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

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Keywords: multiple acyl-CoA dehydrogenase deficiency (glutaric acidemia type II); fatty acid oxidation disorder; adult onset; myopathy; serum acylcarnitine; immunoblotting; in vitro probe acylcarnitine assay

Corresponding Author: Dr. Kenji Yamada, M.D.

Corresponding Author's Institution: Shimane University, Faculty of Medicine

First Author: Kenji Yamada, M.D.

Order of Authors: Kenji Yamada, M.D.; Hironori KOBAYASHI, MD, PhD; Ryosuke BO, MD; Tomoo TAKAHASHI, MD; Jamiyan PUREVSUREN, MD, PhD; Yuki HASEGAWA, MD, PhD; Takeshi TAKETANI, MD, PhD; Seiji FUKUDA, MD, PhD; Takuya OHKUBO, MD, PhD; Takanori YOKOTA, MD, PhD; Mutsufusa WATANABE, MD, PhD; Taiji TSUNEMI, MD, PhD; Hidehiro MIZUSAWA, MD, PhD; Hiroshi TAKUMA, MD, PhD; Ayako SHIOYA, MD; Akiko ISHII, MD, PhD; Akira TAMAOKA, MD, PhD; Yosuke SHIGEMATSU, MD, PhD; Hideo SUGIE, MD, PhD; Seiji YAMAGUCHI, MD, PhD

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.

Suggested Reviewers:

Opposed Reviewers:

Dr. Masashi Mizuguchi
Editor-in-Chief
Brain and Development

August 5, 2015

Dear Dr. Mizuguchi:

We thank the editor and the reviewers for their constructive criticism and suggestions on our manuscript. We have carefully revised our manuscript according to your suggestions. All changes made in the manuscript are highlighted in red color.

Our responses to the reviewers are detailed as below.

We hope that we have adequately responded to the reviewers' comments and that our revised manuscript is now suitable for publication in Brain and Development.

Sincerely yours,

Kenji Yamada, MD
Department of Pediatrics,
Shimane University Faculty of Medicine,
89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan
TEL: +81-853-20-2219
FAX: +81-853-20-2215
E-mail: k-yamada@med.shimane-u.ac.jp

Revisions made according to the Reviewers comments

Reviewer #1:

This article is a report of two cases of adult-onset glutaric acidemia type 2.
As are listed in Table 4, there have already been several reports on similar cases, but it appears that none of them contains more detailed biochemical evaluation than this article.
What is especially informative to me is that serum acylcarnitine anlysis could detect specific abnormalities even in stable conditions, indicating that it is more sensitive than urinary organic acid analysis and other biochemical tests.
For these reasons, I think that this article is worth publishing in Brain & Development.
Revision of a few minor points will make it acceptable to the journal.

Thank you for your comments. We have revised our manuscript according to your comments.

[Abstract]
Type of samples for acylcarnitine analysis should be specifically described as "serum" and "dried blood spots".

We specified the samples used in acylcarnitine analysis as “serum” and “dried blood spots (DBS)”. Thank you for your suggestion.

[Introduction]
L12. sarcosine CoA dehydrogenase >> sarcosine dehydrogenase

We made change according to your comments.

[Results] 3.3 Histological studies
L8. Muscle stained with... >> Muscles stained with..., or Muscle tissues stained with...

"Muscle" was corrected into "Muscle tissues" as you suggested.

Reviewer #2:

The authors described two patients with a milder adult-onset form of GA2 with detailed biochemical and molecular analyses. Then, they proposed that late-onset form of GA2 should be divided into two forms, juvenile form (intermediate form) and myopathic form (adult-onset form). The paper was well-written and informative for neurologists including pediatric neurologists.

Thank you for comments on our manuscript.

Major criticism

They did not mention whether mutations identified in adult onset form are different from mutations identified in juvenile form and severe form (neonatal onset)? Is there clear phenotype-genotype correlation in this disorder?

Our study cannot indicate a clear evidence of phenotype-genotype correlation between adult-onset form and juvenile-onset form. However, both two adult-onset cases were ETFDH deficiency in our study. Additionally, previous reports indicate that almost adult cases were associated with defect of ETFDH. It is considered that GA2 due to defect of ETFDH tend to be milder form in particular Asian peoples, although some patients with defect of ETFDH occasionally exhibited severe clinical features. Because clinical form can't predicted exclusively by the genotype, we think that making diagnosis using IVP assay is useful. These points were discussed in the revised manuscript.

In Japan, GA2 is the second groups of target diseases. Maybe it is useful to

comment on possibility that such adult-onset form can be screened by newborn screening.

We appreciated the reviewer for bringing up this important point, which was briefly discussed in the text.

Minor comments
Abstract
conclusion
Do you mean that late-onset GA2 should be separated into two forms, intermediate form and myopathic form?
The last sentence, "versus the infantile form" or "versus intermediate form"

Yes, “infantile-onset form” represented “intermediate (juvenile-onset) form” in this abstract. Therefore, we changed all “infantile-onset” into “intermediate”. Additionally, we corrected some phrases in conclusion of abstract because of some mistakes in grammar.

Introduction
P6 line 3. develop what?

It was corrected to “to be symptomatic”

Materials and methods
P9 line 5 and others. blood filter paper should be "dried blood spots" Blood filter paper is not common in English.

Thank you for pointing this out. As suggested, “blood filter paper” was changed into “dried blood spots”. Additionally, “dried blood spots” was abbreviated as DBS in our manuscript.

Results

Page 11 line 15 and others
1367C>T (P456L) should be c.1367C>T(p. P456L). The same changes should be made in Table 2.

We made change as you suggested both in the text and tables 2 and 4. Additionally, we made some changes in Tables 2 as below. 1) Italics and underlines were removed from "lipid deposit", 2) "Normal" was changed to "normal". 3) "mild elevation of C4-C18" was added in column of "Blood acylcarnitine analysis" in case 2.

Table 1　underlined values were abnormal findings

"Abnormal findings are underlined." was added in the legend of Table 1. Additionally, we corrected abnormal values without italics.

Discussion
Page 13 line 8-9　No prerentation of unconsciousness was made in Case report section of case 1.

This point has been stated as "Furthermore, he had 3 episodes of unconsciousness after the age of 50." in Case report section of case 1.

Author may make a comment on possible treatment of these adult-onset form patients with bezafibrate.

Possibility of treatment with bezafibrate was added in "Discussion".

Table 3
patient 9 genotype　Please specify the genotype what do you mean by N/A heterozygote?

This patient was reported as myopathic form of GA2 due to a defect of ETFDH, although she had only heterozygous mutation of IVS3+1G>A

(reference # 22). Therefore, we suspected that she might be a carrier of ETFDH deficiency. Moreover, effect of IVS3+1G>A was not described. However, we corrected “N/A heterozygote?” into “IVS3+1G>A heterozygote” as reported by Wen et al.

Figure 2 legend
"Open and closed triangles represent positive and negative, respectively"
This expression sound strange. Please change the sentence.

We changed the sentence into “Black and white triangles indicate a presence and absence of the protein, respectively.”

Figure 3 legend
Do you mean "C, a (representative) patient with severe form of GA2; D, a (representative) patient with intermediate form of GA2; and E, a healthy control"?
If possible, their genotype should be added.

Yes, their genotype was added.

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], and 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, Illinois, 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

*** Corresponding author**

Kenji Yamada

89-1 En-ya-cho, Izumo, Shimane, 693-8501, Japan

TEL: +81-853-20-2219

FAX: +81-853-20-2215

E-mail: k-yamada@med.shimane-u.ac.jp

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.

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 Material 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 (2,903 IU/L, normal 100-215), and CK (2,364 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. 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.

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 hours 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 (Figure 1-A and 1-B).

3.4 Immunoblotting

In both cases 1 and 2, ETFDH protein was not detected, while both ETFα and ETFβ proteins were observed to be normal. These findings strongly suggested that both patients had GA2 due to a defect in ETFDH (Figure 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 (Figure 3A, 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 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 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 to C16) when palmitate is loaded (Figure 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.

Acknowledgements

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and for comments on this study.

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Figure legends

Figure 1. Pathological findings from the muscle biopsy (Oil-red O stain).

A, case 1, and B, case 2. Arrows indicate lipid deposits.

Figure 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.

Figure 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.

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], and 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, Illinois, 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

*** Corresponding author**

Kenji Yamada

89-1 En-ya-cho, Izumo, Shimane, 693-8501, Japan

TEL: +81-853-20-2219

FAX: +81-853-20-2215

E-mail: k-yamada@med.shimane-u.ac.jp

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.

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 Material 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 (2,903 IU/L, normal 100-215), and CK (2,364 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. 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.

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 hours 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 (Figure 1-A and 1-B).

3.4 Immunoblotting

In both cases 1 and 2, ETFDH protein was not detected, while both ETFα and ETFβ proteins were observed to be normal. These findings strongly suggested that both patients had GA2 due to a defect in ETFDH (Figure 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 (Figure 3A, 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 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 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 to C16) when palmitate is loaded (Figure 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.

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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.

Figure 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.

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Tables

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

	Case 1	Case 2	(Reference value*)
Onset Age	40s	31	
Sex	M	M	
Clinical features	myalgia muscle weakness rhabdomyolysis	myalgia muscle weakness	
Routine blood examination			
CBC			
WBC (/μL)	4,800	5,000	(3300-8600)
RBC (x10^4/μL)	370	539	(385-438)
Hb (g/dL)	12.3	16.5	(11.0-14.8)
Plt (x10^4/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)	2,903	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)

* The reference values used at Shimane University. Abnormal findings are underlined.

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.

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		Reference
						Elevated trans-aminase	LDH (IU/L)	CK (IU/L)		DBS	Serum (Plasma)		Allele 1	Allele 2	
Our cases															
1	M	40s	+	+	coma	+	2,903	3,000	normal	normal	C8-C10	*ETFDH*	p.P456L	p.P456L	our case
2	M	31	+	+	no	+	2,860	1,897	normal	C4-C12	C8-C18	*ETFDH*	p.W297L	p.P317R	our case
Previously reported cases															
3	M	42	+	+	no	N/A	942	1,855	GA, 2HG, EMA	C4, C5, C8, C10, C14	N/A	*ETFDH*	p.I243T	p.T294I	Köppel et al, 2006 [10]
4	F	24	+	+	no	N/A	N/A	677	N/A	C8-C12	N/A	*ETFDH*	p.L409F	p.V291G	Wen et al, 2010 [23]
5	F	23	+	+	vomiting	N/A	N/A	513	N/A	C8-C12	N/A	*ETFDH*	p.L409F	p.V291G	Wen et al, 2010 [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, 2010 [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, 2010 [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, 2010 [23]
9	F	63	-	+	no	N/A	N/A	2,120	GA,2HG, EMA, DCA	C0, C5-C14	N/A	*ETFDH*	IVS3+1G>A heterozygote	none	Wen et al, 2010 [23]
10	F	23	+	+	vomiting	N/A	N/A	1,998	GA, 2HG, EMA, DCA, KB	C8-C14	N/A	*ETFDH*	p.M404T	not detected	Wen et al, 2010 [23]
11	F	22	+	-	no	N/A	N/A	339	normal	C0	N/A	*ETFDH*	p.L409F	not detected	Wen et al, 2010 [23]
12	M	46	+	+	difficulty in breathing	+	543	5,995	GA, 2HG, DCA	N/A	N/A	*ETFDH*	p.M404T	p.D596N	Izumi et al, 2011 [11]
13	F	55	+	+	no	N/A	N/A	8,000	normal	N/A	C4-C18	*ETFDH*	p.H293D	not detected	Kaminsky et al, 2011 [22]
14	M	36	-	-	exercise intolerance	N/A	1,161	3,055	2HG, 2-OH adipate	N/A	N/A	*ETFDH*	p.D511N	p.W603X	Sugai et al, 2012 [12]
15	M	53	+	+	osphyalgia, nausea	+	600	571	GA, 2HG, EMA	C8-C12	N/A	*ETFDH*	p.P508T	p.N528KfsX3	Zhao et al, 2012 [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, 2014 [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 to C16
3. Myopathic form (adult-onset)	school-age or adulthood	episodes of myalgia, muscle weakness, fatigue, or liver dysfunction	-	±	almost normal

1

Figure(s)

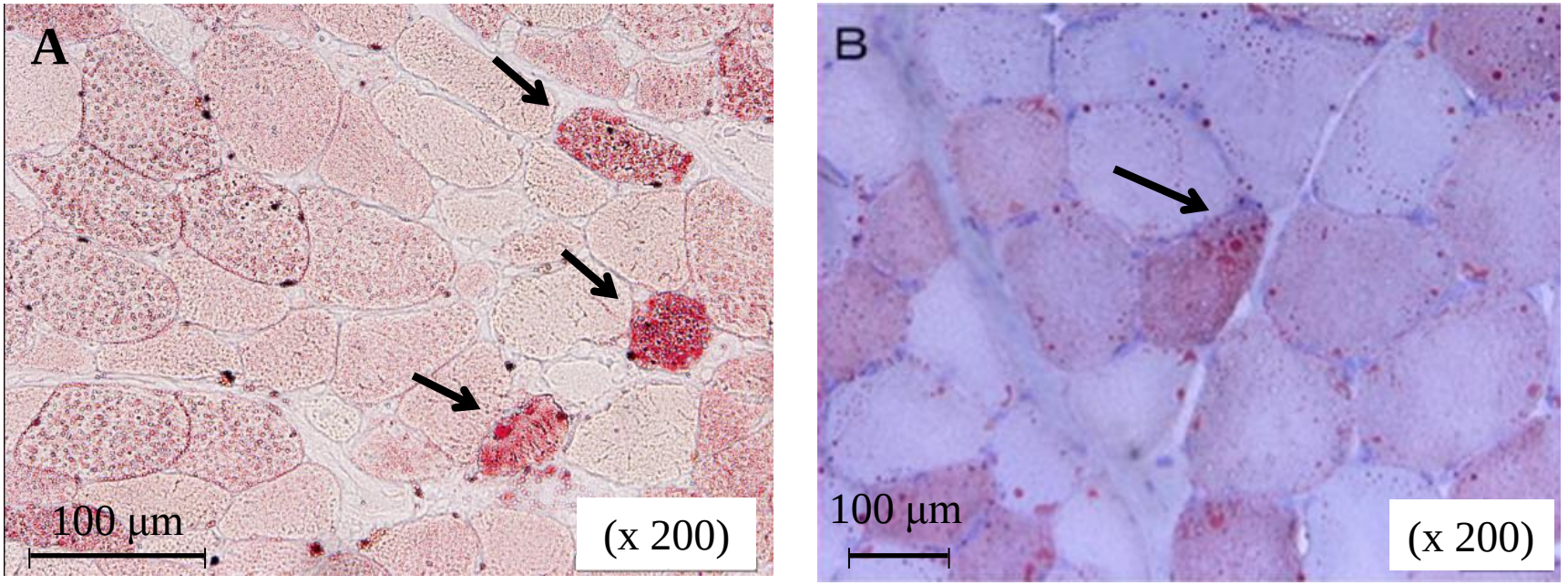

Figure 1. Pathological findings of muscle biopsy (oil red stain)

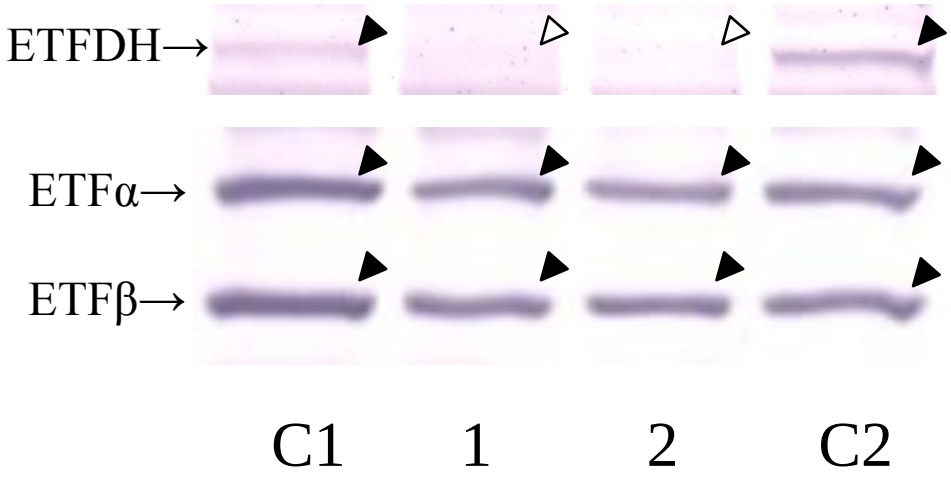

Figure 2. Immunoblotting of fibroblasts

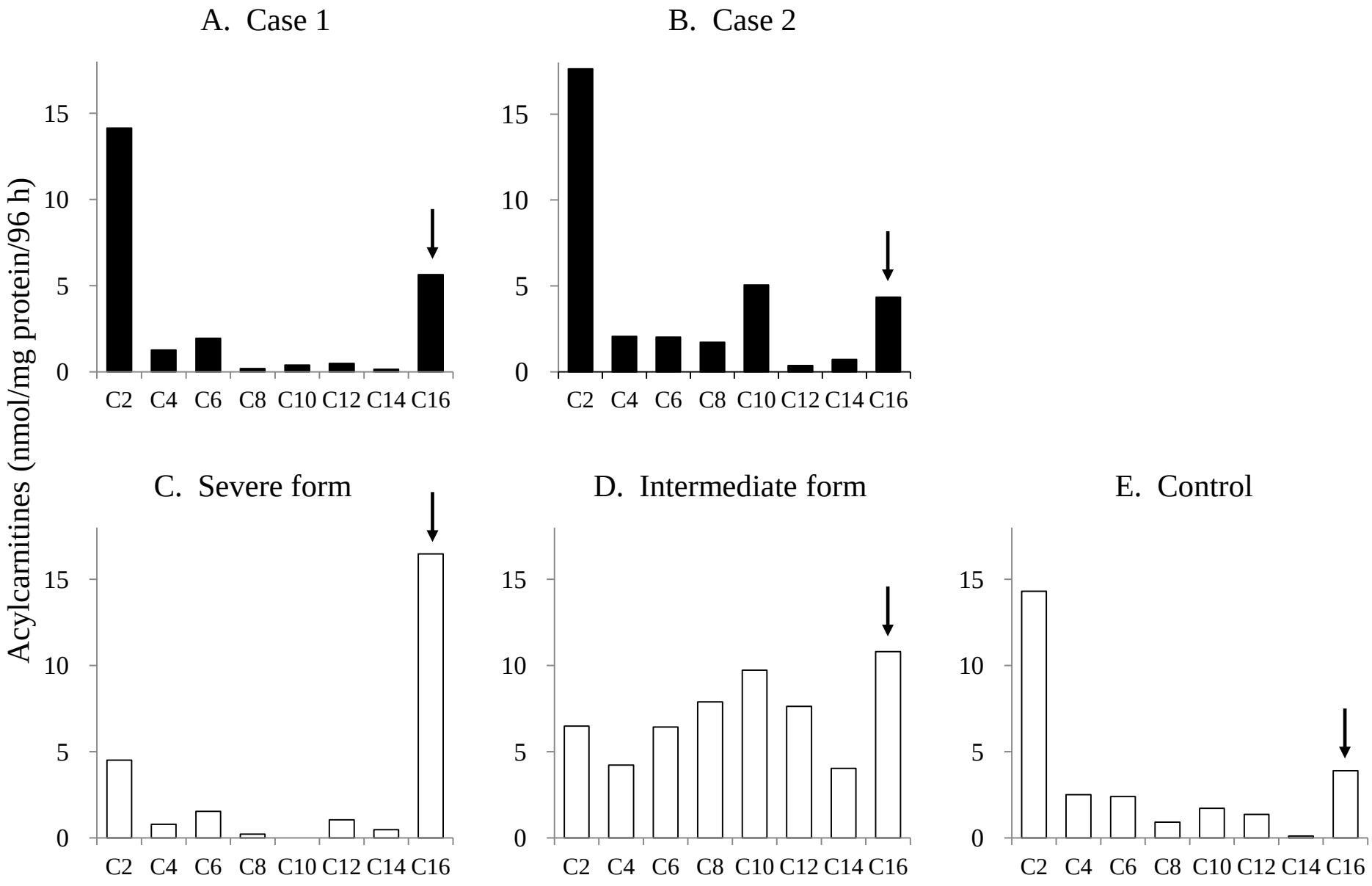

Figure 3. Profiles of *in vitro* probe assay in GA2