所要時間

リンパ球を分離してペレットを作成するのに約2時間を要している。そのまま酵素反応を進める場合、リンパ球の超音波破砕と反応溶液の調製に所要30分程度として、酵素によって10-60分反応させ、停止後に遠心10分間で除タンパクするので、測定サンプルができ上がるまでに1-2時間かかる。1サンプル当たりのHPLC分析時間は酵素により10-40分で、被験者と対照で計6サンプルを測定するには、1時間強で完了するもの（MSUD, MCADD）から4-5時間を要するもの（MMA）まで幅広くなっている。

以上を合計すると、被験児1名を酵素診断する所要時間は5-10時間程度となる。まとまった時間の確保が難しい場合は、リンパ球ペレットを凍結保存して酵素反応を後日に行っている。さらに測定サンプルも凍結保存ができるので、一部のサンプルのHPLC分析を翌日以降に分割するなどして、短い空き時間を有効利用するようにしている。

必要経費

酵素によって様々なビタミン類が必要になるが、金額的には基質となるアシルCoAがほとんど

表2　各種の補酵素A誘導体の試薬価格と1症例の酵素診断に要する経費

疾患	試薬	価格(円)（※Sigma社製品）	（内容量）	1回調製量（100μl）	酵素診断に要する経費(円/1症例)
MSUD	Coenzyme A Li3 salt	67,100	(1g)	75mM	395
PA	Propionyl-CoA	67,800	(25mg)	5mM	1,117
IVA	Isovaleryl-CoA	29,400	(10mg)	10mM	2,503
MCADD	n-Octanoyl-CoA	60,100	(25mg)	20mM	4,297
HMGLD	HMG-CoA	73,700	(25mg)	20mM	5,375
VLCADD	Palmitoyl-CoA	74,100	(25mg)	20mM	5,963
GA1	Glutaryl-CoA	27,800	(10mg)	30mM	7,352
MMA	Methylmalonyl-CoA	137,400	(25mg)	30mM	14,296

MSUD；メープルシロップ尿症，PA；プロピオン酸血症，IVA；イソ吉草酸血症，MCADD；中鎖アシルCoA脱水素酵素欠損症，HMGLD；HMG-CoAリアーゼ欠損症，VLCADD；極長鎖アシルCoA脱水素酵素欠損症，GA1；グルタル酸尿症I型，MMA；メチルマロン酸血症

(1) 分析時間が最も長いメチルマロン酸血症の場合の移動相（Nacarai製）

NaH_2PO_4	¥1,700／500g	0.1M NaH_2PO_4	880mL
蒸留水	¥2,350／3L	＋メタノール	120mL
メタノール	¥3,400／3L	＝¥861／L	

流速 1.5mL/分×40分/サンプル×6サンプル＝360mL → 1例診断当たり¥310

(2) 有機溶媒使用量が最も多いVLCAD欠損症の場合の移動相（Nacarai製）

NaH_2PO_4	¥1,700／500g	0.1M NaH_2PO_4	510mL
蒸留水	¥2,350／3L	＋アセトニトリル	490mL
メタノール	¥6,400／3L	＝¥1,465／L	

流速 1.5mL/分×25分/サンプル×6サンプル＝150mL → 1例診断当たり¥329

図2　酵素反応のHPLC分析で消費する移動相溶液の経費

Nacarai社製品のカタログ価格に基づいた概算例を示す。メチルマロン酸血症とVLCAD欠損症以外の対象疾患の場合は、分析時間・有機溶媒使用量とも、両疾患の場合の中間となるため、必要経費はより低額となる。実際の移動相使用量は、サンプル分析前後の送液分や再測定の必要などから、計算例の2-3倍程度となることが多い。

を占める。Sigma社2009年版カタログ上の価格と、被験児1名の診断のために調製する使用量から求めた必要額を表2に示す。また、HPLC分析用の移動相については、分析時間が最も長いMMAと、有機溶媒濃度が最も高いVLCADDの場合での必要額を図2に示す。以上より、最も高額となるMMAの場合は、分析前後の移動相消費も見込むと以下のようになる。

Methylmalonyl-CoA（Sigma; ¥137,400/25mg）2.6mg　＝約14,000円

Coenzyme B12（Sigma; ¥10,200/100mg）0.5mg　＝約50円

移動相（蒸留水880mL＋メタノール120mL＋NaH_2PO_4 10g）500mL　＝約450円

結局、被験児1名の酵素診断に必要な消耗品の経費のほとんどは、基質となるアシルCoAが占めており、従って概算額はほぼ表2の通りとなる。

他のランニングコストとしては、HPLCカラムの費用がかかる。各酵素に共通して使用する逆相カラムは、移動相の有機溶媒（メタノールまたはアセトニトリル）に応じて2本を交換して使用しており、測定頻度にもよるが、年1回程度更新すると2本で約10万円が必要になる。

有用性と課題

1．除外診断における有用性

スクリーニングで陽性となった新生児の両親・家族には、罹患・非罹患を問わず、できるだけ早期に診断を明確にすることが求められる。その場合、真の罹患児については、異常代謝産物の分析や遺伝子解析などの方法で、陽性所見に基づいて診断することができ、それは必ずしも難しいことではない。一方、異常代謝産物の蓄積が軽度であったり安定状態では消失するような場合や、遺伝子変異が同定困難あるいは新規の1塩基置換である場合などは、これらの方法では確定診断も除外診断も不明確になる。酵素活性測定は、被験児の状態や変異の種類に関係なく罹患・非罹患を区別することが可能で、特に酵素反応産物が十分に生成するという「陽性所見」で偽陽性例を積極的に除外診断できる点が特に優れている。

実際の応用結果として、広島県のタンデムマス新生児スクリーニング陽性例の診断結果を表3に示す。陽性32例中18例を罹患者と診断し、そのうち11例で酵素活性低下を確認した（※SCADDの2例は変異酵素発現系での活性測定結果であり[8]、CPT1D例の酵素活性測定は千葉県こども病院・高柳正樹先生による）。一方、除外診断された14例では6例が正常レベルの酵素活性値に依拠していた。また、グルタル酸尿症Ⅱ型（GA2）疑いで非罹患とした2例中1例と、シトルリン低値でオルニチントランスカルバミラーゼ欠損症（OTCD）罹患者とは判断されなかった1例は、異常代謝産物の陰性所見だけが根拠となっており、完全に除外診断できたとは言い難い。GA2疑いで非罹患とした別の1例は、リンパ球β酸化能測定が実用化されていたため[9]、その正常所見によって除外診断することが可能であった。

広島県および国内各地のタンデムマス新生児スクリーニング陽性例（※およびMSUD疑い例）の酵素診断結果をまとめたものが表4である。52例中35例を酵素欠損症罹患者と判定したが、裏返せば除外診断を要するケースが約1/3もあったことになる。このように、タンデムマス新生児スクリーニングでは非罹患者の除外診断も大きな課題であり、その解決には酵素診断が最適と言える。

2．残存活性の定量的評価

酵素診断では、残存活性の高さによって重症度を推定できることが期待されるところである。当科の酵素診断法のうち、この面で最も有用性が高いと考えられるのはMCADDについてのものである。図3に示すように、急性発症後に診断された罹患児群の残存活性は、1例を除いて正常対照平均値の5%未満に分布しているのに対し、発症前に罹患者と診断された群の残存活性はより高い値まで広がりを示しており、新生児スクリーニングでは多様な重症度の症例が発見されうることが示唆された。

一方、有症状例の診断依頼数が最も多いMMAの場合は、遅発性で最重症型ではないと考えられる症例でも、我々の方法では酵素活性値が測定感

表3　広島県のタンデムマス新生児スクリーニング陽性例の診断結果

	疾患	陽性	罹患	確定診断所見		除外診断所見		除外例に関する備考
有機酸代謝異常症	PA	7	4	尿中有機酸異常 酵素活性低下 遺伝子変異同定	4 4 3	酵素活性正常	3	
	MMA	2	1	尿中有機酸異常 酵素活性低下	1 1	酵素活性正常	1	
	HCSD	1	1	尿中有機酸異常 遺伝子変異同定	1 1			
	MCG	2	0			尿中有機酸正常	2	母体酵素欠損に起因する一過性の異常と判定。
	小計	12	6					
脂肪酸代謝異常症	MCADD	5	3	尿中有機酸異常 酵素活性低下 遺伝子変異同定	2 3 2	酵素活性正常	2	2例中1例は低出生体重児でMCTオイルを投与されていた。
	GA2	4	2	尿中有機酸異常	2	尿中有機酸正常 β酸化能正常	2 1	1例は完全な除外診断には至っていない。
	CTD	3	0			カルニチン補充 →中止後再低下なし	3	2例はNICU管理中の栄養不良による低カルニチン血症と判断。1例はリスクなし。
	SCADD	2	2	尿中有機酸異常 酵素活性低下 遺伝子変異同定	2 2 2			
	CPT1D	1	1	酵素活性低下 遺伝子変異同定	1 1			
	小計	15	8					
尿素回路異常症	OTCD	3	2	血中アミノ酸異常 尿中有機酸異常 遺伝子変異同定	2 2 1	血中アミノ酸正常 尿中有機酸正常	1 1	完全な除外診断には至っていない。
	ASA	1	1	血中アミノ酸異常 尿中有機酸異常	1 1			
	CD	1	1	遺伝子変異同定	1			
	小計	5	4					
合計		32	18	1999年4月—2010年1月 受検総数221,506人(受検率 75.4%)				患者発見率　1/12,305人

PA；プロピオン酸血症, MMA；メチルマロン酸血症, HCSD；ホロカルボキシラーゼ合成酵素欠損症, MCG；3-メチルクロトニルグリシン尿症, MCADD；中鎖アシルCoA脱水素酵素欠損症, GA2；グルタル酸尿症Ⅱ型, CTD；カルニチントランスポーター異常症, SCADD；短鎖アシルCoA脱水素酵素欠損症, CPT1D；カルニチンパルミトイルトランスフェラーゼ-Ⅰ欠損症, OTCD；オルニチントランスカルバミラーゼ欠損症, ASA；アルギニノコハク酸尿症, CD；シトリン欠損症

度以下となるケースが多く、重症度の推定は困難な結果となっている。MMAの場合、放射性同位体標識基質を使用する従来法に比べて、HPLC法では感度が低いものと思われ、基質や酵素量（細胞数）を増やすことで改善できるかも知れないが、費用や採血量の問題があり実現できていない。

表4　国内新生児スクリーニング陽性例の酵素診断結果

疾患	陽性例		酵素活性低下例		正常活性例	
MMA	5	(2)	3	(1)	2	(1)
PA	20	(7)	13	(4)	7	(3)
IVA	2		2		0	
GA1	4		4		0	
MCADD	12	(5)	8	(3)	4	(2)
VLCADD	2		2		0	
MUSD	7	(1)	3	(0)	4	(4)
計	52	(14)	35	(8)	17	(6)

MMA；メチルマロン酸血症, PA；プロピオン酸血症, IVA；イソ吉草酸血症, GA1；グルタル酸尿症Ⅰ型, MCADD；中鎖アシルCoA脱水素酵素欠損症, VLCADD；極長鎖アシルCoA脱水素酵素欠損症, MSUD；メープルシロップ尿症. ( ) は広島県の症例数（内数）.

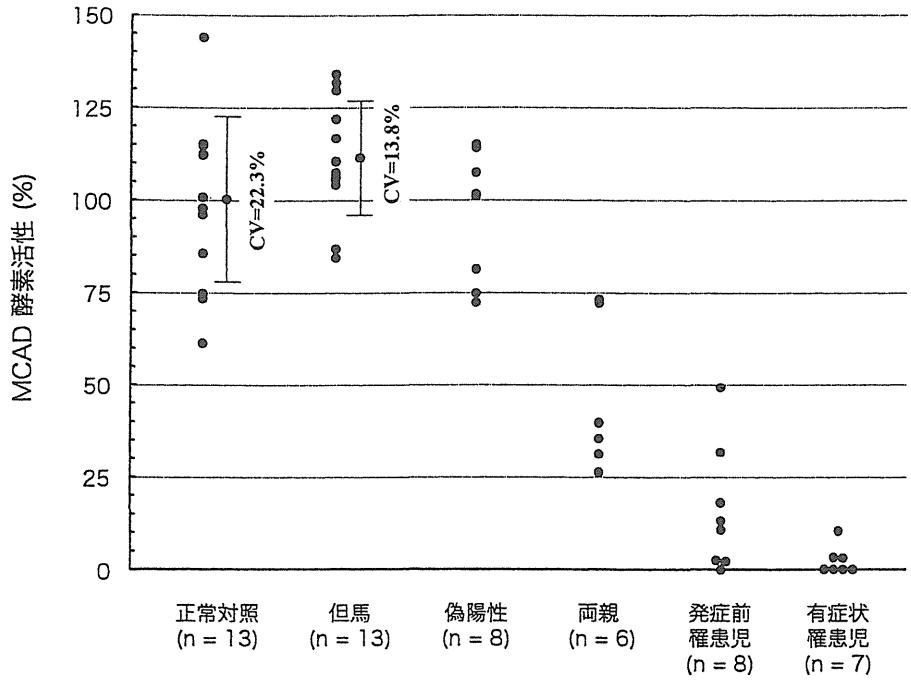

図3　国内のMCAD欠損症疑い例の酵素活性測定結果

血中C8-アシルカルニチン高値からMCAD欠損症が疑われた未発症例（新生児スクリーニング，家族歴からの精査など）および発症後精査例について実施した酵素活性測定値の分布（正常対照群の平均値に対する百分率表示）を示す。罹患者と判定した症例の一部は遺伝子変異も同定されており、正常対照群との分離は良好である。保因者の活性値も、理論的に期待される正常対照群と罹患者群の中間域に分布している。なお、発症前診断例の一部は保因者レベルの活性値を示したが、遺伝子解析結果から罹患者との判断がなされたため、罹患者群に分類している。

測定感度の問題以外にも、HPLC分析時間の短縮（MMA, GA1, VLCAD）や、クロマトグラムにおける反応産物の分離の改善（GA1, HMGLD）など、測定酵素ごとに課題はあるが、解決策の検討は、時間と人員の制約で進んでいないのが実状である。

ま と め

我々の酵素診断法は、汎用HPLCシステムを備えた一般的な検査室で容易に導入できるものとなっている。実際に国内外の数施設で、筆者らの論文参照＋メールによるアドバイスだけで測定できるようになった実績がある[10]。また、工夫次第で、我々が実施している測定系の改善や、その他の疾患への応用もできるはずである。将来的な目標としては、人員不足が深刻な小児科医に依存せず、各地のスクリーニング拠点で臨床検査技師の

手によって管理・運用する体制の構築を期待したい。そのような体制が実現すれば、タンデムマス新生児スクリーニングを長く継続していく上で大いに役立つものと考える。

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血中フリーカルニチン・アシルカルニチンの採血日齢との関連性について

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【要　旨】

タンデム質量分析計による新生児スクリーニングにおいて指標として用いられるアシルカルニチン類の一部は，採血時日齢によって血中濃度が大きく変化する．そのため，検査結果の判定に資する一定のデータを蓄積することが望ましい．

私たちは，札幌市の新生児スクリーニングで2回以上採血を行った児を対象として，採血日齢群別に測定統計値を比較することで，新生児期とそれに続く乳児期のアシルカルニチン類の血中濃度の推移を評価した．

その結果，acetylcarnitine，propionylcarnitine，長鎖アシルカルニチン類は日齢20付近まで減少する傾向があり，C5OHアシルカルニチン，フリーカルニチンは日齢とともに増加する傾向があった．これらの傾向の把握は，精度の高い検査の実施に貢献するものだと考えられる．

【キーワード】

アシルカルニチン，フリーカルニチン，新生児スクリーニング，採血日齢

緒　言

タンデム質量分析計による新生児スクリーニング（以下；タンデム検査）は，2004年から始まった厚生労働科学研究費補助金子ども家庭総合研究事業に研究課題として組み込まれて以降，一部の自治体や検査施設がこれに参加する形でパイロットスタディが開始され，2008年には全国のカバー率が人口比で20%程度に達したとされている[1]．この間，およそ7,600人に1人の割合で患者が発見され，のちの治療による予後の改善とあいまって，タンデム検査の有用性は広く認識されるようになってきた[2]．

タンデム検査における検査施設の問題点として，タンデム検査に対する国内の外部精度管理体制が未整備であることと，カットオフ値について各検査施設の判断に任されていることがある．このうち後者の問題に関しては，採血日齢に基づくアシルカルニチン類の血中濃度の推移を把握した上で，適切なカットオフ値を定めることが望ましい．

そこで，私たちは，いまだ基礎データの蓄積が十分ではない新たな「指標物質」であるアシルカルニチン類について，タンデム検査の測定値を用いて，新生児期とそれに続く乳児期の血中濃度の推移をまとめた．

方　法

タンデム検査により得られたフリーカルニチン及び各アシルカルニチンの血中濃度について，採血日齢によりグループ分けした群毎に，統計量を算出することで行った．また，データが明らかに正規分布できないため，中央値（50パー

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Table. Age-related variations in acylcarnitine and free carnitine concentrations in DBS in infants
Units: μM　＊ molar ratio

Day of birth	4-6	7-16	17-26	27-36	37-46	47-56	57-66	>67
n	809	662	250	288	89	73	72	159
C0								
median	26.7	28.0	30.0	33.1	39.3	41.0	45.3	42.8
the third quantile	33.9	34.1	35.9	40.7	52.0	50.2	55.6	54.0
the first quantile	22.1	23.0	24.3	27.6	31.4	34.2	33.4	33.1
C2								
median	21.9	15.1	14.2	16.2	22.0	26.4	34.9	30.5
the third quantile	27.5	18.7	19.2	22.9	33.5	39.3	34.3	41.5
the first quantile	17.7	12.0	10.9	12.9	15.5	19.2	22.4	22.6
C3								
median	1.68	1.16	1.07	1.28	1.72	1.89	2.12	1.94
the third quantile	2.14	1.42	1.50	1.78	2.46	2.85	3.09	2.86
the first quantile	1.27	0.81	0.69	0.92	1.21	1.45	1.46	1.38
C4								
median	0.20	0.18	0.16	0.15	0.16	0.16	0.16	0.15
the third quantile	0.25	0.21	0.19	0.18	0.18	0.19	0.19	0.19
the first quantile	0.17	0.15	0.13	0.12	0.14	0.13	0.14	0.13
C5:1								
median	0.011	0.011	0.009	0.010	0.010	0.011	0.011	0.011
the third quantile	0.014	0.013	0.012	0.012	0.013	0.014	0.013	0.013
the first quantile	0.008	0.008	0.007	0.007	0.008	0.008	0.009	0.008
C5								
median	0.12	0.15	0.14	0.13	0.13	0.13	0.12	0.10
the third quantile	0.16	0.20	0.19	0.17	0.16	0.16	0.16	0.14
the first quantile	0.10	0.12	0.10	0.10	0.11	0.10	0.09	0.09
C6								
median	0.040	0.037	0.038	0.040	0.042	0.040	0.044	0.042
the third quantile	0.051	0.046	0.049	0.050	0.056	0.051	0.056	0.053
the first quantile	0.031	0.029	0.030	0.031	0.035	0.033	0.036	0.032
C5OH								
median	0.10	0.12	0.14	0.16	0.18	0.21	0.28	0.28
the third quantile	0.13	0.15	0.18	0.20	0.28	0.29	0.38	0.41
the first quantile	0.09	0.12	0.11	0.12	0.14	0.16	0.17	0.19
C8								
median	0.055	0.050	0.049	0.049	0.054	0.045	0.050	0.053
the third quantile	0.072	0.060	0.061	0.060	0.067	0.056	0.062	0.072
the first quantile	0.043	0.039	0.038	0.038	0.042	0.036	0.040	0.040
C10:1								
median	0.087	0.074	0.068	0.066	0.070	0.063	0.069	0.074
the third quantile	0.112	0.091	0.083	0.082	0.086	0.077	0.079	0.102
the first quantile	0.067	0.058	0.055	0.053	0.052	0.050	0.055	0.054
C10								
median	0.10	0.09	0.08	0.08	0.09	0.08	0.09	0.10
the third quantile	0.14	0.11	0.11	0.10	0.11	0.11	0.12	0.13
the first quantile	0.08	0.07	0.06	0.06	0.07	0.06	0.07	0.07
C5DC								
median	0.039	0.037	0.031	0.031	0.033	0.030	0.030	0.031
the third quantile	0.054	0.043	0.039	0.038	0.039	0.036	0.041	0.042
the first quantile	0.030	0.033	0.026	0.024	0.024	0.023	0.026	0.024

Day of birth	4-6	7-16	17-26	27-36	37-46	47-56	57-66	>67
n	809	662	250	288	89	73	72	159
C12								
median	0.11	0.08	0.06	0.06	0.07	0.07	0.08	0.08
the third quantile	0.16	0.10	0.08	0.08	0.09	0.08	0.09	0.10
the first quantile	0.08	0.06	0.05	0.05	0.06	0.05	0.06	0.06
C14:1								
median	0.079	0.050	0.036	0.039	0.048	0.047	0.055	0.059
the third quantile	0.115	0.070	0.048	0.052	0.066	0.060	0.081	0.082
the first quantile	0.057	0.037	0.027	0.028	0.033	0.038	0.038	0.043
C14								
median	0.21	0.15	0.11	0.11	0.14	0.16	0.21	0.19
the third quantile	0.26	0.20	0.15	0.15	0.20	0.22	0.26	0.24
the first quantile	0.17	0.12	0.09	0.08	0.11	0.12	0.16	0.14
C16								
median	2.10	1.05	0.59	0.53	0.69	0.83	1.00	1.05
the third quantile	2.66	1.40	0.76	0.68	0.94	1.07	1.30	1.34
the first quantile	1.56	1.05	0.49	0.42	0.53	0.60	0.78	0.80
C16OH								
median	0.013	0.009	0.008	0.007	0.009	0.009	0.010	0.010
the third quantile	0.017	0.011	0.010	0.009	0.010	0.011	0.012	0.013
the first quantile	0.010	0.007	0.006	0.006	0.007	0.007	0.007	0.008
C18								
median	0.72	0.49	0.32	0.26	0.29	0.37	0.48	0.51
the third quantile	0.91	0.66	0.43	0.34	0.41	0.49	0.61	0.68
the first quantile	0.57	0.39	0.25	0.21	0.22	0.28	0.37	0.39
C18:1OH								
median	0.012	0.008	0.007	0.006	0.007	0.008	0.010	0.010
the third quantile	0.015	0.011	0.009	0.008	0.009	0.011	0.012	0.013
the first quantile	0.012	0.006	0.005	0.005	0.004	0.005	0.007	0.007
C0/(C16+C18)*								
median	9.9	18.2	31.5	40.7	41.4	34.8	31.1	27.8
the third quantile	12.8	23.9	38.9	50.3	49.7	44.1	36.6	35.8
the first quantile	7.2	13.1	25.2	33.4	31.9	26.8	23.7	21.1
C3/C2*								
median	0.077	0.071	0.072	0.078	0.084	0.069	0.064	0.064
the third quantile	0.097	0.093	0.092	0.098	0.105	0.092	0.085	0.080
the first quantile	0.060	0.054	0.053	0.060	0.057	0.057	0.049	0.051
C8/C10*								
median	0.54	0.56	0.59	0.61	0.60	0.57	0.54	0.54
the third quantile	0.62	0.65	0.69	0.70	0.70	0.67	0.60	0.64
the first quantile	0.46	0.49	0.50	0.53	0.54	0.50	0.47	0.49
C8/C2* (x1,000)								
median	2.63	3.33	3.35	2.97	2.69	1.55	1.41	1.76
the third quantile	3.37	4.13	4.57	4.15	3.68	2.66	2.09	2.43
the first quantile	1.93	2.65	2.42	2.21	1.63	1.13	1.18	1.21
C14:1/C2* (x1,000)								
median	3.70	3.37	2.48	2.32	2.10	1.73	1.85	2.04
the third quantile	5.06	4.84	3.52	3.17	2.85	2.74	2.40	2.83
the first quantile	2.69	2.50	1.79	1.67	1.59	1.20	1.14	1.46

C0; Freecarnitine C2; Acetylcarnitine C3; Propionylcarnitine C4; C4acylcarnitine C5; C5acylcarnitine C5:1; C5:1acylcarnitine C6; C6acylcarnitine C5OH; C5OHacylcarnitine C8; C8acylcarnitine C10:1; C10:1acylcarnitine C10; C10acylcarnitine C5DC; C5DCacylcarnitine C12; C12acylcarnitine C14:1; C14:1acylcarnitine C14; C14acylarnitine C16; C16acylcarnitine C16OH; C16OHacylcarnitine C18; C18acylcarnitine C18:1OH; C18:1OHacylcarnitine

センタイル），第3四分位点（75パーセンタイル），第1四分位点（25パーセンタイル）の3つのノンパラメトリックな統計量を使用した[3]．

対象は，札幌市で2005年4月から2007年3月までの2年間で，保護者の希望に基づいて実施したタンデム検査対象者のうち，低出生体重，もしくは新生児スクリーニング対象疾患のどれか1つ以上の項目で要再採血となり，1回以上再採血を行ったものとした．ただし，抗生剤の使用による検査値の異常を認めたもの[1]及びタンデム検査対象疾患の患者は除外した．これらの条件に該当したものは2,402件で，その検査値を今回の検討に用いた．なお再採血の理由は，殆どが低出生体重，もしくは初回内分泌関連項目陽性であった．

グループ分けは，母集団を採血日齢により，4～6日，7～16日，17～26日，27～36日，37～46日，47～56日，57～66日，67日以上の8群に群別することで行った．

検査は既報に従い行った[5]．

結　果

群毎の対象数と，フリーカルニチン，18種のアシルカルニチン，及びタンデム検査の指標として有用な5つのmolar ratioについて各統計値を表にまとめた．

acetylcarnitine（以下；C2），propionylcarnitine（以下；C3），C14:1アシルカルニチン（以下；C14:1），C16アシルカルニチン（以下；C16），C18アシルカルニチン（以下；C18）は日齢4～6に高値を示し，その後日齢20前後まで減少する傾向を示した．

C5OHアシルカルニチン（以下；C5OH）とフリーカルニチン（以下；C0）は日齢とともに増加する傾向を示した．

molar ratio C0/(C16+C18) は日齢20～50にかけて高値のピークを築く傾向を示した．

他のアシルカルニチン，molar ratioは，日齢によらず概ね安定していた．

考　察

これまで，タンデム検査における検査法や検査結果には様々な報告がなされているが，新生児期とそれに続く乳児期のアシルカルニチン類の血中濃度の推移についてはまとまった報告が少ない[6-8]．しかし，いくつかのアシルカルニチンは，疾患の指標として非常に重要であるにも係わらず，日齢によって血中濃度が変化する傾向がある．札幌市では2005年にタンデム検査を開始した際，低出生体重のため2回目採血を行った対象及び他の新生児スクリーニング検査項目で要再採血となった対象について，すべて初回時と同等の検査を実施することとした．そのため，タンデム検査について日齢幅のある測定値が蓄積していることに着目し，今回の検討を行った．なお，今回対象とした範囲では，出生体重による各測定値の変化は少なく，出生体重を挙動の要因に加味する必要はないと考えられた．

札幌市では初回採血を日齢4～6日で行っている．そのため日齢の群別は，日齢4～6日のデータをまず1つの群としてまとめ，その後10日ずつに日齢を区切ってグループ化した．

対象を，再採血を行ったもののみとしたのは，仮に全例を対象として同様の群別集計を行った場合，採血日齢の大きな群では，採血日齢4～6の群から「初回陰性検体」が大幅に除外されることで，初回陽性のものが高い割合で抽出されることになり，各群の性格が著しく異なってしまうためである．また，今回の検討の対象からは，タンデム検査の対象疾患の患者及び抗生剤の影響が認められたものを除外した．これらの処理によって，日齢のみによる濃度変化に近いデータが得られていると考える．

カルニチントランスポータ異常症の指標であるC0の血中濃度は，日齢4～6で平均が28.7μMであるが，日齢とともに上昇し，採血日齢40日前後から，40μM以上で安定した．カルニチントランスポータ異常症の患者の場合，9μM以下の低い濃度のまま経過するため，再採血により大半の偽陽性例を除外することが可能だと考えられた．

メチルマロン酸血症，プロピオン酸血症の指標としてはC3もしくはC3とC2のmolar ratio (C3/C2) が用いられるが，C3の血中濃度が採血

日齢によって大きく変動したのに比較し，C3/C2は採血日齢によらず安定していた．このため，検査施設におけるカットオフ値としてはC3/C2を用いた方が適切な判断ができると考えられる．

マルチプルカルボキシラーゼ欠損症等の指標であるC5OHの血中濃度は，日齢4～6では0.11 μM前後であるが，C0と同様に日齢とともに上昇が続き，日齢60近傍では0.3μM程度に達する傾向にあった．Cavedon CTらはこの加齢に伴う増加が成人期まで継続することを報告しており[8]，今回の私たちの結果も日齢，年齢を鑑みた陽性・陰性の判断が必要なことを示唆するものだった．

C16及びC18の血中濃度は，日齢4～6に高値を示し，その後日齢20前後まで減少する傾向があった．そのため，カルニチンパルミトイルトランスフェラーゼⅠ欠損症の指標であるmolar ratio C0/(C16+C18)は，C0の増加傾向とあいまって，日齢20～50にかけて高値のピークを出現した．採血日齢が当該範囲内であった場合は，これらの推移に配慮した上で，陽性・陰性を判断することが適切であろうと考えられた．

また，C16, C18の他，C14:1をはじめとする脂肪酸β酸化異常症群の指標となる長鎖域のアシルカルニチン類の血中濃度は，全般に日齢4～6で高値を示す傾向があった．これは出生直後の飢餓状況により，脂肪酸β酸化の代謝過程が活発に働いていることを反映したためと考えられる．これらの場合，初回陽性であっても，栄養状態の改善された再採血時の検査データにより，真陽性かどうかを確認することができると思われた．しかし，極長鎖アシルCoA脱水素酵素欠損症の場合，患者であっても再採血時にC14:1が大きく低下する場合があることも報告されており[9]，その取り扱いには慎重を要するだろう．

結　語

データの蓄積が十分ではないと考えられる新生児期とそれに続く乳児期のアシルカルニチン類の血中濃度について，可能な範囲で日齢と関連した推移をまとめた．本来，このような統計は，無作為抽出した正常対象群から，繰り返し採血を行なうことで，日齢との関連性を表した精度の高いものとして算出することできる．その場合，採血日齢による濃度分布の差異について，有意差検定なども有用であろう．しかし，現実的に新生児を対象として，そのようなデータを取得することはきわめて困難である．今回，私たちは出来うる限りの方法で，タンデム検査により得られたデータを採血日齢別に分類し整理した．今後，同様のデータが様々に明らかになれば，より適切なカットオフ値の設定や再採血率の低減に貢献できるであろう．

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The Relationship between Age and the Concentration of Free carnitine and Acylcarnitines

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Clinical and molecular investigation of 19 Japanese cases of glutaric acidemia type 1

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ABSTRACT

Glutaric acidemia type 1 (GA1) is a metabolic disease caused by a deficiency of glutaryl-CoA dehydrogenase (GCDH). Untreated patients mostly develop severe striatal degeneration. More than 200 mutations have been reported in the *GCDH* gene, and common R402W and IVS10-2A>C were found in Caucasian and Chinese/Taiwanese, respectively. However, in Japan, genetic mutations have only been reported in a few cases. Herein, we report the clinical and molecular basis of GA1 in 19 Japanese patients, including six previously reported patients. All cases showed high urinary glutaric acid excretion. Eleven patients were severely impaired (three patients died), three had mild impairment, and five showed normal development. Four of 5 patients that developed normally were detected in the presymptomatic stage by neonatal or sibling screening. Nineteen mutations in 26 alleles were identified, and eight of them (89 or 90delC, Y155C, IVS4+2T C, G244S, Q352X, G354A, K361E, and 1144-1145delGC) were novel. S305L (12.1%, 4/34 alleles) was found in several cases, suggesting that this mutation is a common mutation. In contrast, R402W was not identified and IVS10-2A>C was only found in one allele, suggesting that Japanese patients with GA1 show allelic heterogeneity and have a different genetic background to patients from other countries. One of a pair of sisters with the same mutations (M339V/S305L) lacking residual activity was severely retarded, whereas the older girl remains asymptomatic at 22 years of age, indicating that genotype does not necessarily predict GA1 phenotype. We consistently found that there was no association between genotype and phenotype. However, children with mild impairment were diagnosed and treated earlier than severely impaired cases {4.7±2.5 months (range: 2–8 months) vs. 11.6±12.7 months (range: 4–51 months)}. Our results suggest that early detection and treatment but not genotype are associated with better patient outcome, reinforcing the importance of neonatal screening.

1. Introduction

Glutaric aciduria type 1 (GA1, OMIN 231670) is an autosomal recessive metabolic disorder caused by deficiency of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) [1,2]. GCDH is located in the mitochondrial matrix and acts in the intermediate steps of lysine, hydroxylysine, and tryptophan metabolisms [3]. The clinical manifestations of GA1 include extrapyramidal symptoms, developmental regression, and macrocephaly, appearing most often after acute encephalopathic crises, which are accompanied by bilateral marked enlargement of the sylvian fissure and degeneration of the striatum [1], and in addition, extrastriatal abnormalities [4] and abnormal hemodynamic changes [5]. Its biochemical characteristics include the accumulation of glutaric acid (GA), and 3-hydroxyglutaric acid, which can be detected by gas chromatography (GC/MS), and glutarylcarnitine, which can be identified by electrospray ionization/tandem mass spectrometry (MS/MS) [1,2]. It has been reported that GA1 can be classified into two types based on the level of excreted GA: the high excretion form (GA>100 mmol/mol creatine) and the low excretion form (GA<100 mmol/mol creatine) [6].

Since GA1 was first described in 1975 [3], more than 200 different mutations have been reported [7–9], and its frequency was estimated to be approximately 1 in 100,000 newborns [2]. Although almost all mutations are private, several common mutations have been identified, including A421V in the Amish Community [10], IVS 1+5G T in Canadian Oji-Cree Indians [11], and E365K in Irish travelers [8]. R402W is the most frequent mutation in the European population [6,8], and IVS10-2A C is relatively common in China [12] and Taiwan [13]. In Japan, the frequency of GA1 has been estimated to be approximately 1 in 210, 000 newborns, based on a newborn screening pilot study [14,15]. However, mutations have only been characterized in a few cases [16] since the first description of a Japanese case in 1987 [17]. Herein, we investigated the clinical and molecular aspects of 19 Japanese patients with GA1.

2. Subjects and methods

2.1. Subjects

We studied 19 Japanese patients who were diagnosed with GA1 based on their urinary organic acid profiles and/or blood acylcarnitine

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analysis. The diagnoses were confirmed by analyzing the *GCDH* gene and/or GCDH activity.

The mutations of 6 cases (cases 2–5, 12, and 19) were reported previously (cases 4, 12, and 19: [16], cases 2, 3, and 5: Japanese domestic journal). In this study, we analyzed the mutations in 13 cases (cases 1, 6–11, and 13–18). Among the 13 patients, 4 cases (case 6, 7, 10, and 11) were previously described in case reports [18,19]. No family demonstrated consanguineous marriage.

2.2. DNA sequencing

Genomic DNA was isolated from skin fibroblasts using a Qiamp DNA Microkit (QIAGEN GmbH, Hilden, Germany) and from peripheral blood lymphocytes using the DNA Quick II kit (Dainippon Pharmaceuticals, Osaka, Japan). Each exon of *GCDH* including the intron/exon boundaries was PCR-amplified for 30 cycles using the conditions shown in Supplemental Table 1. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced using the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) or the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA). The structure of the human *GCDH* gene was obtained from the GenBank database (ENSG00000105607). Informed consent to perform DNA analysis was obtained from the parents of the patients. Our study protocol was approved by the Ethics Committee of the Shimane University Faculty of Medicine.

3. Results

3.1. Clinical characteristics

The clinical features of 19 Japanese GA1 patients (10 boys and 9 girls) are summarized in Table 1. Cases 4 and 19 and cases 15 and 18 were siblings. Fifteen of the 19 cases were symptomatic patients. Three (cases 1–3) of 19 cases were detected in a newborn screening pilot study, and one (case 4) was an asymptomatic sibling case that was detected at 2 years of age. To evaluate their outcomes, we classified them into three groups based on disability score [20] that included motor disability, cognitive function, and speech: a) the severe handicap group (disability score 7–9), b) the mild impairment group (disability score 4–6), and c) the normal developmental group (disability score 3) (Supplemental Table 2).

Eleven of the 19 cases were classified into severe handicap group (three of them died), 3 cases belonged to mild impairment group, and 5 cases showed normal development (Fig. 1). The mean age at onset of the symptomatic cases was 5.7 m (range: 4–8 m) in the severe handicap group, 2.3 m (range: 2–3 m) in the mild impairment group, and 6 m in case 4 of the normal development group who suffered from macrocephaly. The mean age at diagnosis was 11.6 m (range: 4–51 m) in the severe handicap group, 4.7 m (range: 2–8 m) in the mild impairment group, and 27 m (range: 24–30 m) in the normal development group, except for the 3 cases diagnosed by newborn screening. Macrocephaly was observed in 31.6% of patients (6/19). All 19 cases showed high urinary glutaric acid excretion. Cranial CT and/or MRI demonstrated frontotemporal atrophy and striatum signal abnormalities in all cases involving mild impairment or severe handicap. In contrast, three of five cases in the normal development group demonstrated mild changes by neuroimaging.

3.2. Clinical manifestations of patients

No cases had a past history except for cases 1, 6, 7, and 9. None of the cases showed abnormal development before the onset of GA1. Immediately after the diagnosis of GA1, all cases were treated with dietary restriction, L-carnitine administration, and prompt intravenous fluid infusions for catabolic states such as recurrent vomiting and diarrhea. In addition, a GABA analogue and vitamin B2 were given to the 14 and 8 cases, respectively.

3.2.1. Normal development group

Cases 1–3 were detected prior to displaying any specific symptoms by a newborn screening program using MS/MS. Case 1 weighed 2952 g when she was born at a gestational age of 39 weeks and 2 days. Abruptio placentae occurred during her birth and she suffered from asphyxia (Apgar score: 3/4). She recovered following hypothermia treatment for hypoxic–ischemic encephalopathy. Cases 2 [21] and 3 [21] had no remarkable delivery events. In these 3 cases, no signs of neurologic complications were evident at 4 months, 5 years, and 7 years old, respectively.

Case 4 was the nonsymptomatic older sister of case 19, who was severely handicapped [16]. She was diagnosed with GA1 by a sibling GC/MS screening in the presymptomatic stage at 2 years old.

Case 5 was hospitalized because of macrocephaly (47.6 cm, +2.5 S.D.) at 6 months. There was no sign of neurologic complications or developmental delay, but cranial CT suggested a subarachnoid cyst and a subdural hematoma. Thereafter, the subarachnoid cyst and subdural hematoma became smaller. At 2.5 years, he was referred to the pediatric department due to progressive macrocephaly (56.5 cm, +3.0 S.D.). Brain CT demonstrated widening of the Sylvian fissures, which in fact had been found by CT at 7 months.

3.2.2. Mild impairment group

Case 6 was treated for initial vomiting and idiopathic hyperbilirubinemia during the neonatal period [18]. Screening by brain echography identified dilated ventricles.

Case 7 was delivered at 27 weeks of dizygotic twin gestation [18]. His birth weight was 998 g. Macrocephaly and convulsions were noticed at 2 and 3 months, respectively. Following treatment, his development caught up.

In case 8, progressive macrocephaly was noticed at 3 months old. Her head circumference was +5.0 S.D. at 7 months old. Her regression and hypotonia, which were accompanied by seizures at 8 months old, improved gradually after treatment.

3.2.3. Severe handicap group

Cases 10, 11, and 13 died. Case 10 displayed a lack of head control at 4 months old [17,18] and irritability and sleeplessness at 5 months old. She died suddenly at 5 years old after developing a common cold. Cases 11 [19] and 13 presented encephalitis-like disease at 5 and 7 months, respectively. Case 11 died suddenly at the age of 3 years. Case 13 died of airway obstruction due to choking after developing an infection at 3 years old.

Similarly, no treatment was effective for the neurological symptoms of the severely handicapped patients that survived, all of whom are bedridden, require tube feeding, and smile spontaneously. Case 9 was born at 35 weeks with an Apgar score of 6/9 by cesarean delivery for premature membrane rupture and breech presentation. His birth weight was 2235 g. He was diagnosed with GA1 at 4 months after an episode of convulsions. He required mechanical ventilation and a tracheostomy for respiratory distress at 10 months old. Case 12 suffered from encephalitis-like symptoms including convulsions, unconsciousness, and rigidity following fever and an upper respiratory tract infection at 5 months old [16]. Case 14 was affected with Kawasaki disease at 5 months old. Intravenous immunoglobulin resulted in rapid defervescence, but his regression, involuntary movement, and irritability accompanied by fever were irreversible. Case 16 was affected by viral encephalitis with hyperpyrexia, consciousness disturbance, and hypertonia at 7 months of age. Case 17 was found to have subependymal pseudocysts and temporal lobe hypoplasia at 1 month. Transient regression was observed at 7 months after gastroenteritis. Thereafter, progressive neurological regression, hypotonia, and rigidity were observed following convulsions associated with pneumonia at 8 months. Case 19 was the younger sister of

Table 1
Clinical manifestations and genetic characteristics of Japanese patients with glutaric acidemia type 1.

Case I.D	Sex	Age at onset	Age at diagnosis	Precipitating factor	Clinical symptoms	Macrocephaly	Treatment	Outcome	Urine GA	C5DC (<0.3)	Neuroimaging	Exon or intron affected	Base change	Effect	GCDH activity
Normal development group															
(Newborn screening cases)															
1	F	–	1m	None	Normal development	–	L-carnitine	Normal (4m)	High	1.08	Typical	**Exon9 / Exon10**	**1064 G>A / 1147 C>T**	**R355H/R383C**	N.D
2	F	–	1 m	None	Normal development	–	L-carnitine	Normal (5y4m)	High	2.22	Mild	Exon6 / Exon8	556 A>T / 914 C>T	S186C / S305L	Deficiency
3	F	–	1 m	None	Normal development	–	L-carnitine	Normal (7y6m)	High	1.95	Mild	Exon3 / Exon10	215 G>T / 1237 T>G	R72L /Y413D	Deficiency
(Sibling screening cases)															
a 4	F	–	2 y 0 m	None	Normal development	–	L-carnitine, GABA analogue	Normal (22y)	High	N.D	Mild	Exon8 / Exon9	914C >T / 1015A> G	S305L / M339V	Deficiency
(other cases except for screening)															
5	M	6	2 y 6 m	None	Normal development	+	L-carnitine, vitamin B2	Normal (6y11m)	High	4.4	Typical	Exon5 / ?	416C >T / ?	S139L / ?	Deficiency
Mild impairment group															
6	M	2 m	2 m	None	Enlargement of ventricles	+	L-carnitine, vitamin B2, GABA analogue	Mild (23y)	High	N.D	Typical	**Exon8 / Exon10**	**914C >T / 1147 C>T**	**S305L / R383C**	Deficiency
7	M	2 m	4 m	None	Seizure	+	L-carnitine, GABA analogue	Mild (25y)	High	N.D	Typical	**Exon5 / Exon5**	**413G>A / 416C>T**	**R138K / S139L**	Deficiency
8	F	3 m	8 m	None	Seizure, regression	+	L-carnitine, GABA analogue, antiepileptic	Mild (3y2m)	High	3.36	Typical	**Exon8/ Exon9**	**914C >T / 1081A> G**	**S305L / K361E**	N.D
Severe handicap group															
9	M	4 m	4 m	None	Seizure, regression	+	L-carnitine, vitamin B2, antiepileptic	Severe (1y4m)	High	N.D	Typical	**Intron4 / Exon6**	**IVS4+2T>C / 532G>A**	**Truncated (Splicing) / G178R**	N.D
10	F	4 m	7 m	None	Regression, irritability, sleeplessness, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (5y:died)	High	N.D	Typical	**Exon9 / Exon9**	**1054C> T / 1054C> T**	**Truncated(Q352stop) / Truncated(Q352stop)**	Deficiency
11	M	5 m	6 m	None	Seizure, regression, hypotonia, dystonia	–	L-carnitine, GABA analogue	Severe (3y:died)	High	N.D	Typical	**Exon3 / Exon7**	**226C>T / 730G>A**	**Truncated (Q76stop) / G244S**	Deficiency
12	M	5 m	6 m	Infection, fever	Seizure, dystonic	–	L-carnitine, vitamin B2, GABA analogue	Severe (14y6m)	High	N.D	Typical	Exon9 / Exon9	1064G >A / 1064G >A	R355H / R355H	N.D
13	F	5 m	7 m	Infection	Seizure, regression, hypertonia	–	L-carnitine, GABA analogue	Severe (3y9m: died)	High	N.D	Typical	**Exon9 / Intron10**	**1061G > C / IVS10-2 A>C**	**G354A / Truncated (splicing)**	Deficiency
14	M	5 m	7 m	Kawasaki disease	Regression, dystonia	–	L-carnitine, GABA analogue, antiepileptic	Severe (7y)	High	1.76	Typical	**Exon5 / Exon7**	**416C >T / 769C>T**	**S139L / R257W**	Deficiency
b 15	M	5 m	4 y 3 m	Fever of unknown origin	Regression, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (5y2m)	High	0.38	Typical	**Exon1 / Exon5**	**89 or 90delC / 461A>G**	**Truncated (frame shift) / Y155C**	N.D
16	M	7 m	7 m	Infection, fever	Unconscious, dystonia	–	L-carnitine, GABA analogue	Severe (1y1m)	High	0.57	Typical	**Exon10 / Exon11**	**1144-1145delGC / 1298C>T**	**Truncated (frame shift) / A433V**	N.D
17	M	7 m	12 m	Gastroenteritis	Seizure, regression, dystonia, hypotonia	+	L-carnitine, GABA analogue, antiepileptic	Severe (2y5m)	High	N.D	Typical	**Exon5 / Exon10**	**383G>A/ 1147C>T**	**R128Q / R383C**	Deficiency
b 18	F	8 m	9 m	Polio vaccine, infection, fever	Regression, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (1y8m)	High	0.41	Typical	**Exon1 / Exon5**	**89 or 90delC / 461A>G**	**Truncated (frame shift) / Y155C**	Deficiency
a 19	F	8 m	12 m	Gastroenteritis	Coma, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (20y)	High	N.D	Typical	Exon8 / Exon9	914C >T / 1015A>G	S305L / M339V	Deficiency

Abbreviations: a,b, siblings; M, male; F, female; Age, y (years); m (months); Treatment, except for dietary restriction ; GA, glutaric acid; C5DC, glutarylcarnitine in dried spots (nmol/ml) when the patient was diagnosed; GCDH, glutaryl-CoA dehydrogenase.
Novel mutations are underlined. The mutations highlighted in bold were identified in this study; Deficiency: GCDH activity ≤5%.
N.D: not determined.

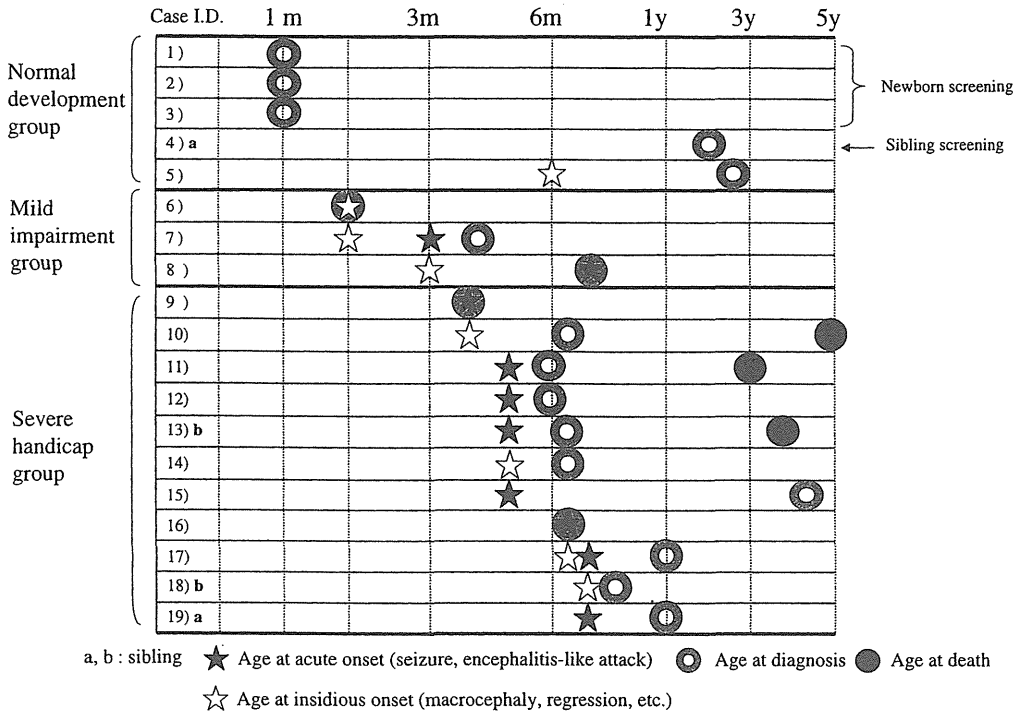

Fig. 1. Age at onset and diagnosis in three groups with different outcomes. The mild impairment group was diagnosed earlier than the severe handicap group {4.7 m (2–8 m) vs. 11.6 m (4–51 m)}. Three cases (cases 10, 11, and 13) died.

case 4. At 8 months old, she suffered an encephalopathic crisis after gastroenteritis, which lasted for several days [16]. Cases 15 and 18 were siblings. Case 15, the older brother, was hospitalized for fever of unknown origin at 5 months of age and treated with antibiotics for 10 days. In addition to hypotonia, which appeared at the time of discharge, his regression, rigidity, and involuntary movement worsened every month that he suffered from fever. Although idiopathic encephalopathy was initially suspected, a diagnosis of GA1 was made in a sibling screening program by GC/MS, and treatment was initiated at 4 years and 3 months. Case 18 suffered from fever after polio vaccination at 8 months. Thereafter, she became unable to support her head and roll over. Her neurological skills deteriorated every month that she suffered from fever. The diagnosis of GA1 was made by GC/MS at 9 months of age.

3.3. Gene mutations in GCDH

Nineteen mutations were identified in 13 cases, and 8 of them were novel. These included four missense mutations (Y155C, G244S, G354A, and K361E), a nonsense change (Q352X), a splice site alteration (IVS4+2T>C), and frame shift mutations (89 or 90delC, and 1144-1145delGC). These novel mutations were not detected in 100 chromosomes from unaffected Japanese individuals.

All mutations are summarized in Table 1 and Supplemental Fig. 1, together with information on 6 cases whose genetic alterations were reported previously ([16] and Japanese domestic journal). Only two unrelated patients out of 19 cases had homozygous mutations (Q352X, R355H). In 34 independent alleles, the frequency of S305L was 12.1% (4/34 alleles), S139L, R355H, and R383C had frequencies of 8.8% (3/34 alleles), respectively and Q352X were found in 2 alleles (5.8%) each. Another 19 mutations were only found in a single allele.

4. Discussion

Since it has been remaining unknown whether there are common mutations and a phenotype/genotype correlation in Japanese GA1 cases, we investigated the relationship between clinical and mutational spectrums of 19 Japanese patients with GA1. Japanese are relatively homogenous ethnic population on islands isolated from other countries. We found a few common mutations distinct from other nations. We also found that mutations in Japanese cases are different from what have been reported in the Caucasian cases, indicating specific genetic information unique for Japanese cases are crucial for their diagnosis in the future. The current study also indicates that earlier detection of the disease followed by appropriate medicare is crucial for the better outcome than the genotype, reinforcing the importance of neonatal screening for GA1. This is a first report that studied the largest cohort of Japanese patients with GA1.

In this study, we identified 19 mutations in 24 independent alleles including eight novel mutations. The amino acids affected by these new mutations are highly conserved among different species (Pan troglodytes, mice, Xenopus, and Bordetella parapepertusis) including humans, suggesting that the region plays an important functional role in GCDH activity. It is highly likely that Q352X, 89 or 90 delC, 1144-1145delGC, and IVS4+2T C abolish GCDH activity, because these mutations result in truncation of the peptide. G354S and Y155H, which affect the same positions as G354A and Y155C, respectively, were reported to have no enzymatic activity [6,7]. The homology of the peptide's structure indicates that G244, G354, and K361 are conserved in the acyl-CoA dehydrogenase group [22]. These findings suggested that all 8 novel mutations in this study have little GCDH activity. In the 19 Japanese cases of GA1 including 6 previously reported patients, Q352X and R355H were homozygous mutations and found in 2 alleles. The frequency of S305L was 12.1% (4/34 alleles), suggesting that this mutation is common in Japanese, in contrast to the very few reports of this mutation from other countries. S139L, R355H, and R383C were also found on 8.8% (3/34 alleles), respectively, implicating that these mutations may be also common, respectively. Additionally, mutations in exon 9 were found more frequently in Japanese GA1 compared with the report by the HGMD (http://www.hgmd.cf.ac.uk/ac/index.php). It is highly likely that understanding common mutations

will facilitate rapid and accurate diagnosis of Japanese cases with GA1. Furthermore, this information may be useful for other Asian countries as well, since some of them are shared with patients from other Asian countries. Newborn screening using MS/MS is becoming popular, and the number of patients will become larger in Asian countries [23] as well as the other countries [24–26]. R402W, the most common mutation in Caucasians, in whom it shows an allele frequency of 12–25% [6,8], was not found in our Japanese cases. IVS10-2A C, a common mutation in China (30%, 3/10 alleles) [10] and Taiwan (66.7%, 4/6 alleles) [13], was also only found in a single allele in our study. Collectively, these findings suggest that Japanese GA1 patients show allelic heterogeneity and have different genetic backgrounds to GA1 patients from other countries. However, S139L, R355H and G178R, in addition to IVS10-2A C, may be common mutations among oriental populations, since S139L have been discovered in 2 of 4 alleles in Korean cases [27], and R355H and G178R were detected in one allele in Chinese case, respectively [10].

All 19 cases demonstrated a high-excretor phenotype in urinary organic acid analysis by GC/MS, suggesting that their mutations resulted in lower enzyme activity (≤5%) [6]. In fact, an enzyme assay confirmed 0–5% residual GCDH activity in 11 cases (Cases 2–7, 10–11, 13–14, and 19) [19,21,28,29]. Furthermore, an *in vitro* probe assay using cultured fibroblasts and MS/MS demonstrated a deficiency of GCDH in 10 cases (cases 4, 6, 7, 10, 11, 13–14, and 17–19) [30]. Although all 19 cases were assumed to have barely detectable enzyme activity, their clinical outcomes were diverse, ranging from normal development, through mild impairment, to severe handicap. This study suggests that the phenotypes of Japanese GA1 patients are not associated with a specific genotype. A previous study also showed that there is no clear correlation between genotype, biochemical phenotype, and the clinical severity of GA1 [6,24]. Frequency (31.6%: 6/19 cases) of macrocephaly of this study is lower than other reports (65–75%) [2,31]. This may represent unique phenotype in Japanese patients with GA1, which have genetic backgrounds distinct from other nations. However, additional case studies are warranted to validate whether this is indeed the cases.

All symptomatic cases except for case 5 had mild impairment or severe handicap indicating that the neurological sequelae of symptomatic cases are poor in Japanese GA1 patients, as reported in previous cases [24,31–33]. With respect to the grounds for the neurological manifestation, we were not able to completely rule out hypoxic–ischemic encephalopathy, hyperbilirubinemia, prematurity, very low birth weight, or encephalitis. However, since there was no sign of neurologic complications or developmental delay before the onset in any cases, we suspect that the neurological symptoms are not a consequence of these conditions. Importantly, the mild impairment group was diagnosed earlier than the severe handicap group {4.7 ± 2.5 m (2–8 m) vs. 11.6 m ± 12.7 m (4–51 m)}, suggesting that a better outcome was induced by early diagnosis. The reason for the better outcome seen in the patients who were diagnosed younger age was considered that early diagnosis led to an earlier initiation of the treatment and/or intervention in a timely manner for any medical conditions, which in turn prevented patients from neurological impairment. The frequency of macrocephaly was higher in the mild impairment group (3/3 cases) than in the severe impairment group (2/11 cases), making it likely that macrocephaly led to an early diagnosis of GA1. Furthermore, there was a notable difference in the phenotypes of siblings with the same mutations: case 4 showed normal development, whereas case 19 showed severe retardation (Supplemental Fig. 2), indicating that genotype does not predict clinical outcome. Taken together, these findings strongly suggest that early diagnosis and treatment but not genotype are associated with a better patient outcome.

Because the diagnosis was made by newborn screening only in 15.8% (3/19 cases), there is no direct evidence that newborn screening has neuroprotective effect for the patients with GA1 in this study. However, our study indicates that genotype does not necessarily predict clinical outcome and that early diagnosis and treatment are critical for a better outcome. While indirect findings, these observations strongly suggest that earliest diagnosis by the newborn screening will also be beneficial for a better outcome. In this regard, it is very important to expand newborn screening by MS/MS to improve the outcome of Japanese GA1 patients.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2010.11.159.

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CASE REPORT

Three Japanese Patients with Beta-Ketothiolase Deficiency Who Share a Mutation, c.431A>C (H144P) in *ACAT1*: Subtle Abnormality in Urinary Organic Acid Analysis and Blood Acylcarnitine Analysis Using Tandem Mass Spectrometry

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Abstract Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency affects both isoleucine catabolism and ketone body metabolism. The disorder is characterized by intermittent ketoacidotic episodes. We report three Japanese patients. One patient (GK69) experienced two ketoacidotic episodes at the age of 9 months and 3 years, and no further episodes until the age of 25 years. She had two uncomplicated pregnancies. GK69 was a compound heterozygote of the c.431A>C (H144P) and c.1168T>C (S390P) mutations in T2 (*ACAT1*) gene. She was not suspected of having T2 deficiency during her childhood, but she was diagnosed as T2 deficient at the age of 25 years by enzyme assay using fibroblasts. The other two patients were identical twin siblings who presented their first ketoacidotic crisis simultaneously at the age of 3 years 4 months. One of them (GK77b) died during the first crisis and the other (GK77) survived. Even during severe crises, C5-OH and C5:1 were within normal ranges in their blood acylcarnitine profiles and trace amounts of tiglylglycine and small amounts of 2-methyl-3-hydroxybutyrate were detected in their urinary organic acid profiles. They were H144P homozygotes. This H144P mutation has retained the highest residual T2 activity in the transient expression analysis of mutant cDNA thus far, while the S390P mutation did not retain any residual T2 activity. The "mild" H144P mutation may result in subtle profiles in blood acylcarnitine and urinary organic acid analyses. T2-deficient patients with "mild" mutations have severe ketoacidotic crises but their chemical phenotypes may be subtle even during acute crises.

Abbreviations

SCOT Succinyl-CoA:3-ketoacid CoA transferase
T2 Mitochondrial acetoacetyl-CoA thiolase

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2, gene symbol ACAT1) deficiency (OMIM 203750) is an autosomal recessive inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. This disorder, first described by Daum et al. (1971), is characterized by intermittent episodes of metabolic ketoacidosis associated with vomiting and unconsciousness often triggered by infections (Fukao et al. 2001). There are no clinical symptoms between episodes. Typical T2 deficiency is easily diagnosed by urinary organic acid analysis, characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate both during ketoacidotic episodes and between episodes (Fukao et al. 2001, 2003). Diagnosis is confirmed by measurement of T2 activity on cultured skin fibroblasts (Robinson et al. 1979; Zhang et al. 2004). T2 deficiency is caused by mutations in the *ACAT1 (T2)* gene located on chromosome 11q22.3-q23.1 (Fukao et al. 1990; Kano et al. 1991). T2 deficiency is very heterogeneous at the genotype level, with at least 50 different mutations described (Fukao et al. 1995, 1997, 1998, 2001, 2002, 2003, 2007, 2008, 2010a, b; Wakazono et al. 1995; Nakamura et al. 2001; Zhang et al. 2004, 2006; Sakurai et al. 2007).

Some T2-deficient patients with mutations which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003). We herein describe three Japanese patients with T2 deficiency whose H144P mutation retains significant residual activity. Their urinary organic acid and blood acylcarnitine profiles were atypical and subtle even during severe ketoacidotic crises.

Materials and Methods

Case Reports

GK69

This Japanese woman (GK69), born in 1984, developed severe metabolic acidosis at the age of 9 months. On admission to a third-level hospital, she was semicomatose, polypneic (48/min), and hypotonic. Laboratory values were: blood glucose 6.8 mmol/L, NH_3 92 μmol/L, blood pH 7.225, pCO_2 7.2 mmHg, bicarbonate 3 mmol/L, base excess −21.3, Na 153 mEq/L (normal range: 139–146), BUN 28.5 mg/dL (normal range: 10–18), and creatinine 1.1 mg/dL (normal range: 0.18–0.46). Metabolic acidosis was refractory to sodium bicarbonate therapy. Peritoneal dialysis was performed for 2 days. On the second hospital day, polypnea and unconsciousness disappeared and the blood gas data improved. Urinary organic acid analysis showed massive amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic aciduria. No increases in 2-methyl-3-hydroxybutyrate or tiglylglycine were noted, although this analysis was performed in an outside laboratory and no urine samples were available for reanalysis. At that time, T2 deficiency was excluded from differential diagnosis based on this organic acid data and the tentative diagnosis was succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. However, an enzyme assay for SCOT was not performed. At the age of 3 years, the patient had a similar but milder episode. Subsequently, she had no further ketoacidotic episodes. Growth and development were normal. She had two uncomplicated pregnancies.

Twin Siblings (GK77b and GK77)

GK77b is a twin Japanese boy. He was born at 36 weeks gestation weighing 2,400 g. His parents had no known consanguinity but both were from a small island in Amami islands in Japan. He experienced several febrile illnesses without ketoacidosis. However, at 3 years 4 months of age, after a 3-day history of fever, cough, and vomiting, he developed anorexia, lethargy, and polypnea. He was admitted to a local hospital. His blood glucose level was 2.3 mmol/L. Blood gas analysis was not performed. Hypoglycemia was corrected with intravenous glucose injection of 20 ml of 20% glucose solution followed by continuous infusion of a 2.6% glucose solution. About 30 h after admission, his condition worsened. Blood gas analysis revealed severe metabolic acidosis showed pH 6.88, pCO_2 6.1 mmHg, and bicarbonate 1.1 mmol/L. He was transferred to a regional hospital. On arrival at the hospital, he was unconscious with a heart rate of 168/min and respiratory rate of 39/min. Blood laboratory data were: WBC 19,050/μL, CRP 0.2 mg/dL (normal values: <0.15), BUN 36.2 mg/dL (normal range: 10–18) creatinine 0.5 mg/dL (normal range: 0.25–0.49), NH3 33.5 μmol/L, glucose 3.8 mmol/L, pH 7.17, pCO_2 20 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 16.3 mmol/L. He received continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.4–0.47 mEq/kg/h. However, unconsciousness and metabolic acidosis did not improve. On the fifth hospital day, he died before being transferred to a third-level hospital.

GK77 is the twin brother of GK77b. Pyloric stenosis was diagnosed at the age of 1 month and corrected surgically;

thereafter, he was well until 3 years 4 months of age. Two days after the onset of his twin brother, he developed frequent repeated vomiting after cough and nasal discharge. Therefore, he was admitted to the regional hospital at the same time as his twin. On admission, he was lethargic. Laboratory findings were: WBC 7,760/μL, CRP 0.5 mg/dL (normal values: <0.15), BUN 20.2 mg/dL (normal range: 10–18), creatinine 0.4 mg/dL (normal range: 0.25–0.49), glucose 3.7 mmol/L, NH3 25 μmol/L, blood pH 7.135, pCO_2 19.5 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 10.1 mmol/L. He received a continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.3 mEq/kg/h. On the third hospital day, his condition worsened and he was transferred to a third-level hospital. On admission, the blood gasses were pH 7.372, pCO_2 21.6 mmHg, bicarbonate 12.2 mmol/L, and base excess −11.2 mmol/L. A glucose infusion rate was further increased to 6.5 mg/kg/min with 10% glucose solution. Acidosis normalized with 9 h (pH 7.399, bicarbonate 21.7 mmol/L, base excess −2.6 mmol/L). Two days later, the urinary ketones became negative and he started eating.

GK77 is now 4 years 8 months and has experienced no further ketoacidotic episodes. The family has been advised to avoid fasting and to come to the local hospital if he has a high fever or appetite loss. His growth and development are within normal ranges.

Urinary Organic Acid Analysis and Acylcarnitine Analysis

Urine samples containing 0.2 mg of creatinine were used for our high risk screening of organic acids. As internal standards, 20 mg each of tropate (TA, C9), margarate (MGA, C17), and tetracosane (C24) were added to these samples. Trimethylsilylated samples were analyzed using capillary gas chromatography-mass spectrometry (QP 5050A, Shimadzu Co. Ltd., Kyoto, Japan), as described earlier (Kimura et al. 1999). The values of organic acids were expressed as the peak area (%) relative to IS-1 (margarate) on the mass chromatogram. Quantification of 2-methyl-3-hydroxybutyrate and tiglylglycine in urine samples from GK77b and GK77 was kindly done by Dr. Sass (Freiburg University) (Lehnert 1994). For comparison, quantification was also done in urine samples from T2-deficient patients whose urinary screening profiles had typical T2 deficient ones. We used urine sample in stable condition from GK01 who is a compound heterozygote of A333P and c.149delC (Fukao et al. 1998) and samples in acute and stable conditions from T2-deficient patients from India (GK(Ind)) in our high-risk screening. Blood spot and serum acylcarnitine analysis using tandem mass spectrometry was also done, as described (Kobayashi et al. 2007), and blood spot samples from GK75 and GK79, who are R208X homozygotes (Fukao et al. 2010b) were used as positive controls.

Enzyme Assay and Immunoblot Analysis Using Fibroblasts

Control and patients' fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described (Robinson et al. 1979; Zhang et al. 2004). We assayed acetoacetyl-CoA thiolase activity in the presence and absence of potassium-ion, since T2 is the only thiolase which is activated by the ion. Immunoblot analysis was done, as described (Fukao et al. 1997). In the cases of the controls, twofold serial dilution samples from 30 to 3.75 μg were electrophoresed together with samples (30 μg) of GK68 and GK77 to determine the amount of T2 protein in the patients' fibroblasts relative to that in the control fibroblasts.

Mutation Analysis

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Genomic DNA was extracted from fibroblasts using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed by PCR and direct sequencing of genomic fragments that included each exon and its surrounding intron sequences (Fukao et al. 1998). For GK77b and the parents, exon 5 was amplified from a dried blood spot 1.25 mm in diameter, which was used for tandem mass spectrometry, using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

Restriction Enzyme Assay to Detect c.431A>C (H144P)

The c.431A>C (H144P) mutation creates a new BmgT120 I site (GGACC). DNAs from 110 Japanese controls were examined using a restriction enzyme assay, as follows.

A fragment (314 bp), including exon 5 and its surrounding introns, was amplified using the following primers:

In4 as (in intron, −69 to −48)5′-CATGCTCTATTAAG-TTCTGCAG-3′

In5 as (in intron, +137 to +119) 5′-ATCCAGACACTCT-TGAGCA-3′

An aliquot of the resulting amplicon was digested with BmgT120 I, then resolved on a 5% polyacrylamide gel. The c.431A fragment (wild-type) is 314-bp long and the c.421C fragment is cut into 162-bp and 152-bp fragments.