

New Mitochondrial DNA Homoplasmic Mutations Associated With Japanese Patients With Type 2 Diabetes

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Epidemiological studies have shown that patients with type 2 diabetes are more likely to have affected mothers than fathers and that the disease is often transmitted in a mode of maternal inheritance (1,2). Because transmission of mitochondria is exclusively maternal (3), mitochondrial DNA (mtDNA) mutations have been implicated in the maternal inheritance of diabetes (4). However, mtDNA mutation at 3243 has been reported in only 1–1.5% of patients with type 2 diabetes (5,6). Therefore we postulated that other mtDNA mutations may be associated with type 2 diabetes. Recently, using polymerase chain reaction (PCR)-restriction fragment (RF)-single-strand conformation polymorphism (SSCP) analysis (7), we confirmed 56 mtDNA mutations in Japanese subjects (8). In this study, we investigated the prevalence of 5 mtDNA mutations, including 3 mutations that we reported (8) in Japanese subjects.

The study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects, and the study design was approved by the Ethical Committee of Yamanashi Medical University. Peripheral blood was obtained from 253 unrelated patients (mean age: 55.7 ± 1.8 years) with type 2 diabetes in the order of their visits to outpatient Clinic of Yamanashi Medical University Hospital. We also recruited 345 healthy control subjects (mean age: 43.6 ± 1.6 years) with no family history of diabetes and normal oral glucose tolerance or HbA_{1c}, who visited the Isawa Curehouse or Koseiren Health Center for medical checkups. Genomic DNA was obtained from peripheral leukocytes using a DNAQuick kit (Dainippon Pharmaceutical, Osaka, Japan).

mtDNA mutations were detected either by PCR-SSCP or PCR-restriction fragment length polymorphism (RFLP) analyses. Two primer pairs were used: primer pair A, for-

ward, 1279–1298, and reverse, 1510–1491; and primer pair B, forward, 11798–11817, and reverse, 12519–12500. The number of nucleotides was described based on the Cambridge Sequence (9). PCR was performed using a Takara PCR kit (Takara Shuzo, Kyoto, Japan) as previously reported (10), and the samples were subjected to SSCP (11) or RFLP analysis. mtDNA mutation at 3243 (12) and deletion of 10.4 kb (13) were detected as previously reported. For direct sequencing, the appropriate locations of the mtDNA were amplified by PCR using another set of primer pairs. The PCR products were recovered using a QIAEX II gel extraction kit (QIAGEN GmbH; Hilden, Germany) for template of the second asymmetric PCR. Sequencing was carried out with the dideoxy termination method (14) using a dsDNA sequencing kit (Life Technologies, Gaithersburg, MD).

PCR-SSCP analysis using primer pair A showed six patterns. Sequencing revealed that the six patterns comprised combinations of four different mutations: C to T at 1310, A to C at 1382, T to C at 1391, and A to G at 1438; two of these mutations have been previously observed (8). Digestion of PCR products using primer pair B by *HincII* (Takara Shuzo) revealed two patterns in RFLP analysis depending on the A-to-G mutation at 12026 (8). All the mutations were homoplasmic.

Fisher's exact test was applied for statistical analysis. As shown in Table 1, the prevalence of the C to T mutation at 1310 was 2.77% (7 of 253) in diabetic patients compared with 0.29% (1 of 345) in control subjects, which was a significant difference ($P = 0.0011$). The prevalence of A at 1438 was 2.37% (6 of 253) in diabetic patients compared with 0.29% (1 of 345) in control subjects, which was, again, statistically significant ($P = 0.0457$). In addition, the prevalence of the A-to-G mutation at 12026 was 3.95% (10 of 253) in diabetic patients compared with 0.87% (3 of 345) in control subjects, which was also a significant difference ($P = 0.0194$). All seven subjects, including one control subject, who had A at 1438 were also associated with the mutation at 12026. Four diabetic patients were associated with three mutations at 1310, 1438, and 12026, whereas no control subject was associated with those three mutations, which was also statistically significant ($P = 0.0319$). Ozawa et al. (15) also observed mtDNA mutations at 1310, 1438, and 12026 in a single individual. Therefore, we evaluated linkage disequilibrium in these three mutations. As shown in Table 1, we confirmed that the mutations at 1310, 1438, and 12026 are in linkage disequilibrium. The prevalence of any one of these three mutations was 5.14% (13 of 253) in diabetic patients compared with 1.16% (4 of 345) in control sub-

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mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; RF, restriction fragment; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

TABLE 1
Prevalence of mtDNA mutations in Japanese

| Nucleotide position | Location | NIDDM Patients (n = 253) | Control subjects (n = 345) | P value |
|-------------------------|------------|-----------------------------|-------------------------------|---------|
| 1310 C/T | 12S rRNA | 7 (2.77%) | 1 (0.29%) | 0.0011 |
| 1382 A/C | 12S rRNA | 17 (6.72%) | 30 (8.69%) | NS |
| 1391 T/C | 12S rRNA | 3 (1.19%) | 1 (0.29%) | NS |
| 1438 G/A | 12S rRNA | 6 (2.37%) | 1 (0.29%) | 0.0457 |
| 12026 A/G | ND 4 (I-V) | 10 (3.95%) | 3 (0.87%) | 0.0194 |
| 1438 and 12026 | | 6 (2.37%)* | 1 (0.29%) | 0.0457 |
| 1310 and 1438 and 12026 | | 4 (1.58%)* | 0 (0%) | 0.0319 |

The Cambridge Sequence (9) at 1438 is A. However, most of the Japanese (591/598) had G at this position. So 1438 A is a reverted mutation. A-to-G mutation at 12026 is a missense mutation from isoleucine to valine in NADH dehydrogenase 4. Fisher's exact test was applied to compare the prevalence between NIDDM patients and control subjects. Linkage disequilibrium was evaluated by χ^2 analysis. * $P < 0.0001$ for linkage disequilibrium.

jects, which was also statistically significant ($P = 0.0133$). Among those subjects with mutations at 1310, 1438, or 12026, no one showed a mutation at 3243 or a 10.4 kb deletion. Thus we think that these are new mtDNA mutations associated with Japanese diabetic patients. Although Thomas et al. (16) reported several mtDNA mutations, they did not observe a significant difference in the prevalence between diabetic patients and control subjects. Our study indicated that mtDNA mutations are more frequently associated with Japanese diabetic patients than has previously been reported (5).

The mutations at 1310, 1438, and 12026 are located in 12S rRNA and in NADH dehydrogenase 4 with an amino acid replacement from isoleucine to valine, respectively (10). Although we do not have functional data at present, we speculate that impairments of mitochondria due to these mutations may render the affected subjects easily susceptible to developing diabetes.

In conclusion, our study clarified that mtDNA mutations at 1310, 1438, and 12026 are new mutations associated with type 2 diabetic patients in Japanese. The prevalence of these mtDNA mutations needs to be examined in other countries or ethnic groups.

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