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Genetic and Biochemical Impairment of Mitochondrial Complex I Activity in a Family with Leber Hereditary Optic Neuropathy and Hereditary Spastic Dystonia

D. D. De Vries,[1] L. N. Went,[3] G. W. Bruyn,[4] H. R. Scholte,[5] R. M. W. Hofstra,[6] P. A. Bolhuis,[7] and B. A. van Oost[2]

Departments of [1]Pediatrics and [2]Human Genetics, University Hospital Nijmegen, Nijmegen; Departments of [3]Human Genetics and [4]Neurology, University of Leiden, Leiden; [5]Department of Biochemistry, Cardiovascular Research Institute, Erasmus University, Rotterdam; [6]Department of Medical Genetics, University of Groningen, Groningen; and [7]Department of Neurology, Academic Medical Center, Amsterdam

Summary

A rare form of Leber hereditary optic neuropathy (LHON) that is associated with hereditary spastic dystonia has been studied in a large Dutch family. Neuropathy and ophthalmological lesions were present together in some family members, whereas only one type of abnormality was found in others. mtDNA mutations previously reported in LHON were not present. Sequence analysis of the protein-coding mitochondrial genes revealed two previously unreported mtDNA mutations. A heteroplasmic A→G transition at nucleotide position 11696 in the ND4 gene resulted in the substitution of an isoleucine for valine at amino acid position 312. A second mutation, a homoplasmic T→A transition at nucleotide position 14596 in the ND6 gene, resulted in the substitution of a methionine for the isoleucine at amino acid residue 26. Biochemical analysis of a muscle biopsy revealed a severe complex I deficiency, providing a link between these unique mtDNA mutations and this rare, complex phenotype including Leber optic neuropathy.

Introduction

Leber hereditary optic neuropathy (LHON) is a maternally inherited form of acute or subacute bilateral adult-onset blindness with a mean age at onset of 27 years, but with a wide range of 6–60 years. Central vision is lost rapidly in both eyes, but peripheral vision is usually retained. Complete blindness rarely results. There is a substantial sex bias, with ~85% of affected persons being males, although in Japan only 60% of the patients are males (Newman 1993; Nikoskelainen 1994). As a general rule, the loss of vision is permanent, but improvement in some affected individuals has been reported (Mackey and Howell 1992; Johns et al. 1993). LHON can be considered as a mitochondrial multisystem disorder (Nikoskelainen 1994), since, in addition to the impairment of vision, 15 other symptoms, due to heart conduction defects, peripheral neuropathy, and/or demyelinating disease, have been reported to be present in addition to LHON in some patients (Newman et al. 1991; Newman 1993).

A number of mtDNA mutations have been associated with LHON. Depending on the ethnic background, ~40%–90% of LHON cases are associated with the 11778 mutation (Wallace et al. 1988; Nakamura et al. 1992). The two other, more common primary mutations are the 3460 mutation and the 14484 mutation, each of which can be found in 15% of the LHON cases (Howell et al. 1991*a*; Huoponen et al. 1991; Johns et al. 1992; Johns and Neufeld 1993*b*).

Hereditary spastic dystonia and LHON, manifested either separately or in combination, have occurred among 24 individuals over seven generations of a kindred of 549 members in a large Dutch family (Bruyn and Went 1964; Went 1964*a*, 1964*b*; Bruyn et al. 1991, 1992). Both the dystonia and LHON show strict maternal inheritance. In this family, 119 males and 123 females are maternal relatives: 12 of them (10 males and 2 females) had optic atrophy only, 4 (2 males and 2 females) exhibited exclusively the neurological disorder (1 with unilateral involvement), and 8 presented with optic atrophy and the neurological disorder; 1 of these 8 had a unilateral neurological deficit. Other pedigrees with a possibly comparable "dystonia–Leber syndrome" have been reported (Marsden et al. 1986; Novotny et al. 1986; Larsson et al. 1991).

It was found in previous studies that the primary 11778 mutation and the 13708 secondary mutation were not present in the Dutch LHON-dystonia family (Bruyn et al. 1992). In view of both the genetic heteroge-

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Address for correspondence and reprints: Dr. Bernard A. van Oost, Faculty of Veterinary Medicine, Universiteit Utrecht, Department of Clinical Sciences of Companion Animals, P.O. Box 80.154, NL-3508 TD Utrecht, The Netherlands. E-mail: B.vanoost@ukg.dgk.ruu.nl

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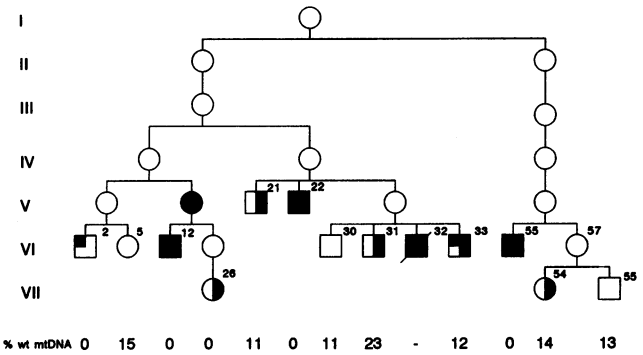

Figure 1 Partial pedigree of the LHON-dystonia family (Bruyn and Went 1964; Went 1964*a*, 1964*b*). The pedigree numbers (adjacent to the symbols) are identical to those presented in the complete pedigree by Went (1964*b*) and Bruyn et al. (1992), with the exception of VII-54, who was VII-18 in the original publication by Went (1964*b*). Squares denote males; and circles denote females; ◨ = unilateral neurological symptoms; ◧ = bilateral neurological symptoms; ◨/◑ = LHON; ◨ = LHON + unilateral neurological symptoms; and ■/● = LHON + bilateral neurological symptoms. The percentages below the symbols are the percentages of the wild-type sequence at nucleotide 11696 in white-blood-cell DNA.

neity in LHON and the unique combination of the clinical symptoms in this family, we set out to sequence all protein-coding genes in this family, to elucidate the molecular basis of hereditary spastic dystonia and LHON.

Subjects and Methods

Of the complete pedigree, which was published by Went (1964*b*) and updated by Bruyn et al. (1992), only a small part is shown in figure 1, and that presents those branches with individuals from whom blood was collected (n = 13). Three had optic atrophy and the complete neurological picture, three (including one female) had optic atrophy only, two had only unilateral neurological abnormalities (one with optic atrophy), and one woman had a bilateral neurological deficit only. Four individuals (two males, of ages 64 and 34 years, and two females, of ages 63 and 58 years) were clinically normal.

Clinical Status of Patients

The optic atrophy was typical for LHON: acute bilateral visual loss with pale optic disks, notably on the temporal side, and variable centrocecal scotomata. Average age at onset of visual loss was 22 years (range 8–36 years). In four individuals with a relatively recent onset of the optic atrophy, fluorescence angiography performed directly after the onset of the visual loss revealed tortuosity of peripapillary vessels. No cardiological abnormalities were observed. The prominent neurological abnormalities (with an onset of symptoms at age 5–9 years) were spasticity, rigidity, bradykinesia, dystonia, dysarthria, athetoid dyskinesias, and occasional amyotrophy (Bruyn and Went 1964). There were no behavioral abnormalities, and intelligence was normal. Nuclear magnetic resonance studies revealed putaminal necrosis (unilateral only in patient VI-2; Bruyn et al. 1991). Neuropathological findings in patient VI-32 revealed complete neuronal depletion in the putamen and lateral part of the caudate (Bruyn et al. 1992).

Isolation of Genomic DNA

White-blood-cell DNA from family members V-21, V-22, VI-2, VI-5, VI-12, VI-30, VI-31, VI-33, VI-55, VI-57, VII-26, VII-54, and VII-55 and fibroblast DNA from patient VI-32 (fig. 1) were prepared according to standard procedures (Miller et al. 1988). Total DNA was extracted from brain of patient VI-32, as described by De Vries et al. (1992) for muscle tissue, with the modification that DNA was concentrated by ethanol precipitation.

PCR and mtDNA Sequence Analysis

The mitochondrial complex I, complex III, and complex IV genes, as well as the ATPase 6 and ATPase 8 genes, were sequenced as described elsewhere (De Vries et al. 1993). In brief, synthetic oligonucleotide primers were used to PCR-amplify fragments spanning protein-coding genes in the mitochondrial genome. The nucleotide sequences from both strands were analyzed by the fluorescent dideoxy chain-termination method, on an Automated DNA fluorescent Sequencer (Applied Biosystems/Perkin-Elmer).

Single-Nucleotide Primer Extension

The detection primers for the single-nucleotide extension reactions of the ND4/11696 mutation and of the ND6/14596 mutation were complementary to np 11676–11695 and np 14574–14595, respectively, terminating immediately adjacent to the mutations. For each subject, the DNA fragment containing the mutation site was amplified by PCR as described above. In the primer-extension reaction, 0.1–1 μg template DNA and 25 ng detection primer in a total volume of 10.5 μl, overlaid with mineral oil, were denatured (96°C for 2 min) in a Perkin-Elmer thermal cycler. Subsequently, after cooling of the samples to 60°C, they were combined with a premix of 1 μl of each of the dideoxy terminators and 4 μl of 5 × TACS buffer (Perkin-Elmer/Applied Biosystems) and 0.5 μl AmpliTaq® DNA polymerase (Cetus and Perkin Elmer/Applied Biosystems). Deionized water was added, to a final volume of 9.5 μl. For primer A, we modified the premix for the extension reaction, with 0.5 μl fluorescently labeled dGTP, 2 mM ddGTP, and 1 μl fluorescently labeled dATP, instead of with 1 μl of each of the fluorescently labeled dNTPs.

The samples were amplified through 25–35 cycles and, after purification by phenol extraction and ethanol precipitation, were analyzed on a 20% polyacrylamide/bisacrylamide gel with 7 M urea in an automated 370A DNA sequencer, by use of Genescan-672 software (Perkin-Elmer/Applied Biosystems). Quantification of the degree of heteroplasmy of the ND4 11696 mutation in tissues was accomplished by constructing a standard curve of known amounts of cloned wild-type and mutated ND4 template.

Biochemical Analysis

Biopsy was taken, under local analgesia, from the musculus triceps, which was less atrophic than the other muscles, by the open procedure. Homogenates from frozen muscle were prepared and assayed as described elsewhere (Scholte et al. 1987, 1995). Complex I was assayed at 37°C in triply frozen-thawed 5% homogenate (20–70 μg protein/ml). The oxidation of 0.1 mM NADH was measured by dual beam (340 and 432 nm) spectroscopy, with 25 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 2 mM KCN, 2 μg antimycin/ml, 2.5 mg bovine serum albumin (fatty acid free)/ml, 12.5 μM horse heart cytochrome c, and 65 μM decylubiquinone. The rotenone-insensitive activity was assayed in another cuvette, which contained 2 μM rotenone, and was ~5% of the rotenone-sensitive activity in controls and 20% of that in the patient. Citrate synthetase was assayed according to the method of Srere (1969). Mitochondria were isolated from 508 mg freshly biopsied muscle and were investigated according to the method described by Scholte et al. (1987, 1995).

Results

Sequence Analysis of the Protein-Coding Mitochondrial Genes

The DNA sequence of the protein-coding mitochondrial genes of family member VI-12 (fig. 1) was determined. Compared with the Cambridge sequence (Anderson et al. 1981), a total of nine nucleotide changes were found. Four of the nucleotide substitutions were silent polymorphisms, three of which (A4769G, G4985A, and T11335C) had been identified in previous studies and one of which (C5228G) had not been reported before; and three common amino acid polymorphisms were caused by the G9559C, the G13702C, and the G14199T substitutions (Marzuki et al. 1991; Wallace et al. 1991*b*; Lertrit et al. 1994). The remaining two nucleotide substitutions caused previously unidentified missense mutations. At np 11696 in the ND4 gene, an A→G transition was found that resulted in substitution of an isoleucine for valine at amino acid position 312, and in the ND6 gene at np 14596 a T→A transition was found that resulted in the substitution of methionine for isoleucine at amino acid position 26.

Patient VI-12 was also screened for other mtDNA mutations. Southern blotting of *Pvu*II-restricted mtDNA from blood did not reveal any deletions or rearrangements. None of the LHON-associated mutations (listed in table 1) were found in the mitochondrial genome of this family. The diagnostic tests described for the np 3243 (Goto et al. 1990) and np 4317 (Tanaka et al. 1990) mtDNA mutations that are found in patients with mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS); the np 8344 (Zeviani et al. 1991) mtDNA mutation found in patients with myoclonus epilepsy and ragged red fibers (MERRF); and the np 8356 (Silvestri et al. 1992) mtDNA mutation found in the MERRF-MELAS syndrome with hyperthyroidy were also absent in patient VI-12.

Estimation of Frequency and Heteroplasmy of the 11696 Mutation and the 14596 Mutation and Correlation with Clinical Phenotype

To discriminate between the mutant and wild-type sequences, we modified the single-nucleotide primer-extension technique described by Kuppuswamy et al. (1991). Both the ND4/11696 substitution and the ND6/14596 substitution were present in all family members who were tested. Neither of these mutations was detected in 100 control subjects, in 10 nonrelated Dutch LHON families, or in 10 complex I–deficient patients with a mitochondrial myopathy.

The ND4 mutation was heteroplasmic in most of the family members. To determine the degree of heteroplasmy of the ND4/11696 mutation, a standard curve was constructed with different mixtures of wild-type A^{11696} and mutant G^{11696} plasmids. Good reproducibility was obtained in several independent primer-extension reactions. The extent of heteroplasmy for the ND4/11696 mutation in blood from the family members is indicated in figure 1. The extent of heteroplasmy in white-blood-cell DNA seemed to be correlated, to a certain extent, with the severity of the disease (fig. 2). For patient VI-32, who died in a nursing home in 1991 at the age of 59 years, no blood was available. In his fibroblasts, no wild-type mtDNA was found, whereas in optic nerve 12% wild-type mtDNA was detected. With the same technique, all family members were found to be homoplasmic for the ND6/14596 mutation.

Biochemical Characterization of the Complex I Defect

A muscle biopsy was obtained from patient VI-12 (fig. 1) and was processed immediately for biochemical analysis. The oxidation of substrates measured, the stimulation of the oxygen-uptake rates by ADP, the moles of high-energy phosphate synthesized divided by 0.5 mol oxygen consumed, the activities of Mg^{2+}-ATPase with

Table 1

mtDNA Mutations Associated with LHON

Gene	Nucleotide Substitution	Amino Acid Replacement	Interspecies Conservation of Substituted Nucleotide[a]	Homoplasmy[b]	Heteroplasmy[b]	Reference(s)
ND1	T3394C	Y→H	H	ND	ND	Johns et al. 1992
	G3460A[c]	A→T	M	+	−	Huoponen et al. 1991, Howell et al. 1991*b*
	T4160C	L→P	H	+	−	Howell et al. 1991*a*
	T4216C	Y→H	L	+	−	Johns and Berman 1991
ND2	A4917G	D→N	H	+	−	Johns and Berman 1991
	G5244A	G→S	H	−	+	Wallace et al. 1991*a*, Brown et al. 1992*b*
COI	G7444A	Term→K[d]	L	+	−	Brown et al. 1992*c*
ATP6	T9101C	I→T	M	+	−	Lamminen et al. 1995
COIII	G9438A	G→S	H	+	−	Johns and Neufeld 1993*a*
	G9804A	A→T	H	+	−	Johns and Neufeld 1993*a*
ND4	A11696G	V→I	L	+	+	Present study
	G11778A[c]	R→H	H	+	+	Wallace et al. 1998
ND5	G13708A	A→T	M	+	−	Johns and Neufeld 1991, Brown et al. 1992*b*
ND6	G14459A	A→V	M	+	+	Jun et al. 1994*a*
	T14484C[c]	M→V	M	ND	ND	Johns et al. 1992, Johns and Neufeld 1993*b*
	T14596A	I→M	M	+	−	Present study
Cytochrome b	G15257A	D→N	H	+	−	Johns and Neufeld 1991, Brown et al. 1992*a*, 1992*b*
	G15812A	V→M	M	+	−	Johns et al. 1992, Johns and Neufeld 1993*a*

[a] H = high; M = medium; and L = low.
[b] ND = not determined.
[c] Established primary LHON mutation.
[d] Term = termination codon.

and without uncoupler, the radiopalmitate oxidation in the presence of carnitine, and the activity and latency of citrate synthethase of the isolated mitochondria were in the range of those in controls (data not shown), except for a decreased stimulation of the respiration of pyruvate plus malate by ADP (1.4 vs. 5.8; range 2.9–9.2 in 10 controls). Another exceptional finding in the isolated mitochondria was that 3 h after biopsy and 2 h after isolation of the mitochondria, the pyruvate+malate oxidation had decreased to 50% of its original level. The activities of the respiratory-chain complexes I, II+III, and IV in the muscle homogenate were decreased to 21%, 76%, and 39%, respectively, of the average activities in controls. The activity of citrate synthetase was 94% of that in controls (table 2). Except for some small and atrophic fibers, the histological and histochemical analysis of the muscle was normal, and, in particular, no ragged red fibers were seen (data not shown).

Discussion

By sequencing the entire region of the mtDNA genes that encode protein, we have found two unique missense mutations in a patient afflicted with LHON and dystonia (VI-12; fig. 1). We could exclude all of the previously reported mutations associated with LHON (table 1). A heteroplasmic transition at position 11696 in the ND4 gene and a homoplasmic 14596 transition in the ND6 gene were found. Neither of these two mutations was found in controls or LHON patients, whereas both were present in the 12 family members who were analyzed. In a muscle biopsy of patient VI-12, a defect in the respiratory chain was found, a result supporting the pathophysiological role of these mutations in mitochondrial complex I genes for the etiology of LHON and dystonia.

We found evidence for correlation between the degree of heteroplasmy of the ND4 mutation and the clinical severity of the disease, considering the most extreme clinical phenotypes: 11%–15% wild-type mtDNA was detected in blood from all four nonaffected family members, whereas *no* wild-type mtDNA was found in blood from the three family members with LHON and bilateral spastic dystonia (fig. 2). For the other clinical phenotypes, no obvious correlation with the degree of hetero-

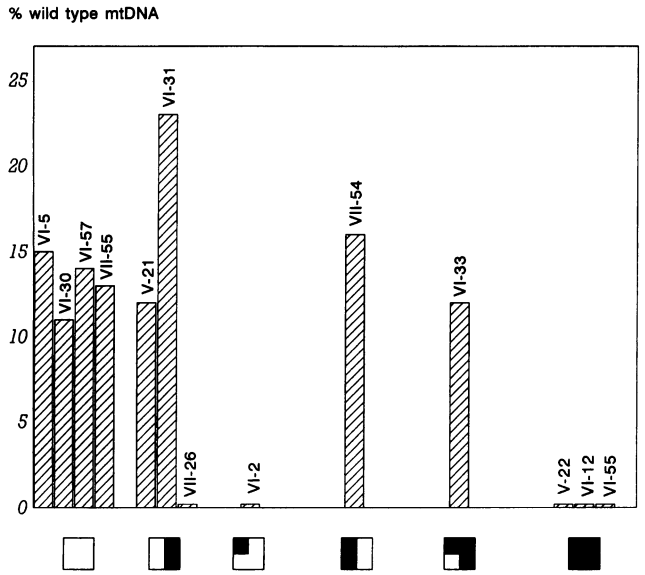

Figure 2 Degree of heteroplasmy for the ND4/11696 mutation, as a function of the clinical phenotypes. Symbols are as in fig. 1.

plasmy was observed, when either gender or age at onset is taken into account. In general, the range in the percentages of wild-type mtDNA in blood of the family members, affected or not, is relatively narrow: 0%–23%. Similar low amounts of wild-type mtDNA have previously been described for the most prevalent LHON mutations, at nt 11778 and 3460 (Holt et al. 1989; Sweeney et al. 1992).

Interestingly, the ND4/A11696G mutation remains heteroplasmic over at least six generations in this family, without any remarkable shift in degree of heteroplasmy, similar to findings described by Howell et al. (1992). This is unexpected, since rapid shifts of human mtDNA heteroplasmy in pedigrees with a mitochondrial disease have been found (Bolhuis et al. 1990; De Vries et al. 1994), and since rapid cellular segregation from the heteroplasmic state to the homoplasmic state takes place in various organisms (Hauswirth and Laipis 1985; King and Attardi 1988; De Stordeur et al. 1989; Birky et al. 1989; Niki et al. 1989; Koehler et al. 1991). On the basis of findings in Finland, the Nikoskelainen group (Villki et al. 1990) has suggested that there might be a selective advantage in the sustaining of heteroplasmy for certain mtDNA mutations (Villki et al. 1990), a selection that might also be operative in our family.

So far, little is known about the tertiary structure of the ND4 and ND6 subunits, in which the 11696 and 14596 mutations were found (Walker 1992). The valine at position 312 in the ND4 protein is located in a predicted transmembrane region, 28 amino acids aminoterminal to the R340H LHON mutation (Fearnley and Walker 1992). Interspecies comparison does not show significant conservation of this valine, and in most mammals a threonine residue is found at this position (table 3). The isoleucine at position 26 is located at the edge of the second predicted membrane-spanning element of the ND6 gene (Fearnley and Walker 1992). This amino acid residue and the surrounding region are well conserved among mammals, but not in more diverse taxa (table 3). Which of the two mtDNA mutations is—or whether both are—responsible for the clinical phenotype cannot be decided at the present time. Given the possible correlation between the severe phenotype and the ND4 mutation, it might be that both act synergistically. Since the ND6/14596 mutation is homoplasmic, this mutation probably arose before the ND4/11696 mutation. It would be of interest to study in vitro both mutations separately in the mtDNA-less (rho zero) cell complementation system (King and Attardi 1989), to analyze their effect on the complex I activity.

Of the respiratory-enzyme complexes measured, the complex I activity was decreased most. The much-less-reduced activities of complex II+III and complex IV are most likely secondary to the complex I deficiency. In two other families with LHON and dystonia, mutations in the mtDNA and complex I deficiency have been reported. Howell et al. (1991*b*; Howell 1994) found the 14484C primary LHON mutation together with a 4160C mutation in the ND1 gene in the "Queensland family," in which a marked complex I deficiency had been found previously (Parker et al. 1989). Jun et al. (1994*a*) found a G→A substitution in the codon for an evolutionary conserved alanine at position 72 in the ND6 gene in a five-generation Hispanic family with LHON and dystonia, which subsequently was found to have a complex I deficiency (Jun et al. 1994*b*). Because a complex I deficiency has been reported in patients with idiopathic dystonia, for which the gene defect is unknown (Benecke et al. 1992), it is likely that LHON and dystonia have a common etiology. It seems that these complex neurological disorders, all clinically similar but not identical, have private mtDNA mutations. Recently, the primary 11778 LHON mutation was found in a family with LHON and cerebellar ataxia, but other pathogenic mtDNA mutations were not excluded (Funakawa et al. 1995).

For LHON, the relation between the defect at the genetic level and the results of the biochemical analyses is not straightforward. Whereas in patients harboring the 3460 mutation a profound complex I deficiency has been observed (Howell et al. 1991*b*; Majander et al. 1991; Smith et al. 1994), the results of biochemical studies in patients harboring the 11778 mutation are more difficult to reconcile (Larsson et al. 1991; Majander et al. 1991; Cornelissen et al. 1993; Smith et al. 1994). Recently, Degli Esposti obtained evidence that the 11778 mutation interfered in the interaction between the ND4 subunit and the ubiquinone substrate, thereby

Table 2

Activities of Respiratory Chain and Citrate Synthetase in Patient VI-12 (Fig. 1) and Controls

	Patient	Control Mean (Range) [*n*]
NADH-decylubiquinone oxidoreductase (μmol NADH)	1.10	5.24 (4.14–8.85) [12]
Succinate–cytochrome c oxidoreductase (μmol cytochrome c)	3.95	5.20 (1.57–11.6) [54]
Cytochrome c oxidase (first-order rate-constant *k*) (g muslce/min)	37	95 (45–161) [49]
Citrate synthetase (μmol)	15	16 (8.6–36) [10]

NOTE.—Measurements were made in a fresh muscle homogenate, as described in the Subjects and Methods section. The activities are expressed in terms of either amount of substrate converted or first-order rate-constant *k*. The protein content was in the normal range, whereas total carnitine was slightly below the lower limit of normal.

providing an explanation for the discrepancy between normal complex I enzyme activity and decreased electron flux with complex I–linked substrates in 11778-mutation patients (Degli Esposti et al. 1994). Further support for the causal relation between impairment of the respiratory chain and mtDNA mutations in LHON comes for mtDNA mutations other than in complex I. Mutations in cytochrome b and cytochrome oxidase have been found (see table 1), and, interestingly, Lamminen et al. (1995) found a homoplasmic T9101C mutation causing a threonine-for-isoleucine substitution in the ATPase 6 subunit gene in a single LHON patient with a partial complex V deficiency.

In conclusion, five arguments can be formulated in favor of the functional importance of the mutations presented here. First, they are the only mutations in the protein-coding sequence of the mtDNA in these patients. In LHON, mtDNA mutations are found exclusively in the protein-coding regions. Second, both mutations do not occur in the normal population, since neither was found in 100 control subjects. This situation makes the probability of both mutations present in the same control individual even more remote. Third, the mutation in the ND6 gene is close to a very well-conserved region of the ND6 gene, in which a different mutation in a clinically similar patient was found (Jun et al. 1994*a*). Fourth, the degree of heteroplasmy of the mutation in the ND4 gene shows a correlation with the severity of the clinical phenotype. Last, the activity of complex I in muscle tissue of the patient analyzed was clearly decreased, suggesting a deleterious effect of the mutations found. As Howell et al. (1991*a*, 1991*b*) have suggested,

Table 3

Amino Acid Conservation of Valine at Position 312 in the ND4 Gene and of Isoleucine at Position 26 in the ND 6 Gene

Organism	ND4	ND6	Reference
Human	W S F T G A V I L M I A H	S S K P S P I Y G G L V L	Anderson et al. 1981
LHON/dystonia	. . . . . I	. . . . . M	Present paper
Cow	. . Y M . . T A	. . . . . G .	Anderson et al. 1982
Mouse	. . . M . . T M	A L . . . . . G .	Bibb et al. 1981
Rat	. . . M . . T M	A L . . . . . F G .	Gadaleta et al. 1989
Whale	. . Y M . . T A	. . . . . G .	Arnason et al. 1991
Seal	. . Y M . . T A	. . . . .	Arnason and Johansson 1992
Chicken	. . . S . . M . . . . S .	A . N . . . Y . . V V G .	Desjardin and Morais 1990
Xenopus	K A L . . . M . . N T S D	A . N . . . F . A A . G .	Roe et al. 1985
Lamprey	. A W S L . L A M	V L S . . . Y F S A . G .	Lee and Kocher 1995
Sea urchin	. G M N . . L M . . V . .	S L – – . . Y . S A . G .	Jacobs et al. 1988
Drosophila	. G L C . S V T	F N M I H . L A L . . T .	Clary and Wolstenholme 1985
Caenorhabditis elegans	S . K I S S . M . . L . .	Y I N I D . M K S S F F .	Okimoto et al. 1992
Ascaris suum	S G K . . G . . . . L . .	Y I N V D . M K S S F F .	Okimoto et al. 1992
Sea anemone	E G L V A . . F M . L . .	I . A L N . V H S V F W .	Pont-Kingdon et al. 1994
Liverwort	L G L N . . I L Q . . S .	L L N A D F V A A A Q I .	Kochi et al. 1988

NOTE.—Deviations from the human sequence are denoted by one-letter amino acid symbols, and gaps are indicated by a dash.

LHON is probably a group of related mitochondrial genetic diseases that share a characteristic pattern of ophthalmological changes. The data presented here provide support for a causal relation between the neurological abnormalities associated with LHON and different mitochondrial gene mutations.

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References

Anderson S, Bankier AT, Barrell BG, De Bruijn MHL, Coulson AR, Drouin J, Eperon IC, et al (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Anderson S, de Bruijn MHL, Coulson AR, Eperon IC, Bankier AT, Sanger F, Young IG (1982) Complete sequence of bovine mitochondrial DNA: conserved features of the mammalian mitochondrial genome. J Mol Biol 156:683–717

Arnason U, Gullberg A, Widegren B (1991) The complete nucleotide sequence of the mitochondrial DNA of the fin whale, *Balaenoptera physalus*. J Mol Evol 33:556–568

Arnason U, Johansson E (1992) The complete mitochondrial DNA sequence of the harbor seal, *Phoca viluina*. J Mol Evol 34:493–505

Benecke R, Strümper P, Weiss H (1992) Electron transfer complex I defect in idiopathic dystonia. Ann Neurol 32:683–686

Bibb MJ, van Etten RA, Wright CT, Walberg MW, Clayton DA (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26:167–180

Birky CW Jr, Feurst P, Maruyama T (1989) Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. Genetics 121:613–627

Bolhuis PA, Bleeker-Wagemakers EM, Ponne NJ, Van Schooneveld MJ, Westerveld A, Van den Bogert C, Tabak HF (1990) Rapid shift in genotype of human mitochondrial DNA in a family with Leber's hereditary optic neuropathy. Biochem Biophys Res Commun 170:994–997

Brown MD, Voljavec AS, Lott MT, MacDonald I, Wallace DC (1992*a*) Leber's hereditary optic neuropathy: a model for mitochondrial neurodegenerative diseases. FASEB J 6:2791–2799

Brown MD, Voljavec AS, Lott MT, Torroni A, Yang C, Wallace DC (1992*b*) Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy. Genetics 130:163–173

Brown MD, Yang C-C, Trounce I, Torroni A, Lott MT, Wallace DC (1992*c*) A mitochondrial DNA variant, identified in Leber hereditary optic neuropathy patients, which extends the amino acid sequence of cytochrome c oxidase subunit I. Am J Hum Genet 51:378–385

Bruyn GW, Bots GTAM, Went LN, Klinkhamer PJJM (1992) Hereditary spastic dystonia with Leber's hereditary optic neuropathy: neuropathological findings. J Neurol Sci 113:55–61

Bruyn GW, Vielvoye GJ, Went LN (1991) Hereditary spastic dystonia: a new mitochondrial encephalopathy? putaminal necrosis as a diagnostic sign. J Neurol Sci 103:195–202

Bruyn GW, Went LN (1964) A sex-linked heredodegenerative neurological disorder, associated with Leber's optic atrophy. I. Clinical studies. J Neurol Sci 1:59–80

Clary DO, Wolstenholme DR (1985) The mitochondrial DNA molecule of Drosophila yakuba: nucleotide sequence, gene organization, and genetic code. J Mol Evol 22:252–271

Cornelissen JC, Wanders RJA, Bolhuis PA, Bleeker-Wagemakers E, Oostra R-J, Wijburg FA (1993) Respiratory chain function in Leber's hereditary optic neuropathy: lack of correlation with clinical disease. J Inherit Metab Dis 16:531–533

Degli Esposti M, Carelli V, Ghelli A, Ratta M, Crimi M, Sangiorgo S, Montagna P, et al (1994) Functional alterations of the mitochondrially encoded ND4 subunit associated with Leber's hereditary optic neuropathy. FEBS Lett 352:375–379

De Stordeur E, Solignac M, Monnerot M, Mounolou JC (1989) The generation of transplasmic *Drosophila simulans* by cytoplasmic injection: effects of segregation and selection on the perpetuation of mitochondrial DNA heteroplasmy. Mol Gen Genet 220:127–132

Desjardins P, Morais R (1990) Sequence and gene organization of the chicken mitochondrial genome. J Mol Biol 212:599–634

De Vries D, De Wijs I, Ruitenbeek W, Begeer J, Smit P, Bentlage H, Van Oost B (1994) Extreme variability of clinical symptoms among sibs in a MELAS family correlated with heteroplasmy for the mitochondrial A3243G mutation. J Neurol Sci 124:77–82

De Vries DD, Ruitenbeek W, van Oost BA (1992) Detection of extremely low levels of wild type mitochondrial DNA in the liver of a patient with Pearson syndrome by a sensitive PCR assay. J Inherit Metab Dis 15:307–310

De Vries DD, Van Engelen BGM, Gabreëls FJM, Ruitenbeek W, Van Oost BA (1993) A second missense mutation in the mitochondrial ATPase 6 gene in Leigh syndrome. Ann Neurol 34:410–412

Fearnley IM, Walker JE (1992) Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. Biochim Biophys Acta 1140:105–134

Funakawa I, Kato H, Terao A, Ichihashi K, Kawashima S, Hayashi T, Mitani K, et al (1995) Cerebellar ataxia in patients with Leber's hereditary optic neuropathy. J Neurol 242:75–77

Gadeleta G, Pepe G, De Candia G, Quagliariello C, Sbisa E,

Saccone C (1989) The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome: cryptic signals revealed by comparative analysis between vertebrates. J Mol Evol 28:497–516

Goto Y, Nonaka I, Horai S (1990) A mutation in the $tRNA^{leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348:651–653

Hauswirth WW, Laipis PJ (1985) Transmission genetics of mammalian mitochondria: a molecular model and experimental evidence. In: Quagliariello E, Slater EC, Palmieri F, Saccone C, Kroon AM (eds) Achievements and perspectives of mitochondrial research. Elsevier, New York, pp 49–59

Holt IJ, Miller DH, Harding AE (1989) Genetic heterogeneity and mitochondrial DNA heteroplasmy in Leber's hereditary optic neuropathy. J Med Genet 26:739–743

Howell N (1994) Primary LHON mutations: trying to separate "fruyt" from "chaf." Clin Neurosci 2:130–137

Howell N, Bindoff LA, McCullough DA, Kubacka I, Poulton J, Mackey D, Taylor L, et al (1991*a*) Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. Am J Hum Genet 49:939–950

Howell N, Halvorson S, Kubacka I, McCullough DA, Bindoff LA, Turnbull DM (1992) Mitochondrial gene segregation in mammals: is the bottleneck always narrow? Hum Genet 90:117–120

Howell N, Kubacka I, Xu M, McCullough DA (1991*b*) Leber hereditary optic neuropathy: involvement of the mitochondrial ND1 gene and evidence for an intragenic suppressor mutation. Am J Hum Genet 48:935–942

Huoponen K, Vilkki J, Aula P, Nikoskelainen EK, Savontaus M-L (1991) A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy. Am J Hum Genet 48:1147–1153

Jacobs HT, Elliott DJ, Math VB, Farquharson A (1988) Nucleotide sequence and gene organization of sea urchin mitochondrial DNA. J Mol Biol 202:185–217

Johns DR, Berman J (1991) Alternative, simultaneous complex I mitochondrial DNA mutations in Leber's hereditary optic neuropathy. Biochem Biophys Res Commun 174:1324–1330

Johns DR, Heher KL, Miller NR, Smith KH (1993) Leber's hereditary optic neuropathy: clinical manifestations of the 14484 mutation. Arch Ophthalmol 111:495–498

Johns DR, Neufeld MJ (1991) Cytochrome b mutations in Leber hereditary optic neuropathy. Biochem Biophys Res Commun 181:1358–1364

——— (1993*a*) Cytochrome c oxidase mutations in Leber hereditary optic neuropathy. Biochem Biophys Res Commun 196:810–815

——— (1993*b*) Pitfalls in the molecular genetic diagnosis of Leber hereditary optic neuropathy (LHON). Am J Hum Genet 53:916–920

Johns DR, Neufeld MJ, Park RD (1992) An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy. Biochem Biophys Res Commun 187:1551–1557

Jun AS, Brown MD, Wallace DC (1994*a*) A mitochondrial DNA mutation at nucleotide pair 14459 of the NADH dehydrogenase subunit 6 gene associated with maternally inherited Leber hereditary optic neuropathy and dystonia. Proc Natl Acad Sci USA 91:6206–6210

Jun AS, Trounce IA, Brown MD, Shoffner JM, Kim YL, Wallace DC (1994*b*) Marked complex I deficiency in patients harboring the np 14459 ND6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy and dystonia. Am J Hum Genet Suppl 55:A225

King MP, Attardi G (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 246:500–503

Kochi T, Shirai H, Fukuzawa H, Sano T, Komano T, Umesono K, Inokuchi H, et al (1988) Structure and organization of *Marchantia polymorpha* chloroplast genome. J Mol Biol 203:353–372

Koehler CM, Lindberg GL, Brown DR, Beitz DC, Freeman AF, Mayfield JE, Myers AM (1991) Replacement of bovine mitochondrial DNA by a sequence variant within one generation. Genetics 129:247–255

Kuppuswamy MN, Hoffmann JW, Kaspar CK, Spitzer SG, Groce SL, Bajaj SP (1991) Single nucleotide primer extension to detect genetic diseases: experimental application to hemophilia B (factor IX) and cystic fibrosis genes. Proc Natl Acad Sci USA 88:1143–1147

Lamminen T, Majander A, Juvonen V, Wikström M, Aula P, Nikoskelainen E, Savontaus M-L (1995) A mitochondrial mutation at nt 9101 in the ATP synthase 6 gene associated with deficient oxidative phosphorylation in a family with Leber hereditary optic neuroretinopathy. Am J Hum Genet 56:1238–1240

Larsson NG, Andersen O, Holme E, Oldfors A, Wahlstrom J (1991) Leber's hereditary optic neuropathy and complex I deficiency in muscle. Ann Neurol 30:701–708

Lee W-J, Kocher TD (1995) Complete sequence of a sea Lamprey (*Petromyzon marinus*) mitochondrial genome: early establishment of the vertebrate genome organization. Genetics 139:873–887

Lertrit P, Kapsa RMI, Jean-Francois MJB, Thyagarajan D, Noer AS, Marzuki S, Byrne E (1994) Mitochondrial DNA polymorphism in disease: a possible contributor to respiratory dysfunction. Hum Mol Genet 3:1973–1981

Mackey D, Howell N (1992) A variant of Leber hereditary optic neuropathy characterized by recovery of vision and by an unusual mitochondrial genetic etiology. Am J Hum Genet 51:1218–1228

Majander A, Huoponen K, Savontaus ML, Nikoskelainen E, Wikström M (1991) Electron transfer properties of NADH:ubiquinone reductase in the ND1/3460 and the ND4/11778 mutations of the Leber hereditary optic neuropathy (LHON). FEBS Lett 292:289–292

Marsden CD, Lang AE, Quinn NP, McDonald WI, Abdallat A, Nimri S (1986) Familial dystonia and visual failure with striatal lucencies. J Neurol Neurosurg Psychiatry 49:500–509

Marzuki S, Noer AS, Lertrit P, Thyagarajan D, Kapsa R, Utthanaphol P, Byrne E (1991) Normal variants of human mitochondrial DNA and translation products: the building of a reference data base. Hum Genet 88:139–145

Miller SA, Dykes DD, Polesky HF (1988) A simple-salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Nakamura M, Ara F, Yamada M, Hotta Y, Hayakawa M, Fujike K, Kanai A, et al (1992) High frequency of mitochondrial ND4 gene mutation in Japanese pedigrees with Leber hereditary optic neuropathy. Jpn J Ophthalmol 36:56–61

Newman NJ (1993) Leber's hereditary optic neuropathy. Arch Neurol 50:540–548

Newman NJ, Lott MT, Wallace DC (1991) The clinical characteristics of pedigrees of Leber's hereditary optic neuropathy with the 11778 mutation. Am J Ophthalmol 111:750–762

Niki Y, Chigusa SI, Matsuura ET (1989) Complete replacement of mitochondrial DNA in *Drosophila*. Nature 341:551–552

Nikoskelainen EK (1994) Clinical picture of LHON. Clin Neurosci 2:115–120

Novotny EJ, Singh G, Wallace DC (1986) Leber's disease and dystonia: a mitochondrial disease. Neurology 36:1053–1060

Okimoto R, Macfarlane JL, Clary DO, Wolstenholme DR (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Genetics 130:471–498

Parker WD, Oley CA, Parks JK (1989) A defect in mitochondrial electron-transport activity (NADH-coenzyme Q oxidoreductase) in Leber's hereditary optic neuropathy. N Engl J Med 320:1331–1333

Pont-Kingdon GA, Beagley CT, Okimoto R, Wolstenholme DR (1994) Mitochondrial DNA of the sea anemone, *Metridium senile* (Cnidaria): prokaryote-like genes for $tRNA^{f\text{-}Met}$ and small-subunit ribosomal RNA, and standard genetic code specificities for AGR and ATA codons. J Mol Evol 39:387–399

Roe BA, Ma D-P, Wilson RK, Wong J F-H (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J Biol Chem 260:9759–9774

Scholte HR, Busch HFM, Bakker HD, Bogaard JM, Luyt-Houwen IEM, Kuyt LP (1995) Riboflavin-responsive complex I deficiency. Biochim Biophys Acta 1271:75–83

Scholte HR, Busch HFM, Luyt-Houwen IEM, Vaandrager-Verduin MHM, Przyrembel H, Arts WFM (1987) Defects in oxidative phosphorylation: biochemical investigations in skeletal muscle and expression of the lesion in other cells. J Inherit Metab Dis 10 Suppl 1:81–97

Silvestri G, Moraes CT, Shanske S, Oh SJ, DiMauro S (1992) A new mtDNA mutation in the $tRNA^{Lys}$ gene associated with myoclonic epilepsy and ragged-red fibers (MERRF). Am J Hum Genet 51:1213–1217

Smith PR, Cooper JM, Govan GG, Harding AE, Shapira AHV (1994) Platelet mitochondrial function in Leber's hereditary optic neuropathy. J Neurol Sci 122:80–83

Srere PA (1969) Citrate synthase. Methods Enzymol 13:3–11

Sweeney MG, Davis MB, Lashwood A, Brockington M, Toscano A, Harding AE (1992) Evidence against an X-linked locus close to DXS7 determining visual loss susceptibility in British and Italian families with Leber hereditary optic neuropathy. Am J Hum Genet 51:741–748

Tanaka M, Ino H, Ohno K, Hattori K, Sato W, Ozawa T, Tanaka T, et al (1990) Mitochondrial mutation in fatal infantile cardiomyopathy. Lancet 336:1452

Vilkki J, Savontaus M-L, Nikoskelainen EK (1990) Segregation of mitochondrial genomes in a heteroplasmic lineage with Leber hereditary optic neuroretinopathy. Am J Hum Genet 47:95–100

Walker JE (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. Q Rev Biophys 25:253–324

Wallace DC, Brown MD, Lott MT, Voljavec AS, Torroni A, Yang C-C (1991*a*) Multiple mitochondrial DNA mutations associated with Leber's hereditary optic neuropathy. Cytogenet Cell Genet 58:2121

Wallace DC, Lott MT, Torroni A, Shoffner JM (1991*b*) Report of the committee on human mitochondrial DNA. Cytogenet Cell Genet 58:1103–1123

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AMS, Elsas LJ II, et al (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242:1427–1430

Went LN (1964*a*) A sex-linked heredodegenerative neurological disorder, associated with Leber's optic atrophy. II. Laboratory investigations. J Neurol Sci 1:81–87

——— (1964*b*) A sex-linked heredodegenerative neurological disorder, associated with Leber's optic atrophy. III. Genetical aspects. Acta Genet (Basel) 14:220–239

Zeviani M, Amati P, Bresolin N, Antozzi C, Piccolo G, Toscano A, DiDonato S (1991) Rapid detection of the $A \rightarrow G^{(8344)}$ mutation of mtDNA in Italian families with myoclonus epilepsy and ragged red fibers (MERRF). Am J Hum Genet 48:203–211