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G7731A mutation in mouse mitochondrial *tRNA^{Lys}* regulates late-onset disorders in transmitochondrial mice

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ABSTRACT

We previously generated mito-mice-tRNA^{Lys7731} as a model for primary prevention of mitochondrial diseases. These mice harbour a G7731A mtDNA mutation in the *tRNA^{Lys}* gene, but express only muscle weakness and short body length by four months. Here, we examined the effects of their aging on metabolic and histologic features. Unlike young mito-mice-tRNA^{Lys7731}, aged mito-mice-tRNA^{Lys7731} developed muscle atrophy, renal failures, and various metabolic abnormalities, such as lactic acidosis and anemia, characteristic of patients with mitochondrial diseases. These observations provide convincing evidence that the respiration defects induced by high G7731A mtDNA levels cause these late-onset disorders that are relevant to mitochondrial diseases.

1. Introduction

The accumulation of mitochondrial DNA (mtDNA) with pathogenic mutations is proposed to be closely associated with mitochondrial diseases, normal aging, and age-associated disorders, including diabetes, because of its induction of significant respiration defects [1–3]. Three most prevalent mitochondrial diseases are chronic progressive external ophthalmoplegia (CPEO), due to mtDNA with large-scale deletions (ΔmtDNA); myoclonic epilepsy with ragged-red fibers (MERRF), due to single-point mutations in the *tRNA^{Lys}* gene; and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS), due to single-point mutations in the *tRNA$^{Leu(UUR)}$* gene [1–3]. Subsequent studies [4–6] have proved the pathogenicities of these mtDNA mutations by inducing respiration defects after the transfer of patient-derived mutated mtDNA into mtDNA-less human cells. However, it has not been established unequivocally whether the respiration defects due to the accumulation of mtDNA with pathogenic mutations in the tissues actually induce the various clinical phenotypes observed in patients with mitochondrial diseases or with age-associated disorders.

This issue was resolved in part by our previous studies [7–11], in which we generated transmitochondrial mice (mito-mice) carrying exogenously introduced mouse mtDNA with mutations orthologous to those found in patients with mitochondrial diseases. For example, mito-mice-Δ, which harbored mouse ΔmtDNA and therefore corresponded to disease models for CPEO, simultaneously expressed respiration defects, which were induced by accumulated ΔmtDNA, and disease phenotypes corresponding to those of CPEO in humans [7,8]. Therefore, experiments in mito-mice could provide convincing evidence that the respiration defects due to mtDNA mutations regulate clinical abnormalities relevant to mitochondrial diseases or age-associated disorders.

We recently generated mito-mice-tRNA^{Lys7731}, which harbour mtDNA that contain a pathogenic G7731A mutation in the *tRNA^{Lys}* gene (G7731A mtDNA), as a model for primary prevention of mitochondrial diseases [12]. Because a G8328A mutation orthologous to the mouse G7731A mutation occurs in patients with mitochondrial diseases [13,14], mito-mice-tRNA^{Lys7731} can be used

Abbreviations: mtDNA, mitochondrial DNA; ΔmtDNA, mtDNA with large-scale deletions; CPEO, chronic progressive external ophthalmoplegia; MERRF, myoclonic epilepsy with ragged red fibers; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome; COX, cytochrome c oxidase; RRFs, ragged red fibers; SDH, succinate dehydrogenase.

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as prospective models for diseases that, like MERRF, are caused by mutations in the mitochondrial *tRNA^{Lys}* gene. Young mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA expressed respiration defects and the resultant phenotypic features characteristic of mitochondrial diseases, such as short body length and muscle weakness, but they did not exhibit ragged red fibers (RRFs) or other traits seen in patients with mitochondrial diseases [12].

Here, to compare the effects of aging on the expression of phenotypes characteristic of mitochondrial diseases and age-associated disorders, we used aged mito-mice-tRNA^{Lys7731} that share the same nuclear background but carry either low or high levels of G7731A mtDNA.

2. Materials and methods

Mice. Inbred B6 mice were obtained from CLEA Japan. Mito-mice-tRNA^{Lys7731} were generated in our previous work [12]. Animal experiments were performed in accordance with protocols approved by the Experimental Animal Committee of the University of Tsukuba, Japan (approval number, 14243).

Genotyping of mtDNA. To detect the G7731A mutation, a 130-bp fragment containing the 7731 site was PCR-amplified by using the nucleotide sequences from 7633 to 7653 (5′-GCCCATTGTCCT AGAAATGGT-3′) and 7762 to 7732 (5′-ACTATGGAGATTTTAAGG TCTCTAACTTTAA-3′) as oligonucleotide primers. The G7731A mutation creates a restriction site for *DraI* and generates 96- and 34-bp fragments on DraI digestion of PCR products. The restriction fragments were separated by electrophoresis in a 3% agarose gel. For quantification of G7731A mtDNA, we used ImageJ (Rasband, WS., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014) software.

Histopathologic analyses. Formalin-fixed, paraffin-embedded sections (thickness, 5 μm) were stained with hematoxylin and eosin (H&E) to identify features characteristic of renal failures. Cryosections (thickness, 10 μm) of skeletal muscle were stained with modified Gomori trichrome for histopathologic analysis to identify RRFs. Cryosections (thickness, 10 μm) of renal tissues were prepared, and histologic analyses of COX and succinate dehydrogenase activities were performed as described previously [15].

Grip strength test. Muscle strength was estimated by using a Grip Strength Meter (Columbus Instruments, Columbus, USA); three sequential trials were performed on each mouse bilaterally.

Measurement of blood glucose, lactate, and BUN. To determine fasting blood lactate and glucose concentrations, peripheral blood was collected from the tail veins of mice after food had been withheld overnight. Glucose (1.5 g/kg body weight) was administered orally, blood was collected 15–120 min after glucose administration, and lactate and glucose concentrations were measured with an automatic blood lactate meter (Lactate Pro 2; Arkray, Kyoto, Japan) and glucose meter (Dexter ZII; Bayer, Leverkusen, Germany), respectively. BUN was measured with a Urea N B test (Wako Pure Chemical, Osaka, Japan) in accordance with the manufacturer's protocol.

Measurement of hematocrit. To determine hematocrit, capillary blood samples were obtained from each mouse by using heparinized capillary tubes, which then were centrifuged at 10,500 × *g* for 5 min. Packed cell volumes were measured by using a hematocrit reader.

Statistical analysis. Data are presented as mean ± SD and were analysed by using Student's *t* test; *P* values less than 0.05 were considered significant. Excel software was used for all statistical analysis.

3. Results

3.1. Late-onset metabolic abnormalities in aged mito-mice-tRNA^{Lys7731}

We used aged (26-month-old) male mito-mice-tRNA^{Lys7731} with low (<5%) or high (70%–75%) levels of G7731A mtDNA in their tails at 4 weeks after birth and age- and sex-matched B6 mice (negative controls). We first evaluated body length (Fig. 1A) and muscle strength (Fig. 1B), because abnormalities in these phenotypes are expressed in young (4-month-old) mito-mice-tRNA^{Lys7731} [12] and can be examined without killing the mice. Short body length (Fig. 1A) and muscle weakness (Fig. 1B), which are closely associated with the clinical abnormalities caused by the orthologous G8328A mutation in the human mitochondrial *tRNA^{Lys}* gene [13,14], occurred exclusively in mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA. These results are consistent with our previous findings obtained from young mito-mice-tRNA^{Lys7731} [12].

We then examined various metabolic parameters relevant to mitochondrial diseases. Whereas these features were normal in our young mito-mice-tRNA^{Lys7731} [12], we expected that abnormalities in these parameters would be expressed as late-onset disorders as the mito-mice-tRNA^{Lys7731} aged. Unlike aged B6 mice and aged mito-mice-tRNA^{Lys7731} with low levels of G7731A mtDNA, aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA exclusively had low hematocrit values (Fig. 1C), lactic acidosis (Fig. 1D),

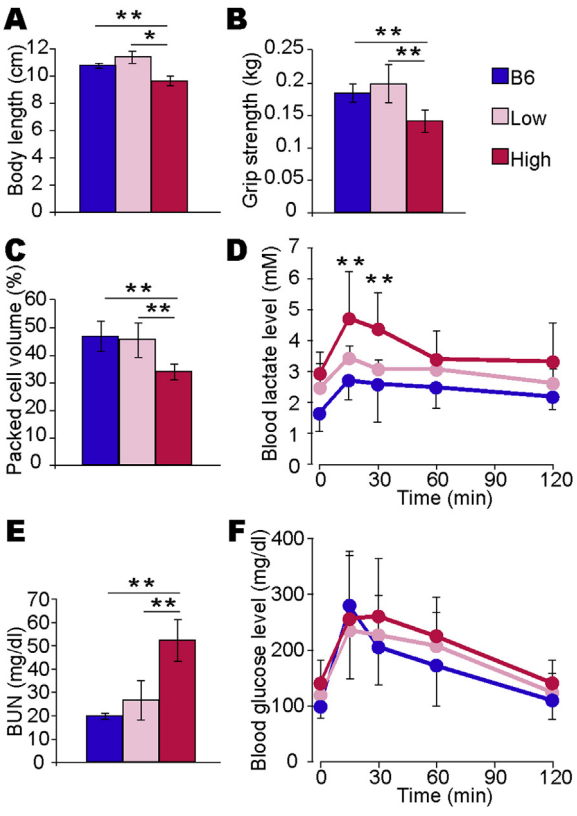

Fig. 1. Mitochondrial disease–related parameters that can be examined without euthanizing aged mito-mice-tRNA^{Lys7731}. Study populations comprised aged B6 mice ($n = 6$) and aged mito-mice-tRNA^{Lys7731} with low (less than 5% in tail tissue; $n = 4$) and high ($n = 4$; 70%, 70%, 72%, and 75% in tail tissue [Fig. S1]) levels of G7731A mtDNA in tails at 4 weeks after birth. Disease-related parameters were compared at 26 months after birth. Intergroup comparison of (A) body length, (B) grip strength, (C) hematocrit, (D) blood lactate level, (E) BUN value, and (F) blood glucose level. Data are presented as means ± 1 SD. *, $P < 0.05$; **, $P < 0.01$.

and increased levels of BUN in the peripheral blood (Fig. 1E). In contrast, although patients with mitochondrial diseases sometimes demonstrate mitochondrial diabetes [1–3], neither group of aged mito-mice-tRNA^{Lys7731} manifested hyperglycemia (Fig. 1F). Therefore, except for hyperglycemia, the metabolic abnormalities seen in patients with mitochondrial diseases were expressed as late-onset disorders in aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA.

3.2. *Lifespan and tissue abnormalities in euthanized moribund mito-mice-tRNA^{Lys7731}*

We then assessed the lifespans of mito-mice-tRNA^{Lys7731} and the tissue abnormalities in euthanized moribund mice. The median survival times in B6 mice and in mito-mice-tRNA^{Lys7731} with low or high levels of G7731A mtDNA were 26, 28, and 27 months, respectively (Fig. 2). Thus, median survival times did not differ significantly between mito-mice-tRNA^{Lys7731} with high and low levels of G7731A mtDNA.

Gross necropsy of euthanized moribund mice showed that muscle atrophy (Fig. 3A) and anemic kidneys (Fig. 4A) occurred exclusively in mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA. Muscle atrophy occurs not only in elderly men and women [16] and patients with mitochondrial diseases [17] but also in mtDNA mutator mice [18,19] and mito-mice-Δ that express significant respiration defects [20]. Moreover, renal abnormalities have been reported to occur occasionally in patients with mitochondrial diseases [21–23] and in mito-mice-Δ that express significant respiration defects [7,8].

We then histologically analyzed these tissues to characterize the macroscopic abnormalities in greater detail. First, we used modified Gomori trichrome staining to reveal RRFs that occur frequently in patients with mitochondrial diseases [2]. However, even aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA lacked RRFs in skeletal muscle (Fig. 3B); this was consistent with our recent findings in mtDNA mutator mice and mito-mice-Δ [20].

Although the skeletal muscle was histologically normal, the anemic kidneys demonstrated multiple abnormalities. The renal cortical tubules of aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA were dilated and contained casts; these changes, indicative of renal failures, did not occur in aged B6 mice or in aged mito-mice-tRNA^{Lys7731} with low levels of mutated mtDNA (Fig. 4B). Given that young mito-mice-tRNA^{Lys7731} lacked similar renal changes [12], these changes correspond to a late-onset disorder. Histologic analysis of mitochondrial respiratory function revealed decreased mitochondrial cytochrome c oxidase (COX) activity in

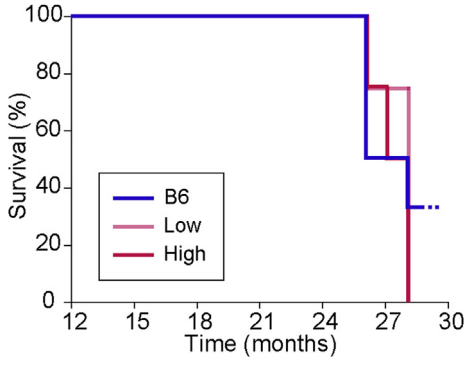

Fig. 2. Kaplan–Meier survival curves of B6 mice ($n = 6$) and mito-mice-tRNA^{Lys7731} with low (less than 5%; $n = 4$) and high (70%–75%; $n = 4$) levels of G7731A mtDNA. Median survival times were 26 months for B6 mice, 28 months for mito-mice-tRNA^{Lys7731} with low levels, and 27 months for mito-mice-tRNA^{Lys7731} with high levels.

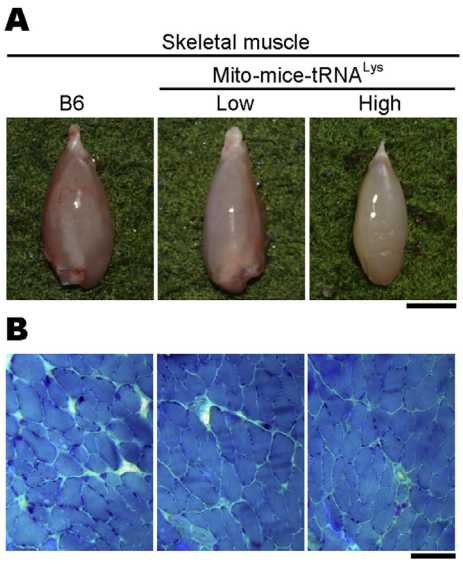

Fig. 3. Skeletal muscle abnormalities in aged mito-mice-tRNA^{Lys7731}. (A) Macroscopic evidence of muscle atrophy in the quadriceps of mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA. Scale bar, 5 mm. (B) Histopathologic analysis of skeletal muscles to identify RRFs. Cryosections (thickness, 10 μm) of soleus muscle fibers were stained by using modified Gomori trichrome to identify RRFs. No RRFs were present even in aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA. Scale bar, 50 μm. Genotyping of skeletal muscle (soleus) showed that the sample from the low group contained 5% G7731A mtDNA, whereas the sample from the high group contained 65% G7731A mtDNA.

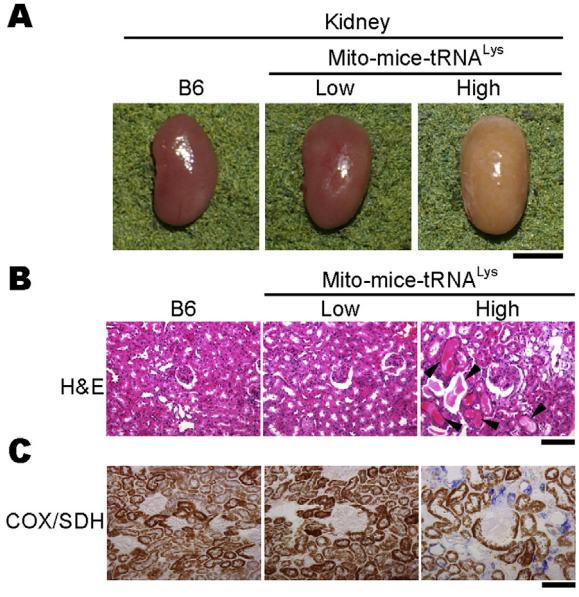

Fig. 4. Renal abnormalities in aged mito-mice-tRNA^{Lys7731}. (A) Macroscopic evidence of anemic kidneys in mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA. (B) Hemotoxylin and eosin (H&E) staining of formalin-fixed, paraffin-embedded sections (thickness, 5 μm) of renal cortex revealed dilated lumens of renal tubules and casts (arrowheads) in mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA. Scale bar, 50 μm. (C) Histochemical analysis of mitochondrial respiratory enzyme activities in the kidney. Tissue sections were stained for COX (brown) and succinate dehydrogenase (SDH) (blue). Cells that had lost COX activity were blue because of the absence of COX activity. Scale bar, 50 μm. Genotyping of kidney tissue showed that the sample from the low group contained 10% G7731A mtDNA, whereas the sample from the high group contained 62% G7731A mtDNA.

the kidney tissue of aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA (Fig. 4C). Therefore, the respiration defects in mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA (Fig. 4C) likely caused their renal failures (Fig. 4B).

3.3. Genotyping of G7731A mtDNA in tissues of mito-mice-tRNA^{Lys7731}

For the purposes of this study, we classified 4-week-old mito-mice-tRNA^{Lys7731} as having low or high amounts of mutated mtDNA by estimating the proportions of G7731A mtDNA isolated from samples of tail tissue. The question that then arises is whether the proportions of G7731A mtDNA differ among tissues or with age.

We estimated G7731A mtDNA proportions in various tissues of euthanized moribund mito-mice-tRNA^{Lys7731}. The results showed that, regardless of whether the mouse was characterized as having low or high amounts of mutated mtDNA, the proportion of G7731A mtDNA in the tail tissue did not differ substantially between samples obtained from the same mito-mouse-tRNA^{Lys7731} at 4 weeks compared with at 26–28 months after birth (Fig. S1). Moreover, G7731A mtDNA levels were similar among all tissue samples from the same individual mito-mice-tRNA^{Lys7731} (Fig. S1).

4. Discussion

Using mito-mice-tRNA^{Lys7731}, we showed that high levels of G7731A mtDNA and the resultant respiration defects did not affect median survival times (Fig. 2), even though they induced the late-onset disorders (Figs. 1, 3 and 4). In contrast, accumulation of pathogenic mutations in human mtDNA and the resultant respiration defects have been proposed to be responsible for features of normal aging as well as of mitochondrial diseases [1–3]. However, our current observations suggest that mtDNA with pathogenic mutations is not necessarily associated with aging processes. This idea is further supported by our previous findings that mito-mice-COI^{6589}, which contain mtDNA with a T6589C mutation in *COI*, and mito-mice-ND6^{13997}, which carry mtDNA with a G13997A mutation in *ND6*, had normal lifespans [11].

In contrast, our mito-mice-tRNA^{Lys7731} developed several phenotypes relevant to mitochondrial diseases, including anemia (Fig. 1C), lactic acidosis (Fig. 1D), and increased BUN (Fig. 1E). Moreover, euthanized moribund mito-mice-tRNA^{Lys7731} showed muscle atrophy (Fig. 3A) and renal failures (Fig. 4). The muscle atrophy in aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA (Fig. 3A) likely is associated with their muscle weakness (Fig. 1B). It is also likely that the anemic kidney tissue in the aged mito-mice-tRNA^{Lys7731} (Fig. 4A) reflects their low hematocrit levels (Fig. 1C). Moreover, the increased BUN values (Fig. 1E) likely reflect the renal failures (Fig. 4B) caused by high levels of G7731A mtDNA and the resultant respiration defects (Fig. 4C). Given that mito-mice-tRNA^{Lys7731} with low and high levels of G7731A mtDNA have the same B6 nuclear genetic background, the respiration defects induced exclusively by the high levels of G7731A mtDNA are responsible for the development of these late-onset disorders.

A question that then arises is why these abnormalities have a late onset in mito-mice-tRNA^{Lys7731}, even though aging did not affect the proportions of G7731A mtDNA in the tissues (Fig. S1). One explanation is the requirement of long-term exposure of the tissues to respiration defects or the requirement of age-dependent development of nuclear abnormalities for the onset of these disorders. It is also likely that each cell in a tissue eventually contains solely mtDNA either with or without the G7731A mutation as a consequence of stochastic segregation over time [24–26], resulting in the induction of the late-onset disorders due to the apoptosis or substantial dysfunction of cells containing only G7731A mtDNA.

In contrast to their other clinically characteristic features, aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA did not demonstrate peripheral hyperglycemia (Fig. 1F) indicative of mitochondrial diabetes; this disorder has been proposed to occur frequently in patients with mitochondrial diseases [1–3]. However, our results are consistent with our previous findings in mito-mice-COI^{6589}, which have normal blood glucose levels (9). In addition, blood glucose levels were low in mito-mice-Δ [27] but high in mito-mice-ND6^{13997} [11], indicating that high levels of pathogenic mtDNA mutations and the resultant respiration defects do not necessarily cause mitochondrial diabetes.

Current study also showed that all groups of our mice—even aged mito-mice-tRNA^{Lys7731} with high levels of G7731A mtDNA (that is, 70%–75%)—lacked RRFs (Fig. 3B). The absence of RRFs may be due in part to the fact that the amount of G7731A mtDNA was insufficient to induce RRFs. Thus, somehow mito-mice-tRNA^{Lys7731} with more than 75% G7731A mtDNA have to be generated for RRF development. However, the death of B6 mouse oocytes that carry levels of G7731A mtDNA in excess of 85% [12] would prevent the generation of mito-mice-tRNA^{Lys7731} with sufficient proportions of G7731A mtDNA to develop RRFs. It is likely that some as-yet unknown nuclear factors in mouse strains other than B6 may allow the survival of oocytes with more than 85% G7731A mtDNA.

To evaluate this idea, we plan to replace the B6 nuclear genetic background of mito-mice-tRNA^{Lys7731} with that from other strains. This replacement may support the survival of oocytes with more than 85% G7731A mtDNA and subsequently the development of skeletal muscle RRFs and peripheral hyperglycemia in the resultant mito-mice-tRNA^{Lys7731}.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.070.

Transparency document

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