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ORIGINAL ARTICLE

Molecular characterization of six Chinese families with m.3460G>A and Leber hereditary optic neuropathy

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Abstract The primary mutation m.3460G>A occurs with a very low frequency (~1%) in Chinese patients with Leber hereditary optic neuropathy (LHON). Up to now, there is no comprehensive study of Chinese patients harboring this mutation. We characterized six unrelated probands with m.3460G>A in this study, which were identified from 1,626 patients with LHON or suspected with LHON. The overall penetrance of LHON (25.6% [10/39]) in four pedigrees with m.3460G>A was substantially lower than those families with m.11778G>A (33.3% [619/1859]) as reported in our previous study. Intriguingly, family Le688 with a heteroplasmic m.3460G>A presented a lower penetrance (12.5%) than the other three families with a homoplasmic mutation. There is an elevated gender bias (affected male to affected female=4:1) in the four families with m.3460G>A compared to those LHON families with m.11778G>A (2.4:1). Complete mtDNA sequencing indicated that the six matrilines belonged to haplogroups B4d1, F2, A5b, M12a, D4b2b, and D4b2, respectively. We did not identify any potential secondary mutation(s) that will affect or be associated with the penetrance of LHON in the six probands by using an evolutionary analysis and protein secondary-structure prediction. Taken together, our results suggested that the m.3460G>A mutation occurred multiple times in Chinese LHON patients. The heteroplasmic status of mutation m.3460G>A might influence the penetrance of LHON in family Le688.

Keywords LHON · m.3460G>A · Chinese · Phylogenetic analysis · Multiple origins

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Introduction

Leber hereditary optic neuropathy (LHON, MIM 535000) is the first proved maternally inherited disease linked with mitochondrial DNA (mtDNA) point mutation [1]. Although the special clinical features of LHON, e.g., incomplete penetrance and gender bias, have been long recognized [2, 3], and extensive molecular studies have been performed in the past two decades, the pathogenesis of this disease has not been well understood [4–7]. Nonetheless, we have now expanded our knowledge about LHON and we know that: (1) over 95% of LHON cases are caused by one of the three primary mtDNA mutations (m.3460G>A in the *MT-ND1* gene, m.11778 G>A in the *MT-ND4* gene, and m. 14484 T>C in the *MT-ND6* gene [1, 5, 8–10]. (2) The spectra of the primary mutations in LHON patients from Europe and East

Asia are remarkably different [11, 12]. Mutation m.11778G>A is highly prevalent in East Asian LHON patients (90.2%) than in European patients (56.6%). In contrast, frequencies of m.3460G>A (22.6%) and m.14484T>C (20.8%) are significantly higher in European than in East Asian LHON patients (m.3460G>A, 1.1%; m.14484T>C, 8.7%) [11, 12]. (3) About 50% of males and 10% of females with the primary mtDNA mutations will finally develop optic neuropathy [5] and mtDNA haplogroup background affects the clinical expression of LHON [11, 13]. (4) There must be other factors that participate in the disease expression of LHON [4–7, 11, 14, 15].

We recently launched a comprehensive survey for mtDNA mutations in Chinese patients with LHON or suspected LHON [12, 13, 16–19] and have collected data from 1,626 patients. The in-depth study of a portion of these Chinese families with m.11778G>A revealed that haplogroups M7b1'2 and M8a affect the disease expression [13]. The overall LHON penetrance of m.11778G>A was 33.3% (619/1859) in our studied families, with an affected male-to-female ratio of 2.4:1 [13]. Among the large cohort of patients, we only identified six families/singleton cases harbored m.3460G>A mutation. In this study, we presented a molecular characterization of these six Chinese families/cases with m.3460G>A.

Materials and methods

Subjects

The six Chinese probands with m.3460G>A were identified in 1,626 patients who undergone a genetic diagnosis for LHON primary mutations at the Zhongshan Ophthalmic Center. The presence of the m.3460G>A mutation was further confirmed by using the allele-specific PCR amplification in the Kunming Institute of Zoology before direct sequencing the entire mtDNA genome. Among them, four probands were from families with a family history of LHON and two probands were sporadic. Five patients were from Guangdong Province and one sporadic case from Henan Province, China. Informed consents conforming to the tenets of the Declaration of Helsinki and following the guidance of sample collection of Human Genetic Disease (863 program) by the Ministry of Public Health of China were obtained from each participant prior to the study. The institutional review boards of the Zhongshan Ophthalmic Center and the Kunming Institute of Zoology approved this study.

Complete mtDNA sequencing

Genomic DNA was extracted from peripheral blood using the standard phenol/chloroform method. The complete mtDNA sequences were amplified by using four overlapping primers according to our previous study [19]. PCR products were then purified on spin columns (Tiangen Biotech Co., Beijing, China) and were directly sequenced by using 66 inner primers described in our previous studies [19, 20] and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) on a 3730 DNA sequencer (Applied Biosystems).

Data analysis

Sequence variations in the proband mtDNA sequences were scored relative to the revised Cambridge Reference Sequence (rCRS) [21]. We followed the recently updated version of East Asian mtDNA tree [22] and the PhyloTree.org database (http://www.phylotree.org) [23] to classify each mtDNA. We defined the uniqueness of mtDNA variant(s) in certain matriline by an exhaustive database search following the available guidelines [24]. A schematic tree was reconstructed to show the relationship among these mtDNAs. Evolutionary conservation analysis for certain mtDNA variant was performed using the same approach as in our previous study [13, 19]. We performed secondary-structure modeling for the mtDNA encoded protein harboring specific mutations and compared the result to the wild-type (rCRS) by using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). This allowed us to analyze the potential change of hydrophobicity or hydrophilicity between the mutant and wild-type protein.

Results

As shown in Fig. 1, four families presented variable level of LHON penetrance, with an overall penetrance rate of 25.6% (10/39). The percentages of affected males and females were 50.0% (8/16) and 8.7% (2/23), respectively. In families Le688 and Le1027, the affected individuals were exclusive to male offspring. We were unable to access to any family members of probands Le228 and Le1441, despite that these two claimed that their families had no family history of LHON. The penetrance of LHON in subjects with m.3460G>A (25.6% [10/39]) was substantially lower than that of subjects with m.11778G>A in our previous study (33.3% [619/1859]) [13], but the difference was not statistically significant (Fisher's exact test, two-tailed test, $P=0.3910$), partially due to the small number of pedigrees with m.3460G>A considered here.

Complete mtDNA genome sequencing of the six probands showed that m.3460G>A mutation in the proband from family Le688 was heteroplasmic, while this mutation was homoplasmic in the other five probands (Fig. 2). This result was well in agreement with the initial genetic

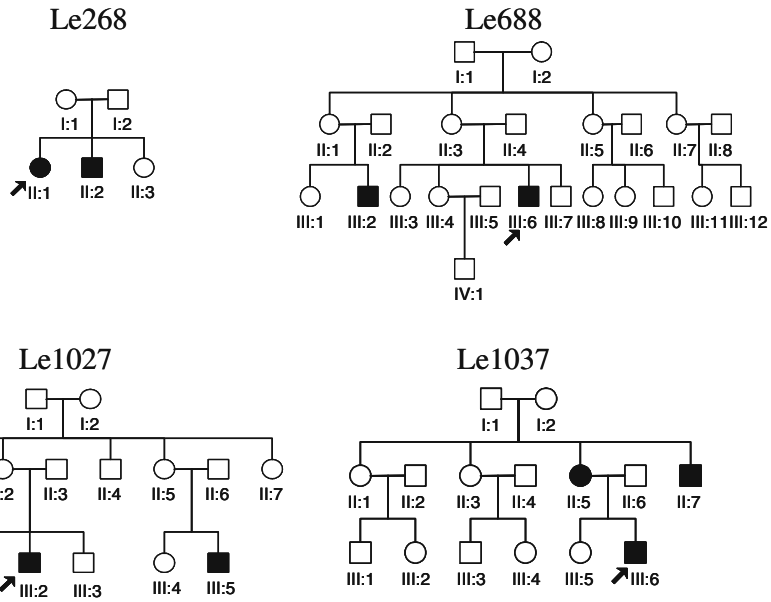

Fig. 1 Pedigree information for four Chinese LHON families with m.3460G>A. Affected individuals are marked by *filled symbols*. *Arrows* indicate probands that were analyzed for complete mtDNA sequences in this study

diagnosis by using the allele-specific PCR amplification and single-strand conformational polymorphism analysis as described in our previous study [12] and the current study. Based on the full array of haplogroup-specific variants in each mtDNA, we classified these six matrilines into haplogroups B4d1, F2, A5b, M12a, D4b2b, and D4b2 (Fig. 3). All of these haplogroups are common in Chinese, albeit with different regional distribution pattern.

The private mutation/variant in each proband may act as a secondary mutation for clinical presentation of the primary mutation m.3460G>A; therefore, we focused on the non-synonymous and transfer RNA (mt-tRNA) variants at the twig level in the tree. A total of 11 different non-synonymous mutations/variants and three mt-tRNA mutations were identified in families Le268 (m.12358A>G [MT-ND5: p.T8A]), Le1027 (m.3866T>C [MT-ND1: p.I187T]; m.4048G>A [MT-

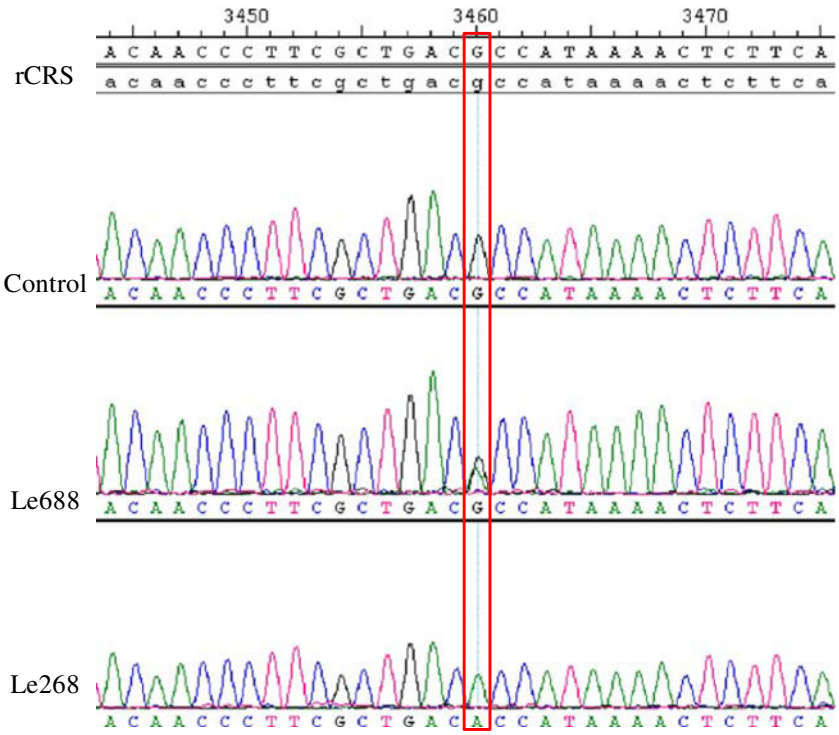

Fig. 2 Sequencing electrogram of Le688 with heteroplasmic m.3460G>A mutation, Le268 with the homoplasmic mutation, and a normal individual without the mutation. The sequences were aligned together with the revised Cambridge reference sequence (*rCRS*) [21]

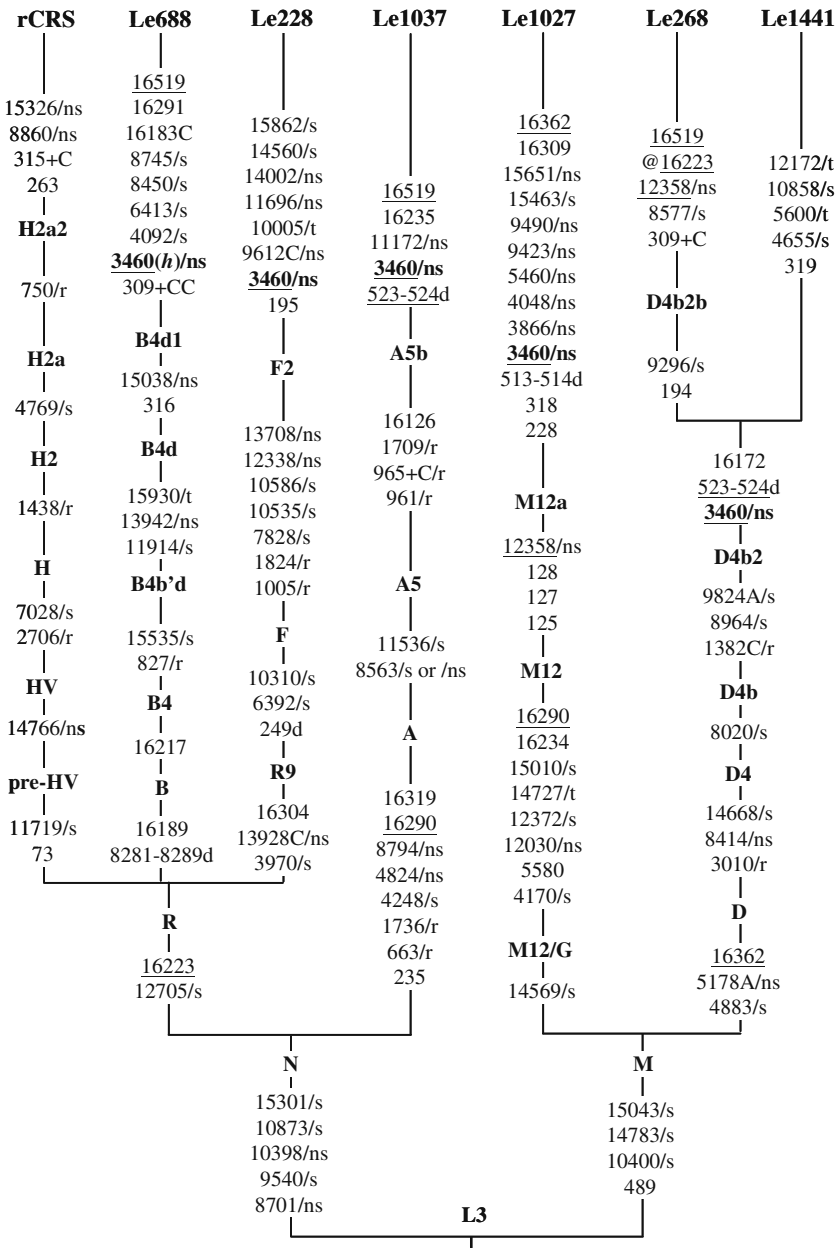

Fig. 3 Classification tree of the entire mtDNA sequences of six matrilines with m.3460G>A, plus the revised Cambridge reference sequence (*rCRS*) [21]. The order of mutations on each uninterrupted branch section is arbitrary. Back mutations are highlighted by the prefix "@", and recurrent mutations are *underlined*. Suffix "+*C*" indicates an insertion of cytosine. The primary mutation m.3460G>A is in *bold*. Proband Le688 had a heteroplasmic status of m.3460G>A and was marked by "(*h*)". The synonymous and non-synonymous coding-region variants in the six mtDNAs are further denoted by "/*s*" and "/*ns*", respectively. Variations in the transfer RNA and the ribosomal RNA genes are denoted by "/*t*" and "/*r*", respectively

ND1:p.D248N]; m.5460G>A [MT-ND2: p.A331T]; m.9423C>T [MT-CO3:p.P73S]; m.9490C>T [MT-CO3: p.A95V]; m.15651C>T [MT-CYB: A302V]), Le1037 (m.11172A>G [MT-ND4: p.N138S]), and sporadic Le228 (m.9612G>A [MT-CO3: p.V136M]; m.10005A>G [MT-TG]; m.11696G>A [MT-ND4: p.V313I]; m.14002A>G [MT-ND5: p.T556A]), Le1441 (m.5600A>G [MT-TA]; m.12172A>G [MT-TH]) (Table 1).With the exception of m.9423C>T, all variants can be found in reported mtDNA samples via standard database and web-based searches [24]. Variant m.12358A>G occurred twice (one time as a haplogroup-specific variant for M12a) in this small data set, consistent with a relatively high mutation rate for this site [25]. None of these mtDNA variants has been reported to be associated with LHON (Table 1), except for m.11696G>A, which was first considered to be a secondary mutation for LHON expression [26]. However, the claimed pathogenic role of m.11696G>A was questioned in later studies, as this variant is one of the haplogroup-specific variants for haplogroup D4j [22, 27, 28]. The newly identified coding-region variant m.9423C>T in subject Le1027 was not conserved according to the evolutionary conservation analysis (Table 1 and Fig. S1). Moreover, the amino acid change (P73S) caused by this nucleotide transition had limited effect on the secondary structure of the MT-CO3 protein (Fig. S2). Therefore, it should be better categorized as a polymorphism.

Table 1 Private non-synonymous and mt-tRNA variants in Chinese LHON mtDNAs with m.3460G>A

Family/ subject[a]	Haplogroup	Nucleotide variant (amino acid change)	Gene	Reported (population context)[b]	Reported (disease context)[b]	Conservation[c]	Haplogroup-specific variant[d]
Le228	F2	m.9612G>A (p.V136M)	*MT-CO3*	Yes	Yes	Yes	Yes (W1b)
		m.10005A>G	*MT-TG*	Yes	Yes	No	Yes (D4a2a)
		m.11696G>A (p.V313I)	*MT-ND4*	Yes	Yes	No	Yes (D4j, M43)
		m.14002A>G (p.T556A)	*MT-ND5*	Yes	Yes	No	Yes (F1a1b, R14, K1c2)
Le268	D4b2b	m.12358A>G (p.T8A)	*MT-ND5*	Yes	Yes	No	Yes (M27, D4b2b2, N9a, etc.)
Le1027	M12a	m.3866T>C (p.I187T)	*MT-ND1*	Yes	Yes	No	Yes (L0a1a)
		m.4048G>A (p.D248N)	*MT-ND1*	Yes	Yes [e]	No	Yes (M7b, B4e, L3d1a)
		m.5460G>A (p.A331T)	*MT-ND2*	Yes	Yes	No	Yes (M7b'd, G4, W, etc.)
		m.9423C>T (p.P73S)	*MT-CO3*	No	No	No	No
		m.9490C>T (p.A95V)	*MT-CO3*	Yes	Yes[e]	No	No
		m.15651C>T (p.A302V)	*MT-CYB*	Yes	Yes[e]	No	No
Le1037	A5b	m.11172A>G (p.N138S)	*MT-ND4*	Yes	No	No	Yes (L0a2, R8a1a2a, K2c)
Le1441	D4b2	m.5600A>G	*MT-TA*	Yes	Yes	Yes	No
		m.12172A>G	*MT-TH*	Yes	Yes	No	Yes (D4b1b1a1, M28b, etc.)

[a] The complete mtDNA genome of Le688 contained no private non-synonymous and mt-tRNA variants and was not included in the table

[b] The search was performed on February 9, 2010 following the same strategy described in Bandelt et al. [24], e.g., both 'G9612A mtDNA' and '9612G>A mtDNA' were queried

[c] The conservation analysis was performed by comparing *Homo sapiens* mtDNA (GenBank accession no. J01415) to nine different vertebrate species, *Gorilla gorilla* (NC_001645), *Mus musculus* (AY466499), *Bos taurus* (AY526085), *Equus caballus* (EF597513), *Canis lupus chanco* (EU442884), *Canis familiaris* (DQ480502), *Balaenoptera musculus* (NC_001601), *Rana nigromaculata* (AB043889), and *Danio rerio* (NC_002333).

[d] The column "Haplogroup-specific variant" refers to the presence or absence of the corresponding variants in the world mtDNA phylogeny displayed at http://www.phylotree.org/tree/main.htm (mtDNA tree Build 7; 10 Nov 2009) [23]. In round brackets we indicate the haplogroup status as it defined in that tree

[e] These variations were detected in a mtDNA reported by Liu et al. [40], which defined an uncharacterized branch within haplogroup M12a

The complete mtDNA sequences generated in this study have been deposited in GenBank under accession numbers GQ999958–GQ999963.

Discussion

The frequency of LHON primary mutation m.3460G>A is significantly lower in Chinese LHON patients than in European patients [11, 12], showing a remarkable racial difference. In this study, we presented molecular characterization of six LHON patients with m.3460G>A mutation that were detected in 1,626 patients with or suspected with LHON, with an intention to learn more about the Chinese LHON patients caused by this mutation. Moreover, a comparison of Chinese LHON families with m.3460G>A to those families with m.11778G>A may offer further insights into understanding the pathogenesis of LHON. We noticed that families with m.3460G>A had a substantially lower penetrance of LHON compared to families with m.11778G>A in Chinese. However, this value should be received with caution as the penetrance rate for m.3460G>A was based only on four families.

One feature for European families with m.3460G>A is a high frequency of heteroplasmy of this mutation. The prevalence of the heteroplasmy has been reported in 40% of 167 genealogically unrelated LHON families harboring the m.3460G>A mutation, which was of significantly higher incidence compared to mutations m.11778G>A (5.6%) and m.14484T>C (36.4%) [29]. In Chinese patients, m.11778G>A was homoplasmic in nearly all patients [12, 13]. Among the six patients with m.3460G>A that were analyzed in this study, one (family Le688) presented a heteroplasmy up to 40% of the mutant allele based on the sequencing electropherogram (Fig. 2). The occurrence of heteroplasmy in Chinese LHON patients with m.3460G>A (16.7% [1/6]) was obviously lower than that of European patients [29]. The potential correlation between the heteroplasmy of the primary mutation and the clinical expression of LHON is still an unresolved question: such a correlation was observed in some studies, but some cases may develop optic neuropathy at a very low level

of heteroplasmy (15%) of m.3460G>A [30–33]. In this study, family Le688 had a lower penetrance rate (12.5%) than the other three Chinese families (66.7%, 30%, 30%) harboring a homoplasmic m.3460G>A. This pattern seems to be consistent with the claim that the mutation load of m.3460G>A influences the clinical expression of LHON. Further study to correlate the mutation load of m.3460G>A with LHON expression in all maternally related members in this family and to recruit more Chinese families with a heteroplasmic m.3460G>A will be essential to solidify our speculation.

Sequence analysis of the entire mitochondrial genomes of the six Chinese LHON probands showed that they belonged to completely different mtDNA haplogroups. This result suggests that m.3460G>A, same to m.11778G>A and other mtDNA pathogenic mutations [13, 22, 34–36], was of multiple origins in Chinese. The exact reason for the low occurrence of m.3460G>A mutation in Chinese is unclear. Apparently, there is no founder effect for these Chinese patients with m.3460G>A.

An association between the expression of LHON in families with the primary mutation and mtDNA haplogroup backgrounds has been well described in western Europeans, and a significant association between haplogroup J and mutations m.11778G>A/m.14484T>C, but not for m.3460G>A, was observed [37–39]. This association was solidified by Hudson et al. [11] and they further showed that the risk of visual failure in European patients with m.3460G>A can be increased in haplogroup K [11]. We recently found that the expression of LHON in Chinese patients with m.11778G>A was also affected by mtDNA background, with an increase in visual loss for haplogroup M7b1'2 and a reduced risk for haplogroup M8a [13]. Because of the small sample size in this study, we were unable to estimate any haplogroup background effect on the clinical expression of m.3460G>A.

The primary mutations are essential for the onset of LHON [7] and the penetrance of LHON is affected by many factors such as mtDNA background, X-chromosome haplotype or other (unknown) nuclear genes, and environmental factors [4–7]. Some mtDNA mutations may enact a synergistic role to the primary mutation during the clinical expression of the disease, whereas others may have a counteractive effect on the primary mutation. We recently found that the deaf-associated mtDNA mutation m.1555A>G might increase the penetrance of LHON in a family with m.11778G>A [17]. Whether there are any mutations/variants that work collectively or counteractively with m.3460G>A in Chinese patients is an intriguing question. In total, we identified 14 private non-synonymous and mt-tRNA mutations/variants in the terminal branches of the classification tree for the six probands. A web-based search for these variants [24] showed that ten of them had been found in reported mtDNAs belonging to different haplogroups (Table 1) and were not related to any disease. Although proband Le1027 carried more private non-synonymous mutations than the other probands at the twig level, four variants in this matriline (m.4048G>A, m.9490C>T, m.15463A>G, and m.15651C>T) have been reported in another mtDNA complete sequence reported by Liu et al. [40], which suggest that they belong to a undefined subhaplogroup of M12a and may be irrelevant to LHON. The newly identified variant m.9423C>T in this family changed amino acid polarity at the 73rd position (p.P73S) of the MT-CO3 protein, but this change did not significantly alter the hydrophilicity of the protein (Fig. S2). Evolutionary conservation analysis indicated that this variant was not conserved in vertebrates. Taken together, it is unlikely that variant m.9423C>T played a synergistic role with m.3460G>A and should be regarded as a rare polymorphism.

In summary, we presented the data for the largest number of Chinese LHON families with mutation m.3460G>A up to now. We found that m.3460G>A mutation arose independently in different mtDNA backgrounds in Chinese families. There is no evidence for a secondary mutation that affect the penetrance of m.3460G>A in the studied families, but the mutation load of m.3460G>A in family Le688 appears to influence the expression of the disease. Further studies should be performed to elucidate the potential role of nuclear genes and/or environmental factors during the pathogenesis of LHON in Chinese patients with m.3460G>A.

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