Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness

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Maternally transmitted non-syndromic deafness was described recently both in pedigrees with susceptibility to aminoglycoside ototoxicity and in a large Arab-Israeli pedigree. Because of the known action of aminoglycosides on bacterial ribosomes, we analysed the sequence of the mitochondrial rRNA genes of three unrelated patients with familial aminoglycoside-induced deafness. We also sequenced the complete mitochondrial genome of the Arab-Israeli pedigree. All four families shared a nucleotide 1555 A to G substitution in the 12S rRNA gene, a site implicated in aminoglycoside activity. Our study offers the first description of a mitochondrial rRNA mutation leading to disease, the first cases of non-syndromic deafness caused by a mitochondrial DNA mutation and the first molecular genetic study of antibiotic-induced ototoxicity.

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Sensorineural deafness, either in conjunction with neuromuscular diseases or with diabetes, has been associated with heteroplasmic mitochondrial DNA (mtDNA) mutations[1–4]. The likelihood of mtDNA mutations has also been suggested in two forms of non-syndromic deafness. A maternal inheritance pattern has been reported in several pedigrees in the Far East with familial aminoglycoside-induced deafness[5,6], and we have described a single large Arab-Israeli pedigree with maternally-inherited congenital deafness[7]. In this paper we have examined the mtDNA sequence in individuals with each type of non-syndromic deafness, and have found a strong candidate for a causative mutation.

Irreversible hearing loss is the main complication of aminoglycoside antibiotics such as streptomycin, gentamicin and kanamycin[8]. In China, due to widespread use of aminoglycosides, nearly 25% of all deaf-mutes in one district of Shanghai could trace the cause of hearing loss to aminoglycoside usage[6]. Of these patients, roughly 1/4 had other relatives with ototoxic deafness. In all of the 22 cases in which vertical transmission of this susceptibility could be traced, the inheritance pattern matched that of a mitochondrially-inherited trait, i.e. being transmitted only through females. A similar situation occurred in Japan, where in all but two of 28 families with streptomycin-induced deafness, the susceptibility trait was maternally-inherited[5]. The majority of familial cases received antibiotics for a much shorter period than the sporadic cases, implying the presence of a predisposing mutation or genetic susceptibility[6].

The mitochondrial ribosome in the cochlea is the most likely target of aminoglycoside ototoxicity, since the "natural target" of aminoglycosides is the evolutionarily-related bacterial ribosome[8]. In bacterial studies, aminoglycosides appear to stabilize mismatched aminoacyl-tRNAs in the 70S ribosome, allowing misreading of the mRNA during translation[9]. In addition to their interactions with ribosomal proteins, aminoglycosides bind to the *Escherichia coli* 16S rRNA, demonstrated by chemical protection and crosslinking experiments[10,11]. These physical experiments predict regions of the small rRNA which are important in translational fidelity. Their relevance has been borne out by the isolation of aminoglycoside-resistance mutations in bacteria, yeast mitochondria, Tetrahymena and chloroplasts which map to the predicted regions of the evolutionarily conserved small rRNA[12–15]. Thus, the mitochondrial rRNA genes, and especially the corresponding 12S rRNA gene, become prime candidates for the site of the mtDNA mutation in maternally-inherited aminoglycoside-induced deafness.

We have also searched for the mtDNA mutation in a large Arab-Israeli pedigree with maternally-inherited sensorineural deafness, which can be traced back through five generations to one common female ancestor[7]. This mutation is likely to be homoplasmic, as family members have either severe hearing loss or normal hearing, without intermediate levels of impairment, and a uniformly early age-of-onset of deafness[7]. Formal segregation analysis predicted that the disease phenotype is caused by the simultaneous inheritance of a homoplasmic mtDNA mutation and an autosomal recessive mutation[7,16,17]. In preliminary studies of the mtDNA in this family, Southern blot analysis revealed no gross deletions, insertions or rearrangements[7]. Our biochemical studies of lymphoblastoid cell lines from this pedigree showed no

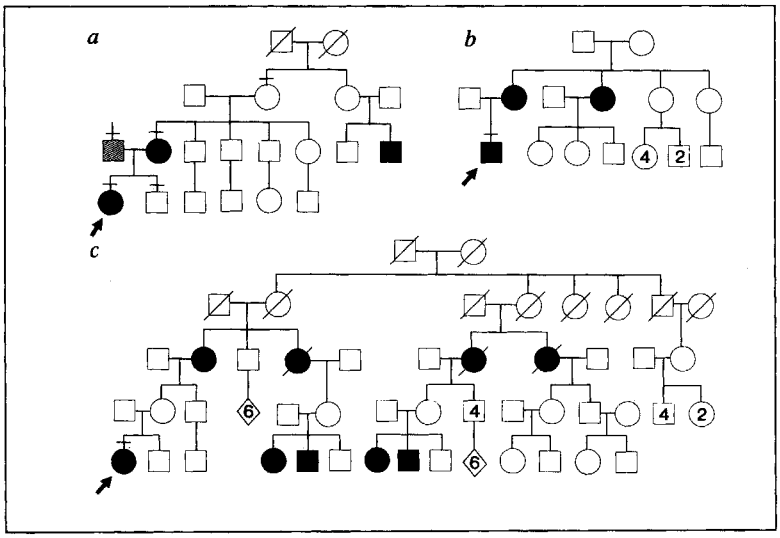

Fig. 1 Three pedigrees with maternally-transmitted aminoglycoside-induced deafness. Solid symbols indicate individuals treated with streptomycin before high frequency sensorineural hearing loss ensued; the father of proband *a* (hatched symbol) was deaf from unknown causes. Horizontal bars indicate individuals whose DNA was sampled. Probands are indicated by arrows. Pedigrees *a* and *c* are from Shanghai, although the proband in *c* is now living in the USA. Pedigree *b* is from Beijing.

gross enzymatic or biosynthetic defects, although two oxidative phosphorylation (OXPHOS) enzyme complexes, III and V, showed increased activities in family members[18]. However, we did not rule out the possibility that these were secondary changes and that biochemical abnormalities contributing to the deafness might only be detectable in cochlear cells. Therefore, mtDNA mutations throughout the mitochondrial genome could be candidates for the pathogenic mutation.

We searched for the proposed mitochondrial deafness mutations in the candidate rRNA genes in two patients with maternally-inherited aminoglycoside-induced deafness, and in the complete mtDNA in two patients from the Arab-Israeli pedigree. Any mutations found in the Arab-Israeli pedigree were also screened in the aminoglycoside-induced deafness families. Although several rare sequence variations (termed "mutations" hereafter) were found in the Arab-Israeli pedigree, only one mutation was found in common with the three families with maternally-inherited ototoxic deafness and not in 278 control individuals. Most importantly, this 1555 A to G mutation occurs at a highly conserved region of the 12S rRNA gene, in which aminoglycosides are known to bind[10,11] and in which aminoglycoside resistance mutations have been described in other species[12,13]. We suggest that this mutation, in conjunction with an otherwise non-toxic dose of aminoglycosides, or a putative mutated autosomal cochlear-specific gene, leads to tissue-specific impairment of the mitochondrial translation system and deafness.

Table 1 MtDNA mutations in the Arab-Israeli pedigree

Gene	Replacement mutation	Conservation[a]	Test[b]	No. of controls with mutation /total controls
12S rRNA	709: G to A	not cons.	ASO	3/19
	769: G to A	not cons.	ASO	4/19
	825: T to A	not cons.	ASO	2/103
	851: A to G	not cons.	ASO	0/107
	930: G to A	not cons.	ASO	5/107
	1018: G to A	not cons.	ASO	4/19
	1555: A to G	=B,M,R	ASO	0/278
16S rRNA	**1822: T to C**	not cons.	ASO	1/110
tRNA asn	**5704: C to T**	=B,M,R	ASO	0/106
tRNA asp	7521: G to A	not cons.	ASO	3/19
ATPase 6	**8582: C to T (Ala to Val)**	not cons.	Fnu4HI(-)	1/109
	8701: A to G (Thr to Ala)	not cons.	TONI assay	4/16
	8860: A to G (Thr to Ala)	not cons.	HhaI (+)	13/17
CO 3	9559: G to C (Arg to Pro)	not cons.	TONI assay	5/5
ND 3	**10143: G to A (Gly to Ser)**	not cons.	AluI (+)	1/107
	10398: A to G (Thr to Ala)	=B,M,R	ASO	15/35
ND 4	**11025: T to C (Leu to Pro)**	=B	ASO	0/109
tRNA ser (AGY)	12236: G to A	=B,M	ASO	6/100
ND 5	**12950: A to G (Asn to Ser)**	=B,M,R,X	HhaI (+)	0/110
	13105: A to G (Ile to Val)	=B,M,R,X	ASO	4/42
Cyt b	**15884: G to A (Ala to Thr)**	not cons.	HaeIII (-)	3/111

[a]Conservation of amino acid for protein-coding genes, or nucleotide for rRNAs and tRNAs, in bovine (B), mouse (M), Rat (R, and Xenopus laevis (X). Not cons, not conserved.
[b]Test methods included ASO hybridizations, RFLP analysis of PCR products, where (–) and (+) indicate loss or gain, respectively, of the restriction site indicated, and TONI assay (a template-specified nucleotide incorporation assay). In addition to the above mutations, we found six sequence changes known to be errors in the Cambridge consensus sequence at nt 1438, 13702, 14199, 14272, 14368 and 15326 (reviewed in refs 20 and 35) and 44 additional neutral mutations in the protein coding genes, plus three new errors in the Cambridge sequence at nt 740, 2706 and 3107 (Prezant *et al.* submitted). Previously described polymorphisms include the mutations at nt 8701, 8860, 9559 and 10398 (refs 20,35,43,47). Mutations in bold occurred in <5% of controls. Controls included 35 Arab-Israelis and Caucasians, Asians and Blacks in equal proportions, except for the 1555 A to G mutations, which was screened in all 278 controls.

The pedigrees

All affected individuals in the three pedigrees with maternally-transmitted aminoglycoside-induced deafness (Fig. 1) developed high frequency sensorineural hearing loss after receiving streptomycin. The five generation Arab-Israeli pedigree, with 55 maternal line family members having severe to profound hearing loss, was described in Jaber *et al.*[7].

MtDNA analysis in familial aminoglycoside ototoxicity

We directly sequenced polymerase chain reaction (PCR)-amplified products encompassing the mitochondrial 12S and 16S rRNA genes, between nucleotides (nt) 648–1601 and 1671–3229, respectively[19]. Sequences were obtained from two independent patients, the proband's mother from pedigree A and the proband from pedigree C (Fig. 1). Both patients have the same four sequence changes in the 12S rRNA gene: 663 A to G, 750 A to G, 1438 A to G, 1555 A to G and three sequence changes in the 16S rRNA gene: 1736 A to G, 2706 A to G and a 1 base deletion at nucleotide 3107. The "mutations" at nt 750, 1438, 2706 and 3107 are actually errors in the Cambridge sequence[20] (Prezant *et al.*,

manuscript submitted). We used allele-specific oligonucleotide hybridization[21–23] to screen another patient with ototoxic deafness (the proband in *b*, Fig. 1), as well as hearing controls, for the three "real" mutations 663 A to G, 1555 A to G and 1736 A to G. Interestingly, all three mutations were also present in the third patient.

The mutation 1555 A to G in the 12S rRNA gene was not found in 278 controls (see methodology) while both the 663 A to G and 1736 A to G mutations occurred in 11% (13 of 115) and 10% (13 of 130) of Asian controls, respectively. The same 13 controls have both the 663 and 1736 mutations. The 1555 A to G mutation is homoplasmic in these three pedigrees (see below).

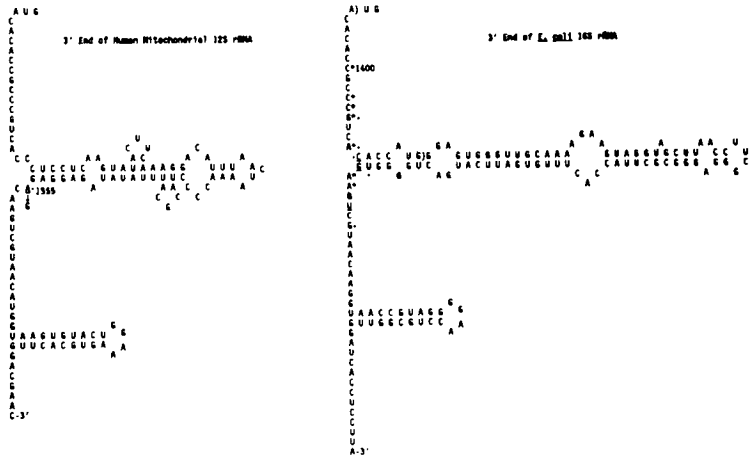

Fig. 2 The 3' end of human mitochondrial 12S rRNA and the corresponding region of *E. coli* 16S rRNA, showing the site of the 1555 A to G mutation in the four pedigrees with maternally-transmitted deafness. The functional regions of the *E. coli* 16S rRNA[28,38] are depicted as follows: the sequence in { } is crosslinked to streptomycin[11]; *, nucleotides that bind tRNA[37]; •, nucleotides at the ribosomal subunit interface[10]; nucleotides in bold, altered reactivity to chemical modification upon aminoglycoside binding[10]; underlined nucleotides in the *E. coli* sequence, sites of aminoglycoside resistance mutations in other species[12,13].

MtDNA analysis in the Arab-Israeli pedigree

In a search for heteroplasmic point mutations, small deletions or insertions, we performed single strand conformation polymorphism (SSCP) analysis of the mitochondrial genome[24], or heteroduplex analysis for fragments >420 basepairs (bp)[25]. Sixty overlapping fragments of the mtDNA were amplified from blood DNA of three maternally-related family members (the deaf proband, a deaf male from an earlier generation with atypical adult onset of deafness and an unaffected female) and an unrelated Arab control. Polymorphic differences were seen for more than half of the family's mtDNA fragments compared to those of the Arab control, but we found no differences between family members which could indicate potential heteroplasmy (data not shown).

We then sequenced the rRNA, tRNA and protein coding genes of the mtDNA in the proband and her maternal cousin to identify disease-related point mutation(s), by sequencing both strands of PCR-amplified mtDNA (proband) and by M13 sequencing (cousin). Both individuals contained the same mutations, compared to the Cambridge sequence[19]. These include 12 rRNA mutations, 3 tRNA mutations and 15 mutations which change amino acids. All of the mutations were verified in 12–20 additional family members, and tested for their frequency in four ethnic groups (Arab-Israelis, Caucasians, Asians and Blacks, Table 1). The test methods included allele-specific oligonucleotide hybridizations (ASO), restriction enzyme analysis of PCR-amplified fragments and trapped oligonucleotide nucleotide incorporation (TONI) assays[21–23,26,27]. An additional 44 neutral mutations were identified which do not produce amino acid substitutions (data not shown).

We found nine mutations in the 12S rRNA gene and three in the 16S rRNA gene, including the four errors in the Cambridge sequence mentioned above. Of the seven "real" mutations in the 12S rRNA gene, four were rarely or never seen in controls (825 T to A, 851 A to G, 930 G to A and 1555 A to G); the single mutation in the 16S rRNA gene (1822 T to C) is also very rare. Except for the 1555 A to G mutation, these residues are not evolutionarily conserved[28–30].

The three tRNA mutations, at nt 5704, 7521 and 12236, have not been described previously. Of these, only the nt 5704 mutation in $tRNA^{Asn}$ is both conserved[31–34] and unique to the family, not being observed in 106 controls.

We found 15 mutations which result in amino acid substitutions. These include four known polymorphisms at nt 8701, 8860, 9559, 10398 and five errors in the Cambridge sequence at nt 13702, 14199, 14272, 14368 and 15326 (reviewed in refs 20 and 35). The high frequencies with which we also observed these polymorphisms in a sampling of our controls suggests that they are unrelated to the disease segregating in this family.

The remaining six mutations are newly described in this family (the mutation at nt 15884, assayed by loss of the *Hae*III restriction site, might correspond to the *Hae*III morph 9 described by Brown[36], but their loss of the restriction site could be a result of substitutions in other positions). These mutations, at nt 8582, 10143, 11025, 12950, 13105 and 15884, affect five different OXPHOS subunits, ATPase 6, ND3, ND4, ND5 and Cyt b, respectively. The 11025 and 12950 mutations alter conserved amino acids[31–34], and are extremely rare (not observed in >100 controls). The mutation at nt 13105, while changing a highly conserved position in ND5[31–34], results in a conservative amino acid change. This mutation was also found in more than 9% of the controls tested, so it is unlikely to be disease-related. The other three mutations do not change evolutionarily conserved amino acids[31–34], but are present in <3% of the controls tested, and so might also have contributory effects to the disease phenotype.

All of the mtDNA mutations appeared homoplasmic upon sequencing both strands of DNA fragments amplified by PCR, and by SSCP/heteroduplex analysis. In addition, the analytic methods used to test for the mutations in at least 12–20 family members would have detected heteroplasmy if the normal sequence was present at levels

of 10% or more. All restriction digests were complete, because the PCR products were chosen to include a control site for each enzyme. Also, no evidence of heteroplasmy was found in the ASO hybridizations[21–23].

We investigated the possibility of heteroplasmy for the 1555 A to G mutation, which abolishes a *BsmA*I restriction site, by performing Southern blot analysis. The normal mtDNA has two *BsmA*I fragments of 1106 and 1460 bp in this region, while the patients have the corresponding mutant fragment of 2566 bp. We compared genomic DNA digests from three members (two deaf and one unaffected) of the Arab-Israeli pedigree, five members of the pedigrees with ototoxic deafness (4 from Family A and the proband from Family B) and a hearing control, and found no evidence of heteroplasmy, since the deaf pedigree members had no normal sized *BsmA*I fragments (data not shown).

The rare mutations from the Arab-Israeli pedigree, highlighted in bold in Table 1, were screened in the three pedigrees with ototoxic deafness. None of these rare mutations was found in any of them, except for the 1555 A to G mutation which was present in all three of the families.

Functional analysis of the 1555 rRNA mutation

The 1555 A to G mutation affects a highly conserved domain of the small rRNA (Fig. 2), which has two single-stranded regions with conserved sequence, separated by two structurally conserved stem-loops in organisms as diverse as bacteria, plants, invertebrates and mammals[28,29,37]. Bacterial studies have shown that this portion of the molecule is part of the aminoacyl site, in which mRNAs are decoded, and lies at the ribosomal subunit interface[9,10,38]. Aminoglycosides affect translational fidelity by binding to this decoding region and stabilizing mismatched aminoacyl tRNAs[9]. Several nucleotides in this region (Fig. 2) are implicated in aminoglycoside binding, as they have altered reactivity to chemical modifying agents when streptomycin, gentamicin, neomycin, kanamycin or hygromycin are bound[10]. Streptomycin also crosslinks this region (Fig. 2) to another part of the *E. coli* small rRNA[11], in which streptomycin resistance mutations are found[39,40].

Lastly, this region is important in aminoglycoside function, because aminoglycoside-resistance mutations (Fig. 2) were found in the analogous sites of small rRNAs in yeast mitochondria[12] and Tetrahymena[13]. Significantly, two of these mutations break the basepair which is adjacent to our mutation site, in effect "widening" the aminoglycoside-binding pocket. In contrast, the 1555 A to G mutation can produce a new C–G basepair, elongating the stem/loop and "tightening" this area, making the secondary structure resemble more closely that of the bacterial small rRNA. We postulate that the 1555 A to G mutation lengthens this helix, resulting in greater aminoglycoside binding and increased susceptibility to the effects of these antibiotics on translational fidelity.

Discussion

We analysed the mtDNA sequence in two different disorders with a maternally-transmitted susceptibility to non-syndromic deafness, one requiring an extrinsic agent and the other requiring a postulated nuclear mutation for phenotypic expression. A mtDNA mutation at nt 1555 in the 12S rRNA gene was identified, that appears to be the pathogenic mutation for the following reasons: (i) it was the only mutation common to all four families; (ii) it was not present in 278 normal hearing controls, most of whom are ethnically matched; (iii) no other rare mutation was found in the candidate genes of the three families with aminoglycoside-induced deafness; (iv) none of the other rare mutations identified in the Arab-Israeli family has been ascribed a pathologic role in any mitochondrial disorder or was found in the families with ototoxic deafness; (v) it changes a nucleotide in a highly conserved region of the 12S rRNA; and (vi) the mutated nucleotide in the 12S rRNA gene is in the region known to bind aminoglycosides[10], and in which aminoglycoside-resistance mutations have been found in other species[12,13].

The 1555 A to G mutation was the only one of the three sequence changes identified in the three families with ototoxic deafness which showed a unique correlation with the disease. The two other mutations, 663 A to G and 1736 A to G, were also found in these pedigrees, but both of these mutations are common Chinese polymorphisms, with a frequency of 10–11%. As both of these mutations occurred in 13 control individuals, the nt 1555 mutation most likely arose on a mitochondrial chromosome having these two mutations. We do not believe the 663 A to G and 1736 A to G by themselves are causative mutations, because the overall incidence of aminoglycoside ototoxic deafness is estimated at 1:10,000 (ref. 6). More importantly, at least four of the control individuals with both of these mutations received streptomycin, with no ill effects. The most immediate clinical implication of these studies is the avoidance of aminoglycosides for any maternal relatives in families with maternally-inherited aminoglycoside-induced deafness, as well as for any member in the maternal line of the Arab-Israeli pedigree. To our knowledge, no member of the Arab-Israeli pedigree has received aminoglycosides in the past.

While the studies of aminoglycoside action in other organisms implicated *a priori* the mitochondrial 12S rRNA gene in maternally-transmitted aminoglycoside-induced deafness, no clear candidate existed for the Arab-Israeli family. MtDNA functional analysis only suggested the mitochondrial ATPase 6, 8 and Cyt b genes as candidates for the mtDNA mutation, because altered enzymatic activities were found for the complexes in which these subunits participate[18]. Among the sequence changes which we identified, those at nt 8582 and 15884 affect the mtDNA genes ATPase 6 and Cyt b, respectively, results theoretically consistent with our biochemical findings[18]. However, as enzymatic increases have not yet been described in any mitochondrial disorder, it is equally possible that the enzymatic changes that we observed are benign variations, possibly related to the mitochondrial mutations which we identified in those genes and the pathogenic mutation is phenotypically silent in lymphoblastoid cells.

We found several family-specific mutations in the Arab-Israeli pedigree, which could contribute to the disease phenotype, or enhance the effect of the 1555 A to G mutation. By analogy to Leber's hereditary optic neuroretinopathy (LHON), a nerve-specific mitochondrial disorder which might also require the involvement of an additional nuclear gene[41,42], any or all of these rare mutations may interact to produce a "pathogenic haplotype." The majority of LHON patients have single mtDNA point mutations[43–46], but some LHON cases appear to be due to a multiplicity of mutations, with their

combinatorial effect resulting in disease[47–49]. Because the 1555 mutation was found in three other pedigrees with maternally-transmitted susceptibility to deafness, we favour this mutation as the causative defect in the Arab-Israeli pedigree. Possibly the additional mtDNA mutations in this family participate to impair oxidative phosphorylation below a threshold required in the developing cochlea. All of the family members tested had the pathogenic haplotype, with each mutation appearing homoplasmic (heteroplasmy, if present, would have been at levels <10% in blood). Therefore, the reason that unaffected family members with the identical mtDNA sequence are not deaf is presumably due to the absence of homozygosity for the putative autosomal recessive mutation.

We propose a two-hit model for the development of non-syndromic deafness in these pedigrees. In addition to the underlying 1555 A to G mutation, the disease can be precipitated by the exogenous administration of aminoglycosides, which accumulate in the cochlea[8], or, as in the Arab-Israeli pedigree, by the interaction of a hypothetical cochlear-specific ribosomal subunit with the 12S rRNA mutation. Either event would disturb or abolish the translational ability of the mitochondrial ribosome.

Methodology

DNA sources. DNA was isolated from blood samples except for the proband in *c*, the cousin of the Arab-Israeli proband, and 33 controls, in which the DNA was isolated from EBV-transformed lymphoblastoid cell lines. DNA was obtained from three Chinese families with aminoglycoside ototoxicity (Fig. 1), as well as from 20 maternally-related members, four "married-in" fathers and an adopted child from the Arab-Israeli pedigree[7]. The 278 controls included 138 Asians (117 Chinese, 21 non-Chinese), 60 Arab-Israelis, 55 Caucasians and 25 Blacks. All samples were obtained with informed consent.

PCR reactions. MtDNA was amplified[26] using 100 ng DNA, 10 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs and 1 U *Taq* DNA polymerase (Perkin-Elmer/Cetus) in a volume of 25–100 μl, with an initial 5 min denaturation at 95 °C, followed by 35 step-cycles of 95 °C, 30 s; 55 °C, 1 min; 65 °C, 1.5 min kb^{-1} in a Perkin-Elmer/Cetus thermal cycler.

SSCP analysis. SSCP analysis was performed as described[24], with the following modifications: MtDNA was amplified[26] in 60 overlapping PCR products (excluding nt 16101–529) which were electrophoresed on nondenaturing gels (0.5×MDE, ATBiochem; 0.6×TBE; 5% glycerol) covalently bound to GelBond PAG (FMC Bioproducts). Single stranded DNAs were visualized by silver staining (Bio-Rad).

Heteroduplex analysis. PCR products >420 bp were denatured and allowed to slowly reanneal, forming heteroduplexes[25] which migrate more slowly than homoduplexes in gels containing 1×MDE, ATBiochem and 0.6×TBE. Double stranded DNAs were visualized by silver staining (Bio-Rad).

M13 sequencing. MtDNA isolated from lymphoblastoid mitochondria of the Arab-Israeli proband's cousin[7,18] was digested with *HpaI*, *HpaI* + *DraI* or *Hin*cII. Restriction fragments were cloned into the *Sma*I-site of M13mp18, and transformed into DH5αF'IQ cells (BRL), with SURE™ cells (Stratagene) in the bacterial lawn to avoid rearrangements of the inserts. Plaques were screened by hybridization with mtDNA PCR products multiprime labelled (Amersham) with [α^{32}P]dCTP (ICN Biomedicals) and confirmed with restriction digestions. Sequencing used the Sequenase 2.0 (USB), and primers from Operon Technologies (purified with oligonucleotide purification cartridges and reagents from Applied Biosystems) or Oligos, etc. (used directly).

PCR sequencing. PCR products were purified from low-melting point agarose gels (GeneLine LMP agarose, Beckman Instruments), with Promega's Magic Prep kit and sequenced with the New England Biolabs cycle-sequencing kit, using [α-^{35}S]dATP (NEN/Dupont).

Mutational analysis. (i) Allele-specific oligonucleotide hybridizations[21,22]: PCR products were boiled, diluted in chilled 18× SSC, and spotted onto magnaNT nylon filters (Micron Separations) with a dot-blot manifold (Schleicher and Schuell). Synthetic 15-mer oligonucleotides (Oligos, etc.), with the mutant or normal nucleotide in the central position, were 3' end-labelled with [α-^{32}P]dCTP (ICN), or the digoxigenin-ddUTP supplied in a 3' end-labelling kit (Boehringer Mannheim). Hybridizations were at 37 °C, in the buffer recommended, in a hybridization incubator (Robbins Scientific). After initial washes in 2× SSC, 0.1% SDS, at room temperature, the stringent wash contained 3 M tetramethyl ammonium chloride (Sigma), 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, at 46 °C[23]. For digoxigenin-labelled probes, the labelled moiety was detected chemiluminescently, according to the manufacturer. (ii) For mutations which altered restriction sites, PCR products were synthesized which included an additional site for the enzyme being tested, as a positive control for complete digestion and heteroplasmy. Reaction products were digested according to manufacturer's instructions (New England Biolabs), with PCR products used directly or first isolated in low-melting point agarose with 50 mM Tris-acetate, pH 8.2 buffer. Restriction digests were electrophoresed and visualized by staining with ethidium bromide.(iii) Trapped-oligonucleotide nucleotide incorporation (TONI) assays were performed as described[27]. Briefly, a 5' biotinylated oligonucleotide, with its 3' end adjacent to the mutation site, was elongated by incorporation of the template-specified next nucleotide. Parallel reactions included only one radioactive [α-^{32}P]dNTP, corresponding to the normal or mutant sequence. The radiolabelled oligonucleotide reaction products were trapped by streptavidin-linked magnetic beads (Advanced Magnetics), washed and quantitated by Cerenkov counts in a liquid scintillation counter.

Southern blot analysis. 1 μg genomic blood DNA was digested with 8 U *BsmA*I (New England Biolabs). After electrophoresis through 1.5% agarose and capillary transfer to a nylon membrane (MagnaNT, Micron Separations), the blot was hybridized with an [α-^{32}P]dCTP-labelled (ICN) mtDNA fragment (a PCR product between nt 319 and 2590), labelled with Amersham's multiprime kit, in Boehringer Mannheim's hybridization buffer, at 55 °C for 16 h. The stringent wash contained 0.1× SSC, 0.1% SDS at 55 °C.

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