The MT-ND1 and MT-ND5 genes are mutational hotspots for Chinese families with clinical features of LHON but lacking the three primary mutations

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1. Introduction

Leber hereditary optic neuropathy (LHON, MIM 535000) is one of the most common causes of blindness occurring in young male adults and is characterized by painless, acute or subacute bilateral visual loss [1–3]. Three primary mutations, m.11778G>A, m.14484T>C, and m.3460G>A, have been identified to be the essential factors for the onset of LHON [2]. Incomplete penetrance and gender bias of the disease expression are two main unsolved problems in understanding the pathogenesis of LHON. Many factors, including mtDNA haplogroup background, nuclear genes, and environmental factors, have been reported to be associated with the penetrance of LHON [1–7].

Compared to these well-characterized LHON patients whose conditions are mainly caused by one of the three primary mtDNA mutations, there is an abundance of patients with clinical features of LHON who present a family history of the disease but none of the three telltale mtDNA mutations [8]. The exact pathogenic mutations in these families suspected of having LHON have not been well examined. Analysis of the complete mtDNA genome sequences in these patients will help us to genetically diagnose LHON and broaden the available pathogenic mutation spectrum. Indeed, two recent studies of Chinese families with (suspected) LHON have confirmed that m.3635G>A in the MT-ND1 gene is a rare pathogenic mutation for LHON, thereby enlarging the primary LHON mutation list for Chinese patients [9,10].

We have started a comprehensive study for Chinese patients with LHON in recent years [5,9,11–13]. Currently, we have collected 1626 records of patients with LHON or suspected LHON. Among them are several patients confirmed to lack the three primary mutations but who have a maternally transmitted family history of optic neuropathy or exhibit a mode of disease inheritance that does not exclude the possibility of maternal transmission. In this study, we performed a molecular characterization of 10 such families, with an intention to learn more about the mtDNA mutation spectrum of Chinese families with suspected LHON.
2. Materials and methods

2.1. Patient collection and mtDNA complete genome sequencing

We selected 10 unrelated probands from 1626 patients with LHON or suspected LHON for study based on three criteria: (1) patients show typical LHON features, (2) patients have a family history of the disease, (3) genetic diagnosis confirmed the absence of the three primary mutations in each family. All samples were collected at the Eye Hospital, Zhongshan Ophthalmic Center. Informed consent conforming to the tenets of the Declaration of Helsinki was 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.

Total genomic DNA was extracted from whole blood by using the standard phenol/chloroform method. The entire mtDNA sequences of 10 probands were amplified and sequenced by the previously described procedure [11].

2.2. Haplogroup classification and evolutionary conservation analysis

mtDNA sequence variations in each mtDNA were scored relative to the revised Cambridge Reference Sequence (rCRS) [14]. We followed the East Asian mtDNA phylogenetic tree [15], with updated information from the bulk tree at the PhyloTree (http://www.phyloTree.org; mtDNA tree Build 8) [16], to classify each lineage. All the sequence variations in the studied families were further presented in a tree profile, as described in our previous studies [5,15,17]. We defined the uniqueness of each private variant that was located in the terminal branch of the tree following the available guideline [18]. Evolutionary conservation analysis for certain mtDNA variant was performed by comparing human mtDNA (GenBank Accession No. J01415) to 43 different vertebrate species (Fig. S1). We followed the reported approach [19] to calculate the conservation index (CI) of each variant, which is defined by the percentage of species for a list of 44 different vertebrate species (including human) that have the wild type allele of human sequence.

2.3. Resampling simulation for private variants

The non-synonymous and synonymous variants that were located in the terminal branches were further compared to those variants in 68 Chinese samples that were taken from Chinese populations [15,20] and one mtDNA generated in this study. We used a resampling strategy to avoid the potential sampling bias in the comparison of the private variants in mtDNAs from general Chinese samples (control) and patients with suspected LHON. We randomly selected 10 mtDNAs from the control mtDNA population and counted the total number of the non-synonymous variants in the terminal branch of the phylogenetic tree for each coding gene. This sampling procedure was repeated 1000 times. The resampling simulation was performed by a program developed using Perl. We employed a box plot to show the difference in the number of private non-synonymous variants of each gene between the compiled simulation result and the counts for the 10 patients. The box plot was produced by using SPSS (version 13; SPSS Inc., Chicago, IL).

2.4. Prediction of secondary structures of the MT-ND1, MT-ND5 proteins and the MT-TL2 gene

We performed a secondary structure modeling for the non-synonymous mutations that are located in the MT-ND1 and MT-ND5 genes. The corresponding genes in the rCRS were regarded as wild type. The TMPred program (http://www.ch.embnet.org/software/TMPRED_form.html) was used to predict the secondary structure changes caused by the variants. For comparison, some reported LHON mutations that are located in the MT-ND1 and MT-ND5 genes (cf. MITOMAP: http://www.mitomap.org/) were also analyzed. The secondary structure of the tRNA ^A^ {L-2} (MT-TL2) gene was performed by using the Mobyle method (http://mobyle.pasteur.fr/cgi-bin/portal.py).

3. Results

3.1. Penetrance rate of optic neuropathy

Total of 10 families analyzed in this study evidently had clinical LHON features and either a maternal transmission family history or an otherwise-inherited pattern (Fig. 1). The average disease penetrance rate of the 10 families is 53.4% (31/58), with an almost equal penetrance between male and female [male, 57.1% (16/28); female, 50.0% (15/30)]. The overall penetrance of LHON in the 10 families without the three primary mutations is significantly higher than that of LHON families with m.11778G>A (33.3% [619/1859]; \[P = 0.0027; \[5\]) or with m.3460G>A mutation (25.6% [10/39]; \[P = 0.0072; \[13\]) (Fisher’s exact test, two-tailed test). The overall ratio of affected male to female patients in the 10 families (1.07:1; 16/15) is significantly lower than the ratio observed in families with m.11778G>A (2.38:1) [5] and m.3460G>A (4:1) [13].

3.2. mtDNA mutation spectrum

Total of 10 complete mtDNA sequences were classified into 10 different haplogroups (Fig. 2). The proband Le370 belonged to the rare haplogroup M74. We defined a subhaplogroup of M74, M74a, according to six shared variants between this mtDNA and the reported M74 lineage (Fig. 2). A total of 68 private variants (these variants that were found in two or more families were counted as one variant) were found in the probands. Most of these variants (53/68) are located in the mtDNA non-coding region or act as synonymous variants in the coding region; 15 variants are non-synonymous or occur in RNA genes and are found in seven families (Table 1). Note that families Le370, Le913, and Le1135 contain no private non-synonymous variants and/or RNA variants. Variant m.12332A>G in family Le256 has not been reported according to our extensive web search [18]. The complete mtDNA sequences of 10 probands have been placed in GenBank under Accession Nos. GU377081–GU377090.

Evolutionary conservation analysis of the 15 private variants showed that the two non-synonymous mutations (m.3395A>G, p.Y30C; m.3736G>A, p.V144I) in the MT-ND1 gene in family Le1235 were highly conserved in 44 different vertebrate species and had a CI value of 100.0, whereas the CI values for the other variants ranged from 2.3 to 95.5 (Table 1 and Fig. S1). There is no significant difference in the number of terminal non-synonymous variants and synonymous variants between the 10 patients and the 69 control mtDNAs (including one M74a sequence reported in this study) [15,20], although we observed a higher non-synonymous mutation rate in the MT-ND1 and MT-ND5 genes in the 10 patients (Table S1 and Fig. S2). Simulation of resampling 10 mtDNAs from the reference population containing 69 individuals showed a relative excess of non-synonymous variants on the terminal branches of the phylogenetic tree for the MT-CO1 and MT-ATP6 genes, whereas the MT-ND1 and MT-ND5 genes had substantially fewer variants compared to the 10 matrices from families with suspected LHON (Fig. 3). Note that such a comparison may have a limitation, given the difficulty to match geographic origin and the criterion of having controls from the same haplogroup.
Most of the non-synonymous MT-ND1 and MT-ND5 variants are located in transmembrane helical structures or along the two outer surfaces of the inner mitochondrial membrane, but they did not significantly change the hydrophobicity of the protein (Fig. 4). The previously unreported mitochondrial tRNALeu variant m.12332A>G is located in acceptor arm of the MT-TL2 gene. This variant was not conserved among the examined vertebrate species and caused no change of the predicted secondary structure of the MT-TL2 gene (Fig. S3).

4. Discussion

Many previous studies have been performed to elucidate the pathogenic mechanism of LHON caused by the three primary mutations, which are not sufficient but essential for the onset of LHON [2]. Among these confirmed LHON patients, over 95% of them harbor one of the three primary mutations [2–4]. Compared with these well-defined LHON patients, mtDNA studies for the families with clinical LHON features but without the three primary mutations are insufficient [8]. Genetic analysis of these patients is of particular importance as these patients account for the majority of patients with blindness caused by unknown reasons [8]. Understanding the role of mtDNA mutation in these patients may identify novel primary mutations and facilitate subsequent genetic diagnosis and consultation.

In this study, we selected 10 Chinese families with typical LHON phenotypes and with a confirmed absence of the three primary mutations. The observed penetrance of bilateral visual loss is significantly higher than that of Chinese LHON families with m.11778G>A [5] or m.3460G>A [13]. Because some of the studied pedigrees were small, we thought this bias would contribute to such an exceptionally high penetrance rate, at least partially. Moreover, the percentage of the affected female subjects in these 10 families is also much higher than that observed for typical LHON families with m.11778G>A or m.3460G>A. All these results suggest that the 10 families with suspected LHON had a different clinical onset pattern compared with those families harboring one of the three primary mutations.

Most of previous studies about LHON patients without the three primary mutations were based on European patients [21,22]. The MT-ND1 and MT-ND6 genes have been reported to be mutational hotspots in these LHON patients, and several pathogenic mutations for LHON were characterized [23,24]. For instance, mutation m.3733G>A (p.E143K) changed a conserved glutamic acid at position 143 to lysine in the MT-ND1 protein and could significantly decrease the rotenone sensitivity of complex I [24]. We found that non-synonymous variants in the MT-ND1 and MT-ND5 genes, especially on the terminal branches of the phylogenetic tree (Fig. S2), were abundant in the 10 Chinese families. Additionally the variants were located either in the same domain as the reported pathogenic mutations or in some domains without pathogenic mutations (Fig. 4). Variant m.3735G>A (p.V144I) of the MT-ND1 gene in proband Le1235, which was just one amino acid from m.3733G>A (p.E143K) [24], changed a conserved valine at the 144th position...
and p.V144I) shared a similar mechanism of pathogenesis, despite

the small physicochemical difference between valine and isoleu-

cine (Grantham value = 29; http://mtnsp.tminig.or.jp/mtnsp/index_e.shtml). Indeed, the predicted structure of the MT-ND1 protein in

to isoleucine and most likely account for the onset of LHON in this

family. We speculated that both the amino acid changes (p.E143K

and p.V144I) shared a similar mechanism of pathogenesis, despite
### Table 1

Private non-synonymous and RNA variants in Chinese families without the three primary mutations.

<table>
<thead>
<tr>
<th>Family†</th>
<th>Haplogroup</th>
<th>Nucleotide variant (amino acid change)</th>
<th>Gene</th>
<th>Conservation index (CI)b</th>
<th>Reported (population context)c</th>
<th>Reported (disorder context)c</th>
<th>Haplogroup specific variantd</th>
<th>Variant frequencye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Le256</td>
<td>B5b2</td>
<td>m.3640G&gt;A (p.A112T)</td>
<td>MT-ND1</td>
<td>65.9</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>1/2196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m.12332A&gt;G</td>
<td>MT-</td>
<td>86.4</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>0/2196</td>
</tr>
<tr>
<td>Le316</td>
<td>D4a3</td>
<td>m.1520T&gt;C</td>
<td>MT-RNR1</td>
<td>19.5</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>2/2196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m.13834A&gt;G (p.T500A)</td>
<td>MT-ND5</td>
<td>6.8</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>2/2196</td>
</tr>
<tr>
<td>Le508</td>
<td>F1a</td>
<td>m.9655G&gt;A (p.S150N)</td>
<td>MT-CO3</td>
<td>95.5</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>0/2196</td>
</tr>
<tr>
<td>Le1065</td>
<td>B5a2</td>
<td>m.3316G&gt;A (p.A4T)</td>
<td>MT-ND1</td>
<td>6.85</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>10/2196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m.3892A&gt;G (p.T196A)</td>
<td>MT-ND1</td>
<td>13.65</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>m.15236A&gt;G (p.I164V)</td>
<td>MT-CYB</td>
<td>77.3</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>6/2196</td>
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<tr>
<td>Le1107</td>
<td>M13a</td>
<td>m.980T&gt;C</td>
<td>MT-RNR1</td>
<td>40.93</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>6/2196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m.9053G&gt;A (p.S176N)</td>
<td>MT-ATP6</td>
<td>25.0</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>8/2196</td>
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<tr>
<td></td>
<td></td>
<td>m.10680C&gt;A (p.A71T)</td>
<td>MT-</td>
<td>95.5</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>0/2196</td>
</tr>
<tr>
<td>Le1207</td>
<td>G2a1</td>
<td>m.12406G&gt;A (p.V24I)</td>
<td>MT-ND5</td>
<td>2.3</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>9/2196</td>
</tr>
<tr>
<td>Le1235</td>
<td>B4a'g</td>
<td>m.3395A&gt;G (p.Y30C)</td>
<td>MT-ND1</td>
<td>100.0</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>3/2196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m.3736G&gt;A (p.V144I)</td>
<td>MT-ND1</td>
<td>100.0</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>3/2196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m.14122A&gt;G (p.J596V)</td>
<td>MT-ND5</td>
<td>20.9</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>0/2196</td>
</tr>
</tbody>
</table>

† The complete mtDNAs of Le370, Le913, and Le1135 contain no private non-synonymous and RNA variants and are not included in Table 1.

b The conservation index is the percentage of species for a list of 44 different vertebrate species that have the wild type human sequence (GenBank Accession No. J01415) [19]. The entire list of the species was shown in Fig. S1. Only 43 and 41 species were considered when we calculated the CI of variants m.14122A>G and m.1520T>C, respectively, because the gaps existed in this amino acid position of a few species.

c The search was performed on 27 April 2010 following the described strategy [18] (e.g. both ‘G3640A mtDNA’ and ‘3640C>A mtDNA’ were queried).

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 8; 21 March 2010) [16]. In round brackets we indicate the haplogroup status as is defined in that tree.

e The variant frequency was adopted from Soares et al. [28], which was based on the number of occurrences for each variant in 2196 complete mtDNA sequences.

f There were many hits when we searched “G3640A” using Google, but all of these hits actually referred to spelling error for “G3460A”.

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**Fig. 3.** Box plot of the number of mtDNA non-synonymous variants on the terminal branches of the phylogenetic tree. The simulation resampling of 10 mtDNAs from the reference populations which was composed of 69 complete mtDNAs ([15,20] and this study) were performed by a program developed using Perl.
the presence of m.3736G>A (p.V144I) was similar to that of m.3733G>A (p.E143K) (Fig. S4). Intriguingly, proband Le1235 contained an additional private variant m.3395A>G (p.Y30C) in the MT-ND1 gene. The Y30C replacement caused a reduction of hydrophilicity (Fig. S4) and might render MT-ND1 protein more susceptible to oxidative stress. This variant was reported in two young obese adults [25], but there was no functional assay to characterize its potentially pathogenic role in obesity. The strongly high conservation of m.3736G>A and m.3395A>G would also suggest importance of both positions in MT-ND1 protein. No private non-synonymous variant was found in the MT-ND6 gene in this study, despite the fact that mutations in this gene were reported in LHON patients outside of East Asia [23,26,27]. This result may suggest a racial difference but need further study due to limited number of families studied.

Haplogroup classification of the 10 families shows that these lineages belonged to a diverse pool and suggests no role of haplogroup background effect. Therefore, the potentially pathogenic mutations in the 10 families are located in the terminal branch of the mtDNA tree, instead of being presented as shared variants on the internal branches. An extensive analysis of the previously published mtDNA complete sequence data by Soares et al. [28] found that non-synonymous mutations are more frequently detected in the new branches of phylogenetic tree of mtDNA. We evaluated the non-synonymous/synonymous ratio between our patients and 69 Chinese samples ([15,20] and this study). Although we found no statistical difference between the two groups, the non-synonymous mutation rate in the MT-ND1 and MT-ND5 genes are substantially higher in our patients than in controls (Table S1). This pattern was further confirmed by our simulation assay (Fig. 3). Evidently, our findings confirmed a notion that mutations in the respiratory chain complex I and the decreased functional activity of this complex may be crucial for the onset of optic neuropathy [29,30].

A rare mtDNA variant m.10680G>A in the MT-ND4L gene was recently reported to have a synergistic role with the primary mutation m.14484T>C because of the complete penetrance of LHON in that family [31]. This variant was found in family Le1107 but showed no co-segregation with a primary mutation. This may explain why only 40% of the members in this family show a penetrance of the disease. Based on the available data, m.10680G>A could well be a primary pathogenic LHON mutation in the pedigree Le1107 that shows a quite clear maternal inheritance. Further functional assays are essential to determine the pathogenicity of this mutation.

The novel tRNA variant m.12332A>G in family Le256 is located in the acceptor arm of the MT-TL2 gene. This variant did not change the predicted secondary structure of this gene (Fig. S3). Two other variants near this location, m.12331A>G and m.12334G>A, express completely contrary functions: m.12331A>G is a polymorphism in world populations, whereas m.12334G>A is claimed to be the pathogenic mutation for muscle disease [32]. Whether
m.12332A>G is a polymorphism or a pathogenic mutation is still an open question.

5. Conclusion

To our knowledge, this is the first study for the penetrance and complete mtDNA genome sequence variation in Chinese patients with LHON features but without the three primary mutations. We found that the MT-ND1 and MT-ND5 genes are mutational hotspots for these patients. In particular, we identified several (presumably) pathogenic mutations, e.g., variants m.3736G>A (p.V144I) and m.10680G>A (p.A71T), in our patients. All these results suggest that the mutation pattern in Chinese patients with the typical LHON phenotype but lacking the three primary mutations is quite different from the pattern observed in western European patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbrc.2010.07.051.

References