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Genetics

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

(heteroplasmy/oxidative phosphorylation/mtDNA haplotype)

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ABSTRACT **A five-generation Hispanic family expressing maternally transmitted Leber hereditary optic neuropathy and/or early-onset dystonia associated with bilateral basal ganglia lesions was studied. Buffy coat mitochondrial DNA (mtDNA) from a severely affected child was amplified by the polymerase chain reaction and greater than 90% sequenced. The mtDNA proved to be a Native American haplogroup D genotype and differed from the standard "Cambridge" sequence at 40 nucleotide positions. One of these variants, a G-to-A transition at nucleotide pair (np) 14459, changed a moderately conserved alanine to a valine at NADH dehydrogenase subunit 6 (ND6) residue 72. The np 14459 variant was not found in any of 38 Native American haplogroup D mtDNAs, nor was it detected in 108 Asian, 103 Caucasian, or 99 African mtDNAs. Six maternal relatives in three generations were tested and were found to harbor the mutation, with one female affected with Leber hereditary optic neuropathy being heteroplasmic. Thus, the np 14459 G-to-A missense mutation is specific to this family, alters a moderately conserved amino acid in a complex I gene, is a unique mtDNA variant in Native American haplogroup D, and is heteroplasmic, suggesting that it is the disease-causing mutation.**

Leber hereditary optic neuropathy (LHON) associated with dystonia (LHON and dystonia) was originally hypothesized to be a mitochondrial disease on the basis of transmission of the clinical symptoms in a five-generation Hispanic family of 79 individuals, including 22 affected (1). In this family, all affected individuals were related through the maternal lineage, the clinical presentation varied from only LHON to severe neurological disease including dystonia, and the severity of the symptoms seemed to increase with successive generations (Fig. 1) (1). Since the mitochondrial DNA (mtDNA) is the only maternally inherited component of the human genome (2), and the mtDNA genotype of heteroplasmic (containing mutant and normal mtDNAs) tissues can shift during cell propagation, yielding variable phenotypes (3, 4), we speculated that LHON and dystonia could be the result of a heteroplasmic mtDNA mutation (1, 5).

The mitochondria of eukaryotic cells generate adenosine triphosphate (ATP) via oxidative phosphorylation, an enzyme pathway consisting of five multisubunit enzyme complexes located within the mitochondrial inner membrane. This pathway is subdivided into the electron transport chain (complexes I to IV) and the ATP synthase (complex V). Complexes I, III, IV, and V are assembled from both nuclear DNA and mtDNA gene products. The mtDNA contributes seven polypeptide subunits (ND1, -2, -3, -4, -4L, -5, and -6) to complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3); one subunit (cytochrome *b*) to complex III (ubiquinol–cytochrome-*c* oxidoreductase, EC 1.10.2.2); three subunits (COI, -II, and -III) to complex IV (cytochrome-*c* oxidase, EC 1.9.3.1); and two subunits (ATPase 6 and 8) to complex V (ATP synthase, EC 3.6.1.34). The mtDNA also encodes the 12S and 16S rRNAs and the 22 tRNAs required for mitochondrial protein synthesis.

LHON is a midlife disease associated with acute or subacute central vision loss and has been shown to result from missense mutations in the mtDNA genes encoding subunits of oxidative phosphorylation complexes I, III, or IV (4). The most common LHON mutations occur in complex I genes and include a G-to-A transition at nucleotide pair (np) 3460 in ND1 (6, 7); a G-to-A transition at np 11778 in ND4 (8); and a T-to-C transition at np 14484 in ND6 (9–13). Leigh syndrome is a rapidly progressive, childhood neurodegenerative disease associated with symmetric basal ganglia lesions that has been shown to result in a number of cases from mutations at np 8993 within the mitochondrial ATPase 6 gene (14–17). Thus, mtDNA missense mutations in complex I and V genes can yield clinical phenotypes similar to those seen in LHON and dystonia.

The mtDNA hypothesis for LHON and dystonia has been further supported by the publication of additional large LHON and dystonia kindreds showing exclusive (18–22) or potential (23) maternal transmission. Moreover, patients with idiopathic dystonia have been shown to have marked respiratory complex I defects when assayed from blood platelets (24), and chronic treatment of mammals with the complex IV inhibitor sodium azide (25) or the complex II inhibitor 3-nitropropanoic acid (26, 27) produces basal ganglia lesions in primates and rodents similar to those seen on computerized tomography (CT) and magnetic resonance imaging analysis of severely affected LHON and dystonia patients (1, 5).

To test the hypothesis that the maternally inherited LHON and dystonia in this Hispanic family was due to a mtDNA mutation, we sequenced the mtDNA of a severely affected individual. A single G-to-A missense mutation was observed in the ND6 gene at np 14459 which was unique to this family. This mutation was heteroplasmic in some maternal relatives and changed a moderately conserved amino acid in the most conserved region of the ND6 polypeptide. Hence, this mutation is likely to cause LHON and dystonia in this family.

MATERIALS AND METHODS

Pedigree and Patients. The pedigree numbers, clinical features, and ages of individuals in this study are taken from our original report (Fig. 1) (1).

The sequenced proband (IV-36) was a 10-year-old female with onset of mild clinical manifestations at 2 years progress-

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Abbreviations: LHON, Leber hereditary optic neuropathy; np, nucleotide pair; CT, computerized tomography.

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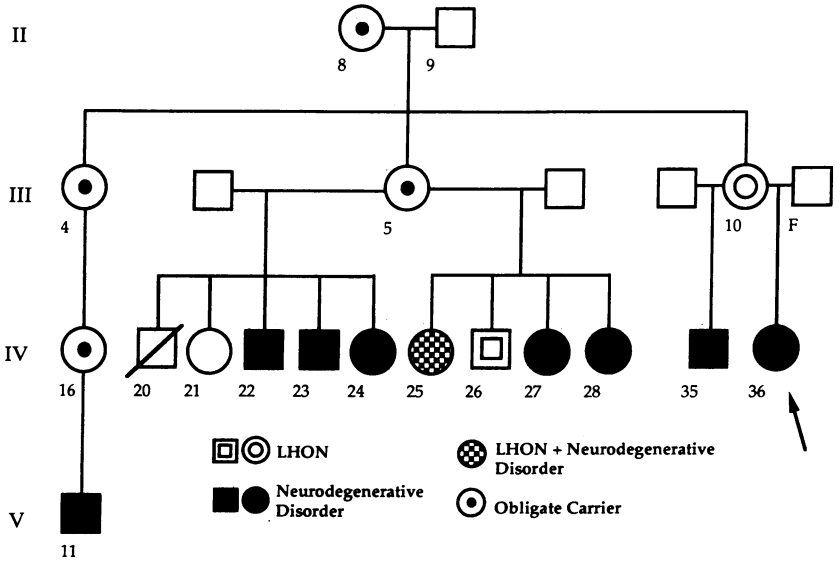

FIG. 1. Partial pedigree of the five-generation LHON and dystonia Hispanic-American family. Individual identification is according to the original report (1).

ing to generalized dystonia at 5 years. By 8 years, corticospinal tract dysfunction, dysarthria, and extraocular muscle involvement were observed. CT and magnetic resonance imaging examination revealed bilateral low-density lesions of the putamina at 2 years which progressed to the caudate nuclei at 8 years, with similar bilateral lesions within the regions of the centromedian nuclei (1).

Patient IV-35 at 13 years had a milder dystonia but greater intellectual impairment. Patient III-10 experienced at 32 years the bilateral optic atrophy of LHON. Individual III-F is the unaffected father of IV-36. Individual III-5 is an obligate carrier and mother of LHON patient IV-26. Patient V-11 presented at 5 years with mild generalized dystonia and abnormal basal ganglia lucencies on CT scan (1).

Tissue Samples and DNA Isolation. Total genomic DNA was extracted from buffy coat (III-10, III-F, IV-26, IV-35, IV-36, and controls), phytohemagglutinin-stimulated lymphocytes (III-5), and platelets (V-11) by standard methods.

mtDNA Haplotype and Phylogenetic Analysis. mtDNA haplotypes were determined as described (28). The phylogenetic relationship between the family's mtDNA haplotype and 120 additional Native American mtDNA haplotypes was determined by parsimony analysis (PAUP version 3.0S) (29). The resulting tree included 508 individuals and 131 character states (28, 30), was 173 steps in length with consistency and retention indices of 0.564 and 0.894, respectively, and was rooted by using a Senegalese mtDNA.

mtDNA Sequence Analysis. mtDNA was asymmetrically PCR-amplified and directly sequenced by using Amplitaq (Perkin–Elmer/Cetus) or Sequenase (United States Biochemical) protocols (12). Ambiguities were resolved by sequencing the opposite DNA strand or by diagnostic restriction endonuclease digestion (11).

Restriction Enzyme Analysis and Population Surveys of mtDNA Variants. Population screens for the np 2092, 3010, 8414, 9966, and 14459 mutations included mtDNAs from 38 clinically normal haplogroup D Native Americans and a large number of unrelated African, Asian, and Caucasian mtDNAs.

Tests for known pathogenic mtDNA mutations included LHON (np 11778, 3460, 4160, 15257, and 14484); myoclonic epilepsy and ragged red fiber disease (MERRF) (np 8344); mitochondrial encephalomyopathy, lactic acidosis, and strokelike syndrome (MELAS) (np 3243, 3271, and 11084); cardiomyopathy plus myopathy (np 3260); mitochondrial myopathy (np 3250); and neurogenic muscle weakness, ataxia, and rentinitis pigmentosum (NARP)/Leigh disease (np 8993). PCR-based restriction enzyme tests for base substitutions at np 2092, 3010, 8414, 9966, and 14459 were designed, using mismatched PCR primers (11). Coordinates of all nucleotide pairs given correspond to the published human mtDNA sequence (31).

For np 2092, the forward primer extended from np 2063 to np 2091 (5′-to-3′) with a mismatched A at np 2087, creating an *Ase* I (New England Biolabs) site in the presence of a C-to-T transition at np 2092. The reverse primer extended from np 2881 to 2900 (3′-to-5′). *Ase* I digestion yielded 684- and 153-bp fragments for normal and 658-, 153-, and 26-bp fragments for mutant mtDNAs.

For np 3010, the forward primer extended from np 2981 to np 3009 (5′-to-3′) with a mismatched G at np 3008, creating a *Bst*UI (New England Biolabs) site in the presence of a G-to-A transition at np 3010. The reverse primer extended from np 3351 to np 3370 (3′-to-5′). *Bst*UI digestion yielded an uncut 389-bp product for normal and 362- and 27-bp fragments in mutant mtDNAs.

For np 8414, the forward primer extended from np 8374 to np 8413 (5′-to-3′) with a mismatched A at np 8410 creating an *Ssp* I (New England Biolabs) site in the presence of a C-to-T transition at np 8414. The reverse primer extended from np 8608 to np 8628 (3′-to-5′). *Ssp* I digestion yielded 180- and 74-bp fragments for normal and 180-, 38-, and 36-bp fragments for mutant mtDNAs.

For np 9966, the forward primer extended from np 9802 to np 9821 (5′-to-3′) and the reverse primer from np 10356 to np 10375 (3′-to-5′). *Bsm*AI (New England Biolabs) digestion yielded 409- and 164-bp fragments for normal and an uncut 573-bp fragment for mutant mtDNAs. All putative np 9966 G-to-A mutations identified by this test were verified by sequencing.

For np 14459, the forward primer extended from np 14430 to np 14458 (5′-to-3′) with a mismatched G at np 14456, creating a *Mae* III (Boehringer Mannheim) site in the presence of a G-to-A transition at np 14459 on the L strand. The reverse primer extended from np 14855 to np 14874 (3′-to-5′). *Mae* III digestion yielded an uncut 444-bp fragment for normal and 419- and 25-bp fragments for mutant mtDNAs. For *Mae* III digestions, 1 μl of enzyme was added to a 20-μl total reaction volume, and the mixture was incubated at 55°C for 12 hr; then another 1 μl of *Mae* III was added and incubation was continued for 4 hr at 55°C. Prior to the addition of agarose gel loading dye, samples were heated to 95°C for 5 min.

For the np 14459 mutation, heteroplasmy was quantitated by one-dimensional densitometry using an Ultroscan XL densitometer equipped with Gel-Scan XL software, version 2.0 (Pharmacia LKB) and type 55 Polaroid negatives of ethidium bromide-stained agarose gels.

RESULTS

Haplotype and Phylogenetic Analysis Reveals a Native American mtDNA. mtDNA haplotypes determined for IV-36, III-10, and IV-35 differed from the standard "Cambridge" sequence (31) by 12 restriction site variants. Seven of these variants, an *Alu* I site loss at np 4769, an *Alu* I site gain at np 7025, a *Hha* I site gain at np 8858, a *Hae* III site loss at np 13702, a *Hinc*II site loss at np 14199, a *Hinf*I site gain at np 14268, and a *Hinf*I site loss at np 14368, have been previously observed in multiple population studies (8, 12, 13, 28, 32–40), indicating that they are very common polymorphisms or "errors" in the published sequence. Four of the site variants, an *Alu* I site loss at np 5176, a *Dde* I site gain at np 10394, an *Alu* I site gain at np 10397, and a *Hae* III site gain at np 16517, are common nonpathogenic polymorphisms characteristic of certain human populations (28, 32, 34, 35). Among these, the site variants at np 5176, 10394, and 10397 define haplogroup D, one of four Native American-specific mtDNA haplogroups designated A, B, C, and D (28, 35). Thus, the mtDNA of this Hispanic family is of Native American origin. The twelfth variant, a *Hae* III site gain at np 11092, has not been observed in previous population surveys (36). No mtDNA length polymorphisms were observed on haplotyping, a result confirmed by Southern analysis (ref. 1 and data not shown).

The close relationship of the LHON and dystonia family's mtDNA haplotype with the other 16 haplotypes (38 individuals) of haplogroup D is shown in the maximum parsimony phylogeny of 120 Native American haplotypes (Fig. 2). The proband's mtDNA represents a new branch of the Native American haplogroup D mtDNA lineage, delineated by a *Hae* III site generated by a silent A-to-G transition at np 11092.

mtDNA Sequence Analysis Reveals a np 14459 Variant Not Described Previously. The mtDNA genome of IV-36 was sequenced to the following extent: D-loop region, 98%; tRNA genes, 92%; rRNA genes, 80%; and protein coding genes, 92%. In addition to the 12 restriction fragment length polymorphism (RFLP) variants discussed above, 28 base substitutions were identified, giving a total of 40 base substitutions relative to the Cambridge sequence (Table 1). No known pathogenic mtDNA mutations were observed, a result confirmed by mutation-specific restriction endonuclease digests (see *Materials and Methods*).

The majority of the 40 variants could be excluded from an etiologic role in this disease. Seventeen variants (np 3423, 4883, 4985, 6179, 7055, 9540, 10163, 10400, 10538, 10873, 11092, 11335, 12705, 14668, 14783, 15043, and 15301) were silent substitutions within protein-coding genes. Ten variants (np 263, 4769, 7028, 8860, 13702, 14199, 14272, 14365, 14368, and 15326) have been observed either in all individuals or in certain ethnic groups and thus are either errors in the Cambridge sequence or ethnic-specific variants. Four variants (np 750, 8701, 10398, and 16519) have been observed at significant polymorphic frequencies in some ethnic groups (8, 11, 12, 28, 32, 35, 37–39). Three variants (np 489, 16223, and 16291) occur in the non-coding D-loop region and have been observed previously (refs. 28, 40, and 42; M.D.B. and D.C.W., unpublished data). One variant (np 5178) is found in all Native American haplogroup D mtDNAs and defines that group (28, 35).

The five remaining variants (np 2092, 3010, 8414, 9966, and 14459) were investigated further. Three of these variants (np 3010, 8414, and 9966) have been observed previously (42–44), while two (np 2092 and 14459) have not. The C-to-T transition at np 2092 alters a nonconserved nucleotide in the 16S rRNA gene (Table 1). Screening for this variant revealed its presence in 35 of 37 haplogroup D controls. Thus, it is a common Native American haplogroup D polymorphism. The G-to-A transition at np 3010 changes a moderately conserved nucleotide in the 16S rRNA gene (Table 1). Screening for this variant revealed that 6 of 36 haplogroup D Native Americans had this variant, suggesting a neutral polymorphism. The C-to-T transition at np 8414 converts a nonconserved leucine to phenylalanine at codon 17 of the ATPase 8 gene (Table 1). All Native American haplogroup D mtDNAs had this variant. Hence, this marker is haplogroup D-specific. The G-to-A transition at np 9966 substitutes an isoleucine for a valine at codon 254 of the COIII gene. This amino acid is moderately conserved among animal species, although an isoleucine is found at the corresponding position in *Drosophila yakuba* (46). Population screening revealed that this variant was absent from 37 Native American haplogroup D mtDNAs and 59 random Asians, but it was found in 1/60 (1.7%) African and 2/65 (3.1%) Caucasian control mtDNAs. Hence, this is a rare variant of possible relevance, but it is unlikely to be the primary disease mutation. The G-to-A transition at np 14459 changes a moderately conserved alanine to valine at codon 72 of the ND6 gene (Table 2). All mammals have an alanine at this position and all reported mtDNAs have either an alanine or serine, with the exceptions of fungus (leucine) and liverwort (phenylalanine) (Table 2).

A population survey of the np 14459 mutation, using mutation-specific primer mismatch PCR and *Mae* III restriction endonuclease digestion, revealed that all 38 Native American haplogroup D mtDNAs analyzed were negative for this mutation. An additional 310 controls, including 99 Africans, 108 Asians, and 103 Caucasians, were also negative for this mutation, yielding a total of 348 controls tested. Hence, the np 14459 variant is unique to this LHON and dystonia family.

Blood cell mtDNAs of five additional maternal relatives (III-5, III-10, IV-26, IV-35, and V-11) plus the proband's

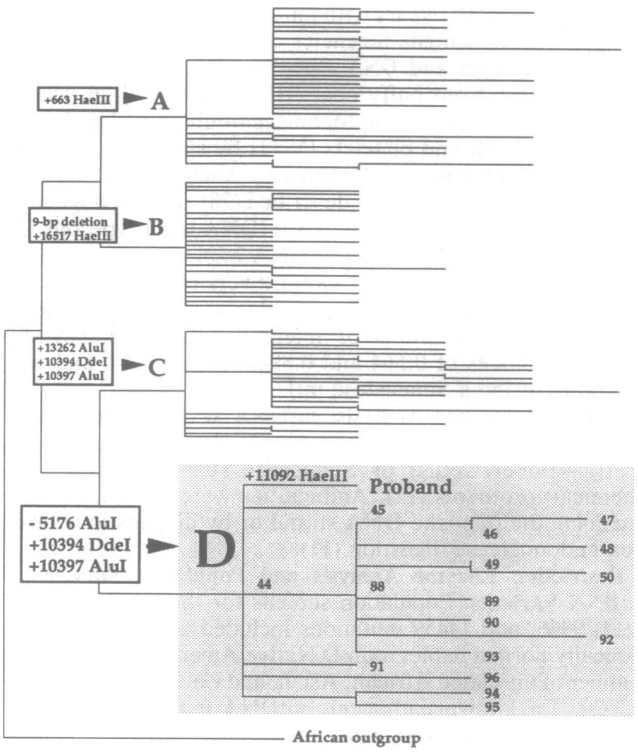

FIG. 2. Native American mtDNA phylogenetic tree including the LHON and dystonia haplotype. The Native American controls represent 508 individuals and 120 distinct haplotypes (28, 30). The haplogroup D samples represent 59 individuals and 16 distinct haplotypes (28). A *Hae* III site loss at np 11092 results in a novel LHON and dystonia haplotype.

Table 1. mtDNA sequence variants in LHON and dystonia proband IV-36

Gene product	np	Base change	Amino acid change	Nucleotide or amino acid conservation (*H/B/M/X*)*
16S rRNA	2092	C to T	—	C/A/A/G
16S rRNA	3010	G to A	—	G/G/A/G
ND2	4883	C to T	None	
COI	6179	G to A	None	
COI	7055	A to C	None	
ATPase 8	8414	C to T	L to F	L/F/M/W
COIII	9966	G to A	V to I	V/V/V/V
ND3	10163	C to A	None	
ND4L	10538	C to T	None	
ND4	11092	A to G	None	
ND6	**14459**	**G to A**	**A to V**	**A/A/A/A**
ND6	14668	C to T	None	
Cyt *b*	14783	T to C	None	
Cyt *b*	15043	G to A	None	
Cyt *b*	15301	G to A	None	

Previously reported polymorphisms or Cambridge sequencing errors found in the proband include mutations at np 263, 489, 750, 3423, 4769, 4985, 5178, 7028, 8701, 8860, 9540, 10398, 10400, 10873, 11335, 12705, 13702, 14199, 14272, 14365, 14368, 15326, 16223, 16291, 16519 (refs. 8, 11–13, 28, 31, 32, 34, 35, 37–39, and 41–45). Mutations are reported here as L-strand base changes.

*The nucleotides (for 16S rRNA only) or amino acid residues (single-letter code) found in this position in *Homo sapiens/Bos bovis/Mus musculus/Xenopus laevis*.

father (III-F) were tested for the np 14459 mutation. The father was negative for the mutation, while all maternal relatives were positive. The buffy coat mtDNA of the proband's mother was heteroplasmic for this mutation, with 73% of her mtDNAs being mutant and 27% being normal (Fig. 3). The remaining five maternal relatives were essentially homoplasmic, with >99% mutant mtDNAs as determined by laser densitometry.

DISCUSSION

Analysis of the mtDNA of a patient with early onset dystonia and bilateral striatal necrosis from a family with maternally inherited LHON and dystonia revealed that a heteroplasmic ND6 mutation at np 14459 is the probable cause of this disease. This G-to-A transition changes a moderately conserved alanine to a valine at codon 72 of the ND6 gene, an amino acid in the "span C" region of ND6, the most

Table 2. Amino acid conservation of alanine-72 in ND6

Species	Amino acid sequence							Ref.
LHON + dystonia	Y	T	T	**V**	M	A	I	
Homo sapiens	Y	T	T	**A**	M	A	I	31
Bos bovis	Y	T	T	**A**	M	A	T	47
Mus musculus	Y	T	T	**A**	M	A	T	48
Rattus norvegicus	Y	T	T	**A**	M	A	T	49
Balaenoptera physalus	Y	T	T	**A**	M	A	T	50
Xenopus laevis	Y	S	A	**A**	R	A	K	51
Strongylocentrotus purpuratus	Y	S	S	**A**	I	S	–	52
Gallus domesticus	Y	S	V	**S**	L	A	A	53
Coturnix japonica	Y	S	V	**S**	L	A	A	54
Drosophila yakuba	Y	V	T	**S**	L	A	S	46
Caenorhabditis elegans	Y	F	S	**S**	L	S	K	55
Ascaris suum	Y	F	S	**S**	L	S	K	55
Aspergillus nidulans	L	T	V	**L**	L	F	Y	56
Marchantia polymorpha	Y	S	N	**F**	F	V	Y	57

Boldface amino acid symbols correspond to codon 72 of the human ND6 polypeptide.

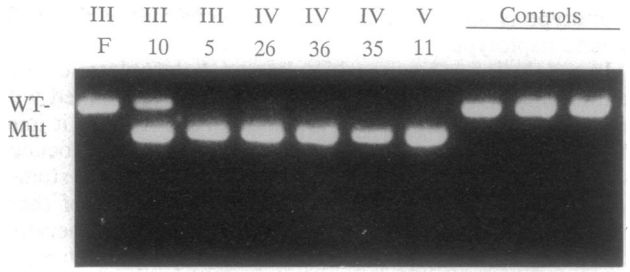

FIG. 3. *Mae* III restriction digest test for the np 14459 mutation in LHON and dystonia family members and unrelated Native American haplogroup D controls. Normal (WT) sequence lacks the *Mae* III site and leaves an uncut 444-bp fragment. Mutant (Mut) sequence generates a *Mae* III site at which the 444-bp fragment is cleaved into 419- and 25-bp fragments.

evolutionarily conserved sequence of this polypeptide (58). Interestingly, the known LHON mutation at np 14484 (9, 10) also changes a span C amino acid only eight residues away from the alanine altered by the np 14459 mutation. Like the np 14459 mutation, the np 14484 mutation changes a moderately conserved amino acid and has been observed in a five-generation, 136-member Australian family exhibiting maternally inherited LHON, a generalized movement disorder, and infantile encephalopathy (7, 59). Thus, mutations in this region of ND6 may contribute to neurological disease.

The np 14459 mutation was not found in any of 348 normal controls representing diverse haplotypes from four major racial/ethnic groups: African, Caucasian, Asian, and Native American. The latter group is of particular importance since the patient's mtDNA genotype belongs to Native American haplogroup D, yet none of the 38 individuals in this control group harbor this mutation. Thus the np 14459 mutation is not a rare, ethnic-specific variant, but instead occurred recently in this mtDNA lineage.

The recent occurrence of the np 14459 mutation is further substantiated by the discovery that the mother of the proband is heteroplasmic for the mutation. This implies that this mutation arose in the family concurrently with the appearance of LHON in generation I of this pedigree. Since many of the individuals in the most recent generations have the more severe pediatric dystonia and are essentially homoplasmic for the mutation, it is tempting to speculate that replicative segregation to homoplasmic mutant mtDNAs in affected tissues accounts for the increasing severity of the disease in more recent generations. Thus, the np 14459 mutation fulfills the criteria that would be expected for a mtDNA mutation causing maternally transmitted LHON and dystonia (1, 5).

While the evidence indicates that the np 14459 mutation is the primary cause of LHON and dystonia, it is possible that other variants associated with this Native American haplogroup D mtDNA might act synergistically to enhance the pathogenicity of the ND6 mutation. This type of genetic interaction has been proposed to account for some occurrences of LHON (12, 60), Alzheimer disease, and Parkinson disease (33, 61), and it may be an important factor influencing disease expression in the Australian LHON and neurological disease family harboring the np 14484 LHON mutation. The Australian pedigree has been found to harbor a second, homoplasmic, mtDNA missense mutation in the ND1 gene at np 4160 (7), which changes a conserved leucine to a proline. Similarly, the Hispanic LHON and dystonia family harbors a homoplasmic COIII variant at np 9966 which alters a moderately conserved amino acid, is not observed in other haplogroup D mtDNAs, and is only rarely seen in the general population (8, 11, 12, 37–39, 42, 44, 45, 62). Thus, it may be that the severity of the Hispanic LHON and dystonia family's

phenotype is influenced by the np 9966 variant in the background haplotype.

In conclusion, a previously unknown, heteroplasmic, ND6 mutation at np 14459 has been found to be associated with maternally inherited LHON and dystonia. As such, it joins the np 14484 variant as one of two ND6 mutations associated with LHON and/or neurological disease. Additional studies will be required, however, to determine if either of these mutations is capable by itself of causing neurodegenerative disease or if both require secondary modifying mutations to give the most severe clinical presentation.

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