Table 1 (*continued*)

Treatment group	Medical group	Resective group	Palliative group
ESES, generalized continuous	1		
Bilateral generalized synchronous epileptic activities	54	3	23
Bilateral multifocal epileptic activities	108	10	13
Lateralized epileptic activities	20	10	
Focal epileptic activities	53	8	1
No epileptic activities	3		
Abnormal background activities	92	16	20
Normal	1		
MRI findings			
Focal - hemispheric MRI lesions	43	25	10
Bilateral MRI lesions	116	4	13
Normal MRI	67	1	6
Uncertain	24	1	7

resective surgery and six required palliative surgery. In the palliative group, one patient required resective surgery, and two required palliative surgery. In the resective group, four resective surgeries and one palliative surgery were performed. These 24 patients were treated as originally classified in subsequent analyses.

3.6. Complications during follow-up

Five deaths were reported by the third year after discharge in the medical group. The causes of death included sudden unexpected death in epilepsy (one case), ventricular tachycardia (one case), asphyxia at home (one case), septic shock (one case), and unknown (one case). No deaths were reported among the patients treated surgically.

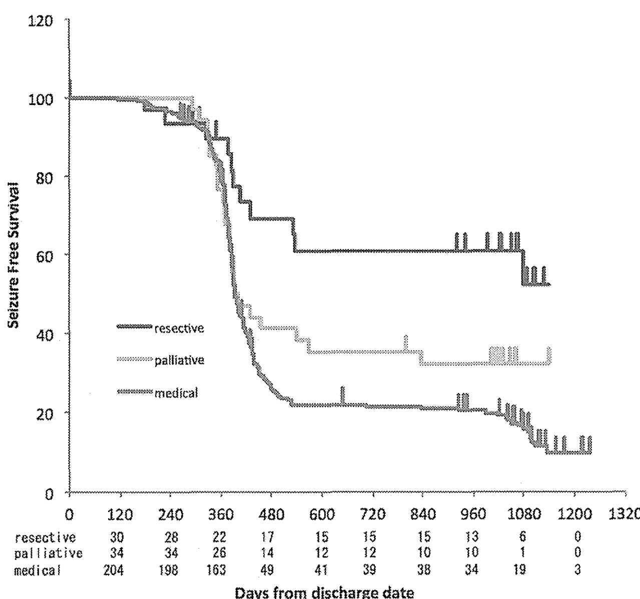

Fig. 1. Kaplan–Meier analysis of seizure-free survival in the medical, palliative, and resective groups. Numbers of children at risk in each of the treatment groups are shown along with their days of discharge date.

3.7. Seizure outcome

3.7.1. Seizure-free survival

Kaplan–Meier analysis of seizure-free survival time (Fig. 1) revealed that the median seizure-free survival was 395 days (95% CI, 388–413) in the medical group, 397 days (95% CI, 370–836) in the palliative group, and 1140 days (95% CI, 428–1140) in the resective group. At 3 years (1080 days) after discharge, the seizure-free survival rate according to Kaplan–Meier analysis was 15.7%, 32.1%, and 52.4% in the medical, palliative, and resective groups, respectively.

3.7.2. Cox proportional hazard analysis of the factors affecting seizure-free survival during the 3-year follow-up period

Cox proportional hazard analysis was undertaken to adjust for the different clinical backgrounds of the treatment groups, including age at seizure onset, age at admission, epilepsy syndrome, major etiology, seizure types, EEG findings on admission, and MRI findings (Table 2). It was revealed that the hazard ratios (HRs) of seizure recurrence in the resective and palliative groups versus the medical group were 0.43 (95% CI, 0.21–0.87, $P = 0.019$) and 0.82 (95% CI, 0. 46–1.46, $P = 0.50$), respectively; the former was statistically significant ($P < 0.025$ by Bonferroni correction).

3.7.3. Patient subgroup correlated with seizure-free outcome by surgery

Table 3 shows the seizure outcome in each patient subgroup divided by epilepsy syndrome and seizure type. It was found that children in the resective group demonstrated better seizure-free outcomes compared to the medical group pertaining to non-syndromic epilepsies [70.0% ($n = 10$) versus 13.3% ($n = 75$), $P < 0.01$, chi-square test]. On the other hand, children in the palliative group demonstrated better seizure-free outcomes compared to the medical group when their most disabling seizure type on admission was epileptic

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Table 2
Results of Cox proportional hazard analysis of the factors that affect seizure-free survival during the 3-year follow-up.

Variables	Reference	Hazard ratio	[95% CI]	p-value
Treatment group				
Palliative group	Medical group	0.819	[0.459–1.462]	$p = 0.50$
Resective group	Medical group	0.43	[0.212–0.872]	$p = 0.019^{*}$
Sex				
Male	Female	0.943	[0.684–1.301]	$p = 0.72$
Age of onset	Continuous variable	0.979	[0.964–0.994]	$p = 0.008$
Age of admission	Continuous variable	1.001	[0.991–1.011]	$p = 0.85$
Epilepsy syndrome on admission				
West syndrome		0.995	[0.496–1.998]	$p = 0.99$
Lennox–Gastaut syndrome		0.772	[0.375–1.588]	$p = 0.48$
Dravet syndrome		1.357	[0.564–3.269]	$p = 0.50$
Rasmussen syndrome		1.362	[0.344–5.386]	$p = 0.66$
Others		1.249	[0.811–1.922]	$p = 0.31$
Unclassified	Reference			
Major etiology				
Cortical dysplasias		1.868	[1.045–3.340]	$p = 0.035$
Genetic/chromosomal abnormalities		1.475	[0.894–2.433]	$p = 0.13$
Vascular lesions		0.685	[0.285–1.644]	$p = 0.40$
Infection		0.568	[0.245–1.318]	$p = 0.19$
Hypoxic encephalopathy		0.738	[0.435–1.255]	$p = 0.26$
Others		0.887	[0.469–1.676]	$p = 0.71$
Unknown	Reference			
Seizure types on admission				
Epileptic spasms	Else	0.985	[0.548–1.773]	$p = 0.96$
Head nodding	Else	0.604	[0.369–0.987]	$p = 0.044$
Generalized tonic	Else	1.16	[0.787–1.710]	$p = 0.45$
Generalized clonic	Else	0.58	[0.242–1.389]	$p = 0.22$
Generalized tonic clonic	Else	1.3	[0.768–2.200]	$p = 0.33$
Generalized absence (atypical/typical)	Else	1.4	[0.873–2.245]	$p = 0.16$
Generalized myoclonic (positive/negative)	Else	1.648	[0.988–2.748]	$p = 0.06$
Generalized atonic	Else	1.699	[0.722–3.999]	$p = 0.23$
Partial simple motor	Else	1.045	[0.618–1.767]	$p = 0.87$
Partial complex motor	Else	1.226	[0.751–2.002]	$p = 0.41$
Hypomotor seizure	Else	0.654	[0.306–1.398]	$p = 0.27$
Gelastic seizures	Else	5.609	[1.473–21.359]	$p = 0.012$
EEG findings on admission				
Hypsarrhythmia	Else	0.692	[0.385–1.242]	$p = 0.22$
Slow spike-wave burst	Else	1.371	[0.796–2.362]	$p = 0.26$
Bilateral generalized synchronous epileptic activities	Else	0.765	[0.500–1.173]	$p = 0.23$
Bilateral multifocal epileptic activities	Else	0.827	[0.561–1.220]	$p = 0.34$
Lateralized epileptic activities	Else	0.401	[0.199–0.807]	$p = 0.010$
Focal epileptic activities	Else	0.895	[0.545–1.469]	$p = 0.66$
Abnormal background activities	Else	1.034	[0.707–1.513]	$p = 0.86$
MRI findings				
Focal – hemispheric MRI lesions	Normal MRI	0.677	[0.369–1.239]	$p = 0.21$
Bilateral MRI lesions	Normal MRI	1.246	[0.818–1.899]	$p = 0.31$
Uncertain	Normal MRI	0.558	[0.302–1.028]	$p = 0.06$

[*] $p < 0.025$.

spasms [70.0% ($n = 10$) versus 30.3% ($n = 66$), $P < 0.05$, chi-square test].

3.8. Developmental outcome

3.8.1. Developmental status before admission

The overall psychomotor development was normal to borderline in 56.4% of children before seizure onset, whereas 17.5% and 26.1% of patients presented with mild to moderate and severe delay, respectively. On admission, 25.8% of children had normal to borderline psychomotor development, whereas 26.4% and 47.8% were characterized with mild to moderate and severe delays, respectively. In fact, 55.9% of children who were considered normal to borderline before seizure onset, developed

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Table 3
Seizure outcome in each patient subgroup divided by clinical background including epilepsy syndrome and seizure type.

(A) Epilepsy syndrome

Group			Epilepsy syndromes																Non-syndromic epilepsy		Total	
			West syndrome		Lennox–Gastaut syndrome		Ohtahara syndrome		Dravet syndrome		Doose syndrome		Rasmussen syndrome		Sturge–Weber syndrome		Other syndromes					
Medical		Sz free	20	31.7%	10	55.6%	0	0.0%	0	0.0%	1	33.3%	0	0.0%	0	0.0%	1	20.0%	10	13.3%	42	23.5%
		Sz not free	43	68.3%	8	44.4%	1	100%	10	100%	2	66.7%	3	100%	1	100%	4	80.0%	65	86.7%	137	76.5%
		Total	63	100%	18	100%	1	100%	10	100%	3	100.0%	3	100%	1	100%	5	100%	75	100%	179	100%
Palliative	VNS	Sz free	0	0.0%	1	100%													0	0.0%	1	16.7%
		Sz not free	2	100%	0	0.0%													3	100%	5	83.3%
	Callosotomy	Sz free	7	53.8%	1	14.3%													1	33.3%	9	39.1%
		Sz not free	6	46.2%	6	85.7%													2	66.7%	14	60.9%
		Total	15	100%	8	100%													6	100%	29	100%
Resective		Sz free	0	0.0%	4	80.0%							3	100%	1	100%	0	0.0%	7	70.0%	15	68.2%
		Sz not free	2	100%	1	20.0%							0	0.0%	0	0.0%	1	100%	3	30.0%	7	31.8%
		Total	2	100%	5	100%							3	100%	1	100%	1	100%	10	100%	22	100%

(B) Seizure type

Group			Most disabling seizure on admission																								Total	
			Epileptic spasms		Head nodding		Generalized tonic		Generalized clonic		Generalized tonic clonic		Generalized absence (atypical/ typical)		Generalized myoclonic (positive/ negative)		Generalized atonic		Partial simple motor		Partial complex motor		Hypomotor seizure		Gelastic seizure			
Medical		Sz free	20	30.3%	3	50%	6	17.6%	0	0%	0	0%	1	12.5%	3	37.5%	1	50%	1	14.3%	6	20%	1	50%	0	0%	42	23.5%
		Not free	46	69.7%	3	50%	28	82.4%	4	100%	11	100%	7	87.5%	5	62.5%	1	50%	6	85.7%	24	80%	1	50%	1	100%	137	76.5%
		Total	66	100%	6	100%	34	100%	4	100%	11	100%	8	100%	8	100%	2	100%	7	100%	30	100%	2	100%	1	100%	179	100%
Palliative	VNS	Sz free					0	0%					0	0%					1	100%							1	16.7%
		Not free					4	100%					1	100%					0	0%							5	83.3%
	Callosotomy	Sz free	7	70.0%	1	25%	1	14%			0	0%											0	0%			9	39.1%
		Not free	3	30.0%	3	75%	6	86%			1	100%											1	100%			14	60.9%
		Total	10	100%	4	100%	11	100%			1	100%	1	100%					1	100%			1	100%			29	100%
Resective		Sz free	2	50%			6	100%	1	50%					0	0%			2	66.7%	2	66.7%	2	100%	0	0%	15	68.2%
		Not free	2	50%			0	0%	1	50%					1	100%			1	33.3%	1	33.3%	0	0%	1	100%	7	31.8%
		Total	4	100%			6	100%	2	100%					1	100%			3	100%	3	100%	2	100%	1	100%	22	100%

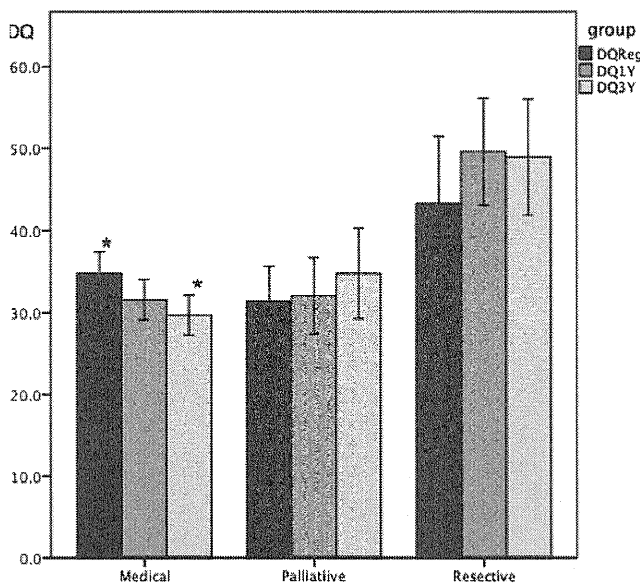

Fig. 2. Sequential DQ changes during the 3-year follow-up in each treatment group. The mean DQ score decreased significantly among the children in the medical group. DQReg: DQ at registration, DQ1Y: DQ at 1-year follow-up, and DQ3Y: DQ at 3-year follow-up. Bars indicate SE. $^{*}P < 0.05$.

some degree of delay by the time of admission (a mean interval of 20.2 months).

3.8.2. Change of DQ in each treatment group during the follow-up period

Changes in the developmental status of each treatment group were assessed in 172 children who obtained complete successive DQs during the 3-year follow-up period [medical group, 138; palliative group, 21 (callosotomy, 19; VNS, 2); resective group, 13]. Among the children in the medical group, the mean DQ score decreased from 34.8 (SD: 30.7) to 31.6 (SD: 29.2) and to 29.7 (SD: 28.7) at the 1-year and 3-year follow-up visits, respectively ($P < 0.05$, paired t-test). As for surgical groups, the mean DQ score changed from 31.4 (SD: 19.5) to 32.0 (SD: 21.2) and to 34.8 (SD: 25.2) in the palliative group, and from 43.2 (SD: 29.6) to 49.6 (SD: 23.6) and to 49.0 (SD: 25.5) in the resective group, respectively (Fig. 2).

The mean DQ was compared among treatment groups using ANCOVA adjusting for DQ on admission. As a result, the mean DQs in the resective group increased significantly compared to those in the medical group at 1- and 3-year follow-ups [17.0 (95% CI, 26.0–7.9, $P < 0.01$) at 1-year follow-up, and 20.0 (95% CI, 31.5–8.5, $P < 0.01$) at 3-year follow-up, respectively].

3.8.3. Patient subgroup correlated with better DQ changes by surgery

The mean DQ was compared among treatment groups using ANCOVA adjusting for DQ on admission according to each epilepsy syndrome. As a result, the mean DQs in the resective group increased significantly compared to those in the medical group in children with non-syndromic epilepsy [24.0 (95% CI, 29.9–18.1, $P < 0.01$) at 1-year follow-up, and 29.7 (95% CI, 36.7–22.7, $P < 0.01$) at 3-year follow-up, respectively]. No specific patient subgroup was identified, in which palliative surgery was significantly related to better ΔDQ during the follow-up (Supplementary Table 2).

4. Discussion

To the best of our knowledge, this is the first international multicenter prospective cohort study of infants and young children with epileptic encephalopathy that compared seizure and developmental outcomes between surgical and medical treatments. Regarding seizure outcome, it was found that seizure-free survival at the 3-year follow-up was 15.7%, 32.1%, and 52.4% in the medical, palliative, and resective groups, respectively. After adjusting clinical background in each treatment group using Cox proportional hazard model, it was revealed that children in the resective group demonstrated significantly better seizure-free outcomes compared with those in the medical group. Regarding the developmental outcome, the mean DQs in the resective group were increased significantly compared to those in the medical group during the follow-up. As for subgroup analysis, better seizure and development outcomes were demonstrated in the resective group compared to the medical group in children with nonsyndromic epilepsies (those to which no known epilepsy syndromes were applicable).

A number of articles have assessed the indications for resective surgery in children with epileptic encephalopathy during infancy and early childhood [5.6.10]. These studies described beneficial outcomes in children with focal or unilateral epileptogenic pathologies, such as cortical dysplasia, dysplastic tumors, vascular lesions, and encephalomalacia. In these conditions, deteriorations in brain function are caused by the epileptic seizures themselves, rather than the direct effects of the underlying pathology. Indeed, early surgical intervention has been recommended to avoid the negative effects on the developing brain. The present observational cohort study supports the findings of previous case studies.

In contrast, the indications for palliative surgery in children of this age with epileptic encephalopathy have been rarely reported [21]. This contrasts starkly with the abundance of literature available for older children with Lennox–Gastaut syndrome, in which callosotomy is reportedly effective for controlling the drop attacks caused by atonic seizures [22,23]. However, Pinard et al. [24] reported that epileptic spasms resolved after

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total callosotomy in 80% of 16 children with post-West syndrome. Other studies [25–27] also reported cases of children with epileptic spasms who achieved complete seizure resolution after callosotomy. In the present study, 70.0% of the 10 children with epileptic spasms treated by callosotomy were seizure free at the 3-year follow-up. These positive effects by callosotomy may be associated with functions of the corpus callosum in epilepsy, i.e., propagation, synchronization, and facilitation of epileptogenic activities between hemispheres [28,29]. However, the small sample size necessitates further investigations to confirm the efficacy of callosotomy in seizure and developmental prognosis. The neurophysiological mechanism of seizure cessation by callosotomy should also be investigated.

A limitation of this observational study is the difference in the patients' clinical background when comparing the outcomes between treatment groups, which is inevitable in a non-randomized cohort study. To control for this difference, we used the Cox proportional hazard model, which is a statistical technique for exploring the relationship between the survival of a patient and several explanatory variables. In this study, the treatment effect on seizure survival (seizure free or not) were compared between treatment groups (3 groups) with such covariables as epilepsy syndrome, etiology, seizure types, EEG findings, and MRI findings. Statistical significance was defined as $P < 0.025$ based on Bonferroni correction.

Regarding developmental prognosis, worse developmental outcomes were reported for children with earlier onset, a longer duration from onset to admission, epileptic spasms, symptomatic epilepsies, and higher number of presurgical AED trials [30,31]. Conversely, postsurgical seizure control was reported to predict higher DQ scores after surgery and correlate with improvements in the DQ [32,33].

Another important and striking finding in the current study is that children with medical treatment demonstrated significant successive developmental deterioration, especially in children with higher baseline DQ. In fact, 55.9% of children having normal to borderline statuses before seizure onset became developmentally delayed at the time of admission, with a mean interval of 20.2 months. Moreover, the mean DQ of children in the medical group decreased successively during the follow-up. Conversely, seizure cessation and surgical interventions for selected patients could reverse this developmental deterioration. However, there may be a relatively narrow time frame in which children with epileptic encephalopathy can recover normal brain function, given that a short treatment delay of months or weeks could affect long-term cognitive prognosis significantly [34,35]. Therefore, it should be stressed that children with frequent seizures require urgent and careful treatment to prevent further deteriorations in their developmental statuses.

The presence of focal, lobar, or unilateral hemispheric MRI pathologies was a key indication for selecting patients for resective surgery. The prognosis of these children is generally worse than those with nonlesional cases, and can be improved by surgery [36]. However, an important feature of epileptic encephalopathy in infancy and early childhood is the similar clinical phenotype that presents despite the wide variety of underlying etiologies. Indeed, the seizure types and EEG findings are often indistinguishable among causes, including genetic abnormalities, cortical malformations, perinatal insults, and tumors [37]. Moreover, cortical dysplasia, which was the most common etiology in the resective group in the current study, often shows very subtle MRI changes; therefore, other multimodal functional imaging studies are needed for an accurate diagnosis [38,39]. Early neuroimaging studies, particularly using high magnetic field MRI, are indispensable in these children to help differentiate the etiology and start the most appropriate treatment.

In conclusion, the present study revealed that epilepsy surgery had a significant effect on the seizure and developmental outcomes in children with epileptic encephalopathy during infancy and early childhood. This was particularly the case for those that underwent resective surgery. The present observations may facilitate the identification of infants and young children with epileptic encephalopathy who could benefit from surgery.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.braindev.2015.11.004.

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Original article

Clinical, biochemical and molecular investigation of adult-onset glutaric acidemia type II: Characteristics in comparison with pediatric cases

Kenji Yamada [a,*], Hironori Kobayashi [a], Ryosuke Bo [a], Tomoo Takahashi [a], Jamiyan Purevsuren [a], Yuki Hasegawa [a], Takeshi Taketani [a], Seiji Fukuda [a], Takuya Ohkubo [b], Takanori Yokota [b], Mutsufusa Watanabe [c], Taiji Tsunemi [d], Hidehiro Mizusawa [e], Hiroshi Takuma [f], Ayako Shioya [f], Akiko Ishii [f], Akira Tamaoka [f], Yosuke Shigematsu [g], Hideo Sugie [h], Seiji Yamaguchi [a]

[a] *Department of Pediatrics, Shimane University Faculty of Medicine, Izumo, Shimane, Japan*
[b] *Department of Neurology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[c] *Department of Internal Medicine, Tokyo Metropolitan Bokutoh Hospital, Sumida-ku, Tokyo, Japan*
[d] *Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA*
[e] *National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan*
[f] *Department of Neurology, University of Tsukuba Faculty of Medicine, Tsukuba, Ibaraki, Japan*
[g] *Department of Pediatrics, University of Fukui Faculty of Medical Sciences, Yoshida-gun, Fukui, Japan*
[h] *Faculty of Health and Medical Sciences, Tokoha University, Hamamatsu, Shizuoka, Japan*

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Abstract

Introduction: An increasing number of adult patients have been diagnosed with fatty acid β-oxidation disorders with the rising use of diagnostic technologies. In this study, clinical, biochemical, and molecular characteristics of 2 Japanese patients with adult-onset glutaric acidemia type II (GA2) were investigated and compared with those of pediatric cases.

Methods: The patients were a 58-year-old male and a 31-year-old male. In both cases, episodes of myopathic symptoms, including myalgia, muscle weakness, and liver dysfunction of unknown cause, had been noted for the past several years. Muscle biopsy, urinary organic acid analysis (OA), acylcarnitine (AC) analysis in dried blood spots (DBS) and serum, immunoblotting, genetic analysis, and an *in vitro* probe acylcarnitine (IVP) assay were used for diagnosis and investigation.

Results: In both cases, there was no obvious abnormality of AC in DBS or urinary OA, although there was a increase in medium- and long-chain ACs in serum; also, fat deposits were observed in the muscle biopsy. Immunoblotting and gene analysis revealed that both patients had GA2 due to a defect in electron transfer flavoprotein dehydrogenase (ETFDH). The IVP assay indicated no special abnormalities in either case.

Conclusion: Late-onset GA2 is separated into the intermediate and myopathic forms. In the myopathic form, episodic muscular symptoms or liver dysfunction are primarily exhibited after later childhood. Muscle biopsy and serum (or plasma) AC analysis allow accurate diagnosis in contrast with other biochemical tests, such as analysis of AC in DBS, urinary OA, or the IVP assay, which show fewer abnormalities in the myopathic form compared to intermediate form.
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* Corresponding author at: 89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan. Tel.: +81 853 20 2219; fax: +81 853 20 2215.
E-mail address: k-yamada@med.shimane-u.ac.jp (K. Yamada).

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Keywords: Multiple acyl-CoA dehydrogenase deficiency (glutaric acidemia type II); Adult onset; Myopathy; Serum acylcarnitine; Immunoblotting; *In vitro* probe acylcarnitine assay

1. Introduction

Many organic acidemias or fatty acid oxidation disorders (FAODs) are often believed to be symptomatic in childhood, especially in early infancy [1]. However, an increasing number of adult patients with inherited metabolic diseases (IMDs) has recently been identified with new developments in diagnostic technologies, including mass spectrometry, and the spread of knowledge regarding IMDs, even in the field of adult neurology.

Glutaric acidemia type II (GA2) is an autosomal recessive disease caused by a defect in electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH), resulting in deficiencies in multiple acyl-CoA dehydrogenases, such as short-, medium-, and long-chain acyl CoA dehydrogenases, as well as isovaleryl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, and sarcosine dehydrogenase [2,3]. GA2 has been clinically classified into 2 types: (1) the neonatal-onset type, which develops during the neonatal period or early infancy and is often severe, and (2) the late-onset type, which develops after the infantile period [4].

Patients with the neonatal-onset type of GA2 develop severe respiratory failure, cardiomyopathy, hypotonia, metabolic acidosis, and profound hypoglycemia soon after birth, and they often have a fatal outcome in early infancy. Some patients with this type have congenital anomalies, including Potter's face or polycystic kidney disease [5,6]. In the late-onset type, intermittent episodic attacks of lethargy, hypoglycemia, and hyperammonemia, or, occasionally, acute encephalopathy or sudden death triggered by infection, diarrhea, or long fasting are seen starting in early childhood [7–9].

Recently, several adult-onset GA2 cases have been reported [10–13]. However, it is not always easy to establish the correct diagnosis. In this study, the clinical, biochemical, and pathological characteristics of 2 cases of adult-onset GA2 were investigated and compared with those of pediatric cases.

2. Materials and methods

2.1. Patients

Case 1 was a 58-year-old male with chief complaints of episodic myalgia and muscle weakness. The clinical course of case 1 has been reported previously [14]. His younger brother died unexpectedly from an unknown cause in his 30s. The patient sometimes had general fatigue, myalgia, or muscle weakness as early as in his 40s. Those symptoms progressively worsened in his 50s, and he began to use a wheelchair because of persistent muscle weakness and myalgia. Furthermore, he had 3 episodes of unconsciousness after the age of 50. Although he was hospitalized at the third episode, there were no obvious abnormalities in routine biochemical tests, including blood sugar and liver function. He visited several neurology clinics and hospitals to undergo a more detailed examination. However, no abnormality was found, except for the occasional elevation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and creatine kinase (CK). The diagnosis was "myopathy of unknown cause". Then, as he repeatedly developed liver dysfunction and rhabdomyolysis, he was hospitalized at age 58 for detailed examination, including muscle biopsy.

On admission, his level of consciousness was normal, and his vital signs and intelligence were normal. No hepatosplenomegaly was noted. Muscle tenderness and atrophy with mild sensory dysfunction were observed in his limbs, especially in the lower limbs, as neurological findings. The deep tendon reflex was normal, and he was able to walk with support. In manual muscle testing, his muscle strength was level 2 for the deltoid and iliopsoas muscles and 3+ to 4 for other upper and lower limb muscles.

Routine blood examination indicated the elevation of liver and muscle enzymes, such as AST (197 IU/L, normal range 10–38), ALT (215 IU/L, normal 5–40), LDH (2903 IU/L, normal 100–215), and CK (2364 IU/L, normal 36–216), as shown in Table 1.

Case 2 was a 31-year-old male with episodic muscle weakness and myalgia similar to case 1. No abnormalities in his past and family history were noted. He was formerly a baseball player on a non-professional team, but he developed muscle weakness after retiring from the baseball team at 29 years of age. Then, his exertional muscle weakness worsened gradually, and he began to experience difficulty in his daily activities. Although he visited several neurology clinics or hospitals, only liver dysfunction of unknown cause was occasionally noted. He was hospitalized to undergo further examination at 31 years of age.

His level of consciousness and his intellectual level were normal. Abnormalities in vital signs and hepatosplenomegaly were not observed. His patellar and Achilles tendon reflexes were slightly reduced, but no pathological reflex or muscle atrophy was observed.

Table 1
Outlines of the patients and results of routine laboratory tests.

	Case 1	Case 2	(Reference value[a])
Onset age	40s	31	
Sex	M	M	
Clinical features			
	Myalgia Muscle weakness Rhabdomyolysis	Myalgia Muscle weakness	
Routine blood examination			
CBC			
WBC (/μL)	4800	5000	(3300–8600)
RBC ($\times 10^4$/μL)	370	539	(385–438)
Hb (g/dL)	12.3	16.5	(11.0–14.8)
Plt ($\times 10^4$/μL)	18.7	20.7	(15.8–35.3)
Biochemical data			
T-Bil (mg/dL)	0.3	0.8	(0.2–1.2)
TP (g/dL)	5.6	7.3	(6.5–8.2)
Alb (g/dL)	3.4	5.1	(3.8–5.1)
AST (IU/L)	197	71	(10–38)
ALT (IU/L)	215	84	(5–40)
LDH (IU/L)	2903	684	(100–215)
ALP (IU/L)	178	152	(110–340)
CK (IU/L)	2364	689	(36–216)
BUN (mg/dL)	7	10.9	(8.0–21.0)
Cre (mg/dL)	0.35	0.5	(0.44–0.83)
Na (mEq/L)	138	139	(137–146)
K (mEq/L)	3.4	4.1	(3.5–4.9)
Cl (mEq/L)	101	103	(98–109)
Ca (mg/dL)	8.8	10.6	(8.6–10.3)
BS (mg/dL)	90	104	(60–109)

[a] The reference values used at Shimane University. Abnormal findings are underlined.

The results of manual muscle testing were also within the normal range.

Blood examination indicated a slight elevation of liver and muscle enzymes (AST 71 IU/L, ALT 84 IU/L, LDH 684 IU/L, and CK 689 IU/L), although no abnormalities were observed in other tests.

This study was conducted with the approval of the Institutional Review Board of Shimane University and consent from the patients.

2.2. Urinary organic acid analysis

The urinary organic acids (OAs) were analyzed using gas chromatography mass spectrometry (GC/MS; QP-2010 plus; Shimadzu, Kyoto, Japan) at Shimane University, Japan, after solvent extraction and oxime-trimethylsilyl derivatization of urine samples as previously described [1,15].

2.3. Blood acylcarnitine analysis

Acylcarnitine (AC) in dried blood spots (DBS) or serum was analyzed using tandem mass spectrometry (MS/MS) (API-3000; Applied Biosystems, Foster City, CA, USA) after butyl-derivatization of samples, as previously described [16,17].

2.4. Histological studies

Muscle biopsies were performed using the rectus femoris muscle and biceps brachii in cases 1 and 2, respectively. The biopsied materials were frozen and cryostat-sectioned for Oil-Red O staining [18].

2.5. Cell culture

Skin fibroblasts were cultured in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 2 mmol/L glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2/95% air incubator until confluence [19,20].

2.6. Immunoblotting

Twenty five micrograms of protein derived from the cellular extract of a pellet of cultured fibroblasts was subjected to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE). Immunoblotting was performed according to a routine protocol using rabbit polyclonal antibodies against ETF, which were a gift from Dr. T. Hashimoto (Professor Emeritus of Shinshu University, Matsumoto, Japan),

and ETFDH, which was purchased from Japan Bio Services Co., Ltd. (Saitama, Japan), as the primary antibodies. Blots were visualized using the Immuno-Pure NBT/BCIP Substrate Kit TM (Promega, Madison WI, USA) [19,21].

2.7. Gene analysis of ETFDH

Genomic DNA was isolated from fibroblasts using a QIAamp DNA Microkit (QIAGEN GmbH, Hilden, Germany). Each exon of *ETFA*, *ETFB*, and *ETFDH*, including intron/exon boundaries, was PCR-amplified for 30 cycles. Primers for *ETFDH* were prepared as previously reported [2,14]. The PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) or CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA).

2.8. In vitro probe acylcarnitine (IVP) assay

An IVP assay to evaluate the β-oxidation capacity was performed as previously described [20]. Briefly, confluent cells were harvested by trypsinization and seeded onto 6-well microplates with fresh medium (described above) until they again reached confluence. Thereafter, cells were washed twice with D-PBS and cultured at 37 °C in 1 mL of experimental MEM containing 0.4% essential fatty acid-free BSA, 0.4 mmol/L L-carnitine, and 1% penicillin/streptomycin with 0.2 mmol/L unlabeled palmitic acid. The concentration of ACs in 10 μL of the culture medium after incubation for 96 h was determined by MS/MS.

3. Results

3.1. Urinary organic acid analysis

No obvious abnormalities were found for urinary OAs under stable conditions for both cases 1 and 2 (Table 2).

3.2. Blood acylcarnitine analysis

In the AC profiles in DBS, there were no obvious abnormalities in case 1, while there was slight elevation from C4 to C18 in case 2 (Table 3).

In contrast, in the serum AC analysis, slight elevation of C8 and C10 was observed, even under the stable conditions of case 1, and remarkable elevation from C8 to C18 was observed in case 2 (Table 3).

3.3. Histological studies

Muscle tissues stained with Oil-Red O revealed abundant fat deposition in both cases 1 and 2, suggesting metabolic myopathy (Fig. 1A and B).

3.4. Immunoblotting

In both cases 1 and 2, ETFDH protein was not detected, while both ETFα and ETFβ proteins were

Table 2
Results of special examinations.

	Case 1	Case 2
Muscle biopsy	Lipid deposit	Lipid deposit
Urinary organic acid analysis	Normal	Non-specific finding
Blood acylcarnitine analysis (dried blood spots)	Normal	Mild elevation of C4-C18
Gene analysis of *ETFDH*	c.1367C>T (p.P456L) (homozygote)	c.890G>T (p.W297L)/c.950C>G (p.P317R)

Table 3
Comparison of free carnitine and acylcarnitine in DBS and serum.

	Dried blood spot			Serum		
	Case 1	Case 2	(Reference)	Case 1	Case 2	(Reference)
C0	37.94	45.37	(20–60)	32.79	52.35	(10–55)
C2	28.07	46.19	(5–45)	11.56	33.02	(4–60)
C4	0.37	1.77	(<1.4)	0.27	0.78	(<1.65)
C8	0.06	0.98	(<0.25)	1.92	1.61	(<0.46)
C10	0.18	2.03	(<0.35)	1.88	4.63	(<0.8)
C12	0.09	0.8	(<0.4)	0.24	1.35	(<0.4)
C14	0.38	1.01	(<0.7)	0.08	3.29	(<0.3)
C16	2.90	3.12	(<7.0)	0.22	1.19	(<0.5)
C18	1.14	2.32	(<2.1)	0.06	0.55	(<0.3)

The reference values reported here are those used at Shimane University. Values judged as abnormal are underlined.

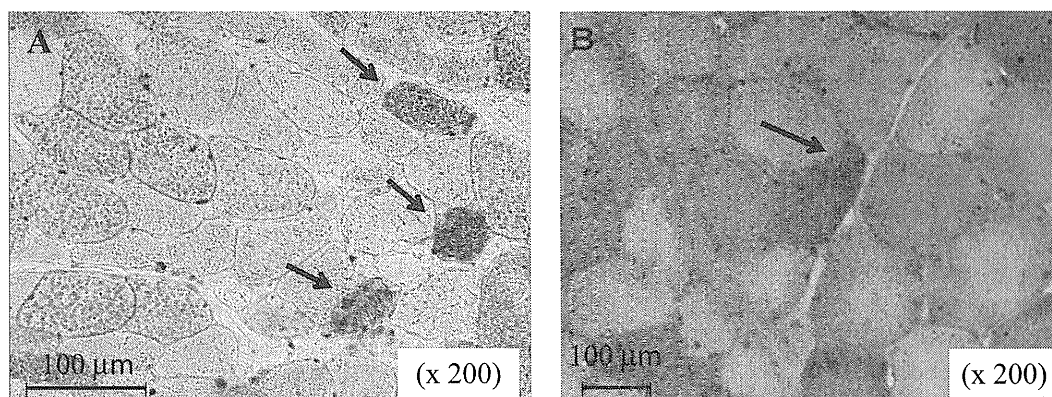

Fig. 1. Pathological findings from the muscle biopsy (Oil-red O stain). (A) Case 1 and (B) case 2. Arrows indicate lipid deposits.

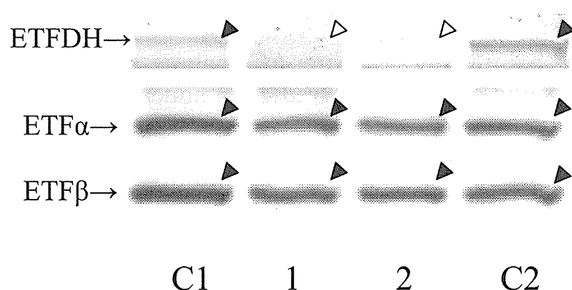

Fig. 2. Immunoblots of ETFDH and ETF proteins using fibroblasts. Lanes C1 and C2, normal controls; lanes 1 and 2, cases 1 and 2, respectively. Black and white triangles indicate a presence and absence of the protein, respectively.

observed to be normal. These findings strongly suggested that both patients had GA2 due to a defect in ETFDH (Fig. 2).

3.5. Gene analysis of ETFDH

Mutation analysis revealed that case 1 was a homozygote of c.1367C>T (p.P456L), and case 2 was a compound heterozygote of c.890G>T (p.W297L) and c.950C>G (p.P317R). Eventually, both cases were diagnosed with GA2 due to a defect in ETFDH (Table 2).

3.6. In vitro probe acylcarnitine assay

Only a slight elevation in C10 was observed in case 2, and the elevation of short- to long-chain ACs, which is a characteristic profile for the IVP assay in pediatric cases of GA2, was not observed in either case (Fig. 3A and B).

4. Discussion

In this study, we report the clinical, biochemical, and molecular aspects of the adult-onset myopathic form of GA2 in 2 cases. Our cases exhibited the following characteristics compared with pediatric cases: (1) repeated episodes of general fatigue, myalgia, or muscular hypotonia after adulthood (approximately 30 or 40 years of age); (2) in routine laboratory findings, slight or moderate elevation of AST, ALT, LDH, and CK; (3) no specific abnormalities for urinary OA analysis under stable conditions; (4) no or barely observable abnormalities in the AC analysis in DBS; (5) significant abnormalities for ACs in the serum; (6) lipid deposition in the muscular biopsy as an initial hint suggesting a GA2 diagnosis; and (7) no abnormalities in the IVP assay for adult-onset cases.

In both cases, few or no abnormalities were detected in several examinations, including urinary OA analysis and AC analysis in DBS. Indeed, cases of adult-onset GA2 with little biochemical abnormality have been previously reported [22,23], suggesting that a biochemical diagnosis of adult-onset GA2 is challenging. Therefore, a number of adult-onset GA2 patients with myopathy of unknown cause might be hidden. Likewise, there is a possibility of overlooking adult-onset GA2 in neonatal mass screening using DBS.

Serum AC analysis appeared to be more informative than DBS for diagnosing adult-onset GA2. There are previous reports that serum or plasma AC analysis could be more useful than DBS for diagnosing long-chain FAODs, such as very long-chain acyl-CoA dehydrogenase deficiency or carnitine palmitoyltransferase-II deficiency [17,24].

The histological findings of lipid deposition provided an initial clue for the diagnosis of GA2 in both of our cases. If fatty degeneration is revealed by muscle biopsy in patients with myopathy of unknown cause, the possibility of FAODs should be considered, even in adult cases.

We previously reported that pediatric cases of GA2 could be classified into the severe or milder form using the results of the IVP assay [25]. However, the profiles for the IVP assay in our cases were different from those of the severe or milder forms. In other words, the biochemical characteristics of adult-onset GA2 are different

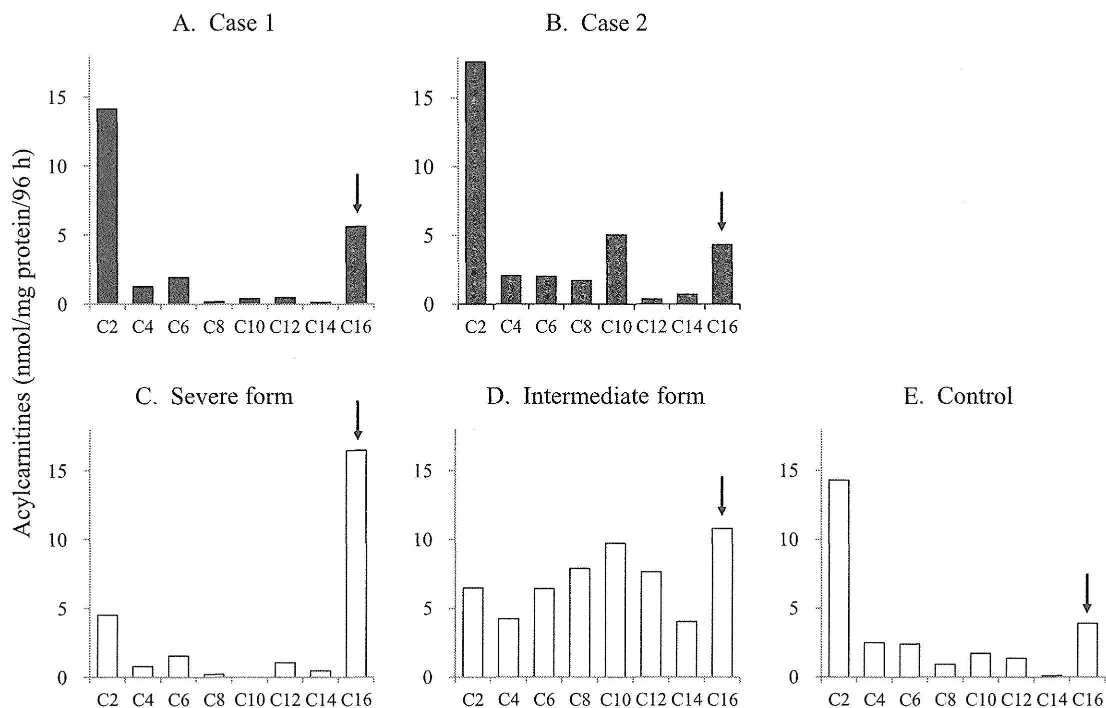

Fig. 3. Profiles of the *in vitro* probe assay. Arrows indicate loaded fatty acid (palmitic acid). The *Y*-axis represents values of acylcarnitines expressed as nmol/mg protein/96 h. (A) Case 1; (B) case 2; (C) patient with a severe form of GA2 due to defect of *ETFA* with homozygote of IVS6-1G>C (frame shift); (D) patient with an intermediate form of GA2 due to defect of *ETFDH* with compound heterozygote of c.G1078C (p.A360P) and c. T1519G (p.Y505D); and (E) healthy controls. Black and white columns indicate our cases and previously tested cases of the severe form, the intermediate form, and the control, respectively.

from those of pediatric cases. Additionally, we determined whether abnormal findings in the IVP assay could be improved by bezafibrate [26], but it may be difficult to evaluate the efficacy of bezafibrate for adult-onset GA2 because the profile of the IVP assay in adult-onset GA2 does not encompass specific abnormalities. However, treating the patients with adult-onset GA2 using bezafibrate may be helpful, even though efficacy of bezafibrate cannot be estimated *in vitro*, because bezafibrate was effective for a pediatric case which is more serious than the adult-onset type [26].

The clinical findings in case 1 included at least three episodes of unconsciousness, which were estimated to be caused by a hypoglycemic attack. Moreover, the younger brother of case 1 had previously died suddenly from an unknown cause in his 30s, suggesting that he might also have had GA2 and then developed profound hypoglycemia or arrhythmia, leading to sudden death. There are previous case reports of adult-onset GA2 cases with serious complications, including a 25-year-old female who was treated with a ventilator due to respiratory muscle failure [27] and a 19-year-old female patient who had repeated hypoglycemic attacks [28]. These cases indicate that critical symptoms can occur in the adult-onset type.

Clinical and biochemical features of adult-onset GA2 have recently been reported, as shown in Table 4. All were myopathic cases associated with ETFDH deficiency. However, there is also a report of a late-onset type other than ETFDH deficiency, although this is very rare [29]. It is considered that GA2 due to defect of ETFDH tend to be milder form in particular in Asian peoples, although some patients with defect of ETFDH occasionally exhibited severe clinical features [14]. The clinical severity varied; severe general symptoms manifested in patients with adult-onset GA2 despite few biochemical abnormalities, as in case 1 reported here and a case reported by Rosenbohm et al. [27], suggesting an unlikely association between the degree of clinical severity and biochemical abnormality.

GA2 has been roughly classified into the neonate-onset and late-onset types [4]. However, the clinical course of the "late-onset type" differs substantially among individuals; some cases have encephalopathy or sudden death during the infantile period, while others may only have muscular symptoms in adulthood, as was the case with the patients reported here. Therefore, we propose to distinguish the late-onset type of GA2 between the intermediate and myopathic forms, as shown in Table 5, according to the results of the IVP assay as well as age at onset, fatality, and clinical characteristics. The intermediate form (juvenile-onset form) exhibits intermittent attacks, including hypotonia, hypoglycemia, hyperammonemia, and acute

Table 4
Recently reported clinical and biochemical features for adult-onset GA2.

No	Sex	Age at onset (year)	Myalgia	Muscle weakness	Other symptoms	Laboratory data			Increased urinary organic acid	Elevated acylcarnitines		Gene	Gene mutation		Refs.
						Elevated trans-aminase	LDH (IU/L)	CK(IU/L)		DBS	Serum (plasma)		Allele 1	Allele 2	
Our cases															
1	M	40s	+	+	Coma	+	2903	3000	Normal	Normal	C8–C10	ETFDH	p.P456L	p.P456L	Our case
2	M	31	+	+	No	+	2860	1897	Normal	C4–C12	C8–C18	ETFDH	p.W297L	p.P317R	Our case
Previously reported cases															
3	M	42	+	+	No	N/A	942	1855	GA, 2HG, EMA	C4, C5, C8, C10, C14	N/A	ETFDH	p.I243T	p.T294I	Köppel et al. [10]
4	F	24	+	+	No	N/A	N/A	677	N/A	C8–C12	N/A	ETFDH	p.L409F	p.V291G	Wen et al. [23]
5	F	23	+	+	Vomiting	N/A	N/A	513	N/A	C8–C12	N/A	ETFDH	p.L409F	p.V291G	Wen et al. [23]
6	F	48	+	+	Vomiting	N/A	N/A	128	N/A	C0 (↓), C8–C10	N/A	ETFDH	p.Y257C	Not detected	Wen et al. [23]
7	F	22	–	+	No	N/A	N/A	478	GA, 2HG, EMA, DCA, KB	C4-OH, C10–C14	N/A	ETFDH	p.Y257C	p.V291G	Wen et al. [23]
8	F	33	+	+	No	N/A	N/A	352	GA, 2HG, EMA, DCA, KB	C0 (↓), C12–C14	N/A	ETFDH	p.Y257C	p.325del48	Wen et al. [23]
9	F	63	–	+	No	N/A	N/A	2120	GA,2HG, EMA, DCA	C0, C5–C14	N/A	ETFDH	IVS3+1G>A heterozygote	None	Wen et al. [23]
10	F	23	+	+	Vomiting	N/A	N/A	1998	GA, 2HG, EMA, DCA, KB	C8–C14	N/A	ETFDH	p.M404T	Not detected	Wen et al. [23]
11	F	22	+	–	No	N/A	N/A	339	normal	C0	N/A	ETFDH	p.L409F	Not detected	Wen et al. [23]
12	M	46	+	+	Difficulty in breathing	+	543	5995	GA, 2HG, DCA	N/A	N/A	ETFDH	p.M404T	p.D596N	Izumi et al. [11]
13	F	55	+	+	No	N/A	N/A	8000	normal	N/A	C4–C18	ETFDH	p.H293D	Not detected	Kaminsky et al. [22]
14	M	36	–	–	Exercise intolerance	N/A	1161	3055	2HG, 2-OH adipate	N/A	N/A	ETFDH	p.D511 N	p.W603X	Sugai et al. [12]
15	M	53	+	+	Osphyalgia, nausea	+	600	571	GA, 2HG, EMA	C8–C12	N/A	ETFDH	p.P508T	p. N528KfsX3	Zhao et al. [13]
16	F	24	+	+	Vomiting, respiratory insufficiency	+	N/A	20,000	2HG, EMA, DCA, HG, SG	N/A	C2 (↓), C14:1	ETFDH	p.S515I	p.S515I	Rosenbohm et al. [27]

LDH: lactate dehydrogenase, CK: creatine kinase, DBS: dried blood spot, N/A: not available, GA: glutarate, HG: 2-hydroxyglutarate, EMA: ethylmalonate, DCA: dicarboxylate, KB: ketone body, HG: hexanoylglycine, SG: suberylglycine, and (↓): decreased.

Table 5
Classification of glutaric acidemia type II based on the severity and IVP assay results.

Clinical form	Age at onset	Clinical course	Mortality	Biochemical abnormality	*In vitro* probe assay with C16 loaded
1. Severe from (neonatal-onset)	Soon after birth	Rapid onset and early death after birth hyperammonemia, hypoglycemia, or cardiomyopathy	++	++	Marked elevation of C16
2. Intermediate form (juvenile-onset)	Infantile or childhood	Episodes of lethargy, liver dysfunction, or hypoglycemia occasionally encephalopathy or even sudden death	+	+	Elevation of C4–C16
3. Myopathic form (adult-onset)	School-age or adulthood	Episodes of myalgia, muscle weakness, fatigue, or liver dysfunction	−	±	Almost normal

encephalopathy-like attack, with typical biochemical abnormalities and relatively high mortality following metabolic stress from an infection or diarrhea in infancy or young childhood. The IVP assay for the intermediate form reveals the elevation of broad ranges in acylcarnitine (C4–C16) when palmitate is loaded (Fig. 3D) [25]. The myopathic form (adult-onset form), in which the patients primarily present with intermittent muscular symptoms after adolescence or adulthood with normal intelligence, offers a favorable life prognosis in many cases. However, it should be noted that muscle symptoms are sometimes exhibited during the infantile period even in the myopathic form [30].

The above classification based on the IVP assay can also be used for preclinical risk control of GA2 detected in neonatal mass screening. Moreover, it is considered that making diagnosis using IVP assay is useful because clinical form cannot be predicted only by the genotype. It is expected that, with the spread of knowledge regarding the clinical characteristics of adult-onset GA2, such a form of GA2 will be found among patients with "myopathy of unknown origin" in the future.

Conflict of interest

The authors indicate no potential conflict of interest.

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Case Report

A fetus with mitochondrial trifunctional protein deficiency: Elevation of 3-OH-acylcarnitines in amniotic fluid functionally assured the genetic diagnosis

Ryosuke Bo [a,b,*,1], Yuki Hasegawa [a], Kenji Yamada [a], Hironori Kobayashi [a], Takeshi Taketani [a], Seiji Fukuda [a], Seiji Yamaguchi [a]

[a] *Department of Pediatrics, Shimane University Faculty of Medicine, 89-1, Ennya-cho, Izumo, Shimane 6938501, Japan*
[b] *Department of Pediatrics, Kobe University Graduate School of Medicine, 7-5-1, Kusunokicho, Chuo, Kobe, Hyogo 6500017, Japan*

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ABSTRACT

Mitochondrial trifunctional protein (TFP) is a multienzyme complex that catalyzes the last three steps of the β-oxidation cycle of long-chain fatty acids. In the prenatal diagnosis of TFP deficiency, acylcarnitine (AC) analysis has been considered difficult because of limited excretion of long-chain ACs into the fetal urine and hence into the amniotic fluid. Here, we report our experience with prenatally diagnosing TFP deficiency using AC analysis of amniotic fluid. The index case was a boy born at 38 weeks gestation and weighing 2588 g. He suddenly became unconscious and hypoglycemic and died on day 6 of life. Postmortem blood AC analysis and gene sequencing revealed TFP deficiency. Therefore, the parents underwent prenatal diagnoses for their subsequent 2 pregnancies. Mutation analysis suggested that one (Case 1) was affected and the other (Case 2) was not. AC analysis also demonstrated identical results, with significantly elevated 3-hydroxy-AC levels in the amniotic fluid of the affected pregnancy compared with those of heterozygotes and normal controls ($n = 2$ for heterozygotes and $n = 8$ for normal controls). Our findings suggest that AC analysis can functionally confirm results even in families with unidentified mutations, without raising issues related to maternal cell contamination. During prenatal diagnosis, misdiagnosis has to be avoided, and combining AC analysis with gene sequencing may result in more accurate prenatal diagnosis of TFP deficiency.

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1. Introduction

Mitochondrial trifunctional protein (TFP) is a multienzyme complex consisting of trans-2,3-long-chain enoyl-CoA hydratase (LCEH, EC 4.2.1.74), long-chain 3-OH-acyl-CoA dehydrogenase (LCHAD, EC 1.1.1.211) located in the TFP α-subunit (HADHA, OMIM: 600890), and long-chain 3-ketoacyl-CoA thiolase (LCKT, EC 2.3.1.16) located in the TFP β-subunit (HADHB, OMIM: 143450). These enzymes catalyze the last three steps of the β-oxidation cycle of long-chain fatty acids [1,2]. TFP deficiency is clinically classified into three types: 1) lethal type (neonatal-onset form), which includes the development of profound hypoglycemia, lactic acidosis and cardiomyopathy during the neonatal period; 2) intermediate type (infant-onset form), which is accompanied by hypoketotic hypoglycemia that is generally observed following infection or long periods of fasting during the infantile period; and 3) myopathic type (adult-onset form), which includes muscular symptoms, such as intermittent myalgia or rhabdomyolysis, that are associated with prolonged exercise after adolescence. The neonatal form is normally lethal during the neonatal period, irrespective of any intensive treatments [3]. Therefore, families who have had such an affected child often undergo genetic counseling for prenatal diagnosis during subsequent pregnancies.

TFP deficiency is usually diagnosed based on increased levels of long-chain 3-OH-acylcarnitines (3-OH-ACs), such as C16-OH or C18:1-OH, which can be measured by blood acylcarnitine (AC) analysis using tandem mass spectrometry (MS/MS). However, instead of AC analysis, gene analysis is usually performed for the prenatal diagnosis of TFP deficiency [4]. Herein, we report our experience with prenatally diagnosing TFP deficiency using AC analysis and gene analysis. Our data indicate that AC analysis of amniotic fluid is useful for the prenatal diagnosis of TFP deficiency.

* Corresponding author at: Department of Pediatrics, Shimane University Faculty of Medicine, 89-1, Ennya-cho, Izumo, Shimane 6938501, Japan.
E-mail address: ryobo@med.shimane-u.ac.jp (R. Bo).
[1] Present/permanent address: Kobe University Graduate School of Medicine, 7-5-1, Kusunokicho, Chuo, Kobe, Hyogo 6500017, Japan.

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2. Materials and methods

The protocol for this study was approved by the Ethical Committee of Shimane University Faculty of Medicine.

2.1. Case

The index case was a boy born at 38 weeks gestation via vaginal delivery and weighing 2588 g. He was the second child of non-consanguineous parents, and his elder brother was healthy (Fig. 1).

His mother had no abnormalities during the pregnancy, including no HELLP (hemolysis, elevated liver enzymes, and low platelet counts) syndrome or AFLP (acute fatty liver of pregnancy). On the 2nd day after birth, the boy suddenly became unconscious and hypotonic, accompanied by severe hypoglycemia and lactic acidosis. Despite various treatments, including continuous hemodiafiltration that was performed to address the potential of septic shock, his clinical condition deteriorated, and he died of heart failure on the 6th day. Postmortem blood AC analysis revealed the accumulation of 3-OH-ACs, suggesting that the boy had the lethal type of TFP deficiency. Gene analysis and Western blotting confirmed the diagnosis. Therefore, the parents underwent prenatal diagnoses for their subsequent 2 pregnancies (Cases 1 and 2).

After obtaining informed consent from the parents, AC analysis of the amniotic fluid was performed as well as gene analysis and Western blotting.

2.2. Amniotic fluid

Amniotic fluid was collected at 16 and 18 weeks of gestation for Cases 1 and 2, respectively. For comparison purposes, amniotic fluid samples were obtained after 15 weeks from 8 normal controls and from 2 heterozygotes for TFP deficiency who had both undergone prenatal genetic testing (heterozygote-A: c.442+614 A>G and heterozygote-B: c.1364T>G in the *HADHB* gene).

2.3. Gene analysis

Genomic DNA was extracted from the pellets of centrifuged amniotic fluid using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Both the *HADHA* and the *HADHB* genes, which encode TFP, were sequenced as previously reported [5].

2.4. Western blot analysis

Western blot analysis of cultured fibroblasts or amniocytes was performed using a rabbit polyclonal antibody raised against both the α- and β-subunits of TFP; this antibody was kindly provided by Dr. T. Hashimoto, Professor Emeritus, Shinshu University, Matsumoto, Japan. The signals were visualized using the ImmunoPure NBT/BCIP Substrate Kit™ (Promega, Madison, WI, USA), as previously described [6].

2.5. Acylcarnitine analysis

AC analysis was performed according to the modification of the method as reported [7]. Briefly, 10 μL of amniotic fluid supernatant was obtained after centrifugation at 3000 rpm for 5 min. The sample was then subjected to butyl derivatization using API 3000 triple–quadrupole tandem mass spectrometer (MS/MS) in combination with an SIL-HTc autosampler (Shimadzu, Kyoto, Japan).

3. Results

3.1. Gene analysis

Direct DNA sequencing of the index case revealed the compound heterozygote mutations c.1392+1G>A and c.1689+2T>G in the *HADHA* gene, both of which induce mRNA splicing errors. The same mutations were identified in Case 1, whereas no mutations were identified in Case 2.

3.2. Western blot analysis

Western blot analysis of TFP in the cultured amniotic cells showed that both the α- and β-subunits were detected in Case 2, but not in Case 1, whereas the very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.8.9) protein was expressed normally in both cases (Fig. 2).

3.3. Acylcarnitine analysis

AC analysis of the amniotic fluid of Case 1 demonstrated significant elevations of 3-hydroxy-ACs: C14-OH measured at 55 nmol/L (control, 3.9 ± 5.0; +10.3 SD), C16-OH measured at 120 nmol/L (control, 0.8 ± 1.5; +77.6 SD), C18-OH measured at 31 nmol/L (control, 3.2 ±

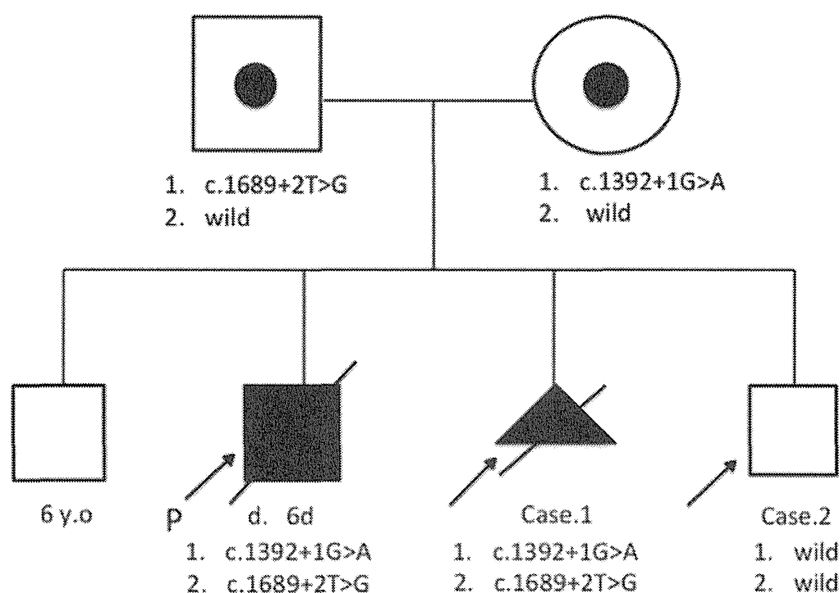

Fig. 1. Family tree The age of each patient and sibling is described for the time at which the amniotic fluid of Case 2 was obtained.

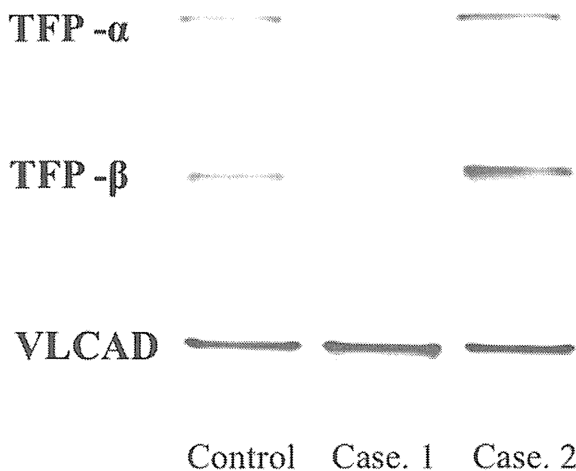

Fig. 2. Western blot analysis of mitochondrial trifunctional protein in the cells in the amniotic fluid. TFP-α and TFP-β were not detected in Case 1. Abbreviations: TFP-α and TFP-β, α- and β-subunits of TFP, respectively; VLCAD, very-long-chain acyl-CoA dehydrogenase.

7.4; +3.5 SD), and C18:1-OH measured at 44 nmol/L (control, 4.6 ± 4.9; +7.9 SD), as shown in Table 1. Conversely, 3-hydroxy-AC levels were not elevated in Case 2 compared with the normal control. There was a marginal increase in the amounts of C14-OH (24 nmol/L; control 3.9 ± 5.0; +1.6 SD) and C16-OH (13 nmol/L; control 0.9 ± 1.5; +4.6 SD) in heterozygote-A, although these values were lower than those in Case 1. Meanwhile, the AC values of the specimen from heterozygote-B overlapped with those of the normal controls.

3.4. Follow-up

It was concluded that Case 1 was affected but that Case 2 was normal. Eventually, Case 1 was artificially aborted at 21 weeks of gestation after obtaining consent from the parents. The diagnosis was validated by AC analysis of umbilical cord blood. In Case 2, the pregnancy was continued, and a boy was born healthy at 38 weeks and 5 days of gestation.

4. Discussion

Our study clearly demonstrates that AC analysis using amniotic fluid can distinguish a fetus affected with TFP deficiency from a healthy fetus. In certain organic acidemias, such as methylmalonic acidemia, propionic acidemia and glutaric acidemia type 1, prenatal diagnosis by gas chromatography/mass spectrometry (GC/MS) and MS/MS using amniotic fluid has been reported [8–11]. In contrast, the prenatal diagnosis of long-chain fatty acid oxidation disorders (FAODs) using MS/MS has not been considered as an optimal approach because ACs derived from long-chain fatty acids do not seem to accumulate in the fetus in response to the inhibition of β-oxidation via malonyl-CoA-mediated suppression of carnitine palmitoyltransferase (CPT)-1 activity and seem to have limited excretion into the fetal urine and hence into the amniotic fluid due to poor solubility [12,13].

Table 1
Acylcarnitines in amniotic fluid of Case 1, Case 2 and heterozygotes.

	Amniotic fluid					Serum
	Case 1	Case 2	Hetero zygote-A	Hetero zygote-B	Control	Index case
C14	27	0	7.0	0	4.6 ± 3.4	1160
C14:1	21	0	3.0	0	5.8 ± 6.5	430
C14-OH	55	0	24	0	3.9 ± 5.0	310
C16	52	0	17	4.0	6.9 ± 8.6	6300
C16-OH	120	0	13	3.0	0.9 ± 1.5	3100
C18	14	10	0	0	2.9 ± 4.6	780
C18-OH	31	0	0	0	3.6 ± 7.8	760
C18:1-OH	44	0	0	8.0	5.1 ± 5.0	1900

Normal control values are the mean ± SD (n = 9).
Unit of AC values: nmol/L.
Abbreviations: C14, myristoylcarnitine; C14:1, tetradecenoylcarnitine; C14-OH, hydroxy-tetradecanoylcarnitine; C16, palmitoylcarnitine; C16-OH, hydroxy-hexadecanoylcarnitine; C18, stearoylcarnitine; C18-OH, hydroxy-octadecanoylcarnitine; C18:1-OH, hydroxy-octadecenoylcarnitine.

However, a recent report demonstrated that fatty acid oxidation is activated and plays a significant role even during the fetal periods [14–16], suggesting that the potential use of AC analysis to detect fetal FAODs is feasible. Although long-chain ACs are considered to be hydrophobic, the presence of a polar hydroxyl group can make ACs (C14:1, C16-OH, C18:1-OH) more soluble in amniotic fluid, thus making their detection in amniotic fluid feasible. Moreover, as identified in Case 1, unsaturated ACs such as C14:1 was elevated. Although it has been suggested that AC analysis using MS/MS is not appropriate for the prenatal diagnosis of long-chain FAODs, the results presented here show that ACs specific for other long-chain FAODs, such as VLCAD deficiency, could be detected during the prenatal period.

AC analysis of amniotic fluid has several advantages over gene analysis and Western blotting. AC analysis allows for the rapid detection of accumulated ACs in a small volume of amniotic fluid. Moreover, this analysis makes prenatal diagnosis feasible, even when the mutation of the index case cannot be identified and there are no issues related to contamination by maternal cells, in contrast to gene analysis. In our study, the AC values overlapped between heterozygotes and normal controls, although those with affected specimens were undoubtedly distinguishable from the heterozygotes and the controls. These findings suggest that the accurate diagnosis of milder cases may be complicated, although prenatal diagnosis may not be necessary in these cases.

Because only one case was studied in this report, additional studies need to be performed. Nevertheless, our study demonstrates that AC analysis can be used for prenatal diagnosis of TFP deficiency.

5. Conclusion

During prenatal diagnosis, misdiagnosis has to be avoided in all cases. Therefore, multiple approaches from several angles are desirable, and their results should be evaluated comprehensively. In this regard, combining AC analysis with genetic testing (gene sequencing) may result in more effective prenatal diagnoses of TFP deficiency and may avoid misleading results.

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