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

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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 (O_L) 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[1]. 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[1]. 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[2–6] of a spectrum of DM patients has 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[7]. 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[8–12]. In man, the importance of OXPHOS to pancreatic islet cell function is emphasized by the occurrence of DM in patients with mtDNA mutations[13–15]. 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)[16–18].

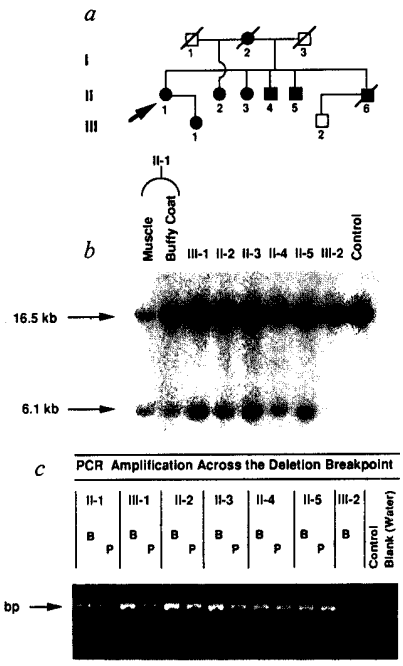
We now report that a unique mtDNA deletion can cause familial DM and premature deafness without ophthalmoplegia and mitochondrial myopathy, the hallmarks of mtDNA deletion syndromes[17]. This deletion and the associated disease are maternally inherited, supporting the concept that mtDNA deletions can have tissue-specific manifestations such as DM, comparable to the optic atrophy of mtDNA missense mutations[19]. 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. 1*a*; a fourth brother of the proband is not shown). The pedigree matriarch (I-2) had affected offspring by two husbands (I-1 and I-3),

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Patient Phenotypes	I-2	II-1	II-2	II-3	II-4	II-5	II-6	III-1	III-2
Deletion (%)	nd	44	39	42	38	43	nd	44	0
Diabetes	+	+	+	+	+	+	+	-	-
Deafness	+	+	+	+	+	+	+	+	-
Strokes	+	+	-	-	-	-	-	-	-
Ophthalmoplegia	-	-	-	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-	-	-	-
Mt myopathy	nd	-	nd	nd	nd	nd	nd	nd	nd
Age	*	49	53	51	46	45	*	25	29

Fig. 1 *a*, 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 (*Pvu*II digest) of the maternally inherited 10.4 kb mt DNA 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 $tRNA^{Ile}$ and cytochrome b genes. Each pair of lanes show the PCR products from the buffy coat (B) 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[20], he expressed pancytopenia. No pedigree member had ptosis, ophthalmoplegia, or muscle weakness.

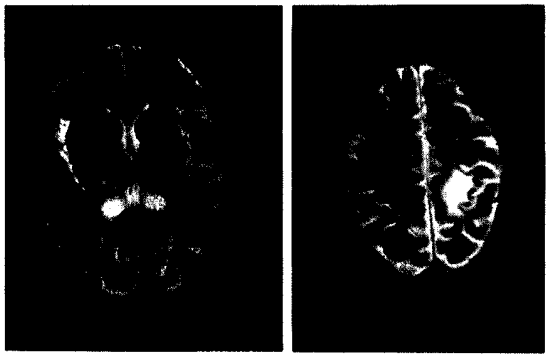


Fig. 2 T2-weighted axial MRI images of the proband's brain demonstrating lacunar infarcts involving the thalamus bilaterally (left panel) and a left parietal infarct (right panel) (TR, 2270 ms; TE, 90 ms).

The only significant disease manifestations other than DM and deafness were the occurrence of strokes in the pedigree matriarch (I-2) and the proband (II-1) (Fig. 1*d*) 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%

Table 1 Oxidative phosphorylation enzyme analysis

	Proband II-1	Controls Mean	sd	5% level
Complex I	37	194	60	54
Complex I+III	60	229	149	0
Complex II+III	138	605	164	223
Complex III	289	1952	700	323
Complex IV (FT)	147	1426	433	373
Complex IV (SON)	194	1615	309	896
Fragility index	1.3	1.24	0.35	0.44

OXPHOS defect in the proband's muscle mitochondria. Complex I-NADH-n-decyl coenzyme Q oxidoreductase; Complex I + III-NADH-cytochrome c oxidoreductase (rotenone-sensitive fraction); Complex III-reduced n-decyl coenzyme Q-cytochrome c oxidoreductase; Complex II + III-succinate-cytochrome c oxidoreductase; Complex IV-cytochrome c oxidase; FT, freeze thaw; SON, sonicated; Fragility index= complex IV (sonicated)/complex IV (freeze thaw).

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 *Xho*I or *Pvu*II, and hybridized to a mtDNA probe. This analysis indicated that the proband had two different mtDNAs in skeletal muscle consisting of 31% normal mtDNA of 16.5 kb, and 69% deleted mtDNA of 6.1 kb. Further restriction endonuclease mapping of the deleted mtDNA with *Hin*dIII and *Apa*I showed that all three *Hin*dIII sites at nps 6203, 11680, and 12570 and three of the five *Apa*I 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 $tRNA^{Gln}$ gene and 14822 in the cytochrome b gene (Fig. 3). The deletion removed 2 nps at the 5' end of the $tRNA^{Gln}$ gene, 11 of 13 OXPHOS subunit genes, 15 of 22 tRNAs, the O_L, 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_H), 6 tRNAs (Phe, Val, Leu (UAA/G), Ile, Thr and Pro), the 12S and 16S 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 *Pvu*II (Fig. 1*a* and 1*b*). 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 mtDNA (Fig. 1*b* and 1*d*). 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. 1*c*), 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. 1*b*) or PCR (Fig. 1*c*) 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 fulfils the criteria for maternal inheritance.

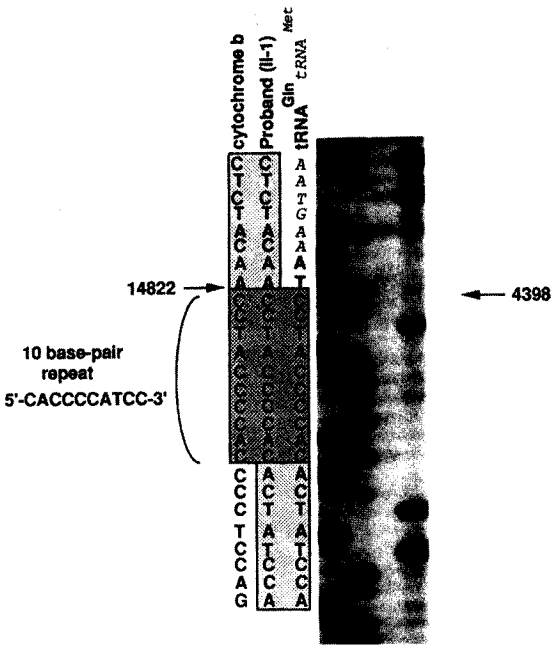


Fig. 3 Direct sequencing across the deletion breakpoint at nps 4398:14822. This region was sequenced in maternal relatives II-1 to -5, and III-1 to verify that all family members harbour the same deletion. The heavily shaded area defined by the brackets shows the 10 np direct repeat associated with the breakpoint. The 5' A and T of $tRNA^{Gln}$ removed by the deleion are shown in bold type on the right sequence above the repeat.The adjacent $tRNA^{Met}$ sequence is shown in italics. A portion of the deleted 5' end of the cytochrome b gene is shown in bold type below the left direct repeat sequence. The cytochrome b and $tRNA^{Gln}$ regions retained in the proband's sequence are highlighted by light shading.

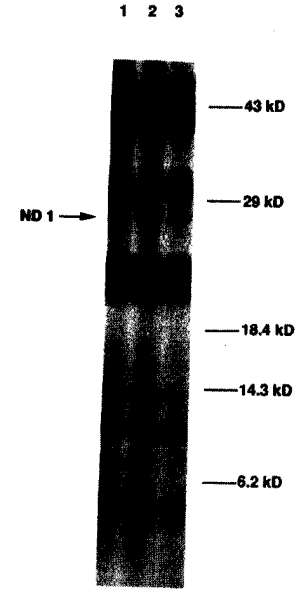
Mitochondrial protein synthesis

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 mtDNAs and had a marked impairment of mitochondrial protein synthesis, incorporating only 52% of the control ^{35}S-methionine into mitochondrial translation products (Fig. 4*a* and *b*). By contrast, the proband's myoblast cell lines, which had partially segregated the deleted mtDNAs 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 mtDNAs 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 mtDNA 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/CPEO[16,18,20], or due to a nuclear mutation, as in autosomal dominant KSS/CPEO[22].

Second, both the DM and deafness and the mtDNA deletion are associated with the same biochemical defect.



b

	% deleted mtDNA	% labeling
Controls (lanes 1, 2)	0	100 (89.4, 110.6)
Proband Myoblasts (not shown)	17	116
Proband Lymphoblasts (lane 3)	53	52 (47, 57)

Fig. 4 Mitochondiral protein synthesis of the proband and control cell lines. *a*, Polyacrylamide gel showing the mitochondrial translation products of EBV-transformed lymphoblasts from two controls (lane 1 and 2) and the proband (lane 3), revealing an overall reduction in labelling of all products in the proband. *b*, Table comparing the percent deleted mtDNA to the percent ^{35}S-methionine incorporated in all translation products for the proband, two control lymphoblastoid cell lines, and for the proband and a control myoblast cell line.

The proband's muscle mitochondria contained 69% deleted mtDNA and showed a severe reduction in all of the enzyme complexes of the respiratory chain (Complexes I, III, IV). This pleiotrophic OXPHOS defect is a consistent feature of mtDNA mutations which alter protein synthesis genes such as the $tRNA^{Lys}$ mutation causing myoclonic epilepsy and ragged red fibre disease[23,24], and the $tRNA^{Leu}$ mutation causing maternally inherited myopathy and cardiomyopathy[25]. Similarly, in cultured cells from the proband harbouring 53% deleted mtDNAs, a severe deficiency in mitochondrial protein synthesis was observed. This defect was linked to the deleted mtDNA by showing that it was lost when the deleted molecules segregated, which is consistent with somatic cell fusion studies which show that deleted mtDNAs inhibit mitochondrial protein synthesis when present above a certain percentage of molecules[26]. Hence, DM and deafness are caused by the 10.4 kb deletion in this pedigree because they are linked and share a common biochemical defect.

In addition to causing DM and deafness, the 10.4 kb deletion is unique for several reasons. It is the first mtDNA deletion that has been found to be inherited through multiple generations at high levels. Until now, essentially all mtDNA deletions have been spontaneous[17]. Those few that have been reported to be maternally inherited either had different deletion breakpoints[27] or, could be detected only in first degree relatives by PCR analysis of the deletion junction fragment[28]. Second, this deletion lacks O_L. All other deletions to date retain both origins of replication. This is true for those which occur spontaneously and are associated with KSS/CPEO[16-18], and those resulting from a nuclear mutation which generates mtDNA deletions[22]. The maternal inheritance of the 10.4 kb deletion and hence its clear replication, is strongly at variance with biochemical studies which indicate that O_L is essential for mammalian mtDNA replication[29,30].

The pathology resulting from the 10.4 kb mtDNA deletion is also unique. Virtually all deletions are associated with ptosis, ophthalmoplegia, and mitochondrial myopathy[17], 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 O_L. It has been proposed that the mtDNA replication rates are proportional to the molecular length of the deleted molecules when both replication origins are present[31]. 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 O_L 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 rRNAs, tRNAs, and mRNAs[26,32,33]. Alternatively, the 10.4 kb deletion might inhibit protein synthesis through the presence of a mutant $tRNA^{Gln}$ 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). Histochemical processing included haematoxylin–eosin, Gomori modified trichrome, NADH-tetrazolium, ATPase (pH 9.2), oil-red-O, and myophosphorylase[21]. Portions 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 nmoles of substrate min^{-1} mg^{-1} mitochondrial protein[34]. Control mean, standard deviation and 5% confidence levels were calculated from 11 normal muscle biopsies (controls 5–7 and 9–16), ages 26–59 years[35]. 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, ~3μg of buffy coat or muscle (proband) genomic DNA were digested with *Xho*I, *Pvu*II, *Apa*I or *Hin*dIII and loaded onto 0.8% or 1.5% SeaKem

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agarose gels (FMC BioProducts), electrophoresed, blotted and hybridized with linearized HeLa mtDNA labelled by primer extension[36]. After localizing the deletion breakpoint, PCR primers nps 4308–4325 and nps 14874–14855 were used to amplify 5 ng of blood cell DNA across the breakpoint, resulting in a 150 bp fragment. PCR conditions for 35 cycles were 1 min at 94 °C, 45 s at 51 °C, 1 min at 72 °C. Approximately 5 μl of each amplification product were then electrophoresed on 2.0% SeaKem agarose gels. The percentage of the deleted mtDNAs was estimated densitometrically (Pharmacia LKB Ultroscan XL Densitometer) from a *Pvu*II digest Southern blot hybridized to a common 12S and 16S rRNAs gene mtDNA probe generated by PCR (nps 534–3001).

Direct DNA sequencing. PCR primers nps 3598–3615 and nps 15360–15343 were used to amplify across the deletion breakpoint using the following PCR conditions: 35 cycles, 1 min at 94 °C, 45 s at 51 °C, 1 min at 72 °C. Asymmetric amplification used 20 pmoles of primer nps 15226–15207 and 0.2 pmoles of primer nps 3951–3970 under the following PCR conditions: 40 cycles, 45 s at 94 °C, 45 s at 53 °C, 45 s at 72 °C. The product of the asymmetric PCR was then precipitated with 4M ammonium acetate/isopropanol and used as a template for dideoxy-sequencing using Sequenase (2.0; United States Biochemical Corporation), according to the manufacturers specifications. The sequencing primer covered nps 4308–4325.

Mitochondrial protein synthesis. Approximately 2.5×10^6 cells were washed with methionine-free medium and incubated for 2 hours with 400 μCi ml^{-1} ^{35}S-methionine in the presence of 100 μg ml^{-1} emetine, followed by a 20 min chase with 0.1 mM cold L-methionine. Cell pellets were solubilized with 4% SDS, sonicated and 50 μg of sample protein run on a 15% polyacrylamide gel[37]. Gels were dried onto filter paper and exposed to Kodak XAR film at –80 °C. The percent labelling of the translation products was determined by densitometry of all bands for the patient and controls. The sum of the labelling of the bands for each sample was used to calculate the relative levels of incorporation.

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