Mutation Screening of Mitochondrial DNA as Well as OPA1 and OPA3 in a Chinese Cohort With Suspected Hereditary Optic Atrophy

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PURPOSE. Leber’s hereditary optic atrophy (LHON) and autosomal dominant optic atrophy (DOA) are the two most common forms. The objective of this study was to define the fractional prevalence of LHON and DOA in a cohort of Chinese patients with suspected hereditary optic neuropathy.

METHODS. We recruited 520 unrelated patients with bilateral optic atrophy for genetic analysis: 174 patients had a positive family history of visual failure and 346 were sporadic cases. A total of 14 primary LHON-causing mtDNA mutations were screened by PCR-based sequencing methods for all patients except the individuals with a paternal family history. All coding exons and exon-intron boundaries of the OPA1 and OPA3 gene were screened for mutations by PCR-based DNA sequencing for all patients with paternal family history and for the LHON-negative patients. A large genomic DNA arrangement of the OPA1 gene was detected further by multiplex ligation probe amplification (MLPA) assay for the patients with paternal family history, but results were negative for the OPA1 and OPA3 mutation screenings.

RESULTS. We found molecular defects in 523 (62%) of the 520 probands screened. Among these, 271 patients (83.9%) had an mtDNA mutation, 50 patients (15.5%) carried an OPA1 mutation, and 2 patients (0.6%) had an OPA3 mutation. Coexistence m.3460 G>A and m.11778G>A was found in one patient. We identified 40 intragenic mutations and six large genomic DNA arrangements of the OPA1 gene, 23 of which were novel.

CONCLUSIONS. The LHON-mtDNA mutations are the most common genetic defects, followed by the OPA1 mutations, in this Chinese cohort.

Keywords: LHON, ADOA, mtDNA, OPA1, OPA3

Hereditary optic neuropathy refers to a genetically heterogeneous group of disorders that involve the optic nerve and retinal ganglion cells (RGC), causing variable visual loss. These kinds of disorders have several modes of inheritance, but autosomal dominant optic atrophy (DOA, OMIM165500) and Leber’s hereditary optic atrophy (LHON, OMIM535000) are the two most common forms.1

Autosomal DOA, with prevalence between 1:12000 and 1:50000, is a disorder characterized by early onset bilateral visual loss, defects in color vision, paracentral or central scotomata, and typical temporal optic nerve pallor.2–4 The condition of DOA is genetically heterogeneous and five chromosome loci: OPA1, OPA3, OPA4, OPA5, and OPA8, have been mapped for it.3 So far, only the OPA1 gene located on 3q28–29 and the OPA3 gene located on 19q13 have been identified as the causative genes for DOA.5–7 Previous studies have shown that the majority (60%–80%) of DOA patients are caused by mutations identified in the OPA1 gene.1–6,8–15 However, less than 2% of the patients with DOA usually associated with cataract are found carrying mutations of the OPA3 gene.7,15 The OPA1 and OPA3 genes encode mitochondrial proteins, which are mostly anchored on the mitochondrial inner membrane and ubiquitously expressed.5,16,17 The OPA1 protein is involved in several cellular functions, including the maintenance of structural integrity of the cristae, control of the programmed cell death through the regulation of cytochrome C redistribution, and regulation of mitochondrial oxidative phosphorylation.18 To date, over 200 mutations have been identified in the OPA1 gene and over 70% of these are splicing mutations (27%), small deletions or insertions (23.5%), nonsense mutations (16.5%), and genomic DNA arrangements, including large deletions or duplications (6%). As the majority of these mutations create premature stop codons or exons skipping, which would finally cause truncated proteins, the disease mechanism for OPA1 evolvement may be related with haploinsufficiency.8–15

The condition of LHON is one of the most widely-studied maternally-inherited disorders, with a prevalence of 1:50,000.19,20 Typically, LHON presents with acute/subacute loss of central vision usually in patients 18 to 35 years old and most frequently affects males.20–25 The LHON is caused by mutations of the mitochondrial DNA (mtDNA), which consists of 13 protein coding genes, two ribosomal RNAs, and 22 tRNAs. Over 90% of LHON is due to one of three common mutations, m.3460G>A, m.11778G>A, and m.14484T>C, which individually involve the MFN2, MFN4, and MFN6 subunits of the complex I, which is the largest of the respiratory multiprotein complexes situated in the inner mitochondrial
membrane.\textsuperscript{19–22} The RGC death caused by LHON-mtDNA mutations may be related to the biochemical impairment of the complex I, increased generation of reactive oxygen species (ROS), and activation of apoptosis, however, the real pathogenesis of LHON still is unclear.\textsuperscript{19,20}

Although the genetic and clinical features of hereditary optic atrophy have been described in several Caucasians case series, the fractional prevalence of LHON and DOA mutations in Chinese patients with suspected hereditary optic atrophy still remains to be established. In this study, we report the results of a comprehensive molecular screening of 520 probands referred to our laboratory for the genetic analysis of hereditary optic atrophy.

**Materials and Methods**

**Patients**

This study included 520 unrelated patients with bilateral optic atrophy referred for molecular diagnosis to the Genetics Laboratory of Beijing Institute of Ophthalmology, Beijing Tongren Ophthalmic Center, during the period of 2006 to 2012. In this cohort, 174 (33%) had a family history of optic atrophy, while the remaining 346 patients (67%) did not show an evident family history related with optic neuropathy (Table 1). Of the 174 probands with family history, only 24 families showed an autosomal dominant pattern of inheritance. The clinical criteria for patients attending this study included decreased central visual acuity not associated with ametropia and opacity of refracting medium, variant extent optic atrophy observed by fundus examination, and exclusion of all known reasons, such as compressive, inflammatory, glaucoma, and infiltrative, which could cause optic atrophy. Clinical examinations, which include best-corrected visual acuity (BCVA) using E decimal charts, slit-lamp biomicroscopy, and fundus examination, were performed on all probands after getting their informed consent. Over 60% of the probands had fundus photography and less than half of the patients had visual field examinations by static (Octopus) perimetry or kinetic (Goldmann) perimetry. All probands were assessed by qualified neuro-ophthalmologists or ophthalmologists. This study, permitted by the Beijing Tongren Hospital Joint Committee on Clinical Investigation, was performed in agreement with the creeds of the Statement of Helsinki.

**PCR-Based Sequencing of LHON-mtDNA, OPA1, and OPA3**

Genomic DNA of all probands and their relatives was isolated from fresh peripheral blood leukocytes using the genomic DNA extraction and purification kit (Vigorous Whole Blood Genomic DNA extraction kit; Vigorous, Beijing, China), following the protocol of the manufacturer. A total of 14 primary LHON-causing mtDNA mutations (m.3460 G>A, m.3635G>A, m3700 G>A, m.3735G>A, m.3736G>A, m.3866T>C, m.11696A>G, m.11778 G>A, m.14459G>A, m.14482C>G, m.14484 T>C, m.14493A>G, m.14502T>C, and m.14568C>T) was screened initially by PCR-based sequencing methods with three pairs of primers for all patients except for the 24 individuals with a male-to-male transmission family history. The coding regions and exon-intron boundaries of the OPA1 and OPA3 gene were amplified by the PCR in the patients who had a family history suggesting an inheritance form of autosomal dominant and in the patients with a negative result for the LHON-mtDNA mutation screening. Detailed information about the primers used in this study is available on request. The PCR assays were done using standard reaction mixtures and purified amplicons were sequenced by an ABI Prism 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing results were compared to the published mtDNA sequence (AC_000021.2), and cDNA sequence of OPA1 (GenBank NM_155660) and OPA3 (GenBank NM_025136.2). For the OPA1 and OPA3 gene, cDNA numbering +1 refers to A in the initiation AUG translation codon in OPA1 and OPA3.

Allele-specific PCR analysis (AS-PCR) was done in 100 normal controls and in the available family members and to confirm the variations found in the sequencing. The PolyPhen (Polymorphism Phenotyping) program was used to predict the potential functional impact of an amino acid change.\textsuperscript{24}

**Multiplex Ligation-Dependent Probe Amplification (MLPA) Analysis**

The patients with an autosomal dominant pattern of family history, but whose PCR-based sequencing did not detect a disease-causing OPA1 or OPA3 variant were screened further for large genomic DNA deletions or duplications of the OPA1 gene by MLPA analysis. As previously described, MLPA assay was performed with a SALSA MLPA Kit P229-B2 (MRC-Holland, Amsterdam, The Netherlands), following the manufacturer’s protocols; this kit contains at least one probe for each exon of OPA1 and OPA3.

**Statistical Analysis**

The 1-sample t-test, the independent sample t-test, and the nonparametric test, homogeneity of variance test, Fisher’s least significant difference t-test, Dunnett t-test, Students-Newman-Keuls test, and the 1-way ANOVA were used for group comparisons with Statistical Product and Service Solutions version 13.0. For the convenience of statistical analysis, Snellen visual acuity numbers were changed into logMAR decimal values. A value of 0, 1.0, and 2.0 in logMAR, respectively, corresponds to 1.0, 0.1, and counting fingers in Snellen visual acuity.\textsuperscript{20}

**Results**

**mtDNA LHON Mutations**

We identified seven LHON-mtDNA mutations in 271 (52%) of 520 probands (Table 1). The seven mutations included the three common and four rare primary mutations. Among the 271 LHON-positive probands, nine patients (3.3%, 9/271) carried two primary mtDNA mutations (Table 2). The three
The 271 LHON-mtDNA positive probands included 241 males and 30 females and the male-to-female ratio was 8:1 (Table 1). The mean onset age of visual loss was 19.25 years (SD, 8.42 years; range, 3–52 years). The mean onset age was statistically younger (P = 0.021) in females (15.44 years; SD, 12.11) than in males (19.68 years; SD, 7.82; Fig. 1); however, no statistically significant difference was observed in mean logMAR visual acuity between the male and female patients (P = 0.131). The visual acuity impairment was statistically more severe (P = 0.003) for the patients carrying one of the three common mutations than for the patients carrying one rare mutation (P < 0.003) for the patients carrying one of the three common mutations.

### LHON Clinical Profile

The 271 LHON-mtDNA positive probands included 241 males and 30 females and the male-to-female ratio was 8:1 (Table 1). The mean onset age of visual loss was 19.25 years (SD, 8.42 years; range, 3–52 years). The mean onset age was statistically younger (P = 0.021) in females (15.44 years; SD, 12.11) than in males (19.68 years; SD, 7.82; Fig. 1); however, no statistically significant difference was observed in mean logMAR visual acuity between the male and female patients (P = 0.131). The visual acuity impairment was statistically more severe (P = 0.003) for the patients carrying one of the three common mutations than for the patients carrying one rare mutation (Table 2). Of the 271 LHON-mtDNA positive probands, 18% clearly experienced acute or subacute vision decreases, and 96% had bilateral visual dysfunction, with the second eye becoming affected either at the same time (70%) or successively (30%), with an intereye interval that ranged from 1 week to 13 years.

Fundus abnormalities were observed in 93% of the eyes, with normal fundus appearance in 93% of the eyes, while normal fundus appearance was observed in the remaining 7%. Of the eyes with fundal abnormalities, acute fundal appearance presenting optic disc hyperemia, swelling of the parapapillary retinal nerve fiber layer (RNFL), and retinal vascular tortuosity was observed in 13%, temporal pallor of the disc with parapapillary RNFL defect in 68%, and total disc pallor in 19% (Fig. 2A). Asymmetry in the fundal appearance was observed in almost 20% of the probands.

The phenotype of one patient was worth depicting in detail. A 7-year-old girl was identified as harboring mutations homoplasmic m.11778 G>A and heteroplasmic m.3460 G>A. She had experienced a subacute visual loss in her right eye 1 month before she had her eyes examinations in our hospital. Her BCVA was 0.05 for the right eye and 0.8 for the left eye. Her fundus examinations revealed mild optic disc hyperemia, remarkable swelling of the parapapillary RNFL, and retinal vascular tortuosity in her right eye, and clear optic disc hyperemia and mild swelling of the parapapillary RNFL in her left eye. Her family history could be traced to four generations. Her mother, grandmother, and several other mother-side relatives had been diagnosed with optic atrophy. No additional neurological signs were observed in the affected individuals in this family.

### OPA1 Mutations Identified

We identified 46 different mutations of the OPA1 gene in 9.6% (50/520) of all probands and 20.1% (50/249) of the LHON-negative patients (Tables 1, 3). The mutations included missense (12/46, 26.1%), nonsense (10/46, 21.7%), splicing defect (7/46, 15.2%), small deletion or insertion (11/46, 23.9%), and large genomic arrangement (6/46, 13.0%; Table 3). One reported mutation c.2708_2711del TAG (p.V903GfsX907) in exon 27 was identified in five unrelated probands; the remaining 45 mutations were found only once.

Of the 40 intragenic mutations, 23 were detected for the OPA1 gene in 9.6% (50/520) of all probands and 20.1% (50/249) of the LHON-negative patients (Tables 1, 3). The mutations included missense (12/46, 26.1%), nonsense (10/46, 21.7%), splicing defect (7/46, 15.2%), small deletion or insertion (11/46, 23.9%), and large genomic arrangement (6/46, 13.0%; Table 3). One reported mutation c.2708_2711del TAG (p.V903GfsX907) in exon 27 was identified in five unrelated probands; the remaining 45 mutations were found only once.

Of the 40 intragenic mutations, 23 were detected for the first time in this study. The AS-PCR analyses showed that 15 novel mutations co-segregated with the phenotype in the cohort patients, and none of the mutations was detected in 100 normal controls. The PolyPhen program analysis predicted that seven of the novel missense mutations were probably damaging. Large genomic arrangements, including three different exon deletions (exon 1, exon 13, and exon 22) and three duplications (exon 2, exon 18-22, and 23-26 duplication), were identified in six unrelated patients (Table 3).
visual acuity for the probands carrying the OPA1 mutations was 0.76 (SD, 0.38; mean Snellen equivalent, 0.18; range, 0.8–0.03), which was statistically significantly better ($P = 0.0001$) than the value for the LHON-positive patients (Fig. 3). No statistically significant difference was seen in mean onset age ($P = 0.415$) or mean logMAR visual acuity ($P = 0.185$) between the various OPA1 mutational subtypes. The appearance of the optic nerve head was abnormal in 49 probands (98%, 49/50), with a prominent temporal wedge of pallor in 84 of 98 eyes (86%) and total disc pallor observed in 14 of 98 eyes (14%, Fig. 2B). One patient (2%, 1/50) showed normal optic nerve head appearance on his fundus examination. Symmetry fundal appearances were observed in all the probands.

The clinical features of two unrelated families deserved to be described in detail. In the first case, a 36-year-old male was identified as harboring a novel missense heterozygous mutation p.C435Y in exon 14 of the OPA1 gene. He had progressive bilateral visual defect and hearing loss since his early childhood. His BCVA was 0.1 in both eyes, with bilateral optic disc pallor and diffused RNFL defects in his fundus examination. His son, nine years old, also had bilateral visual defect; however, he had no hearing loss complaints at the time of examination.

In the second case, a 12-year-old boy with a male-to-male transmission family history of optic atrophy was identified to harbor one reported missense heterozygous mutation p.R445H. The boy had bilateral poor vision noted at age six and underwent neuro-ophthalmo logic examination at age 11, when his BCVA was 0.5 in his right eye and 0.4 in his left eye, with bilateral central scotomas on Octopus perimetry. Fundus examination shown typical temporal pallor of the disc, with parapapillary RNFL defect in both eyes. He had normal hearing, and normal ocular motility and alignment at that time. One year later, the boy complained of hearing loss as well as muscle pain and fatigue in both lower limbs after long walks. His aunt, carrying the same mutation, only had vision defect, but his father and grandfather had neither vision defect nor hearing loss complaint.

**OPA3 Mutations Identified**

Three different mutations of the OPA3 gene were identified in 2 of 199 probands who were negative for the LHON and OPA1 mutation screening (Table 1). The mutation detection rate was 0.38 % (2/520) for all screened patients and 0.80 % (2/249) for the LHON-negative patients (Table 1). All mutations were identified for the first time in the current study. A 9-year-old girl was found to harbor one heterozygous OPA3 missense mutation c.209C>T (p.P70L) and one mutation involving the first initial codon c.1A>G (p.M1?). Unfortunately, we could not obtain her parent’s DNA to do further co-segregation analysis. The BCVA of the girl was 0.2, with bilateral temporal pallor of the disc and parapapillary RNFL defect in her fundus examination (Fig. 2C). The other patient who carried a heterozygous missense mutation c.123C>G (p.I41M) was a 12-year-old male. His BCVA was 0.1 and fundus examination shown bilateral optic disc pallor and diffused RNFL defects. His twin brother, harboring the same mutation, also had visual defects and optic atrophy. No congenital cataract was found in these three patients.
To our knowledge, this is the first comprehensive molecular analysis for LHON and DOA in a large cohort of Chinese patients with suspected hereditary optic neuropathy. The relatively carefully selected nature of the patients tested might contribute to the high overall mutation detection rate (62%, 323/520). Our results suggested that LHON-mtDNA mutations were definitely the most common cause for Chinese patients, as these were found in 52% of probands. This was more frequent than \( OPA1 \) mutations, which were identified in less than 10% of cases. These findings contrasted with the results from a similar large French cohort with 980 unrelated patients, which showed \( OPA1 \) mutations to be the most frequent mutations, identified in 30% of probands, while LHON-mtDNA mutations were detected in only 13% of patients.\(^{15}\) This study further confirmed the \( OPA1 \) gene is the main disease-causing gene for Chinese DOA patients. Consistent with several previous studies,\(^ {15,28,29} \) the \( OPA3 \) gene mutations were rare and only detected in two patients in this cohort study.

For the LHON-mtDNA mutations, the frequency for mutation m.11778 G\( > \)A was not as high as that previously reported in Chinese and Japanese patients (90%).\(^ {22,27} \) In contrast, the frequency for mutations m.11778 G\( > \)A and m.14484 T\( > \)C was rather similar to that found in Caucasian patients.\(^ {21,25} \) Only the frequency for mutation m.3460 G\( > \)A was as low as previously reported in Chinese patients.\(^ {27} \) Except for the three common primary LHON-mtDNA mutations, four rare mutations were identified in almost 10% of patients and over 3% of patients were found simultaneously harboring two primary mutations. The presence of the two primary LHON mutations in one patient was very rare in many early studies,\(^ {16} \) however, in a recent large cohort study which including 1218 Han Chinese patients, 6 unrelated patients were found harboring m.14484 T\( > \)C and m.14502T\( > \)C mutations.\(^ {30} \) In the current study, except for one patient carrying two common primary mutations (m.3460 G\( > \)A and m.11778 G\( > \)A), the remaining eight patients carry one of the three common LHON mutations combined with one of the two

**FIGURE 2.** Fundal appearance of patients with LHON or DOA. (A) Optic disc appearance of three LHON-positive patients showing bilateral disc hyperemia, swelling of the parapapillary RNFL and retinal vascular tortuosity (upper), temporal pallor of the disc with parapapillary RNFL defect (middle), and total disc pallor (lower) respectively. (B) Optic disc appearance of \( OPA1 \)-positive two patients showing bilateral prominent temporal wedge of pallor (upper) and total disc pallor (lower). (C) Optic disc appearance of \( OPA3 \)-positive patient showing bilateral temporal pallor of the disc with parapapillary RNFL defect.
### Table 3. Molecular and Clinical Features of OPA1-Positive Probands Identified in this Study

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M, male; F, female; S, sporadic; EC, early childhood; Fa, family; NA, not available.

* Reference number.

The 11 rare mutations screening in this study have been confirmed to be clearly pathogenic in LHON and do not belong to the category of secondary mutations (available in the public domain at www.mitomap.org).1 To our knowledge, the family carrying the combination of mutation m.11696A>G and m.14502T>C. The 11 rare mutations in this study were found to be clearly pathogenic in LHON and do not belong to the category of secondary mutations (available in the public domain at www.mitomap.org).31 To our knowledge, the family carrying the combination of mutation m.11696A>G and m.14502T>C. The 11 rare mutations in this study were found to be clearly pathogenic in LHON and do not belong to the category of secondary mutations (available in the public domain at www.mitomap.org).31
sporadic cases might be related with the fact that over 90% of patients in this study were not local residents since they came from all over the country and it was very difficult to trace their distant relatives in a very large country like China. In this study, the male-to-female ratio (8:1) in the LHON-positive cases was much higher than the normal male-to-female ratio (5:1); this might be due to the initial higher composition of male cases (416/520) in our cohort, which was a limitation of the current study. As expected, the 40 different OPA1 intragenic mutations identified in this study were not distributed randomly over the gene. The majority of them were located in the GTPase, dynamin central region, and terminal effector domains (Fig. 4).

Figure 3. Comparison of the logMAR vision between the LHON-positive patients and the OPA1-positive patients in this study ($P = 0.0001$).

Figure 4. Distribution and frequency of the OPA1 mutations identified in this study. e, exon; del, deletion; dul, duplication.
Consistent with several other studies, the mutation c.2708_2711delTTAG in exon 27 was identified in five unrelated patients and was the most common mutation of the OPA1 gene in this study.8,10-15 The four other exons, where mutations had been identified 4 times or more, were exons 2, 8, 26, and 28. These exons, supposed to be mutational hot regions, should be firstly sequenced in the OPA1 mutation analysis for Chinese patients. No mutation was identified in the exon 15 in our cohort and in several other Chinese studies.28,33-35 This may be related to an ethnic difference. Other than the 40 intragenic OPA1 mutations, six OPA1 genomic DNA rearrangements were identified, giving an overall frequency of 12% (6/50) in the OPA1 mutations. Our result is in agreement with the previous findings that indicated a high frequency of mutations involving genomic rearrangements.25,36 Since we only conducted MLPA analysis in the patients with prenatal family history, the rate of OPA1 genomic DNA rearrangements might be underestimated.

The observations of two previous studies indicated that 10% to 20% of the patients carrying OPA1 mutations showed additional neuromuscular features except optic atrophy, which is called DOA plus syndrome (DOA+).15-17 Two patients (4%) of the probands carrying the OPA1 mutations displayed the so-called “DOA+” clinical manifestation. One patient carried a reported mutation p.R445H, the other patients harbored a novel mutation p.C435Y. To date, the mutation p.R445H has been identified in several families with the DOA+ phenotype.15,37-39

Mutations of the OPA3 gene can cause either autosomal dominant or autosomal recessive optic atrophy.7,40 For the autosomal recessive optic atrophy, patients usually have later onset spasticity, extrapyramidal signs, and cognitive deficit known as the Costeff syndrome in addition to early onset optic atrophy.38 In this study, three OPA3 mutations were identified in two unrelated probands. One patient carried one missense mutation and one mutation involving the first OPA3 initiation codon (c.1A > G). Abrogation of the highly conserved AUG code would affect mRNA translation, either using no-AUG start codon or skipping to the second AUG in the downstream.41 Translation from the second AUG, located in 21 bases downstream, would yield a mutant protein lack of the first seven wild type amino acids. The first 18 amino acids of the OPA3 have been predicted to constitute an N-terminal mitochondrial leader sequence, which is crucial for sorting the mature OPA3 to mitochondrion.42 The rare initiation codon mutation also has been reported in the OPA1 c.1A > T mutation.39 Other than the optic atrophy, the patient showed no other neuromuscular manifestation.

The clinical features of Chinese patients with either one primary LHON mutation or two mutations were similar to the studies reported in the Caucasian patients.21,22 As expected, the mean onset age is significantly younger for DOA patients than for LHON patients, while the visual impairment is milder for DOA patients than for LHON patients. Almost all DOA patients had a relatively symmetric fundus appearance; however, up to 20% of the LHON patients showed asymmetry in their fundal appearances. Except for the distinct clinical features and different mode of inheritance between LHON and DOA, there still is some extent of phenotypic overlap, especially in the atrophic phase. Therefore, molecular analysis is very crucial in the diagnosis of patients especially for sporadic cases with unknown reason for optic neuropathy.

In this study, there still are 197 probands (38%, 197/520) whose disease-causing mutations were not identified. The remaining probands are likely to harbor other rare mutations of unscreened LHON-mtDNA, mutations of the OPA1 or OPA3 either in the promoter or intronic regions, or mutations in other unidentified nuclear genes. Moreover, as most of the negative probands were sporadic, the disease-causing reasons for some patients may be related to nongenetic factors.

In conclusion, LHON-mtDNA mutations are the most common genetic defects, followed by the OPA1 mutations in this Chinese cohort. Analysis of genomic rearrangements is mandatory in OPA1 mutation screening, especially for patients with an autosomal dominant pattern of family history.

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Fractional Prevalence of LHON and DOA in Chinese Patients


