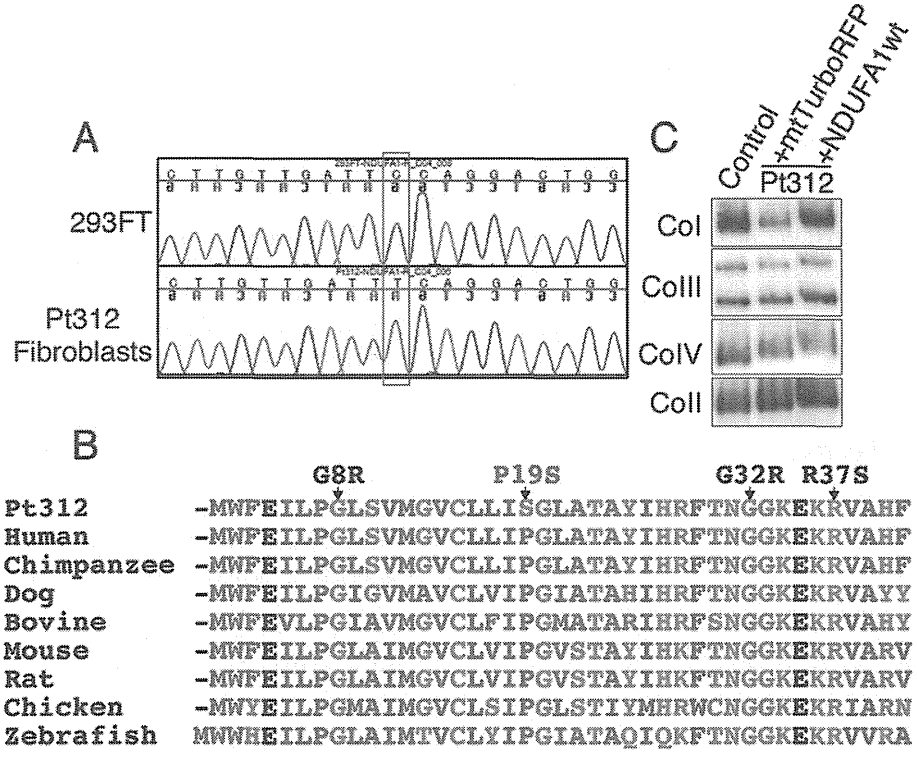

Figure 5. The novel nDNA mutation c.55C>T in *NDUFA1*. (A) Sequence chromatograms showing the c.55C>T (*NDUFA1* p.P19S) mutation in Pt312 and 293FT genomic DNA as a wild-type control. (B) Alignment of amino acid sequences of NDUFA1 subunit between different species shows the high conservation of amino acid Proline 19. G8R, G32R, and R37S show reported pathogenic mutations in *NDUFA1*. (C) Blue native polyacrylamide gel electrophoresis for CI, CII, CIII, and CIV following lentiviral transductions. Transduction of wild-type *NDUFA1* cDNA into Pt312 fibroblasts using recombinant lentivirus rescued complex I assembly levels of the fibroblasts, similar to the transduction of mtTurboRFP into normal fibroblasts (fHDF). As control gene of candidate genes, mtTurboRFP was used which inserted mitochondrial targeting signal sequence to N terminal of TurboRFP protein. By contrast, lentiviral transduction of control mtTurboRFP into Pt312 fibroblasts decreased the assembly level of complex I.

ously found to be associated with Leigh syndrome[20] and MELAS,[21] and this gene region is also reported to be a hot spot for LHON mutations.[22] Mitochondrial *12S rRNA* is a hot spot for mutations associated with aminoglycoside ototoxicity and non syndromic hearing loss, although mutations in this gene have not been reported to cause syndromic mitochondrial disorders.[23] We found that the m.14439G>A mutation altered an evolutionarily conserved proline to a serine in the hydrophilic inner membrane space of the ND6 protein[22] (Fig. 3C). As this mutation was homoplasmic in the patient's fibroblasts and absent from the blood of unaffected parents (Fig. 3A and B), this suggests that it developed de novo.

Exome sequencing in this study identified a single hemizygous change (c.55C>T, p.P19S) in exon 1 of the X-linked *NDUFA1* gene. To date, three missense mutations (G8R,[10] G32R,[24] and R37S[10]) have been reported in *NDUFA1* that are associated with neurological symptoms. *NDUFA1* was shown to interact with the subunits encoded by mtDNA during the complex I assembly process.[11]

Cybrid study is a powerful tool for detecting pathogenicity of either mtDNA or nDNA origin, although patients' cells showing RC enzyme deficiency are inevitable. Nevertheless, a major limitation of this technique is the length of time to establish transmitochondrial cybrids. We would, therefore, propose a systematic approach for diagnosing MRCD that starts with a biochemical enzyme assay and is followed by whole mtDNA sequencing. For patients with no apparent putative mtDNA mutations, whole exome sequencing is a powerful tool to diagnose nuclear gene mutations especially in cases when molecular diagnosis leads to appropriate genetic counseling.

Acknowledgments

We thank T. Hirata and Y. Yatsuka for their technical assistance. This study was supported in part by a grant from the Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation), a Grant-in-Aid for the Development of New Technology from The Promotion and Mutual Aid Corporation for Private

Schools of Japan from MEXT (to Y. O.), a Grant-in-Aid research grants for Scientific Research (A-22240072, B-21390459, A-25242062) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan to M. T., and a Grant-in-Aids (H23-016, H23-119, and H24-005) for the Research on Intractable Diseases (Mitochondrial Disease) from the Ministry of Health, Labour and Welfare (MHLW) of Japan to M. T. and A. O., and a Grant-in-Aids (H23-001, H24-017, H24-071) for the Research on Intractable Diseases from the Ministry of Health, Labour and Welfare (MHLW) of Japan to A. O.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary methods.

Biochemical and Biophysical Research Communications 448 (2014) 175–181

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Functional analysis of iPSC-derived myocytes from a patient with carnitine palmitoyltransferase II deficiency

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ARTICLE INFO

Article history:
Received 13 April 2014
Available online 26 April 2014

Keywords:
Carnitine palmitoyltransferase II deficiency
iPSC
Disease modeling
Rhabdomyolysis
Bezafibrate

ABSTRACT

Introduction: Carnitine palmitoyltransferase II (CPT II) deficiency is an inherited disorder involving β-oxidation of long-chain fatty acids (FAO), which leads to rhabdomyolysis and subsequent acute renal failure. The detailed mechanisms of disease pathogenesis remain unknown; however, the availability of relevant human cell types for investigation, such as skeletal muscle cells, is limited, and the development of novel disease models is required.

Methods: We generated human induced pluripotent stem cells (hiPSCs) from skin fibroblasts of a Japanese patient with CPT II deficiency. Mature myocytes were differentiated from the patient-derived hiPSCs by introducing myogenic differentiation 1 (*MYOD1*), the master transcriptional regulator of myocyte differentiation. Using an *in vitro* acylcarnitine profiling assay, we investigated the effects of a hypolipidemic drug, bezafibrate, and heat stress on mitochondrial FAO in CPT II-deficient myocytes and controls.

Results: CPT II-deficient myocytes accumulated more palmitoylcarnitine (C16) than did control myocytes. Heat stress, induced by incubation at 38 °C, leads to a robust increase of C16 in CPT II-deficient myocytes, but not in controls. Bezafibrate reduced the amount of C16 in control and CPT II-deficient myocytes.

Discussion: In this study, we induced differentiation of CPT II-deficient hiPSCs into mature myocytes in a highly efficient and reproducible manner and recapitulated some aspects of the disease phenotypes of CPT II deficiency in the myocyte disease models. This approach addresses the challenges of modeling the abnormality of FAO in CPT II deficiency using iPSC technology and has the potential to revolutionize translational research in this field.

1. Introduction

β-Oxidation of long-chain fatty acids (LCFA) occurs in the mitochondria with the activity of carnitine palmitoyltransferase II (CPT II; EC2.3.1.21), carnitine-acylcarnitine translocase (CACT), CPT I, and acyl-coenzyme A (CoA) synthetase. These enzymes mediate LCFA transport from the cytosol into the mitochondria. In response to conditions with a high-energy demand, such as intensive exercise, severe infection, and fasting, LCFA transfer is promptly activated [1–4]. *CPT2* maps to chromosome 1p32, spans 20 kb, contains five exons, and encodes the CPT II enzyme. Defects in CPT II enzymatic activity are classified into three clinical categories in humans: lethal neonatal (MIM #608836), severe infantile (MIM #600649), and mild adult-onset (MIM #255110) types. Due to the low enzymatic activity of CPT II, the neonatal and infantile forms result in liver failure, hypoketotic hypoglycemia, and cardiomegaly. The neonatal form causes death within several months. The infantile form has been implicated in cases of sudden infant death syndrome. On the other hand, the adult-onset type

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http://dx.doi.org/10.1016/j.bbrc.2014.04.084
0006-291X/

manifests as recurrent myalgia (muscle pain), rhabdomyolysis, and myoglobinuria, which can cause acute renal failure. CPT II deficiency is generally considered an autosomal recessive disease; however, many cases of symptomatic carriers have been reported [5]. Individuals who carry a *CPT2* mutation [6–9] may develop the clinical features of CPT II deficiency when treated with medications that affect the activity of the remaining wild-type CPT II enzyme.

In this study, we successfully derived human induced pluripotent stem cells (hiPSCs) from a patient with CPT II deficiency, differentiated them into a mature myocyte lineage within 2 weeks in a highly efficient and reproducible manner, and recapitulated some of the disease phenotypes associated with CPT II deficiency. We discuss the opportunities to use iPSC technology for modeling defects in FAO and for evaluating therapeutic regimens for CPT II deficiency.

2. Patient and methods

2.1. Patient

The subject of the current study was a 24-year-old Japanese man whose genetic and clinical presentation has already been described [10]. The patient suffered from acute renal failure induced by rhabdomyolysis and was diagnosed as having adult-onset CPT II deficiency. Skin biopsy samples were obtained from the patient with his written informed consent. This study was approved by the Ethics Committee on hereditary disease, Research of the Graduate School of Medical Sciences, Fukuoka University, and by the Ethics Committee of Kyoto University. The dermal fibroblasts were expanded from skin biopsy explants in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Japan Bioserum, Hiroshima, Japan). Control iPSCs (201B7) were previously established from the facial dermis of a 36-year-old Caucasian woman at the Center for iPS Cell Research and Application (CiRA), Kyoto University [11].

2.2. Methods

2.2.1. Generation of hiPSCs from the patient

CPT II deficiency-specific hiPSCs were derived from the patient by transducing the four reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) or three factors (excluding c-MYC) into skin fibroblasts with retrovirus vectors as previously described [11,12]. In brief, fibroblasts derived from the CPT II-deficient patient were maintained and expanded in DMEM containing 10% fetal bovine serum. The patient fibroblasts were seeded in 6-well plates at 1.0×10^5 cells/well. The next day, the cells were infected with Slc7al lentiviruses with 4 μg/mL polybrene (Nacalai Tesque). Fibroblasts expressing the mouse Slc7al were seeded in 6-well plates at 1.0×10^5 cells/well 1 day before transduction. Equal amounts of four retrovirus-containing supernatants were mixed and supplemented with 4 μg/mL polybrene. Six days after transduction, the fibroblasts were replated onto mitomycin C-treated SNL feeder cells. Thirty days after transduction, iPSC colonies were selected for expansion.

2.2.2. Cell culture

CPT II-deficient hiPSCs were cultured as previously described [11]. The hiPSCs were grown on mitomycin C-treated SNL feeder cells in Primate ES medium (ReproCELL, Kanagawa, Japan) supplemented with 500 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 4 ng/mL recombinant human basic fibroblast growth factor (bFGF, Wako, Osaka, Japan). For routine passaging, hiPSC colonies were dissociated by an enzymatic method with CTK dissociation solution consisting of 0.25% trypsin (Invitrogen), 0.1% collagenase IV (Invitrogen), 20% knockout serum replacement (KSR, Invitrogen), and 1 mM $CaCl_2$ in PBS (Nacalai Tesque) and split at a ratio between 1:3 and 1:6.

2.2.3. Embryoid body (EB) formation

For EB formation, a 10-cm plate containing hiPSCs was rinsed with PBS and treated with 1 mg/mL type IV collagenase (Invitrogen) in DMEM for 10 min at 37 °C. The collagenase was rinsed away with PBS and replaced with undifferentiation medium. The cells were then scraped off with a cell scraper (IWAKI, Tokyo, Japan), dissociated by pipetting, and distributed into a low attachment 6-well plate (Corning, Tokyo, Japan) containing knockout-DMEM (Invitrogen) supplemented with 20% KSR, 0.1 mM non-essential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 500 U/mL penicillin/streptomycin, and 0.55 mM 2-mercaptoethanol (Invitrogen). After 8 days as a floating culture, the EBs were transferred to gelatin-coated plates and cultured in the same medium for another 8 days.

2.2.4. Teratoma formation

The undifferentiated iPSCs were harvested using CTK dissociation solution, collected, and centrifuged, and the pellets were resuspended in DMEM/F12 (Invitrogen). A quarter of the iPSCs from a confluent 10-cm plate was injected into the testes of a non-obese diabetic/severe combined immunodeficient (NOD-SCID mouse, CLEA, Tokyo, Japan). Nine to 12 weeks after injection, the tumors were dissected and fixed with PBS containing 4% paraformaldehyde (PFA). Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin.

2.2.5. Mutational analysis of the CPT2 in patient-derived iPSCs

Overlapping PCR primers that targeted CPT2 exons were designed to cover the entire coding region (Table 1; GenBank accession No. M58581). The PCR protocol was as follows: 30 cycles of 1 min at 94 °C for denaturation, 1 min at 60 °C for annealing, and 1 min at 72 °C for extension, followed by 1 cycle of 10 min at 60 °C for completion. Each PCR product was sequenced on an automated DNA sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems Hitachi, Tokyo, Japan) by using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA) and the sequencing primers listed in Table 1.

2.2.6. Induction of hiPSCs into skeletal muscle cells

We used our previously reported method in which *MYOD1* overexpression in undifferentiated hiPSCs efficiently and reproducibly induces differentiation into mature skeletal muscle cells within 10 days [13]. Briefly, we transduced a self-contained Tet-inducible MYOD1 expressing *piggyBac* vector (Tet-MYOD1 vector) and transposase into CPT II-deficient iPSCs by lipofection. This system allows the indirect monitoring of induced *MYOD1* expression in response to doxycycline (Dox) by co-expression of a red fluorescent protein (mCherry). It was also reported that low glucose culture conditions purified the cardiomyocytes from mouse and human iPSC differentiation cultures by selecting only cardiomyocytes, based on the findings of the substantial biochemical differences in glucose and lactate metabolism between cardiomyocytes and undifferentiated iPSCs [14]. We used a similar strategy to increase the purity of generated myocytes and cultured the hiPSC-derived differentiated cells with low glucose media (1.0 g/L) for an additional day after 10 days of myocyte induction by *MYOD1* overexpression. The low-glucose medium was composed of MEM (Sigma, St. Louis, MO) containing 0.4% bovine serum albumin (Sigma), 0.4 mM L-carnitine (Sigma), 0.2 mM unlabeled palmitic acid (Nacalai Tesque), and 500 U/mL penicillin/streptomycin. For

Table 1
Oligonucleotide sequences, related to Fig. 1. Sequences of primers used in this study.

Gene	Forward primer: 5′ to 3′	Reverse primer: 5′ to 3′
hOCT4Tg	GCTCTCCCATGCATTCAAACTGA	CCCTTTTTCTGGAGACTAAATAAA
hSOX2 Tg	TTCACATGTCCCAGCACTACCAGA	GACATGGCCTGCCCGGTTATTATT
hKLF4 Tg	CCACCTCGCCTTACACATGAAGA	GACATGGCCTGCCCGGTTATTATT
hcMYC Tg	ATACATCCTGTCCGTCCAAGCAGA	GACATGGCCTGCCCGGTTATTATT
hOCT4 Total	CCCCAGGGCCCCATTTTGGTACC	ACCTCAGTTTGAATGCATGGGAGAGC
hSOX2 Total	TTCACATGTCCCAGCACTACCAGA	TCACATGTGTGAGAGGGGCAGTGTGC
hKLF4 Total	GATTACGCGGGCTGCGGCAAAACCTACACA	TTAAAAATGTCTCTTCATGTGTAAGGCGAG
hcMYC Total	ATACATCCTGTCCGTCCAAGCAGA	TCACGCACAAGAGTTCCGTAGCTGTTCAAG
CPT2 exon1	CGGCCTTGTGTTTAGACTCC	CTTCCAGATTAGGGGCTGTG
CPT2 exon2	GCCTTACACTGACCCTGCTT	AGGTTCTGGGTTCCTGGAGA
CPT2 exon3	TTCCAGGTTTTAGGGCTATG	GGAGGATGAGACGTTACTTC
CPT2 exon4	TAGGGACAGCATTAACATTT	TGGCCTTGTCATCAGTGAAG
CPT2 exon4	GTCCCAGTATTTTCGGCTTT	TGTGGGACAAGTGGACAAGG
CPT2 exon4	GAGTTTCCCCTGGCATACCT	GCCTCCTCTCTGAAACTGGA
CPT2 exon4	ACAGCTGCTAAGGAAAAGTT	CAAGACCCAAGGGCATGCTC
CPT2 exon5	CTGAGACGCTGGTTTTCCA	GGTAGCTTTTCATCTGCCCA

immunostaining analyses of hiPSC-derived myocytes, human myosin heavy chain (MHC) antibody (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Samples were observed under an inverted type fluorescence phase-contrast microscope (BZ-9000E; Keyence, Osaka, Japan).

2.2.7. In vitro probe assay of AC profiles

hiPSC-derived myocytes were cultured in a 6-well plate for 96 h with 1 mL medium A composed of MEM, 0.4% bovine serum albumin, 0.4 mM L-carnitine, 0.2 mM unlabeled palmitic acid, and 1% penicillin/streptomycin without L-glutamine, or medium B composed of medium A supplemented with 0.4 mM bezafibrate (Sigma) [15]. Cultured cells were incubated with medium A or B at 38 °C for 96 h to determine the effects of heat stress on mitochondrial FAO.

2.2.8. Quantitative acylcarnitine analysis

Acylcarnitine in the culture supernatant was analyzed by MS/MS (API 3000; Applied Biosystems).

2.2.9. Heat stimulation

Differentiated myocytes on culture day 9 were subjected to heat stress at 38 °C on a hot plate.

2.2.10. Regulated PCR array for skeletal muscle-related genes

To analyze the expression of skeletal muscle-related genes, we performed a regulated PCR array. For first-strand cDNA synthesis, 1 μg total RNA was reverse-transcribed in a 20-μl reaction mix and the RT^2 First Strand Kit (RT^2 Profiler PCR Array, SuperArray Bioscience, Frederick, MD) according to the manufacturer's instructions. qRT-PCR was performed with a CFX96 (Bio-Rad, Hercules, CA) and universal cycling conditions (10 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C for 40 cycles). The fold change in gene expression was determined by the comparative cycle Ct (ΔΔCt) method. Statistical calculations were based on the web-based RT^2 Profiler PCR Array Data Analysis (SuperArray Bioscience).

2.2.11. Microarray analysis

aRNA preparation, fragmentation, hybridization, and scanning of the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) were performed according to manufacturer's protocols. Labeled aRNA was prepared with the GeneChip 3′IVT Express Kit (Affymetrix). Briefly, cDNA was generated from total RNA (100 ng), using reverse transcriptase and a T7-oligo (dT) primer. After second-strand cDNA synthesis, the cDNA was converted to aRNA by an *in vitro* transcription reaction with biotin-labeled ribonucleotides and T7 RNA polymerase. After synthesis, the aRNA was purified to remove enzymes, salts, and unincorporated nucleotides. The concentration of cRNA was determined from the absorbance at 260 nm in a UV spectrophotometer. The aRNA was fragmented at 94 °C in fragmentation buffer (Affymetrix). The samples were hybridized to the GeneChip(R) Human Genome U133 Plus 2.0 Arrays at 45 °C for 16 h with rotation (60 rpm) in an oven. The arrays were automatically washed and stained with the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The Probe Array was scanned using a GeneChip Scanner 3000 7G (Affymetrix). Intensity data and the CHP files were generated by Affymetrix GeneChip Command Console Software and Affymetrix Expression Console Software.

3. Results

3.1. Generation of CPT II-deficient iPSCs (CPTIID-iPSCs) from patient fibroblasts

The skin fibroblasts were converted into iPSCs after transduction with four retroviral vectors encoding OCT4, SOX2, KLF4, and c-MYC, or with three vectors (excluding c-MYC). Quantitative reverse-transcription PCR was used to evaluate the CPTIID-iPSC clones with repression of the exogenously introduced genes analyzed as the ratio of transgene (Tg) expression to total (endogenous and transgene) expression (Table 1). Based on these analyses, the iPSC clone with the highest level of repression was selected for further experiments. This clone exhibited characteristic human embryonic stem cell (ESC) morphology (Fig. 1A), expressed pluripotency markers, including OCT4, NANOG, SOX2, SSEA4, TRA-1-60, TRA-1-81, and alkaline phosphatase (AP) activity (Fig. 1B), and had a normal karyotype (Fig. 1C). The pluripotent properties of CPTIID-iPSCs were also assessed using embryoid body (EB) and teratoma formation upon intratesticular injection of undifferentiated CPTIID-iPSCs into NOD-SCID mice (Fig. 2A and B). Genetic identity was confirmed by STR analyses of the patient fibroblasts and iPSCs (data not shown). Mutation analysis of the causative gene revealed that the patient had compound heterozygous mutations in the *CPT2* [10]. Genomic analysis showed that both CPTIID-iPSCs and their parental fibroblasts possessed mutant CPT2 alleles (Fig. 1D). Sequencing from the 5′ and 3′ ends showed a CT deletion in the TCT at codon 408 (1223delCT), resulting in a stop signal at codon 420, and a sense mutation of arginine to cysteine at codon 631 (1891C→T; R631C). These results suggest that disease-specific iPSCs can be generated from the skin fibroblasts of a CPT II-deficient patient.

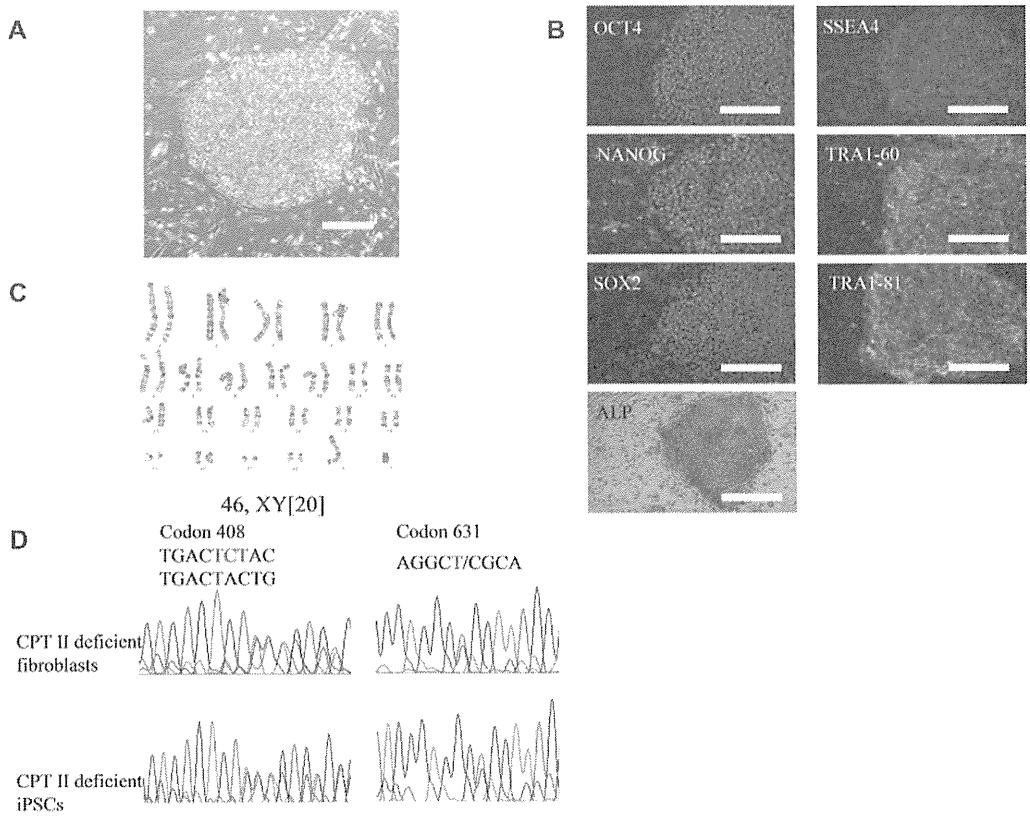

Fig. 1. Generation of iPSCs from a patient with CPT II deficiency. (A) Typical image of human embryonic stem cell (ESC)-like colony. Scale bars: 50 μm. (B) Immunocytochemistry for OCT4, NANOG, SOX2, SSEA4, TRA1-60 and TRA1-81, and the examination of alkaline phosphatase (AP) enzyme activity. Nuclei were stained with Hoechst 33342 (blue). Scale bars: 100 μm. (C) Karyotype analyses of CPT II-deficient iPSCs. (D) Mutational analyses of CPT II-deficient iPSCs and their parental fibroblasts. Sequencing from the 5′ and 3′ ends reveals a CT deletion from the TCT at codon 408, and an arginine to cysteine substitution at codon 631.

3.2. Differentiation of CPTIID-iPSCs into mature myocytes

We next examined whether the patient-derived iPSCs could be differentiated into myocytes, the target cell type of CPT II deficiency. We recently reported a highly efficient myocyte differentiation method based on overexpression of the *MYOD1* gene, a master regulator of myocyte lineage differentiation, in undifferentiated hiPSCs [13]. Tohyama et al. reported a non-genetic method for purifying cardiomyocytes in mouse and human iPSC differentiation cultures [14]. Their strategy is based on the substantial biochemical differences in glucose and lactate metabolism between cardiomyocytes and non-cardiomyocytes, including undifferentiated iPSCs. We used a combination of these strategies to generate myocytes from CPTIID-iPSCs. We transduced a Tet-MYOD1 vector and transposase into CPTIID-iPSCs by lipofection. We forced expression of *MYOD1* with Dox in undifferentiated CPTIID-iPSCs for 10 days. We then used low glucose medium for an additional day to select myocytes. After culture in low glucose medium, the remaining undifferentiated cells disappeared, and the differentiated cells survived, yielding myocyte generation at 50%–60% induction efficiency.

We confirmed the presence of mature myocytes by staining with anti-human MHC antibody (Fig. 3A). Electron microscopy revealed that differentiated myocytes derived from CPTIID-iPSCs had myofibrils containing mature myosin fibers and Z line-like structures (Fig. 3B). We also performed a PCR array and unsupervised clustering to generate myogenic gene profiles for myocytes differentiated from CPTIID-iPSCs, myocytes from control iPSCs (201B7), undifferentiated CPTIID-iPSCs, and undifferentiated 201B7 cells (Fig. 3C, Table 2). We confirmed the upregulation of markers of skeletal muscle contractility, skeletal myogenesis, and skeletal muscle autocrine signaling in myocytes differentiated from CPTIID-iPSCs compared to undifferentiated CPTIID-iPSCs. The expression patterns of muscle-related genes also differed between myocytes derived from CPTIID-iPSCs and control myocytes. These results suggest that mature myocytes can be efficiently generated from CPTIID-iPSCs by introducing a master transcriptional regulator of myocyte differentiation, MYOD1, and culturing in low glucose conditions.

3.3. Acylcarnitine (AC) profiles of the CPT II-deficient myocytes

An acylcarnitine profile determined by tandem mass spectrometry is essential for the definitive diagnosis of CPT II deficiency [15]. We thus examined the profile in myocytes differentiated from CPTIID-iPSCs. CPT II-deficient myocytes accumulated more C16 (palmitoylcarnitine) than did control myocytes (Fig. 4). Results were similar in myocytes differentiated from other iPSC clones from the same patient in this study (data not shown). These data indicated that patient-derived iPSCs recapitulated one of the clinical features of CPT II deficiency.

We previously reported that fibroblasts from patients with LCFA β-oxidation disorders, including CPT II deficiency, were more susceptible to heat stress in comparison to the fibroblasts of patients with medium-chain fatty acid β-oxidation disorders or healthy controls [15]. We thus investigated the effects of heat stress on myocytes derived from CPTIID-iPSCs and found that this treatment significantly increased C16 in CPT II-deficient cells, but not in controls (Fig. 4). We also demonstrated that bezafibrate, an agonist of peroxisome proliferator-activated receptor (PPAR), restores FAO activity in fibroblasts

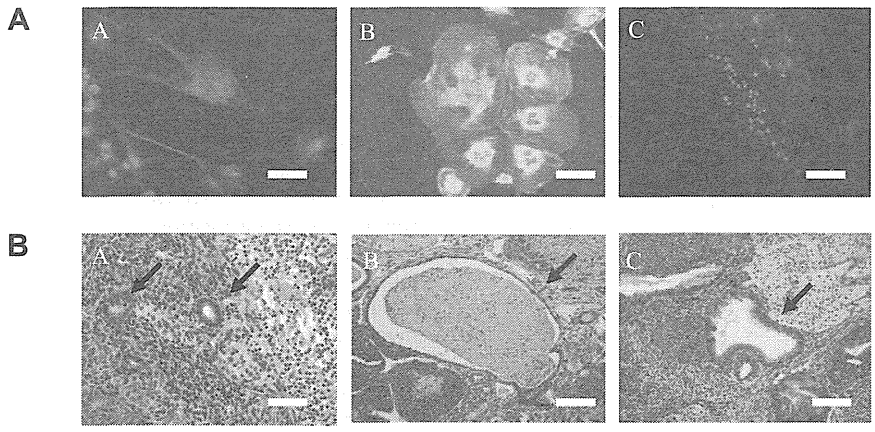

Fig. 2. Embryoid body (EB)- and teratoma-mediated differentiation of CPT II-deficient iPSCs. (A) Immunostaining of EBs generated from CPT II-deficient iPSCs for TUJ1 (ectoderm, A), α-SMA (mesoderm, B), and SOX17 (endoderm, C). Nuclei were stained with Hoechst 33342 (blue). Scale bars: 100 μm. (B) Hematoxylin and eosin staining of histological sections of teratomas derived from CPT II-deficient iPSCs. Neural tissues (ectoderm, A), cartilage (mesoderm, B), gut-like epithelia (endoderm, C). Scale bars: 100 μm.

with CPT II deficiency [15]. We investigated the effects of bezafibrate on myocytes derived from CPTIID-iPSCs. At 37 °C and 38 °C, bezafibrate decreased C16 levels in controls and CPT II-deficient myocytes. In the CPT II-deficient myocytes, bezafibrate at 37 °C reduced C16 levels to those observed in controls (Fig. 4). These results suggest that mature myocytes derived from CPTIID-iPSCs recapitulate some of the phenotypes associated with CPT II deficiency.

4. Discussion

Rhabdomyolysis occurs after exhaustive exercise or severe infection without trauma or drugs in CPT II deficiency. The adult-onset form presents with myoglobinuria and myalgia, frequently leading to acute renal failure. Rhabdomyolysis may repeatedly develop within the same families. Hereditary rhabdomyolysis is often caused by compromised enzymatic activity associated with LCFA metabolism.

Accurate experimental modeling of the disease and its response to clinical intervention are hampered due to a lack of appropriate animal models. To address this issue, we sought to derive iPSCs from a patient with CPT II deficiency, who had a history of repetitive rhabdomyolysis and acute renal failure. There have been no reports of derivation of iPSCs from patients with FAO disorders, including CPT II deficiency, and to the best of our knowledge, we are the first to report the successful generation of iPSCs from a CPT II-deficient patient.

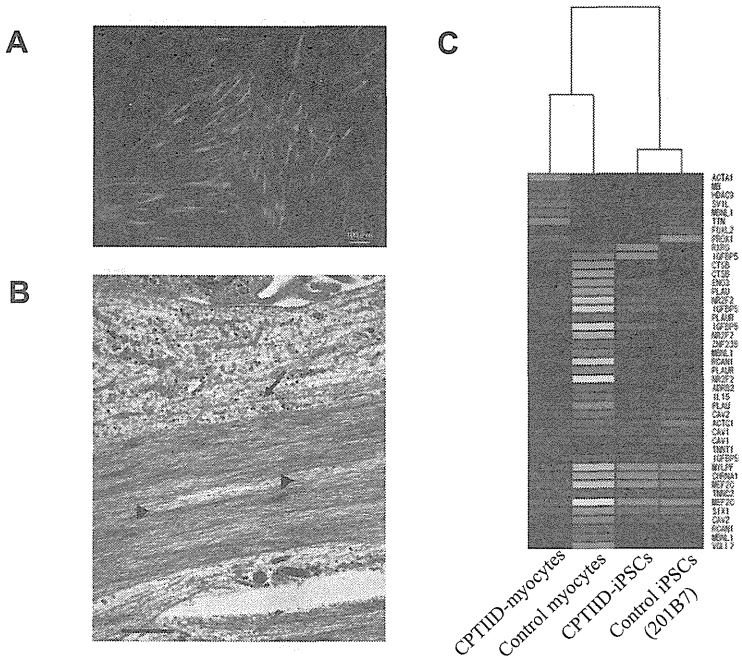

Fig. 3. Directed differentiation of CPT II-deficient iPSCs into skeletal muscle cells. (A) Immunocytochemistry of the myocytes derived from CPT II-deficient iPSCs with anti-human Myosin Heavy Chain (MHC) antibody. Nuclei were stained with Hoechst 33342 (blue). Scale bar: 100 μm. (B) Structural analysis of myocytes differentiated from CPT II-deficient iPSCs by electron microscopy. A red arrow indicates myofibrils. Black arrowheads indicate immature Z lines. A black arrow indicates myosin fibers. Scale bar: 500 nm. (C) Characterization of iPSC-derived myocytes. Myogenic gene profiles and unsupervised clustering based on markers associated with myocytes in undifferentiated iPSCs and differentiated myocytes. CPTIID-myocytes, control myocytes, CPTIID-iPSCs, and control iPSCs. Green indicates up-regulated genes and red indicates down-regulated genes. Up-regulated genes were identified by changes of at least 2-fold. CPTIID-myocytes; Myocytes differentiated from CPTIID-iPSCs.

Table 2
The expression of markers for skeletal muscle contractility, skeletal myogenesis, and skeletal muscle autocrine signaling in the myocytes differentiated from CPT II-deficient iPSCs. Gene expression was evaluated using quantitative real time RT-PCR as described in Materials and Methods; GAPDH was the internal control. Results are shown as fold change relative to control samples of undifferentiated CPT II-deficient iPSCs.

	Symbol	Fold change
Skeletal muscle contractility	ATP2A1	4.6
	CAV3	22.0
	DES	33.5
	DMPK	2.1
	DYSF	2.2
	LMNA	3.9
	MB	8.3
	MYH1	86.5
	MYOT	4.0
	NEB	4.3
	SGCA	4.1
	TNNC1	4.1
	TNNI2	8.4
	TNNT1	2.2
	TNNT3	35.7
	TTN	6.4
Skeletal myogenesis	ACTA1	7.4
	CAPN2	3.1
	CAV1	3.8
	IGF1	5.7
	IGFBP3	5.9
	IGFBP5	15.1
	MEF2C	43.3
	MSTN	74.8
	MUSK	11.0
	MYOG	477.3
	PAX3	2.7
Skeletal muscle autocrine signaling	IGF1	5.7
	IGF2	59.7
	IL6	3.2

Many somatic cell types have been generated from iPSCs, but there have been limited reports describing directed differentiation into myocyte lineages [13]. Our protocol was used to generate CPT II-deficient myocytes from CPTIID-iPSCs [13]. These processes were validated by the detection of genes involved in skeletal muscle development and function in CPT II-deficient myocytes. Culture in low glucose caused the death of undifferentiated iPSCs, which require large quantities of glucose, while the differentiated cells require limited glucose and produce lactic and pyruvic acids to more effectively obtain energy by mitochondrial oxidative phosphorylation. Thus, by using low-glucose medium, we increased the differentiation efficiency of iPSC-derived myocytes.

Yamaguchi et al. showed that an *in vitro* AC profiling assay could reliably detect various FAO disorders, consistent with reports from other groups [16–18]. In particular, C16 accumulation was found to be a reliable biomarker that could be used to diagnose CPT II deficiency. Consistent with these findings, our hiPSC-derived myocytes mimicked the metabolic characteristics of the disease.

The effects of PPAR agonists on mitochondrial FAO have been extensively examined [19]. Bezafibrate has proven efficacy in the treatment of long-chain FAO disorder [20–24]. We found that bezafibrate reduced long-chain ACs more effectively in myocytes from patient-derived iPSCs than in those from control hiPSCs. These findings indicate that bezafibrate could be a therapeutic drug for CPT II deficiency and other mitochondrial FAO disorders.

In conclusion, we successfully derived disease-specific iPSCs from a patient with CPT II deficiency and differentiated them into myocytes. Our results suggest that cellular models using patient-derived iPSCs will be of significant benefit for research groups studying CPT II deficiency-related diseases, and that these iPSC disease models may be a valuable resource for testing novel therapeutic strategies for these disorders.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We thank Ms. Y Sasaguri for technical and secretarial assistance and Dr. S Mae for technical support and helpful discussion. This work was supported in part by research funds no. 101001 (H.K.) and no. 122502 (T.Y.) from the Central Research Institute of Fukuoka University, a Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science (23791200, 25870995

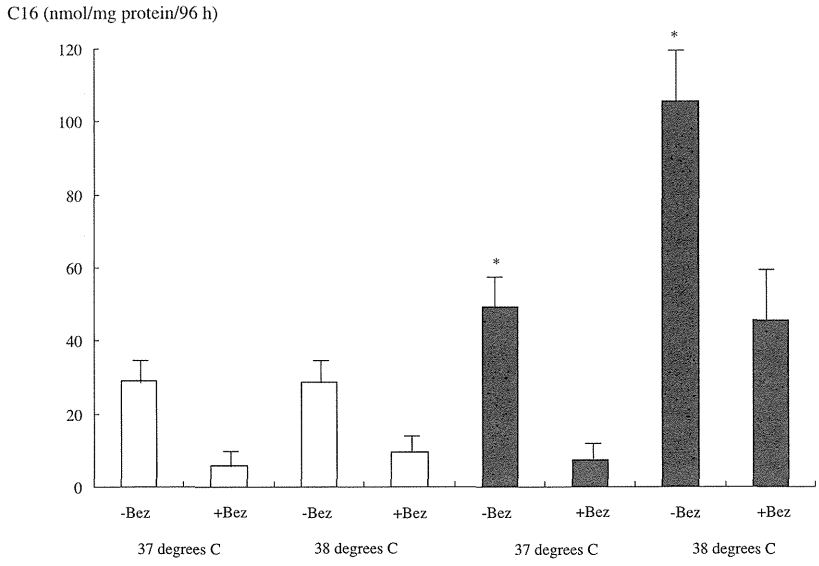

Fig. 4. Acylcarnitine (AC) profiles in culture medium of iPSC-derived myocytes loaded with palmitic acid after bezafibrate (Bez) treatment. Data are shown as mean ± SD (nmol/mg protein/96 h) ($n = 3$) White bars: Myocytes from control iPSCs; Black bars: Myocytes from CPT II-deficient iPSCs. Statistically significant differences between 37 °C Bez (-) of control and 37 °C or 38 °C Bez (-) of patients shown as $^*p < 0.05$.

to T.Y.), Takahashi Industrial and Economic Research Foundation, and Kaibara Morikazu Medical Science Promotion Foundation. This work was also supported by the Japan Society for the Promotion of Science (JSPS) through its "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)" to K.O. and by the Japan Science and Technology Agency (JST) through its research grant "Core Center for iPS Cell Research, Research Center Network for Realization of Regenerative Medicine" to K.O.

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Biochimica et Biophysica Acta 1840 (2014) 1355–1359

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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Diagnosis and molecular basis of mitochondrial respiratory chain disorders: Exome sequencing for disease gene identification ☆,☆☆

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ARTICLE INFO

Article history:
Received 30 September 2013
Received in revised form 13 January 2014
Accepted 14 January 2014
Available online 24 January 2014

Keywords:
Mitochondrial respiratory chain disorder
Blue native polyacrylamide gel
Electrophoresis
Exome sequencing
Narrowing down protocol

ABSTRACT

Mitochondrial disorders have the highest incidence among congenital metabolic diseases, and are thought to occur at a rate of 1 in 5000 births. About 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities, while the rest occur due to defects in genes encoded in the nucleus. The most important function of the mitochondria is biosynthesis of ATP. Mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorder, as respiratory chain complexes serve a central role in ATP biosynthesis. By next-generation sequencing of the exome, we analyzed 104 patients with mitochondrial respiratory chain disorders. The results of analysis to date were 18 patients with novel variants in genes previously reported to be disease-causing, and 27 patients with mutations in genes suggested to be associated in some way with mitochondria, and it is likely that they are new disease-causing genes in mitochondrial disorders. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

1.1. Mitochondrial disorders

Mitochondrial disorders have the highest incidence among congenital metabolic disorders, and are thought to occur at a rate of 1 in 5000 births [1]. The common view of mitochondrial disorders is that they include mitochondrial encephalopathy and myopathy, with onset due to mitochondrial DNA defects inherited through the maternal line. In fact, however, only about 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities [2,3], while the rest occur due to defects in genes encoded in the nucleus. Most cases are sporadic (do not have a clear genetic association), and a majority of cases resulting from nuclear gene abnormalities are autosomal recessive. Mitochondrial DNA has a circular structure with a length of 16.6 kbp, and encodes only 13 proteins [4]. These 13 proteins are part of the structural composition of complex I (7 proteins), complex III (1 protein), complex IV (3 proteins) and complex V (2 proteins) in the respiratory chain. They do not include any complex II structural proteins. The remaining genes encoded in mitochondrial DNA are 22 tRNAs and two ribosomal RNAs, and mitochondrial disorders due to defects in these RNAs have also been reported. Meanwhile, a certain amount of the gene products encoded in the nucleus exists in the mitochondria, and roughly 1500 are thought to serve important roles in mitochondrial function [5]. In this analysis, we focused on mitochondrial disorders thought to occur due to defects in genes encoded in the nucleus. Mitochondria have many functions, one of the most important being biosynthesis of energy (ATP), and we assume for the following discussion that mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorders (MRCD), as respiratory chain complexes [6] serve a central role in ATP biosynthesis.

1.2. Mitochondrial disorders of nuclear origin

As stated above, of the approximately 1500 genes encoded in the nucleus that are thought to be involved in biosynthesis and mitochondrial function, more than 100 have been reported to be causes of mitochondrial disorders [7–9] (Table 1). Among these, about 90% of genes have an autosomal recessive inheritance pattern, and only a small portion

Abbreviations: MRCD, mitochondrial respiratory chain disorder; BN-PAGE, blue native polyacrylamide gel electrophoresis; iPS, induced pluripotent stem cells; LIMD, lethal infantile mitochondrial disease; LCSH, Long Contiguous Stretch of Homozygosity

☆☆ This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

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http://dx.doi.org/10.1016/j.bbagen.2014.01.025

Table 1
The genetic basis of MRCD.

mtDNA mutations: 35/37 genes	
tRNAs, subunits, rRNAs, and deletions & duplications	
Nuclear mutations: 117 genes	
Nuclear-encoded subunits: 27/~80 genes	**mtDNA replication: 5 genes**
Complex I: *NDUFV1, 2, NDUFB3, 9 NDUFA1, 2, 9, 10, 11, 12, NDUFS1, 2, 3, 4, 6, 7, 8*	*POLG, POLG2, C10orf2, MPV17, AGK*
Complex II: *SDHA, SDHB, SDHC, SDHD*	**mtDNA expression: 24 genes**
Complex III: *UQCRB, UQCRQ*	*LRPPRC, TACO1, MTPAP, MRPS16, MRPS22,MRPL3, GFM1,TSFM,TUFM,TRMU,C12orf65,MTFMT, DARS2, RARS2,YARS2,SARS2,AARS2,HARS2,MARS2,EARS2, RMND1, MTO1, FARS2, GFM2*
Complex IV: *COX6B1, COX4I2, COX7B*	
Complex V: *ATP5E*	
Import, processing, assembly: 38 genes	**Nucleotide transport, synthesis: 9 genes**
Complex I: *C8orf38, C20orf7, NDUFAF1, F2, F3, F4, FOXRED1, NUBPL, ACAD9, AIFM1*	*SLC 25A4, SLC25A3, TYMP, DGUOK, TK2, PUS1, SUCLA2, SUCLG1, RRM 2B*
Complex II:*SDHAF1, SDHAF2*	**Membrane composition: 14 genes**
Complex III:*BCS1L, HCCS, TTC19*	*COQ2, COQ6, COQ9, PDSS1, PDSS2, CABC1, SERAC1, MPC1, NMT, TAZ, CYCS, OPA1, MFN2, DNM1L*
Complex IV:*SURF1, SCO2, SCO1, COX10, COX15, ETHE1, FASTKD2, C2orf64, C12orf62*	
Complex V:*ATPAF2, TMEM70*	
Multiple:*TIMM8A, SPG7, HSPD1, AFG3L2, DNAJC19, GFER*	
Iron/FeS: *FXN, ISCU, GLRX5, ABCB7, NFU1, BOLA3*	
117 nuclear gene defects	Categories are based on D.R Thorburn's paper[7]

95: autosomal recessive·
10: autosomal dominant·
5: recessive or dominant·
7: X-linked·

have a dominant inheritance pattern [10]. There have also been seven reported cases of mitochondrial disorders from defects in genes encoded by the X chromosome. By function, these include genes involved in the structural composition of the complexes and mitochondrial biosynthesis, genes involved in membrane composition, genes involved in the synthesis and transport of nucleic acids, genes involved in regulating the expression of mitochondrial DNA, and genes involved in mitochondrial DNA replication.

We have actively analyzed the exomes of patients with MRCD in order to identify the cause. Here, we briefly describe our project and discuss the results of exome analyses performed to date, touching on some of the problems that have been encountered.

2. Outline of exome analysis project for MRCD patients

Fig. 1 outlines our current project. It is supported by the Ministry of Education, Culture, Sports, Science and Technology's Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) (http://www.cell-innovation.org/english/html/program/theme_010_okazaki.html). First, analyses of enzyme activity [11], quantity and size were performed using fibroblasts from patient skin or biopsy specimens from diseased organs of patients suspected of having MRCD in clinical practice [12]. Quantity and size were analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE) [13]. Next, among patients in whom decreased enzyme activity or complex formation abnormalities were seen biochemically, whole exome analysis was performed in those with no known mitochondrial DNA abnormalities, and the obtained candidate causal genes were confirmed at the cellular level by rescue experiment or other methods, such as siRNA experiment. Many patients with mitochondrial disorders have primary symptoms in the central nervous system, but brain biopsy in these patients is untenable. Therefore, induced pluripotent stem (iPS) cells were created using fibroblasts from the skin of patients from whom informed consent was obtained. These iPS cells were then differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction that occurs specifically in the nervous system, based on the notion that this may lead to treatment at the cellular level and ultimately to treatment in humans.

3. Clinical diagnosis of MRCD

Mitochondria exist in all tissues, and symptoms are presented in various organs and/or pathological entities. In pediatric MRCD, symptoms are broadly divided into: (1) encephalomyopathy symptoms; (2) gastrointestinal/hepatic symptoms; and (3) myocardial symptoms [14]. So-called "mitochondrial encephalomyopathy," which has traditionally been considered the main form of mitochondrial disease, belongs among the relatively mild mitochondrial diseases and occurs mostly in older people. Fig. 2 shows a breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013 [15]. Patients with the traditionally described nerve and muscle symptoms numbered 111 in total, including 50 with Leigh syndrome, 11 with neurodegenerative disorders for which no clear cause could be identified, and 50 with so-called "mitochondrial encephalomyopathy." These 111 patients accounted for 40% of the total of 275 patients. Conversely, other forms accounted for two-thirds of cases, among which were 49 cases of lethal infantile mitochondrial disease (LIMD). Together with non-lethal infantile mitochondrial disease (NLIMD), which follows the same course but in which patients survive beyond 1 year of age, the number reached 71, and was by far the most common clinical diagnosis. LIMD encompasses hyperlactacidemia occurring in the neonatal period together with multiple organ failure. Most cases have poor outcomes, and it is thought that most of these patients died with the cause remaining unknown and no diagnosis established. Next were mitochondrial disorders showing single organ dysfunction only, such as mitochondrial hepatopathy (12%) and cardiomyopathy (7%).

4. Exome analysis of MRCD patients

As most mitochondrial diseases occur sporadically with only a few cases discovered in one family line, linkage analysis using a large pedigree cannot be applied, thus suggesting that we cannot use information on chromosomal localization for causal gene identification. When identifying disease-causing genes using bioinformatics analysis for exome data, knowledge of the inheritance patterns is very important [16]. As approximately 90% of MRCD-causing genes show a recessive mode of

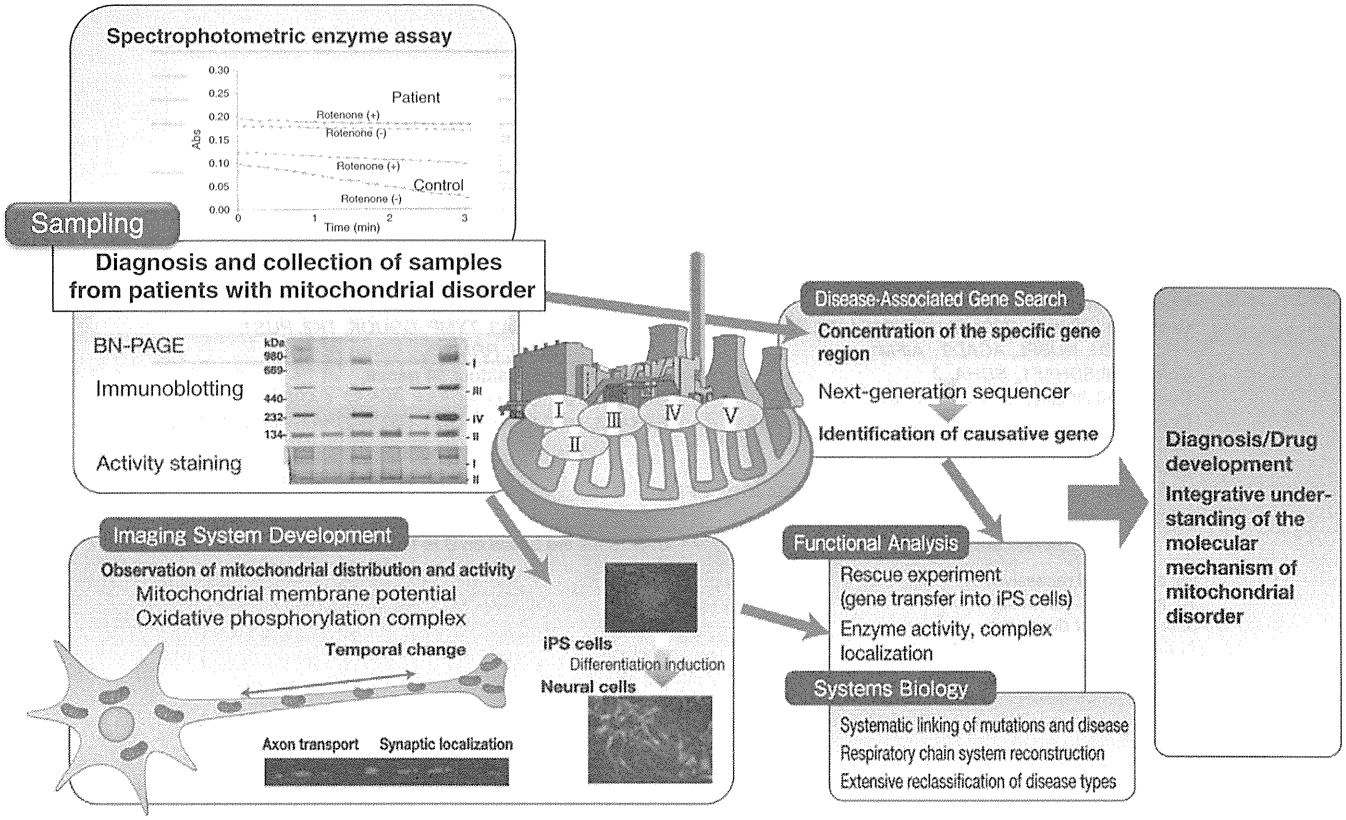

Fig. 1. Outline of exome analysis project for MRCD patients. The first step is 'Sampling', which refers to diagnosis and collection of samples from patients with mitochondrial disorders using both spectrophotometric enzyme assay [11] and BN-PAGE [13]. The next step is 'Disease-Associated Gene Search' using exome analysis. In 'Functional Analysis' and 'System Biology', candidate causal genes are confirmed at the cellular level by rescue experiment or other means. In 'Imaging System Development', induced pluripotent stem cells are created using fibroblasts and differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction. The final purpose of our project is integrative understanding of the molecular mechanisms of mitochondrial disorders.

inheritance (as shown in Table 1), we prioritized such genes as harboring rare variants in a homozygous or compound heterozygous fashion. Low priority is given to the analysis of genes showing mutation in only one allele because patients and healthy control individuals harbored a comparable number of rare heterozygous alleles; we were unable to prioritize dominant-acting genes.

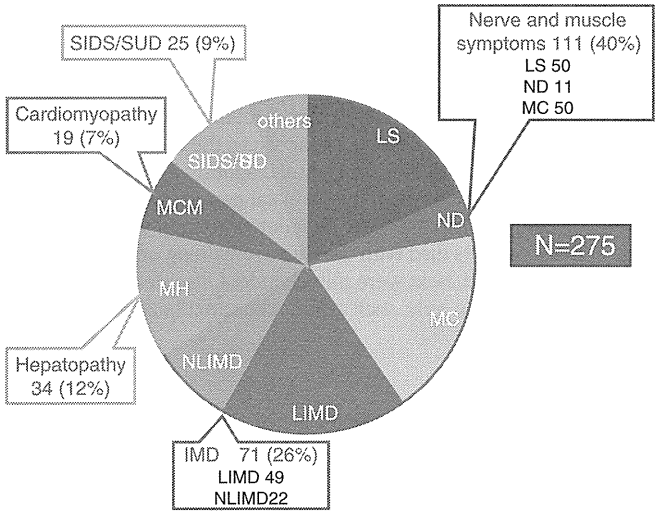

Fig. 2. Breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013. LS, Leigh syndrome; ND, neurodegenerative disorder; MC, mitochondrial cytopathy; IMD, infantile mitochondrial disease (lethal and non-lethal); MH, mitochondrial hepatopathy; MCM, mitochondrial cardiomyopathy; SIDS, sudden infant death syndrome; SUD, sudden unexpected death.

Our current bioinformatics analysis pipeline is as follows: read alignment was performed with a Burrows–Wheeler Aligner (BWA, version 0.7.0) [17] using the 1000 Genomes project phase II reference genome (hs37d5.fa). PCR duplicate reads were removed using Picard (version 1.89) (http://picard.sourceforge.net) and non-mappable reads were removed using SAMtools (version 0.1.19) [18]. After filtering out these reads, the Genome Analysis Toolkit (GATK) version 2.4-9-nightly-2013-04-12-g3fc5478 [19] was used to realign insertions and deletions, and for quality recalibration and variant calling (UnifiedGenotyper). Detected variants were annotated using ANNOVAR (version 2013Feb21) [20] and custom ruby scripts. The effect of the mutations on protein function was assessed by SIFT and GERP using dbNSFP [21]. The positions of mutations were based on RefSeq transcript sequences. Variants were assessed by comparing allele frequencies in the dbSNP135, Exome Sequencing Project (ESP5400) data set, and 1000 Genomes Projects (based on phase 1 release v3 called from 20101123 alignments). As mitochondrial disorders are rare, we excluded variants present in dbSNP with a frequency > 0.1%. After filtering out these variants, the VAAST program [22] was used to create a candidate gene list in each patient showing recessive characteristics.

As stated above, because mitochondrial disease patients have very high heterogeneity, the number of patients sharing the same gene mutation is quite low. Hence, attention should be directed towards removing these mutations from the disease candidates when the same amino acid substitutions are shared among multiple patients in our study, because these variants are highly likely to be SNPs unique to the Japanese population. Using these criteria, we are able to narrow down the number of variants to a mean of several genes for each patient. After listing

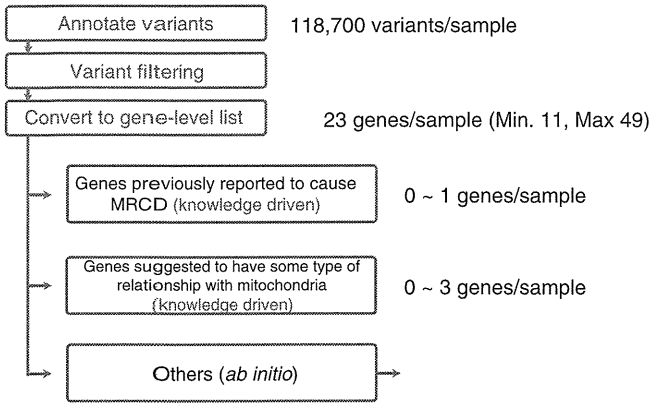

Fig. 3. Narrowing down of gene mutations discovered by exome analysis. After filtering out variants with the methods described in the 'Exome analysis of MRCD patients' section, genes were divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (*ab initio*).

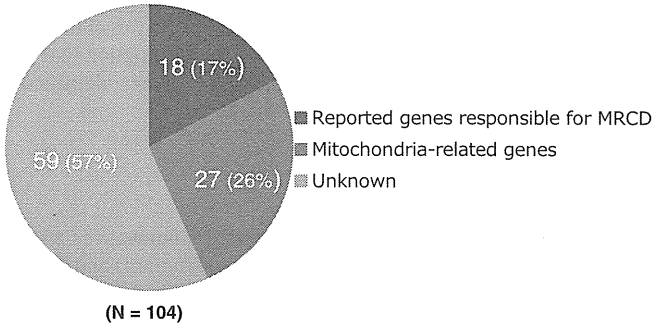

Fig. 4. Candidate genes with exome analysis for MRCD patients. Results of analysis for 104 patients to date (as of January 2013) are shown. Eighteen patients (17%) had variants previously reported to be disease-causing. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria. The remaining 59 patients (57%) are highly likely to contain completely novel disease-causing mutations for which no data have been obtained to date.

these candidate variants, we further investigated whether these variants are located within genes related to mitochondrial function. When genes overlapped with those reported to be related to mitochondrial function, we found that they were likely to be causative genes and were further subjected to experimental analysis such as haplotype phasing or functional assay including rescue experiments. To prepare a list of genes reported to be related to mitochondria, we included genes annotated as somehow related to mitochondria in the UniProt (http://www.uniprot.org/) [23] database, as well as the MitoCarta database (http://www.broadinstitute.org/pubs/MitoCarta/index.html) [24], which includes approximately 1000 gene products listed with the use of shotgun proteomics and mitochondrial localization analysis.

We also investigated whether there is Long Contiguous Stretch of Homozygosity (LCSH) using Affymetrix SNP arrays in a majority of patients. Although no cases of consanguineous marriage were reported in the interviews with the primary physician, about 5% of cases harbor LCSH proven by SNP arrays. When homozygous mutations are localized in these LCSH regions, the mutations are highly likely to be causative of disease.

5. Results of exome analysis for MRCD patients

The variants (mutations) found in the process of narrowing down the gene mutations discovered to date are shown in Fig. 3. These genes were narrowed down to the final candidate genes and divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (*ab initio*). The results of analysis of 104 patients to date (as of January 2013) are shown in Fig. 4. Eighteen patients (17%) had variants previously reported to be disease-causing. Among these 18 patients, one had a homozygote of a previously reported mutation and two had a compound heterozygote of a reported and a novel mutation (data not shown). All other mutations found in this study were new. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria, and it is likely that they are novel disease-causing genes in mitochondrial disorders. Table 2 lists the functions of the genes in these 27 cases. For the remaining 59 cases, each patient has about 20 gene variants that are unique to each patient, and it is necessary to confirm whether any of these mutations can actually cause the disease. These 59 patients are highly likely to contain completely novel disease-causing mutations for which no clues have been obtained to date. The biggest issue we currently face is how to confirm the disease-causing gene from these 20 gene variants for each patient.

6. Conclusion and future prospects

The above describes the progress we have made in exome analysis of neonatal or infantile MRCD patients. While we have identified many candidate genes, the causes of MRCD are extremely diverse and heterogeneous. Thus, in many cases, it is difficult to demonstrate conclusively that a mutation in a candidate gene is the true cause. We have performed analyses focusing on cases in which a biochemical diagnosis was established at the cellular level in addition to clinical symptoms such as enzyme activity and complex formation abnormalities. Nonetheless, confirmation of the causal genes with rescue experiments or other means is difficult. In the future, it will be necessary to increase the case number or search for patients with similar symptoms and similar gene mutations in collaboration with researchers throughout the world. We are currently conducting analyses of pediatric patients with a focus on MRCD, and gene mutations (amino acid substitutions) harbored by patients of the childhood onset type are probably variants conferring major damage on enzyme activity or protein function. Onset is also thought to occur in adulthood rather than in childhood in some cases of milder (hypomorphic: partial loss of function) variants with the same gene defect. As these are thought to include nerve diseases,

Table 2
Functions of new disease-causing candidate genes for MRCD.

MtoX#1	Non-receptor tyrosine kinase
MtoX#2	Acyl-CoA thioesterase
MtoX#3	Fatty acid β oxidation
MtoX#4	tRNA synthetase
MtoX#5	ABC transporter superfamily
MtoX#6	ATR-dependent AMP-binding enzyme family
MtoX#7	Heme biosynthesis
MtoX#8	AAA ATPase family
MtoX#9	Pre-mRNA splicing factor
MtoX#10	Creatine kinase
MtoX#11	Synaptic transmission
MtoX#12	Synthesis of Coenzyme Q
MtoX#13	Heme biosynthetic process
MtoX#14	Citrate synthase family.
MtoX#15	Cholesterol metabolism
MtoX#16	Mitochondrial fission
MtoX#17	Muscle organ development
MtoX#18	Cholesterol biosynthetic process
MtoX#19	Ribosomal protein
MtoX#20	Tumor suppressor
MtoX#21	A component of complex I
MtoX#22	A protease, located in inner membrane
MtoX#23	Regulation of PDH
MtoX#24	Mitochondrial translation
MtoX#25	Queuosine biosynthetic process
MtoX#26	Mitochondrial carrier family
MtoX#27	Methyltransferase superfamilya

mental disorders, and diabetes or other metabolic diseases of unknown cause, we plan to conduct research based on the assumption that such cases include those caused by abnormalities in genes identified in MRCD patients.

Acknowledgements

This work was supported in part by a grant for Innovative Cell Biology by Innovative Technology (Cell Innovation Program) and Support Project from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, by a grant for Strategic Research Centers in Private Universities from MEXT, Japan to Saitama Medical University Research Center for Genomic Medicine, and by Grants-in-Aid for Research on Intractable Diseases (Mitochondrial Disorder) from the Ministry of Health, Labor and Welfare of Japan. Dr. Murayama was supported by the Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics. The authors would also like to thank Dr. Ayako Fujinami, Dr. Kaori Muta, Dr. Emi Kawachi, Dr. Takuya Fushimi, Dr. Keiko Ichimoto, Dr. Tomoko Tsuruoka, Ms. Keio Baba and Ms. Masami Ajima at Chiba Children's Hospital for their support.

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BJD
British Journal of Dermatology

The novel *SLCO2A1* heterozygous missense mutation p.E427K and nonsense mutation p.R603* in a female patient with pachydermoperiostosis with an atypical phenotype

DOI: 10.1111/bjd.12790

Dear Editor, Pachydermoperiostosis (PDP), or primary hypertrophic osteoarthropathy (PHO: MIM 167100), is a rare genetic disease affecting the skin and bones. The major diagnostic criteria include finger clubbing, periostosis, pachydermia and cutis verticis gyrata (CVG). Additional symptoms, including sebaceous hyperplasia, hyperhidrosis and arthropathy, have been reported.[1,2]

Uppal *et al.*[3] discovered that a homozygous mutation in *HPGD*, which encodes 15-hydroxyprostaglandin dehydrogenase (15-PGDH), causes PHO and PDP. However, PHO and PDP are genetically heterogeneous. Exome analysis of PDP in Japanese, Chinese, Caucasian and other races has revealed homozygous mutations in the solute carrier organic anion transporter family member 2A1 (*SLCO2A1*) gene, which encodes prostaglandin transporter (PGT).[4–8] Increased levels of prostaglandin E2 (PGE2) resulting from defective degradation contribute to the pathogenesis of PHO and PDP. A genetic defect in either *SLCO2A1* or *HPGD* can cause PHO and PDP.

In this study, we describe the first observation of a *SLCO2A1* mutation in a female patient.

A 67-year-old woman was referred for *SLCO2A1* mutation analysis. At the age of 43 years, she developed myelopathy of unknown aetiology. She received rehabilitation therapy without medication. A neurologist had examined her muscle weakness at the T-helper 7 level on the right side following a diagnosis of suspected multiple sclerosis. At the age of 64 years, she had multiple seronegative arthralgias but no serious problems. She was referred to Tohoku Kouseinenkin Hospital because of recurring arthralgia and was treated with methotrexate and prednisone. She responded favourably to the medication with alleviation of the pain and decreased serum levels of C-reactive protein. Physical examination revealed finger clubbing and swelling of the large joints, as seen in Figure 1b,c. No skin manifestations, including facial coarseness or greasiness, and no hyperhidrosis were observed. Marked thickening of the scalp (CVG) was not evident. Radiological examination showed the presence of periostosis of the diaphysis of the tibia and fibula (Fig. 1d). No hydrarthrosis was evident. A diagnosis of possible incomplete type of PDP or PHO was made because of minimal pachydermia. She had no history of peptic ulcers or anaemia. Diagnostic imaging and laboratory data revealed no evidence of secondary PDP. She has a healthy son and daughter.

This study was approved by the ethics committee of the National Centre for Child Health and Development and Keio University School of Medicine. The participants provided written informed consent. All exons of *HPGD* and *SLCO2A1* along with sequences adjacent to the exon–intron borders were amplified, sequenced and screened for mutations.[4] Serum and urinary levels of PGE2 were measured with a commercial enzyme immunoassay kit (Cayman, Cayman Biochemical, Ann Arbor, MI, U.S.A.).[4]

We identified compound heterozygous novel mutations c.1279G>A/p.E427K and c.1807C>T/p.R603* in *SLCO2A1* (Fig. 2). We also detected a heterozygous mutation c.1279G>A in her daughter, but she has not developed any triad of PDP.

In her seventh decade, the patient's atypical history showed minimal impact of pachydermia. Serum PGE2 was not detected and her urinary PGE2 was within normal limits (372 pg mL^{-1}). One of the mutations, c.1279G>A (p.E427K), is included within the region of a previously reported deletion, c.1279_1290del12 (p.E427_P430del).[4] Another mutation, c.1807C>T/p.R603*, is detected close to the C-terminus of PGT, resulting in a shortened predicted protein. The loss of function in truncated PGT is consistent with the presence of the p.R603* mutation in another patient, who had the complete type of PDP (manuscript in preparation).

This patient is the first woman with PDP who had an *SLCO2A1* gene mutation. It is unlikely that the mild phenotype of P1 was due to the missense mutation p.E427K. A recent report on PDP in a Chinese family described a homozygous p.A286Qfs*35 frameshift mutation in a male proband who had PDP.[7] Two of the proband's sisters were also homozygous for p.A286Qfs*35, but at ages 42 years and 47 years, they had neither history nor findings suggestive of PDP.[7] Diggle *et al.*[6] reported that two women in two PDP families were homozygous for pathogenic *SLCO2A1* mutations. One had mild finger clubbing but no musculoskeletal or skin symptoms at 34 years of age. The other was asymptomatic at 19 years of age. Taken together, we propose that PDP resulting from *SLCO2A1* defects is a sex-dependent autosomal recessive disease, and women homozygous for pathogenic *SLCO2A1* mutations may develop late-onset PDP symptoms. The explanation of mild- and low-frequency disease in women remains unclear. Hatano *et al.*[9] suggested that reactivity to prostaglandin was milder in women than in men. Ospina *et al.*[10]

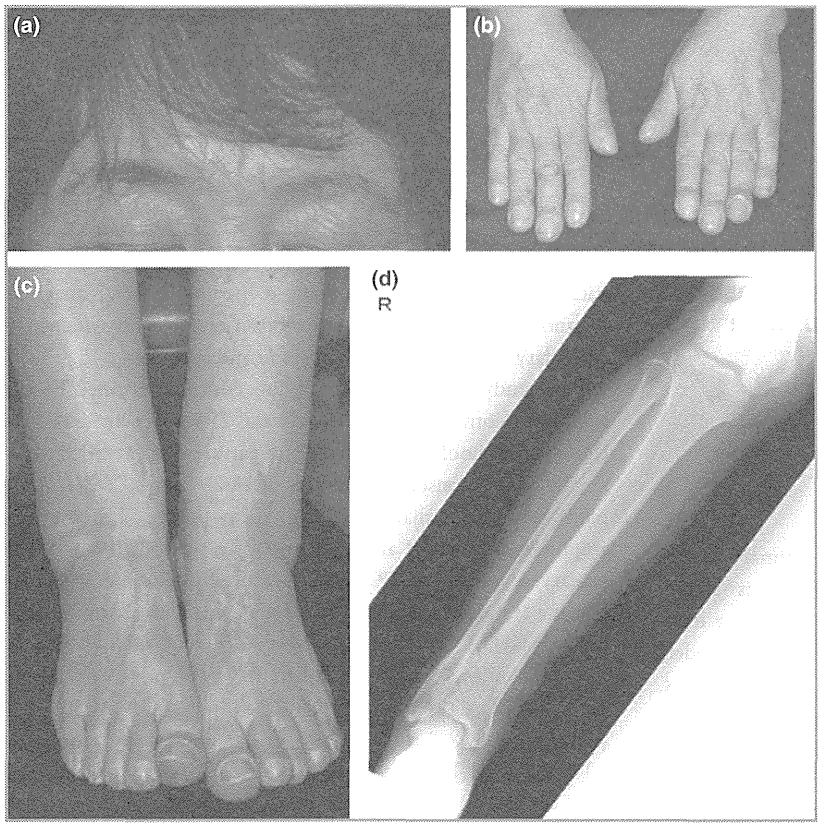

Fig 1. Clinical features including radiograph. (a) The facial appearance of the patient. Furrowing of the forehead and greasiness of facial skin are negligible. (b) Digital clubbing. (c) Clubbing of toes and cylindrical enlargement of the legs. (d) Periosteal hyperostosis of the tibia and fibula.

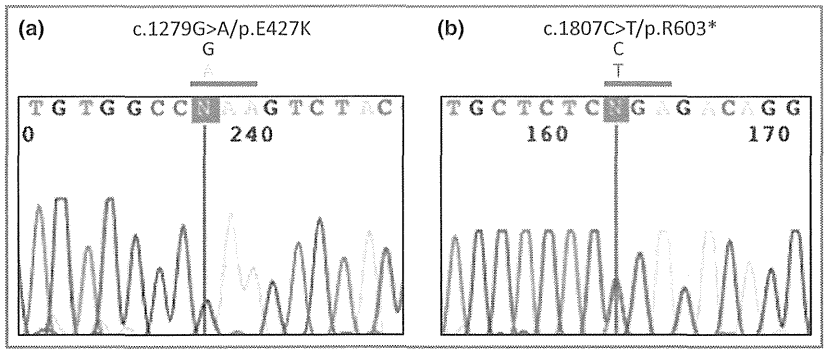

Fig 2. Two novel mutations in *SLCO2A1* were identified by the Sanger method. CodonCode Aligner (CodonCode Corporation, Centerville, MA, U.S.A.) was used to assemble sequences and detect mutations. (a) One nonsynonymous mutation: c.1279G>A/p.E427K (P1). (b) The premature stop codon mutation: c.1807C>T/p.R603*.

reported that oestrogen suppressed interleukin-1β-mediated induction of the COX-2 pathway in rat cerebral blood vessels. These data imply that a decreased level of oestrogen plays a role in the sex-dependent pathogenesis. Further analyses will clarify this issue.

In conclusion, we have described the first female case of PDP with compound heterozygous *SLCO2A1* mutations. Her atypical history shows minimal pachydermia impact.

Acknowledgments

We thank the patient and her family members for their generous cooperation. We also thank Ms A. Maruta for secretarial assistance and Ms M. Furuhashi for her excellent technical assistance.

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Funding sources: The Ministry of Health, Labour and Welfare of Japan to H.N. Research for Intractable Diseases and a grant from Keio University to J.K.

Conflicts of interest: None declared.

Urticaria-like neutrophilic dermatosis in association with IgA gammopathy: a new entity

DOI: 10.1111/bjd.12771

Dear Editor, IgA gammopathy and multiple myeloma have been associated with multiple neutrophilic dermatoses including subcorneal pustular dermatosis,[1–4] erythema elevatum diutinum,[5] urticarial vasculitis,[6] Sweet syndrome[7] and pyoderma gangrenosum.[8] We report an unusual urticaria-like neutrophilic dermatosis as the presenting sign of IgA myeloma. An 84-year-old woman with cardiomyopathy, atrial fibrillation, rectal cancer in remission, and hypertension was admitted for syncope. She reported bone pain and an intermittent rash over the preceding months. Laboratory studies were notable for normocytic anaemia, normal white blood cell count, negative serologies for HTLV-1, RPR, hepatitis and HIV, widespread osteolytic lesions on skeletal survey, elevated serum IgA (1080 mg dL^{-1}, normal: 70–350), decreased serum IgG (599 mg dL^{-1}, normal: 700–1700) and IgM (< 6·5 mg dL^{-1}, normal: 50–300) and a monoclonal IgA kappa on serum protein electrophoresis consistent with plasma cell dyscrasia and confirmed with bone marrow biopsy, demonstrating 15% atypical plasma cells.

The patient had noticed a mildly pruritic rash on the back and legs for the preceding 2 months; individual lesions lasted 3–4 days. On examination, she had annular and round erythematous plaques, some with central clearing, on the back, buttocks and proximal extremities, as well as pink-brown patches, corresponding to resolved lesions (Fig. 1). Several lesions were marked in pen and noted to persist for a minimum of 3 days.

Skin biopsy demonstrated a moderate superficial interstitial infiltrate of predominantly neutrophils with some nuclear deb-

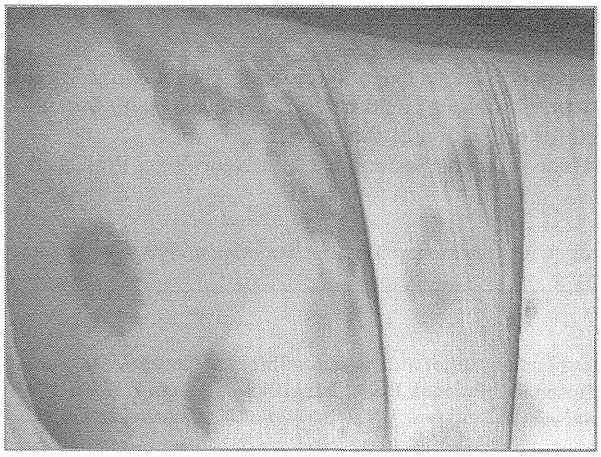

Fig 1. Clinical image showing lesions.

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Brain & Development xxx (2014) xxx–xxx

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

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Original article

Carnitine–acylcarnitine translocase deficiency: Two neonatal cases with common splicing mutation and *in vitro* bezafibrate response

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Received 28 July 2014; received in revised form 7 October 2014; accepted 8 October 2014

Abstract

Background: Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of acute encephalopathy- or myopathy-like illness. Carnitine–acylcarnitine translocase (CACT) deficiency is a rare FAO disorder, which represent an energy production insufficiency during prolonged fasting, febrile illness, or increased muscular activity. CACT deficiency is caused by mutations of the *SLC25A20* gene. Most patients developed severe metabolic decompensation in the neonatal period and died in infancy despite aggressive treatment.

Patients and methods: We herein report the clinical findings of two unrelated cases of CACT deficiency with mutation confirmation, and *in vitro* bezafibrate responses using *in vitro* probe acylcarnitine (IVP) assay. Patients 1 and 2 are products of nonconsanguineous parents. Both patients developed cardiac arrest at day 3 of life but survived the initial events. Their blood chemistry revealed hypoglycemia and metabolic acidosis. The acylcarnitine profiles in both patients demonstrated increased long-chain acylcarnitines, suggesting CACT or carnitine palmitoyltransferase-2 (CPT2) deficiency.

Results: The mutation analysis identified homozygous IVS2-10T>G in the *SLC25A20* gene in both patients, confirming the diagnosis of CACT deficiency. The IVP assay revealed increased C16, C16:1, but decreased C2 with improvement by bezafibrate in the cultured fibroblasts. The short-term clinical trial of bezafibrate in Patient 1 did not show clinical improvement, and died after starting the trial for 6 months.

Conclusion: This splicing mutation has been identified in other Asian populations indicating a possible founder effect. IVP assay of cultured fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial of more enrolled patients is required for evaluation of this therapy.

Keywords: CACT deficiency; *SLC25A20* mutation; IVP assay; Bezafibrate

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http://dx.doi.org/10.1016/j.braindev.2014.10.005
0387-7604/

Please cite this article in press as: Vatanavicharn N et al. Carnitine–acylcarnitine translocase deficiency: Two neonatal cases with common splicing mutation and *in vitro* bezafibrate response. Brain Dev (2014), http://dx.doi.org/10.1016/j.braindev.2014.10.005

1. Introduction

Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of neuromuscular symptoms as well as acute encephalopathy or even sudden death. In particular, the carnitine cycle is important in energy-producing pathway for cardiac and skeletal muscle and for preventing from hypoglycemia especially during prolonged fasting or increased muscular exercise. Carnitine–acylcarnitine translocase (CACT, EC 2.3.1.21) is one of the enzymes in the carnitine cycle, which catalyzes the transfer of the long-chain fatty acylcarnitines across the inner mitochondrial membrane in exchange of free carnitine. CACT deficiency (OMIM 212138) was first described in 1992 [1]. It is an autosomal-recessive disease caused by mutations of the *SLC25A20* gene located in chromosome 3p21.31 [2]. The gene consists of 9 exons and encodes protein comprising 301 amino acids [3]. CACT deficiency is a very rare disorder with so far as approximately 30 patients have been described, and accounted for 10% of patients with FAO disorders in French population [4]. However, it might be a common FAO disorder in some East Asian countries such as Hong Kong with the estimated incidence of 1 in 60,000 live births, and accounted for 33% of patients with FAO disorders [5]. Most patients develop neonatal-onset encephalopathy with nonketotic hypoglycemia, hyperammonemia, and hypothermia, or sudden death from cardiac arrhythmias. Cardiomyopathy and hepatic dysfunction may be the associated complications. CACT deficiency could be detected by elevations of C16 and C18 acylcarnitines, and low free carnitine in acylcarnitine profiles. However, the same profile could be found in neonatal carnitine palmitoyltransferase-2 (CPT2) deficiency. Therefore, confirmation of diagnosis requires CACT enzyme assay or molecular analysis of the *SLC25A20* gene [6]. Treatment includes intravenous glucose for acute decompensation, and avoidance of long fasting with frequent meals. Long-chain fatty acids may be restricted in diet, but medium-chain triglyceride (MCT) oil is supplemented instead. Carnitine therapy is still controversial. Despite aggressive treatment, most patients still died in infancy [7]. However, there have been some patients who received early treatment with good outcomes [8,9]. Novel therapy for FAOD using bezafibrate, which is a hypolipedimic drug acting as a peroxisome proliferator-activated receptor (PPAR) agonist has been reported. The clinical trials of bezafibrate showed clinical improvement in adult patients with CPT2 deficiency [10], and a child with glutaric acidemia type 2 (GA2) [11]. *In vitro* probe acylcarnitine (IVP) assay can be used to evaluate FAO disorders [12], and determine the effect of bezafibrate [13]. We herein report the clinical findings of two unrelated cases with neonatal-onset CACT deficiency, and *in vitro* bezafibrate response using the IVP assay.

2. Patients and methods

2.1. Patients

2.1.1. Case 1

This patient was the first child of possibly consanguineous parents from the southern province of Thailand. He was born at 37 weeks of gestation with birth weight of 2460 g (25th percentile), length 48 cm (3rd percentile), and head circumference 30 cm (<3rd percentile). He developed hypothermia at 10 h of age. Sepsis was suspected, but the patient rapidly responded to rewarming treatment. However, after rooming-in with the mother, he developed hypothermia again. At 60 h after birth, he had cardiac arrest. On physical examination, no abnormalities were found. Serum glucose was 1.2 mmol/L and acetoacetate was 0 mmol/L. Venous blood pH was 7.24 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 471 μmol/L (normal, <110 μmol/L). There were mildly elevated liver enzymes aspartate aminotransferase (AST) (97 U/L; normal, 0–32) and alanine aminotransferase (ALT) (78 U/L; normal, 0–33). Serum creatine kinase was 4439 U/L (normal, <190). He had a good response to treatment with intravenous glucose administration. Urine organic acids were unremarkable. A dried blood spot acylcarnitine profile by tandem mass spectrometry (MS/MS) showed free carnitine (C0), 5.26 μM (10–60); C16-acylcarnitine, 14.14 μM (0.6–7); C18-acylcarnitine, 2.71 μM (0.15–2.1); C18:1-acylcarnitine, 4.3 μM (0.3–3.2); and a (C16 + C18)/C0 ratio, 3.21 (0.007–0.5). The profile was consistent with CPT2 or CACT deficiency. The patient has been treated with a modular medical formula, which has been composed of modified fats (long-chain fatty acid restriction along with supplementation of 83% of fat as medium-chain triglyceride oil), protein, maltodextrins, minerals, and fat-, and water-soluble vitamins. L-Carnitine at a daily dosage of 100–150 mg/kg has been supplemented. Thereafter, he has had several episodes of hypoglycemia, hyperammonemia, and metabolic acidosis following infections. At 8 months of age, he developed cholestasis and hepatomegaly. At 9 months of age, an echocardiogram revealed hypertrophic cardiomyopathy. At the age of 15 months, he had mild developmental delay and generalized hypotonia. He could stand with support, put block in cup, and say one word. Then he had a metabolic crisis, and developed generalized weakness. After he recovered from encephalopathy, neurologic examination revealed normal cranial nerves, muscle weakness (grade 3/5), and decreased muscle tone and deep tendon reflexes (1+) in all extremities. A brain computed tomography scan was normal. Serum creatine kinase was elevated (1419 U/L). A nerve conduction study showed no evidence of demyelination. He had been ventilator-dependent since then. At 2½ years of

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age, he had several complications including chronic liver disease, upper gastrointestinal bleeding, and osteoporosis. He died at the age of 2 years and 8 months from upper gastrointestinal bleeding and metabolic decompensation.

2.1.2. Case 2

The patient was the first child of nonconsanguineous parents. She was born at 35 weeks of gestation with a birth weight of 2.3 kg (50th percentile), length 44 cm (25th percentile), and head circumference 30 cm (10th percentile). At 2 days after birth, she developed lethargy, poor feeding, and cardiac arrest. Blood glucose was 0.56 mmol/L. She responded to cardiac resuscitation and intravenous glucose infusion. Serum acetoacetate was 0 mmol/L. Venous blood pH was 7.39 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 157 μmol/L (normal, <110 μmol/L). There were elevated liver enzymes AST (638 U/L; normal, 0–32) and ALT (83 U/L; normal, 0–33). Plasma lactate dehydrogenase (LDH) was 522 U/L (normal, 240–480). An echocardiogram revealed no cardiomyopathy. A dried blood spot acylcarnitine profile by MS/MS analysis showed C0, 13.8 μM (10–60); C16-acylcanitine, 15 μM (0.6–7); C18-acylcarnitine, 4.3 μM (0.15–2.1); C18:1-acylcarnitine, 5.9 μM (0.3–3.2); and a (C16 + C18)/C0 ratio, 1.4 (0.007–0.5). The profile was consistent with either CPT2 or CACT deficiency. The patient had been treated with a high-MCT formula (Portagen®, Mead Johnson Nutritionals), and 100 mg/kg/day of L-carnitine. At 1 month of age, she developed anemia from Hb AE Bart's disease – a thalassemia intermedia resulting from the interaction between α-thalassemia and heterozygous Hb E, which required monthly blood transfusion. At the age of 4 months, she had poor feeding and cardiac arrest. Blood glucose was 0.5 mmol/L. The patient died without any response to resuscitation. An autopsy revealed left ventricular hypertrophy, micro/macrovesicular steatosis of the liver with focal areas of bridging fibrosis, and abnormal lipid accumulation in skeletal muscles and the proximal renal tubules.

2.2. Materials and methods

This study was approved by the Siriraj Institutional Review Board. The written informed consents for the mutation analysis, IVP assay, and bezafibrate trial were obtained from the parents. Genomic DNA was extracted from leukocytes. Mutation analyses of the *CPT2* and *SLC25A20* genes were performed in case 1, and only *SLC25A20* gene in case 2. All coding exons and their flanking intron sequences (up to 20 bases for both sides) of the *CPT2* and *SLC25A20* genes were PCR-amplified and directly sequenced according to the previously described method [14]. The IVP assay was performed using the skin fibroblasts in the absence and presence of bezafibrate according to the previously described method [11].

3. Results

3.1. Mutation analysis and IVP assay

Mutation analysis of the *SLC25A20* gene identified homozygous c.199-10T>G (IVS2-10T>G) mutation in both patients, and heterozygous mutation in their parents (Fig. 1). Mutation analysis of the *CPT2* gene revealed no pathogenic mutation in Case 1. The IVP assay profiles revealed increased C16, C16:1 acylcarnitines, and decreased C2 (acetylcarnitine) indicating a typical pattern of CPT2 or CACT deficiency, with substantial reduction of long-chain acylcarnitines by the presence of bezafibrate in the cultured fibroblasts from both patients (Fig. 2). However, C2 acylcarnitine did not increase as expected.

3.2. Clinical trial of bezafibrate

We started a clinical trial of bezafibrate in case 1 at age of 2 years and 2 months, after the IVP assay which

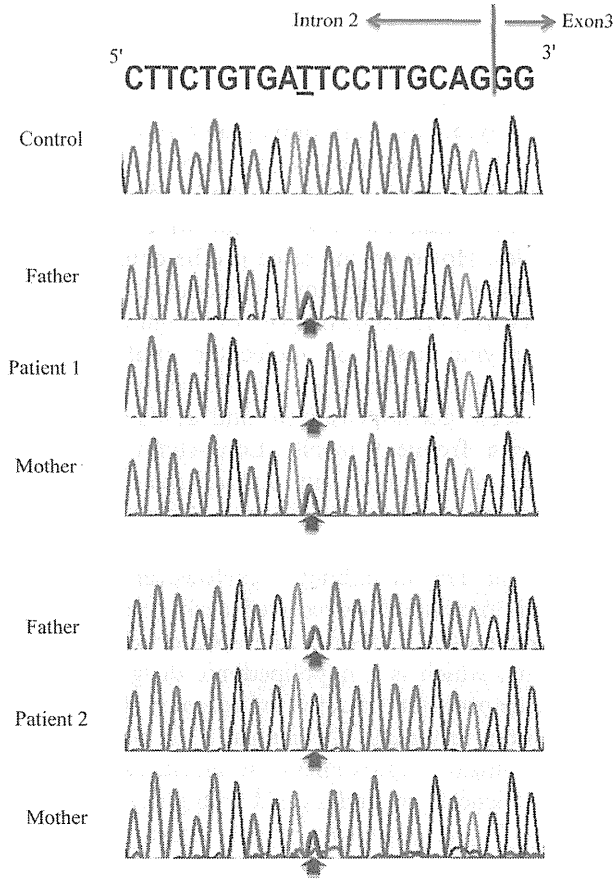

Fig. 1. The reference DNA sequence of an intron 2/exon 3 boundary of the *SLC25A20* gene, and the IVS2-10T>G mutation identified in both patients and their parents denoted by black arrows and the underlined letter.

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