Brief Genetics Reports

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

Masato Tawata, Masayuki Ohtaka, Eiichi Iwase, Yukinobu Ikegishi, Kaoru Aida, and Toshimasa Onaya

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, forward, 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 *Hinc*II (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-

From the Third Department of Internal Medicine, Yamanashi Medical University, Tamaho, Yamanashi, Japan.

Address correspondence and reprint requests to Dr. Masato Tawata, Third Department of Internal Medicine, Yamanashi Medical University, Tamaho, Yamanashi 409-38, Japan. E-mail: tawatam@res.yamanashi-med.ac.jp.

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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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REFERENCES

1. Alcolado JC, Alcolado R: Importance of maternal history of non-insulin dependent diabetic patients. *BMJ* 302:1178–1180, 1991
2. Thomas F, Balkau B, Vauzelle-Kervroedan F, Papoz L, CODiAB-INSERM-Zeneca Study Group: Maternal effect and familial aggregation in NIDDM: the CODIAB Study. *Diabetes* 43:63–67, 1994
3. Giles RE, Blanc H, Cann HM, Wallace DC: Maternal inheritance of human mitochondrial DNA. *Proc Natl Acad Sci USA* 77:6715–6719, 1980
4. Gerbitz KD: Does the mitochondrial DNA play a role in the development of diabetes? *Diabetologia* 35:1181–1186, 1992
5. Katagiri H, Asano T, Ishihara H, Inukai K, Anai M, Yamanouchi T, Tsukuda K, Kikuchi M, Kitaoka H, Ohsawa N, Yazaki Y, Oka Y: Mitochondrial diabetes mellitus: prevalence and clinical characterization of diabetes due to mitochondrial tRNA$^{Leu(UUR)}$ gene mutation in Japanese patients. *Diabetologia* 37:504–510, 1994
6. van den Ouweland JM, Lemkes HH, Gerbitz KD, Maassen JA: Maternally inherited diabetes and deafness (MIDD): a distinct subtype of diabetes associated with a mitochondrial tRNA$^{Leu(UUR)}$ gene point mutation. *Muscle Nerve Suppl* 3:S124–S130, 1995
7. Tawata M, Iwase E, Aida K, Onaya T: A mass screening device of genome by polymerase chain reaction-restriction fragment-single-strand conformation polymorphism analysis. *Genet Anal Biomol Eng* 12:125–127, 1996
8. Tawata M, Ikegishi Y, Iwase E, Aida K, Onaya T: Mitochondrial DNA mutations in Japanese detected by polymerase chain reaction-restriction fragment-single-strand conformation polymorphism analysis. *Genet Anal Biomol Eng* 14:17–19, 1997
9. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG: Sequence and organization of the human mitochondrial genome. *Nature* 290:457–465, 1981
10. Tawata M, Kurihara A, Gan N, Iwase E, Ohtaka M, Inoue M, Onaya T: Variant forms of glucokinase gene in Japanese patients with late-onset type 2 diabetes. *Acta Diabetol* 31:238–241, 1994
11. Orita M, Suzuki Y, Sekiya T, Hayashi K: Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5:874–879, 1989
12. van den Ouweland JM, Lemkes HH, Ruitenbeek W, Sandkuijl LA, de Vijlder MF, Struyvenberg PA, van de Kamp JJ, Maassen JA: Mutation in mitochondrial tRNA$^{Leu(UUR)}$ gene in a large pedigree with maternally transmitted type 2 diabetes mellitus and deafness. *Nat Genet* 1:368–371, 1992
13. Ballinger SW, Shoffner JM, Hedaya EV, Trounce I, Polak MA, Koontz DA, Wallace DC: Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial deletion. *Nat Genet* 1:11–15, 1992
14. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 7:A5463–A5467, 1977
15. Ozawa T, Tanaka M, Ino H, Ohno K, Sano T, Wada Y, Yoneda M, Tanno Y, Miyatake T, Tanaka T, Itoyama S, Ikebe S, Hattori N, Mizuno Y: Distinct clustering of point mutations in mitochondrial DNA among patients with mitochondrial encephalomyopathies and with Parkinson's disease. *Biochem Biophys Res Commun* 176:938–946, 1991
16. Thomas AW, Edwards A, Sherratt EJ, Majid A, Gagg J, Alcolado JC: Molecular scanning of candidate mitochondrial tRNA genes in type 2 (non-insulin dependent) diabetes mellitus. *J Med Genet* 33:253–255, 1996