Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial DNA deletion

Scott W. Ballinger1,2, John M. Shoffner1,2, Ellis V. Hedaya4,3, Ian Trounce1, Meraida A. Polak1,2, Deborah A. Koontz1 & Douglas C. Wallace1,2,3

Diabetes mellitus (DM) is one of the most common chronic disorders of children and adults. Several reports have suggested an increased incidence of maternal transmission in some forms of DM. Therefore, we tested a pedigree with maternally transmitted DM and deafness for mitochondrial DNA mutations and discovered a 10.4 kilobase (kb) mtDNA deletion. This deletion is unique because it is maternally inherited, removes the light strand origin (Ol) of mtDNA replication, inhibits mitochondrial protein synthesis, and is not associated with the hallmarks of mtDNA deletion syndromes. This discovery demonstrates that DM can be caused by mtDNA mutations and suggests that some of the heterogeneity of this disease results from the novel features of mtDNA genetics.

Diabetes mellitus is a genetically heterogeneous group of disorders that share the common feature of glucose intolerance. Collectively, DM is one of the most common chronic disorders, afflicting up to 5–10% of the individuals in the Western world. DM is generally divided into idiopathic DM and hereditary DM. Various criteria have been proposed for subclassification of DM, but the absence of simple models for inheritance has been a significant impediment to molecular genetic investigations.

Idiopathic DM is further subdivided into an insulin dependent type (IDDM or type I) and a non-insulin dependent type (NIDDM or type II). Type I DM is a chronic autoimmune disorder with early onset that is characterized by low or absent circulating insulin levels, pancreatic islet cell antibodies, HLA DR3 and/or DR4 linkage, and episodes of ketoacidosis. By contrast, type II DM usually appears after age 40, has normal to low insulin levels, is ketoacidosis-resistant, and is frequently associated with obesity. Hereditary DM encompasses a wide spectrum of clinical presentations and possible inheritance patterns, with more than 60 hereditary syndromes having been described. Although these classification schemes are clinically useful, they provide limited insight into the genetic heterogeneity of DM. Therefore, genetic markers that further divide DM into identifiable subgroups are needed.

One subgroup of DM may be caused by defects in mitochondrial oxidative phosphorylation (OXPHOS). Epidemiologic studies of a spectrum of DM patients have shown that for patients with an age of onset of 25 years or older, DM is transmitted 2–3 times more frequently from the mother than the father, and in some instances is inherited through multiple maternal generations. While this has been consistently attributed to gestational effects, maternal inheritance of mtDNA mutations are also possible. The deleterious effect of OXPHOS inhibition on pancreatic islet cell function has been shown in rats by administration of the antibiotic streptozotocin, which impairs mtDNA replication, transcription, and OXPHOS function. In man, the importance of OXPHOS to pancreatic islet cell function is emphasized by the occurrence of DM in patients with mtDNA mutations. DM in these patients is also associated with a variety of severe disease manifestations affecting other organ systems as seen in Kearns Sayre Syndrome/Chronic Progressive External Ophthalmoplegia (KSS/CPEO).

We now report that a unique mtDNA deletion can cause familial DM and premature deafness without ophthalmoplegia and mitochondrial myopathy, the hallmarks of mtDNA deletion syndromes. This deletion and the associated disease are maternally inherited, supporting the concept that mtDNA deletions can have tissue-specific manifestations such as DM, comparable to the optic atrophy of mtDNA missense mutations. Since the distinctions between idiopathic and hereditary DM are poorly defined, this observation suggests that mtDNA mutations could help explain the increased incidence of maternal transmission in certain cases of idiopathic and hereditary DM.

Clinical description
The pedigree showed the maternal transmission of DM and/or deafness over three generations, with a total of nine individuals being affected (Fig. 1a; a fourth brother of the proband is not shown). The pedigree matrix (I-2) had affected offspring by two husbands (I-1 and I-3),
Fig. 1a. Three generation pedigree showing maternal inheritance of diabetes and/or deafness. Open symbols represent non-affected individuals and solid symbols indicate affected pedigree members with deafness (III-1) or diabetes and deafness (II-1 to II-6 and I-2). An affected brother with deafness and diabetes who died of AIDS is not shown. b, Southern blot (PvuII digest) of the maternally inherited 10.4 kb mtDNA deletion. Each lane contains buffy coat or muscle cell DNA from the indicated pedigree member. c, PCR amplification across the deletion breakpoint using primers in the IRNAS and cytochrome b genes. Each pair of lanes shows the PCR products from the buffy coat (S) and platelets (P) of the indicated family member. d, Table presenting patient phenotypes. +, present; -, absent; nd, no data; asterisk, deceased. Deletion (%) is for buffy coat mtDNA.

confirming that she transmitted the disease. Her son (II-6) did not transmit the disorder to his son (III-2); while her daughter, the proband (II-1), did transmit it to her daughter (III-1). Thus the pedigree fulfills the clinical genetic criteria for maternal inheritance of DM and deafness.

All individuals with DM required normal dosages of insulin, but only individuals II-1, II-4 and II-5 experienced episodes of diabetic ketoacidosis. The age of onset for diabetes was between 20 and 30 years of age, except for individual II-4, who was first diagnosed with DM at about 43 years of age. A fourth brother (not shown in Fig. 1) had DM and deafness but died of AIDS. When treated with 400–500 mg day of Zidovudine, a known inhibitor of mitochondrial replication, he expressed pancytopenia. No pedigree member had prostatitis, ophthalmoplegia, or muscle weakness.

Fig. T2-weighted axial MRI images of the proband's brain demonstrating lacunar infarcts involving the thalamus bilaterally (left panel) and a left parietal infarct (right panel) (TR, 2270 ms; TE, 90 ms).

The only significant disease manifestations other than DM and deafness were the occurrence of strokes in the pedigree members (I-2) and the proband (II-1) (Fig. 1d) and mild cardiomyopathy in II-5. The proband, who had mild hypertension, experienced both a left parietal infarct as well as multiple lacunar infarctions involving basal ganglia, thalamus, and internal capsules (Fig. 2). Urine organic and amino acids were normal. Quadriceps muscle biopsy of the proband (II-1) revealed only a mildly increased variability in fibre diameter. No evidence of ragged-red muscle fibres or mitochondrial accumulation were detected by modified Gomori trichrome or other standard histochemical stains. Ultrastructural study showed normal muscle fibre and mitochondria structure and no paracrystalline inclusions.

Biochemical analysis
Mitochondria were isolated from the proband's quadriceps muscle immediately after biopsy and were assayed for OXPHOS defects with NADH-n-decyl coenzyme Q oxidoreductase (Complex I), reduced n-decyl coenzyme Q-cytochrome c oxidoreductase (Complex III), and cytochrome c oxidase (Complex IV) assays. All three electron-transport chain enzymes were below the 5%
confidence limits of our control group (see Table 1). The reduced activity of the individual respiratory chain complexes was also reflected in the assays for Complexes I + III and II + III, which require intact electron flow within and between enzyme complexes. Thus, the proband had a generalized OXPHOS defect in skeletal muscle.

**MtdNA analysis**

Total genomic DNA was isolated from the proband's skeletal muscle, digested with *XhoI* or *PvuII*, and hybridized to a mtdNA probe. This analysis indicated that the proband had two different mtdDNAs in skeletal muscle consisting of 31% normal mtdDNA of 16.5 kb, and 69% deleted mtdDNA of 6.1 kb. Further restriction endonuclease mapping of the deleted mtdNA with *HindIII* and *ApaI* showed that all three *HindIII* sites at nps 6203, 11680, and 12570 and three of the five *ApaI* sites at nps 4427, 8249, and 9265 were absent. Polymerase chain reaction (PCR) analysis further localized the deletion breakpoint to a 150 np region between nps 4308 and 14874.

Direct DNA sequencing across the deletion breakpoint revealed that a 10,423 np segment had been removed from the mtdNA between nps 4398 in the tRNA^Glu^ gene and 14822 in the cytochrome b gene (Fig. 3). The deletion removed 2 nps at the 5’ end of the tRNA^Glu^ gene, 11 of 13 OXPHOS subunit genes, 15 of 22 tRNAs, the OX, and 75 nps at the 5’ end of cytochrome b. This mutant mtdNA retained complete sequences for the D-loop including the heavy strand replication origin (O), 6 tRNAs (Phe, Val, Leu (UAA/G), Thr and Pro), the 125 and 165 rRNA genes, and the ND1 gene. The deletion was flanked by two 10 np direct repeats (5’-CACCCCATCC-3’) at nps 14812–14821 and nps 4389–4398, one of which was lost.

To evaluate the maternal transmission of the mutant mtdNA, total genomic DNA was isolated from the buffy coat of all available family members (II-1 through II-5, III-1, III-2) and digested with *PvuII* (Fig. 1a and 1b). All maternal lineage family members (II-1 through II-5, III-1) were heteroplasmic for the deleted mtdNA at levels ranging from 38% to 44% of the total mtdDNA (Fig. 1b and 1d). A 150 np PCR fragment (nps 4308–14874), which was specific for the deletion breakpoint, was amplified from both buffy coat and platelets of these individuals (Fig. 1c), and direct sequencing revealed exactly the same deletion breakpoint in the six maternal relatives examined (II-1 to 5, and III-1). By contrast, the paternal lineage relative, III-2, did not inherit the deleted mtdNA as assessed by Southern blot (Fig. 1b) or PCR (Fig. 1c) analysis. Therefore, this novel 10.4 kb mtdNA deletion was transmitted exclusively along the maternal lineage, and was present at high frequencies in several tissues. Hence, it fulfills the criteria for maternal inheritance.

**Mitochondrial protein synthesis**

To further assess the effects of the deleted mtdNA on mitochondrial function, both Epstein-Barr virus transformed lymphoblast and primary myoblast cell lines were established from the proband (II-1). Mitochondrial protein synthesis was quantitated in each cell line. The proband's lymphoblast cell line contained 53% deleted mtdDNAs and had a marked impairment of mitochondrial protein synthesis, incorporating only 52% of the control 5’-methionine into mitochondrial translation products (Fig. 4a and b). By contrast, the proband's myoblast cell lines, which had partially segregated the deleted mtdDNAs to 17%, had a normal level of mitochondrial protein synthesis. Thus, this deletion is associated with severe inhibition of mitochondrial protein synthesis and the inhibition is lost when the deleted mtdDNAs segregate.

**Discussion**

In this pedigree, DM and deafness were shown to be caused by a 10.4 kb mtdNA deletion through two lines of evidence. First, DM and deafness was linked to the mtdDNA deletion by showing that both were maternally inherited. Maternal inheritance of the disease was demonstrated by showing that all maternal relatives were affected, and that the disease was consistently transmitted through affected females but not through affected males. Maternal inheritance of the 10.4 kb deletion was proven by showing that all maternal lineage relatives harboured a high percentage of the deletion, while it could not be detected in paternal lineage relatives, even when using PCR amplification. Moreover, direct PCR sequencing confirmed that all maternal relatives had exactly the same deletion breakpoint, rendering it extremely unlikely that the deletion could have arisen spontaneously within each individual, as is seen in KSS/CPEO16,20, or due to a nuclear mutation, as in autosomal dominant KSS/CPEO22.

Second, both the DM and deafness and the mtdDNA deletion are associated with the same biochemical defect.
This is true for those which occur spontaneously and are associated with KSS/CPEO16,18, and those resulting from a nuclear mutation which generates mtDNA deletions21. The maternal inheritance of the 10.4 kb deletion and hence its clear replication, is strongly at variance with biochemical studies which indicate that O2 is essential for mammalian mtDNA replication29,30.

The pathology resulting from the 10.4 kb mtDNA deletion is also unique. Virtually all deletions are associated with ptosis, ophthalmoplegia, and mitochondrial myopathy37, yet these features were conspicuously absent in the current pedigree. Perhaps the maternal transmission and the unusual pathology associated the 10.4 kb deletion is a product of its lacking O2. It has been proposed that the mtDNA replication rates are proportional to the molecular length of the deleted molecules when both replication origins are present31. Thus, deleted molecules could have a replicative advantage and become progressively enriched in stable tissues or along maternal lineages until cellular energy metabolism fails and the cells with high levels of deleted mtDNA die. The loss of O2 in the current mutant mtDNA might reduce replication efficiency, thus preventing its progressive enrichment and avoiding the muscle pathology and toxicity to the maternal lineage.

While the 10.4 kb deleted molecules clearly inhibit mitochondrial protein synthesis, the mechanism by which protein synthesis is inhibited remains unclear. MtDNA deletions in KSS/CPEO have been proposed to inhibit mitochondrial protein synthesis by altering the ratios of tRNAs, tRNAs, and mRNAs22,23,24. Alternatively, the 10.4 kb deletion might inhibit protein synthesis through the presence of a mutant tRNA50 which lacks the first two 5′nps (Fig. 3). Such a tRNA could occupy the ribosomal binding site but would not be charged, thus inhibiting nascent polypeptide chain elongation. Alternatively, inhibition of mitochondrial protein synthesis by deleted mtDNAs might be the result of other as yet unidentified factors.

Analysis of this pedigree has shown that diabetes and deafness can be caused by defects in mitochondrial protein synthesis due to mtDNA mutations. This surprising observation suggests that mtDNA mutations might also be important in other late-onset degenerative diseases. Hence, it may be important to consider the mitochondrial paradigm when investigating other diseases with complex inheritance patterns.

**Methodology**

Muscle histology and OXPHOS enzyme analysis. After informed consent, quadriceps muscle was obtained under local anaesthesia (2% lidocaine-HCl). Histological processing included haematoxylin–eosin, Gomori modified trichrome, NADH-tetrazolium, ATPase (pH 9.2), oil-red-O, and myophosphorylase27. Sections were fixed in glutaraldehyde, sectioned, stained and examined with the electron microscope. Muscle mitochondria were isolated by differential centrifugation immediately after biopsy and the OXPHOS enzyme activities determined and reported in mmoles of substrate min⁻¹ mg⁻¹ mitochondrial protein. Control mean, standard deviation and 5% confidence levels were calculated from 11 normal muscle biopsies (controls 5–7 and 9–16), ages 26–59 years29. OXPHOS determinations using rat mitochondria were performed along with the patient samples to provide a positive control for reduced OXPHOS activities.

Southern blot and PCR analysis. For Southern blot analysis, 3 μg of buffy coat or muscle (proband) genomic DNA were digested with Xhol, PvuII, Apal or HindIII and loaded onto 0.8% or 1.5% SeaKem...
The assistance of the Clinical Research Center of Emory University School of Medicine (supported by a grant from NIH) is gratefully acknowledged. This work was supported by the Emory Neuromuscular Center, by an NIH clinical investigator grant awarded to J.M.S., and NIH grants and a Muscular Dystrophy Clinical grant awarded to D.C.W.

Received 25 November 1991; accepted 21 January 1992.