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A New Mitochondrial Disease Associated with Mitochondrial DNA Heteroplasmy

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Summary

A variable combination of developmental delay, retinitis pigmentosa, dementia, seizures, ataxia, proximal neurogenic muscle weakness, and sensory neuropathy occurred in four members of a family and was maternally transmitted. There was no histochemical evidence of mitochondrial myopathy. Blood and muscle from the patients contained two populations of mitochondrial DNA, one of which had a previously unreported restriction site for *AvaI*. Sequence analysis showed that this was due to a point mutation at nucleotide 8993, resulting in an amino acid change from a highly conserved leucine to arginine in subunit 6 of mitochondrial H^+-ATPase. There was some correlation between clinical severity and the amount of mutant mitochondrial DNA in the patients; this was present in only small quantities in the blood of healthy elderly relatives in the same maternal line.

Introduction

Human mitochondrial DNA (mtDNA) is a closed circular molecule 16,569 bp in length which is exclusively maternally inherited (Giles et al. 1980). The mitochondrial genome encodes two rRNAs, 22 tRNAs, and 13 polypeptides of the respiratory chain and energy-transducing system; seven subunits of complex I (NADH CoQ reductase), apocytochrome b; cytochrome oxidase subunits I, II, and III; and ATPase subunits 6 and 8 (Yatscoff et al. 1978; Chomyn et al. 1985*a*, 1985*b*). Defects of the mitochondrial genome have recently been identified in association with human disease, in mitochondrial myopathies and in Leber optic atrophy, both of which may exhibit maternal inheritance. Substantial deletions of a proportion of muscle mtDNAs have been demonstrated in 30%–40% of cases of mitochondrial myopathy (Holt et al. 1988*a*, 1988*b*; Zeviani et al. 1988; Moraes et al. 1989), and two patients with tandem duplications of one population of mtDNAs from leukocytes have subsequently been reported (Poulton et al. 1989). In both these instances, there was heteroplasmy—i.e., two different populations of mtDNA—within tissues. More recently, Wallace et al. (1988) observed a homoplasmic point mutation at position 11778 in mtDNA from all maternally related individuals in nine of 11 families with Leber optic atrophy.

The present paper reports a maternally inherited neurologic disease with some clinical features similar to those found in cases of mitochondrial myopathy, but this diagnosis was not confirmed histologically (Petty et al. 1986). All affected individuals had mtDNA heteroplasmy, a mixture of normal mtDNA, and a population containing a nucleotide change at position 8993, in blood and muscle.

Patients and Methods

Patients

The pedigree of the family is shown in figure 1. The index case (case 1) was a 47-year-old female who developed night blindness at the age of 12 years and was diagnosed as having retinitis pigmentosa. She was almost blind by the age of 30 years. At the age of 24 years she had a generalized seizure and was treated with phenytoin. In her early thirties she suddenly noticed unsteadiness on walking, which subsequently progressed; there had been no evidence of anticonvulsant toxicity.

On examination she could just perceive light, and

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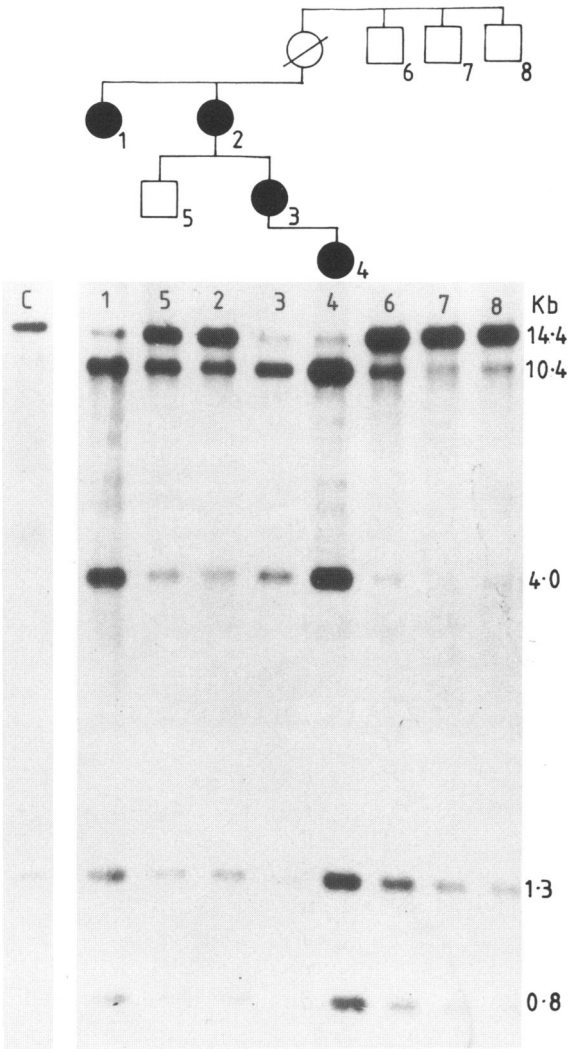

Figure 1 Pedigree of family (filled circles) [● = clinically affected; see text) with (*below*) mtDNA fragments after digestion with *Ava*I. In the control subject (C; leftmost lane), there were fragments 14.4, 1.3, and 0.8 kb in length (the last of these fragments is not visible in figure). In all the patients and their maternal relatives, a variable proportion of the 14.4-kb fragments was cleaved into two, one of 10.4 kb and one of 4.0 kb.

there were clumps of pigment in both retinae with bone spicule formation, typical of retinitis pigmentosa, and optic atrophy. Ocular movements were full, and there was no ptosis. There was no muscle weakness, but she had marked limb and gait ataxia. The ankle jerks were absent. Proprioception was mildly impaired at the toes, and pain appreciation was reduced in a stocking distribution. Hematologic and biochemical tests were normal, including creatine kinase and serum lactate concentrations. Electroencephalography (EEG) showed a low-amplitude record. Electromyography (EMG) was normal, as were motor-nerve conduction velocities (MNCV), but sensory action potentials (SAPs) were reduced in amplitude, indicating an axonal sensory neuropathy. Computerized tomography (CT) of the brain showed mild cerebral and cerebellar atrophy. Psychometry suggested cognitive deterioration, with a verbal IQ of 69. Quadriceps-muscle biopsy showed mild chronic partial denervation with collateral reinnervation. Sections stained by the Gomori trichrome method and for succinic dehydrogenase and cytochrome oxidase activity were normal. Electron microscopy showed occasional mitochondria with abnormal cristae, but there were no mitochondrial aggregates or paracrystalline inclusions.

The patient's sister, case 2, was asymptomatic at the age of 52 years but had a few clumps of retinal pigment and proximal muscle weakness. Her daughter, case 3, noticed reduced peripheral vision at the age of 25 years and was diagnosed as having retinitis pigmentosa. She was a poor athlete as a child. On examination at the age of 29 years she had retinitis pigmentosa with slightly constricted visual fields, mild proximal muscle weakness, extensor plantar responses, and mild gait ataxia. Serum lactate concentrations were normal. EMG showed an excess of short-duration spikey units but no clear evidence of myopathy. SAP amplitudes were at the lower end of the normal range, and NCVs were normal. Muscle biopsy was normal histochemically. In occasional fibers, electron microscopy showed small subsarcolemmal aggregations of mitochondria, some of which contained abnormal branching cristae. There were no paracrystalline inclusions.

Case 4 was delivered at term by cesarean section because of breech presentation. Fetal movements were reduced. She sat at the age of 13 mo and walked at the age of 24 mo. At the age of 28 mo she had an episode of fever, irritability, and lethargy and was generally unwell for 1 mo. She stopped walking for 5 mo. On examination at the age of 3 years, her height was 88 cm (fifth centile), her head circumference was 47 cm (third centile), and her weight was 11.5 kg (third centile). She only spoke single words. There was a pigmentary retinopathy. Tone was increased in the limbs, and the tendon reflexes were increased with extensor plantar responses. She had limb and gait ataxia. Serum lactate concentrations were normal. EEG showed an excess of irregular posterior slow components. There was cerebellar and brain-stem atrophy on CT scan. Electroretinogram showed small retinal responses. Muscle biopsy showed minor variation in fiber diameter, and fine

lipid droplets were seen in type 1 fibers; there were no ragged red fibers, and activities of succinic dehydrogenase and cytochrome oxidase were normal. No abnormal mitochondria were seen on electron microscopy.

The parents of cases 1 and 2 both died at the age of 72 years and were apparently free of neurologic disease or visual problems. Cases 6–8, age 76–88, and the brother of case 3 (case 5), age 29 years, had no clinical evidence of neurologic or retinal disease.

Methods

DNA was extracted from 10 ml blood from each subject by standard techniques (Old 1986). DNA from muscle was extracted as described elsewhere (Holt et al. 1988*a*). Five-microgram samples were digested with 10 units each of 28 restriction endonucleases (Holt et al. 1988*c*) under conditions recommended by the manufacturers (Bethesda Research Laboratories and Northumbria Biologicals Limited), with the addition of BSA and spermidine. The digested DNA fragments were separated by horizontal agarose gel (0.8%–1.6%) electrophoresis for 16 h at 45 V and then transferred to nylon membrane (Hybond-N; Amersham, U.K.) by Southern blotting. Purified Hela cell mtDNA was oligolabeled (Feinberg and Vogelstein 1983) with ^{32}P to a specific activity of $>1 \times 10^8$ cpm/μg. Prehybridization and hybridization were as recommended for Hybond-N. mtDNA fragments were visualized by autoradiography for 24–28 h at −70°C. Densitometry was performed using an LKB Ultroscan densitometer.

mtDNA in the region 8646–9199 was amplified by means of the polymerase chain-reaction (PCR) (Saiki et al. 1988), using thermostable *Taq* polymerase (Perkin-Elmer Cetus), primers CCGACTAATCACCACCCAAC (8648–8665) and TGTCGTGCAGGTAGAGGCTT (9180–9199), and a Hybaid Intelligent Heating Block. DNA was amplified in 36 three-step cycles: denaturation (91.6°C, 80 s), annealing (56°C, 100 s), and extension (71.5°C, 120 s). The amplified DNA was purified and concentrated twofold in Centricon 30 microconcentrators (Amicon). Ragged ends were filled using 1 unit Klenow (Northumbria Biologicals, Ltd) and 0.5 mM dNTPs (Pharmacia) at 37°C for 30 min. Blunt-ended ligation into *Sma*I-cut M13mp18 was mediated by T4 DNA ligase (Amersham) at 17°C overnight. The ligation reaction was transformed into $CaCl_2$-treated *Escherichia coli* JM 109 cells, clear colonies were cultured, and single-stranded M13 was purified by polyethylene glycol precipitation. Clones were sequenced by the dideoxy method of Sanger et al. (1977) by using a Sequenase kit (USB) and alpha ^{35}S-dATP (Amersham). Labeled oligonucleotides were resolved on polyacrylamide gels (8%) by elecrophoresis at 2,500 V (2–4 h).

Results

In all members of this family, digestion of leukocyte mtDNA with the restriction endonuclease *Ava*I resulted in an unusual pattern of fragments (fig. 1). Other digests suggested no variation from the published sequence of mtDNA (Anderson et al. 1981). Digestion with *Ava*I normally yields one large fragment of 14.4 kb and two small fragments, one each of 1.35 and 0.8 kb. Cases 1–3 were investigated in an earlier study (Holt et al. 1988*c*), and all showed two extra bands of approximately 10 and 4 kb, implying a restriction-site gain within the 14.4-kb fragment. This was initially interpreted as a normal polymorphism with partial digestion. Double digestion with *Ava*I and *Pvu*II cleaved the 10-kb fragment into two, one each of 6 and 4 kb, localizing the *Ava*I site gain to the ATPase 6 reading frame. Analysis of blood mtDNA from cases 4 and 8 and three distant maternal relatives (cases 5–7) indicated the presence of the additional 10- and 4-kb fragments, in very small amounts compared with the normal fragments in the last three (fig. 1 and table 1). Muscle from cases 1, 3, and 4 contained both populations of mtDNA, in proportions similar to those seen in blood.

PCR using primers at 8648–8665 and 9180–9199 produced a single fragment (300–400 bp) of amplified DNA from blood and muscle in cases 1 and 4 and in two control subjects. This was cleaved by *Ava*I in samples from both patients but not in those from controls. Analysis of the sequence 8665–9180 (Anderson et al. 1981) identified four potential sites—8784, 8900, 8993, and 9062—at which a single base change could generate an *Ava*I recognition site. Sequencing of two clones from case 1 and of four clones from case 4 (all from muscle mtDNA) identified a thymine-to-guanine change at 8993 in all instances, creating the sequence CCCGGG recognized by *Ava*I. This sequence includes the recognition site of *Hpa*II. As expected, digestion of the patients' PCR product with *Hpa*II gave the same result as *Ava*I. None of the other potential *Ava*I sites could have resulted in an accompanying *Hpa*II site gain.

The base substitution at position 8993 observed here leads to an amino acid change from hydrophobic leucine to hydrophilic arginine at position 156 in subunit 6 of mitochondrial H^+-ATPase. This leucine codon is

Table 1

Proportions of Normal and Abnormal Leukocyte mtDNA in Eight Members of a Family with a Novel Mitochondrial Disease

Case (sex)	Age (years)	Age at Onset (years)	% Normal mtDNA	% Abnormal mtDNA
1 (F)	47	12	18	82
2 (F)	52	Asymptomatic	77	23
3 (F)	29	25	12	88
4 (F)	3	Prenatal	3	97
5 (M)	29	Normal	66	34
6 (M)	76	Normal	86	14
7 (M)	78	Normal	94	6
8 (M)	88	Normal	91	9

conserved in bovine, mouse, sea urchin, *Xenopus laevis,* and *Drosophila* mtDNAs (table 2; Bibb et al. 1981; Anderson et al. 1982; Jacobs et al. 1988).

Discussion

The patients reported here exhibited a variable neurologic syndrome comprising typical retinitis pigmentosa, ataxia, seizures, dementia, proximal neurogenic muscle weakness, sensory neuropathy, and developmental delay. Although all of these features may be observed in cases of morphologically defined mitochondrial myopathy (DiMauro et al. 1985; Petty et al. 1986), classical retinitis pigmentosa with bone spicule formation, as opposed to a salt-and-pepper retinopathy, is very unusual (Mullie et al. 1985), and muscle weakness is nearly always myopathic in origin, as opposed to neurogenic as was the case in case 1. The presence of ragged red fibers in skeletal muscle is not essential for the diagnosis of mitochondrial myopathy, as these have been absent both in some cases (van Erwen et al. 1987) and in affected relatives of others (Rosing et al. 1985). Nevertheless, such patients have usually had symptoms and signs confined to the central nervous system, whereas two of the patients reported here had muscle weakness. The minor ultrastructural changes in the muscle of cases 1 and 3 were suggestive of mitochondrial dysfunction.

The abnormality of the mitochondrial genome identified in this kindred is unique. Despite the fact that the patients do not appear to have mitochondrial myopathy as usually defined, there are a number of reasons for suspecting that the mtDNA abnormality is related to their disease. Although the pedigree is small, inheritance appears to be maternal. There was a correlation between the amount of mutant mtDNA and both the presence and severity of neurologic disease in affected members of this kindred. The mutation at position 8993 observed here has not been reported in over 600 normal subjects from a wide range of racial backgrounds whose mtDNAs were studied for polymorphisms with *Hpa*II (Bonne-Tamir et al. 1986; Brega et

Table 2

Amino Acids 153–160 of ATPase Subunit 6 in Patients, Normal Humans, and Other Species

Source	Amino Acids							
Patients	Pro	Met	Ala	Arg[a]	Ala	Val	Arg	Leu
Normal humans	Pro	Met	Ala	Leu	Ala	Val	Arg	Leu
Bovine	Pro	Met	Ala	Leu	Ala	Val	Arg	Leu
Mouse	Pro	Met	Ala	Leu	Ala	Val	Arg	Leu
Sea urchin	Pro	Iso	Ala	Leu	Gly	Val	Arg	Leu
Xenopus laevis	Pro	Leu	Ala	Leu	Ala	Val	Arg	Leu
Drosophila	Pro	Gly	Thr	Leu	Ala	Val	Arg	Leu

[a] Amino acid substitution in abnormal proportion of ATPase subunit 6; see text for references.

al. 1986*a*, 1986*b*; Horai and Matsunuga 1986; Cann et al. 1987; Holt et al. 1988*c*). This mutation results in a substitution of a hydrophilic amino acid for the normal hydrophobic leucine which is highly conserved in subunit 6 of ATPase in other species. This leucine normally occurs in a consistently hydrophobic region of the polypeptide (table 2), and therefore the substitution might be expected to affect the structure and function of the enzyme in these patients. Polarographic studies of muscle mitochondria were not performed.

The presence of two populations of mtDNA (heteroplasmy), one normal and one mutant, in this maternal lineage is also of interest. It is not surprising that all six clones sequenced demonstrated the 8993 mutation, given that the mutant mtDNA was present in such high proportions in cases 1 and 4. mtDNA heteroplasmy has never been described in control subjects (Horai et al. 1984; Horai and Matsunuga 1986; Cann et al. 1987; Holt et al. 1988*c*), despite the high mtDNA mutation rate which implies a need for heteroplasmy during the transition from one genotype to another. Hauswirth and Laipis (1982) demonstrated heteroplasmy in a single maternal line of Holstein cows. They later suggested that mtDNA could switch completely from one genotype to another in a single generation if the number of mtDNAs is greatly reduced at some point in oogenesis (Hauswirth and Laipis 1985). This would have to take place prior to mtDNA amplification in maturing ova (Clayton 1982).

The patients reported here represent the third example of mtDNA heteroplasmy associated with disease in man. We have previously observed a deleted population of muscle mtDNA in 40% of patients with mitochondrial myopathies (Holt et al. 1988*a*, 1988*b*). Leber hereditary optic neuropathy, a maternally transmitted disease causing blindness, is often associated with an mtDNA mutation at position 11778 (Wallace et al. 1988). In a number of patients and their maternal relatives, we have observed a mixture of mutant and normal mtDNAs (Holt et al. 1989). These observations suggest that persistent heteroplasmy and deleterious mtDNA mutations are related in some way. It is probable that the rapid switch from one mtDNA type to another—a switch which seems to occur during the evolution of harmless, and possibly advantageous, polymorphisms—does not take place when mtDNA mutations are harmful, because of selection in favor of normal mtDNAs. Survival would be less likely if these mutations were homoplasmic.

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