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Dr. Yau-Huei Wei graduated in June 1974 from the Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan. He joined the laboratory of the late Professor Tsoo E. King in 1976 and earned his PhD degree in 1980 from the Department of Chemistry, State University of New York at Albany, New York, USA. He returned to Taiwan after one year of postdoctoral training at the Departments of Chemistry and Physics, SUNY-Albany. He was appointed as an associate professor during 1981–1985 at the Department of Biochemistry, National Yang-Ming Medical College, Taipei, Taiwan. He was promoted to full professor in 1985 and served as the chairman of the department until 1991. He also served as the Director, Common Instrumentation Center (1986–1989) and the Dean of Student Affairs (1989–1991) of the College. Dr. Wei was appointed as the Director General, Department of Life Sciences, National Science Council of Taiwan, 2001–2005. He served as the Dean of Academic Affairs (2006–2008) and was a Distinguished Professor (2007–2009), National Yang-Ming University. In August 2009, Dr. Wei was appointed the founding President of Mackay Medical College. He has actively participated in the promotion of international collaboration in biomedical research and mitochondrial medicine. Dr. Wei was one of the founding members of Asian Society for Mitochondrial Research and Medicine, and was the Vice-President (2002–2005) and President (2005–2008) of the Society. He has been the President of Taiwan Society for Mitochondrial Research and Medicine (2006–2012). Since 2006, Dr. Wei has served on the editorial board of Biochimica et Biophysica Acta-General Subjects. Dr. Wei's major research has focused on "Molecular and cellular biology studies of mitochondrial diseases, cancer and age-related diseases" and "The cross-talk between mitochondria and the nucleus and metabolic shift in the differentiation of stem cells". He was among the few investigators to show that mitochondrial function decline and mitochondrial DNA mutations are important contributory factors of human aging. His research team was one of the earliest groups to demonstrate that oxidative stress and oxidative damages elicited by mitochondrial DNA mutations contributes to the pathophysiology of many mitochondrial disorders. In the past few years, Dr. Wei and his students have established that mitochondrial biogenesis and respiratory function as well as antioxidant enzymes are upregulated in a coordinate manner in the process of differentiation of stem cells. Dr. Wei and his students have published in SCI journals ~300 research papers and ~30 review articles and book chapters in the fields of bioenergetics, mitochondrial medicine, free radical biology and medicine, molecular and cellular biology, male infertility, and aging research.

Yasutoshi Koga
Guest editor
Kurume University Graduate School of Medicine, Japan
Corresponding author. Tel.: +81 942 31 7565; fax: +81 942 38 1792.
E-mail address: yasukoga@med.kurume-u.ac.jp

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Co-editor
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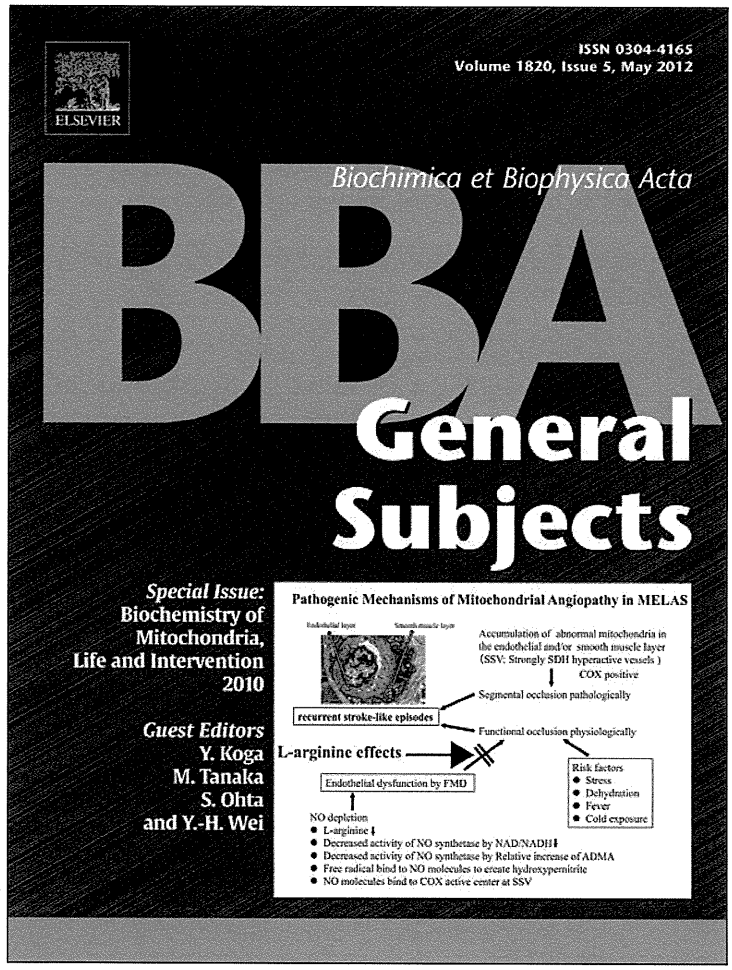

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Review

Molecular pathology of MELAS and L-arginine effects☆,☆☆

Yasutoshi Koga [a,*], Nataliya Povalko [a], Junko Nishioka [a], Koujyu Katayama [a], Shuichi Yatsuga [a,b], Toyojiro Matsuishi [a]

[a] Department of Pediatrics and Child Health, Kurume University Graduate School of Medicine, Kurume, Japan
[b] Research Program of Molecular Neurology, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

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ABSTRACT

Background: The pathogenic mechanism of stroke-like episodes seen in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) has not been clarified yet. About 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, which is the base change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$ gene.
Scope of review: This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes at the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.
Major conclusions: Molecular pathogenesis is mainly demonstrated using ρ^0 cybrid system. The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of $tRNA^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-$tRNA^{Leu(UUR)}$ versus uncharged $tRNA^{Leu(UUR)}$ molecules; 3) the accumulation of aminoacylation with leucine without any misacylation; 4) accumulation of processing intermediates such as RNA 19, 5) wobble modification defects. All of these loss of function abnormalities are created by the threshold effects of cell or organ to the mitochondrial energy requirement when they establish the phenotype. Mitochondrial angiopathy demonstrated by muscle or brain pathology, as SSV (SDH strongly stained vessels), and by vascular physiology using FMD (flow mediated dilation). MELAS patients show decreased capacity of NO dependent vasodilation because of the low plasma levels of L-arginine and/or of respiratory chain dysfunction. Although the underlying mechanisms are not completely understood in stroke-like episodes in MELAS, L-arginine therapy improved endothelial dysfunction.
General significance: Though the molecular pathogenesis of an A3243G or T3271C mutation of mitochondrial $tRNA^{Leu(UUR)}$ gene has been clarified as a mitochondrial cytopathy, the underlying mechanisms of stroke-like episodes in MELAS are not completely understood. At this point, L-arginine therapy showed promise in treating of the stroke-like episodes in MELAS. This article is part of a Special Issue entitled Biochemistry of Mitochondria.

1. Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (OMIM 540000), characterized by an early onset of stroke-like episodes, was first described by Pavlakis and colleagues in 1984 [1]. At least 39 distinct mitochondrial DNA mutations have been associated with MELAS [2], about 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (OMIM 590050) [3–5]. Although more than 25 years have passed since MELAS was first defined clinically and pathologically, the pathogenesis of the stroke-like episodes is still uncertain. Mitochondrial angiopathy with degenerative changes in small arteries and arterioles, which has been reported in many MELAS patients [6,7], is suggested by the observation of strong succinate dehydrogenase activity in the wall of blood vessels (SSVs) [8]. In spite of the fact that many therapeutic trials have been conducted to cure mitochondrial disorders, no trial has been successful, though several clinical trials are still on-going. Based on the hypothesis that stroke-like episodes in MELAS are caused by segmental impairment of vasodilatation in intracerebral arteries, we use L-arginine in MELAS patients during the acute phase to cure the symptoms or to

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* Corresponding author at: Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-Machi, Kurume City, Fukuoka 30-0011, Japan. Tel.: +81 942 31 7565; fax: +81 942 38 1792.
E-mail address: yasukoga@med.kurume-u.ac.jp (Y. Koga).

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decrease the frequency and/or the severity of the stroke-like episodes [9,10,11]. This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes in the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.

2. Molecular pathophysiology of mitochondrial cytopathy in MELAS

2.1. Characteristics of $tRNA^{Leu(UUR)}$ gene and structure stabilization of mutant

A point mutation in the structural gene for a tRNA may be expected to result in a deficiency in translation. However, inhibition of translation due to a mutated tRNA gene may occur at several levels. The base change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$ is an invariant A in bacterial and cytosolic eukaryotic tRNAs and is typically involved in the tertiary folding of classical tRNAs (Fig. 1A) [12]. Because of above reason, the A3243G mutation is primarily thought to disrupt the tertiary interaction between the highly conserved np A14 (>90% for adenine) and U8, a binding that stabilizes the L-shaped tertiary fold [13,14], which results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [15]. The mutant tRNA is able to adapt to the synthetase, but results in incorrect tRNA processing and enzyme maturation and accordingly defects in a variety of biochemical pathways. The mutation may directly affect the mitochondrial tRNA function in translation, such as structure stabilization, methylation, amino-acylation, and codon recognition, or alternatively, may affect recognition of the tRNA by an enzyme not directly involved in translation, such as the enzymes which process the large polycistronic transcripts of the mtDNA.

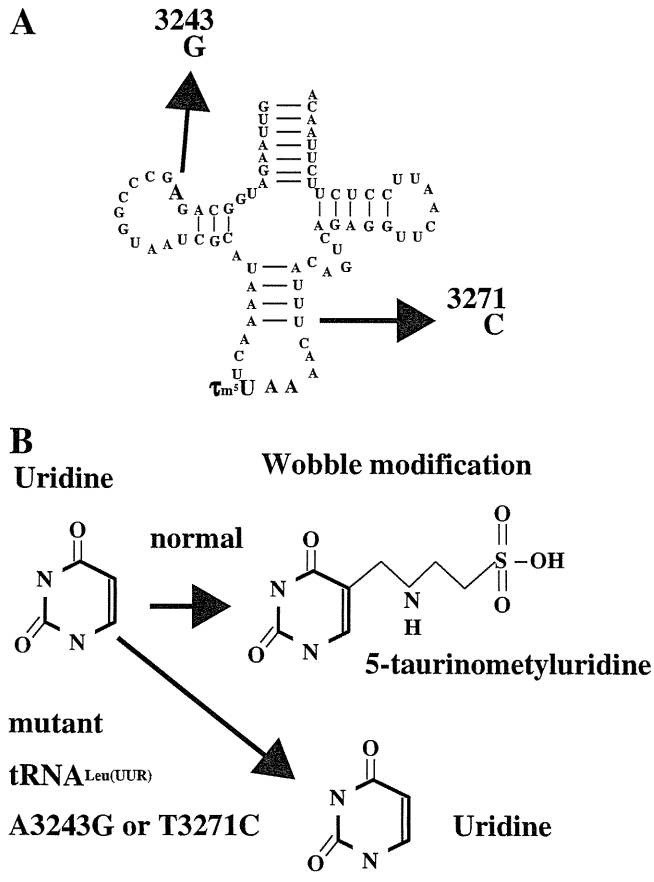

Fig. 1. $tRNA^{Leu(UUR)}$ structure and wobble modification. $tRNA^{Leu(UUR)}$ structure. An A to G change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$, which is thought to disrupt the tertiary folding of classical tRNAs [12], results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [14,15]. B. Wobble modification The wild-type $tRNA^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base [35], however its modification is absent in the $tRNA^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. The wobble modified uridine in the wild-type $tRNA^{Leu(UUR)}$ is 5-taurinomethyluridine (sm5U). The U on the bold indicates the unmodified uridine present in the mutant tRNAs.

2.2. ρ^0 cybrid system in MELAS

King et al. developed the technologies whereby the mitochondria from cells derived from patients are transferred to a cell line lacking mtDNA (so called ρ^0 cybrid system), which allowed to conduct the study of the genotype-phenotype relationships in mitochondrial function [16,17]. In this manner, it is possible to create trans-mitochondrial cell lines containing different proportions of mutated mtDNA from 0% to 100%, and to study the effects of a given mutant load on the activity of respiratory chain complexes, mitochondrial respiration and cell growth, as well as mitochondrial tRNA stability, methylation, aminoacylation, codon recognition and threshold effects. First application of this technique to an A3243G mutation related to molecular basis of MELAS, has been reported by Chomyn et al. [18], and King et al. [19] independently. Mutant transformants showed protein synthesis defects clearly, and demonstrated that there was the direct evidence between single nucleotide change at 14th position of an A to G transition in the mitochondrial $tRNA^{Leu(UUR)}$ gene and mitochondrial dysfunction. However, the reduction in labeling of the various mitochondrial translation products in mutant was not correlated with their UUR-encoded leucine content. King also reported the similar effects in transformants having a T3271C mutation [19]. This ρ^0 cybrid system becomes the orthodox and powerful tool when one evaluates the pathogenicity of any nucleotide changes in the mitochondrial DNA.

2.3. Transcription termination of mitochondrial RNAs in MELAS

The mammalian mitochondrial tRNAs are transcribed as part of larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences (Fig. 2). The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. In these polycistronic molecules, the tRNA structures are believed to act as recognition signals for the processing enzymes which make precise endonucleolytic cleavages at the 5' and 3' ends of the tRNA sequences in the primary transcripts, yielding the mature rRNAs, mRNAs, and tRNAs [21]. The ribosomal DNA transcription unit, one of three polycistronic transcription units of human mtDNA, terminates at the 3'-end of the 16S rRNA gene just before the $tRNA^{Leu(UUR)}$ gene. This transcript, corresponding to the ribosomal genes, is processed to yield the mature rRNAs and, due to its very high rate of synthesis, is responsible for the bulk of the rRNA formation [22]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the $tRNA^{Leu(UUR)}$ gene, and which promotes termination of transcription (Fig. 3A) [22,23]. Since this mutation is located exactly in the middle of termination protein binding domain, the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene has been shown in vitro to impair the binding of this protein factor and to affect the efficiency of transcription termination at the end of the 16S rRNA gene [23]. However, in vivo analysis using ρ^0 cybrid system provided no evidence to support above data. There were no alterations of size of the $tRNA^{Leu(UUR)}$ or of the immediately downstream-encoded ND1 mRNA or of the 16S rRNA, as detectable by changes in their electrophoretic mobility [18]. The steady-state amounts of mitochondrial rRNAs, mRNAs, and $tRNA^{Leu(UUR)}$ are not significantly affected by the MELAS mutation in ρ^0 cybrid system. The discrepancy of the data described above may be explained by the possibility that the

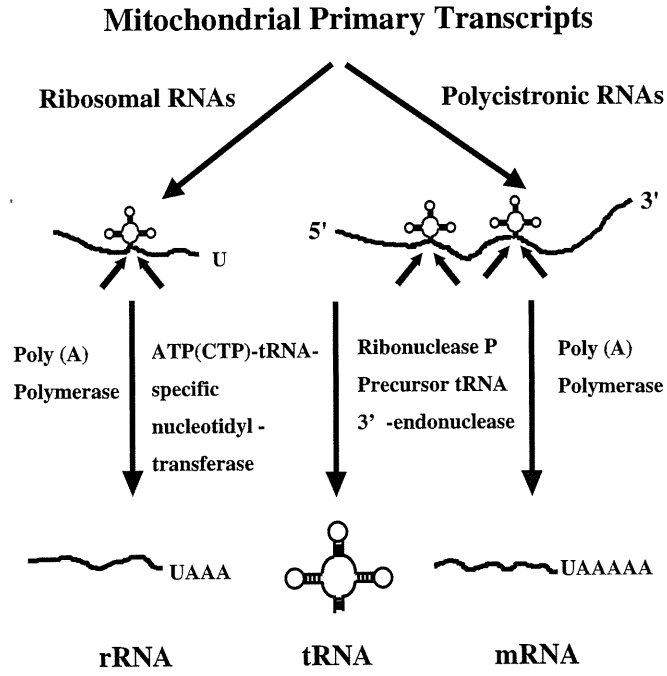

Fig. 2. Mammalian mitochondrial transcription system. The human mitochondrial RNAs are transcribed as a larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences.

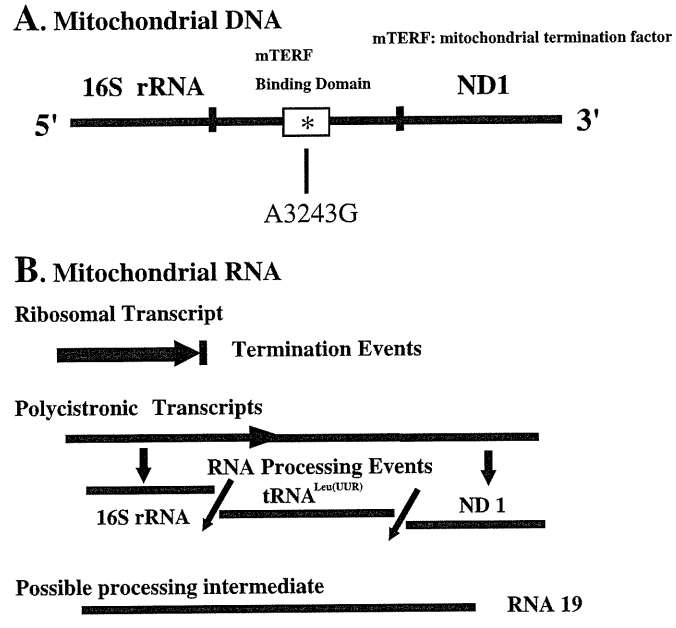

Fig. 3. Post-transcriptional modification. A. Transcription termination. The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the $tRNA^{Leu(UUR)}$ gene, and which promotes termination of ribosomal transcription. B. Post-transcriptional modification and RNA 19 The increase of RNA 19, corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes, found in mutant $tRNA^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids as efficiently as in wild-type cybrids [19]. RNA 19 is also accumulated in muscle specimens from 8 MELAS patients [26]. The proportion of mutated RNA in RNA 19 fraction is always higher than those in the percentage of mutation in mitochondrial DNA, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30].

reduction in affinity of mTERF for the mutated target sequence is compensated by hyper-expression of the protein. Anyway, using genetic, biochemical, and morphological techniques, it was found that the mutant, but not wild-type cybrids, displayed quantitative deficiencies in cell growth, protein synthesis, and respiratory chain activity [19].

2.4. Processing of polycistronic transcripts in MELAS

It was found that there was an accumulation of a previously unidentified RNA transcript in mutant cybrids (A3243G or T3271C), designated as RNA 19, corresponds to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND1 genes, which are contiguous in the mtDNA (Fig. 3B) [19]. The ratios of mtDNA-encoded rRNAs to mRNAs were not found to be altered in these in vitro experiments. In order to analyze whether the MELAS mutation is associated with errors in transcription termination and processing of the polycistronic transcripts in the region of the mutation, it was performed fine mapping of the mature transcripts derived from the 16S rRNA, $tRNA^{Leu(UUR)}$, and ND 1 genes in both wild-type and mutant cybrids. It was also analyzed the steady-state levels of $tRNA^{Leu(UUR)}$ by high-resolution RNA transfer hybridizations. It was found that mutation has no effect in vivo on the accuracy of transcription termination at the end of the ribosomal RNA genes, on the precise endonucleolytic cleavage of the polycistronic RNA at $tRNA^{Leu(UUR)}$, or on the post transcriptional addition of -CCA at the 3′ end of $tRNA^{Leu(UUR)}$ [24]. On the other hand, the experiments using plasmids carrying $tRNA^{Leu(UUR)}$ inserts (wild type, as well as A3243G) which designated to evaluate the endonucleolytic 3′-end processing and CCA addition at the tRNA 3′ terminus, showed that A3243G mutation reduced 2.2 fold of the efficiency of 3′-end cleavage, and almost has no abnormal effects on CCA addition [25].

2.5. Accumulation of RNA 19 in MELAS cybrids and organs from patients

The increased amounts of the transcript corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes, designated as RNA 19, found in mutant $tRNA^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids (A3243G or T3271C) as efficiently as in wild-type cybrids [19]. It was demonstrated that RNA 19 is accumulated in muscle specimens from 8 MELAS patients who have a heterogeneous percentage of mutation (58% to 99%) in the A3243G of $tRNA^{Leu(UUR)}$ gene [26]. An increase in the levels of RNA 19 was observed in nearly all tissues examined from these patients, which do not provide evidence for tissue-specific differences in mitochondrial RNA processing. The elevation of steady-state levels of RNA 19 have also reported in skeletal muscle and fibroblasts of a patient with mitochondrial myopathy and a complex I deficiency who harbored an A to G transition in $tRNA^{Leu(UUR)}$ gene at position 3302 [27]. Thus, altered RNA processing may be associated with other point mutations in $tRNA^{Leu(UUR)}$ gene associated with MELAS. It also analyzed a mutated proportion of RNA 19 in an RNA fraction obtained from sampled skeletal muscles from 6 unrelated patients with MELAS. The proportion of mutated RNA in RNA 19 fraction exceeded 95% in all patients, although the percentage of mutation in mitochondrial DNA ranged from 54 to 92, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30]. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243G, T3271C and A3302G mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the $tRNA^{Leu(UUR)}$ (Fig. 4) [31]. Though the reason why RNA 19 was elevated in patients who have the point mutation of $tRNA^{Leu(UUR)}$ gene is unknown, we believe that elevated levels of RNA 19 may play an important role in the pathogenesis of this disorder.

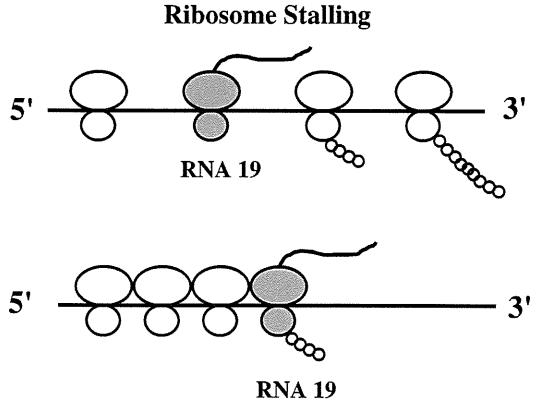

Fig. 4. xRibosomal stalling. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243 mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the $tRNA^{Leu(UUR)}$ [31].

2.6. Aminoacylation

The decrease in level of total $tRNA^{Leu(UUR)}$ observed in the mutant cell lines (46–62% of the control values) could arise either from decreased rate of formation from the corresponding primary heavy strand transcript or from a decreased metabolic stability [32]. The increased amount of RNA 19, which may be a precursor of $tRNA^{Leu(UUR)}$, was demonstrated in ρ^0 cybrid system as well as somatic tissues in MELAS patients. RNA 19 may suggest the former possibility. On the other hand, the A3243G mutation could perfectly destabilize the tertiary structure of the molecule, and mutant $tRNA^{Leu(UUR)}$ becomes more susceptible to nucleolytic attack [13,33]. It is proposed that mutant tRNA induces the misincorporation of amino acids in mitochondrial DNA encoded polypeptides. However, the demonstration of aminoacylation by mutant tRNA has been little pursued because a chemical amount of the mutant tRNA has not been purified, probably due to technical difficulties. In 2000, Yasukawa succeeded in purifying the mutant $tRNA^{Leu(UUR)}$ molecules in a chemical amount by taking advantage of the solid phase probing method [34], and clearly demonstrated that the mutant $tRNA^{Leu(UUR)}$ is aminoacylated with leucine only. However the extent of aminoacylation of the mutant tRNAs was relatively low. The total amounts of leucyl-$tRNA^{Leu(UUR)}$ with the mutations were estimated to be less than 30% that of the wild-type counterpart [35]. To determine if the decreased fraction of aminoacylated $tRNA^{Leu(UUR)}$ in mutant cells was due to a defect in the ability of mutant tRNA to be aminoacylated by the human mitochondrial leucyl-tRNA synthetase, Park et al. examined the aminoacylation kinetics of wild-type and mutant $tRNA^{Leu(UUR)}$, using both native and in vitro transcribed $tRNA^{Leu(UUR)}$ [36]. An A3243G mutant $tRNA^{Leu(UUR)}$ was 25-fold less efficiently aminoacylated in vitro, compared to the wild-type $tRNA^{Leu(UUR)}$. There are many evidences that aminoacylation capacities in $tRNA^{Leu(UUR)}$ gene mutations are reduced [37]. The reduced amount of aminoacyl-$tRNA^{Leu(UUR)}$ with the A3243G mutation could explain the reduction in protein synthesis.

2.7. Modified defects at wobble position in mitochondrial tRNA gene

A number of reports suggest that a decrease of protein synthesis cannot explain the decline in respiratory enzyme activity or in oxygen consumption [38,39]. Even when the mitochondrial protein synthesis rate was normal, the enzymatic activity of complex I was observed to be significantly affected in cybrid clones containing 60% to 95% mutant mtDNA. The muscle form of Complex I deficiency turned out to be MELAS clinically and was confirmed to have an A3243G mutation in all patients [40]. Thus, the decrease in protein synthesis may not itself contribute directly to the pathogenesis caused by mitochondrial dysfunction. Some unusual mobilities of proteins in SDS-polyacrylamide gel electrophoresis have been reported [18,38], which strongly suggest that amino acids were misincorporated into the proteins synthesized in the mitochondria with the mutant mtDNA. The steady-state amounts of $tRNA^{Leu(UUR)}$ with the A3243G or the T3271C mutation in the respective cybrid clones were about 30% that of the wild-type in the control cybrid clones with wild type mtDNA [35]. In contrast, the steady-state amounts of $tRNA^{Phe}$ and $tRNA^{Ile}$ (encoded upstream and downstream of the $tRNA^{Leu(UUR)}$gene) remained unchanged in both the mutant and control cybrid cells. The life span of the mutant $tRNA^{Leu(UUR)}$ is significantly shortened. The half-life of the wild-type $tRNA^{Leu(UUR)}$ was estimated to be about 56 h, whereas those of the A3243G and T3271C mutants were only about 6 and 12 h. Therefore the reduced steady-state levels were due to the shortened life spans of the mutant tRNAs. Yasukawa found that the wild-type $tRNA^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base (Fig. 1B). In contrast, this uridine modification is absent in the $tRNA^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. It is interesting to note that both of the mutant $tRNA^{Leu(UUR)}$ are deficient in the modification at the wobble position despite having mutations at different positions. Modified defects at wobble position in mitochondrial tRNA gene are also demonstrated by primer extension methods [41]. The deficiency in uridine modification at the wobble position in the mutant $tRNA^{Leu(UUR)}$ strongly suggests mistranslation by these mutant tRNAs according to the mitochondrial wobble rule, which is also demonstrated in other tRNA mutation in MERRF (myoclonus epilepsy with ragged-red fibers) [42–45]. Although mutant $tRNA^{Leu(UUR)}$ does not follow the wobble rule, the mutant $tRNA^{Leu(UUR)}$ is aminoacylated with only leucine, not with other aminoacids. The stability and aminoacylation of the mutant $tRNA^{Leu(UUR)}$ were found to be decreased, suggesting that the molecular pathogenesis of MELAS could be a combination of a lowered availability of aminoacyl $tRNA^{Leu(UUR)}$ and defective translation. This is the first observation of a common modification defect affected by different point mutations within a single tRNA gene.

2.8. Threshold effects in various steps in the cell or in the organs

The phenotypic threshold effect observed at the single-cell level could arise when the products of the wild-type mtDNA can no longer "complement" the effects of the mutated ones [46,47]. For instance, a heteroplasmic mutation in mtDNA will result in the co-existence of mutated mRNAs, mutated tRNAs and defective respiratory chain subunits along with their wild-type homologues. These wild-type molecules may be sufficient to support normal function of the organelle until their levels fall below a critical value (threshold), at which point they can no longer compensate for the effect of the mutation, leading to impairment of mitochondrial function. The phenotypic threshold effect is based on this reserve of different macromolecules (mRNAs, tRNAs, subunits), and can then be considered as a protective mechanism providing a safety margin against the effects of deleterious mutations. Above complementation can occur at different levels of mitochondrial gene expression, such as 1) gene transcription, 2) structural stability of the tRNAs, 3) maturation process of the tRNAs, ribosomal RNA, and mRNAs, 4) wobble modification of tRNAs, 5) aminoacylation, 6) translation, 7) molecular assembly of the active form of enzyme complexes in harmony with mitochondrial and nuclear-encoded polypeptides, 8) locate to the mitochondrial inner membrane, 9) biochemical overall function of mitochondria in the cell, 10) biochemical overall function of mitochondria in the organ, 11) original threshold of organ to the mitochondrial energy requirement. The cells which require high energy states, such as neurons, muscles, heart, and kidneys, may be more severely affected by the threshold level of mutation than cell that require low energy levels. The phenotypes in the severity of the disease may influence various factors listed above and are more complicated to elucidate.

2.8.1. Summary of molecular mechanisms of mitochondrial cytopathy

The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of $tRNA^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-$tRNA^{Leu(UUR)}$ versus uncharged $tRNA^{Leu(UUR)}$ molecules; 3) accumulation of processing intermediates such as RNA 19, 4) wobble modification defects leading to translation defect. The A3243G mutation shows dominant negative effects in the processing system of mitochondrial transcription seen in both trans-mitochondrial cell and muscles in MELAS patients. Molecular mechanisms described above may contribute to respiratory chain enzyme defects, especially complex I, and lead to the mitochondrial cytopathy seen in the MELAS patients. Moreover the A3243G mutation affects the nuclear background [46,47], resulting in a high glycolytic rate, increased lactate production, reduced glucose oxidation, impaired NADH-response, reduced mitochondrial membrane potential, markedly reduced ATP production, deranged cell calcium handling with an increased cytosolic calcium handling with an increased cytosolic calcium load, an increased amount of reactive oxygen species in cybrid cells, reduced insulin secretion, premature aging, and deregulation of genes involved in the metabolism of amino groups and urea genesis. The above mechanism may lead to the cytotoxic edema seen in stroke-like episodes in MELAS.

3. Pathophysiology of mitochondrial angiopathy in MELAS

3.1. Hypotheses of stroke-like episodes in MELAS

The primary cause for stroke-like episodes in young MELAS patients—whether 1) mitochondrial cytopathy, 2) mitochondrial angiopathy, 3) non-ischemic neurovascular cellular mechanism, or combined—remains controversial. Mitochondrial cytopathy is caused by an oxidative phosphorylation defect in neurons, glia, or both as supported by evidence of an oxidative phosphorylation defect described by molecular pathogenesis section. Mitochondrial angiopathy is caused by the endothelial dysfunction evidenced by pathological, vascular physiological [11], or therapeutic findings [9,10]. Finally, the non-ischemic neurovascular cellular mechanism has been recently proposed by the clinical and neuroimaging data by Iizuka et al. [3].

3.2. Mitochondrial angiopathy in MELAS

Mitochondrial angiopathy with degenerative changes in small arteries and arterioles in the brain has been reported in autopsy cases of MELAS patients [6,7]. The mitochondria in the endothelium and smooth muscle cells of cerebral arterioles and capillaries also proliferate in a similar fashion as an area of ragged-red fibers (RRFs). Abnormal accumulation of mitochondria in vascular endothelial cells and smooth muscle cells is responsible for the infarct-like lesions [48]. These blood vessels have been designated as strongly succinate dehydrogenase-reactive vessels (SSVs), since they are rich in abnormal mitochondria [8]. Unlike RRFs and SSVs seen in MERRF and Kearns–Sayre syndrome (KSS), RRFs and SSVs seen in MELAS are typically cytochrome c oxidase (COX) positive, while those seen in MERRF or KSS are mostly COX negative, what is known as the "MELAS paradox"[49]. Since nitric oxide (NO) can bind to the active site of COX and displace heme-bound oxygen, hyperactive COX may decrease the regional NO concentration and lead to the segmental vasodilatation defect in SSV regions. Although infarct-like lesions histopathologically and stroke-like episodes clinically may not be caused simply by occlusion or obliteration of small vessels, this mitochondrial angiopathy, which can be severe in pial arterioles and small arteries, seems to explain the distribution of multiple areas of necrosis [50]. Since MELAS was associated with respiratory dysfunction, accumulated superoxide radical anion may react with nitric oxide to create the powerful oxidant hydroxypernitrite which may induce the neuronal apoptosis or cell damage [51]. All findings, described here, suggest that mitochondrial angiopathy is a unique and common change in all MELAS brains examined. This pathological abnormality, called mitochondrial angiopathy, may lead to the vasogenic edema seen in stroke-like episodes in MELAS.

3.3. Non-ischemic neurovascular cellular mechanism

Iizuka et al. proposed that the stroke-like episodes in MELAS may reflect neuronal hyperexcitability (epileptic activity), which increases energy demand and creates an imbalance between energy requirements and the adequate availability of ATP due to an oxidative phosphorylation defect, particularly in the susceptible neuronal population [3,52]. The generalized cytopathic mechanism and non-ischemic neurovascular cellular mechanism reflect the so-called mitochondrial cytopathy theory.

3.4. Neuro-imaging analysis in stroke-like episodes

Unlike thrombotic or embolic stroke usually seen in adult patients, the stroke-like episodes in MELAS are atypical because they affect young people and are often triggered by febrile illnesses, migraine-like headaches, seizure, psychological stress, and dehydration. Many neuro-imaging studies have been reported at different phases of onset from stroke-like episodes in MELAS through the use of computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), single emission computed tomography (SPECT), and positron emission tomography (PET). Calcification of the basal ganglia is frequently observed in MELAS by CT even before starting the stroke-like episodes. MRI scans of acute stroke-like events show an increased signal on T2-weighted or on fluid attenuation inversion recovery (FLAIR). The regions do not conform to the territories of large cerebral arteries but rather affect the cortex and subjacent white matter with sparing of deeper white matter. Acute changes in these regions may fluctuate, migrate, or even disappear during the time course. Cerebral angiograms in MELAS patients have confirmed absence of large-vessel pathology by demonstrating normal results, increased size of caliber arteries, veins, or capillary blush with early venous filling, with the exception of several case reports [53,54]. MRS studied revealed that the decrease in N-acetylaspartate (NAA), which is thought to be an amino acid specific to neurons, and an increase of lactate, which is reflected of anaerobic metabolism by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS), were in evidence in the affected areas at acute stroke-like episodes. Kubota et al. reported that L-arginine infusion protect the accumulation of lactate by MRS analysis in stroke-like episodes in MELAS [55]. The increased level of lactate on ^{1}H-MRS is also recognized even in normal appearing regions [56]. Phosphorus MRS studies have shown decreased levels of high-energy phosphate compounds in the brains of MELAS patients [57], showing that mitochondrial cytopathy constantly exists in the MELAS patient. SPECT studies have generally revealed that the increased tracer accumulation was reported in acute (several days) and subacute stage (month) from the onset of stroke-like episodes and lasted for several months. In the chronic stage (several months or years later), the decreased tracer accumulation was reported. However, in the hyperacute stage (3 h after the onset of stroke-like episodes), we observed hypoperfusion by SPM-SPECT analysis [58]. Moreover, the hypoperfusion and the hyperperfusion areas are both demonstrated in the MELAS patients not only at an acute phase but at an interictal phase, showing that MELAS has inappropriate cerebral circulation [54]. Moreover, MELAS showed hypoperfusion in the posterior cingulated cortex by SPM-SPECT, which is the common finding in Alzheimer disease, and may be related to the dementia state usually seen in the progressive stage of MELAS. There are several PET studies using ($rCMRO_2$), [^{62}Cu]-diacetyl bis (N4-methylthiosemicarbazone) (^{62}Cu-ATSM), and [^{18}F]-fluorodeoxyglucose (^{18}FDG) in stroke-like regions [59,60]. All of the PET studies of

patients have revealed decreased oxygen consumption relative to glucose utilization, further confirming the impairment of oxidative phosphorylation [61]. The dissociation in PET findings between cerebral glucose and oxygen metabolism may be the characteristic feature of MELAS, suggesting the mitochondrial cytopathy theory or non-ischemic neurovascular cellular mechanism. Diffusion-weighted (DWI) imaging is a new MRI technique for detecting diffusion of water molecules. Using DWI, local water mobility can be assayed as the absolute value of tissue water and expressed as the apparent diffusion coefficient (ADC). It has been shown using a stroke model in rats that ADC (a marker for cytotoxic brain edema) significantly declined within the first 5–10 min after stroke onset, while T2-relaxation time (a marker for vasogenic brain edema) increased as early as at the first T2-imaging time-point (20–35 min after embolization) [62]. The acute phase of stroke-like lesions in MELAS appear as a high signal on DWI with normal or increased ADC values, suggesting vasogenic edema which support the mitochondrial angiopathy theory [63,64]. On the contrary, many case reports found a decrease in ADC, which suggests mitochondrial cytopathy theory [65]. Recently, it was reported that increased and decreased ADC portions are mixed in stroke-like lesions, in which the increased ADC portion showed disappearance of the lesions thereafter, and the decreased portion showed persistent lesions. They suggested that there might be different levels of mitochondrial energetic transport impairment, correlated with cellular dysfunction. Specifically, this would be a mild energy failure resulting in moderate cellular dysfunction, responsible for vasogenic edema (high ADCs) and a severe energy failure resulting in irreversible cellular failure with cytotoxic edema (low ADCs) [66].

3.5. Endothelial dysfunction in MELAS

Physiologically, MELAS patients have a decreased vasodilation capacity in small arteries examined by flow mediated vasodilatation (FMD) methods, sized from 3 to 5 mm in their diameter [11]. MELAS patients have significantly decreased levels of L-arginine at acute phase of stroke-like episodes, which plays an important role in endothelial-dependent vascular relaxation [67], vasodilatation may be more severely affected in MELAS. Since MELAS patients have defective respiratory chain enzyme activities, a high NADH/NAD + ratio inhibits the NO synthetase reaction to cause a decreased production of NO at the endothelial cells or smooth muscle cells in the artery. In addition, ADMA (asymmetrical dimetyl-arginine), a risk factor of ischemic heart disorders, was relatively increased in MELAS patients [10], which may lead to a negative effect on the endothelial NO synthetase activity. If hyperactive COX may decrease the regional NO concentration as described in "MELAS paradox" [49], all of the above scenarios lead to the segmental vasodilatation defect especially in the segment of SSV regions in the cerebral artery or arterioles. The investigator-mediated clinical trial of L-arginine on MELAS (Dr. Koga as a principle investigator) to cure the symptoms of stroke-like episodes at acute phase, and to prevent or decrease the severity of stroke-like episodes at interictal phase of MELAS are on-going at 15 institutions of university hospital in Japan.

3.5.1. Summary of mitochondrial angiopathy and L-arginine effects

Pathophysiological mechanisms of mitochondrial angiopathy and the effects of L-arginine are summarized in Fig. 5.

4. Conclusion and future direction

The possible pathogenic mechanism of stroke-like episodes in MELAS may not be simple but complicated as described by the mechanisms in mitochondrial cytopathy and in mitochondrial angiopathy. Mitochondrial cytopathy has been demonstrated clearly as molecular and cellular defects by trans-mitochondrial cellular models. Mitochondrial angiopathy also has been demonstrated in brain and muscle pathology and vascular physiology. Although the results of

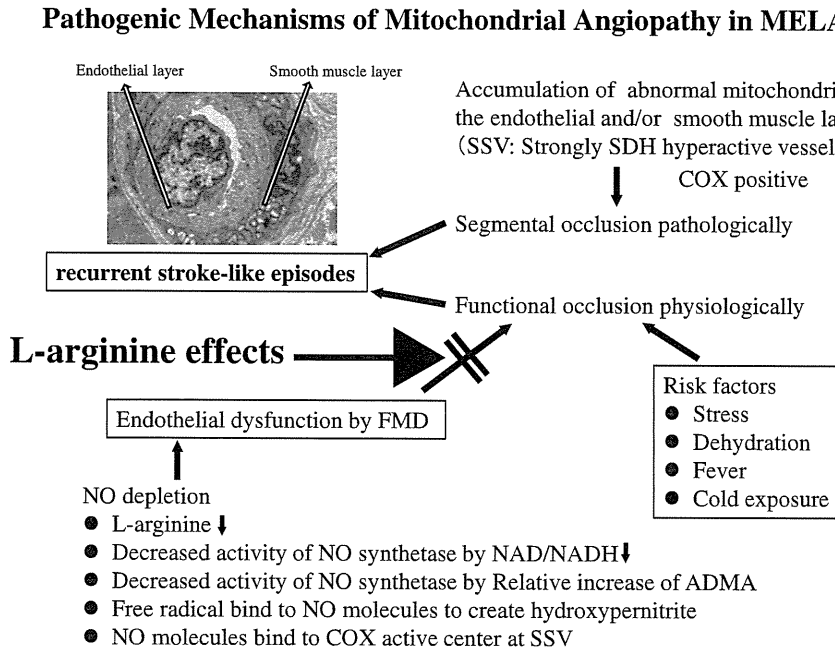

Fig. 5. Pathogenic mechanisms of mitochondrial angiopathy in MELAS. Segmental occlusions of small artery or arteriolen are evident in brain as well as in muscle pathology, in which abnormal accumulations of mitochondria have seen in endothelial and smooth muscle layers [6,7]. This phenomenon is recognized as SSVs in muscle and brain in MELAS [8], whereby mitochondrial function is more profoundly defective than the rest of the vessels, and demonstrated as endothelial dysfunction by FMD physiologically [11]. In MELAS patients, decreased levels of L-arginine is reported at acute phase of stroke-like episodes, a potent donor of NO, is also responsible for NO-dependent vascular dilatation defect. The decreased NAD/NADH ratio and accumulation of superoxide come from respiratory chain deficiency results in the inhibition of NO synthetase at generation process and decrease NO molecules by binding to create hydroxypernitrite, also contribute to the NO-dependent vasodilation abnormality. Since SSVs has usually high COX-positive feature histochemically, high COX activity decrease the residual NO molecules by binding to COX reactive center. The mental stress, dehydration, fever and cold exposure are also very important factors to increase the risk of the stroke-like episodes in MELAS.

neuro-imaging studies are controversial and are difficult to evaluate, there are several specific findings which may lead to the pathophysiology of stroke-like episodes in MELAS. We have to elucidate what is the trigger of stroke-like episodes in MELAS in future. Currently L-arginine therapy, to cure the symptoms of stroke-like episodes at acute phase, and to prevent or decrease the severity of stroke-like episodes at interictal phase of MELAS, is the most promising therapy for this incurable disorder. Global clinical trial of L-arginine on MELAS using randomized double blind placebo control protocol may be done in the nearer future.

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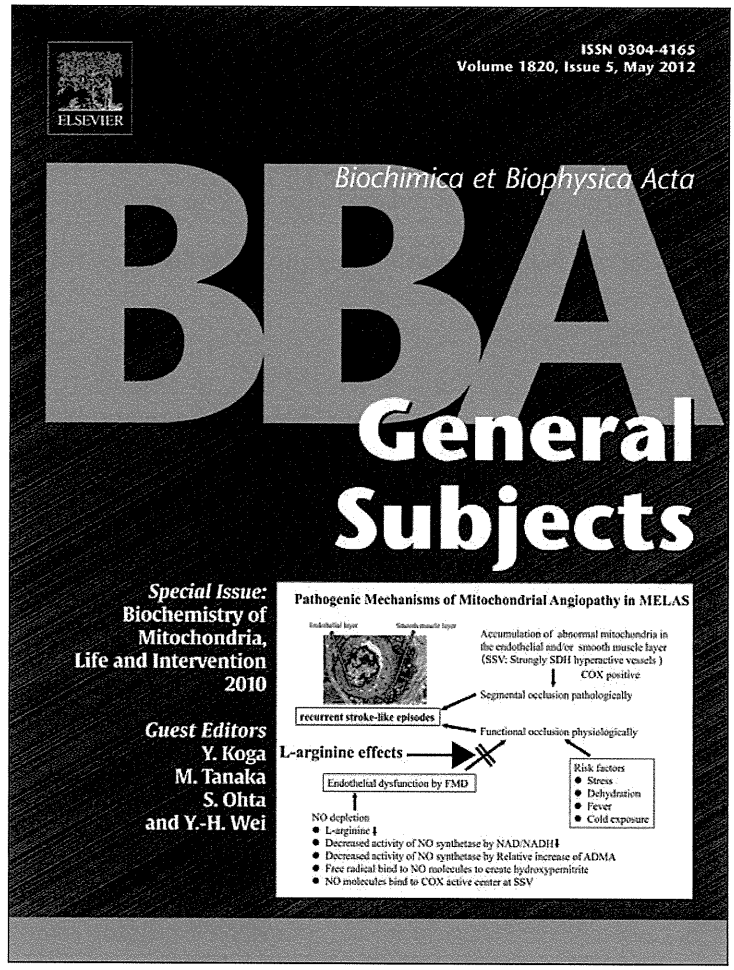

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MELAS: A nationwide prospective cohort study of 96 patients in Japan ☆

Shuichi Yatsuga [a,d], Nataliya Povalko [a], Junko Nishioka [a], Koju Katayama [a], Noriko Kakimoto [a], Toyojiro Matsuishi [a], Tatsuyuki Kakuma [b], Yasutoshi Koga [a,*] and Taro Matsuoka for MELAS Study Group in Japan [c]

[a] Department of Pediatrics and Child Health, Kurume University Graduate School of Medicine, Kurume, Japan
[b] Department of Biostatistics, Kurume University Graduate School of Medicine, Kurume, Japan
[c] MELAS study group in Japan
[d] Research Program of Molecular Neurology, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

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ABSTRACT

Background: MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) (OMIM 540000) is the most dominant subtype of mitochondrial myopathy. The aim of this study was to determine the prevalence, natural course, and severity of MELAS.
Methods: A prospective cohort study of 96 Japanese patients with MELAS was followed between June 2003 and April 2008. Patients with MELAS were identified and enrolled based on questionnaires administered to neurologists in Japan. MELAS was defined using the Japanese diagnostic criteria for MELAS. Two follow-up questionnaires were administered to neurologists managing MELAS patients at an interval of 5 years.
Results: A prevalence of at least 0.58 (95% confidential interval (CI), 0.54–0.62)/100,000 was calculated for mitochondrial myopathy, whereas the prevalence of MELAS was 0.18 (95%CI, 0.02–0.34)/100,000 in the total population. MELAS patients were divided into two sub-groups: juvenile form and adult form. Stroke-like episodes, seizure and headache were the most frequent symptoms seen in both forms of MELAS. Short stature was significantly more frequent in the juvenile form, whereas hearing loss, cortical blindness and diabetes mellitus were significantly more frequent in the adult form. According to the Japanese mitochondrial disease rating scale, MELAS patients showed rapidly increasing scores (mean ± standard deviation, 12.8 ± 8.7) within 5 years from onset of the disease. According to a Kaplan–Meier analysis, the juvenile form was associated with a higher risk of death than the adult form (hazard ratio, 3.29; 95%CI, 1.32–8.20; $p = 0.0105$).
Conclusions and General Significance: We confirmed that MELAS shows a rapid degenerative progression within a 5-year interval and that this occurs in both the juvenile and the adult forms of MELAS and follows different natural courses. This article is part of a Special Issue entitled: Biochemistry of Mitochondria.

1. Introduction

Mitochondrial dysfunction increases the risk of developing various human diseases, including degenerative neuromuscular disorders, diabetic or metabolic conditions, and cancer; it also affects the aging process [1]. The classical clinical entity in this category is the so-called mitochondrial myopathy, in which mitochondrial dysfunction is caused by mitochondrial or nuclear genetic abnormalities. The disease, which encompasses mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (OMIM 540000), is characterized by the early onset of stroke-like episodes and was first described by Pavlakis and colleagues in 1984 [2]; it is thought to be the most dominant subtype of mitochondrial dysfunction. At least 39 distinct mitochondrial DNA mutations have been associated with MELAS [3]; however, approximately 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (OMIM 590050) [4] and [5]. Because this mutation was also found to be a major genetic abnormality in diabetes mellitus, it may be a particularly common genetic variant in human populations [6]. Although more than 26 years have passed since the clinical and pathological definition of MELAS, there are few reports on its prevalence and epidemiology, and no reports exist on the natural course, survival rate or severity of the disease in a cohort study, meta-analysis, or nationwide survey [7] and [8]. In this study, we determined the prevalence, clinical symptoms, natural course, severity, and survival rate of MELAS patients in a nationwide Japanese

Abbreviations: JMDRS, Japanese mitochondrial disease rating scale; NPMDS, Newcastle pediatric mitochondrial disease scale; NMDAS, Newcastle mitochondrial disease adult scale

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* Corresponding author. Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-Machi, Kurume City, Fukuoka 30-0011, Japan. Tel.: +81 942 31 7565; fax: +81 942 38 1792.
E-mail address: yasukoga@med.kurume-u.ac.jp (Y. Koga).

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cohort study. Additionally, we also evaluated the clinical rating scale that may be a very useful tool for the assessment of efficacy of therapeutic approach for mitochondrial myopathy.

2. Materials and methods

2.1. Study design, patients, and data collection for the Japanese cohort study

The cohort study was performed using questionnaires. To determine the prevalence of mitochondrial myopathies throughout the country, the first questionnaire was mailed in 2001 to 2236 neurology departments within Japan (1474 departments with pediatric neurologists and 762 departments with adult neurologists, including governmental, public, private and university hospitals with 50 beds or more). Patients' medical records were evaluated using MELAS diagnostic criteria (Table 1) and adequately screened. In 2003, after compiling the results of the first questionnaire, we mailed a second questionnaire to the neurologists who had examined MELAS patients in 2001. In 2008, we mailed a third questionnaire to the same group of neurologists. The second and third questionnaires included a Japanese mitochondrial disease rating scale (JMDRS) (Supplemental Table 1). Relevant information from the medical records of eligible patients was transcribed onto case report forms by neurologists, who were later interviewed by telephone if ambiguous data or unsatisfactory descriptions were found in the case report forms. Detailed documentation of the patients' clinical status was compiled by the same neurologists. The case report form was originally constructed according to the JMDRS and was updated whenever the scores were altered. Written informed consent was obtained from the patients or their legal guardians. The study protocol was approved by the Institutional Review Board (Kurume University #9715).

2.2. Diagnostic criteria for MELAS

The nationwide survey of MELAS in this study is based on the definitive diagnosis of MELAS presented in Table 1.

Table 1
Diagnostic criteria for MELAS (MELAS study committee in Japan).

Category A. Clinical findings of stroke-like episodes
1. Headache with vomiting
2. Seizure
3. Hemiplegia
4. Cortical blindness or hemianopsia
5. Acute focal lesion observed via brain imaging[a]
Category B. Evidence of mitochondrial dysfunction
1. High lactate levels in plasma and/or cerebral spinal fluid or deficiency of mitochondrial-related enzyme activities[b]
2. Mitochondrial abnormalities in muscle biopsy[c]
3. Definitive gene mutation related to MELAS[d]
Definitive MELAS
Two items of Category A and two items of Category B (four items or more)
Suspicion of MELAS
One item of Category A and two items of Category B (at least three items)

[a] Focal brain abnormalities in CT and/or MRI.
[b] 2 mmol/L (18mg/dl) or more lactate in plasma at rest or in cerebral spinal fluid and/or deficiency of electron transport chain enzyme, pyruvate-related, TCA cycle-related enzymes or lipid metabolism-related enzymes in somatic cells (desirable for muscle cells).
[c] RRF (ragged-red fiber) in modified Gomori's trichrome stain and/or SSV (strongly SDH-reactive blood vessels) in succinate dehydrogenase stain, cytochrome c oxidase-deficient fibers or abnormal mitochondria in electron microscopy.
[d] Definitive mitochondrial gene mutations reported in the literature (G583A, G1642A, G1644A, A3243G, A3243T, A3252G, C3256T, A3260G, T3271C, T3291C, G3481A, G3697A, T3949C, G4332A, G5521A, A5814G, G7023A, T7512C, A8296G, T8316C, T9957C, A12299C, A12770G, G13042A, A13084T, G13513A, A13514G, A13528G, and G14453A) as of 2010 [3].

2.3. Japanese Mitochondrial Disease Rating Scale (JMDRS)

We prospectively analyzed the clinical progress of MELAS using the JMDRS (Supplementary Table 1), which was revised following the European NeuroMuscular Conference (ENMC) in 2003 [9]. The second and the third questionnaires were also based on the JMDRS and enabled longitudinal analysis of disease progression. We established a rating score for each patient in 2003 and 2008, and these values were used to analyze the clinical severity of MELAS.

2.4. Statistical analysis

Demographic and clinical data for the juvenile and adult forms of MELAS were summarized using descriptive statistics. An unpaired *t*-test was used to test for any differences in the death rates of juvenile and adult forms. Differences between the juvenile and adult forms in the symptoms at onset and throughout the entire follow-up period were evaluated by chi-square tests or Fisher's exacts test when the criteria for the chi-square test were not fulfilled. Alterations in the JMDRS scores between 2003 and 2008 were evaluated using unpaired *t*-tests alone or combined with a Welch correction when variances were significantly different. Survival rates were compared between juvenile and adult forms using the log-rank test. Statistical analyses were performed with the SPSS 11.0 J software package for Windows. $p<0.05$ was considered statistically significant.

3. Results

3.1. Questionnaire responses from the Japanese cohort

We received 1051 responses to the first questionnaire (total 47.0% response rate, 1051/2236); among them, 756 were from pediatric neurology departments(51.3% of responses) and 295 were from adult neurology departments(38.7% of responses). We identified 741 patients with mitochondrial myopathies and of these, 233 were MELAS patients (31.4% of total mitochondrial myopathy patients, 233/741), as described by 105 pediatric neurologists and 29 adult neurologists. We received 64 responses to the second questionnaire (total 47.8% response rate, 64/134): 36 from pediatric neurologists (34.3% response rate, 36/105) and 28 from adult neurologists (96.6% response rate, 28/29). We received 64 responses to the third questionnaire (100% response rate, 64/64); only 96 MELAS patients completed the 5-year cohort study.

3.2. Prevalence of MELAS in Japan

We found 741 cases of mitochondrial myopathy in our cohort study. Based on the MELAS diagnostic criteria (Table 1), we found 233 MELAS patients (juvenile/adult = 111/122) among the Japanese population of approximately 127,434,000 (approximately 22,275,000 under 18 years of age and approximately 105,159,000 over 18 years of age, adult form, according to census data from 2001). The prevalence of mitochondrial myopathy in Japan is therefore at least 0.58 (95% confidence interval (CI), 0.54–0.62)/100,000 in the total population. The prevalence of MELAS is at least 0.18 (95%CI, 0.17–0.19)/100,000 in the total population, 0.50 (95%CI, 0.41–0.59)/100,000 in children under 18 years of age, and 0.12 (95%CI, 0.10–0.14)/100,000 in the population over 18 years of age.

3.3. Demographic and pathological findings of MELAS in the cohort study

Our cohort study included 96 MELAS patients who were followed prospectively for 5 years. A histogram and a density plot showing the various ages of onset in MELAS in these patients indicate an approximately bimodal distribution (Fig. 1). We therefore divided the MELAS patients into two sub-groups to determine whether MELAS

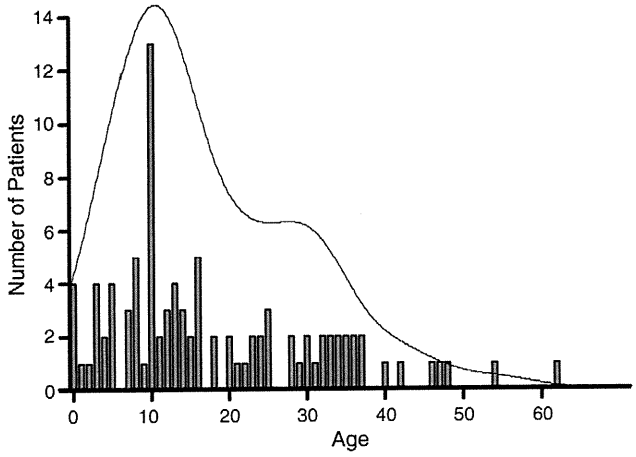

Fig. 1. Histogram detailing age of onset. A histogram and density plot of the various ages of MELAS onset is shown. In total, 96 Japanese patients were identified as having definitive MELAS as determined by diagnostic criteria (Table 1). Given the approximately bimodal distribution of patient age, MELAS patients were divided into two subgroups by the age of onset. Patients with an age of onset less than 18 years old were defined as having the juvenile form of MELAS, and patients with an age of onset greater than 18 years old were defined as having the adult form of MELAS.

has different features depending on the age of onset. Patients with an age of onset under 18 years were defined as having the juvenile form of MELAS, whereas patients with an age of onset above 18 years were defined as having the adult form of MELAS. A summary of the indexed MELAS patients is shown (Table 2). The ages of onset, diagnosis, and death were determined for both juvenile and adult forms. During this study, 17 of the 20 deceased MELAS patients presented with the juvenile form. Causes of death were cardiac insufficiency (7), severe respiratory infection (6), multiple organ insufficiency (3), and unknown causes (4).

Seventy-eight patients received muscle biopsies and 71 patients (91.0%) showed positive findings, including ragged-red fibers (RRF), SDH strongly reactive blood vessels (SSV), or both. However, seven patients presented normal features in the muscle biopsy.

3.4. Symptoms at onset and during the entire course

We evaluated the symptoms at onset in 96 MELAS patients (Table 3). The first sign of any symptoms or events such as seizure, stroke-like episode, or severe headache, which were associated with a neuroimaging-abnormality, was defined as the onset of MELAS. Symptoms at onset and during the entire course were similar as follows; seizure, stroke-like episode, and headache were the most frequent symptoms. Short stature and failure to thrive were significantly more prevalent in the juvenile form than in the adult form. However, hearing loss, diabetes mellitus and hemiplegia were significantly more frequent in the adult form than in the juvenile form.

Table 2
Demographic findings for MELAS cases.

	All form	Juvenile form	Adult
Patient[b] (male/female)	96 (52/44)	58 (35/23)	38 (17/21)
Age of onset, years[a]	17.7 ± 13.6	9.0 ± 4.7	32.2 ± 10.0
Age of diagnosis, years[a]	19.9 ± 13.5	11.0 ± 5.0	33.6 ± 10.6
Age of death, years[a]	18.8 ± 11.5	15.0 ± 7.9	40.0 ± 3.6
Death (%)[b,c]	20 (20.8 %)	17 (29.3 %)	3 (7.9 %)
Time from diagnosis to death[a]	7.3 ± 5.0	6.4 ± 4.5	10.2 ± 8.3
Positive family history[b] (%)	23 (24.0)	13 (22.4)	10 (26.3)
Muscle biopsy examination[b]	78	42	36
Positive findings[b] (%)	71 (91.0)	36 (85.7)	35 (97.2)
RRF[b]	56	24	32
SSV[b]	2	2	0
RRF + SSV[b]	13	10	3
A3243G mutation positive[b] (%)	75 (78.1)	46 (79.3)	29 (76.3)
Other mutation found in mtDNA[b]	4	4	0
Mutation not found[b]	17	8	9

[a] Mean ± SD.
[b] Number.
[c] Death ratio was higher for the juvenile form than for the adult form ($p = 0.0115$).

Table 3
Symptoms.

	Total (%) (n = 96)	Juvenile (%) (n = 58)	Adult (%) (n = 38)
Symptoms at onset			
Seizure	54/96 (56.3)	36/58 (62.1)	18/38 (47.4)
Stroke-like episode	53/96 (55.2)	29/58 (50.0)	24/38 (63.2)
Headache	48/96 (50.0)	27/58 (46.6)	21/38 (55.3)
Short stature[a]	46/96 (47.9)	35/58 (60.3)	11/38 (28.9)
Muscle weakness	36/96 (37.5)	26/58 (44.8)	10/38 (26.3)
General fatigue	30/96 (31.3)	20/58 (34.5)	10/38 (26.3)
Cortical blindness	26/96 (27.1)	15/58 (25.9)	11/38 (28.9)
Failure to thrive[a]	25/96 (26.0)	23/58 (39.7)	2/38 (5.3)
Vomiting/nausea	23/96 (24.0)	17/58 (29.3)	6/38 (15.8)
Hearing loss[a]	21/96 (21.9)	6/58 (10.3)	15/38 (39.5)
Unconsciousness	19/96 (19.8)	10/58 (17.2)	9/38 (23.7)
Teichopsia	18/96 (18.8)	12/58 (20.7)	6/38 (15.8)
Diabetes mellitus[a]	12/96 (12.5)	2/58 (3.4)	10/38 (26.3)
Symptoms in the entire course			
Stroke-like episode	81/96 (84.4)	49/58 (84.5)	32/38 (84.2)
Seizure	68/96 (70.8)	42/58 (72.4)	26/38 (68.4)
Short stature[a]	53/96 (55.2)	37/58 (63.8)	16/38 (42.1)
Headache	52/96 (54.2)	30/58 (51.7)	22/38 (57.9)
Cortical blindness[a]	43/96 (44.8)	21/58 (36.2)	22/38 (57.9)
Muscle weakness	40/96 (41.7)	27/58 (46.6)	13/38 (34.2)
General fatigue	38/96 (39.6)	26/58 (44.8)	12/38 (31.6)
Mental regression	38/96 (39.6)	20/58 (34.5)	18/38 (47.4)
Gait disturbance	37/96 (38.5)	23/58 (39.7)	14/38 (36.8)
Unconsciousness	36/96 (37.5)	20/58 (34.5)	16/38 (42.1)
Teichopsia	31/96 (32.3)	20/58 (34.5)	11/38 (28.9)
Cardiac dysfunction	29/96 (30.2)	18/58 (31.0)	11/38 (28.9)
Failure to thrive[a]	27/96 (28.1)	24/58 (41.4)	3/38 (7.9)
Speech disturbance	22/96 (22.9)	16/58 (27.6)	6/38 (15.8)
Memory loss	20/96 (20.8)	12/58 (20.7)	8/38 (21.1)
Diabetes mellitus[a]	20/96 (20.8)	5/58 (8.6)	15/38 (39.5)

[a] Significant difference between juvenile and adult forms. $p<0.05$ was considered statistically significant. At onset: short stature ($p = 0.0026$), failure to thrive ($p = 0.0001$), hearing loss ($p = 0.0007$), diabetes mellitus ($p = 0.0014$). During follow-up: short stature ($p = 0.0366$), hearing loss ($p = 0.0012$), cortical blindness ($p = 0.0366$), failure to thrive ($p = 0.0004$), diabetes mellitus ($p = 0.0006$).

3.5. Disease progression monitored with the JMDRS

MELAS was monitored in 2003 and 2008 with the JMDRS, which covers (1) activities of daily living, (2) motor functions, (3) special sensory functions, (4) endocrine functions, (5) cardiac functions, (6) renal functions, and (7) cognitive functions. Though JMDRS has not yet been validated, all MELAS patients had a significantly higher JMDRS score in 2008 than in 2003 (Table 4). Although no differences in the 2003 JMDRS scores were observed between the juvenile and adult forms, the 2008 scores revealed that the juvenile form was associated with a more aggressive deterioration than the adult form. The variation in scores between 2003 and 2008 was much larger in the juvenile form than in the adult form (Table 4).

3.6. Survival curve

Fig. 2 shows the Kaplan–Meier survival curve for MELAS patients. The log-rank analysis showed significant differences in survival between the juvenile and adult forms ($p = 0.0105$). The juvenile

Table 4
Variations of the JMDRS score in the 5-year interval.

	2003	2008	*p* Value
Raw score (minimum = 0, maximum = 81)			
Total ($n=96$)*	4.4 ± 3.2	16.1 ± 9.2	0.0001
Juvenile onset ($n=58$)*	4.9 ± 3.0	19.1 ± 9.7[a]	0.0001
Adult onset ($n=38$)*	3.6 ± 3.5	11.5 ± 6.1[a]	0.006
Score variances between 2003 and 2008			
Total ($n=96$)*	11.8 ± 8.3		
Juvenile onset ($n=58$)*	14.5 ± 8.8[b]		
Adult onset ($n=38$) *	7.8 ± 5.6[b] *Mean ± SD.		

[a] $p=0.0001$, raw scores between the juvenile and adult forms in 2008.
[b] $p=0.0001$, score variances between the juvenile and adult forms.

form had a higher rate of mortality than the adult form (hazard ratio, 3.29; 95%CI, 1.32–8.20).

4. Discussion

In this nationwide, multicenter, 5-year prospective Japanese cohort study, we determined the prevalence of mitochondrial myopathies such as Kearns Sayre syndrome (KSS)/progressive external ophthalmoplegia (PEO), Leigh syndrome, and MELAS. In our study, the prevalence of mitochondrial myopathy was at least 0.58/100,000 in the total population, with MELAS as the most common subtype (data not shown). Although the reported prevalence of mitochondrial disease varies depending on methodology, geography, ethnic group, and subject group, the population-based prevalence of mitochondrial disease risk was 9.18 to 12.48/100,000 in the total population of northeast England [10,11] and [12], 16.5/100,000 in the pediatric population of northeast England [10], 4.7 (95%CI, 2.8–7.6)/100,000 in the pediatric population of western Sweden [13], and 5.0 (95%CI, 4.0–6.2)/100,000 to 13.1/100,000 at birth in Victoria, Australia [14] and [15]. In general, epidemiological studies have estimated that the minimum prevalence of mitochondrial disease is 1/5000 in the general population [16]. The aforementioned prevalence estimates are approximately 10- to 34-fold higher than our estimate (0.58 (95%CI, 0.54–0.62)/100,000 in the total population).

This discrepancy can be explained partly by methodological differences. Because all previously reported prevalence data are based on estimations of risk of mitochondrial diseases extrapolated from the mutation or disease frequency in a limited population or region with regional mitochondrial research institutes or mitochondrial specialists, these values are likely to overestimate the prevalence in the entire population. Whether carriers of pathogenic mitochondrial DNA develop severe mitochondrial disorders depends on the degree and distribution of the mutation in important somatic organs. Although all prevalence studies can contain methodological bias, the prevalence of mitochondrial myopathy should be confirmed by a meta-analysis or nationwide cohort studies in other countries. The discrepancy between our prevalence estimate and previously reported data might also be attributable to a number of additional factors. First, we might have missed some patients due to the imperfect response rate (47%), even though our study included almost all of the main hospitals and institutes in Japan. There was no tendency with respect to region for the lack of responses. However, the response rate for the second questionnaire was significantly different between pediatric and adult neurologists. Pediatric neurologists may not have examined MELAS patients in 2003 although they had examined MELAS patients in 2001; because juvenile MELAS develops at a faster rate than adult MELAS, patients may have died or been referred to an inpatient hospital during 2-year interval. All non-responsive hospitals had less than 300 beds. Generally in Japan, MELAS that is very rare and multi-systematic diseases are monitored in large hospitals that have many departments and beds. Second, it is possible that the mitochondrial myopathies may have been misdiagnosed due to their rarity. Finally, given that most of the prior reports described Caucasian populations, it is possible that the disparity may derive from racial differences. In 2010, the Ministry of Health, Welfare and Labour, Japan has newly approved the mitochondrial myopathy as a supported disorders for their medical expenses, and started to collect the application for such privilege in entire Japan. In above situation, only 100 applications have been collected, to date (personal communication). Because our first questionnaire is also including death case of mitochondrial myopathies, our result come from disease-based prevalence study may be more realistic date at least in Japan.

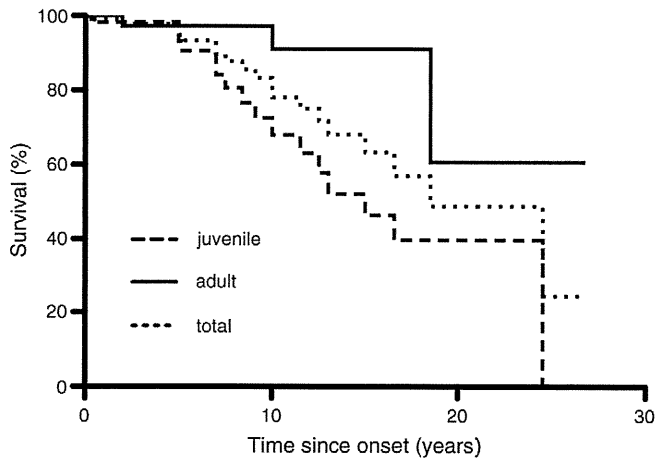

Fig. 2. MELAS survival curve. A Kaplan–Meier survival curve is shown. The dashed line indicates the juvenile form and the solid line indicates the adult form. The results of the log-rank analysis were significant. The juvenile form was associated with a higher risk of mortality than the adult form (hazard ratio, 3.29; 95%CI, 1.32–8.20).

Because MELAS is the most dominant subtype of mitochondrial myopathy and has been associated with an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, several studies have reported the prevalence of the A3243G mutation. The absolute prevalence of this mutation has been estimated to be as high as 1.41 (95%CI, 0.83–1.20)/100,000 individuals in northern England [12] and [17], 16.3 (95%CI, 11.3–21.4)/100,000 in the adult population of northern Finland [18], and 18.4 (95%CI, 10.9–29.1)/100,000 in the Finnish pediatric population [19]. With the exception of a report from Australia in a large Caucasian population that showed the highest prevalence of 236/100,000 [20], the prevalence of MELAS in Japan (0.18 (95%CI, 0.17–0.19)/100,000) seems to be quite reasonable, given the previously reported prevalence of the A3243G mutation. Because this mutation has been found in association with various clinical conditions, including subclinical asymptomatic carriers and patients with short stature, diabetes mellitus, migraine headache, PEO, MELAS, and/or Leigh syndrome with cardiomyopathy, only individuals whose mutation load in important organs is 50% or more may present with MELAS or a more severe phenotype [21]. Multiple medical problems, including various neurological, cardiological, endocrinological, gastroenterological, and psychiatric symptoms, were reported in 45 families with 45 MELAS patients and 78 carrier relatives in a regional cohort study in the USA [22]. Accordingly, the actual prevalence of the A3243G mutation in human populations may be much higher than previously thought, if we take into account not only the individuals who showed full symptoms of mitochondrial disorders, but also those who show minimal symptoms, even when they were not followed up at a hospital. Nevertheless, MELAS is a clinically and histopathologically defined entity, and the prevalence of MELAS (0.18/100,000 in the total population) in Japan is unlikely to change drastically given more information.

To identify the various symptoms associated with MELAS, we defined the diagnostic criteria for MELAS in Japanese patients. The diagnostic criteria were constructed on the basis of the information provided by Hirano [23] and Hirano and Pavlakis [7]. Category A contains clinical findings of stroke-like episodes, while category B contains evidence of mitochondrial dysfunction. We evaluated only 96 out of 233 MELAS patients. The other patients were excluded for the following reasons; 1) non-response, 2) failure to receive informed consent, 3) patients/neurologists moved to other regions, and 4) other unknown reasons.

According to our data, MELAS can be divided into two subgroups: juvenile and adult forms. This distinction is warranted because of an approximately bimodal distribution of the age of onset, different manifestations of MELAS symptoms in pediatric and adult patients, and differences in the progression of disease as monitored by JMDRS scores. The juvenile form is more severe than the adult form (Tables 2 and 3). No differences in family history were noted between these two forms. However, the juvenile form was associated with significantly higher mortality and a more rapid disease progression than the adult form. We believe that this discrepancy arises because (1) children require more energy to complete their development and maintain their physicality and (2) juvenile patients may have a higher mutation load in mitochondrial genes than in adult patients. Almost all patients with the adult form have a normal life until onset despite having some kind of mitochondrial dysfunction. Therefore, it appears that the adult form requires a longer time for significant symptoms to develop and for the disease to worsen. Because the juvenile form has a greater mutation load than the adult form, it can present more severe complications such as the cardiac and/or renal failure, and patients with the juvenile form are at increased risk of multiple organ failure. Patients with the adult form of MELAS are more likely to have diabetes mellitus and to have a more gradual disease progression. Given these differences in disease progression, our 5-year cohort study may not have provided sufficient time to identify the chronic negative effects of diabetes mellitus especially in the adult form.

Among the clinical symptoms at onset or during the entire course of the disease, seizure and headache were very common and associated with stroke-like episodes in both the juvenile and the adult form. However, of the symptoms present at onset, short stature and failure to thrive were significantly more common in the juvenile form than in the adult form. In contrast, patients with the adult form presented with symptoms such as diabetes mellitus and hearing loss significantly more often than patients with the juvenile form, perhaps because these symptoms are more chronic and maturity (age)-related and can be induced by the accumulation of abnormal mitochondria in low-turnover environments such as pancreatic beta-cells or hearing organs. Of the symptoms encountered during the entire course of the disease, stroke-like episodes were noted in more than 84% of juvenile and adult form patients. Seizure and headache, which are the main symptoms associated with stroke-like episodes, were also common in both juvenile and adult forms. Interestingly, hearing loss, cortical blindness and diabetes mellitus, which are not recognized as main symptoms, were seen significantly more often in the adult form than in the juvenile form. The symptoms listed in our study are consistent with those of previous reports, including the American cohort study [7,8] and [24], the Finnish cohort study [19], and the Japanese muscle biopsy registry of MELAS [5].

We used JMDRS scores to evaluate the progression of MELAS over a 5-year interval. The validated mitochondrial disease rating scale was published in 2006 [25] and [26]. This scale has four classifications, which are age group classification of 0–24 months, 2–11 years, and 12–18 years from the Newcastle pediatric mitochondrial disease scale (NPMDS) [25], and an adult age group classification from the Newcastle mitochondrial disease adult scale (NMDAS) [26]. We had to use the JMDRS although it had not yet been validated because this study started in 2001, and the rating scale was initially mailed to the neurologists in 2003. Contents and indexed factors are similar between NPMDS, NMDAS, and JMDRS. However, NPMDS and NMDAS include contents from patient interviews. This feature is quite different between the Newcastle scales and the JMDRS. In all other respect, the JMDRS is thought to be a comprehensive, quantitative, reproducible, and sensitive monitoring system to detect the progression of disease severity in MELAS. We aimed to use and analyze JMDRS as a pilot study in the present work. According to this analysis, all MELAS patients (both juvenile and adult forms) showed an increased score and worsening of their condition during the 5-year interval. The progression of dysfunction in section 1 (activity of daily living), section 2 (motor activity) and section 7 (cognition and impairment) occurred more rapidly than that in other sections, and it was more pronounced in the juvenile form than in the adult form (data not shown). Patients with more rapidly increasing scores were more commonly found in the group with the juvenile form and had a higher risk of death than those with a more mild disease. This result indicates that the juvenile form progresses more rapidly and is more severe than the adult form. Despite the lack of validation, in this study the JMDRS produced findings that were consistent with a previous study [27] and we believe that the JMDRS is a useful scoring system that allows sensitive and reproducible monitoring of the progression of MELAS. In the future, we will more explicitly validate the JMDRS scoring system for MELAS.

In conclusion, given that no drugs have yet been approved for MELAS, we believe it is important to develop efficacious treatments for MELAS. L-arginine therapy, which is currently in development for MEALS [28], might be a promising drug for the future, and we believe that the results from this study will be helpful for the development of new therapeutic interventions aimed at MELAS.

5. Conclusions

We determined that MELAS occurs into two forms; adult and juvenile, and that the juvenile form is more severe than the adult form. Although our results may contain several biases, including limited information from neurologists, our data highlight new and important information for both pediatric and adult neurologists who are assessing MELAS patients. JMDRS is a useful scoring system for evaluating disease progression in MELAS.

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Pyruvate therapy for mitochondrial DNA depletion syndrome☆

Keiko Saito [a,*], Nobusuke Kimura [a,1], Nozomi Oda [a], Hideki Shimomura [a], Tomohiro Kumada [a], Tomoko Miyajima [a], Kei Murayama [b], Masashi Tanaka [c], Tatsuya Fujii [a]

[a] Department of Pediatrics, Shiga Medical Center for Children, Moriyama-City, Shiga 524-0022, Japan
[b] Department of metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan
[c] Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi, Tokyo 173-0015, Japan

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ABSTRACT

Background: Mitochondrial DNA depletion syndromes are a group of heterogeneous autosomal recessive disorders associated with a severe reduction in mitochondrial DNA in the affected tissues. Sodium pyruvate has been reported to have a therapeutic effect in mitochondrial diseases.
Methods: We analyzed the effects of 0.5 g/kg of sodium pyruvate administered through a nasogastric tube in a one-year-old patient with myopathic mitochondrial DNA depletion syndrome. To evaluate the improvement, we used the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) and manual muscle testing. As the improvement of motor functions in this severely disabled infant could not be comprehensively detected by NPMDS, we also observed the infant's ability to perform several tasks such as pouting, winking, and number of times she could tap a toy xylophone with a stick. Blood lactate and pyruvate levels were also monitored.
Results: After one month's treatment, the NPMDS score in section IV, the domain for the quality of life, improved from 17 to13. The infant became capable of raising her forearm, lower leg and wrist against gravity. The maximum number of times she could repeat each task increased and the movements became brisker and stronger. No significant change of the blood lactate level or lactate-to-pyruvate ratio, both of which were mildly increased at the initiation of the therapy, was observed despite the clinical improvement.
Conclusion: Sodium pyruvate administered at 0.5 g/kg improved the muscle strength and the NPMDS score of an infant with myopathic mitochondrial DNA depletion syndrome.
General significance: Sodium pyruvate may be effective for ameliorating the clinical manifestations of mitochondrial diseases. This article is part of a Special Issue entitled: Biochemistry of Mitochondria.

1. Introduction

Mitochondrial DNA depletion syndromes (MDSs) are a heterogeneous group of autosomal recessive disorders manifesting mainly in infancy and childhood that are associated with a severe reduction of the mitochondrial DNA (mtDNA) copy number in the affected tissues [1]. Three different clinical forms of MDSs have been described: myopathic, encephalomyopathic, and hepatocerebral MDSs [2–6]. The clinical phenotypes can overlap and patients with myopathic MDS could develop encephalomyopathic MDS at a later date. Several causes of MDSs, which affect the mtDNA replication and maintenance, have been reported. These include defects of enzymes affecting the nucleotide pools (mitochondrial thymidine kinase, deoxyguanosine kinase, ribonucleotide reductase p53-R2 subunit and thymidine phosphorylase), defects of mtDNA replication proteins (mtDNA polymerase gamma and Twinkle), defects of succinyl-CoA ligase, which interacts with mitochondrial nucleotide diphosphate kinase, and defects of proteins of unknown function, including MPV 17 [5].

Like all of the other mitochondrial respiratory-chain disorders, there are no curative therapies for MDSs. For mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), however, which is associated with disturbance of the nucleotide pools, treatments that reduce the circulating levels of nucleotides can improve the symptoms, including peritoneal dialysis [7], enzyme replacement therapy [8] and allogenic stem cell transfusions [9]. Unfortunately however, such treatments cannot be applied to other types of MDSs. Treatments with vitamins, cofactors and respiratory substrates may improve some symptoms, however, the efficacy is limited.

Tanaka et al. recently reported the therapeutic promise of pyruvate for mitochondrial diseases [10]. According to their theory, pyruvate supplementation would improve the intracellular redox state by

Abbreviations: MDS, mitochondrial DNA depletion syndrome; L/P, lactate-to-pyruvate

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* Corresponding author at: 5-7-30, Moriyama, Moriyama City, Shiga 524-0022, Japan. Tel.: +81 77 582 6200; fax: +81 77 582 6304.
E-mail address: ksaitou-kyt@umin.ac.jp (K. Saito).
[1] Present address: National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders, Shizuoka 420-8688, Japan.

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providing NAD^+ and reducing the lactate-to-pyruvate (L/P) molar ratio, which is high in cells with mitochondrial respiratory deficiency; as a result, ATP production by the glycolytic pathway would improve. In a preliminary study, they administered 5 g of sodium pyruvate to an adult patient with chronic progressive external ophthalmoplegia associated with mtDNA deletion. At 30 min after the administration of pyruvate, the blood lactate level decreased from 2.42 mM to 2.10 mM and the L/P ratio decreased from 25.65 to 16.29. No clinical improvement was, however, described in this report. So far, case reports on pyruvate therapy for mitochondrial diseases are very limited, and the efficacy of this treatment still remains inconclusive.

In the present report, we describe the clinical course of a one-year-old patient with myopathic MDS who was treated with sodium pyruvate, and discuss the efficacy of this newly proposed therapy for amelioration of the clinical manifestations of mitochondrial disorders.

2. Patients and methods

2.1. Patient

A one-year-old girl was born by Cesarean section (indication: breech presentation and placenta previa) to non-consanguineous parents at 37 weeks of gestation; the birth weight was 2970 g and the Apgar scores were 8 and 9. The family history was non-contributory. The infant began to have feeding difficulty on postnatal day 3 and developed respiratory failure and lactic acidosis (11.3 mM; normal range, 0.33–1.9 mM) on 10 days of age. She has been on a respirator ever since. The blood level of creatine phosphokinase was 3158 IU/L on postnatal day 3, but normalized later. There was no evidence of hepatomegaly and the blood levels of aspartate amino trasferase and alanine transaminase were mildly elevated (50 and 30 IU/L, respectively). Blood ammonia levels, acylcarnitine profile and urinary organic acids were normal. With improvement of the respiratory failure by mechanical ventilation, the blood lactate levels decreased, but remained between 3.0 mM and 6.5 mM, with high L/P ratios (between 36 and 97; normal <15), consistent with the diagnosis of a mitochondrial respiratory chain disorder. The lactate and pyruvate levels in the cerebrospinal fluid (CSF) were 4.2 mM and 0.18 mM, respectively with an L/P ratio of 23. Brain MRI at the age of 7 months showed mild dilatation of the lateral ventricles without any abnormal signals in the parenchyma. Treatment with coenzyme Q, thiamine, ascorbic acid and l-carnitine at the age of 3 months decreased the blood lactate levels (to between 1.4 mM and 3.1 mM), however, the L/P ratios remained high (between 16 and 45). The severe motor weakness and respirator dependence did not improve with this treatment.

Muscle biopsy performed at 10 months of age showed mild variations of the fiber size and predominance of the type 2A/2B fibers, comprising 71% of the fibers. A significant number of type 2C fibers were also found (22%). All fibers showed lipid droplets and glycogen accumulation. Ragged red fibers were found, however, strongly succinate dehydrogenase-positive vessels were not found. Cytochrome c oxidase staining was decreased, but not absent, in most fibers (Fig. 1).

Biochemical analysis of the respiratory chain enzymes in the muscle specimen revealed deficiencies of complex I (CoI), III (CoIII) and IV (CoIV), that were confirmed by the assay against citrate synthase (CS) or complex II (CoII) [11]: the activities of CoI, CoIII and CoIV relative to the activity of CS were 10.6%, 26.7% and 14.1%, respectively, and those relative to the activity of CoII were 6.5%, 16.4% and 8.8%, respectively (definite deficiency; <30% of CS or CoII).

Quantitative analysis of the mitochondrial DNA by real-time PCR [12] revealed that the ratio of the copy number of the mitochondrial NDI subunit relative to the nuclear CETR gene was 35.3% (normal; >40%), indicative of mitochondrial DNA depletion. Mutation analysis is underway.

The patient showed slowly progressive motor regression despite the treatment; by the age of 12 months, she lost the ability to smile, hold her arms above her chest against gravity or raise the lower legs, all of which she had been able to do until 8 months of age. At the age of 12 months, the patient was referred to our hospital for further treatment. Physical examination on admission showed severe

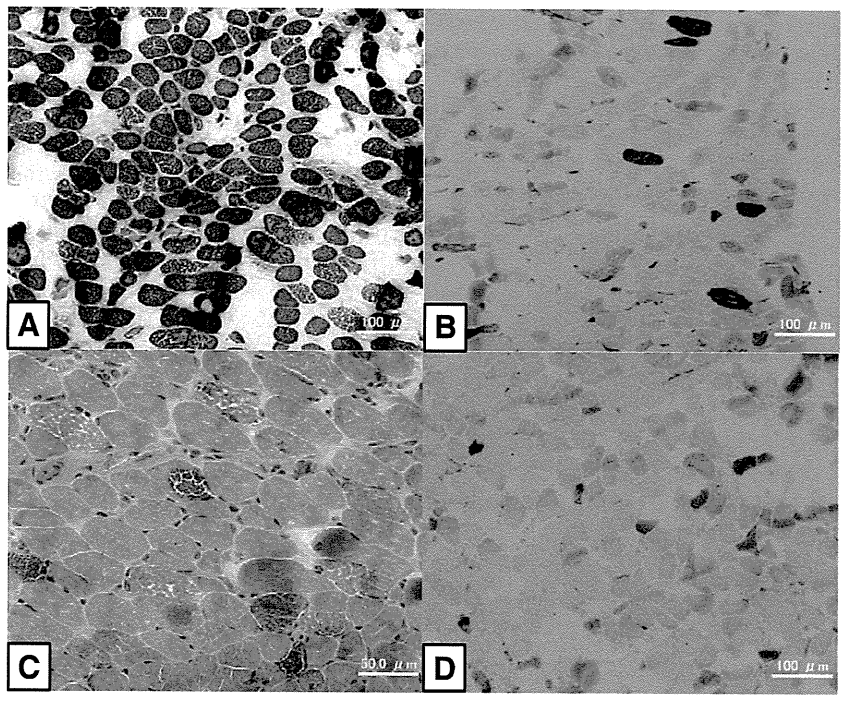

Fig. 1. Histochemistry of the biopsied muscle. ATPase staining shows type 2 fiber predominance at pH 10.6 (A) and an increased number of type 2C fibers at pH4.2 (B). The percentages of type 1, 2A/B and 2C fibers were 7%, 71% and 22%, respectively. Modified Gomori trichrome staining (C) shows scattered ragged-red fibers. Cytochrome *c* oxidase staining (D) shows decreased, but not absent, staining in most fibers.

generalized hypotonia and muscle weakness. Echocardiography was normal. She had dysphagia and was fed via a nasogastric tube. Her cognition ability seemed normal despite the mild ventricular dilatation on MRI. Her hepatic dysfunction was limited to mild elevation of the serum transaminases. Therefore, the infant was diagnosed as having myopathic-type MDS.

2.2. Pyruvate therapy

The pyruvate treatment was approved by the ethics committee of Shiga Medical Center for Children and written informed consent was obtained from the parents. Sodium pyruvate (Musashino Chemical Laboratory, Tokyo), dissolved at 0.5 g/kg in water at the concentration of 0.06 g/ml was given through a nasogastric tube in three divided doses (although the recommended concentration of sodium pyruvate to avoid osmotic diarrhea is about 0.02 g/ml, we chose the higher concentration to avoid water overload). During the pyruvate therapy, other treatments, including vitamins and coenzyme Q, remained unchanged. Pyruvate was administered throughout the study period and the effects of the therapy were examined one month and two months after the initiation of the therapy.

2.3. Evaluation of the treatment effect

To evaluate the treatment effect, we used the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) for 0–24 months [13]. The measurements were performed on the day of the start of treatment before taking the first dose of pyruvate and one month and two months after the initiation of therapy with pyruvate. Considering that the motor disabilities of this patient were probably too severe for any changes to be detected by this scale, we also tried to evaluate the changes in the motor activities or muscle power by performing manual muscle testing (MMT) on the extremities as well as observing the patient's ability to perform tasks including pouting, pulling the corner of the mouth laterally, winking repeatedly, and tapping a toy xylophone with a stick by rotating the wrist while resting the arm on the floor. These tasks were the ones which her mother had let her do almost daily either as play or as a communication tool for more than two months before the initiation of the pyruvate therapy. We coaxed her to repeat the movements as many times as possible and counted the number of times she could repeat them. The measurement of each task was conducted only once because of development of fatigue. The examination was done on the day of initiation of the pyruvate therapy and one month and two months after the treatment initiation. During the treatment, the frequency of performance of the tasks which the infant's mother let her do almost daily was the same as that before the treatment, and the patient was not particularly trained to show better performance of the tasks.

2.4. Results

The pyruvate therapy did not cause any side effects, including diarrhea. The overall NPMDS score before the treatment initiation was 35, which decreased (improved) to 31 after one month of pyruvate therapy (Table 1). However, the improvement was only observed in the domain of the quality of life (section IV of the scale), which reflects the parent's subjective opinion. The scale measured two months after the initiation of therapy was the same as the one measured after one month of therapy. We also found that the patient became able to raise her forearms briefly by about 30° after one month of treatment, and by almost 90° after two months. She regained the ability to raise and hold the lower legs briefly by 2 months after the start of the therapy. She could move the wrist only horizontally before the treatment, but became able to also move it vertically after 1 month of the treatment. These observations indicated that the power of the biceps brachii, quadriceps femoris and brachioradialis muscles increased from grade 2 to grade 3 on MMT (Table 2). One month after the start of the pyruvate therapy, the number of times of pouting increased from 6 times to 15, winking from 6 times to 10, and tapping a xylophone from 5 times to 7. She could barely move the mouth corner before and until one month after the start of the therapy; however, she could move it 8 times by the second month (Table 2). Some other improvements which we observed, but could not measure quantitatively, included extended duration of each movement such as pouting and stretching of the mouth corner, increase in the speed and strength of the tapping, as well as more vivid facial expressions.

The blood lactate levels and L/P ratios did not change with the therapy. The lactate levels measured twice on separate days before the start of the treatment were 2.1 mM and 2.5 mM, with L/P ratios of 18 and 18, respectively. The lactate levels after one month and two months of pyruvate treatment were 2.7 mM and 2.3 mM, with L/P ratios of 18 and 18, respectively.

Table 1
Changes of the NPMDS scores with pyruvate therapy.

Section	Before Tx	1 month after Tx	2 months after Tx
I	7	7	7
II	6	6	6
III	5	5	5
IV	17	13	13
Overall	35	31	31

NPMDS, Newcastle Paediatric Mitochondrial Disease Scale; Tx, treatment.

3. Discussion

Tanaka et al. proposed several possible mechanisms by which pyruvate may improve the energy metabolism in respiratory chain-deficient mitochondria (Fig. 2) [10]: (a) Pyruvate reacts non-enzymatically with hydrogen peroxide to yield acetate, carbon dioxide and water, thereby eliminating hydrogen peroxide which is increased due to leakage of reactive oxygen species from the respiratory-chain deficient mitochondria. (b) In the presence of lactate dehydrogenase, pyruvate provides NAD^+ from NADH. NAD^+ is essential for oxidation of glyceraldehyde 3-phosphate by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to form 1,3-bisphosphoglycerate, which donates a phosphate group to ADP to produce ATP. Mitochondria with respiratory-chain disturbance are deficient in NAD^+, causing inhibition of the glycolytic pathway via GAPDH and an increase in the NADH-to-NAD^+ ratio, which is equivalent to the L/P ratio. Pyruvate supply reactivates the glycolysis which is impaired secondarily due to disturbance of the respiratory chain, and lowers the NADH/NAD^+ and L/P ratio. (c) Pyruvate dehydrogenase kinase (PDK) inhibits pyruvate dehydrogenase (PDH) activity, and pyruvate inhibits PDK activity. As a result, pyruvate activates PDH.

Table 2
Changes in motor function and lactate levels with pyruvate therapy.

	Before Tx	1 month	2 months
Lip pouting	6	15	ND
Winking	6	10	11
Pulling the mouth corner	None	None	8
Tapping a xylophone with a stick	5	7	ND
Raising the forearms from the bed floor	None	30°	90°
Raising the lower legs against gravity	Barely	Possible	Can hold
Flexing the wrists against gravity	Impossible	Possible	Possible
Blood lactate level	2.5 mM	2.7 mM	2.3 mM
Lactate-to-pyruvate ratio	18	18	18

The patient was asked to repeat the tasks as many times as possible. The number of times she could repeat the tasks was observed before, one month and two months after the start of the treatment. For raising the forearms, angles from the floor at which the arms could be raised were measured. Tx, treatment; ND, not done because the patient was not willing to perform.

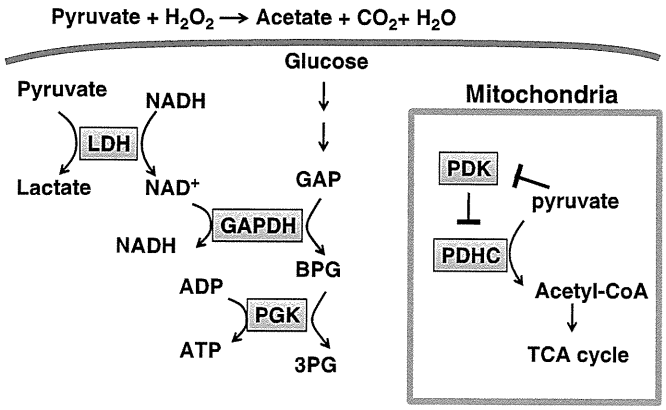

Fig. 2. Effects of pyruvate on energy metabolism and cell injury. Pyruvate eliminates hydrogen peroxide by a non-enzymatic reaction. Pyruvate provides NAD^+ from NADH with lactate dehydrogenase (LDH). In the presence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NAD^+ oxidizes glyceraldehyde 3-phosphate (GAP) to form 1,3-bisphosphoglycerate (BPG). BPG then provides its phosphate group to ADP to form ATP by phosphoglycerate kinase (PGK), and becomes 3-phosphoglycerate (3PG). In the mitochondrial matrix, pyruvate inhibits pyruvate dehydrogenase kinase (PDK) which inactivates pyruvate dehydrogenase complex (PDHC). As a result, PDH activates PDHC and provides acetyl-CoA, which enters TCA cycle.

The efficacy of pyruvate in improving the energy metabolism was observed in ρ^0 cells, which lack mtDNA. By adding pyruvate to the culture media, the ρ^0 cells survived, probably because of improved ATP production by pyruvate [14]. As the cells of the affected tissues in MDS are similar to the ρ^0 cells, it is reasonable to assume that pyruvate may be effective for ameliorating the clinical manifestations of MDS.

The weakness of the present study lies in the incomplete quantitative analysis of the treatment effect. Because of the patient's age and the severe weakness, it was not possible to measure the muscle strength accurately. As we anticipated, the NPMDS did not show any changes in scores in the domains that can show improvement in the muscle power, because the disability was too severe to allow detection of any improvement using this scale; in the domain for the current clinical assessment (section III), for example, the severity of myopathy is rated as severe when a patient is wheelchair dependent and the grade is defined as moderate when a patient has proximal weakness limiting functional movement. The improvement of the motor weakness in our patient was not sufficient to cause the rating to change from severe to moderate. However, even under this situation, the score for the quality of life showed improvement. One can argue that the improvement in the NPMDS score was due to the normal developmental process with age. However, the patient showed motor regression during the 11 months prior to the start of the treatment, and the parents noticed improvement by one month after the start of the treatment.

The tasks we chose to evaluate the muscle function can be influenced by skill rather than muscle strength. Therefore, the improvement in the performance of tasks could be simply due to a training effect, as the patient had been doing the same tasks daily. However, the patient had started to perform the tasks at least two months before the start of treatment, and no improvement was noticed during this pre-treatment period. On the other hand, improvement began to be noticed within a few weeks after the start of pyruvate therapy. Besides the improvement noted in the performance of these tasks which need skill, and may, therefore, be influenced by training, a significant increase in the muscle power in the biceps brachii, quadriceps femoris and brachioradialis muscles was observed; the patient became able to raise her forearms, lower legs and wrists against gravity, all of which she had become unable to do during the course of illness since 8 months of age. Our findings therefore suggest that the pyruvate therapy significantly improved the muscle strength and quality of life of the patient by a month after the start of treatment.

Contrary to the observed clinical improvement and the theory proposed by Tanaka et al., no significant changes of the blood lactate levels and L/P ratio were observed in this patient. One explanation for this discrepancy is that the blood lactate levels at the time of the therapy were too low (although higher than normal) to allow detection of any changes; the lactate level and the L/P ratio shortly before the start of pyruvate therapy were 2.5 mM and 18, respectively while those at the age of 3 months, by which time the patient was more active, were between 3.0 mM and 6.5 mM and 36 and 97, respectively. This apparent improvement in the blood lactate levels even before the start of pyruvate therapy might be due to the decrease in the muscle bulk as well as the severely weak muscle activity, which decreased the lactate production. Another factor which may have contributed to this discrepancy is the normal mitochondrial function in the liver. In myopathic MDS, mtDNA in the liver is not depleted; therefore, lactate released from the muscle might be metabolized in the liver, causing the blood lactate levels and L/P ratios to become near normal. On the other hand, when the lactate levels were very high at the age of 3 months, this factor did not contribute significantly. To prove that pyruvate does decrease the lactate levels and L/P ratios and increases the ATP production within the muscles, changes in these parameters in the muscles must be shown *in vivo*, possibly by magnetic resonance spectroscopy. We conducted no such evaluation in this study.

Thus, more clinical studies are necessary to precisely evaluate the efficacy of pyruvate therapy in patients with MDSs. However, there is only one published report, and several unpublished case reports on pyruvate therapy for mitochondrial diseases so far. Komaki et al. reported that an 11-year-old patient with Leigh syndrome associated with cytochrome c oxidase deficiency, who had easy fatigability and ataxic gait, became capable of participating in athletic games after treatment with oral sodium pyruvate at 0.5 g/kg [15]. They reported decrease of the blood lactate level from 2.3 mM to 1.1 mM and decrease of the L/P ratio from 18.1 to 11.7 in this patient. They also found an improvement in the cardiac dysfunction in the patient after one year's treatment. Other unpublished case reports include improvements in the MRI findings and cardiac dysfunction in a patient with Leigh syndrome (Wakamoto et al.) [15], cardiac improvement in another patient with Leigh syndrome (Koga et al.) [15], and activation of PDH activity which was estimated by measuring the $^{13}CO_2$ in exhaled air per unit time after administration of [1–^{13}C] pyruvate in two patients with PDH deficiency (Hamada et al. presented at the 52nd annual meeting of Japanese Society for Inherited Metabolic Diseases). We also treated a one-year old patient with Leigh syndrome associated with T9176C mutation in the mtDNA. The patient was severely disabled with tetraplegia at the time of the therapy, and showed no clinical improvement with pyruvate therapy. The severity of the symptoms at the time of pyruvate therapy may have differed between the patient treated by Komaki et al. and our own patient with Leigh syndrome. This finding highlights the limitation of this therapy and the possibly superior effects of the therapy in patients with an earlier stage of the disorder. No case reports on MDS are available.

Unlike non-physiological chemical drugs, such as dicholoroacetate, which can have some serious adverse effects, pyruvate is a physiological metabolite. The only possible side effects are sodium overload and osmotic diarrhea. Our patient did not develop diarrhea even though we did not dilute the sodium pyruvate as recommended, to avoid water overload. No serious adverse effects have been reported so far.

4. Conclusions

Oral (through a nasogastric tube) administration of 0.5 g/kg of sodium pyruvate improved the muscle power and quality of life of our patient with myopathic MDS. There are some case reports describing