High Mitochondrial DNA Copy Number Is a Protective Factor From Vision Loss in Heteroplasmic Leber's Hereditary Optic Neuropathy (LHON)

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Purpose. Leber's hereditary optic neuropathy (LHON) is a mitochondrial disease that typically causes bilateral blindness in young men. It is characterized by as yet undisclosed genetic and environmental factors affecting the incomplete penetrance.

Methods. We identified 27 LHON subjects who possess heteroplasmic primary LHON mutations. Mitochondrial DNA (mtDNA) copy number was evaluated.

Results. The presence of centrocecal scotoma, an edematous, hyperemic optic nerve head, and vascular tortuosity, as well as telangiectasia was recognized in affected subjects. We found higher cellular mtDNA content in peripheral blood cells of unaffected heteroplasmic mutation carriers with respect to the affected.

Conclusions. The increase of cellular mtDNA content prevents complete loss of vision despite the presence of a heteroplasmic state of LHON primary mutation, suggesting that it is a key factor responsible for penetrance of LHON.

Keywords: LHON, mitochondrial DNA, mtDNA copy number, heteroplasmy

Leber's hereditary optic neuropathy (LHON) is the most common of all of the mitochondrial diseases. Approximately 90% of individuals harbor one of three 'primary' mutations in the mitochondrial genome (i.e., m.3460G>A, m.11778G>A and m.14484T>C). Each mutation affects subunits of NADH-ubiquinone oxidoreductase (EC 1.6.5.3).[1] The presence of primary mutations is necessary, but not sufficient alone, to cause optic neuropathy, because disease penetrance can vary in different families harboring the same mutation.[2,3] Thus, exists the implication that environmental and/or other genetic factors affect its penetrance. Mitochondrial DNA (mtDNA), a multicopy genome, is essential for 13 subunits of the oxidative phosphorylation system. Recognized as one of the genetic factors contributing to disease penetrance, mtDNA itself may contribute in one of three ways: (1) by adding 'secondary' mtDNA mutations acting in synergy with the primary ones, (2) by adjusting the cellular quantity of mtDNA, known as 'the copy number',[4] and (3) by modifying the relative proportion of mutants versus wild-type mitochondrial genotypes that may occur as a mixture in a cell or a tissue, which is known as 'heteroplasmy'. In most known mitochondrial neurologic diseases, the mutated versus wild-type ratio impinges on the penetrance, or the severity, of the condition.[5] Among the LHON primary mutations, m.11778G>A, being the most frequently occurring, is typically homoplasmic (all the molecules have the same genotype)[6] but heteroplasmy, predominantly for the m.3460G>A, has been detected as well.[7–11]

We determined the presence as well as the heteroplasmic status of primary LHON mutations in a cohort of patients with a clinical diagnosis of LHON; we additionally determined such in their relatives. We evaluated the contribution of the mutational load to the penetrance of LHON disease and the mtDNA copy number in all the heteroplasmic subjects to assess whether it can discriminate between affected and unaffected subjects as it happens in large groups of homoplasmic LHON individuals.[4,12]

Materials and Methods

LHON Patients and Family Members

After receiving informed consent, we enrolled 30 subjects in the study at three sites: Ophthalmology Clinic, Policlinico Bari Hospital, Italy; Hospital IRCCS 'Casa Sollievo della Sofferenza', Italy; and the University of Zaragoza, Spain. Several examinations were performed: slit-lamp biomicroscopy, fundal and optic nerve head stereoscopy, fluorescein angiography, optical coherence tomography, and visual field testing. The control group consisted of 90 unrelated subjects. Research adhered to the tenets of the Declaration of Helsinki.

Molecular Genetics and Statistics

Total genomic DNA was extracted by standard methods from participants' peripheral blood. Using the PCR-restriction-fragment length polymorphism (PCR-RFLP) method,[13] we

determined the primary LHON mutations, as well as the relative quantification of mtDNA copy number was performed.[14] The distances between different population distributions were calculated by the Kolmogorov–Smirnov (KS) statistic on two samples and implemented in the Scipy numerical software library.[15] Data were also analyzed applying the ANOVA test in conjunction with Bonferroni test. Statistical significance was set at $P < 0.05$.

Results

Clinical Features and Genetic Diagnosis

We included 30 subjects in the study cohort: 28 participants belonged to 10 families; the remaining 2 participating were unrelated (Supplementary Fig. S1). Clinical findings (when available) including sex, molecular genetic testing results, LHON risk factors, recovery of vision and response to Idebenone therapy, are reported in Table 1. In 12 subjects (male:female ratio, 10:2) the diagnosis of LHON was based on unilateral and severe visual decline followed, within a few weeks, by declining vision in the contralateral eye. Ophthalmologic examinations revealed the presence of a centrocecal scotoma, the presence of an edematous, hyperemic optic nerve head, tortuosity and telangiectasia of vasculature and, finally, the absence of leakage and staining using fluorescein angiography. Visual acuity ranged between light perception and 20/25 (Table 1). Patients with these features comprised the Affected cohort; those patients lacking these features were Carriers. The 90 unrelated subjects (male:female ratio, 47:43), the Control group, exhibited no history of retinal disease, eye trauma or surgery, nor any evidence of systemic or neurologic disease. The diagnosis of LHON was confirmed by mitochondrial genetics testing: the m.11778G>A mutation was identified in 14 subjects (2 homoplasmic; 12 heteroplasmic) and the m.3460G>A in 16 subjects (1 homoplasmic and 17 heteroplasmic) (Table 1; Supplementary Fig. S2). None carried the m.14484T>C primary mutation. The mean frequency of the appearance of mutant alleles for both mutations of heteroplasmic subjects was 70% for the Affected (range, 15%–95%) and 53% for the Carriers (8%–95% range) (Table 1). The penetrance value of both LHON mutations was 40% (12/30) in our LHON cohort, 71% (10/14) in male and 12% (2/16) in female. Among the 14 subjects (8 males; 6 females) who harbored the m.11778G>A mutation, six (5 male; 1 female) developed typical optic neuropathy with a total of 43% phenotype penetrance. In this group, the penetrance value of 83% in men was five times higher than that for woman. Among the 16 subjects (6 males; 10 females) who harbored m.3460G>A mutation, six (5 males; 1 female) were affected with a total of 37% phenotype penetrance. In this group, the penetrance value of 83% in men was eight times higher than that for women. One m.11778G>A patient recovered vision without Idebenone treatment (Table 1). The determination of mtDNA copy numbers was performed independently from the type of LHON primary mutations in all the heteroplasmic subjects (5 Affected and 18 Carriers) and all compared with Controls. We could not quantify the mtDNA content for three homoplasmic Affected (II-1 FAM-A2; III-2 FAM-A4; III-1 FAM-B4; Table 1) and for four heteroplasmic Affected (II-1 FAM-A1; II-3 FAM-A3; I-1 FAM-A5; II-1 FAM-B3; Table 1) due to the insufficient quantity of useful genetic material. The mtDNA copy number distribution was highly variable between the Affected and Carriers. Using very strict criterion, the Carriers showed a different distribution with respect to either the Affected or the Controls who could not be distinguished (i.e., the probability of the null hypothesis < 0.001) (Table 2). Conversely, when the distribution of the two LHON populations was compared with the heteroplasmy of mutations, no statistically significant differences were observed (not shown). Panel A of the Figure shows that all the Affected fall in the same range of mtDNA copy number as the Controls. The mean value of the mtDNA copy number showed that the peak of mtDNA content shifted progressively toward higher values from Controls (210 ± 66) to Affected (293 ± 85) to Carriers (488 ± 140) (Controls versus Carriers $P < 0.001$; Affected versus Carriers $P < 0.001$; Controls versus Affected did not reach statistical significance; ANOVA test) (Fig. panel B). It is important to note that two affected subjects (II-5 FAM-A4; I-1 FAM-B7) having the lowest quantities of mtDNA had been exposed to ethanol, tobacco, and controlled substances, all of which have been shown to decrease mtDNA amounts and considered as risk factors for the development of LHON.[1,4,16,17]

Discussion

The aim of this study was to identify factors controlling LHON penetrance. In our cohort of 30 LHON subjects, we found the two most common primary LHON mutations, are heteroplasmic in peripheral blood cells of 27 subjects. Heteroplasmy was more frequent for m.3460G>A than for m.11778G>A in our cohort, as has been reported by other investigators.[18,19] When we looked at the segregation of heteroplasmy along the maternal lineages in those eight families with two or three successive generations available (Table 1), we found in seven families an increase of mutant alleles, which suggests a positive selection; conversely, in one family we found a decrease in the percentage of mutant alleles. Our sample size was too small to draw conclusions, yet our data agree with a random drift model for LHON mutations segregation.[8,20] We found no correlation between the clinical manifestations of LHON and the percentage of mtDNA mutation: one patient harbored 15% and another 55% heteroplasmic mutant alleles in their peripheral blood, which is below the threshold value of 60% proposed in a retrospective analysis of 17 families.[10] On the other hand, the presence of 95% and even 100% homoplasmic mutant alleles are not unequivocally the cause of LHON clinical manifestations, as reported.[8,19,21] We report a unique finding in a heteroplasmic cohort: a significantly higher level of mtDNA copy number, which characterized the Carriers respect to the Affected. However, the Affected fall in the same range of mtDNA copy number as the Controls, suggesting that for subjects carrying LHON mutations, a smaller amount of mtDNA vis-à-vis the Carriers is a risk factor for vision loss and the development of LHON. It is of note that three subjects who have been exposed to trigger environmental factors like smoke, illicit drugs, and alcohol have developed the disease. On the other hand, among the Carriers those subjects with quantities of mtDNA similar to that of Controls may be considered at high risk for developing the disease and must be observed over time. Our results are in line with previous reports that high levels of mtDNA are found in blood cells of LHON homoplasmic asymptomatic subjects.[5,13] The greater number of mtDNA copies found in Carriers versus the Affected indicates that the increase in the number of mtDNA molecules occurs despite the homo- or heteroplasmy of LHON mutations. All of these findings suggest that cells carrying LHON primary homoplasmic and heteroplasmic mutations trigger a compensatory response depending on the increase of mitochondrial biogenesis, as evidenced by mtDNA copy number. The ability to produce such increased copy numbers is significantly more efficient in those individuals who remain Carriers. The failure of a strategy to compensate for mitochondrial dysfunction is commonly observed in severe pathogenic heteroplasmic

Investigative Ophthalmology & Visual Science

Table 1. Clinical Data and Genetic Findings in LHON Subjects

Subject ID	Family ID	Sex	Relation	Visual Condition at the First Examination		Currently Visual Condition		Recovery	Idebenone Treatment	Risk Factors	Clinical Features	Genotype	HET, %mut	Copy Number, mtDNA/nDNA
				RE	LE	RE	LE							
II-1	FAM-A1	M	Proband	20/200	20/200	20/200	20/200	n.a.	n.a.	–	Bilateral optic atrophy	m.11778G>A	75	n.a.
II-2		M	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	40	460 ± 93
II-3		M	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	30	364 ± 7
I-1	FAM-A2	F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	75	413 ± 110
I-2		M	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	95	670 ± 2
I-3		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	95	569 ± 15
II-1		M	Proband	20/200	20/200	20/200	20/200	n.a.	n.a.	–	Bilateral optic atrophy	m.11778G>A	100	n.a.
I-1	FAM-A3	F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	40	782 ± 13
II-1		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	40	482 ± 65
II-2		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.11778G>A	90	481 ± 58
II-3		F	Proband	LP	LP	LP	LP	n.a.	n.a.	–	Bilateral optic atrophy	m.11778G>A	90	n.a.
II-5	FAM-A4	M	Proband	n.a.	n.a.	20/20	20/20	Y	–	Alcohol abuse	Bilateral optic subatrophy	m.11778G>A	60	149 ± 40
III-2		M	Proband	n.a.	n.a.	LP	LP	n.a.	–	–	Bilateral optic subatrophy	m.11778G>A	100	n.a.
I-1	FAM-A5	M	Proband	20/300	20/300	LP	LP	N	+	Alcohol abuse	Bilateral optic atrophy	m.11778G>A	75	n.a.
I-1	FAM-B1	F	Relative	20/20	20/20	20/20	20/20	–			Normal vision	m.3460G>A	5	574 ± 45
II-1		F	Proband	LP	LP	LP	LP	N	n.a.	n.a.	Bilateral optic atrophy	m.3460G>A	55	351 ± 48
I-1	FAM-B2	F	Relative	20/20	20/20	20/20	20/20				Normal vision	m.3460G>A	55	231 ± 33
II-1		M	Proband	20/200	20/200	20/200	20/200	N	n.a.	n.a.	Bilateral optic atrophy	m.3460G>A	80	331 ± 24
I-1	FAM-B3	F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	60	519 ± 23
II-1		M	Proband	LP	LP	LP	LP	n.a.	n.a.	–	Bilateral optic atrophy	m.3460G>A	90	n.a.
II-2		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	50	404 ± 67
II-3		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	60	511 ± 163
I-1	FAM-B4	F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	20	453 ± 36
I-2		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	8	481 ± 169
II-2		M	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	8	649 ± 86
III-1		M	Proband	20/200	20/200	20/200	20/200	n.a.	n.a.	–	Bilateral optic atrophy	m.3460G>A	100	n.a.
III-2		F	Relative	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	70	520 ± 126
I-2	FAM-B5	F	Unrelated	20/20	20/20	20/20	20/20	–	–	–	Normal vision	m.3460G>A	75	217 ± 11
I-1	FAM-B6	M	Proband	20/200	20/200	20/200	20/200	n.a.	n.a.	–	Bilateral optic atrophy	m.3460G>A	95	347 ± 17
I-1	FAM-B7	M	Proband	20/32	20/25	20/32	20/25	N	+	Alcohol, tobacco, and drug abuse	Bilateral optic subatrophy with hemeralopia	m.3460G>A	15	285 ± 24

Ophthalmologic findings, possible exposure to environmental triggers (i.e., alcohol, smoking, and illicit drugs) and mtDNA LHON mutations, percentage of heteroplasmy and copy number are indicated. M, male; F, female; RE, right eye; LE, left eye; LP, light perception; N, no; Y, yes; n.a., not available; HET, heteroplasmic.

Table 2. mtDNA Copy Number Distribution in LHON Affected, Carriers, and Controls

KS Test	D	$D_{0.001}$	*P* Value
All-LHON vs. Controls	0.7816	0.4556	8.07×10^{-11}
Affected vs. Controls	0.7000	0.8960	0.009
Carriers vs. Controls	0.8556	0.5035	1.05×10^{-10}
Carriers vs. Affected	0.8889	0.9858	0.0013

Differences in the cumulative distribution of mtDNA copy number of Affected, Carriers, and Controls were assessed in a pairwise manner via a 2-sample KS test. D, calculated KS distance; $D_{0.001}$, critical value for the KS test statistic at $\alpha = 0.001$.

mutations such as those leading to 'ragged red fibers'. Interestingly, in the case of LHON mutations, which have weak pathogenic potential, mitochondrial mass may represent a compensatory mechanism for the development of complex I deficiency, as well as a possible molecular explanation for the variable penetrance of the LHON primary mutations.[4] Thus, the mitochondrial proliferation in Carriers appears to be a consequence of less-severe deleterious mutations leading to alterations in mitochondrial activity. This mechanism, for unknown reasons, does not occur, or may be interrupted at a certain time in subjects who manifest the disease. The molecular pathway and the nature of the retrograde signaling generated by the less performing complex I to boost mitochondria proliferation in LHON Carriers are not known.

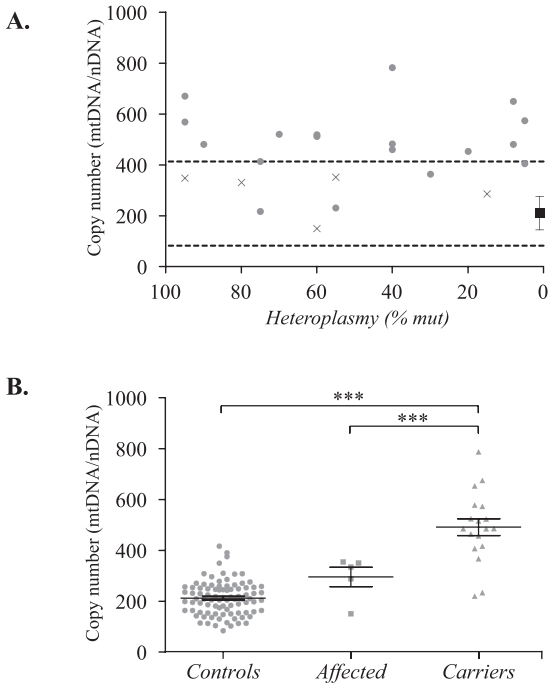

Figure. Analysis of mtDNA content in LHON heteroplasmic subjects. (**A**) Scatter plot showing the heteroplasmy levels versus the mtDNA copy number for the heteroplasmic LHON subjects. The mean ± SD of mtDNA copy number for the Controls are reported as a *black square* at 0% heteroplasmy. The minimum and maximum number of mtDNA copy number observed in the Controls is indicated as *dashed black lines*. Carriers are indicated as *gray circles*; Affected are indicated as black crosses. (**B**) mtDNA copy numbers with mean ± SD were evaluated in Affected, Carriers, and Controls. Experiments were performed in triplicates for all the samples. Comparison of mtDNA levels among the cohorts of heteroplasmic subjects was performed by ANOVA test after the Bonferroni correction for multiple comparisons. *Asterisks* indicate statistical significance: Controls versus Carriers, $P < 0.001$; Affected versus Carriers, $P < 0.001$.

Overall, despite the small number of subjects, our results suggest that mtDNA content may be a determining factor in LHON onset. Higher mtDNA content is possibly involved in protecting from or promoting the disease process, an observation that may have important implication for our understanding of the mechanisms involved in the development of LHON, and most importantly, for the potential to use this information as a prognostic biomarker and a therapeutic strategy.

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