Brief Genetics Report

Mitochondrial Diabetes

Investigation and Identification of a Novel Mutation

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Defects of mitochondrial DNA (mtDNA) have recently been recognized as a cause of diabetes (1,2). The mitochondrial genome is the only extrachromosomal DNA in human cells. It encodes for 13 essential proteins of the mitochondrial respiratory chain and the RNAs necessary for intramitochondrial protein synthesis. Both mtDNA point mutations and rearrangements have been identified in families with diabetes (1,2). The most commonly described mutation is the point mutation A3243G in the tRNA$^{Leu(UUR)}$ gene, and the prevalence of this mutation may be as high as 1–1.5% in certain diabetic populations (3).

The true incidence of mtDNA defects in diabetes is unknown, and a major factor limiting the identification of patients is the complexity of mitochondrial genetics. The mitochondrial genome is strictly maternally inherited, and there are multiple copies of mtDNA in an individual mitochondrion and potentially several thousand copies in an individual cell. In the majority of patients, there is a mixture of both mutated and wild-type mtDNA, a situation termed heteroplasmy. The proportion of mutated to wild-type mtDNA (% heteroplasmy) can vary markedly between tissues with high levels in postmitotic tissues, such as muscle and β-cells, and those with very low levels in rapidly dividing tissues, such as blood. This has implications for the detection of novel mutations, since sequencing can only reliably detect mutations at a high level of heteroplasmy, i.e., >40%.

In the search for novel mutations, it is important to investigate a tissue that is likely to have high levels of mutant mtDNA. Therefore, in appropriate patients, our strategy is to screen the complete mitochondrial genome by DNA sequencing from skeletal muscle.

Following from a previous study of the incidence of the A3243G tRNA$^{Leu(UUR)}$ mutation (4), we gave a questionnaire to all our patients about a possible maternal family history of diabetes and/or neuromuscular disease. Our studies have identified a number of families with clear maternal inheritance of diabetes who do not have the A3243G mutation or the new mutation described in this report. Here we present the detailed molecular studies of one of these families, in which we have identified a new tRNA mutation. In the family, there is extensive maternal transmission of cerebellar ataxia, cataracts, and diabetes. The index case is a 61-year-old woman who developed cataracts at 30 years, deafness at 39 years, and cerebellar ataxia at 57 years of age. Diabetes was diagnosed at 60 years and is currently treated with gliclazide 160 mg b.i.d. She is lean, with a BMI of 18 kg/m^2. Her daughter is 30 years old and developed deafness and cataracts in her early 20s. She underwent a 75-g oral glucose tolerance test and was found to have impaired glucose tolerance (2-h plasma glucose 10 mmol/l). Acute insulin response (Δ 30–0 min insulin/Δ 30–0 min glucose [5]) was low (5 mU/mmol; reference range for normal subjects 5–120), while basal insulin sensitivity (6) was normal (homeostasis model assessment [HOMA] index = 0.9; reference value for normal insulin sensitivity = 1.0).

Muscle biopsies were frozen in isopentane cooled to –150°C with liquid nitrogen. Sections were cut routinely at 8 μm (for measurement of cytochrome c oxidase [COX] activity and succinate dehydrogenase [SDH] activity) and 30 μm sections for single fiber polymerase chain reaction (PCR) studies. The entire mitochondrial genome was amplified using 25 overlapping oligonucleotide primer pairs that incorporated M13 tails. The PCR products were column purified (Qiagen PCR purification columns; Qiagen, Crawley, U.K.). The PCR products were sequenced by dye primer cycle sequencing using an ABI 373 Sequencer (Perkin-Elmer, Warrington, U.K.). Analysis was performed using the software programs Factura and Sequence Navigator (Perkin-Elmer).

A mismatch PCR was designed to screen for the novel C12258A mutation, creating a restriction enzyme site for *Cla*I in mutant DNA only. The forward mismatch primer was 12235–12257 TGCCCCCATGTCTAACAACATCG (the mismatch nucleotide is underlined), and the reverse primer was 12361–12341 TGGTTATAGTAGTGTGCATGG. Conditions for the mismatch PCR were as follows: one cycle of 94°C for 3 min, followed by 35 cycles of 92°C for 30 s, 50°C for 30 s,

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bp, base pair; COX, cytochrome c oxidase; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; SDH, succinate dehydrogenase.

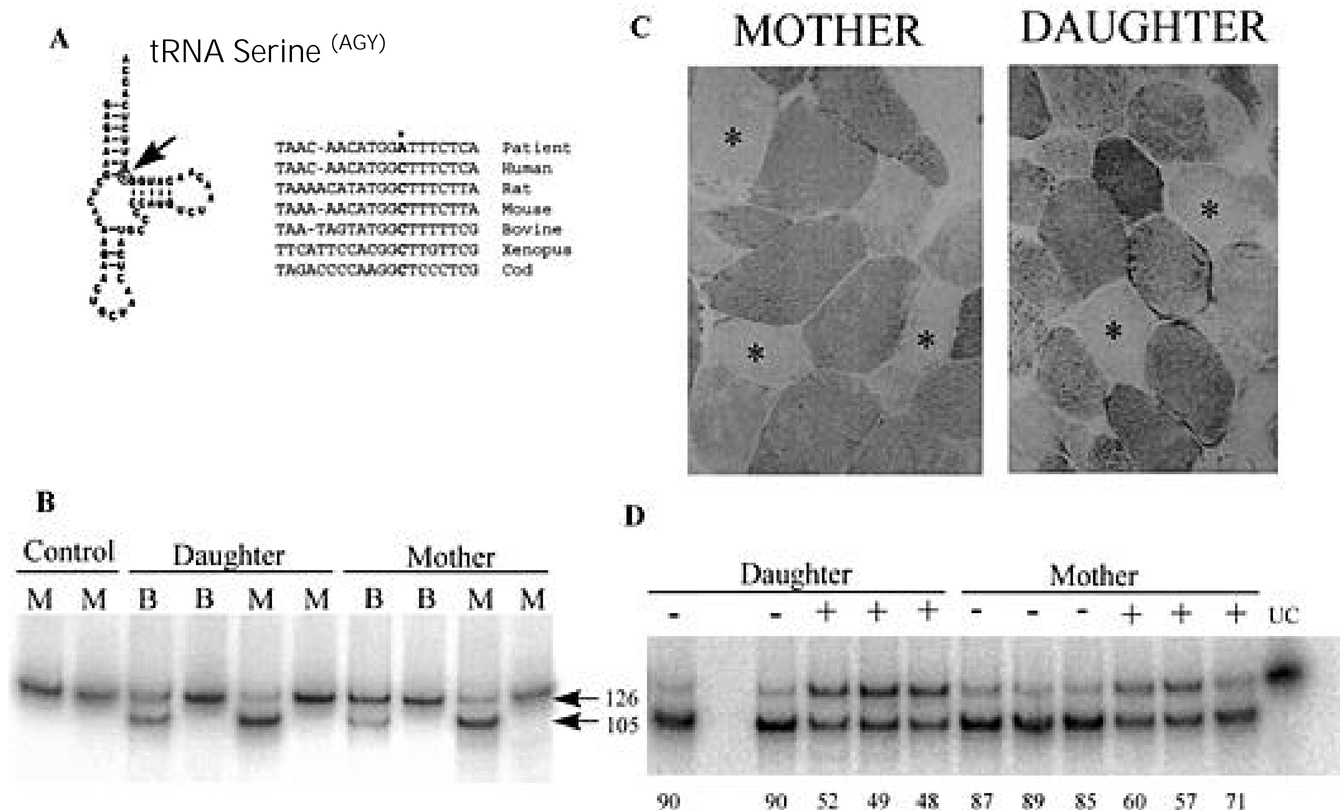

FIG. 1. Investigation of the C12258A mutation. *A*: Schematic representation of the $tRNA^{Ser(AGY)}$ gene indicating the mutated base (arrow) and alignment of the patient sequence with human, rat, mouse, bovine, Xenopus, and Atlantic cod sequences. This clearly demonstrates the conserved nature of this particular residue (*). *B*: Mismatch PCR analysis of muscle (M) and blood (B) mtDNA from both mother and daughter. The C12258A mutation introduces a restriction site, and the band at 126 bp is digested to give an additional band at 105 bp. The 105-bp band was not detected in a control muscle sample. The undigested product is given in the right hand lane for each tissue. *C*: Histochemical staining of muscle sections from both mother and daughter indicated COX-negative fibers (*). *D*: COX-negative (–) and -positive (+) fibers were isolated from the muscle sections and analyzed by mismatch PCR. The percentage of mutation in each fiber is indicated below each lane. The uncut product is indicated (UC).

and 72°C for 30 s, and finally, one cycle of 72°C for 10 min. After digestion with *Cla*I, fragments were separated on a 3% agarose gel and stained with ethidium bromide. The 126–base pair (bp) product was digested into two fragments of 105 and 21 bp in mutant mtDNA, but not in wild-type mtDNA.

To quantify the 12258 mutation, the mismatch PCR was carried out as described, and one extra cycle was then performed (8 min at 94°C, 2 min at 50°C, and 8 min at 72°C) after the addition of 5 µCi of [α-^{32}P]dCTP, 10 pmol of each primer, and 2.5 U of *Taq* polymerase. Labeled fragments were digested with *Cla*I and electrophoresed through a 12% nondenaturing polyacrylamide gel. The radioactive fragments were quantitated using a PhosphorImager screen (Molecular Dynamics, Chesham, U.K.) and appropriate software.

For analysis of DNA from single fibers, sections were stained for both SDH and COX. Single fibers that stained COX-positive and -negative were isolated and placed in an individual microfuge tube containing 10 µl of water. After centrifugation and removal of the water, each fiber was lysed by the addition of 10 µl of lysis buffer containing 200 mmol/l KOH and 50 mmol/l dithiothreitol (DTT) and incubated for 1 h at 65°C. Lysis was neutralized with 10 µl of buffer containing 900 mmol/l Tris-HCl and 200 mmol/l HCl. Last hot cycle PCR analysis was then carried out directly using 10 µl samples.

Southern blot analysis showed no evidence of a major rearrangement in the mtDNA. Sequencing of the mitochondrial genome demonstrated a number of changes when compared with the published mtDNA sequence (7). The sequence changes were as follows: T6776C, C11953T, C12258A, T13404C, and T14766C. The sequence changes at 11953, 12258, and 13404 have not been previously reported. The differences at 6776, 11953, 13404, and 14766 are neutral polymorphisms and are homoplasmic, while that at 12258 occurs in a tRNA coding region (Fig. 1*A*). The C A 12258 (tRNA serine) was detected in both mother and daughter. This base is highly conserved between species during evolution, and the mutated nucleotide at 12258 does not occur at this position in a number of species examined. The change at 12258 was heteroplasmic in both mother and daughter (Fig. 1*B*). The proportion of the 12258 mutant mtDNA was highest in the muscle of the daughter (85%) compared with that of the mother (68.4%). Using the mismatch PCR technique, the 12258 mutation was also quantified in blood from both individuals (19.4% in mother, 45% in daughter). The mutation was not detected in 100 unrelated nondiabetic control subjects. Muscle histochemistry showed the presence of COX-negative muscle fibers from both individuals (Fig. 1*C*). To correlate genotype to the biochemical phenotype, COX-positive and -negative single fibers were isolated from muscle sections, and PCR was

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carried out using the mismatch primer technique. The level of mutation in the COX-positive fibers ranged from 23 to 71%, while the COX-negative fibers were shown to be >85% mutant (Fig. 1*D*).

In this family, we have identified a novel mutation at position 12258. We believe this mutation is pathogenic for the following reasons. First, it is heteroplasmic and has higher levels in muscle compared with blood. Second, the C A alters a highly conserved base pair in the acceptor stem of $tRNA^{Ser}$ that is likely to effect both secondary and tertiary structure and the function of this tRNA. The structure of this tRNA is atypical in that it lacks the whole D arm, has an unusual T loop, and possibly demonstrates the minimal conserved requirements for protein synthesis. The position at which the C12258A mutation occurs in the tRNA molecule would create a molecule with a shorter acceptor stem and so reduce the dimensions of the molecule (8). Therefore, the loss of the base pair from the amino acid acceptor stem may influence the successful tertiary interactions of the mature tRNA. Third, the mutated DNA is at much higher levels in the COX-negative compared with the COX-positive fibers. This evidence confirms that this mutation is causing the observed biochemical defect in the muscle and presumably the pancreatic β-cells, since insulin secretion is heavily dependent on oxidative metabolism and mitochondrial dysfunction will impair insulin secretion.

This newly identified point mutation highlights the difficulty in assessing the true incidence of mtDNA defects in diabetes. Most studies to date have focused on the A3243G $tRNA^{Leu(UUR)}$ mutation in diabetes and have shown that in many cases it is associated with a distinct subtype of maternally inherited diabetes and deafness (MIDD) (3). However, at least 30 different point mutations and 100 different rearrangements of mtDNA have been described so far in relation to human disease (9), and it is a daunting task to consider the true incidence of these mutations in diabetes.

Identifying diabetic patients with mtDNA defects is important, since definition of the clinical phenotype and the natural history will allow focused clinical management. We were fortunate in that our patients also had neuromuscular features that encouraged us to investigate in detail. However, not all diabetic patients with mtDNA defects have neurological features. A maternal family history is helpful in suggesting that there may be an mtDNA defect. However, there are many patients described in whom there is either a new mutation or the patient's mother is clinically unaffected despite carrying the mutation. Thus, one cannot depend solely on the presence of a family history.

Our studies have confirmed not only a new mtDNA mutation causing diabetes, but also the value of investigating muscle in our search for mtDNA mutations. One major advantage of muscle is that in some patients with mtDNA disease there is the important histochemical finding of COX-negative fibers (10). The presence of these fibers not only highlights the likelihood of an mtDNA defect, but also allows direct correlation of genotype to biochemical phenotype. We believe muscle is a much better tissue to investigate than blood, since even in the presence of clinical disease there may be very low or undetectable levels of mutant mtDNA in blood (11). Also, because there are multiple polymorphisms in mtDNA, this limits the value of rapid screening methods, such as single-strand conformation polymorphism. Automated sequencing of the mitochondrial genome is a valuable approach, since the whole genome is only 16.5 kb, and can easily be performed once the system is established.

In conclusion, we have identified a novel mtDNA mutation at C12258A in the tRNA serine gene that is associated with diabetes and deafness. We believe screening muscle mtDNA from patients with diabetes and associated problems will help to define the true incidence of mtDNA mutations in diabetes.

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