Strikingly different penetrance of LHON in two Chinese families with primary mutation G11778A is independent of mtDNA haplogroup background and secondary mutation G13708A

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The penetrance of Leber’s hereditary optic neuropathy (LHON) in families with primary mitochondrial DNA (mtDNA) mutations is very complex. Matrilineal and nuclear genetic background, as well as environmental factors, have been reported to be involved in different affected pedigrees. Here we describe two large Chinese families that show a striking difference in the penetrance of LHON, in which 53.3% and 15.0% of members were affected (P < 0.02), respectively. Analysis of the complete mtDNA genome of the two families revealed the presence of the primary mutation G11778A and several other variants suggesting the same haplogroup status G2a. The family with higher penetrance contained a previously described secondary mutation G13708A, which presents a polymorphism in normal Chinese samples and does not affect in vivo mitochondrial oxidative metabolism as described in a previous study. Evolutionary analysis failed to indicate any putatively pathogenic mutation that cosegregated with G11778A in these two pedigrees. Our results suggest that the variable penetrance of LHON in the two Chinese families is independent of both their mtDNA haplotype background and a secondary mutation G13708A. As a result, it is likely that unknown nuclear gene involvement and/or other factors contribute to the strikingly different penetrance of LHON.

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1. Introduction

Leber’s hereditary optic neuropathy (LHON; MIM 535000) is the first disease to be linked with mitochondrial DNA (mtDNA) point mutation [1] and is one of the most studied mitochondrial genetic diseases [2–4]. It is characterized by painless, acute or subacute bilateral visual loss, predominantly affecting young men [2–4]. The majority of LHON cases (over 95%) are caused by one of the three primary mtDNA point mutations (G3460A, G11778A, and T14484C) that affect the function of complex I in the mitochondrial respiratory chain [2–4]. However, not all individuals harboring one of the three primary mutations will develop LHON. The penetrance and phenotypic expression of LHON is complicated by additional genetic factors, such as nuclear genes and mtDNA haplogroup background, as well as environmental factors [2–9]. The etiology and pathophysiology of LHON has not been fully understood despite numerous studies on this topic [2–4].

More than a decade ago, researchers noticed that mtDNA haplogroup (which is comprised of a group of mtDNAs that shared some ancestral mutations and clustered into the same clade in a phylogenetic tree), e.g. haplogroup J in western Eurasian, can contribute to increased penetrance of LHON pathogenic mutations [8,10–12]. The potential association between haplogroup J and LHON has been recently addressed in two studies: Carelli et al. [13] analyzed the entire mtDNA genome of LHON patients and narrowed the association to subhaplogroups J1c and J2b, in which two specific combinations of amino acid changes in the cytochrome b gene (L236I-F19L in J1c and L236I-D171N-V356M in J2b) contribute to the mtDNA background effect. Hudson et al. [6] provided the first clear evidence that the clinical expression of the three primary LHON mutations was increased when G11778A was present in haplogroup J2 and T14484C in haplogroup J1; these authors also found
a reduced penetrance of G1778A on haplogroup H background and an increased penetrance of G3460A on haplogroup K background [6]. We investigated the epidemiology of the three primary LHON mutations in a large cohorts of probands with (suspected) LHON and found mutations of G1778A, T14484C, and G3460A in 34.6%, 3.3% and 0.4% of a total 903 Chinese families, respectively [14]. Further dissection of the matrilineal genetic components in 41 Han Chinese LHON families with G1778A showed that the majority of samples belonged to haplogroups M7, D, B, and A [15]. The frequency of haplogroup F in the patient group was significantly lower than that of control Han samples, suggesting that this haplogroup might confer resistance against the expression of LHON in Chinese [15].

In this study, we characterized two large Chinese pedigrees with strikingly different penetrance patterns of LHON and G1778A to answer two questions: (1) does mtDNA haplogroup background contribute to the observed different penetrance? (2) If haplogroup effect is nonexistent, is there any other pathogenetic mutation which when present with G1778A has a synergistic effect that can account for the higher penetrance of LHON in one family?

2. Materials and methods

2.1. Patients

Two large families with LHON were collected from the Genetic Clinic of the Eye Hospital, Zhongshan Ophthalmic Center. 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 Running Institute of Zoology approved this study.

2.2. mtDNA genome sequencing and data quality control

Total genomic DNA was isolated from whole blood with the standard phenol/chloroform method. The entire mtDNA sequence was amplified by using four overlapping primer pairs (Table 1). PCR reactions were performed in 50 μL of reaction mixture containing 5 μL of 10× LA PCR Buffer II (Mg2+ Plus), 2.5 units of TaKaRa LA Taq (TaKaRa Bio Inc., Dalian, China), 400 μM of each dNTP, 0.2 μL of each primer, and 50 ng DNA. The amplification was run on the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following procedures: one denaturation cycle of 94°C for 1 min; 30 amplification cycles of 94°C for 30 s and 65.6°C for 45 s; and one full extension cycle of 72°C for 10 min. The PCR products were purified on spin columns (Watson Biotechnologies Inc., Shanghai, China) and were directly sequenced by using 66 inner primers (Table 1) described in our previous studies [16–18] and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 3730 DNA sequencer according to the manufacturer’s manual.

To avoid sequencing errors prevalent in medical field [5,19–21], we followed a stringent procedure for data quality. First, each amplified fragment has a length around 4800 bp and overlaps with the neighboring fragments by more than 500 bp, such an amplification strategy can efficiently reduce the risk of artificial recombination caused by sample crossover and help to avoid the amplification of pseudo-mitochondrial gene from the nuclear genome. Second, each uncertain variation, as identified by a phylogenetic approach [5,17,19], was checked by independent PCR and sequencing. Third, we sequenced two maternally related individuals from the family to confirm the sequence variation. The three complete mtDNA sequences determined in this study were deposited in GenBank under accession numbers EU545470–EU545472.

2.3. Haplogroup classification, database comparison and statistical analysis

Sequence variation in the complete mtDNA sequence was scored relative to the revised Cambridge Reference Sequence (rCRS) [22]. We classified the mtDNAs from the two families according to the most recently updated East Asian mtDNA phylogeny [17]. The classification tree was drawn following the same procedure as described in our previous studies [17,19]. We searched the presence of the private mutations, which occurred at the tip level of the mtDNA tree, in >4300 available (near) complete mtDNAs across world (including those presented at the mtDB [http://www.genpat.uu.se/mitDB] and MITMAP website [www.mitomap.org]), to discern the novelty and recurrent status of the variants, as described in our recent study [23]. Evolutionary analysis was used to estimate the conservation of a particular

Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For amplification</td>
<td>ACTCTGAGACTATCACCATCACAT/ATTGGTCGTGGTTGTAGTCCGTGCGAGAA</td>
</tr>
<tr>
<td>L13884/H12181</td>
<td>TGCAGCGCTGTGCACTCCTCA/ATTGGTCGTGGTTGTAGTCCGTGCGAGAA</td>
</tr>
<tr>
<td>H1677/H6505</td>
<td>TCTTGTGAGTGTGGGGAGGAA</td>
</tr>
<tr>
<td>L5868/H10718</td>
<td>ATGGTTATGTACGTAGTCTAGGCCATATGTG</td>
</tr>
<tr>
<td>H9198</td>
<td>AGCCTCTACCTGCACGAC</td>
</tr>
<tr>
<td>L9794</td>
<td>GACGGCATCTACGGCTCAACA</td>
</tr>
<tr>
<td>L10170</td>
<td>ACATGAAAATCACAACCCCTACCAG</td>
</tr>
<tr>
<td>H10356</td>
<td>TCACTCATAGGCCACCTAG</td>
</tr>
<tr>
<td>H14586</td>
<td>TCTGGTGCTGCTGATATTGTAT</td>
</tr>
<tr>
<td>H11081</td>
<td>CAATGTGCGAGGAAAAGCAAC</td>
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<td>H11338</td>
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<td>H11718</td>
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<tr>
<td>H12028</td>
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</tr>
<tr>
<td>H12334</td>
<td>TAGATGGCGGCCCTTGCC</td>
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<td>H12572</td>
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<td>H15996</td>
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<tr>
<td>H16209</td>
<td>CCCATGCTTACAAGCAGAT</td>
</tr>
<tr>
<td>H16347</td>
<td>GGGACGAGAAGGGATTTGA</td>
</tr>
</tbody>
</table>

ular human mtDNA variant in several vertebrate species including zebrafish (Danio rerio, GenBank accession number NC_002333), frog (Rana nigromaculata, AB043889), blue whale (Balaenoptera musculus, NC_001601), mouse (Mus musculus, AY466499), cattle (Bos Taurus, AY526085), horse (Equus caballus, EF597513), dog (Canis lupus familiaris, DQ480502), and gorilla (Gorilla gorilla, NC_001645). Fisher’s exact test and Chi-square test were used to quantify the different penetrance of LHON between the two families.

3. Results

3.1. Clinical features

Families A (Le1269) and B (Le1244) were from Henan Province and Jiangxi Province, respectively, of central China. Affected individuals in both families had a history of subacute visual loss in both eyes and bilateral optic atrophy. Average age at onset of LHON was 12.5 and 18 years old in families A and B, respectively. These two families presented a dramatic difference in the penetrance of LHON: the overall penetrance of LHON in whole family members reached 53.3% (16/30) in family A, while in family B, only 15.0% (3/20) of individuals had the clinical presentation of LHON (Fig. 1). The difference of LHON penetrance between the two families was statistically significant (Fisher’s exact test, two-tailed test, $P = 0.008$; Chi-square with Yates’ correction, 5.946, $P = 0.015$). When we only counted the male individuals, 64.3% (9/14) of males in family A had LHON compared to 27.3% (3/11) of those in family B were affected, but the difference was not statistically significant (for both Fisher’s exact test and Chi-square test, $P > 0.05$). The frequency of affected female individuals in family A (7/16) was significantly higher than that of family B (0/9) (Fisher’s exact test, two-tailed test, $P = 0.027$; Chi-square with Yates’ correction, 3.514, $P = 0.061$). Note that the statistical tests for the difference linked to gender in the two families should be received with caution due to the small sample size.

3.2. mtDNA tree and evolutionary analysis

Analysis of the entire mtDNA genomes of both families showed the presence of the primary mutation G11778A, thus confirming the clinical diagnosis. Both pedigrees shared a string of variants that are characteristic of haplogroup G2a (Fig. 2). The mtDNAs of the two LHON matrilines differed from each other by five coding region transitions C3351T (NADH dehydrogenase 1 [ND1] gene), A4721G (ND2 gene), G7604A (cytochrome c oxidase II [COII]), G13708A (ND5), and T14200C (ND6), one C to A transversion at position 2357 in 16S rRNA, as well as four transitions in the control region. Among these variants, only variant C2357A was not found in the published mtDNA sequences (note that a transition at this position was reported in a L3a mtDNA by Torroni et al. [24]), the other variants could be found in published mtDNA sequences with different haplogroup status. Variants C3351T, A4721G, and T14200C did not cause an amino acid change, whereas both variants G7604A and G13708A occurred at the first base pair of the codon and caused an amino acid change from valine to methionine and from alanine to threonine, respectively. A comparison of 9 different vertebrate species showed that sites 2357, 7604, and 13708 were not conserved. The two amino acids that were defined by the two codons containing variants G7604A and G13708A, respectively, were also varied in different species (Fig. 3).

To further define whether the primary mutation G11778A occurred independently in the two Chinese families, we included the reported samples TCsq0042 (accession number AP008301) from Tanaka et al. [25] and XJ8416 (AY255157) from Kong et al. [16] for comparison. Samples Le1244 and the two reported mtDNAs could be further grouped into a subhaplogroup G2a1 of haplogroup G2a, whereas Le1269 turned out to be a basal lineage of G2a. This
The pathogenesis and etiology of LHON is very complex [2–4]. Interaction between mtDNA primary mutations and nuclear genetic background, in particular with the X-chromosome, has long been claimed to be involved in the pathogenesis of LHON, although the exact loci for disease susceptibility are still controversial.

**Discussion**

Recent studies have identified several mtDNA mutations that may contribute to the pathogenesis of LHON. Among these, G11778A mutation has been extensively studied due to its high prevalence in LHON patients. The pathogenic role of G11778A has been supported by various lines of evidence, including family studies, population-based studies, and functional analyses in cell and animal models. However, the exact mechanism by which G11778A leads to LHON remains elusive.

The synergistic effect of G11778A with other mutations or genetic backgrounds has been a subject of intense research. Interaction between mtDNA primary mutations and nuclear gene(s) may influence the penetrance and expressivity of LHON. For instance, a recent study by Hudson et al. identified an X-chromosomal haplotype (DXS8090 [186]-DXS1068 [258]) that interacts with the primary mtDNA mutations to cause LHON [7]. This interaction suggests the importance of considering nuclear gene(s) in the pathogenesis of LHON.

Future studies should focus on identifying additional genetic factors that interact with primary mtDNA mutations to cause LHON. The integration of functional and evolutionary approaches, along with the use of advanced computational tools, will be crucial in this endeavor. Moreover, the identification of additional genetic factors will help in understanding the heterogeneity of LHON, which may vary across different populations and within families.

In conclusion, the pathogenesis of LHON is a complex interplay between mtDNA mutations and nuclear genetic background. The identification of additional genetic factors and their interactions with mtDNA mutations will be essential in advancing our understanding of LHON and developing effective therapeutic strategies.

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**References**


background can be excluded. Seemingly, the recurrent nature of G7604A undermines a speculation for a beneficial role of this variant in ameliorating the deterioration effect of G11778A that led to a low penetrance of LHON in family B.

In summary, the analysis of complete mtDNA sequence variation in the two Chinese LHON families with G11778A failed to identify additional mtDNA putatively pathogenic mutations based on the canonical criteria [35,38]. The striking difference in penetrance of LHON in these two families was independent of their mtDNA background and mutation G11778A was most likely of multiple origins. Unknown nuclear gene involvement and/or other factors should account for the variable penetrance of LHON. Further study on cosegregation of X-chromosomal haplotype with LHON in these families may provide valuable information on nuclear gene involvement.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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