Bernier FP, et al：Neurology 2002；59：1406-1411, Walker UA, et al：Eur Neurol 1996；36：260-267

I 臨床症状
以下の3項目(①～③)をすべて満たす臨床的に確定診断されたミトコンドリア脳筋症またはミトコンドリアサイトパチー☆
①他の病因では説明できない多臓器にまたがる症状が存在する：少なくとも以下の3系統以上の臓器にまたがること
1)神経系，2)筋肉，3)心臓，4)腎臓，5)消化器系，6)肝臓，7)内分泌，8)造血器，9)耳科，10)眼科，11)皮膚科，12)奇形症候群
②発作性進行性経過：しばしば感染を機に増悪するまたは
母系遺伝を思わせる家族歴：母方の親戚の1人以上にミトコンドリア呼吸鎖異常症の疑い例または可能性例が存在する
③代謝性あるいは非代謝性の除外診断を確実に行うこと

II 病理組織像
骨格筋 2%以上のragged red fiber(赤色ぼろ線維)

III 酵素活性
①抗体染色：COX(－) fiber 50歳以下の場合2%以上
50歳以上の場合5%以上
② In vitro 呼吸鎖酵素活性☆☆
1つの臓器で20%以下または2つ以上の臓器にまたがって30%以下
1つの培養細胞で30%以下

IV 機能解析
線維芽細胞のATP合成能：平均マイナス3 SD以下

V 分子生物学
核またはミトコンドリアの明らかな病原遺伝子異常がみつかること

I 臨床症状
1つでもミトコンドリア脳筋症に合致した症状#があること

II 病理組織像
骨格筋：ragged red fiber 30～50歳 1～2%
30歳以下 少しでもあればよい
筋線維膜下のミトコンドリアの異常集積
16歳以下で2%以上
臓器は問わず：ミトコンドリアの電顕的異常

III 酵素活性
①抗体染色による呼吸鎖酵素欠損の証明
② In vitro 呼吸鎖酵素活性☆☆
1つの臓器で20～30%または2つ以上の臓器にまたがって30～40%
1つの培養細胞で30～40%

IV 機能解析
①線維芽細胞のATP合成能：平均マイナス2-3 SD
② Galactose medium中で成育できない線維芽細胞

V 分子生物学
核またはミトコンドリアの可能性のある遺伝子異常がみつかること

VI 呼吸鎖異常を示唆する1つ以上の検査所見
①血中，髄液中乳酸・ピルビン酸・アラニン高値
②髄液中タンパクの増加(KSS疑いのとき)
③ ^{31}P-MRSまたはPETの異常所見(筋肉or脳)
④エルゴメター異常所見(VO_2max，AVO_2D，乳酸閾値の低下)

Definite：確定例
大基準2つ又は，大基準1つプラス小基準2つ
Probable：疑い例
大基準1つプラス小基準1つ又は，小基準3つ
Possible：可能性例
大基準1つ又は，小基準のI(臨床症状)プラス他の小基準もう1つ

下線は埼玉医大小児科で解析できるものを表す．
(Blue Native電気泳動は，この診断基準には含まれていない．)

補足

☆ミトコンドリア脳筋症またはミトコンドリアサイトパチー
① Leigh disease ② Alpers 症候群 ③ LIMD 致死型乳児ミトコンドリア病 ④ Pearson 症候群 ⑤ KSS (Kearns-Sayre 症候群) ⑥ MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) ⑦ MERRF (myoclonic epilepsy with ragged-red fibers) ⑧ NARP (neuropathy, ataxia, and retinitis pigmentosa) ⑨ MNGIE (mitochondrial neuro-gastro-intestinal encephalomyopathy) ⑩ LHON (Leber's hereditary optic neuropathy)

☆☆呼吸鎖酵素活性は，クエン酸合成酵素 (CS) 又は，complex II との比を正常対照平均に対する % で表す．

☆ミトコンドリア脳筋症に一般的な症状：

①筋肉
1) 眼筋症：外眼筋麻痺，眼けん下垂
2) 運動不耐症，筋力低下，脱力感
3) 心筋伝導障害
4) 肥大型 or 拡張型 (まれ) 心筋症
5) 筋痛症，横紋筋融解症

②神経
1) 失調症
2) 感音性難聴
3) 認知症 or 精神遅滞
4) 網膜色素変性症
5) 視神経萎縮
6) 癲癇／ミオクローヌス
7) 卒中様発作
8) 末梢性神経症

③その他
1) 成長障害
2) De Toni-Fanconi-Debre 症候群
3) 糖尿病
4) 肝障害，肝不全
5) 消化管運動障害 and/or 吸収不良
6) 多発性対称性脂肪腫症
7) 汎血球減少症

これらに小児科的には以下の症状を加える
①胎動が乏しく流産に至った既往
②新生児死亡
③動きの乏しい児
④重症体重増加不良
⑤新生児筋緊張の低下
⑥新生児筋緊張の亢進
(成人基準では筋または神経症状が必須であるが，小児基準には必ずしも適切ではない)

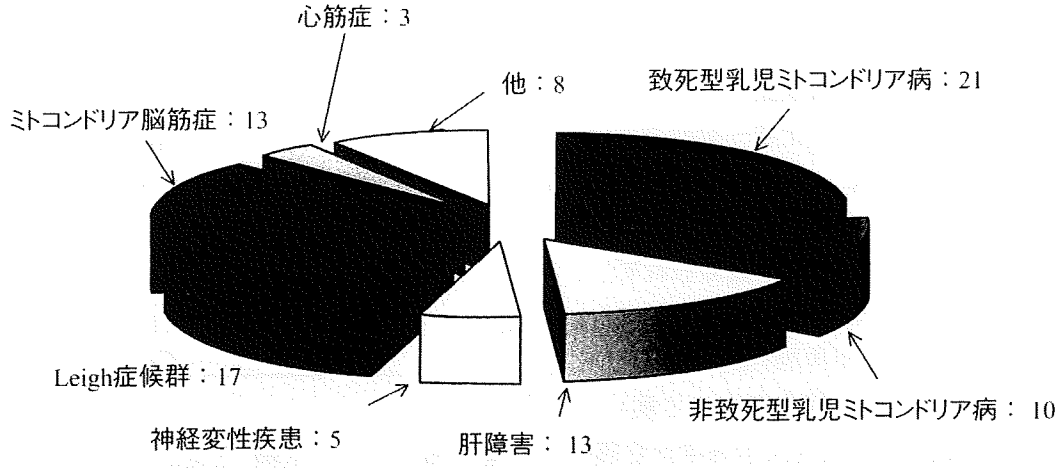

図3 ミトコンドリア病と診断された患者の臨床診断

埼玉医科大学小児科 大竹 明

27 新生児タンデムマスパイロット研究にて異常を指摘された新生児例

1 初診から精査開始までの経過

2002年12月5日生まれ．女児．当院初診時日齢20．

主　訴：タンデムマス新生児スクリーニング異常の精査．

既往歴：在胎39週1日，2,822 gにて出生．生後経過に著変なし．

家族歴：血族婚なし．両親・二人の兄は健康．

現病歴：日齢5の濾紙血をタンデムマス新生児スクリーニング(福井大学医学部看護学科　重松陽介先生に依頼)へ提出したところ，炭素鎖数4～14のアシルカルニチンが増加していたことから，精査のため当科受診を指示された．

初診時現症：

身長：50 cm(−0.6 SD)．

体重：3,376 g(−0.9 SD)．

頭囲：35.5 cm(+0.5 SD)．啼泣良好，活気あり．

顔貌：異常なし．

胸部：心音・呼吸音に異常所見なし．

腹部：軟，腹満なし，肝2 cm触知，脾は触知せず．

外性器：異常なし．

神経・筋・骨格系：変形，筋緊張低下等なし．

表1　初診時検査所見

〈末梢血液検査〉		〈血液化学〉	
WBC	12,080/μL	T.Bil	16.9 mg/dL
RBC	427 × 10^4/μL	D.Bil	0.7 mg/dL
Hb	15.1 g/dL	AST	33 IU/L
Hct	103.20%	ALT	15 IU/L
Plt	50.77 × 10^4/μL	LDH	228 IU/L
〈メタボリックプロフィール〉		ChE	319 IU/L
glucose	83 mg/dL	ALP	1,069 IU/L
NH_3	150 μg/dL	LAP	55 IU/L
		γ-GTP	98 IU/L
遊離カルニチン	55.4 μmol/L	CK	167 IU/L
アシルカルニチン	13.3 μmol/L	TP	5.8 g/dL
		ALB	4.1 g/dL
		T.Cho	151 mg/dL
		TG	151 mg/dL
		BUN	7 mg/dL
		CRE	0.23 mg/dL
		UA	1.4 mg/dL
		Na	138 mEq/L
		K	5.0 mEq/L
		Cl	105 mEq/L
		Ca	10.5 mg/dL

初診時および入院時検査：

末梢血液像・血液生化学一般検査では著変を認めず(表1)．血清カルニチン分画測定でも，遊離カルニチン低下やアシルカルニチン増加は認められず，正常パターンであった．血清でのアシルカルニチン分析再検(福井大学医学部看護学科　重松陽介先生に依頼)，尿中有機酸分析(福井大学医学部看護学科　重松陽介先生に依頼)を提出のうえ，精査のため入院．胸部単純X線撮影，心電図，心臓超音波検査，頭部MRIのいずれも著変なし．腹部超音波検査では両側卵巣に複数の嚢腫像を認めたが，血清LH, FSH, HCG, AFPはいずれも正常レベルであった．

2 確定診断までの経過

a. 鑑別診断 ……

a)タンデムマス新生児スクリーニング陽性例

b)濾紙血中の C4 ～ C14 アシルカルニチンが増加

c)臨床症状なし

短鎖から長鎖にわたる複数の炭素鎖長のアシルカルニチンが増加する特徴的なパターンから，グルタル酸尿症 II 型が疑われた．

b. 確定診断のための検査 ……

1)血清でのアシルカルニチン分析(表 2，図 1)

初回スクリーニング時の濾紙血と同様，C4 ～C14 のアシルカルニチン増加が認められた．

2)尿中有機酸分析(図 2)

短鎖～長鎖脂肪酸のアシル CoA 脱水素反応の障害を反映するエチルマロン酸(C5)，アジピン酸(C6)，ズベリン酸(C8)，ズベリルグリシン(C8)の増加と，有機酸代謝経路の脱水素反応の障害を反映するグルタル酸，2-ヒドロキシグルタル酸の増加が認められ，グルタル酸尿症 II 型と化学診断された．

3)他の確定検査法

本疾患では典型的な尿中有機酸異常が確定的所見となるが，分子生物学的な確定検査法としては electron transfer flavoprotein(ETF) および ETF dehydrogenase のイムノブロットや遺伝子解析があげられる．本症例では検索していない．

診断名

グルタル酸尿症 II 型

3 確定診断後の治療と経過

グルタル酸尿症 II 型の一部にはリボフラビン反応型の症例があることから，初診時の検体採取後よりリボフラビン投与を開始．精査入院中に行った経時的血糖測定では，授乳後 5 時間での血糖 60 mg/dL が最低値であった．その後のアシルカルニチン分析再検ではリボフラビンによる改善効果を認めず，生後 2 ヶ月で投与中止．以後はカルニチン内服，低脂肪ミルクによる頻回授乳，在宅自己血糖測定，体調不良時の早めの受診を指示した．

生後 7 ヶ月時，感染徴候などの誘因なく，早朝にけいれん発作を起こして直近の小児科医院を救急受診．血糖 0 mg/dL となっていたが，直ちに輸液療法開始のうえ，二次救急病院小児科へ転送され，後遺症なく回復した(図 3)．このほか，生後 6 ヶ月以降の約 3 年間に，気道感染症状や胃腸炎症状などに際して輸液療法が計 9 回行われたが，いずれも低血糖は観察されなかった．3 歳 6 ヶ月より保育所に入所したところ，昼食前の血糖低下傾向(40 ～ 60 mg/dL 程度)が繰り返し観察されたが，3 歳 9 ヶ月以降はみられなくなった．以後，6 歳 3 ヶ月現在まで急性代謝不全のエピソードはなく，身体発育・精神運動発達いずれも良好である．

表 2　血中アシルカルニチン分析結果

	アシルカルニチン(nmol/mL)									
	F-carnitine	C2	C4	C5	C6	C8	C10	C12	C14	diC5*
正常参照値	36 ～ 74	10.7 ± 8.8	＜ 0.2	＜ 0.5	＜ 0.2	＜ 0.2	＜ 0.2	＜ 0.2	＜ 0.2	＜ 0.5
初回濾紙血	37.2	16.7	0.48	0.5	0.49	0.75	1.01	1.42	1.32	0.44
再検血清	48.0	13.0	0.49	1.1	0.42	0.74	1.36	0.79	0.32	0.85

＊：diC5 ＝グルタル酸(炭素鎖数 5 のジカルボン酸)のアシルカルニチン．

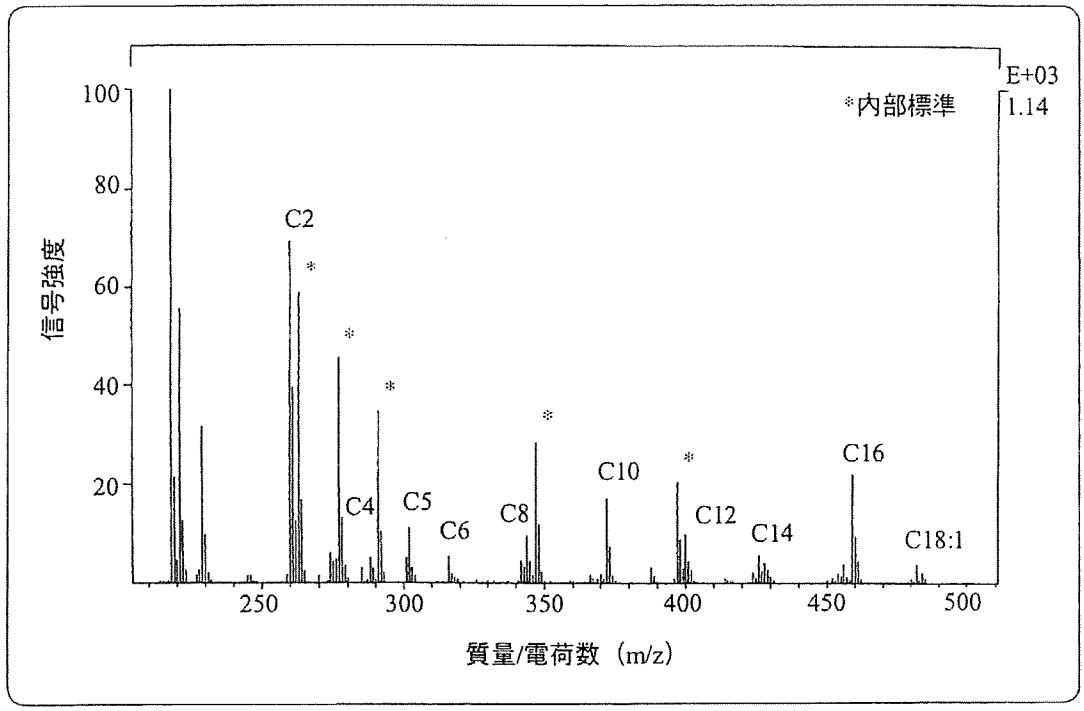

図1　血清アシルカルニチン分析

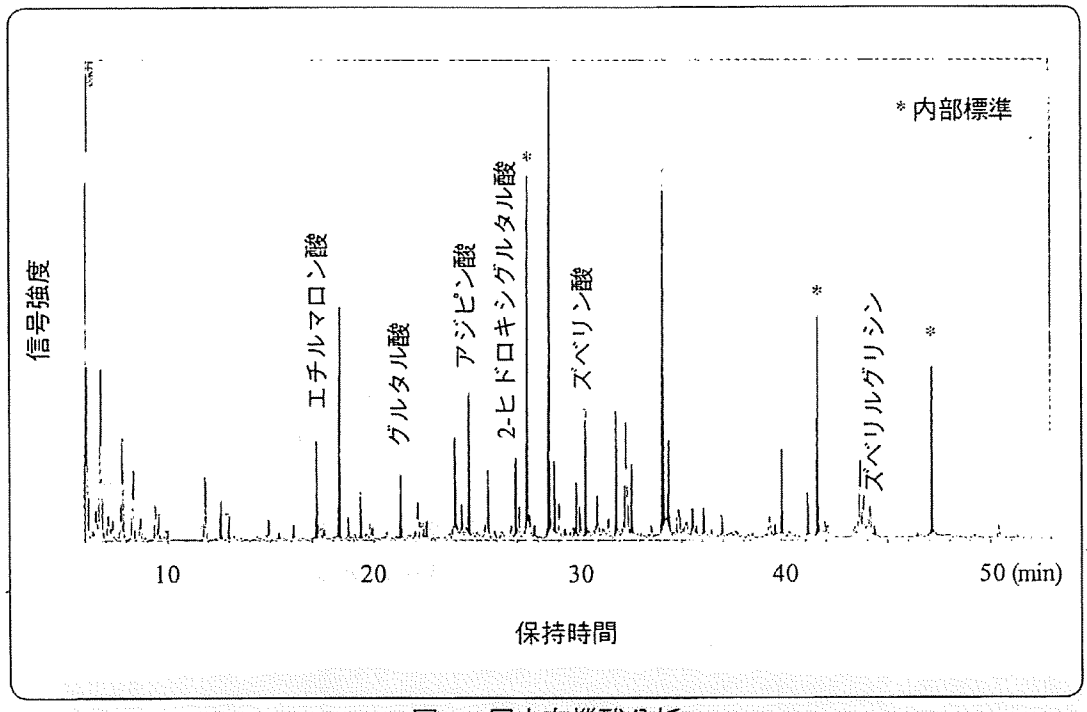

図2　尿中有機酸分析

4 本症例を経験して

この女児はタンデムマス新生児スクリーニング試験研究で発症前診断されたグルタル酸尿症II型のわが国での初例であり，代謝不全によるSIDS(sudden infant death) / ALTE(apparent life-threatening event)を起こさせないために，考えられる一通りの方策を講じた．これまでのところ，感染症罹患時などの低血糖症は予防できたといえる経過だが，本疾患をはじめ，脂肪酸β酸化異常症の急性発症として典型的な，夜間の授乳間隔が延びてくる乳児期後半の早朝低血糖発作を完全に防ぐことはできなかった．しかし，このエピソードが後遺症なくレスキューさ

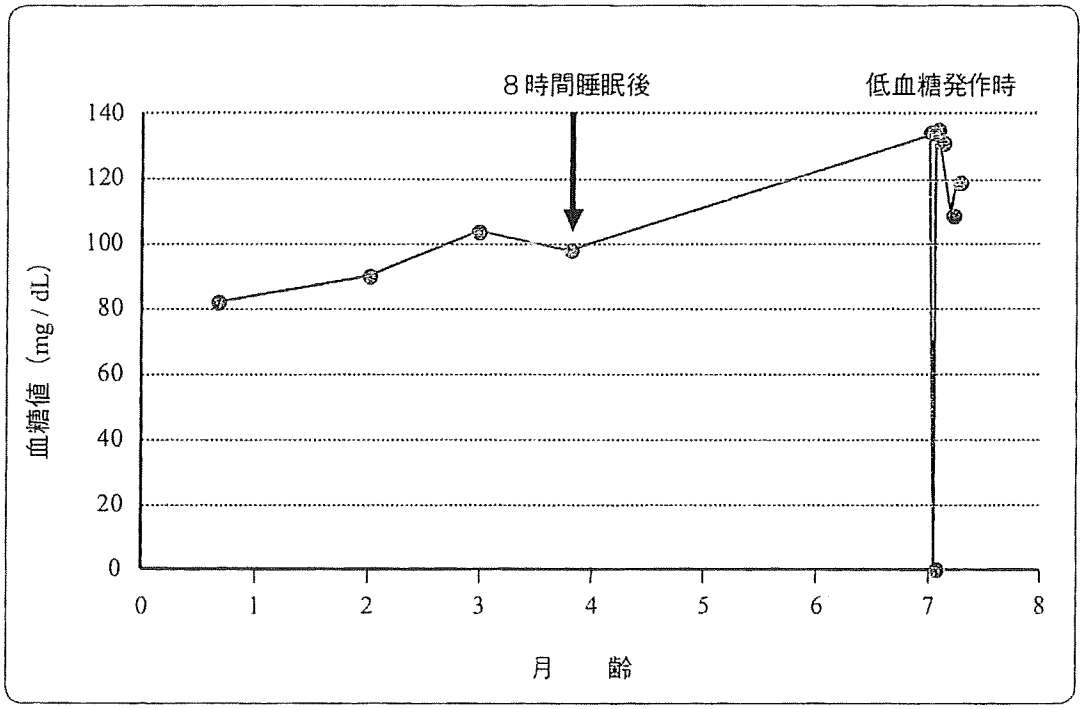

図3　乳児期の血糖測定値

れたのには，急性発症の危険性とその際の対応について，保護者・直近の小児科医院・二次救急病院小児科にあらかじめ十分な説明を行い，スムーズな連携をとれたことが大きく寄与しているものと考えられる．

発症後に診断された日本人グルタル酸尿症II型15症例に関する文献によると，新生児期発症の重症型4例全例とリボフラビン反応型2例を除く遅発型9例のうち3例が死亡し，後遺症なく生存しているのは5例と報告されており[1]，対応の遅れは重篤な転帰に直結するものと考えておかなければならない．2005年よりタンデムマス新生児スクリーニングの施行地域が拡大し，同じ疾患の罹患児でも，出生する地域によって待ち受ける運命が全く異なる，という事態が現実化している[2,3]．運よく発症前に診断された症例については，まずリボフラビン投与を試み，効果が認められなければ頻回食による飢餓状態の回避(特に夜間に注意)と，体調不良時の速やかな病院受診・輸液療法について厳重に指導することで，急性代謝不全による悲惨な結果を確実に防ぎたい．また，急性脳症や低血糖発作，SIDS/ALTEなどの症例に遭遇した場合は，血中アシルカルニン分析と尿中有機酸分析を必ず行い，診断漏れのないようにすることが求められる．

1) Yotsumoto Y, *et al.*: Clinical and molecular investigation of Japanese cases of glutaric acidemia type 2. *Mol Genet Metab* 2008；**94**：61-67
2) 重松陽介：広がりはじめたタンデムマス・スクリーニングの現況．日本マス・スクリーニング学会誌 2007；**17**：19-24
3) 北川照男，他：タンデムマススクリーニングで発見される有機酸・脂肪酸代謝異常症の早期診断・治療ステムの検討．特殊ミルク情報 2008；**44**：35-59

1) 山口清次：グルタル酸尿症2型．松本勇(監修)，臨床化学診断学．ソフトサイエンス社，1995；108-112

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ORIGINAL PAPER

Intractable secretory diarrhea in a Japanese boy with mitochondrial respiratory chain complex I deficiency

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Abstract The etiology of secretory diarrhea in early life is often unclear. We report a Japanese boy who survived until 3 years of age, despite intractable diarrhea commencing soon after birth. The fecal sodium content was strikingly high (109 mmol/L [normal range, 27–35 mmol/L]) and the osmotic gap was decreased (15 mOsm/kg), consistent with the findings of congenital sodium diarrhea. We examined the mitochondrial respiratory chain function by blue native polyacrylamide gel electrophoresis (BN-PAGE) in-gel enzyme staining, BN-PAGE western blotting, respiratory chain enzyme activity assay, and immunohistochemistry. Liver respiratory chain complex (Co) I activity was undetectable, while other respiratory chain complex activities were increased (Co II, 138%; Co III, 153%; Co IV, 126% versus respective control activities). Liver BN-PAGE in-gel enzyme staining and western blotting showed an extremely weak complex I band, while immunohistochemistry showed extremely weak staining for the 30-kDa subunit of complex I, but normal staining for the 70-kDa subunit of complex II. The patient was, therefore, diagnosed with complex I deficiency. The overall complex I activity of the jejunum was substantially decreased (63% of the control activity). The immunohistochemistry displayed apparently decreased staining of the 30-kDa complex I subunit, together with a slightly enhanced staining of the 70-kDa complex II subunit in intestinal epithelial cells. These data imply that intestinal epithelial cells are also complex I-deficient in this patient. Complex I deficiency is a novel cause of secretory diarrhea and may act via disrupting the supply of adenosine triphosphate (ATP) needed for the maintenance of ion gradients across membranes.

Keywords Intractable secretory diarrhea · Mitochondrial respiratory chain disorder · Complex I deficiency

Abbreviations

BN-PAGE	Blue native polyacrylamide gel electrophoresis
Co	Complex
CS	Citrate synthase

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CMVA Congenital microvillous atrophy
ATP Adenosine triphosphate

Introduction

Congenital secretory diarrhea with an onset in early life is almost always severe and is often life threatening [1, 3, 6–8, 11, 14]. To date, mutations in the $Cl^-/HCO3^-$ exchanger gene (SLC26A3) are the only documented genetic cause of congenital chloride diarrhea [7, 8]. On the other hand, the number of newborns or early infants who present with secretory sodium diarrhea with voluminous alkaline stools containing high concentrations of sodium leading to hyponatremia and profound metabolic acidosis has been increasing [3, 8, 11]. However, the mechanisms underlying the development of sodium diarrhea have not been fully elucidated, with the exception of congenital microvillous atrophy (CMVA), which is histologically characterized by microvillous inclusion bodies and periodic acid-Schiff (PAS)-positive granules in intestinal epithelial cells [11, 14].

Here, we report a boy with mitochondrial complex I deficiency who presented with sodium diarrhea directly after birth. The possible contribution of mitochondrial respiratory chain disorders to the development of secretary diarrhea is discussed.

Case report

The patient, a Japanese boy, was born at term weighing 3,202 g as the second child to healthy parents with no consanguinity. His elder sister is entirely healthy. There is no family history of bowel disease or neurological disease. The pregnancy was not complicated by polyhydramnios. Soon after birth, he developed severe watery diarrhea and was admitted to our hospital at the age of 4 days. On admission, he was drowsy and his weight was strikingly decreased (−23.4% of birth weight). Blood gas analysis showed profound metabolic acidosis, together with high plasma concentrations of sodium, potassium, and chloride (Table 1). Blood pH and electrolytes were corrected within a few days by an intravenous drip infusion of the appropriate solution. Thereafter, despite the discontinuation of enteral nutrition, the severe diarrhea with voluminous stools never improved, and the plasma sodium level continued to decrease. To maintain a plasma sodium level in the normal range, 11–19 mmol/kg/day of sodium as a solution was administered intravenously. Stool analysis at 1 month after the admission revealed high sodium and chloride contents, high pH, and low osmotic gap. On the other hand, the sodium content of his urine was somewhat low, while the chloride content was high (Table 1). These findings were compatible with secretory sodium diarrhea [3, 8, 11]. The pathogens that possibly cause diarrhea were repeatedly negative in stools. Endocrinological disorders or tumors associated with diarrhea were also never detected. Microscopic and electron microscopic examinations revealed villous atrophy, but did not show any other findings suggesting the etiology of the secretary diarrhea [14]. At the age of 3 months, he underwent total parenteral nutrition, leading to weight gain. Nevertheless, sufficient weight gain was not achieved (Fig. 1). After the age of 1 year, multiple bone deformities, such as oar-shaped ribs and egg-shaped vertebrae became apparent, and, simultaneously, he began to develop recurrent bone fractures in the lower limbs. From the age of 2 years, he developed involuntary horizontal and vertical movements of the bilateral eyeballs, so-called opsoclonus. At the age of 3 years, he suddenly died of septic shock resulting from catheter infection and subsequent adrenal hemorrhage.

Throughout the course of the patient, his blood lactate level was almost normal (11–20 mg/dl; normal 7–16 mg/dl) and other metabolic screening tests, such as plasma amino acid profiles, never showed findings suggestive of congenital metabolic disorders. Nevertheless, the clinical manifestations involving multiple organs led us to suspect a mitochondrial disorder [12].

Methods

Expression of the mitochondrial respiratory chain complex I, II, III, and IV proteins was examined by in-gel enzyme staining and western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to methods described previously [5, 16]. Gels were loaded with 10 μg of protein from enriched mitochondria of the liver and intestine. Concurrently, activities of respiratory chain complexes I, II, III, and IV were assayed as described previously [9, 15]. In these assays, citrate synthase (CS) was employed as a mitochondrial enzyme marker. This method was performed using crude post-600xg supernatants from each tissue.

Immunohistochemical staining was performed on paraffin-embedded sections. After microwave treatment at 95°C for 20 min in citrate buffer pH 6.0, endogenous peroxidase activity was blocked with 3% H_2O_2 for 20 min. Sections were first incubated with a primary antibody against complex I 30-kDa subunit (MS110, MitoSciences, Eugene, OR, USA) (diluted 200-fold) and complex II 70-kDa subunit (MS204, MitoSciences) (diluted 1,000-fold) for overnight incubation at 4°C and visualized by a labeled streptavidin-biotin method with LSAB2 kit and chromogen diaminobenzidin tetrahydrochlorid (Dako, Carpinteria, CA, USA).

Table 1 Blood, fecal, and urinary electrolytes on admission and gastrointestinal hormones

Characteristic	Value	Characteristic	Value
Weight (kg)*	2.43	**Urinary electrolytes****	
Height (cm)*	50	sodium (meq/L) (2–26)	32
Biochemistry*		potassium (meq/L) (1–16)	51
pH	7.105	chloride (meq/L) (3–39)	46
bicarbonate (meq/L) (3.6–5.0)	11		
pCO_2 (mmHg) (35–45)	30	**Fecal electrolytes****	
sodium (meq/L) (136–147)	151	sodium (mmol/L) (27–35)	109
potassium (meq/L) (3.6–5.0)	5.8	potassium (mmol/L) (71–79)	25
chloride (meq/L) (98–109)	111	chloride (mmol/L) (14–18)	68
calcium (mg/dl) (8.7–10.1)	9.6	bicarbonate (mmol/L) (36–44)	53
phosphate (mg/dl) (2.4–5.3)	5.9	osmotic gap (mOsm/kg)†	15
plasma osmolality (mOsm/kg/m) (276–292)	287	pH (6.5–7.4)	7.6
blood urea nitrogen (mg/dl) (8–16)	33		
creatinine (mg/dl) (0.1–0.4)	0.77	**Gastrointestinal hormones****	
aspartate aminotransferase (IU/L) (12–44)	20	serum gastrin (pg/mL) (40–140)	64
alanine aminotransferase (IU/L) (2–37)	21	serum VIP (pg/mL) (50 – 100)	16
total protein (g/dl) (6.4–7.6)	6.7	serum glucagon (75–125)	77
albumin (g/dl) (4.2–5.1)	4.7	prostaglandin (pmol/L) (59–280)	229
blood sugar (mg/dl) (60–105)	144	urinary VMA (mg/day) (1.3–5.7)	1.7
C-reactive protein (mg/dl) (<0.3)	0.1	urinary HVA (mg/day) (1.5–6.6)	3.1

Normal ranges in parentheses
VIP=vasoactive intestinal polypeptide; VMA=vanillyl mandelic acid; HVA=homovanillic acid
†Osmotic gap was calculated as 290-2(Na+K)
*Data on admission
**Data after the correction of electrolytes by intravenous treatment

Total DNA was extracted from both liver and intestine samples using standard procedures. The direct sequencing of all seven mitochondrial DNA-encoded complex I genes was performed as previously described [17], using M13-tagged polymerase chain reaction (PCR) primers and BigDye terminator cycle sequencing chemistries (Applied Biosystems, Foster City, CA, USA).

Results

Liver respiratory chain complex I was shown to be deficient in this patient by four independent methods: (i) BN-PAGE in-gel enzyme staining showed decreased protein levels of complex I in the regular (900-kDa) and supercomplex forms (Fig. 2A); (ii) BN-PAGE western blotting showed findings consistent with those of BN-PAGE in-gel enzyme staining (Fig. 2B); (iii) respiratory chain enzyme assay showed that complex I activity was undetectable in this patient, while activities of complexes II, III, IV, and citrate synthase were normal or somewhat increased (Table 2); (iv) the immunohistochemistry showed substantial steatosis and a decreased amount of complex I 30-kDa subunit but a normal amount of complex II 70-kDa subunit (Fig. 3A–D).

In contrast to liver complex I activity, the jejunum complex I activity was only moderately decreased (Table 2). The immunohistochemistry of the jejunum suggested a slightly increased amount of complex II staining in epithelial cells and moderately decreased staining of complex I protein, implying a relative decrease in complex I protein (Fig. 3E–J).

Discussion

Mitochondrial respiratory chain disorders present an extraordinary diversity of clinical manifestations, affecting most organ systems, alone or in combination, and with almost any age of onset [12]. Accordingly, it is not surprising that these disorders could be associated with gastrointestinal diseases presenting as diarrhea. However, there are only limited reports describing the associations between mitochondrial disease and chronic diarrhea. Cormier-Daire et al. reported a patient with a mitochondrial DNA deletion presenting as chronic secretory diarrhea, in whom villous atrophy was present without the histological features of CMVA, such as microvillous inclusion bodies [4].

Congenital sodium diarrhea has been postulated to result from a defective sodium/proton exchanger localized in the apical membrane of small intestinal cells [3, 8, 11]. However, no mutation could be identified in genes

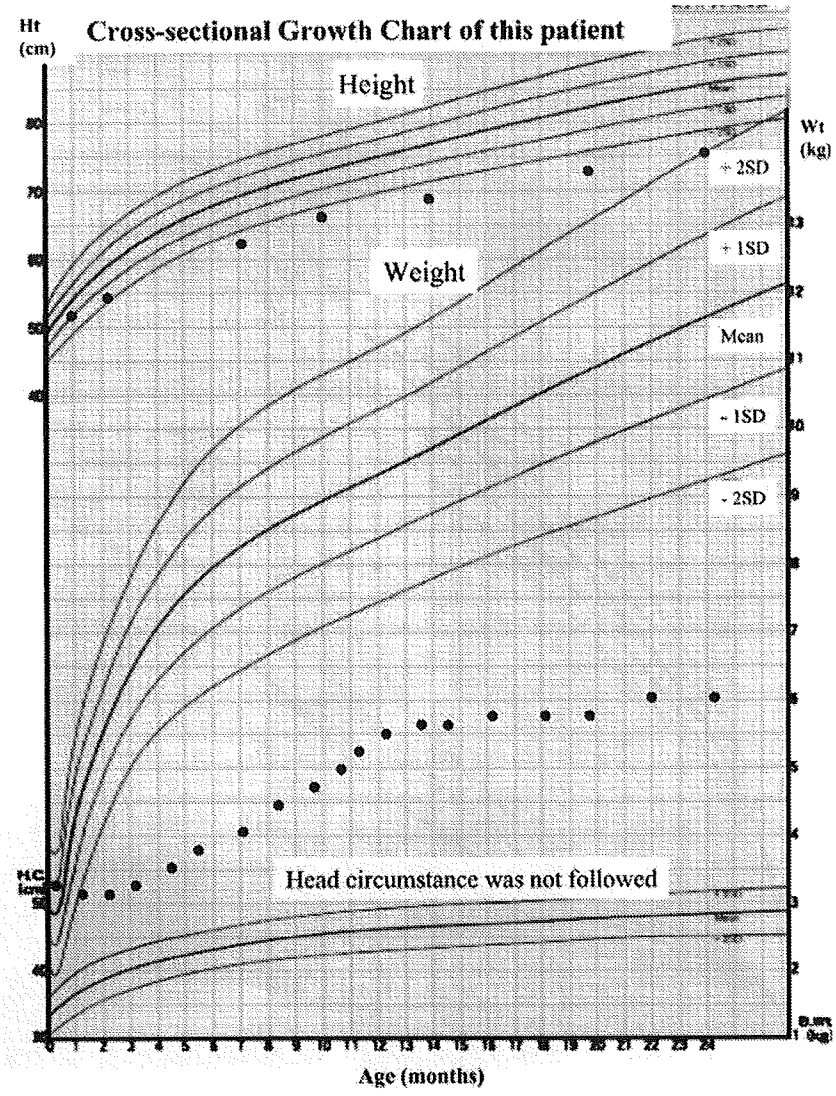

Fig. 1 Growth chart of the patient

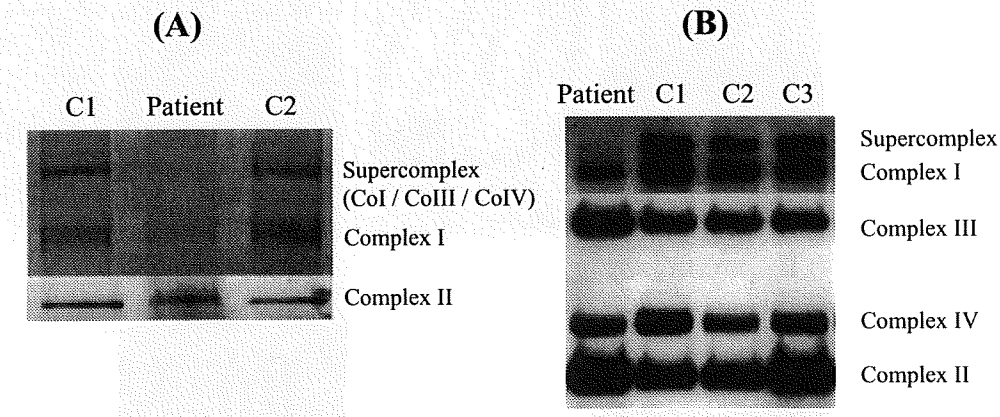

Fig. 2 Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of liver respiratory chain enzymes by: **a** in-gel enzyme staining of complexes I and II, and **b** immunoblot analysis of complexes I, II, III, and IV. In-gel enzyme staining showed markedly decreased complex I activity in both the complex I and supercomplex bands compared with complex II in this patient. Western blotting used antibodies specific to the complex I 30-kDa subunit, complex II 70-kDa subunit, complex III core subunit, and complex IV COX1 subunit. This showed markedly decreased protein levels of complex I and supercomplex, while the protein levels of complex II, III, and IV were comparable to control samples

Table 2 Respiratory chain enzyme assay of this patient

%	Co I	Co II	Co III	Co IV	CS
Liver					
% of normal	0	138	153	126	321
CS ratio	0	43	47	39	–
Co II ratio	0	–	112	91	–
Intestine					
% of normal	63	162	76	118	98
CS ratio	64	165	78	120	–
Co II ratio	39	–	47	73	–

Co I=complex I; Co II=complex II; Co III=complex III; Co IV=complex IV; CS=citrate synthase
Enzyme activities are expressed as % of mean normal control activity relative to protein, relative to CS, and relative to Co II

encoding known sodium/proton exchangers [11]. Adenosine triphosphate (ATP) is required either directly or indirectly to drive sodium/proton exchange. The patient described here suggests that a defect of the respiratory chain generating ATP via oxidative phosphorylation in the intestine might cause impairment of sodium/proton exchangers.

Our patient had a definite diagnosis of complex I deficiency based on four independent analyses of complex I activity and amount in the liver, but complex I deficiency in the intestine was not so dramatic. Intuitively, one might expect that complex I deficiency should be markedly deficient in the intestine if it is causing the secretory diarrhea. However, such a discrepancy could be explained by the tissue-specificity of complex I activity. The intestine consists of many components, such as smooth muscle, that may have normal complex I activity. In addition, villous atrophy is a consequence of the loss of the most distal villous epithelial cells, which may have had lower complex I activity. Finally, the immunohistochemistry measures the amount of only one of the 45 complex I subunits and may not reflect the amount of other subunits and overall activity in intestinal epithelial cells. For these reasons, such results could be consistent with complex I deficiency being causative.

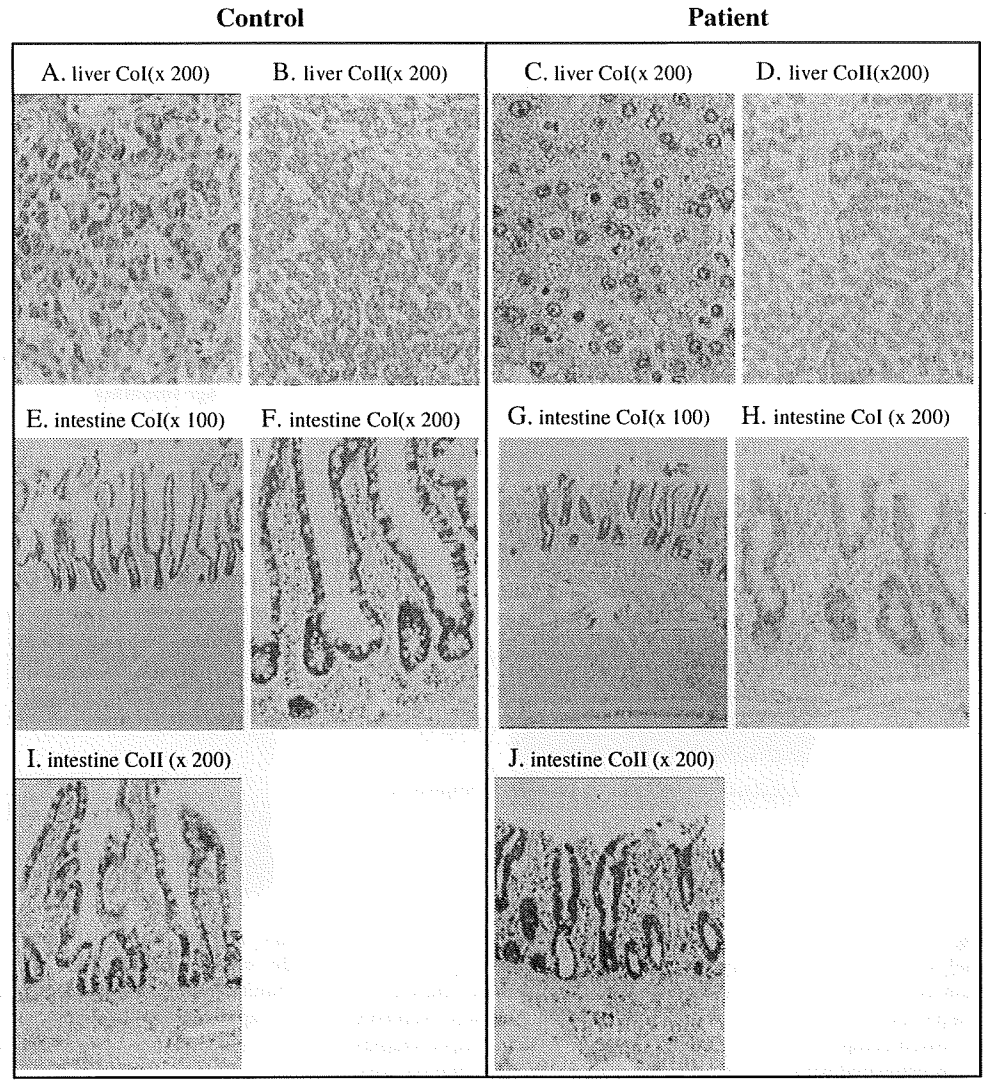

Fig. 3 Immunostaining of 30-kDa complex I and 70-kDa complex II subunits in the liver and small intestine. Patient and control liver and intestine samples were immunostained with either 30-kDa complex I or 70-kDa complex II antibodies. The patient liver shows markedly decreased complex I and moderately increased complex II staining. The patient intestine shows villous atrophy and moderately decreased complex I staining in epithelial cells, with slightly increased complex II staining

Lactic academia is the classic biochemical finding of mitochondrial disease, but Kirby et al. reported that the blood lactic acid level was normal in about 20% of patients with complex I deficiency [9]. Our patient usually showed normal blood lactate level, and the major symptom in the first year of life was only secretary diarrhea. Nevertheless, the additional appearance of other clinical manifestations, such as bone deformity and opsoclonus, prompted us to suspect mitochondrial disease.

The mitochondrial respiratory chain consists of many complex subunits, and, therefore, the molecular diagnosis of respiratory chain disorders is often very difficult. For example, complex I has 45 subunits, and 17 of these subunit genes plus two complex I assembly genes have been shown to have mutations causing human disease [2, 13, 18]. The known genes only appear to account for perhaps half of all patients with complex I deficiency, so further disease genes remain unknown [10]. We could not find any mutations in this patient in the seven complex I subunits encoded by mitochondrial DNA, so the complex I defect is likely to be caused by a mutation in a nuclear gene.

Recently, BN-PAGE immunoblotting and enzyme staining have been shown to be powerful diagnostic tools for mitochondrial disorders. We confirmed their utility in studying this patient, whose secretory sodium diarrhea appears to be caused by respiratory chain complex I deficiency. More investigation is required in order to acquire a better understanding of gastrointestinal disease involving sodium diarrhea in mitochondrial respiratory chain disorders.

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Fluctuating liver functions in siblings with MPV17 mutations and possible improvement associated with dietary and pharmaceutical treatments targeting respiratory chain complex II

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ABSTRACT

Background/aims: To describe the clinical and biological findings of two Japanese siblings with novel MPV17 gene mutations (c.451insC/c.509C > T) manifesting hepatic mitochondrial DNA depletion syndrome.
Methods: We observed these brothers and sought to determine the efficacy of treatment targeting respiratory chain complex II for the younger brother.
Results: A 3-month-old boy had presented with profound liver dysfunction, failure to thrive, and watery diarrhea. Although he was then placed on a carbohydrate-rich diet, his liver function thereafter fluctuated greatly in association with viral infections, and rapidly deteriorated to liver failure. He underwent liver transplantation at 17 months of age but died at 22 months of age. The younger brother, aged 47 months at the time of this writing, presented with liver dysfunction from 8 months of age. His transaminase levels also fluctuated considerably fluctuations in association with viral infections. At 31 months of age, treatment with succinate and ubiquinone was initiated together with a lipid-rich diet using ketone milk. Thereafter, his transaminase levels normalized and never fluctuated, and the liver histology improved.
Conclusions: These cases suggested that the clinical courses of patients with MPV17 mutations are greatly influenced by viral infections and that dietary and pharmaceutical treatments targeting the mitochondrial respiratory chain complex II may be beneficial in the clinical management of MPV17 mutant patients.

Introduction

MPV17 is a mitochondrial gene encoded by a nuclear gene [1]. Its mutations cause mitochondrial DNA depletion syndrome (MDS)[2], presenting multiple mitochondrial respiratory chain depletions in the manner of autosomal recessive inheritance [1–5]. Recently, the occurrence of patients with MPV mutations has been increasing; the majority of patients have developed liver disease within a few months after birth, with rapid deterioration to liver failure, while the remaining patients have shown relatively slow progression of liver disease or neurological regression [1–5].

However, the number of patients with MPV17 mutations is still small, and the clinical courses according to the mutations or the genotype- phenotype correlation remain unclear. Further, the appropriate internal therapy has yet to be established, although Parini and colleagues recently reported that glucose administration to avoid hypoglycemia is efficient in slowing the progression of liver disease [5].

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[2] *Abbreviations used:* MDS, mitochondrial DNA depletion syndrome; RC, respiratory chain; Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.

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In this report, we present the clinical courses of two siblings with novel mutations, c.451insC and c.509C > T, whose liver functions greatly fluctuated according to their respective viral infections. The beneficial effect of treatment targeting mitochondrial respiratory chain (RC) complex (Co) II, including succinate and coenzyme Q, is also described.

Patients and methods

Cases

Case 1: A Japanese boy born as the second child to unrelated healthy parents. Their first son is healthy. The second son, on the other hand, was born without any complications at 37 weeks of gestation age, and weighed 3060 g. At 3 months of age, he was referred to our hospital to receive precise examinations for failure to thrive, hypotonia, mild jaundice, and creamy stools. He had moderate head lag and incomplete head control. He maintained good eye contact and had a sociable smile; he had no seizures or clinical signs of peripheral neuropathy except for moderate hypotonia. Findings of brain computed tomography were normal. The liver was soft and palpated at 4.5 cm below the costal margin with no splenomegaly. Laboratory tests then showed elevated levels of serum bilirubin, total bile acid (TBA), transaminases, and gamma-glutamyl transpeptidase (GGT) as well as prolonged coagulation time (total bilirubin 4.2 mg/dl; direct bilirubin 2.7 mg/dl; TBA 362 μmol/L; GGT 178 IU/L; AST 173 IU/L; ALT 58 IU/L; hepaplastin time 38%). He had no episode of hypoglycemia (his blood glucose level was 65 mg/dl). Simultaneously, low body weight and height were prominent (body weight 4610 g, −2.7 SD; height, 59 cm, −1.8 SD). His plasma amino acid profile and urinary organic acid profile did not suggest any etiology for liver disease, but viral examinations detected an IgM cytomegalovirus (CMV)-specific antibody in the plasma. His liver functions thereafter are shown in Fig. 1 (upper panel).

A liver biopsy specimen obtained at 4 months of age showed moderate inflammatory cell infiltration with destroyed limiting plates and fibrosis in the portal tracts (Fig. S1A1). Two different types of degenerated hepatocytes were found: swollen hepatocytes containing lipid droplets of various sizes with occasional formation of multinuclear giant cells, and small, concentrated acidophilic hepatocytes. Bile plugs were noticed in the cytoplasm of hepatocytes and dilated canaliculi, consistent with the findings of cholestasis (Fig. S1A2).

Immediately during his first visit to our hospital, he was fed with medium-chain triglyceride (MCT) milk (100–105 kcal/kg/day; lipid 25%, carbohydrate 56.6%, protein 13.2%, eight times per day) and received fat-soluble vitamins. His liver dysfunction improved at 7 months of age when the cytomegalovirus-IgM antibody became undetectable. However, 1 month later, he began to frequently vomit, and tube feeding was initiated. Thereafter, he developed recurrent bouts of jaundice and elevations of transaminases accompanied by flu-like signs such as nasal discharge and cough, and his liver dysfunction deteriorated to liver failure with cirrhosis (Fig. S1B shows his liver histology at 15 months of age). He underwent a liver transplantation at 17 months of age but died of recurrent peritonitis and the resultant sepsis at 22 months of age. Afterward, the liver specimens obtained through the explantation, which histologically showed cirrhosis, were subjected to mitochondrial RC examinations when his younger brother received RC examinations.

Case 2: The younger brother of case 1 was born, 15 months after his elder brother died, without any complications at 40 weeks of gestation, with a weight of 3260 g. At the age of 8 months he was referred to our hospital with failure to thrive (height, 62 cm, −3.5 SD; weight 5.5 kg, −3.1 SD) and mild cholestasis. He could sit up alone, but could not crawl yet. He had no seizure and no clinical sign of peripheral neuropathy except for mild hypotonia. He could appropriately respond to changes in emotional content of social interaction. His liver and spleen were not palpated below the costal margin. Liver function tests then showed mild or moderate elevations of serum AST, ALT, GGT, and TBA levels (AST, 150 IU/L; ALT 40 IU/L; GGT, 82 IU/L; TBA 40 μmol/L). The coagulation tests had normal results. He had no episode of hypoglycemia (his blood glucose level was 64 mg/dl). His plasma lactate and pyruvate levels were slightly elevated (lactate, 2.2–3.3 mmol/L; pyruvate 0.1–0.2 mmol/L), but his cerebrospinal fluid lactate and pyruvate levels were entirely normal. His amino acid profile in blood and organic acid profile in urine showed no finding suggesting an etiology, and serological viral tests were all negative. Brain magnetic resonance imaging (MRI) was normal at 9 months of age. His liver functions thereafter are shown in Fig. 1 (lower panel).

Liver histology at 8 months of age displayed mild infiltration of inflammatory cells, fibrosis in the portal tracts, and various-sized lipid droplets in hepatocytes, comparable to the histological findings of his elder brother. He was placed on a carbohydrate-rich, lipid-poor diet using MCT milk (100 kcal/kg/day; lipid 25%, carbohydrate 56.6%, protein 13.2%, eight times per day by nasogastric tube) as his elder brother had been, and was treated with fat-soluble vitamins and ursodeoxycholic acid.

His liver dysfunction thereafter fluctuated as a result of upper respiratory infections, probably due to some viruses, and diarrhea due to rotavirus infection (Fig. 1, lower panel).

Liver histology at 30 months of age showed a rather progressive fibrosis with bridging formation and inflammatory cell infiltration compared with that of the previous biopsy (Fig. S1C). The activity levels of mitochondrial RC Co I and III were decreased in this liver sample, whereas Co II activity remained normal (Table 1). Quantitative PCR revealed a decrease in the amount of mitochondrial DNA, and the boy was diagnosed with MDS. Gene analysis revealed that he is a compound heterozygote for mutations in the MPV17 gene responsible for MDS.

At 31 months of age, he began medication with carnitine (300 mg/day), succinate (2 g/day), and ubiquinone (coenzyme Q10: 30 mg/day). Simultaneously, the carbohydrate-rich, lipid-poor diet using MCT milk was changed to a lipid-rich, carbohydrate-poor diet using ketone milk (lipid 71.8%, carbohydrate 8.8%, protein 15%) and MCT milk (total: 90–100 kcal/kg/day; lipid 56.3–60.1%, carbohydrate 20.8–24.6%, protein 14.4–14.6%, eight times per day by nasogastric tube). After the initiation of these dietary and pharmaceutical treatments, his transaminases decreased to normal (Fig. 1, lower panel).

At the age of 37 months, a liver needle biopsy was again performed; infiltration by inflammatory cells decreased and fibrosis was suppressed, but fatty degeneration remained unchanged (Fig. S1D).

Recently, his body weight and height have increased gradually but are still low (body weight 10.3 kg, −2.6 SD; height 81.5 cm, −4.5 SD). Brain MRI was normal at 42 months of age. At the time of this writing, he is 47 months old, and shows normal psychomotor development.

Materials and methods

Samples for the examination of respiratory chains

The liver sample from the elder brother was the excised part of the liver transplantation at 17 months of age. Liver samples from the younger brother were obtained by liver biopsies at 30 and 37 months of age.

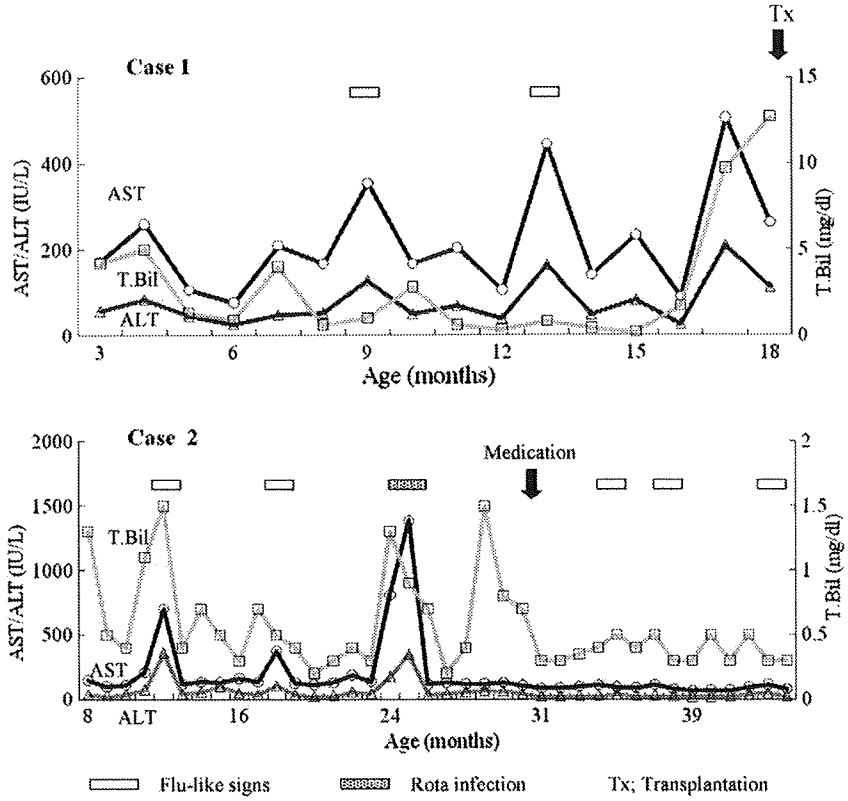

Fig. 1. Clinical courses of cases 1 (upper panel) and 2 (lower panel).

Determination of enzyme activities

Activities of RC Co I, II, III, and IV were assayed for the crude post-600 g supernatant of the liver samples as described previously [6,7]. The activity of each complex was presented as a percentage of the mean value obtained from 35 healthy controls. For each patient, the percentages of Co I, II, III, and IV activities relative to that of citrate synthase (CS) as a mitochondrial enzyme marker or Co II activity were calculated [6].

BN-PAGE Western blotting

Expression levels of the mitochondrial RC Co I, II, III, and IV proteins in the liver were examined by Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to the methods described previously [8,9]. Ten micrograms of the protein in the mitochondria-enriched fraction was separated by BN-PAGE. Immunostaining was performed using a monoclonal antibody specific for the 39 kD subunit of Co I, the 70 kD subunit of Co II, the core 1 subunit of Co III, and the subunit 1 of Co IV (Molecular Probes, Eugene, OR).

Quantitative PCR

mtDNA was quantitatively estimated by the real-time amplification of fragments of ND1 in the mtDNA genome, as previously described [10,11]. To determine the overall abundance of mtDNA, we compared the real-time amplification of ND1 with a single-

Table 1
Enzyme assay of respiratory chain and quantitative mtDNA evaluation by qPCR.

%	Co I	Co II	Co III	Co IV	CS	mtDNA/nDNA (%)
Elder Brother (17 months)						7.8
% of normal	0	80	13	41	300	
CS ratio	0	27	4	14	–	
Co II ratio	0	–	16	50	–	
Younger Brother (30 months)						6.6
% of normal	22	80	34	83	397	
CS ratio	6	36	9	21	–	
Co II ratio	15	–	24	57	–	
Younger Brother (37 months)						
% of normal	23	170	28	75	254	
CS ratio	9	67	11	29	–	
Co II ratio	13	–	16	43	–	–

Co I; complex I, Co II; complex II, Co III; complex III, Co IV; complex IV, CS; citrate synthase.
Enzyme activities are expressed as % of mean normal control activity relative to protein, relative to CS, and relative to Co II.

copy nuclear reference gene (exon 24 of the CFTR gene, chosen because it lacks single-nucleotide polymorphisms). For both experiments, DNA from six adult liver samples (from needle biopsies, obtained with informed consent) was used as controls. The results presented were the means of four independent runs, with samples assayed in triplicate in each run.

Mutation detection

Genomic DNA was extracted from liver or peripheral blood leukocyte according to the standard procedures. Detailed sequencing methods appear in the supplemental materials.

Results

Enzyme activities

Both affected siblings showed low activity levels of RC Co I, III, and IV. In particular, their Co I activities were strikingly low. In contrast, their Co II activities were maintained at normal, and those of citrate synthase were greatly elevated (Table 1). Co III and Co IV activity levels were higher in the younger brother than those in the elder brother.

BN-PAGE Western blot analysis

Fig. S2 shows the RC Co amounts by BN-PAGE in each brother. In both brothers, the band corresponding to either assembled Co I or assembled Co IV was invisible, and the band corresponding to the assembled Co III was strikingly weak. On the other hand, the intensity of the Co II band remained normal in both brothers.

Quantitative PCR

Quantitative PCR revealed that liver mtDNA was markedly decreased in both brothers (Table 1). The ratio of ND1 to CFTR in the liver of each brother was lower than those of the six controls (mean ± SD: 7.8 ± 4.6% for the elder brother, 6.6 ± 1.5% for the younger brother).

Mutations in MPV17

Both brothers were confirmed to be compound heterozygotes for c.451insC/c.509C > T (Fig. S3). c.451insC in exon 6 causes a frame-shift predicting an elongated gene product p.Leu151fsX189 (p.Leu151PhefsX39, according to the standard mutation nomenclature guidelines at http://www.genomic.unimelb.edu.au/mdi/mutnomen/). The c. 509C > T in exon 7 causes an amino acid substitution (Ser170Phe). These variations had not registered as genetic polymorphisms in the ensembl_mart_47 database (martdb.ensembl.org) and had not been reported as disease-causing mutations. Moreover, the alignment shows that both amino acid residues (Leu151 and Ser170) mutated in the affected siblings are absolutely conserved in all species (Fig. S4). Therefore, we consider these variations to be novel mutations. A single allele of c.451insC was present in all three siblings and their mother, whereas c. 509C > T was detected in both affected siblings and their father (Fig. S3). The fact that two such mutations were inherited from each parent independently indicated that these mutations were compound heterozygous in both affected siblings. The parents and the unaffected sibling had only one mutation, and had no obvious phenotype (Fig. S3). These observations support an autosomal recessive manner of inheritance for the hepatic dysfunction phenotype segregating within this family.

Discussion

Both brothers had novel compound heterozygous mutations, c.451insC/c.509C > T, but their clinical courses differed greatly. The phenotype of the elder brother was classified as possibly the infantile form, characterized by early onset liver disease that rapidly progresses to liver failure within the first few years of life [4,5].

In contrast, the younger brother exhibited a rather mild course. His liver damage was relatively mild, and he did not show any apparent neurological abnormality.

Such a great difference in the clinical courses between these brothers might be explained, in part, by the differences in their RC activity levels. The degree of reduction in RC activity was generally milder in the younger brother than in the elder. However, several studies have shown that RC activities were not correlated with the clinical course [4,5].

In our MPV17 mutant patients, the fluctuations in liver function were associated with infections that may cause oxidative stress. It was likely that cytomegalovirus infection promoted the onset and progression of liver disease in the elder brother, and that the liver dysfunctions in these siblings were greatly exacerbated by viral infections, in particular rotavirus infection.

Taken together, our experiences with these cases allowed us to assume that the clinical course and prognosis of MDS caused by MPV17 mutations were determined not only by the mutation but also by other factors. We postulated that many complicating factors may arise, including infection.

An effective treatment for mitochondrial RC disorders involving MDS has yet to be established. Liver transplantation is not so promising [6,12–14]. Besides the surgical complication, neurological regression after transplantation has been reported. Collectively, the survival rate is less than 50% [14].

For the younger brother, we tried to administer medications targeting the RC system, including succinate and coenzyme Q. Simultaneously, a lipid-rich carbohydrate-restricted diet using ketone milk was initiated. This combined treatment improved his liver disease biochemically and histologically. However, his liver RC activities did not improve.

Initially, he received a carbohydrate-rich, lipid-restricted diet and fat-soluble vitamins, together with UDCA, as had his elder brother. Recently, Parini et al. reported that glucose administration to avoid hypoglycemia is efficient in slowing the progression of liver disease [5]. However, this dietary treatment did not achieve favorable effects for our patients. Therefore, we resorted to another treatment for the younger brother.

The efficacy of medications with succinate and coenzyme Q, together with a lipid-rich diet, was possibly explained by their biochemical features. The mitochondrial RC system comprises Co I, II, III, and IV. Co I activity was markedly reduced in the younger brother, while his Co III activity remained mildly or moderately decreased. On the other hand, his Co II activity was entirely normal. Co I, an electron and proton acceptor from NADH and H^+, respectively, is the most important reduction-type hydrogen carrier, generating ATP by glucose oxidation [13]. From this context, glucose should hardly have been used as an energy source in the liver of the younger brother. On the other hand, succinate might donate electrons and protons to Co II connected to ubiquinone via $FADH_2$ [15]. In addition, a lipid-rich diet was expected to donate electrons and protons to ETF (electron-transfer flavoprotein): QO (ubiquinone oxidoreductase) connected to ubiquinone by promotion of $FADH_2$ production.

In summary, these cases suggested that the clinical course of MPV17 mutation is not determined solely by the mutation but rather is greatly influenced by viral infection, and that medications targeting Co II, together with a lipid-rich diet, may be beneficial in the clinical management of patients with MDS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymgme.2009.04.014.

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Pyruvate therapy for Leigh syndrome due to cytochrome *c* oxidase deficiency

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ABSTRACT

Background: Recently we proposed the therapeutic potential of pyruvate therapy for mitochondrial diseases. Leigh syndrome is a progressive neurodegenerative disorder ascribed to either mitochondrial or nuclear DNA mutations.
Methods: In an attempt to circumvent the mitochondrial dysfunction, we orally applied sodium pyruvate and analyzed its effect on an 11-year-old female with Leigh syndrome due to cytochrome *c* oxidase deficiency accompanied by cardiomyopathy. The patient was administered sodium pyruvate at a maintenance dose of 0.5 g/kg/day and followed up for 1 year.
Results: The exercise intolerance was remarkably improved so that she became capable of running. Echocardiography indicated improvements both in the left ventricle ejection fraction and in the fractional shortening. Electrocardiography demonstrated amelioration of the inverted T waves. When the pyruvate administration was interrupted because of a gastrointestinal infection, the serum lactate level became elevated and the serum pyruvate level, decreased, suggesting that the pyruvate administration was effective in decreasing the lactate-to-pyruvate ratio.
Conclusions: These data indicate that pyruvate therapy was effective in improving exercise intolerance at least in a patient with cytochrome *c* oxidase deficiency.
General significance: Administration of sodium pyruvate may prove effective for other patients with cytochrome *c* oxidase deficiency due to mitochondrial or nuclear DNA mutations.

1. Introduction

Mitochondrial diseases are intractable disorders, including encephalomyopathy, cardiomyopathy, hearing or visual loss, and diabetes; and they are caused by either mitochondrial or nuclear DNA mutations. In spite of the research efforts for gene therapy aiming at removal of a specific mitochondrial DNA mutation by use of restriction enzymes, *e.g.*, SmaI or XmaI for the m.8993T>G mutation [1], definite therapies have not been established for mitochondrial diseases. The supplementation of vitamins and cofactors are not satisfactory except for a limited number of patients, such as those with thiamine-responsive pyruvate dehydrogenase complex deficiency [2] or those with defects in the biosynthetic pathway of coenzyme Q [3, 4]. Earlier we proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm, (b) pyruvate can activate the pyruvate dehydrogenase complex (PDHC) by inhibiting pyruvate dehydrogenase kinase, and (c) pyruvate can scavenge hydrogen peroxide by a non-enzymatic reaction [5].

Leigh syndrome (LS) is an early-onset progressive neurodegenerative disorder characterized by developmental delay or regression, lactic acidosis, and bilateral symmetrical lesions in the basal ganglia, thalamus, and brainstem [6, 7]. The disease is caused by mutations in both nuclear and mitochondrial genes involved in energy metabolism; however, the underlying gene defects remain unidentified in nearly half of the patients [8, 9]. Because of the clinical and genetic heterogeneity of the disorder, there is no established treatment for patients with LS.

Our recent trial showed that sodium pyruvate produced a slightly favorable change in the plasma lactate and pyruvate levels for the treatment of mitochondrial disease [5]. This preliminary result prompted us to apply sodium pyruvate to a patient with LS due to cytochrome *c* oxidase. In the present report, we describe our clinical experience with pyruvate therapy in an adolescent with cytochrome *c* oxidase deficiency.

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2. Administration of pyruvate to a patient with cytochrome *c* oxidase deficiency

An 11-year-old female complained of frequent falls during walking and slowness in running. This patient was born with a weight of 3590 g after a normal pregnancy. At the age of 6 years, she complained of double and blurred vision. Neurological examinations revealed gaze nystagmus and bilateral paresis of the abducens nerve. Cranial magnetic resonance imaging (MRI) demonstrated bilateral lesions in the putamen (Fig. 1). The lactate level was elevated in the cerebrospinal fluid (31 mg/dL, normal 10–20 mg/dL). Histopathological study of the skeletal muscle revealed the presence of diffuse cytochrome *c* oxidase-negative fibers (Fig. 2) without ragged-red fibers (RRF) or strongly succinate dehydrogenase-reactive blood vessels (SSV). Biochemical analysis of the mitochondria isolated from the skeletal muscle indicated a marked deficiency of cytochrome *c* oxidase activity (17% of the normal control value). The sequencing of the entire mtDNA identified no pathogenic mutations either in the protein-coding regions or in the ribosomal and transfer RNA genes. From these findings she was diagnosed as having LS due to cytochrome *c* oxidase deficiency. From the age of 8 years oral administration of coenzyme Q was started, but her motor dysfunction became gradually aggravated and her easy fatigability, enhanced. Neurological examination revealed dystonia and an ataxic gait. She sometimes needed assistance in walking, and her speech became gradually slurred. At the age of 10 years, echocardiography revealed mild cardiac dysfunction: her left ventricular ejection fraction was 52% (normal 55%–80%), and the fractional shortening was 26% (normal>28%). An electrocardiogram revealed inverted T waves in leads V3 and V4, suggesting cardiac muscle involvement. The blood lactate and pyruvate levels were 20.5 mg/dL and 1.13 mg/dL, respectively, with a lactate-to-pyruvate ratio of 18.1. The levels of lactate and pyruvate in the cerebrospinal fluid were 32.4 mg/dL and 1.21 mg/dL, respectively, giving a lactate-to-pyruvate ratio of 26.8.

At the age of 11 years, administration of sodium pyruvate (0.5 g/kg/day) was started. After the administration both the blood lactate and pyruvate levels decreased to 10.3 mg/dL and 0.88 mg/dL, respectively, with a reduction in the lactate-to-pyruvate ratio to 11.7.

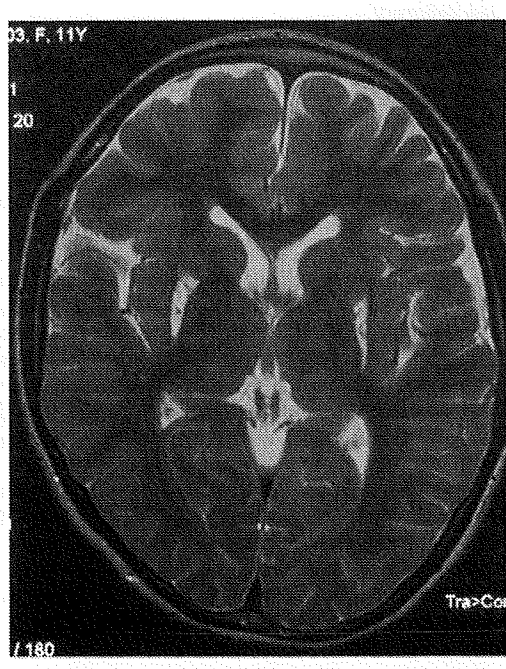

Fig. 1. T2-weighed magnetic resonance imaging (MRI) of the brain of the patient at 11 years of age.

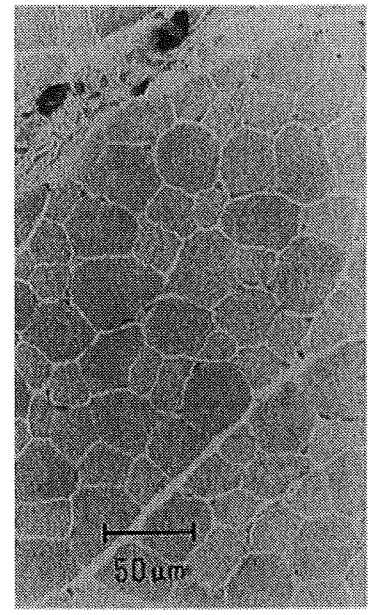

Fig. 2. Histochemical staining for cytochrome *c* oxidase in the biopsied skeletal muscle of the patient. Diffuse deficiency of cytochrome *c* oxidase is to be noted.

Interestingly, the exercise tolerance of the patient improved after the start of pyruvate administration; and she became capable of participating in athletic games in school. One year after the start of pyruvate administration, although none of the neurological symptoms or signs had significantly improved, her cardiac function returned to within the normal ranges: left ventricular ejection fraction of 58% and fractional shortening of 30%. Inverted T waves in leads V3 and V4 of the electrocardiogram were diminished. These findings suggest that pyruvate administration might have beneficial effects on mitochondrial cardiomyopathy.

When pyruvate administration was interrupted because of a gastrointestinal infection, the serum lactate level of the patient increased from 11.3 mg/dL to 14.3 mg/dL; and her serum pyruvate level decreased from 0.96 mg/dL to 0.94 mg/dL, suggesting that the pyruvate administration was effective in decreasing the lactate-to-pyruvate ratio.

The present observations suggest that oral administration of sodium pyruvate at a dose of 0.5 g/kg/day had no harmful effects, although diarrhea was sometimes observed when the pyruvate was administered at a high concentration. We therefore recommend administering sodium pyruvate at 16.5 g/L (150 mM) diluted in either water, milk or fruit juice.

3. Discussion

In the present study, we reported a patient with LS who responded to pyruvate administration. The histochemical finding of diffuse cytochrome *c* oxidase deficiency indicated that this condition was distinct from the benign infantile mitochondrial myopathy due to reversible cytochrome *c* oxidase deficiency [10]. The sustained levels of blood lactate and pyruvate suggested that the enzyme defect itself was persisting in the present patient.

We also administered sodium pyruvate to several patients with mitochondrial encephalomyopathies in advanced stages. In such patients having respiratory disturbance necessitating artificial ventilation, dysphagia requiring tube feeding or a gastric fistula, severe psychomotor developmental delay, and/or multiple organ failure, we were unable to assess the efficacy of pyruvate administration. Considering the progressive nature of LS, and given that the pyruvate

administration is efficacious in preventing neurodegeneration, therapeutic intervention should be started in the early stage of disease progression.

There are some limitations in the present study. First, despite vigorous analysis of mitochondrial DNA mutations, we were unable to identify the causative etiology of LS in the present patient. Further survey for nuclear DNA mutations is needed. Second, this was a clinical study on one patient, in which the results must be interpreted with caution. For validation of our findings, multi-institutional research including the present case should be conducted.

In a patient with LS associated with cardiomyopathy examined previous to the present one (Wakamoto et al., unpublished observation), MRI conducted 1 year after the start of pyruvate therapy demonstrated remarkable improvement with distinct decreases in the size and intensity of the lesions located in the basal ganglia. Echocardiography also demonstrated marked improvements in the values of left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fractional shortening, and left ventricular ejection fraction; although the degree of hypertrophy of the heart muscle was not influenced by the pyruvate administration. These observations indicated improved cardiac function after the treatment of this LS patient.

In another patient with LS, a marked improvement in the electroencephalographic findings was noticed after administration of sodium pyruvate (Koga et al., unpublished). Because LS is caused by a wide variety of the molecular and genetic defects, we need to identify the specific subtypes that are responsive to pyruvate therapy. For this purpose, we have started constructing a rapid and comprehensive detection system for pathogenic mutations of mitochondrial DNA by use of the Luminex suspension array technology (Nishigaki et al., in preparation). Efficient and systematic screening for nuclear DNA mutations should be also established.

Hermann et al. investigated the effect of intracoronary pyruvate in 8 patients with congestive heart failure, and concluded that pyruvate had a favorable inotropic effect [11]. Pyruvate affects energy metabolism by its input into the tricarboxylic-acid (TCA) cycle in 2 ways. First, pyruvate enters the TCA cycle as acetyl-CoA after decarboxylation via pyruvate dehydrogenase. Second, pyruvate enriches the TCA cycle after carboxylation to oxaloacetate and/or malate via pyruvate carboxylase and/or malic enzyme. Actually the ^{13}C NMR spectroscopic study by Weiss et al. demonstrated that the addition of 0.8 mM pyruvate significantly increased in the levels of citrate in the rat heart perfused with 5 mM [2-^{13}C]acetate [12]. This anaplerotic effect of pyruvate would increase the flux through the TCA cycle, supplementing oxidative phosphorylation. The exact mechanisms by which pyruvate improved the exercise intolerance in the present patient with cytochrome *c* oxidase deficiency should be further investigated.

We previously demonstrated that pyruvate infusion lowered the lactate-to-pyruvate ratio and corrected the deficit in ureogenesis in the liver of citrin-knockout ($Ctrn^{-/-}$) mice, a model of adult-onset type II citrullinaemia [13]. Recently, Mutoh et al. reported the use of arginine and sodium pyruvate for the treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia [14]. Oral administration of arginine and sodium pyruvate for over 3 years improved the clinical symptoms and almost completely normalized the laboratory findings of the patient. The authors concluded that the administration of arginine and sodium pyruvate with low-carbohydrate meals may be an effective therapy for patients with citrin deficiency in order either to prolong metabolic normalcy or to provide a safer and more affordable alternative to liver transplantation [14]. Thus, extended studies are needed to confirm the therapeutic potential of pyruvate for both citrin deficiency and mitochondrial respiratory defects.

In conclusion, although the pathogenic mutation causing the mitochondrial dysfunction was not determined, our results suggest that exercise intolerance, mild cardiac dysfunction, and lactic acidosis were ameliorated by the pyruvate administration. Administration of sodium pyruvate may prove effective for other patients with cytochrome *c* oxidase deficiency due to mitochondrial or nuclear DNA mutations. Validation of our findings will require their replication with additional patients having different mitochondrial abnormalities confirmed by genetic or biochemical analysis.

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