In this population-based (also a descriptive), retrospective study, we aimed to investigate the intrapartum FHR monitoring patterns preceding the onset of terminal bradycardia in infants born at >34 weeks of gestation who developed subsequent brain damage. The findings of this study may help to anticipate terminal bradycardia, and to avoid fetal catastrophe during labor and delivery. They could also contribute to the development of potential prevention strategies.

Methods

In 1998, we initiated a regional population-based study on perinatal deaths and neurological damage in Miyazaki Prefecture, Japan, which has a population of 1 million, with 10 000 deliveries annually.[3–6] Miyazaki has 34 primary obstetric clinics, seven secondary-level perinatal centers, and one tertiary-level perinatal center spanning four medical districts. Each district has at least one secondary perinatal center, and all but two of the primary clinics are located within 30 min of the nearby centers. Approximately 80% of the women were low-risk and delivered mainly in the primary clinics, while the remaining 20% were referred to secondary or tertiary centers depending on their risk factors. The details for the high-risk pregnancies and newborns requiring referral to the regional secondary centers are given in a previous report.[3] Those with high-risk factors are typically treated in secondary or tertiary centers.

Additionally, in 1998, we initiated a peer-review audit conference where perinatal and neonatal specialists from eight perinatal centers attend in order to determine the causes and associated clinical factors for perinatal deaths and neurological complications. Our inclusion criteria for registering neurologically high-risk infants are listed in Table 1. We excluded fetal and neonatal deaths and congenital anomalies from the present study.

The registered infants were followed by pediatric neurologists who examined them for CP, mental retardation, and/or epilepsy at ≥2 years of age. The causes and clinically relevant factors of neurological complications were classified at the peer-review audit conferences and categorically applied to infants born at >34 weeks of gestation.

Intrapartum FHR monitoring charts from at least 1 h prior to the onset of bradycardia were retrospectively analyzed and classified according to the guidelines of the National Institute for Child Health and Human Development.[7] Variable decelerations were classified as mild, moderate, or severe according to the classification of Kubli *et al.*[8] Prolonged decelerations were defined as decelerations of >2 to <10 min with a decrease to <100 b.p.m. Late decelerations were defined as recurrent if they occurred during >50% of the uterine contractions in a 1-h segment. Bradycardia was defined as a baseline FHR <110 b.p.m. for >10 min. Baseline FHR, baseline variability, accelerations, and decelerations were interpreted and recorded on an hourly basis. Reassuring FHR was defined in the presence of normal baseline FHR and moderate baseline variability with accelerations, but without late, moderate to severe, variable, or prolonged decelerations. Non-reassuring FHR patterns were defined as those not meeting the reassuring pattern definition.

Table 1 Inclusion criteria for the neurologically high-risk infants

Umbilical arterial pH < 7.0 or base deficit > 12 mmol/L.
Umbilical arterial pH < 7.0 or base deficit > 12 mmol/L.
Abnormal neurological findings during the neonatal period
a. Seizure activity
b. Hypertonia or hypotonia
c. Abnormal reflex
d. Irritability or hyperexcitability
e. Poor sucking and swallowing reflexes
f. Shallow, irregular respirations
g. Apnea (not caused by prematurity)
Abnormal neurological images during the neonatal period
a. Intraventricular hemorrhage (grades 3–4)
b. Periventricular leukomalacia
c. Hydrocephalus
d. Congenital CNS anomalies
e. Hypoxic–ischemic encephalopathy
Congenital infection that may cause neurological damage
Severe IUGR (>3SD)

CNS, central nervous system; IUGR, intrauterine growth restriction; SD, standard deviation.

According to our previous findings,[3,9] the worst pattern immediately preceding delivery was taken as the FHR pattern. If one or more prolonged decelerations occurred, we defined the pattern as prolonged deceleration. The FHR monitoring charts were reviewed by three investigators (Y.K., H.S., T.I.). Intrauterine infection was defined clinically according to the criteria by Lencki *et al.*,[10] which were maternal temperature > 38°C and at least one of the following four criteria: maternal tachycardia > 100 b.p.m., uterine tenderness, white blood cell count > 15 000/mm^3, and foul-smelling vaginal discharge. If no temperature elevation was present, all four of the other criteria had to be present.

Imaging studies were performed for 11 infants with brain damage, but the timing and modality, computed

tomography (CT) or magnetic resonance imaging (MRI) varied. In general, the first CT scan was performed when the infant was successfully stabilized. MRI was performed in nine of the 11 infants and the findings were interpreted by radiologists blinded to the neurological results.

The neurological outcome (CP and its type, mental retardation, and epilepsy) on the last examination by pediatric neurologists, independent of the present results, was used for the analysis.

The study protocol was approved by the institutional review board of the Faculty of Medicine, University of Miyazaki.

Results

Of the 65 197 births from 1998 to 2003, 190 (0.29%) were stillbirths, 115 (0.18%) were neonatal deaths, and 136 (0.21%) infants had neurological high-risk factors. The high-risk infants were as follows: severe metabolic acidosis ($n=17$), abnormal neurological signs ($n=52$), abnormal neurological images ($n=68$), infectious disease ($n=10$), and severe intrauterine growth restriction ($n=18$). Several patients had multiple factors (Fig. 1).

Among the neurologically high-risk infants, 15 showed intrapartum terminal bradycardia. After a follow-up period of at least 2 years, 13 infants were diagnosed as having neurological damage, such as CP, mental retardation, or epilepsy, and two developed normally (Fig. 1).

Cases of brain damage in these 13 infants were spastic or mixed types of CP ($n=11$), or mental retardation with epilepsy ($n=2$). Two infants had bradycardia on admission associated with placental abruption ($n=1$) and massive bleeding due to placental previa ($n=1$). In the remaining 11 infants, the preceding FHR patterns were reassuring or only mild variable decelerations in six (55%) and non-reassuring in five (45%), including baseline tachycardia ($n=2$), late decelerations ($n=4$), and moderate to severe variable decelerations ($n=2$) (Table 2).

The clinical factors relevant to the non-reassuring FHR patterns included intrauterine infection ($n=3$), malpresentation with umbilical cord coiling ($n=1$), and unknown causes ($n=1$). The factors relevant to the reassuring FHR patterns included umbilical cord prolapse ($n=1$), vaginal breech delivery ($n=1$), shoulder dystocia ($n=1$), rupture of membranes ($n=1$), and unknown causes ($n=2$). Seven infants were delivered by emergent cesarean section, four by vacuum extraction, and the remaining two vaginally without any instruments.

Neonatal encephalopathy

Immediately after birth, 12 infants were transferred to the neonatal intensive care unit due to respiratory problems and all showed early-onset seizure before 24 h of

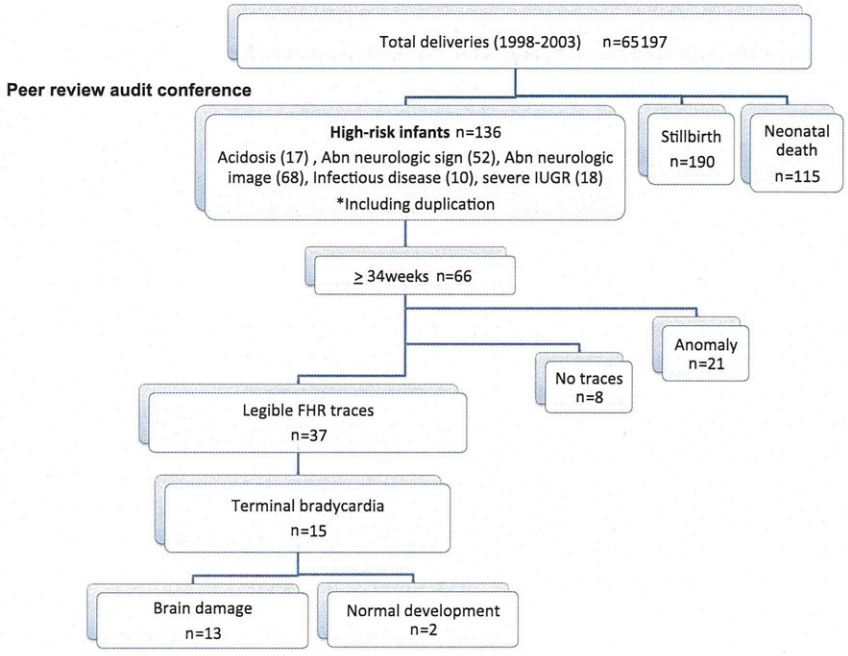

Figure 1 Flow diagram for the study population. Abn, abnormal; FHR, fetal heart rate; IUGR, intrauterine growth restriction; ND, neonatal death.

Table 2 Brain damaged infants with intrapartum terminal bradycardia

Case no.	pH	BE	Apgar score (1 min/5 min)	Cause	FHR patterns prior to bradycardia	Bradycardia	Outcome
Bradycardia on admission							
1	6.64	–27	1/3	Placental abruption	NA	<80 b.p.m., 120 min	CP
2	6.80	–21	1/3	Placenta previa	NA	<70 b.p.m., 30 min	CP
Non-reassuring pattern preceding bradycardia							
3	6.91	–20	4/6	Intrauterine infection	rLD, tachycardia	50-70 b.p.m., 40 min	CP
4	6.69	–23	0/0	Intrauterine infection	oLD, VD	<80 b.p.m., 40 min	CP
5	7.20	– 6.8	3/4	Intrauterine infection	rLD, tachycardia	90-120 b.p.m., 15 min	MR, Epi
6	6.80	–23	2/5	Malpresentation, cord coiling	oLD	<80 b.p.m., 14 min	CP
7	6.75	–18	2/2	Unknown	VD	<100 b.p.m., 20–40 min	CP
Reassuring pattern preceding bradycardia							
8	NA	NA	1/1	Breech delivery	Reassuring	<70 b.p.m., 20 min	CP
9	7.09	–11	2/3	Cord prolapse	Reassuring	<100 b.p.m., 30 min	CP
10	6.86	–21	2/2	Shoulder dystocia	Mild VD	60–90 b.p.m., 13 min	CP
11	6.87	–18	1/3	ROM	Reassuring	60–80 b.p.m., 40 min	CP
12	6.75	–19	3/4	Unknown	Mild VD	<80 b.p.m., 40 min	CP
13	7.00	–15	8/9	Unknown	Mild VD	90 b.p.m., 10 min	MR, Epi

BE, base excess; CP, cerebral palsy; Epi, epilepsy; FHR, fetal heart rate; MR, mental retardation; NA, not available; oLD, occasional late deceleration; rLD, recurrent late deceleration; ROM, rupture of menbrane; VD, variable deceleration.

age requiring anticonvulsants. One infant (case 13), with Apgar scores of 8 and 9 at 1 and 5 min, respectively, had stable respiration but experienced a seizure on the first day of life and was transferred to the neonatal intensive care unit. Specifically, the infant had intracranial hemorrhage of the left thalamus and later required a ventricular peritoneum shunt for progressive hydrocephalus. All 13 infants survived to more than 2 years of age; 11 developed severe encephalopathy with feeding intolerance and two (cases 5 and 13) had mental retardation with epilepsy (Table 2).

Imaging

MRI scans were performed in nine infants. The other two (cases 4 and 6) were examined by CT only. White matter and/or cortex damage were the most common abnormal findings (both in 9/11, 82%), visualized as low-density areas on CT and/or abnormal signals on MRI. Thalamic damage was observed in seven (64%) infants and basal ganglia damage in five (45%). Only one infant (case 3) had diffuse atrophy of the brain stem with high-intensity areas on T1-weighted scans of the basal ganglia and thalamus (Table 3).

Discussion

The intrapartum charts we interpreted were at least 1 h and 2 h at the longest. The length of the chart was determined by how long the chart left before the onset of bradycardia. In our previous study, we determined that fetal bradycardia with a nadir < 80 b.p.m. and lasting ≥13 min was associated with acidemia-related CP.[3] The aim of the present study was to ascertain the FHR patterns that occur prior to the onset of terminal bradycardia, which causes brain injury due to intrauterine hypoxic–ischemic insult. In this unselected population-based study, the prevalence of brain injury at >34 weeks due to terminal bradycardia was 2.0/10 000 (13/65 197).

Except for two infants with bradycardia on admission, the FHR pattern was reassuring until the onset of terminal bradycardia in six. The prevalence of brain damage at >34 weeks due to sudden onset terminal bradycardia without any preceding non-reassuring FHR patterns was 0.92/10 000. In this group, the known causes were vaginal breech delivery, umbilical cord prolapse, shoulder dystocia, and rupture of membranes. Aside from vaginal breech delivery, whether the other factors can serve as reliable predictors of terminal bradycardia to save an infant from brain damage remains unclear. Pasternak *et al.*[11] also reported 11 infants with unrecoverable terminal bradycardia, seven of whom exhibited completely unremarkable FHR monitoring prior to the onset of bradycardia.

In the present study, intrauterine infection was present in three of five (60%) patients with non-reassuring patterns preceding the bradycardia. Several recent studies have implicated maternal infection as a potential cause

Table 3 Location of the injury on imaging and outcomes for the 11 infants

Case no.	Outcome	Imaging results for the brain				
		Brain stem	Thalamus	Basal ganglia	Cortex	White matter
1	CP (spastic)	—	O	O	O	O
2	CP (spastic)	—	O	O	O	O
3	CP (spastic)	O	O	O	O	O
4	CP (mixed)	—	—	—	O	O
6	CP (spastic)	—	O	O	O	—
7	CP (spastic)	—	—	—	O	O
8	CP (spastic)	—	O	—	—	O
9	CP (spastic)	—	—	—	O	O
11	CP (mixed)	—	O	O	O	O
12	CP (spastic)	—	—	—	O	O
13	MR, Epi	—	O	—	—	—

CP, cerebral palsy; MR, mental retardation; Epi, epilepsy.

of CP. Bax *et al.*[12] reported that 39.5% (158/400) of infants with CP were born to a mother with a history of infection during pregnancy. Similarly, Neufeld *et al.*[13] also reported maternal infection as a risk factor for CP in both term and preterm infants in a population-based case–control study. Wu *et al.*[14] revealed an increased prevalence of infection in infants born with CP at or near term, where 14% of the mothers had chorioamnionitis compared to 4% of the controls. Sameshima *et al.* reported an increased prevalence of non-reassuring FHR patterns in patients with intrauterine infection,[15] and suggested that mature infants might require exposure to both infection and hypoxia in order for CP to occur.[16] Our present study emphasizes the important association between intrauterine infection and terminal bradycardia preceded by non-reassuring FHR patterns.

Two cases (case 5 and 13) turned out to be mental retardation, not CP. This may be because the damages caused by hypoxic–ischemic insult were not so severe as to develop CP. The bradycardia was less severe in nadir and duration, and the umbilical cord blood pH was 7.20 and 7.00, respectively.

Imaging studies have demonstrated that hypoxic–ischemic changes in the cortex and white matter are the predominant lesions in infants with brain damage caused by terminal bradycardia during delivery. Animals subjected to hypoxic–ischemic insult demonstrate four patterns of damage in response to different conditions of oxygen deprivation.[17] However, the clinical syndromes corresponding to these patterns have not been fully defined. Okumura *et al.*[18] reported two cases of severe bradycardia lasting more than 20 min in infants with basal ganglia and thalamic damage in the absence of extensive cortical changes. In our study, damage to the basal ganglia, thalamus, or both was accompanied by extensive damage to the cerebral cortex and/or cerebral white matter, except in one infant (case 13). This pattern of brain damage is expected in infants who have experienced severe prolonged asphyxia with terminal bradycardia. Roland *et al.*[19] suggested that intrauterine asphyxia in term infants preferentially damages the cerebral cortex and underlying white matter.

One limitation of this study is its retrospective design. In particular, management, in terms of expeditious delivery and neonatal resuscitation, was not thoroughly taken into account, and due to the size of the task, we were unable to follow all 65 197 infants to search for neurological sequelae. Although almost all of the high-risk pregnancies and newborns were transferred to secondary or tertiary centers in our district, a few might not have been registered in our peer-review audit conferences. Furthermore, even though some neonates exhibited no apparent abnormalities during the neonatal period, they may develop CP later.[20] In order not to miss these cases, we cooperated with our public health service centers. The registration of severely handicapped infants ($n = 142$) during the study period also supports our assumption that we were able to enroll most of the high-risk infants for neurological damages (registered cases were 136 in this study), which means that this study covers 96% of them.

Nonetheless, we conducted this study in an unselected, population-based setting. To the best of our knowledge, our study is one of very few unselected population-based investigations documenting FHR patterns prior to terminal bradycardia in infants (>34 weeks of gestation) who developed subsequent central nervous system sequelae. The cause of fetal bradycardia was not determined in some cases, but more than half of the cases showed a reassuring FHR pattern until a

sudden decrease in the fetal heart rate. Our findings could be useful not only in obstetrical lawsuit cases but also in the intrapartum management of intrauterine infection. Once such a situation occurs, it can be difficult to save the fetus from irreversible brain injury. Intrauterine infection was a major sentinel event in non-reassuring FHR patterns preceding terminal bradycardia, suggesting that patients with intrauterine infection need continuous FHR monitoring and preparation for a possible emergency event.

Acknowledgments

We thank Drs K. Kai, K. Michikata (Miyazaki Municipal Hospital), H. Urabe, H. Taniguchi (Fujimoto-Hayasuzu Hospital), A. Yamauchi (Miyazaki Prefectural Hospital, Nichinan), K. Doi (Miyazaki Prefectural Hospital, Nobeoka), and Y. Takasaki (Miyakonojyo Hospital) for their help in reviewing the medical records.

Disclosure

We have no conflicts of interest.

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PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2015) **57**, 348–353 doi: 10.1111/ped.12660

Review Article

Metabolic disease in 10 patients with sudden unexpected death in infancy or acute life-threatening events

Tomoo Takahashi, Kenji Yamada, Hironori Kobayashi, Yuki Hasegawa, Takeshi Taketani, Seiji Fukuda and Seiji Yamaguchi

Department of Pediatrics, Shimane University Faculty of Medicine, Izumo, Shimane, Japan

Abstract In order to determine the associations between sudden unexpected death in infancy (SUDI) or acute life-threatening events (ALTE) and inborn errors of metabolism, particularly organic acidemia and fatty acid oxidation disorders, we evaluated clinical features in patients with SUDI or ALTE. The subjects were infants between the ages of 7 days and 3 years who developed SUDI or ALTE between January 2004 and December 2013. They were then diagnosed as having inborn errors of metabolism on gas chromatography–mass spectrometry (GC/MS) and/or tandem mass spectrometry (MS/MS). The age distribution, onset forms, and clinical findings were evaluated during the acute phase. Inborn errors of metabolism were detected in three of 196 patients with SUDI, and in seven of 167 patients with ALTE. Of these 10 patients, nine had a history of poor feeding and somnolence during the neonatal period, and symptoms of infection such as cough, fever or vomiting during infancy. Routine laboratory tests during an acute phase indicated hyperammonemia, liver dysfunction, increased blood creatine kinase, acidosis, positive ketone bodies in urine or blood, or hypoglycemia. When SUDI or ALTE are encountered in the emergency unit, it is essential that a detailed medical history is taken, particularly with regard to the neonatal period, and that specific abnormalities are investigated on routine laboratory tests. Moreover, samples such as urine, serum, and filter paper blood specimens should be collected for GC/MS and/or MS/MS of organic acids and acylcarnitines, to identify inborn metabolic disorders.

Key words apparent life-threatening event, gas chromatography, inborn error of metabolism, sudden unexpected death in infancy, tandem mass spectrometry.

Sudden infant death syndrome (SIDS) is defined as the sudden, unexpected death of an infant that cannot be explained based on previous medical history or symptoms. The cause of the death cannot be specified on autopsy. A recent study found SIDS to be the third leading cause of overall infant mortality in Japan, following congenital anomalies, and perinatal disorders. A sudden death may occur not only in infants with no prodromal symptoms but also in previously healthy infants who develop infections or diarrhea.[1–6] The latter cases are not considered to represent SIDS in a strictly defined sense, but the broad term "sudden unexpected death in infancy" (SUDI) is applied. An apparent life-threatening event (ALTE) is defined as a life-threatening episode in an infant that does not lead to death and is characterized by sudden apnea resulting in skin color change, muscle tone change, coughing, and so on. ALTE includes a condition that was previously called near-miss SIDS.

Newborn mass screening on tandem mass spectrometry (MS/MS) has been widely used and newborns can now be screened for organic acid and fatty acid disorders in addition to amino acidemias. Some infants with organic acid and fatty acid disorders are known to develop symptoms similar to those of SUDI or ALTE.

Correspondence: Tomoo Takahashi, MD, Department of Pediatrics, Shimane University Faculty of Medicine, 89-1, Enya, Izumo, Shimane 693-8501, Japan. Email: tomoo@yahoo.co.jp

Received 11 November 2014; revised 18 February 2015; accepted 2 March 2015.

Given that newborn mass screening on MS/MS would allow early detection and intervention prior to the development of symptoms of organic acid and fatty acid disorders, this form of mass screening is expected to prevent sudden deaths due to such disorders.

We have been analyzing metabolic disorders using gas chromatography–mass spectrometry (GC/MS) and/or MS/MS in children who present with symptoms similar to those of acute encephalopathy, SUDI, or ALTE. Thus, we conducted the present study with the aims of improving the early detection and the treatment of inborn metabolic disorders underlying SUDI or ALTE. We thus evaluated detection frequency, age distribution, and clinical features in infants <3 years of age who presented with symptoms similar to those of SUDI or ALTE, and were diagnosed as having organic acid and fatty acid disorders on GC/MS or MS/MS.

Method

Subjects

Among infants who were referred to the Department of Pediatrics, Shimane University for GC/MS of urinary organic acids or MS/MS of acylcarnitines during the 10 year period between January 2004 and December 2013, those who met the following criteria were included: (i) age between 7 days and 3 years; (i) clinical diagnosis of SUDI or ALTE; and (iii) established diagnosis of organic acid or fatty acid disorders.

Neonates before day 7 of birth were excluded because congenital abnormalities and perinatal disorders might have contributed to their condition.[7,8]

Sample preparation and GC/MS

Organic acid analysis

Urine samples for GC/MS of urinary organic acids were pretreated as described previously. Briefly, to an aliquot of urine 0.2 mg equivalent of creatinine, 20 μg heptadecanic acid, 20 μg tetracosane (C24), and 40 μg tropic acid were added as internal standards. Distilled water was added to yield 2.0 mL of the mixture, and solvent extraction, oximation, and trimethylsilyl derivatization were performed.[9,10]

GC/MS was performed using GCMS QP2010 Plus (Shimadzu, Kyoto, Japan). The column (30 m × 1.0 mm i.d.) was DB-5 (J&W Scientific, Folsom, CA, USA). The oven temperature was initially 100°C and was then raised to 290°C at a rate of 4°C/min.

Glycerol metabolite analysis

In 30 patients with residual urine samples, glycerol-3-phosphate was measured according to a method described previously.[11,12] Briefly, to achieve urea decomposition, 20 μg tropic acid and 20 units of urease were added to an aliquot of urine equivalent to 0.1 mg creatinine. In total, 500 μL ethanol was then added for deproteinization, and the solution was dried under a nitrogen stream at 50°C. The dried residue of organic acids was trimethylsilylated. GC/MS was performed under the same conditions as aforedescribed.

Quantitative acylcarnitine analysis

Acylcarnitines were analyzed on MS/MS after butyl derivatization had been performed in serum sample aliquots of 10 μL according to a method described previously.[13] MS/MS was carried out using an API 3000 (Applied Biosystems, Foster City, CA, USA). Data were analyzed with ChemoView™ (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Results

Age distribution

We studied a total of 363 infants, including 196 with SUDI (GC/MS and MS/MS in 57, GC/MS only in 10, MS/MS only in 129), and 167 with ALTE (GC/MS and MS/MS in 95, GC/MS only in 13, MS/MS only in 59).

Figure 1 shows the age distribution. The numbers of infants in each age group were as follows: 7–28 days (neonates), n = 45 (SUDI, n = 20; ALTE, n = 25); 1–6 months, n = 177 (SUDI, n = 91; ALTE, n = 86); 6–12 months, n = 55 (SUDI, n = 39; ALTE, n = 16); 1–2 years, n = 66 (SUDI, n = 37; ALTE, n = 29); 2–3 years, n = 20 (SUDI, n = 9; ALTE, n = 11).

Confirmed metabolic disorders

The GC/MS of urinary organic acids, MS/MS of acylcarnitines, and genetic testing yielded a diagnosis of inborn errors of metabolism in 10 (2.7%) of the total 363 infants. Among these 10 patients, three were newborns, three were 1 month–1 year of age, and four were ≥1 year of age.

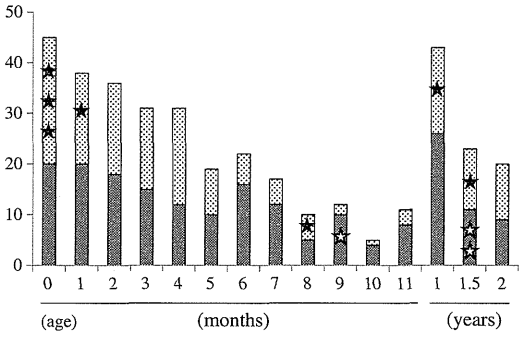

Fig. 1 Age distribution in infants with (▨) acute life-threatening events (n = 167) or (■) sudden unexpected death (n = 196). ★ or ☆, inborn error of metabolism.

Among these 10 patients, two had medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, one had carnitine palmitoyltransferase type 2 (CPT2) deficiency presenting with SUDI, four had methylmalonic acidemia (MMA), two had urea cycle disorders, and one had mitochondrial trifunction protein (TFP) deficiency with ALTE (Table 1).

Clinical findings

Poor feeding and somnolence were common in three neonates (patients 1–3) as prodromal symptoms. Of the seven patients between the ages of 1 month and 3 years (patients 4–10), six had cold symptoms such as fever, cough, rhinorrhea, and vomiting as prodromal symptoms. Of these seven patients, three had intractable vomiting; one had a medical history of transient hypoglycemia; and one had an episode of cyanosis during the neonatal period.

Laboratory results during an acute phase included acidosis (pH, 6.9–7.3) in seven of 10 infants; positive ketone bodies (in urine or blood) in four of six infants who received ketone testing; liver dysfunction (hyper blood aspartate aminotransferase (AST; 52-3144IU/L); hyper blood alanine aminotransferase (ALT; 43-1712IU/L) in eight of 10 infants; high blood creatine kinase (CK; 203–8077 IU/L) in eight of 10 infants; hyperammonemia (147–2006 μg/dL) in seven of eight whose data were available; and hypoglycemia (blood glucose, ≤17–30 mg/dL) in three of 10 infants (Table 2).

Two of the three newborns (patients 1,3) had loss of the Moro reflex. Only one infant (patient 8) had a family history of abnormality (acute encephalopathy).

In addition to the aforedescribed 10 infants, there were at least 10 other infants who lacked definitive diagnosis but were strongly suspected to have inborn errors of metabolic disease (Table 3). The results were suggestive of glutaric acidemia type 2, very long-chain acyl-CoA dehydrogenase deficiency, and primary carnitine deficiency, but a definitive diagnosis could not be obtained.

Table 1 Organic or fatty acid disorder patient profiles

Patient ID no.	Age at onset	Sex	Diagnosis	ALTE/SUDI	GC/MS (abnormal OA)	MS/MS (elevated AC)	Abnormality in neonatal period	Prodrome
1	7 days	F	MMA	ALTE	MM, MC, 3HP	C3, C3/C2	Poor sucking	Poor sucking
2	8 days	F	UCD	ALTE	Orotic, uracil	Cit	Lethargy	Lethargy
3	8 days	F	UCD	ALTE	Normal	Cit	Poor sucking	Poor sucking
4	1 month	M	TFP def.	ALTE	NKDA, LA, PA	C14-OH, C16-OH, C18-OH, C18:1-OH	Cyanosis	Non-specific
5	8 months	M	MMA	ALTE	MM, MC, 3HP	WNL	WNL	Vomiting
6	9 months	M	CPT2 def.	SUDI	NKDA	C14,C16,C18	WNL	Fever
7	1 year 1 month	F	CPT2 def.	SUDI	WNL	C16	WNL	Cough
8	1 year 8 months	F	MMA	ALTE	MM, MC, 3HP, PG	C3, C3/C2	WNL	Cough, vomiting
9	1 year 8 months	M	MCAD def.	SUDI	NKDA, HG, SG	C6, C8, C10	Hypoglycemia	Fever, cough
10	1 year 10 months	M	MMA	ALTE	MM, MC, 3HP, PG	C3, C3/C2	ND	Fever, vomiting

3HP, 3-OH-propionate; AC, acylcarnitine; ALTE, acute life threatening event; CPT2, carnitine palmitoyltransferase type 2; def., deficiency; GA2, glutaric acidemia type 2; GC/MS, gas chromatography–mass spectrometry; HG, hexanoylglycine; KDA, ketotic dicarboxylic aciduria; LC, long-chain; MC, methylcitrate; MCA, medium chain acylcarnitine; MCAD, medium chain acyl-CoA dehydrogenase; MM, methylmalonate; MMA, methylmalonic acidemia; MS/MS, tandem mass spectrometry; NA, not analyzed; ND, no data; NKDA, non-ketotic dicarboxylic aciduria; OA, organic acid; PG, propionylglycine; SG, suberylglycine; SUDI, sudden unexpected death in infancy; TFP, trifunctional protein; UCD, urea cycle disorder; WNL, within normal limits.

Table 2 Acute stage laboratory data at admission

Patient ID no.	Age at onset	Diagnosis	pH	Ketosis	AST (IU/L)	ALT (IU/L)	CK (IU/L)	NH_3 (μg/dL)	Glucose (mg/dL)	Gene analysis
1	7 days	MMA	7.3	–	65	53	245	674	143	NA
2	8 days	UCD	Acidosis	NA	55	33	308	2006	79	NA
3	8 days	UCD	WNL	+	36	16	284	1035	78	NA
4	1 month	TFP def.	WNL	NA	153	71	8077	WNL	71	c.1364T>G (homo)
5	8 months	MMA	7.1	+	WNL	WNL	1084	ND	63	NA
6	9 months	CPT2 def.	7.2	NA	3144	1712	1100	ND	17	c.520G>A (homo)
7	1 year 1 month	CPT2 def.	7.37	–	353	178	203	147	98	c.745delG/ c.1148T>A
8	1 year 8 months	MMA	7.2	+	40	43	560	162	30	NA
9	1 year 8 months	MCAD def.	7.2	NA	80	40	107	1640	Not detected	c.449_452 delCTGA(homo)
10	1 year 10 months	MMA	6.9	+	52	17	100	188	88	NA

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CPT2, carnitine palmitoyltransferase type 2; def., deficiency; MCAD, medium chain acyl-CoA dehydrogenase; MMA, methylmalonic acidemia; NA, not available; ND, no data; TFP, trifunctional protein; UCD, urea cycle disorder; WNL, within normal limits.

Table 3 Suspected inherited metabolic disease in SUDI/ALTE patients

Patient ID no.	Age	Sex	ALTE or SUDI	Suspected disease	GC/MS (OA abnormality)	MS/MS (AC abnormality)
11	14 days	F	ALTE	GA2	LA, PA, KDA	C4, C6, C8, C10
12	20 days	M	SUDI	VLCAD def. or GA2	NA	LC AC
13	28 days	F	ALTE	PCD	PA,KDA	Low C0 (6.8)
14	1 month	M	ALTE	PCD	NA	Low C0 (13.97)
15	1 month	M	ALTE	GA2	LA, KDA	MC–LC AC
16	4 months	F	SUDI	PCD	NA	Low C0 (17.27)
17	4 months	M	SUDI	GA2 or MCAD def.	NA	MC AC
18	5 months	M	ALTE	VLCAD def. or GA2	NA	LC AC
19	1 year 2 months	M	SUDI	GA2	LA, PA, KDA	SC–LC AC
20	1 year 4 months	M	ALTE	GA2	NA	SC–LC AC

AC, acylcarnitine; ALTE, acute life threatening event; def., deficiency; GA2, glutaric acidemia type 2; GC/MS, gas chromatography–mass spectrometry; KDA, ketotic dicarboxylic aciduria; LA, lactic acid; LC, long-chain; MC, medium-chain; MS/MS, tandem mass spectrometry; NA, not analyzed; OA, organic acid; PA, pyruvic acid; PCD, primary carnitine deficiency; SC, short-chain; SUDI, sudden unexpected death in infancy; VLCAD, very long chain acyl-CoA dehydrogenase.

Step 1: Case reports

Case 1

Patient 1 was a 7-day-old girl who had been admitted to hospital on the seventh day after birth because of poor feeding noticed on the fifth day of life. She was born at 40 weeks 4 days of gestation without asphyxia and with a birthweight of 2770 g. She showed closing of her eyes, poor response to painful stimuli, asterixis, loss of the Moro reflex, and marked dehydration. GC/MS and MS/MS yielded a diagnosis of MMA. Her condition improved with arginine treatment and exchange transfusion.

Case 2

Patient 2 was an 8-day-old girl, born at 39 weeks 2 days of gestation with a birthweight of 3170 g. Apgar scores were 9 at 1 min and 10 at 5 min. Reduced activity and somnolence developed from the second day of life. On the seventh day of life, marked somnolence recurred, and grunting plus a low body temperature (34.0°C) were also noticed. GC/MS of organic acids showed elevated orotic acid and uracil. MS/MS indicated citrulline elevation. Eventually, a diagnosis of citrullinemia type 1 was made. She was treated with continuous hemodiafiltration, and survived.

Case 3

Patient 3 was an 8-day-old girl. She was born at 38 weeks 6 days of gestation without asphyxia and the birthweight was 2350 g. Poor feeding and weak crying were apparent on the fourth day of life. Shallow and superficial respiration developed on the eighth day of life and the Moro reflex could not be elicited. GC/MS showed no special abnormalities whereas MS/MS indicated elevated citrulline. Citrullinemia type 1 was thus diagnosed. Her condition improved with dialysis.

Case 4

Patient 4 was a 1-month-old boy. Cyanosis developed on the fifth day of life but then improved. Respiratory failure suddenly developed on the 32nd day of life. GC/MS showed hypoketotic dicarboxylic aciduria. MS/MS indicated increased C14-OH, C16-OH, C18-OH and C18:1-OH. Genetic tests showed homozygosity for the c.1364T>G mutation on *HADHB* [Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit] gene (Table 2). Thus, diagnosis of mitochondrial TFP deficiency was made. His condition improved with appropriate treatment.

Case 5

Patient 5 was an 8-month-old boy. His growth and development had been normal until the sudden occurrence of vomiting, tachypnea, somnolence and impaired consciousness. Magnetic resonance imaging (MRI) of the brain showed a high-intensity area in the globus pallidus. GC/MS yielded a diagnosis of MMA. His condition improved with appropriate treatment.

Case 6

Patient 6 was a 9-month-old boy. No abnormalities in his growth and development had been noted. He was taken to a nearby physician because of fever, and influenza A was diagnosed. Shallow respiration rapidly worsened thereafter, and cardiopulmonary arrest occurred. MS/MS showed an increase in C14, C16 and C18. Genetic testing indicated homozygosity for the 520G>A (E174K) mutation on *CPT2*. Thus, CPT2 deficiency was diagnosed. The infant had no response to the treatments given, and died.

Case 7

Patient 7, a girl, was 13 months old. She had received ambulatory medical care for the common cold because of cough and rhinorrhea. Her mother found that she was lethargic at 06:00 hours on the eighth day of this illness. The infant was transported to a hospital by ambulance. Convulsions developed, and the anterior fontanelle bulged. MRI of the brain showed brain edema. MS/MS indicated elevation of C14, C16 and C18. Genetic testing of *CPT2* indicated compound heterozygous mutations of c.745G/c.1148T>A. Thus, diagnosis of CPT2 deficiency was made. She died 10 h after admission, showing no response to resuscitation.

Case 8

Patient 8, a girl, was 1 year 8 months old. She first presented with cough and vomiting. Somnolence appeared and gradually

worsened. Decreased consciousness and cyanosis were apparent on the fourth day of this illness, and she was admitted to hospital. GC/MS and MS/MS yielded a diagnosis of MMA. After admission, consciousness improved with appropriate treatment.

Case 9

Patient 9 was a 1-year and 8-month-old boy. He had cold-like symptom of cough and rhinorrhea, with pyrexia developing on day 3–5 of this illness. During a daytime nap on the sixth day of illness, disturbance of consciousness suddenly developed. He was transported to the emergency department, where he died 2 h later. His sibling had a psychosomatic disorder that had developed after acute encephalopathy of unknown cause. Organic acid included in the urine analysis showed hypoketotic dicarboxylic acids, uric with evaluation of hexanoylglycine, and suberylglycine. Blood filter paper acylcarnitines analysis showed elevation of C8 and C10 acylcarnitines. Genetic testing indicated homozygosity for c.449_452delCTGA of acyl-Coenzyme A dehydrogenase (*ACADM*). Thus, postmortem diagnosis of MCAD deficiency was made.

Case 10

Patient 10 was a 1-year and 10-month-old boy. Pyrexia had developed, but resolved 3 days later. Vomiting and diarrhea occurred on the fifth day after pyrexia onset. On the seventh day of illness, tachypnea and retractive breathing developed, and he was taken to a clinic. GC/MS and MS/MS yielded a diagnosis of MMA. His condition improved with appropriate treatment.

Discussion

The Japanese Ministry of Health, Labour and Welfare started a campaign to prevent SIDS in 1999 through the implementation of three key intervention strategies: (i) avoidance of prone sleeping; (ii) cessation of family smoking; and (iii) breast-feeding to the greatest extent possible. Consequently, the reported number of SIDS cases was reduced from 412 to 148 per year, in 1999–2011, respectively.[14] SIDS in infants aged less than 1 year has received attention but there are also some cases attributable to heart disease and infection among infants aged more than 1 year who had no prodromal symptoms.[15]

In the present study, clinical features were investigated in 10 infants with inborn errors of metabolism, in which symptoms similar to those of SUDI/ALTE allowed confirmation of the diagnoses of metabolic disorders. Three newborns aged between 7 and 28 days were identified as having inborn errors of metabolism. Inborn errors of metabolism that manifest during the neonatal period generally have a high mortality rate.[16] These three infants included one with MMA and two with urea cycle disorders, but they all survived with emergency treatment despite disease onset being a few days after discharge from obstetric institutions. This suggests that an early diagnosis can be life saving, even after the development of symptoms.

Two of seven infants aged more than 1 month had prodromal episodes including transient hypoglycemia during the neonatal period. Meticulous history taking to document even minor episodes during the neonatal period, including transient cyanosis and hypoglycemia, may be diagnostically useful in the search for the cause of SUDI or ALTE.

The most common disease was MMA, which was detected in four infants. This disease commonly manifests during early infancy. Some infants have onset at ≥1 year of age.[17] In addition to the four infants with MMA, there were two with urea cycle disorder, and four with fatty acid oxidation disorder.

Features during the clinical course included poor feeding and somnolence during the neonatal period, as well as acute onset patterns with symptoms such as intractable vomiting, convulsions, and impaired consciousness following fever, cough, and diarrhea, in most of the infants. Given that hypercatabolism of proteins and fatty acids frequently accelerates during these illnesses, prevention of the hypercatabolism with hypertonic glucose solution at a very early stage is essential for prevention of SUDI and ALTE.

Only one patient (patient 8) with MCAD deficiency was found to have a family history of an abnormality. A meticulously taken family history may provide important information when dealing with a potential case of SUDI or ALTE.

Routine laboratory findings, characteristically observed during an acute phase of SUDI or ALTE in cases of underlying inborn errors of metabolism, included ketoacidosis and hyperammonemia in organic acidemia patients. In the patients with fatty acid oxidation disorder, increased blood CK, AST, or ALT reflect abnormalities in the skeletal muscles, myocardium, and liver, because these are β-oxidation-dependent organs.

Chace *et al.* reported that inborn errors of metabolism were identified in 66 (0.9%) of 7058 infants with SUDI, while Boles *et al.* identified such inborn errors in 27 (6.4%) of the 418 infants in their study.[16,18–21] In the present study, three (1.5%) of 196 infants with SUDI were diagnosed as having inborn errors of metabolism.

A definitive diagnosis could not be made in at least another nine infants due to reasons such as shortage of specimens, although urinary organic acid and blood acylcarnitine analyses were suggestive of inborn errors of metabolism. This indicates that infants with organic acid and fatty acid disorders may account for at least 1.5% of those presenting with SUDI. Neither blood ammonia nor urinary ketone bodies were measured in the clinical setting in many cases. Moreover, in a number of infants, urinary organic acids could not be analyzed due to difficulty in collecting urine, thus only MS/MS of blood acylcarnitines could be performed.

When SUDI or ALTE is encountered, it is necessary to measure blood glucose, blood gases, ammonia, and CK as well as liver function. At the same time, urine and blood specimens should be routinely collected for GC/MS and MS/MS. For such analyses, urine, serum and filter paper blood (blood spots on Guthrie cards) samples are useful. In the event of a patient's death, urine should be obtained by even suprapubic aspiration of the bladder and bile should be reserved because it contains high levels of acylcarnitines.

Metabolic disorders were diagnosed more frequently in ALTE than SUDI. If diagnosis in ALTE is delayed, the patient is more likely to die, therefore it is important to distinguish metabolic disease in patients with ALTE.

In addition, if peripheral lymphocytes or skin biopsy specimens (for the purpose of culturing skin fibroblasts) are cleanly obtained and sent to a specialized center, then measurement of enzyme

activity[22] or *in vitro* probe assay using cultured cells and MS/MS,[23,24] as well as gene analysis, can be performed.

Detection of inborn errors of metabolism using next-generation sequencing technology has become widespread. Unlike routine genetic analysis, this technology requires the DNA of parents and siblings to be analyzed simultaneously because the aim is a comprehensive genetic analysis for causative genes. This genetic technology is especially worth using in the case of siblings of patients with ALTE or SUDI. MS/MS newborn mass screening was useful in identifying diseases in the present subjects. Benefits of MS/MS screening are expected, in terms of the prevention of SUDI or ALTE, with more extensive newborn mass screening in the future.

Acknowledgments

This study was supported in part by grants from the Ministry of Health, Labor and Welfare, and from the Ministry of Education, Science, and Culture, Further, we thank Professor S. Fukuda, Department of Pediatrics, Shimane University for comments on this manuscript, and Ms M. Furui, N. Tomita, Y. Ito, and E. Mizuno, Department of Pediatrics, Shimane University, for technical assistance.

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Molecular Genetics and Metabolism Reports 5 (2015) 26–32

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Metabolic autopsy with next generation sequencing in sudden unexpected death in infancy: Postmortem diagnosis of fatty acid oxidation disorders

Takuma Yamamoto [a,*], Hiroyuki Mishima [b], Hajime Mizukami [c,d], Yuki Fukahori [a], Takahiro Umehara [a], Takehiko Murase [a], Masamune Kobayashi [e], Shinjiro Mori [d], Tomonori Nagai [d], Tatsushige Fukunaga [d], Seiji Yamaguchi [f], Koh-ichiro Yoshiura [b], Kazuya Ikematsu [a]

[a] Division of Forensic Pathology and Science, Unit of Social Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki, Nagasaki 852-8523, Japan
[b] Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8523, Japan
[c] Department of Legal Medicine, Kanazawa Medical University, Ishikawa 920-0293, Japan
[d] Tokyo Medical Examiner's Office, Tokyo Metropolitan Government, Tokyo 112-0012, Japan
[e] Department of Forensic Medicine, Tokyo Medical University, Tokyo 160-8402, Japan
[f] Department of Pediatrics, Shimane University Faculty of Medicine, Shimane 693-8501, Japan

ARTICLE INFO

Article history:
Received 24 September 2015
Accepted 24 September 2015
Available online xxxx

Keywords:
Sudden unexpected death in infancy
Metabolic autopsy
Next generation sequencing
Fatty acid oxidation disorder
Carnitine palmitoyltransferase II deficiency

ABSTRACT

The recent introduction of metabolic autopsy in the field of forensic science has made it possible to detect hidden inherited metabolic diseases. Since the next generation sequencing (NGS) has recently become available for use in postmortem examinations, we used NGS to perform metabolic autopsy in 15 sudden unexpected death in infancy cases. Diagnostic results revealed a case of carnitine palmitoyltransferase II deficiency and some cases of fatty acid oxidation-related gene variants. Metabolic autopsy performed with NGS is a useful method, especially when postmortem biochemical testing is not available.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as a term that has been variably used to refer to all cases of sudden and unexpected deaths in infancy, and not just to those where the death has been attributed to sudden infant death syndrome [1]. The common causes of SUDI are accidental death, infection, cardiovascular anomaly, child abuse and metabolic diseases.

Fatty acid oxidation disorder (FAOD) is one type of the inherited metabolic diseases that can lead to patient death during situations such as long fasting or infection. Before the introduction of expanded newborn screening by tandem mass spectrometry, FAODs account for approximately 5% of sudden infant deaths [2–7] (Table 1). Some patients can now be screened before the symptom is apparent, others still may remain undetected because the screening has just started nationwide in Japan and some disorders including carnitine palmitoyltransferase (CPT) II deficiency are not included in the first target disease [8]. Since FAODs are functional diseases, they are not easy to diagnose, especially during postmortem examinations. Therefore, some cases may remain undiagnosed or may be incorrectly classified as sudden infant death syndrome.

The recent introduction of metabolic autopsy in the field of forensic science has made it possible to detect hidden inherited metabolic diseases [7,9]. Metabolic autopsy is the autopsy which focuses on the inherited metabolic diseases [10]. The term metabolic autopsy includes microscopic examination of the liver, postmortem blood acylcarnitine analysis and genetic analysis [7]. Since liver steatosis is often seen as a nonspecific change in SUDI and the results of postmortem blood acylcarnitine analysis is often modified by postmortem changes, it is not easy to make a biochemical diagnosis [11]. Although successful genetic analysis has been reported in many cases, there are about 20 FAOD-related genes and thus, surveying all of the genes using Sanger's traditional method of "one gene, one exon at a time" is not an effective way for analyzing these types of cases.

The next generation sequencing (NGS) has recently become available for use in postmortem examinations [12,13]. This technique makes it possible to examine a much larger number of genes and exons at a lower cost in addition to requiring less time than that for the conventional Sanger's method. However, most of the studies using these new techniques have focused on cardiac diseases and thus, there has yet to be metabolic autopsy performed with NGS [13].

* Corresponding author.
E-mail address: t-yamamoto@nagasaki-u.ac.jp (T. Yamamoto).

http://dx.doi.org/10.1016/j.ymgmr.2015.09.005

Table 1
Proportion of inherited metabolic disorders among sudden unexpected death in infancy.

Number of cases	Number of diagnosis	Reference
58	6 (10.3%)	Bennett et al. [2]
79	3 (3.8%)	Lundemose et al. [3]
418	14 (3.3%)	Boles et al. [4]
7058	66 (0.93%)	Chace et al. [5]
247	3 (1.2%)	Wilcox et al. [6]
30	1 (3.3%)	Yamamoto et al. [7]

Therefore, the aim of our current study is to detect FAOD-related gene variants among sudden death cases. As far as we know, this is the first metabolic autopsy performed with NGS.

2. Materials and methods

2.1. Subjects and target sequence

A total of 15 SUDI cases, all of which did not have any characteristic appearance and remained undiagnosed after macroscopic examination, were analyzed at the Department of Forensic Pathology at Nagasaki University (Table 2; 11 males, 4 females, age range; 0 days to 11 months). All cases were born before 2014 and none of them were screened by tandem mass spectrometry.

2.2. Extraction of genomic DNA and genetic analysis

Genomic DNA was isolated from blood leukocytes with the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan) in accordance with the manufacturer's standard methods. A custom-made HaloPlex Target Enrichment System (Agilent Technologies, Santa Clara, CA) was designed to capture the coding exons of the 13 genes targeted to the FAODs (Table 3). The sequencing was performed on an Illumina MiSeq (Illumina, San Diego, CA).

The sequencing reads were mapped on the hg19 human genome sequence using Novoalign version 3.02.12 software (Novocraft, Petaling Jaya, Selangor, Malaysia). Single nucleotide variations and small insertions/deletions were detected using the Genome Analysis Toolkit [14] and annotated using the ANNOVAR software package [15].

Filtering of the variant data was performed as described below. Using the gene information of the GENCODE version 19 [16], single nucleotide variations causing non-synonymous, splice site, or nonsense substitutions and insertions/deletions occurring in the coding regions or the splice sites were retrieved. To identify putatively pathogenic variants, we retained variants with allele frequencies equal to or less than 0.5% in any of the ethnic subgroups found in the various databases. These included variants listed in the Japanese 1200 exomes from the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/), the 1000 Genomes Project [17] and the 6500 control exomes from the NHLBI GO Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/EVS/).

The detected variants were confirmed by the traditional Sanger's method. Each of the exons was amplified, and then examined by polymerase chain reaction (PCR). All reactions were performed in a 25-μL volume containing 12.5 μL of PrimeSTAR Max Premix (2×) (Takara, Otsu, Japan), 0.4 μM each of the primers and 200 ng of template DNA under the following conditions: 98.0 °C for 1 min, (98.0 °C for 10 s, 54.0 °C for 5 s, 72.0 °C for 30 s) for 30 cycles, and 72.0 °C for 5 min. The products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on Applied Biosystems 3130 DNA Analyzer (Applied Biosystems) in accordance with the manufacturer's instructions. The sequence from both strands was visually inspected in order to confirm the substitution.

Table 2
Case summary.

Case	Age	Sex	Diagnosis	Postmortem acylcarnitine analysis	Fat staining
1	0 d	F	Unknown	None specific change	Negative
2	0 d	M	Unknown	None specific change	Negative
3	2 d	M	Pneumonia	Not analyzed	Negative
4	22 d	M	Unknown	None specific change	Negative
5	2 m	M	Acute respiratory infection	Not analyzed	Negative
6	3 m	M	SIDS	None specific change	Negative
7	4 m	M	SIDS	None specific change	Moderate
8	5 m	M	Pneumonia	Not analyzed	Negative
9	5 m	M	Unknown	Not analyzed	Negative
10	7 m	M	SIDS	Not analyzed	Negative
11	8 m	M	Reye's-like syndrome	Not analyzed	Moderate
12	8 m	M	SIDS	Not analyzed	Negative
13	10 m	F	Unknown	None specific change	Moderate
14	11 m	F	Acute encephalopathy	Not analyzed	Negative
15	11 m	F	Reye's-like syndrome	Long-chain fatty acid defect	Distinctive

SIDS; sudden infant death syndrome, d; day, m; month, M; male, F; female.

Table 3
Gene summary.

Gene	OMIM	Exon	Disease	OMIM
SLC22A5	603377	10	Primary carnitine deficiency	212140
CPT1A	600528	19	CPT I deficiency	255120
CPT2	600650	5	CPT II deficiency	608836
				600649
				255110
SLC25A20	613698	9	CACT deficiency	212138
ACADVL	609575	20	VLCAD deficiency	201475
ACADM	607008	12	MCAD deficiency	201450
ACADS	606885	10	SCAD deficiency	201470
HADHA	600890	20	LCHAD, MTP deficiency	609016 (LCHAD)
HADHB	143450	16	MTP, LCKAT deficiency	609015 (MTP)
HADH	601609	8	HAD deficiency	231530
ETFA	608053	12	MAD deficiency	231680
ETFB	130410	6		
ETFDH	231675	13		

CPT; carnitine palmitoyltransferase, CACT; carnitine-acylcarnitine translocase, VLCAD; very-long-chain acyl-CoA dehydrogenase, MCAD; medium-chain acyl-CoA dehydrogenase, SCAD; short-chain acyl-CoA dehydrogenase, LCHAD; long-chain 3-hydroxyacyl-CoA dehydrogenase, MTP; mitochondrial trifunctional protein, LCKAT; long-chain 3-ketoacyl-CoA thiolase, HAD; 3-hydroxyacyl-CoA dehydrogenase, MAD; multiple acyl-CoA dehydrogenase.

2.3. Analysis of the amino acid residues

SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml/) were used to predict whether each amino acid substitution would affect the function of each protein. These substitutions were also aligned across species (https://genome.ucsc.edu/cgi-bin/hgGateway/).

2.4. Postmortem blood acylcarnitine analysis by tandem mass spectrometry

Whole blood samples were blotted onto one spot on a Guthrie card and then subjected to acylcarnitine analysis by tandem mass spectrometry.

2.5. Extraction of genomic DNA and performance of mutational analysis in the other family members

Genomic DNA was purified from buccal cell swabs in accordance with standard methodology. PCR was performed using the previously discussed method. Genomic DNA samples from the parents and the sister were examined for the presence or absence of the *CPT2* mutation.

2.6. Ethics

This study was approved by the Ethics Committee of the Nagasaki University Graduate School of Medicine.

3. Case history in Case 15

The patient was a female carried to full-term and delivered via cesarean section. Her Apgar score was 6/9, birth weight was 2870 g, height was 49.0 cm and head circumference was 34.0 cm.

At 11 months of age, she was taken to a doctor's office because of fever and vomiting that had continued for several days. After examination, she was prescribed cephem antibiotics. During the morning of the next day, however, she suddenly lost consciousness and an ambulance was requested. By the time the emergency service personnel arrived, she had already suffered cardiopulmonary arrest. Although cardiopulmonary resuscitation was initiated immediately and continued until she reached the hospital, she was pronounced dead.

The patient had never been ill until the time of her death. Family history was negative for seizures, arrhythmias and sudden death. Her parents were not consanguineous.

Histological examination of the patient led to the diagnosis of Reye's-like syndrome. The liver showed diffuse and distinctive vacuoles, which Oil-red O staining subsequently confirmed to be the accumulation of fatty acids. Oil-red O-positive vacuoles were also detected in the kidney and heart (Fig. 1).

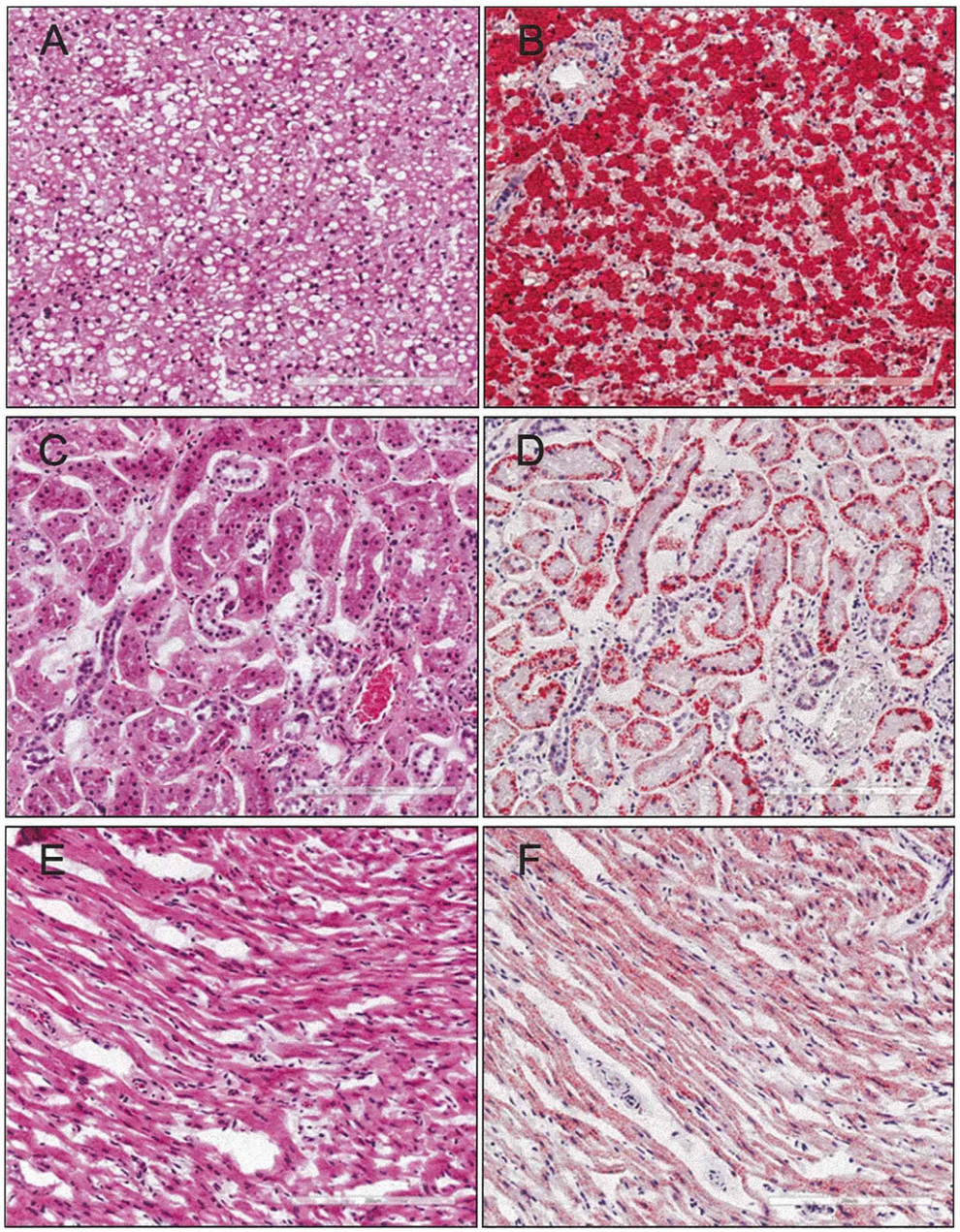

Fig. 1. Histological examination of Case 15. Steatosis was detected in the liver (A; hematoxylin–eosin (H.E.), B; Oil-red-O), kidney (C; H.E., D; Oil-red-O) and heart (E; H.E., F; Oil-red-O).

Table 4
Detected variants.

Case	Gene	AA	Substitution		SIFT		PolyPhen-2		HGVB	EXAC
5	*ACADS*	P55L	164C>T	Heterozygote	Damaging	0.0	Probably D	0.993	8/8654	3/1346
6	*CPT2*	F383Y	1148T>A	Heterozygote	Tolerated	0.61	Possibly D	0.932	3/8644	1/600
7	*SLC22A5*	D487N	1459G>A	Heterozygote	Tolerated	1.0	Benign	0.001	0/121,410	1/600
14	*ACADS*	V84M	250G>A	Heterozygote	Damaging	0.02	Probably D	0.997	0/6496	0/600
15	*CPT2*	F323fs	968_969 del TC	Heterozygote						
15	*CPT2*	V605L	1813G>C	Heterozygote	Damaging	0.004	Possibly D	0.885	3/8654	1/600

W; wild type, M; mutation type, AA; amino acid change, probably D; probably damaging, possibly D; possibly damaging, HGVB; Human Genetic Variation Browser, EXAC; The Exome Aggregation Consortium.

4. Results

4.1. Target sequence of sudden death cases

Targeted resequencing revealed the mean coverage of the coding sequence in the target genes was 155.4 reads, with an overall average gene level coverage at ≥10 reads of 90.9%.

Table 4 shows the detected variants found during the filtering steps. After the filtering, only six variants remained.

4.2. Genetic analysis in Case 15

Among the detected variants, only Case 15 was found to have two pairs of heterozygous deletion (c.968_969 del TC, p.F323fs) and substitution (c.1813G>C, p.V605L) in the *CPT2* gene, all of which were confirmed by Sanger's sequencing (Fig. 2). Pedigree analysis confirmed that the deletion was transmitted from her father while the substitution was from her mother (Fig. 2). The c.1813G>C (p.V605L) substitution has been previously reported in a Japanese CPT II deficiency patient [21]. This was detected in 3 alleles among the 8654 East Asian control alleles (minor allele frequency: 0.0004, The Exome Aggregation Consortium) and in 1 allele out of the 600 Japanese control alleles (minor allele frequency: 0.002, Human Genetic Variation Browser). Results also predicted there would be an effect on the function of the protein, with a SIFT of 0.004, which indicated damaging and a PolyPhen-2 of 0.885, which indicated possibly damaging. An examination of species ranging from zebrafish to human showed the substitution was conserved (Fig. 3).

Analysis also showed there were two homozygous substitutions (c.1055T>G, p.F352C and c.1102G>A, p.V368I) in the *CPT2* gene. Since these two substitutions are common genetic polymorphisms [18–22], they were excluded from the targeted NGS analysis.

4.3. Other genetic substitutions

There were four other substitutions found in four different cases. Case 5 had the substitution *ACADS*-P55L (c.164C>T), Case 6 had *CPT2*-F383Y (c.1148T>A), Case 14 had *ACADS*-V84M (c,250G>A), and Case 7 had *SLC22A5*-D487N (c.1459G>A) (Table 4). The former three were heterozygous substitutions that according to SIFT or PolyPhen-2 were predicted to affect the function of the protein. An examination of species ranging from zebrafish to human showed the substitution was conserved (Fig. 3).

4.4. Acylcarnitine analysis in Case 15

We performed acylcarnitine analysis of the postmortem whole blood samples using tandem mass spectrometry. Free carnitine was 181.24 μM, which was increased as compared to the normal range (<60 μM), but which was within the postmortem reference value

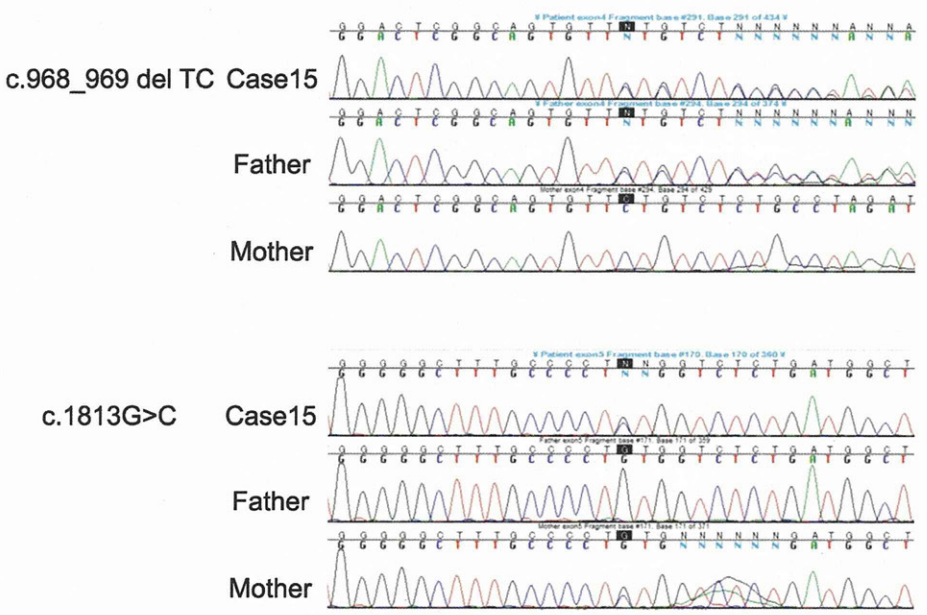

Fig. 2. Sequencing analysis in Case 15. Case 15 had two pairs of heterozygous deletions (c.968_969 del TC) and substitutions (c.1813G>C) in the *CPT2* gene. The father had a heterozygous deletion (c.968_969 del TC) while the mother had a heterozygous substitution (c.1813G>C).

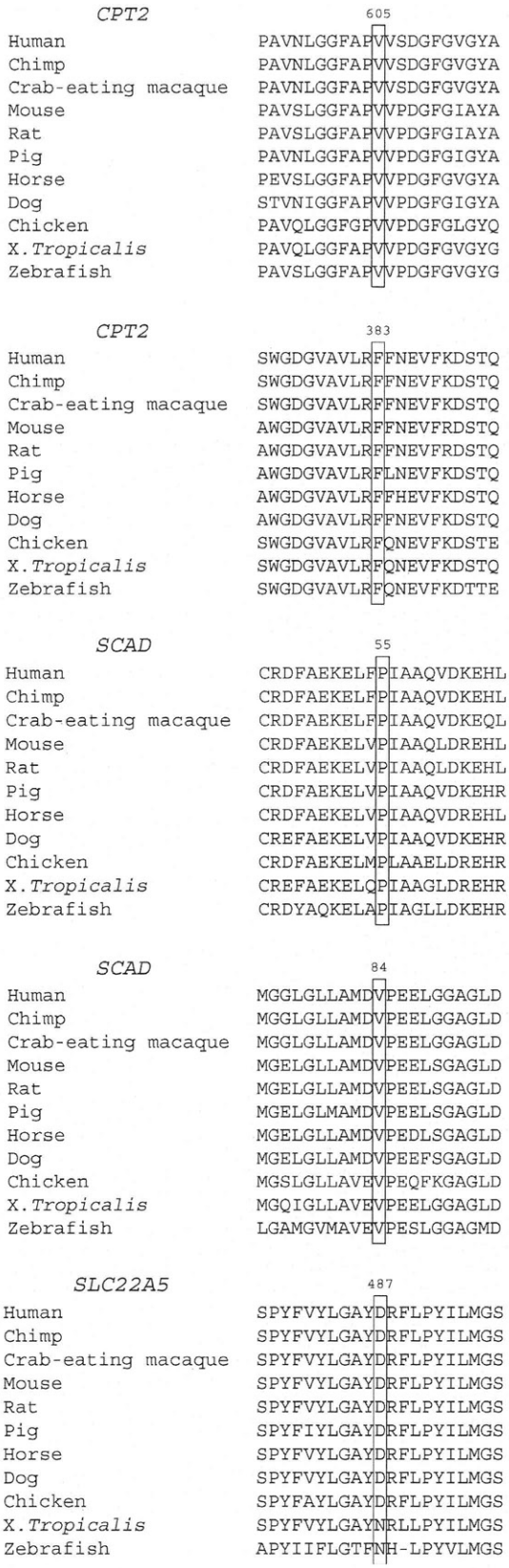

Fig. 3. Sequence alignment between species. Except for *SLC22A5*-D487N, the substitutions were highly conserved between the species.

Table 5
Acylcarnitine profile in Case 15.

	C0	C2	C5	C16	C18	C18:1	C18:2
Case 15	181.24	4.65	1.51	4.13	2.3	4.39	0.97
PRV	422.59	147.13	1.5	3.495	2.495	3.095	0.925
NR	60.0	45.0	1.0	7.0	2.1	3.2	0.8

PRV; postmortem reference value, NR; normal range.

(<422 μM). C5-acylcarnitine was increased to 1.51 μM (normal range: <1.0 μM, postmortem reference value: <1.5 μM). There was also an increase in the C16, C18, C18:1 and C18:2-acylcarnitine (Table 5). The ratio of [C16 + C18:1] to C2 was as high as 1.83, which suggests that Case 15 had either a CPT II or carnitine–acylcarnitine translocase deficiency.

5. Discussion

After many research studies and case reports examined postmortem samples [2–4,23–28], in 2001, Chace et al. performed a large-scale study of FAODs in which they analyzed postmortem blood samples by tandem mass spectrometry [5]. In our previous study, we also performed postmortem acylcarnitine analyses, which subsequently led to our discovery of other FAODs cases [7]. Due to postmortem changes, the interpretation of the postmortem acylcarnitine analysis is sometimes not easy. Table 6 shows our in-house data for our postmortem acylcarnitine analysis. Based on our analytical findings, it was possible to definitively diagnose a CPT II deficiency case, which we reported in our previously published study [7]. In the two false-positive cases that we observed, we found there were increases in the long-chain acylcarnitine in the absence of any genetic abnormality. The C16, C18, C18:1 and C18:2-acylcarnitine values in the present case were much lower than that of not only our definitively diagnosed case, but also the two false-positive cases.

Definite diagnoses of FAODs can be made by either a genetic or an enzyme analysis. The relationship between SUDI and FAODs was first discussed in detail in the 1990's [24,29,30]. After Bennett and Powell reported finding SUDI cases with an A985G mutation of the medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, they recommended genetic analysis for SUDI be performed [2]. Unfortunately, this study only targeted one particular substitution and did not cover any of the other candidate genes. However, it should be noted that the number of candidate genes for FAODs is approximately 20 genes and thus, postmortem genetic screening is not really practical.

Recently, NGS has been developed and is more widely used, especially in the field of sudden cardiac death [12,13]. This technique makes it possible to examine a larger number of genes and exons at a lower cost, in addition to requiring a smaller amount of time compared to the conventional Sanger's method [13]. Unlike that for clinical patients, there is a lack of information and no definitive clinical symptoms for sudden death cases. Thus, there are numerous candidate genes that could be responsible for sudden death cases.

In our current study, we used NGS to perform target exon sequencing, in addition to conducting postmortem comprehensive genetic screening for FAODs. As far as we know, this is the first study to examine metabolic autopsy performed with NGS.

In most of the cases, the sequence covered more than 90% of the targeted exons. False-positives and false-negatives were rare. After excluding the variants that have been reported elsewhere, there were six substitutions and deletions remaining. Because the majority of the FAODs are inherited in an autosomal recessive manner, we chose the one case that had at least two pairs of the heterozygous variant (Case

Table 6
The comparison with other postmortem samples.

	C0	C2	C5	C16	C18	C18:1	C18:2
Case 15	181.24	4.65	1.51	4.13	2.3	4.39	0.97
DD	69.46	8.5	0.13	7.41	5.29	6.79	1.64
FP 1	147.82	96.45	4.29	13.65	6.74	8.99	1.23
FP 2	127.4	35.25	1.97	6.13	3.74	3.87	1.55

DD, definitively diagnosed case, FP; false-positive case.

15). This patient had a compound heterozygous deletion (c.968_969 del TC, p.F323fs) and substitution (c.1813G>C, p.V605L) in the *CPT2* gene. Sanger's method was used to confirm these variants. The former variant was inherited from her father and caused a frameshift, thereby resulting in an immature protein. The latter variant was inherited from her mother and has been previously found and reported in a Japanese CPT II deficiency patient [21]. SIFT and PolyPhen-2 classified the function of the protein as damaging. Our analyses additionally showed that the amino acid was conserved between the species examined. Thus, when taken together, these genetic analyses demonstrated that this patient had a CPT II deficiency.

There were four cases with other variants, each of which had a heterozygous substitution. The substitution in Case 5 was *ACADS*-P55L (c.164C>T), in Case 6 was *CPT2*-F383Y (c.1148T>A), in Case 14 was *ACADS*-V84M (c.259G>A) and in Case 7 was *SLC22A5*-D487N (c.1459G>A). Because the substitution of *CPT2*-F383Y has also been shown to cause decreases in the CPT II activity and been reported in a CPT II deficiency patient [8,19–21,31], even in the heterozygous F383Y mutation state [21,32,33], the heterozygous *CPT2*-F383Y mutation could be a cause of sudden death. While the postmortem acylcarnitine analysis in our current case did not suggest any CPT II deficiency, it is possible that Case 6 might have been affected by the mutation.

The substitutions of *ACADS* are controversial. *ACADS*-P55L was detected in 8 alleles among the 8654 East Asian control alleles (minor allele frequency: 0.0009, The Exome Aggregation Consortium) and in 3 alleles out of the 1346 Japanese control alleles that were examined (minor allele frequency: 0.002, Human Genetic Variation Browser). *ACADS*-V84M was neither detected in the 6496 East Asian control alleles (The Exome Aggregation Consortium) nor in the 600 Japanese control alleles (Human Genetic Variation Browser). The SIFT scores for these two *ACADS* substitutions were classified as damaging, with both substitutions conserved between species. It has been reported that *ACADS*-P55L decreases the enzyme activity of SCAD [34]. Although the final diagnoses in Case 14 and Case 5 were acute encephalopathy and respiratory infection, respectively, it cannot be denied that these substitutions could have potentially affected the enzyme activity to a greater or lesser extent. Thus, the possibility exists that individuals with rare variants are susceptible to environmental stress. However, MCAD can compensate for the SCAD enzyme deficiency because of the overlap in the substrate specificity [35] and the substitution may not have a major effect to the death. Further accumulation of the genetic data will be necessary in order to link these rare variants and SUDI.

Even though *SLC22A5*-D487N was not detected in the 121,410 control alleles (The Exome Aggregation Consortium), it was detected in 1 allele out of the 600 Japanese control alleles (minor allele frequency: 0.002, Human Genetic Variation Browser). The SIFT score was classified as tolerated while the PolyPhen-2 was classified as benign. Since amino acid position 487 is asparagine in *X. tropicalis* and zebrafish, this substitution might not affect the activity of this gene.

In the current study, our use of NGS led to finding a case of compound heterozygote CPT II deficiency. Furthermore, we additionally found a case of heterozygote CPT II deficiency-related gene variant and two cases of SCAD deficiency-related variants. It is true that the cost of NGS is still higher than postmortem blood acylcarnitine analysis, but these variants would not have been detected without the use of NGS. Thus, metabolic autopsy performed with NGS is a useful method, especially when postmortem blood acylcarnitine analysis is not available. These findings suggest that metabolic autopsy should be performed in all cases of sudden death.

Acknowledgments

We would like to thank Norimasa Kageyama, Fumi Kuriiwa, Yuko Moriyama and Yutaka Araki for their technical advice. This study was supported in part by a grant from the Ministry of Health, Labour and Welfare of Japan, and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (25860490).

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and drawbacks of both treatment strategies, we performed PAB as the initial surgery, and intended to perform the VSD closure and TAPVC repair concomitantly as the subsequent surgery.

Stenting of the ductus venosus, which has been reported to be effective in alleviating PVO in low-birthweight infants with infracardiac TAPVC,[8] was another treatment option in the present case. We assumed, however, that the adjustment of pulmonary blood flow by PAB would have priority over the relief of PVO in this patient, who had a left-to-right shunt lesion and did not have clinically apparent PVO during the early neonatal period.

Anatomic repair for ccTGA is now considered preferable to conventional repair, in which systemic right ventricular dysfunction could cause long-term mortality and morbidity.[9] In the present patient, however, performing TAPVC repair and anatomic repair for ccTGA simultaneously was considered highly invasive and technically demanding. As a result, the patient has a systemic right ventricle with a non-systemic ventricular paced rhythm, which puts her at high risk of future heart failure.[10] The patient should be meticulously followed up for signs of systemic right ventricular failure.

Acknowledgment

The authors state no conflicts of interest.

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Combination of flecainide and propranolol for congenital junctional ectopic tachycardia

Tomohiko Imamura, Yuji Tanaka, Yumiko Ninomiya and Masao Yoshinaga

Department of Pediatrics, National Hospital Organization Kagoshima Medical Center, Kagoshima, Japan

Abstract Congenital junctional ectopic tachycardia is a rare tachyarrhythmia with high mortality. A pharmacological approach in early infancy is regarded as the first-line therapeutic option. Pharmacologically, amiodarone alone or in combination with other drugs is the most commonly reported effective agent for congenital junctional ectopic tachycardia, but it has many adverse effects. Here we report the case of a 40-day-old infant. The clinical course suggests that combined oral flecainide and propranolol is an effective alternative therapy for early infants. Esophageal lead electrocardiography may give a clear diagnosis of junctional ectopic tachycardia.

Key words amiodarone, congenital junctional ectopic tachycardia, esophageal lead, propranolol, tachyarrhythmia.

Correspondence: Yuji Tanaka, MD PhD, Department of Pediatrics, National Hospital Organization Kagoshima Medical Center, Shiroyama-cho 8-1, Kagoshima 892-0853, Japan. Email: yutanaka@kagomc2.hosp.go.jp

Received 19 May 2014; revised 10 October 2014; accepted 10 November 2014.
doi: 10.1111/ped.12573

The congenital form of junctional ectopic tachycardia (JET) usually occurs in the first 6 months of life and can present as incessant, sustained, or sporadic forms.[1] It is believed to result from abnormal automaticity at, or close to, the His bundle, and may accelerate or decelerate in response to autonomic tone.[2] Electrocardiography (ECG) typically shows a narrow QRS

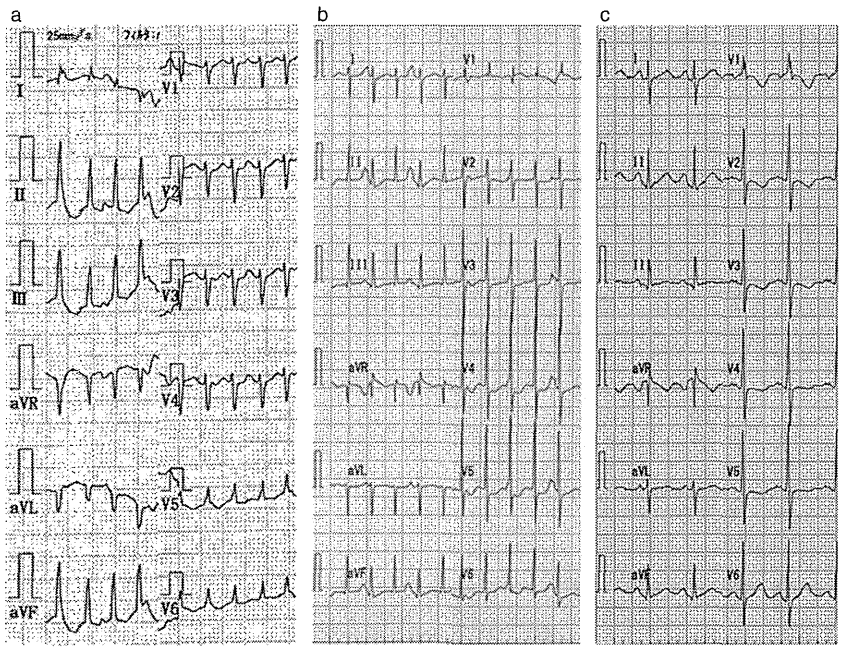

Fig. 1 Twelve-lead electrocardiogram at (a) 1 month medical check (29th day), (b) first visit to hospital (40th day), and (c) outpatient clinic (72nd day). QRS morphology during JET is the same as sinus capture QRS morphology.

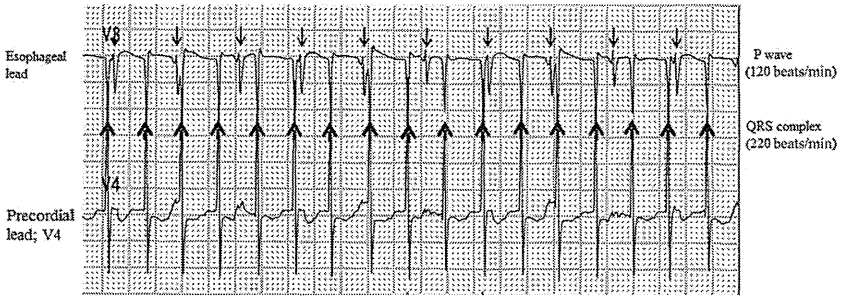

Fig. 2 Esophageal lead electrocardiogram (ECG): 12-lead ECG was recorded with the precordial lead V3 at the esophagus, showing clear P waves and atrioventricular dissociation.

complex with variable RR intervals, and either atrioventricular dissociation or, less commonly, 1:1 ventriculoatrial (VA) conduction.[2] Mortality from the congenital form of JET ranges from 35% (9/26 infants)[3] in an early report to 4% (4/94 infants) in a relatively recent report.[1] All four deaths in the latter report occurred at age ≤6 months, indicating that JET in early infancy is accompanied by refractoriness to treatment and high mortality.

Case report

A 40-day-old infant was referred to hospital with tachycardia. The tachycardia was observed at 1 month medical check on the 29th day of life, on 12-lead ECG, which showed a wide QRS tachyarrhythmia (Fig. 1). At the first visit to hospital at 40 days of age, however, 12-lead ECG including an esophageal lead (Fig. 2) showed narrow QRS tachycardia with a P rate of 120 beats/min and a QRS rate of 220 beats/min. He was diagnosed with JET. Echocardiography showed an anatomically normal heart except for a small patent foramen ovale; left ventricular ejection fraction was 60%. I.v. ATP twice and digoxin failed to stop the tachyarrhythmia; furthermore, the QRS wave did not change at all after injection, suggesting that it was not of supraventricular origin. Combination of oral propranolol and flecainide gradually decreased the heart rate to around 120 beats/min on the third day of admission. Atrioventricular (AV) dissociation remained and premature beats with wide QRS configuration appeared on 24 h Holter ECG on the fifth day. Mexiletine was added instead of flecainide to exclude the possibility of tachyarrhythmia of ventricular origin. The heart rate again increased to as high as 155 beats/min (Fig. 3). The combination of propranolol (1.9 mg/kg/day) and flecainide (4.6 mg/kg/day) was re-started. He was discharged from hospital with rate control. Holter ECG 1 week after discharge showed sinus rhythm with infrequent short runs of JET and AV dissociation. Thereafter, JET recurred a few times during outpatient clinics, possibly due to a shortage of the drug in his system because of weight gain. Spontaneous resolution of JET can be expected up to the age of approximately 3 years.[1] Therefore we now plan to continue combined propranolol and flecainide during infancy, on the expectation of spontaneous resolution. If the tachyarrhythmia does not resolve, radiofrequency catheter ablation or catheter cryoablation will need to be considered.