TABLE I. Neurological and Molecular Findings of Our Patient and 18 Previously Reported Patients With Microdeletion Encompassing the 2q23.1 Region

			Deleted region						Seizures					
Patient [reference]	Sex	Array	Start	End	Size (Mb)	Deleted genes	OFC (centile)	MR	Severity	Type	Onset	AEDs	EEG	MRI
Our patient	M	Agilent Oligo 1M	148.8	149.8	0.992	*MBD5, EPC2, KIF5C, LYPD6B*	<3rd (−3.6 SD)	Severe	Severe	CPS	10M	CBZ, ZNS, CLB, TPM	$F^{\#1}$	Myelination delay, brain atrophy in $F^{\#1}$ and $T^{\#2}$
1 [Wagenstaller et al., 2007, Patient 27737]	M	Affimetrix SNP 100K	149.0	149.2	0.2	*MBD5*	ND	Severe	Drug resistant	ND	1Y4M	ND	ND	ND
2 [Jaillard et al., 2009, Subject 1]	M	Agilent Oligo 44K	148.8–149.1	149.3–149.4	0.3	*MBD5, EPC2*	<3rd (−3 SD)	Severe	ND	ND	3M	ND	Non-specific	Small cerebellar vermis
3 [van Bon et al., 2010, Patient 8a]	M	Agilent Oligo 244K	148.5	148.9	0.4	*ORC4L, MBD5*	25th	Moderate	—	—	—	—	ND	Normal
4 [van Bon et al., 2010, Patient 8b]	F	Agilent Oligo 244K	148.5	148.9	0.4	*ORC4L, MBD5*	25th	Moderate	ND	ND	10Y	ND	ND	Ventricular asymmetry
5 [van Bon et al., 2010, Patient 7]	M	Agilent Oligo 44K	148.7	149.2	0.5	*MBD5, EPC2*	<3rd	ND	Severe epileptic encephalopathy	GTCS, GTS, AS, Ab	3Y	Multiple	Multiregion	Normal
6 [Williams et al., 2010, Case 1]	F	Agilent Oligo 244K	148.4	149.4	~0.93	*ORC4L, MBD5, EPC2, KIF5C*	<3rd	ND	Well controlled	CPS	8Y	OXC	Mild diffuse encephalopathy' changes	Normal
7 [van Bon et al., 2010, Patient 10]	M	Agilent Oligo 244K	148.1	149.2	1.1	*ACVR2A, ORC4L, MBD5, EPC2*	<3rd	Severe	ND	PS, Secondary GS	Newborn	ND	$F^{\#1}$ and $C^{\#3}$	Focal cortical abnormalities in right $T^{\#2}$
8 [van Bon et al., 2010, Patient 9]	F	Affymetrix SNP 250K	148.8	150	1.2	*MBD5, EPC2, KIF5C, LYPD6B, LYPD6*	10th	Severe	Drug resistant	ND	10M	ND	Right $T^{\#2}$ and $O^{\#5}$	Wide frontal ventricles and myelination delay
9 [Koolen et al., 2004]	F	BAC 3.6K	145.4–146.7	148.7–151.1	2.0	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B*	<3rd (−2 SD)	Severe	ND	GS	12Y	ND	ND	Cortical atrophy
10 [van Bon et al., 2010, Patient 5]	F	Agilent Oligo 244K	148.4	151.1	2.7	*ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3*	50th	Severe	Drug resistant, and died after several seizures	ND	9M	ND	ND	ND
11 [van Bon et al., 2010, Patient 2]	F	Affymetrix SNP 250K	148.7	151.5	2.8	*MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3*	10th	ND	ND	ND	3Y10M	ND	ND	Normal

12 [van Bon et al., 2010, Patient 3]	F	Agilent Oligo 244K	148.1	151	2.9	*ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC*	50th	Moderate	—	—	—	—	ND	Normal
13 [van Bon et al., 2010, Patient 4]	M	HumanCNV370 CNV-SNP 370K	147.2	150.1	2.9	*ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6*	16th	Severe	ND	AS	1Y5M	ND	ND	Thinnng of PCC[#4]
14 [Jaillard et al., 2009, Subject 2]	M	IntegraChips BAC 3K	145.3–146.9	149.3–150.7	2.4–5.4	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC*	Median	Severe	ND	ND	3Y	VPA	Normal	Hypoplasia of F[#1]
15 [Williams et al., 2010, Case 2]	F	Agilent Oligo 244K	146.8	150.3	3.51	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC*	15th	ND	Well controlled	GTCS	8M	VPA	Light F[#1]	Normal
16 [Chung et al., 2011]	F	Agilent Oligo 105K/244K	148.9	152.9	3.986	*MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3, RBM43, NMI, TNFAIP6, RIF1, NEB, ARL5A, CACNB4, STAM2*	<3rd	Moderate, regression at age 6 years	Well controlled	ND	3Y	ND	ND	Normal
17 [van Bon et al., 2010, Patient 6]	M	Cytochip v 3.01 BAC >5K	146.7	151.8	5.2	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3*	<3rd	ND	ND	Ab, Fs	2Y	ND	Normal	Normal
18 [van Bon et al., 2010, Patient 1]	F	Affymetrix SNP 500K	146.6	152.2	5.5	*PABPCP2, ACVR2A, ORC4L, MBD5, EPC2, KIF5C, LYPD6B, LYPD6, MMADHC, RND3, RBM43, NMI, TNFAIP6, RIF1, NEB*	2nd	ND	ND	ND	ND	ND	MD	White matter abnormalities

MR, mental retardation; AEDs, antiepileptic drugs; ND, not described; CPS, complex partial seizure; AS, atonic seizure; Ab, absence seizure; Fs, febrile seizure; GTCS, generalized tonic-clonic seizure; GTS, generalized tonic seizure; GS, generalized seizure; Secondary GS, secondary generalized seizure; PS, partial seizure; CBZ, carbamazepine; ZNS, zonisamide; CLB, clobazam; TPM, topiramate; VPA, valproate; OXC, oxcarbazepine.
$F^{\#1}$, frontal region; $T^{\#2}$, temporal region; $C^{\#3}$, central region; $PCC^{\#4}$, posterior corpus callosum; $O^{\#5}$, occipital region.

repetitive behavior, disturbed sleep pattern, and broad-based gait [van Bon et al., 2010].

There have been no reports reviewing neurodevelopmenal features in 2q23.1 microdeletion syndrome. We here present the detailed clinical features and course of a boy with the syndrome who had severe psychomotor developmental delay and ID, progressive microcephaly, and intractable epilepsy that was improved by multi-drug therapy including topiramate (TPM). High-resolution array CGH demonstrated a 992-kb deletion at 2q23.1–q23.2 involving four genes including *MBD5*, and the breakpoint-junction sequencing revealed microhomology of three nucleotides at the distal and proximal breakpoints, suggesting that the deletion might have been mediated by recently delineated genomic rearrangement mechanism Fork Stalling and Template Switching (FoSTeS)/microhomology-mediated break-induced replication (MMBIR).

CLINICAL REPORT

The patient is the third child, with two healthy brothers, of a healthy non-consanguineous 33-year-old mother and 34-year-old father. He was born at 38 weeks of gestation by spontaneous vaginal delivery. His birth weight was 2,960 g (−0.1 SD), length 48.2 cm (−0.2 SD), and OFC 31.5 cm (−1.0 SD). He has suffered from bronchial asthma since infancy. At the age of 10 months, he was referred to our hospital for afebrile clonic seizures involving alternating sides with impaired consciousness. The patient's seizures tended to occur episodically and in clusters. Although he had one episode of status epilepticus for 60 min, most of his seizures were not prolonged. On presentation, his weight, height, and OFC were 9.1 kg (± 0 SD), 71 cm (−0.7 SD), and 42.5 cm (−2.0 SD, Fig. 1a), respectively. He showed psychomotor delay, with a developmental quotient (DQ) of 57 on the Kinder Infant Development Scale (KIDS) [Cheng et al., 2010]. His craniofacial features included brachycephaly, strabismus, a short nose with anteverted nostrils, a short philtrum, macroglossia, a high palate, a bifid uvula, and a submucous cleft palate. Additionally, he had short and curved 5th fingers, a single transverse crease on the right palm, and bilateral undescended testes. Blood levels of lactate and pyruvate and serum levels of thyroid hormones were within normal ranges. Amino acid and organic acid disorders were excluded. A cardiac ultrasonography showed mild supravalvular pulmonary artery stenosis. An interictal EEG showed sporadic spikes in the frontal region during sleep with normal background activity (Fig. 1b). A brain MRI showed no obvious abnormalities and a normal myelination pattern.

The patient's clinical course of epilepsy is shown in Fig. 1c. Carbamazepine (CBZ) was started at the age of 10 months. Seizure frequency and duration decreased, but he began exhibiting stereotypical characteristics of frontal lobe epilepsy (FLE): motion and speech arrested with mild rigidity of the upper limbs and incomplete loss of consciousness for 40–60 sec. Zonisamide (ZNS) resulted in a slight reduction of seizure frequency and clobazam (CLB) controlled his condition for several months, although the seizures restarted with cluster attacks. At the age of 4 years, TPM altered from ZNS reduced his seizure frequency and intensity, disappeared cluster attacks, and shortened (<30 sec) the durations. An interictal EEG showed many paroxysmal discharges with slow wave in the frontal region (Fig. 1d). A brain MRI at the age of 5 years showed delayed myelination and mild brain atrophy (Fig. 1e).

Microcephaly was evident after age 1 (Fig. 1a). The patient could roll over and sit without support at the age of 9 months, walk independently at 21 months, and jump at 4 years. When last examined at the age of 5 years, he weighed 15.2 kg (−1.0 SD) and had a height of 98.3 cm (−2.0 SD), and OFC of 45.3 cm (−3.4 SD). He could produce several words but no sentences. Although he had no regressive psychomotor changes, his DQ as evaluated by KIDS had dropped to 24 from 46 at age 2.

CYTOGENETIC AND MOLECULAR ANALYSIS

G-banded chromosomal analysis (550 bands level) using the patient's peripheral blood leukocytes showed a normal karyotype (46,XY). Array CGH analysis with Agilent 1M array (Agilent Technologies, Inc., Santa Clara, CA) demonstrated a 992-kb heterozygous deletion at 2q23.1–q23.2 (USCS hg18, Mar. 2006, chromosome 2: 148,830,937–149,823,345 bp) (Fig. 2a). The deletion was confirmed with FISH using probes originated from four BACs (RP11-295N18 at 2q22.3, RP11-375H16 at 2q23.1, RP11-1005D13 at 2q23.2, and RP11-714O10 at 2q23.3): RP11-375H16 and RP11-1005D13 were deleted, whereas RP11-295N18 and RP11-714O10 were present (Fig. 2b). Subsequently, the deletion junction was amplified by a long PCR (Fig. 2c; sequences of the primer set are available on request) and its product was directly sequenced (Fig. 2d). Three nucleotides (CTG) were shared by sequences at the proximal breakpoint and the distal breakpoint in normal chromosome 2 (Fig. 2d). FISH analysis using the four BAC probes (data not shown) and the junction PCR on parental samples (Fig. 2c) showed that the deletion had occurred de novo. Therefore, the karyotype was concluded as 46,XY.arr 2q23.1q23.2-(148,830,937–149,823,345) × 1 dn, and he was diagnosed with 2q23.1 microdeletion syndrome involving four genes: *MBD5*, enhancer of polycomb, drosophila, homolog of 2 (*EPC2*), kinesin family member 5C (*KIF5C*), and LY6/PLAUR domain containing 6B (*LYPD6B*).

DISCUSSION

The patient we have described had severe psychomotor developmental delay and ID with progressive microcephaly and intractable seizures improved by multi-drug therapy including TPM. Roughly 1 Mb region at 2q23.1–q23.2, which involved four genes including *MBD5*, was found to be deleted and both the proximal and the distal breakpoints were demonstrated to share microhomology of three nucleotides.

The neurological and molecular cytogenetic findings of 19 patients with the syndrome, including the present patient, are shown in Table I. Microcephaly (OFC < 3rd centile) was evident in 9 of 18 patients (50%), whereas younger patients with OFC > 3rd centile might exhibit microcephaly thereafter as demonstrated in the present patient. Moderate to severe ID was noted in all patients whose data were available. Seventeen patients (89%) had seizures: generalized seizures in five patients, partial seizures in three, and seizures of unspecified nature in nine. Median age of seizure onset

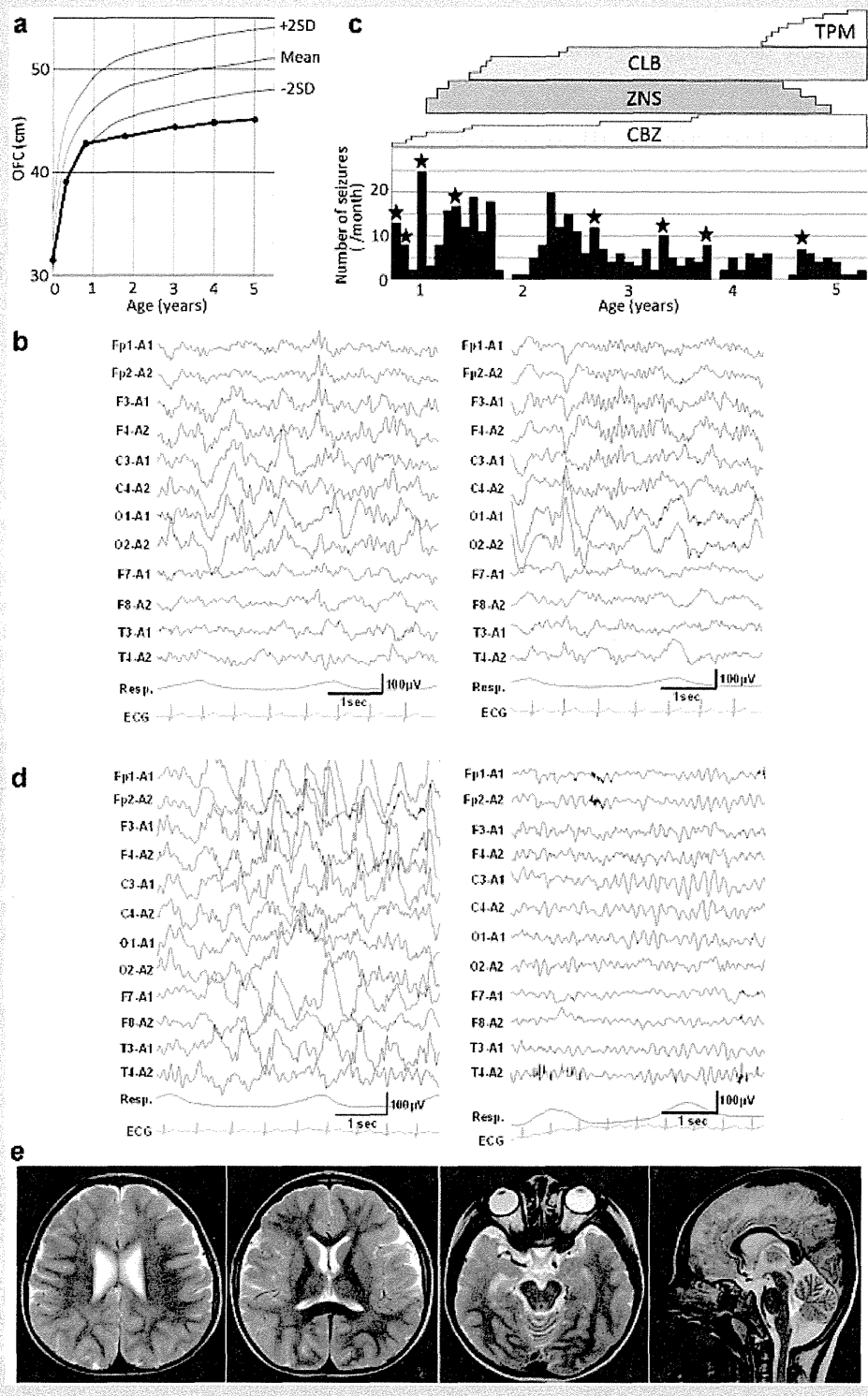

FIG. 1. Clinical findings. a: OFC growth curve. b: An interictal EEG during sleep at age 10 months. Sporadic spikes over the frontal region during sleep records are observed (left). Background activity is consistent with his age (right). c: Therapeutic course of seizures. CBZ, carbamazepine; ZNS, zonisamide; CLB, clobazam; TPM, topiramate. ★ indicates seizures with cluster attacks. d: An interictal EEG at age 4 years. Many paroxysmal discharges including not only sporadic spikes but also slow wave bursts in the bilateral frontal region during sleep are observed (left). A lower frequency theta rhythm in the front-central region during wakefulness is recorded (right). e: Brain MRI at age 5 years. Delayed myelination and mild brain atrophy mainly in the front-temporal lobe are noted.

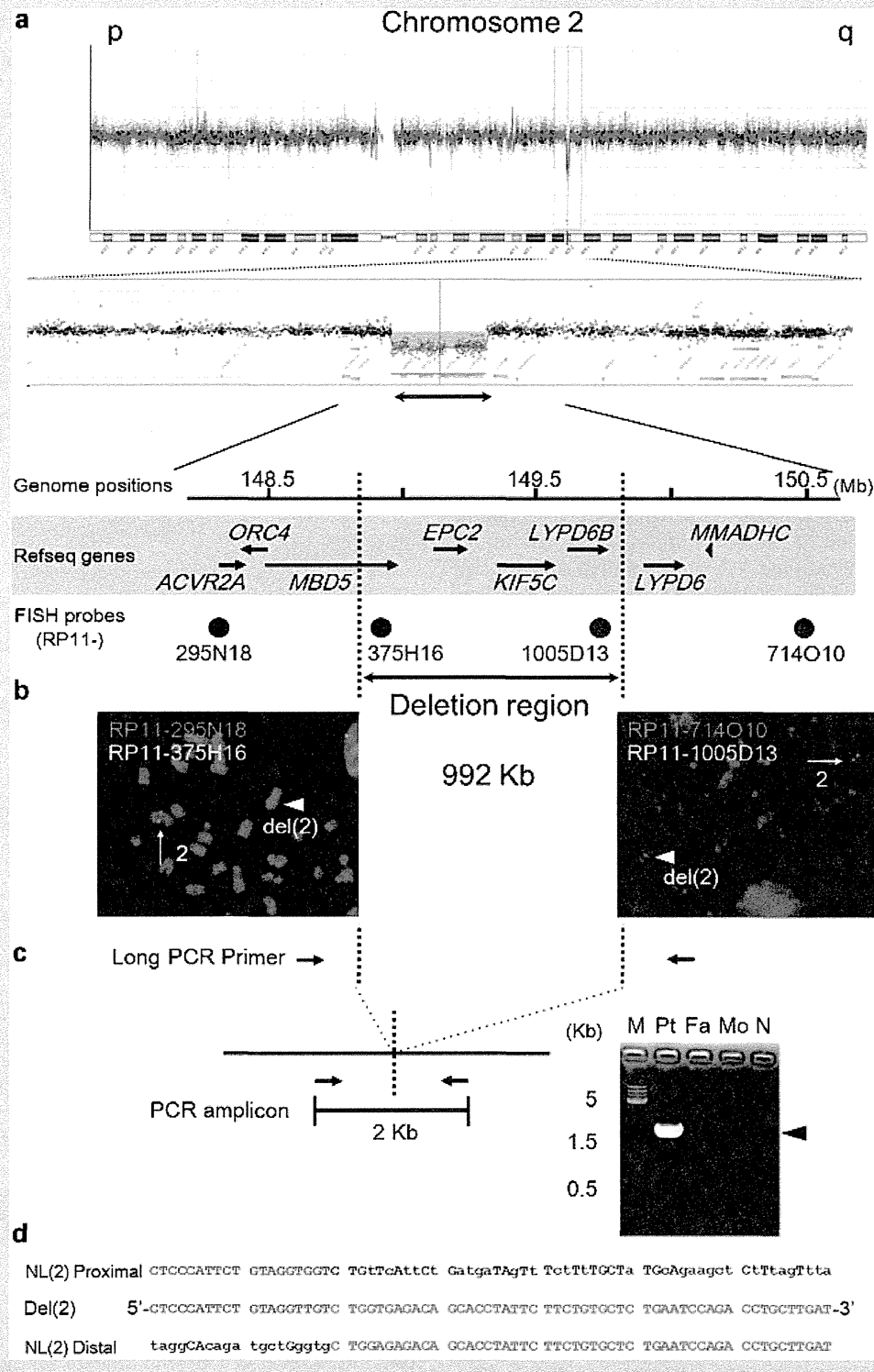

FIG. 2. Cytogenetic and molecular findings. a: High-resolution oligo array [Agilent 1M array] showing the 992 Kb deletion at 2q23.1–q23.2 that contains three Refseq genes [*EPC2*, *KIF5C*, *LYPD6B*] and a disrupted *MBD5*. b: FISH analyses using probes for four BACs [RP11-295N18, RP11-375H16, RP11-1005D13, RP11-714O10]. A normal chromosome 2 [2] is indicated by white arrows and a deleted chromosome 2 [del(2)] is indicated by white arrowheads. Signals for the RP11-375H16 and RP11-1005D13 probes are not detected on deleted chromosome 2. c: Deletion junction PCR showing that an aberrant 2 Kb-PCR amplicon is detected only in the patient [black arrowhead]. M, size markers; Pt, the patient's sample; Fa, the father's sample; Mo, the mother's sample; N, negative control. d: Deletion junction sequence. The normal upper and lower sequences are seen around the proximal [2q23.1] and the distal [2q23.2] deletion breakpoints, respectively. Homologous sequences are indicated by capital letters. The middle sequence is the deletion junction of the patient. NL(2), normal chromosome 2; Del(2), deleted chromosome 2.

was 1 years and 8.5 months (range, neonate to 12 years old). Although the seizures of three patients were well controlled, those of five patients were intractable. In particular, patient 10 began suffering from seizures at the age of 9 months and died at 26 years because of seizures. Patient 5 developed severe epileptic encephalopathy. EEG abnormalities were found in seven of nine patients (78%). The present patient was considered to have FLE with complex partial seizures. His seizures were multi-drug resistant and EEG findings progressively worsened. The effect of CLB was transient, and TPM appeared to be most effective at present to control seizures. MRI abnormalities were detected in 9 of 17 patients (53%) and included cortical atrophy, small vermis, cortical or subcortical lesion, ventricular asymmetry, thinning of the posterior corpus callosum, and delayed myelination. Interestingly, the present patient had no obvious abnormalities during infancy, but later showed brain atrophy and delayed myelination in the front-temporal region at the age of 5 years that were consistent with EEG findings. Considering such a progressive course, careful longitudinal observation of neurodevelopmental features would be necessary for this syndrome. The chromosomal deletion size in the syndrome ranged from 200 kb to 5.5 Mb, but did not seem to correlate with severity of ID, seizures, or MRI abnormalities. Since deleted regions comprised only a part of *MBD5* to 15 genes including *MBD5*, *MBD5* is considered to be responsible for moderate to severe ID and well-controlled to intractable epilepsy.

The deletions in the patients with this syndrome have not been considered to be mediated by NAHR, because the reported deletions did not have common breakpoints [van Bon et al., 2010]. In this study, we sequenced the deletion junction for the first time. The proximal breakpoint was located within a LINE element (LIP3) included in the genomic region of *MBD5*, whereas the distal breakpoint was not located within a LINE element, according to UCSC genome browser. BLAST search found no significant sequence similarity between LIP3 and a sequence around the distal breakpoint in normal chromosome 2. For NAHR to take place, there must be segments of a minimal length sharing extremely high similarity or identity between the low copy repeats, with 10–400 kb in length and >96% sequence identity [Gu et al., 2008]. We, therefore, have concluded the deletion in the present patient not to be mediated by NAHR. Furthermore, we have identified a consensus sequence CTG both at the proximal and the distal breakpoints. This sequence microhomology might have resulted in the deletion in the present patient, via the recently delineated genomic rearrangement mechanism FoSTeS/MMBIR. The mechanism has been reported to mediate genomic rearrangements in Pelizaeus-Merzbacher disease [Lee et al., 2007], Potoki-Lupski microduplication syndrome [Zhang et al., 2009], Smith-Magenis microdeletion syndrome [Zhang et al., 2009], and Charcot-Marie-Tooth disease type 1A duplication/hereditary neuropathies with liability to pressure palsies [Zhang et al., 2009], as well as in 87% of patients with rare pathologic copy number variations [Vissers et al., 2009]. Thorough investigation of breakpoint sequences in the other patients would uncover the etiology of deletions of 2q23.1 microdeletion syndrome.

In conclusion, the present study described detailed neurodevelopmental features including the therapeutic course for intractable epilepsy in a patient with 2q23.1 microdeletion syndrome. Review of neurological and molecular features in previously reported patients demonstrated that *MBD5* would be responsible for ID and epilepsy. Microhomology of three nucleotides, identified at the distal and proximal breakpoints, suggested that the deletion might have been mediated by recently delineated genomic rearrangement mechanism FoSTeS/MMBIR.

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Bezafibrate can be a new treatment option for mitochondrial fatty acid oxidation disorders: Evaluation by in vitro probe acylcarnitine assay

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ABSTRACT

Background: The number of patients with mitochondrial fatty acid oxidation (FAO) disorders is recently becoming larger with the spread of newborn mass screening. Despite the advances in metabolic and molecular characterization of FAO disorders, the therapeutic studies are still limited. It was reported recently that bezafibrate (BEZ), an agonist of peroxisome proliferating activator receptor (PPAR), can restore FAO activity in cells from carnitine palmitoyltransferase-2 (CPT2) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiencies as well as clinical symptoms in the adult patients.
Methods: In this study, the therapeutic effect of BEZ was determined by in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry on various FAO disorders. The clinical trial of BEZ treatment for a boy with the intermediate form of glutaric acidemia type 2 (GA2) was also performed.
Results: The effect of BEZ was proven in cells from various FAO disorders including GA2, deficiencies of VLCAD, medium-chain acyl-CoA dehydrogenase, CPT2, carnitine acylcarnitine translocase and trifunctional protein, by the IVP assay. The aberrantly elevated long- or medium-chain acylcarnitines that are characteristic for each FAO disorder were clearly corrected by the presence of BEZ (0.4 mmol/L) in culture medium. Moreover, daily administration of BEZ in a 2-year-old boy with GA2 dramatically improved his motor and cognitive skills, accompanied by sustained reduction of C4, C8, C10 and C12 acylcarnitines in blood, and normalized the urinary organic acid profile. No major adverse effects have been observed.
Conclusion: These results indicate that BEZ could be a new treatment option for FAO disorders.

1. Introduction

Mitochondrial β-oxidation (FAO) is an essential energy producing pathway, particularly during the reduced energy supply from carbohydrate due to prolonged starvation or low caloric intake during infection, diarrhea or febrile illness. A number of FAO disorders have been recognized with the spread of tandem mass spectrometry (MS/MS) in the field of study of inborn metabolic disease as well as neonatal mass screening [1,2]. Many of them show episodic attacks like lethargy, acute encephalopathy or even sudden death due to energy production insufficiency.

It is considered that the FAO system consists of the following four groups : 1) carnitine cycle, which activates long-chain fatty acids for undergoing β-oxidation, including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 or -2 (CPT1 or CPT2, respectively, EC 2.3.1.21), or carnitine acylcarnitine translocase (CACT, EC 2.3.1.21); 2) long-chain FAO, whose enzymes are connected to the mitochondrial inner membrane, including very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) deficiency, and trifunctional protein (TFP, EC 1.1.1.211 and EC 2.3.1.16); 3) medium-chain FAO, whose enzymes are located in the mitochondrial matrix, including medium- and short-chain acyl-CoA dehydrogenases (MCAD, EC 1.3.99.3 and SCAD, EC 1.3.8.1) respectively), enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, or medium- and short-chain 3-ketothiolase (MCKAT and SCKAT, respectively); and 4) electron transfer system, from the dehydrogenases to respiratory chain, including electron transferring flavoprotein (ETF, EC 1.5.8.2) and ETF dehydrogenase (ETFDH, EC 1.5.5.1) [3–5].

Clinical features of FAO disorders can be roughly divided into the following three types: 1) severe form (neonatal form): patients present life-threatening illness with profound hypoglycemia, liver failure or hyperammonemia, and are often fatal in early infancy; 2) intermediate

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form (juvenile form): patients have intermittent episodic attacks like lethargy, encephalopathy, or even sudden death often onset in infancy or young childhood; 3) mild form (myopathic form): the patients may often show late onset after school ages or adulthood with episodes of hypotonia, myalgia, lethargy, myopathy-like symptoms, or liver dysfunction [6].

In vitro probe acylcarnitine profiling (IVP) assay was developed to evaluate FAO disorders recently [7,8]. Acylcarnitine (AC) profiles in the special culture medium as below after incubating with fatty acids as substrates are determined by MS/MS. Bezafibrate (BEZ) is a hypolipidemic drug, which is an agonist of peroxisome proliferating activator receptor (PPAR), and is claimed to act for induction of several FAO enzymes [9–11].

In this study, the effect of BEZ on various FAO disorders was evaluated using the IVP assay. Furthermore, we report an in vivo trial of BEZ on a boy with the intermediate form of GA2, presenting dramatic improvement with BEZ.

2. Materials and methods

2.1. Subjects and skin fibroblasts

Fibroblasts from 10 Japanese children with FAO disorders, one each of severe and intermediate forms of GA2, 2 each of severe and myopathic (mild) forms of VLCAD deficiency, one each of deficiencies of MCAD, CPT2, CACT, and TFP as well as 6 controls (healthy volunteers, passages 3 to 16) were used. The clinical types and genotypes are shown in Table 1. The child with MCAD deficiency was detected in a newborn mass screening and non-symptomatic, while one with the intermediate form of CPT2 deficiency had liver dysfunction in infancy. The child with the intermediate form of CACT deficiency had two life-threatening episodes in infancy, and after that no episodes were noted with normal development [12]. The child with TFP deficiency had an episode of liver failure in infancy, and then intermittent episodes of myalgia or hypotonia particularly following infection.

The clinical types and genotypes are shown in Table 1. In all cases, at least one allele has missense mutation, although the other alleles had missense or truncated mutations. In CACT deficiency (case 9), a missense mutation in an initiation codon (c.3G>A) in SLC25A29 was detected, but this could harbor a residual activity (Fukao et al., unpublished data).

Table 1
Clinical types and genotypes of patients with mitochondrial fatty acid oxidation disorders investigated.

Disease & case No.	Phenotype	Gene	Genotype, nucleotides (amino acids)	
			Allele 1	Allele 2
GA2				
1 (B)	Severe	*ETFA*	c.799G>A (G267R)	c.7C>T (R3X)
2 (C)	Intermediate	*ETFDH*	c.1217G>A (S406N)	c.1675C>T (R559X)
VLCAD deficiency				
3 (D)	Severe	*ACADV*	c.553G>A (G185S)	IVS9+1g>c
4 (E)	Severe	*ACADV*	c.454G>A (G152S)	c.997insT (A333fsX358)
5 (F)	Myopathic	*ACADV*	c.790A>G (K264E)	c.997insT (A333fsX358)
6 (G)	Myopathic	*ACADV*	c.1144A>C (K382Q)	c.1339G>A (G447R)
MCAD deficiency				
7 (H)	Non-symptomatic	*ACADM*	c.134A>G (Q45R)	c.449delCTGA (T150fsX153)
CPT2 deficiency				
8 (I)	Intermediate	*CPT2*	c.151A>G (R51G)	c.520G>A (E174K)
CACT deficiency				
9 (J)	Intermediate	*SLC25A29*	c.3G>A (M1I)	IVS4+1g>t
TFP deficiency				
10 (K)	Intermediate	*HADHB*	c.739C>T (R247C)	c.817delG (D273fsX292)

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; GA2, glutaric acidemia type 2; VLCAD, very-long-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase-2; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase. Case 2 (C) is a boy with GA2 who underwent the clinical trial of BEZ. Non-symptomatic case 7 (H) was detected in the newborn mass screening. Severe, intermediate, and myopathic forms are mentioned in the text. (B) to (K) correspond to those of Fig. 1.

2.2. In vitro probe assay with BEZ

Fibroblasts were cultured in 75 cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2/95% air incubator [13].

Fibroblasts harvested by trypsinization were seeded onto 6-well microplates (35 mm i.d., Iwaki, Japan) with the fresh above medium (2 mL/per well) until they reached confluence. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate (experimental medium). The experimental medium is MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabeled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, in the presence or absence of BEZ (0.4 mmol/L; Sigma). AC profiles in the culture medium were analyzed after 96 h. The experiments for each case were performed in triplicate.

2.3. Quantitative acylcarnitine analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) as described previously [13]. Briefly, methanol (200 μL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of the supernatant from culture medium. The portions were placed on ice for 30 min, and centrifuged at 1000×g for 10 min. Then, 150 μL of the supernatant was dried under a nitrogen stream, and butyl-derivatized with 50 μL of 3N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1 v/v). The ACs in 10 μL of the resultant aliquots were analyzed using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. The AC concentrations are expressed as nmol/mg protein.

2.4. Organic acid analysis using GC/MS

Urinary organic acids were analyzed according to the previous method [14]. Briefly, 40 μg of tropate (IS-2) and 20 μg each of heptadecanoate (IS-1) and tetracosane (C24) as internal standards were added to a urine specimen containing 0.2 mg creatinine. The samples were oxime-derivatized, and solvent extracted with ethylacetate, and trimethylsilylated (TMS-derivatization). The resultant aliquots were subjected to GC/MS (Shimadzu GC/MS QP2010 Plus, Kyoto, Japan), with a DB-5 column of 0.25 mm I.D×30 m, 1 μm film thickness (J&W, Folsom, CA). The temperature program was from 100 °C to 290 °C at a rate of 4 °C/min).

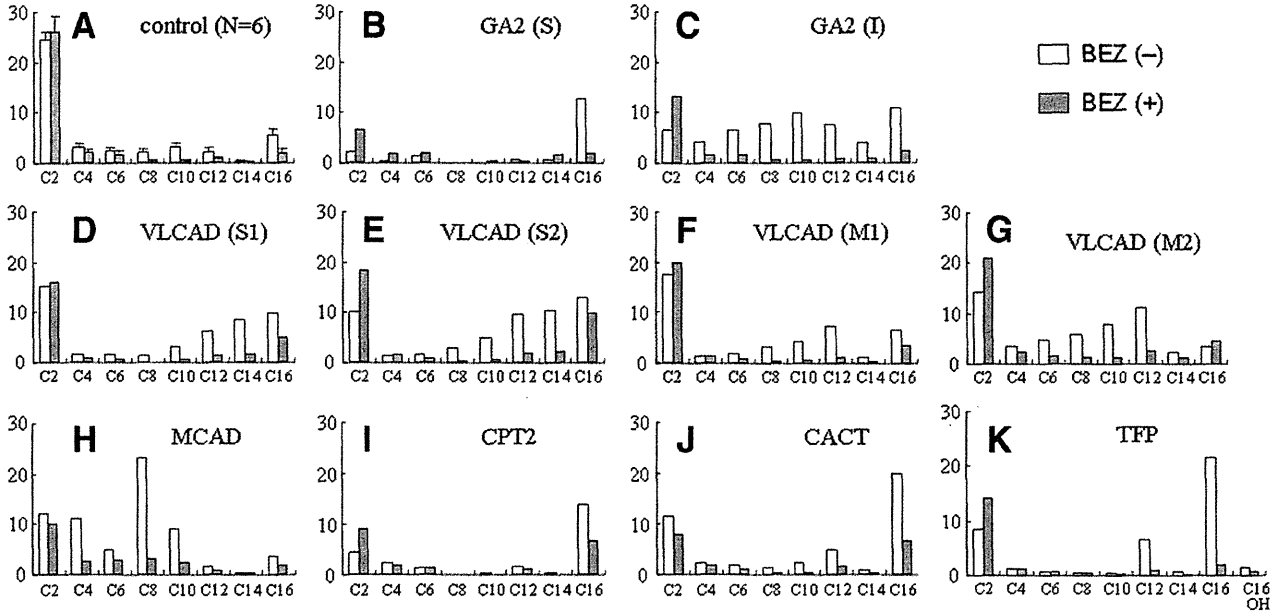

Fig. 1. Acylcarnitine profiles of in vitro probe assay in the presence and absence of bezafibrate. A, normal control; B, severe form of GA2; C, intermediate form of GA2 (the boy who underwent the clinical trial) (S and I, the clinically severe and intermittent form, respectively); D and E, severe form of VLCAD deficiency (S1 and S2, two cases, respectively); F and G: myopathic (mild) form of VLCAD deficiency (M1 and M2, two cases, respectively); H, I, J, and K: deficiencies of MCAD, CPT, CACT, and TFP, respectively. Unit of vertical lines, nmol /mg protein of acylcarnitines; the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C10, C12, C14, C16, and C16-OH. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars. In control (A), the mean plus SD values of 6 controls are shown.

2.5. BEZ trial on a child with the intermittent form of GA2

A Japanese boy with GA2 was detected in the newborn mass screening using MS/MS, and had no special symptoms in infancy with therapies of special formula and carnitine (approximately100 mg/kg/day, div. 3). After 1 year of age, however, he sometimes experienced episodes of hypotonia or lethargy following infection, and muscle weakness, often falling. At the age 2 years and 1 month, he was hospitalized for 2 and a half months, because of infection and lethargy, receiving treatments including artificial respiration to repeated aspiration pneumonia and unconsciousness in intensive care unit (ICU). At discharge, he could not walk alone, and could speak only a few words. So, his family consulted us, and strongly expressed a desire for any new therapies that might help their son.

Thereafter, under the approval by the ethical committee of Shimane University, we started a clinical trial of BEZ, continuing the dietary and carnitine therapies as before, since 2 years and 9 months of his age. His body weight ranged from 12 to 14 kg during the treatment, and 200 to 300 mg/day (approximately 17 to 25 mg/kg/day, div. 3) of BEZ was used in the trial. BEZ was purchased from Kissei Co Ltd, Tokyo, Japan. The study had no potential conflicts of interest (COI) to the authors.

3. Results

3.1. Effects of BEZ on FAO disorders by IVP assay

The AC profiles in the culture medium of fibroblasts from various FAO disorders in the presence and absence of BEZ are illustrated in Fig. 1. In control cells, C2 (acetylcarnitine) is the only prominent peak, and many of ACs further decreased in the presence of BEZ (Fig. 1A).

In the severe form of GA2 (Fig. 1B/S), C16 was apparently decreased, and C2 increased in the presence of BEZ, while C16 was extremely high before BEZ addition. The increase of C2 may indicate the acceleration of FAO, namely an increase of acetyl-CoA production. In the intermediate form of GA2 (Fig. 1C/I), all elevated ACs clearly decreased and normalized in the presence of BEZ, although broad ranges of ACs from C4 to C16 were extremely high before adding BEZ. This patient is the case 3 in Table 1, who underwent the clinical trial of BEZ treatment as illustrated in Fig. 2.

In 2 cases of the severe form of VLCAD deficiency (Figs. 1D/S1, and 1E/S2), elevation of C14 and C16 was larger, compared with that in 2 cases of the mild form (Figs. 1F/M1, and 1G/M2). The elevated ACs

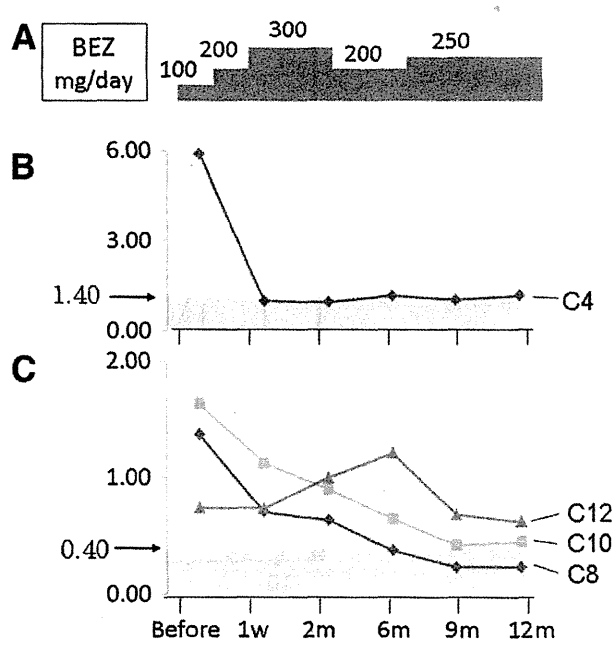

Fig. 2. Bezafibrate administration and changes in blood acylcarnitines. A, dose of bezafibrate, mg/day (approximately 17 to 25 mg/kg/day, div. 3); B, change of C4 acylcarnitine; C, changes in C8, C10, and C12. Arrows with the 1.40 and 0.40 indicate the cutoff values of blood acylcarnitines. Unit of acylcarnitine is nmol/mg protein.

Table 2
Time course of biochemical findings after initiation of bezafibrate administration.

	(Unit)	Before	After the start of BEZ treatment					Reference value*
			1w	2 m	6 m	9 m	12 m	
AST	(IU/L)	47	35	44	43	26	42	10–38
ALT	(IU/L)	27	17	22	24	20	21	5–40
LDH	(IU/L)	448	426	392	384	341	371	100–215
CK	(IU/L)	496	185	187	324	174	207	36–216
TChol	(mg/dL)	161	127	117	141	127	140	150–219

* : used in Shimane University Hospital. Abbreviations: AST, aspartate amino transferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; and TChol, total cholesterol.

such as C10, C12, C14, or C16 in both the severe and mild forms apparently decreased in the presence of BEZ.

In MCAD deficiency (Fig. 1H), the AC peaks of C4 to C10 were significant, but in the presence of BEZ, these AC peaks were almost normalized. In cases of CPT2 deficiency (Fig. 1I), CACT deficiency (Fig. 1J) and TFP deficiency (Fig. 1K), the extremely high AC peaks of C16 and/or C12 apparently decreased to an almost normal level, in the presence of BEZ.

3.2. Clinical trial of BEZ to a GA2 patient

Since the start of BEZ treatment, his motor and social development, and languages remarkably improved, and no metabolic episodes were noted. He became able to walk alone, showed improved muscle strength, and could speak markedly more words in a few weeks. Furthermore, several months later, he could ride a kid's tricycle by himself, although his intellectual ability was on the borderline for entrance into a kindergarten. For at least 1 year of the administration, no adverse effects of BEZ such as hypolipidemia or rhabdomyolysis have been observed.

The routine laboratory data such as blood AST, ALT, LDH or CK were in normal or subnormal ranges as shown in Table 2, showing stable levels of each test, although these laboratory data had sometimes fluctuated, in particular, when his condition was unstable before the initiation of BEZ. For example, during the stay in the ICU at the age of 2 years, the maximum levels of AST, ALT, LDH or CK were 1450 IU/L, 825 IU/L, 5200 IU/L, or 10,750 IU/L, respectively. The maximum level of blood ammonia at the ICU was 126 μg/dL, while no significant elevation was observed after that. Hypoglycemic attacks have not been noted.

BEZ is a hypolipidemic drug, and we have paid attention to the blood level of Cholesterol (TChol), because of the potential adverse effects. The dose of BEZ was 100 mg/day for the first 3 days, 200 mg/day for 4 days, and 300 mg/day for 2 months, respectively, as shown in Fig. 2A. At 2 months after starting BEZ of 300 mg/day, TChol level was a bit low, 117 mg/dL. Since then the dose has been lowered to 200 or 250 mg/day, and the TChol level has ranged between around 130 to 150 mg/mL, as shown in Table 2.

The changes in the AC levels of C4, C8, C10, and C12 are illustrated in Figs. 2B and C, respectively. All the increased ACs returned to approximately normal levels with the administration of BEZ after several months. In particular, C4 decreased to the normal range within a few weeks. Urinary organic acid analysis showed remarkable increases of ethylmalonate, methylsuccinate, adipate, 2-hydroxyglutarate, hexanoylglycine, suberate, and suberylglycine, before the BEZ treatment as shown in Fig. 3. The abnormalities in urinary organic acids were markedly corrected as early as 2 weeks after the initiation of BEZ therapy. The profile was almost normal but for a slight increase of ethylmalonate, and/or hexanoylglycine as illustrated in Fig. 3B.

4. Discussion

The treatments for FAO disorders have generally been described as follows: 1) avoiding a "long fasting": it prevents the increased requirement of fuel from FAO; 2) early infusion of glucose: it should be performed during the metabolic stress resulting from infection, diarrhea or overexercise, to prevent hypercatabolism; 3) carnitine therapy: it may be effective in many cases, although controversy

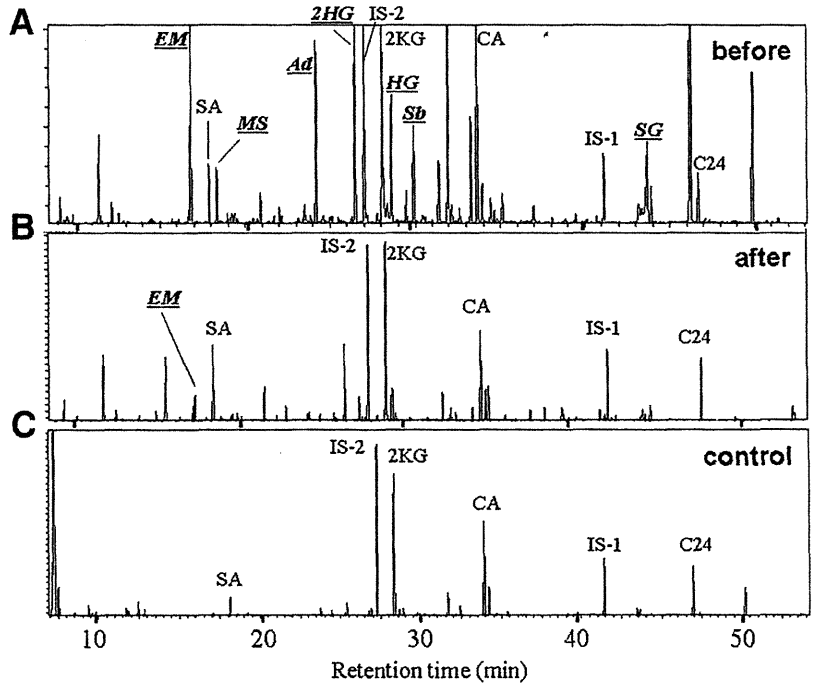

Fig. 3. Urinary organic acid profiles before and after bezafibrate administration. A, The total ion chromatogram (GC/MS) of urinary organic acids just before the start of BEZ; B, One year after the treatment; C, Normal control. Abbreviations: IS-2, IS-1 and C24 are tropate, heptadecanoate, and tetracosane, respectively, as internal standards; EM, ethylmalonate; SA, succinate; MS, methylsuccinate; Ad, adipate; 2HG, 2-hydroxyglutarate; 2KG, 2-ketoglutarate; HG, hexanoylglycine; Sb, suberate; CA, citrate; SG, suberylglycine. Metabolites judged as abnormal are shown in bold letters underlined.

remains in some cases; and 4) dietary therapy, including high carbohydrate/low lipid diet: Dietary restriction in FAO disorders may be less strict [15–18].

In this study, we demonstrated the effect of BEZ on various FAO disorders at both in vitro and in vivo levels. It was indicated by the IVP assay that FAO capacity was corrected by BEZ in various FAO disorders, and a clinical trial of BEZ in a boy with the intermediate form of GA2 showed a favorable consequence. Bastin, Djourdi and their colleagues reported the potential effect of BEZ for FAO disorders showing the increase of enzyme activity and mRNA production in several FAO enzymes from normal individuals, or reduced ACs in cells from VLCAD deficiency by the IVP assay using stable isotope-labeled palmitate [19]. Furthermore, they are performing a clinical trial on adult cases of mild form of CPT2 deficiency [20,21]. We should continue to pay attention to potential adverse effects of BEZ, including hypolipidemia or rhabdomyolysis, although such signs have never seen up to now.

We used the IVP assay to investigate the effect of BEZ in the other FAO disorders including GA2, deficiencies of MCAD, CACT, and TFP as well as CPT2 or VLCAD deficiencies. The beneficial effect of BEZ was clearly demonstrated in all these cases tested in this study, which included the clinically intermediate or severe forms as well as the mild form, having missense mutation of at least one allele. However, it is not yet clear whether the effect of BEZ is due to induction of mutant enzyme itself, or due to stimulation of the other FAO enzymes. If the effect is due to the latter mechanism, BEZ could potentially induce a "high pressure" on the FAO pathway, even resulting in devastating outcomes. We should further investigate the effect on the other severe forms of FAO disorders, the relation with the genotypes, or the dose dependency.

BEZ is an agonist of PPAR, which facilitates transcription of genes encoding FAO enzymes, and subsequently induces FAO enzyme production. Eventually, it can be considered to correct the FAO capacity in FAO disorders. Recently, it was reported that resveratrol which is a natural polyphenol and an activator of Sirtuin 1, is also expected to be a novel treatment option for FAO disorders [22]. The effect of resveratrol on FAO capacity can also be evaluated by the IVP assay like this study.

In conclusion, BEZ could be a new promising treatment option for FAO disorders. Many of patients with FAO disorders, particularly children with the milder form or adult cases, are intellectually normal, and their life prognosis is favorable if they can be prevented from severe episodes like encephalopathy. Symptoms or severity of FAO disorders are very heterogeneous depending on the disease, genetic background or lifestyle. Additional clinical studies of BEZ treatment will be essential for confirmation of its safety and practical utility.

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ORIGINAL PAPER

Intracellular in vitro probe acylcarnitine assay for identifying deficiencies of carnitine transporter and carnitine palmitoyltransferase-1

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Abstract Mitochondrial fatty acid oxidation (FAO) disorders are caused by defects in one of the FAO enzymes that regulates cellular uptake of fatty acids and free carnitine. An in vitro probe acylcarnitine (IVP) assay using cultured cells and tandem mass spectrometry is a tool to diagnose enzyme defects linked to most FAO disorders. Extracellular acylcarnitine (AC) profiling detects carnitine palmitoyltransferase-2, carnitine acylcarnitine translocase, and other FAO deficiencies. However, the diagnosis of primary carnitine deficiency (PCD) or carnitine palmitoyltransferase-1 (CPT1) deficiency using the conventional IVP assay has been hampered by the presence of a large amount of free carnitine (C0), a key molecule deregulated by these deficiencies. In the present study, we developed a novel IVP assay for the diagnosis of PCD and CPT1 deficiency by analyzing intracellular ACs. When exogenous C0 was reduced, intracellular C0 and total AC in these deficiencies showed specific profiles clearly distinguishable from other FAO disorders and control cells. Also, the ratio of intracellular to extracellular C0 levels showed a significant difference in cells with these deficiencies compared with control. Hence, intracellular AC profiling using the IVP assay under reduced C0 conditions is a useful method for diagnosing PCD or CPT1 deficiency.

Keywords Fatty acid oxidation · Carnitine cycle disorder · Acylcarnitine profile · ESI-MS/MS

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Introduction

L-Carnitine plays an essential role in the transfer and activation of long-chain fatty acids across the outer and inner mitochondrial membranes during which it is acted upon by enzymes including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 (CPT1), carnitine palmitoyltransferase-2 (CPT2), and carnitine acylcarnitine translocase (CACT) (Fig. 1) [1, 2]. Carnitine penetrates into cells across the plasma membrane against a high concentration gradient of free carnitine with the aid of the plasma membrane OCTN2 protein encoded by the SLC22A5 gene [3]. Deficiency of OCTN2 causes primary carnitine deficiency (PCD, OMIM 212140), which is characterized by systemic carnitine deficiency in tissues and blood but in concord with increased excretion of free L-carnitine in the urine [4–6]. Clinical symptoms in patients with PCD such as cardiomyopathy,

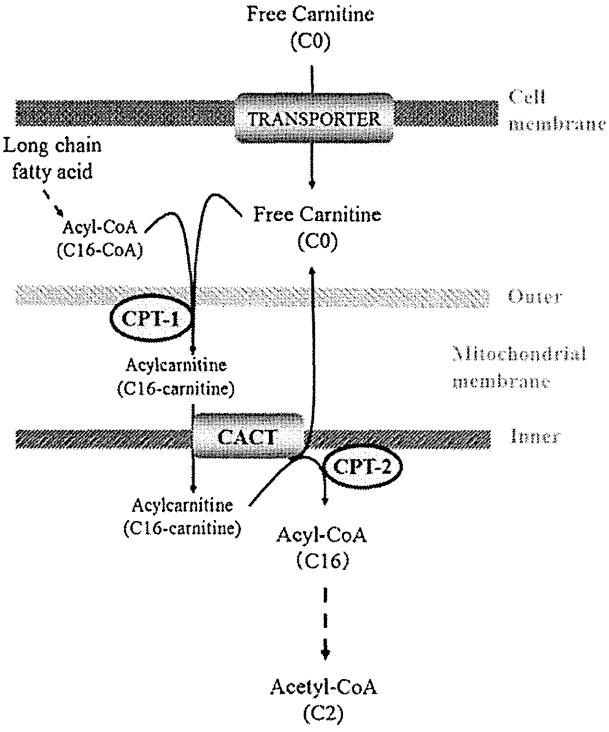

Fig. 1 Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. *Solid arrows* indicate single reactions; *dashed arrows* indicate multiple reactions or steps

encephalopathy, hepatomegaly, myopathy, hypoglycemia, and hyperammonemia, mainly result from low carnitine concentration in the tissues. On the other hand, secondary carnitine deficiency occurs in some conditions such as organic acidemias, renal dialysis, long-term medication (antiepileptic drugs or some antibiotics), and alimentary deficiency of L-carnitine [7–9].

It is necessary to make a differential diagnosis of PCD from the secondary carnitine deficiency or other false-positive cases, and diagnosis is confirmed by demonstrating reduced transport in skin fibroblasts from the patients. Until now, cluster-tray method using radioisotope-labeled substrate was used for the diagnosis of PCD [4, 10–12]. However, such a diagnostic method requires handling of radioactive substrates and focused only on diagnosis of PCD. Gene sequencing in SLC22A5 is one diagnostic method for PCD. However, it is molecularly heterogenous, and around 50 different mutations have been identified [6]. After acylcarnitine analysis using tandem MS analysis became available in the worldwide, blood acylcarnitine analysis was used as an initial method for diagnosis of FAO disorders and a detection of FAO disorders has been increased. However, it is necessary to confirm the diagnosis of the diseases with detailed analysis. The in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry (MS/MS) has been used to evaluate FAO capacity in the cultured cells and make a diagnosis of FAO disorders [13–15]. However, conventional IVP assay is not feasible to diagnose PCD or CPT1 deficiency, because excess amount of free carnitine is added to the experimental medium at the beginning. Estimation of free carnitine, which is the key marker for the above diseases, in experimental medium was nonsense for diagnosis of these disorders. We developed a novel functional assay for PCD and CPT1 deficiency using the IVP assay, with some modifications. This method uses different concentrations of exogenous free carnitine and measures intracellular as well as extracellular acylcarnitine (AC) levels, which overcomes the disadvantage of the conventional IVP assay in the diagnosis of carnitine cycle disorders.

Materials and methods

Materials

Hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), and palmitoylcarnitine (C16) were purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). As an internal standard, a labeled carnitine standard kit (NSK-B), which contains $^{2}[H]_{9}$-carnitine, $^{2}[H]_{3}$-acetylcarnitine, $^{2}[H]_{3}$-propionylcarnitine, $^{2}[H]_{3}$-butyrylcarnitine, $^{2}[H]_{9}$-isovalerylcarnitine, $^{2}[H]_{3}$-octanoylcarnitine, $^{2}[H]_{9}$-myristoylcarnitine, and $^{2}[H]_{3}$-palmitoylcarnitine, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Preparation of standard solutions of ACs

Standard solutions containing 1, 10, 25, and 50 μmol/L each of C6, C8, C10, and C16 were used to validate the recovery and determine linear concentration range of ACs after extraction by the Folch method [16]. The ACs were dissolved in methanol (99.8 %), and the prepared standard solution was analyzed directly and after extraction by the Folch method.

Subjects

Human skin fibroblasts from six healthy controls (volunteers) and seven patients with various carnitine cycle disorders—three each with PCD and CPT2 deficiency and one with CPT1 deficiency—were analyzed. In all cases, diagnoses were confirmed by mass spectrometric analyses (gas chromatography-mass spectrometry and MS/MS), enzyme assay, and protein or mutational analyses. Informed consent was obtained from the patients or their families. This study was approved by the Ethical Committee of the Shimane University School of Medicine.

In vitro probe acylcarnitine (IVP) assay using MS/MS

An IVP assay was performed, as described, with some modifications [13, 15, 17], and principle of IVP assay was shown Fig. 2. Briefly, 3×10^6 cells were seeded in triplicate onto a six-well microplate (35 mm i.d.; Iwaki) and cultured until confluent. After washing twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA), the cells were subsequently cultured for 96 h in 1 ml of a special experimental minimal essential medium (MEM) containing bovine serum albumin (0.4 % essential fatty acid-free BSA; Sigma), two different concentrations of C0 (Sigma)—10 μmol/L (reduced level, lower compared with physiological level) and 400 μmol/L (excess level)—and unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque). C0 and AC levels in the culture medium (extracellular fraction) and in the intracellular extract were analyzed after a 96-h incubation period using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA), as described [18].

Intracellular acylcarnitine extraction

Intracellular C0 and ACs were extracted using the Folch method, with some modification [16]. Briefly, harvested cells were washed twice with DPBS buffer. The cell pellet was resuspended in 100 μl volume of DPBS buffer and immediately frozen in liquid N_2. In order to separate phospholipids and cell debris, 250 μl of Folch reagent (chloroform/methanol, 2:1) was added to the resuspended cell pellet. After vigorous mixing using a vortex mixer, the solution was centrifuged for 10 min at 15,000 rpm at 4 °C. The debris layer around the interface between the aqueous and lipid phases was removed, and the extracted aqueous and lipid phases were mixed and thereafter dried under a nitrogen stream at 50 °C. ACs in culture medium supernatants and extracted intracellular ACs lysate were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Briefly, methanol (200 μl) including an isotopically labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of supernatant from culture medium and extracted intracellular ACs, for 30 min. Portions were centrifuged at 1,000×*g* for 10 min, and then 150 μL of supernatant was dried under a nitrogen stream and butylated with 50 μL of 3 N *n*-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80 % acetonitrile/water (4:1 *v/v*), and then the ACs in 10 μL of the aliquots were determined using MS/MS [18] and quantified using Chemo-View™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

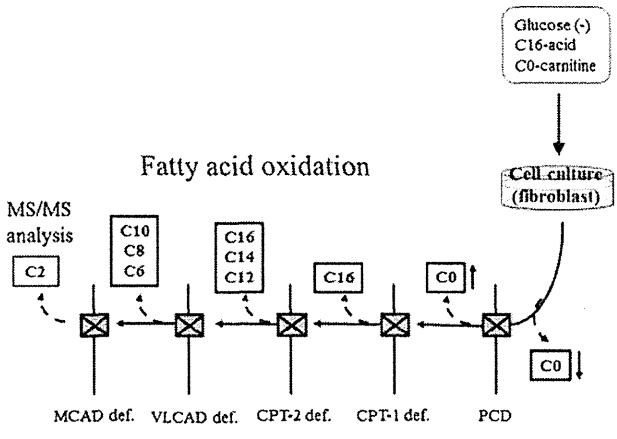

Fig. 2 Principle of in vitro probe acylcarnitine assay. *C2, C4, C6, C8, C10, C12, C14*, and *C16* represent acylcarnitines

Protein concentration and cell viability

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) [19]. The percentage of viable cells was determined at 24, 48, 72, and 96 h of incubation using the modified 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay [20].

Data and statistical analysis

The results are expressed as mean±SD from at least three independent experiments for IVP assay in each cultured cell and three intra-assays and three inter-assays for recovery of standard AC solutions, and statistical significance was evaluated using Student's *t*test in Microsoft Excel. The AC concentrations were expressed as nanomoles per milligram protein.

Results

Recovery of ACs during Folch extraction

The AC standards in the aqueous or lipid fraction were analyzed separately using MS/MS, after extraction by the Folch procedure, and compared with direct analysis of the total mixed standard solutions using three inter-assays and three intra-assays of analysis of standard AC solution. As shown in Fig. 3, most of the C6 and C8-carnitines fractionated to the aqueous phase, while almost all C16-carnitine was exclusively retained in the lipid phase. The amount of C10-carnitine was comparable in both aqueous and lipid phases.

To determine the loss of C0 and ACs during Folch extraction, the standard AC solution was analyzed directly after routine sample preparation for MS/MS and compared with that after Folch extraction. The recovery of ACs in the

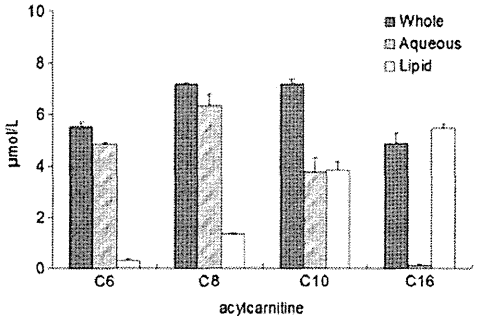

Fig. 3 Recovery of ACs during extraction using the Folch method. Standard solutions of 10 μmol/L each of C6-, C8-, C10-, and C16-carnitine were used to determine the recovery of ACs in the aqueous and lipid fractions during extraction using the Folch method. *Grey column*: ACs in the whole extract after Folch method; *striped column*: ACs in the aqueous fraction of Folch extraction; *open column*: ACs in lipid fraction of Folch extraction. Data are expressed as mean±SD (micromoles per liter) from three intra-assays and three inter-assays, and statistical significance was evaluated using Student's *t*test in Microsoft Excel

standard solutions after direct analysis and Folch extraction procedure was analyzed three times by inter-assay. The inter-assay CV of acylcarnitines ranged from 3.21 to 8.33 %. No statistical difference was seen between direct analysis and after Folch extraction.

Acylcarnitine profile in extracellular medium of cultured fibroblasts with excess and reduced concentrations of free carnitine

Using fibroblasts from various carnitine cycle disorders, AC profiles were determined in the extracellular medium with reduced or excess concentration of C0. Reported conventional IVP assay used excess levels of C0 (400 μmol/L) [14, 15, 17, 21]. With excess amount of C0 (Table 1, "Medium (C0-excess, 400 μM)"), a selective increase in C16 and a decrease in acetylcarnitine (C2) was observed in cases of CPT2-deficient fibroblasts. AC profiles in media from PCD- and CPT1-deficient fibroblasts were similar to that of healthy controls. In PCD fibroblasts, C2 was 53.1 % of the normal control while C2 in CPT1-deficient fibroblasts was 140 % of the normal control. No statistical difference in C0 level was observed among CPT2-, PCD-, and CPT1-deficient fibroblasts and a healthy control.

In the extracellular medium containing reduced C0, C16 remains higher in cells with CPT2 deficiency, while AC profiles were similar to those observed in C0-excess for PCD- and CPT1-deficient cells and the healthy controls (Table 1, "Medium (C0-reduced, 10 μM)").

Acylcarnitine profile in intracellular lysate with various concentrations of free carnitine

The intracellular C0 and ACs were measured after AC extraction using the Folch method. C16 in the intracellular lysate from CPT2-deficient fibroblasts was significantly elevated in both reduced and excess C0 conditions similar to those in extracellular medium, and diagnostic significant was kept. In the excess C0 condition, CPT1- and PCD-deficient fibroblasts could not be distinguished clearly; based on the C0 levels, even C16 level was relatively low (Fig. 4a). On the other hand, the intracellular C0 under conditions with reduced C0 was 41.78±1.47 and 6.31± 2.88 nmol/mg protein/96 h in the normal controls (n=6) and patients with PCD (n=3), respectively, and the C0 levels of PCD cells were significantly lower (p<0.001) as shown in Fig. 4b. This indicated that the C0 uptake was significantly decreased in PCD compared with control in

Table 1 Acylcarnitine profiles of in vitro probe acylcarnitine assay

	Acylcarnitines, nmol/mg protein/96 h						
	C0	C2	C6	C8	C12	C14	C16
Medium (C0 excess, 400 μM)							
Control (n=6)	411.74±23.08	11.80±1.54	2.60±0.09	1.70±0.47	0.79±0.22	0.34±0.19	2.06±0.77
PCD (n=3)	432.18±18.76	6.25±0.96	2.09±0.40	0.94±0.54	0.41±0.33	0.20±0.10	1.72±0.57
CPT-1 (n=1)	357.69±34.16	16.52±5.60	1.73±0.87	0.54±0.94	0.18±0.14	0.17±0.16	1.36±0.98
CPT-2 (n=3)	376.56±42.71	6.88±0.72	0.94±0.65	0.41±0.22	1.70±0.35	0.80±0.05	18.73±1.07
Medium (C0 reduced, 10 μM)							
Control (n=6)	9.85±0.30	1.70±0.74	0.78±0.30	0.18±0.09	0.10±0.08	0.03±0.01	0.51±0.11
PCD (n=3)	10.03±0.71	0.74 ±0.33	0.75±0.31	0.06±0.04	0.03±0.01	0.01±0.01	0.20±0.08
CPT-1 (n=1)	11.06±0.75	7.56±3.10	0.98±0.30	0.55±0.62	0.09±0.09	0.08 ±0.07	0.01 ±0.02
CPT-2 (n=3)	9.73±1.94	0.64±0.23	0.54±0.20	0.11±0.03	0.22±0.06	0.04±0.01	2.79±0.38

The results are expressed as mean±SD from three independent experiments with triplication in each cell line. The AC concentration was expressed as nanomoles per milligram protein. *C0* free carnitine, *C2* acetylcarnitine, *C6* hexanoylcarnitine, *C8* octanoylcarnitine, *C12* dodecanoylcarnitine, *C14* myristoylcarnitine, *C16* palmitoylcarnitine

C0-reduced condition. Concentration of C16 was also significantly low in PCD in C0-reduced condition. Under the C0-reduced condition, intracellular C0 was much higher, but C16 was much lower in CPT1-deficient fibroblasts, compared with the levels in controls (Fig. 4b).

The ratio of intracellular C0 to extracellular C0 in PCD was significantly lower than that of the controls ($p<0.001$) in the C0-reduced condition, while that in C0-excessive condition was not significantly different (Fig. 5). Cell viability was measured using the MTT assay under reduced or excess concentrations of C0. The percentage of viable cells cultured in C0-reduced medium was equivalent to that in C0-excess media (data not shown).

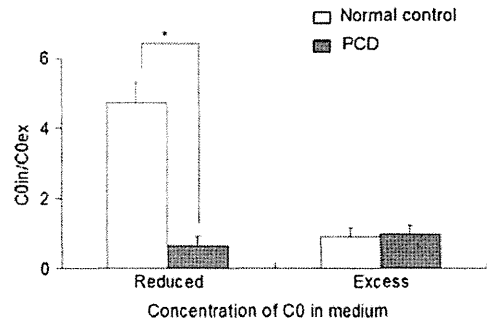

Fig. 5 Ratio of intracellular C0 to extracellular C0. *Open square*: normal control ($n=6$); *closed square*: PCD ($n=3$). Extra- and intracellular C0 of cells with normal control and PCD were measured in C0-reduced (10 μmol/L) and C0-excess (400 μmol/L) conditions using MS/MS. Data are expressed as mean±SD of six normal controls and three patients with PCD. Experiment in each cell line was repeated twice with triplications. Significant differences between normal control and PCD are shown as $*p<0.001$

Discussion

The present study developed a novel IVP assay for the accurate diagnosis of PCD and CPT1 deficiency. Although previous studies reported that IVP assay was a powerful method for the diagnosis of most FAO disorders [13, 14, 21], this assay turned out to be unable to identify PCD and CPT1 deficiencies. At first, we used a C0-excess experimental medium, which contained 400 μmol/L of C0, according to previous reports [13, 14, 21]. Extracellular AC profiles of patients with PCD and CPT1 deficiency showed a pattern similar to that of normal controls by the conventional assay that contains excessive C0 (400 μmol/L) in the culture medium, since C0 moves across the cell membrane down its concentration gradient by passive diffusion. Long-chain fatty acids are transferred across the inner mitochondrial membrane with the assistance of carnitine and carnitine cycle enzymes. The subsequent FAO functions normally even in PCD, and AC profile in PCD is similar to that in normal FAO. Next, we used 50 μmol/L of C0 because the normal range of free carnitine in human plasma was approximately 25 to 50 μmol/L [6]. However, there was no diagnostic difference compare with C0-excess condition, and data are not shown. We analyzed IVP assay in C0-deficient condition (10 μmol/L of C0).

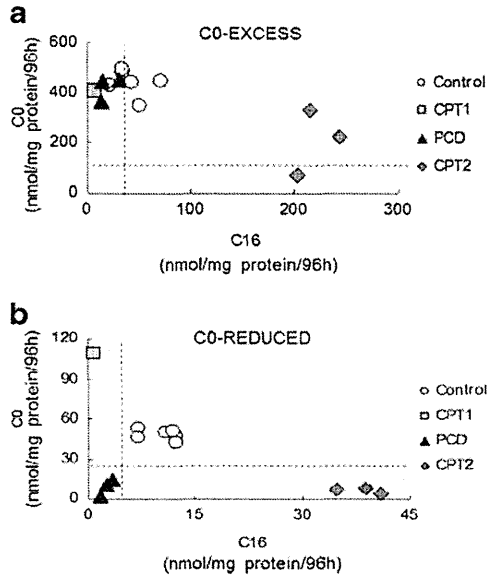

Fig. 4 Intracellular C0 and C16 correlation in patients with carnitine cycle disorders. **a** C0-excessive condition (−*E*); **b** C0-reduced condition (−*R*). *open circle*: healthy control ($n=6$); *closed triangle*: PCD ($n=3$); *closed square*: CPT1 deficiency ($n=1$); *closed diamond*: CPT2 deficiency ($n=3$). Cells were incubated in experimental medium with 400 or 10 μmol/L of free carnitine and 200 μmol/L of palmitic acid. After 96-h incubation, cells were harvested, and intracellular free carnitine (C0) and palmitoylcarnitine (C16) were extracted using Folch method and measured using MS/MS. Data of mean values of triplicates are presented (nanomoles per milligram protein per 96 h)

It is known that fibroblasts and muscle and cardiac cells have a high-affinity, low-capacity transporter system [22], and carnitine concentrations in the tissues are much higher than those in serum [23]. Analysis of intracellular C0 and ACs is more relevant for the diagnosis of PCD and CPT1 deficiency because it was shown that C0 was decreased in PCD and increased in CPT1 deficiency in those tissues. When we analyzed cell lysates with MS/MS after direct sonication, artificial peaks of ACs were detected, and the background peaks of mass spectrum were high and hampered the subsequent analyses (data not shown). Hence, we extracted intracellular ACs using a modified Folch method and analyzed both the intracellular lysate and the extracellular medium. This allowed visualization of clear peaks of C0 and ACs in the intracellular lysate, validating that the Folch extraction can be used for simultaneous quantitation of intracellular C0 and a wide range of ACs (short- to long-chain AC).

Uptake of C0 and abnormalities in ACs were associated with the concentration of C0 in culture medium. In the C0-excess condition, it was hard to differentiate PCD from control

cells. Levels of C0 and C16 were overlapped with those of normal control. On the other hand, in the C0-reduced condition, intracellular C0 was significantly decreased in PCD while being increased in CPT1 deficiency, compared with that in normal control. C0-reduced medium was changed after fibroblasts equilibrated in MEM, and normal control could force to uptake free carnitine in C0-deficient condition while cells with PCD could not uptake sufficiently in that condition. Furthermore, the following fatty acid oxidation cycle interrupted, and C16 also decreased in PCD. This correlation of C0 and C16 in the C0-reduced condition is more informative for the diagnosis of carnitine cycle disorders (Fig. 4b). Since cells with PCD cannot uptake C0 via the cell membrane, the finding of reduction of both C0 and C16 is specific for PCD. In case of CPT1 deficiency, C0 uptake is normal, but it cannot bind acyl-CoA ester, resulting in reduced long-chain acylcarnitine production, and FAO is disturbed. Therefore, the stored intracellular ACs were consumed by FAO, and intracellular C16 as well as total ACs were decreased, and C0 was accumulated in intracellular lysate. In contrast, the AC profile of low level of C0 and high level of C16 is diagnostic for CPT2 deficiency. In this disease, normally transferred long-chain AC cannot be converted back from ACs to acyl-CoA esters and C0, the substrate for FAO. Additionally, the ratio of intracellular and extracellular C0s can sensitively distinguish PCD from control in the C0-reduced medium because carnitine transporter of normal cells was forced to uptake C0 up to physiological level in C0-reduced condition while cells with PCD failed for it. In excessive C0 condition, ratio of intracellular and extracellular C0 was similar to that in normal control and PCD since C0 transfer by passive diffusion across the cell membrane.

In conclusion, the simultaneous analysis of intracellular and extracellular C0 and ACs under the various concentrations of free carnitine in the culture medium is useful for diagnosis of FAO, especially carnitine cycle disorders. This study confirms that the newly modified IVP assay is an easy and safe method to diagnose PCD and CPT1 deficiency.

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ORIGINAL ARTICLE

Missense mutations in the DNA-binding/dimerization domain of *NFIX* cause Sotos-like features

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Sotos syndrome is characterized by prenatal and postnatal overgrowth, characteristic craniofacial features and mental retardation. Haploinsufficiency of *NSD1* causes Sotos syndrome. Recently, two microdeletions encompassing *Nuclear Factor I-X (NFIX)* and a nonsense mutation in *NFIX* have been found in three individuals with Sotos-like overgrowth features, suggesting possible involvements of *NFIX* abnormalities in Sotos-like features. Interestingly, seven frameshift and two splice site mutations in *NFIX* have also been found in nine individuals with Marshall–Smith syndrome. In this study, 48 individuals who were suspected as Sotos syndrome but showing no *NSD1* abnormalities were examined for *NFIX* mutations by high-resolution melt analysis. We identified two heterozygous missense mutations in the DNA-binding/dimerization domain of the NFIX protein. Both mutations occurred at evolutionally conserved amino acids. The c.179T>C (p.Leu60Pro) mutation occurred *de novo* and the c.362G>C (p.Arg121Pro) mutation was inherited from possibly affected mother. Both mutations were absent in 250 healthy Japanese controls. Our study revealed that missense mutations in *NFIX* were able to cause Sotos-like features. Mutations in DNA-binding/dimerization domain of NFIX protein also suggest that the transcriptional regulation is abnormally fluctuated because of *NFIX* abnormalities. In individuals with Sotos-like features unrelated to *NSD1* changes, genetic testing of *NFIX* should be considered.
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INTRODUCTION

Sotos syndrome (MIM #117550) is an overgrowth syndrome characterized by tall stature and/or macrocephaly, distinctive facial appearance and mental retardation.[1] A *de novo* t(5;8)(q35;q24.1) translocation in a patient with Sotos syndrome revealed disruption of *NSD1* at 5q35. Subsequent identification of nonsense, frameshift and submicroscopic deletion mutations of *NSD1* in patients with Sotos syndrome clearly showed that haploinsufficiency of *NSD1* causes Sotos syndrome.[2] *NSD1* encodes nuclear receptor-binding SET domain protein 1, which functions as a histone methyltransferase that activates and represses transcription through chromatin modification.[3] The diagnosis of Sotos syndrome is established by confirming *NSD1* abnormalities,[4] and abnormalities of *NSD1* causes up to 90% of Sotos syndrome cases. However, a part of patients with suspected Sotos syndrome are known to show no abnormalities in *NSD1*,[5] suggesting involvement of another gene.

Recently it was reported that two patients with Sotos-like overgrowth features possessed microdeletions encompassing *Nuclear Factor I-X (NFIX)* at 19p13.2. In addition, a nonsense mutation in *NFIX* was identified in one patient with Sotos-like features.[6] Interestingly, frameshift and donor-splice site mutations were also identified in Marshall–Smith syndrome (MIM 602535) that is osteochondysplasia syndrome characterized by accelerated skeletal maturation, relative failure to thrive, respiratory difficulties, mental retardation and unusual facial features.[7] Therefore, *NFIX* mutations could cause either Sotos-like features or Marshall–Smith syndrome. Whereas the transcripts possessing the nonsense mutation in a patient with Sotos-like features suffered from the nonsense-mediated mRNA decay, transcripts of mutated alleles (by a donor-spice site and two frameshift mutations) in patients with Marshall–Smith syndrome escaped from the nonsense-mediated mRNA decay surveillance and could be translated, suggesting that haploinsufficiency of *NFIX* leads to

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