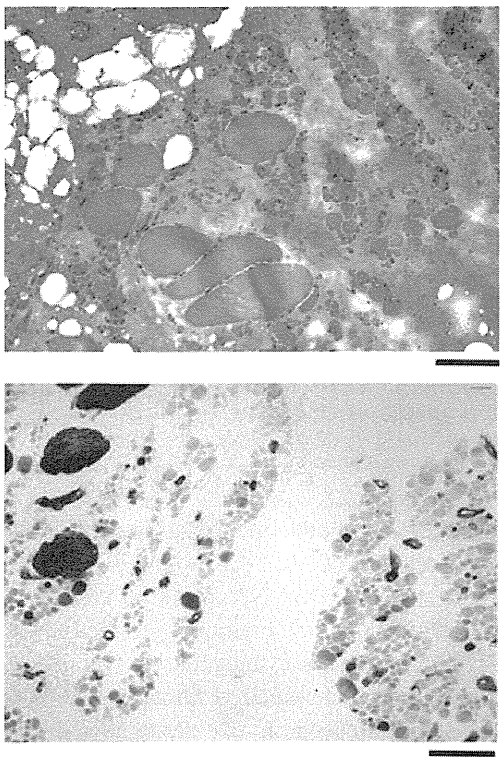

Fig. 2 Histological study. Hematoxylin–eosin staining (*upper*) and ATPase staining (pH=4.6, *lower*) of the quadriceps femoris muscle of patient II-1. The *scale bar* indicates 100 μm in length

SeattleSeq Annotation website (http://gvs.gs.washington.edu/SeattleSeqAnnotation/).

Priority scheme and capillary sequencing

We adopted a prioritization scheme used in recent studies to identify the pathogenic mutation [5–7]. Called variants found by each informatics method filtered into unregistered variants (excluding registered dbSNP131 and 1,000 genomes), overlapping variants called in common by NextGENe and MAQ, nonsynonymous changes (NS), splice site mutations (±2 bp from the exon–intron junctions) (SS), small insertions or deletions (indels), and overlapping variants called in II-1 and II-2 were checked, and variants found in our 33 in-house exomes derived from 19 healthy individuals and 14 individuals with unrelated diseases were excluded (Table 1). An online human genome mutation database (HGMD, https://portal.biobase-international.com/hgmd/pro/start.php) was referred to as a reference for disease-causing mutations. The variants were confirmed as true positives by Sanger sequencing of polymerase chain reaction products amplified using genomic DNA as a template. Sanger sequencing was performed on an ABI3500XL or ABI3100 autosequencer (Life Technologies, Carlsbad, CA). Sequencing data were analyzed using Sequencer software (Gene Codes Corporation, Ann Arbor, MI).

A total of 177 Japanese control samples (354 alleles) were check by high-resolution melting analysis using a LightCycler 480 (Roche Diagnostics, Otsu, Japan) to see the variant frequency. The reaction was performed in 10 μl containing 10 ng of genomic DNA, 0.2 mM dNTPs,

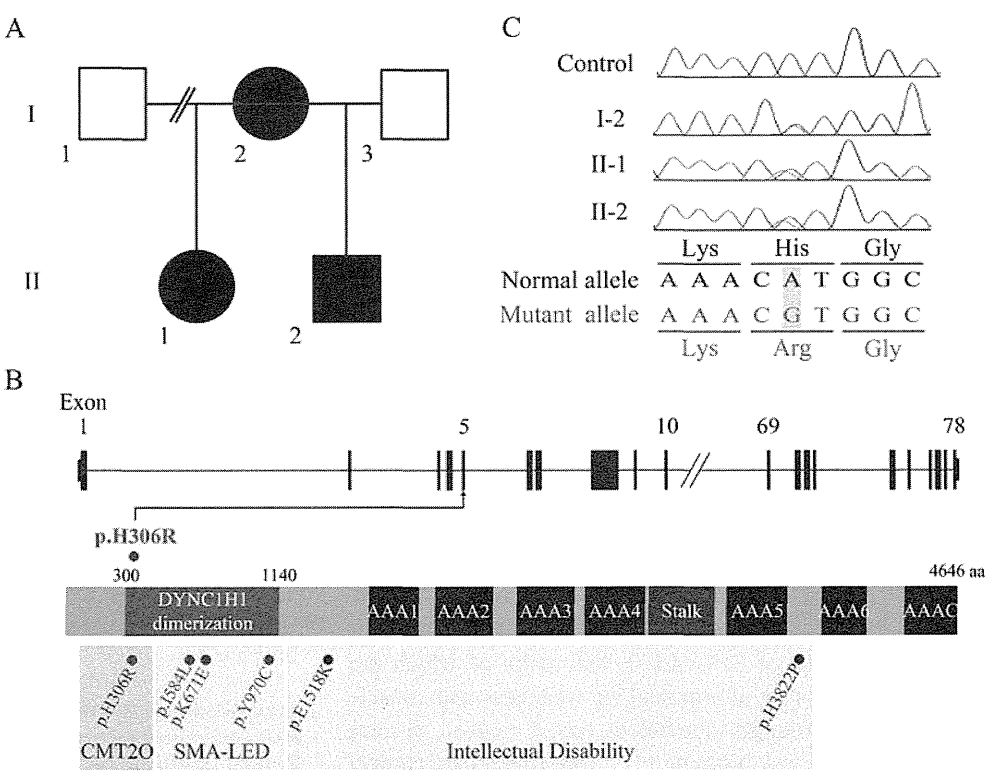

Fig. 3 Genetic study. **a** Familial pedigree. **b** The gene structure of *DYNC1H1* with the mutation [p.H306R (c.917A>G)] (*upper*), the protein structure with functional domains (*middle*), and reported mutations corresponding to respective diseases (*lower*). *AAA* ATPase domains (AAA 1 to 6), *AAAC* unrelated seventh domain. **c** Sequences of a control and the family members are displayed. Heterozygous mutations are observed in patients I-2, II-1, and II-2

Table 1 Variant priority scheme of exome sequencing data

	Half sister		Half brother	
	NextGENe	MAQ (SeattleSeq)	NextGENe	MAQ (SeattleSeq)
Total variants called	73,966	174,757	73,370	163,707
Autosomal+chr X	71,522	174,165	70,068	163,013
Unknown SNP variants (dbSNP, 1,000 genomes)	11,132	21,284	10,857	19,858
Overlap of NextGENe and MAQ	1,598		1,482	
NS/SS/I	426		411	
Overlapping in half sibs	135			
Unknown variants (in-house database)	62			

MAQs were annotated using SeattleSeq annotation. The annotation includes gene names, dbSNP rs IDs, and SNP functions (e.g., missense), protein positions and amino acid changes. *NS* nonsynonymous, *SS* splice site (±2 bp), *I* indels

0.125 U of ExTaq (Takara Bio, Inc., Otsu, Japan), 1× buffer, and 1.5 μM SYTO9 (Invitrogen, Carlsbad, CA).

Results

Approximately 9.8 and 9.5 Gb of sequence data were generated for II-1 and II-2, respectively. This approach resulted in more than 85.8 % (II-1) and 86.5 % (II-2) of the target regions being covered by ten reads or more. Two informatics methods identified 62 potentially pathogenic changes (Table 1). We found a missense mutation [p.H306R (c.917A>G)] in *DYNC1H1* from among 62 variants using the HGMD as a reference; this mutation has been reported as a causative mutation for CMT disease [2]. The heterozygous missense mutation was confirmed in I-2, II-1, and II-2 (Fig. 3b). This missense mutation was not found in 177 control samples.

Discussion

The identical *DYNC1H1* mutation (p.H306R) found in a large pedigree with axonal type of CMT disease was detected by exome sequencing in a family with a unique form of quadriceps-dominant neurogenic muscular atrophy [2]. Three members of the family demonstrated very similar clinical features, which were distinct from CMT disease. The most striking feature was a unique distribution of muscle involvement. The quadriceps femoris muscle was almost selectively involved in the early course of the disease, and the proximal lower limb was predominantly involved throughout the disease course. Recently, three other missense mutations were detected in families with SMA-LED. Clinical features of the current family are essentially consistent with those of SMA-LED, hallmarks of which are early childhood onset of proximal leg weakness with muscle atrophy and nonlength-dependent motor neuron disease without sensory involvement [3]. Nonprogressive clinical course despite early childhood onset as in our family should be another hallmark of SMA-LED. These cumulative data clearly indicate that *DYNC1H1* plays an essential role in maintenance of spinal motor neurons and their axon.

Thus far, four missense mutations (p.H306R, p.I584L, p.K671E, p.Y970C) identified in human cases of CMT or SMA-LED are located in the same tail domain for DYNC1H1 dimerization. It is of note that three missense mutations (p.F580Y, p.G1042A, p.T1057C) found in mouse models are also located in the tail domain [8–10]. These mice involve not only spinal motor neurons but also sensory and cortical neurons. The tail domain is thought to be essential for dimerization of dynein heavy chains, and thus, missense mutations in the tail domain may disrupt function of dynein complex formation in a dominant negative manner. Two distinct de novo mutations (p.E1518K, p.H3822P) identified in patients with severe intellectual disability and variable neuronal migration defects were located outside of the tail domain. These patients also showed possible peripheral nerve involvement, but formal neurophysiological investigation was not available. Since mice with *Dync1h1* abnormality show broad central nervous system involvement, *DNYC1H1* is likely to cause a wide range of neuronal migration disorders.

CMT disease with the p.H306R mutation has been designated as CMT2O (OMIM 614228). Most members of the pedigree with p.H306R reported by Weedon et al. demonstrated distal dominant muscle weakness, while one patient showed proximal lower limb-dominant muscle atrophy as in our family [2]. Therefore, the same missense mutation in the tail domain could cause CMT2O phenotype and SMA-LED phenotype even within the same pedigree. It is hard to explain the underlying mechanism of pleotropic effects of the mutation. Further studies are absolutely necessary to elucidate phenotype–genotype correlation and pleiotropic mutational consequences.

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RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Tissue-Limited Ring Chromosome 18 Mosaicism as a Cause of Pitt–Hopkins Syndrome

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TO THE EDITOR:

We wish to congratulate the authors on their detailed review of Pitt–Hopkins syndrome (PTHS, [OMIM 610954]) published in a recent issue of this journal [Marangi et al., 2011]. PTHS represents a rare disorder with characteristic facial gestalt, episodic hyperventilation, and severe developmental delay with absent speech [Pitt and Hopkins, 1978]. Haploinsufficiency of transcription factor 4 (TCF4) gene at chromosome 18q21.2 is responsible for PTHS [Brockschmidt et al., 2007; Zweier et al., 2007]. The comprehensive review by Marangi et al. illustrated that TCF4 point mutations, balanced translocations spanning the TCF4 locus, and even very large 18q deletions can result in the distinctive PTHS phenotype as long as the TCF4 locus is deleted. Here, we wish to demonstrate that ring (18) mosaicism represents yet another mechanism leading to the classic PTHS phenotype.

The propositus was born to unrelated Japanese parents. Her family history was non-contributory, with one older sister who is developing normally. The propositus was born at 40 and 1/7 weeks gestation via normal spontaneous vaginal delivery. Her birth weight was 2,805 g, and her head circumference was 32.3 cm. Soon after birth, she exhibited frequent episodes of projective vomiting caused by severe gastroesophageal reflux. Subsequently, she developed recurrent aspiration pneumonia requiring multiple hospital admissions. A magnetic resonance imaging of the brain at the age of 23 months revealed "delayed myelination" consistent with an age of 15 months but no other major structural abnormalities. A G-band chromosome analysis performed at that time was reportedly normal. She was first presented to us at the age of 3 years because of severe developmental delays. Her weight was 9.8 kg (−2.1 SD), height 83.8 cm (−2.1 SD), and head circumference 44.4 cm (−3.2 SD). Upon examination, she had a happy disposition with unexpected laughing, clapping of her hands and absent speech, microcephaly, global hypotonia, scoliosis, a short neck and syndactyly, and bilateral single palmar creases. Her finger pads were not prominent. She had characteristic facial features, that is, midfacial hypoplasia, a short philtrum, separated incisors, fleshy ears, downward slanting eyes, a pointed chin, macroglossia, and a prominent lower lip (Fig. 1). There was no apparent brachycephaly (Fig. 1). The serum IgA level was within the normal range, that is, 95 mg/dl [age reference 60–354 mg/dl]. She started to have generalized seizures at the age of 3 years. She is currently 11 years old and is unable to sit without support or to communicate verbally. She has been severely constipated requiring a daily enema. She exhibits episodic apnea-hyperpnea, often provoked by emotional excitement.

A FISH analysis using a BAC probe spanning the TCF4 locus (RP11-1079G18) was performed using a buccal swab specimen, and a mosaic deletion in 97.7% of the cells was revealed (Fig. 2a). An extensive chromosomal G-band analysis of a peripheral blood sample showed 5 ring (18) cells out of the 110 cells that were studied, yielding an average prevalence of one abnormal copy in 22 cells (Fig. 2b). An array comparative genomic hybridization (CGH)

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FIG. 1. Facial appearance of the propositus. Note midfacial hypoplasia, a short philtrum, separated incisors, fleshy ears, downward slanting eyes, a pointed chin, macroglossia, and a prominent lower lip.

analysis of her peripheral blood that was performed concurrently with the FISH analysis revealed a 29.12-Mb deletion from position 46,962,563 to 76,083,258 in 18q21.2-q23 and a 1.5-Mb deletion from position 108,560 to 1,617,028 in 18p11.32. The mosaic ratio estimated from an array CGH analysis was 26% according to the method described by Cheung et al. [2007].

The patient reported herein suggests that a ring (18) mosaicism can cause PTHS. A mosaic ring chromosome has not been recognized as a cause of PTHS, although there have been two PTHS patients due to mosaic deletion of TCF4 [Giurgea et al., 2008; Stavropoulos et al., 2010]. It was difficult to ascertain whether PTHS has been overlooked in patients with ring (18) mosaicism, since cytogenetic confirmation of TCF4 involvement has never performed in reported patients with ring (18) mosaicism [Lo-Castro et al., 2011].

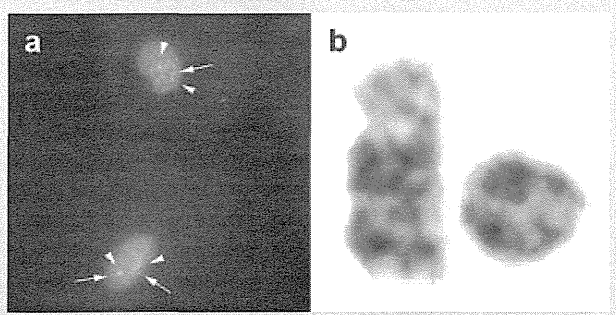

FIG. 2. Cytogenetic analyses of the propositus. a: A interphase FISH analysis of buccal swab specimen using TCF 4 probe (arrows, red) and CEP 18 probe (arrowheads, green) demonstrated 87 cells with one TCF4 probe (top) and two cells with two TCF4 probes (bottom) out of 89 cells that were studied. b: A G-band analysis of a peripheral blood sample revealed presence of ring chromosome 18 (right) in addition to normal chromosome 18 (left), consistent with ring (18) mosaicism.

In retrospect, reaching a correct diagnosis in the propositus was not a straightforward process: a pointed chin in the presence of autism, epilepsy, and a happy disposition with unexpected laughing was suggestive of Angelman syndrome [MIM 105830], whereas hand clapping in the presence of autism and epilepsy was reminiscent of Rett syndrome [MIM 312750], especially in females. Indeed, we had to perform a round of methylation studies and a UBE3A mutation analysis for Angelman syndrome as well as MECP2 mutation analysis before reaching a correct diagnosis. Because PTHS is gradually gaining recognition as differential diagnosis of Angelman syndrome and Rett syndrome among pediatric neurologists, we did perform a FISH for TCF4 on peripheral blood cells, but the test was interpreted as negative apparently because of the extremely low level of mosaicism of the ring (18). Her diagnosis would have been easily missed without performing a FISH using a buccal mucosa sample or an array CGH.

Indeed, the large discrepancy in the level of mosaicism in two aspects of the propositus hindered the diagnosis: first, there was a discrepancy between the mosaic ratio in the peripheral blood obtained from a G-band analysis (5/110) and that estimated from an array CGH analysis (26%). This discrepancy reflects the preferential selection of normal cells (46, XX cells) during the PHA stimulation of T cells required in the G-banding analysis. The array CGH analysis, which does not depend on the PHA stimulation process, is not subject to such an artifactual bias in the assessment of the mosaic rate [Ballif et al., 2006]. Not unexpectedly, in retrospect, the initial G-band analysis failed to detect the presence of cells with ring (18) chromosomes. Second, there was a discrepancy between the mosaic ratio in the peripheral blood evaluated using the array CGH analysis and that obtained from an interphase FISH study using the buccal smear. Since buccal smear FISH is not subject to the selection bias discussed above, the difference in the mosaic rate likely reflects a true difference in the level of mosaicism among tissues (i.e., peripheral blood vs. buccal cells). It is not clear whether the high percentage of abnormal cells in the buccal smear represents the situation in her central nervous system. Given her profound neurological disability, we suspect that her brain tissue may contain a very high percentage of abnormal cells. The situation is quite comparable to Pallister–Killian syndrome (tetrasomy 12p mosaicism), in which a diagnosis is dependent on a FISH study of non-blood tissues [Manasse et al., 2000] or an array CGH study of the blood [Theisen et al., 2009].

Clearly, the haploinsufficiency of deleted genes on chromosome 18q other than TCF4 has contributed to the phenotype of the propositus. The propositus exhibited macroglossia and delayed myelination. Macroglossia has been described in patients with Beckwith–Wiedemann like phenotype and 18q deletion [Brewer et al., 1998; Lirussi et al., 2007]. However, the propositus did not have other features of Beckwith–Wiedemann syndrome, such as overgrowth. The initial brain MRI of the propositus reported delayed myelination, which could be attributable to the deletion of myelin basic protein (MBP, OMIM #159430) located in 18q23 [Popko et al., 1987]. Although severe mental retardation with autistic features can be seen both in 18q deletion syndrome and in PTHS, episodic hyperventilation observed in the propositus is a distinctive feature of PTHS [Ouvrier, 2008]. On the other hand,

some commonly observed features of PTHS were not apparent. The propositus did not have characteristic facial features such as a pointed nasal tip, flaring nostrils, or brachycephaly, which are known features of PTHS [Zweier et al., 2007]. Moreover, Lehalle et al. [2011] reported that several individuals with PTHS have prominent finger pads, which were not apparent in the propositus. The absence of these features might be a consequence of the haploinsufficiency of genes other than TCF4.

In summary, an extensive and thorough investigation of the TCF4 locus, including that on a mosaic ring (18), should be performed in patients with a high clinical suspicion of PTHS.

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Case report

Late-onset Leigh syndrome with myoclonic epilepsy with ragged-red fibers

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Abstract

We report the case of a boy with myoclonic epilepsy with ragged-red fibers (MERRF) who had astatic seizures since 2 years of age and later developed ataxia, absence seizures, and myoclonus. Almost homoplasmic A8344G mutation of mitochondrial DNA (m.8344A>G mutation) was detected in lymphocytes. He developed late-onset Leigh syndrome (LS) when he contracted pneumonia at 6 years. He developed bulbar palsy and deep coma. MRI demonstrated lesions in the brainstem, basal ganglia, and cerebral cortex. Three similar cases have been reported; two carried the almost-homoplasmic m.8344A>G mutation in muscle tissue. These suggested that almost homoplastic m.8344A>G mutation developed clinical phenotype of MERRF in the early stage and late-onset Leigh syndrome in the late course of the disease.

Keywords: Leigh syndrome; MERRF; m.8344A>G

1. Introduction

Slowly progressive myoclonic epilepsy, ataxia, and myopathy are the main clinical features of myoclonic epilepsy with ragged-red fibers (MERRF) (OMIM #545000) [1]. MERRF onset varies from childhood to adulthood, after normal early development. The A → G mutation at nucleotide 8344 of mitochondrial DNA (m.8344A>G mutation) accounts for 80–90% of MERRF cases [2]. Biochemically, enzyme complexes of the respiratory chain, mainly NADH-CoQ reductase (complex I) and cytochrome c oxidase (complex IV), are deficient [3].

Leigh syndrome (LS; OMIM #256000) is a rapidly progressive neurodegenerative disorder characterized by necrotizing changes in the basal ganglia and brainstem. Psychomotor retardation, seizures, nystagmus, ophthalmoplegia, optic atrophy, ataxia, dystonia, and respiratory failure are the main clinical features [4]. Most patients developed LS until 2 years of life and diseased in several days to months after the onset. LS has a heterogeneous genetic background, and mitochondrial and nuclear genes coding respiratory chain complexes or the pyruvate dehydrogenase complex are responsible for this disease [5].

The m.8344A>G mutation may rarely be a cause for LS [6]. The development of LS in a patient with the MERRF phenotype is very rare. To our knowledge, only three cases have been reported [1,2,7]. We report the case of a boy diagnosed with LS at 6 years who showed the MERRF phenotype from 2 years of age.

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2. Case report (Fig. 1)

The patient was born at term by normal delivery from nonconsanguineous parents. There was no family history of neurological disorders. Developmental milestones were normal until 1 year of age, when he could walk on his own. At 2 years, he developed astatic seizures well-controlled by valproic acid (VPA). At 3 years, he developed cerebellar ataxia with dysarthria and wide-based gait. At 4 years, he presented with atypical absence and myoclonic seizures, well-controlled with ethosuximide and clonazepam in addition to VPA.

Laboratory data at 5 years were as follows: Blood gas analysis (in vein revealed the following: pH, 7.405; pCO_2, 41.0 mmHg; pO_2, 87.7 mmHg; HCO_3^- 19.1 mmol/l; and base excess, −4.7 mmol/l. Lactate and pyruvate levels in serum were elevated to 33.2 mg/dl and 1.89 mg/dl, respectively. In the cerebrospinal fluid (CSF), lactate and pyruvate levels were 22.3 mg/dl and 1.33 mg/dl, respectively. Ictal EEG during astatic seizures at 2 years showed bilateral occipital-dominant, 3–4 Hz diffuse spike and wave complexes (Fig. 2). Visual evoked potentials (VEPs) demonstrated high amplitude. Somatosensory evoked potentials were normal. MRI revealed cerebral and cerebellar cortex atrophy. Molecular genetic analysis examined the A → G transition at position 8344 in the $tRNA^{Lys}$ gene of mtDNA. The mtDNA mutation in the investigated lymphocytes were demonstrated by the method of Yoneda et al. [8] (Fig. 4). PCR products were digested by Nae 1. The RELP analysis of PCR products generated from wild type and mutant mtDNAs. The mutation band was evaluated by measuring scanned photographs in NIH image J software (available at http://rsb.info.nih.gov/nihimage/Default.html) to determine the relative intensity. The wild type mutation was not detected in the bands, which was considered to be almost homoplasmic in this case.

After MERRF diagnosis at 4 years, VPA was discontinued and CoQ_{10} and Vit B1 were administered. At

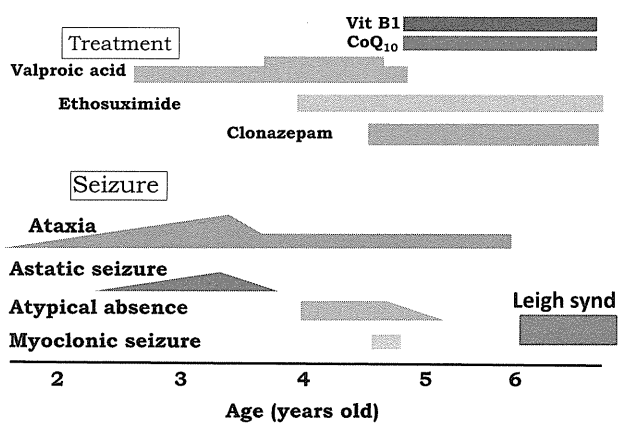

Fig. 1. Clinical course.

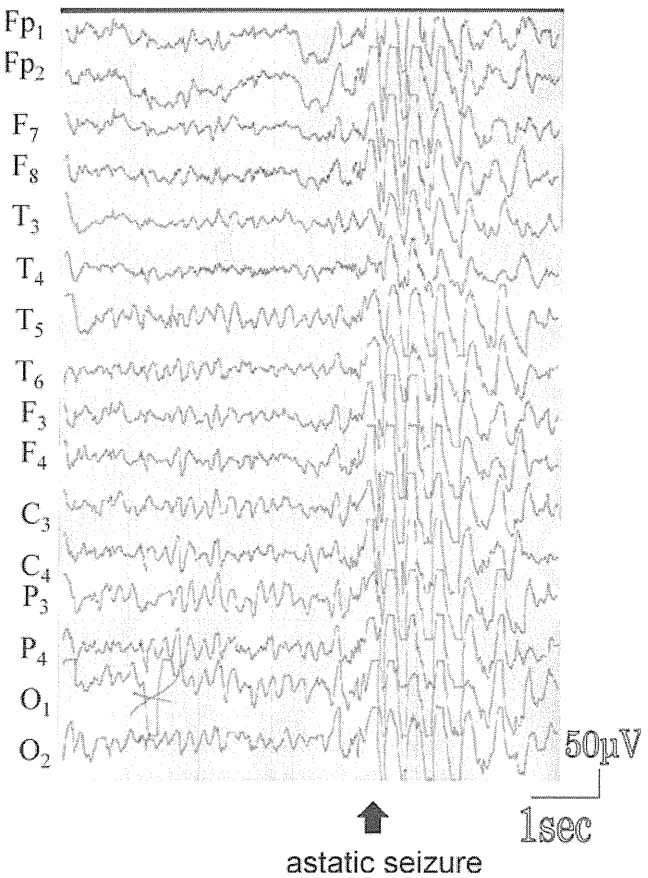

Fig. 2. Ictal EEG in astatic seizure.

6 years, he developed bacterial pneumonia with slight fever (from 98 to 100 °F) for 10 days. He did not have any neurological disturbance at that time. However, he suddenly presented with progressive bulbar palsy and marked generalized hypotonia two days after the body temperature was elevated above 102 °F. Brain T2-weighted MRI demonstrated high-signal bilateral lesions from the pons to the medulla. His consciousness slowly deteriorated, and lesions extending to the thalamus and bilateral cerebral hemispheres were noted on MRI (Fig. 3 (A)–(C)) 4–6 weeks after the initial episode. Within 6 weeks, he went into deep coma without spontaneous movements and was placed on permanent mechanical ventilation.

3. Discussion

Age of onset was 2 years; the male patient had astatic seizures with 3–4 Hz diffuse spike and wave complexes on EEG. The initial differential diagnosis was myoclonic astatic epilepsy, Dravet syndrome and Lennox–Gastaut syndrome. At 4 years, he had absences and myoclonic seizures with elevated lactate and pyruvate levels in serum and CSF and high-amplitude VEPs, and the patient was diagnosed with MERRF with almost

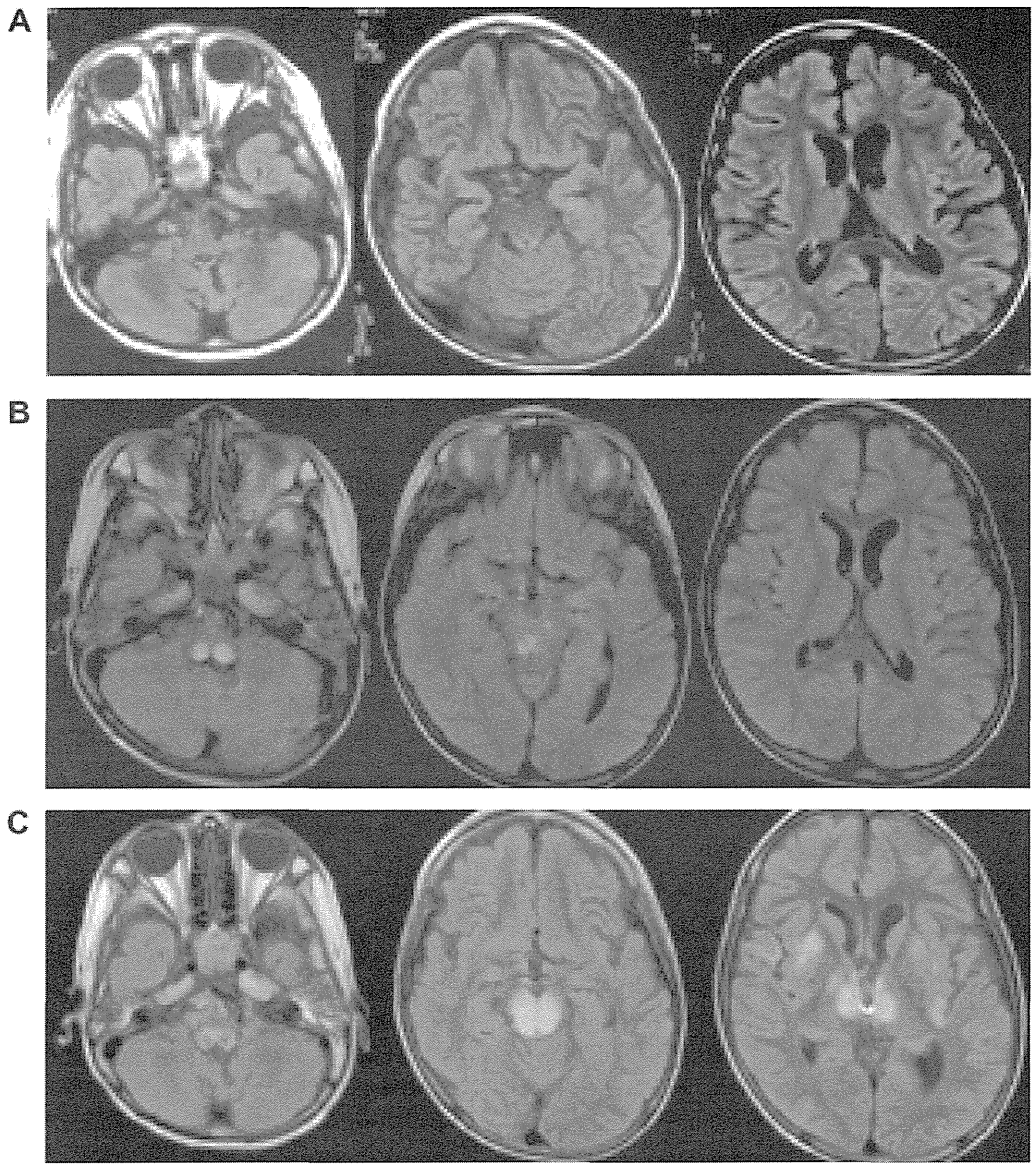

Fig. 3. Brain MRI (FLAIR images) (A) At 4 years of age, when MERRF was diagnosed: diffuse cerebral cortex atrophy. (B) At 6 years of age, when LS was diagnosed: Bilateral, symmetric, high-intensity lesions are seen in the brainstem from the pons to medulla. (C) Six weeks after diagnosis: the lesions extend to the thalamus and bilateral cerebral hemispheres.

homoplastic m.8344A>G mutation in lymphocytes. At 6 years, he rapidly developed LS after pneumonia was developed with high grade fever.

MERRF with LS and m.8344A>G mutation is very rare; only three cases have been reported [1,2,7]. Sweeney et al. [1] reported MERRF with ataxia and myoclonic epilepsy in a 7-year-old boy who developed LS at 18 years when he contracted bacterial pneumonia. The m.8344A>G mutation rates were detected in the lymphocytes (81%), skeletal muscle (99%), cerebellum (97%), cerebral cortex (97%), cardiac muscle (97%), liver (99%), and kidney (98%). Silvestri et al. [2] reported that only two of 150 cases with m.8344A>G mutation developed LS. High mutation rates were detected in muscle (100%) and lymphocytes (93%), but detailed clinical information was not written. Berkovic et al. [7] reported the case of a 4-year-old boy diagnosed with MERRF who died at 9 years, after contracting measles and pneumonia. Brain autopsy demonstrated LS but the mtDNA mutation rate was not investigated.

Including our patient, three of four reported cases developed MERRF before 7 years of age and LS 5–11 years later. Therefore, early-onset MERRF patients should be closely monitored for LS symptoms.

Factors affecting the LS progression have not been clearly described, but the mechanism is considered to be the compromised oxidative phosphorylation (OXPHOS) function due to mutations in nuclear or

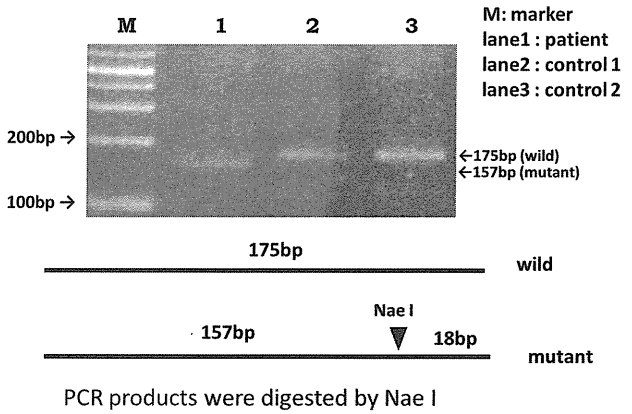

Fig. 4. Restriction fragment length polymorphism (RFLP) analysis for the A → G transition at position 8344. Mutated PCR products were digested by Nae 1. A 175-bp band corresponds to the wild-type sequence; 175- and 18-bp bands correspond to the Nae 1 digested fragments derived from the mutated mtDNA sequence. The patient demonstrated homoplasmic mutation.

mitochondrial genes encoding respiratory chain components or their assembly factors. In addition, sudden clinical and metabolic deterioration can happen with additional stress, such as fever, resulting in the pathological picture of LS [7]. Our case also suggested the affecting factor was high fever with pneumonia. Two of the three cases also described the development of LS after pneumonia was diagnosed, though these cases did not describe the febrile status or special events in infection.

The relationship between the percentage of mtDNA mutation rate and the severity of phenotype has been controversial. Piccolo et al. [9] reported that both severe MERRF phenotype and unaffected cases showed a high percentage of m.8344A>G. On the other hand, Tatuch et al. [10] reported that the high rate of LS m.8933T>G was related with the risk of LS. The mutation present in the ATP 6 gene was considered to cause failure of ATP synthesis. Including our case, the three cases reported that mtDNA mutation homoplasmy was detected in lymphocytes or muscle. The result suggests that the m.8344A>G mutation was potentially indicative of a broad expressive spectrum, and mtDNA homoplasmy or high mutation of heteroplasmy in lymphocytes or muscle may be a risk factor for LS. However, the matter of small sample size needs further investigation.

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Metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy: Diagnosis of mitochondrial respiratory chain disorders

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ABSTRACT

Mitochondrial respiratory chain disorders are the most common disorders among inherited metabolic disorders. However, there are few published reports regarding the relationship between mitochondrial respiratory chain disorders and sudden unexpected death in infancy. In the present study, we performed metabolic autopsy in 13 Japanese cases of sudden unexpected death in infancy. We performed fat staining of liver and postmortem acylcarnitine analysis. In addition, we analyzed mitochondrial respiratory chain enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. In heart, 11 cases of complex I activity met the major criteria and one case of complex I activity met the minor criteria. In liver, three cases of complex I activity met the major criteria and four cases of complex I activity met the minor criteria. However, these specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. In cultured fibroblasts, only one case of complex I activity met the major criteria and one case of complex I activity met the minor criteria. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. These cases might not have been identified without postmortem cultured fibroblasts. In conclusion, we detected one probable case and one possible case of mitochondrial respiratory chain disorders among 13 Japanese cases of sudden unexpected death in infancy. Mitochondrial respiratory chain disorders are one of the important inherited metabolic disorders causing sudden unexpected death in infancy. We advocate metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy cases.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as sudden unexpected death occurring before 12 months of age. If SUDI remains unexplained after thorough investigations, it is classified as sudden infant death syndrome (SIDS). The more common causes of SUDI are infection, cardiovascular anomaly, child abuse, and metabolic disorders. However, the many potential inherited metabolic disorders are more difficult to diagnose at autopsy as compared to cardiovascular defects and serious infection. Inherited metabolic disorders may, therefore, be underdiagnosed as a cause of SUDI or misdiagnosed as SIDS. Fatty acid oxidation disorders (FAODs) are one type of the inherited metabolic disorders and may cause as much as 5% of SUDI cases after thorough investigations including metabolic autopsy [1–5]. In a review of SUDI cases with respect to potential FAODs, we found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we performed fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis, advocating the importance of metabolic autopsy in SUDI cases.

Mitochondrial respiratory chain (MRC) disorders were first identified in 1962 [7]. MRC disorders have a frequency of about at least 1:5000 newborns and are the most common disorders among inherited metabolic disorders [8]. However, there are few published reports regarding the relationship between MRC disorders and SUDI. Studies of MRC disorders have not progressed because of technical difficulties or variability in clinical manifestations [9]. In sudden death cases especially, clinical features are unclear and postmortem changes complicate molecular analysis.

In the present study, we performed metabolic autopsy in 13 Japanese cases of SUDI in order to determine whether MRC disorders could be detected or not. We performed fat staining of liver and postmortem

Abbreviations: CS, citrate synthetase; FAODs, fatty acid oxidation disorders; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; SIDS, sudden infant death syndrome; SUDI, sudden unexpected death in infancy.

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acylcarnitine analysis according to the previous methods. In addition, we analyzed MRC enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. With such metabolic autopsy, we were able to detect one probable case and one possible case of MRC disorders. These cases might not have been identified without metabolic autopsy. MRC disorders are important diseases causing SUDI and metabolic autopsy might be helpful for forensic scientists and pediatricians to diagnose MRC disorders that might not otherwise be identified.

2. Materials and methods

2.1. Subjects

Between October 2009 and September 2011, forensic autopsy was performed on 588 cases at our institute, 22 of whom were under 12 months of age. Following macroscopic examination, nine cases could be diagnosed but 13 cases (Table 1) did not have any characteristic appearance and remained undiagnosed. In this study, we reviewed these 13 undiagnosed cases (8 males, 5 females) with age ranging from 1 to 10 months.

2.2. Autopsy

Autopsies were performed within 24 h following death. Blood was obtained from the femoral vein. Heart and liver specimens were immediately cut and frozen at −80 °C. Dermis, which was cut and sterilized, was cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 1% penicillin streptomycin glutamine, and 2.5% amphotericin B (Life Technologies, Indianapolis, IN). Once cultures were established, fibroblasts were frozen at −80 °C.

2.3. Sudan III staining

Liver samples preserved in 4% phosphate-buffered formaldehyde solution were frozen, cut into 10-μm sections, and stained by the Sudan III method for fat staining.

2.4. Postmortem blood acylcarnitine analysis by tandem mass spectrometry

Whole blood samples obtained at autopsy were blotted onto one spot on Guthrie cards. They were subjected to acylcarnitine analysis by tandem mass spectrometry and compared with the previously determined normal range [6].

Table 1
SUDI cases.

Case no.	Age/sex	Height/weight (cm/kg)	Circumstances	Fever	Remarks
1	4 mo/M	68/7.5	Sleeping	−	
2	10 mo/F	70/8.8	Sleeping	−	Sister: undiagnosed encephalitis
3	10 mo/F	71/7.7	Sleeping	+	Cesarean section
4	9 mo/M	67/7.5	Sleeping	−	
5	4 mo/M	60/5.7	Sleeping	−	Hydrocephalia
6	6 mo/M	68/8.0	Sleeping	−	
7	1 mo/F	51/3.6	Sleeping	−	Twins, preterm birth
8	10 mo/M	72/9.9	Sleeping	−	Developmental disease (right side of the body paralysis)
9	6 mo/F	64/8.9	Sleeping	−	Bronchitis
10	4 mo/M	65/7.4	Sleeping	−	Cesarean section
11	1 mo/M	58/4.8	Sleeping	−	
12	5 mo/M	59/4.2	Sleeping	−	Preterm birth
13	2 mo/F	53/3.9	Sleeping	−	Low-birth-weight infant

Abbreviations: F, female; M, male; mo, month; SUDI, sudden unexpected death in infancy.

2.5. Enzyme analysis

The activity of mitochondrial respiratory chain complexes I, II, III, and IV was assayed in the crude post-600-g supernatant of heart and liver, and in isolated mitochondria from skin fibroblasts as described previously [10]. The activity of each complex was presented as a percent ratio relative to the mean value [9]. The activity of complexes I, II, III, and IV was also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker or complex II activity [10].

2.6. Ethics

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

3. Results

3.1. Microscopic examination

One of the common features in diagnosing MRC disorders is hepatic steatosis. We therefore performed Sudan III staining to examine whether vacuoles caused by fatty degeneration were present in hepatocytes. Diffuse microvesicular steatosis was detected in case 5 (Fig. 1A). No Sudan III-positive vacuole was detected in case 13 (Fig. 1B) and the other cases, for example, case 2 (Fig. 1C).

3.2. Postmortem blood acylcarnitine analysis

We performed acylcarnitine analysis by tandem mass spectrometry using whole blood samples. In all samples, data were within the normal range. These data suggested that no case was affected by FAODs (data not shown).

3.3. Enzyme analysis of MRC complexes in heart, liver, and cultured fibroblasts

The enzyme activity of each complex was compared with the CS ratio and complex II ratio. Lower than 20% activity of any complex in a tissue or lower than 30% activity of any complex in a cell line meets the major criteria. Lower than 30% activity of any complex in a tissue or lower than 40% activity of any complex in a cell line meets the minor criteria according to Bernier et al. [11].

In heart, 11 cases of complex I activity met the major criteria of MRC disorders and one case of complex I activity met the minor criteria (Fig. 2A). In liver, three cases of complex I activity met the major criteria of MRC disorders and four cases of complex I activity met the minor criteria (Fig. 2B). In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria of MRC disorders and one case (case 13) of complex I activity met the minor criteria (Fig. 2C, Table 2). The activity of complexes II, III, and IV was maintained in almost all cases.

3.4. Diagnosis

A definite diagnosis is defined as the identification of either two major criteria or one major plus two minor criteria. A probable diagnosis is defined as either one major plus one minor criterion or at least three minor criteria. A possible diagnosis is defined as either a single major criterion or two minor criteria, one of which must be clinical [11].

All the cases had a clinical symptom of sudden death, meeting one minor criterion. In the enzyme activity, eleven cases (cases 2, 4–13) met the major criteria and we could make a probable diagnosis in these 11 cases. The other two cases (cases 1 and 3) met the minor criteria and we could make a possible diagnosis.

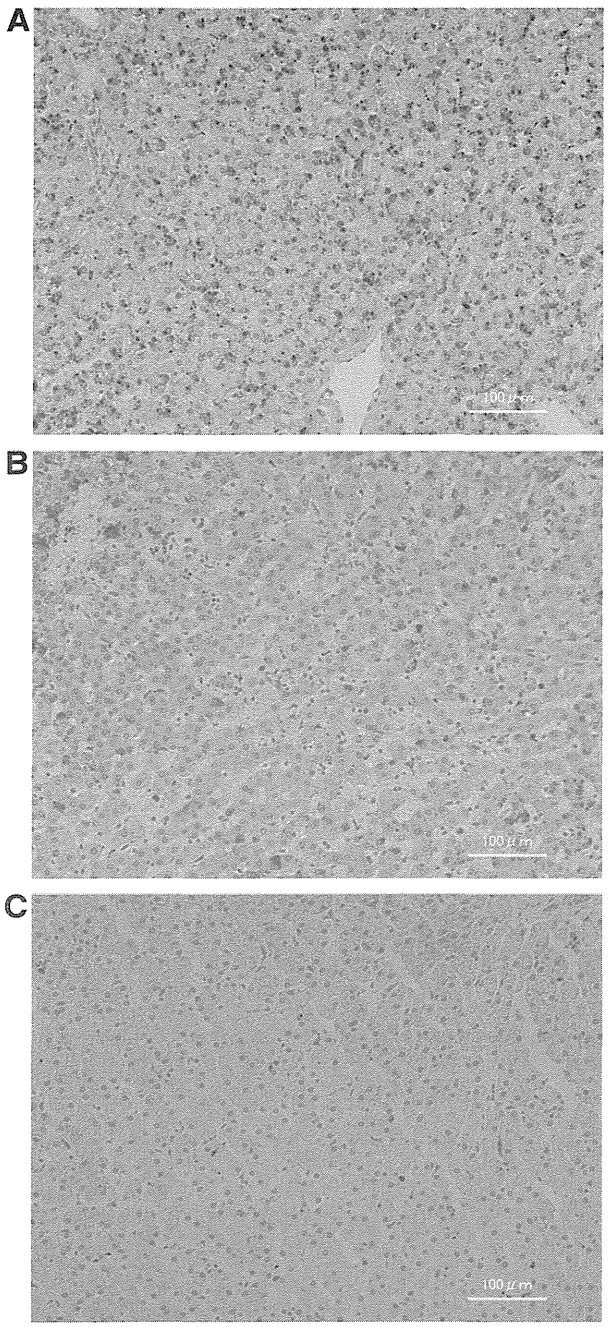

Fig. 1. Microscopic examination of liver (Sudan III staining): (A) case 5, (B) case 13, and (C) case 2. Diffuse microvesicular steatosis was detected in case 5 (A). No Sudan III-positive vacuole was detected in case 13 (B) and the other cases, for example, case 2 (C).

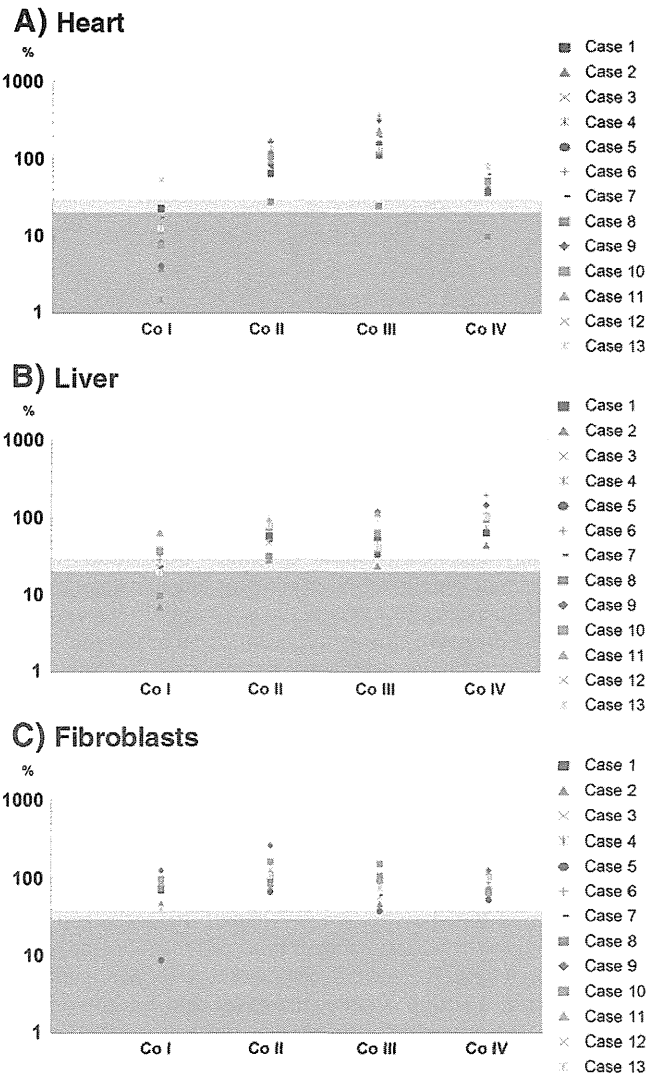

Fig. 2. Enzyme activity of MRC complexes in heart (A), liver (B), and cultured fibroblasts (C). In heart, 11 cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and one case of complex I activity was under 30% of the CS ratio, meeting the minor criteria (A). In liver, three cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and four cases of complex I activity were under 30% of the CS ratio, meeting the minor criteria (B). In cultured fibroblasts, one case (case 5) of complex I activity was under 30% of the CS ratio, meeting the major criteria and one case (case 13) of complex I activity was under 40% of the CS ratio, meeting the minor criteria (C). The activity of complexes II, III, and IV was maintained in almost all cases. The enzyme activity of each complex was compared with the CS ratio. Lower than 20% activity in a tissue or lower than 30% activity in a cell line (dark blue) meets the major criteria. Lower than 30% activity in a tissue or lower than 40% activity in a cell line (light blue) meets the minor criteria.

4. Discussion

Mitochondria are essential organelles that exist in all nucleated mammalian cells. They provide the energy required for normal cell function through oxidative phosphorylation (OXPHOS). OXPHOS includes MRC complexes (complexes I, II, III, and IV) and ATP synthase (complex V) [12], which use reduced coenzymes from the tricarboxylic acid cycle and molecular oxygen, generating cellular energy in the form of ATP [13].

The infantile or early neonatal period demands high energy. Patients with MRC disorders are unable to produce adequate energy, which may thus compromise them in the first days of life or during infancy. MRC disorders affect most organ systems and present variable clinical manifestations from prenatal complications through acute neonatal decompensation and death to adult-onset disorders. Therefore, it is not surprising that MRC disorders are also one of the causes of SUDI. However, there are few reports on a relationship between MRC disorders and SUDI [12,14].

We have previously reviewed SUDI cases with respect to FAODs and found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we advocated the importance of metabolic autopsy [15], including fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis. Using this protocol, most FAODs, some amino acid oxidation disorders, and some organic acid oxidation disorders could be diagnosed.

However, MRC disorders are difficult to diagnose. First, they present variable clinical manifestations and non-specific features such as failure to thrive or hepatic, cardiac, renal, gastrointestinal, endocrine, hematological, or other symptoms [10,16]. Second, although blood

Table 2
Enzyme assay of mitochondrial respiratory chain complexes in cultured fibroblasts.

	Enzyme activity (%)[a]			
	Co I	Co II	Co III	Co IV
Case 5				
CS ratio	9	66	38	53
Co II ratio	13	—	71	58
Case 13				
CS ratio	39	106	76	98
Co II ratio	37	—	73	92

Abbreviations: Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthetase.
[a] Relative to mean CS and Co II of the normal controls.

lactate levels and muscle morphology can be used as a screening test, some confirmed patients were normal [10]. Third, genomic mutational analysis is difficult because MRC complexes are composed of 13 subunits encoded by mitochondrial DNA and over 70 subunits encoded by nuclear genes. In addition, nuclear genes are related to many assembly factors, membrane dynamics, nucleotide transport synthesis, and mitochondrial DNA replication and expression. Therefore, enzyme analysis still remains the most significant diagnostic tool. A definite diagnosis thus requires enzyme analysis [8].

In the present study, we performed enzyme analysis in frozen heart, frozen liver, and cultured fibroblasts. Eleven cases were supposed to be a probable diagnosis and two cases were supposed to be a possible diagnosis. However, it seemed unlikely that such a high proportion would have real MRC disorders. Did we have to take the effect of postmortem changes into consideration?

For forensic autopsy, organ specimens are often preserved in formaldehyde solution and sometimes frozen. These specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. Based on the previous report that artifactual loss of complex II activity in autopsy samples preceded that of complex I and the data that complex II activity in the present study was maintained, this low complex I activity might be decreased before death. However, postmortem changes cannot be completely ruled out and this low complex I activity may not therefore be consistent with premortem activity.

We therefore analyzed activity in cultured fibroblasts. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria and one case (case 13) of complex I activity met the minor criteria. In case 5, complex I activity was distinctively decreased. Sudan III staining of the case revealed hepatic steatosis, consistent with Reye-like syndrome. Reye-like syndrome is one of the characteristic features of MRC disorders [9]. We could therefore make a probable diagnosis (case 5) and a possible diagnosis (case 13) from metabolic autopsy with postmortem cultured fibroblasts.

Case 5 had hydrocephalia and case 13 was a low-birth-weight infant. However, neither was severe. Macroscopic examination did not reveal any abnormal appearance and microscopic examination showed no pathological findings except for steatosis. These cases might not have been identified without postmortem cultured fibroblasts. As with such cases, some MRC disorders reveal no clinical manifestation and no pathological characteristic. We believe it is important to perform metabolic autopsy with postmortem cultured fibroblasts when encountering SUDI cases.

We emphasized the advantage of metabolic autopsy with cultured fibroblasts. First, despite lacking obvious preceding symptoms, MRC disorders could be diagnosed. Second, cultured cells are the only method to retrieve premortem information from the deceased. Third, even frozen samples are affected by postmortem changes and may lead to a false positive diagnosis. However, we have to discuss the disadvantage. MRC disorders showed tissue specificity and the activity of cultured fibroblasts represent normal in some cases. Some of the low complex I activity in heart or liver could represent premortem MRC disorders despite normal activity in cultured fibroblasts. Thus, other molecular investigations may well be added to enzyme analysis. Recently, systematic gene analysis using next-generation sequencing has been reported for the diagnosis of patients with MRC disorders [17]. Further investigations are thus needed.

In conclusion, we detected one probable case and one possible case of MRC disorders among 13 Japanese cases of SUDI. MRC disorders are one of the important inherited metabolic disorders causing SUDI. We advocate metabolic autopsy with postmortem cultured fibroblasts in SUDI cases.

Acknowledgments

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Impact of enzyme activity assay on indication in liver transplantation for ornithine transcarbamylase deficiency

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ABSTRACT

There are no objective and concrete guidelines for the management of Ornithine transcarbamylase deficiency (OTCD). Based on previous findings, we hypothesized that patients with OTCD have a low Ornithine transcarbamylase (OTC) activity in the liver, and therefore it would be better to determine the appropriate indications and optimal timing for liver transplantation (LT) based on the OTC activity. However, few data have so far been accumulated on the OTC activity in cases that are indicated for LT. The purpose of the present study was to clarify the OTC activity in cases that were indicated for LT. This study involved thirteen children with OTCD (8 males and 5 females) who underwent LT, and two females with OTCD who did not require LT. The OTC activity of the neonatal onset type ranged from 0% to 7.2%, while that of the late onset type who underwent LT ranged from 4.4% to 18.7%. The OTC activity of the late onset type which did not require LT was 33–38% based on a preoperative needle liver biopsy. Some late onset patients that underwent LT, showed an activity that was as low as that observed in the neonatal onset cases. This is the first report to show the results of measuring the OTC activity for serial OTCD cases indicated for LT. OTC activity might be an indicator to determine the indications for and the timing of LT in the late onset type, however, further investigations are necessary.

1. Introduction

Ornithine transcarbamylase deficiency (OTCD) is the most common urea cycle disorder seen in approximately one in every 40,000 to 80,000 births. OTCD is an X chromosome-linked disorder. Patients with OTCD have low Ornithine transcarbamylase (OTC) activity in the liver and thus experience repeated hyperammonemic episodes, leading to disorders of the central nervous system. It is not rare for impairment to be seen after such central nervous system disorders. This disease is occasionally fatal [1,2]. If OTCD is treated medically, many cases require dietary restrictions and oral medications for the rest of their lives. In recent years, liver transplantation (LT) began to be adopted as a radical treatment for OTCD, yielding favorable outcomes [3-10].

Based on previous findings, we hypothesized that patients with OTCD have low OTC activity in the liver, and therefore it would be better to determine the indications for and timing of LT in cases of OTCD based on the OTC activity. However, few data have so far been accumulated on OTC activity in cases that indicated for LT and no report has so far evaluated the clinical findings. Establishing the relationship between the OTC activity and long-term prognosis could provide a more objective and concrete treatment. In addition, it might increase patient survival without neurological impairment. The purpose of the present study was to clarify the OTC activity in cases that are indicated for LT.

2. Patients and methods

Between May 2001 and November 2010, 190 children underwent LDLT at our facility. Fourteen patients were indicated for LDLT because of OTCD, and 13 of the 14 patients who had their OTC activity measured were retrospectively analyzed in this study. Their postoperative follow-up period ranged from 7 to 72 months (median: 26 months). In addition, 2 patients who did not require LT were also retrospectively analyzed.

Abbreviations: OTCD, Ornithine transcarbamylase deficiency; OTC, Ornithine transcarbamylase; LT, liver transplantation; LDLT, living donor liver transplantation; HPS, hemophagocytic syndrome.

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We reviewed these 13 cases (Table 1) to collect the following data: gender, age at onset, peak plasma ammonium level (peak NH3), mutation type of OTCD, pre-transplant clinical status, age at LDLT, body weight at LDLT, donor, and the postoperative outcome of LDLT. Mutation type was classified into inherited mutations and sporadic mutations. We performed a mutation analysis of the OTC gene in all recipients and their mothers. We classified the mutations as inherited when there was a common gene mutation in both the mother and the child. Conversely, when only the recipient had the mutation, we classified it as being sporadic. The pre-transplant clinical status, including the severity of disease (DS), metabolic status (MS) and neurological status (NS), was assessed by accepted grading scales essentially following Morioka et al.'s [3] study. The grading scale for severity of disease was as follows: Grade 4, many episodes of severe hyperammonemia coma, some with NH3 > 300 μmol/l; 3, one to several episodes of hyperammonemic coma, no more than one with NH3 > 300 μmol/l; 2, one to few episodes of hyperammonemic coma, none with NH3 > 300 μmol/l; 1, only one episode of hyperammonemic coma, with NH3 < 300 μmol/l; 0, no episodes of hyperammonemic coma, and no NH3 > 100 μmol/l. The grading scale for metabolic status was: 4, no improvement, severe hyperammonemia, need for constant, full doses of medication; 3, some improvement moderate hyperammonemia, need for constant medication; 2, major improvement, mild hyperammonemia, need for some medication for control; 1, almost complete correction, occasional mild hyperammonemia, with or without need for medication; 0, complete correction, no hyperammonemia, no need for medication. The grading scale for neurological status is as follows: grade 5, persistent coma or vegetative state; grade 4, responds to noxious stimuli, but no social interaction, no ambulation, no communication; grade 3, limited social interaction, no bipedal ambulation, limited communication through gestures; grade 2, definite social interaction, fair ambulation though possibly limited by spasticity, some use of language; grade 1, good social interaction, full ambulation but possible partially impaired gross and fine motor skills, use of language, mildly delayed development, only modest learning deficits; grade 0, seems to be within the normal spectrum for social interaction, motor skills, language development and learning.

The operative procedures and immunosuppression therapy of our institution have been described elsewhere [10].

2.1. Enzymatic assay

The OTC activity was measured in the OTCD liver tissue that had been removed at LT. Moreover, the OTC activity of graft liver tissue that had been taken immediately before abdominal wall closure was measured as a normal control. The liver tissue was gathered from the right and left lobe of the removed OTCD liver, and the specimen was frozen −80 °C and used for the assay. Similarly, the activity in the liver tissue of transplanted graft was measured as a control. In cases which did not require LT, the OTC activity was measured in the two liver specimens that had been obtained during a preoperative needle biopsy.

A fixed colorimetric quantity of citrulline was extracted and calculated using the crude extract of frozen livers. The DAMO/Antipyrine method [11] was employed, with some modifications to the original method by Brown and Cohen [12]. Cary300 (Varian company) was used for the absorption spectrometer. Each liver tissue specimen was measured twice. The OTC activity of the OTCD liver is the average of four measurements and those of the graft liver is the average of two measurements.

The enzyme assay was done by the same two technical experts, so the quality of the assay was consistently stable. In addition, more than three samples of normal control liver were included in every assay to confirm the stability of each assay. Our data showed that the sensitivity was 94.3% and the selectivity was 96.3%.

2.2. Statistical analysis

The difference between two groups was determined using Mann-Whitney's *U* test. The $P<0.05$ was considered to be significant throughout the study. All calculations were performed using StatView (SAS Institute, Cary, NC).

3. Results

Table 1 shows a profile of the recipients in this study. This study involved 8 boys and 5 girls with OTCD. The cases with neonatal onset type of OTCD included 6 boys, while the late onset type who underwent LT consisted of 2 boys and all 5 of the examined girls. In the neonatal onset patients, the age at disease onset was 2–8 days, and LT was performed between 9 months and 3 years 4 months of age. In the late onset cases that underwent LT, the age at disease onset was 7 months to 7 years, and LT was performed between 3 years 6 months and 15 years of age. The mean preoperative peak plasma ammonium level of neonatal onset type was 958 μmol/l (range: 212–1632 μmol/l), which was significantly high compared with 324 μmol/l (range: 191–413 μmol/l) of late onset type who underwent LT ($P<0.05$). Before LT, all cases had developed hyperammonemia and medical treatment (oral medications and dietary restrictions) had been administered always. Of the six males who presented in the neonatal period, three (cases 2, 4 and 5) had preoperatively shown neurological impairment

Table 1
Summary of LDLT for OTCD.

Case	Gender	Age at onset	Peak NH3 (μmol/l)	Mutation type	DS/MS/NS	Age at LT	BW at LT (kg)	Donor	Survival outcome
Neonatal onset									
1	M	2 d	212	Inherited	1/3/0	1 y 10 m	10.0	Father	72 m, alive
2	M	2 d	1394	Inherited	3/3/2	1 y 1 m	11.0	Father	63 m, alive
3	M	8 d	901	Inherited	3/3/0	3 y 4 m	16.0	Mother*	35 m, alive
4	M	3 d	1136	Inherited	4/3/2	1 y 7 m	7.8	Father	26 m, alive
5	M	5 d	473	Inherited	3/3/1	1 y 2 m	9.0	Father	15 m, alive
6	M	2 d	1632	Inherited	4/3/0	9 m	8.6	Father	14 m, alive
Late onset									
7	F	3y	234	Inherited	2/3/0	8 y 2 m	22.7	Father	3 m, died of HPS
8	F	3y	191	Sporadic	2/3/0	3 y 10 m	15.0	Mother	47 m, alive
9	F	10 m	400	Sporadic	3/3/0	3 y 7 m	19.0	Mother	34 m, alive
10	F	7y	302	Inherited	3/3/0	11 y 7 m	38.5	Father	34 m, alive
11	F	7 m	320	Sporadic	4/3/0	3 y 6 m	16.0	Mother	21 m, alive
12	M	3y	413	Inherited	3/3/0	5 y 3 m	20.9	Father	l0m, alive
13	M	9 m	411	Sporadic	4/3/0	15 y	49.7	Mother	7 m, alive

DS, severity of the disease; MS, metabolic status; NS, neurological status; HPS, hemophagocytic syndrome.
d, days; m, months; y, years.
* Donor with heterozygotes

Table 2
The OTC activity of the OTCD liver and graft liver.

Case	OTCD liver (%)	Graft liver (%)
Neonatal onset		
1	7.2	123.0
2	0	130.7
3	0	97.2*
4	0	41.7
5	0.7	88.6
6	0.3	106.3
Late onset		
7	18.7	75.0
8	11.8	91.4
9	4.5	79.2
10	10.0	90.7
11	6.5	46.0
12	4.4	89.5
13	6.1	90.3
Average	5.4	88.4

* Donor with heterozygotes.

secondary to hyperammonemic encephalopathy. The donor was the father in 8 cases and the mother in 5 cases. Of the 5 patients with the mother serving as the donor, one (the mother of Case 3) was an asymptomatic carrier having no history of hyperammonemic episodes and whose OTC activity was preserved based on preoperative needle liver biopsy.

The postoperative follow-up period ranged from 7 to 72 months (median: 26 months). The postoperative survival rate was 92.3%, with one patient (Case 7) dying of hemophagocytic syndrome (HPS). No patient developed hyperammonemic attack during the postoperative follow-up period. No case required a resumption of either dietary restriction or oral medications. In the three cases in which a neurological impairment secondary to hyperammonemic encephalopathy had been observed preoperatively, this impairment did not subside after LT. All cases, except for the one patient who died, are currently doing well with a normal graft function. The donors had no re-operation and no mortality and all of the donors are currently doing well without any complications.

Table 2 shows the results of the enzymatic assay in cases where LT was performed. The OTC activity of OTCD patients who needed LT ranged from 0% to 18.7% (mean ± SD: 5.4 ± 5.6%). The OTC activity of the neonatal onset type ranged from 0% to 7.2% (mean ± SD: 1.4 ± 2.9%). There was almost no activity, excluding case 1 that showed 7.2%. The OTC activity of the late onset type in patients who underwent LT ranged from 4.4% to 18.7% (mean ± SD: 8.8 ± 5.1%). The mean OTC activity of the neonatal onset type patients was significantly lower than in those with the late onset type who underwent LT ($P<0.05$). The OTC activity of graft liver except for carrier liver (Case 3) ranged from 41.7% to 130.7% (mean ± SD: 87.7 ± 26.2%). There was a significant difference between each group (Fig. 1).

Table 3 shows a profile of two late onset patients who did not require LT. Their son developed hyperammonemia and was diagnosed with OTCD and was indicated for LT. They were diagnosed to have OTCD during a screening examination for potential donor candidates. They sometimes had headaches and required temporary medication. Their enzyme activities were 33–38% based on a preoperative needle liver biopsy, and thus they were not employed as a donor. The mean OTC activity of the late onset type for patients who did not require LT was higher than that in those with the late onset type who underwent LT (Fig. 1).

Fig. 2 shows that there was a negative correlation between the OTC activities of the livers of OTCD patients and the preoperative peak plasma ammonium level.

4. Discussion

OTCD is clinically classified into two types: neonatal onset and late onset. The neonatal onset type of OTCD develops within several days after birth and advances rapidly thereafter. The disease is severe in an overwhelming majority of cases with this type of OTCD, and many of these patients die within one year despite intensive care. In Japan, the 5-year survival rate is reportedly about 8.8% for patients with this type of OTCD, and moderate to severe neurological impairment are often seen in long-term survivors [13,14].

The late onset type develops with initial symptoms resembling encephalopathy during infancy through adulthood following hyperammonemia triggered by infection, hunger or fatigue. Unless treated immediately, encephalopathy advance, sometimes leading to death. When treated only medically, patients with this type of OTCD are likely to experience repeated cycles of remission and relapse of hyperammonemic episodes and the prognosis is poor, with the 5-year survival rate being 45% [13]. At present, it is desirable to establish a valid strategy for treating OTCD (including radical treatment methods), to improve the survival of patients as well as QOL for patients and their family members.

Regarding the indications and timing of LT, a consensus is being reached over the view that the neonatal onset type of OTCD is absolutely indicated for LT and that early LT should be performed for cases with this type of OTCD [4,8,9]. As Campeau et al. recommended, early transplantation in the first year of age has an excellent outcome [15]. In the neonatal type, it is unlikely that the OTC activity will influence that clinical decision making.

On the other hand, no widely accepted view has yet been accepted regarding the late onset type of OTCD. The indications for and the timing

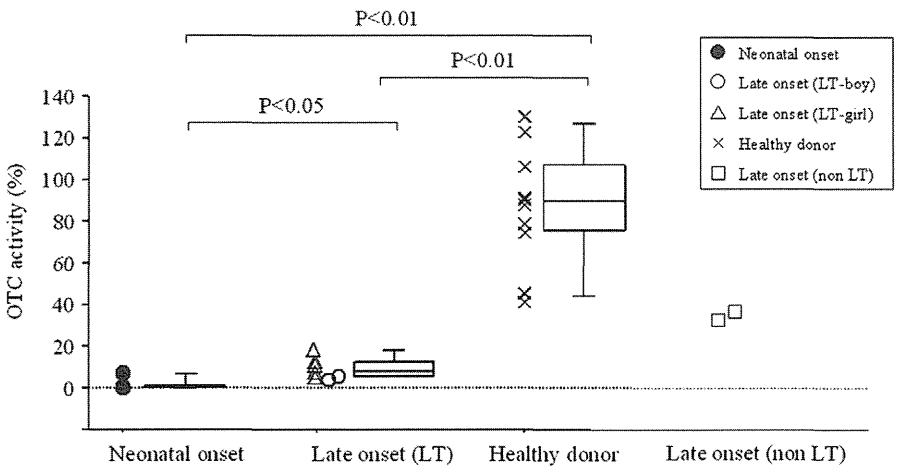

Fig. 1. Comparison of the OTC activity between patients.

Table 3
Profiles of the late onset patients who did not require LT.

Case	Gender	Age at diagnosis	Peak NH3 (μ mol/l)	Mutation type	OTC activity (%)	Medication
14	F	30 y	61	Sporadic	33.8	Temporary
15	F	30 y	41	Sporadic	38.4	Temporary

y, years.

of LT for the late onset type still remain controversial. At present, the indications for and the timing of LT in cases of late onset type OTCD are determined based only on information regarding the clinical symptoms and the course of the disease. In current practice, the indications for and timing of LT in these cases are determined based on a general assessment of the presence/absence of repeated hyperammonemic episodes resistant to medical treatment, presence/absence of marked growth and/or developmental retardation, presence/absence of QOL reduction due to intense dietary restriction, presence/absence of signs of advanced disease revealed by diagnostic imaging (head MRI, etc.), and so on. In our facility, LT in late onset type was determined based on these clinical factors, and not based biochemical or enzymatic factors. It is desirable henceforth to establish more objective and concrete guidelines for the management of this disease to facilitate appropriate determination of the indications for and timing of LT, to reduce the risk of hyperammonemic episodes and improve the survival of patients while avoiding neurological impairment.

In this study, the OTC activity of the late onset patients who underwent LT ranged from 4.4% to 18.7%. The mean OTC activity of the late onset patients who underwent LT was lower than that in those with the late onset who did not require LT. Some patients (Cases 9, 11, 12 and 13) showed an activity that was as low as that observed in neonatal onset cases. Late onset cases with very low OTC activity are thought to comparable risk of repeated hyperammonemic attack to those with the neonatal onset type. It would therefore be better to manage such late onset cases by the same strategy as that used for neonatal onset type cases.

Although the current study is small, the results suggest that the OTC activity might be an objective and concrete indicator to determine the indications for and timing of LT in late onset type of OTCD. Despite the fact that metabolic diseases are a less common indication for LT than other chronic liver diseases in children, further investigations will need to be conducted worldwide to clarify this point.

5. Conclusion

This is the first report to show the results of measuring the OTC activity for serial OTCD cases requiring LT. The OTC activity of the neonatal onset type was very low, and therefore this type is absolutely indicated for early LT. Some late onset patients that underwent LT showed an activity that was as low that observed in neonatal onset cases. The OTC activity might therefore be an objective and concrete indicator to determine the indications for and the timing of LT in late onset type of OTCD. However, further investigations are necessary before any definitive conclusions can be made.

Financial disclosure and conflict of interest

None.

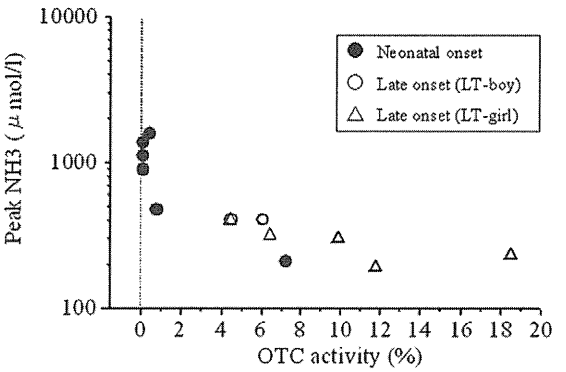

Fig. 2. The correlation between the OTC activities of the OTCD liver and the preoperative peak plasma ammonium level.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ymgme.2011.12.019.

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Original article

Efficacy and tolerability of modified Atkins diet in Japanese children with medication-resistant epilepsy

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Abstract

Ten Japanese patients aged 1.5–17 years with medication-resistant epilepsy were placed on the modified Atkins diet (MAD) for 3 weeks during admission to our hospital. Dietary carbohydrate was restricted to 10 g per day. We studied the efficacy of the diet regarding the seizure frequency and tolerability of the diet at the end of the 3 weeks on the diet. Those who decided to continue the MAD at the time of discharge were followed up in the out-patient clinic to observe the effect of the diet on the seizure frequency. Three of the 10 patients could not continue the diet during the 3-week admission; one had rotavirus enterocolitis and the other 2 disliked the diet. Among the remaining 7 patients who could continue the diet for 3 weeks, 3 achieved the seizure reduction; 2 became seizure-free and 1 showed about 75% reduction in the seizure frequency within 10 days on the diet. All of these 3 patients continued the diet after the 3-week admission. The other 4 patients did not show a reduction of the seizure frequency by the end of the 3 weeks on the diet. Two of them discontinued the diet on discharge. The remaining 2 still continued the diet at home and one became seizure-free 3 months after the start of the diet. In total, 4 of 10 patients achieved >75% reduction in the seizure frequency, although relapse occurred in 2 of the patients, at 5 months and 2 years after seizure reduction, respectively. The MAD was effective and well-tolerated in children with medication-resistant epilepsy in Japan.

Keywords: Modified Atkins diet; Ketogenic diet; Epilepsy; Children; Japan

1. Introduction

The ketogenic diet (KD) has been used worldwide for the treatment of medication-resistant epilepsy. For the implementation of the KD, palatability is a main issue particularly in Japan where the traditional diets contain less fat than the Western diets whereas the KD is fat-dominant [1,2].

The modified Atkins diet (MAD) was developed at Johns Hopkins Hospital as a more palatable and less restrictive dietary treatment for intractable epilepsy than the classical KD. The diet does not impose a restriction of protein or calories, therefore, parents of the patients have to weigh out carbohydrates only [3]. Since the first report of the successful use of the MAD in children with intractable epilepsy by Kossoff and McGrogan [4,5], several reports on the MAD for patients with epilepsy have been published from many countries including South Korea, another of the Asian countries where carbohydrate-rich foods are the main dietary source of energy [6,7]. Ito et al. reported the first successful use of the MAD in Japanese patients with glucose transporter type 1 deficiency syndrome [8].

These suggested that the MAD may be well-tolerated and have a similar therapeutic efficacy in Japanese

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children with medication-resistant epilepsy as well. To investigate the tolerability and efficacy of the MAD, we started a 3-week MAD program with hospitalization for children with medication-resistant epilepsy in 2007. The program consisted of a trial of the MAD therapy and nutritional education by our dietitians to the parents during the 3-week stay in the hospital. Although the MAD is usually initiated in the out-patient clinic in Western countries, we hospitalized the patients for initiating the diet because the parents of the patients would have had difficulty in preparing the MAD menu with no available textbooks or information about the MAD written in Japanese. Three weeks after admission, we let the patients and their parents decide whether to continue or discontinue the diet. If they decided to continue the diet, we gave them dietary support in the outpatient clinic.

2. Methods

2.1. Subjects

From November 2007 until March 2010, we initiated the MAD on admission for children with epilepsy aged under 18 years old who had been treated with at least 3 AEDs and were still having at least 3 seizures per week. Those who were not capable of oral intake of solid foods, for example, milk-feeding infants and tube-feeding children, were excluded.

2.2. Diet protocol

Two dietitians were responsible for all the contacts with the families throughout the hospitalization. Before admission, the dietitians estimated the caloric requirements of each of the patients and identified their favorite foods by interviewing the parents.

The carbohydrate content of the diet was restricted to 10 g per day at the initiation of the MAD, and the total calorie content was adjusted to the routine caloric intake. If the patients suffered from hunger, an increase of the total daily calories in the diet was permitted without exceeding the carbohydrate restriction of 10 g daily. Fibers were included in the 10 g of carbohydrates. No restrictions were imposed with regard to the intake of fluids and protein. No changes in the AED regimen were made during the 3 weeks on the diet.

After the 3 weeks on the diet, we let the parents decide whether or not to continue the diet. We added calcium and water-soluble vitamins for those who continued with the diet therapy after discharge. Carnitine supplementation was undertaken for those who were taking valproate.

2.3. Tolerability and side effects

We assessed the tolerability and side effects of the diet during the 3-week hospitalization. Intolerance was defined as discontinuing the MAD before the end of the 3 weeks. We investigated the side effects of the MAD on the clinical findings and examined the effects of the MAD on the laboratory test findings. Laboratory values were evaluated at the baseline and after 3 weeks on the diet. These included the serum levels of liver enzymes, total cholesterol, low-density lipoprotein (LDL)-cholesterol, blood urea nitrogen, uric acid, and beta hydroxybutyrate. All the samples were obtained 2 h after breakfast. Median data were compared using Wilcoxon's two-sample test.

2.4. Efficacy on seizures

We assessed the efficacy of the diet for reducing the seizure frequency at the end of the 3-week diet program. Those who decided to continue were followed up in the out-patient clinic to observe the longer-term effect of the diet on the seizure frequency.

3. Results

3.1. Patients' profile

We enrolled 10 patients with epilepsy (7 female, 3 male) aged 1–17 years old (median: 4 years). Classification of the type of epilepsy and the underlying diseases in the patients are listed in Table 1. Five patients had symptomatic generalized epilepsy including infantile spasms and Lennox-Gastaut syndrome, and one had Doose syndrome. The remaining 5 had symptomatic localization-related epilepsy. The seizure types were variable, and 6 patients had multiple seizure types. All but 1 of the patients had daily seizures. The median number of past and present AEDs per child was 7.5 (range, 3–11). All the children had some degree of mental retardation, with seven being severely or profoundly retarded.

3.2. Diet compositions

The diet compositions for each patient are shown in Table 2. The mean ketogenic ratio, calculated retrospectively as the weight (gram) of fat divided by that of the carbohydrate plus protein, was in the range of 1.44–2.04 (median: 1.62). As shown in Fig. 1(a) and (b), which are examples of the lunches of pt-6, the dietitians designed Japanese-style menus to suit the palate of the Japanese children.

3.3. Tolerability and side effects

Fig. 2 shows a flow chart of the process of the MAD therapy in all the patients. The diet program could be maintained as scheduled for three weeks in seven of the 10 patients. The 3-week program could not be completed in 3 of the patients (pt-1, 4, and 9). Pt-1 was