Correlation between the Clinical Symptoms and the Proportion of Mitochondrial DNA Carrying the 8993 Point Mutation in the NARP Syndrome

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ABSTRACT

We describe a four-generation family with a maternally inherited mitochondrial disorder. The symptoms were restricted to the CNS and muscle, the most common features being subacute necrotizing encephalomyopathy, cognitive impairment, ataxia, retinitis pigmentosa, infantile spasms, and optic atrophy. A point mutation at the nucleotide 8993 of the gene encoding subunit 6 of the ATP synthase, associated with the neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP) syndrome, was shown to be inherited maternally in this family, and a clear correlation was found between the clinical severity of the disease and the proportion of mutant mtDNA. Analysis of oxidative phosphorylation in mitochondria carrying 80% mutant mitochondrial DNA showed a reduction of the ATP generation rate coupled to substrate oxidation. (*Pediatr Res* **37: 634–639, 1995**)

Abbreviations
mtDNA, mitochondrial DNA
NARP, neurogenic muscle weakness, ataxia, retinitis pigmentosa
nt, nucleotide
LHON, Leber hereditary optic neuroretinopathy
MELAS, mitochondrial encephalomyopathy, lactic acidosis and strokelike episodes
CT, computerized tomography
PCR, polymerase chain reaction

Point mutations of mtDNA are associated with a number of maternally inherited diseases. The most frequent of these diseases are LHON (1), MELAS (2, 3), myoclonus epilepsy and ragged-red fibers (4), and NARP syndromes (5). The point mutations are usually heteroplasmic; both normal and mutant mtDNA are present in the same subject. In LHON, the majority of the families have been reported to be homoplasmic for the two most common mutations, but low-level heteroplasmy has also been described (6, 7).

The proportion of mutant mtDNA may vary in the offspring of one mother as well as in different tissues of the same individual. This is most probably caused by different proportions of mutant mtDNA in the mitochondria of ova and random distribution of these organelles into different cell types in embryogenesis.

NARP syndrome is associated with a heteroplasmic mtDNA point mutation, a T→G change at nt 8993 of mtDNA (5) of the gene encoding the subunit 6 of F_1F_0 ATP synthase. Two patients with Leigh's syndrome have been described with a T→C change (8) at the same nucleotide. These nucleotide changes substitute arginine (5) or proline (8) for leucine. The former amino acid change has been shown to inhibit H^+ translocation in *Escherichia coli* ATP synthase (9).

The 8993 point mutation has also been found to be associated with phenotypes other than NARP itself, including Leigh's syndrome, *i.e.* subacute necrotizing encephalomyopathy (10), psychomotor retardation, seizures, infantile spasms, and dementia (8, 11–16). Morphologic findings typical of mitochondrial myopathy, such as ragged-red fibers, have not been found in association with this point mutation (5, 11–13, 17).

We studied a four-generation family with the mtDNA 8993 T→G point mutation. The proportion of mutant mtDNA was accurately determined using the solid-phase minisequencing method (18–21), and the symptoms of the patients correlated with the proportion of mutant mtDNA. The effect of mutant

mtDNA on oxidative phosphorylation was analyzed in mitochondria isolated from cultured patient lymphoblasts.

METHODS

Subjects

Figure 1 shows the pedigree with the maternally inherited mitochondrial disorder, and Table 1 summarizes the clinical findings in the family members.

Subjects with no or mild symptoms. II/1, 5–8, 10, and 11, and III/7 and 9 had no record of ill health. Two of them, II/6 and 8, underwent ophthalmologic examination, and no abnormality was found. Subjects I/1–2, II/2–4, III/1 and 8, and IV/1 were examined by us. An ophthalmologic examination was performed on subjects I/2, III/1, and IV/1, and neither retinitis pigmentosa nor optic atrophy was present, although subject III/1, a 37-y-old woman, had recently noticed visual impairment in the dark. Subjects I/2 and II/8 had migraine. The other subjects were either healthy or suffered from diseases that were probably acquired (cancer of the ovary, glaucoma, high blood pressure).

Patients. II/9, III/2 and 6, and the two deceased infants, IV/2 and 3, were examined by us. Results from previous examinations, including ophthalmologic follow-up, were available from hospital records. Records were also available for the three other deceased patients, III/3–5.

Patient II/9 was a mildly mentally retarded, visually handicapped 50-y-old man at the time of examination. He had had migraine since childhood and learning problems at school. Retinitis pigmentosa and cognitive impairment were diagnosed at the age of 30 y. He was slender, and his height was +0.5 SD. He had neither ataxia nor muscle weakness. Cerebral and cerebellar atrophy was detected by CT scanning, and the sensory potentials could not be elicited in electroneuromyography.

Patient III/2 was a visually handicapped 35-y-old woman with cognitive impairment and a mild sensorineural hearing deficit. She had learning difficulties at school. Retinitis pigmentosa was diagnosed at the age of 14, after which her vision had gradually deteriorated. She was slender, but had neither muscle weakness nor ataxia. Her height was +1 SD.
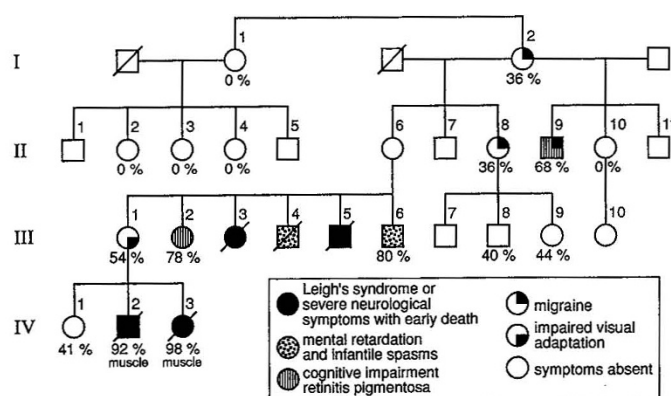
Patient III/3 developed normally until the age of 2.5 mo, when she began to have seizures. A small ventricular septal defect was suspected because of a cardiac murmur. She died suddenly of pneumonia at the age of 3.5 mo. An autopsy was refused.

Patient III/4 developed normally until the age of 6 mo, when infantile spasms manifested themselves. Hypsarrhythmia was detected in an EEG, and he was treated with ACTH, followed by phenobarbital and phenytoin, but without response. At the age of 8 y he was severely mentally retarded and hypotonic with brisk tendon reflexes and an unsteady gait, his vision and the optic fundi were normal, but visual impairment developed later. He died of pneumonia at the age of 17. An autopsy was refused.

Patient III/5 was hypotonic and had a high-pitched cry after birth. He needed to be tube-fed and began to have seizures with apneic spells at the age of 6 mo. At that time he was severely retarded and floppy. Optic atrophy and retinal degeneration were noted on neuroophthalmologic examination. His electroneuromyographic examination was normal. He developed poorly and died of pneumonia at the age of 1 y. An autopsy was refused.

Patient III/6 developed normally until the age of 5 mo when hypotonia was detected. Infantile spasms with hypsarrhythmia manifested themselves in EEG at the age of 9 mo, and frequent seizures continued until 2 y of age in spite of anticonvulsive medication. He learned to speak and walk after the age of 2 y, but suffered from ataxia and vomiting. Retinitis pigmentosa was discovered at the age of 10. Muscle power and tendon reflexes were normal, but he fatigued easily during sustained physical activity. At present he is 20 y of age, mentally retarded, and visually handicapped.

Patient IV/2 was examined after birth owing to reduced responsiveness. He was tube-fed and began to have extension fits with apneic spells at the age of 4 mo. His EEG was progressively abnormal with periodic spike and wave bursts and low-voltage background activity. Generalized atrophy was seen in CT scanning. He had optic atrophy and retinal degeneration. The visual evoked potentials could not be elicited. At the age of 7 mo he was floppy and severely mentally retarded with poor reaction to sounds. He died of pneumonia at the age of 2 y. An autopsy was refused.

Patient IV/3 was hypotonic after birth, and her motor development was slow. From the age of 6 mo she had extension fits and apneic spells, and on examination she was floppy and retarded with poor visual contact. Her condition worsened rapidly, and she died of respiratory failure at the age of 7 mo. General and neuropathologic autopsy studies were performed, and all the structural alterations detected were compatible with the Leigh's syndrome.

Tests of sera and cerebrospinal fluid. Tests revealed occasionally elevated blood lactate values, up to 5.2 mmol/L (normal, <1.6 mmol/L) in the severely affected infants. Cerebrospinal fluid was studied in six patients, and it did not contain increased amounts of protein. The cerebrospinal fluid lactate value of patient II/9 was within the normal range.

Figure 1. Family pedigree showing the symptoms of the subjects with 8993 T->G mutation and the proportion of mutant mtDNA in leukocytes, except in patients IV/2,3 of whom the proportion of mutant mtDNA was determined from muscle.

Table 1. *Clinical findings in the symptomatic members of the family*

Findings	Patient								
	III-3	IV-3	III-5	IV-2	III-4	III-6	III-2	II-9	III-1
Age at present/at death †(y)	0.25†	0.6†	1†	2†	17†	20	34	50	37
Age of onset (y)	0.15	Birth	Birth	Birth	0.5	0.5	<15	<15	36
Optic atrophy	n.d.	+	+	+	n.d.	−	−	−	−
Retinal degeneration	n.d.	+++	+++	+++	n.d.	++	++	++	(+)
Ataxia	−	−	−	−	++	+	−	−	−
Seizures	+++	+++	+++	+++	++	++	−	−	−
Cognitive impairment	n.d.	+++	+++	+++	++	++	+	+	−
Migraine	n.d.	n.d.	n.d.	n.d.	−	−	−	+	−
Birth weight (g)	3950	4630	4130	4690	4400	4890	4300	n.d.	n.d.
Height (cm)	n.d.	55	50	55	52	54	n.d.	n.d.	n.d.
Apgar score	n.d.	8/9	7/7	7/9	9/10	10	n.d.	n.d.	n.d.

n.d. = no data, (+) = impaired vision in the dark with normal neuroophthalmologic examination.

Tissue Samples and Cell Cultures

Subject III/6 underwent needle muscle biopsy. One part of the specimen was frozen at −70°C for mtDNA studies, and another part was fixed in a 2.5% glutaraldehyde solution for electron microscopic studies. A surgical muscle biopsy specimen from patients IV/2 and 3 was available for light microscopic study. Peripheral venous blood samples with EDTA as the anticoagulant were stored at −20°C.

Lymphoblast cell lines were established from two control individuals and patient III/6 by EBV transformation of peripheral lymphocytes. Cells were cultured in RPMI 1640 medium supplemented with 25 mmol/L Hepes, 20 mmol/L sodium bicarbonate, 17% FCS, 3.7 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma Chemical Co.).

DNA Analysis

Total DNA was isolated from leukocytes and tissue samples in accordance with standard procedures (22). The paraffin-embedded tissue samples had originally been prepared for histologic studies and had been stored at room temperature for up to 8 y. The samples were deparaffinized (23), and DNA was released from cells by a rapid lysis method (24).

The oligonucleotides. Oligonucleotides for PCRs (25) and solid-phase minisequencing reaction (18) were synthesized on a 382A Applied Biosystems DNA/RNA synthesizer (Applied Biosystems Inc., Foster City, CA). The upstream primer, equal to the sequence between nt 8967 and 8988 of mtDNA, was biotinylated. The downstream primer was complementary to the mtDNA sequence between nt 9027 and 9008. The detection primer for the minisequencing reaction was designed to hybridize adjacent to the nt 8993 mutation site and was homologous to nt 9013–8994. The specific hybridization temperatures for the primers were calculated as per McGraw *et al.* (26).

PCR. DNA (5 ng) was amplified with the biotinylated upstream primer at 0.2 μmol/L, the downstream primer at 1 μmol/L, and the dNTPs at 0.2 mmol/L concentrations using 1.25 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega, Madison WI) in 50 μL of its buffer on a programmable heating block (MJ Research Inc.). The conditions were as follows: 95°C 5 min, followed by addition of the *Taq* polymerase, then 20 cycles as follows: 94°C, 1 min; 60°C, 1 min; and 72°C, 1 min.

Solid phase minisequencing reactions. Reactions were performed essentially as previously described (20). The detection step was modified as follows. The nucleotide-specific minisequencing reaction mixture contained 10 pmol detection primer and 0.25 U *Taq* DNA polymerase in its buffer and 15 pmol of [^{3}H]dATP (Amersham International plc, Amersham, UK), 2.78 or 2.59 TBq/mmol, to detect the normal T^{8993}, or 15 pmol of [^{3}H]dCTP (Amersham), 2.26 TBq/mmol, to detect the mutant G^{8993}. The eluted radioactivity was measured in a liquid scintillation counter (1209 Rackbeta, LKB). The result of the assay is expressed as the ratio between the incorporated [^{3}H]dCTP and the total radioactivity incorporated in each sample. The percentage of mutant mtDNA was calculated from this ratio taking into account the specific activities of the [^{3}H]dNTPs used.

Analysis of Oxidative Phosphorylation

Mitochondria were isolated from the EBV-transformed lymphoblastoid cells derived from patient III/6 as previously described (27), with two modifications: the cell pellet after the first centrifugation at 10 000 × *g* was resuspended and gently homogenized in 0.25 mol/L sucrose and 0.2 mmol/L EGTA; the final mitochondrial pellet was also resuspended in this buffer.

Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone-sensitive ATP generation coupled to substrate oxidation was analyzed fluorometrically in isolated mitochondria with hexokinase and glucose-6-phosphate dehydrogenase (28). Rotenone-sensitive α-ketoglutarate:ferricyanide reductase activity was determined in 0.25 mol/L mannitol, 10 mmol/L KCl, 10 mmol/L potassium phosphate, pH 7.2, 0.2 mmol/L EDTA, 1 mmol/L $MgCl_2$, 0.5 mmol/L ADP, 10 mmol/L α-ketoglutarate, 0.5 mM L-malate, 0.7 U/mL hexokinase, 20 mM D-glucose, 2 mmol/L KCN, and 0.1 mmol/L ferricyanide with and without 0.5 μmol/L carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. Ferricyanide reduction was followed at 420–500 nm, and samples for glucose-6-phosphate determination were acidified with perchloric acid. The glucose-6-phosphate concentration was determined as described elsewhere (28). Protein

concentrations were assayed with BCA protein assay reagent (Pierce Chemical Co, Oud Beijerland, The Netherlands).

RESULTS

Clinical examination. The proportion of mutant mtDNA in the leukocytes or muscle of each individual studied is indicated in the pedigree (Fig. 1) and shown in Table 2. The subjects were divided into four groups based on the severity of the clinical symptoms: *1*) The most severe form of the disease was a lethal encephalomyopathy giving symptoms immediately after birth and leading to death during infancy or early childhood (subjects III/3 and 5, and IV/2 and 3). The infants had seizures, EEG abnormality, apneic spells, and feeding problems. Optic atrophy and retinal degeneration were present and neuropathologically verified Leigh's syndrome was found in the autopsied case (subject IV/3). Light microscopic examination of the muscle biopsy specimens of patients IV/2 and 3 was normal. *2*) In the second group the disease manifested itself during the first year of life as severe mental retardation, ataxia, EEG-verified infantile spasms and retinitis pigmentosa (subjects III/4 and 6). *3*) The patients of the third disease group had cognitive impairment and retinitis pigmentosa leading to a severe visual handicap (II/9 and III/2). A mild sensorineural hearing deficit and early-onset migraine were also present. The slowly progressive symptoms manifested themselves during the second or third decade. *4*) The most mildly affected subjects had subtle symptoms possibly associated with the disease, *e.g.* migraine (I/2 and II/8) and slight impairment of visual

adaptation in the dark (III/1), although the neuroophthalmologic findings were normal. Some of the subjects were completely asymptomatic.

DNA analysis. The results of mtDNA analysis revealing the proportion of the mutant mtDNA in patients' samples based on the solid-phase minisequencing method are shown in Table 2 and in Figure 1.

Morphologic analysis. Light microscopic examination of the muscle of patient IV/2 or IV/3 did not reveal any significant morphologic changes, but an accumulation of large mitochondria was seen in the electron microscopic examination of the muscle of patient III/6 (Fig. 2).

Analysis of oxidative phosphorylation. Table 3 shows the results of the biochemical analysis of isolated mitochondria. In mitochondria from lymphoblast cells carrying 80% mutant mtDNA uncoupler-sensitive ATP generation rate coupled to substrate oxidation was 64% of the control values.

DISCUSSION

The point mutation at nt 8993 of mitochondrial DNA results in a variety of clinical symptoms ranging from Leigh's syndrome, mental retardation, and ataxia to milder manifestations, such as retinitis pigmentosa (5, 11–17). We studied a large

Table 2. *Results of the quantification of the 8993 T→G point mutation in the samples of the family members, obtained by the solid-phase minisequencing method*

Samples	CPM_C	CPM_A	$CPM_C/CPM_A + CPM_C$	Percent of mutant mtDNA*
I/1$_{leukocyte}$	33	4780	0.007	0
I/2$_{leukocyte}$	4550	9320	0.33	36
II/2$_{leukocyte}$	41	5420	0.0075	0
II/3$_{leukocyte}$	52	4480	0.011	0
II/4$_{leukocyte}$	90	6420	0.014	0
II/8$_{leukocyte}$	2230	4670	0.32	36
II/9$_{leukocyte}$	5620	3210	0.64	68
II/10$_{leukocyte}$	30	4140	0.007	0
III/1$_{leukocyte}$	3820	3990	0.49	54
III/1$_{muscle}$	2140	1790	0.54	60
III/2$_{leukocyte}$	7170	2420	0.75	78
III/6$_{leukocyte}$	2540	781	0.76	80
III/6$_{muscle}$	3980	863	0.82	85
III/8$_{leukocyte}$	3020	5640	0.35	40
III/9$_{leukocyte}$	1400	2210	0.39	44
IV/1$_{leukocyte}$	2010	3500	0.36	41
IV/2$_{muscle}$	922	96.1	0.91	92
IV/3$_{muscle}$	5140	119	0.98	98
IV/3$_{brain}$	3350	176	0.95	96
IV/3$_{heart}$	1310	165	0.88	91
IV/3$_{liver}$	6350	1290	0.83	86
IV/3$_{kidney}$	2230	81.6	0.96	97
No DNA	43	15		

* Calculated from the specific activities of the [^{3}H]dNTPs used, as described in "Methods."
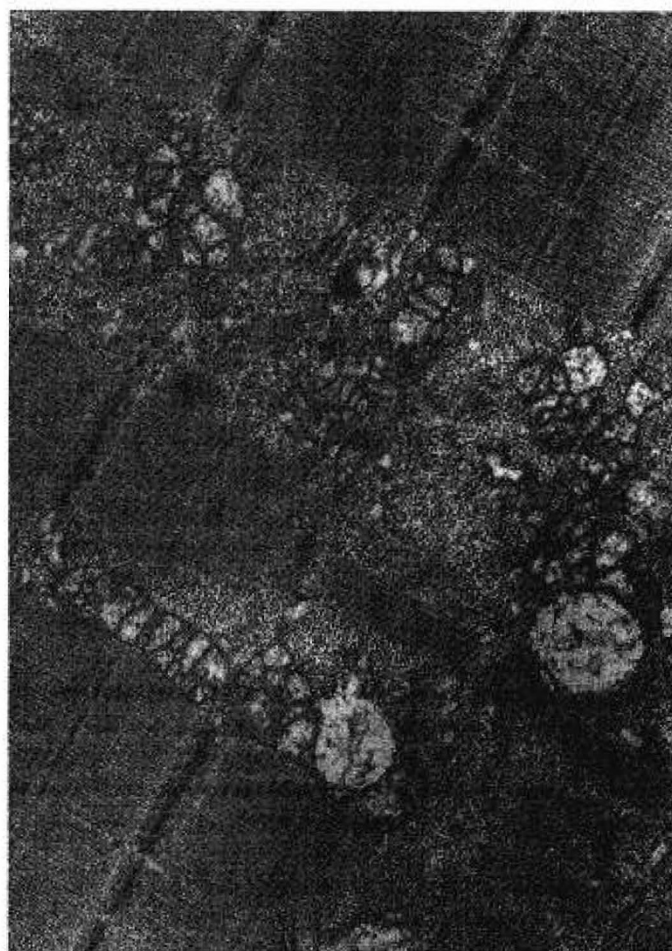

Figure 2. Electron micrograph of the muscle specimen of patient III/6 showing large interfibrillar accumulations of enlarged and vacuolated mitochondria. Magnification × 14,000.

Table 3. *Phosphorylation coupled to the oxidation of*
α-ketoglutarate (α-KG) by ferricytochrome c (cyt c) in
mitochondria isolated from lymphoblasts of patient III/6 and two
control individuals

Cell line	α-KG:cyt c (nmol/min mg)	ATP:2e$^-$
Patient III/6 ($n = 5$)	114.4 ± 33.9	0.89 ± 0.08
Controls ($n = 7$)	97.1 ± 42.6	1.38 ± 0.14

Values for α-KG:cyt c correspond to the rate of reduction of ferricyanide
expressed as nmol/min mg. Mean ± SD, number of experiments on separate
mitochondrial preparations in parentheses.

family in which the 8993 mutation was present in four gener-
ations. The clinical symptoms included those mentioned above,
as well as infantile spasms and migraine. A remarkable phe-
notypic variation is a well-known feature of the disorders
caused by mtDNA mutations. In our family, the clinical vari-
ation was tightly connected with the proportion of mutant
mtDNA in the tissues of the patients. Four infants died, of
whom two had over 90% of mutant mtDNA, and one had
Leigh's syndrome verified by autopsy. The patient who had
85% mutant mtDNA in the muscle had severe mental retarda-
tion, ataxia, retinitis pigmentosa, and infantile spasms. The
patients with 60–80% mutant mtDNA had cognitive impair-
ment and retinitis pigmentosa, and those with less than 60%
mutant mtDNA had migraine, impairment of visual adaptation
in the dark, or were completely asymptomatic. These results
are consistent with previous results (11–13, 15). However,
Shoffner *et al.* (14) and Yoshinaga *et al.* (17) found Leigh's
syndrome in patients with only 77% of mutant mtDNA in the
brain or 56% in the muscle, respectively. The variation in the
proportion of mutant mtDNA in patients with Leigh's syn-
drome in different reports cannot be explained by method-
ologic differences only. The proportion of mutant mtDNA
resulting in a specific phenotype may be family specific, *e.g.*
the result of other inherited traits either protecting or making
cells more susceptible to mitochondrial dysfunction.

In previous studies the proportion of mutant mtDNA has
been determined using electrophoretic separation of restriction
fragments after PCR amplication, followed by densitometry.
This method has been shown to be semiquantitive because of
heteroduplex formation in the last cycle of PCR and overesti-
mation of undigested fragments (29). This is avoided by adding
a radioactive nucleotide in the last cycle of PCR (29) or by
using the solid-phase minisequencing method, in which the
result obtained reflects directly the original proportions of
mutant and wild-type mtDNA (19, 20).

Of the patient I/2 offspring, one lineage resulted in severely
affected individuals including four severely affected infants,
who died in early infancy, another lineage was homoplasmic
for normal mtDNA, and a third lineage included asymptomatic
carriers of the mutation. This finding in this largest family so
far described with the 8993 point mutation differs from the
previous studies (5, 11, 14–16) that suggested increasing se-
verity of clinical symptoms and proportion of mutant mtDNA
in subsequent generations, *i.e.* anticipation. Our pedigree
shows anticipation-like phenomenon only in one branch,
whereas in the others the mutant mtDNA population seems to

be disappearing. Possibly a tight "bottleneck" of mtDNA exists
in the early phases of embryogenesis, *i.e.* the number of
mtDNA molecules becomes dramatically reduced, and the
mtDNA molecules of the fetus originate from a small number
of mtDNA copies (30). In Holstein cows it has been shown that
a nucleotide at a polymorphic site may change into another
reaching homoplasmy in only two generations (30), thus sup-
porting the reduction theory, whereas Howell *et al.* (31) have
demonstrated the conservation of heteroplasmy of a neutral
polymorphism over three generations in a LHON family,
suggesting the opposite.

Mutant mtDNA has been shown to accumulate in certain
cells of affected tissues, whereas others may harbor less mutant
mtDNA (32). It has therefore been argued that conclusions
about the correlation between a certain phenotype and the
proportion of mutant mtDNA cannot be reliably drawn by
summing the information of a large number of cells, but should
rather be determined by single-cell PCR or *in situ* hybridization
of affected tissue (32). In our patients the severity of the
clinical symptoms clearly correlated with the proportion of
mutant mtDNA in the leukocytes and muscle samples, and the
proportion of mutant mtDNA in the leukocytes was very close
to that in the muscle. This finding differs from that in, for
example MELAS, in which it has been suggested that selection
in rapidly diving tissues is the cause of large differences in the
proportion of mutant mtDNA in the muscle and blood (20).
The preservation of the 8993 mutation in the leukocytes sug-
gests that the mutation, affecting a protein-coding gene, does
not cause very severe dysfunction of the mitochondria.

Inasmuch as the dysfunction of cells in mitochondrial dis-
eases is caused by energy deficiency, direct evidence of dis-
turbed energy metabolism is necessary in each syndrome to
demonstrate the pathogenity of a certain mutation. The patho-
genic effect of the 8993 mutation has been studied in *Esche-
richia coli*, in which the corresponding amino acid substitution
was constructed and expressed. The mutation inhibited nearly
totally the reverse proton translocation by the F_1F_0 ATPase (9).
A decreased ATP synthesis was also previously detected in
mitochondria from lymphoblastoid cells derived from a patient
carrying the 8993 point mutation in mtDNA. Because respira-
tion rate was not studied in the corresponding conditions the
defect could not be assigned to ATP synthase activity alone
(33), although the reduction also in the reverse ATPase activity
suggested it. We studied ATP synthesis and oxidation activity
in lymphoblasts carrying the point mutation and detected a
partial reduction in the phosphorylation coupled to substrate
oxidation, locating the defect into the ATP synthase specifi-
cally. However, the biochemical defect was not as severe as
might be expected from the high percentage of mutant mtDNA,
or from the profound inhibition of proton translocation activity
demonstrated in the *E. coli* construct (9). Such a difference may
be explained by differences in experimental conditions and by
different organisms and further studies on the effect of the
mutation on oxidative phosphorylation are warranted.

Syvänen and the assistance of Dr. Ilkka Tarvainen in the investigation of patient II/9.

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