Defining the Importance of Mitochondrial Gene Defects in Maternally Inherited Diabetes by Sequencing the Entire Mitochondrial Genome

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For any mitochondrial DNA (mtDNA) mutation, the ratio of mutant to wild-type mtDNA (% heteroplasmy) varies across tissues, with low levels in leukocytes and high levels in postmitotic tissues (e.g., skeletal muscle). Direct sequencing is the gold-standard method used to detect novel mutations, but can only reliably detect % heteroplasmy >25%, which is rare in leukocytes. Therefore, we investigated the role of mtDNA defects in maternally inherited diabetes by first screening for the A3243G tRNALeu(UUR) mutation by restriction assay, followed by sequencing of the entire mitochondrial genome using skeletal muscle derived mtDNA. A total of 28 patients had maternally inherited diabetes either alone (group 1, n = 17) or with one or more additional features of mitochondrial disease, including bilateral sensori-neural deafness and neuromuscular disease (group 2, n = 11). Three patients (all from group 2) carried the A3243G mutation. Skeletal muscle mtDNA from eight group 1 patients and six more group 2 patients was sequenced. No pathogenic mutations were found in the group 1 patients, while two patients from group 2 had mutations at positions 12258 and 14709 in the tRNA serine and glutamic acid genes, respectively. We conclude, therefore, that screening for mtDNA mutations should be considered in patients with maternally inherited diabetes, but only when additional features of mitochondrial disease are present. Diabetes 51:2317–2320, 2002

It is well recognized that in some families diabetes follows a maternal inheritance pattern, and that there is an excess of maternal transmission in type 2 diabetes. As mitochondrial DNA (mtDNA) is passed exclusively down the maternal line, it was postulated that mtDNA defects might contribute to the excess maternal transmission. This was subsequently confirmed, and a number of mtDNA defects have been implicated in the development of diabetes, as recently reviewed (1–3). An A to G substitution at position A3243G in the tRNALeu(UUR) gene is the most commonly reported defect associated with diabetes. This mutation was first described in relation to the neuromuscular metabolic MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes) in which diabetes is a common feature, and was more recently recognized as a cause of the diabetic subtype of maternally inherited diabetes and deafness (MIDD) (1). This is characterized by bilateral sensori-neural deafness, and diabetes that tends to develop during middle age and frequently progresses to insulin therapy. However, autoimmune markers of type 1 diabetes are nearly always negative. MIDD therefore shares some of the clinical features of both type 1 and type 2 diabetes.

The prevalence of the A3243G mutation in diabetes has been comparatively easy to ascertain and has been shown to account for ~1–2% of cases in the general diabetic population (3). However, a much more important question is what is the prevalence of all forms of mtDNA defects in the diabetic population? This has been difficult to address because of several problems related to the complexity of mtDNA biology. First, pathogenic mutations can occur at almost any site throughout the mitochondrial genome, and hence comprehensive screening requires analysis of the entire mtDNA molecule. Second, nonfunctional homoplasmic variants are common and must be distinguished from functional heteroplasmic defects. Third, mutations may be missed because of variable tissue expression. This is because the level of the mutated mtDNA in relation to the wild-type mtDNA (% heteroplasmy) varies between tissues, being high in postmitotic tissues, such as skeletal muscle and brain, and low in rapidly dividing tissues, such as blood leukocytes. As blood leukocytes are the usual

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% heteroplasmy, ratio of mutant to wild-type mtDNA; ICA, islet cell antibody; MIDD, maternally inherited diabetes and deafness; mtDNA, mitochondrial DNA; SSCP, single-strand conformational polymorphism.
source of DNA for study, this has important implications for mtDNA mutation detection. There is usually no problem detecting known mutations such as the A3243G mutation, as restriction assays can detect levels of heteroplasmy down to ~1%. However, the situation is different for the detection of novel mutations, as the methods are not as sensitive. Direct sequencing is the gold-standard method for mutation detection, but this can only reliably detect levels of heteroplasmy >25%. Therefore, a novel mtDNA mutation could be present at levels of anything up to 25% in leukocyte DNA and go undetected. As a consequence, this would lead to an underestimation of the true prevalence of mtDNA defects in conditions such as diabetes. The solution to this problem is either to develop more sensitive methods to detect novel mutations or to screen a postmitotic tissue in which the levels of mutation will be much higher and therefore amenable to detection by direct sequencing. In this study, we pursued the second approach to define the role of mtDNA defects in maternally inherited diabetes. The specific aim of the study was to search for novel mtDNA mutations in patients with evidence of maternally inherited diabetes, and to see if there were differences between patients with diabetes alone and those with additional features of mitochondrial disease.

A questionnaire was used to screen patients attending the Newcastle Diabetes and Audiometry Services, and 28 of 595 patients were identified with clear evidence of maternal transmission of diabetes. This was defined as transmission through three consecutive generations or through two consecutive generations with at least three affected individuals. In addition, there had to be no evidence of paternal transmission of diabetes. Patients were subdivided into those with maternally transmitted diabetes and no other features of mitochondrial disease (group 1, n = 17) and those with maternally inherited diabetes plus one or more features of mitochondrial disease (group 2, n = 11); these features were defined as bilateral sensori-neural deafness, myopathy, cerebellar ataxia, non-diabetic retinopathy, premature cataracts, stroke-like episodes before the age of 50 years, and encephalopathy. Subjects were first screened by restriction digest assay for the A3243G mutation, and three patients from group 2 were identified with the mutation and excluded from further study. A further 11 patients were either excluded because of contraindications to needle muscle biopsy (n = 7) or declined to have the procedure (n = 4). Of the remaining 14 patients, 8 from group 1 and 6 from group 2 underwent needle muscle biopsy. Skeletal muscle samples were prepared for histology and mtDNA analysis. The entire mitochondrial genome, including the D-loop, was sequenced using 28 overlapping primer pairs (see Table A1: online appendix at http://diabetes.diabetesjournals.org).

Table 1 summarizes the clinical and molecular findings for the group 1 patients who underwent screening of the entire mitochondrial genome. All patients had developed diabetes in adulthood; all but one was insulin treated, but all patients were islet cell antibody (ICA) negative. While these features are consistent with mitochondrial-related diabetes, sequencing of the entire mitochondrial genome in each patient did not reveal any pathogenic point mutations. The common deletion was detected in muscle mtDNA from three patients, but at very low levels and compatible with age-related changes.

Table 2 summarizes the clinical and molecular findings for the group 2 patients. Like the group 1 patients, those in group 2 displayed many of the clinical features associated
with mitochondrial-related diabetes, except one of the six patients was ICA positive. None of the patients carried the common deletion or major rearrangements. However, we did identify pathogenic point mutations in two patients from group 2. The first functional mutation was a C to A substitution at position 12258 in a tRNA serine gene. This mutation has been previously reported (4,11), and was associated with maternally inherited diabetes, sensorineural deafness, cataracts, and cerebellar ataxia. The second mutation was the previously reported T to C substitution at position 14709 in the tRNA glutamic acid gene (5). As observed in our pedigree, previous reports have linked the mutation with the development of diabetes and myopathy (5,6,12). However, in an Italian pedigree, the clinical expression was heterogeneous with some affected individuals showing just the MIDD phenotype (6).

This is the first systematic search for novel inherited mtDNA mutations in maternally transmitted diabetes using mtDNA derived from postmitotic tissue. As mentioned above, an alternative approach would be to develop a more sensitive system for mutation detection that could be used to screen peripheral leukocyte DNA samples. Such a system has been reported based upon single-strand conformational polymorphism (SSCP) analysis (7), but the sensitivity of SSCP for mutation detection ranges between 75 and 97%, even under optimal conditions (8). The problem is further compounded by the fact that some mtDNA mutations may simply not be expressed in leukocyte DNA. For example, we investigated a patient with a mitochondrial point mutation (A to G at position 12320) that was present at high levels in muscle but was undetectable in leukocyte DNA by the gold-standard method of direct sequencing of the mitochondrial genome. However, it is worth noting that the same comprehensive search failed to identify pathogenic mtDNA defects in a further four group 2 patients with the MIDD phenotype (Table 2).

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Diabetes</th>
<th>Type and duration (years)</th>
<th>Current diabetes therapy</th>
<th>Age deafness (years)</th>
<th>Criteria for recruitment</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td>53</td>
<td>M</td>
<td>Diabetes</td>
<td>37</td>
<td>Insulin (7)</td>
<td>32</td>
<td>Diabetes in 2 generations, BND in 1 generation</td>
</tr>
<tr>
<td>10</td>
<td>51</td>
<td>M</td>
<td>Diabetes</td>
<td>38</td>
<td>Insulin (5)</td>
<td>46</td>
<td>Diabetes in 2 generations, BND in 2 generations</td>
</tr>
<tr>
<td>11</td>
<td>65</td>
<td>M</td>
<td>Diabetes</td>
<td>35</td>
<td>Insulin (14)</td>
<td>44</td>
<td>Diabetes in 1 generation, BND in 2 generations</td>
</tr>
<tr>
<td>12</td>
<td>54</td>
<td>M</td>
<td>Diabetes</td>
<td>22</td>
<td>Insulin (17)</td>
<td>38</td>
<td>Diabetes in 3 generations, BND in 1 generation</td>
</tr>
<tr>
<td>13</td>
<td>68</td>
<td>F</td>
<td>Diabetes</td>
<td>45</td>
<td>No deafness</td>
<td>39</td>
<td>Diabetes in 2 generations, Myopathy in 3 generations</td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td>F</td>
<td>Diabetes</td>
<td>60</td>
<td>OHA (1)</td>
<td>39</td>
<td>Diabetes, CA, BND and cataracts in 3 generations</td>
</tr>
</tbody>
</table>

CA = cerebellar ataxia; BND = bilateral nerve deafness; OHA = oral hypoglycaemic agent; T14709C = mutation in tRNA glutamic acid gene; C12258A = mutation in tRNA serine gene; ND = not detected; N/A = not available.
common condition and can arise from a large number of autosomal-inherited gene defects (10). It is quite possible, therefore, that these four patients with the MIDD phenotype were from pedigrees that carried separate defects, one causing maternally inherited diabetes and the other deafness.

In conclusion, we believe that these observations and those of previous studies allow us to provide the following guidance for clinical practice. First, based on our own observations, there is really no case to search for inherited mtDNA defects in pedigrees with maternally inherited diabetes alone. Conversely, we recommend that patients with maternally inherited diabetes plus one or more additional features of mitochondrial disease should be referred to a specialist center as they are likely to need more extensive investigation for mtDNA defects.

RESEARCH DESIGN AND METHODS

Subjects. Patients were recruited from the Newcastle Diabetes and Newcastle Audiology Services. A questionnaire was sent to patients requesting information about personal and family history of diabetes and features of mitochondrial disease, as described in the text. From a total of 1,045 mailed questionnaires, 595 replies were received, and patients with evidence of maternal transmission of diabetes were identified. From this initial screen, 28 patients agreed to undergo further clinical assessment. Between 100 and 150 mg tissue was obtained by quadriceps needle biopsy. Exclusion criteria for muscle biopsy included anticoagulant therapy, extensive peripheral vascular disease and/or neuropathy, and marked obesity. The presence or absence of sensori-neural deafness independent of age was assessed by pure tone audiometry. The study was approved by the Newcastle and North Tyneside Joint Ethics Committee.

Methods. Frozen sections of muscle were cut at 8 μm and stained for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activity, as previously described (4). The remainder of the muscle sample was used for molecular analysis. The mitochondrial genome, including the noncoding D-loop, was amplified using 28 overlapping primer pairs using the PCR (primers listed in Table A1; online appendix at http://diabetes.diabetesjournals.org). The PCR reaction (50 μl) was set up as follows: 1.5 μl of each primer pair (20 μmol/l), 1 μl DNA (200 ng/μl), 5 μl 10 × PCR buffer (500 mmol/l KCl, 100 mmol/l Tris-HCl, pH 8.3, 15 mmol/l MgCl₂, and 0.01% (wt/vol) gelatin), and 0.25 μl AmpliTaq Gold DNA Polymerase (5 units/μl; Applied Biosystems). The reaction conditions were one cycle at 94°C for 12 min, followed by 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 8 min. The amplified fragments were column purified using the QIA Quick PCR Purification Kit (Qiagen Ltd, Crawley, U.K.) and then sequenced using a Big Dye Terminator cycle sequencing kit on a ABI Prism 377 DNA sequencer (Applied Biosystems). The sequence data were analyzed using the software packages Factura and Sequence Navigator (Applied Biosystems), and were compared with the Cambridge sequence and MITOMAP (MITOMAP 1999).

Long PCR was used to detect large scale deletions. This used the Expand Long Template PCR System (Roche Diagnostics, Lewes, U.K.), and products were run out on a 1% agarose gel. Southern Blot analysis was carried out to detect rearrangements. DNA (3 μg) was added to 2 μl 10 × NEBuffer (New England Biolabs) and 2 μl PfuUl (10 units/ml; New England Biolabs), and was made up to 20 μl with dH₂O. The DNA was digested for 1.5 h at 37°C using PfuUl. Rearrangements are detected by the generation of different length fragments. After digestion, the sample was run out on a 0.8% agarose gel, transferred onto a nitrocellulose membrane, and probed using a labeled probe against the O₁ region (nucleotide position 15782–1289).

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REFERENCES


