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Leber Hereditary Optic Neuropathy: Involvement of the Mitochondrial ND1 Gene and Evidence for an Intragenic Suppressor Mutation

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Summary

A large Queensland family has an extreme form of Leber hereditary optic neuropathy (LHON) in which several neurological abnormalities and an infantile encephalopathy are present in addition to the characteristic ophthalmological changes. Sequence analysis of the seven mitochondrial genes encoding subunits of respiratory chain complex I (NADH–ubiquinone oxidoreductase) reveals two novel features of the etiology of this mitochondrial genetic disease. The first conclusion from these studies is that the ophthalmological and neurological deficits in this family are produced by a mutation at nucleotide 4160 of the ND1 gene. This nucleotide alteration results in the substitution of proline for the highly conserved leucine residue at position 285 of the ND1 protein. Secondary-structure analysis predicts that the proline replacement disrupts a small *alpha* helix in a hydrophilic loop. All nine family members analyzed were homoplasmic for this mutation. The second major result from these studies is that the members of one branch of this family carry, at nucleotide 4136 of the same gene, a *second* mutation, also homoplasmic, which produces a cysteine-for-tyrosine replacement at position 277. The clinical and biochemical phenotypes of the family members indicate that this second nucleotide substitution may function as an intragenic suppressor mutation which ameliorates the neurological abnormalities and complex I deficiency.

Introduction

Leber hereditary optic neuropathy is now recognized to be a mitochondrial genetic disease. It is most broadly characterized as a maternally inherited late-onset form of blindness in which there is a bilateral retinal degeneration generally leading to a permanent loss of central vision (Lundsgard 1944; van Senus 1963; Seedorff 1970; Nikoskelainen et al. 1987). Among the LHON families who have been screened (Holt et al. 1989; Vilkki et al. 1989), approximately one-half carry the mitochondrial ND4 gene mutation which was initially identified by Wallace and coworkers (Wallace et al. 1988; Singh et al. 1989). The ND4 gene is one of the seven mitochondrial loci which encode subunits of respiratory chain complex I, NADH–ubiquinone oxidoreductase (Chomyn et al. 1985, 1986).

A large Queensland LHON family has been described by Wallace (1970). The optic neuropathy in this family is relatively severe, as indicated by its high proportion of afflicted family members. In addition, family members frequently display severe neurological complications (including dysarthria, ataxia, tremors, posterior column signs, and skeletal deformities) and, also, a severe infantile encephalopathy which has been fatal in at least three cases. Parker et al. (1989) have shown that there is a marked decrease in the specific activity of complex I in platelet mitochondria from four family members. This remains the only LHON family for which a biochemical defect in the mitochondrial respiratory chain has been identified.

Previous studies from this laboratory have established that in this LHON family the mitochondrial ND3, ND4L, and ND4 genes have not undergone any muta-

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tions which could cause the biochemical and clinical abnormalities (Howell and McCullough 1990). The sequence analysis of the ND1, ND2, ND5, and ND6 genes is the subject of the present investigation. The results of these studies demonstrate that the disease in this family is caused by a mutation in the mitochondrial ND1 gene. Moreover, there is evidence that members of one branch of this family carry, in this same gene, a second mutation, which may act as a partial intragenic suppressor. Both the complexity of the genetic etiology and the expression of neurological abnormalities in some LHON families are discussed in relation to the optic neuropathy.

Methods

Cloning and Sequencing Strategy

The nucleotide sequencing strategy for the mitochondrial ND1, ND2, ND5, and ND6 genes is the same as that used elsewhere (Howell and McCullough (1990). In brief, total DNA was isolated from the white-blood-cell/platelet fraction. Overlapping subfragments of the mitochondrial genes were PCR amplified by using synthetic oligonucleotide primers into which *Sau*3A restriction sites had been introduced by changing one or two basepairs. Thus, the sequence of the mitochondrial ND1 gene (nucleotides 3305 to 4262) was spanned entirely by amplifying four subfragments. The nucleotide coordinates and the number of amplified subfragments for the other ND genes are as follows: ND2 (4470 to 5511), four; ND5 (12,337 to 14,148), seven; ND6 (−14,673 to −14,148), two. It should be noted that the ND6 gene is transcribed from the strand *opposite* that of the other complex I genes and that negative coordinate numbers are used to denote this fact. In addition, 30–40 bp each of the upstream and downstream regions were sequenced for the mitochondrial ND genes; in the human mitochondrial genome, these regions are either tRNA or other ND genes. Nucleotides are numbered according to the Cambridge human mtDNA sequence (Anderson et al. 1981).

For each mitochondrial gene subfragment, at least eight independent PCR amplification reactions were pooled. This was done to ensure that any replication errors occurring during the early stages of amplification would be diluted and, therefore, would not be scored as mitochondrial gene mutations. In these studies, the PCR amplification error rate (mainly transitions) was about 1/4,500 bp (Howell and McCullough 1990). More rarely, single base deletions and truncated gene fragments are observed among the products. The same frequency of amplification errors is found for DNA from both LHON and non-LHON individuals.

The amplified gene subfragments were digested with *Sau*3A and then electrophoresed through agarose slab gels. After excision from the gel matrix, the subfragments were ligated into *Bam*HI-cleaved M13 mp18/19 by using an in-gel procedure. Recombinants were selected after transformation into *Escherichia coli* DH5*alpha* F′. Single-stranded recombinant plasmid molecules were isolated and used for DNA sequencing using the dideoxy chain termination procedure.

The hydropathic index of the ND1 protein was calculated by using the procedure of Kyte and Doolittle (1982) with a window size of 11 residues. Secondary structure was predicted by using the method of Garnier and Robson (1990). Both programs were part of the PCGENE software package (Intelligenetics, Inc.).

Clinical Status of Family Members

The clinical status of this family has not been comprehensively reevaluated since the original studies by Wallace (1970). Visual problems were present in IV-32, V-8, V-12, V-13, V-14, V-17, and V-18; neurological abnormalities were found in IV-28, IV-30 (infantile encephalopathy), IV-32, V-7 (infantile encephalopathy), V-9 (infantile encephalopathy), V-11 (infantile encephalopathy), and V-14 (infantile encephalopathy). This summary of which family members express the optic neuropathy and of which have neurological abnormalities includes information supplied by Drs.Christine Oley (Mater Mother's Hospital) and Davis Parker (University of Colorado Health Sciences Center). The clinical status of members of generation VI is reportedly normal at the present time, but these individuals are just now at the age when the disease manifests itself in this family.

Results

Sequence Analysis of the Mitochondrial ND1 Gene

A portion of the pedigree of the large Queensland LHON family, including the family members from whom DNA has been isolated, is shown in figure 1. The DNA sequence of the entire mitochondrial ND1 gene from three members of this family was determined. At least 30 independent clones of each gene

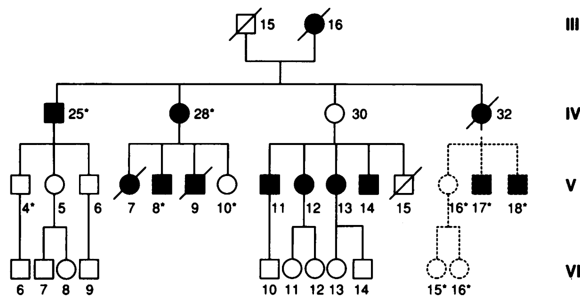

Figure 1 Partial pedigree of Queensland LHON family. The blackened symbols indicate family members who are afflicted visually and/or neurologically, and the unblackened symbols denote asymptomatic individuals. It should be noted that the clinical status of the family members shown here is that as of 1970. In this pedigree, family member IV-30 is denoted as clinically normal as an adult; however, she was afflicted with the infantile encephalopathy and recovered. Asterisks indicate family members from whom DNA has been isolated for nucleotide sequencing of the mitochondrial ND1 gene. The dashed line delineates the branch of the family that carries *both* the L285P and Y277C mutations.

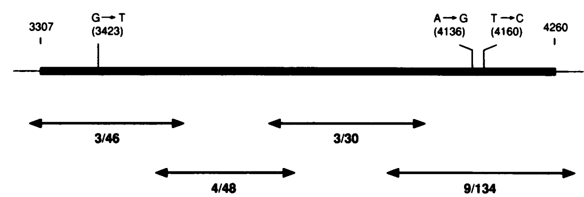

Figure 2 Nucleotide sequencing analysis of mitochondrial ND1 gene from Queensland LHON family. The arrows below the solid bar (ND1 gene) indicate the PCR fragments; the first number below each arrow indicates the number of LHON family members for whom DNA sequence was obtained, and the second number denotes the total number of independent M13 clones analyzed. Sites of nucleotide alteration are shown above the solid bar.

fragment were sequenced (fig. 2). By comparison with the prototype human ND1 sequence (Anderson et al. 1981), two nucleotide sequence alterations were found which altered the predicted amino acid sequence. This region of the ND1 locus was then analyzed in another six members of the Queensland LHON family. In all nine LHON family members, there was, at nucleotide 4160 of the noncoding strand, a T-to-C transition which results in the substitution of proline for leucine at amino acid position 285 (a mutation hereafter designated ND1/L285P). This transition occurred in 133 of 134 independent M13 subclones. As the remaining clone contained three other transitions, its significance is questionable. A minimum of 12 independent clones of this region of the ND1 gene have been sequenced in each of the nine family members. On the basis of the available evidence, therefore, all family members appear to be homoplasmic for the mutation at nucleotide 4160. It should be noted that four of the family members used for the sequencing studies were those used for the biochemical assays (Parker et al. 1989).

The second mutation, at nucleotide 4136, was an A-to-G transition which results in the substitution of cysteine for tyrosine at amino acid residue 277 (a mutation hereafter designated ND1/Y277C). Unlike the L285P mutation, however, the sequence alteration at nucleotide 4136 was not found in all nine family members analyzed. As shown in figure 1, the second mutation occurs only in the branch comprising individuals V-16, V-17, V-18, and the daughters (VI-15 and VI-16) of V-16. This mutation was found in all 78 independent clones sequenced, so that it also appears to be homoplasmic among the members of this branch of the LHON family. Neither the L285P nor the Y277C mutation was found in the ND1 gene of 18 control subjects (a total of 239 independent clones were analyzed); these controls included clinically normal individuals and patients with non-LHON mitochondrial or neurological diseases.

Evolutionary Conservation and Secondary-structural Effects of the L285P and Y277C Substitutions

The LEU285 residue of the human ND1 protein is highly conserved during evolution. Table 1 shows that the leucine at this position is invariant in ND1 proteins—with the exception of that from *Chlamydomonas,* where there is a conservative replacement with phenylalanine. Not only is the LEU285 residue highly conserved, but the entire region equivalent to residues 270–300 is one of the four segments of the ND1 protein which are conserved during evolution (Boer and Gray 1988; N. Howell, unpublished results), a finding supporting the importance that this residue has in structure or function. There are two other ND1 proteins, which are not included in table 1. A liverwort chloroplast-encoded protein may be homologous to ND1, although the overall sequence conservation is very low (about 20%); according to the alignment with the human sequence, there is a phenylalanine residue at the position equivalent to LEU285 (Kohchi et al. 1988). The amino acid sequence of the ND1 protein from *Paramecium* is also poorly conserved. Moreover, it is apparently truncated and contains only 261 amino acid residues, thus completely lacking the C-terminal region (Pritchard et al. 1990). Firm bio-

Table 1

ND-1 Protein Sequence Conservation among Various Organisms

Organism	ND1 Amino Acid Sequence[a]	277		285		Reference
Human	LWIRTA	Y	PRFRYDQ	L	MHLLWK	Anderson et al. 1981
Lhon	LWIRTA	C	PRFRYDQ	P	MHLLWK	Present paper
Rat	LWIRAS	Y	PRFRYDQ	L	MMLLWK	Gadaleta et al. 1989
Mouse	LWIRAS	Y	PRFRYDQ	L	MHLLWK	Bibb et al. 1981
Cow	LWIRAS	Y	PRFRYDQ	L	MHLLWK	Anderson et al. 1982
Chicken	LWIRAS	Y	PRFRYDQ	L	MHLLWK	Desjardins and Morais 1990
Xenopus	LWVRAS	Y	PRFRYDQ	L	MHLVWK	Roe et al. 1985
Drosophila	IWVRGT	L	PRFRYDK	L	MYLAWK	Clary and Wolstenholme 1985
Sea urchin	LWVRAA	Y	PRFRYDQ	L	MFLTWK	Jacobs et al. 1988
Aspergillus	IWVRAS	F	PRIRFDQ	L	MSVCWT	Brown et al. 1983
Podospora	IWVRAS	F	PRIRFDQ	L	MAFCWT	Cummings et al. 1988
Neurospora	ILGRAS	F	PRIRYDQ	L	MGFCWT	Burger and Werner 1985
Chlamydomonas	VWTRGT	L	PRYRYDQ	F	MRLGWK	Boer and Gray 1988
Trypanosome	FLI---	-	PRVICCR	L	KITTAQ	Hensgens et al. 1984

[a] Alignments are relative to that of human. Residues at positions equivalent to TYR277 and LEU285 of human ND1 are separated for emphasis.

chemical evidence for a mitochondrial-type complex I in either of these organisms is lacking.

The hydropathy profile of the human ND1 protein is shown in figure 3. The conserved segment containing the TYR277 and LEU285 residues is one of the few hydrophilic regions of the protein. Secondary-structure modeling of this hydrophilic region predicts that a turn sequence precedes a short *alpha* helix spanning residues 282–291. This short helix is the proximal boundary of a long hydrophobic stretch which is predicted to be transmembrane. Replacing the LEU285 residue with proline disrupts this conserved, surface-exposed *alpha* helix, leaving this region as a hydrophilic random coil.

The TYR277 residue is also conserved during evolution, although not as stringently as the LEU285 residue (table 1). However, it is always tyrosine, phenylalanine, or leucine, except in *Paramecium* and trypanosomes. In the latter organism, a small deletion creates in the protein sequence a four residue "gap" which includes the residue equivalent to TYR277. It is interesting to note that this residue immediately precedes an evolutionarily invariant proline-asparagine doublet. The TYR277 residue occurs in the turn region preceding the *alpha* helix. Replacing tyrosine with cysteine has no major impact on the predicted secondary structure, although the small volume of the cysteine side-chain might increase flexibility in this region of the ND1 protein.

Sequence Analysis of the ND2, ND5, and ND6 Genes

To confirm that the mutation at nucleotide 4160 is the mitochondrial complex I gene mutation producing the biochemical and clinical abnormalities in this family, the DNA sequences of the mitochondrial ND2, ND5, and ND6 genes were determined. As the ND3, ND4, and ND4L genes were sequenced earlier (Howell and McCullough 1990), all seven of the mitochondrial complex I genes from members of this family

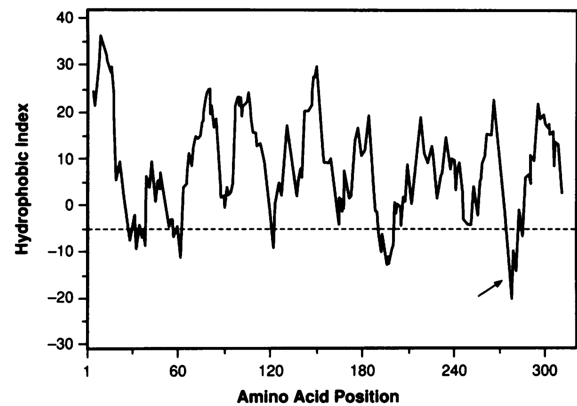

Figure 3 Hydrophobicity profile of human mitochondrial ND1 gene. The hydropathic index was calculated by using the procedure of Kyte and Doolittle (1982) with a window size of 11 residues. The arrow indicates the conserved hydrophilic region which includes the TYR277 and LEU285 residues.

Table 2

Nucleotide Sequence Changes in Mitochondrial Complex I Genes of Queensland LHON Family

Gene and Nucleotide[a]	Sequence Change	Amino Acid Residue
ND1:		
3423[b]	GC→TA	VAL39 unchanged
ND2:		
4646	TA→CG	TYR59 unchanged
4769	AT→GC	MET100 unchanged
4985	AT→GC	GLN172 unchanged
ND4:		
11,332	CG→TA	ALA191 unchanged
11,335[b]	TA→CG	ASN192 unchanged
11,467	AT→GC	LEU238 unchanged
11,719[b]	GC→AT	GLY320 unchanged
ND5:		
12,372	GC→AT	LEU12 unchanged
12,937	AT→GC	MET201→VAL
ND6:		
−14,199[b]	CG→AT	PRO159→THR
−14,272[b]	CG→GC	PHE134→LEU
−14,365[b]	CG→GC	VAL103 unchanged
−14,368[b]	CG→GC	PHE102→LEU
−14,484	AT→GC	MET64→VAL
−14,620	GC→AT	GLY18 unchanged

[a] Coordinates of sites within the ND6 gene are preceded by negative signs to denote that this gene is transcribed from the opposite strand.

[b] Also found in ND genes of normal control subjects (data not shown).

[c] Results are from Howell and McCullough (1990).

have now been analyzed. The ND2, ND5, and ND6 genes from two family members (IV-25 and V-17) were sequenced; the former carried only the ND1/L285P substitution, whereas the second carried both the L285P and Y277C changes. Other than the mutation producing the Y277C substitution, the sequences of the other ND genes in these two individuals were identical. Compared with the Cambridge sequence (Anderson et al. 1981), a total of 16 nucleotide changes were found (table 2), 11 of which produce silent polymorphisms. For the sake of completeness, the four sequence changes observed in the previous study of this family (Howell and McCullough 1990) are also included in table 2.

Five nucleotide changes produce amino acid substitutions. Four of the amino acid changes occur in the ND6 protein, which, in most comparisons, is the least evolutionarily conserved of the mitochondrially encoded complex I subunits (e.g., see Clary and Wolstenholme 1985). However, three of these changes have been found also in the ND6 genes of the two *control* subjects in which sequencing studies have been carried out (data not shown). Thus, aside from the L285P/Y277C mutations, the seven ND genes from the Queensland LHON family contain only two nucleotide changes which result in amino acid changes not found also in normal control subjects. In both instances, the substitutions are chemically conservative and occur in residues which are not evolutionarily invariant, even among the ND1 proteins of vertebrates. For example, the ND5/MET201 residue (VAL in the Queensland LHON individuals) is isoleucine in rat, cow, and mouse ND5 proteins and is valine in that of *Xenopus*. The ND6/MET64 residue (also VAL in the Queensland family) is methionine in the cow ND6 protein and is leucine in that from mice, rats, and *Xenopus* (references for the mtDNA sequences are given in table 1).

In addition to the nucleotide changes listed in table 2, it was observed that there is a GC:CG transversion at nucleotide 13702. The Cambridge sequence predicts that there is a glycine residue at amino acid position 456 of the ND5 protein, whereas an arginine residue was found in these studies, in both LHON and non-LHON subjects. Since there is an invariant arginine at this position in all other vertebrate ND1 proteins, it is likely that the Cambridge sequence is in error for this nucleotide. A similar conclusion has been reached by Wallace et al. (1988). The transversion at nucleotide 3423 has also been observed in the ND1 genes of all LHON and non-LHON individuals who have been analyzed.

Discussion

The sequence analysis of the mitochondrial complex I genes from the Queensland LHON family members reveals new features of this disease and, at the same time, underscores unresolved aspects. The major finding is that the clinical (Wallace 1970) and biochemical (Parker et al. 1989) deficiencies in this family are produced by a mutation in the ND1 gene, a mutation which results in the replacement of the LEU285 residue by PRO. The only other LHON mutation identified is that occurring in the ND4 gene of about 50% of all families with this disease (Wallace et al. 1988; Holt et al. 1989; Singh et al. 1989; Vilkki et al. 1989).

The ND1 protein plays an important role in the molecular enzymology of complex I. It has been identified as the subunit which binds rotenone (Earley et al. 1987), an inhibitor which blocks the quinone reduc-

tase site (Ragan 1987). It has recently been concluded that the quinone reductase site is formed, at least in part, by stretches of residues toward the middle of the ND1 protein (Friedrich et al. 1990). The conserved hydrophilic loop containing the LEU285 residue is distal to this region. Therefore, the effect that the L285P substitution has on complex I function may be indirect, perhaps allosterically deranging the quinone reductase site or, alternatively, altering the association of the ND1 protein with other subunits of complex I.

The disease in the Queensland family is extreme in its clinical features (Wallace 1970). Thus, while the ophthalmological features and strict maternal inheritance are typical, the proportion of affected family members—both in males and in females—is unusually high, and onset can be at early adolescence. More important, there are in this family neurological abnormalities, including a severe infantile encephalopathy, tremors, ataxia, dysarthria, posterior column signs, and spasticity (Wallace 1970). It is interesting that there are other LHON families with marked neurological abnormalities. There appear, however, to be differences among these LHON families—the infantile encephalopathy apparently being specific to the Queensland family, for example. In a LHON family studied by Bruyn and Went (1964), the neurological abnormalities were predominantly in males and included spasticity with rigidity, athetosis, and marked dysarthria but no ataxia. The abnormalities in another LHON family were described by Lees et al. (1964) as a disseminated sclerosis.

The molecular genetic and clinical features of the Queensland family support the suggestion put forward elsewhere (Howell and McCullough 1990)—i.e., that LHON is a set of related mitochondrial genetic diseases which share a characteristic pattern of ophthalmological changes. It is unclear, however, how many distinct clinical and/or genetic subgroups of LHON occur. The reason for this uncertainty is that studies have tended to focus on a single type of clinical abnormality. Thus, individuals from several LHON families display neurological abnormalities at a higher-than-expected frequency (Adams et al. 1966; de Weerdt and Went 1971; Palan et al. 1989). Another approach has shown that at least some LHON patients show evidence of *auditory* nerve damage (Mondelli et al. 1990). Finally, cardiovascular abnormalities have been reported in some LHON families (Rose et al. 1970).

When these various studies are considered together, one unanswered question is whether (*a*) the differences *among* LHON families in their array of neurological abnormalities (or lack thereof) are a function of the mitochondrial gene mutation or, alternatively, (*b*) whether there are additional genetic factors, nuclear or mitochondrial. The recent report by Holt et al. (1989) is interesting in this regard. They noted that visually affected males from LHON families carrying the ND4 mutation showed fewer instances of improvement than did males from LHON families not carrying this mutation. It should be noted that *within* LHON families there is also a marked variation in the neurological and ophthalmological changes (discussed below); whether the interfamilial and intrafamilial variation are determined by the same mechanisms is not known. As more of the proximal mitochondrial gene mutations in LHON families are identified, these issues can be addressed in a more definitive manner.

Another striking result to arise from these studies was the finding of a putative intragenic suppressor mutation in one branch of the family. The ND1/Y277C mutation was homoplasmic in the three offspring of IV-32 but has not yet been detected in other family members. This is another example of the rapidity with which mitochondrial genes can segregate in LHON families (also see Bolhuis et al. 1990). In addition to its remarkable proximity to the proline substitution at position 285, there are two sets of observations suggesting that the Y277C replacement has a suppressor effect. First, among the five family members carrying both ND1 mutations, only two males (V-17 and V-18) are clinically abnormal, and these have impaired vision (fig. 1). There is no history of the infantile encephalopathy or severe neurological complications. In contrast, in another branch, V-7 and V-9 were afflicted with the infantile encephalopathy, whereas their mother (IV-28) has neurological abnormalities (fig. 1). Both she and two other children (V-8 and V-10) carry only the L285P mutation.

Second, the preliminary biochemical studies of Parker et al. (1989) showed that the complex I defect was less severe in the two family members carrying the ND1/Y227C substitution. The specific activity of complex I in platelet mitochondria from normal controls was 17.9 nmol NADH oxidized/min/mg protein. The activities for family members IV-25 and V-8 (L285P mutation only) were 2.2 and 2.5, respectively. In contrast, the corresponding values for V-17 and V-18 (both carry the L285P and Y277C mutations) were 5.0 and 5.6. While the available data thus support a role for the Y277C mutation as a partial suppressor, this conclusion requires further biochemi-

cal and molecular genetic analysis of this family. Screening for the Y277C mutation will be greatly facilitated by the fact that the nucleotide change at coordinate 4136 generates a new *Sph*I site.

The results presented here have provided preliminary evidence for a mitochondrial intragenic suppressor. Even if this suggestion is verified, it cannot explain the clinical variability among other branches of the family. For example, individual V-10 is clinically normal and yet was shown to carry only the L285P mutation. It is characteristic of LHON that there is a marked intrafamilial variability in the expression of the neurological or ophthalmological abnormalities. This was first noted as a marked preponderance of males afflicted with the optic neuropathy (Lundsgard 1944; van Senus 1963; Seedorf 1970; Nikoskelainen et al. 1987), although this trend is less marked in the Queensland LHON family (Wallace 1970). There is another level of complexity in those LHON families in which there are neurological abnormalities: discordancy (Bruyn and Went 1964; Wallace 1970). Thus, within the Queensland LHON family, IV-28 (the mother of asymptomatic V-10) has normal vision but displays neurological abnormalities, whereas her son (V-8) has optic atrophy but no neurological changes. These individuals are known to carry the ND1/L285P mutation. Another family member displays both visual and neurological problems (IV-32), and there is at least one family member (IV-30) who recovered from the infantile encephalopathy but is clinically normal as an adult. At least in this LHON family, there is no evidence that heteroplasmy contributes to this phenomenon.

It thus appears that a mutation in one of the mitochondrially encoded subunits of complex I may be necessary—but not sufficient—for development or expression of this disease. The next challenge is to develop testable models of the interfamilial and intrafamilial variation of LHON. The role of mitochondrial genes in human disease is just beginning to be understood.

Acknowledgments

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