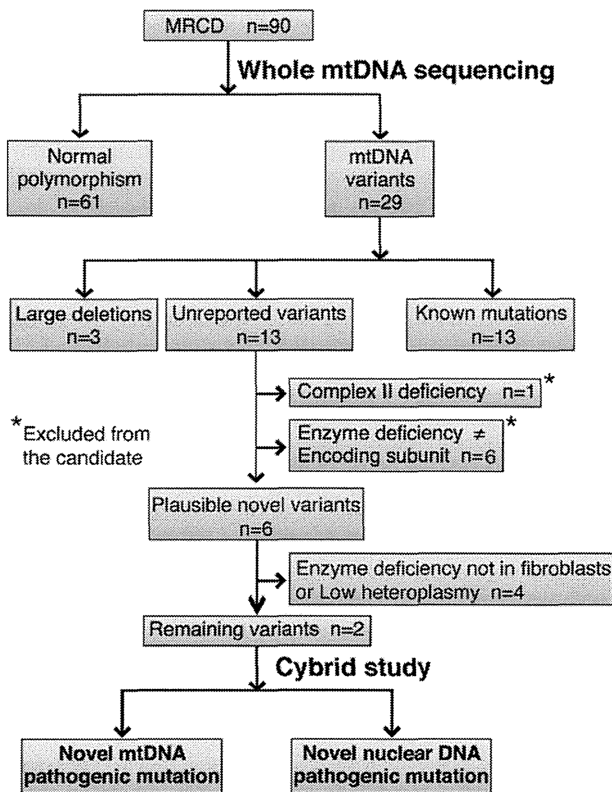

Figure 1. Flow diagram of study analysis. Ninety MRCD patients were analyzed in this study. Sixty-one patients had normal polymorphisms and 29 had mtDNA variants. Of these variants, 13 patients had MRCD causative mutations that had been previously described. We identified three novel large deletions and 13 unreported variants. Of the unreported variants, one patient with complex II deficiency was excluded because complex II is not encoded by mtDNA. Six patients were excluded because their enzyme deficiency pattern did not coincide with the variants found in mtDNA. Four patients were excluded because of the lack of fibroblast enzyme deficiency or low heteroplasmy. The remaining two cases were analyzed by cybrid study.

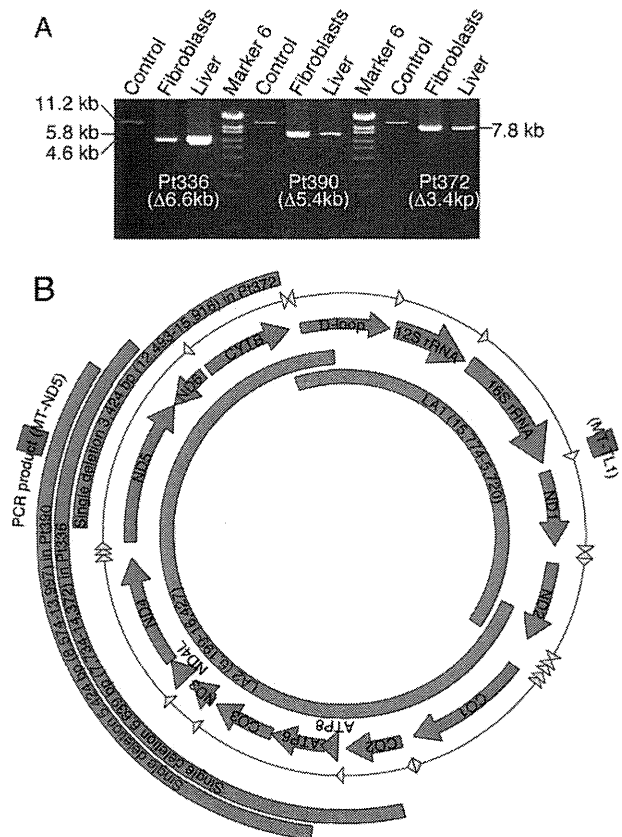

Figure 2. Identification of three large deletions. (A) Characterization of the three novel mtDNA deletions using agarose electrophoresis. First-round PCR products amplified from patient fibroblast and liver DNA clearly showed the presence of mtDNA deletions in Pt336, 390, and 372. Normal mtDNA from an MRCD patient was used as a positive control. (B) Positions of the novel mtDNA deletions are shown in blue. LA1 and LA2 amplification is shown in green. Two red squares represent real-time PCR amplicons MT-ND5 and MT-TL1.

analyzed for an mtDNA deletion. Second-round PCR was performed using fewer (25–26) PCR cycles to avoid untargeted DNA amplification. To identify the location of the deletion, we first compared the density of bands and screened the faint bands with agarose electrophoresis. The precise deletion boundaries were confirmed by sequencing analysis with primers used for second-round PCR that were close to the probable deletion region.

Results

Patient characteristics and their mtDNA mutations

A total of 90 patients (49 were men and 41 were women) with MRCD were subjected to whole mtDNA sequencing analysis (Table 1). Eighty-four subjects (93%) were nonconsanguineous. Seventy-six subjects (84%) were aged 1 year or younger. We identified 13 previously reported mtDNA mutations, 13 unreported variants, and three novel deletions (Fig. 1). The remaining 61 subjects had normal polymorphisms in their mtDNA (Fig. 1).

Large mtDNA deletions were identified in three patients

Agarose gel electrophoresis of first-round PCR from fibroblast and liver mtDNA clearly showed the presence of mtDNA deletions in Pt336, 390, and 372 (Fig. 2A). The precise deletion sites were confirmed by sequencing analysis. The expected size of the first-round PCR LA2 product in wild-type mtDNA from an MRCD patient was 11.2 kb, which enabled us to estimate the deletion sizes

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of Pt336, 390, and 372 as 6639, 5424, and 3424 bp, respectively (Fig. 2A and B). In Pt336, the 6639-bp deletion was located between nucleotides 7734 and 14,372 and was flanked by 5-bp perfect direct repeats. This deletion results in the loss of 15 genes (*CO2, ATP8, ATP6, CO3, ND3, ND4L, ND4, ND5, ND6*, and six *tRNA* genes). The heteroplasmy ratio of this deletion was 9.2% in the fibroblasts (Fb) and 92.6% in the liver (Hep) (Table 2 and Data S1). In Pt390, the 5424-bp deletion was located between nucleotide positions 8574 and 13,997 and was flanked by 11-bp imperfect direct repeats. This deletion results in the loss of 11 genes (*ATP6, CO3, ND3, ND4L, ND4, ND5*, and five *tRNA* genes). The heteroplasmy ratio of this deletion was 44.9% (Fb) and 86.4% (Hep) (Table 2). In Pt372, the 3424-bp deletion was located between nucleotides 12,493 and 15,916 and was flanked by 6-bp imperfect direct repeats. This deletion results in the loss of five genes (*ND5, ND6, CYB*, and two *tRNA* genes). The heteroplasmy ratio of this deletion was 65.7% (Fb), and 89.9% (Hep) (Table 2).

Unreported variants of mtDNA detected in 13 patients

We identified 13 unreported mtDNA variants. Of these, seven were excluded by manual curation (Fig. 1). One of these was excluded because the enzyme deficiency was specific to complex II, which is not encoded by mtDNA. The other six were excluded because their enzyme deficiency pattern did not coincide with the variants found in mtDNA. From the remaining six plausible mtDNA variants, we determined whether they were causative using the following inclusion criteria for further analysis: (1) cells were viable for further assay, (2) mtDNA variants corresponded to the enzyme assay data in the RC subunit, (3) enzyme deficiency was observed in the fibroblasts, and (4) variants had high heteroplasmy ratios (Fig. 1 and Table 2). On the basis of these criteria, we selected two patients whose mtDNA variants (m.14439G>A in *MT-ND6* and m.1356A>G in *12S rRNA*) were suitable for further analysis as shown in Figure 1. The other four patients were excluded because they did not show enzyme deficiency in their fibroblasts or because of low heteroplasmy ratios (Table 2).

m.14439G>A (*MT-ND6*), but not m.1356A>G (12S rRNA), is a causative mutation

The m.14439G>A (*MT-ND6*) variant was observed in fibroblasts from Pt377 (Fig. 3A). PCR- restriction fragment length polymorphism (RFLP) analysis with the *Hpy*188I restriction enzyme found Pt377 fibroblasts to be homoplasmic, and the m.14439G>A variant was not detected in the blood of the patient's parents (Fig. 3A and B). This mutation changes the proline to a serine at amino acid position 79, which is highly conserved among vertebrates (Fig. 3C). ND6 is one of the mtDNA-encoded complex I subunits and alignment of the ND6 protein in different species revealed conservation of amino acids. The activity level of the RC complex I was coincidentally reduced in the patient's fibroblasts (Fig. 4A). To further confirm whether this mutation was causative of mitochondrial dysfunction, we performed cybrid analysis (Data S1). The cybrids showed a reduction in the complex I activity level consistent with the respiratory enzyme assay in the patient's fibroblasts (Fig. 4B). These data strongly support the idea that the m.14439G>A (*ND6*) mutation detected in Pt377 is responsible for the complex I deficiency.

The m.1356A>G (*12S rRNA*) variant was observed in fibroblasts from Pt312, which showed reduced activity levels of RC complex I (Fig. 4A). By mismatch PCR-RFLP-analysis using the *Sty*I restriction enzyme, this variant was determined at a heteroplasmy ratio of 66% in the patient's fibroblasts (Table 2). The cybrids harboring this variant showed a recovery of complex I enzyme activity compared with the original patient's fibroblasts (Fig. 4B). These data suggest that reduced complex I enzyme activity was rescued by nuclear DNA and that this mtDNA variation is not causative. This further indicates that the nuclear gene mutation is the cause of MRCD in this patient.

Identification of the c.55C>T (*NDUFA1*) mutation in Pt312 by whole exome sequencing

To search for the causative nuclear gene mutation in Pt312, we performed whole exome sequencing (Data S1). This identified a single hemizygous mutation (c.55C>T) in exon 1 of the *NDUFA1* gene, which altered the amino acid residue at position 19 from proline to serine (p. P19S). The mutation was confirmed by Sanger sequencing (Fig. 5A). This conserved proline residue lies within the hydrophobic N-terminal side constituting a functional domain that is involved in mitochondrial targeting, import, and orientation of *NDUFA1*.[10,11] SIFT and PolyPhen, which predict the function of non-synonymous variants (http://genetics.bwh.harvard.edu/pph/), also revealed that the p.P19S mutation "probably" damages the function of the NDUFA1 protein (damaging score, 0.956). Alignment of the NDUFA1 protein between different species revealed the conservation of three amino acids, including the proline at position 19, which is highly conserved among vertebrates (Fig. 5B). To further confirm if the complex I deficiency in Pt312 occurred because of the mutation in *NDUFA1*, we overexpressed *NDUFA1* cDNA to determine if the enzyme deficiency

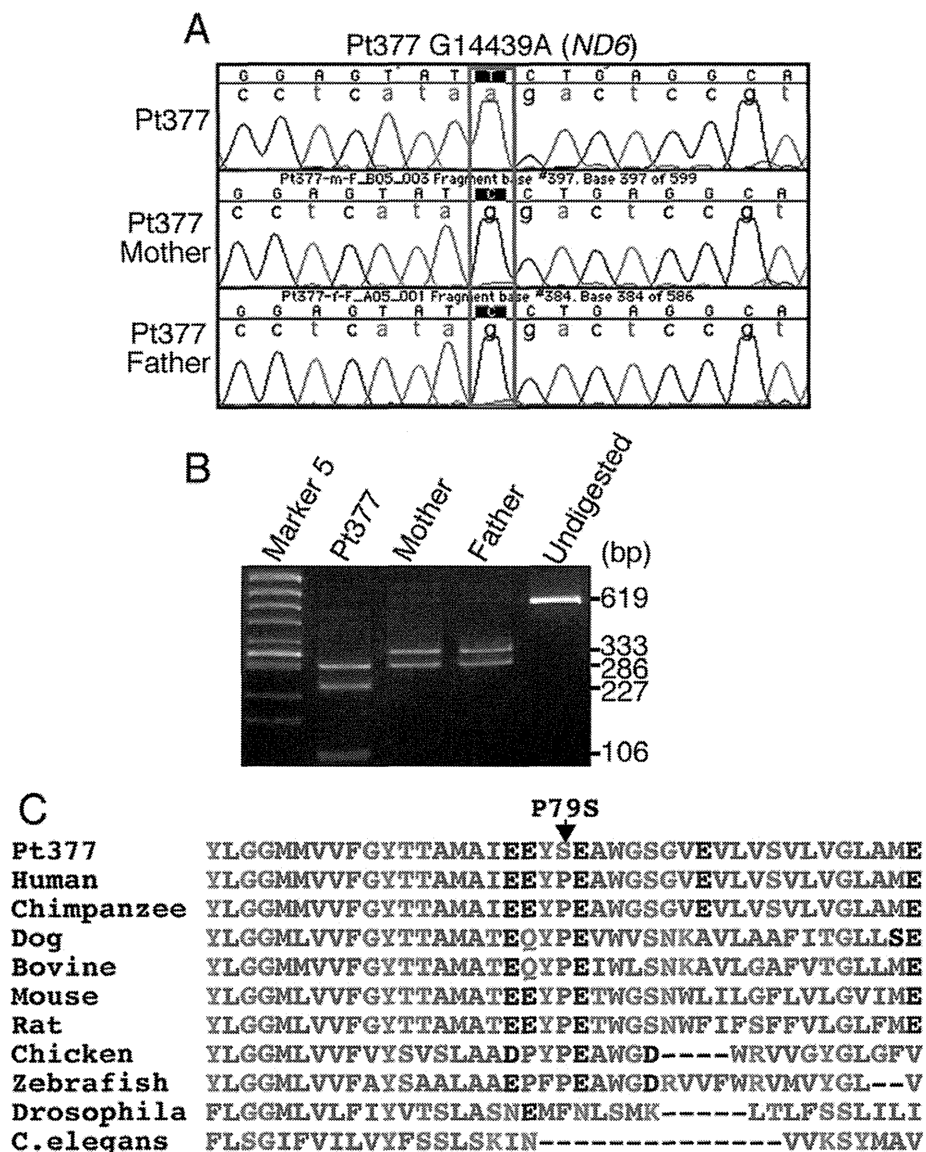

Figure 3. Novel mutation m.14439G>A in Pt377 mtDNA. (A) Trio-sequencing analysis of m.14439G>A (*MT-ND6* p.P79S) change in Pt377 family. Sequence chromatograms show that the m.14439G>A is detectable only in Pt377. (B) PCR-RFLPanalysis using fibroblast mtDNA from Pt377 and blood from both parents. A 619-bp PCR fragment was digested with *Hpy*188I. Wild-type mtDNA was cleaved into two fragments of 333 and 286 bp as shown in "Mother" and "Father", whereas the PCR product containing the m.14439G>A mutation was cleaved into three fragments: 286, 227, and 106 bp ("Pt377"). Undigested = undigested PCR product. (C) Alignment of MT-ND6 protein between different species shows the conservation of amino acid Proline 79. Amino acid sequences of *MT-ND6* gene products were aligned by ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and NCBI/homologene (http://www.ncbi.nlm.nih.gov/homologene).

could be recovered (Data S1). Lentiviral transfection of NDUFA1 resulted in a significant increase in complex I assembly level as determined by blue native polyacrylamide gel electrophoresis. By contrast, lentiviral transfection of control mtTurboRFP did not rescue the phenotype (Fig. 5C). These data indicate that the c.55C>T mutation in *NDUFA1* is responsible for the complex I deficiency in Pt312.

Discussion

MRCD is particularly difficult to diagnose in pediatric cases as the clinical features are highly variable. We, therefore, propose a systematic approach for diagnosing MRCD that starts with a biochemical enzyme assay and is followed by whole mtDNA sequencing. In this study, we performed whole mtDNA sequencing for 90 children with

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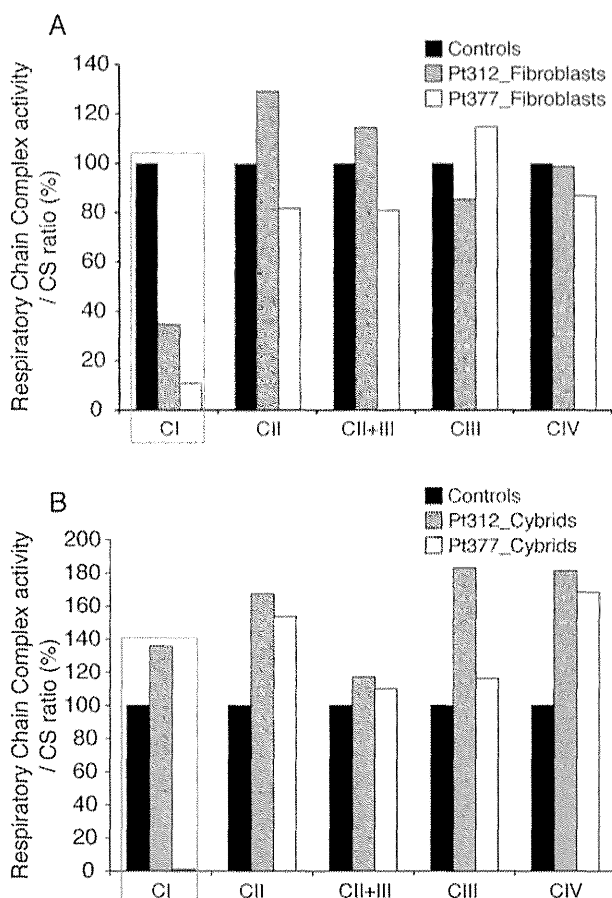

Figure 4. Biochemical assay for respiratory chain enzyme activity in fibroblasts and cybrid cells from Pt377 and Pt312. (A) Respiratory chain complex enzyme activity for CI, CII, CII + III, and CIV in skin fibroblast mitochondria from Pt312 and Pt377 compared with normal controls. The activity of each complex was calculated as a ratio relative to citrate synthase (CS). CI showed a reduction in enzyme activity in Pt312 and 377 fibroblasts. (B) Respiratory chain complex enzyme activity of cybrids established from Pt312 and Pt377 fibroblasts. Cybrids were established from rho0-HeLa cell and Pt312 or Pt377 fibroblasts. The activity of each complex in these cybrids was calculated as a ratio relative to that of citrate synthase (CS).

MRCD, and identified 29 mtDNA variants. Of these, we identified 13 known causative mutations, three large deletions, and further confirmed that m.14439G>A (*MT-ND6)* and c.55C>T (*NDUFA1*) are new causative mutations for MRCD from the results of a cybrid assay, whole exome sequencing, and a complementation study. The diagnosis of MRCD was then confirmed as definite by molecular analysis in these 18 cases.

Whole mitochondrial DNA sequencing identified 13 cases (14%) harboring known causative mtDNA mutations. mt. 10191T>C (*ND3*) and mt. 8993T>C or G (*ATP6*) mutations were detected in three and two patients, respectively (data not shown). Both are common causative mutations of infantile Leigh syndrome. Previous reports found that most common MRCD causative mutations are primarily responsible for adult-onset disease, whereas few are responsible for childhood-onset MRCD;[12,13] only 14% of our cases were attributed to known mtDNA mutations.

Most patients in this study were 1-year old or younger at the onset of disease, with no family history. We used the RC complex enzyme assay to diagnose pediatric patients who had not been diagnosed with MRCD in a clinical setting. Several MRCD cases in children were previously reported to be difficult to diagnose with nonspecific clinical presentations in contrast to the characteristic clinical syndromes such as MELAS and MERRF caused by common mtDNA mutations.[6,12]

We identified three novel deletions that we concluded were causative because they include several genes that could explain the deficiency of the RC enzymes. Generally, most mtDNA deletions share similar structural characteristics, are located in the major arc between two proposed origins of replication (OH and OL; Mitomap), and are predominantly (~85%) flanked by short direct repeats.[14,15] Single mtDNA deletions are reported to be the common causes of sporadic MRCD such as Kearns-Sayre syndrome (KSS), CPEO, and Pearson's syndrome. In this study, all three deletions were located in the major arc and were flanked by repeat sequences, similar to previous studies. Although Pt390 was diagnosed with Pearson's syndrome, the other two patients (Pt336 and Pt372) did not show a common phenotype caused by a single deletion such as KSS, CPEO, or Pearson's syndrome. Therefore, screening by mtDNA size differences is important even in those patients not clinically suspected to have mtDNA deletions.

Manual curation identified six plausible mtDNA variants that had not previously been reported (Fig. 1). We attempted to carry out a functional assay of the two patients whose fibroblasts are enzyme deficient, although it was difficult to apply this strategy to those fibroblasts with normal enzyme activity. In this sense, it is important to collect patients with similar phenotypes and carrying the same mtDNA variants to accurately diagnose the causal mutation. Thus, this study of patients harboring unreported mtDNA variants will be useful in a clinical situation. Of these, the m.14439G>A (*MT-ND6)* variant was experimentally confirmed to be a novel causative mtDNA mutation, while 1356A>G (*12S rRNA*) was confirmed to be non-pathogenic by a cybrid assay. The remaining four novel variants have yet to be experimentally elucidated, but m.5537A>G (*mt-tRNA trp*) in Pt004 is likely to be causative because m.5537AinsT was reported to be disease causing.[16]

ND6 is an mtDNA-encoded complex I subunit that is essential for the assembly of complex I and the maintenance of its structure.[17–19] *ND6* mutations were previ-

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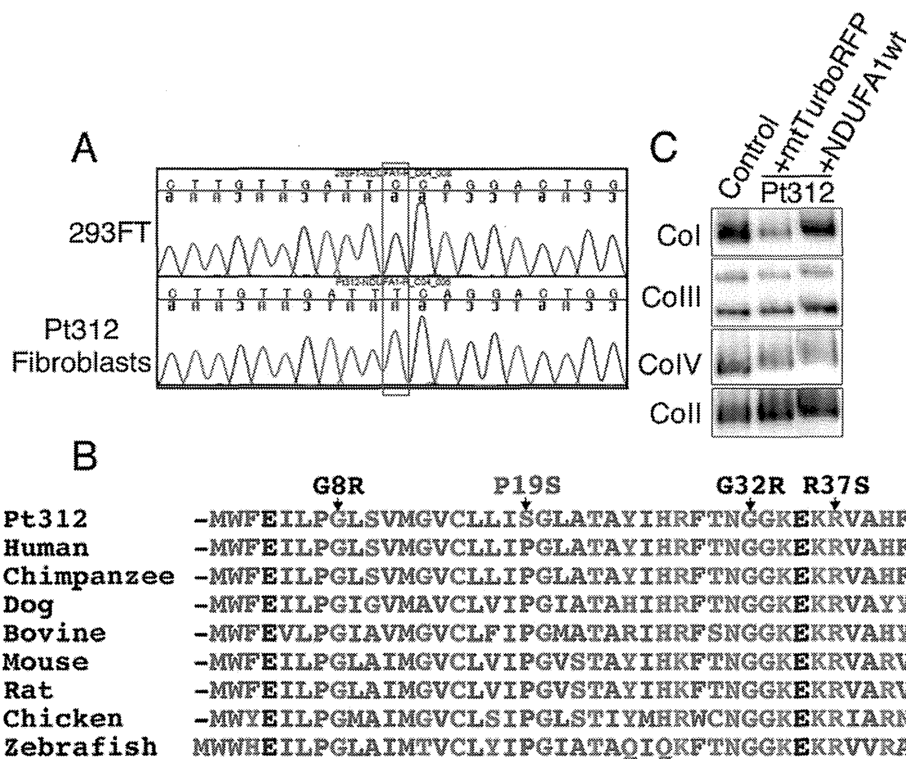

Figure 5. The novel nDNA mutation c.55C>T in *NDUFA1*. (A) Sequence chromatograms showing the c.55C>T (*NDUFA1* p.P19S) mutation in Pt312 and 293FT genomic DNA as a wild-type control. (B) Alignment of amino acid sequences of NDUFA1 subunit between different species shows the high conservation of amino 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.

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Conflict of Interest

None declared.

References

1. Calvo S, Jain M, Xie X, et al. Systematic identification of human mitochondrial disease genes through integrative genomics. Nat Genet 2006;38:576–582.
2. Zeviani M, Di Donato S. Mitochondrial disorders. Brain 2004;127(Pt 10):2153–2172.
3. Munnich A, Rötig A, Chretien D, et al. Clinical presentation of mitochondrial disorders in childhood. J Inherit Metab Dis 1996;19:521–527.
4. Skladal D, Halliday J, Thorburn DR. Minimum birth prevalence of mitochondrial respiratory chain disorders in children. Brain 2003;126(Pt 8):1905–1912.
5. Kisler JE, Whittaker RG, McFarland R. Mitochondrial diseases in childhood: a clinical approach to investigation and management. Dev Med Child Neurol 2010;52: 422–433.
6. Thorburn DR. Mitochondrial disorders: prevalence, myths and advances. J Inherit Metab Dis 2004;27:349–362.
7. Zeviani M, Bertagnolio B, Uziel G. Neurological presentations of mitochondrial diseases. J Inherit Metab Dis 1996;19:504–520.
8. Bernier FP, Boneh A, Dennett X, et al. Diagnostic criteria for respiratory chain disorders in adults and children. Neurology 2002;59:1406–1411.
9. Akanuma J, Muraki K, Komaki H, et al. Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene. J Hum Genet 2000;45:337–341.
10. Potluri P, Davila A, Ruiz-Pesini E, et al. A novel NDUFA1 mutation leads to a progressive mitochondrial complex I-specific neurodegenerative disease. Mol Genet Metab 2009;96:189–195.
11. Yadava N, Houchens T, Potluri P, Scheffler IE. Development and characterization of a conditional mitochondrial complex I assembly system. J Biol Chem 2004;279:12406–12413.
12. Elliott HR, Samuels DC, Eden JA, et al. Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet 2008;83:254–260.
13. Shoffner JM. Oxidative phosphorylation disease diagnosis. Ann N Y Acad Sci 1999;893:42–60.
14. Bua E, Johnson J, Herbst A, et al. Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. Am J Hum Genet 2006;79:469–480.
15. Krishnan KJ, Reeve AK, Samuels DC, et al. What causes mitochondrial DNA deletions in human cells? Nat Genet 2008;40:275–279.
16. Tulinius M, Moslemi AR, Darin N, et al. Leigh syndrome with cytochrome-c oxidase deficiency and a single T insertion nt 5537 in the mitochondrial tRNATrp gene. Neuropediatrics 2003;34:87–91.
17. Bai Y, Attardi G. The mtDNA-encoded ND6 subunit of mitochondrial NADH dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme. EMBO J 1998;17:4848–4858.
18. Cardol P, Matagne RF, Remacle C. Impact of mutations affecting ND mitochondria-encoded subunits on the activity and assembly of complex I in Chlamydomonas. Implication for the structural organization of the enzyme. J Mol Biol 2002;319:1211–1221.
19. Ugalde C, Triepels RH, Coenen MJ, et al. Impaired complex I assembly in a Leigh syndrome patient with a novel missense mutation in the ND6 gene. Ann Neurol 2003;54:665–669.
20. Kirby DM, Kahler SG, Freckmann ML, et al. Leigh disease caused by the mitochondrial DNA G14459A mutation in unrelated families. Ann Neurol 2000;48:102–104.
21. Ravn K, Wibrand F, Hansen FJ, et al. An mtDNA mutation, 14453G–>A, in the NADH dehydrogenase subunit 6 associated with severe MELAS syndrome. Eur J Hum Genet 2001;9:805–809.
22. Chinnery PF, Brown DT, Andrews RM, et al. The mitochondrial ND6 gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy. Brain 2001;124 (Pt 1):209–218.
23. Prezant TR, Agapian JV, Bohlman MC, et al. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet 1993;4:289–294.
24. Fernandez-Moreira D, Ugalde C, Smeets R, et al. X-linked NDUFA1 gene mutations associated with mitochondrial encephalomyopathy. Ann Neurol 2007;61:73–83.

Supporting Information

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

Data S1. Supplementary methods.

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The first case in Asia of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (HSD10 disease) with atypical presentation.

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Abstract

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (2M3HBD) deficiency (HSD10 disease) is a rare inborn error of metabolism, and <30 cases have been reported worldwide. This disorder is typically characterized by progressive neurodegenerative disease from 6 to 18 months of age. Here, we report the first patient with this disorder in Asia, with atypical clinical presentation. A 6-year-old boy, who had been well, presented with severe ketoacidosis following a 5-day history of gastroenteritis. Urinary organic acid analysis showed elevated excretion of 2-methyl-3-hydroxybutyrate and tiglylglycine. He was tentatively diagnosed with β-ketothiolase (T2) deficiency. However, repeated enzyme assays using lymphocytes showed normal T2 activity and no T2 mutation was found. Instead, a hemizygous c.460G>A (p.A154T) mutation was identified in the HSD17B10 gene. This mutation was not found in 258 alleles from Japanese subjects (controls). A normal level of the HSD17B10 protein was found by immunoblot analysis but no 2M3HBD enzyme activity was detected in enzyme assays using the patient's fibroblasts. These data confirmed that this patient was affected with HSD10 disease. He has had no neurological regression until now. His fibroblasts showed punctate and fragmented mitochondrial organization by MitoTracker staining and had relatively low respiratory chain complex IV activity to those of other complexes.

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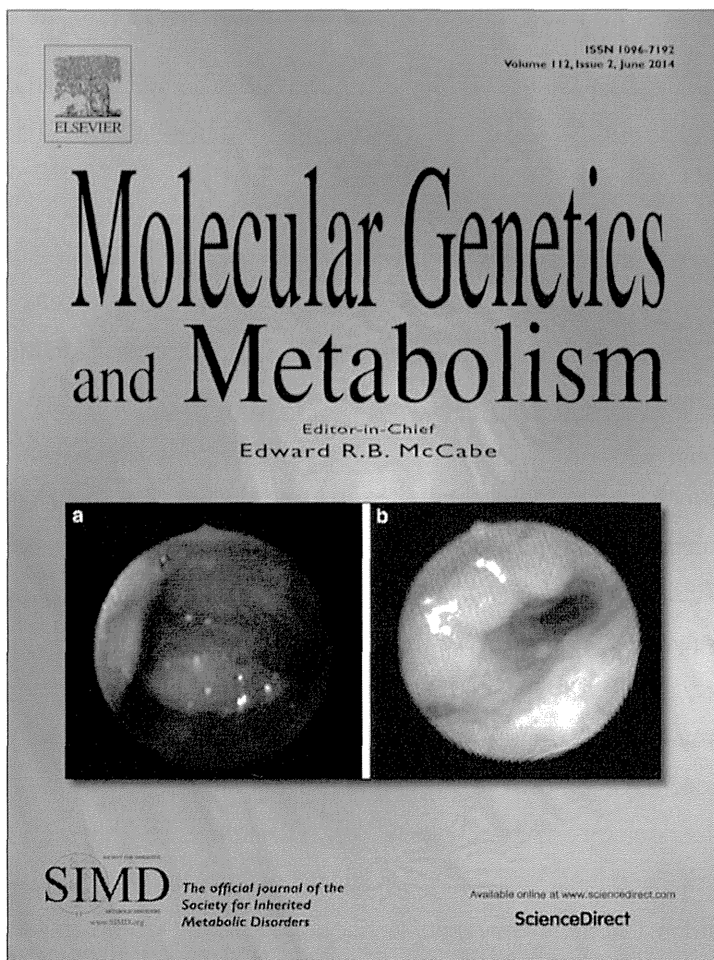

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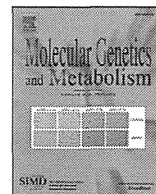

Efficacy of pyruvate therapy in patients with mitochondrial disease: A semi-quantitative clinical evaluation study

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ABSTRACT

Background: Disorders of oxidative phosphorylation (OXPHOS) cause an increase in the NADH/NAD^+ ratio, which impairs the glycolysis pathway. Treatment with pyruvate is expected to decrease the ratio and thereby restore glycolysis. There are some case reports on the efficacy of pyruvate treatment for mitochondrial diseases. However, few of these reports assessed their results using a standardized scale.
Methods: We monitored 4 bedridden patients with OXPHOS disorders who continued therapies of 0.5–1.0 g/kg/day of sodium pyruvate for more than 12 months. The efficacies of these treatments were evaluated with the Newcastle Pediatric Mitochondrial Disease Scale and the Gross Motor Function Measure with 88 items.
Results: The ages of the patients at the treatment initiation ranged from 8–100 months. Of the 4 patients, 3 exhibited improvements within 1–3 months from the initiation of treatment. Among these 3 patients, one maintained the improvement for over 2 years. The remaining 2 regressed 3–6 months after the initiation of treatment. The blood lactate/pyruvate ratios did not correlate with the efficacy of treatment.
Conclusion: Pyruvate was effective even in bedridden patients with OXPHOS disorders, at least in the short term. Clinical trials with more patients and less severe disabilities are necessary to evaluate the long-term efficacy of this treatment. Biomarkers other than lactate and pyruvate need to be identified to biochemically monitor the efficacy of this treatment.

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1. Introduction

Tanaka et al. [1] proposed that pyruvate has therapeutic potential for patients with oxidative phosphorylation (OXPHOS) disorders in which the intracellular NADH/NAD^+ ratio is increased. Such an increased ratio impairs the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the glycolysis pathway. Theoretically, with lactate dehydrogenase, pyruvate provides NAD^+ and decreases this ratio and thereby restores the activity of GAPDH, which produces ATP. Additionally, pyruvate activates pyruvate dehydrogenase and non-enzymatically eliminates hydrogen peroxide.

There are several case reports on the efficacy of pyruvate in patients with OXPHOS disorders [2–4]. However, few of these reports have evaluated the clinical outcomes using a standardized clinical assessment scale. We semi-quantitatively evaluated the efficacy of pyruvate therapy in 4 patients with OXPHOS disorders using standardized scales. This study was approved by the Ethical Committee of our institution. Written informed consent was obtained from the parents of every patient.

2. Patients and methods

2.1. Patients

Four patients who had been on pyruvate for more than 12 months were studied (Table 1). Two patients had Leigh syndrome associated with m.8993 T>G or m.9176 T>C mutations. One patient had non-specific encephalomyopathy associated with complex I and IV combined deficiency. Another patient had myopathic mitochondrial DNA depletion syndrome. All patients were bedridden, and all but one

Abbreviations: NPMDS, Newcastle Pediatric Mitochondrial Disease Scale; GMFM-88, Gross Motor Function Measure with 88 items; JMDRS, Japanese Mitochondrial Disease Rating Scale; OXPHOS, Oxidative phosphorylation; MELAS, Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; FGF-21, Fibroblast growth factor 21.

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Table 1
Profiles of the patients.

Patients	Clinical Dx	Molecular or biochemical Dx	Age at the start of the Tx	ADL at the start of the Tx	Dose of sodium pyruvate (g/kg/day)	Duration of the Tx
Patient 1	Leigh syndrome	m.8993 T>G	8 y 4 m	Bedridden Unable to roll over Tube fed	0.5	27 m
Patient 2	Leigh syndrome	m.9176 T>C	8 m	Bedridden Unable to roll over Tube fed	0.5	66 m
Patient 3	Non-specific encephalomyopathy	Complex I + IV deficiency	1 y 8 m	Able to roll over to one direction Unable to creep Orally fed	0.5 then 1.0	17 m
Patient 4	Myopathic mitochondrial depletion syndrome	mtDNA depletion	1 y 7 m	Bedridden Unable to roll over On a respirator Tube fed	0.5	41 m

Dx, diagnosis; Tx, treatment; mt, mitochondrial; ADL, activities of daily living.

(namely, the patient with combined deficiencies of complex I and IV) were tube fed. The ages at the initiation of pyruvate therapy were 8–100 months (median 20 months). The durations of therapy were 17–66 months (median 34 months). During the pyruvate therapy monitoring period, all other concomitant mitochondrial disease medications were maintained unchanged.

2.2. Pyruvate

Sodium pyruvate was obtained from Musashino Chemical Laboratory (Tokyo). Sodium pyruvate was administered at 0.5 g/kg/day orally or through a feeding tube in 2 divided doses. This dose was increased to 1.0 g/kg/day in one patient. To avoid osmotic diarrhea, the pyruvate was dissolved in water at concentrations of approximately 2%–10%. Higher concentrations were utilized if the dilution caused overhydration or the volume was too large to drink.

2.3. Clinical evaluation

The efficacy of the pyruvate therapy was clinically evaluated with 3 standard scales: the Newcastle Pediatric Mitochondrial Disease Scale (NPMDS) [5], the Gross Motor Function Measure with 88 items (GMFM-88) [6], and the Japanese Mitochondrial Disease Rating Scale (JMDRS) [7]. The NPMDS is composed of 4 domains: Section I, current function; Section II, systemic specific involvement; Section III, current clinical assessment; and Section IV, quality of life. Sections I–III are scored based on objective observations, and Section IV takes the subjective views of the parents into account. Higher scores indicate more severe clinical situations. There are 3 sets of age-specific NPMDSs. Depending on the patient's age at the time of the evaluation, the NPMDS for 0–24 months or that for 2–11 years was used. The GMFM-88 is composed of 5 dimensions: A, lying and rolling; B, sitting; C, crawling and kneeling; D, standing; and E, walking, running and jumping. The scores are expressed in percentages relative to the maximum score in each dimension. The total score is expressed as the mean of percentages across all 5 dimensions. As the patients were bedridden, only dimensions A and B could be assessed, and the scores for the dimensions C to E were considered to be zero %. Higher scores indicate better motor abilities. The JMDRS is the modified Japanese version of the European Neuromuscular Conference (ENMC) Mitochondrial Disease Rating Scale [8]. Higher scores in this scale indicate more severe symptoms. With the exception of Patient 4, who was only assessed with the NPMDS, all other patients were evaluated with the NPMDS and the GMFM at the same time. Patient 2 was initially monitored with the JMDRS. Then, after a 4-week-washout period, the patient was reassessed with the NPMDS and GMFM. Changes in motor functions that were too subtle to be detected with these scales were descriptively recorded. Serum lactate and pyruvate levels as well as plasma amino acids were monitored.

2.4. Statistical analysis

Statistical analysis of the biochemical data was performed using Mann–Whitney *U*-test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

The changes in motor function and assessment scores are summarized in Table 2.

3.1. Patient 1 (m.8993 T>G Leigh syndrome)

The therapy was initiated at the age of 8 years and 4 months, and at this time, this female patient was unable to roll over. In the supine position, she could not raise her legs more than 45 degrees from the floor (as measured at the hip joint). One month after the initiation of therapy, the patient gained the abilities to roll over and raise her legs vertically from the floor. The movement of her arms became more active and rapid. The overall NPMDS score changed from 42.3 to 38.6. The sum of the scores for sections I–III changed from 31 to 29, which indicates that the objective findings improved by 2 points over one month. Dimension A of the GMFM-88 also changed from 31.4% to 47.1%, which resulted in a change from 6.3% to 9.4% in the total score. Thus, this patient's improvement was confirmed semi-quantitatively with 2 scales. Next, pyruvate was withdrawn to confirm the effect of the pyruvate treatment. Within 1 to 2 weeks, the patient became lethargic and less active. After 19 days of washout, she developed status epilepticus. Resumption of pyruvate therapy restored her clinical status to the pre-washout state. Upon re-evaluation at the age of 10 years and 7 months (after 26 months of treatment excluding the washout period), the patient exhibited maintained improved motor ability as confirmed by the unchanged GMFM-88 score. The NPMDS was not administered at this point.

Blood lactate levels and lactate/pyruvate ratios measured twice during the pre-treatment period and once after the 19-day-washout were from 1.2 mM to 1.5 mM (median 1.2 mM), and from 14.2 to 25.6 (median 19.7), respectively. Those measured at 1, 4, 18 and 20 months after the treatment resumption following the washout period ranged from 0.81 mM to 1.2 mM (median 0.85 mM), and from 15.7 to 27.3 (median 20.0), respectively (Table 3). Thus, lactate levels decreased with pyruvate therapy, but the difference was not significant. Lactate/pyruvate ratio was not reduced. Plasma alanine, valine and lysine levels were measured after the washout and 1 month after the treatment resumption. None of these decreased with the therapy (Table 3).

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Table 2
Clinical effects of pyruvate therapy.

Patient 1, Leigh syndrome with m.8993 T>G				
		At the Tx initiation (Age 8 y 4 M)	1 month Tx (Age 8 y 5 m)	26 months Tx (Age 10 y 7 m)
ADL		Unable to roll over Unable to raise the legs > 45° in supine position	Able to roll over Able to raise the legs 90° Moves arms more rapidly	The same as the ADL at 8 y 5 m
NPMDS	I	18	18	ND
	II	2	1	ND
	III	11	10	ND
	IV	11.3	9.6	ND
	Overall	42.3	38.6	ND
GMFM	A	31.4%	47.1%	47.1%
	Total	6.3%	9.4%	9.4%
Patient 2, Leigh syndrome with m.9176 T>C. First treatment				
		At the Tx initiation (Age 8 m)	1-month Tx (Age 9 m)	12-month Tx (Age 20 m)
ADL		Unable to roll over Partially tube-fed	Unable to roll over Partially tube-fed	Able to roll over Orally fed
JMDRS		52	52	53
Patient 2. Second treatment after washout.				
		After 4-week washout (Age 5 y 3 m)	2 months after the Tx resumption (Age 5 y 5 m)	11 months after the resumption (Age 6 y 5 m)
ADL		Unable to roll over Tube-fed	Unable to roll over Tube-fed	Unable to roll over Tube-fed
NPMDS	I	13	13	15
	II	3	3	5
	III	14	14	17
	IV	4.2	4.2	16.7
	Overall	34.2	34.2	53.7
GMFM	A	5.9%	5.9%	3.9%
	Total	1.2%	1.2%	0.8%
Patient 3, complex I + IV deficiency				
		At the Tx initiation (Age 1 y 8 m)	2-month Tx (1 y 10 m)	12-month Tx (2 y 8 m)
ADL		Roll over one direction Head control fair Mild dysphagia	Roll over bilaterally Head control fair No dysphagia	Roll over bilaterally Head control poor
NPMDS	I	7	6	6
	II	6	6	2
	III	15	13	13
	IV	16.7	7.3	7.3
	Overall	44.7	32.3	28.3
GMFM	A	54.9%	66.7%	60.8%
	B	13.3%	13.3%	3.3%
	Total	13.6%	16.0%	12.8%
Patient 4, mitochondrial DNA depletion syndrome				
		At the Tx initiation (Age 1 y 7 m)	2-month Tx (Age 1 y 9 m)	41-month Tx (Age 5 y 0 m)
ADL		On respirator Unable to raise the forearm above the floor Myopathy only	On respirator Able to raise the forearm 90° at the elbow. Myopathy only	On respirator Unable to raise the forearm Encephalomyopathy
NPMDS	I	7	7	15
	II	6	6	15
	III	5	5	24
	IV	17	13	10.8
	Overall	35	31	64.8

Tx, treatment; ADL, Activities of daily living; NPMDS, Newcastle Pediatric Mitochondrial Disease Scale; GMFM, Gross Motor Function Measure; JMDRS, Japanese Mitochondrial Disease Rating Scale; I–IV, Sections I–IV of NPMDS; A and B, Dimensions A and B of GMFM; ND, not done.

3.2. Patient 2 (m.9176 T>C Leigh syndrome)

Pyruvate therapy was initiated at the age of 8 months for this male patient who was unable to roll over and had poor head control. Oral feeding was partially possible. After one-month of treatment, motor function was not altered and neither was the JMDRS score, which was 52. After 12 months of treatment, at the age of 1 year and 8 months, the patient was able to roll over and full oral feeding became possible. However, these subtle changes were not detected by JMDRS. The JMDRS score actually increased by 1 point due to seizures. At 3 years

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Table 3
Changes in blood lactate and amino acids levels with pyruvate therapy.

	Lactate (mM) Median (range) (n) (normal: 0.3–1.9)		Lactate/Pyruvate ratio Median (range) (n) (normal: 10–15)		Alanine (μM) Median (range) (n) (normal: 209–523)		Valine (μM) Median (range) (n) (normal: 148–307)		Lysine (μM) Median (range) (n) (normal: 109–242)	
	Before	After	Before	After	Before	After	Before	After	Before	After
Patient 1	1.2 (1.2–1.5) (3)	0.85 (0.81–1.2) (4)	19.7 (14.2–25.6) (3)	20.0 (15.7–27.3) (4)	256 (1)	439 (1)	165 (1)	263 (1)	104 (1)	200 (1)
Patient 2	2.8 (1.2–4.4) (2)	2.4 (0.9–3.1) (5)	23.2 (19.2–27.2) (2)	23.1 (14.7–30.5) (5)	402 (360–443) (2)	340 (320–428) (5)	173 (172–174) (2)	168 (135–171) (5)	139 (96.6–180) (2)	112 (96.2–172) (5)
Patient 3	3.9 (2.5–8.0) (4)	5.6 (3.7–9.3) (7)	25.0 (14.7–35.3) (4)	30.5 (17.7–45.9) (7)	543 (427–659) (2)	729 (549–840) (7)	171 (154–188) (2)	219 (149–280) (7)	117 (87.8–146) (2)	122 (88.7–172) (7)
Patient 4	2.3 (2.1–2.7) (4)	2.5 (2.3–2.7) (5)	16.9 (14.9–18.7) (4)	17.3 (14.1–21.2) (5)	350 (1)	384 (381–386) (2)	140 (1)	187 (182–191) (2)	108 (1)	158 (157–158) (2)

Mann–Whitney U-test did not show any significant differences.

of age, the patient developed acute encephalopathy associated with a viral infection and lost the abilities of oral feeding and rolling over. To re-evaluate the efficacy of pyruvate, the patient was reassessed with the NPMDS and GMFM-88 at the age of 5 years and 3 months after a 4-week pyruvate washout period. The washout did not cause any deterioration. Two months after the resumption of the pyruvate therapy, neither the NPMDS (overall score, 34.2) nor the GMFM-88 (total score 1.2%) scores changed. After 11 months of therapy after the washout, the scores for all sections of the NPMDS increased, and the overall score increased by 19.5 points. The total GMFM-88 score decreased from 1.2% to 0.8%. Thus, pyruvate was not effective for this patient.

Blood lactate levels and lactate/pyruvate ratios measured twice during 2 months before the first pyruvate therapy at the age of 8 months were 1.2 mM and 4.4 mM (median, 2.8 mM), and 19.2 and 27.2 (median, 23.2), respectively. Those at 1, 2, 3, 4 and 12 months after the therapy ranged from 0.9 mM to 3.1 mM (median, 2.4 mM) and from 14.7 to 30.5 (median, 23.1), respectively. Lactate levels and lactate/pyruvate ratios did not change significantly with the therapy (Table 3). Plasma alanine, valine and lysine levels measured twice before and at 1, 2, 3, 4 and 12 months after the therapy showed a mild but non-significant decrease with the therapy (Table 3).

3.3. Patient 3 (combined deficiencies of complex I and IV)

This male patient presented with developmental delay, nystagmus, hypertrophic cardiomyopathy and mild hearing disturbance (38 dB). At the age of 11 months, he developed status epileptics followed by regression. Increased lactate levels and lactate/pyruvate ratio in the cerebrospinal fluid (CSF) (lactate:5.2 mM, lactate/pyruvate ratio: 20.0) and blood (lactate: 12.3 mM, lactate/pyruvate ratio: 41.6) led to a skin biopsy, which revealed deficiencies in complexes I and IV: the activities of complex I and IV relative to the activity of citrate synthase were 24.7% and 22.9% of normal controls ($n = 12$), respectively, and those relative to the activity of complex II were 33.5% and 31.4% of normal, respectively. Muscle biopsy could not be obtained. The clinical signs and symptoms fulfilled the mitochondrial disease criteria for definite mitochondrial disorder proposed by Morava et al. [9]. No mutation was revealed in the mitochondrial DNA. Molecular analysis of the nuclear genes is under way. Treatment with coenzyme Q_{10} at the age of 1 year and 6 months did not produce any improvement. Pyruvate therapy was initiated at the age of 1 year and 8 months, and at this time the patient had mild dysphagia and incomplete head-control. He could roll over only in one direction. After 2 months of pyruvate therapy with a maintenance dose of 1.0 g/kg/day, he gained the ability to roll over bilaterally and the dysphagia disappeared. The total scores for sections I–III decreased from 28 to 25, and the score for IV also decreased from 16.7 to 7.3. The GMFM-88 score increased from 13.6% to 16.0%. Thus, the efficacy of the 2-month pyruvate therapy was confirmed by both scales. However, over the next 10 months, a slow regression in motor function was observed, and at 2 years and 8 months of age (after 12 months of treatment), this patient's GMFM-88 score decreased from 16.0% to 12.8%. However, the scores for section II of the NPMDS (the version for 2–11 year-olds was used) decreased by 4 points due to improvements in seizures and gastrointestinal and hepatic function. The regression of motor function that was evident in the GMFM-88 was not detected by the NPMDS (the scores for sections I and III were unchanged).

Blood lactate levels and lactate/pyruvate ratios measured 4 times during the 9-month pre-treatment period ranged from 2.5 mM to 8.0 mM (median, 3.9 mM), and from 14.7 to 35.3 (median, 25.0), respectively. Those measured 1, 2, 3, 4, 6, 9 and 12 months after the therapy ranged from 3.7 mM to 9.3 mM (median, 5.6 mM), and from 17.7 to 45.9 (median 30.5), respectively (Table 3). Thus, neither the blood lactate levels nor the lactate/pyruvate ratios decreased with the pyruvate therapy. Among the measurements, those measured twice during the first 2-month treatment, which was clinically effective, did not show any decrease either. Plasma alanine, valine and lysine levels were

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measured twice before the treatment and 7 times after the therapy. None of these decreased significantly with the therapy (Table 3).

Throughout the therapy, the patient exhibited chronic diarrhea that seemed to be a side effect of the treatment.

3.4. Patient 4 (myopathic form of the mtDNA depletion syndrome)

The short-term efficacy of pyruvate therapy for this female patient and her clinical and biochemical profile have been reported in detail elsewhere [3]. Briefly, the patient developed severe generalized weakness including facial muscles and respiratory failure during the neonatal period. The patient had a tracheostomy and was on a respirator. She had lactic acidosis (3.0 mM to 6.5 mM) with high lactate/pyruvate ratio (36 to 97). Muscle biopsy revealed ragged red fibers and decreased cytochrome c oxidase staining. The activities of complex I, III and IV relative to the activity of citrate synthase in the muscle were 10.6%, 26.7% and 14.1% of the control, respectively. Those relative to the activity of complex II were 6.5%, 16.4% and 8.8%, respectively. Quantitative analysis of the mtDNA revealed that the copy number of the mitochondrial ND1 subunit relative to the nuclear CFTR gene was 35.3% (normal: >40%). Exome sequencing is under way to detect a mutation in causative genes. The clinical signs and symptoms were compatible with Morava et al.'s criteria for definite mitochondrial disease [9]. As reported elsewhere, after 2 months of pyruvate therapy, the patient exhibited a mild improvement in the movement of her extremities at the age of 1 year and 9 months [3]. The overall NPMDS scores decreased from 35 to 31, but this decrease was limited to section IV. As the patient was not assessed with the GMFM, we were unable to semi-quantitatively demonstrate the improvement in motor function. One month later (after 3 months of treatment), the patient developed status epilepticus. An MRI revealed lesions in the occipital areas, which indicated a progression from the myopathic form to the encephalomyopathic form. At 5 years of age, after 41 months of treatment, scores in all sections of the NPMDS increased, and the increase in overall NPMDS score was 33.8 points compared to the score at the 2-month treatment.

Blood lactate and lactate/pyruvate ratios measured 4 times during the 2-month pre-treatment period ranged from 2.1 mM to 2.7 mM (median, 2.3 mM), and from 14.9 to 18.7 (median, 16.9), respectively. Those measured 1, 4, 6, 8 and 13 weeks after the therapy ranged from 2.3 mM to 2.7 mM (median, 2.5 mM), and from 14.1 to 21.2 (median, 17.3), respectively (Table 3). Plasma alanine, valine and lysine levels were measured once before the therapy and 4 and 8 weeks after the therapy. None of these decreased with the pyruvate therapy (Table 3).

4. Discussion

All 4 of the treated patients were severely disabled and bedridden. Therefore, objective and semi-quantitative assessments of the outcomes were difficult because the expected improvements were subtle. The NPMDS is a scale that was designed to specifically monitor mitochondrial disease, which results in a variety of multi-organ symptoms. Therefore, the scale encompasses all aspects of mitochondrial disease. Consequently, this scale cannot detect small changes in motor function. The logic applies to the JMDRS. In contrast, the GMFM-88 evaluates motor function with as many as 88 items; therefore, this assessment may detect small changes in motor abilities. However, the GMFM was designed to assess cerebral palsy, and its reliability in monitoring mitochondrial disease has not been validated. In contrast to the GMFM-66, which can only be used for cerebral palsy, the GMFM-88 has been validated for the monitoring of motor functions in disorders other than cerebral palsy, such as spinal muscular atrophy, Down syndrome and traumatic brain injuries. [10–12] Therefore, we assumed that the GMFM-88 could also be used to monitor motor functions in mitochondrial disease. Nevertheless, given that the GMFM-88 has not been validated for using in mitochondrial disease, we assessed the outcomes via a combination of the GMFM-88 and NPMDS scores with the exception of patient 4, who was assessed only with the NPMDS. We also tried using other scales including Pediatric Evaluation of Disability Inventory (PEDI) [13] and Functional Independence Measure for Children (Wee-FIM) [14]. Our preliminary study, however, showed that these could not detect clinical changes in our patients.

Patients 3 and 4 were assessed with 2 different sets of age-specific NPMDSs as they matured into ages suitable for the application of the older age-specific NPMDSs during the monitoring period. The number of items scored in each section of the NPMDS for 2–11-year-olds is greater than that of the NPMDS for 0–24-month-olds. Therefore, it is possible that total NPMDS scores may increase when the version for older patients is used even if clinical severity remains unchanged. In Patient 3, the score for section II as assessed 2 years and 8 months decreased compared to the score assessed at 1 year and 10 months, whereas the scores for the other sections remained unchanged. Thus, a "pseudo-increase" in the score due to the use of a different set of NPMDS scales did not occur in this patient. In Patient 4, the scores for sections I, II and III increased by 8, 9 and 19 points, respectively, at 5 years of age compared to the scores observed at 21 months of age. Given that the maximum scores for sections I, II and III are higher by 6, 3 and 6 points, respectively, in the NPMDS for 2-11 year-olds than in the NPMDS for 0–24 month-olds, the increases in the scores that were higher than the maximum possible increases due to the differences in the versions of the NPMDS indicated that the increases were real.

The most noteworthy result of this study was that 3 of the 4 severely disabled patients (Patients 1, 3 and 4) exhibited improvement within 1 to 2 months of the initiation of pyruvate therapy. These improvements were confirmed by both the NPMDS and GMFM-88 (Patients 1 and 3) or the NPMDS only (Patient 4). The semi-quantitative improvement observed in Patient 4 was limited to section IV of the NPMDS, which accounts for the parents' subjective assessments. However, a descriptive observation record also revealed improvement in muscle power. [3] Given that no improvements were observed prior to pyruvate therapy in these patients and that the improvements were observed with 1–2 months of the initiation of pyruvate therapy, it is unlikely that the observed ameliorations were simply due to natural motor development rather than the effects of the therapy. The efficacy was particularly evident in Patient 1 who had m.8993 T>G and exhibited improvements in motor function that were maintained for over 2 years. The worsening of symptoms during pyruvate withdrawal also supported the efficacy of pyruvate treatment in this patient. In contrast, 2 of the 3 responsive patients did not maintain the improvements for longer than several months. Notably however, the overall NPMDS score for Patient 3 decreased (i.e., symptoms improved) after 12 months of therapy compared to this patient's score after 2 months of the therapy despite the worsening of the GMFM score. These findings indicated that the patient's overall health improved during long-term therapy, although this patient's motor abilities regressed. In Patient 4, the disease progression overwhelmed the effect of the pyruvate therapy shortly after the responsiveness was confirmed after 2 months of therapy; this finding indicated a limitation of this therapy. We could not explain why Patient 2, who had m.9176 T>C, did not respond to pyruvate therapy. Given the age of this patient, the mild improvements in motor function after 12 months of pyruvate therapy, which could not be detected with the JMDRS, seemed to be due to natural motor development rather than resulting from the treatment.

The only adverse effect of pyruvate therapy was the mild but chronic diarrhea that was observed in one patient who was on 1.0 g/kg/day of sodium pyruvate.

An *in vitro* study that utilized cybrid cells harboring MELAS m.3243A>G mutant mitochondria found that pyruvate treatment facilitates the pyruvate-to-lactate conversion, decreases the lactate/pyruvate ratio, normalizes the NADH/NAD^+ ratio, and enhances ATP production and energy charge without significantly altering the intracellular lactate level. [15] These data support the theory that the effects of pyruvate

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therapy are mediated via the normalization of the $NADH/NAD^+$ ratio, which provides the NAD^+ that is deficient in OXPHOS disturbances. In contrast to the theory and the result of this *in vitro* study, none of our responsive patients exhibited decreases in blood lactate/pyruvate ratios, which are equivalent to the $NADH/NAD^+$ ratios, during the effective short-term therapy. Blood lactate levels decreased in 2 patients, especially in Patient 1, but the differences were non-significant. Thus, the blood lactate/pyruvate ratios and blood lactate levels of our patients could not be used as biochemical markers to monitor the effects of the therapy. The discrepancy between the clinical data from our patients and the *in vitro* data may be partly explained by the fact that blood lactate levels vary depending on the physical activity of the patient at the time of blood sampling, the interval between meal and sampling, as well as on the time required for the blood sampling procedure. However, all of our patients were bedridden and the data were from multiple samplings in different days. The blood samplings were done either after overnight-fast or several hours after a meal. Therefore, it is unlikely that the discrepancy was artifactual. Still, monitoring the lactate levels and lactate/pyruvate ratios in the CSF rather than in the blood would further reduce the possible artifact. Komaki et al. treated an ambulatory patient with Leigh syndrome associated with cytochrome c oxidase deficiency [2]. With pyruvate therapy, blood lactate level and lactate/pyruvate ratio decreased from 2.3 mM to 1.1 mM, and from 17.7 to 11.4, respectively. However, the measurements were done only once before and after the therapy, so the statistical significance could not be evaluated. Koga et al. found statistically significant decreases in blood lactate, pyruvate and alanine levels with pyruvate therapy in a non-ambulatory patient with pyruvate dehydrogenase (PDH) deficiency [4]. Blood lactate/pyruvate ratio in this patient also decreased, but the difference was non-significant (the ratios in PDH deficiency are generally normal). Differences between Komaki et al. and Koga et al.'s patients from ours were that 1) Komaki et al.'s patient was ambulatory, and 2) the pre-treatment blood levels of lactate and alanine in Koga et al.'s patient were much higher than those in our patients: the blood lactate and alanine levels in this patient were 9.6 ± 0.54 mM ($n = 8$) and 1700 ± 280 μM ($n = 8$), respectively, while the median values of pre-treatment lactate levels in our 4 patients ranged from 1.2 to 3.9 mM and those of alanine were from 256 to 543 μM. This may indicate that the blood lactate and alanine levels and lactate/pyruvate ratio are not sensitive biochemical markers to monitor the pyruvate therapy unless the patients are ambulatory or their pre-treatment blood levels of lactate and alanine are very high.

If the blood lactate/pyruvate ratio does not necessarily reflect the intracellular $NADH/NAD^+$ ratio, the identification of a marker other than blood lactate and pyruvate is crucial. Kami et al. found that the lysine and valine levels in media in which MELAS-mutant cybrid cells were incubated with 10 mM lactate were higher than those of controls. These increases may be because catabolisms of lysine to acetyl CoA and valine to succinyl CoA require NAD^+, which is deficient due to the imbalance in the $NADH/NAD^+$ ratio [15]. Plasma levels of lysine and valine in our patients, however, did not decrease with the therapy. We do not know if the levels of these amino acids may decrease with pyruvate therapy in patients with very high blood lactate levels: Koga et al. did not measure valine and lysine levels in their responsive patient [4]. Fibroblast growth factor 21 (FGF-21), a circulating hormone-like cytokine, is reported to be one of the best biomarker with high sensitivity and specificity for detecting muscle-manifesting mitochondrial respiratory chain deficiencies [16]. Although FGF-21 has higher sensitivity than lactate or lactate/pyruvate ratio to diagnose mitochondrial disease, its utility in monitoring the disease is unknown. Further study is necessary to find biomarkers to monitor the effect of pyruvate therapy biochemically.

In conclusion, as confirmed by the GMFM-88 and/or NPMDS, pyruvate therapy was safe and effective even in severely disabled patients with OXPHOS disorders, at least in the short-term. Further studies utilizing greater numbers of patients with less severe disabilities are necessary to evaluate the long-term efficacy of this treatment. The blood lactate and pyruvate levels did not correlate with the efficacy of the pyruvate therapy in our patients as has been reported in *in vitro* studies. The identification of more sensitive biomarkers that reflect the intracellular $NADH/NAD^+$ ratio or improvements in ATP production is crucial for monitoring the clinical and biochemical efficacy of this therapy.

Conflict of interest

The authors have no conflicts of interest to disclose.

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References

[1] M. Tanaka, Y. Nishigaki, N. Fuku, T. Ibi, K. Sahashi, Y. Koga, Therapeutic potential of pyruvate therapy for mitochondrial diseases, Mitochondrion 7 (2007) 399–401.
[2] H. Komaki, Y. Nishigaki, N. Fuku, H. Hosoya, K. Murayama, A. Ohtake, Y. Goto, H. Wakamoto, Y. Koga, M. Tanaka, Pyruvate therapy for Leigh syndrome due to cytochrome c oxidase deficiency, Biochim. Biophys. Acta 1800 (2010) 313–315.
[3] K. Saito, N. Kimura, N. Oda, H. Shimomura, T. Kumada, T. Miyajima, K. Murayama, M. Tanaka, T. Fujii, Pyruvate therapy for mitochondrial DNA depletion syndrome, Biochim. Biophys. Acta 1820 (2012) 632–636.
[4] Y. Koga, N. Povalko, K. Katayama, N. Kakimoto, T. Matsuishi, E. Naito, M. Tanaka, Beneficial effect of pyruvate therapy on Leigh syndrome due to a novel mutation in PDH E1alpha gene, Brain Dev. 34 (2012) 87–91.
[5] C. Phoenix, A.M. Schaefer, J.L. Elson, E. Morava, M. Bugiani, G. Uziel, J.A. Smeitink, D.M. Turnbull, R. McFarland, A scale to monitor progression and treatment of mitochondrial disease in children, Neuromuscul. Disord. 16 (2006) 814–820.
[6] M. Alotaibi, T. Long, E. Kennedy, S. Bavishi, The efficacy of GMFM-88 and GMFM-66 to detect changes in gross motor function in children with cerebral palsy (CP): a literature review, Disabil. Rehabil. 36 (2014) 617–627.
[7] S. Yatsuga, N. Povalko, J. Nishioka, K. Katayama, N. Kakimoto, T. Matsuishi, T. Kakuma, Y. Koga, M.S.G.i.J. Taro Matsuoka for, MELAS: a nationwide prospective cohort study of 96 patients in Japan, Biochim. Biophys. Acta 1820 (2012) 619–624.
[8] P.F. Chinnery, L.A. Bindoff, 116th ENMC international workshop: the treatment of mitochondrial disorders, 14th–16th March 2003, Naarden, the Netherlands, Neuromuscul. Disord. 13 (2003) 757–764.
[9] E. Morava, L. van den Heuvel, F. Hol, M.C. de Vries, M. Hogeveen, R.J. Rodenburg, J.A. Smeitink, Mitochondrial disease criteria: diagnostic applications in children, Neurology 67 (2006) 1823–1826.
[10] M. Linder-Lucht, V. Othmer, M. Walther, J. Vry, U. Michaelis, S. Stein, H. Weissenmayer, R. Korinthenberg, V. Mall, Gross motor function measure-traumatic brain injury study, Validation of the gross motor function measure for use in children and adolescents with traumatic brain injuries, Pediatrics 120 (2007) e880–e886.
[11] L. Nelson, H. Owens, L.S. Hynan, S.T. Iannaccone, S.G. Am, The gross motor function measure is a valid and sensitive outcome measure for spinal muscular atrophy, Neuromuscul. Disord. 16 (2006) 374–380.
[12] D. Russell, R. Palisano, S. Walter, P. Rosenbaum, M. Gemus, C. Gowland, B. Galuppi, M. Lane, Evaluating motor function in children with Down syndrome: validity of the GMFM, Dev. Med. Child Neurol. 40 (1998) 693–701.
[13] L.V. Iyer, S.M. Haley, M.P. Watkins, H.M. Dumas, Establishing minimal clinically important differences for scores on the pediatric evaluation of disability inventory for inpatient rehabilitation, Phys. Ther. 83 (2003) 888–898.
[14] M.E. Msall, K. DiGaudio, B.T. Rogers, S. LaForest, N.L. Catanzaro, J. Campbell, F. Wilczenski, L.C. Duffy, The Functional Independence Measure for Children (WeeFIM): conceptual basis and pilot use in children with developmental disabilities, Clin. Pediatr. 33 (1994) 421–430.
[15] K. Kami, Y. Fujita, S. Igarashi, S. Koike, S. Sugawara, S. Ikeda, N. Sato, M. Ito, M. Tanaka, M. Tomita, T. Soga, Metabolomic profiling rationalized pyruvate efficacy in cybrid cells harboring MELAS mitochondrial DNA mutations, Mitochondrion 12 (2012) 644–653.
[16] A. Suomalainen, J.M. Elo, K.H. Pietilainen, A.H. Hakonen, K. Sevastianova, M. Korpela, P. Isohanni, S.K. Marjavaara, T. Tyni, S. Kiuru-Enari, H. Pihko, N. Darin, K. Ounap, L.A. Kluijtmans, A. Paetau, J. Buzkova, L.A. Bindoff, J. Annunen-Rasila, J. Uusimaa, A. Rissanen, H. Yki-Jarvinen, M. Hirano, M. Tulinius, J. Smeitink, H. Tyynismaa, FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a diagnostic study, Lancet Neurol. 10 (2011) 806–818.

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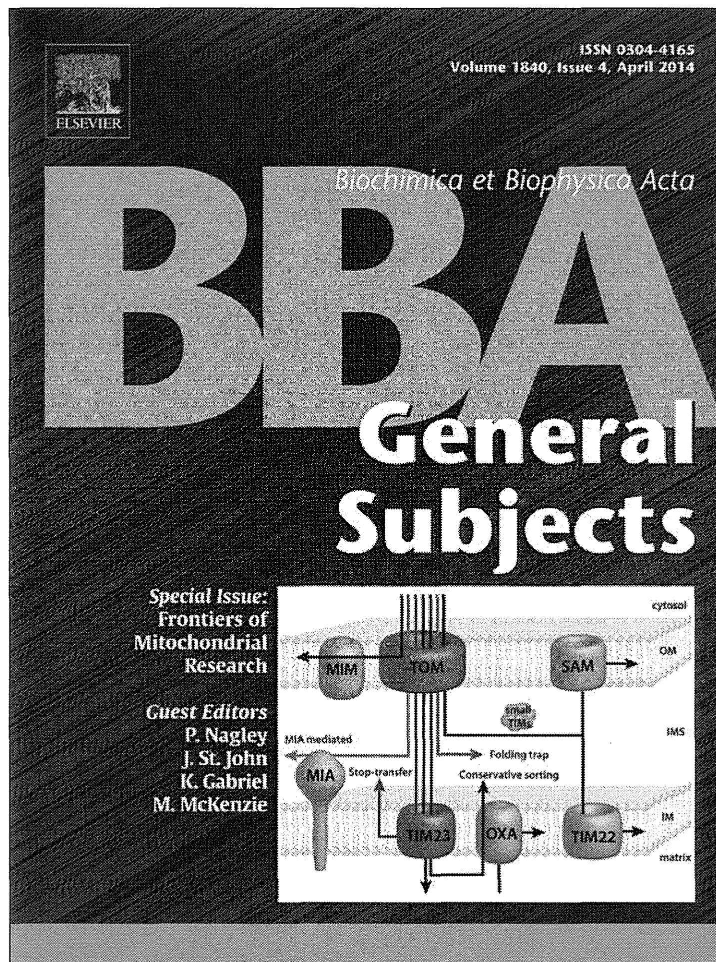

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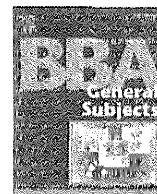

Diagnosis and molecular basis of mitochondrial respiratory chain disorders: Exome sequencing for disease gene identification ☆,☆☆

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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.

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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

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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*	*POLG, POLG2, C10orf2, MPV17, AGK*
NDUFA1, 2, 9, 10, 11, 12, NDUFS1, 2, 3, 4, 6, 7, 8	**mtDNA expression: 24 genes**
Complex II: *SDHA, SDHB, SDHC, SDHD*	*LRPPRC, TACO1, MTPAP, MRPS16, MRPS22,MRPL3,*
Complex III: *UQCRB, UQCRQ*	*GFM1,TSFM,TUFM,TRMU,C12orf65,MTFMT, DARS2,*
Complex IV: *COX6B1, COX4I2, COX7B*	*RARS2,YARS2,SARS2,AARS2,HARS2,MARS2,EARS2,*
Complex V: *ATP5E*	*RMND1, MTO1, FARS2, GFM2*
Import, processing, assembly: 38 genes	**Nucleotide transport, synthesis: 9 genes**
Complex I: *C8orf38, C20orf7, NDUFAF1, F2, F3, F4, FOXRED1, NUBPL, ACAD9, AIFM1*	*SLC25A4, SLC25A3, TYMP, DGUOK, TK2, PUS1, SUCLA2, SUCLG1, RRM2B*
Complex II:*SDHAF1, SDHAF2*	**Membrane composition: 14 genes**
Complex III:*BCS1L, HCCS, TTC19*	*COQ2, COQ6, COQ9, PDSS1, PDSS2, CABC1,*
Complex IV:*SURF1, SCO2, SCO1, COX10, COX15, ETHE1, FASTKD2, C2orf64, C12orf62*	*SERAC1, MPC1, NMT, TAZ, CYCS, OPA1, MFN2, DNM1L*
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

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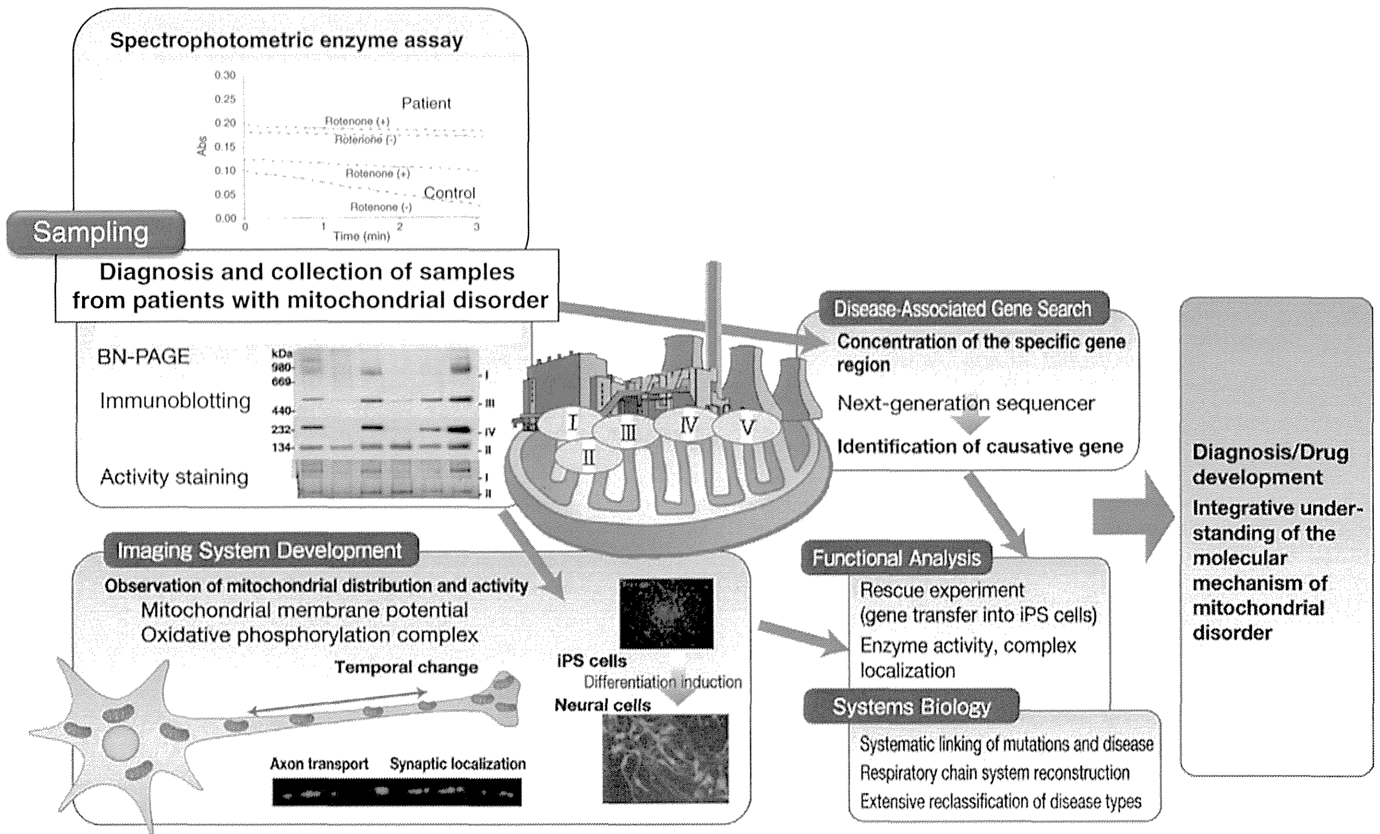

Fig. 1. Outline of exome analysis project for MRCD patients. The first step is 'Sampling', which refers to diagnosis and collection of samples from patients with mitochondrial disorders using both spectrophotometric enzyme assay [11] and BN-PAGE [13]. The next step is 'Disease-Associated Gene Search' 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.

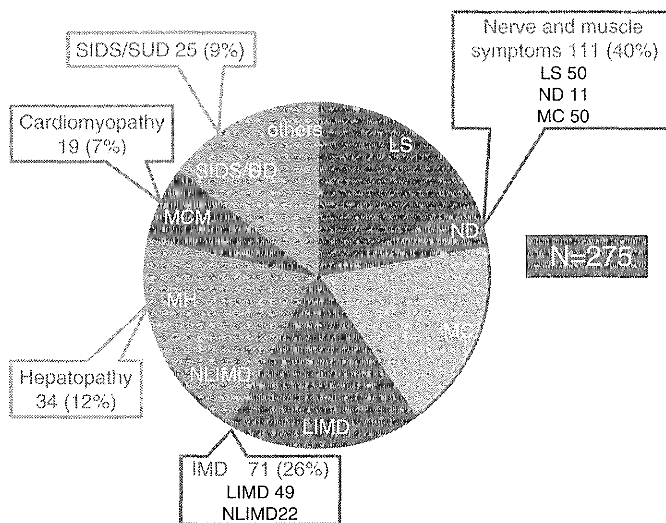

Fig. 2. Breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013. LS, Leigh syndrome; ND, neurodegenerative disorder; MC, mitochondrial cytopathy; IMD, infantile mitochondrial disease (lethal and non-lethal); MH, mitochondrial hepatopathy; MCM, mitochondrial cardiomyopathy;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

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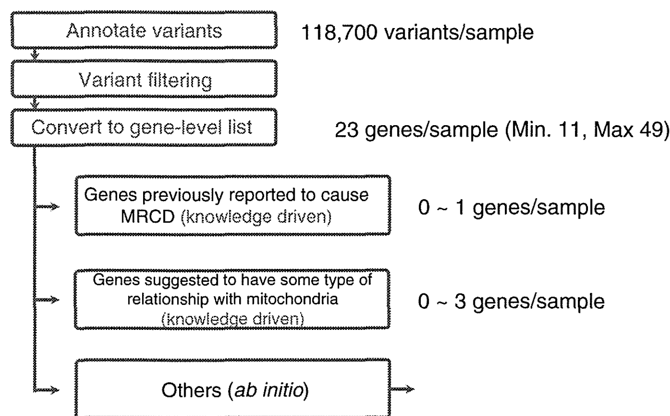

Fig. 3. Narrowing down of gene mutations discovered by exome analysis. After filtering out variants with the methods described in the 'Exome analysis of MRCD patients' section, genes were divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some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

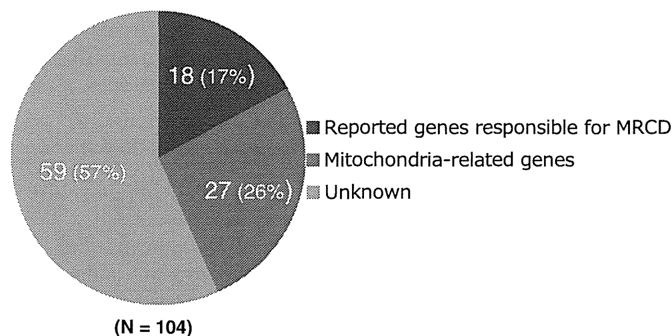

Fig. 4. Candidate genes with exome analysis for MRCD patients. Results of analysis for 104 patients to date (as of January 2013) are shown. Eighteen patients (17%) had variants previously reported to be disease-causing. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow 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

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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.

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References

[1] D. Skladal, J. Halliday, D.R. Thorburn, Minimum birth prevalence of mitochondrial respiratory chain disorders in children, Brain 126 (2003) 1905–1912.
[2] S. Dimauro, G. Davidzon, Mitochondrial DNA and disease, Ann. Med. 37 (2005) 222–232.
[3] D.M. Kirby, D.R. Thorburn, Approaches to finding the molecular basis of mitochondrial oxidative phosphorylation disorders, Twin Res. Hum. Genet. 11 (2008) 395–411.
[4] D.C. Wallace, Mitochondria and cancer, Nat. Rev. Cancer 12 (2012) 685–698.
[5] M.F. Lopez, B.S. Kristal, E. Chernokalskaya, A. Lazarev, A.I. Shestopalov, A. Bogdanova, M. Robinson, High-throughput profiling of the mitochondrial proteome using affinity fractionation and automation, Electrophoresis 21 (2000) 3427–3440.
[6] H. Schägger, K. Pfeiffer, The ratio of oxidative phosphorylation complexes I–V in bovine heart mitochondria and the composition of respiratory chain supercomplexes, J. Biol. Chem. 276 (2001) 37861–37867.
[7] D.R. Thorburn, Mitochondrial disorders: prevalence, myths and advances, J. Inherit. Metab. Dis. 27 (2004) 349–362.
[8] W.J. Koopman, P.H. Willems, J.A. Smeitink, Monogenic mitochondrial disorders, N. Engl. J. Med. 366 (2012) 1132–1141.
[9] S.B. Vafai, V.K. Mootha, Mitochondrial disorders as windows into an ancient organelle, Nature 491 (2012) 374–383.
[10] E.J. Tucker, A.G. Compton, D.R. Thorburn, Recent advances in the genetics of mitochondrial encephalopathies, Curr. Neurol. Neurosci. Rep. 10 (2010) 277–285.
[11] D.M. Kirby, M. Crawford, M.A. Cleary, H.H. Dahl, X. Dennett, D.R. Thorburn, Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder, Neurology 52 (1999) 1255–1264.
[12] F.P. Bernier, A. Boneh, X. Dennett, C.W. Chow, M.A. Cleary, D.R. Thorburn, Diagnostic criteria for respiratory chain disorders in adults and children, Neurology 59 (2002) 1406–1411.
[13] H. Schägger, G. von Jagow, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, Anal. Biochem. 199 (1991) 223–231.
[14] K. Gibson, J.L. Halliday, D.M. Kirby, J. Yaplito-Lee, D.R. Thorburn, A. Boneh, Mitochondrial oxidative phosphorylation disorders presenting in neonates: clinical manifestations and enzymatic and molecular diagnoses, Pediatrics 122 (2008) 1003–1008.
[15] T. Yamazaki, K. Murayama, A.G. Compton, C. Sugiana, H. Harashima, S. Amemiya, M. Ajima, T. Tsuruoka, A. Fujinami, E. Kawachi, Y. Kurashige, K. Matsushita, H. Wakiguchi, M. Mori, H. Iwasa, Y. Okazaki, D.R. Thorburn, A. Ohtake, Molecular diagnosis of mitochondrial respiratory chain disorders in Japan: focusing on mitochondrial DNA depletion syndrome, Pediatr. Int. 56 (2014)(in press).
[16] C. Gilissen, A. Hoischen, H.G. Brunner, J.A. Veltman, Disease gene identification strategies for exome sequencing, Eur. J. Hum. Genet. 20 (2012) 490–497.
[17] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows–Wheeler transform, Bioinformatics 25 (2009) 1754–1760.
[18] S.B. Ng, E.H. Turner, P.D. Robertson, S.D. Flygare, A.W. Bigham, C. Lee, T. Shaffer, M. Wong, A. Bhattacharjee, E.E. Eichler, M. Bamshad, D.A. Nickerson, J. Shendure, Targeted capture and massively parallel sequencing of 12 human exomes, Nature 461 (2009) 272–276.
[19] A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, K. Garimella, D. Altshuler, S. Gabriel, M. Daly, M.A. DePristo, The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, Genome Res. 20 (2010) 1297–1303.
[20] K. Wang, M. Li, H. Hakonarson, ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data, Nucleic Acids Res. 38 (2010) e164.
[21] X. Liu, X. Jian, E. Boerwinkle, dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions, Hum. Mutat. 32 (2011) 894–899.
[22] M. Yandell, C. Huff, H. Hu, M. Singleton, B. Moore, J. Xing, L.B. Jorde, M.G. Reese, A probabilistic disease-gene finder for personal genomes, Genome Res. 21 (2011) 1529–1542.
[23] The UniProt Consortium, Update on activities at the Universal Protein Resource (UniProt) in 2013, Nucleic Acids Res. 41 (2013) D43–D47.
[24] D.J. Pagliarini, S.E. Calvo, B. Chang, S.A. Sheth, S.B. Vafai, S.E. Ong, G.A. Walford, C. Sugiana, A. Boneh, W.K. Chen, D.E. Hill, M. Vidal, J.G. Evans, D.R. Thorburn, S.A. Carr, V.K. Mootha, A mitochondrial protein compendium elucidates complex I disease biology, Cell 134 (2008) 112–123.