Research Paper

Prenatal diagnosis of severe metabolic disorders in diseases targeted by newborn screening in Japan

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Abstract The introduction of tandem mass spectrometry (TMS) analysis into newborn screening (NBS) has improved the prognosis of patients with organic acidemias and fatty acid oxidation disorders through early detection and timely intervention. However, some severe phenotypes remain unresponsive to treatment and often result in death during early infancy. Genetic counseling and a prenatal diagnosis may be the only options for the families who have newborns with such forms of the disease and strongly wish to have another baby. Prenatal diagnostic evaluations were conducted in 134 cases, using gas chromatography/mass spectrometry (GC-MS), TMS analysis, and/or genetic testing, at the request of families with an affected proband. Of the 134 fetuses, 46 were diagnosed as being affected, and all 46 pregnancies were therefore terminated. All cases diagnosed as unaffected were confirmed to have no postnatal abnormalities. Given the direct impact of the prenatal diagnosis on the continuation of pregnancy, it is essential that the analytical methods used are reliable. A mass spectrometry (GC-MS and TMS) analysis of amniotic fluid was found to be highly sensitive for prenatal diagnosis. This article suggests that mass spectrometry, combined with genetic testing, significantly improves the accuracy of prenatal diagnosis.

Key words: organic acidemia, fatty acid oxidation disorder, prenatal diagnosis, gas chromatography mass spectrometry, tandem mass spectrometry, newborn screening

Introduction

Nationwide newborn screening using tandem mass spectrometry (TMS)[1–3] was introduced in Japan in 2014. This screening program expanded the target diseases beyond amino acid metabolism disorders to include organic acidemias (OAs) and fatty acid oxidation disorders (FAODs). Consequently, many patients identified early through this screening can be protected from the onset of disease and disability through the timely initiation of treatment.

However, some of the OAs and FAODs present as a lethal neonatal-onset phenotype, which remains resistant to all available treatment. These cases often result in death within days to weeks of birth or lead to severe neurological sequelae in survivors. Genetic counseling is crucial for families with a history of such disorders who are considering subsequent pregnancies, and a prenatal diagnosis (PND) may be the only viable option.

The prenatal diagnosis of OAs and FAODs can be performed through the analysis of metabolites in amniotic fluid, enzyme activity assays evaluating amniotic cells, or genetic analyses[4–8]. However, genetic analysis requires prior knowledge of the proband's genotype.

At the Faculty of Medicine, Shimane University, we provided genetic counseling and a prenatal diagnosis for a family with a history of a proband exhibiting severe pheno-

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types. In 2005, we reported on 28 cases of PND for OAs[5]. Since then, including cases of FAODs, we have conducted a total of 134 PNDs through 2016. In this report, our diagnostic experience and the clinical outcomes with PND of these cases are described.

In particular, in a family affected by mitochondrial trifunctional protein (TFP) deficiency, a type of long-chain FAOD, PND based on acylcarnitine analysis was highly effective in distinguishing affected from unaffected fetuses. Therefore, the details of this representative case are presented with supporting data.

Materials and Methods

This study was conducted in accordance with the ethical principles outlined in the Guidelines for Genetic Testing and Diagnosis in Medical Practice[9] and was approved by the Ethics Committee of Shimane University Faculty of Medicine (formerly Shimane Medical University) under the title "Prenatal Diagnosis of Severe Inborn Errors of Metabolism" (Research Management Number: 20150227-6).

1. Subjects

This study included 134 cases of PND performed at the Faculty of Medicine, Shimane University, between 1998 and 2016. The cases met the following criteria:

1) The proband had a severe OA or FAOD that resulted in death during the neonatal or early infantile period or led to severe sequelae.
2) The family expressed a strong desire for PND in genetic counseling, and the diagnosis was deemed appropriate based on the Guidelines for Genetic Tests and Diagnosis in Medical Practice[9].
3) In cases involving genetic analysis, pathogenic variants in the proband and parents were identified.

2. Sample collection

Amniotic fluid samples were collected via amniocentesis between the 12th and 16th weeks of pregnancy at designated medical facilities. The samples were transported at <4°C and subsequently centrifuged to separate the supernatant and sediment fractions.

3. Amniotic fluid analysis

The supernatant fraction was used for an organic acid analysis by gas chromatography-mass spectrometry (GC-MS) and an acylcarnitine analysis by tandem mass spectrometry (TMS). For FAODs, in addition to mass spectrometry, genomic DNA was extracted from the pellet fraction of centrifuged amniotic fluid for genetic analysis.

(i) Mass spectrometry

Sample preparation, reagent use, and analytical procedures for GC-MS and TMS followed established methodologies[5,10,11]. The GC-MS system consisted of a Shimadzu GCMS-QP 5050 Model (Shimadzu, Kyoto, Japan) until 2007, followed by a GCMS-QP 2020plus Model (Shimadzu), and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (t-BDMS) derivatization was used for the organic acid analysis. Quantification was carried out in the selective ion monitoring (SIM) mode. The detection of methylmalonic acid using this method is illustrated in Fig. 1.

The TMS system used for acylcarnitine analysis was an API 3000 triple-quadrupole tandem mass spectrometer in combination with an SIL-HTc autosampler (Shimadzu).

(ii) Genetic analysis

Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Target genes identified in the proband were sequenced according to previously established methods[12].

(iii) Individual identification in genetic analysis

In cases in which a maternal-origin mutation was identified by genetic analysis, a microsatellite analysis was performed to confirm individual identification by comparing DNA from amniotic cells and maternal blood.

Results

1. Proportion of affected fetuses

Of the 134 fetuses considered at risk for OA and FAODs, 118 were categorized as at risk for OAs and 16 for FAODs, based on the diagnostic information of the probands (Table 1). The key diagnostic markers (organic acids and acylcarnitines) used for each disorder are summarized in Table 2.

Of the OA cases that asked for PND, methylmalonic acidemia (MMA) was the most common disease (85 cases), followed by propionic acidemia (PPA; 17 cases), glutaric acidemia type I (GA1; 11 cases), and multiple carboxylase deficiency (MCD; 5 cases). Forty-six of these cases were ultimately diagnosed as affected. Thirty-five cases were diagnosed as MMA, 8 as PPA, 2 as GA1, and 1 as MCD (Table 1).

Regarding FAODs, 8 cases with mitochondrial trifunc-

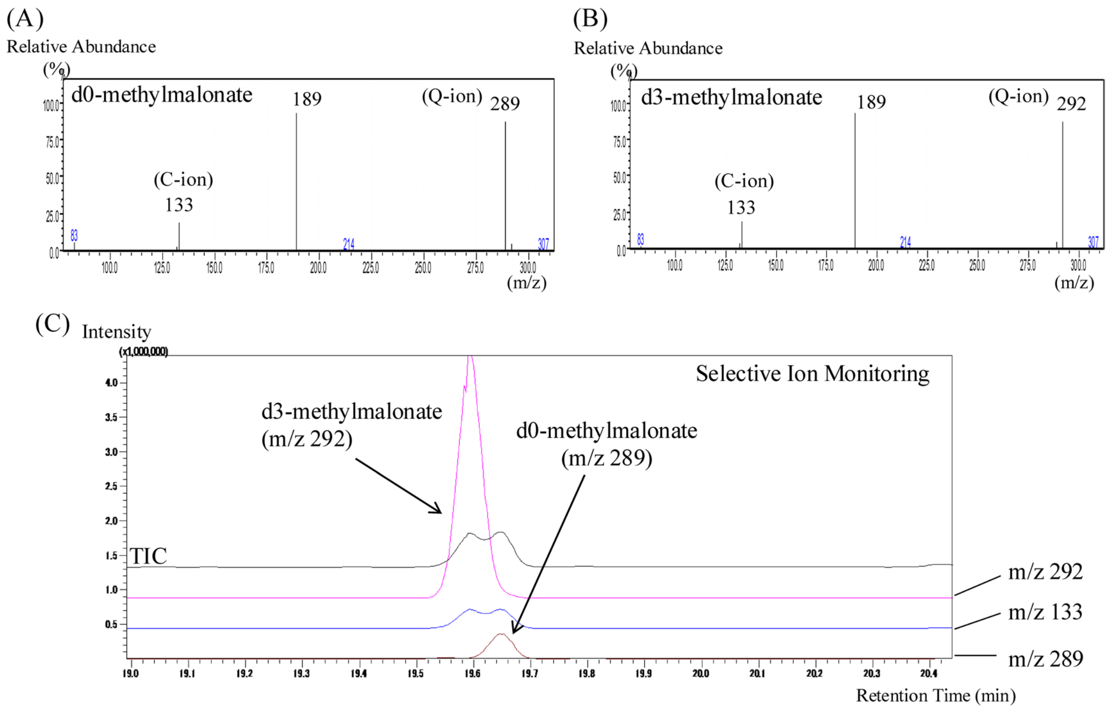


Fig. 1 Extracted ion chromatogram and mass spectrum of t-BDMS derivatives of native (d0) and stable-isotope-labeled (d3) methylmalonate in a case at risk for methylmalonic acidemia.

(A) Mass spectrum of d0-methylmalonate. (B) Mass spectrum of d3-methylmalonate. (C) Extracted ion chromatogram of d0-methylmalonate (*m/z* 289) and d3-methylmalonate (*m/z* 292).

Due to differences in m/z values, retention times in mass spectrometric detection may vary, and quantification is therefore performed based on the ratio of peak areas.

Abbreviations: Q- and C-ion; selected ion for quantification and confirmation, respectively.

Table 1. Number of prenatal diagnoses

Disease	Cases	Affected	Analytical method
1) Organic acidemias	118	46	
Methylmalonic acidemia	85	35	GC-MS TMS
Propionic acidemia	17	8	
Glutaric acidemia type I	11	2	
Multiple carboxylase deficiency	5	1	
2) Fatty acid oxidation disorders	16	3	
Mitochondrial trifunctional protein (TFP) deficiency	8	1	Gene TMS GC-MS
Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	3	0	
Glutaric acidemia type II	3	1	
Carnitine acylcarnitine translocase deficiency	2	1	
Total	134	49	

tional protein (TFP) deficiency, 3 cases each of very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and glutaric acidemia type II (GA2), and 2 cases of carnitine acylcarnitine translocase (CACT) deficiency were evaluated. Of these cases, one each of TFP deficiency, GA2, and CACT deficiency were judged to be affected (Table 1).

All fetuses diagnosed as unaffected were confirmed to have no metabolic abnormalities by a postnatal biochemical analysis. The unaffected individuals have been followed for 8 to 20 years postnatally; however, as of the end of 2024, none has developed any clinical symptoms suggestive of late-onset disease.

Table 2. Target diagnostic compounds for organic acidemias and fatty acid oxidation disorders

Disease	Target compounds	
	Organic acids	Acylcarnitines
1) Organic acidemias		
Methylmalonic acidemia	Methylmalonate and Methylcitrate	C_3, C_3/C_2
Propionic acidemia	Methylcitrate (without Methylmalonate)	C_3, C_3/C_2
Glutaric acidemia type I	Glutarate	C_5DC
Multiple carboxylase deficiency	Methylcitrate and Methylcrotonylglycine	C_3, C_3/C_2, C_5-OH
2) Fatty acid oxidation disorders		
TFP deficiency	—	C_{14}-OH, C_{16}-OH, C_{18}-OH, $C_{18:1}$-OH
VLCAD deficiency	—	$C_{14:1}$
Glutaric acidemia type II	—	C_6, C_8, C_{10}, C_{12}
Carnitine acylcarnitine translocase deficiency	—	$C_{14:1}$, C_{16}, $(C_{16}+C_{18})/C_0$

Abbreviations: C_0: free carnitine, C_2: Acetylcarnitine, C_3: Propionylglycine, C_5DC: Glutarylcarnitine, C_5-OH: 3-Hydroxyisovalerylcarnitine, C_6: Hexanoylcarnitine, C_8: Octanoylcarnitine, C_{10}: Decanoylcarnitine, C_{12}: Dodecanoylcarnitine, $C_{14:1}$: Tetradecenoylcarnitine, C_{14}-OH: 3-Hydroxymyristoylcarnitine, C_{16}: Palmitoylcarnitine, C_{16}-OH: 3-Hydroxypaimitoylcarnitine, C_{18}: Stearoylcarnitine, $C_{18:1}$: Oleoylcarnitine, C_{18}-OH: 3-Hydroxystearoylcarnitine, $C_{18:1}$-OH: 3-Hydroxyoleoylcarnitine.

In all cases in which a fetus was diagnosed as affected, the pregnancy was terminated. In cases in which a mass spectrometry analysis was performed using cord blood or other postnatal samples, the prenatal and postnatal diagnoses were consistent in all instances.

Furthermore, in cases in which genetic analysis was performed, the results were fully consistent with the classification of fetuses as affected or unaffected based on mass spectrometry.

2. Comparison between methylmalonic acidemia (MMA) and propionic acidemia (PPA)

In both MMA and PPA, organic acid analyses demonstrated increased concentrations of methylcitrate, whereas acylcarnitine analyses showed elevated concentrations of propionylcarnitine (C3). However, MMA exhibited a marked increase in methylmalonate, which facilitated the differentiation between the two diseases (Table 3). The methylcitrate and C3 concentrations were generally higher in the PPA group than in the MMA group. As illustrated in Fig. 2, this reflects the metabolic pathway position of propionyl-CoA carboxylase upstream of methylmalonyl-CoA mutase.

3. Prenatal diagnosis of TFP deficiency

A case of TFP deficiency, one of the FAODs, was analyzed in a family with a history of this disease. The proband was born via a normal delivery but died on day 6 of life due to cardiomyopathy that did not respond to treatment. A postmortem acylcarnitine analysis using dried blood spots strongly suggested a TFP deficiency, and genetic analysis confirmed the presence of compound heterozygous mutations in *HADHA* (c.1392+1G>A and c.1689+2T>G).

Subsequently, two PND tests were performed in subse-

Table 3. Concentrations of methylcitrate, methylmalonate, and propionylglycine (C3) in amniotic fluid from the index cases

Disease	Organic acid (GC-MS)		Acylcarnitine (TMS)
	Methylcitrate	Methylmalonate	C3
Propionic acidemia	13.31	0.37	11.62
	32.94	2.44	13.85
	10.10	0.41	5.74
	26.66	0.24	16.75
	18.72	2.28	19.72
Methylmalonic acidemia	9.52	43.09	7.86
	7.01	36.91	8.10
	3.18	28.20	7.39
	9.18	42.37	10.74
	10.28	45.89	8.26
Control	0.18−1.56	0.30−3.70	0.43−1.72

(Unit: μmol/L)

The values of five cases are shown for each disease. Underlined values indicate elevated concentrations.

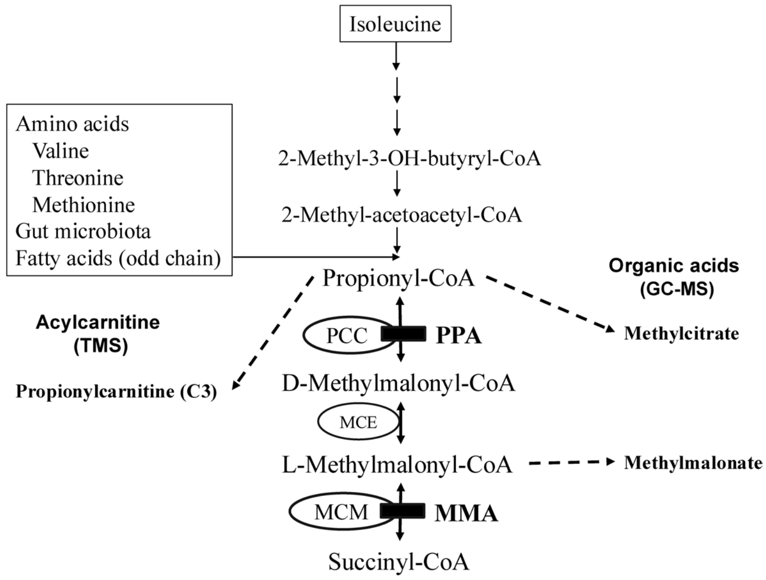
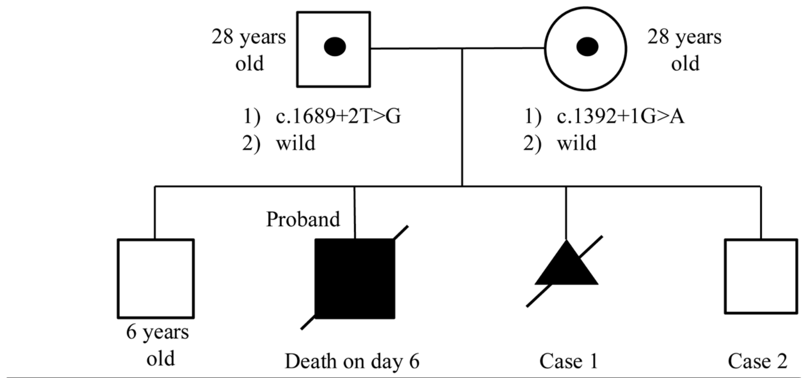


Fig. 2. Metabolic pathway of propionic acidemia (PPA) and methylmalonic acidemia (MMA).
Abbreviations: PCC: Propionyl-CoA carboxylase, MCE: Methylmalonyl-CoA epimerase, MCM: Methylmalonyl-CoA mutase.



Genotype	N.A N.A	1) c.1392+1G>A 2) c.1689+2T>G	1) c.1392+1G>A 2) c.1689+2T>G	1) wild 2) wild

Fig. 3. Family tree and genotypes with TFP deficiency.
Abbreviation: TFP: mitochondrial trifunctional protein, N.A: not analyzed.

quent pregnancies, which identified 1 affected fetus (Case 1) and 1 unaffected fetus (Case 2) (Fig. 3).

*Case 1 (Affected)

The same pathogenic variants as those in the proband (c.1392+1G>A and c.1689+2T>G) were identified. An acylcarnitine analysis of the amniotic fluid supernatant showed markedly elevated concentrations of 3-hydroxypalmitoylcarnitine (C16-OH) and 3-hydroxystearoylcarnitine (C18-OH), which are characteristic markers of TFP deficiency (Table 4). The final diagnosis of the affected status was confirmed by individual identification using a microsatellite analysis.

*Case 2 (Unaffected)

No abnormalities were detected in either the genetic analysis of the amniotic cells or the acylcarnitine analysis of the amniotic fluid supernatant. Therefore, the fetus was diagnosed as unaffected. In addition, a postnatal acylcarnitine analysis of the blood showed no abnormalities (no data available).

Discussion

Severe forms of OAs and FAODs often lead to rapid deterioration in the neonatal period, resulting in death or severe sequelae despite medical interventions. These devastating outcomes cause profound grief and distress in affected families. According to the "Guidelines for Genetic Tests and Diagnosis in Medical Practice" issued by the Japanese Association of Medical Science[9], PND of these

Table 4. Concentrations of acylcarnitines in amniotic fluid for prenatal diagnosis of TFP deficiency

Acylcarnitine	Amniotic fluid (Unit: nmol/L)			Dried blood spots (Unit: nmol/mL)	
	Case 1	Case 2	Control (n=9)	Proband	Cut-off
C14	27	0	(4.6±3.4)	1.16	(<0.4)
C14:1	21	0	(5.8±6.5)	0.43	(<0.3)
C14:1-OH	50	0	(5.0±4.8)	0.31	(<0.1)
C16	52	0	(6.9±8.0)	6.26	(<3.0)
C16-OH	120	0	(0.9±1.5)	3.1	(<0.1)
C18	14	10	(2.9±4.6)	0.78	(<2.0)
C18-OH	31	0	(3.6±7.8)	0.76	(<0.08)
C18:1-OH	44	0	(5.1±5.0)	1.90	(<0.08)

Underlined values indicate elevated concentrations.
Compared with the fetus unaffected by TFP deficiency, the concentration of C16-OH in the affected fetus was approximately 133 times higher, and C18-OH showed about an 8.6-fold increase.
Abbreviations: C14: Tetradecanoylcarnitine, C14:1: Tetradecenoylcarnitine, C14:1-OH: 3-Hydroxytetradecenoylcanitine, C16: Palmitoylcarnitine, C16-OH: 3-Hydroxypalmitoylcarnitine, C18: Stearoylcarnitine, C18-OH: 3-Hydroxystearoylcarnitine, C18:1-OH: 3-Hydroxyloleoylcarnitine.

inborn errors of metabolism is considered ethically permissible with careful consideration. When a fetus is diagnosed as affected, it is highly likely to follow the same clinical course as the proband, making the continuation of pregnancy potentially unbearable to the family. In this study, all families opted for termination in cases where the fetus was diagnosed as affected. Conversely, of the cases diagnosed as unaffected, all neonates were confirmed to be postnatally healthy. These findings underscore the critical importance of making an accurate PND, which necessitates comprehensive and multifaceted testing.

In the present study, multiple diagnostic methods were used whenever possible to enhance diagnostic accuracy, including organic acid and acylcarnitine analyses, genetic testing, and enzyme activity assays.

In a previous study, we demonstrated the utility of amniotic fluid mass spectrometry for the PND of OAs[5]. Other studies have similarly reported that the combination of multiple diagnostic tests (e.g., concurrent measurement of enzyme activity and metabolites[7], the simultaneous analysis of chorionic villi and amniotic fluid[13], and the use of multiple mass spectrometry techniques[8]) improves diagnostic reliability.

In addition, the previous study confirmed that acylcarnitines in amniotic fluid become unstable at room temperature[5]. Therefore, in addition to organic acid and acylcarnitine analyses, genetic testing was performed concurrently to enhance diagnostic accuracy and prevent false-positive results. Special attention was paid to the storage and transportation conditions of the specimens, and genetic analyses were conducted when applicable.

The present study also demonstrated that the combination of an acylcarnitine analysis and genetic testing of amniotic fluid is an effective approach for diagnosing FAODs. The prenatal diagnosis of FAODs has been reported since 1996. It has been conducted using methods such as characteristic acylcarnitine profiling and enzyme activity assays using amniotic cells[11,14]. Similarly, the PND of VLCAD deficiency has been achieved through an acylcarnitine analysis of amniotic fluid supernatants and genetic testing[15], as demonstrated in the present study. Although amniocentesis is an invasive procedure, it remains an essential and widely used prenatal diagnosis technique.

In conclusion, a mass spectrometry-based metabolite analysis using amniotic fluid supernatant with genetic analysis is a valuable tool for the PND of OAs and FAODs. An acylcarnitine analysis provides rapid and reliable results if specimens can be stored and transported in a frozen state to prevent degradation.

When pathogenic variants in the proband are identified, the integration of genetic analysis with other diagnostic methods enhances diagnostic reliability. Furthermore, individual identification through a chimerism analysis is essential for differentiating maternal cell contamination and further improving the accuracy of PND.

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Dr. Hasegawa summarized the overall analysis, interpreted and verified the organic acid analysis, and contributed to the composition of the manuscript. Dr. Kobayashi was responsible for the acylcarnitine analysis. Dr. Bo made significant contributions to the analysis of patients with TFP deficiencies. Prof. Taketani provided review and editing. Furthermore, Dr. Hasegawa is grateful to Emeritus Prof. Yamaguchi for providing critical insights into analytical methods and research design, and for his supervision of this study.

Conflict of Interest

The authors declare no conflicts of interest in association with the present study.

Abbreviations

NBS	newborn screening
TMS	tandem mass spectrometry
GC-MS	gas chromatography/mass spectrometry
t-BDMS	N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide
OAs	organic acidemias
FAODs	fatty acid oxidation disorders
PND	prenatal diagnosis
TFP	mitochondrial trifunctional protein
MMA	methylmalonic acidemia
PPA	propionic acidemia
GA	glutaric acidemia
MCD	multiple carboxylase deficiency
VLCAD	very long-chain acyl-CoA dehydrogenase
CACT	carnitine acylcarnitine translocase

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