okuyama T, Takeuchi H, Kroos A, Verheijen FW, Reuser AJ, Okumiya T.	Improved assay for differential diagnosis between Pompe disease acid α-glucosidase pseudodeficiency on dried blood spots.	Molecular genetics and Metabolism	103	12-17	2011

中村公俊	Fabry病の疫学と診断	神経内科	73(2)	168-172	2010
中村公俊	アミノ酸代謝異常症、尿素サイクル異常症、糖原病の新しい治療法	小児内科	42(7)	1191-1194	2010
中村公俊、遠藤文夫	先天性アミノ酸代謝異常症—高チロシン血症1型—	日本臨床　新領域別症候群シリーズ	肝胆道症候群I肝臓編	486-489	2010
Nakamura K,Hattori H, Endo F	Newborn screening for lysozomal storage disorders	American journal of medical genetic partC	157	63-71	2011
Okuyama T, SUehiro Y, Imada H, Shimada A Naruse K, Terada H, Kobo T, Takeuchi H.	Induction of c-fos transcription in the medaka brain in response to mating stimuli	Biochemical and biophysical research communication	404	453-457	2011
Arai H, Noguchi A, Goto R, Matsuda T, Nakajima H, Takahashi T.	Liver fibrosis in an extremely small infant for gestational age	Tohoku J Exp Med	221	181-185	2010
Hori T, Fukao T, Kobayashi H, Teramoto T, et al.	Carnitine palmitoyltransferase 2 deficiency: the time-course of blood and urinary acylcarnitine levels during initial L-carniti	Tohoku J Exp Med.	221(3)	191-195	2010
Fukao T, Horikawa R, Naiki Y, et all.	A novel mutation (c.951C>T) in an exonic splicing enhancer results in exon 10 skipping in the human mitochondrial acetoac	Mol Genet Metab	100(4)	339-44	2010
Kimura A, Kage M, Nagata I, et al.	Histological findings in the livers of patients with neonatal intrahepatic cholestasis caused by citrin deficiency.	Hepatol Res	40(3)	295-303	2010
Fukao T, Ishii T, Amano N, Kursula P, et al.	A neonatal-onset succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patient with T435N and c.658-666dupAACGTGA	J Inherit Metab Dis	Jul 21.	online	2010

IV.　研究成果の刊行物・別冊

Molecular Genetics and Metabolism 103 (2011) 12–17

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

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

Improved assay for differential diagnosis between Pompe disease and acid α-glucosidase pseudodeficiency on dried blood spots

Shohei Shigeto [a], Tatsuya Katafuchi [a], Yuya Okada [a], Kimitoshi Nakamura [b], Fumio Endo [b], Torayuki Okuyama [c], Hiroaki Takeuchi [d], Marian A. Kroos [e], Frans W. Verheijen [e], Arnold J.J. Reuser [e], Toshika Okumiya [a,*]

[a] Department of Biomedical Laboratory Sciences, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
[b] Department of Pediatrics, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
[c] Department of Laboratory Medicine, National Center for Child Health and Development, Tokyo, Japan
[d] Department of Clinical Laboratory Medicine, Kochi Medical School, Kochi Japan
[e] Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 24 December 2010
Received in revised form 18 January 2011
Accepted 18 January 2011
Available online 22 January 2011

Keywords:
Pompe disease
Glycogen storage disease type II
Acid maltase
Acid alpha-glucosidase
Dried blood spot
Newborn screening
Genetic polymorphism

ABSTRACT

The high frequency (3.3–3.9%) of acid α-glucosidase pseudodeficiency, c.[1726G>A; 2065G>A] homozygote (AA homozygote), in Asian populations complicates newborn screening for Pompe disease (glycogen storage disease type II or acid maltase deficiency) on dried blood spots, since AA homozygotes have a considerably low enzyme activity. We observed that hemoglobin in the enzyme reaction solution strongly interferes with the fluorescence of 4-methylumbelliferone released from 4-methylumbelliferyl α-D-glucopyranoside (4MU-αGlc) by acid α-glucosidase. Therefore, we have searched for a method to effectively eliminate hemoglobin in the reaction solution. Hemoglobin precipitation with barium hydroxide and zinc sulfate (Ba/Zn method) carried out after the enzyme reaction considerably enhances the fluorescence intensity while it does not reduce the intensity to any extent as can occur with conventional deproteinization agents like trichloroacetic acid. The Ba/Zn method greatly improved the separation between 18 Japanese patients with Pompe disease and 70 unaffected AA homozygotes in a population of Japanese newborns in the assay with 4MU-αGlc on dried blood spots. No overlap was observed between both groups. We further examined acid α-glucosidase activity in fibroblasts from 11 Japanese patients and 57 Japanese unaffected individuals including 31 c.[1726G; 2065G] homozygotes, 18 c.[1726G; 2065G]/[1726A; 2065A] heterozygotes and 8 AA homozygotes to confirm that fibroblasts can be used for definitive diagnosis. The patients were reliably distinguished from three control groups. These data provide advanced information for the development of a simple and reliable newborn screening program with dried blood spots for Pompe disease in Asian populations.

1. Introduction

Early diagnosis is a critical issue for effective enzyme replacement therapy in lysosomal storage diseases. To this end, efforts have been made to develop methods for newborn screening. Most methods are based on the direct measurement of lysosomal enzyme activities in dried blood spots (DBSs) [1–6]. Other procedures include antibodies to increase the specificity of the assay, or to determine the amount of enzyme protein rather than activity, or to probe lysosomal disease markers [7–10]. Multiplex assays with the parallel measurement of several lysosomal enzyme activities are aimed to improve the cost effectiveness of newborn screening [11–14].

Using DBSs a first large scale newborn screening program in Taiwan was shown to improve clinical outcomes for patients with Pompe disease [4], also known as glycogen storage disease type II or acid maltase deficiency (OMIM No. 232300). Pompe disease is an autosomal recessive disorder of glycogen metabolism resulting from a generalized deficiency of the lysosomal enzyme acid α-glucosidase (AαGlu; EC 3.2.1.20/3). The enzyme deficiency causes intralysosomal glycogen storage in numerous tissues, but predominantly in muscle. The disorder exhibits a broad clinical spectrum with regard to age of onset, cardiac involvement and progression of skeletal muscle dysfunction. Since 1999, several clinical trials have shown that patients with Pompe disease can benefit from enzyme replacement

Abbreviations: AαGlu, acid α-glucosidase; AA homozygote, c.[1726A 2065A] homozygote acid α-glucosidase pseudodeficiency; GG/AA heterozygote, c.[1726G 2065G]/c.[1726A 2065A] heterozygote; GG homozygote, c.[1726G 2065G] homozygote; 4MU, 4-methylumbelliferone; 4MU-αGlc, 4-methylumbelliferyl α-D-glucopyranoside; DBS, dried blood spot; TCA, trichloroacetic acid; Ba/Zn method, barium hydroxide and zinc sulfate method.

* Corresponding author at: Department of Biomedical Laboratory Sciences, Faculty of Life Sciences, Kumamoto University, 4-24-1, Kuhonji, Kumamoto 862-0976, Japan. Fax: +81 96 373 5492.
E-mail address: okumiyat@kumamoto-u.ac.jp (T. Okumiya).

1096-7192/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2011.01.006

therapy [15–20]. The effect of enzyme therapy in severely affected infants is readily recognized by regression of the cardiomegaly, prolonged survival and acquirement of motor skills. Beneficial effects of enzyme replacement therapy in children, adolescents and adults with Pompe disease also have been reported and are promising, but the crucial outcome of long term treatment has still to be awaited [18–22]. Further, it appears that infants with rather well preserved muscle morphology respond better to therapy than those who are diagnosed late and have severe muscle damage at start of treatment. Early diagnosis seems a must in Pompe disease to optimize any form of therapeutic intervention [23].

Previously, we examined AαGlu activity in 715 apparently healthy Japanese newborns with DBSs and showed that the distribution of the activity was bimodal. The median activity of the minor group (31 individuals, 4.3% of the samples) was 6.5 times lower than that of major group [6]. Genetic analysis revealed that 28 individuals of the minor group were homozygous for c.[1726G>A; 2065G>A], also known as pseudodeficiency (AA homozygote). Four of the AA homozygotes had activities in the patients' range (the AαGlu activity range of the AA homozygotes is 1.4–10.1 pmol/h/disk; the activity range of the Pompe patients is 0–2.8 pmol/h/disk). The AαGlu pseudodeficiency allele has a much higher frequency in the Asian compared to the Caucasian populations [6,24]. Substitution p.E689K caused by c.2065G>A characterizes the "GAA4" allozyme, which is found in Chinese and Japanese populations with frequencies of 0.27–0.28 and 0.27–0.31, respectively, and reduces the AαGlu activity by 50–60% of average normal [25–27; JSNP, http://snp.ims.u-tokyo.ac.jp/]. On the contrary, substitution p.G576S caused by c.1726G>A reduces the AαGlu activity to such extent that it may overlap with the patient range [24]. Thus, to achieve reliable newborn screening for Pompe disease in Asian populations, sensitivity and selectivity of the method should be improved to distinguish pseudodeficiency from pathologic deficiency. We were informed that hemoglobin precipitation with trichloroacetic acid (TCA) improves 4MU-based diagnostic assays for lysosomal storage disease in DBSs because it eliminates quenching of the fluorescence signal [28].

In this study, we have looked for the most effective method to eliminate hemoglobin from the reaction solution in order to maximize the separation between newborns with Pompe disease and AA homozygotes in Asian populations. We here describe that hemoglobin precipitation with barium hydroxide and zinc sulfate after the enzyme reaction considerably improves the 4MU fluorescence intensity and circumvents the potential problem of signal reduction by TCA precipitation.

2. Subjects, materials and methods

2.1. Subjects and DBS collection

DBSs from 252 Japanese newborns (second to fifth day postpartum) and 18 Japanese patients with Pompe disease were used in this study. The patient group included one child with classic infantile Pompe disease, 6 juveniles, 10 adults, and one patient with unknown phenotype. The DBSs on filter paper were obtained with the standard heel-stick for collecting newborn screening samples, or prepared by drop-wise application of EDTA-blood samples on the filter paper (filter paper #510AD01, Advantec, Tokyo, Japan) that is routinely used for newborn screening in Japan. DBSs were dried at room temperature for at least 3 h but no more than 16 h, and were subsequently stored at −20 °C in sealed plastic bags until use. Written informed consent was obtained from all subjects, and all samples from these subjects were prepared and analyzed in accordance with the protocols approved by the Ethics Committee for Gene Analysis and Genome Research of Kumamoto University.

Fibroblasts from 57 Japanese unaffected individuals (controls) and 11 patients with Pompe disease were used for this study. Fibroblasts were cultured under standard conditions in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (50 kU/L penicillin, 50 mg/L streptomycin). After reaching confluency, the fibroblasts were harvested and washed with phosphate-buffered saline. The cell pellets were stored at −40 °C until use. The pellets ($2–4\times10^6$ cells) were homogenized in 500 μL water by sonicating on ice for two times 10 s, using a UP50 ultrasonic processor (Hielscher, Teltow, Germany) with a 2 mm diameter tip size, set at 100 μm amplitude. The protein concentration of the cell homogenates was measured using the Pierce BCA protein assay reagent kit (Rockford, IL) with bovine serum albumin as a calibrator. The protein concentration of the homogenates was adjusted to 0.6–1.2 mg/mL unless otherwise indicated.

2.2. Chemicals, reagents and instrument

4-Methylumbelliferyl α-D-glucopyranoside (4MU-αGlc), glycogen (type III, from rabbit liver) and glucose were purchased from Sigma-Aldrich (St. Louis, MO). Acarbose and 4-methylumbelliferone (4MU) were from Toronto Research Chemicals (North York, Canada) and Nacalai Tesque (Kyoto, Japan), respectively. The chromogen, 10-N-methylcarbamoyl-3,7-bis(dimethylamino)phenothiazine, for highly sensitive detection of glucose from glycogen were provided from Kyowa medix (Tokyo, Japan). Other chemicals were of reagent grade and from Sigma-Aldrich or Nacalai Tesque. The fluorescence intensity of 4MU liberated from 4MU-AαGlu by AαGlu was measured with the CORONA spectrofluorometer (MTP-800AFC, Colona Electric, Hitachinaka, Japan) at excitation and emission wavelengths of 360 nm and 450 nm, respectively. COBAS MIRA automatic analyzer (Roche, Basel, Switzerland) was used to measure AαGlu activity with glycogen as a substrate.

2.3. Effect of hemoglobin elimination on 4MU detection

A solution of 60 μmol/L 4-methylumbelliferone in a buffer consisting of 0.2 mol/L citrate/0.4 mol/L potassium-phosphate at pH 4.0 with different concentrations of hemoglobin added to it (0, 225, 450, 900 and 1800 mg/L in the final reaction mixture) was used to compare two different methods for hemoglobin elimination. For the hemoglobin precipitation with TCA (TCA method), 60 μL of the sample solution in a 1.5 ml reaction tube was incubated at 37 °C for 120 min, and then 20 μL of 16% chilled TCA was added. After vortex mixing and incubation at 4 °C for 10 min, the sample solution was centrifuged at 10,000 *g* at 4 °C for 5 min. 60 μL of the supernatant was transferred to a 96-well black microwell-plate (PerkinElmer, Boston, MA), and then 190 μL of 0.5 mol/L sodium-carbonate/sodium-bicarbonate buffer at pH 10.7 containing 0.1% Triton X-100 was added for measurement of fluorescence intensity. For the hemoglobin precipitation with barium hydroxide and zinc sulfate (Ba/Zn method), 60 μL of the sample solution in a 1.5 mL reaction tube was treated with the Ba/Zn method as described below and the fluorescence intensity was measured. As a control, 60 μL of the sample solution was treated as in the TCA method except that distilled water was used instead of 16% TCA.

2.4. Measurement of AαGlu activity in DBSs

We assayed AαGlu activity in DBSs with two different methods. One method was without hemoglobin elimination, as previously published (previous method) [6]. In the other we used the Ba/Zn method to eliminate hemoglobin. For the previous method, a 3.2-mm diameter disk punched from the DBSs was incubated in a well of a 96-well clear microwell-plate (Corning, New York, NY) with 100 μL distilled water for 1 h at room temperature while mixing gently. A 20 μL aliquot of the water extract was then added to 40 μL of 2.0 mmol/L 4MU-AαGlc in 0.2 mol/L citrate/0.4 mol/L potassium-phosphate buffer at pH 4.0 containing 4.5 μmol/L acarbose (3.0 μmol/L in final concentration) in a 96-well black microwell-plate

(PerkinElmer). The reaction mixture was incubated at 37 °C for 24 h, and the reaction was stopped by addition of 190 μL of 0.2 mol/L glycine/NaOH buffer at pH 10.7 containing 0.1% Triton X100 to measure fluorescence intensity. For the Ba/Zn method, a similar 3.2-mm diameter disk punched from the DBSs was placed in a 1.5 mL reaction tube and gently mixed for 10 min at room temperature in 60 μL of 0.2 mol/L citrate/0.4 mol/L potassium-phosphate buffer at pH 4.0 containing 2.0 mmol/L 4MU-AαGlc and 3.0 μmol/L acarbose. The reaction mixture was then incubated at 37 °C for 24 h. After this period, 30 μL of 0.15 mol/L barium hydroxide was added and, after vortex mixing, the reaction tube was left at room temperature for 5 min. Thereafter, 30 μL of 0.15 mol/L zinc sulfate was added and again, after vortex mixing, the tube was left for 10 min at room temperature. The tube was then centrifuged for 5 min at 10,000 *g* and 4 °C. Finally, 90 μL of the supernatant was transferred to a 96-well black microwell-plate (PerkinElmer), and 160 μL of 0.4 mol/L glycine/NaOH buffer at pH 10.7 containing 0.1% Triton X-100 was added to measure fluorescence intensity. We used stock solutions of 0, 6.25, 12.5, 25, 50 and 100 μmol/L 4MU in 20 mmol/L sodium-phosphate buffer at pH 7.0 to calibrate the measurement of liberated 4MU. The enzyme activity was expressed as picomoles 4MU released per hour per 3.2 mm diameter disk (pmol/h/disk). Each assay was performed in duplicate.

2.5. Measurement of AαGlu activity in fibroblasts

AαGlu activity in fibroblasts was measured with 4MU-αGlc as substrate as described [29] with minor changes. Briefly, 10 μL of the cell homogenate was added to 40 μL of the substrate solution containing 2.0 mmol/L of 4MU-αGlc in 0.2 mol/L citrate/0.4 mol/L potassium-phosphate buffer at pH 4.0 with 3.75 μmol/L acarbose (3.0 μmol/L in the final concentration) in a well of a 96-well black microwell-plate (PerkinElmer). The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by addition of 200 μL of 0.2 mol/L glycine/NaOH buffer at pH 10.7 containing 0.1% Triton X100 to measure fluorescence intensity, and corrected for substrate blank. We used a stock solution of 250 μmol/L 4-methylumbelliferone in 20 mmol/L sodium-phosphate buffer at pH 7.0 to calibrate the measurement of liberated 4MU. Each assay was performed in duplicate. The enzyme activity was expressed as nanomoles 4MU released per hour per milligram cellular protein (nmol/h/mg protein).

AαGlu activity in fibroblasts was also measured with glycogen as substrate followed by an enzymatic determination of liberated glucose. Briefly, 12 μL of the cell homogenates was added to 48 μL of the substrate solution containing 62.5 mg/mL glycogen in 0.1 mol/L citrate/0.2 mol/L sodium-phosphate buffer at pH 4.0 with 3.75 μmol/L Acarbose (3.0 μmol/L in the final concentration) and incubated for 1 h at 37 °C in a 1.5 mL reaction tube. The reaction was terminated by heating at 95 °C for 5 min. Then the reaction tube was immediately cooled on ice and centrifuged at 10,000 *g* for 3 min. An aliquot of the supernatant was subjected to quantitative analysis for liberated glucose with two reagents on COBAS MIRA automatic analyzer. Reagent 1 consisted of 0.15 mmol/L of 10-N-methylcarbamoyl-3,7-bis(dimethylamino)phenothiazine, 0.38 kU/L mutarotase (from porcine kidney, Wako, Osaka, Japan), 0.77 mmol/L triethylenetetraminehexaacetic acid, 0.2% Triton X100 and 0.23 mol/L Tris/0.36 mol/L sodium-phosphate buffer at pH 7.0. Reagent 2 consisted of 86 kU/L of glucose oxidase (from *Aspergilles niger*, Sigma-Aldrich), 3.8 kU/L peroxidase (from horseradish, TOYOBO, Tokyo, Japan), 70 μmol/L potassium ferrocyanide and 0.23 mol/L Tris/0.36 mol/L sodium-phosphate buffer at pH 7.0. The analytical conditions on the COBAS MIRA automatic analyzer were as follows: sampling volume, 15 μL (washing distilled water volume, 35 μL); the reagent 1 volume, 130 μL; the reagent 2 volume, 70 μL; wavelength, 660 nm; temperature, 37 °C; and calculation mode, endpoint assay with a reagent blank. Timing (25 s per one cycle) for sample and reagent additions was: sample and the reagent 1, cycle 1; the reagent 2, cycle 5. Timing for readings was: first, cycle 4; and last, cycle 30. The reaction time after the addition of the reagent 2 was 10 min 50 s. The enzyme activity was expressed as nanomoles glucose released per hour per milligram cellular protein (nmol/h/mg protein). To avoid erroneous results attributable to turbidity of the glycogen solution and the free cellular glucose, we also performed the assay without adding the cell homogenate (glycogen blank) and without adding the glycogen (sample blank). Each assay was performed in duplicate. The activity was calculated after correcting for glycogen and sample blanks. As a calibrator for the measurement of glucose, we used 400 μmol/L glucose dissolved in distilled water.

3. Results and discussion

The high frequency of c.[1726G>A;2065G>A] homozygotes (3.3–3.9%; AA homozygotes) with a very low AαGlu activity (AαGlu pseudodeficiency) critically complicates newborn screening for Pompe disease in Asian populations [6,24,30,31]. Complete separation between affected infants with hardly any residual AαGlu activity and AA homozygotes demands a very sensitive assay. We have followed up on the observation that elimination of hemoglobin by TCA precipitation greatly improves the measurement of the AαGlu activity with 4MU-αGlc substrate [28]. To this end we have compared two different methods to precipitate hemoglobin from the reaction mixture. Fig. 1 shows the results obtained with either TCA or barium hydroxide/zinc sulfate precipitation. While hemoglobin greatly decreases the fluorescence intensity in a dose-dependent manner, both precipitation methods significantly restore the loss of fluorescence intensity. However, we noticed an important difference between the two methods: over the whole range of hemoglobin concentrations the actual fluorescence intensities obtained with the TCA method were 28–34% lower than that with Ba/Zn method. This counter effect of TCA on the free 4MU fluorescence intensity proved highly dependent on the precise analytical conditions (*e.g.*, wavelength and band-pass) and type of the spectrofluorometer (data not shown). Based on these results, we chose the hemoglobin precipitation method with barium hydroxide/zinc sulfate to measure the AαGlu activity in DBSs from 18 Pompe patients, 70 AA homozygotes, 70 c.[1726G; 2065G]/c.[1726A; 2065A] heterozygotes (GG/AA heterozygotes) and 112 c.[1726G; 2065G] homozygotes (GG homozygotes). Comparison of Figs. 2A and B shows that the separation between the patient group and the control groups is greatly improved by application of the Ba/Zn method. The overlap between 11 of the 70 AA homozygotes (15.7%) and the patient group (Fig. 2A + inset) in our previously used procedure was virtually resolved using the Ba/Zn method (Fig. 2B + inset). These data suggest that newborn screening in Asian populations can be improved by applying Ba/Zn precipitation of hemoglobin.

With regard to the definitive diagnosis, it is true that the activities of some AA homozygotes remained very close to the patient range despite the Ba/Zn method. Hence, if large numbers of newborns are subjected to the screening program, some AA homozygotes will still be scored false positive. Fig. 3 illustrates that measuring the activity of AαGlu in cultured fibroblasts using 4MU-αGlc and glycogen as substrates can make the final diagnosis. Others have used a lymphocyte assay for this purpose [31]. Examining 11 Pompe patients and 57 unaffected individuals with three different genotypes including 8 AA homozygotes, 18 GG/AA heterozygotes and 31 GG homozygotes, we found that the three subgroups of unaffected individuals were completely separated from the patient group in both assay methods using either 4MU-αGlc or glycogen as substrate (Fig. 3).

Any attempt to compare the results of existing methods for measuring the activity of AαGlu in DBS using 4MU-αGlc as substrate is complicated by the fact that each laboratory uses its preferred DBS extraction methodology, substrate concentration, incubation time, measuring procedure and activity units. However, *a priori* it is evident

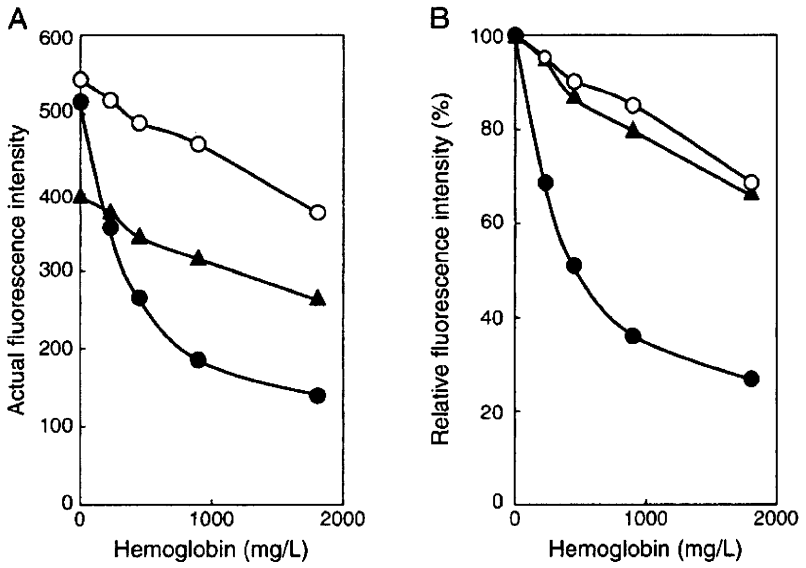


Fig. 1. Effect of hemoglobin elimination on the fluorescence intensity of 4MU. 4MU solution in the presence of different concentrations of hemoglobin (0–1800 mg/L in final reaction mixture) was subjected to two different treatments for hemoglobin elimination, and the fluorescence intensity was assayed as described in the subjects, materials and methods. The symbols represent as follows; open circles, treatment with barium hydroxide and zinc sulfate; closed triangles, treatment with TCA; closed circles, without treatment. The detected fluorescence intensity was expressed as actual reading value (A) and % of those without hemoglobin (B).

that the highest 4MU fluorescence intensities will be measured and the best separation between affected and unaffected individuals will be obtained using the most concentrated DBS samples with nevertheless the lowest hemoglobin concentration. A substrate concentration of at least 2 times the Km of AαGlu for 4MU-αGlc (>2 mmol/L) will further optimize the separation between affected and unaffected individuals. Our new method approaches these ideal conditions since the DBS is not extracted in water like in other procedures and the AαGlu extract is not diluted prior to the incubation with substrate. Instead, the DBS as a whole is immediately immersed in the substrate solution containing a final 4MU-αGlc concentration of 2 mmol/L. All other published procedures use lower substrate concentrations ranging from 0.7 to 1.47 mmol/L. The Ba/Zn method eliminates the negative effect of the relatively high hemoglobin concentration in the DBS extract obtained with our procedure. In other procedures the negative effect of hemoglobin is partially eliminated by the water extraction and sample dilution, but that action leads to reduction of the final output signal in terms of fluorescence units and negatively affects the separation between the activity ranges of affected and unaffected newborns.

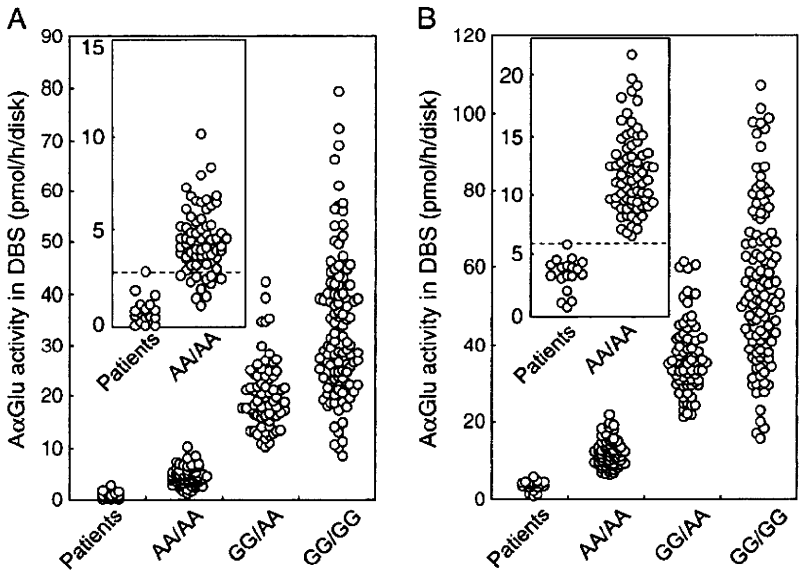


Fig. 2. AαGlu activity in DBSs with the previous and Ba/Zn methods. The AαGlu activity was measured with the previous (A) and Ba/Zn (B) methods with DBSs from 18 Japanese Pompe patients and 252 Japanese healthy newborns [112 GG homozygotes (GG/GG), 70 GG/AA heterozygotes (GG/AA), 70 AA homozygotes (AA/AA)]. The enzyme activities (mean ± SD, pmol/h/disk) with the previous method were 33.3 ± 13.7 for the GG/GG, 20.1 ± 6.7 for the GG/AA, 4.5 ± 1.7 for the AA/AA and 0.77 ± 0.75 for the patients, and the range of the activities were 8.5–79.3 for the GG/GG, 10.2–42.3 for the GG/AA, 1.2–10.2 for the AA/AA and 0–2.8 for the patients. The enzyme activities with the Ba/Zn method were 55.1 ± 20.3 for the GG/GG, 36.6 ± 9.8 for the GG/AA, 11.8 ± 3.4 for the AA/AA and 3.4 ± 1.3 for the patients, and the range of the activities were 15.4–106.9 for the GG/GG, 20.9–61.1 for the GG/AA, 6.4–21.4 for the AA/AA and 0.9–5.9 for the patients. The measurement was performed as described in the subjects, materials and methods, and the data were expressed as an average of duplicate determinations. The inset shows the enlarged distribution of the activities for the patients and AA homozygotes.

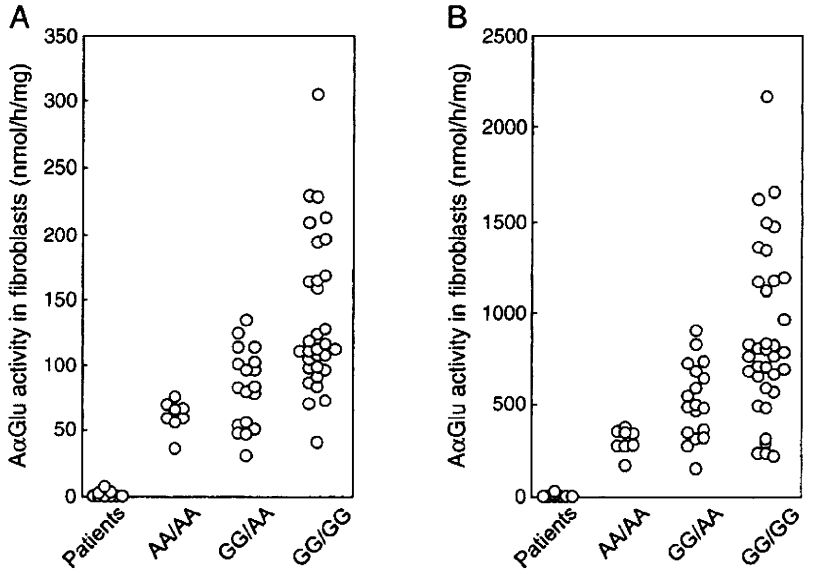


Fig. 3. AαGlu activity in fibroblasts with 4MU-αGlc and glycogen as substrate. The AαGlu activity was measured with 4MU-αGlc (A) and glycogen (B) as substrate in fibroblasts from 11 Japanese Pompe patients and 57 Japanese unaffected individuals (31 GG/GG, 18 GG/AA, 8 AA/AA). The enzyme activities (mean ± SD, nmol/h/mg protein) with 4MU-αGlc were 136 ± 58 for the GG/GG, 82 ± 30 for the GG/AA, 60 ± 12 for the AA/AA and 1.2 ± 2.0 for the patients, and the range of the activities were 40–304 for the GG/GG, 30–134 for the GG/AA, 35–75 for the AA/AA and 0.1–6.6 for the patients. The enzyme activities with glycogen were 954 ± 419 for the GG/GG, 515 ± 207 for the GG/AA, 294 ± 65 for the AA/AA and 3.5 ± 8.4 for the patients, and the range of the activities were 282–2163 for the GG/GG, 146–900 for the GG/AA, 164–364 for the AA/AA and 0–26 for the patients. The measurement was performed as described in the subjects, materials and methods, and the data were expressed as an average of duplicate determinations.

4. Conclusion

We have demonstrated that the elimination of hemoglobin with barium hydroxide/zinc sulfate greatly improves the enzymatic diagnosis of Pompe disease in DBSs. This new method provides the solution for the critical issue of newborn screening for Pompe disease in Asian populations due to high incidence of AA homozygotes with a very low AαGlu activity.

Acknowledgments

We wish to thank Shingo Kumamoto, Mitsuyasu Ikeda and Yasushi Ueyanagi of Kumamoto University for their excellent technical supports, and Akira Ohtake and Hiriko Harashima of Saitama Medical University for supplying the fibroblast samples. This work was supported by grants from The Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research C, T.O.), and is connected to the Dutch TI Pharma initiative to develop a project on Sustainable Orphan Drug Development through Registries and Monitoring (T6-208).

References

[1] N.A. Chamoles, G. Niizawa, M. Blanco, D. Gaggioli, C. Casentini, Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper, Clin. Chim. Acta 347 (2004) 97–102.
[2] M. Spada, S. Pagliardini, M. Yasuda, T. Tukel, G. Thiagarajan, H. Sakuraba, A. Ponzone, R.J. Desnick, High incidence of later-onset fabry disease revealed by newborn screening, Am. J. Hum. Genet. 79 (2006) 31–40.
[3] H. Zhang, H. Kallwass, S.P. Young, C. Carr, J. Dai, P.S. Kishnani, D.S. Millington, J. Keutzer, Y.T. Chen, D. Bali, Comparison of maltose and acarbose as inhibitors of maltase-glucoamylase activity in assaying acid α-glucosidase activity in dried blood spots for the diagnosis of infantile Pompe disease, Genet. Med. 8 (2006) 302–306.
[4] Y.H. Chien, S.C. Chiang, X.K. Zhang, J. Keutzer, N.C. Lee, A.C. Huang, C.A. Chen, M.H. Wu, P.H. Huang, F.J. Tsai, Y.T. Chen, W.L. Hwu, Early detection of Pompe disease by newborn screening is feasible: results from the Taiwan screening program, Pediatrics 122 (2008) e39–e45.
[5] A. Dajnoki, A. Muhl, G. Fekete, J. Keutzer, J. Orsini, V. Dejesus, X.K. Zhang, O.A. Bodamer, Newborn screening for Pompe disease by measuring acid α-glucosidase activity using tandem mass spectrometry, Clin. Chem. 54 (2008) 1624–1629.
[6] S. Kumamoto, T. Katafuchi, K. Nakamura, F. Endo, E. Oda, T. Okuyama, M.A. Kroos, A.J.J. Reuser, T. Okumiya, High frequency of acid α-glucosidase pseudodeficiency complicates newborn screening for glycogen storage disease type II in the Japanese population, Mol. Genet. Metab. 97 (2009) 190–195.
[7] K. Umapathysivam, A.M. Whittle, E. Ranieri, C. Bindloss, E.M. Ravenscroft, O.P. van Diggelen, J.J. Hopwood, P.J. Meikle, Determination of acid α-glucosidase protein: evaluation as a screening marker for Pompe disease and other lysosomal storage disorders, Clin. Chem. 46 (2000) 1318–1325.
[8] K. Umapathysivam, J.J. Hopwood, P.J. Meikle, Determination of acid α-glucosidase activity in blood spots as a diagnostic test for Pompe disease, Clin. Chem. 47 (2001) 1378–1383.
[9] P.J. Meikle, E. Ranieri, H. Simonsen, T. Rozaklis, S.L. Ramsay, P.D. Whitfield, M. Fuller, E. Christensen, F. Skovby, J.J. Hopwood, Newborn screening for lysosomal storage disorders: clinical evaluation of a two-tier strategy, Pediatrics 114 (2004) 909–916.
[10] P.J. Meikle, D.J. Grasby, C.J. Dean, D.L. Lang, M. Bockmann, A.M. Whittle, M.J. Fietz, H. Simonsen, M. Fuller, D.A. Brooks, J.J. Hopwood, Newborn screening for lysosomal storage disorders, Mol. Genet. Metab. 88 (2006) 307–314.
[11] Y. Li, C.R. Scott, N.A. Chamoles, A. Ghavami, B.M. Pinto, F. Turecek, M.H. Gelb, Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening, Clin. Chem. 50 (2004) 1785–1795.
[12] M.H. Gelb, F. Turecek, C.R. Scott, N.A. Chamoles, Direct multiplex assay of enzymes in dried blood spots by tandem mass spectrometry for the newborn screening of lysosomal storage disorders, J. Inherit. Metab. Dis. 29 (2006) 397–404.
[13] X.K. Zhang, C.S. Elbin, W.L. Chuang, S.K. Cooper, C.A. Marashio, C. Beauregard, J.M. Keutzer, Multiplex enzyme assay screening of dried blood spots for lysosomal storage disorders by using tandem mass spectrometry, Clin. Chem. 54 (2008) 1725–1728.
[14] V.R. De Jesus, X.K. Zhang, J. Keutzer, O.A. Bodamer, A. Muhl, J.J. Orsini, M. Caggana, R.F. Vogt, W.H. Hannon, Development and evaluation of quality control dried blood spot materials in newborn screening for lysosomal storage disorders, Clin. Chem. 55 (2009) 158–164.
[15] H. Van den Hout, A.J. Reuser, A.G. Vulto, M.C. Loonen, A. Cromme-Dijkhuis, A.T. Van der Ploeg, Recombinant human α-glucosidase from rabbit milk in Pompe patients, Lancet 356 (2000) 397–398.
[16] L. Klinge, V. Straub, U. Neudorf, T. Voit, Enzyme replacement therapy in classical infantile Pompe disease: results of a ten-month follow-up study, Neuropediatrics 36 (2005) 6–11.
[17] P.S. Kishnani, D. Corzo, M. Nicolino, B. Byrne, H. Mandel, W.L. Hwu, N. Leslie, J. Levine, C. Spencer, M. McDonald, J. Li, J. Dumontier, M. Halberthal, Y.H. Chien, R. Hopkin, S. Vijayaraghavan, D. Gruskin, D. Bartholomew, A. van der Ploeg, J.P. Clancy, R. Parini, G. Morin, M. Beck, G.S. De la Gastine, M. Jokic, B. Thurberg, S. Richards, D. Bali, M. Davison, M.A. Worden, Y.T. Chen, J.E. Wraith, Recombinant human acid α-glucosidase: major clinical benefits in infantile-onset Pompe disease, Neurology 68 (2007) 99–109.
[18] M. Rossi, G. Parenti, R. Della Casa, A. Romano, G. Mansi, T. Agovino, F. Rosapepe, C. Vosa, E. Del Giudice, G. Andria, Long-term enzyme replacement therapy for Pompe disease with recombinant human α-glucosidase derived from Chinese hamster ovary cells, J. Child Neurol. 22 (2007) 565–573.

[19] C.I. van Capelle, L.P. Winkel, M.L. Hagemans, S.K. Shapira, W.F. Arts, P.A. van Doorn, W.C. Hop, A.J. Reuser, A.T. van der Ploeg, Eight years experience with enzyme replacement therapy in two children and one adult with Pompe disease, Neuromuscul. Disord. 18 (2008) 447–452.
[20] A.T. van der Ploeg, P.R. Clemens, D. Corzo, D.M. Escolar, J. Florence, G.J. Groeneveld, S. Herson, P.S. Kishnani, P. Laforet, S.L. Lake, D.J. Lange, R.T. Leshner, J.E. Mayhew, C. Morgan, K. Nozaki, D.J. Park, A. Pestronk, B. Rosenbloom, A. Skrinar, C.I. van Capelle, N.A. van der Beek, M. Wasserstein, S.A. Zivkovic, A randomized study of alglucosidase alfa in late-onset Pompe's disease, N. Engl. J. Med. 362 (2010) 1396–1406.
[21] L.P. Winkel, J.M. Van den Hout, J.H. Kamphoven, J.A. Disseldorp, M. Remmerswaal, W.F. Arts, M.C. Loonen, A.G. Vulto, P.A. Van Doorn, G. De Jong, W. Hop, G.P. Smit, S.K. Shapira, M.A. Boer, O.P. van Diggelen, A.J. Reuser, A.T. Van der Ploeg, Enzyme replacement therapy in late-onset Pompe's disease: a three-year follow-up, Ann. Neurol. 55 (2004) 495–502.
[22] T. Merk, T. Wibmer, C. Schumann, S. Kruger, Glycogen storage disease type II (Pompe disease) – influence of enzyme replacement therapy in adults, Eur. J. Neurol. 16 (2009) 274–277.
[23] A.T. van der Ploeg, A.J. Reuser, Pompe's disease, Lancet 372 (2008) 1342–1353.
[24] M.A. Kroos, R.A. Mullaart, L. Van Vliet, R.J. Pomponio, H. Amartino, E.H. Kolodny, G.M. Pastores, R.A. Wevers, A.T. Van der Ploeg, D.J. Halley, A.J. Reuser, p.[G576S; E689K]: pathogenic combination or polymorphism in Pompe disease? Eur. J. Hum. Genet. 16 (2008) 875–879.
[25] B.E. Nickel, P.J. McAlpine, Extension of human acid α-glucosidase polymorphism by isoelectric focusing in polyacrylamide gel, Ann. Hum. Genet. 46 (1982) 97–103.
[26] M.L. Huie, M. Menaker, P.J. McAlpine, R. Hirschhorn, Identification of an E689K substitution as the molecular basis of the human acid α-glucosidase type 4 allozyme (GAA*4), Ann. Hum. Genet. 60 (1996) 365–368.
[27] J.J. Shieh, C.Y. Lin, Frequent mutation in Chinese patients with infantile type of GSD II in Taiwan: evidence for a founder effect, Hum. Mutat. 11 (1998) 306–312.
[28] LF Oemardien, AM Boer, GJ Ruijter, AT van der Ploeg, JB de Klerk, AJ Reuser, FW Verheijen, Hemoglobin precipitation greatly improves 4-methylumbelliferone-based diagnostic assays for lysosomal storage diseases in dried blood spots. Mol. Genet. Metab. 102 (2011) 44–48.
[29] T. Okumiya, J.L. Keulemans, M.A. Kroos, N.M. Van der Beek, M.A. Boer, H. Takeuchi, O.P. Van Diggelen, A.J. Reuser, A new diagnostic assay for glycogen storage disease type II in mixed leukocytes, Mol. Genet. Metab. 88 (2006) 22–28.
[30] Y. Tajima, F. Matsuzawa, S. Aikawa, T. Okumiya, M. Yoshimizu, T. Tsukimura, M. Ikekita, S. Tsujino, A. Tsuji, T. Edmunds, H. Sakuraba, Structural and biochemical studies on Pompe disease and a "pseudodeficiency of acid α-glucosidase", J. Hum. Genet. 52 (2007) 898–906.
[31] P. Labrousse, Y.H. Chien, R.J. Pomponio, J. Keutzer, N.C. Lee, V.R. Akmaev, T. Scholl, W.L. Hwu, Genetic heterozygosity and pseudodeficiency in the Pompe disease newborn screening pilot program, Mol. Genet. Metab. 99 (2010) 379–383.

神経内科，73(2)：168-172，2010

特集 Fabry病—update—

Fabry病の疫学と診断*

中村公俊**

Key Words : Fabry disease, α-galactosidase, enzyme replacement therapy, epidemiology, heterozygous

はじめに

Fabry病はリソゾーム内の加水分解酵素であるα-ガラクトシダーゼ(α-galactosidase, EC 3.2.1.22)の活性の低下によって糖脂質が組織や体液中に蓄積し，心不全，腎不全，脳血管障害など多彩な臨床症状を呈する遺伝性疾患である[1)2)]．イギリスのAndersonとドイツのFabryが，1898年にそれぞれ別個に初めて報告している[3)4)]．α-ガラクトシダーゼ遺伝子はXq22に存在し，全長約12kb，7個のエクソンからなり，その遺伝形式はX染色体連鎖性である．Fabry病の男性はヘミ接合体，Fabry病女性はヘテロ接合体の患者となる．ヘテロ接合体女性も発症する例が少なくないことは，一般のX連鎖性遺伝性疾患と異なり注意が必要である[5)]．Fabry病の男性には，古典型と亜型(遅発型)の二つの病型が存在する[6)7)]．どちらもα-ガラクトシダーゼの酵素活性の低下によって発症し，酵素活性の低下の程度によって無症状から亜型，古典型までさまざまな臨床症状を示す．古典型Fabry病では小児期から四肢末端痛，発汗低下，被角血管腫，角膜混濁などの症状がみられ，思春期以降に循環器症状，腎障害，脳血管障害などの主要臓器の重篤な障害が明らかとなる．また，遅発型の経過をとる亜型では，小児期の症状はないか，あってもごく軽微であり，成人期以降に心不全または腎不全などの臓器障害が現れ重篤な障害へと進行する．ヘテロ接合体の女性ではX染色体不活化の程度の違いによって，無症状から心臓，腎臓などの単一臓器の障害，古典型と同様の複数の臓器にわたる障害までさまざまな病型を示す．近年，Fabry病に対して酵素補充療法が行われるようになり，患者のQOLの改善が可能となったため早期診断が重要である[8)9)]．しかし，Fabry病の症状は多様であり，発症から診断まで長期にわたる症例も認められる．Fabry病が念頭にない場合には診断は困難であることが多い．診断には白血球や血液中のα-ガラクトシダーゼ酵素活性の測定や遺伝子解析が用いられており，確定診断に有効である[10)]

Fabry病の疫学

Fabry病の頻度は人口約3～4万人に1人とされてきた[1)2)]．これは古典型のFabry病の頻度であると考えられる．このほかに亜型である心型Fabry病や腎型Fabry病の存在が明らかとなってきた．成人発症の特発性肥大型心筋症の約3～6％，腎不全の透析患者の約0.2～1.2％，潜在性脳卒中患者の約1.2％がFabry病であるとされる[6)7)11)](表1)．また，ヘテロ女性ではこれまで考えられていたよりも高い頻度で発症すると考えられている．新生児のスクリーニングによると，Fabry病の頻

* Epidemiology and diagnosis of Fabry disease.

** Kimitoshi NAKAMURA, M.D.: 熊本大学医学部附属病院小児科〔〒860-8556 熊本県熊本市本荘1-1-1〕; Department of Pediatrics, School of Medicine, Kumamoto University Hospital, Kumamoto 860-8556, Japan.

表1　Fabry病スクリーニングにおける頻度

地域	対象者	頻度	報告者
イタリア	男性透析患者	0.25%	Spada M et al. (J Inhr Metab Dis 2003)
オランダ	男性透析患者	0.22%	Linrhoear GE et al. (Nephrol Dial Transplant 2003)
日本	男性透析患者	0.1%	Tanaka M et al. (Clin Nephrol 2005)
	女性透析患者	0.3%	
チェコ	男性透析患者	0.26%	Merta M et al. (NephrolDial Transplant 2007)
	女性透析患者	0.16%	
日本	男性左室肥大患者	3.0%	Nakao S et al. (N Eng J Med 1995)[6]
イギリス	男性心肥大患者	3.9%	Sachdev B et al. (Circulation 2002)
ドイツ	男性脳梗塞患者	4.9%	Rolfs A et al. (Lancet 2005)
	女性脳梗塞患者	2.4%	
イタリア	新生児男児	0.03%	Spada M et al. (Am J Hum Genet 2006)[12]
台湾	新生児男児	0.06%	Hwu WL et al. (Hum Mutat 2009)[13]
台湾	新生児男児	0.09%	Lin HY et al. (Circ Cardiovasc Genet 2009)[14]

度は男児約3,000名に1人[12]，また，台湾では男児1,100～1,600名に1人[13][14]という高い頻度が報告されている．Fabry病は決して稀な疾患ではなく，これまで考えられていたより頻度の高い疾患である．

1．古典型Fabry病

小児期に四肢末端痛，発汗の低下，被角血管腫，角膜混濁などの症状がみられる[1][2]．とくに疼痛発作は古典型患者の約80%以上にみられる特徴的な症状である．Burning painと呼ばれる手掌や足底の焼けつくような痛みが数分から数日続く．運動や入浴によって誘発されることがあり，幼児期においても運動を嫌がる，入浴させると機嫌が悪くなる，などを契機に気づかれることがある．関節痛や四肢の広範な痛みを訴え，関節炎や成長痛と診断されている例もある．X染色体連鎖性遺伝であるため母親にも同様の四肢痛を認めることがあり，児の痛みに早期に気づくことがある．また，発汗障害があるため体温が上昇しやすく，うつ熱となり運動を嫌がる，真夏でも汗をかかないなどの症状がみられる．皮膚エクリン汗腺への糖脂質の蓄積による障害が原因とされる．被角血管腫はFabry病の最初の報告にも記載されている特徴的な症状である．真皮の毛細血管拡張が表皮層に突出したものである．外陰部，下腹部から腰，大腿などに粟粒大の丘疹を多数認める．加齢によっても出現するため，若年で認める場合に診断的価値がある．眼症状としては，細隙灯顕微鏡検査で80～90%の患者に渦巻き状角膜混濁を認める．50%以上の患者に白内障や毛細血管の異常拡張や蛇行を認める．

古典型Fabry病の男性では思春期以降に腎障害，循環器症状，消化器症状，脳血管障害などを認める．腎障害は蛋白尿によって気づかれることが多く，約10年の経過で透析導入が必要となる．腎生検組織の電子顕微鏡検査において糸球体上皮や血管内皮細胞などに層状蓄積物を認める．また，循環器症状は小児期には稀で，一般に30歳以降に出現する．左室肥大，不整脈，狭心症や心筋梗塞などを発症する．洞機能不全や房室ブロックのためペースメーカーが必要となることもある．心筋生検を行うことによって組織への糖脂質の蓄積が証明される．消化器症状としては下痢，嘔吐，腹痛，イレウスなどがあげられ，10歳代からこれらの症状を繰り返していることがある[15]．脳血管障害では椎骨脳底動脈系の梗塞が起こりやすい[16]．多発性微小梗塞を認め，脳血管造影では脳底動脈の拡張や蛇行を認める．脳出血よりも脳梗塞の頻度が高い．また，MRI検査で脳梗塞を認めた症例の約3分の1は無症状である．稀に10歳代で脳梗塞を発症することがあり，早期からMRIによる微小梗塞の検索が重要と考えられる．このほかの中枢神経症状として，めまいや感音性難聴が起こることが知られている．無治療の古典型Fabry病の平均寿命は40～50歳程度とされている．

2．亜型Fabry病

病変がほぼ特定の臓器のみに限って発症する遅発型のFabry病が存在し，亜型Fabry病と呼ば