Molecular characterization of two pedigrees with maternally inherited diabetes mellitus

Akira Miyamoto*, Ueda Tomotaka**, Kubo Takaaki†, Mori Kenichi‡ and Miyamoto Chimi

*Faculty of Rehabilitation, Kobe International University, Hyogo, Japan; †Faculty of Rehabilitation, Nishikyushu University, Saga, Japan; ‡Faculty of health science, Kumamoto Health Science University, Kumamoto, Japan; and **Department of Occupational Therapy, Faculty of Health Science, Aino University, Osaka, Japan

ABSTRACT
Mutations in mitochondrial DNA (mtDNA), especially in mitochondrial tRNA (mt-tRNAs) genes, play important roles in maternally inherited type 2 diabetes mellitus (T2DM), but the molecular mechanism remains unclear. In this study, two families with maternally transmitted T2DM are underwent clinical, genetic and molecular assessments. The mtDNA mutations are screened by direct sequencing. Furthermore, the phylogenetic conservation analysis and pathogenicity scoring system were used to evaluate the pathogenic status of mt-tRNA mutations. Interestingly, matrilineal relatives exhibit variable severity of DM, in particular, the age at onset of DM varies from 39 to 60 years, with an average of 50 years. Screening for the entire mitochondrial genomes identifies the existence of tRNA$^\text{Thr}$ A15901G and C15926T mutations, as well as 59 variants belonging to mtDNA haplogroups D2 and C4c. Notably, the m.A15901G mutation is located at D-arm of tRNA$^\text{Thr}$, whereas the m.C15926T mutation resides in the anticodon loop of tRNA$^\text{Thr}$, both of these positions are well conserved and critical for tRNA functions. Thus, the m.A15901G and m.C15926T mutations may impair mitochondrial translation and lead to mitochondrial dysfunctions. However, the fail to identify any other functional variants indicate that mitochondrial haplogroup may not play a role in T2DM. Hence, tRNA$^\text{Thr}$ A15901G and C15926T may be the novel mutations associated with T2DM.

Introduction
DM is a serious public health problem which remains a big challenging for physicians. Clinically, DM and its complications such as metabolic syndromes, cardiovascular diseases and foot problems contribute to its high mortality (Oguntibeju 2019; Lovic et al. 2020). In fact, T2DM is the most frequent type of DM worldwide (Magliano et al. 2019). Although great progress has been made toward T2DM, its pathogenicity is still elusive.

Recently, growing number of evidences indicate that T2DM is caused by genetic and environmental factors (Maassen et al. 2004, 2007). Some case-control studies implicate that T2DM could be maternally inherited (Avital et al. 2012; Fang et al. 2018), emphasizing the central roles of mitochondrial dysfunctions in this disease (Lin et al. 2021). Actually, mitochondria are found in most eukaryotic cells and involved in several biochemical processes, especially in generating ATP via oxidative phosphorylation (OXPHOS) (Wescott et al. 2019). Furthermore, as mtDNA lacks histones protection and is sensitive to oxidative stress, mtDNA has higher mutation rates than nuclear DNA (Madsen-Bouteur et al. 2010). There are several forms of T2DM-related mtDNA mutations: rearrangements such as large deletions (Ballinger et al. 1992) and point mutations in tRNAs or OXPHOS genes (Dabravolski et al. 2021). Among these, ND1 T3394C and T42116C, ND2 C5178A, ND3 A10398G, ND4L T10609C and C10676G, tRNA$^\text{Leu(UUR)}$ A3243G and T3264C, tRNA$^{Ile}$ T4291C, tRNA$^\text{Glu}$ T14709C and A14692G, tRNA$^\text{Gly}$ T10003C have been identified as pathogenic mutations affecting T2DM predisposition (van den Ouweland et al. 1992; Suzuki et al. 1997; Damore et al. 1999; Wilson et al. 2004; Tang et al. 2006; Liu et al. 2015; Wang et al. 2016; Destiarani et al. 2020; Lalarhoului et al. 2020; Jiang et al. 2021). However, current knowledge of molecular mechanisms by which these mutations contribute to the pathogenesis of T2DM is widely unclear.

To explore the roles of mtDNA mutations for T2DM, this study described two families with T2DM, through PCR-Sanger sequencing, two tRNA$^\text{Thr}$ mutations: m.A15901G and m.C15926T were identified. We noticed that all of these mutations occur at evolutionary conserved positions, which may have functional impact on tRNA$^\text{Thr}$ (Suzuki et al. 2011).

Materials and methods
Subjects and clinical assessments
Two families with DM (Family A and B) were ascertainment in the Department of Occupational Therapy, Faculty of Health...
The diagnosis of T2DM is based on the criteria proposed by American Diabetes Association (2010): Hemoglobin A1c (HbA1c) ≥6.5%; fasting plasma glucose (FPG) ≥126 mg/dl; an oral glucose tolerance test (OGTT) with a plasma glucose ≥200 mg/dl 2 hr after a 75-g glucose load.

Blood sample from each affected subject was collected in the morning between 7:00 AM and 10:00 AM after an overnight fast. The HbA1c level was measured by high-performance liquid chromatography (Bio-Rad, CA). While the serum FPG was determined by the regular laboratory methods (Beckman Coulter AU5800, Tokyo, Japan).

Mutational analysis of mitochondrial genomes
The genomic DNA of matrilineal relatives (Family A: II-5 and III-2; Family B: II-1, II-8 and III-4) were isolated from blood by using phenol-chloroform standard procedures (Lewin and Stewart-Haynes 1992). The individuals’ DNA fragments spanning the complete mtDNA genes were PCR amplified by 24 primers as described previously (Ding et al. 2020). After PCR amplification, the product was purified and sequenced by ABI PRISM 3100-Avant automated DNA sequencer using the BigDye Terminator Cycle reaction kit (version 1.1). The mtDNA mutations were screened by comparing the revised Cambridge reference sequence (rCRS, GenBank accession number: NC_012920.1) (Andrews et al. 1999).

Analysis of mt-tRNA secondary structure
MitotRNAdb, a database for analyzing mt-tRNA genes was employed to determine the stem and loop structure (Jühling et al. 2009). The interactions of tertiary structure for tRNA were based on the report by Suzuki et al. (2011).

Assigning the pathogenicity
Due to the high mutation rate, distinguishing pathogenic mtDNA mutations from polymorphisms remained challenging (Yarham et al. 2010). The adjusted scoring system was used to determine the pathogenicity of m.A15901G and m.C15926T mutations (Yarham et al. 2011). Notably, if the total scores of a mutation ≤6, it was classified as ‘neutral polymorphism,’ if the scores were 7 ~ 10, it belonged to ‘possibly pathogenic,’ if the scores ≥11, it was ‘definitely pathogenic.’

Results
Clinical presentations
Both the families had relatively high penetrances of T2DM (33.3% for Family A and 42.9% for Family B). In Family A, the
### Table 1. Summary of clinical and biochemical data for several members in two families with DM.

| Subjects | Gender | Age at onset (yrs) | Age at test (yrs) | Glucose (0-h) (mmol/L) | Glucose (2-h) (mmol/L) | HbA1c (%) |
|----------|--------|--------------------|------------------|------------------------|------------------------|-----------|
| Family A: II-5 | Female | 55 | 65 | 7.9 | 12.5 | 6.9 |
| Family A: III-2 | Male | 39 | 40 | 7.2 | 13.7 | 7.0 |
| Family B: II-1 | Male | 60 | 62 | 9.1 | 14.6 | 7.7 |
| Family B: II-8 | Female | 58 | 66 | 7.0 | 12.0 | 8.5 |
| Family B: III-4 | Male | 41 | 44 | 6.6 | 12.8 | 8.1 |
| Family B: II-7 | Male | / | 66 | 4.8 | 6.9 | 5.3 |

### Table 2. mtDNA mutations in two families with T2DM.

| Gene | Position | Mutation (amino acid changes) | Conservation (H/B/M/X) | Cl (%) | rCRS | Family A | Family B | Previously reported |
|------|----------|------------------------------|------------------------|--------|------|---------|---------|---------------------|
| D-loop | 73 | A to G | NA | A | G | G | Yes |
| 146 | T to C | NA | T | C | NA | Yes |
| 153 | A to G | NA | A | NA | G | Yes |
| 195 | T to C | NA | T | C | NA | Yes |
| 263 | A to G | NA | A | G | G | Yes |
| 310 | T to TC | NA | T | TC | NA | Yes |
| 489 | T to C | NA | T | NA | C | Yes |
| 514 | DelC | NA | C | DelC | NA | Yes |
| 1611 | C to T | NA | C | T | NA | Yes |
| 16129 | G to A | NA | G | A | A | Yes |
| 16185 | C to T | NA | C | NA | G | Yes |
| 16189 | T to C | NA | T | C | NA | Yes |
| 16223 | C to T | NA | C | T | T | Yes |
| 16319 | G to A | NA | G | A | A | Yes |
| 16362 | T to C | NA | T | C | NA | Yes |
| 16569 | T to C | NA | T | NA | C | Yes |
| 12S rRNA | 709 | G to A | G/G/A/- | 40.0 | G | A | NA | Yes |
| 750 | A to G | A/A/A/- | 97.78 | A | G | G | Yes |
| 1438 | A to G | A/A/A/G | 86.67 | A | G | G | Yes |
| 1598 | G to A | G/A/T/T | 15.56 | G | NA | A | Yes |
| 16S rRNA | 2626 | T to C | T/T/A/G | 68.89 | T | NA | C | Yes |
| 2706 | A to G | A/G/A/A | 84.44 | A | G | G | Yes |
| 3010 | G to A | G/G/A/A | 20.0 | G | A | NA | Yes |
| 3107 | DelN | NA | 2.22 | DelN | DelN | Yes |
| ND1 | 1611 | C to T (Ala to Val) | T/V/V/S | 15.56 | C | NA | T | Yes |
| 3970 | C to T | NA | 80.0 | C | T | T | Yes |
| 4216 | T to C (Tyr to His) | Y/Y/H/H | 24.44 | T | C | NA | Yes |
| ND2 | 4491 | G to A (Val to lie) | V/I/I/V | 4.44 | G | A | NA | Yes |
| 4883 | C to T | NA | 55.5 | C | NA | T | Yes |
| 5417 | G to A | NA | 64.44 | G | A | NA | Yes |
| ND | 6599 | A to G | NA | 100 | A | G | G | Yes |
| 7028 | C to T | NA | 100 | C | T | T | Yes |
| 7270 | T to C | NA | 15.56 | T | NA | C | Yes |
| ND2 | 7785 | T to C | NA | 62.78 | T | C | NA | Yes |
| 8020 | G to A | NA | 97.78 | G | NA | A | Yes |
| 9896 | A to G | NA | 24.44 | A | G | NA | Yes |
| A8 | 8414 | C to T (Leu to Phe) | NA | 31.11 | C | T | T | Yes |
| A6 | 8701 | A to G (Thr to Ala) | T/S/L/Q | 42.22 | A | G | G | Yes |
| 8860 | A to G (Thr to Ala) | T/A/A/T | 71.11 | A | G | G | Yes |
| CO3 | 9296 | C to T | NA | 100 | C | T | NA | Yes |
| 9540 | T to C | NA | 97.78 | T | NA | C | Yes |
| 9896 | A to G | NA | 24.44 | A | G | NA | Yes |
| ND3 | 10310 | G to A | NA | 66.67 | G | NA | A | Yes |
| 10398 | A to G (Thr to Ala) | T/T/T/A | 51.11 | A | G | NA | Yes |
| 10400 | C to T | NA | 51.11 | C | T | NA | Yes |
| ND4 | 10873 | T to C | NA | 20.0 | T | NA | C | Yes |
| 11719 | G to A | NA | 97.78 | G | A | A | Yes |
| ND5 | 12361 | A to G | NA | 13.33 | A | NA | G | Yes |
| 12705 | C to T | NA | 28.89 | C | T | T | Yes |
| 13708 | G to A | NA | 33.33 | G | NA | A | Yes |
| 13928 | G to C (Ser to Thr) | S/T/S/T | 11.11 | G | NA | C | Yes |
| ND6 | 14311 | T to C | NA | 24.44 | T | C | NA | Yes |
| 14569 | A to G | NA | 80.0 | A | NA | G | Yes |
| 14668 | C to T | NA | 24.44 | C | T | T | Yes |
| ND6 | 14766 | C to T (Thr to lie) | T/S/T/S | 48.89 | C | T | T | Yes |
| 14783 | T to C | NA | 17.78 | T | NA | C | Yes |
| 15043 | G to A | NA | 97.78 | G | A | A | Yes |
| 15301 | G to A | NA | 95.56 | G | NA | A | Yes |
| 15326 | A to G (Thr to Ala) | T/M/T/T | 17.78 | A | G | G | Yes |
| ND5 | 15901 | A to G | A/A/A/A | 100 | A | G | NA | Yes |
| 15926 | C to T | C/C/C/C | 100 | C | NA | T | Yes |

aConservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and Xenopus laevis (X).

bconservation index.

crevised Cambridge Reference Sequence.
dPlease see online mitochondrial database (www.mitomap.org).
proband (II-5) was a 65-year-old woman who went to Department of Occupational Therapy, Faculty of Health Science, Aino University for treatment of T2DM. As shown in Table 1, the HbA1c level, as well as the OGTT results strongly indicated that she was a diabetic carrier, further genetic counseling suggested that she developed T2DM 10 years ago, and the family history indicated a maternal inheritance. Moreover, we noticed that the family member (III-3) and the proband (II-5) had glucose intolerance.

In Family B, the proband (II-8) was a 66-years-old woman who went to the Department of Occupational Therapy, Faculty of Health Science, Aino University for regular treatment of DM. She suffered from DM when she was 58. Among 7 matrilineal relatives, 3 subjects developed DM. The age at onset of DM ranged from 41 to 60 years (mean age: 53). Moreover, these members showed no other clinical abnormalities, including cancer, hearing loss, neurological or infection disorders.

Screening for mitochondrial mutations

To explore the molecular basis of T2DM, we screened the mtDNA mutations of matrilineal relatives (Family A: II-5 and III-2; Family B: II-1, II-8 and III-4) by using PCR and sequencing. As shown in Table 2, compared with the rCRS, which allowed to be assigned to haplogroup D2 for Family A and haplogroup C4c for Family B (van Oven and Kayser 2009). Among these, there were 16 known variants in D-loop, four variants in 12S rRNA, four variants in 16S rRNA, two mutations in tRNA<sup>Thr</sup> (m.A15901G and m.C15926T), while others were localized in OXPHOS-genes (Owen and McCarthy 2007). Moreover, ten missense mutations were identified: ND1 C3497T (Ala to Val) and T4216C (Tyr to His), ND2 G4491A (Val to Ile), A8 C8414T (Leu to Phe), A6 A8701G (Thr to Ala) and A8860G (Thr to Ala), ND3 A10398G (Thr to Ala), ND5 G13928C (Ser to Thr), CytB C14766T (Thr to Ile) and A15326G (Thr to Ala). These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis and sequences from other 16 vertebrates, including mouse (Bibb et al. 1981), bovine (Gadaleta et al. 1989) and Xenopus laevis (Roe et al. 1985). We found that except for the m.A15901G and m.C15926T mutations (Figure 2), other variants were not well conserved. We also screened the presence of m.A15901G or m.C15926T mutation in 330 controls, as shown in Figure 3, after electrophoresis, the size of PCR product spanning the entire tRNA<sup>Thr</sup> gene was 1,126-bp. However, none of the healthy subjects harbored these mutations.

As shown in Figures 4 and 5, the homoplasmic m.A15901G mutation occurred at position 14 in the D-arm of tRNA<sup>Thr</sup>, which was extremely conserved from different species. Moreover, the m.C15926T mutation was located at position 41 in the anticodon stem of tRNA<sup>Thr</sup>. It was interesting to note that the m.C15926T mutation created a novel base-pairing (29 A-41T) and may result a failure in tRNA metabolism (Figure 5).

M.A15901G and m.C15926T mutations were ‘possibly pathogenic’ for T2DM

According to the revised pathogenicity scoring system (Yarham et al. 2011), the total scores of m.A15901G and m.C15926T mutations were both 10 points and belonged to ‘possibly pathogenic’ for T2DM (Table 3).

Discussion

In the present study, two families with maternally inherited T2DM were underwent clinical and molecular evaluations. T2DM was only presented in matrilineal lineage of these pedigrees, suggesting that mtDNA mutations were the molecular basis for this disorder. The age at onset of DM varied from 39 to 60 years, with an average of 50 years. The variable age at onset of DM in these pedigrees suggested that nuclear genes, environmental factors and epigenetic modifications may contribute to T2DM progression, as in the case of m.T3394C mutation (You et al. 2021).
Sequence analysis of the mitochondrial genomes in Family A and B revealed the presence of two tRNA<sup>Thr</sup> mutations, together with sets of polymorphisms belonging to mitochondrial haplogroup D2 and C4c, respectively (van Oven and Kayser 2009). In fact, the m.A15901G mutation resided at position 14 in the D-arm of tRNA<sup>Thr</sup>, which was conserved from various species. Furthermore, m.A15901G mutation occurred at 14 A-8T interaction, which was served as one of determinants for tRNA recognition by cognate aminoacyl-tRNA synthetases (Figuccia et al. 2021). Hence, this mutation may affect aminoacylation ability, as well as the steady-state level of tRNA<sup>Thr</sup> (Zheng et al. 2020). Notably, the m.A5814G which occurred at the same position of tRNA<sup>Cys</sup> had been regarded as a pathogenic mutation for mitochondrial encephalopathy (Santorelli et al. 1997; Sternberg et al. 1998). Importantly, biochemical analysis of the muscle derived from the patients carrying the m.A5814G mutation revealed significant reductions in the activities of complexes I and IV (Scuderi et al. 2007). Thus, it can be speculated that the m.A15901G, which was similar to the m.A5814G mutation, may also impair respiratory chain functions and lead to mitochondrial dysfunctions.

Furthermore, the homoplasmic m.C15926T mutation created a new base-pairing (29 A-41T) in the anticodon stem of tRNA<sup>Thr</sup>. Interestingly, the 3272delT which occurred at the same position of tRNA<sup>Leu(UUR)</sup> had been found to be associated with mitochondrial encephalomyopathy (Shoffner et al. 1995). In fact, nucleotide at position 41 of tRNAs was often modified, thereby contributing to the structural formation and stabilization of tRNAs (Ding et al. 2021). Therefore, the m.C15926T mutation may alter the tRNA<sup>Thr</sup> post-transcriptionally modification and lead to the failure in tRNA metabolism (Wong et al. 2020).

Heteroplasmic mtDNA mutation had been traditionally considered as an important factor for pathogenicity. However, there were some pathogenic mtDNA mutations that were in homoplasmic forms (McFarland et al. 2004; Jia et al. 2019; Zhao et al. 2019). These mutations were considered relatively mild and may require additional factors to produce a clinical phenotype (Cai et al. 2021; Cataldi et al. 2021; Dixon et al. 2021).

We believed that m.A15901G and m.C15926T were pathogenic mutations on the following lines of evidence: (1) these mutations presented only in DM patients but were absent in controls; (2) the A-to-G and C-to-T at positions 15901 and 15926 were extremely conserved from different species and predicted to affect the secondary structure of tRNA<sup>Thr</sup>; (3) these mutations segregated in the maternal lineage and healthy subjects.

Based on these observations, the molecular mechanisms underlying m.A15901G and m.C15926T mutations may be as follows: these mutations influenced the structure of tRNA<sup>Thr</sup> and caused the failure in tRNA metabolism, which may subsequently affect mitochondrial translation and respiratory chain functions (Wallace 2018). These events would lead to mitochondrial dysfunctions including the drop in ATP and mitochondrial membrane potential, increasing ROS.
generation, which will cause β-cell apoptosis and contributed to T2DM progression (Pinti et al. 2019).

In conclusion, this report suggested that m.A15901G and m.C15926T mutations were associated with DM. The limitation of current study was the relatively small sample size, further studies including more patients were needed to verify this conclusion.

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Authors contribution
MA and MC designed the study, UT and KT performed the clinical and molecular assessment, MK analyzed the data, MA and MC wrote the draft and revised it critically for intellectual content, MC approved the final version of this manuscript, all authors agreed to be accountable for all aspects of the work.

Disclosure statement
No potential conflict of interest was reported by the authors.

Data availability statement
The genome sequence data that support the findings of this study are openly available in GenBank of NCBI at (https://www.ncbi.nlm.nih.gov/) under the accession NC_012920.1.

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