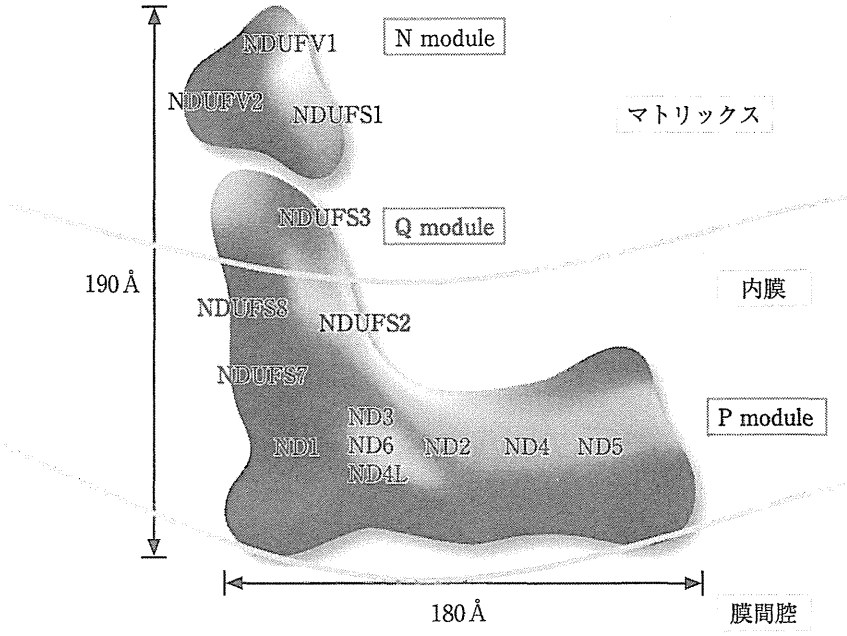

図1　哺乳類呼吸鎖複合体Iの模式図
(文献[2]より改変)

マトリックスアーム(Nモジュール，Qモジュール)と内膜アーム(Pモジュール)が100°の角度でL字型構造を形作る．Nモジュール：NADH脱水素酵素(NADH dehydrogenase)モジュール，Qモジュール：電子伝達(electron transfer)モジュール，Pモジュール：プロトンポンピング(proton translocation)モジュール．コアサブユニットのみサブユニット名を記す(紫は核遺伝子由来，緑はミトコンドリア遺伝子由来)．

ATPaseの構造変化を引き起こしATPが生成される．

complex Iは980 kDaにもなる大分子複合体であり，mtDNA由来サブユニットが7個，nDNA由来サブユニットが38個であることは上述したが，欠損症の病因として報告のあるサブユニット遺伝子異常を**表1**に示す．コアサブユニット以外のnDNA由来サブユニットはいまだ機能不明のものも多いが，今後これらの欠損症患者の解析を通して機能が明らかになるものも多いであろう．最終的に構造単位に含まれるサブユニット以外にも，complex Iは多数のアセンブリー因子の助けを借りて生合成される．これらアセンブリー因子のうち，病因として報告のあるもの[5-21]を**表2**に示した．アセンブリー因子の詳細については字数の関係で本稿では触れないが，文献2)に詳しいので参照されたい．

更に注意すべきは，complex Iには最も多くのmtDNA由来サブユニットタンパクが含まれているため，mtDNAの複製・転写障害(mt tRNA遺伝子異常や核由来の複製・転写調節遺伝子の異常)でも，特に初期はcomplex I単独欠損の場合も多いことである．これらの多くは病期が進めばその多くは複合型欠損症に変化する．

4. 病　　態

complex I欠損症は，エネルギー産生系の臓器障害を中心に極めて多彩な症状・病型を示す．多数の臓器が同時に犯されることも，単一臓器のみの場合(心筋症や視神経症など)もある．代表的病型としては，致死型乳児ミトコンドリア病(lethal infantile mitochondrial disease: LIMD)，Leigh脳症，白質脳症，ミトコンドリア脳筋症，高乳酸血症，卒中様発作を伴う症候群(mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes: MELAS)，心筋症などであるが，その中からここでは乳児期の

表1 ミトコンドリア病を引き起こす呼吸鎖複合体Iサブユニット異常症(文献[2]より改変)

	ヒトサブユニット名	ウシホモログ	モジュール	臨床病型
ミトコンドリア遺伝子由来	ND1	ND1	P	LHON[a], MELAS[b], LS[c]
	ND2	ND2	P	LS
	ND3	ND3	P	LS, LIMD[d]
	ND4	ND4	P	LHON, LS
	ND4L	ND4L	P	LHON
	ND5	ND5	P	LS, MELAS, LHON
	ND6	ND6	P	LS, LHON, ジストニア
核遺伝子由来	NDUFA1	MWFE		LS, ミトコンドリア脳筋症
	NDUFA2	B8		LS
	NDUFA10	42 kDa		LS
	NDUFA11	B14.7		LIMD, ミトコンドリア脳筋症, ミトコンドリア心筋症
	NDUFA12	B17.2		LS
	NDUFS1	75 kDa	N	LS, 白質ジストロフィー
	NDUFS2	49 kDa	Q	LS, LIMD, ミトコンドリア脳筋症, ミトコンドリア心筋症
	NDUFS3	30 kDa	Q	LS
	NDUFS4	18 kDa	N	LS
	NDUFS6	13 kDa	N	LIMD
	NDUFS7	PSST	Q	LS
	NDUFS8	TYKY	Q	LS, ミトコンドリア脳筋症, ミトコンドリア心筋症, 白質ジストロフィー
	NDUFV1	51 kDa	N	LS, ミトコンドリア脳筋症
	NDUFV2	24 kDa	N	ミトコンドリア脳筋症, ミトコンドリア心筋症

[a]LHON: Leber遺伝性視神経症(Leber hereditary optic neuropathy).
[b]MELAS: ミトコンドリア脳筋症, 高乳酸血症, 卒中様発作を伴う症候群(mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes).
[c]LS: Leigh脳症(症候群).
[d]LIMD: 致死型乳児ミトコンドリア病(lethal infantile mitochondrial disease).

表2 呼吸鎖Iアセンブリー因子異常症(文献[2]より改変)

アセンブリー因子	臨床病型	文 献
C20orf7	LIMD[a], LS[b]	5-7)
Ndufaf3(C3orf60)	LIMD	8)
Ndufaf4(C6orf66)	LIMD, ミトコンドリア心筋症	9)
Ndufaf1(CIA30)	ミトコンドリア心筋脳症	10, 11)
ACAD9	ミトコンドリア心筋症, ミトコンドリア脳筋症, 運動不耐症, 難聴, 低身長	12-14)
Ndufaf2(B17.2L, NDUFA12L)	LS, ミトコンドリア脳筋症	15-18)
NUBPL(Ind1)	ミトコンドリア脳筋症	18, 19)
C8orf38	LS	20)
FOXRED1	LS	18, 21)

[a]LIMD: 致死型乳児ミトコンドリア病(lethal infantile mitochondrial disease).
[b]LS: Leigh脳症(症候群).

表3　ミトコンドリア遺伝子異常による Leigh 脳症

遺伝子シンボル	診断材料	MRCD による Leigh 脳症中の割合
MT-ATP6 m.8993T>G or C	WBC DNA	10-20 %
ATP6, TL1, TK, TW, TV, ND1, ND2, ND3, ND4, ND5, ND6, CO3	muscle DNA (hair follicles, urine sediment cells)	10-20 %

表4　呼吸鎖複合体欠損を伴う核遺伝子異常による Leigh 脳症
(MRCD による Leigh 脳症中の約 70 % に相当する)

欠損する呼吸鎖複合体	病　名	遺伝子シンボル
I	呼吸鎖 I 欠損を伴う Leigh 脳症	*NDUFV1, FS1, FS2, FS3, FS4, FS7, FS8, FA1, FA2, FA10, FAF2, C8orf38, C20orf7, FOXRED1,* ほか未知遺伝子
II	呼吸鎖 II 欠損を伴う Leigh 脳症	*SDHA*
IV	シトクローム c オキシダーゼ (呼吸鎖 IV) 欠損を伴う Leigh 脳症	*SURF1, COX10, COX15,* ほか未知遺伝子
	French-Canadian または Saguenay-Lac Saint Jean 型	*LRPPRC*
II+III	コエンザイム Q_{10} 欠損症	*PDSS2,* ほか未知遺伝子
I, III+IV	ミトコンドリア DNA 枯渇症候群	*POLG, SUCLG1,* ほか未知遺伝子
I, III+IV	mtDNA 転写・翻訳障害	*C12orf65,* ほか未知遺伝子

代表的なミトコンドリア病である Leigh 脳症についてまとめてみる.

Leigh 脳症とは狭義には以下の 4 つの条件を満たす疾患と定義される[22]. ①精神運動発達の退行を伴った進行性の神経疾患, ②不随意運動, 哺乳嚥下障害, 呼吸障害, 眼球運動障害, 失調などの, 脳幹 and/or 大脳基底核症状を伴う, ③血中 and/or 髄液中の乳酸濃度の上昇, ④次のうちの 1 つ以上：(i) 画像上の対称性基底核・脳幹病変, (ii) 典型的神経病理学的変性 (海綿状壊死), (iii) 同様症状の同胞の存在.

病因としては MRCD 以外にピルビン酸脱水素酵素欠損症, ピルビン酸カルボキシラーゼ欠損症, コエンザイム Q 欠損症などが挙げられるが, ここでは Leigh 脳症の病因としての MRCD[23] を説明する. MRCD による Leigh 脳症中, **表3** に示す mtDNA 異常が全体の約 30 % で, **表4** に示す nDNA 異常が 70 % である. 病因検索においては, まず血液を用いて m.8993 変異の有無を確認し, それに異常のない場合には mtDNA 全周塩基配列決定を行う. 更にこれと並行して, 低下した呼吸鎖複合体活性に基づき, 解析する nDNA 候補を推測する. しかしそれでも病因のわからない場合も多く, 概論の稿に示した次世代シークエンサーを用いた全エキソーム解析結果に待つ場合も多い.

5. 診断と鑑別診断

1) 酵素診断

complex I欠損症の診断はまずは疑うことに始まる．持続する高乳酸血症を伴う場合はもちろんであるが，高乳酸血症がなくても単一病因では説明のできない多臓器にまたがる症状が存在する場合は，まずは呼吸鎖酵素複合体活性を測定すべきである．

材料としては筋肉や心筋，肝臓などを中心とする罹患臓器の解析が最も望ましい．特に心筋症では，心筋のみで活性が低下し他の筋肉では活性が正常のこともあるので注意して欲しい．皮膚線維芽細胞は異常の検出率は落ちるが，診断確定後の分子生物学的検討や出生前診断のためにはその解析は必要である．また，Leigh脳症を中心とする神経症状中心のミトコンドリア病では，皮膚線維芽細胞における異常の検出率はほぼ筋肉に匹敵することもわかってきた．

NADH酸化に伴う吸光度の変化を測定するが，生体内には多数のNADH酸化還元酵素があるので，complex Iの特異的阻害剤であるrotenoneを加える前の活性から加えた後の活性を差し引き，それをcomplex I活性としている．各臓器・組織におけるミトコンドリア量の違いを補正するため，単独活性よりもクエン酸合成酵素やコハク酸脱水素酵素(complex II)活性で除した比活性で表すことが多い．酵素診断は決して簡単な作業ではなく，また全施設を網羅するような正常値もない．各施設の壁を越えた検定システムの構築とともに，施設自身での自助努力が今後益々必要になってくる．

更に注意が必要なのは，この酵素活性はNADH酸化能を測っていることである．つまり**図1**に示すNモジュールの活性を測っていることになり，遠く離れたPモジュールなどの異常では活性低下のないこともある．これを補うために，blue native電気泳動を用いて酵素複合体量の測定を行ったり，ポラログラフィー(ATP産生能)やオキシグラフィー(酸素消費量)を組み合わせることも必要になる．

病因から考えるとcomplex I単独欠損であるはずの場合でも，複合型欠損になることがよく観察される．complex I欠損により産生される活性酸素が他の呼吸鎖複合体活性を阻害する，またはcomplex Iの異常が超複合体(概論の稿参照)全体の安定化障害を引き起こし他の呼吸鎖も破壊される，などの説明がなされているが，詳細はわかっていない．

2) 画像診断

complex I欠損症では脳幹部の画像異常が高率に観察されるとの報告もある．しかし今のところ，酵素診断に代わる確定診断法にはなり得ないのが現状である．

3) 組織診断

筋生検所見としては，軽度の脂肪蓄積や筋線維不均衡(fiber type disproportion)などの非特異的変化がほとんどで，赤色ボロ線維(ragged red fiber)などの特異的変化は，mtDNA異常や，その複製・転写障害の場合などに限られる．

4) 生化学診断

通常の代謝病スクリーニング法(有機酸分析，タンデムマス分析など)に特異的マーカーはなく，高乳酸血症の存在も程度も病因や重症度に直結するものはない．

5) 診断のまとめと鑑別診断

症状・所見よりMRCDを疑ったら呼吸鎖酵素複合体活性を測定し，異常が認められたらmtDNAと既報告のnDNA異常を組み込んだキャプチャーアレイを挟み，それでも異常の認められない場合は次世代シークエンサーによるエキソーム解析を行う(概論の稿の図7)．最近はコストパフォーマンスの上からもキャプチャーアレイのステップは省くことも多い．鑑別については'疑ったら酵素活性を測る'の一言である．

6. 治療と予後

complex I欠損症に有効であると定まった治療法はない．検討中の薬物の中で有望なものはリボフラビンであろう[14]．

おわりに

ミトコンドリア病はすべての科の医師がその存在を知っておくべき病気であることは概論の

稿にも述べた．単一病因では説明のできない多臓器にまたがる症状の存在するときには，たとえ高乳酸血症が存在しなくともcomplex Iを中心とするミトコンドリア病＝MRCDを常に鑑別に入れておく必要があることを最後に再度強調し，本稿の締めくくりとしたい．

本稿完成後にRahmanらのcomplex Iに関する優れた総説[24]が出版された．幾つかの新しい病因遺伝子も加わっており，一読されることをお勧めする．

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Case report

Liver-specific mitochondrial respiratory chain complex I deficiency in fatal influenza encephalopathy

Chikako Arakawa [a,*], Ayumi Endo [a], Ryutaro Kohira [a], Yukihiko Fujita [a], Tatsuo Fuchigami [a], Hideo Mugishima [a], Akira Ohtake [b], Kei Murayama [c], Masato Mori [d], Rie Miyata [e], Yoshiho Hatai [e]

[a] *Department of Pediatrics and Child Health, Nihon University School of Medicine, Tokyo, Japan*
[b] *Department of Pediatrics, Saitama Medical University, Saitama, Japan*
[c] *Department of Metabolism, Chiba Children's Hospital, Chiba, Japan*
[d] *Department of Pediatrics, Jichi Medical University, Japan*
[e] *Department of Pediatrics, Tokyo-Kita Social Insurance Hospital, Tokyo, Japan*

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Abstract

We report on a 4-year-old boy who died from influenza encephalopathy. The clinical course and microscopic findings of the autopsied liver were compatible with Reye's syndrome. We examined the mitochondrial respiratory chain function by blue native polyacrylamide gel electrophoresis (BN-PAGE), western blotting, and respiratory chain enzyme activity assays. The activity of liver respiratory chain complex (CO) I was markedly decreased (7.2% of the respective control activity); whereas, the other respiratory chain complex activities were substantially normal (CO II, 57.9%; CO III, 122.3%; CO IV, 161.0%). The activities of CO I–IV in fibroblasts were normal (CO I, 82.0%; CO II, 83.1%; CO III, 72.9%; CO IV, 97.3%). The patient was diagnosed with liver-specific complex I deficiency. This inborn disorder may have contributed to the fatal outcome. We propose that relying only on fibroblast respiratory chain complex activities may lead to the misdiagnosis of liver-specific complex I deficiency.

Keywords: Influenza encephalopathy; Reye's syndrome; Mitochondria; Complex I deficiency; Liver-specific

1. Introduction

Influenza encephalopathy is a critical complication of influenza infection. Although the pathological mechanism is poorly understood, mitochondrial malfunction is suggested to play a role in the pathogenesis [1]. We describe a boy with liver-specific mitochondrial respiratory chain complex I deficiency who developed fatal encephalopathy associated with influenza A infection. The possible contribution of the mitochondrial respiratory chain disorder to the clinical course is discussed.

2. Case report

A 4-year-old Japanese boy developed pyrexia. He was treated with acetaminophen once and visited the family doctor. Influenza A infection was diagnosed by nasal antigen test in a clinic and he was treated with oseltamivir. He was admitted to a nearby hospital due to a generalized seizure in the evening; then, he was transferred to our institute because of highly elevated serum transaminase. He was the first child born to healthy parents with no consanguinity. No other child had died in early

* Corresponding author. Tel.: +81 3 3972 8111x2442; fax: +81 3 3957 6186.
E-mail address: chi-ka@sage.ocn.ne.jp (C. Arakawa).

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infancy within three degrees of relationship. He had normal psychomotor development and had not been vaccinated against influenza.

On arrival, he was comatose and had a temperature of 38.9 °C, heart rate of 136 beats per minute, and blood pressure of 106/62 mm Hg. Neither arrhythmia nor cardiac hypertrophy was seen in the electrocardiogram or echocardiography. Blood examination showed marked liver dysfunction and ammonemia (aspartate aminotransferase, 4282 IU/l; alanine aminotransferase, 1750 IU/l; ammonia, 156 μg/dl). Blood gas analysis showed marked acidosis (pH 6.964, pCO_2 59.6 mm Hg, HCO_3 11.2 mol/l, BE −23.7 mmol, and lactate 9.0 mmol/l). Blood glucose was 128 mg/dl under intravenous infusion. Influenza encephalopathy was diagnosed and intensive therapy, including mechanical ventilation, steroid, and heart stimulants, was started. A few hours later, he developed cardio-pulmonary arrest and died 36 h after developing pyrexia. This clinical course led us to suspect Reye's syndrome and mitochondrial disorders. The parents consented to resection of the patient's liver and skin fibroblasts. Urine organic acid analysis, blood amino acid profile, and carnitine profile did not show any findings suggestive of congenital metabolic disorders. Microscopical finding showed microvesicular fatty droplets in hepatic cytoplasm in hematoxylin-eosin and oil red O staining (Fig. 1), that was compatible with Reye's syndrome. The grade of histological hepatic changes was milder than the fulminant clinical course.

The activities of respiratory chain complexes (Co) I, II, III, and IV were assayed in the crude post-600 g supernatant of the liver and in isolated mitochondria from skin fibroblasts as described previously [2]. The activity of each complex was presented as a percent ratio relative to the mean value obtained from 12 healthy controls. The activities of Co I, II, III, and IV were also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker, or Co II activity [2]. Liver respiratory chain complex I activities were very low, but CS, Co II, III, and IV activities were normal. In contrast to the liver, the fibroblast complex I activity was normal (Table 1).

The expression of the mitochondrial respiratory chain Co I, II, III, and IV proteins in the liver and fibroblasts were examined by Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to methods described previously [3]. The results of BN-PAGE are shown in Fig. 2. The band corresponding to Co I was not visible; while, the intensities of the Co II, III, and IV bands remained normal. Several base substitutions were detected by polymerase chain reaction, but there was no pathogenic mutation in the genomic DNA extracted from the autopsied liver tissue.

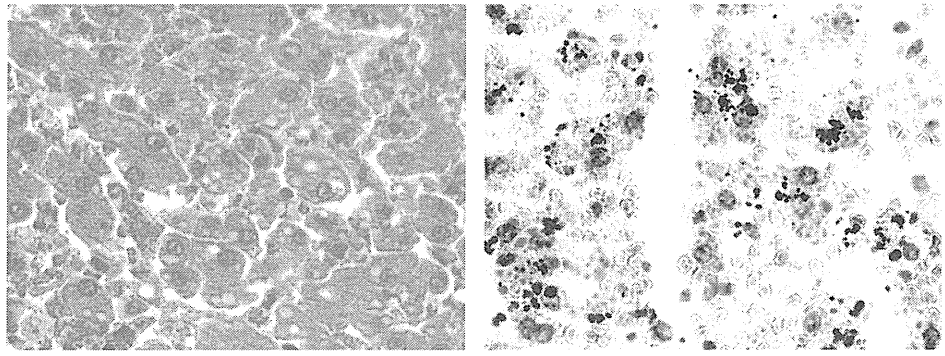
3. Discussion

Mitochondrial malfunction has been described in influenza encephalopathy. There are no reports of mitochondrial respiratory chain diseases, although disorders of fatty acid oxidation have been discussed [1]. Complex I deficiency was first recognized in 1979 by Morgan-

Table 1
Enzyme assay of respiratory chain complexes.

%	Co I	Co II	Co III	Co IV	CS
Liver					
% of normal	7.2	57.9	122.3	161.0	78.1
CS ratio	9.2	74.1	155.0	203.8	–
Co II ratio	12.3	–	212.2	272.2	–
Fibroblast					
% of normal	82.0	83.1	72.9	97.3	120.4
CS ratio	66.2	66.8	56.5	76.3	–
Co II ratio	98.2	–	83.7	112.5	–

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



A. Hematoxylin-Eosin staining (× 400) B. Oil Red O staining (× 400)

Fig. 1. Autopsy liver samples show preserved hepatic architecture with scattered distribution of micro-vesicular fatty droplets in the hepatic cytoplasm (A). Marked congestion, focal necrosis, and mild inflammatory cellular infiltration without fibrosis were noted. Fat deposition was also suggested with oil red O staining (B). The grade of histological hepatic changes was milder than the fulminant clinical course.

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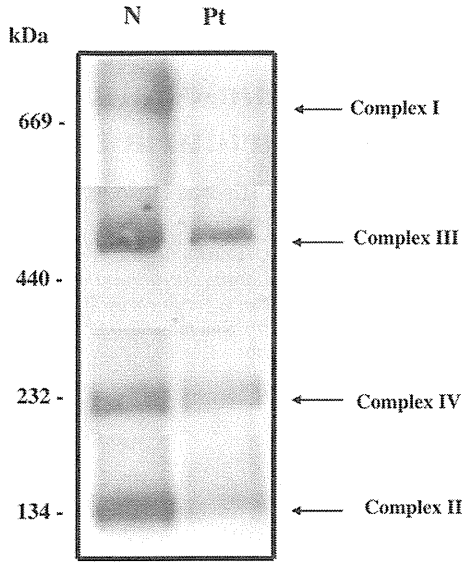

Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of liver respiratory chain enzymes showed markedly decreased protein expression of complex I, while the protein bands of complex II, III, and IV were comparable to the control (N) samples.

Hughes; yet, studies have not progressed because of technical difficulties. More recently, complex I deficiency was regarded as the most common energy generation disorder. The manifestations range from typical mitochondrial diseases, such as Leigh syndrome, to obscure conditions such as slow regression or intractable secretory diarrhea [4].

Complex II activity has been shown to be more labile than complex I when measuring respiratory chain enzymes in patients with a wide range of metabolic disorders, liver failure, or liver disease [5]. In the present case, only complex I activity was very low; this indicates primary complex I deficiency rather than a secondary effect of influenza A infection. Complex I includes seven mitochondrial DNA-encoded subunits and at least 39 nuclear-encoded subunits. In our case, no mutation was detected in the mitochondrial DNA (mtDNA). The detection rate for mutations in mitochondrial or nuclear DNA in complex I deficiency is as small as 20% [6,7].

In the present case, complex I was deficient only in the liver, not in fibroblasts. Mitochondrial respiratory complex disorders can show clinical and biochemical tissue specificity [2,4,6,8,10]. For this reason, it is difficult to diagnose by suspension cells or serum enzyme assays. The possible mechanisms of tissue specificity are tissue-specific subunits of complex I [9], the ratio between normal and mutant mtDNA in a specific tissue [7], and tissue differences in RNA processing [10]. To our knowledge, very few cases with liver-specific complex I deficiency have been reported [2,8]. These reported cases had chronic neurological symptoms such as epilepsy, hypotonia, or developmental regression, with the exception of one case that had severe cardiomyopathy in early infancy [2]. There was one case without evidence of liver dysfunction [8]. Clinically there was no definite difference from usual Co I deficiency. One reason for the small number of cases is that the liver is not the prime diagnostic tissue. Respiratory chain complex deficiency is usually confirmed by tissue biopsy. Muscle is usually the prime diagnostic tissue, and cultured skin fibroblasts are also often analyzed [10]. False-negative diagnostic results may occur because the liver is not examined.

This case was determined to be complex I deficiency by BN-PAGE Western blotting and determination of enzyme activities. This is the first report of respiratory chain complex I deficiency in influenza encephalopathy. We suggest there may be many undiagnosed cases of this metabolic disorder. Here, we described a healthy child, who had never been suspected of having any disease, diagnosed with a metabolic disorder after acute encephalopathy with subsequent death. Future studies are needed to focus on the development of a method to detect this inborn metabolic disorder before onset.

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CLINICAL STUDY

Analysis of plasma ghrelin in patients with medium-chain acyl-CoA dehydrogenase deficiency and glutaric aciduria type II

Takashi Akamizu[1,2], Nobuo Sakura[3], Yosuke Shigematsu[4], Go Tajima[3], Akira Ohtake[5], Hiroshi Hosoda[6], Hiroshi Iwakura[2], Hiroyuki Ariyasu[2] and Kenji Kangawa[6]

[1]*The First Department of Medicine, Wakayama Medical University, 811-1 Kimi-idera, Wakayama 641-8509, Japan,* [2]*Ghrelin Research Project, Department of Experimental Therapeutics, Faculty of Medicine, Translational Research Center, Kyoto University, Kyoto, Japan,* [3]*Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8551, Japan,* [4]*Department of Health Science, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan,* [5]*Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan and* [6]*Department of Biochemistry, National Cerebral and Cardiovascular Center Research Institute, Osaka 565-8565, Japan*

(Correspondence should be addressed to T Akamizu at The First Department of Medicine, Wakayama Medical University; Email: akamizu@wakayama-med.ac.jp)

Abstract

Objective: Ghrelin requires a fatty acid modification for binding to the GH secretagogue receptor. Acylation of the Ser3 residue of ghrelin is essential for its biological activities. We hypothesized that acyl-CoA is the fatty acid substrate for ghrelin acylation. Because serum octanoyl-CoA levels are altered by fatty acid oxidation disorders, we examined circulating ghrelin levels in affected patients.
Materials and methods: Blood levels of acyl (A) and des-acyl (D) forms of ghrelin and acylcarnitine of patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and glutaric aciduria type II (GA2) were measured.
Results: Plasma acyl ghrelin levels and A/D ratios increased in patients with MCAD deficiency or GA2 when compared with normal subjects. Reverse-phase HPLC confirmed that *n*-octanoylated ghrelin levels were elevated in these patients.
Conclusion: Changing serum medium-chain acylcarnitine levels may affect circulating acyl ghrelin levels, suggesting that acyl-CoA is the substrate for ghrelin acylation.

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Introduction

Ghrelin, an endogenous ligand for the GH secretagogue receptor, is an acylated peptide produced by gastrointestinal endocrine cells (1). Ghrelin is the only peptide known to require a fatty acid modification. Octanoylation of the Ser3 residue is essential for ghrelin-mediated stimulation of GH secretion and regulation of energy homeostasis via increased food intake and adiposity (2, 3). Other than octanoylation (C8:0), the hormone is subject to other types of acyl modification, decanoylation (C10:0), and possibly decenoylation (C10:1) (4, 5). Recently, ghrelin *O*-acyltransferase (GOAT), which octanoylates ghrelin, was identified (6, 7). The fatty acid substrate that contributes to ghrelin acylation, however, has not been clarified, although the presumed donor is acyl-CoA.

Mitochondrial fatty acid oxidation (FAO) disorders result from genetic defects in transport proteins or enzymes involved in fatty acid β-oxidation (8, 9). The clinical phenotypes have recently been associated with a growing number of disorders, such as Reye syndrome, sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications during pregnancy (10). Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, the most common inherited defect in FAO, causes elevated serum octanoylcarnitine levels (11), reflecting elevated octanoyl-CoA levels. Glutaric aciduria type II (GA2), which is caused by defects in electron transfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase, or other unknown abnormalities in flavin metabolism or transport, is characterized by elevated serum acylcarnitine levels, including octanoylcarnitine (8, 9). In carnitine palmitoyltransferase II (CPT II) deficiency and very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, serum octanoyl-CoA levels do not increase, but at times actually decrease (8, 9).

We hypothesized that octanoyl-CoA is the fatty acid substrate for ghrelin acylation. To examine this hypothesis, we measured circulating ghrelin levels in patients with MCAD deficiency (MCADD) and GA2.

Materials and methods

Subjects

Five female patients with FAO deficiency (two with MCADD one with GA2, one with CPT II deficiency (12),

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and one with VLCAD deficiency) were recruited for this study. The study protocol was approved by the ethics committee on human research at the Kyoto University Graduate School of Medicine. Written informed consent was obtained prior to enrollment.

Measurement of plasma ghrelin concentrations

Because FAO patients tend to develop hypoglycemia by fasting, it was difficult to do overnight fasting. Therefore, blood samples for ghrelin analyses were drawn from a forearm vein in the morning after fasting as long as possible. Plasma samples were prepared as described previously (13). Blood samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 1000 kallikrein inactivator units/ml = 23.6 nmol/ml (23.6 pM)) and centrifuged at 4 °C. One-tenth volume of 1 M HCl was immediately added to the separated plasma. The acylated and desacylated forms of ghrelin were measured using a fluorescence enzyme immunoassay (FEIA; Tosoh Corp. Tokyo, Japan). The minimal detection limits for acyl and des-acyl ghrelin in this assay system were 2.5 and 10 fmol/ml respectively. The interassay coefficients of variation were 2.9 and 3.1% for acyl and des-acyl ghrelins respectively.

Reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) was performed as described previously (4, 5, 14). Briefly, plasma diluted 50% with 0.9% saline was applied to a Sep-Pak C18 cartridge pre-equilibrated with 0.9% saline. The cartridge was washed with saline and 10% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Adsorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA. The eluate was evaporated and separated by RP-HPLC. All HPLC fractions were quantified using RIAs for ghrelin (4, 14, 15, 16). RIAs for a ghrelin C-terminal region (C-RIA) and a ghrelin N-terminal region (N-RIA) measure des-acyl ghrelin and octanoyl-ghrelin respectively (15). A RIA for N-terminal ghrelin showed ~20–25% cross-reactivity values for the *n*-decanoylated and *n*-decenoylated forms (16). Authentic human ghrelin-(1–28) was chromatographed with the same HPLC system.

Tandem mass spectrometry

Acylcarnitines in sera and dried blood spots were measured according to previously reported methods (17, 18), without derivatization. Briefly, 3 μl serum and 110 μl methanol solutions (99%) with deuterium-labeled acylcarnitines as internal standards were mixed and centrifuged, and 5 μl of the supernatant was introduced into liquid chromatography flow of methanol/acetonitrile/water (4:4:2) with 0.05% formic acid using a SIL-20AC autoinjector (Shimadzu, Kyoto, Japan). Flow injection and electrospray ionization tandem mass spectrometric (MS/MS) analyses were performed using an API 4000 LC/MS/MS system (AB Sciex, Tokyo, Japan). Positive ion MS/MS analysis was performed in precursor ion scan mode with an m/z value of 85 for the product ion. Data were recorded for 0.7 min after every sample injection and the recorded intensities of the designated ions were averaged using Chemoview Software (Foster City, CA, USA). All samples were measured serially within 1 day.

Results

We measured plasma ghrelin concentrations in patients with MCADD and GA2 (Table 1) and also in patients with CPT II and VLCAD deficiency. Elevated C8-acylcarnitine serum levels were observed in MCADD and GA II, whereas they were unchanged or lower in CPT II or VLCAD deficiency (Table 1). Levels of acyl ghrelin but not des-acyl ghrelin appeared to be elevated in patients with MCADD or GA2 in comparison with those in patients with CPT II or VLCAD deficiency, or those in female normal subjects from a previous study.

We then performed RP-HPLC analysis of ghrelin using plasma from patient 1 with MCADD. It demonstrated an eluted peak that corresponded to *n*-octanoylated human ghrelin-(1–28) in an N-RIA and a C-RIA, indicating that the detected acyl ghrelin was octanoylated (Fig. 1A). When plasma from patient 3 with GA2 was examined using the same method, the N-RIA revealed that the major peak corresponded to *n*-octanoylated human ghrelin-(1–28) (Fig. 1B). In addition, a small peak, which corresponded to decanoylated ghrelin, was observed in fraction 16 (arrow c), reflecting that serum C10-acylcarnitine levels were also elevated in patient 3 (Table 1).

Discussion

Ghrelin is the sole peptide hormone known to have a fatty acid modification. When we started this study in 2007, the catalytic enzyme and fatty acid substrate that mediate ghrelin acylation had not been identified. During this study, the GOAT enzyme was shown to be essential for ghrelin acylation (6, 7). Octanoic acid and octanoyl-CoA were candidates for the fatty acid substrate. We hypothesized that octanoyl-CoA was the substrate, because acylation of ghrelin should be an intracellular process. In fact, Ohgusu *et al.* (19) showed that acyl-CoA can be the substrate for ghrelin acyl-modification using the *in vitro* assay system. We tested this hypothesis in patients with MCADD and GA2,

Table 1 Clinical features, serum acylcarnitine levels, and plasma ghrelin concentrations in female patients with FAO disorders.

Subjects	Disease	Age (years)	BMI	Height (cm)	Acylcarnitine (nmol/ml)									AG (fmol/ml)	DAG (fmol/ml)	A/D ratio
					C4	C6	C8	C10:1	C10	C12	C14	C16	C18			
Patients (n=5)																
1	MCAD	6	15.1	119.5	0.30	0.55	4.61	0.95	0.29	0.04	0.01	0.05	0.01	45.09	57.23	0.79
2	MCAD	11	16.0	125.3	0.07	0.36	2.26	0.40	0.20	0.02	0.02	0.07	0.01	30.11	40.83	0.74
3	GA2	6	15.8	116.1	0.39	0.31	1.24	0.32	1.86	0.35	0.12	0.16	0.05	56.55	50.80	1.11
4	CPT II def.	10	17.8	141.5	0.10	0.06	0.21	0.20	0.40	0.15	0.03	0.08	0.02	19.76	34.49	0.57
5	VLCAD def.	5	14.8	109.5	0.07	0.09	0.07	0.08	0.28	0.42	2.17	2.00	0.87	27.02	113.07	0.24
Normal subjects (n=20; mean±S.D.)[a]		32.6±10.3	20.3±1.9											19.66±11.26	47.71±43.71	0.48±0.17
Reference range (n=34; mean±S.D.)					0.25±0.09	0.04±0.02	0.07±0.06	0.08±0.05	0.13±0.12	0.06±0.05	0.03±0.02	0.09±0.04	0.04±0.02			

C8, octanoyl acylcarnitine; C10, decanoyl acylcarnitine; C10:1, decenoyl acylcarnitine; AG, acyl ghrelin; DAG, des-acyl ghrelin; def., deficiency.
[a]See reference 13. All samples were reassayed using the FEIA.

which are characterized by higher intracellular octanoyl-CoA levels. Indeed, plasma A/D ratios tended to be elevated in these FAO deficiencies. A relationship between age and ghrelin levels may exist (20, 21). Concerning children, Ikezaki reported that the circulating ghrelin levels tended to correlate negatively with age in children and adolescents, but the correlation was not significant (22). Thus, the relationship has not been confirmed yet. Although we did not compare them directly with those in age- and body mass index (BMI)-matched normal children, they appeared to be higher than those in children with CPT II and VLCAD deficiencies with similar BMIs. BMIs of these patients were comparable to those of normal Japanese female children (23). These findings support the hypothesis that octanoyl-CoA is a primary substrate for ghrelin, although medium-chain triglyceride dietary lipids are a direct source for ghrelin acylation (7, 16, 24). Moreover, GOAT is a membrane-bound molecule in the endoplasmic reticulum (ER). Although how octanoyl-CoA gets into the ER lumen is unclear, Yang *et al.* (6) speculated that GOAT might mediate the transfer of octanoyl-CoA from the cytosol to the ER lumen. Although serum acylcarnitine levels tended to correlate with acyl ghrelin levels, further studies using more patients with FAO disorders are needed to confirm this relationship.

In addition to *n*-octanoylated ghrelin, other molecular forms of the ghrelin peptide exist, including des-acyl ghrelin lacking an acyl modification and such minor acylated ghrelin species as *n*-decanoylated ghrelin (Ser3 is modified by *n*-decanoic acid) (4, 5). Serum from a patient with GA2 showed the presence of acylated ghrelin that was not octanoylated and was possibly decanoylated (16). In a patient with GA2, intracellular levels of a variety of acyl-CoAs, including octanoyl- and decanoyl-CoAs, were increased, whereas MCADD was associated with specific elevation of octanoyl-CoA levels. In fact, the patient with GA2 had elevated octanoylcarnitine and decanoylcarnitine levels: 1.24 and 1.86 nmol/ml respectively. Nonetheless, the HPLC peak representing *n*-decanoylated ghrelin was much smaller than that representing *n*-octanoylated ghrelin. Although this is possibly because GOAT acylates ghrelin more efficiently with octanoyl-CoA than decanoyl-CoA, it is more likely because the cross-reactivity between *n*-octanoylated and *n*-decanoylated ghrelins is 20–25% in the N-RIA. In fact, the HPLC peaks of fraction 15–17 in the C-RIA, which detects similarly both *n*-octanoylated and *n*-decanoylated ghrelins, were large, strongly suggesting that a substantial amount of *n*-decanoylated ghrelin comparable to the elevated decanoylcarnitine level was present. Our observation that acyl ghrelin levels were not elevated in VLCAD and CPT II deficiencies, in which medium-chain acyl-CoAs levels are not higher, supported the idea that GOAT specifically acts on medium-chain acyl-CoAs. Although C16 and C18 levels were not increased in the patient with CPT II deficiency (Table 1), they may be normalized during

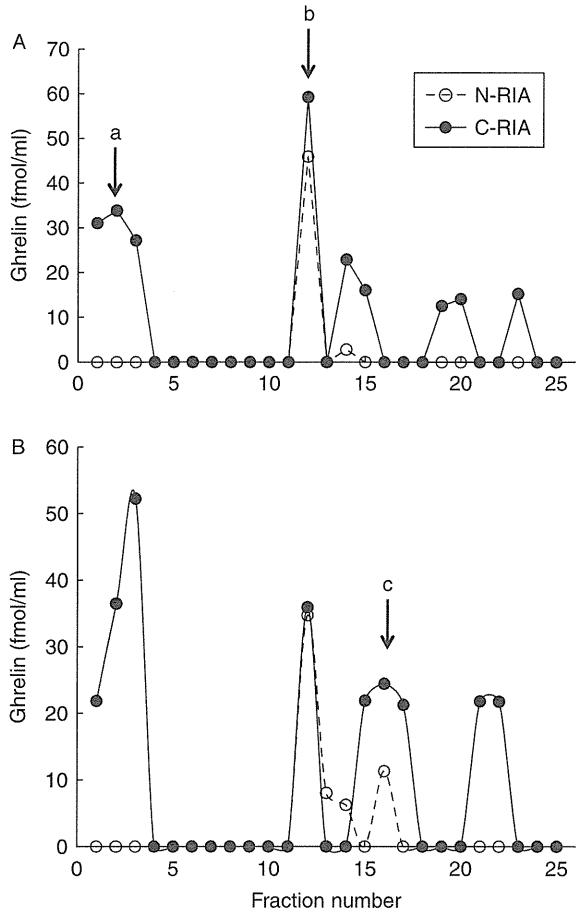

Figure 1 Representative RP-HPLC profiles of ghrelin immunoreactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from a Sep-Pak C18 cartridge were fractionated using a Symmetry300 C18 column (5 mm packing, 3.9×150 mm, Waters). A linear gradient of 10–60% CH_3CN containing 0.1% TFA was passed over the column for 40 min at 1.0 ml/min. The fraction volume was 1.0 ml. Arrows indicate the elution positions of des-acyl human ghrelin-(1–28) (a), *n*-octanoylated human ghrelin-(1–28) (b), and *n*-decanoylated ghrelin (c).

a stable period in a mild form of CPT II deficiency (25). In fact, this patient did not manifest any marked signs or symptoms at the measurement.

Ghrelin modification with the fatty acid is essential for its biological action. Octanoylation of ghrelin may also be linked to energy homeostasis and fat metabolism. For instance, when serum *n*-octanoic acid levels increase following fat degradation, ghrelin octanoylation is enhanced, resulting in stimulation of fat synthesis. Thus, ghrelin may play an important role in energy homeostasis through its own fatty acid metabolism. Related to this concept, Kirchner *et al.* (24) speculated that signaling via GOAT and ghrelin might act as a fat sensor for exogenous nutrients and support fat storage as nutrients are ingested.

FAO deficiency contributes to such clinical problems as sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications (8, 9). Early diagnosis and appropriate management are required to reduce mortality and morbidity associated with this class of disorders. Recently, newborn screening has been expanded in this area. Measuring plasma ghrelin levels may support a diagnosis of MCADD or GA2, for example. Moreover, our results have pathophysiological implications for these disorders. Plasma ghrelin levels are changed by energy demands and food intake (e.g. glucose and fat), and ghrelin affects appetite and adiposity (2, 3). Alterations of plasma ghrelin levels in FAO disorders may reflect and/or influence the patient's metabolic status. In addition, higher acyl ghrelin levels may affect the GH/insulin-like growth factor 1 (IGF1) system. There are reports that higher AG levels would increase GH and IGF1 levels (26, 27, 28, 29) and thereby linear growth could be affected. Although none of our patients manifested markedly abnormal growth velocity, we did not measure their serum GH/IGF1 levels. Thus, further studies are warranted to detail a variety of metabolic parameters in this setting.

There are several limitations in this study. At first, the number of FAO patients tested is small. Unfortunately, the incidence of FAO patients in the Japanese population is much smaller than that in Caucasians. Although we asked pediatricians on a nationwide scale, we could successfully collect only five female patients. No adult case has yet been reported in Japan. Secondly, as mentioned above, the normal female subjects were not matched in age or BMI, although patients with MCADD and GA2 exhibited higher plasma A/D ratios than those in child CPT II and VLCAD deficiencies with similar BMIs. To supplement the correlation study, we performed RP-HPLC analysis to prove the increased octanoylation of ghrelin in MCADD and GA2 directly. Further, the presence of *n*-decanoylated ghrelin is also demonstrated in GA2. Thirdly, the disturbance in the hepatic carbohydrate regulation and the altered peripheral glucose uptake may occur in FAO patients. Hence, abnormal carbohydrate regulation could influence acyl ghrelin levels. Since none of our patients manifested abnormal fasting glucose and HbA1c levels, we speculated that no significant effects occurred.

In summary, we have demonstrated increased levels of acyl ghrelin in patients with MCADD or GA2, which are also characterized by increased intracellular octanoyl-CoA levels. These findings provide mechanistic insights into the biosynthesis of ghrelin. Furthermore, analyzing plasma ghrelin levels may help elucidate pathophysiological processes in FAO deficiencies and aid in the diagnosis of these disorders. Detailed studies using more patients are certainly needed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Case report

Two Japanese patients with Leigh syndrome caused by novel *SURF1* mutations

Junpei Tanigawa [a], Kaori Kaneko [c], Masakazu Honda [d], Hiroko Harashima [d], Kei Murayama [e], Takahito Wada [a], Kyoko Takano [a], Mizue Iai [a], Sumimasa Yamashita [a], Hiroko Shimbo [a], Noriko Aida [b], Akira Ohtake [d], Hitoshi Osaka [a,*]

[a] *Division of Neurology, Kanagawa Children's Medical Center, Yokohama 232-8555, Japan*
[b] *Division of Radiology, Kanagawa Children's Medical Center, Yokohama 232-8555, Japan*
[c] *Division of Pediatric Neurology, Yokohama Ryoiku-iryo Center, Yokohama 241-0014, Japan*
[d] *Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama 350-1241, Japan*
[e] *Department of Metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan*

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Abstract

We report two patients with Leigh syndrome that showed a combination of facial dysmorphism and MRI imaging indicating an *SURF1* deficiency, which was confirmed by sequence analysis. Case 1 is a 3-year-old girl with failure to thrive and developmental delay. She presented with tachypnea at rest and displayed facial dysmorphism including frontal bossing, lateral displacement of inner canthi, esotropia, maxillary hypoplasia, slightly upturned nostril, and hypertrichosis dominant on the forehead and extremities. Case 2 is an 8-year-old boy with respiratory failure. He had been diagnosed as selective complex IV deficiency. Case 2 displayed facial dysmorphism and hypertrichosis. Since both patients displayed characteristic facial dysmorphism and MRI findings, we sequenced the *SURF1* gene and identified two heterozygous mutations; c.49+1 G>T and c.752_753del in Case 1, and homozygous c.743 C>A in Case 2. For patients with Leigh syndrome showing these facial dysmorphism and hypertrichosis, sequence analysis of the *SURF1* gene may be useful.

Keywords: Leigh syndrome; *SURF1* deficiency; Facial dysmorphism; Hypertrichosis

1. Introduction

Leigh syndrome (OMIM 256000) is a progressive neurodegenerative disorder with the usual onset in infancy or early childhood. It is a genetically heterogeneous disease and the most common cause is a molecular defect in mitochondrial energy production system, including the respiratory chain complexes and pyruvate dehydrogenase complex. An isolated generalized defect of complex IV, (Cytochrome C oxidase) is the most common biochemical abnormalities found in Leigh syndrome [1]. Leigh syndrome with *SURF1* mutations, which encode the putative assembly protein of complex IV, have been reported [2] with specific clinical features of facial dysmorphism [3], hypertrichosis [4], and MRI findings [5]. Here, we report two patients with these clinical features and novel *SURF1* mutations.

* Corresponding author. Address: Division of Neurology, Kanagawa Children's Medical Center, 2-138-4 Mutsukawa, Minami-ku, Yokohama 232-8555, Japan. Tel.: +81 45 711 2351; fax: +81 45 721 3324.
E-mail address: hosaka@kcmc.jp (H. Osaka).

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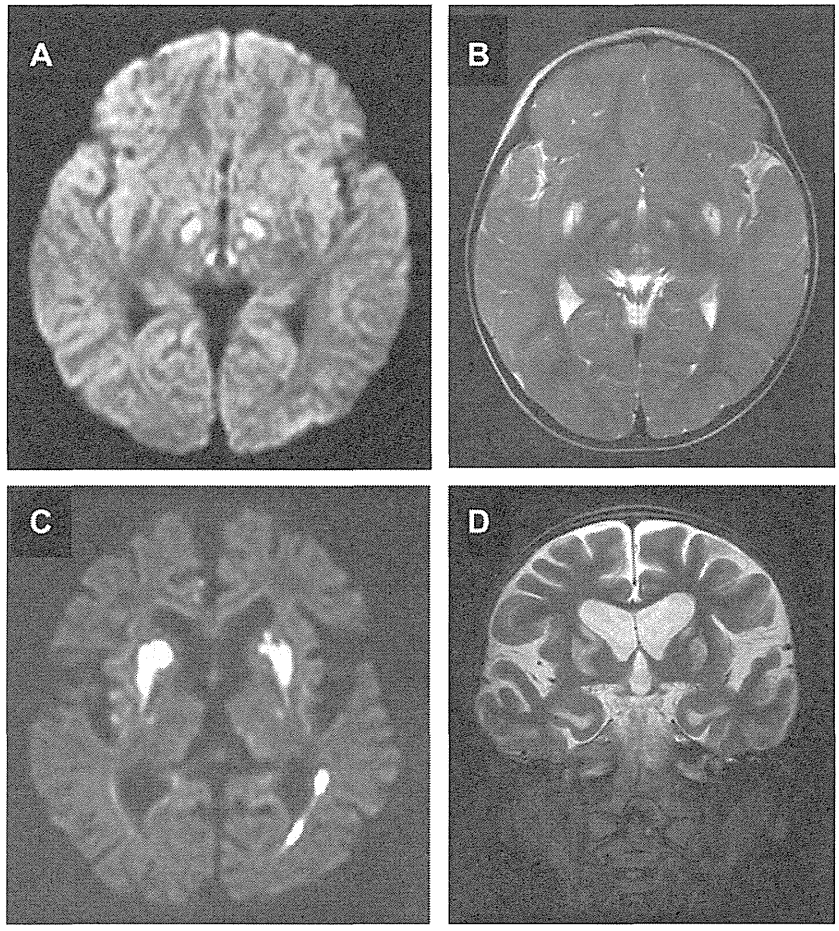

Fig. 1. Diffusion-weighted (A and C) and T2-weighted (B and D) magnetic resonance imaging of the brain in Case 1 at 2 years and 5 months of age (A and B), and in Case 2 at 7 years and 6 months of age (C and D). In Case 1, the bilateral substantia nigra (A), subthalamic nucleus (A and B), red nucleus (A), medial parts of the midbrain (A and B) and putamen (B) show the signal hyperintensity. In Case 2, bilateral striatum reveal hypeintensity (C and D). The left optic radiation is also involved in Case 2 (C) and the global cerebral hemisphere is atrophic (C and D).

2. Case reports

2.1. Case 1

Case 1 is 3-year-old female that was referred to our hospital for an evaluation of failure to thrive and developmental delay at 2 years. She was born to healthy nonconsanguineous Japanese parents. The neonatal period was unremarkable. She held her head upright at 3 months of age, and sat at the 6 months. At the 9 months, she was able to walk independently while holding on to furniture. Her development did not progress thereafter, and she has not walked alone and only speaks using jargon. She was conscious, alert and presented with tachypnea at rest. She displayed facial dysmorphism including frontal bossing, lateral displacement of inner canthi, esotropia, maxillary hypoplasia, slightly upturned nostril, and hypertrichosis dominant on the forehead and extremities. Mild opthalmoplegia and ptosis were noted. She manifested generalized mild hypotonia, truncal ataxia and normal deep tendon reflexes with negative Babinski's signs. Serum lactate was elevated at 35.7 mg/dl. MRI showed signal hyperintensity of the bilateral putamen, subthalamic nucleus, red nucleus and brain stem on T2-weighted images (T2WI) and diffusion-weighted images (DWI) (Fig. 1). The enzyme analysis of the respiratory chain complexes were not performed in this patient.

2.2. Case 2

Case 2 is 8-year-old male on ventilation that was transferred to our hospital for tracheostomy. He was born at term to healthy, nonconsanguineous parents. He had been able to get cruising by 12 months. At 19 months, he presented with neurodevelopmental regression and ataxia. Laboratory investigation revealed elevated cerebrospinal fluid lactate and pyruvate. Brain MRI showed signal hyperintensity of the bilateral basal ganglia, midbrain and medulla oblongata on T2WI. Fibroblast analysis confirmed a decreased amount and activity of complex IV in the respiratory chain complexes

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(Fig. 2). He displayed facial dysmorphism including synophrys and micrognathia, hypertrichosis, thoracic deformity and generalized hypotonia and elevated deep tendon reflexes with positive Babinski's signs. MRI showed that the bilateral cerebral hemisphere were globally atrophic and signal hyperintensity of the bilateral optic radiation, putamen, basal ganglia including subthalamic nucleus, and brain stem on T2WI. The left optic radiation, bilateral putamen and globus pallidus also showed high signal intensity on DWI (Fig. 1).

3. Genomic DNA sequencing, RT-PCR and sequencing

Genomic DNA was prepared from white blood cells using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). PCR of all exons and exon–intron boundaries of the *SURF1* gene was performed with specific primers using Ex Taq PCR version 1.0 kit (Takara, Shiga, Japan) according to the manufactures instruction (Suppl. Table 1). Total RNA was extracted from leukocytes using Trizol reagent and amplified with the SMART™ mRNA amplification method (Clontech, Mountain View, CA). The amplified mRNA was subjected to reverse transcription with Prime Script reverse transcriptase (Takara, Shiga, Japan) using Oligo (dT) primers. RT-PCR was performed using primers at exons 1 and 9 of the *SURF1* gene, according to the manufacture's instruction (Suppl. Table 1). Patients and families participating in the gene analysis gave written informed consent to the gene analysis, which was approved by the ethical committee of Kanagawa Children's Medical Center.

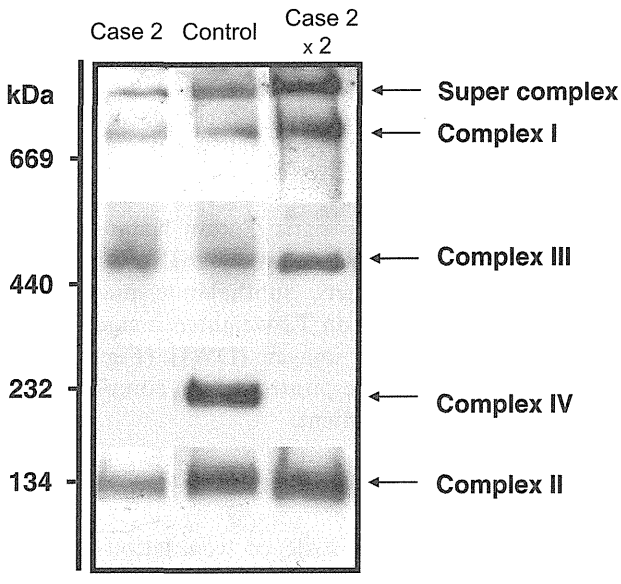

Fig. 2. Analysis of respiratory chain complex amount by blue native polyacrylamide gel electrophoresis in Case 2. Mitochondria isolated from Case 2 and normal control fibroblasts were solubilized in dodecyl maltoside and subjected to BN-PAGE and Western blotting [9]. In x 2 lane, the amount of protein loaded was twice. The amount of fully assembled complex IV was shown to be dramatically decreased in Case 2. The amount of complexes I, II, and III were all comparable to those in the normal control. In vitro enzyme assay [10] also revealed deficiencies of complex IV: the activities of complex I, II, III and IV relative to that of citrate synthase were 137%, 238%, 124% and 12%, respectively.

4. Results

4.1. Case 1

We identified two novel heterozygous mutations: a maternal c.49+1 G>T splice site mutation in intron 1 and a paternal c.752_753del in exon 8. This deletion resulted in a frame shift at amino acid 251(Gln251) causing a stop codon in exon 8 (Fig. 3). The c.49+1 G>T splice site mutation changes the highly conserved G nucleotide at position +1 of the donor splice site (5′ss) in intron 1. We attempted to characterize the splicing outcome of this sequence variation by RT-PCR analysis from patient's blood. Sequence analysis of the RT-PCR reaction detected only the allele with the c.752_753delAG mutation, which implies the presence of a nonsense mediated decay or instability of mRNA from the allele with the c.49+1 G>T splice site mutation.

4.2. Case 2

Sequence analysis of the *SURF1* gene revealed a novel homozygous c.743 C>A, p.Ala248Asp in exon 7. Both parents of this patient were heterozygous for this mutation (Fig. 3). This mutation changes highly conserved Alanine to Aspartate. This mutation was not found in 100 control alleles.

5. Discussion

Molecular elucidation of Leigh syndrome is challenging since many enzymes are involved, such as mitochondrial respiratory chain complexes I, II, III, IV, and V, and components of the pyruvate dehydrogenase complex. Mutation analysis in DNA is more complicated, even after focusing on respiratory complex IV. Mitochondrial-encoded *MTCO3* and nuclear-encoded *COX10*, *COX15*, *SCO2*, and *SURF1*, have been reported as the cause of Leigh syndrome [6,7]. Our two cases presented with mental retardation, failure to thrive, respiratory dysfunction, facial dysmorphism and hypertrichosis. Facial dysmorphism including micrognathia and hypertrichosis especially in the extremities have been reported to be distinctive and characteristic feature of *SURF1* gene mutation [3,4]. Our two cases underscore the importance of *SURF1* analysis in Leigh syndrome with facial dysmorphism and hypertrichosis. However, not all patients with this gene mutation carry these symptoms. Although facial dysmorphism has been also

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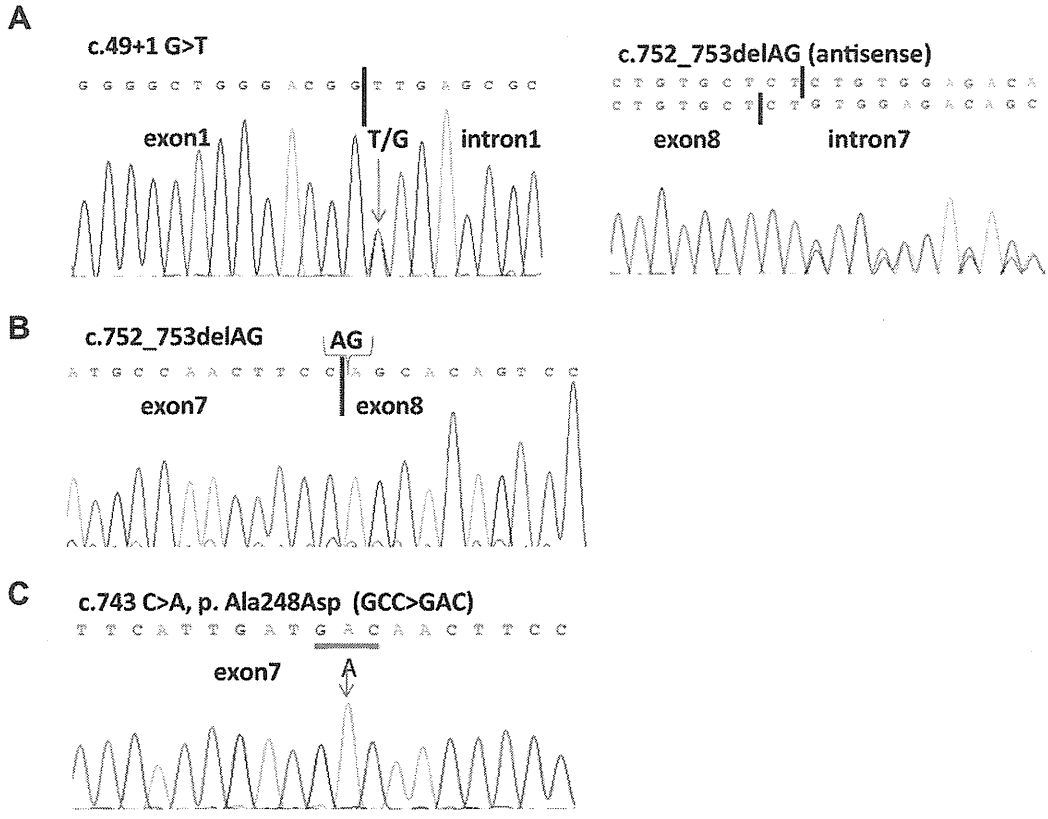

Fig. 3. Analysis of the *SURF1* gene. A chromatogram of the two novel heterozygous mutations; c.49+1 G>T and c.752_753del in Case 1 (A) and homozygous c.743 C>A in Case 2 (C). Panel B shows the chromatogram of cDNA from Case 1. The mutations are shown on the sense strand except for the right panel of A (antisense).

reported in Leigh syndrome with pyruvate dehydrogenase complex, hypertrichosis has not been described [8].

To date, more than 100 patients of Leigh disease with *SURF1* mutations have been reported [6,7]. To our knowledge, this is the first report of a mutation in intron 1, suggesting the need to scan whole exons and exon/intron boundaries.

Common MRI findings of Leigh syndrome are symmetric lesions in the brainstem, basal ganglia, thalamus and spinal cord, Leigh syndrome with *SURF1* mutation have been reported to involved the subthalamic nuclei, medulla, inferior cerebellar peduncles, and substantia nigra [5]. In addition, Case 2 showed signal hyperintensities in bilateral optic radiation on T2WI and DWI, which has not been reported previously in Leigh syndrome with *SURF1* mutations. Since Case 2 had never shown severe hypoxemia, this finding may be significant in patients with *SURF1* mutation or may appear in a progressed stage of disease.

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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.braindev.2012.02.007.

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