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Mitochondrial changes in leukocytes of patients with optic neuritis

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Purpose: Optic neuritis (ON) is a demyelinating disorder affecting optic nerves. It has certain similarities to Leber hereditary optic neuropathy (LHON) and other spontaneous optic neuropathies known to be associated with mitochondriopathies. We evaluated patients with optic neuritis for evidence of systemic mitochondrial abnormalities.
Methods: Patients were selected who had ON affecting one or both eyes. We performed clinical examinations and neuroimaging on the participants. We sequenced the entire mitochondrial DNA (mtDNA) genome except for the D-loop in leukocytes of all patients; assessed relative mtDNA content; measured mitochondrial respiratory function in 15 patients; and sequenced OPA1 and OPA3 genes, where mutations have been associated with dominant and recessive optic nerve atrophy, respectively.
Results: Twenty-six patients (11 males and 15 females; average age at onset 23.4±8.1 years) met inclusion and exclusion criteria. Eleven patients had neuroimaging evidence of disseminated demyelination, and six had clinically definite multiple sclerosis. No patient had a primary LHON mutation or a pathologic sequence change in OPA1 or OPA3 genes. Sixteen patients had potentially pathologic mtDNA changes, and after recovery these patients had significantly worse visual acuity ($p=0.002$) and color vision ($p = 0.009$) than other patients. Mean relative mtDNA content was significantly increased in ON patients compared to controls (2.39 versus 1.03; $p<0.001$), while mitochondrial respiratory activity was significantly decreased (16.78 versus 22.53; $p<0.001$).
Conclusions: The presence of these systemic mitochondrial abnormalities in patients with ON suggests that mitochondrial abnormalities may constitute risk factors for the occurrence and severity of ON.

Optic neuritis (ON) is a spontaneous optic neuropathy characterized by demyelination that can occur in isolation or as part of multiple sclerosis (MS) [1]. Mitochondria may play a role in MS [2], and mitochondrial abnormalities have been recognized in association with a number of spontaneous optic neuropathies, including Leber hereditary optic neuropathy (LHON) [3], dominant optic atrophy [4,5], and hereditary motor sensory neuropathy type VI [6]. Some women with MS and bilateral, relatively severe optic nerve disease have the nt 11778 primary LHON mutation and meet diagnostic criteria for LHON [7]. Parent-child concordance studies in MS have also demonstrated that females are more likely to have affected children than males [8], possibly indicating inheritance of a mitochondrial risk factor.

In this study, we investigated whether ON is associated with systemic mitochondrial abnormalities by evaluating several mitochondrial parameters in leukocytes of patients with ON, including sequencing the entire mitochondrial DNA (mtDNA) genome, estimating relative mtDNA content [9], and assessing mitochondrial respiratory function [10]. We also sequenced OPA1 and OPA3 in order to exclude both dominant and recessive optic nerve atrophy genetically as well as clinically.

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METHODS

Patient enrollment: Patients were eligible for inclusion in this study if they met standard clinical criteria for ON, including age greater than 10 years and less than 50 years, acute or subacute decline in vision of one or both eyes due to optic nerve dysfunction, sometimes with a papillitis as well as pain on eye movement, and with at least modest recovery of vision over a period of weeks [1].

Exclusion criteria included exposure to a known optic nerve toxin; evidence on history, examination, or neuroimaging of an alternative medical or surgical cause of optic nerve damage affecting either eye; a cause of significant visual loss in either eye independent of optic neuropathy; or refusal to participate. Patients were not excluded because of clinical or neuroimaging evidence of disseminated demyelination. They were selected from neuro-ophthalmology clinics at the King Khaled Eye Specialist Hospital and the King Faisal Specialist Hospital and Research Centre. The study was approved by institutional review boards at both hospitals, and informed consent was obtained from each patient.

Hospital records were reviewed, and full neuro-ophthalmologic examinations were performed on all patients. Color vision (CV) was assessed with Ishihara pseudoisochromatic

plates. Patients had either Goldman manual kinetic perimetry (Haag Streit International, Koeniz-Bern) or Humphrey automated, white on white stimulus, static perimetry (Humphrey Field Analyzer II, Carl Zeiss Meditec, Inc, Oberkochen, Germany), or both. Poser criteria [11], and where possible McDonald criteria [12], were applied for the diagnosis of MS.

Brain neuroimaging was obtained on a Siemens Magnetom Allegra 3.0 Tesla MRI Scanner or a Siemens Somatom Sensation 4 CT Scanner (Siemens Medical Systems, Germany). MRI images were assessed using Analyze (Biomedical Imaging Resources, Mayo Foundation, Rochester, MN) and custom-written software [13]. The presence of disseminated demyelination was graded as "none", "possible" (one lesion >3 mm not involving the optic nerves), "probable" (2 lesions >3 mm outside the optic nerve), and "consistent", fulfilling the Fazekas criteria for MS [14].

Control subjects: All control subjects were King Faisal Specialist Hospital and Research Center blood donors who represented the spectrum of Saudi Arabs and who reported no symptomatic metabolic, genetic, or ocular disorders on an extensive questionnaire regarding family history, past medical problems, and current health. The control group for mtDNA sequencing consisted of 159 individuals (106 males and 53 females, mean age 46.3±3.8 years); for relative mtDNA content, 50 different individuals (27 males and 23 females, mean age of 26.8±5.2 years); and for mitochondrial functional testing, 62 different individuals (39 males and 23 females, mean age 30.1±7.5 years). Family information was obtained by history. All patients and control subjects were Saudi Arabs. An attempt was made to match ages of controls for relative mtDNA content and mitochondrial functional testing to those of ON patients because both of these parameters can be affected by age [15].

Sample collection and DNA extraction: Blood samples were collected when patients were in remission. DNA was extracted from whole blood samples of all ON patients and controls using the PureGene DNA isolation kit from Gentra Systems (Minneapolis, MN).

Isolation of lymphocytes from peripheral blood and preparation of cell suspension: Blood (5 ml) was diluted with phosphate buffered saline (PBS) at a ratio of 1:1 within 1 h of extraction and slowly layered onto a 15 ml screw cap tube containing 4.5 ml Ficoll-Hypaque separating solution. The tubes were centrifuged for 20 min at 1000xg after which the lymphocyte-containing layer was collected into a new centrifuge tube using a sterile pipette. The lymphocytes mix was then diluted in 10 ml PBS and centrifuged for 10 min at 660xg. The supernatant was discarded, 5 ml of hypotonic PBS lysing buffer was added, the pellet was mixed gently in this buffer, and the mixture was allowed to sit for about 45 s. Five ml of 2x NaCl solution was added. The mixture was gently pipetted and then centrifuged at 600xg for 10 min. The supernatant was discarded, and the pellet was suspended in RPMI 1640 medium (Gibco, Invitrogen Corporation) supplemented with L-glutamine. The optical density (OD 660) of the lymphocyte suspension was adjusted to 0.20, which is equivalent to a cell density of approximately $5x10^5$ cells/ml. Using this protocol, cell viability assessed by 0.2% trypan blue was 96±2%. These cells were used for mitochondrial respiration testing.

DNA amplification and sequencing: The entire coding region of the mitochondrial genome was amplified in all patients and controls in 24 separate polymerase chain reactions (PCRs) using single set cycling conditions as detailed elsewhere [16]. Primers were used to amplify the entire coding region of the mitochondrial genome except the D-loop [17]. PCRs were run under the following PCR conditions: 20 ng of each DNA sample in a 50 ml PCR reaction mixture containing 200 mM dNTP, 0.2 m M of each primer-pair, 1 unit of Taq DNA polymerase, 50 mM KCL, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). Polymerase chain reaction was performed for 35 cycles and 55 °C annealing temperature in a GeneAmp 9700 PCR system (Perkin-Elmer, Foster City, CA). PCR-Primers were designed to avoid amplifying mtDNA-like sequences in the nuclear genome. Each successfully amplified fragment was directly sequenced using the same primers used for amplifications and the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Samples were run on the ABI prism 3100 sequencer (Applied Biosystems).

Sequence analysis of the mitochondrial DNA coding region: The full mtDNA genome was sequenced except for the D-loop. Sequencing results were compared to the corrected Cambridge reference sequence [17]. All fragments were sequenced in both forward and reverse directions at least twice for confirmation of any detected variant. All sequence variants from both ON patients and controls were compared to the Mitomap database [18], the Human Mitochondrial Genome Database, GenBank, and Medline listed publications. Reported homoplasmic synonymous or non-synonymous (NS; resulting in amino acid change) polymorphisms used predominantly for haplogroup analysis were excluded from further consideration [19].

Prediction of pathogenicity: Characteristics of each remaining nucleotide change in both ON patients and controls were assessed in order to estimate the likelihood that individual NS mtDNA changes might be pathogenic. We used a combination of: (1) standard criteria [20]; (2) an evaluation of interspecies conservation using the PolyPhen database and the Mamit-tRNA website, when necessary; (3) assessment of the possible impact of an amino acid substitution on three-dimensional protein structure using the Protean program, part of the LASERGENE V.6 software (DNASTAR, Inc. Madison, WI), which predicts and displays secondary structural characteristics; and (4) assessment of the possible effect of the mtDNA change on protein function using PolyPhen [21] and the SIFT (Sorting Intolerant From Tolerant) program, which predicts whether protein substitutions are tolerated [22]. The same criteria were applied to both recognized NS mtDNA changes (e.g., reported in Mitomap and other databases) and to novel sequence changes.

An NS nucleotide change was considered potentially pathologic if it met all of the following criteria, when applicable: (1) it changed a moderately or highly conserved amino acid; (2) Protean predicted an alteration of protein structure; (3) it was predicted by SIFT to have an effect on protein func-

tion; and (4) it was assessed as possibly or probably pathologic by PolyPhen. For previously reported NS nucleotide changes, consideration was given to pathologic status determined by others and by mitochondrial databases in addition to the criteria described above.

Quantification of heteroplasmy: Heteroplasmy level was determined for each heteroplasmic sequence variant by the primer extension assay described previously [23]. Primer extension reactions were performed in a total volume of 8 ml. The Ul™ DNA polymerase-catalyzed reactions contained template, 20 fmol fluorescein-labeled primer, 400 m M ddNTPs/ 25 mM dNTPs of the appropriate nucleotide combination and 0.6 U enzyme in buffer containing 10 mM Tris-HCl, pH 8.8, 10 mM KC1, 0.002% Tween 20,2 mM $MgCl_2$. Thermo Sequenase DNA polymerase-catalyzed reactions were performed with 20 fmol fluorescein-labeled primer, 25 mM each of the appropriate ddNTP/dNTP combination and 0.64 U enzyme in buffer containing 10 mM Tris-HCl, pH 9.5, 5 mM KC1, 0.002% Tween 20, 2 mM $MgCl_2$. Each set of primer extension assays included control template preparations that had been amplified from homoplasmic wild-type and mutant DNA. After an initial denaturation step at 95 °C for 2 min, the reaction conditions comprised 20 cycles of 95 °C for 20 s and 55 °C for 40 s. The samples were concentrated to about 1 ml by heating open reaction tubes at 94 °C for 7 min. After the concentration step, 8 ml loading dye (0.5% blue dextran in 83% formamide, 8.3 mM EDTA, pH 8.0) were added. Heteroplasmy level was quantified from fluorescence intensities associated with electrophoretically resolved mutant and wild-type peaks using the GeneScan™ 3.7 software program (Applied Biosystems Division, Perkin Elmer, Foster City, CA). Percentage of heteroplasmy was calculated using the following equation: [fluorescent band intensity for the mutant/(fluorescent band intensity for the wild-type+fluorescent band intensity for the mutant)]x100.

Determination of relative mitochondrial DNA content: A competitive multiplex PCR was carried out with two simultaneous primer sets as described previously [9], a technique that has been applied successfully to a variety of tissues [24,25], including blood of patients with LHON [26] and a number of other optic neuropathies [27-29]. One primer-pair was designed to amplify a 450 bp fragment of the ND1 mitochondrial gene (forward primer sequence 5'-ACA TAC CCA TGG CCA ACC TC-3' and reverse primer sequence 5'-AAT GAT GGC TAG GGT GAC TT-3') and the other pair to amplify a 315 bp fragment of the β-actin nuclear gene (forward primer sequence 5'-ATG TTT GAG ACC TTC AAC AC-3' and reverse primer sequence 5'-CAT CTC TTG CAC GAA GTC GA-3'), which served as an internal control. Control and patient PCRs were run simultaneously under the following PCR conditions: 20 ng of each DNA sample in a 50 ml PCR reaction mixture containing 200 mM dNTP, 0.2 mM of each of the ND1 primer-pair, 0.6 mM of each of the b -actin primer-pair, 1 unit of Taq DNA polymerase, 50 mM KCL, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). Polymerase chain reaction was performed for 25 cycles in a GeneAmp 9700 PCR system (Perkin-Elmer). One ml of SYBR® Green I stain was added to the reaction mixture in the last cycle to label the PCR products. PCR products were separated on 1% agarose gel at 100 V for 1 h, and intensity of the two bands was quantified by the use of gel imager (Typhoon 9410; GE Amersham Biosciences, Schenectady, NY). The ratio of ND1 to β-actin was determined for each patient and control by dividing the fluorescence intensity of the ND 1 band by the intensity of the β-actin band.

Measurement of mitochondrial respiration: Resazurin is a redox-active blue dye that becomes pink and highly fluores-

Table 1. Clinical characteristics of optic neuritis patient

Patient	Age at onset	Sex	Pain	Papil lopathy	Initial VA OD	Initial VA OS	Treatment	Final VA OD	Final VA OS	Final CV OD	Final CV OS	MRI	Final diagnosis
1	38	F	Yes	No	20/20	20/400	IFN	20/20	20/40	9	3	Consistent	MS
2	29	F	Yes	Yes	HM	20/20	IV steroids	20/20	20/20	9	9	Possible	Papillitis OD
3	18	F	Yes	Yes	20/20	CF 1'	IV steroids	20/20	20/20	9	9	Consistent	MS
4	18	F	No	Yes	20/30	20/30	None	20/20	20/25	9	9	Normal	Papillitis OS
5	30	M	Yes	Yes	20/30	20/40	IV steroids	20/25	20/30	9	9	Normal	Sequential papillitis
6	34	F	No	No	NLP	20/20	IV steroids	20/20	20/20	9	9	Consistent	Optic neuritis OS
7	20	F	Yes	No	20/25	CF 1'	IV steroids	20/25	20/40	9	9	Consistent	Optic neuritis OS
8	14	M	No	Yes	20/25	20/50	None	20/25	20/30	9	9	Normal	Papillitis OS
9	24	F	Yes	Yes	CF 1'	CF 1'	IV steroids	20/20	20/20	9	9	NA	Papillitis OU
10	32	F	Yes	Yes	CF 3'	20/40	None	20/30	20/30	9	9	Probable	Papillitis OU
11	16	F	Yes	Yes	LP	20/20	IV steroids	20/25	20/20	9	9	Possible	Papillitis OD
12	14	M	Yes	Yes	HM	HM	IV steroids	20/40	20/40	8	9	Normal	Papillitis OU
13	17	F	Yes	Yes	20/100	20/200	IV steroids then IFN	20/30	20/20	9	9	Consistent	MS
14	20	F	Yes	No	CF	20/20	PO steroids	20/20	20/20	9	9	Consistent	MS
15	21	M	No	No	CF 3'	CF 3'	None	20/200	20/400	9	4	Consistent	MS
16	21	M	No	No	CF 1'	20/400	IV steroids then IFN	20/80	20/100	1	1	Consistent	MS
17	17	M	Yes	No	CF 5'	20/20	PO steroids	20/20	20/20	1	2	Consistent	Recurrent optic neuritis OD
18	34	F	Yes	No	20/200	20/200	IV steroids	20/60	20/40	3	7	Possible	Optic neuritis OU
19	13	M	Yes	Yes	HM	HM	None	20/30	20/25	5	5	Possible	Papillitis OU
20	45	M	No	No	CF 3'	CF 3'	None	20/30	20/30	9	6	Consistent	Sequential optic neuritis
21	17	F	Yes	No	HM	CF 1'	None	20/50	20/30	1	3	Possible	Recurrent optic neuritis OU
22	21	F	No	No	20/20	CF 5'	IV steroids	20/30	20/30	9	9	NA	Sequential optic neuritis
23	30	M	Yes	No	20/20	20/200	None	20/20	20/20	9	7	Consistent	Optic neuritis OS
24	22	F	No	No	CF	CF	IV steroids	20/25	20/20	9	9	Normal	Optic neuritis OU
25	22	M	Yes	Yes	CF 5'	20/20	IFN	20/100	20/20	0	9	Possible	Optic neuritis OD
26	21	M	Yes	Yes	LP	20/20	None	20/80	20/20	9	9	Normal	Papillitis OD

Age at onset represents age at diagnosis in years. The following abbreviations were used: male (M); female (F); visual acuity (VA) by Snellen plates; color vision (CV); right eye (OD); left eye (OS); counting fingers (CF); hand motions (HM); light perception (LP); no light perception (NLP); multiple sclerosis (MS); intravenous (IV); by mouth (PO); interferon (IFN); not available (NA); Final diagnosis combines neuro-ophthalmologic evaluation, Poser criteria [2], and McDonald criteria [3] when possible, MRI interpretation (see Methods) according to Fazekas criteria [1].

cent when reduced. It competes with oxygen for electrons in a standard preparation of circulating lymphocytes, and change in fluorescence (corrected for background and protein concentration) reflects respiration. Lymphocytes from 15 patients and 62 controls were incubated with 6 μM resazurin without and with mitochondrial inhibition by amiodarone 200 μM, and the fluorescence intensity resulting from resazurin reduction was monitored spectrofluorimetrically over time. Inhibited and uninhibited samples were assessed at 240 min, and the inhibited sample reading was subtracted from the uninhibited sample reading. This value was normalized for protein concentration and background reading. The procedure was performed three times for each patient and control, and the results were averaged, yielding the final MRA value.

Sequence analysis of OPA1 and OPA3 genes: The 31 coding exons, exon-intron boundaries, and promoter regions of the OPA1 gene were amplified by PCR from genomic DNA for all patients and subjected to direct sequencing as described

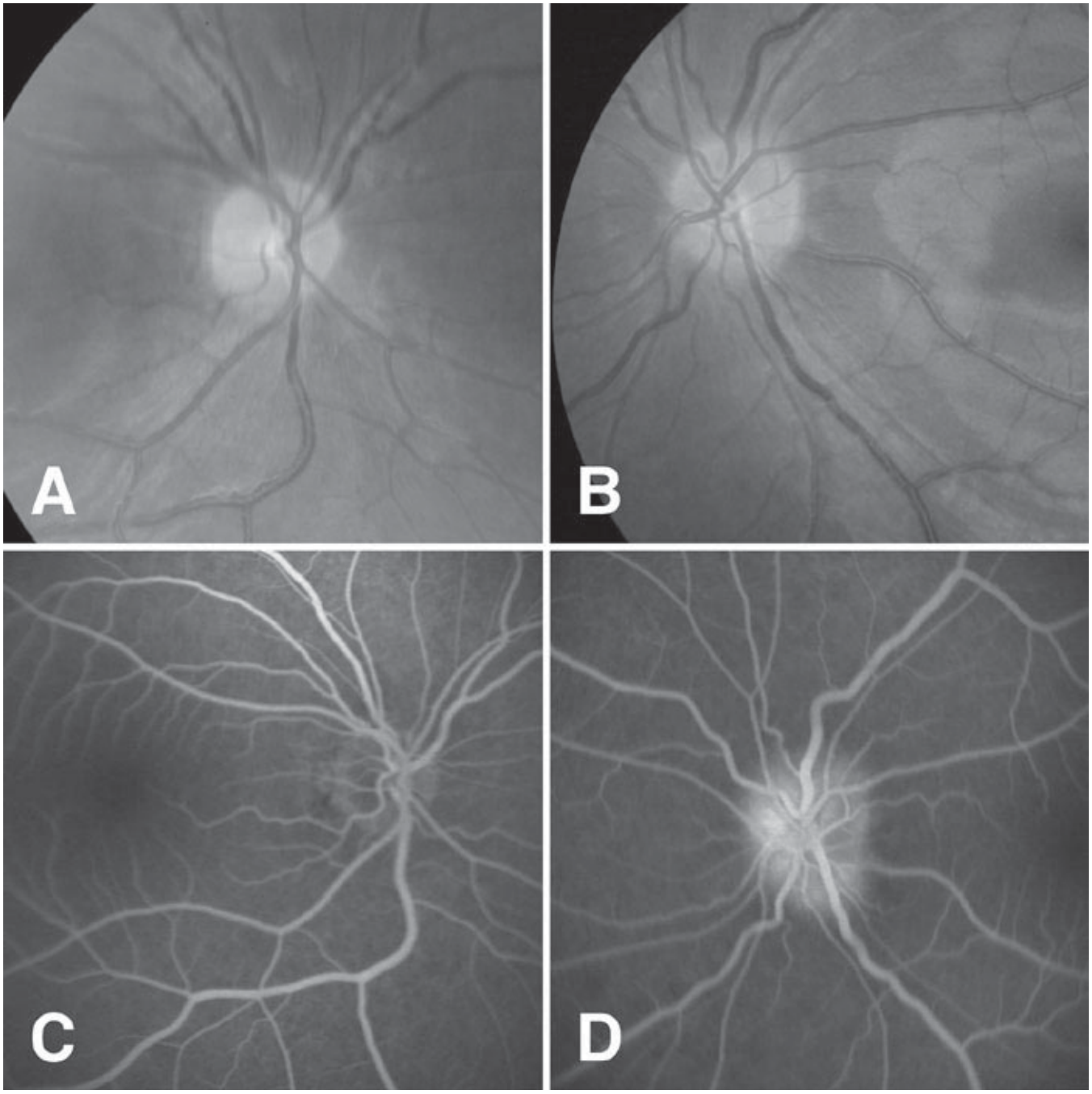

Figure 1. Mild papillitis in a patient with optic neuritis. (**A**) right eye (OD) and (**B**) left eye (OS) fundus photos of Patient 4, who had optic neuritis OS with mild swelling of the optic disc. Fluorescein angiogram OD (**C**; at 5:56 min) and OS (**D**; at 5:31 min) revealed staining of the left optic disc typical of papillitis but not compatible with the pseudoedema seen with Leber hereditary optic neuropathy [54].

previously [33]. Similarly, the whole coding region and exon-intron boundaries for the OPA3 gene were sequenced in all patients using the protocol described previously [34]. 20 ng of each DNA sample in a 50 ml PCR reaction mixture containing 200 m M dNTP, 0.2 mM of each of the primers (detailed sequences are available in the references provided), 1 unit of Taq DNA polymerase, 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). Polymerase chain reaction was performed for 25 cycles in a GeneAmp 9700 PCR system (Perkin-Elmer). PCR products were separated on 1% agarose gel at 100V for 1 h. The successfully amplified PCR products were sequenced using the forward primers used in the PCR utilizing the sequencing protocol we described earlier (see DNA amplification and sequencing section).

Statistical methods: All statistical analyses were performed using SPSS for Windows version 15.0 (SPSS Inc, Chicago, Illinois). Snellen visual acuities were converted to ordinal values, and CV was quantified on an equal interval scale as the number of Ishihara color plates identified (out of 10) with each eye. Statistical comparisons included bivariate correlation, independent samples t-test, Fisher exact analysis, and receiver operator curve (ROC).

RESULTS

Clinical characteristics: Table 1 details the clinical characteristics of all 26 ON patients (mean age at onset 23.4±8.2 years; 11 male and 15 female) from 26 different families who met inclusion and exclusion criteria. No patient reported a dietary abnormality or notable medication around the time of visual loss. Patients 16 and 26 had diabetes mellitus, but no patient had pigmentary retinopathy, ptosis, restricted ocular motility, deafness, ataxia, or diffuse weakness. None of the patients reported myotonia, exercise intolerance, palpitations, syncope, cardiac conduction abnormalities, oral or genital ulcers, erythema nodosum, or somatic anomalies. All patients had normal erythrocyte sedimentation rates, antinuclear antibodies, and syphilis serology. Patients were followed in a neuro-ophthalmology clinic between six months and five years after diagnosis. Four patients reported a family history of poor vision, but none had an obvious multigenerational maternal

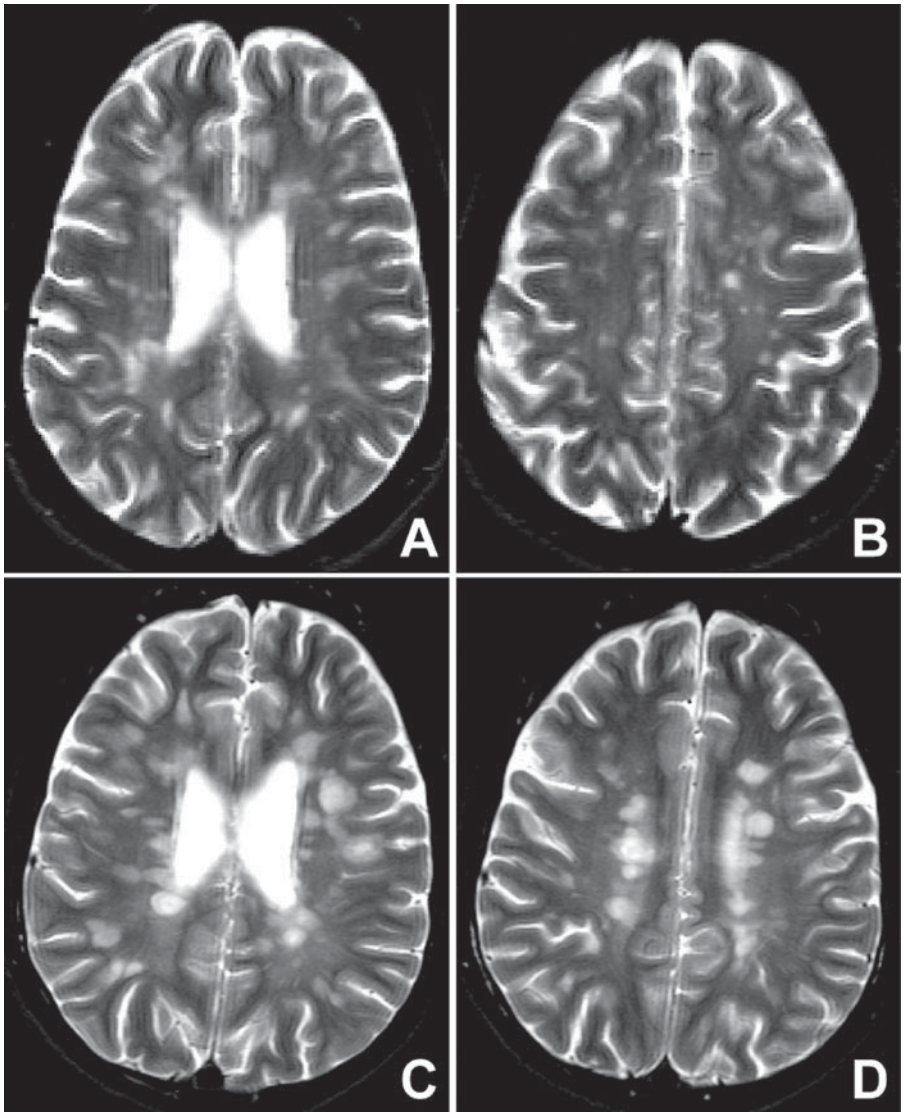

Figure 2. Disseminated demyelination in patients with optic neuritis. Montage of representative T2W MRI images of patients without (**A** and **B**; Patient 6) and with (**C** and **D**; Patient 16) potentially pathologic mitochondrial DNA changes showing a similar neuroimaging appearance typical of disseminated demyelinating disease that fulfills Fazekas criteria [14] despite the absence of clinically definite multiple sclerosis. MRI studies of optic neuritis patients showed a spectrum of demyelinating changes (see Table 1) that did not differ significantly according to mitochondrial parameters.

inheritance pattern. Family members were not examined or evaluated genetically.

Mean visual acuity (VA) at nadir of ON was 20/400, while mean recovery VA was approximately 20/40. Eighteen patients (69%) reported pain at the onset of visual loss, and papillitis (Figure 1) was observed in 13 (50%). Six patients fulfilled both Poser [11] and McDonald [12] criteria for MS. These patients had worse final vision (mean VA 20/100) than patients not having MS (mean VA 20/30; $p<0.001$). A total of 13 patients received IV steroids, and two were prescribed oral steroids at other institutions. Three of the six patients with MS were treated with beta-interferon after enrollment in this study.

All patients had brain neuroimaging, including magnetic resonance imaging in 25 and computed tomography in six. No scan revealed a mass or an alternative cause of optic neuropathy, but 11 participants had MRI scans consistent with demyelinating disease outside of the optic nerve (Table 1 and Figure 2). All six patients with MS had neuroimaging evidence of disseminated demyelination. Patients with and without disseminated demyelination did not differ with regard to clinical characteristics (age, gender, initial or final VA, or final CV).

Mitochondrial analyses: Table 2 lists the 22 NS mtDNA sequence variants detected in ON patients that were not previously reported haplogroup-specific polymorphisms [19]. Table 3 includes additional detail regarding predicted changes in protein structure and function supporting pathogenicity analyses for these sequence changes. No primary LHON mutation was present in patients or controls. Eighteen NS mtDNA nucleotide changes in ON patients were considered potentially pathologic because they changed moderately or highly conserved amino acids and were predicted to alter the corresponding protein structure and/or function (see Methods). Ten of these potentially pathologic changes were among 12 novel mtDNA nucleotide alterations. Seven sequence changes (four of which were novel) were present in a heteroplasmic state with heteroplasmy levels generally above 60%, and all of these were considered potentially pathologic [35].

Table 4 presents a similar pathogenicity analysis of the 26 NS mtDNA nucleotide changes in controls that met the same criteria. No potentially pathologic mtDNA variant and

Table 2. Non-synonymous mitochondrial DNA sequence changes detected in optic neuritis patients

Nucleotide substitution	AA change	Location	Base substitution type	Controls (%)	Hetero-plasmy (%)	Novel	Interspecies conservation	Protean	PolyPhen	SIFT	Summary
3580 C>A	P92T	Functional domain of ND1 gene	Transversion	0	70	Yes	High	Yes	Probably damaging	Yes	Pathologic
4136 A>G	Y277C	Outside the TM domain of ND1 gene	Transition	0	NA	No	High	Yes	Possibly damaging	Yes	Pathologic
4370 T>C	N/A	Anticodon loop of tRNA glutamine	Transition	0	65	No	Moderate	NA	NA	NA	Pathologic
5089 T>G	I207S	TM domain of ND2 gene	Transversion	0	NA	Yes	High	Yes	Probably damaging	Yes	Pathologic
5516 A>C	N/A	Acceptor stem in tRNA tryptophan	Transversion	0	63	No	High	NA	NA	NA	Pathologic
6261 G>A	A120T	TM domain of COI gene	Transition	0	NA	No	Moderate	Yes	Possibly damaging	Yes	Pathologic
6880 C>A	T326K	TM domain of COI gene	Transversion	0	65	Yes	High	Yes	Probably damaging	Yes	Pathologic
8813 C>A	T96N	Outside the TM domain of ATPase 6 gene	Transversion	0	50	Yes	High	Yes	Probably damaging	Yes	Pathologic
9104 T>C	F193S	Outside the TM domain of ATPase 6 gene	Transition	0	NA	Yes	High	No	Benign	No	Non-pathologic
9300 G>A	A32T	TM domain of COIII gene	Transition	1.2	NA	No	Moderate	No	Benign	No	Non-Pathologic
9904 T>G	F233C	Outside the TM domain of COIII gene	Transversion	0	NA	Yes	High	Yes	Probably damaging	Yes	Pathologic
9948 G>A	V248I	TM domain of COIII gene	Transition	0	NA	No	High	Yes	Probably damaging	Yes	Pathologic
10946 A>G	T63A	TM domain of ND4 gene	Transition	0	NA	Yes	Moderate	No	Benign	No	Non-pathologic
11865 T>G	L369W	In the TM functional domain of ND4	Transversion	0	NA	Yes	Moderate	Yes	Probably damaging	Yes	Pathologic
13253 C>G	T306S	In the TM functional domain of ND5	Transversion	0	NA	Yes	High	Yes	Probably damaging	Yes	Pathologic

This table assesses each nucleotide change in patients not previously found to be haplogroup-specific polymorphism as described in Methods. "Transversion" is a mutation in which a purine/pyrimidine replaces a pyrimidine/purine base pair or vice versa (G:C>T:A or C:G, or A:T>T:A or C:G), and "Transition" is a mutation in which a purine/pyrimidine base pair is replaced with a base pair in the same purine/pyrimidine relationship (A:T>G:C or C:G>T:A). "Controls (%)" represents percent of controls with this nucleotide substitution. "Heteroplasmy (%)" represents percent of mutant DNA present. "NA" represents not applicable because the nucleotide change was always found in homoplasmic state. To determine novelty, previous reports of sequence variants were found in the MITOMAP database, the human mitochondrial Genome database, GenBank, and Medline listed publications. Interspecies conservation was assessed using PolyPhen, which determines interspecies conservation for an altered amino acid by performing alignment with all available amino acid sequences for other species, and the Mamit-tRNA website when necessary. PolyPhen predicts and displays secondary structural characteristics. "Yes" indicates nucleotide change will alter protein secondary structure; "No" indicates change will not alter secondary structure. PolyPhen prediction of pathogenicity was assessed utilizing the PolyPhen database. "Probably damaging" constitutes a high confidence of affecting protein function or structure. "Possibly damaging" reflects a likelihood of affecting protein function or structure, while "Benign" changes most likely lack phenotypic effect. "Unknown" means that PolyPhen could not make a prediction due to lack of data. The Sorting Intolerant from Tolerant website (SIFT) returns predictions for which amino acid substitutions will affect protein function. "Yes" means an amino acid change is predicted to affect protein function, while "No" means the amino acid change is not predicted to affect protein function. Summary: A sequence variant was considered potentially pathologic if it satisfied all the following conditions, where possible: it changed a moderately or highly conserved amino acid; Protean predicted an alteration of protein structure; it was predicted by SIFT to have an effect on protein function; and it was assessed as possibly or probably pathogenic by PolyPhen.

TABLE 3. PATHOGENICITY ANALYSIS FOR NON-SYNONYMOUS SEQUENCE CHANGES

Nucleotide substitution	Analysis
3580 C->A	This sequence change is located at codon 92 in the functional domain of the ND1 gene and changes a highly conserved Proline, a hydrophobic AA, to Threonine, a neutral AA, altering the hydropathy index from - 0.42 to - 0.52. Protean predicted a change of protein structure, and SIFT predicted that it should affect protein function. This mutation was not found in controls, and PolyPhen predicted that it should be probably damaging. It was considered potentially pathologic.
4136 A->G	This sequence change is located at codon 277, outside the functional domain of the ND1 gene, and changes a highly conserved Tyrosine, a hydrophilic AA, to Cysteine, a neutral AA, altering the hydropathy index from -1.3 to 2.5. Protean predicted change of the protein structure, and SIFT predicted that it should affect protein function. This mutation was previously reported in Leber hereditary optic neuropathy [4]. It was not found in controls, and PolyPhen predicted that it should be possibly damaging. It was considered potentially pathologic.
4370 T->C	This sequence change is located in the anticodon loop of tRNA glutamine and was predicted to change the shape of this conserved region. It was not found in local controls but was present in a heteroplasmic state in one patient with a level of 65%. It was previously reported in association with mitochondrial myopathy [5]. It was considered potentially pathologic.
5089 T->G	This sequence change is located at codon 207 in the transmembrane domain of the ND2 gene. It changes a highly conserved Isoleucine, a hydrophobic AA, to Serine, a neutral AA, and alters the hydropathy index from -2.16 to -1.57. Protean predicted changes to the protein structure, and SIFT predicted that it should affect protein function. This sequence change was not found in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
5516 A->C	This sequence change is located in a highly conserved region of the acceptor stem of tRNA tryptophan. It was not detected in controls and was not previously reported as a polymorphism. It was considered potentially pathologic.
6261 G->A	This sequence variant is located at codon 120 in transmembrane domain of the COI, which forms part of the catalytic subunit of the enzyme. It changes a moderately conserved Alanine, a hydrophobic AA, to Threonine, a neutral AA, altering the hydropathy index from -0.72 to -0.44. Protean predicted the introduction of a flexible region, and SIFT predicted that it should affect protein function. This sequence change was not found in controls, and PolyPhen predicted it to be possibly damaging. It was reported in lymphocytes of six patients with prostate cancer, where elevated levels of reactive oxygen species implied possible pathogenicity [6]. It was considered potentially pathologic.
6880 C->A	This sequence variant is located at codon 326 in COI. It changes a highly conserved Threonine, a neutral AA, to Lysine, a hydrophilic AA, and alters the hydropathy index from -0.29 to -0.07. Protean predicted a change to the protein structure, and SIFT predicted that it should affect protein function. This mutation was not present in controls, but it was heteroplasmic in one patient with a heteroplasmy level of 65%. PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
8813 C->A	This sequence variant is located at codon 96, outside the transmembrane domain of ATPase 6 gene. It changes a highly conserved Threonine, a neutral AA, to Asparagine, another neutral AA, and alters the hydropathy index from -0.06 to 0.26. Protean predicted a change to the structure of the protein, and SIFT predicted that it should affect protein function. Its heteroplasmy level was 50%, which is close to the accepted threshold of 60% [7]. This sequence change was absent in controls, and PolyPhen predicted it to be probably damaging. Given these factors, it was considered potentially pathologic.
9104 T->C	This sequence variant is located at codon 193, outside the transmembrane domain of ATPase 6 gene. It changes a highly conserved Phenylalanine, a hydrophobic AA, to Serine, a neutral AA, which alters the hydropathy index from -30 to -2.60. Protean predicted no change to the secondary structure of the protein, and SIFT predicted that it should not affect protein function. This sequence change was absent in controls, but PolyPhen predicted it to be benign. It was considered non-pathologic.
9300 G->A	This sequence variant is located at AA position 32 in the transmembrane domain of COIII gene. It changes a moderately conserved Alanine, a hydrophobic AA, to Threonine, a neutral AA, which alters the hydropathy index from -0.48 to -0.20. Protean predicted no change in the protein structure, and SIFT predicted that it should not affect protein function. This sequence change was found in 1.2% of controls, and PolyPhen predicted it to be Benign. It was considered non-pathologic.
9904 T->G	This sequence variant is located at AA position 233 in the outside transmembrane domain of COIII gene. It changes a highly conserved Phenylalanine, a hydrophobic AA, to Cysteine, a neutral AA, which changes the hydropathy index from 0.84 to 0.88. Protean predicted a change in protein structure, and SIFT predicted that it should affect protein function. This sequence alteration was absent in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
9948 G->A	This sequence variant is located at AA position 248 of the transmembrane domain of the COIII gene and changes a highly conserved Valine, a hydrophobic AA, to Isoleucine, a hydrophobic AA. Protean predicted a change in the protein structure, and SIFT predicted that it should affect protein function. This mutation was previously reported as a somatic mutation in tissues from a patient with papillary thyroid carcinoma [8]. It was not found in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
10946 A->G	This sequence variant is located at AA position 63 in the transmembrane domain of the ND4 gene and changes a moderately conserved Threonine, a neutral AA, to Alanine, a hydrophobic AA, altering the hydropathy index from -0.58 to -0.86. Protean predicted no change in the protein structure, and SIFT predicted that it should not affect protein function. This sequence variant was not found in controls, and PolyPhen predicted it to be benign. It was considered non-pathologic.
11865 T->G	This sequence variant is located at AA position 369 in the transmembrane functional domain of ND4 gene. It changes a moderately conserved Leucine, a hydrophobic AA, to Tryptophan, another hydrophobic AA, altering the hydropathy index from 0.84 to 0.88. Protean predicted a change in protein structure, and SIFT predicted that it should affect protein function. This sequence alteration was absent in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.

TABLE 3. CONTINUED.

Nucleotide substitution	Analysis
13253 C->G	This sequence variant is located at AA position 306 in transmembrane functional domain of ND5 gene. It changes a highly conserved Threonine, a neutral AA, to Serine, another neutral AA, altering the hydropathy index from - 0.7 to - 0.8. Protean predicted a change in protein structure, and SIFT predicted that it should affect protein function. This sequence alteration was absent in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
13936 C->A	This sequence variant is located at AA position 534 outside the transmembrane domain of the ND5 gene. It changes a highly conserved Histidine, a hydrophilic AA, to Asparagine, a neutral AA, altering the hydropathy index from 0.46 to 0.49. Protean predicted a change of protein structure, and SIFT predicted that it should affect protein function. This sequence change was absent in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
14516 A->G	This sequence variant is located at AA position 53 in the transmembrane domain of the ND6 gene. It changes a highly conserved Leucine, a hydrophobic AA, to Serine, a neutral AA, altering the hydropathy index from -1.81 to -1.36. Protean predicted a change of protein structure, and SIFT predicted that it should affect protein function. This sequence change was absent in controls, and PolyPhen predicted it to be Probably Damaging. It was considered potentially pathologic.
14525 T->G	This sequence variant is located at AA position 50, outside the transmembrane domain of the ND6 gene. It changed a moderately conserved Tyrosine, a hydrophilic AA, with Serine, a neutral AA, altering hydropathy index from -0.83 to -0.38. Protean predicted significant changes to the protein structure, and SIFT predicted that it should affect protein function. This sequence alteration was absent in controls, and PolyPhen predicted it to be probably damaging. It was considered potentially pathologic.
14831 G->A	This sequence variant is located at AA position 29, outside the transmembrane domain of the CYTB gene. It changed a moderately conserved Alanine, a hydrophobic AA, to Threonine, a neutral AA, altering the hydropathy index from 0.14 to 0.42. Protean predicted no change in protein structure, and SIFT predicted that it should not affect protein function. This sequence change was found in 1.9% of controls, and PolyPhen predicted it to be Benign. This sequence variant was reported in the setting of Leber hereditary optic neuropathy [9] but is listed on the Mitomap database as a polymorphism. It was considered non-pathologic.
15674 T->C	This sequence variant is located at AA position 310 in the C-terminal domain of CYTB, where a pathologic mutation may affect ubiquinol/ubiquinone binding activity. It changes a highly conserved Serine, a neutral AA, to Proline, a hydrophobic AA, altering the hydropathy index from 0.61 to 0.70. Protean predicted no change to the protein structure, and SIFT predicted that it should affect protein function. This sequence alteration was absent in controls, and PolyPhen predicted it to be possibly damaging. It was considered potentially pathologic.
15924 A->G	This sequence variant is located in the anticodon loop of tRNA threonine, a highly conserved nucleotide region. Although, this sequence change was not detected in controls, it is now thought to be a haplogroup specific polymorphism [10]. It was considered non-pathologic.
15968 T->C	This sequence variant is located at the D-stem loop of tRNA proline, a highly conserved region. It was not detected in controls or reported previously as a polymorphism. It was considered potentially pathologic.

Previous reports of sequence variants were found in the Mitomap database, the Human Mitochondrial Genome Database, GenBank, and Medline listed publications. Interspecies conservation was assessed using the Polymorphism phenotyping (PolyPhen) database, which determines interspecies conservation for an altered amino acid by performing alignments with all available amino acid sequences for other species, and when necessary, using the Mamit-tRNA website. PolyPhen pathogenicity prediction was assessed utilizing the PolyPhen database. “Probably damaging” constitutes a high confidence of affecting protein function or structure. “Possibly damaging” reflects a likelihood of affecting protein function or structure, while “Benign” changes most likely lack phenotypic effect. “Unknown” means that PolyPhen could make no prediction due to lack of data. Sorting Intolerant From Tolerant (SIFT) returns predictions for which amino acid substitutions will affect protein function given a particular protein sequence. Protean (Protein Structure Prediction and Annotation) is part of the Lasergene V.6 software (DNASTAR, Inc. Madison, WI). It displays patterns, secondary structural characteristics, and physiochemical properties (hydropathy index, flexibility index and antigenic index). The Hydropathy Index was measured by Protean according to the Kyte-Doolittle method [11], which predicts the regional hydropathy of proteins from their amino acid sequence. Hydropathy values were assigned for all amino acids and were then averaged over a window size equal 7. Results below 0 are hydrophobic and above 0 are hydrophilic. Abbreviations include, amino acid (AA), transcription RNA (tRNA), cytochrome oxidase subunit 1 (COI), cytochrome B (CYTB), cytochrome c oxidase subunit III (COIII).

only one novel mtDNA sequence change were present in controls. The prevalence of both synonymous (25/26 ON patients versus 78/159 controls; $p<0.0001$) and NS (20/26 ON patients versus 63/159 controls; $p=0.0003$) mtDNA variants was greater in patients than in controls.

Table 5 details by patient all NS mtDNA nucleotide changes in Table 2 together with relative mtDNA content and MRA. Ten patients had no mtDNA sequence abnormalities that were likely to be pathologic (see supporting analysis in Table 2 and Table 3). This group included six patients with no mtDNA sequence change other than reported polymorphisms important for haplogroup analysis (Patients 1-6); three patients with only previously reported benign NS mtDNA sequence changes (Patients 7, 8, and 9); and one patient with a novel mtDNA sequence change predicted to be non-pathologic (Patient 10). The remaining 16 patients (Patients 11-26) had one or more nucleotide changes that were predicted to be pathologic. After recovery, these 16 patients with potentially patho-

logic mtDNA changes had worse VA (mean less than 20/40 versus mean greater than 20/25; p=0.002) and color vision (mean approximately 6/10 versus 9/10; p=0.009) than other patients. Other clinical parameters did not differ between these two groups of patients.

Table 5 also details relative mtDNA content and MRA for each patient. Mean relative mtDNA content was significantly greater in patients (2.39±1.19; 95% CI 1.91-2.87) than in controls (1.03±0.27; 95% CI 0.96-1.11; p<0.001). The optimal relative mtDNA content level to distinguish between

Table 4. Non-synonymous mitochondrial DNA sequence changes detected in control subject

Nucleotide substitution	AA change	Location	Base substitution type	Controls (%)	Heteroplasmy (%)	Novel	Interspecies conservation	Protean	Polyphen	SIFT	Summary
4012 A>G	T236A	Functional domain of ND1 gene	Transition	0.6	NA	No	High	Yes	Benign	No	Non-Pathologic
4013 C>G	T236S	Functional domain of ND1 gene	Transversion	0.6	NA	No	High	Yes	Benign	No	Non-Pathologic
4734 A>G	T89A	Outside the TM domain of ND2 gene	Transition	0.6	NA	No	Low	Yes	Benign	No	Non-Pathologic
4904 C>A	I145M	Outside the TM domain of ND2 gene	Transversion	0.6	NA	No	Low	No	Benign	No	Non-Pathologic
5263 C>T	A265V	Outside the TM domain of ND2 gene	Transition	4.4	NA	No	Low	Yes	Benign	Yes	Non-Pathologic
5913 G>A	D4N	Outside the TM domain of COI gene	Transition	3.1	NA	No	Low	No	Benign	No	Non-Pathologic
6040 A>G	N46S	Outside the TM domain of COI gene	Transition	1.9	NA	No	Low	No	Benign	No	Non-Pathologic
7278 T>C	F459L	Outside the TM domain of COI gene	Transition	0.6	NA	No	High	No	Benign	No	Non-Pathologic
7369 C>G	S489C	Outside the TM domain of COI gene	Transversion	0.6	NA	No	Moderate	Yes	Benign	NA	Non-Pathologic
7646 A>C	I21L	Outside the TM domain of COII gene	Transversion	0.6	NA	No	Low	No	Benign	No	Non-Pathologic
8587 G>A	V21M	TM domain of ATPase 6 gene	Transition	0.6	NA	No	Low	No	Benign	No	Non-Pathologic
9104 T>C	F193S	Outside the TM domain of ATPase 6 gene	Transition	0.6	NA	Yes	Moderate	No	Benign	Yes	Non-Pathologic
9300 G>A	A32T	TM domain of COIII gene	Transition	3.1	NA	No	Moderate	Yes	Benign	Yes	Non-Pathologic
9337 T>C	M44T	TM domain of COIII gene	Transition	3.8	NA	No	Low	Yes	Benign	No	Non-Pathologic
9539 A>T	Q111H	Outside TM domain of COIII gene	Transversion	1.3	NA	No	Low	Yes	Benign	No	Non-Pathologic
9667 A>G	N154S	Outside TM domain of COIII gene	Transition	6.9	NA	No	Low	No	Benign	No	Non-Pathologic
9822 C>T	L206F	TM domain of COIII gene	Transition	1.3	NA	No	High	No	Benign	Yes	Non-Pathologic
9966 G>A	V254I	TM domain of COIII gene	Transition	0.6	NA	No	Moderate	No	Benign	No	Non-Pathologic
10079 A>C	L7F	TM domain of ND3 gene	Transversion	1.3	NA	No	Low	No	Benign	No	Non-Pathologic
13811 C>G	A492G	TM domain of ND5 gene	Transversion	0.6	NA	No	Low	Yes	Benign	No	Non-Pathologic
14562 C>T	V38I	TM domain of ND6 gene	Transition	0.6	NA	No	High	No	Benign	No	Non-Pathologic
14582 A>G	V31A	TM domain of ND6 gene	Transition	8.2	NA	No	Low	No	Benign	No	Non-Pathologic
14831 G>A	A29T	Outside the TM domain of CYTB gene	Transition	1.9	NA	No	Low	No	Benign	No	Non-Pathologic
14862 C>T	A39V	TM domain of CYTB gene	Transition	0.6	NA	No	Low	Yes	Benign	No	Non-Pathologic
15267 C>A	T174N	Outside TM domain of CYTB gene	Transversion	0.6	NA	No	High	Yes	Benign	Yes	Non-Pathologic
15617 G>A	V291I	TM domain of CYTB gene	Transition	1.3	NA	No	High	No	Benign	Yes	Non-Pathologic

This table assesses each nucleotide change in controls not previously found to be haplogroup-specific polymorphism as described in Methods. “Transversion” is a mutation in which a purine/pyrimidine replaces a pyrimidine/purine base pair or vice versa (G:C>T:A or C:G, or A:T>T:A or C:G), and “Transition” is a mutation in which a purine/pyrimidine base pair is replaced with a base pair in the same purine/pyrimidine relationship (A:T>G:C or C:G>T:A). “Controls (%)” represents percent of controls with this nucleotide substitution. “Heteroplasmy (%)” represents percent of mutant DNA present. “NA” represents not applicable because the nucleotide change was always found in homoplasmic state. To determine novelty, previous reports of sequence variants were found in the MITOMAP database, the human mitochondrial Genome database, GenBank, and Medline listed publications. Interspecies conservation was assessed using PolyPhen which determines interspecies conservation for an altered amino acid by performing alignment with all available amino acid sequences for other species, and the Mamit-tRNA website when necessary. Protean predicts and displays secondary structural characteristics. “Yes” indicates nucleotide change will alter protein secondary structure; “No” indicates change will not alter secondary structure. PolyPhen prediction of pathogenicity was assessed utilizing the PolyPhen database. “Probably damaging” constitutes a high confidence of affecting protein function or structure. “Possibly damaging” reflects a likelihood of affecting protein function or structure, while “Benign” changes most likely lack phenotypic effect. “Unknown” means that PolyPhen could not make a prediction due to lack of data. The Sorting Intolerant from Tolerant website (SIFT) returns predictions for which amino acid substitutions will affect protein function. “Yes” means an amino acid change is predicted to affect protein function, while “No” means the amino acid change is not predicted to affect protein function. Summary: A sequence variant was considered potentially pathologic if it satisfied all the following conditions, where possible: it changed a moderately or highly conserved amino acid; Protean predicted an alteration of protein structure; it was predicted by SIFT to have an effect on protein function; and it was assessed as possibly or probably pathogenic by PolyPhen.

controls and patients was 1.15 as calculated by ROC (ROC curve not shown; area under ROC curve 0.86; 95% CI 0.77 - 0.95), and 20 patients had relative mtDNA content greater than this value. Mean MRA was significantly less in patients (16.78±3.18; 95% CI 15.02-18.54) than in controls (22.53±0.95; 95% CI 22.26-22.80; p<0.001). The optimal MRA to distinguish between controls and patients was 21.22 (ROC curve not shown; area under ROC curve 0.95; 95% CI 0.88-1.01), and 13 of the 15 studied patients had MRA less than this value.

Sequence analysis of OPA1 and OPA3 genes: No polymorphisms or mutations were found in either the OPA1 or the OPA3 gene in any ON patient.

DISCUSSION

This study included 26 patients from 26 different families who had historical and clinical features of ON [36]. Patients were generally young (average age 23.4 years) and mostly female (15/26), and all had recovery of vision documented on repeat examinations. Eighteen had periorbital pain at onset, 13 had resolving papillopathy, and nine had involvement of both eyes simultaneously or sequentially. Clinical characteristics of these patients were subtly different from the Optic Neuritis Treatment Trial [37] (Participants in that trial were somewhat younger and had somewhat worse vision) and perhaps more comparable to other non-Caucasian groups [38]. Eleven patients had neuroimaging evidence of probable or definite disseminated demyelination, and six had MS [11,12]. None had an obvious alternative cause of optic nerve injury on history, examination, or neuroimaging at follow up of more than six months.

Table 5. Mitochondrial DNA and respiratory changes in optic neuritis patients

Patient	Nucleotide change(s)	Relative mtDNA content	MRA
1	None	2.41	21.93
2	None	2.22	ND
3	None	1.80	14.67
4	None	3.31	22.43
5	None	1.60	ND
6	None	1.03	ND
7	14831	1.94	ND
8	9300	1.24	ND
9	15924	4.10	ND
10	9104	1.08	ND
11	11865	1.32	16.57
12	4136	0.96	21.07
13	10946, 13253, 14831	0.85	ND
14	11865	0.92	17.17
15	4370	0.95	ND
16	15674	1.22	15.87
17	9948	3.51	13.67
18	5089	3.95	14.30
19	9904	4.25	ND
20	9948, 14516, 14525	3.92	19.87
21	8813	3.75	11.77
22	6261, 13936	3.10	16.13
23	6880, 15674	3.10	16.80
24	5516, 15674	2.92	14.40
25	3580	3.68	ND
26	15968	2.95	15.03

Shown are nucleotide change(s) from Table 2 for each patient. The "Relative mtDNA Content" column represents the ratio of NADH dehydrogenase subunit 1 to β-actin (see Methods). MRA represents Mitochondrial Respiratory Activity (see Methods). "ND" means not done.

These patients did not have the LHON phenotype or primary LHON mutations, and all experienced resolution of visual deficit in a fashion characteristic of ON but not typical of LHON [3] or LHON-like optic neuropathies [28]. None reported a maternal family history, and most did not develop bilateral, symmetric optic nerve injury typical of LHON [3] even though follow up generally lasted more than one year. Age at onset and substantial improvement in vision were atypical for other types of optic neuropathy that have been linked to mitochondrial abnormalities, such as dominant optic atrophy [39], hereditary motor sensory neuropathy type VI [6], non-arteritic ischemic optic neuropathy (NAION) [29,40], and primary open angle glaucoma (POAG) [27]. Sequencing OPA1 and OPA3 yielded no genetic evidence of dominant or recessive optic nerve atrophy.

These ON patients had evidence of significant mitochondrial abnormalities in leukocytes. Mitochondrial parameters were evaluated in leukocytes because the target organ was not available and because assessment of peripheral blood has proven to be relevant in studies of patients with mitochondrial disease [41] and optic nerve disease [42,43], including ON [44]. Most patients had novel as well as potentially pathologic mtDNA nucleotide changes not found in controls. As a group, ON patients had significantly more synonymous and NS mtDNA nucleotide changes than controls, which may reflect oxidative stress. They had significantly greater relative mtDNA content and significantly less MRA than controls. Every patient assessed with all three mitochondrial measures had abnormalities in one parameter or more. Patients with MS were indistinguishable from other ON patients on mitochondrial studies, suggesting that these mitochondrial abnormalities are characteristic of ON rather than, or in addition to, MS.

The 16 ON patients with potentially pathologic mtDNA sequence changes had significantly worse vision after resolution of ON than the 10 other patients. These two groups were defined only by the prediction of pathogenicity of certain nucleotide changes because no biochemical tests were performed to prove a difference in mitochondrial function. Nevertheless, the prediction identified groups that differed significantly in visual outcome, suggesting that the prediction criteria have clinical relevance. Harding and her colleagues reported primary LHON mutations in certain women with relatively severe bilateral ON [7]. Results reported here expand those observations and imply a broader association between potentially pathologic mtDNA sequence changes and severity of optic nerve injury in ON.

Similar mitochondrial alterations have been reported in other spontaneous optic neuropathies, including NAION [29,40], POAG [27], and LHON-like optic neuropathies [28].

However, these optic neuropathy syndromes are distinct from each other in mitochondrial characteristics as well as clinical phenotype. MtDNA changes in LHON-like optic neuropathy [28] and NAION [40] were mostly transitions, while those in POAG were predominantly transversions [27]. Primary and provisional LHON mutations were found only in patients with LHON-like optic neuropathies [28]. Relative mtDNA content was not significantly different between controls and POAG patients [27], but it was different between controls and patients with NAION [29], LHON-like optic neuropathies [28], and ON. The presence of several types of mitochondrial abnormalities in several optic neuropathy phenotypes implies that the association between mitochondrial abnormalities and spontaneous optic nerve disease may be more broadly based than previously thought, although these associations do not prove causation. It is not yet clear whether specific combinations of environmental and genetic mitochondrial characteristics are predictive of the type of optic neuropathy that occurs in a particular patient.

ON is clinically different from other optic neuropathies associated with mitochondrial abnormalities [3,45], in part because of a prominent inflammatory component and frequent recovery of vision. Neurons may not be the sole target of mitochondrial abnormalities in acute ON because mitochondrial dysfunction in oligodendrocytes of both environmental [46] and genetic [47] origin can lead to demyelination. Recent pathological studies of acute MS lesions have identified extensive oligodendrocyte apoptosis [48] that may be mitochondrial in origin.

This study includes a relatively small number of individuals from one ethnic group. However, if confirmed in other populations, these results add to evidence implying that mitochondrial disease may play a role in central nervous system demyelination [2,48,49]. It is possible that mitochondria-induced apoptosis of oligodendrocytes contributes to demyelination [50], while apoptosis [49] or elevated reactive oxygen species [51] play a role in chronic axonal loss and atrophy of optic nerve and brain. Immune therapy of ON [37] and MS [52] have been only partially effective, and recognition of additional pathophysiologic mechanisms may lead to other investigative and therapeutic opportunities. A novel in vitro treatment has been devised for the metabolic defect of one primary LHON mutation [53], and other metabolic or genetic interventions might be applicable to correct mitochondrial abnormalities in patients with ON.

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