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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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ABSTRACT

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.

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 composed 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

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a reduced penetrance of G11778A 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 G11778A, T14484C, and G3460A in 34.6%, 3.3%, and 0.4% of a total of 903 Chinese families, respectively [14]. Further dissection of the matrilineal genetic components in 41 Han Chinese LHON families with G11778A 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 G11778A 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 pathogenic mutation which when present with G11778A has a synergistic effect that can account for the higher penentrance 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 Kunming 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 pairs of primers (Table 1). PCR reactions were performed in 50 μL of reaction mixture containing 5 μL 10 × LA PCR Buffer II (Mg^{2+} Plus), 2.5 units of TaKaRa LA Taq (TaKaRa Bio Inc., Dalian, China), 400 μM of each dNTP, 0.2 μM 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 6 min; 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 [5,17,19,21], was checked by independent PCR and sequencing. Third, we sequenced two maternally related individuals from family A 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/mtDB/] 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 partic-

Table 1
Primer pairs for entire mtDNA genome amplification and sequencing

Primer name	Sequence (5′–3′)
For amplification	
L13894/H2187	ACTTAAAATAAAATCCCCACTATGCACAT/ TGTTGAGCTTGAACGCTTTCTTAATTGGTG
L1677/H6505	TTAACTTGACCGCTCTGAGCTAAAC/ AGTAGTATAGTGATGCCAGCAGCTAGGA
L5868/H10718	CTAACCCCTGTCTTTAGATTTACAGTCCA/ GGTTATGTACGTAGTCTAGGCCATATGTG
L9877/H14676	CTATCTGCTTCATCCGCCAACTAAT/ ATTGGTCGTGGTTGTAGTCCGTGCGAGAA
For sequencing	
L29	GGTCTATCACCCTATTAACCAC
L333	GCTTCTGGCCACAGCACT
L713	TGCAAGCATCCCCGTTCC
H902	GACTTGGGTTAATCGTGTGAC
L923	GTCACACGATTAACCCAAGTCA
L1156	GAACACTACGAGCCACAGC
H1172	GATATGAAGCACCGCCAGG
L1466	GAGTGCTTAGTTGAACAGGGCC
L1776	AATTGAAACCTGGCGCAATAG
L2025	GCCTGGTGATAGCTGGTTGTCC
H2053	TTAGAGGGTTCTGTGGGCAAA
L2415	ACCAACAAGTCATTATTACCC
H2426	TGAGCATGCCTGTGTTGGG
L2796	GTCCTAAACTACCAAACCTG
H3274	GGAATTGAACCTCTGACTGT
L3179	AGCGCCTTCCCCCGTAAATG
L3644	GCCACCTCTAGCCTAGCCGT
L4210	CCACTCACCCTAGCATTACTTA
H4227	ATGCTGGAGATTGTAATGGGT
L4499	TGGCCCAACCCGTCATCTAC
H4792	ACTCAGAAGTGAAAGGGGGCTA
L4887	TGACAAAAACTAGCCCCCATCT
L5278	TGGGCCATTATCGAAGAATT
H5442	GCGATGAGTGTGGGGAGGAA
L5781	AGCCCCGGCAGGTTTGAAGC
L6337	CCTGGAGCCTCCGTAGACCT
H6367	TGGCCCCTAAGATAGAGGAGA
L6869	CCGGCGTCAAAGTATTTAGC
L7356	GAAGCGAAAAGTCCTAATAG
H7406	GGGTTCTTCGAATGTGTGGTAG
L7882	TCCCTCCCTTACCATCAAATCA
H7990	CTCGATTGTCAACGTCAAGG
L8215	ACAGTTTCATGCCCATCGTC
H8345	TTTCACTGTAAAGAGGTGTTGG
L8581	ACAATCCTAGGCCTACCCG
H8861	GAGCGAAAGCCTATAATCACTG
L9198	AGCCTCTACCTGCACGAC
H9212	GATAGGCATGTGATTGGTGG
L9580	CAACAGGCATCACCCCGCTAAA
L9794	GACGGCATCTACGGCTCAACA
L10170	ACATAGAAAAATCCACCCCTTACG
H10356	TCACTCATAGGCCAGACTTAG
L10519	TAGCATTTACCATCTCACTTCT
H10660	TTCGCAGGCGGCAAAGACTA
L11004	ACAATCATGGCAAGCCAACG
H11081	TCTGTGGCTGTGAATGTTAT
L11338	CAAACTCCTGAGCCAACAAC
L11718	CGCAGTCATTCTCATAATCGCCCACGG
L12028	GGCTCACTCACCCACCACATT
L12334	GGTGCAACTCCAAATAAAAG
L12572	ACAACCCAGCTCTCCCTAAG
L13049	GACTCCCCTCAGCCATAGA
H13124	ATTTTCTGCTAGGGGGTGGA
L13612	AAGCGCCTATAGCACTCGAA
H13666	AGGGTGGGGTTATTTTCGTT
L14054	TCACAGCACCAAATCTCCAC
H14186	TGGTTGAACATTGTTTGTTGG
L14575	ACCCGACCACACCGCTAACA
L14989	ATGGCTGAATCATCCGCTAC
H15086	AGGAGGATAATGCCGATGTT
L15391	TAGGAATCACCTCCCATTCC
L15598	ACACAATTCTCCGATCCGTC
H16048	GTCAATACTTGGGTGGTACC
L15996	CTCCACCATTAGCACCCAAAGC
L16209	CCCCATGCTTACAAGCAAGT
H16347	GGGGACGAGAAGGGATTTGA

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 methoionine 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

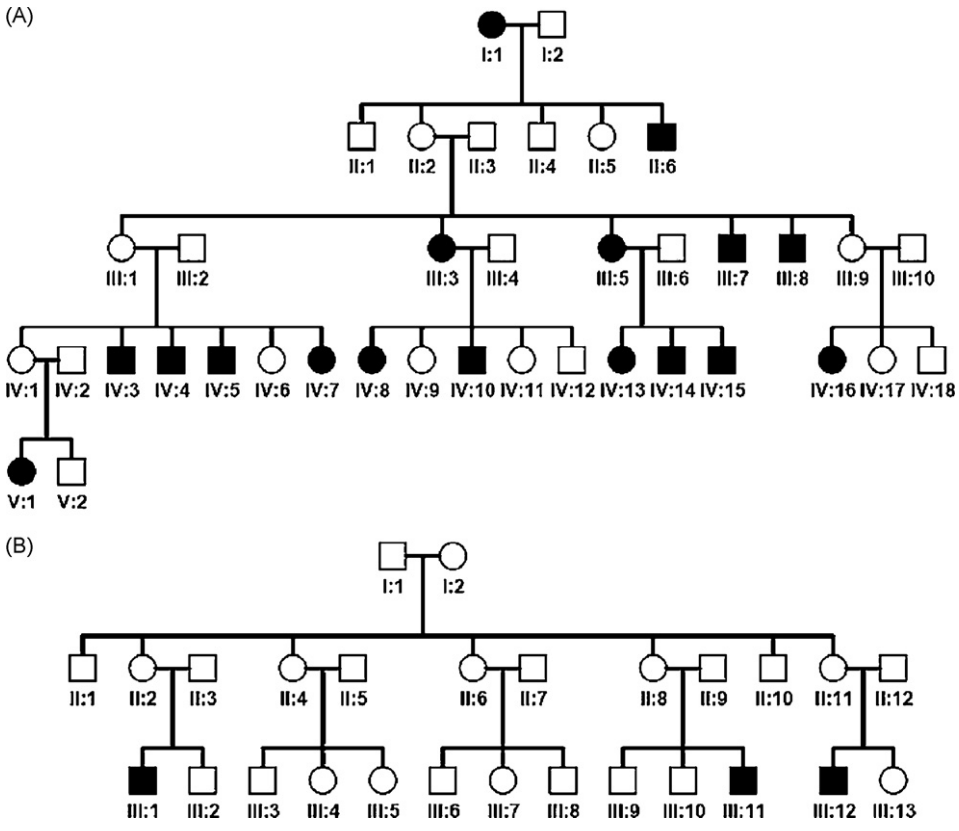

Fig. 1. Striking difference in penetrance of LHON in two Chinese families A (Le1269) and B (Le1244) with G11778A. Affected individuals are marked by filled symbols.

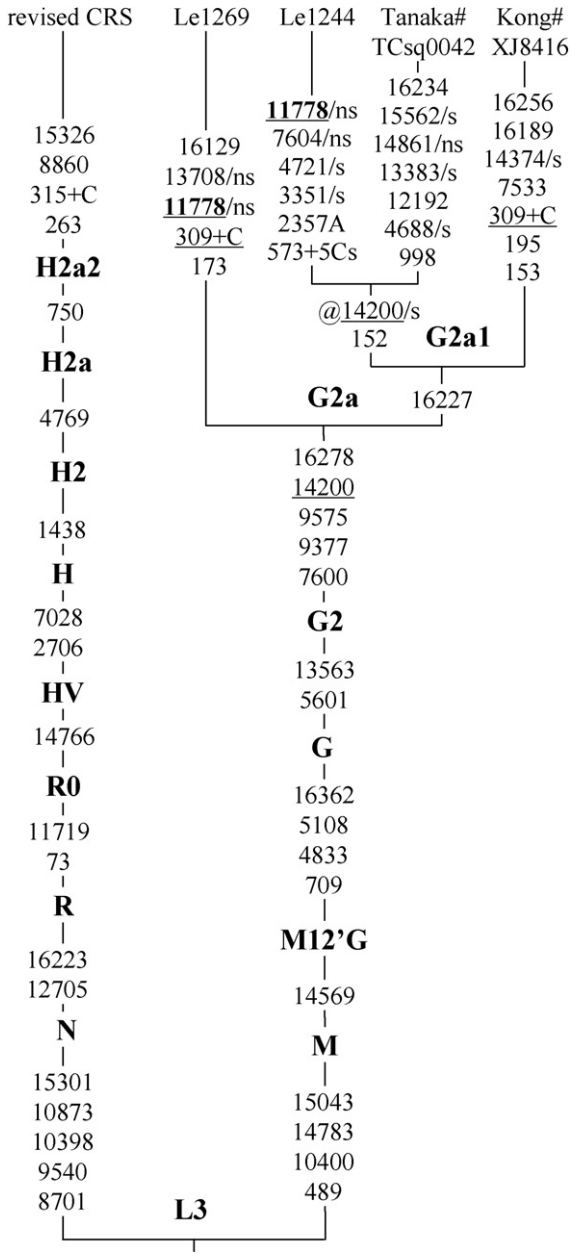

Fig. 2. Classification tree of two complete mtDNAs with G11778A and two reported mtDNAs [16,25] with the same haplogroup status, plus the revised Cambridge Reference Sequence [22]. Haplogroup names are inserted along the branches that determine the locations of the corresponding ancestral haplotypes, following the most recent version of East Asian mtDNA phylogeny [17]. The order of mutations on each uninterrupted branch section is arbitrary. Recurrent mutations are underlined and a back mutation at site 14200 was marked by "@". Suffix "A" refers to transversion and '+C' indicates an insertion of cytosine. The private synonymous and non-synonymous coding-region variants in the four mtDNAs are further denoted by "/s" and "/ns", respectively.

phylogenetic pattern suggested that G11778A in the two LHON families was most likely of independent origin.

4. Discussion

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 [7,9,26–32]. Recently, Hudson et al. identified an X-chromosomal haplotype (DXS8090 [186]-DXS1068 [258]) that interacts with the primary mtDNA mutations to cause LHON [7]. Analysis for a candidate gene close to this region, NDUFB11, failed to show any positive influence on risk and age at onset of visual loss in Italian LHON families [32]. Until now, there has not been a report focused on the influence of nuclear genes in Chinese LHON families. In this study, we analyzed two large Chinese LHON families, which turned out to share the same mtDNA haplogroup background but presented strikingly different penetrance patterns of the disease, to discern genetic effect in the penetrance of LHON. Our hypothesis was that the statistically different penetrance of LHON in the two Chinese families was not caused by other pathogenic (or beneficial) mtDNA mutation(s) that cosegregated (or counteracted) with G11778A, thus supporting a modulation from the nuclear gene(s).

To define the status of a mtDNA mutation in regard to its potential pathogenicity is not easy, especially without evidence from functional experiments [5,19,33]. Evolutionary analysis could serve as a reference to evaluate the potential pathogenicity of mutation [17,19,34], although we should keep in mind that merely evolutionary information may not be always enough [35]. With a considerably large high quality dataset for crosscomparison and a fine-grained phylogenetic tree to start with, one could at least avoid hasty claims for the pathogenicity, as has been exemplified in our previous studies [5,17,19,23].

The direct comparison of the entire mtDNA sequences from families A and B showed that both families belonged to the East Asian specific haplogroup G2a [16,17]. Family B, which had a lower level of penetrance of LHON, harbored more private variants compared with family A. However, the variability of the number of private variants in each mtDNA is within the range that was observed in normal samples. For instance, the Japanese sample TCsq0042 [25] harbored six private coding-region variants, even higher than that of family B (Fig. 2). Conservation analysis and database search indicated that all the variants observed in the two LHON families have been found in published mtDNA sequences (not including transversion C2357A in the 16S rRNA gene) and were not conserved (Fig. 3), therefore should be rather categorized as polymorphisms.

It should be mentioned that variant G13708A detected in family A has previously been described as a secondary mtDNA mutation for LHON, as this variant is one of the specific mutations for haplogroup J, which affects the expression of LHON in western Eurasians [6,8,10,13,36]. Whether this variant (which caused a change from the hydrophobic alanine to the hydrophilic threonine) played a synergistic role with G11778A in family A is an open question. In a previous study, Lodi et al. showed that G13708A did not further impair *in vivo* mitochondrial oxidative metabolism and did not increase the defect in metabolism caused by G11778A [37]. Most recently, the association of haplogroups J1c and J2b with LHON in western Eurasian patients harboring G11778A has been narrowed to specific combinations of amino acid changes in the cytochrome *b* gene and has nothing to do with G13708A [13]. Moreover, G13708A occurs frequently in published East Asian mtDNAs and together with other variants define haplogroups F2, D4b2a1, and D5a1 [17]. In western Eurasians, south Asians, Africans, and aboriginal Australians, this variant has been sporadically found in mtDNAs belonging to haplogroups T2, H5, H2, X2, R5, M5, L3a, L3x, L3h, L2c, L2d, L1c3, L0f, R12, and S (cf. the mtDB database), besides its presence as a haplogroup specific variant in haplogroup J. Evidently, even in humans, this variation is highly recurrent and is unlikely to be a pathogenic mutation. Based on the remarkably different penetrance of LHON in both families, it is apparent that haplogroup G2a might not play an active role in the expression of mutation G11778A in families A and B. The possibility for a synergistic effect between G13708A and a predisposing haplogroup

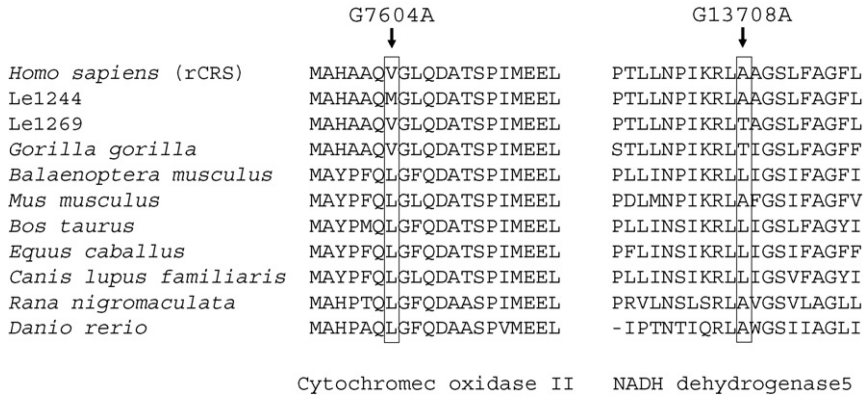

Fig. 3. Evolutionary conservation analysis of the amino acid changes caused by the private nonsynonymous mtDNA variants G7604A and G13708A identified in the two Chinese LHON pedigrees. mtDNAs of eight different vertebrate species, including zebrafish (GenBank accession number NC_002333), frog (AB043889), blue whale (NC_001601), mouse (AY466499), cattle (AY526085), horse (EF597513), dog (DQ480502), and gorilla (NC_001645) were compared to human (J01415).

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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