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Mutation Screening of mtDNA Combined Targeted Exon Sequencing in a Cohort With Suspected Hereditary Optic Neuropathy

Jian-Kang Li1−3,*, Wei Li1,3,*, Feng-Juan Gao5,6,*, Shou-Fang Qu7, Fang-Yuan Hu5,6, Sheng-Hai Zhang5,6, Li-Li Li7, Zi-Wei Wang1,3, Yong Qiu3,4, Lu-Sheng Wang2,3, Jie Huang7, Ji-Hong Wu5,6, and Fang Chen3,4

1 BGIEducationCenter, UniversityofChineseAcademyofSciences, Shenzhen, China
2 Department of Computer Science, City University of Hong Kong, Kowloon, Hong Kong
3 BGI-Shenzhen, Shenzhen, China
4 MGI, BGI-Shenzhen, Shenzhen, China
5 Eye Institute,Eye, Ear, Nose and Throat Hospital, College of Medicine, Fudan University, Shanghai, China
6 Shanghai Key Laboratory of Visual Impairment and Restoration, Science and Technology Commission of Shanghai Municipality, Shanghai, China
7 National Institutes for Food and Drug Control, Tiantan Xili Dongcheng District, Beijing, China

Correspondence: Fang Chen, BGI-Shenzhen, Shenzhen 518083, China. e-mail: fangchen@genomics.cn
Ji-Hong Wu, Eye Institute, Eye and ENT Hospital, College of Medicine, Fudan University, Shanghai, China. e-mail: jihongwu@fudan.edu.cn
Jie Huang, National Institutes for Food and Drug Control, No. 2, Tiantan Xili Dongcheng District, Beijing 100050, China. e-mail: jhuang5522@126.com

Purpose: Leber hereditary optic neuropathy (LHON) and autosomal dominant optic atrophy (ADOA) are the two commonest forms of hereditary optic neuropathy. The aim of this study was to comprehensively investigate the incidence and spectrum of mutations in patients with suspected hereditary optic neuropathy by combining mitochondrial DNA (mtDNA) genome-wide and targeted exon sequencing.

Methods: A cohort of 1101 subjects were recruited to participate in the study, comprising 177 families (177 probands and their family members, a total of 537 subjects, including 254 patients) and 164 sporadic cases with suspected hereditary optic neuropathy, and 400 unrelated control subjects for genetic analysis: all subjects (including control subjects) underwent a comprehensive ophthalmologic examination and were subjected to sequencing analysis of mtDNA genome-wide and targeted exon. Overall, targeted exon sequencing was used to screen 792 genes associated with common hereditary eye diseases, and the mtDNA genome-wide were screened by next-generation sequencing.

Results: We found variants detected in 168 (40.2%, 168/418) of the 418 patients screened. Among these, 132 cases (78.6%, 132/168) were detected with known LHON disease-causing mtDNA variants; 40 cases (23.8%, 40/168) were detected with nuclear DNA (ntDNA) variants, which included 36 cases (21.4%, 36/168) with detected OPA1 mutations, 4 patients (2.4%, 4/168) with detected OPA3 mutations, and 2 patients (1.2%, 2/168) with detected TMEM126A homozygous mutation. Coexistence variation (mtDNA/mtDNA [n = 16], ntDNA/ntDNA [n = 4], mtDNA/ntDNA [n = 7]) was found in 27 patients (16.4%, 27/165), including mtDNA/ntDNA coexistence variation that was detected in seven patients. Among these ntDNA mutations, 38 distinct disease-causing variants, including autosomal recessive heterozygous mutations, were detected, which included 22 novel variants and two de novo variants. Total haplogroup distribution showed that 34.5% (29/84) and 28.6% (24/84) of the affected subjects with m.11778G>A belonged to haplogroup D and M, with a high frequency of subhaplogroups D4, D5, and M7.

Conclusions: The LHON-mtDNA mutations are the commonest genetic defects in this Chinese cohort, followed by the OPA1 mutations. To our knowledge, this is the first comprehensive study of LHON, ADOA, and autosomal recessive optic atrophy combined with mtDNA genome-wide and targeted exon sequencing, as well as haplogroup...
analysis, in a large cohort of Chinese patients with suspected hereditary optic neuropathy. Our findings provide a powerful basis for genetic counseling in patients with suspected hereditary optic neuropathy.

**Translational Relevance:** We applied mtDNA genome-wide sequencing combined with panel-based targeted exon sequencing to explore the pathogenic variation spectrum and genetic characteristics of patients with suspected hereditary optic neuropathy, providing a comprehensive research strategy for clinical assistant diagnosis, treatment, and genetic counseling.

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**Introduction**

Hereditary optic neuropathy is a group of hereditary heterogeneous diseases involving function change in the optic nerve and retinal ganglion cells (RGC), which causes variable vision decline. There are several modes of inheritance for these disorders, including Leber hereditary optic neuropathy (LHON, OMIM535000) and autosomal dominant optic atrophy (ADOA, OMIM165500), which are the two commonest forms of these disorders caused by mutations in mitochondrial DNA (mtDNA) and nuclear DNA (ntDNA). Autosomal recessive optic atrophy (AROA) is a rare mode of inheritance, and several reports of this recessive form have been linked to TMEM126A (OMIM612988), RTN4IP1 (OMIM610502), ACO2 (OMIM100850), and YME1L1 (OMIM617302) genes.5–7,22

LHON is the commonest type of maternal hereditary disorder caused by structural and functional abnormalities of mtDNA, with an estimated prevalence of 1:50000 worldwide.8,9 It was first described by Theodor Leber as a maternally hereditary disorder of optic nerve degeneration that is usually occurring in patients between the age of 18 and 35, with acute or subacute visual loss of the central region and preferentially affects men.10,11 LHON is a heterogeneous disorder caused by mtDNA mutations. The composition of mtDNA includes 13 protein-coding genes, 2 ribosomal RNAs, and 22 tRNAs. Over 90% of LHON is caused by one of three primary disease-causing variants, m.3460G>A, m.11778G>A, and m.14484T>C, which involve the MT-ND1, MT-ND4, and MT-ND6 gene encoding three subunits of the respiratory chain complex I, respectively.8,11,12

Secondary mutations (such as MT-ND1 m.3394T>C and MT-ND4 m.11696G>A) often interact with primary mutations to affect the phenotypic expression of LHON, which is present in the LHON family, but at the same time appears in the control subjects at a lower frequency than LHON patients.13,14 These mtDNA mutations associated with LHON occurred in heterogeneous (mixed wild-type and mutant) or homogenous (mutant-only) forms.

ADOA is a disorder characterized by degenerative optic nerve fibers, mainly involving RGCs and axons of optic nerve formation. The estimated prevalence of the disease ranges from 1:12000 to 1:50000, characterized by early-onset loss of bilateral vision, defects in color vision, and typical temporal pale optic nerve.15 Although several genes have been reported to be associated with ADOA, most studies are still associated with genetic mutations in OPA1 (OMIM605290) and OPA3 (OMIM606580) gene encoding mitochondrial proteins, most of which are anchored to the mitochondrial inner membrane and are ubiquitously expressed.16–18 Previous studies of patients with ADOA have shown that most are caused by mutations in the OPA1 gene, accounting for approximately 60% to 80%. The OPA1 gene encodes mitochondrial proteins located in the mitochondrial inner membrane and regulates the stability of the mitochondrial network, mitochondrial bioenergetic output, and isolation of proapoptotic cytochrome c oxidase molecules in the mitochondrial cristae spaces, and more than 300 mutations have been detected in the previous reports.15,19–21

AROA is a rare disease that affects RGCs and the nervous system. To date, it has been reported that this form of AROA is related to 4 causative genes, including 3 variants detected in the transmembrane protein 126A gene (TMEM126A, OMIM612988) in 8 families, 14 variants detected in the reticulon 4-interacting protein 1 gene (RTN4IP1, OMIM610502) in 17 families, 7 variants detected in the aconitase 2 gene (ACO2, OMIM100850), and 1 variant detected in the YME1L1-like 1 ATPase gene (YME1L1, OMIM617302) in 1 family.5,7,22–24 There are overlapping clinical and pathological features in almost all affected patients, in which RGCs are prone to mitochondrial dysfunction.25 Among these causative genes, the TMEM126A gene has been reported to be specifically associated with nonsyndromic AROA. The TMEM126A gene spans a genomic region of 8.5kb and consists of five exons, and it has been mapped to chromosome 11q14.1-q21.
within a critical interval associated with optic atrophy identified by homozygosity mapping. In contrast to the \( OPA1 \) (OMIM605290) mutations, Hanein et al.\(^5\) found no fragmentation of the mitochondrial network and/or depletion of the mtDNA in patient fibroblasts caused by the \( TMEM26A \) mutation, which indicates that there may be no functional correlation between \( TMEM26A \) and \( OPA1 \). Thus far, three variants of the \( TMEM26A \) gene have been associated with AROA, including a recurrent R55X homozygous nonsense mutation that has been detected in multiple families. Among these families includes four families with autosomal recessive nonsyndromic optic atrophy and one family with optic atrophy combined auditory neuropathy, which suggestion that auditory neuropathy might be a key feature of \( TMEM126A \) associated optic neuropathy.\(^5,26\)

Despite several previous studies that have described the genetic and clinical features of hereditary optic neuropathy, the overall status of LHON, ADOA, and AROA mutations remains to be determined in Chinese patients with suspected hereditary optic neuropathy. In this study, we report the results of a comprehensive molecular analysis of 418 patients with suspected hereditary optic neuropathy and detected molecular variability in nuclear and mitochondrial genomes based on next-generation sequencing.

**Materials and Methods**

**Subjects and Ethics Statement**

A cohort of 1101 subjects, including 177 families (177 probands and their relatives) and 164 sporadic subjects with suspected hereditary optic neuropathy (total patients: 418; total probands: 341) and 400 unrelated control subjects were recruited at the Eye and ENT Hospital of Fudan University from February 2016 to June 2019. The study was permitted by the ethics committee of the Eye and ENT Hospital of Fudan University and adhered to the tenets of the Declaration of Helsinki. Individuals participating in the study signed informed consent.

**Clinical Assessment**

The clinical criteria for the inclusion of patients in this study included a decrease in central vision unrelated to refraction, and a different degree of optic nerve atrophy observed through fundus examination. We eliminated all known causes, such as oppression, inflammation, glaucoma, and drug-induced optic nerve atrophy. All participants in the study underwent complete ophthalmologic examinations, comprising best-corrected visual acuity (BCVA) measurement, slit-lamp biomicroscopy examination, intraocular pressure detection (IOP, Goldmann tonometry; Haag-Streit, UK), full-field electroretinography, fundus photograph (Canon CR6-45NM fundus camera; Canon, Tokyo, Japan), and swept-domain optical coherence tomography (Heidelberg Engineering Inc., Heidelberg, Germany). In addition, information associated with the disease, including family history, age of onset, and decreased subjective visual acuity, were collected. The diagnosis of suspected hereditary optic neuropathy was assessed by professional neuroophthalmologists.

**Targeted Exon Sequencing and Next-Generation Sequencing**

Peripheral blood samples from all participants were collected at the Eye and ENT Hospital of Fudan University. The mtDNA and ntDNA were extracted from whole blood using the MGIEasy Mitochondrial Whole Genome Amplification Kit (MGI, Inc., Shenzhen, China) and FlexiGene DNA Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocols, respectively. In this study, customized panel-based next-generation sequencing (NGS) and mtDNA genome-wide sequencing were performed for all participants. We collaborated with MGI-Shenzhen (Shenzhen, Guangdong, China) to design the Target_Eye_792_V2 chip, which has exon capture and untranslated regions of 792 genes associated with common hereditary eye diseases (Supplementary Table S1).\(^27,28\) On average, the mean coverage depth was more than 300X for ntDNA, the mean coverage depth was more than 30X for mtDNA, and the coverage of target region was close to 99.9% using the MGISEQ-2000 (DNBSEQ-G400) platform (MGI, Inc., Shenzhen, China).

**Bioinformatics Analysis**

The target exon sequencing reads are aligned to the reference human genome (USCS hg38) using the Burrows–Wheeler Aligner (BWA) version 0.7.10 (for more information, see github.com/lh3/bwa). All the mutations detected by sequencing were annotated using the 1000 Genomes Project (http://1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), ExAC (http://exac.broadinstitute.org), and ESP6500 (http://evs.gs.washington.edu/EVS/) databases, screening for mutations with minor allele frequency <0.1% to detect possible deleterious variations. In addition, four online tools, Sorting Intolerant from Tolerant (http://sift.jcvi.org/), MutationTaster
Figure 1. Basic information of clinical presentation and genetic finding of the mtDNA in the patients. (A) The age distribution of the total patients, including age ≤10 years (n = 99), 10 to 20 years (n = 86), 20 to 30 years (n = 78), 30 to 40 years (n = 67), and >40 years (n = 88). (B) The patients received a confirmed genetics diagnosis in this cohort, including mtDNA (n = 132), OPA1 (n = 36), OPA3 (n = 4), and homozygous TMEM126A (n = 2) variants. (C) Thirty-eight different pathogenic/likely pathogenic nuclear variants were detected in this cohort, including OPA1 gene (n = 30), TMEM126A gene (n = 4), RTN4IP1 gene (n = 3), and OPA3 gene (n = 1). (D) Thirty different OPA1 pathogenic/likely pathogenic variants were identified, including missense (n = 11), splicing (n = 7), nonsense (n = 5), frameshift (n = 5), and intron (n = 2) variants. (E, F) Multidimensional comparison between detection of variability in patients and age distribution.

software (http://www.mutationtaster.org/), FATHMM software (http://fathmm.biocompute.org.uk/), and LRT (http://www.genetics.wustl.edu/jflab/) were used to predict potential pathogenic variants. Then, the Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/), HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), and Online Mendelian Inheritance in Man (OMIM, http://www.omim.org/) were used to interpret and prioritize the variants, combined with potential deleterious impacts, genotype-phenotype relationships, and variants report. According to the American College of Medical Genetics (ACMG) and genomics guidelines, variants were classified into five categories, including pathogenic, likely pathogenic, novel variants of uncertain clinical significance, likely benign, and benign. Sanger sequencing was performed to confirm the candidate variants.29,30

The mtDNA genome-wide sequencing results were compared with the published mtDNA sequences (GenBank Accession No. NC_012920), and the mtDNA haplogroup status and potential pathogenic variation were both determined. The “Top 19” primary LHON mutations reported by MITOMAP (https://www.mitomap.org/ioswiki/bin/view/MITOMAP/MutationsLHON) in the literature were screened in all subjects, as well as 19 other candidate LHON mutations and 21 secondary LHON mutations. Conservatism of mitochondrial genome variation was obtained by analysis using ClustalX (see http://www.clustal.org/clustal2/).

Results

Clinical Presentation and Genetic Findings

In this cohort, a total of 418 patients with hereditary optic neuropathy were recruited in the study, of whom most were unrelated probands (341/418). The average age of the patients was 25.7 ± 16.5 years (range, 0.83–66; median, 23), most of whom were under 30 years old, accounting for (63%; 263/418) of the total number of patients (Fig. 1A), and all participants were from all over the Chinese region. Among these patients, 263 were men and 155 were women, with an average age of 23.9 ± 15.0 years and 28.6 ± 18.3 years, respectively (Table 1). The average ages of the two groups of dominant optic atrophy and LHON patients were calculated, and the results showed that...
Table 1. The Demography and the Genetic Screening Results of this Study

<table>
<thead>
<tr>
<th>Patients (%)</th>
<th>Total (Families/Sporadic)</th>
<th>Male (Families/Sporadic)</th>
<th>Female (Families/Sporadic)</th>
<th>Ratio (Male to Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total screened (100%)</td>
<td>418 (254/164)</td>
<td>263 (154/109)</td>
<td>155 (100/55)</td>
<td>1.7:1</td>
</tr>
<tr>
<td>LHON mutation (31.6%, 132/418)</td>
<td>132 (84/48)</td>
<td>90 (55/35)</td>
<td>42 (29/13)</td>
<td>2.1:1</td>
</tr>
<tr>
<td>OPA1 mutation (8.6%, 36/418)</td>
<td>36 (30/6)</td>
<td>22 (18/4)</td>
<td>14 (12/2)</td>
<td>1.6:1</td>
</tr>
<tr>
<td>OPA3 mutation (1.0%, 4/418)</td>
<td>4 (3/1)</td>
<td>3 (3/0)</td>
<td>1 (0/1)</td>
<td>3.0:1</td>
</tr>
<tr>
<td>TMEM126A mutation (0.5%, 2/418)</td>
<td>2 (2/0)</td>
<td>1 (1/0)</td>
<td>1 (1/0)</td>
<td>1.0:1</td>
</tr>
<tr>
<td>Total detected (40.2%, 168/418)</td>
<td>168 (113/55)</td>
<td>111 (72/39)</td>
<td>57 (41/16)</td>
<td>2.0:1</td>
</tr>
</tbody>
</table>

the age of onset of dominant optic atrophy in patients was younger than with LHON, with an average age of 19.8 ± 14.9 (range, 3–54; median, 12) years and 27.1 ± 15.7 (range, 1–66; median, 25) years, respectively. In addition, 400 unrelated control subjects were included from different regions of China, with an average age of 48.5 ± 15.0 (range, 22–92; median, 48) years, who showed no abnormalities through physical, neurologic, and ophthalmic examinations.

Overall, a total of 168 patients (40.2%; 168/418) received confirmed mtDNA or ntDNA variants, and the percentage of patients diagnosed with confirmed genetic variants in the families (44.5%; 113/254) was significantly higher than that of sporadic patients (33.5%; 55/164) (Table 1). As shown in Figure 1B, a total of 132 individuals (76%; 132/174) were detected with LHON-mtDNA mutations, 36 individuals were detected with OPA1 gene mutations, 4 individuals were detected with OPA3 gene mutations, and 2 patients were detected with homozygous TMEM126A gene mutations. We performed a multidimensional comparison between the type of variation and the age distribution of the patients examined. The results showed that the detection rate of mtDNA mutations was almost the same in patients of different ages, but OPA1 mutations were mainly concentrated in children under 10 years of age (Figs. 1E, 1F). Coexisting variants were detected in 27 patients (16.1%; 27/168), including 16 mtDNA/mtDNA variants (59.2%; 16/27), 4 ntDNA/ntDNA variants (14.8%, 4/27), and 7 mtDNA/ntDNA variants (25.9%, 7/27). Among these coexisting variations, the commonest form was ND4 m.11778G>A+ND6 m.14502T>C, followed by ND4 m.11778G>A+ND1 m.3394T>C, or ND6 m.14484T>C+ND1 m.3394T>C, all three forms have been reported.

mtDNA LHON Mutations

As shown in Table 1, LHON-mtDNA mutations were detected in 132 patients (31.6%, 132/418), of which 84 were families subjects (63.6%, 84/132) and 48 were sporadic subjects (36.3%, 48/132); the ratio of male to female was 2:1. Molecular and clinical features of LHON-positive individuals identified in the study are shown in Table 2. The top 3 primary LHON disease-causing mutation were detected in 96 patients, of which 84 patients detected m.11778G>A mutation, 7 patients detected m.3460G>A mutation, and 5 patients detected m.14484T>C mutation. In addition, 11 other candidate LHON disease-causing mutations were found in 52 patients, and 5 putative LHON mutations were detected in 40 patients. The primary top 3 LHON mutations accounted for 72.7% (96/132) of the patients with LHON mutations, and 16 mtDNA/mtDNA coexisting mutations accounted for 12.1% (16/132) (Table 3).

The m.11778G>A mutation was detected in 114 individuals, including 84 patients and 30 unaffected family members. The affected individuals presented bilateral painless visual impairment along with scotomas (central or cecocentral) and pale optic disc. Family members who harbor the primary m.11778G>A mutation were considered as unaffected individuals when they did not show any symptoms of visual impairment at the time of sample collection. Among these patients who harbor the primary m.11778G>A mutation, 56 patients were male (66.7%, 56/84) and 28 were female (33.3, 28/84); the ratio of male to female was 2:1. When compared with the females, the penetrance of the disease was much higher in males, with 91.8% of males (56/61) who carried the primary m.11778G>A mutation developing blindness, whereas 52.8% of females (28/53) presented with loss of vision. The observation was consistence with previous European and Chinese studies, indicating that gender factors have a strong impact on the increased risk of visual impairment in males compared with females who harbor the m.11778G>A mutation in Chinese cohort.31,32

OPA1 and OPA3 Mutations

Thirty-eight distinct pathogenic/likely pathogenic nuclear variants were detected in this cohort.
Table 2. Molecular and Clinical Features of LHON-Positive Patients Identified in this Study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Conservation Index</th>
<th>N of 418 Patients</th>
<th>N of 400 Controls</th>
<th>Clinvar</th>
<th>Polymorphism Phenotyping</th>
<th>Clinical Significance</th>
<th>Age of Diagnosed Mean ± Standard Deviation (Range; Median)</th>
<th>The Most Common Haplogroup (Num; %)</th>
<th>Previous Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND4</td>
<td>11778G&gt;A</td>
<td>1</td>
<td>84</td>
<td>2</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Pathogenic</td>
<td>28.7 ± 16.0 (0.83–66; 25.5)</td>
<td>D4 (20; 23.8%); M7 (12; 14.3%)</td>
<td>Yes</td>
</tr>
<tr>
<td>ND1</td>
<td>3460G&gt;A</td>
<td>0.9111</td>
<td>7</td>
<td>0</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Pathogenic</td>
<td>21.6 ± 11.0 (8–36; 23)</td>
<td>F1 (3; 42.9%); M7 (2; 28.6%)</td>
<td>Yes</td>
</tr>
<tr>
<td>ND6</td>
<td>14484T&gt;C</td>
<td>0.83</td>
<td>5</td>
<td>0</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Pathogenic</td>
<td>36.8 ± 17.5 (12–55; 40)</td>
<td>M9 (2; 40%)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total of the “top 3” primary LHON-disease causing mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND1</td>
<td>3394T&gt;C</td>
<td>0.9333</td>
<td>8</td>
<td>2</td>
<td>Pathogenic</td>
<td>Benign</td>
<td>Risk factors; Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
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<tr>
<td>ND1</td>
<td>3635G&gt;A</td>
<td>0.9333</td>
<td>2</td>
<td>0</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>ND1</td>
<td>4136A&gt;G</td>
<td>0.9778</td>
<td>2</td>
<td>0</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>ND3</td>
<td>10197G&gt;A</td>
<td>0.9556</td>
<td>1</td>
<td>0</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>ND4</td>
<td>10680G&gt;A</td>
<td>0.9333</td>
<td>1</td>
<td>0</td>
<td>Pathogenic</td>
<td>Benign</td>
<td>Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
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<tr>
<td>ND4</td>
<td>11253T&gt;C</td>
<td>0.4222</td>
<td>1</td>
<td>0</td>
<td>Pathogenic</td>
<td>Benign</td>
<td>Pathogenic</td>
<td>–</td>
<td>–</td>
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<tr>
<td>ND4</td>
<td>11696G&gt;A</td>
<td>0.25</td>
<td>2</td>
<td>0</td>
<td>Pathogenic</td>
<td>Benign</td>
<td>Pathogenic</td>
<td>–</td>
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<tr>
<td>ND5</td>
<td>12338T&gt;C</td>
<td>0.83</td>
<td>16</td>
<td>2</td>
<td>Pathogenic</td>
<td>Unknown</td>
<td>Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
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</table>
Table 2. Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Conservation Index</th>
<th>N of 418 Patients</th>
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<th>The Most Common Haplogroup (Num; %)</th>
<th>Previous Report</th>
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<tr>
<td>ND6</td>
<td>14502T&gt;C</td>
<td>0.7778</td>
<td>11</td>
<td>1</td>
<td>Pathogenic</td>
<td>Benign</td>
<td>Risk factors; Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>COX3</td>
<td>9438G&gt;A</td>
<td>0.9333</td>
<td>1</td>
<td>0</td>
<td>Pathogenic</td>
<td>–</td>
<td>Risk factors; Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>tRNA-Thr</td>
<td>15951A&gt;G</td>
<td>0.78</td>
<td>7</td>
<td>0</td>
<td>Pathogenic</td>
<td>Probably damaging</td>
<td>Risk factors; Pathogenic</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Total of the other candidate LHON disease-causing mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Conservation Index</th>
<th>N of 418 Patients</th>
<th>N of 400 Controls</th>
<th>Clinvar</th>
<th>Polymorphism Phenotyping</th>
<th>Clinical Significance</th>
<th>Age of Diagnosed Mean ± Standard Deviation (Range; Median)</th>
<th>The Most Common Haplogroup (Num; %)</th>
<th>Previous Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND1</td>
<td>4216T&gt;C</td>
<td>0.2444</td>
<td>4</td>
<td>3</td>
<td>Conflicting of pathogenicity</td>
<td>Benign</td>
<td>Risk factors</td>
<td>24.5 ± 14.8 (3–55; 22)</td>
<td>M10 (11; 21.2%); F2 (11; 21.2%)</td>
<td>Yes</td>
</tr>
<tr>
<td>ND2</td>
<td>4917A&gt;G</td>
<td>0.9111</td>
<td>1</td>
<td>2</td>
<td>Uncertain significance</td>
<td>Benign</td>
<td>Risk factors</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>ND5</td>
<td>12811T&gt;C</td>
<td>0.5556</td>
<td>16</td>
<td>17</td>
<td>Uncertain significance</td>
<td>Benign</td>
<td>Risk factors</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>ND5</td>
<td>13708G&gt;A</td>
<td>0.3333</td>
<td>17</td>
<td>17</td>
<td>Conflicting of pathogenicity</td>
<td>Benign</td>
<td>Risk factors</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>COX3</td>
<td>9804G&gt;A</td>
<td>0.9333</td>
<td>2</td>
<td>1</td>
<td>Conflicting of pathogenicity</td>
<td>–</td>
<td>Risk factors</td>
<td>–</td>
<td>–</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Total of the putative LHON mutation.
Table 3. Coexistence Variation Identified in the Patients

<table>
<thead>
<tr>
<th>Mutation</th>
<th>N of 418 Patients (%)</th>
<th>Age of Diagnosed Mean ± Standard Deviation (Range; Median)</th>
<th>Haplogroup (num; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND4 m.11778G&gt;A+ND4 m.11696G&gt;A</td>
<td>1</td>
<td>25</td>
<td>D4j</td>
</tr>
<tr>
<td>ND4 m.11778G&gt;A+ND6 m.14502T&gt;C</td>
<td>5</td>
<td>30.8 ± 17.6 (9–54; 30)</td>
<td>M10a1</td>
</tr>
<tr>
<td>ND4 m.11778G&gt;A+rrRNA-Thr m.15951A&gt;G</td>
<td>3</td>
<td>19.3 ± 15.5 (8–37; 13)</td>
<td>D4h</td>
</tr>
<tr>
<td>ND4 m.11778G&gt;A+ND4 m.11696G&gt;A</td>
<td>3</td>
<td>26 ± 14.8 (16–43; 19)</td>
<td>M9a1a1 (2); M7b1a1 (1)</td>
</tr>
<tr>
<td>ND6 m.14484T&gt;C+ND1 m.3394T&gt;C</td>
<td>5</td>
<td>55</td>
<td>N9a7</td>
</tr>
<tr>
<td>ND4 m.11778G&gt;A+ND4 m.11696G&gt;A</td>
<td>1</td>
<td>25</td>
<td>M7/9/10 (11; 68.8%); D4 (4; 25%); N9 (1; 6.2%)</td>
</tr>
<tr>
<td>Coexistence variation of mtDNA/mtDNA</td>
<td>16(3.8%)</td>
<td>28.7 ± 16.1 (8–55; 26)</td>
<td></td>
</tr>
<tr>
<td>OPA1 c.1682-1G&gt;A+OPA3 c.123C&gt;G</td>
<td>2</td>
<td>13 ± 4.2 (10–16; 13)</td>
<td>D4a (1); B5a2a2 (1)</td>
</tr>
<tr>
<td>OPA1 c.1471dup(p.ile491Asnfs*9) /c.1472T&gt;A+TMEM126A c.236C&gt;G,Het</td>
<td>2</td>
<td>25 ± 19.8 (11–39; 25)</td>
<td>B4h1</td>
</tr>
<tr>
<td>Coexistence variation of mtDNA/ntDNA</td>
<td>4 (1%)</td>
<td>19 ± 13.6 (10–39; 13.5)</td>
<td>B (3; 75%); D4 (1; 25%)</td>
</tr>
<tr>
<td>ND1 m.12338T&gt;C+OPA1 c.2203G&gt;T</td>
<td>2</td>
<td>29 ± 26.9 (10–48; 29)</td>
<td>F2a1 (1); H5g (1)</td>
</tr>
<tr>
<td>ND1 m.12338T&gt;C+RTN4IP1 c.308G&gt;A, Het</td>
<td>1</td>
<td>10</td>
<td>F2</td>
</tr>
<tr>
<td>ND1 m.4136A&gt;G+OPA1 c.1835_1836delGA</td>
<td>1</td>
<td>4</td>
<td>G2a1</td>
</tr>
<tr>
<td>ND4 m.10680G&gt;A+OPA1 c.2661+1G&gt;A</td>
<td>1</td>
<td>5</td>
<td>A17</td>
</tr>
<tr>
<td>ND4 m.11778G&gt;A+TMEM126A c.163C&gt;T,Het</td>
<td>1</td>
<td>16</td>
<td>D5a2a</td>
</tr>
<tr>
<td>ND4 m.11253T&gt;C+RTN4IP1 c.1015C&gt;T,Het</td>
<td>1</td>
<td>38</td>
<td>G1</td>
</tr>
<tr>
<td>Coexistence variation of ntDNA/ntDNA</td>
<td>7(1.7%)</td>
<td>18.7 ± 17.3 (4–48; 10)</td>
<td>F2 (2; 28.6); G (2; 28.6); D (1; 14.3%); A (1; 14.3%); H (1; 14.3%)</td>
</tr>
<tr>
<td>Total patients of coexistence variation</td>
<td>27(6.5%)</td>
<td>24.7 ± 16.3 (4–55; 19)</td>
<td>Top 3 haplogroup: M10a (5; 18.5%); M9a (5; 18.5%); D4 (5; 18.5%)</td>
</tr>
</tbody>
</table>

(Supplementary Table S2), including OPA1 gene (n = 30, 79%), TMEM126A gene (n = 4, 10%), RTN4IP1 gene (n = 3, 8%), and OPA3 gene (n = 1, 3%) (Fig. 1C). We identified 30 distinct pathogenic/likely pathogenic variants of the OPA1 gene in 8.6% (36/418) of patients, including missense (n = 11, 36%), splicing (n = 7, 23%), nonsense (n = 5, 17%), frameshift (n = 5, 17%), and intron (n = 2, 7%) variants (Fig. 1D). Among the 30 OPA1 mutations, we classified 16 variants as novel variants because these had not been registered in the Human Gene Mutation Database or Clinvar database and have not been reported in the literature. In addition, when the variants were not detected in the patient’s biological parents, we defined these as de novo variants. In this cohort, two de novo missense variants were detected in the OPA1 gene (1.43%, n = 2/140 families of trios), c.320C>A (p.S107X), and c.1499G>A (p.R500H) were detected in unrelated patients, respectively.

One recurrent OPA3 mutation was detected in four (1.0%) of the 418 patients screened. Of these four patients, two of them were from the same family (F74), and the other two were from independent families (F159, F305). The variant c.123C>G (p.I41M) was reported to be detected in a 12-year-old boy with a BCVA of 0.1. Ophthalmoscopy examination
revealed bilateral optic disc pallor and diffuse defects in the retinal nerve fiber layer (RNFL). His twin brothers also harbors the same mutation, as well as visual acuity defects and optic atrophy.\(^{33}\) Moreover, the clinical significance of these variants was defined according to ACMG and genomics guidelines; the bioinformatics tools analysis results of the 17 missense variants are demonstrated in Supplementary Figure S1.

**Figure 2.** Detection and evaluation of mutations in the *TMEM126A* gene. (A) Multiple sequence alignment of candidate missense mutations from different species to explore the conservation of these mutations. The red arrow represents variants states. (B) SWISS-MODEL was used to predict the three-dimensional structure of both the mutant and wild-type proteins. (C) Pedigrees of the families with mutations. Squares indicate men and circles women; black and white symbols represent affected and unaffected individuals, respectively. The proband is marked with an arrow, and the asterisks indicate those members enrolled in this study.

**TMEM126A and RTN4IP1 Mutations**

One missense mutation (c.107C>T, p.S36L) of the *TMEM126A* gene was identified in five members of family 98 (F98), the probands and his affected biological brother harbor homozygous state, and his parents and sister harbor heterozygous state (Fig. 2C). This missense variant affects a highly evolutionarily conserved region (Fig. 2A) and changes the amino acid
from serine to leucine, and the variant segregates with the disease within the family. The SWISS-MODEL was used to predict the three-dimensional structure of this amino acid in the mutant and wild-type proteins (Fig. 2B), and we observed a difference between the mutated and wild-type structures of transmembrane protein 126A. There have not been any other LHON-mtDNA and ADOA and AROA genes detected in the two affected subjects of this family. Studies have reported that the two affected siblings from Iraqi descent detected the p.S36L variant and categorized the variant as unclear significance. However, according to the ACMG and genomics guidelines, the p.S36L variant is cosegregated with the disease in the family and affects evolutionary conserved amino acid residues. Therefore it should be categorized as pathogenic or likely pathogenic variants, so we regard it as a candidate causative factor for the patients with optic atrophy in this family.

Moreover, we detected three additional heterozygous states of the TMEM126A gene in affected and unaffected individuals (c.163C>T, p.Arg55Ter; c.62T>G, p.Ile21Ser; c.236C>G, p.Thr79Arg), including the detection of a recurrent nonsense mutation (p.Arg55Ter) in three individuals of a family (F147). Among the three mutation carriers, the proband was accompanied by an acute onset of optic neuropathy, and the other two carriers were asymptomatic fathers and aunts. Recurrent nonsense mutations (p.Arg55Ter) detected in the proband were inherited from his father. At the same time, LHON-mtDNA mutation m.11778G>A was also detected in the proband and his four family members, and the proband’s mtDNA mutation was inherited from his mother (Fig. 2C). The proband showed more severe clinical manifestations than the infected mother, and the onset age was earlier (acute onset at the age of 16 years), and the vision dropped sharply in early childhood. However, the affected mother did not have a similar history of the disease; she was late onset and vision decrease was mild. The effect of the coexistence of mtDNA variants (m.11778G>A) and TMEM126A heterozygous variants (c.163C>T, p.Arg55Ter) on clinical manifestation is unknown, and its underlying mechanism remains to be explored.

In three families with early-onset recessive optic neuropathy, we identified three heterozygous missense states in the RTN4IP1 gene (c.1015C>T, p.R339C; c.308G>A, p.R103H; c.962G>C, p.G321A), which encodes a mitochondrial ubiquinol oxydo-reductase. Among these three mutations, two of them were novel heterozygous mutations for the c.1015C>T and c.962G>C substitution on a different haplotype F and C7 (F120 and F148), another one is a heterozygous variant of haplotype F (F8), which has been reported to detect homoyzgy and heterozygous variation of the mutation in four families.

### Haplogroup Distribution

Our comprehensive study of Chinese LHON patients confirmed that LHON individuals with m.11778G>A variants had different mtDNA haplotypes, which is different from previously reported observations that preferentially associate with Western European continent haplogroup J and increase clinical penetrance of the J2 subhaplogroup, indicating their possible influence on clinical expression. Comprehensive haplogroup analysis showed that 34.5% (29/84) and 28.6% (24/84) of the affected subjects belonged to haplogroup D and M, with a high frequency of subhaplogroups D4, D5, and M7 (Supplementary Table S3). These data suggest that such haplotypes are more likely to produce similar clinical manifestations in East Asian populations, and the same situation may exist in other mitochondrial diseases among Chinese populations. Moreover, we performed mitochondrial haplogroup comparison between the 400 control subjects and 418 patients, the result indicating that there is no difference between these two cohorts (Supplementary Table S4).

### Discussion

To our knowledge, this is the first study screening for LHON, ADOA, and AROA in a large cohort of Chinese patients with suspected hereditary optic neuropathy. Comprehensive molecular screening for patients with suspected hereditary optic neuropathy might contribute to the high overall mutation detection rate (40.2%, 168/418). Our results indicate that LHON-mtDNA mutations are definitely the most common among Chinese populations, and the same situation may exist in other mitochondrial diseases among Chinese populations. Moreover, we performed mitochondrial haplogroup comparison between the 400 control subjects and 418 patients, the result indicating that there is no difference between these two cohorts (Supplementary Table S4).
population is due to different ethnic backgrounds, and the difference in the detection rate between studies based on the Chinese population is due to different entry criteria. Our enrollment included a broader group of patients with suspected optic neuropathy, which may lead to a low detection rate, but the results show that mtDNA or OPA1 mutations are indeed detected in some patients with clinical symptoms that are not obvious. In clinical applications, a comprehensive examination helps us to diagnose patients with suspected optic neuropathy. The prevalence of LHON-mtDNA and OPA1, OPA3 mutations was rare in Chinese cohort, OPAL and we only detected one missense variant c.123C

gene mutations were rare in Chinese cohort study, with several previous reports, of all identified LHON-mtDNA mutations. Consistent with LHON from 903 Chinese families. G11778A, T14484C, and G3460A mutations were detected in 312 (90.2%), 30, and 4 probands, respectively.36,37 However, the top three LHON-mtDNA mutations were detected in 28.2% (96/341) of the screened patients who were suspected to harbor m.11696A>G, m.14484T>C, and m.11778G>A mutations. Compared with the mother who only detected m.11778G>A and c.163C>T mutations, the probands who detected m.11778G>A and c.163C>T mutations showed an earlier acute onset.

### Conclusions

LHON-mtDNA mutations are the commonest genetic defects in this Chinese cohort with suspected hereditary optic neuropathy, followed by the OPA1 and OPA3 mutations with ADOA, and TMEM126A mutations with the rare AROA. Comprehensive molecular screening for patients with suspected hereditary optic neuropathy is essential to assist in diagnosing the patient’s disease categories and customize molecular therapies. Three forms of coexisting variation may have potential effects on disease manifestation and disease progression, a task that requires further exploration and in-depth research.

### Acknowledgments

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mtDNA Combined ntDNA Screening for LHON

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FC, J-HW, JC, KL, WL, and F-JG conceived and designed this study. FJG, FYH, SHZ, and JHW recruited patients, performed clinical examinations and interpretation. FJG, FYH, SHZ, JHW, S-FQ, JC, LL, and L-SW collected the clinical samples and clinical data. WL, J-KL, FC, YQ, and Z-WW analyzed the sequencing data. J-KL, WL, and F-JG wrote and revised the manuscript.

Data availability: The data that support the findings of this study have been deposited in the CNSA (https://db.cnbg.org/cnsa/) of CNGBdb with accession number CNP0000453. The datasets are available from the corresponding author on reasonable request.

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* J-KL, WL, and F-JG contributed equally as first authors.

References

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