Number of text pages: 14 pages.

Number of words: 2868 words in main text.

Reference pages: 23 in 4 pages.

Table: 1.

Figures: 7.

Legends to figures: 4 pages

Acknowledgements: 1 page

Abstract

Background: Although mitochondrial respiratory chain disorders (MRCDs) are one of the most common congenital metabolic diseases, there is no cumulative data of enzymatic diagnoses and clinical manifestations about MRCDs in Japan and Asia.

Methods: We evaluated 675 Japanese patients having profound lactic acidemia, or patients having symptoms or signs of multiple-organ origin simultaneously without lactic acidemia by respiratory chain enzyme activity assay and blue native polyacrylamide gel electrophoresis. Quantitative PCR was used to diagnose mitochondrial DNA depletion syndrome (MTDPS). Mutation analysis of several genes responsible for MTDPS was also performed.

Results: A total of 232 patients were diagnosed with a probable or definite MRCDs. MRCDs are common, afflicting one in every several thousand people in Japan. More than one in ten of the patients diagnosed lacked lactic acidemia. A subsequent analysis of the causative genes of MTDPS revealed novel mutations in six of the patients. A 335-base pair deletion in *DGUOK* (g.11692_12026del335 (p.A48fsX90)) was noted in two unrelated families, and may therefore be a common mutation in Japanese. The proportion of all patients with MTDPS, and particularly those with recessive *POLG* mutations, appears to be lower in Japan than in other studies. This is most likely due to the relatively high prevalence of

ancient European *POLG* mutations in Caucasian populations. No other significant differences were identified in a comparison of the enzymatic diagnoses, disease classifications or prognoses in Japanese and Caucasian patients of MRCDs.

Conclusion: MTDPS and other MRCDs are common, but serious, diseases that occur across all races.

Key words:

DGUOK deletion mutation, enzymatic diagnosis, mitochondrial DNA depletion syndrome, mitochondrial respiratory chain disorder, racial difference.

Abbreviations: MRCD, mitochondrial respiratory chain disorder; MTDPS, mitochondrial DNA depletion syndrome; BN-PAGE, blue native polyacrylamide gel electrophoresis; qPCR, quantitative PCR; DGUOK, deoxyguanosine kinase; POLG, DNA polymerase γ.

Introduction

Mitochondrial respiratory chain disorders (MRCDs) are disorders of the oxidative phosphorylation system, which is responsible for ATP production. MRCDs are the most common congenital metabolic diseases, afflicting at least 1 in 5000 persons.[1] Mitochondrial DNA depletion syndrome (MTDPS), in which mitochondrial DNA levels are lower than normal, is one of the major MRCDs. A number of responsible genes of MTDPS have been identified, and the pathophysiology of this disease is partially characterized at the molecular level.[2-5] We have previously diagnosed and characterized MRCD cases in Japan using respiratory chain enzyme analysis.[6-9] Having recently analyzed the molecular diagnoses and clinical manifestations of MRCD in Japanese patients, and analyzing several genes responsible for hepatocerebral MTDPS, we herein discuss and compare the collected data to those reported for MRCD cases outside of Japan.

Materials and Methods

Patients and samples

The study population comprised patients clinically suspected to have MRCD. We measured respiratory chain enzyme activity and quantity for patients having profound lactic acidemia,

or patients having symptoms or signs of multiple-organ origin simultaneously without lactic acidemia. Other metabolic disorders were excluded with plasma tandem mass spectrometry and urine organic acid analyses. About half of candidates were less than 1 year old, and near 90 % were less than 10 years old. In total, 1051 samples from 675 patients in 657 families were analyzed. Of the samples, 479 were cultured skin fibroblast cells, 239 were liver samples, 208 were muscle samples, 84 were myocardial samples, and 41 were other samples (including 25 kidney and 7 brain samples).

Respiratory chain enzyme analysis

Both an *in vitro* respiratory chain enzyme activity assay[10] and blue native polyacrylamide gel electrophoresis (BN-PAGE)[11-13] were used to quantify the activity and amount of respiratory chain enzyme complexes. A diagnosis of MRCD was made when the results from the enzyme activity or BN-PAGE analyses raised the diagnostic criteria assessment to definite or probable for MRCD according to the diagnostic criteria of Bernier et al.[14]

Entire mitochondrial DNA analysis

DNA of patients was purified according to standard methods. The mitoSEQr™ system (Applied Biosystems, CA, USA) was used for entire mitochondrial DNA analysis in each patient diagnosed with MRCD.

Quantitative PCR (qPCR) for a diagnosis of MTDPS

Quantitative PCR (qPCR)[15] was used to determine whether mtDNA depletion was present in patients showing decreased activity levels of multiple respiratory chain enzymes (the mtDNA gene *MT-ND1* was compared against a nuclear gene (*CFTR* exon 24). A diagnosis of MTDPS was made when the relative copy number of mtDNA to nuclear DNA was less than 35% of that in healthy control tissue using 4 independent experiments.

Mutation analysis of genes responsible for MTDPS

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon-intron boundaries of *POLG* (NM_002693.2), *DGUOK* (NM_080916.1 and NM_080918.1), and *MPV17* (NM_002437.4)[16]. Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Melbourne, Australia). Long range PCR encompassing the 335-base pair deletion was performed using primers shown in Fig. 5A legend.

DNA from healthy Japanese controls

A PSC Cell Line Purified DNA 100 set (Japan Health Sciences Foundation, Tokyo, Japan) was used as control DNA of healthy Japanese.

Statistics

The log-rank test and Gehan-Breslow-Wilcoxon test were used to test for statistically

significant differences.

Ethics

This study was approved by the Institutional Review Board in Saitama Medical University.

Case reports of Japanese three deoxyguanosine kinase (DGUOK) deficiency

Patient 1

This Japanese girl was the first child to unrelated healthy parents and was born without any complications at 40 weeks of gestational age, weighing 2510 g. At 3 months of age, she was referred to hospital because of failure to thrive, nystagmus and incomplete head control. Laboratory tests showed mild liver dysfunction of unknown etiology. She was suspected to have hereditary tyrosinemia because her blood tyrosine level was 800 nmol/mL [cut off 500 nmol/mL], but urinary succinylacetone was not detected. At the age of 18 months, her liver dysfunction deteriorated to the level of liver failure with prolonged coagulation time (hepaplastin time 39%), and she underwent a liver transplantation, but died of cardiac tamponade at 19 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (0%, 9%, and 28% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (74% and 308%, respectively). In BN-PAGE analysis, the band corresponding to assembled complex I

was invisible and those of complex III and IV were strikingly weak (data not shown). qPCR revealed that mtDNA of liver was markedly decreased (3%) confirming a diagnosis of hepatocerebral MTDPS.

Patient 2

A healthy sister of Patient 1 was born two years after her elder sister died. A third girl was born four years after her eldest sister died, without any complications at 40 weeks of gestation, with a weight of 2750 g. At 2 days of age, she was referred to hospital due to tachypnea, hypoglycemia, and metabolic acidosis. After that, mild liver dysfunction was found (total bilirubin 4.2 mg/dL; direct bilirubin 1.4 mg/dL; AST 215 IU/L; ALT 49 IU/L; γGTP 842 IU/L) with hyperammonemia (180 μg/dL). Blood levels of lactate and pyruvate were 20.9 mmol/L, and 0.27 mmol/L, respectively. Because of her eldest sister's course, she did not undergo liver transplantation and she died of liver failure at 9 months of age. The liver showed low activity of complexes I, III, and IV (0%, 6%, and 17% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (105% and 281%, respectively), like the eldest sister. qPCR revealed that mtDNA of liver was markedly decreased (6%) and she was diagnosed to have hepatocerebral MTDPS.

Patient 3

A Japanese girl, unrelated to patients 1 and 2, was born as the third child to unrelated healthy parents at 37 weeks of gestational age weighing 1688 g. Symmetrical IUGR was noted from 30 weeks gestation. Her eldest brother died at 1 year and 4 months old with a hepatic disorder of unknown origin. Her elder sister was healthy. At 8 days of age, she was suffering from feeding difficulty with liver dysfunction and nystagmus. Developmental delay and failure to thrive gradually progressed. At the age of 8 months, her liver dysfunction deteriorated to the level of liver failure, and she underwent a liver transplantation, but died at 18 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (12%, 12%, and 16% of normal control, respectively). In contrast, complex II and citrate synthase activity were normal (68% and 106%, respectively). qPCR revealed that mtDNA of liver was markedly decreased (2%) and she was diagnosed with hepatocerebral MTDPS.

Results

Characteristics of Japanese children diagnosed with an MRCD

In total, we diagnosed MRCDs in 232 patients; these patients made up 34% of the study population. The age distribution of these patients is as follows; near 40% before 1 month of

age, three fourth by age 1 year, and >90% by age 7 years. One hundred and twenty (52%) patients were male, and about half of the diagnosed patients were deceased. Diverse clinical diagnoses are shown in Fig. 1. Eighty-seven patients (38%) had neurological disorders consisting of Leigh syndrome, neurodegenerative disorders, and so-called mitochondrial cytopathy. Fifty-nine (25%) had a lethal or non-lethal infantile mitochondrial disorder. Twenty-nine (13%) had mitochondrial hepatopathy, and 17 (7%) had mitochondrial cardiomyopathy. Among all MRCDs, 28 (12%) patients lacked lactic acidemia, a feature that traditionally prompted the suspicion of an MRCD. The entire mitochondrial DNA sequence was determined for 139 patients, but a causative genetic abnormality was found in only 34 (24%) of these patients (data will be published elsewhere); indicating that, in most cases, the causative gene or genes may be present in nuclear DNA.

The enzymatic diagnoses were compared with Australian data[17] (Fig. 2). In Japanese patients, a respiratory chain complex I abnormality was most common (105 patients, 45%), followed, in decreasing order of prevalence, by respiratory chain abnormalities in multiple complexes (80 patients, 34%), a complex IV abnormality (33 patients, 13%), and a complex III abnormality (10 patients, 4%). No patient was given a probable or definitive diagnosis of a complex II abnormality. Similarly, according to the Australian data, the most common abnormality was in complex I (45%), followed by abnormalities in multiple complexes (28%),

complex IV (21%), and complex III (5%); only 1 patient had a complex II abnormality.

Manifestations, Genetic Diagnoses, and Prognoses of MTDPS patients

A qPCR-based diagnosis of MTDPS was made for 16 of the 80 patients with an enzymatic diagnosis of a multiple complex abnormality and 7 of the 105 patients with an enzymatic diagnosis of a respiratory chain complex I abnormality. Three of these 23 patients died with the definition of sudden infant death syndrome and thus had no available records of clinical findings; the clinical findings from the remaining 20 patients were further analyzed.

The disease types among these 20 patients were compared with those reported by Sarzi et al.[4] (Fig. 3). Among the Japanese patients, 13 (65%) had acute hepatocerebral MTDPS, 2 (10%) had Alpers-like syndrome (delayed-onset hepatocerebral MTDPS), and 5 (25%) had encephalomyopathic MTDPS. This distribution is similar to that reported by Sarzi et al. We must note here that "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS. This is because no true case of Alpers syndrome has yet been identified in Japan. The results of analyses of the three main genes responsible for MTDPS are shown in Fig. 4. Causative genetic anomalies were identified in 6 of the 20 Japanese patients (30%). No abnormality was identified in the three genes of the remaining 14 patients (70%). The responsible genes were deoxyguanosine kinase (*DGUOK*) in 3 patients whose clinical reports were shown above, *MPV17* in 2 patients,[7] and DNA polymerase γ (*POLG*) in 1

patient whose clinical report will be published elsewhere. The individual genetic abnormalities are listed with the clinical findings of the patients in Table 1. Although three of them underwent liver transplantations during the infantile period, five of them died before 2 years of age. Patient 5 lived longer than others because of dietary and pharmaceutical treatments targeting the mitochondrial respiratory chain complex II.[7]

The *DGUOK*-related cases were two sisters, with a homozygous 335-base pair deletion (Fig. 5A; g.11692_12026del335; encompassing 308 base pairs of intron 1 and 27 base pairs at the start of exon 2), and a compound heterozygote patient, genetically unrelated to these sisters, with the same deletion and a c.743T>C (p.L248P) missense mutation. The large 335-base pair deletion encompassing from intron 1 to exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). Each parent and healthy sister is heterozygous for this mutation (Fig. 5B). The p.L248P variation is not listed as a polymorphism in the ensembl_mart_47 database (martdb.ensembl.org) and has not been reported as a disease-causing mutation. Moreover, the alignment shows that Leu248 is absolutely conserved in all species (Fig. 6).

The *MPV17* cases were previously reported compound heterozygote siblings.[7] The *POLG* case was a compound heterozygote patient. The genetic mutations noted in these six patients were confirmed to be absent in DNA of 100 healthy Japanese controls (data not

shown).

Like Sarzi et al., who did not find the responsible gene or genes in 60% of the patients, we were unable to identify the responsible gene or genes in a majority of the cases. We sequenced the whole exome of all the MTDPS patients to reveal the underlying nuclear disease genes using next-generation sequencing system (data not shown). This did not identify pathogenic mutations in any of the known genes associated in MTDPS (*TK2, SUCLA2, RRM2B, SUCLG1, MGME1, C10orf2, TYMP, and AGK*) in our MTDPS patients.

Of the genetic mutations identified, *POLG* mutations were less prevalent than in Caucasians. Only 1 of our 15 patients with Alpers syndrome or hepatocerebral MTDPS were caused by recessive POLG mutations, compared with 8 of 39 such cases diagnosed in France.

Sixteen of the 20 Japanese MTDPS patients were deceased. Sarzi et al. reported that 29 of the 50 MTDPS patients they analyzed were deceased. The data of the deceased patients were plotted to obtain curves of the ages of death (in months) in the two populations for comparison (Fig. 7). MTDPS patients had a short life in both study populations; many died during or before reaching early childhood. The log-rank test and Gehan-Breslow-Wilcoxon test revealed no significant differences between the survival data.

Discussion

We started an enzyme diagnosis referral service for children suspected of MRCDs in 2007 and have diagnosed MRCDs in 30 to 40 patients from around Japan each annually since then. In the last year we have made over 100 new MRCD diagnoses. About half of the diagnoses are for neonates. There are about one million births in Japan annually. Under the assumption that the patients referred for enzyme diagnosis to our institute represent about half of all Japanese MRCD patients, the prevalence of neonatal-onset MRCD becomes 50 × 2 / 1,000,000 = 1 / 10,000. When patients with juvenile-onset and adult-onset mitochondrial diseases are factored in, the prevalence of these diseases in Japan becomes one in several thousand, which is comparable to the prevalence in Western countries.[1]

It is noteworthy that more than 10% of the patients lacked lactic acidemia, which many physicians still regard as synonymous with mitochondrial disease. Hence, mitochondrial disease must also be considered in lactic acidemia-free patients with unexplained signs and symptoms in multiple organs.

The enzymatic diagnosis of MRCD showed similar trends in prevalence between Japanese and Australian patients, with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare in both populations.

Twenty percent of the patients with multiple respiratory chain disorders in our population and 50% of the patients in the population of Sarzi et al.[4] had MTDPS. Although MTDPS was the leading cause of MRCDs in both groups, MTDPS represented a smaller proportion of the MRCDs in Japan. According to the Online Mendelian Inheritance in Man database, MTDPS can be classified as encephalomyopathic, hepatocerebral, or specific (a classification that includes mitochondrial neurogastrointestinal encephalopathy (MNGIE) and Sengers syndrome). Encephalomyopathic MTDPS features respiratory failure and myopathy. Hepatocerebral MTDPS is characterized by liver disorders, growth disorders, and hypoglycemia. The distribution of the disease type classifications of Japanese population did not differ from the distribution reported by Sarzi et al.

Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities in the study population of Sarzi et al., while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. However, the causative gene was not identified in the majority of patients in each population. The six Japanese hepatocerebral MTDPS patients in whom the responsible gene was identified are listed in Table 1. The serious nature of this disease is evident, as all six experienced onset as neonates or infants and died during or before reaching early childhood.

DGUOK deficiency was originally described as the cause of an infantile onset

hepatocerebral mitochondrial disease, typically featuring hepatic failure, nystagmus and hypotonia[18]. Recently it has been shown that patients with *DGUOK* mutations may present with neonatal hemochromatosis[19] or adult onset myopathy and mitochondrial DNA multiple deletions, with or without liver involvement.[20,21] We found two novel *DGUOK* mutations in two apparently unrelated Japanese families. Three patients in two families had typical signs and symptoms of hepatocerebral MTDPS, and both parents in each family were heterozygous for these mutations. A 335-base pair deletion in *DGUOK* was found in both families, and may therefore be a common mutation in the Japanese population.

Our analysis of MTDPS patients concluded with a comparison of the ages of death (in months) in the two populations (Figure 7). A commonality between the Japanese patients and the patients described by Sarzi et al. was the early age of death; most patients died during or before reaching early childhood. *DGUOK* deficiency was most serious in both study populations. Likewise, many patients in each population experienced onset as neonates or infants. No significant difference in disease severity was identified between the populations.

Our results indicate a lower prevalence of *POLG* mutations in the Japanese population, which is likely attributable to several common mutations found in Caucasians that appear to be ancient European founder mutations (p.A467T, p.G848S, and p.W748S).[22] In children

with recessive *POLG* mutations, these 3 mutations represented 7 of 16 mutant alleles reported by Sarzi et al.[4] A recent study collated the prevalence of these 3 mutations in 10 studies reporting a total of 249 POLG patients and found they represented 49% of mutant alleles in predominantly Caucasian patients.[23] Most Caucasian POLG patients will thus have at least one allele carrying one of these 3 founder mutations and Hakonen et al. suggested that they may have been spread during Viking times. The carrier frequency of these mutations is as high as 2% in some European countries. Their expected absence in Asian patients likely explains a lower prevalence of recessive POLG disease in Asian populations.

Conclusion

Mitochondrial DNA depletion syndrome and other mitochondrial respiratory chain disorders are common, but serious, diseases that occur across all races.

Figure Legends

Fig. 1. Clinical diagnoses of MRCD in Japan. `Neurodegenerative disorders` meant neurodegenerative disorders unclassified to specific diseases. Patients with `Non lethal infantile mitochondrial disorder` started symptoms like `Lethal infantile mitochondrial disorder` but survived beyond 1 year old. SIDS, sudden infant death syndrome; SUD, sudden unexplained death.

Fig. 2. Percentage distribution of enzymatic diagnoses of MRCD in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare among the two populations.

Fig. 3. Percentage distribution of disease types of MTDPS patients in Japan and those reported by Sarzi et al. "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS, because no true case of Alpers syndrome has yet been identified in Japan. The distribution of disease types in our population did not differ from that reported by Sarzi et al.[4]

Fig. 4. Percentage distribution of responsible genes for MTDPS patients in Japan and those reported by Sarzi et al. The causative gene was not identified in the majority of patients in each population. Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities by Sarzi et al.,[4] while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients.

Fig. 5. Genomic sequence determination of 335-base pair *DGUOK* deletion in the family of patients 1 and 2.

(A) The sequence of exon 2 is capitalized. Long range PCR primer sets are surrounded by two squares and the depicted 335-base pair deletion is underlined. The large 335-base pair deletion encompassing from the end of intron 1 to the beginning of exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90).

(B) Lane1, the father; lane 2, the mother; lane 3, the middle healthy sister; lane 4, normal control; lane 5, patient 1; lane 6, patient 2; lane 7, no sample; lane 8, molecular weight marker. The 1310-base pair band represents the normal sized PCR product. The 975-base pair band represents the PCR product with 335-base pair *DGUOK* deletion in this family.

Fig. 6. ClustalW multiple sequence alignment of DGUOK orthologs. The alignment shows that amino acid 248Leu mutated in the patient is absolutely conserved in all species.

Reference (ClustalW)

Higgins D., Thompson J., Gibson T. Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research 22: 4673-4680.*

URLs

HomoloGene, http://www.ncbi.nlm.nih.gov/homologene (for the DGUOK ortholog amino acid sequences of human [accession number NP_550438.1], Pan [accession number XP_001153473.1], Canis[accession number XP_533001.2], Bos[accession number NP_001014888.2], Mus[accession number XP_001107072.1], Rat[accession number NP_001100072.1], Danio[accession number XP_001093561.1], Arabidopsis[accession number NP_565032.2], Oryza[accession number NP_001044956.1].)

ClustalW, http://www.ebi.ac.uk/Tools/clustalw/