

# Prevalence of primary mutations in Leber hereditary optic neuropathy: A six-year report from a tertiary eye care center in India

Srilekha Sundaramurthy,<sup>1</sup> Ambika Selvakumar,<sup>2</sup> Vidhya Dharani,<sup>2</sup> Nagasamy Soumitra,<sup>3</sup> Jayaprakash Mani,<sup>1</sup> Karthiyayini Thirumalai,<sup>1</sup> Porkodi Periyasamy,<sup>1</sup> Sinnakaruppan Mathavan,<sup>1</sup> Sarangapani Sripriya<sup>1</sup>

<sup>1</sup>SN ONGC Department of Genetics & Molecular Biology, Vision Research Foundation, Chennai, India; <sup>2</sup>Department of Neuro Ophthalmology, Medical Research Foundation, Chennai, India; <sup>3</sup>Formerly at SN ONGC Department of Genetics & Molecular Biology, Vision Research Foundation, Chennai, India

**Purpose:** Genetic testing for primary mutations m.3460G>A, m.11778G>A, and m.14484T>C in *ND1*, *ND4*, and *ND6* genes of mitochondrial DNA is the recommended assay for Leber hereditary optic neuropathy (LHON; OMIM 535000). This report discusses the outcome of molecular genetic screening for these three primary mutations in suspected LHON cases in India.

**Methods:** Two hundred and seventy-eight unrelated presumed LHON patients who were seen at the neuro-ophthalmology clinic of a tertiary eye care center from 2014–2018 were analyzed. They were genotyped for the three common variants by polymerase chain reaction–based direct sequencing, and their plasmid status was also determined by restriction enzyme digestion.

**Results:** Eighty two of 278 patients were positive for one of the 3 common mutations with m.11778G>A in *ND4* gene more frequently distributed (N=72) in homoplasmic state (N=59/82). The mean onset age of visual loss was 21.1 years (SD, 9.8 years; range, 5–58 years) in patients harboring the primary mutation. The most common clinical presentation was bilateral sequential painless vision loss with central and cecentral scotomas in the visual field due to optic disc atrophy.

**Conclusions:** The study subjects are a sample of a much larger number of suspected LHON cases tested for primary mutations in India. (N= 278) and 29.4% (82/278) of patients harbour one of the 3 common mutations. Screening the entire mitochondrial genome and the other nuclear genes encoding mitochondrial protein, would probably aid in identifying the other less common mtDNA mutations causing LHON in Indian population.

Leber hereditary optic neuropathy (LHON; OMIM 535000) is a blinding disorder seen predominantly in young men and characterized by selective degeneration of retinal ganglion cells (RGCs) and optic atrophy that results in maximal vision loss [1]. The clinical features include loss of visual acuity, dyschromatopsia, large central scotomas, and bilateral subacute loss of central vision due to the degenerating subset of macular RGCs that provide axons for the papillomacular bundle [2]. A method of molecular confirmation would be useful in diagnosing LHON, alongside observation of clinical features such as onset of the disease (subacute) and maternal history of visual loss (if available).

Mutations causing LHON are identified in the single subunit of mitochondrial NADH dehydrogenase genes in the complex I subunit, which upon dysfunction result in increased production of Reactive oxygen species and decreased production of ATP. The three common mutations:

m.3460G>A (13%), m.11778G>A (70%) and m.14484T>C (14%) are observed in 90% of people affected by LHON. The recommended guidelines for LHON diagnosis include targeted genetic testing, primarily for the 3 common mutations [3–5], followed by sequencing of the other mitochondrial / nuclear genome based on history, evaluation history of key structural and functional visual parameters [6]. Genetic counseling of the patient and family and lifestyle changes for preventable risk factors, such as smoking, secondary smoke, alcohol consumption, and exposure to mitotoxic agents, are recommended in the management of the disease [7]. In this context, of disease management and diagnosis confirmation, screening the common mutations has proven cost effective for molecular confirmation of LHON. We report here and discuss the molecular diagnosis results for the 3 common primary mutations in suspected LHON cases referred to our genetic clinic for a period of 5 years.

*Clinical examination:* The study was approved by the institutional review board (IRB) and ethics committee, and all procedures were performed according to institutional guidelines and the Declaration of Helsinki.

Correspondence to: Sarangapani Sripriya, SNONGC Department of Genetics and Molecular Biology, Vision Research Foundation, Chennai, India; Phone: 91-44-42271807; FAX: 91-44-2825418; email: [vatsanpriya@gmail.com](mailto:vatsanpriya@gmail.com) and [drss@snmail.org](mailto:drss@snmail.org)

LHON cases were referred for genetic screening after detailed ophthalmic examination that included examination by Snellen chart, slit lamp examination, biomicroscopy and indirect ophthalmoscopy. Visual field analysis by Humphrey automated visual field analyzer Swedish Interactive Threshold Algorithm (SITA) 30-2 standard is a program used in the Humphrey visual field analyzer used for assessing the field of vision in patients. The diagnosis of LHON was based on clinical features like young patient with, acute, to subacute progressive central visual loss, with color vision defect, optic disc pallor and central/centro cecal scotoma.

*Sample collection and DNA isolation:* Detailed pedigree data were documented for patients recommended for genetic testing, followed by blood collection for DNA extraction. Genomic DNA was extracted using a Nucleospin Blood XL kit (Macherey-Nagel, GmbH, Düren, Germany) according to the manufacturer's instructions.

*Screening for primary mutations:* The primers were designed for the three common mutations using Primer 3 (v. 0.4.0) software; m.3460G>A (FP: TAC TTC ACA AAG CGC CTT CC, RP: ATG AAG AAT AGG GCG AAG GG); m.11778G>A (FP: GCT CCC TTC CCC TAC TCA TC, RP: AGG GGT CGT AAG CCT CTG TT); and m.14484T>C (FP: GCA TAA TTA AAC TTT ACT TC, RP: AGA ATA TTG AGG CGC CAT TG) [8]. Direct sequencing was performed using an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). The mitochondrial DNA sequences from LHON cases were compared with the Revised Cambridge Reference Sequences (rCRs; [NC\\_012920](https://www.ncbi.nlm.nih.gov/nuccore/NC_012920)) and analyzed using the online [MITOMAP](https://mitomap.org/) tool. PCR was performed using 50 ng genomic DNA in a 20 µl reaction consisting of 10 µM primers (Sigma-Aldrich, St. Louis, MO), 500 µM dNTPs (Applied Biosystems), and 0.2 µM of Taq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) for an initial denaturation of 5 min at 94 °C, extended for 30 s at 72 °C, followed by a final extension of 7 min at 72 °C. The annealing temperature varied for the three genes (61 °C for 45 s [*ND1*], 61 °C for 30 s [*ND4*], 49 °C for 30 s [*ND6*]). Restriction with BsaHI, Tsp45I, and BstNI (for m.3460G>A, m.11778G>A, and m.14484T>C, respectively) was performed using a reaction containing 5 µl of PCR product, 2 µl of 2X buffer, and 0.2 µl (1 unit) of the respective buffers for a period of 16 h at 60 °C. Restriction products were detected by electrophoresis on a 4.0% agarose gel (Seakem® LE Agarose, Lonza, Rockland, ME).

PCR-based restriction digestion was performed to determine the plasmid status of the primary mutations. The restriction enzymes used, along with the restriction sites, are listed in Appendix 1.

Mutations m.3460G>A and m.11778G>A had naturally occurring restriction sites, and a restriction site for m.14484T>C was induced using a modified mismatched reverse primer with a 3' end adjacent to 14,484 bp. A mismatch of cytosine was introduced at 14,487 bp and 14,488 bp (FP: TCA ACG CCC ATA ATC ATA CAA AG; RP: CCA GGC CCC CTA AAT AAA TTA AAA AA). One hundred unrelated healthy samples over 40 years of age were included as controls for the epidemiological study after undergoing detailed ophthalmic evaluation for any inherited retinal dystrophies [9].

## RESULTS

Two hundred and eighty-two patients were referred for genetic counseling over a period of five years (2014–2018), of which 278 agreed to undergo genetic testing. Pedigree analysis showed a positive family history in six patients (Figure 1). Twenty-nine percent of the study cohort (82 out of 278) were positive for one of the primary mutations that were, distributed among patients in the following order: m.11778G>A (~85%; n = 70), m.14484T>C (~13%; n = 11), and m. 3460G>A (~2%; n = 1; Figure 2A–D, Figure 3A,B). PCR-based restriction enzyme digestion showed heteroplasmy (Figure 2E, Figure 3C) in 12 samples, including some m.11778G>A (n = 11) and m.14484T>C (n = 1). Appendix 2 describes the clinical details of the LHON patients' positive results for the primary mutations. Strong family history was observed for m.11778G>A (N=5) and m.14484T>C (N=1) mutation and all the available affected family members were positive for the mutations in homoplasmy state (Table 1).

In addition to the 3 primary mutations, we identified other missense variants: m.11447G>A; (p.Val230Met) *ND4* and m.3316G>A; (p.Ala4Thr), m.3644T>C; (p. Val113Ala) in the *ND1* gene. The m.3316G>A (N=2) mutation were also observed in the unrelated healthy controls. Distribution of non-synonymous and synonymous coding region variants are as given in Table 2 and Appendix 3. The patients in the current study were from different geographical locations (north India (N=200), south India (N=74), Srilanka (N=2) and one each from Andaman and Nepal).

## DISCUSSION

Two hundred and seventy eight LHON suspects were referred for genetic testing and further proceeded for PCR based direct sequencing and restriction enzyme digestion after their informed consented for the test. History of smoking and alcohol consumption was noted only in 2 patients. The mean onset age of visual loss was 21.1years (SD, 9.8 years; range, 5-58 years) in patients harboring any one of the primary

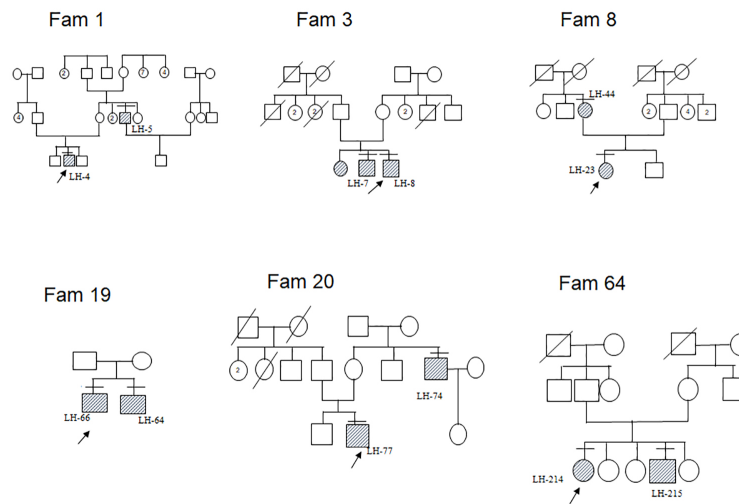


Figure 1. Pedigree information showed a positive family history in six families. The arrow indicates the index case. The solid circles and squares represent affected females and males, respectively. Lines above the individuals indicate the availability of genotype.

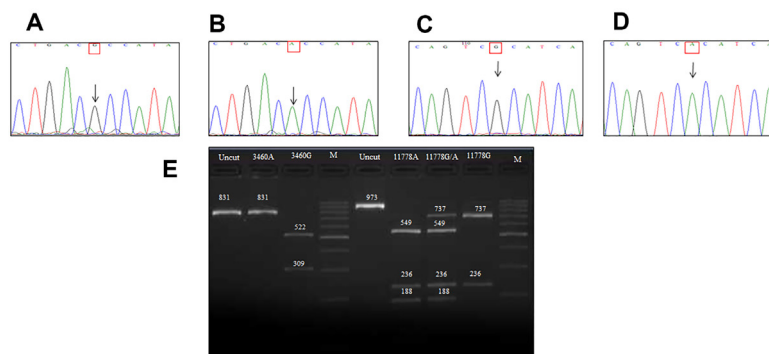


Figure 2. Electropherogram and PCR-RE for m.3460G>A; m.11778G>A. **A** and **B**: Electropherogram of wild-type and mutant m.3460G>A. **C** and **D**: Wild-type and mutant m.11778G>A. **E**: 4% agarose gel electrophoresis of PCR-RE showing the plasmid status of the m.3460G>A and m.11778G>A mutations, respectively; M: size marker; Uncut: non-restricted PCR product.

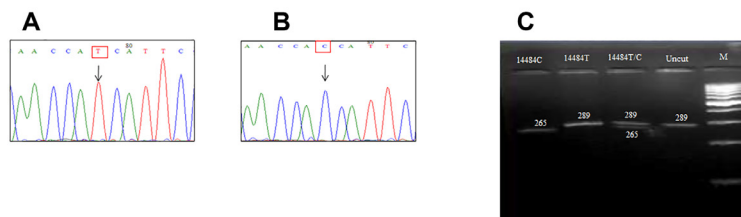


Figure 3. Electropherogram and PCR-RE for m.14484T>C. **A** and **B**: Electropherogram of Wild-type and Mutant m.14484T>C. **C**: 4% agarose gel electrophoresis of PCR-RE showing the plasmid status of m.14484T>C mutation. M: size marker; Uncut: non-restricted PCR product.

**TABLE 1. LHON PRIMARY MUTATION POSITIVE FAMILIAL CASES ALONG WITH CLINICAL CHARACTERISTICS.**

Fam ID	Proband ID / Relation	Primary mutation	Plasmy status	Age	Sex	Age of onset	MRI	Visual acuity		Fundus
								OD	OS	
Fam-1	LH-4	m.11778G>A	Homo	25	M	16	Hyperintense signals in optic nerve	0.47	0.47	Temporal disc pallor
Fam-1	LH-5 / Maternal uncle of LH-4	m.11778G>A	Homo	37	M	18	Thinned out optic nerve	2.07	0.47	Temporal disc pallor
Fam-3	LH-7	m.11778G>A	Homo	33	M	21	Hyperintense signals in optic nerve	2	HM	Disc pallor
Fam-3	LH-8 / sibling of LH-7	m.11778G>A	Homo	31	M	23	Hyperintense signals in optic nerve	CF	CF	Disc pallor
Fam-8	LH-44 mother of LH-23	m.11778G>A	Homo	45	F	16	Not performed	1	1	Disc pallor
Fam-8	LH-23	m.11778G>A	Homo	28	F	18	Not performed	1.77	1.47	Disc pallor
Fam-19	LH-66 / sibling of LH-64	m.11778G>A	Homo	28	M	13	Not performed	1.30	1	Disc pallor
Fam-19	LH-64	m.11778G>A	Homo	37	M	37	Not performed	1.47	1	Temporal disc pallor
Fam-20	LH-77 / maternal uncle of LH-74	m.14484T>C	Homo	36	M	26	Not performed	Not performed	Not performed	Not performed
Fam-20	LH-74	m.14484T>C	Homo	19	M	15	Hyperintense signals in optic nerve	1.30	1.77	Temporal disc pallor
Fam-64	LH-215 / sibling of LH-214	m.11778G>A	Homo	17	M	17	Normal	CF	CF	Temporal pallor
Fam-64	LH-214	m.11778G>A	Homo	20	F	18	Thinned out left optic nerve with increased T2 signal	3/60	3/60	Temporal pallor

OD-Right eye OS-Left eye HM-Hand motion vision CF-Counting Fingers MRI-Magnetic Resonance Imaging

mutations and 22.5 years (SD,12.4 years; range, 1-70 years) for the patients without primary mutations. We did not observe any association with history of smoking and alcohol consumption in those patients positive for primary mutations (Appendix 2). Comparison of the clinical features among the patients did not show any significant correlation with primary mutations (Appendix 2). There weren't any significant differences in age of onset, plasmy status and visual prognosis (between and within homoplasmic and heteroplasmic individuals). Male predominance observed in our study (89.9% (250/278)) is similar to that reported in other population [10–12]. LHON is described as maternally inherited [13]; however pedigree analysis of the samples (96%) in the current study did not show such typical inheritance pattern.

However six families showed family history of LHON with more than one affected member as described in Table 1. There wasn't any difference in the plasmy state and clinical features between the affected family members harboring the primary mutations.

Genetic testing of the other unaffected family members is further suggested to better understand the level of penetrance in this cohort.

Case reports and cohort based studies in LHON patients from India, has shown that frequency distribution of primary mutation ranges from 15% - 27.5% in North India [14–16] and 12%–43% in South India [17–19]; our results are comparable with the reported frequencies. In the current study,

29.4% (82/278) of the study cohort had the primary mutations. Screening 40 LHON patients (within the age group of 10-50) for primary mutation, Mishra et al reported a frequency of 27.5% for m.G11778A mutation in North Indian families [16]. Screening 55 with LHON patients from south India, in a span of 5 years, Gowri et al reports a high frequency of (43.6%) primary mutations [19]. Screening 2 LHON families, Verma et al reports G3460A and G11778A mutation in 2 families [14].

Characterization of 8 unrelated probands with m.14484T>C mutation, has shown an overall penetrance of 19.75% (16/81) but was not associated with any specific haplogroup within the Indian population. Similarly the m.11778G>A mutation was also reported to coexist with different haplogroup Background with variable penetrance. [20,21].

The current study results also suggest the putative involvement of other mitochondrial mutations / nuclear genome / Copy number variations (CNVs) in our population similar to other studies. The prevalence (29.4%: 82/278) of primary mutations observed in this study is similar to the reported from the Chinese population (29.3%: 89/304) [22] and in the majority of worldwide reports (95%) [23].

In addition to primary mutations, we have also identified other mutations (m.3644 T>C and m.11696G>A) in the current study. The m.3644T>C [N=1] variation in *ND1* gene was earlier reported in patients with bipolar disease and correlated with increased risk of bipolar disease with syndromic comorbidity [24,25]; however, the index case in this study did not report any such history in the study subjects.

The other mutation, m.11696G>A [N=1] was observed in LHON patient with additional features of dystonia, hypertension and muscle weakness [26], which are observed in patients diagnosed with Leber optic neuropathy and dystonia (LDYT).

Whole mitochondrial genome sequencing and phylogenetic analysis in 75 LHON patients and 40 healthy controls from south India showed the M group as the most commonly observed haplogroup in LHON patients among the 12 different haplogroups (M, R, U, HV, H, N, C, P, L, D, K and W) [17]. In our earlier studies we did not observe significant association with haplogroup or clinical penetrance in LHON patients with m.14484T>C and m.11778G>A mutation [20,21]. However, we are unable to make any correlation with specific mutations and haplogroup.

Heteroplasmy (males: ~30–50%; females: ~10–15%) was associated with incomplete penetrance in LHON [13]. In this study, we observed heteroplasmy in 16% and 9% of the patients with the m.11778G>A and m.14484T>C mutations, respectively, as compared to other Indian studies (20% for m.11778G>A [21] and nil for m.14484T>C [20]). Interestingly, homoplasmy was observed in the family members of the index cases harboring the m.11778G>A (n = 5) and m.14484T>C (n = 1) mutations. The m.11778G>A mutation was associated with 0.39 fold lesser risk of poor vision prognosis in heteroplasmy when compared to homoplasmy status [21]. In our study we did not find any significant association with the risk of visual failure. The role of other risk factors like alcohol consumption, tobacco usage and drug toxicity [like anti-tubercular treatment (ATT) – ethambutol] in addition to the mitochondrial variations makes the patients more susceptible for the disease [27]. We did not screen the other family members to assess the role of incomplete penetrance in this study cohort. The current study represents large LHON cohort (N=258) from a single ophthalmic center (both north and south India) for primary mutation screening when compared to the other reports from India (sample size N<100). In addition to the larger sample size, the study reports the plasmid status by polymerase chain reaction - restriction enzyme (PCR-RE) method which is not mentioned in other studies from India.

TABLE 2. DISTRIBUTION OF NONSYNONYMOUS CODING REGION VARIANTS IN LHON CASES.

S.No	Family ID	Nucleotide substitution	Protein change	Codon change	Amino acid change	Novel/ Reported	Locus change	Poly-phen-2 score	SIFT score	Disease associated
1.	LH-11, LH-24, LH-37	m.3316G>A	p.Ala4Thr	GCC>ACC	Ala>Thr	Reported	<i>ND1</i>	0 (Benign)	0.18 (Tolerated)	LHON
2.	LH-1	m.3644T>C	p.Val113Ala	TAC>CAC	Val>Ala	Reported	<i>ND1</i>	0.111 (Benign)	0.02 (Deleterious)	B associated D
3.	LH-18	m.11447G>A	p.Val230Met	GTG>ATG	Val>Met	Reported	<i>ND4</i>	0.009 (Benign)	0 (Deleterious)	Nil

**Conclusion:** We screened 278 suspected LHON cases for primary mutations in *ND1*, *ND4* and *ND6* genes, and identified the same in 29.4% (82/278) with m.11778G>A in *ND4* genes. We also report here heteroplasmy for m.11778 G>A and m.14484T<C mutations in 16% and 9% of the families respectively. Whole mitochondrial genome screening would be ideal to reveal the frequency of secondary LHON mutations and other novel mutation(s) which can be pathogenic for the disease process and be specific to Indian population.

### APPENDIX 1. RESTRICTION ENZYMES AND RECOGNITION SITES NCBI REFERENCE SEQUENCE

To access the data, click or select the words “[Appendix 1.](#)” (NC\_012920.1) along with expected digestion product length (bp) for the 3 primary mutations as analyzed in NEBCUTTER v2.0.

### APPENDIX 2. MOLECULAR AND CLINICAL DATA FOR THE PRIMARY MUTATION POSITIVE CASES.

To access the data, click or select the words “[Appendix 2.](#)” OD-Right eye OS-Left eye CF-Counting Fingers HM-Hand motion vision MRI-Medical Resonance Imaging Nil-No habits of Smoking and Alcohol consumption.

### APPENDIX 3. DISTRIBUTION OF SYNONYMOUS CODING REGION VARIANTS IN LHON CASES.

To access the data, click or select the words “[Appendix 3.](#)”

### ACKNOWLEDGMENTS

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