New sensitive method for the detection of the A3243G mutation of human mitochondrial deoxyribonucleic acid in diabetes mellitus patients by ligation-mediated polymerase chain reaction

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An adenine-to-guanine mutation at nucleotide position (np) 3243 in the mitochondrial tRNALeu(UUR) gene is closely associated with various clinical phenotypes of diabetes mellitus. Because the mutation creates a new restriction site for the restriction enzyme ApaI, the mutation is usually detected and quantified by ApaI cleavage of the PCR products including np 3243. The sensitivity of the conventional method is, however, 5–10% heteroplasmy. The percentage of heteroplasmy of the mutation is usually highest in the affected tissues and is much lower in peripheral blood cells, which are used most frequently for the analysis. The sensitivity of the conventional method, however, is not sufficient to detect the mutation from peripheral blood cells. Utilizing ligation-mediated polymerase chain reaction, we have developed a feasible and sensitive method to detect 0.01% heteroplasmy of the 3243 mutation in peripheral leukocytes.

Mitochondria have extranuclear genes, common to all vertebrates. The mitochondrial DNA (mtDNA)4 codes for 13 subunits of the mitochondrial respiratory chain, 22 tRNAs, and 2 rRNAs, all of which are essential for the proper function of the respiratory chain. To date, many mutations of mtDNA involved in mitochondrial disorders have been reported, and the number is still increasing. A cell contains 103–106 molecules of mtDNA. Wild-type and mutant mtDNA can co-exist in a single cell, which is known as heteroplasmy. Mitochondria are functionally unaffected until the percentage of mutant mtDNA exceeds a particular value (threshold). Therefore, affected tissues show the highest heteroplasmy, whereas other apparently healthy cells are very low in heteroplasmy.

Oxidative phosphorylation in mitochondria is considered to be important for the secretion of insulin from pancreatic beta cells. Consistent with this, diabetic symptoms are common features observed in various types of mitochondrial encephalomyopathies, including mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) (1, 2). An A-to-G mutation at nucleotide position (np) 3243 in the human mitochondrial tRNALeu(UUR) gene has been described in a subtype of MELAS (3). This subtype accounts for ~80% of MELAS occurrences. Among many mutations in mtDNA, the A3243G mutation is known to somatically accumulate with age. The accumulation of the mutation in pancreatic beta cells may cause adult-onset diabetes mellitus (DM). In fact, the A3243G mutation is found in patients with DM who were not previously diagnosed as MELAS (4). Although many of these patients exhibit a variety of neurological disorders, typically deafness, the mutation is often found in DM patients with few neuromuscular symptoms. Hence, the detection of the A3243G mutation and quantification of the heteroplasmy are required for definitive diagnosis (5).

The percentage of mtDNA with the mutation varies from tissue to tissue. The heteroplasmy of the mutation is

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4 Nonstandard abbreviations: mtDNA, mitochondrial DNA; MELAS, mitochondrial encephalomyopathy; np, nucleotide position; DM, diabetes mellitus; and LMPCR, ligation-mediated polymerase chain reaction.

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considered to be highest in the affected tissues, as described above. In this regard, the pancreas is the best source of tissue for the examination of the A3243G mutation in patients with diabetes (6). However, it is virtually impossible to include a pancreatic biopsy as a part of the routine screening. Unfortunately, the heteroplasmy is much lower in peripheral leukocytes, which incidentally are the most convenient sample source and which are used more frequently for the screening of the A3243G mutation than muscle or other affected tissues. The A3243G mutation creates a new restriction site for the restriction enzyme Apal. Surveys of the mutation by the conventional PCR method, in which the region including np 3243 is PCR-amplified, digested with Apal, and then stained with ethidium bromide, continue to be performed despite the fact that the method detects only 5–10% heteroplasmy (5). The prevalence of the A3243G mutation in DM patients is estimated to be 1–2% (2). Given that the A3243G mutation may be missed in some DM patients by this method and found 5 subjects carrying 0.01% heteroplasmy in only the DM patients. The prevalence of the A3243G mutation in DM patients remains to be clarified. To determine the true prevalence of the mutation in DM patients, more sensitive and specific methods are required.

In this study to estimate the prevalence of the mutation in patients with only DM, we have developed a new sensitive method for the detection of the 3243 mutation by taking advantage of ligation-mediated polymerase chain reaction (LMPCR). We could detect >0.01% heteroplasmy in peripheral leukocytes by this new method. We examined 233 DM patients and 136 apparently healthy control subjects by this method and found 5 subjects carrying >0.01% heteroplasmy in only the DM patients.

**Materials and Methods**

BamHI, Apal, and T4 DNA ligase were purchased from Takara. RNase A, Vent DNA polymerase, and AmpliTaq Gold™ were from Boehringer Mannheim, New England Biolabs, and Perkin-Elmer, respectively. SepaGene™ was from Sanko Junyaku. Other reagents were of analytical grade.

**Blood Donors and Cell Lines**

Blood was collected in tubes containing 31.3 g/L sodium citrate from randomly selected DM outpatients who visited the Kyushu University Hospital and from healthy donors who were mainly workers in the Kyushu University Hospital. All patients and healthy donors gave informed consent, according to the ethics rules of the Kyushu University Hospital. Two hybrid cell lines carrying 100% wild-type and 100% A3243G mutant mtDNA (2SA and 2SD, respectively) were made by the fusion of human rho0 206 cells that lacked mtDNA and enucleated fibroblasts derived from a patient with A3243G MELAS (8).

**Preparation of DNA**

Total DNA was extracted from peripheral leukocytes with SepaGene according to the manufacturer’s instructions. The DNA (~2 μg) was treated with 1.0 μg of RNase A and 20 U of BamHI in 100 μL of the reaction mixture (200 mmol/L Tris-HCl, pH 8.5, 10 mmol/L MgCl2, 1 mmol/L dithiothreitol, and 100 mmol/L KCl), extracted with phenol–chloroform (1:1, by volume), precipitated with ethanol, resolubilized in 20 μL of distilled water, and quantified by measuring the absorbance at 260 nm. One microgram of the BamHI-digested DNA was cleaved with 12 U of Apal in 10 μL of the reaction mixture for 1 h at 37 °C. The mixture was then diluted twofold to 50 mg/L (50 ng/μL) with distilled water and stored at −20 °C until use.

**LMPCR**

A unidirectional linker was prepared by hybridizing LMPR1 (5’-gcggtgaccgggagatctgtattc-3’) and LMPR2 (5’-gaatatgac-3’). LMPCR was performed essentially as described previously (9–13).

**Primer extension.** The first strand synthesis reaction mixture (60 μL) consisted of 40 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.9, 5 mmol/L MgSO4, 100 mg/L gelatin, 0.3 pmol of primer 1, 0.2 mmol/L each dNTP, 0.5 units of Vent DNA polymerase, 0.6 pmol of RL3534 (np 3534–3553 of the H strand for the 3243 site), 0.6 pmol of L4127 (np 4127–4146 of the L strand for the 4427 site), and 0.5 μg of DNA. The DNA was denatured at 95 °C for 5 min, and the primer was annealed at 60 °C for 30 min, after which the polymerase reaction was performed at 76 °C for 10 min.

**Ligation.** After cooling on ice, 40 μL of the primer extension mixture was mixed with 60 μL of the ligation mix to yield 3.33 mg/L (3.33 ng/μL) of DNA. The reaction was performed at 16 °C for >6 h. Sixty microliters of the ligation mix consisted of 50 mmol/L Tris-HCl, pH 7.5, 13.3 mmol/L MgCl2, 33.3 mmol/L dithiothreitol, 8.5 g/L bovine serum albumin, 1.6 mmol/L ATP, 100 pmol of the unidirectional linker, and 3 Weiss units of T4 DNA ligase.

**PCR amplification.** After the ligation reaction, the reaction mixture was diluted 10-fold with distilled water for amplification of the 3243 site, 105-fold for amplification of the 4427 site for the 0.1% control, and 106-fold for amplification of the 4427 site for the 0.01% control, respectively. A 3-μL aliquot of each diluted sample was used in 25 μL of the PCR mixture, yielding 1.0 ng, 1.0 pg, and 0.1 pg DNA for the 3243 site, the 0.1% control, and the 0.01% control, respectively. The PCR mixture consisted of 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L MgCl2, 10 mg/L gelatin, 50 mL/L dimethyl sulfoxide, 2 mmol/L each dNTP, 1.25 U of AmpliTaq Gold, 5 pmol of the specific primer (RL3516, covering np 3516–3539 of the H strand for the 3243 site, or L4146, covering np 4146–4165 of the L strand for the 4427 site), and 5 pmol of the
linker primer (3243LMPR1 for the 3243 site or LMPR1 for the 4427 site). The linker primer 3243LMPR1 has four nucleotides overlapping the sequence of the 3243 site at its 3’ side for increasing the specificity (5’-tgacccgggagatctg-3’).

The DNA was initially denatured at 94 °C for 10 min and subjected to 38 PCR cycles of 94 °C for 30 s, 69 °C for 30 s, and 72 °C for 30 s. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

Results

PRINCIPLE OF THE HIGHLY SENSITIVE DETECTION

In the conventional PCR method, both the wild-type and the mutation-carrying chains are amplified, and then the PCR products are digested with Apal. The large amount of uncut DNA with the wild-type sequence interferes with the detection of the cleaved DNA on agarose gel electrophoresis, particularly in the case of the low heteroplasmy. To overcome this limitation and to improve the sensitivity, we needed to selectively amplify only the DNA strands harboring the A3243G mutation. For this purpose, the total DNA extracted from peripheral leukocytes was digested with Apal before amplification. Then a primer extension reaction was performed toward the free 5’ end of the DNA strand cleaved at the np 3243 site, using primer 1 (Fig. 1). The unidirectional linker was ligated to the resulting blunt end. PCR amplification was then carried out using the nested primer 2 and the linker. Thus, we could specifically amplify the Apal-cleaved DNA, i.e., DNA carrying the 3243 mutation. In wild-type mtDNA, an authentic restriction site for Apal exists at np 4427. This site is also amplified by LMPCR in parallel as a control of the copy number of mtDNA. By using this 4427 site as a standard, we can correct for the difference in the Apal digestion efficiency between preparations.

PCR EFFICIENCY OF 3243 AND 4427 SITES

Measuring an absolute amount of template DNA by PCR is laborious and time-consuming. In contrast, comparing the quantity of DNA in two samples by PCR is much easier and more reliable. To estimate the degree of heteroplasmy of the 3243 mutation, 1000-fold more of the Apal-digested DNA was used to amplify the DNA strands cleaved at np 3243 than was used to amplify the DNA strands cleaved at 4427. If the efficiency of PCR for the 3243 site is the same as that for the 4427 site, we can then ascertain whether the heteroplasmy is less than or greater than 0.1% by simply comparing the amount of the two PCR products.

To examine PCR efficiency, we used two hybrid cell lines that have the same nuclear background, 2SA and 2SD, harboring 100% wild-type mtDNA and 100% A3243G mutation, respectively. The mitochondrial genotypes of the two cell lines were confirmed as follows. When the region including np 3243 was amplified, the PCR product of the 2SD was completely digested with Apal (Fig. 2, lanes 1 and 2). In contrast, the PCR product of the 2SA cells was completely resistant to Apal digestion (Fig. 2, lanes 3 and 4).

We first performed the LMPCR amplification of the 3243 and 4427 sites, using the Apal-digested total DNA of the 2SD cells. Both the 3243 and 4427 sites of the DNA should be cleaved with Apal to the same extent because
the DNA has 100% mutation at np 3243. The signal intensity of the 3243 LMPCR product was essentially the same as that of the 4427 LMPCR product (Fig. 3A), indicating that the 3243 site was amplified as efficiently as the 4427 site.

We then examined whether the existence of 1000-fold more DNA interferes with the LMPCR amplification of the 3243 site. For that purpose, we performed the LMPCR amplification of the 3243 site using 1.0 pg of the total DNA of the 2SD cells in the absence or presence of 1.0 ng of the total DNA of the 2SA cells. The amount of LMPCR product was not affected by the presence of 1000-fold more DNA (Fig. 3B). From the results shown in Fig. 3, we concluded that it was safe to amplify the 3243 site in the presence of excess DNA to estimate the heteroplasmy.

**CLASSIFICATION OF LMPCR AMPLIFICATION OF THE 3243 SITE**

In practice, we used 1.0 ng of the *Apa*I-digested total DNA extracted from peripheral leukocytes for the amplification of the 3243 site. For the amplification of the 4427 site, we used 1.0 and 0.1 pg of the DNA as controls for 0.1% and 0.01% heteroplasm, respectively. We classified the results into four patterns. The first pattern was that the PCR product of the 3243 site was apparently invisible (Fig. 4, lane 1). The second was that the 3243 signal was weaker than the 4427 signal for the 0.01% heteroplasm control (Fig. 4, lane 4). The third pattern was that the 3243 signal was weaker than the 4427 signal for the 0.1% heteroplasm control (Fig. 4, lane 7). The fourth pattern was that the 3243 signal was stronger than the 4427 signal for the 0.1% heteroplasm control (Fig. 4, lane 10). The relative relationship between the 3243 and 4427 signals was determined with samples taken at 36–40 PCR cycles (results not shown), indicating that the amplification of LMPCR was not saturated (that is, had not reached plateau), and therefore, the comparison between the 3243 and 4427 signals was reliable and valid.

**SCREENING OF THE 3243 MUTATION**

We randomly selected 233 patients with DM who visited the Kyushu University Hospital. For the healthy controls, blood was collected from 136 healthy volunteers. The age distribution of each group was closely matched, as shown in Table 1.

We found two patients who carried >0.1% heteroplasmy and three patients who carried >0.01% hetero-

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**Fig. 3.** The efficiency of LMPCR amplification for the 3243 site is the same as that for the 4427 site.

(A) The 3243 (lane 1) and 4427 (lane 2) sites were amplified by LMPCR as described in Materials and Methods, using 1.0 pg of the ApaI-digested DNA prepared from 2SD cells. (B) The 3243 site was amplified by LMPCR using 1.0 pg of the 2SD ApaI-digested DNA without (lane 1) or with (lane 2) 1.0 ng of the 2SA ApaI-digested DNA.

**Fig. 4.** The four patterns of LMPCR amplification for the 3243 site.

The 3243 site was amplified using 1.0 ng of the *Apa*I-digested DNA (lanes 1, 4, 7, and 10). The 4427 site was amplified as 0.1% (lanes 2, 5, 8, and 11) and 0.01% controls (lanes 3, 6, 9, and 12), using 1.0 and 0.1 pg of the *Apa*I-digested DNA, respectively.
Table 1. Age distribution of the subjects.

<table>
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<th>Age</th>
<th>DM patients</th>
<th>Healthy controls</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>≤20</td>
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<tr>
<td>21–30</td>
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<td>6</td>
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<td>51–60</td>
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<td>46</td>
<td>40</td>
</tr>
<tr>
<td>71–80</td>
<td>17</td>
<td>31</td>
</tr>
<tr>
<td>≥81</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>127</td>
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</table>

Table 2. Screening of the A3243G mutation.

<table>
<thead>
<tr>
<th>Heteroplasmy, %</th>
<th>DM patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND</td>
<td>73 (32.0)</td>
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<tr>
<td>≤0.01</td>
<td>156 (68.0)</td>
<td>93 (68.4)</td>
</tr>
<tr>
<td>0.01–0.1</td>
<td>3 (1.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>≥0.1</td>
<td>2 (0.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>233</td>
<td>136</td>
</tr>
</tbody>
</table>

* The A3243G mutation was examined by LMPCR. The values in parentheses represent the percentage of subjects.

In control from sample to sample or from day to day must be repeatable and specific. Otherwise, a false amplification could occur. In this regard, allele-specific amplification may be suited for research but not for the diagnostic clinical laboratory. In addition, we are unable to obtain information about the extent of the heteroplasmy by allele-specific amplification.

The LMPCR method in this study detects the 3243 mutation signal weaker than the 0.01% control (Fig. 4). However, we found the 3243 signal weaker than the 0.01% control in ~70% of both healthy subjects and DM patients (Table 2). At present, we are not aware whether many people in fact carry a very low percentage of the 3243 mutation or whether the <0.01% heteroplasmy detected here is simply an artifactual event, which, as such, must be taken into account particularly in the case of highly sensitive PCR detection (15, 16). In any case, the <0.01% heteroplasmy detected by this LMPCR method should be considered diagnostically negative from the standpoint that a large segment of healthy control subjects show such weak signals. We did not, however, find any healthy control subjects carrying >0.01% heteroplasmy (Table 2), supporting the assumption that subjects harboring >0.1% heteroplasmy (i.e., 10-fold more than 0.01%) can be regarded as positive for the mutation. We tentatively regarded heteroplasmy between 0.01% and 0.1% as a borderline region. We need to examine more subjects and perform other confirming studies, such as muscle biopsy, to determine the importance of this borderline region.

Although semiquantitative, the estimation of heteroplasmy by the LMPCR method in this study should be reliable for the following reasons: (a) this method overcomes the problem associated with heteroduplex formation (5) because we performed PCR after ApaI-digestion, and (b) the authentic ApaI restriction site is used as an internal standard, which corrects for the differences in digestion efficiency between samples. In this study, we found 5 of 233 patients carrying >0.01% heteroplasmy (2 carrying >0.1% and 3 carrying >0.01%). Even if all five patients are DM patients with the 3243 mutation, the prevalence is 2.1%. This value is within the range reported previously (2). A larger study of known DM patients is needed to establish the prevalence of this mutation to DM.

In conclusion, we have developed a more sensitive method for the quantitative detection of the mitochondrial A3243G mutation. This method should be useful for the screening of the mutation, using peripheral blood cells.

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8. Yoneda M, Miyatake T, Attardi G. Complementation of mutant and wild-type human mitochondrial DNAs coexisting since the mutation event and lack of complementation of DNAs introduced separately into a cell within distinct organelles. Mol Cell Biol 1994;14:2699–712.