3. 剖検方法

SIDSの診断には頭部を含む全身の剖検が必須である．現在登録されている症例の中に，警察の死亡状況調査および検案時の外表所見ではまったくわからなかった頭蓋内損傷が剖検により明らかとなり，殺人事件として立件された事例があり，頭部を除外した剖検ではSIDSと診断してはならない．

外表所見では，外傷や出血斑の有無などの注意深い観察を行う．脱水の有無の観察として眼球の状態，皮膚の性状などの観察，必要に応じて，電解質の測定のための硝子体液の採取(外眼角から注射針で採取)などを施行する．代謝異常や感染の有無の確認のためには血液や脳脊髄液・尿・各種臓器・組織の採取が有用である．

剖検のための画一的なフォーマットは作成していない．これまでに提案されたものは，あまりに詳細すぎて現実的でないものが多い．剖検時の主要な所見は写真撮影を行うことが望ましい．

固定法は緩衝ホルマリン液が推奨される．臓器が比較的新鮮なものでは，肺，肝臓，脳を瞬間凍結(イソペンタンあるいはアセトンとドライアイスまたは液体窒素)させた後に，－80 ℃のディープフリーザーにて保存することが望ましい．凍結切片用はコンパウンドで作製する．

顕微鏡標本は肉眼的に異常が疑われる臓器・組織からはもちろん作製するが，異常所見が見られなくても以下の部位からは作製する：心臓(左右心室)，肺(各葉1か所ずつ，中枢と末梢が観察できるように)，肝，腎(肉眼的に異常がなければ左右いずれかでよい)，脾，副腎(左右)，膵臓(1か所なら膵尾部)，リンパ節(たとえば腸間膜)，腸管，骨髄，胸腺．

次の組織については，少なくともパラフィンブロックの保存が望ましい：下垂体，甲状腺，頸動脈小体，刺激伝導系，舌，扁桃，気管，腸管(各部位)，骨格筋(たとえば腸腰筋)，大動脈，性器(男性では精巣・精巣上体，女性では子宮・卵巣)，皮膚．

染色は基本的にはヘマトキシリン・エオジン(H.E.)染色を行うが，肺・心血管系の病変を観察するには，弾性線維のためのエラスチカ・ワン・ギーソン(EVG)染色，膠原線維のためのアザン染色が用いられる．また，他の病変の観察にも必要に応じて，鍍銀染色，PAS染色，PAM染色や免疫組織化学染色(上皮性マーカーとしてサイトケラチン，白血球共通抗原＝LCAなど)を行うことも可能である．細菌の同定にはギムザ染色とグラム染色がよい．真菌の同定にはPAS染色あるいはグロコット染色を用いる．

主な特殊染色の用途を表6に示す．

SIDSにおける中枢神経病変には，軽微であるが，様々な病変または形成異常などが認められることがある．したがって，中枢神経を観察する場合には，肉眼的・組織学的に，小さな異常にも注意すべきである．参考までに，切り出し時に使用できる参考図を示す

表6　特殊染色の意義と突然死症例における適応

染色	意義・適応
EVG染色	弾性線維を黒褐色に染色する．肺の血管壁の肥厚など肺高圧症（PH）の診断や，肺胞のフレームワークの観察に用いる．
アザン染色	膠原線維を青く染める．種々の臓器での線維化の程度を見る．
マッソン・トリクローム染色	アザン染色とほぼ同意義で利用する．
鍍銀染色	結合組織線維の1つである細網線維を染色する．肺や脾臓のフレームワークの観察に優れている．
ギムザ染色	血球成分の観察や一般細菌の染色に用いる．
グラム染色	ギムザで同定された細菌のグラム陽性・陰性を判定する．
グロコット染色	多糖類を染色するが，真菌の同定に優れている．
PAS染色	グリコーゲンや糖蛋白など広く糖質を染色するので，代謝疾患のスクリーニングあるいは真菌の同定などに用いられる．
PAM染色	糖蛋白や腎臓の糸球体病変に応用される．
脂肪染色	肝臓の代謝性疾患に有用である．ズダンⅢやオイル赤O染色が一般的である．
クリューバー・バレラ染色	髄鞘とニッスル顆粒を選択的に染色する．
サイトケラチン（各種）	上皮性のマーカーとして用いられる．
LCA	白血球共通抗原．浸潤細胞の同定に用いることもできる．
GFAP	神経膠細胞（グリア細胞）を選択的に染色する．
β-amyloid precursor protein	軸索変性の確認に用いられる．

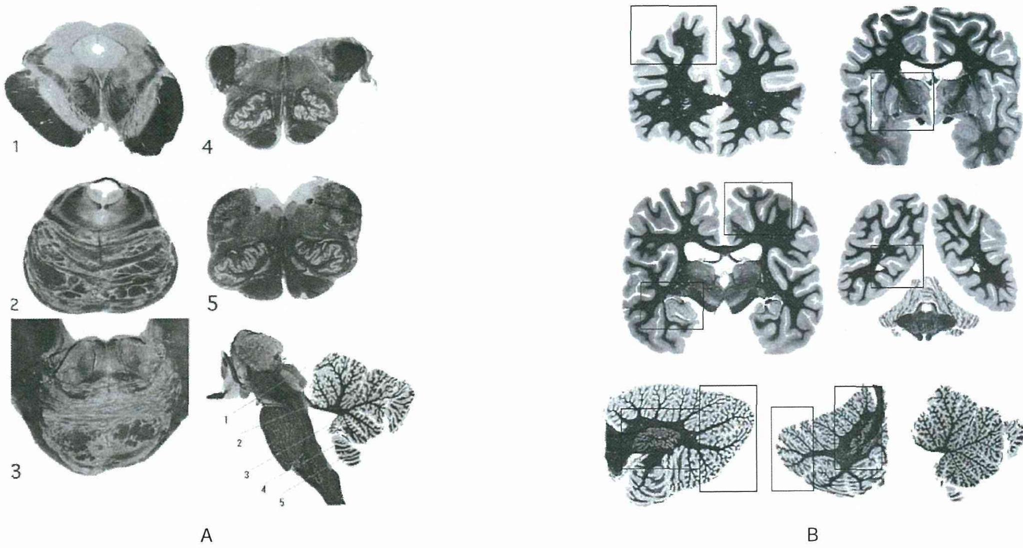


図10　脳の切り出し図．Ａ：脳幹部の切り出し部位．Ｂ：大脳・小脳の切り出し部位．

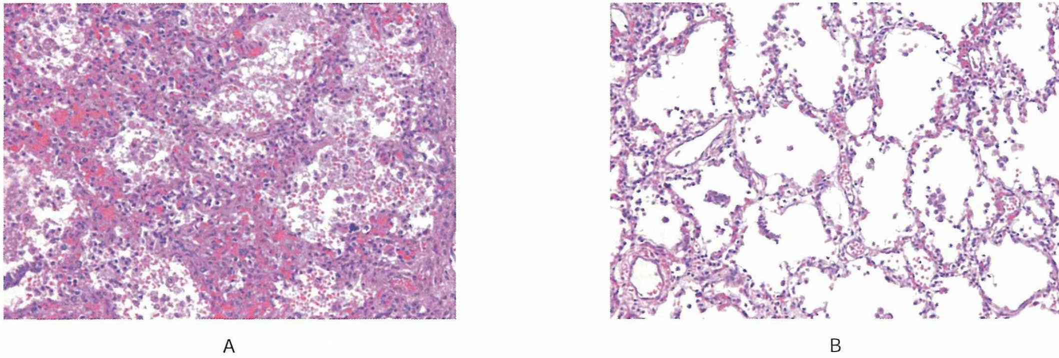


図11　Ａ：ホルマリン未注入肺．Ｂ：ホルマリン注入肺．

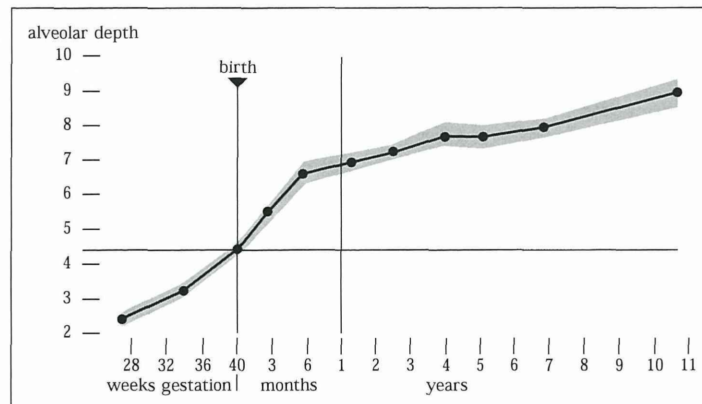
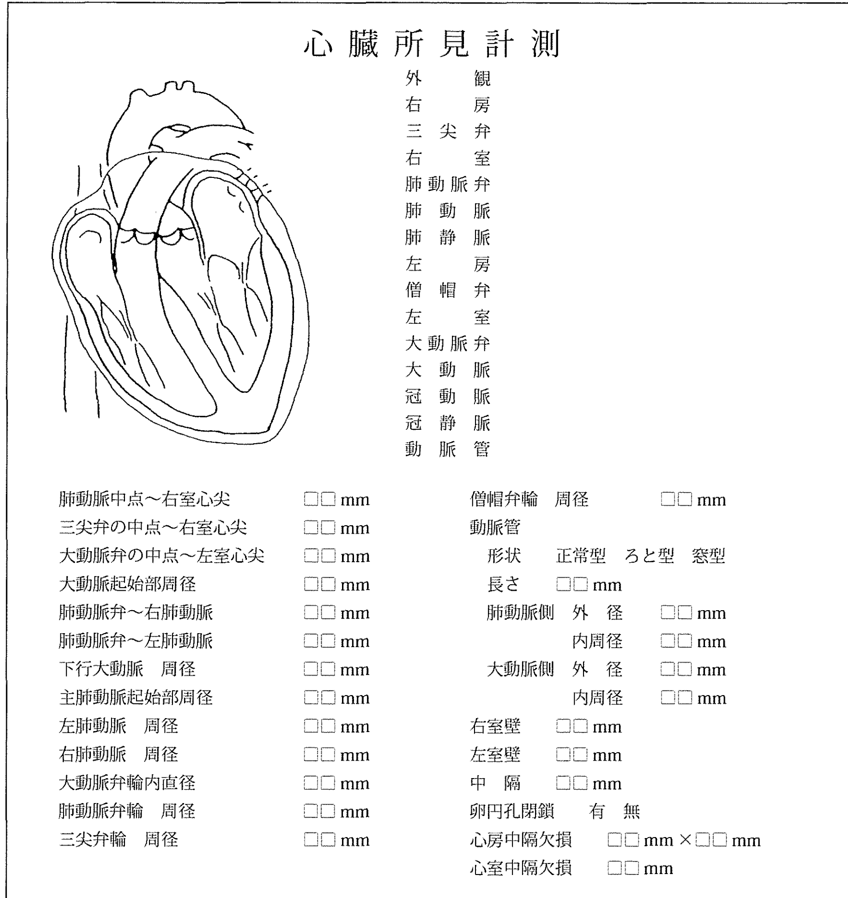


図12　年齢別 RAC の正常値（文献41より引用）



心臓所見計測

外　　観
右　　房
三 尖 弁
右　　室
肺動脈弁
肺 動 脈
肺 静 脈
左　　房
僧 帽 弁
左　　室
大動脈弁
大 動 脈
冠 動 脈
冠 静 脈
動 脈 管

肺動脈中点～右室心尖　□□ mm
三尖弁の中点～右室心尖　□□ mm
大動脈弁の中点～左室心尖　□□ mm
大動脈起始部周径　□□ mm
肺動脈弁～右肺動脈　□□ mm
肺動脈弁～左肺動脈　□□ mm
下行大動脈　周径　□□ mm
主肺動脈起始部周径　□□ mm
左肺動脈　周径　□□ mm
右肺動脈　周径　□□ mm
大動脈弁輪内直径　□□ mm
肺動脈弁輪　周径　□□ mm
三尖弁輪　周径　□□ mm

僧帽弁輪　周径　□□ mm
動脈管
　形状　正常型　ろと型　窓型
　長さ　□□ mm
　肺動脈側　外　径　□□ mm
　　　　　　内周径　□□ mm
　大動脈側　外　径　□□ mm
　　　　　　内周径　□□ mm
右室壁　□□ mm
左室壁　□□ mm
中　隔　□□ mm
卵円孔閉鎖　有　無
心房中隔欠損　□□ mm ×□□ mm
心室中隔欠損　□□ mm

図13　心臓計測所見のモデル

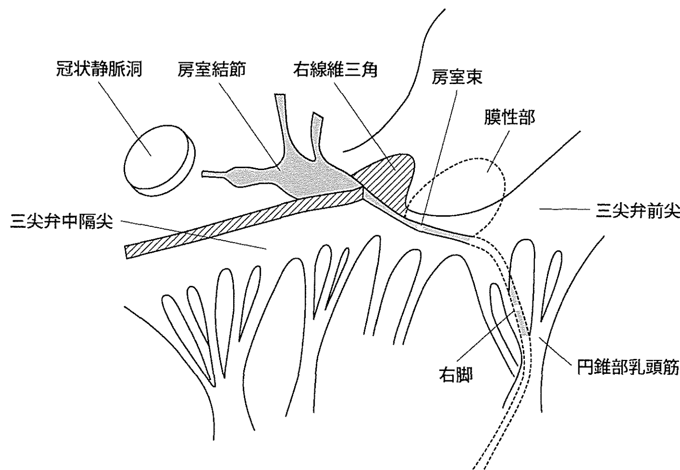


図14　心臓伝導系（房室結節）の模式図

（図10A・B）．

大脳は少なくとも1cm毎の割面を肉眼的に詳細に観察した後に，可能であれば大切片を作製する．

小脳では，半球および虫部をそれぞれに観察する．

脳幹部では，中脳では上丘と下丘の中間のレベル，橋では上部（滑車神経交差のレベル），下部（台形体交差のレベル），延髄では上オリーブ核のレベルとオリーブ核の中央のレベルでそれぞれ標本を作製して観察するのが望ましい．

中枢神経系の染色法として，H.E.染色および髄鞘化を観察するためにクリューバー・バレラ（KB）染色を行うことも可能である．グリオーシスを判定するため，GFAP（glial fibrillary acidic protein）等の免疫組織化学染色を行うことも可能である．

肺は，気管支からホルマリンを注入固定するのが望ましい．気管分岐の近くまで気管支を付けて肺切除を行い，注射器等でホルマリンを肺が適度な大きさになるまで注入する．ホルマリン注入を行わないと，肺胞が重なって固定され，一見肺胞壁が厚く見えたり，細胞密度が高く見えたりするため，間質性肺炎や肺の未熟性と誤診する原因となりやすい（図11A・B）．

肺組織の成熟度を見る簡易な方法として radial alveolar count（RAC）法がある．これは終末細気管支または呼吸細気管支の中央から最短の胸膜あるいは小葉間中隔へ垂線をおろし，その直線上に含まれる肺胞数が，終末気道の分岐数を表すことで，成熟度を推定するものである（図12）[40]．SIDSのRACの検討では，正常の肺の発達と変わりがないという結果が得られている[41]．

ホルマリンが注入されていない場合に，EVG染色や鍍銀染色あるいはサイトケラチンなどの免疫染色で肺胞壁のフレームワークを観察すれば，肺胞壁の構造の理解が容易になる．

ホルマリンを注入すると気管支内の吐乳吸引の有無が観察できないことを危惧する意見もあるが，肺が気腫状に膨脹していない症例（SIDSでは肺がやや虚脱することが多い）では，吐乳吸引を考慮する必要はない．また，ホルマリンが適切に注入されれば，肺の組織学的構造が破壊されたり，肺胞内の浸出物などが失われることはない．

細菌学的検査あるいは免疫組織学的検査のための凍結標本などは必要時にサンプルする必要があるが，残りの肺にホルマリンを注入することも可能である．例えば，右上葉を凍結し，残りの中葉・下葉と左肺にホルマリン注入する．

心臓は，可能な限り心室の大きさ，壁の厚さ，大血管や弁の直径・周径の計測及び冠状動脈の走行の確認などを行うべきである（図13）．細かい計測を行うことにより，見逃していた異常を発見することもある．

刺激伝導系の検索について最も重要な房室結節は，右心房の心房中隔よりの下部で，冠状静脈洞の前方にあり（図14），心内膜直下に認められる．Kochの三角形（Torado腱，三尖弁付着縁，冠状静脈洞）に囲まれた部分にある．詳しい検索については専門医へのコンサルトが望ましい[42]．

4．正常値（基準臓器重量）

乳幼児の剖検では，正確な重量を測ることは非常に重要であり，それとともに，基準値と比較検討することも重要である．表7，8の基準値は，大阪府立母子保健総合医療センターと大阪府監察医事務所の剖検データを解析したものである．脳重量は大脳・小脳・脳幹をあわせた全体の重量である．小脳の重量は，ホルマリン固定後に脳幹部から切り離して測定したものでやや例数は少ないが，所見として示す．心臓の計測値を表8に示す．心肥大や拡張，弁の狭窄や閉鎖不全の診断等に利用可能である．

［謝辞］

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表7　乳幼児の体重別諸計測値（臓器重量）

項目名	体 重 (g, kg)	3,000～3,499	3,500～3,999	4,000～4,499	4,500～4,999	5,000～5,999	6,000～6,999	7,000～7,999	8,000～8,999	9,000～9,999	10kg～15kg未満
	平均(mm)	22. 6	21	33. 2	22. 2	33. 6	27. 7	21. 1	19	19	22
大泉門1	S. D.	13. 1	14. 9	24. 9	6. 6	12. 6	13. 8	13. 2	5. 4	1. 4	8. 9
	計測数	54	33	11	15	16	18	6	5	2	4
	平均(mm)	21. 3	18. 8	31. 6	21. 4	31. 5	24. 7	18	18. 8	16. 5	20. 2
大泉門2	S. D.	11. 7	7. 1	24. 5	7. 1	14. 5	10. 5	10. 1	5. 2	2. 1	8. 6
	計測数	54	33	11	15	16	18	6	5	2	4
	平均(cm)	51. 3	53. 5	56. 3	58. 7	61. 4	67. 7	70. 9	73. 1	82. 2	88. 9
身 長	S. D.	4. 2	3. 2	5. 8	5. 2	6. 3	5	4. 9	6. 5	10. 4	9. 8
	計測数	71	53	22	17	28	29	19	12	16	31
	平均(cm)	35. 7	37	39. 8	40. 5	43. 5	46. 9	48. 8	50. 7	53. 5	56. 1
C-R長	S. D.	1. 5	2	5. 2	3. 2	4. 9	2. 6	2. 2	2. 4	3. 9	4. 1
	計測数	69	50	21	5	20	20	12	7	13	21
	平均(cm)	34. 3	34. 7	35. 6	38. 3	41. 6	40. 5	43. 8	44. 5	46. 4	47. 1
頭 囲	S. D.	2. 2	2	4	2. 5	5. 4	6. 5	2. 8	1. 7	2	3
	計測数	70	52	22	18	23	25	17	8	15	23
	平均(cm)	32. 1	34. 1	34. 9	37. 3	38. 9	40. 1	43. 1	43. 1	46. 3	50. 7
胸 囲	S. D.	2. 2	1. 4	2. 3	1. 8	2. 1	1. 5	1. 7	2. 6	2	2. 7
	計測数	69	52	22	18	22	24	15	8	15	22
	平均(cm)	31. 8	33. 8	34. 7	38. 4	37. 7	37. 9	42. 5	43	42. 5	49. 2
腹 囲	S. D.	4. 9	2. 8	7. 5	3. 8	7	3. 8	3. 7	4. 9	3. 7	8. 8
	計測数	69	52	22	18	22	24	14	7	15	21
	平均(cm)	7. 7	7. 8	7. 6	8. 3	8. 7	9. 5	9. 4	10. 5	11. 4	12. 2
足 長	S. D.	0. 5	0. 4	1. 8	0. 6	0. 5	0. 5	0. 5	0. 9	1. 4	1. 4
	計測数	66	43	19	17	17	21	14	6	9	12
	平均(cm)	0. 4	0. 7	0. 7	0. 7	0. 6	0. 8	0. 8	0. 4	0. 8	0. 7
皮下長	S. D.	0. 2	0. 3	0. 2	0. 5	0. 2	0. 2	0. 3	0. 3	0. 4	0. 5
	計測数	25	28	7	6	14	10	7	5	8	7
	平均(g)	3,243	3,705	4,200	4,672	5,549	6,452	7,472	8,428	9,527	12,035
体 重	S. D.	140	120	139. 1	148. 4	287. 3	271. 1	320. 6	315. 7	290. 2	1365. 5
	計測数	72	54	22	20	28	30	19	12	16	35
	平均(g)	9. 6	18. 3	5. 9	8. 3	15. 5	20. 3	18. 4	22. 5	13. 8	18. 6
胸 腺	S. D.	10. 9	64. 3	4. 6	7. 1	17. 7	14. 3	16. 3	13. 1	12. 3	13
	計測数	61	44	17	17	22	22	19	9	13	23
	平均(g)	37. 9	46. 9	52. 7	63. 1	69	87. 4	89. 6	107. 3	115. 4	130. 2
右 肺	S. D.	23. 4	20. 6	29	26. 2	26. 3	33. 6	50. 3	60. 7	49. 1	44. 8
	計測数	59	42	17	11	25	24	17	11	14	23
	平均(g)	28. 9	35. 3	50	53. 9	56. 2	74. 4	76. 7	88. 8	96. 5	108. 3
左 肺	S. D.	22. 8	18. 5	25	28. 5	20. 4	25. 3	44. 9	45. 8	40. 1	42. 8
	計測数	59	41	18	11	25	23	17	11	14	24

表7（つづき）　乳幼児の体重別諸計測値（臓器重量）

項目名	体　重 (g, kg)	3,000～ 3,499	3,500～ 3,999	4,000～ 4,499	4,500～ 4,999	5,000～ 5,999	6,000～ 6,999	7,000～ 7,999	8,000～ 8,999	9,000～ 9,999	10kg～ 15kg未満
心　臓	平均(g)	28.9	32.3	43.5	45.5	52.5	69.1	66.6	66.5	99.7	95
	S. D.	22	13.2	24.9	24.3	27	37.1	38.2	39.4	36.5	43.1
	計測数	58	40	18	12	26	23	17	11	14	24
肝　臓	平均(g)	134.3	161	186.4	198.8	226.2	244.4	296.7	299.5	380.3	575
	S. D.	52.1	70.5	195.1	118.2	99.2	59.9	122.5	131.6	143	228.5
	計測数	68	50	21	18	24	25	18	10	13	28
脾　臓	平均(g)	10.5	13.8	16.5	20	22.3	22.4	26.4	32.4	37.6	47.5
	S. D.	7.6	9.6	8.9	9.4	8	6.8	11.8	13.7	18.8	23.7
	計測数	64	46	19	16	21	19	15	10	12	26
甲状腺	平均(g)	4	1.1	2.7	0	1.3	1.5	1.6	0	2.2	2.8
	S. D.	0.2	0.2	0	0	0	0.6	0.5	0	0	0.9
	計測数	2	2	1	0	1	2	4	0	3	4
副腎右	平均(g)	3.4	3.3	2.7	2.4	2.5	1.8	2.3	2.1	2.3	2.8
	S. D.	1.7	2.1	1.7	1.1	1.4	0.5	0.9	0.8	1.4	1.3
	計測数	68	49	21	16	24	22	18	10	13	22
副腎左	平均(g)	3.7	3.5	2.7	3.2	2.8	2.2	2.2	2.1	2.8	3.1
	S. D.	1.6	2.2	1.1	1.5	1.5	1.2	0.9	1.1	1	1.4
	計測数	66	48	20	17	24	22	18	10	13	24
膵　臓	平均(g)	3.6	3.9	7.5	6.6	10.1	10.7	11.1	15.7	21.8	24.1
	S. D.	2.1	1.5	3.6	3.3	4.2	3.5	3.9	4.2	9	11
	計測数	54	39	12	17	22	17	15	8	12	22
右　腎	平均(g)	17.9	19.5	24.7	24	32.9	34.3	34.2	35.5	46.5	58.5
	S. D.	9.6	7.7	10.1	6.2	19.9	28.4	112	18.6	10.4	25.7
	計測数	68	47	22	17	23	21	18	10	13	25
左　腎	平均(g)	17.9	19.4	24.9	23.5	34.8	28.3	34.5	33.7	47.9	59.4
	S. D.	9.7	8.7	11.1	6.7	18.5	9.1	9.8	16.7	13.3	23.5
	計測数	65	47	40	18	24	21	18	9	13	24
横隔膜	平均(g)	20.3	21.9	20.2	26.3	28.4	38.3	36.3	53.8	54.1	72.3
	S. D.	7.1	7.6	4.5	9.9	9.7	9.5	10.4	14.6	14.6	22.5
	計測数	36	23	9	7	15	11	7	2	8	11
脳	平均(g)	395.4	396.5	441.3	463.8	737.1	797.2	796	824.4	845.1	936.1
	S. D.	93.9	130.2	201.4	187.8	143.4	145.4	124.4	158.8	390.7	355.1
	計測数	36	22	8	5	11	13	7	8	6	17
小　脳	平均(g)	21.9	21.7	58.3	0	77.1	85.1	0	80.3	0	96.6
	S. D.	3.6	3.5	9.4	0	30.6	0	0	0	0	0
	計測数	7	5	2	0	3	1	0	1	0	1

表8　心臓の諸計測値

項目名	体　重 (g, kg)	3,000〜 3,499	3,500〜 3,999	4,000〜 4,499	4,500〜 4,999	5,000〜 5,999	6,000〜 6,999	7,000〜 7,999	8,000〜 8,999	9,000〜 9,999	10kg〜 15kg未満
肺動脈弁	平均(mm)	32. 2	32. 9	35. 7	37. 7	44	44. 6	44. 8	46	52. 7	49. 7
中点〜	S. D.	7	5. 7	6. 1	6. 7	10. 4	9. 3	14. 1	5. 2	7. 8	5. 8
右室心尖	計測数	64	52	19	15	21	16	9	6	10	16
三尖弁	平均(mm)	24. 6	24. 1	26. 8	26. 2	32. 7	31. 8	30. 5	33. 2	40	36. 4
中点〜	S. D.	5. 5	5. 2	8. 1	3. 3	8. 3	8. 7	12. 8	8. 6	7. 4	8. 5
右室心尖	計測数	64	53	20	15	21	15	11	7	11	17
大動脈弁	平均(mm)	29. 2	29. 9	32. 4	33	36. 6	43. 7	42. 3	407	45. 1	46. 1
中点〜	S. D.	6. 2	6. 8	6. 2	5	8. 8	11. 8	5	5. 4	10. 8	8. 7
左室心尖	計測数	63	50	19	14	20	15	12	7	11	18
大動脈	平均(mm)	19. 1	19. 1	20. 1	24. 5	24. 5	29. 3	30. 5	27. 5	28. 7	37. 3
起始部	S. D.	8	5. 5	5. 7	5. 5	6. 6	5. 9	5. 7	7. 1	11. 4	9. 2
周径	計測数	66	52	19	17	22	19	14	8	12	20
右肺動脈	平均(mm)	6. 3	8. 1	7. 5	10	9. 1	10	14. 7	6	10. 3	9. 3
高さ	S. D.	1. 1	2. 9	3. 5	5. 6	5. 6	0	6. 5	1. 4	2. 5	2. 3
	計測数	9	16	2	2	6	3	4	2	3	3
左肺動脈	平均(mm)	10. 2	11	12	13	12. 5	12. 3	17. 2	12. 5	15. 6	14. 3
高さ	S. D.	2. 2	2. 3	4. 2	5. 6	3. 8	4	2. 9	0. 7	4	1. 5
	計測数	9	17	2	2	6	3	4	2	3	3
下行	平均(mm)	15. 2	16. 1	16. 3	16. 1	18. 4	18. 7	18. 3	20. 2	21. 7	23. 7
大動脈	S. D.	2. 1	1. 5	2. 3	1. 3	1. 6	2. 9	1	2. 6	4. 7	3. 8
周径	計測数	61	49	20	16	21	15	13	4	10	17
主肺動脈	平均(mm)	21. 2	21. 5	25. 3	27. 5	28	31. 3	27. 5	26. 5	34. 2	36. 1
起始部	S. D.	5. 5	5. 9	7. 1	6. 5	9. 2	10. 3	13. 6	4. 8	4. 7	16. 8
周径	計測数	60	49	18	15	22	18	12	7	12	19
左肺動脈	平均(mm)	11. 4	11. 9	13. 5	16. 2	17. 7	18. 8	19. 8	15	20. 2	19. 7
周径	S. D.	6. 4	3. 6	3	4. 6	5. 2	4. 7	7. 4	3	4. 1	7. 5
	計測数	62	50	19	16	16	13	12	5	12	17
右肺動脈	平均(mm)	12. 5	12. 9	13. 8	18. 5	18. 1	20. 5	19. 3	16. 8	21. 6	18. 8
周径	S. D.	6. 1	3. 9	3. 4	6. 2	5. 1	5. 9	6. 4	4. 1	5. 2	6. 7
	計測数	62	49	18	17	15	14	12	7	13	17
大動脈弁輪	平均(mm)	6	6. 2	8. 3	7. 1	7. 3	8. 8	8. 2	10. 7	9. 8	10. 1
内直径	S. D.	3	3. 2	6. 8	1. 6	2	2. 9	1. 6	7. 9	2. 9	2. 5
	計測数	63	51	20	17	19	19	12	9	11	18
肺静脈弁輪	平均(mm)	21. 3	22. 7	23. 3	27. 3	27. 7	32. 5	28. 7	27. 5	33. 8	33. 8
周囲	S. D.	5. 6	5. 1	6. 9	4. 2	8. 6	5. 9	8. 9	8. 4	8. 7	9. 3
	計測数	62	50	19	15	21	14	11	9	10	16
三尖弁輪	平均(mm)	36. 6	39. 1	38. 9	40. 8	49. 1	50. 4	51. 7	45. 5	57. 7	54. 6
周径	S. D.	6. 9	9. 6	8. 3	10. 9	11	14	12. 2	11. 2	8. 9	10. 9
	計測数	63	53	19	15	21	18	10	9	11	17

表8(つづき)　心臓の諸計測値

項目名	体　重 (g, kg)	3,000～3,499	3,500～3,999	4,000～4,499	4,500～4,999	5,000～5,999	6,000～6,999	7,000～7,999	8,000～8,999	9,000～9,999	10kg～15kg未満
僧帽弁輪周径	平均(mm)	29	29.7	29.6	34.5	38.8	40.3	45.4	41.3	44	46.4
	S. D.	6.8	7.1	4.9	6	7.7	8.9	7.9	6.7	11.9	11.8
	計測数	61	48	20	15	19	13	11	9	11	17
動脈管長さ	平均(mm)	9.1	10	8.1	8.6	9	5	9.2	0	8.5	7
	S. D.	2.3	3.1	1.7	2.8	1.7	0	4.3	0	1.9	1
	計測数	41	34	11	8	6	1	5	0	4	3
動脈管肺動脈側外径	平均(mm)	4.2	4.9	4.5	4	3.6	3	3.3	0	5.2	3.6
	S. D.	1.9	1.7	1.7	1	0.5	0	0.5	0	0.9	1.1
	計測数	9	11	4	5	3	1	3	0	4	3
動脈管肺動脈側内周径	平均(mm)	11.3	10.7	9.4	3.5	5.6	0	6.5	3	0	0
	S. D.	2.8	3	3.8	1.7	2.5	0	0.7	0	0	0
	計測数	35	24	7	4	3	0	2	1	0	0
動脈管大動脈側外径	平均(mm)	5.2	4.6	4.5	3.8	3.6	3	3.3	0	4.7	4.6
	S. D.	3.6	1.3	1.2	0.8	0.5	0	0.5	0	2.2	2.8
	計測数	10	10	4	5	3	1	3	0	4	3
動脈管大動脈側内周径	平均(mm)	11.6	11.3	9.1	4	6	0	9.5	2	0	0
	S. D.	2.3	3.6	3.2	2.1	2.6	0	3.5	0	0	0
	計測数	33	24	7	4	3	0	2	1	0	0
右室壁	平均(mm)	5	5.4	6.5	6.2	5.4	6.5	5.4	3.6	5.5	6
	S. D.	2.6	1.9	3	2.3	3.6	3.4	3.6	1	2.5	3.8
	計測数	66	52	20	17	23	22	14	8	14	23
左室壁	平均(mm)	6.2	6.5	7.7	7.9	8.4	9	8.2	8.8	9.2	9.5
	S. D.	2	1.5	2.3	1.3	2	1.8	1.7	1.5	1.7	1.8
	計測数	65	48	21	15	22	19	14	8	14	23
中隔	平均(mm)	6.3	7	8.5	8.2	8.8	9.8	10.5	9	9.2	9.7
	S. D.	1.5	1.6	2.8	1.6	1.9	1.8	3.5	1	1.4	2.6
	計測数	59	45	18	14	21	15	14	8	11	21

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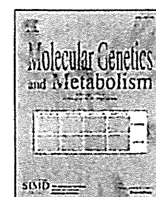

Brief Communication

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

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ABSTRACT

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

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

2. Subjects and methods

2.1. Subjects

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

2.2. Mass spectrometric analysis

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

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

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

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

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

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

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

3. Results

3.1. Clinical features of patients

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

3.2. Biochemical results of patients

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

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

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

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

4. Discussion

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

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

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

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

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ORIGINAL PAPER

Intracellular in vitro probe acylcarnitine assay for identifying deficiencies of carnitine transporter and carnitine palmitoyltransferase-1

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Abstract Mitochondrial fatty acid oxidation (FAO) disorders are caused by defects in one of the FAO enzymes that regulates cellular uptake of fatty acids and free carnitine. An in vitro probe acylcarnitine (IVP) assay using cultured cells and tandem mass spectrometry is a tool to diagnose enzyme defects linked to most FAO disorders. Extracellular acylcarnitine (AC) profiling detects carnitine palmitoyltransferase-2, carnitine acylcarnitine translocase, and other FAO deficiencies. However, the diagnosis of primary carnitine deficiency (PCD) or carnitine palmitoyltransferase-1 (CPT1) deficiency using the conventional IVP assay has been hampered by the presence of a large amount of free carnitine (C0), a key molecule deregulated by these deficiencies. In the present study, we developed a novel IVP assay for the diagnosis of PCD and CPT1 deficiency by analyzing intracellular ACs. When exogenous C0 was reduced, intracellular C0 and total AC in these deficiencies showed specific profiles clearly distinguishable from other FAO disorders and control cells. Also, the ratio of intracellular to extracellular C0 levels showed a significant difference in cells with these deficiencies compared with control. Hence, intracellular AC profiling using the IVP assay under reduced C0 conditions is a useful method for diagnosing PCD or CPT1 deficiency.

Keywords Fatty acid oxidation · Carnitine cycle disorder · Acylcarnitine profile · ESI-MS/MS

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Introduction

L-Carnitine plays an essential role in the transfer and activation of long-chain fatty acids across the outer and inner mitochondrial membranes during which it is acted upon by enzymes including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 (CPT1), carnitine palmitoyltransferase-2 (CPT2), and carnitine acylcarnitine translocase (CACT) (Fig. 1) [1, 2]. Carnitine penetrates into cells across the plasma membrane against a high concentration gradient of free carnitine with the aid of the plasma membrane OCTN2 protein encoded by the SLC22A5 gene [3]. Deficiency of OCTN2 causes primary carnitine deficiency (PCD, OMIM 212140), which is characterized by systemic carnitine deficiency in tissues and blood but in concord with increased excretion of free L-carnitine in the urine [4–6]. Clinical symptoms in patients with PCD such as cardiomyopathy,

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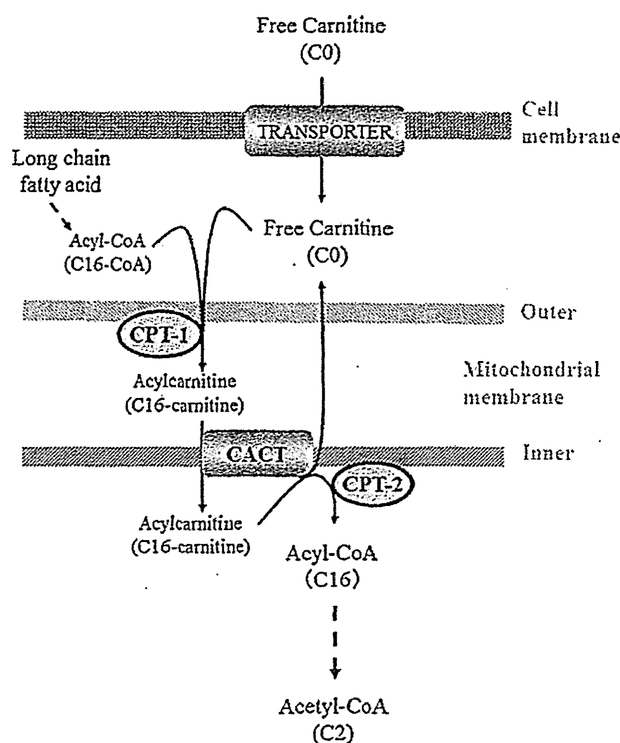

Fig. 1 Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. *Solid arrows* indicate single reactions; *dashed arrows* indicate multiple reactions or steps

encephalopathy, hepatomegaly, myopathy, hypoglycemia, and hyperammonemia, mainly result from low carnitine concentration in the tissues. On the other hand, secondary carnitine deficiency occurs in some conditions such as organic acidemias, renal dialysis, long-term medication (antiepileptic drugs or some antibiotics), and alimentary deficiency of L-carnitine [7–9].

It is necessary to make a differential diagnosis of PCD from the secondary carnitine deficiency or other false-positive cases, and diagnosis is confirmed by demonstrating reduced transport in skin fibroblasts from the patients. Until now, cluster-tray method using radioisotope-labeled substrate was used for the diagnosis of PCD [4, 10–12]. However, such a diagnostic method requires handling of radioactive substrates and focused only on diagnosis of PCD. Gene sequencing in SLC22A5 is one diagnostic method for PCD. However, it is molecularly heterogenous, and around 50 different mutations have been identified [6]. After acylcarnitine analysis using tandem MS analysis became available in the worldwide, blood acylcarnitine analysis was used as an initial method for diagnosis of FAO disorders and a detection of FAO disorders has been increased. However, it is necessary to confirm the diagnosis of the diseases with detailed analysis. The in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry (MS/MS) has been used to evaluate FAO capacity in the cultured cells and make a diagnosis of FAO disorders [13–15]. However, conventional IVP assay is not feasible to diagnose PCD or CPT1 deficiency, because excess amount of free carnitine is added to the experimental medium at the beginning. Estimation of free carnitine, which is the key marker for the above diseases, in experimental medium was nonsense for diagnosis of these disorders. We developed a novel functional assay for PCD and CPT1 deficiency using the IVP assay, with some modifications. This method uses different concentrations of exogenous free carnitine and measures intracellular as well as extracellular acylcarnitine (AC) levels, which overcomes the disadvantage of the conventional IVP assay in the diagnosis of carnitine cycle disorders.

Materials and methods

Materials

Hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), and palmitoylcarnitine (C16) were purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). As an internal standard, a labeled carnitine standard kit (NSK-B), which contains $^{2}[H]_{9}$-carnitine, $^{2}[H]_{3}$-acetylcarnitine, $^{2}[H]_{3}$-propionylcarnitine, $^{2}[H]_{3}$-butyrylcarnitine, $^{2}[H]_{9}$-isovalerylcarnitine, $^{2}[H]_{3}$-octanoylcarnitine, $^{2}[H]_{9}$-myristoylcarnitine, and $^{2}[H]_{3}$-palmitoylcarnitine, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Preparation of standard solutions of ACs

Standard solutions containing 1, 10, 25, and 50 μmol/L each of C6, C8, C10, and C16 were used to validate the recovery and determine linear concentration range of ACs after extraction by the Folch method [16]. The ACs were dissolved in methanol (99.8 %), and the prepared standard solution was analyzed directly and after extraction by the Folch method.

Subjects

Human skin fibroblasts from six healthy controls (volunteers) and seven patients with various carnitine cycle disorders—three each with PCD and CPT2 deficiency and one with CPT1 deficiency—were analyzed. In all cases, diagnoses were confirmed by mass spectrometric analyses (gas chromatography-mass spectrometry and MS/MS), enzyme assay, and protein or mutational analyses. Informed consent was obtained from the patients or their families. This study was approved by the Ethical Committee of the Shimane University School of Medicine.

In vitro probe acylcarnitine (IVP) assay using MS/MS

An IVP assay was performed, as described, with some modifications [13, 15, 17], and principle of IVP assay was shown Fig. 2. Briefly, 3×10^6 cells were seeded in triplicate onto a six-well microplate (35 mm i.d.; Iwaki) and cultured until confluent. After washing twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA), the cells were subsequently cultured for 96 h in 1 ml of a special experimental minimal essential medium (MEM) containing bovine serum albumin (0.4 % essential fatty acid-free BSA; Sigma), two different concentrations of C0 (Sigma)—10 μmol/L (reduced level, lower compared with physiological level) and 400 μmol/L (excess level)—and unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque). C0 and AC levels in the culture medium (extracellular fraction) and in the intracellular extract were analyzed after a 96-h incubation period using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA), as described [18].

Intracellular acylcarnitine extraction

Intracellular C0 and ACs were extracted using the Folch method, with some modification [16]. Briefly, harvested cells were washed twice with DPBS buffer. The cell pellet was resuspended in 100 μl volume of DPBS buffer and immediately frozen in liquid N_2. In order to separate phospholipids and cell debris, 250 μl of Folch reagent (chloroform/methanol, 2:1) was added to the resuspended cell pellet. After vigorous mixing using a vortex mixer, the solution was centrifuged for 10 min at 15,000 rpm at 4 °C. The debris layer around the interface between the aqueous and lipid phases was removed, and the extracted aqueous and lipid phases were mixed and thereafter dried under a nitrogen stream at 50 °C. ACs in culture medium supernatants and extracted intracellular ACs lysate were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Briefly, methanol (200 μl) including an isotopically labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of supernatant from culture medium and extracted intracellular ACs, for 30 min. Portions were centrifuged at 1,000×*g* for 10 min, and then 150 μL of supernatant was dried under a nitrogen stream and butylated with 50 μL of 3 N *n*-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80 % acetonitrile/water (4:1 *v/v*), and then the ACs in 10 μL of the aliquots were determined using MS/MS [18] and quantified using ChemoView^{TM} software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

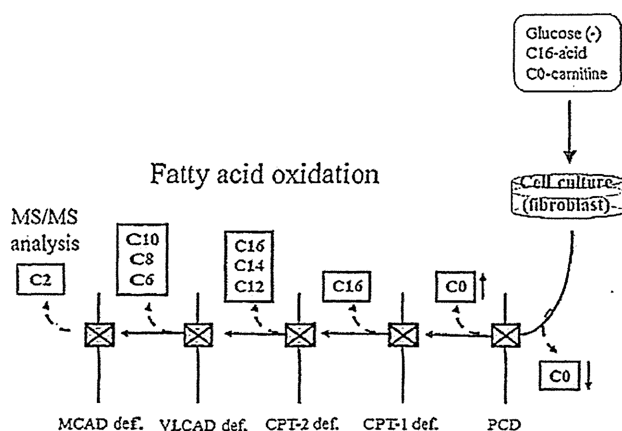

Fig. 2 Principle of in vitro probe acylcarnitine assay. *C2, C4, C6, C8, C10, C12, C14,* and *C16* represent acylcarnitines

Protein concentration and cell viability

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) [19]. The percentage of viable cells was determined at 24, 48, 72, and 96 h of incubation using the modified 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay [20].

Data and statistical analysis

The results are expressed as mean±SD from at least three independent experiments for IVP assay in each cultured cell and three intra-assays and three inter-assays for recovery of standard AC solutions, and statistical significance was evaluated using Student's *t*test in Microsoft Excel. The AC concentrations were expressed as nanomoles per milligram protein.

Results

Recovery of ACs during Folch extraction

The AC standards in the aqueous or lipid fraction were analyzed separately using MS/MS, after extraction by the Folch procedure, and compared with direct analysis of the total mixed standard solutions using three inter-assays and three intra-assays of analysis of standard AC solution. As shown in Fig. 3, most of the C6 and C8-carnitines fractionated to the aqueous phase, while almost all C16-carnitine was exclusively retained in the lipid phase. The amount of C10-carnitine was comparable in both aqueous and lipid phases.

To determine the loss of C0 and ACs during Folch extraction, the standard AC solution was analyzed directly after routine sample preparation for MS/MS and compared with that after Folch extraction. The recovery of ACs in the

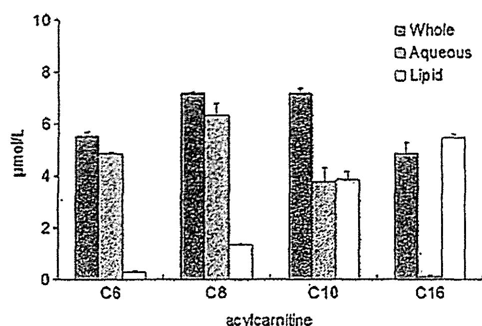

Fig. 3 Recovery of ACs during extraction using the Folch method. Standard solutions of 10 μmol/L each of C6-, C8-, C10-, and C16-carnitine were used to determine the recovery of ACs in the aqueous and lipid fractions during extraction using the Folch method. *Grey column*: ACs in the whole extract after Folch method; *striped column*: ACs in the aqueous fraction of Folch extraction; *open column*: ACs in lipid fraction of Folch extraction. Data are expressed as mean±SD (micromoles per liter) from three intra-assays and three inter-assays, and statistical significance was evaluated using Student's *t*test in Microsoft Excel

standard solutions after direct analysis and Folch extraction procedure was analyzed three times by inter-assay. The inter-assay CV of acylcarnitines ranged from 3.21 to 8.33 %. No statistical difference was seen between direct analysis and after Folch extraction.

Acylcarnitine profile in extracellular medium of cultured fibroblasts with excess and reduced concentrations of free carnitine

Using fibroblasts from various carnitine cycle disorders, AC profiles were determined in the extracellular medium with reduced or excess concentration of C0. Reported conventional IVP assay used excess levels of C0 (400 μmol/L) [14, 15, 17, 21]. With excess amount of C0 (Table 1, "Medium (C0-excess, 400 μM)"), a selective increase in C16 and a decrease in acetylcarnitine (C2) was observed in cases of CPT2-deficient fibroblasts. AC profiles in media from PCD- and CPT1-deficient fibroblasts were similar to that of healthy controls. In PCD fibroblasts, C2 was 53.1 % of the normal control while C2 in CPT1-deficient fibroblasts was 140 % of the normal control. No statistical difference in C0 level was observed among CPT2-, PCD-, and CPT1-deficient fibroblasts and a healthy control.

In the extracellular medium containing reduced C0, C16 remains higher in cells with CPT2 deficiency, while AC profiles were similar to those observed in C0-excess for PCD- and CPT1-deficient cells and the healthy controls (Table 1, "Medium (C0-reduced, 10 μM)").

Acylcarnitine profile in intracellular lysate with various concentrations of free carnitine

The intracellular C0 and ACs were measured after AC extraction using the Folch method. C16 in the intracellular lysate from CPT2-deficient fibroblasts was significantly elevated in both reduced and excess C0 conditions similar to those in extracellular medium, and diagnostic significant was kept. In the excess C0 condition, CPT1- and PCD-deficient fibroblasts could not be distinguished clearly; based on the C0 levels, even C16 level was relatively low (Fig. 4a). On the other hand, the intracellular C0 under conditions with reduced C0 was 41.78±1.47 and 6.31± 2.88 nmol/mg protein/96 h in the normal controls (n=6) and patients with PCD (n=3), respectively, and the C0 levels of PCD cells were significantly lower (p<0.001) as shown in Fig. 4b. This indicated that the C0 uptake was significantly decreased in PCD compared with control in

Table 1 Acylcarnitine profiles of in vitro probe acylcarnitine assay

	Acylcarnitines, nmol/mg protein/96 h						
	C0	C2	C6	C8	C12	C14	C16
Medium (C0 excess, 400 μM)							
Control (n=6)	411.74±23.08	11.80±1.54	2.60±0.09	1.70±0.47	0.79±0.22	0.34±0.19	2.06±0.77
PCD (n=3)	432.18±18.76	6.25±0.96	2.09±0.40	0.94±0.54	0.41±0.33	0.20±0.10	1.72±0.57
CPT-1 (n=1)	357.69±34.16	16.52±5.60	1.73±0.87	0.54±0.94	0.18±0.14	0.17±0.16	1.36±0.98
CPT-2 (n=3)	376.56±42.71	6.88±0.72	0.94±0.65	0.41±0.22	1.70±0.35	0.80±0.05	18.73±1.07
Medium (C0 reduced, 10 μM)							
Control (n=6)	9.85±0.30	1.70±0.74	0.78±0.30	0.18±0.09	0.10±0.08	0.03±0.01	0.51±0.11
PCD (n=3)	10.03±0.71	0.74 ±0.33	0.75±0.31	0.06±0.04	0.03±0.01	0.01±0.01	0.20±0.08
CPT-1 (n=1)	11.06±0.75	7.56±3.10	0.98±0.30	0.55±0.62	0.09±0.09	0.08 ±0.07	0.01 ±0.02
CPT-2 (n=3)	9.73±1.94	0.64±0.23	0.54±0.20	0.11±0.03	0.22±0.06	0.04±0.01	2.79±0.38

The results are expressed as mean±SD from three independent experiments with triplication in each cell line. The AC concentration was expressed as nanomoles per milligram protein. *C0* free carnitine, *C2* acetylcarnitine, *C6* hexanoylcarnitine, *C8* octanoylcarnitine, *C12* dodecanoylcarnitine, *C14* myristoylcarnitine, *C16* palmitoylcarnitine

C0-reduced condition. Concentration of C16 was also significantly low in PCD in C0-reduced condition. Under the C0-reduced condition, intracellular C0 was much higher, but C16 was much lower in CPT1-deficient fibroblasts, compared with the levels in controls (Fig. 4b).

The ratio of intracellular C0 to extracellular C0 in PCD was significantly lower than that of the controls ($p<0.001$) in the C0-reduced condition, while that in C0-excessive condition was not significantly different (Fig. 5). Cell viability was measured using the MTT assay under reduced or excess concentrations of C0. The percentage of viable cells cultured in C0-reduced medium was equivalent to that in C0-excess media (data not shown).

Discussion

The present study developed a novel IVP assay for the accurate diagnosis of PCD and CPT1 deficiency. Although previous studies reported that IVP assay was a powerful method for the diagnosis of most FAO disorders [13, 14, 21], this assay turned out to be unable to identify PCD and CPT1 deficiencies. At first, we used a C0-excess experimental medium, which contained 400 μmol/L of C0, according to previous reports [13, 14, 21]. Extracellular

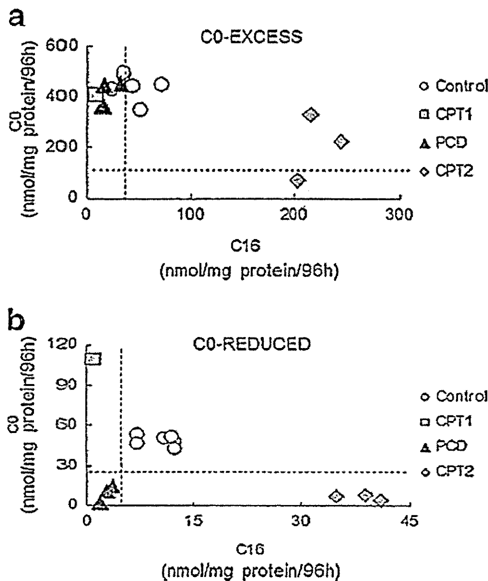

Fig. 4 Intracellular C0 and C16 correlation in patients with carnitine cycle disorders. **a** C0-excessive condition ($-E$); **b** C0-reduced condition ($-R$). *open circle*: healthy control (n=6); *closed triangle*: PCD (n=3); *closed square*: CPT1 deficiency (n=1); *closed diamond*: CPT2 deficiency (n=3). Cells were incubated in experimental medium with 400 or 10 μmol/L of free carnitine and 200 μmol/L of palmitic acid. After 96-h incubation, cells were harvested, and intracellular free carnitine (C0) and palmitoylcarnitine (C16) were extracted using Folch method and measured using MS/MS. Data of mean values of triplicates are presented (nanomoles per milligram protein per 96 h)

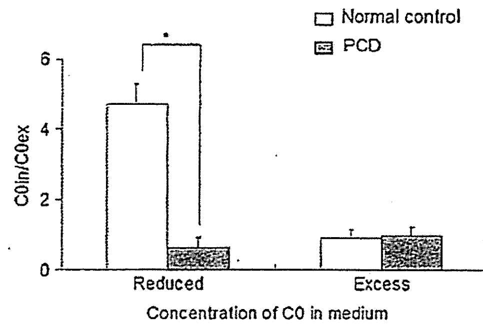

Fig. 5 Ratio of intracellular C0 to extracellular C0. *Open square*: normal control (n=6); *closed square*: PCD (n=3). Extra- and intracellular C0 of cells with normal control and PCD were measured in C0-reduced (10 μmol/L) and C0-excess (400 μmol/L) conditions using MS/MS. Data are expressed as mean±SD of six normal controls and three patients with PCD. Experiment in each cell line was repeated twice with triplications. Significant differences between normal control and PCD are shown as $^*p<0.001$

AC profiles of patients with PCD and CPT1 deficiency showed a pattern similar to that of normal controls by the conventional assay that contains excessive C0 (400 μmol/L) in the culture medium, since C0 moves across the cell membrane down its concentration gradient by passive diffusion. Long-chain fatty acids are transferred across the inner mitochondrial membrane with the assistance of carnitine and carnitine cycle enzymes. The subsequent FAO functions normally even in PCD, and AC profile in PCD is similar to that in normal FAO. Next, we used 50 μmol/L of C0 because the normal range of free carnitine in human plasma was approximately 25 to 50 μmol/L [6]. However, there was no diagnostic difference compare with C0-excess condition, and data are not shown. We analyzed IVP assay in C0-deficient condition (10 μmol/L of C0).

It is known that fibroblasts and muscle and cardiac cells have a high-affinity, low-capacity transporter system [22], and carnitine concentrations in the tissues are much higher than those in serum [23]. Analysis of intracellular C0 and ACs is more relevant for the diagnosis of PCD and CPT1 deficiency because it was shown that C0 was decreased in PCD and increased in CPT1 deficiency in those tissues. When we analyzed cell lysates with MS/MS after direct sonication, artificial peaks of ACs were detected, and the background peaks of mass spectrum were high and hampered the subsequent analyses (data not shown). Hence, we extracted intracellular ACs using a modified Folch method and analyzed both the intracellular lysate and the extracellular medium. This allowed visualization of clear peaks of C0 and ACs in the intracellular lysate, validating that the Folch extraction can be used for simultaneous quantitation of intracellular C0 and a wide range of ACs (short- to long-chain AC).

Uptake of C0 and abnormalities in ACs were associated with the concentration of C0 in culture medium. In the C0-excess condition, it was hard to differentiate PCD from control

cells. Levels of C0 and C16 were overlapped with those of normal control. On the other hand, in the C0-reduced condition, intracellular C0 was significantly decreased in PCD while being increased in CPT1 deficiency, compared with that in normal control. C0-reduced medium was changed after fibroblasts equilibrated in MEM, and normal control could force to uptake free carnitine in C0-deficient condition while cells with PCD could not uptake sufficiently in that condition. Furthermore, the following fatty acid oxidation cycle interrupted, and C16 also decreased in PCD. This correlation of C0 and C16 in the C0-reduced condition is more informative for the diagnosis of carnitine cycle disorders (Fig. 4b). Since cells with PCD cannot uptake C0 via the cell membrane, the finding of reduction of both C0 and C16 is specific for PCD. In case of CPT1 deficiency, C0 uptake is normal, but it cannot bind acyl-CoA ester, resulting in reduced long-chain acylcarnitine production, and FAO is disturbed. Therefore, the stored intracellular ACs were consumed by FAO, and intracellular C16 as well as total ACs were decreased, and C0 was accumulated in intracellular lysate. In contrast, the AC profile of low level of C0 and high level of C16 is diagnostic for CPT2 deficiency. In this disease, normally transferred long-chain AC cannot be converted back from ACs to acyl-CoA esters and. C0, the substrate for FAO. Additionally, the ratio of intracellular and extracellular C0s can sensitively distinguish PCD from control in the C0-reduced medium because carnitine transporter of normal cells was forced to uptake C0 up to physiological level in C0-reduced condition while cells with PCD failed for it. In excessive C0 condition, ratio of intracellular and extracellular C0 was similar to that in normal control and PCD since C0 transfer by passive diffusion across the cell membrane.

In conclusion, the simultaneous analysis of intracellular and extracellular C0 and ACs under the various concentrations of free carnitine in the culture medium is useful for diagnosis of FAO, especially carnitine cycle disorders. This study confirms that the newly modified IVP assay is an easy and safe method to diagnose PCD and CPT1 deficiency.

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