

# Haplogroup Heterogeneity of LHON Patients Carrying the m.14484T>C Mutation in India

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**PURPOSE.** To investigate the clinical and mitochondrial DNA (mtDNA) haplogroup background of Indian Leber hereditary optic neuropathy (LHON) patients carrying the m.14484T>C mutation.

**METHODS.** Detailed clinical investigation and complete mtDNA sequencing analysis was carried out for eight Indian LHON families with the m.14484T>C mutation. Haplogroup was constructed based on the evolutionarily important mtDNA variants.

**RESULTS.** In the present study, we characterized eight unrelated probands selected from 187 LHON cases. The overall penetrance of the disease was estimated to be 19.75% (16/81) in eight pedigrees with the m.14484T>C mutation and showed substantially higher sex bias (male:female = 13:3). The mtDNA haplogrouping revealed that they belong to diverse haplogroups; i.e., F1c1, M31a, U2a, M\*, I1, M6, M3a1, and R30a. Interestingly, we did not find an association of the m.14484T>C mutation with any specific haplogroup within the Indian population. We also did not find any secondary mutation(s) in these pedigrees, which might affect the clinical expression of LHON.

**CONCLUSIONS.** Contrary to earlier reports showing preferential association of the m.14484T>C mutation with western Eurasian haplogroup J and increased clinical penetrance when present in J1 subhaplogroup background, the present study shows that m.14484T>C arose independently in a different mtDNA haplogroup and ethnic background in India, which may influence the clinical expression of the disease.

Keywords: LHON, mutations, mtDNA, m.14484T>C, haplogroup

Mitochondrial DNA (mtDNA) mutations have been reported to cause a large number of genetic disorders.<sup>1,2</sup> During the last two decades there has been major progress in identification of disease-causing mtDNA mutations. Leber hereditary optic neuropathy (LHON, OMIM 535000) is one of the first diseases to be characterized primarily by the missense mutations in mtDNA. LHON is a maternally inherited disorder clinically characterized as a slow and progressive loss of central vision due to degeneration of the optic nerve and retinal ganglion cells in young adults, predominantly affecting males.<sup>3-5</sup>

Mutations in different subunits of mitochondrial complex I genes (m.3460G>A in MT-ND1, m.11778G>A in MT-ND4, and m.14484T>C in MT-ND6) have been reported in more than 95% of LHON cases across the world.<sup>6-8</sup> A number of other mutations have been reported in complex I genes in the pedigrees negative for the three primary mutations.<sup>9</sup> However, the phenotypic expression of these primary mutations have been found to vary in different families and even in different

populations, with only one-third of all individuals inheriting these mutations developing visual failure or blindness. This incomplete penetrance in different pedigrees suggests that other factors, such as mtDNA haplogroup background, nuclear genetic background, and environmental factors, may have an important role in modulating the phenotypic expression and severity of the disease.<sup>10-14</sup> In addition to the primary mutations, many other mtDNA sequence variants have been reported along with the primary LHON mutations, suggesting that these variants have possible synergistic roles in modulating clinical expression.<sup>15-18</sup>

Various studies performed on LHON suggest that the primary mutations m.11778G>A and m.14484T>C are strongly associated with western Eurasian haplogroup J.<sup>18-21</sup> These observations also implicate the involvement of other J haplogroup defining mutations (m.4216T>C and m.13708G>A) in increasing the risk of disease expression.<sup>19</sup> Further studies on pooled European LHON samples showed

that the haplogroups J1, J2, and K increase the risk of vision loss in LHON patients with m.14484T>C, m.11778G>A, and m.3640G>A mutations, respectively, while haplogroup H has been found to have a protective role for LHON patients with the m.11778G>A mutation.<sup>10</sup> These findings about LHON in association with haplogroups have led to a substantial increase in the number of studies to unravel the role of mtDNA sequence variations in various disorders and phenotypes. However, other than LHON, most of the reports showing association between haplogroups and diseases remain provisional. Most of the mtDNA haplogroup association studies have given conflicting results in different populations because the findings of one study have rarely been replicated by studies in other populations.<sup>22</sup>

Studies on the m.14484T>C mutation in a European population showed that more than 75% of LHON patients belonged to haplogroup J,<sup>18</sup> while another study showed low penetrance of LHON when present in non-J haplogroup background.<sup>17</sup> Subsequently, a study by Hudson et al.<sup>10</sup> in a European population showed increased penetrance of the disease when present in a J haplogroup background, specifically in the J1 subhaplogroup. Another study with French Canadian LHON patients revealed that the m.14484T>C mutation is predominantly associated with the LHON phenotype.<sup>23,24</sup> However, studies on Asian families with this mutation did not show a similar pattern of association with any specific haplogroup.<sup>25,26</sup> Until now there was only one study from India, which reported three individuals with the m.14484T>C mutation; however, the haplogroup of these individuals was not established.<sup>27</sup> Hence, we have characterized 8 out of 187 suspected Indian LHON families carrying the primary m.14484T>C mutation to study the clinical, genetic, and phylogenetic background of m.14484T>C positive individuals in the Indian population.

## MATERIALS AND METHODS

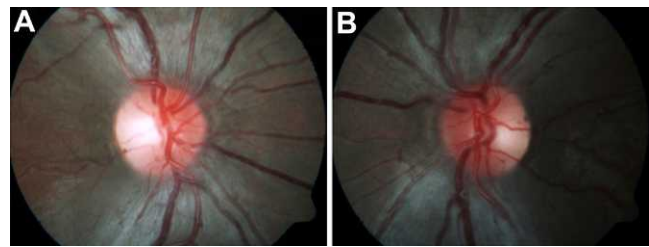
### Patients and Subjects

We screened a total of 187 individuals with LHON for pathogenic mutations and found eight families carrying the primary mutation m.14484T>C. These cases were collected over a period of 7 years at the Sankara Nethralaya Eye Hospital, Chennai, India; National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India; and Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India. Clinical examinations were conducted in all patients and assessed presentation of primary features including acute, gradual, and progressive loss of central vision; color vision defect; and centrocecal scotomas. All patients also underwent complete ophthalmic examination, including evaluation by Snellen chart, visual acuity measurement, slit lamp biomicroscopy, indirect ophthalmology, and Humphrey perimetry analysis. Visual field testing was performed in all patients with good fixation.

The degree of visual impairment was defined according to the visual acuity as follows: normal > 0.3, mild = 0.3 to 0.1; moderate < 0.1 to 0.05 and severe < 0.05 to 0.02. Informed written consent was obtained prior to sample collection from all individuals who participated in this study. The study adhered to the tenets of the Declaration of Helsinki and was approved by Institutional Ethical Committees (IECs) of all the participating institutions.

### Genetic Analysis

Genomic DNA was extracted from peripheral blood samples using a standard protocol.<sup>28</sup> Overlapping DNA fragments to



**FIGURE 1.** (A) Right and (B) left eye of a 20-year-old man with the m.14484T>C mutation in the MT-ND6 gene, presenting with bilateral vision loss. The right eye almost appears normal, but the left eye shows mild peripapillary nerve fiber layer (ppNFL) elevation and microtelangiectasia of vessels. He had bilateral centrocecal field defects.

cover the entire mtDNA were amplified using 24 sets of primers.<sup>29,30</sup> The overlapping amplicons were directly sequenced (both forward and reverse, separately) using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Extended products were precipitated with ethanol and 5 M sodium acetate (5:1) and then dissolved in Hi-Di formamide and analyzed in ABI 3730 DNA Analyzer (Applied Biosystems). All 48 sequences (24 forward and 24 reverse) were edited using Sequence Analysis and assembled with the revised Cambridge Reference Sequence (rCRS; NC\_012920)<sup>31,32</sup> using AutoAssembler software (Applied Biosystems). Quantification of the m.14484T>C mutation was done by PCR restriction length polymorphism analysis (RFLP) as described earlier.<sup>33</sup> The mtDNA sequence of eight patients included in this study has been submitted to GenBank with the following accession numbers: P18 (JX462682), P24 (JX462688), P26 (JX462690), P33 (JX462697), P35 (JX462699), P41 (JX462705), P48 (JX462712), and P51 (JX46271).

### Data Analysis

All mismatched nucleotides along with their position were noted and searched in human mitochondrial genome databases such as Mitomap (<http://www.mitomap.org>), mtDB (<http://www.genpat.uu.se/mtDB>), and HmtDB (<http://www.hmtdb.uniba.it:8080/hmtdb>) for their significance. The data obtained were also compared with 300 ethnically matched control samples from endogamous Indian populations. Haplogroup was constructed based on the available literature ([www.phylotree.org](http://www.phylotree.org); mtDNA tree Build 15 [September 30, 2012]). All data analyses were carried out using MEGA 5 software ([www.megasoftware.net](http://www.megasoftware.net)) to evaluate the conservation of amino acids in mitochondria-encoded protein subunits and nucleotide conservation in genes encoding MT-tRNA and MT-rRNA.

## RESULTS

### Clinical Evaluation

All eight LHON pedigrees included in this study were selected from 187 families after confirming the m.14484T>C mutation in the MT-ND6 gene. Detailed clinical analysis of these families did not reveal any other abnormalities, such as neurological disorders, hearing impairment, muscular diseases, or exercise intolerance, which are frequently associated with mitochondrial dysfunctions. The most common clinical presentation in the patients was bilateral loss of vision along with scotomas in the visual field (central and cecocentral) and optic disc atrophy (Fig. 1). All eight pedigrees consisted of 16 affected members (13 males and 3 females), their mean age of onset was  $23.25 \pm$

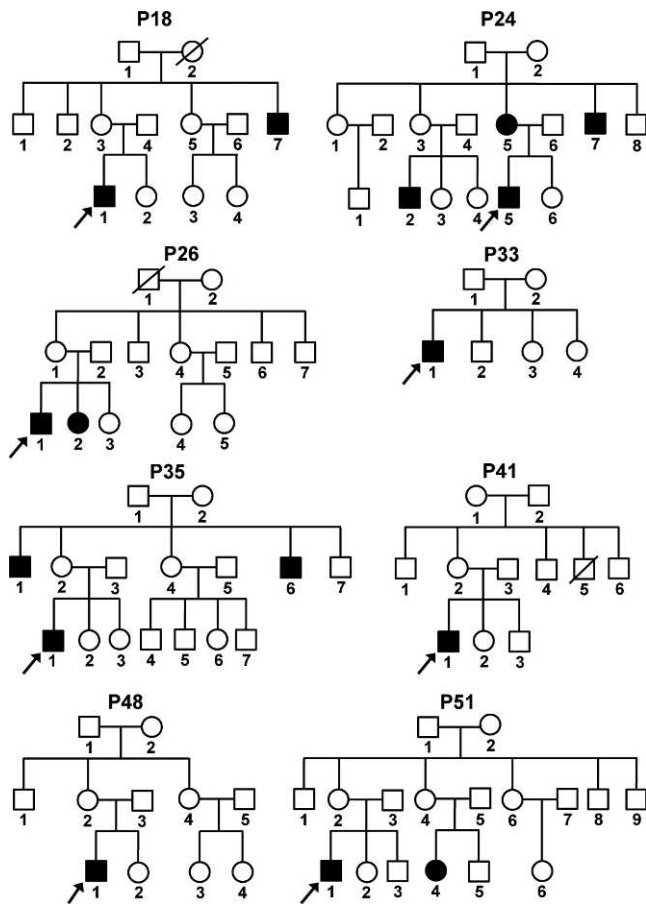


FIGURE 2. Pedigrees of Indian LHON families with the m.14484T>C mutation in the MT-ND6 gene. The affected individuals are marked with filled symbols, and the arrow indicates the probands for whom the complete mtDNA was sequenced.

4.02 years, and they showed variable penetrance of the disease (Fig. 2). The ratio of affected male versus female was 13:3, which is higher than the earlier studies reported with the m.14484T>C mutation. Three pedigrees (P33, P41, and P48) had only one affected male in each, while the penetrance was higher in P18 and P35 with more than one male member affected by vision loss. Both male and female affected members were seen in three pedigrees (P24, P26, and P51). Among these pedigrees, the highest clinical presentation was observed in P24 with three males and one female being affected. Total penetrance was 16 out of 81 individuals (19.75%) showing clinical phenotype of LHON and vision loss.

### Genetic Analysis

Complete mtDNA was sequenced for all probands from each family and confirmed the presence of the m.14484T>C mutation, which was found in homoplasmic state in all the probands and their maternal relatives who were subjected to the analysis. The mtDNA haplogroup was constructed based on the complete mtDNA sequences of eight probands who carried the m.14484T>C mutation, and revealed eight different haplogroups, namely F1c1, M31a, U2a, M\*, I1, M6, M3a1, and R30a (Fig. 3).

The occurrence of the m.14484T>C mutation in a different haplogroup background in the present study is in complete contrast to European pedigrees, which showed strong association of haplogroup J with the m.14484T>C mutation.<sup>8</sup>

The effect of haplogroup on clinical expression is mostly explained by the private nonsynonymous variations observed in haplotypes, which influences the effect of primary mutations and may result in variable clinical expression.<sup>10,11</sup> We observed 22 nonsynonymous variants and four mutations in MT-tRNA genes of the probands analyzed (Table). The variants observed are in pedigrees P18 (m.9053G>A, m.10398A>G, m.10609T>C, m.12406G>A, m.13759G>A, m.13928G>C) and one MT-tRNA mutation m.10454T>C; P24 (m.8701A>G, m.10084T>C, and m.15258A>G); P26 (m.3316G>A, m.7859G>A, m.13708G>A); P33 (m.11447G>A, m.13879T>C); P35 (m.9194A>G, m.9966G>A, m.13780A>G) and one tRNA glycine mutation m.10034T>C; P41 (m.5301A>G) and one tRNA methionine mutation m.4418T>C; P48 (m.12842T>C); and P51 (m.4232T>C, m.5442T>C, m.8584G>A). Among these variants, three MT-tRNA variants (m.10454T>C in tRNA arginine, m.4418T>C in tRNA methionine, and m.15931A>T in tRNA threonine) were found to be conserved across species. In the protein-coding region, m.4232T>C in MT-ND1 (I309T) in pedigree P51, m.9966G>A in MT-COIII (V254I) and m.9194G>A in MT-ATP6 (H223A) in pedigree P35, and m.15258A>G in MT-CYB (D171G) in pedigree P24 were found to be conserved across the species (Table).

### DISCUSSION

The mtDNA mutations m.3460G>A, m.11778G>A, and m.14484T>C have been known as the primary causes for LHON. However, the frequency of m.14484T>C has always been reported to be less than that of m.11778G>A in different populations,<sup>8,15,22,34</sup> the only exception being Canadian population in which m.14484T>C was reported to be the major cause for LHON.<sup>23,24</sup> Other important characteristics of the m.14484T>C mutation is its preferential association with western Eurasian haplogroup J<sup>19,21</sup> and increased clinical penetrance when present in the J1 subhaplogroup background.<sup>10</sup>

In the present study, we found 8 out of 187 LHON individuals carried the m.14484T>C mutation, and we performed clinical, genetic, and molecular characterization of these families. We sequenced the complete mtDNA of the probands to study the matrilineal genetic structure and to look for the potential role of specific haplotype's association with LHON. Painless loss of central vision was the common clinical feature shared by all the individuals. Since all pedigrees were homoplasmic for the m.14484T>C mutation, heteroplasmy cannot be the reason for variable expression of the disease. Frequency of m.14484T>C in the present study was 4.2%, which is similar to another study (3.33%) from India,<sup>27</sup> suggesting a much lower percentage of m.14484T>C among Indian LHON individuals compared with European<sup>10,22</sup> and Chinese populations, in which the frequency was approximately 10.0%.<sup>25</sup> However, considering the population size and heterogeneity, more studies are required to determine the exact frequency of this mutation in the Indian population.

Dissection of matrilineal genetic structure of patients revealed that four out of the eight patients belonged to the major haplogroup M (M31a, M\*, M3a1, and M6); the remaining four haplogroups were I1, F1c1, R30a, and U2a (Fig. 3). Presence of m.14484T>C in such a diverse haplogroup background indicated independent occurrence of this mutation in different ethnic populations in India. Interestingly, we found one family (P18) belonged to the F1c1 haplogroup, having two out of the eight affected individuals (25%). Its penetrance was in contrast to earlier reports from China and

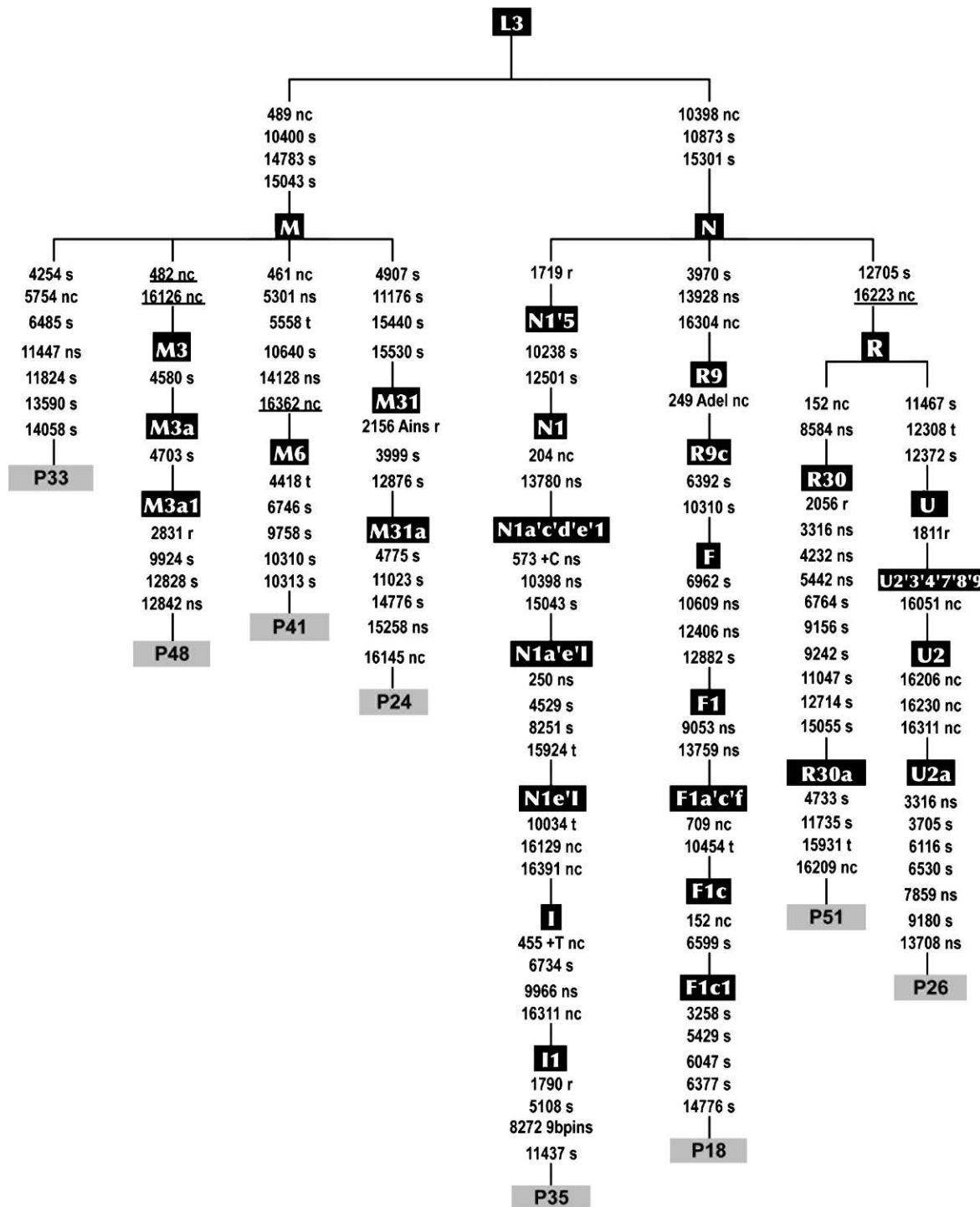


FIGURE 3. Phylogenetic tree constructed based on the variation in complete mtDNA sequences of eight pedigrees with the m.14484T>C mutation. The synonymous and nonsynonymous coding-region variants are denoted by “s” and “ns,” respectively. Variations in the transfer RNA and the ribosomal RNA genes are denoted by “t” and “r,” respectively. The insertions and deletions are denoted by “ins” and “del,” respectively, and underlined mutations are recurrent.

Thailand, which implied a protective role of this haplogroup due to its very low occurrence.<sup>11,25,35</sup> This haplogroup was defined by several nonsynonymous motifs (Table) including tRNA arginine mutation m.10454T>C, which has been reported to influence hearing impairment when co-occurring with the m.1555A>G mutation<sup>36</sup> and might explain the increased penetrance in this family, in contrast to earlier reports.

Pedigrees P26, P35, and P51 belonged to haplogroups U2a, II, and R30a, respectively. It is compelling to think that the clinical penetrance in these families should be affected by nonsynonymous mutations present in the mtDNA background. All three pedigrees have several nonsynonymous motifs (Table). Pedigree P26 possessed three variants (m.3316G>A-MT-ND1; m.7850G>A-MT-COII; and m.13708G>A-MT-ND5), of which m.3316G>A and m.13708G>A have been found to

TABLE. Private Nonsynonymous and MT-tRNA Variants in Indian LHON Families With m.14484T&gt;C Mutation

| Subjects | Haplogroup | Ethnicity      | Nucleotide Position | rCRS | Base Change | Gene        | Position | Amino Acid Change | Conservation | Reported |
|----------|------------|----------------|---------------------|------|-------------|-------------|----------|-------------------|--------------|----------|
| P18      | F1c1       | West Bengal    | 9053                | G    | A           | MT-ATPase6  | 176      | Ser>Asn           | No           | Yes      |
|          |            |                | 10454               | T    | C           | MT-tRNA Arg | —        | —                 | Yes          | Yes      |
|          |            |                | 10398               | A    | G           | MT-ND3      | 114      | Thr>Ala           | No           | Yes      |
|          |            |                | 10609               | T    | C           | MT-ND4L     | 47       | Met>Thr           | No           | Yes      |
|          |            |                | 12406               | G    | A           | MT-ND5      | 24       | Val>Ile           | No           | Yes      |
|          |            |                | 13759               | G    | A           | MT-ND5      | 475      | Ala>Thr           | No           | Yes      |
| P24      | M31a       | West Bengal    | 13928               | G    | C           | MT-ND5      | 531      | Ser>Thr           | No           | Yes      |
|          |            |                | 8701                | A    | G           | MT-ATPase6  | 59       | Thr>Ala           | No           | Yes      |
|          |            |                | 10084               | T    | C           | MT-ND3      | 9        | Ile>Thr           | No           | Yes      |
| P26      | U2a        | Madhya Pradesh | 15258               | A    | G           | MT-Cytb     | 171      | Asp>Gly           | Yes          | Yes      |
|          |            |                | 3316                | G    | A           | MT-ND1      | 4        | Ala>Thr           | No           | Yes      |
|          |            |                | 7859                | G    | A           | MT-COII     | 92       | Asp>Asn           | No           | Yes      |
| P33      | M*         | Tamil Nadu     | 13708               | G    | A           | MT-ND5      | 458      | Ala>Thr           | No           | Yes      |
|          |            |                | 11447               | G    | A           | MT-ND4      | 230      | Val>Met           | No           | Yes      |
|          |            |                | 13879               | T    | C           | MT-ND5      | 515      | Ser>Pro           | No           | Yes      |
| P35      | I1         | Bihar          | 9194                | A    | G           | MT-ATPase6  | 223      | His>Arg           | Yes          | Yes      |
|          |            |                | 9966                | G    | A           | MT-COIII    | 254      | Val>Ile           | Yes          | Yes      |
|          |            |                | 10034               | T    | C           | MT-tRNA Gly | —        | —                 | No           | Yes      |
|          |            |                | 13780               | A    | G           | MT-ND5      | 482      | Ile>Val           | No           | Yes      |
| P41      | M6         | Tamil Nadu     | 4418                | T    | C           | MT-tRNA Met | —        | —                 | Yes          | Yes      |
|          |            |                | 5301                | A    | G           | MT-ND2      | 278      | Ile>Val           | No           | Yes      |
| P48      | M3a1       | Uttar Pradesh  | 12842               | T    | C           | MT-ND5      | 169      | Ile>Thr           | No           | Yes      |
| P51      | R30a       | Andhra Pradesh | 4232                | T    | C           | MT-ND1      | 309      | Ile>Thr           | Yes          | Yes      |
|          |            |                | 5442                | T    | C           | MT-ND2      | 325      | Phe>Leu           | No           | Yes      |
|          |            |                | 8584                | G    | A           | MT-ATPase6  | 20       | Ala>Thr           | No           | Yes      |
|          |            |                | 15931               | A    | T           | MT-tRNA Thr | —        | —                 | Yes          | No       |

The conservation analysis was performed by comparing human mtDNA sequence with 21 different species (rCRS NC\_012920, *Mus musculus* NC\_005089, *Rattus norvegicus* NC\_001665, *Bos taurus* NC\_006853, *Danio rerio* NC\_002333, *Macaca mulatta* NC\_005943, *Canis lupus familiaris* NC\_002008, *Felis catus* NC\_001700, *Equus caballus* NC\_001640, *Cavia porcellus* NC\_000884, *Pan troglodytes* NC\_001643, *Sus scrofa* NC\_000845, *Oryctolagus cuniculus* NC\_001913, *Capra hircus* NC\_005044, *Camelus dromedarius* NC\_009849, *Panthera tigris* NC\_010642, *Elephas maximus* NC\_005129, *Cervus elaphus* NC\_007704, *Canis lupus* NC\_008092, *Giraffa camelopardalis* NC\_012100, *Bufo gargarizans* NC\_008410, *Delphinus capensis* NC\_012061).

be associated with LHON.<sup>19,37</sup> In pedigree P35, three variants were seen (m.9194A>G- MT-ATP6; m.9966G>A- MT-COII; and m.13780A>G- MT-ND5), of which m.9194A>G has been found to be highly conserved and might influence the expression of the m.14484T>C mutation in this pedigree. However, the role of other nonsynonymous variants cannot be completely ignored. Pedigree P51 was found to have three variants in the protein-coding genes (m.4232T>C- MT-ND1; m.5442T>C- MT-ND2; m.8584G>A- MT-ATP6) and one mutation (m.15931A>T) in tRNA threonine, which might modify the effect of m.14484T>C. Among the families falling in the macro haplogroup M, only pedigree P24 (M31a haplogroup) possessed the highest clinical penetrance out of eight pedigrees (34%). Three nonsynonymous variants (m.8701A>G- MT-ATP6; m.10084T>C- MT-ND3; and m.15258A>G- MT-CYB) have been observed in this pedigree. Of these m.8701A>G has been reported to alter mitochondrial matrix Ph and intracellular calcium dynamics.<sup>38</sup> Additional MT-CYB motifs on the J haplogroup is reported to be one of the main reasons for their association and increased clinical expression,<sup>10</sup> and hence it is plausible that the additional variant m.15258A>G, which changes highly conserved aspartic acid to glycine in cytochrome b, might influence the severity of disease expression in this pedigree.

The main interesting observation in this study was the existence of the pathogenic mutation m.14484T>C in different

haplogroup backgrounds. This is in fact not surprising to us because we have demonstrated that India is not inhabited by a single large population, but rather by a large number of small populations that have been maintaining strict endogamy marriage practices over tens of thousands of years.<sup>39–41</sup> Moreover, the finding that four out of eight patients with haplogroup M can be explained by the existence of high frequency of M haplogroup in India.<sup>40</sup> We have also demonstrated that the genetic etiology of various diseases is unique to Indian populations.<sup>28,42</sup> Hence, it would be interesting to see the effect of Indian haplogroups on the LHON-causing m.14484T>C mutation.

This is the first detailed report about the m.14484T>C mutation and its haplogroup association in the Indian population. The distinct set of sequence variations observed in these Indian pedigrees points toward m.14484T>C arising independently in different mitochondrial haplogroup backgrounds in the Indian population. Further, our study explains that there is no association of any specific haplogroup with the m.14484T>C mutation. This is in complete contrast to European pedigrees, which show a strong association of haplogroup J. However, considering the small sample size, more LOHN samples with m.14484T>C mutations need to be analyzed to support our findings. We observed differential penetrance of LHON against different Indian haplogroup backgrounds, thus indicating their possible influence on the

clinical expression of disease. However, further studies on more patients are required for new insights about the m.14484T>C mutation and its haplogroup association with LHON in the Indian population.

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