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Leber's hereditary optic neuropathy is associated with the mitochondrial ND4 G11696A mutation in five Chinese families

Xiangtian Zhou [a,b,1], Qiping Wei [c,1], Li Yang [d,1], Yi Tong [a,e,1], Fuxin Zhao [b], Chunjie Lu [a], Yaping Qian [d], Yanghong Sun [c], Fan Lu [a], Jia Qu [a,b,*], Min-Xin Guan [b,d,f,*]

[a] School of Ophthalmology and Optometry, Wenzhou Medical College, Wenzhou, Zhejiang 325003, China
[b] Zhejiang Provincial Key Laboratory of Medical Genetics, School of Life Sciences, Wenzhou Medical College, Wenzhou, Zhejiang 325003, China
[c] Department of Ophthalmology, Dongfang Hospital, Beijing University of Chinese Medicine and Pharmacology, Beijing 100078, China
[d] Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA
[e] The First Affiliated Hospital, Fujian Medical University, Fuzhou, Fujian 350005, China
[f] Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA

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Abstract

We report here the clinical, genetic, and molecular characterization of five Chinese families with Leber's hereditary optic neuropathy (LHON). Clinical and genetic evaluations revealed the variable severity and age-of-onset in visual impairment in these families. Strikingly, there were extremely low penetrances of visual impairment in these Chinese families. Sequence analysis of the complete mitochondrial genomes in these pedigrees showed the distinct sets of mtDNA polymorphism, in addition to the identical ND4 G11696A mutation associated with LHON. Indeed, this mutation is present in homoplasmy only in the maternal lineage of those pedigrees but not other members of these families. In fact, the occurrence of the G11696A mutation in these several genetically unrelated subjects affected by visual impairment strongly indicates that this mutation is involved in the pathogenesis of visual impairment. Furthermore, the N405D in the ND5 and G5820A in the $tRNA^{Cys}$, showing high evolutional conservation, may contribute to the phenotypic expression of G11696A mutation in the WZ10 pedigree. However, there was the absence of functionally significant mtDNA mutations in other four Chinese pedigrees carrying the G11696A mutation. Therefore, nuclear modifier gene(s) or environmental factor(s) may play a role in the phenotypic expression of the LHON-associated G11696A mutation in these Chinese pedigrees.

Keywords: Leber's hereditary optic neuropathy; Mitochondrial DNA; ND4 G11696A mutation; Penetrance; Vision loss; Chinese

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disorder leading to rapid, painless, bilateral loss of central vision [1–4]. The maternal transmission of visual dysfunction in families with LHON indicates that mutations in mitochondrial DNA (mtDNA) are the molecular basis for this disorder. The sequence analysis of the mitochondrial genome of families with LHON led to the landmark discovery of the ND4 G11778A mutation associated with LHON [5]. Up to date, approximately 25 LHON-associated mtDNA mutations have been identified in various ethnic populations [6–8]. Of these, the ND1 G3460A, ND4 G11778A, and ND6 T14484C mutations, in the genes encoding the subunits of respiratory chain complex I, are the most commonly LHON-associated mtDNA mutations, accounting for ~80–95% of LHON pedigrees in different ethnic backgrounds [2,9–11].

Thus, it is anticipated that additional mutations causing LHON can be found in the mitochondrial genome. To further elucidate the molecular basis of LHON in the Chinese

* Corresponding authors. Fax: +1 513 636 3486 (M.-X. Guan).
E-mail addresses: jqu@wzmc.net (J. Qu), min-xin.guan@cchmc.org (M.-X. Guan).
[1] These authors have equally contributed to this work.

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population, a systematic and extended mutational screening of mtDNA has been initiated in the large clinical population of Ophthalmology Clinic at the Wenzhou Medical College, China [12–14]. In the previous investigation, we showed that the LHON was associated with the ND4 G11778A mutation in five Chinese families with variable penetrance and severity and age-at-onset of visual impairment [12–14]. In the present study, we performed the clinical, genetic, and molecular characterization of another five Chinese families with maternally transmitted LHON. Molecular analysis has led to the identification of G11696A mutation in the ND4 gene in these Chinese families. To elucidate the role of mitochondrial haplotype in the phenotypic manifestation of the G11696A mutation, we performed a PCR-amplification of fragments spanning the entire mitochondrial genome and subsequent DNA sequence analysis in the matrilineal relatives of those Chinese families.

Materials and methods

Patients. As the part of the genetic screening program for visual impairment, five Chinese families (Fig. 1) were ascertained through the School of Ophthalmology and Optometry, Wenzhou Medical College, and Ophthalmology Clinic, Beijing Dongfang Hospital, respectively. Informed consent, blood samples, and clinical evaluations were obtained from all participating family members, under protocols approved by the Cincinnati Children's Hospital Medical Center Institute Review Board and the Wenzhou Medical College Ethics Committee. Members of those pedigrees were interviewed at length to identify both personal and family medical histories of visional impairments and other clinical abnormalities. The 167 control DNA samples used for screening for the presence of mtDNA mutations were obtained from a panel of unaffected individuals from Chinese ancestry.

Ophthalmologic examinations. The ophthalmologic examinations of probands and other members of these families were conducted, including visual acuity, visual field examination (Humphrey Visual Field Analyzer II*i*, SITA Standard), visual evoked potentials (VEP) (Roland Consult RETI port gamma, flash VEP), and fundus photography (Canon CR6-45NM fundus camera). The degree of visual impairment was defined according to the visual acuity as follows: normal > 0.3, mild $= 0.3$–0.1; moderate < 0.1–0.05; severe < 0.05–0.02; and profound < 0.02.

Mutational analysis of the mitochondrial genome. Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kits (Gentra Systems). The presence of G3460A, G11778A, and T14484C mutations was examined as detailed elsewhere [2]. Briefly, affected individuals' DNA fragments spanning these mtDNA mutations were amplified by PCR using oligodeoxynucleotides corresponding to mtDNA at positions 3108–3717 for the G3460A mutation, 11654–11865 for the G11778A mutation, and 14260–14510 for the T14484C mutation [15], respectively. For the detection of G3460A mutation, the PCR-amplified segments were digested with a restriction enzyme *Bsa*HI [2], while the presence of the T14484C mutation was examined by digesting PCR products with a restriction enzyme *Mva*I [2]. For the examination of the G11778A mutation, the PCR-amplified segments were digested with the restriction enzyme *Mae*III [12–14].

The entire mitochondrial genome of five probands was PCR amplified in 24 overlapping fragments using sets of light (L) strand and the heavy (H) strand oligonucleotide primers as described previously [16]. Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3100 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. These sequence results were compared with the updated consensus Cambridge sequence [15]. DNA and protein sequence alignments were carried out using seqweb program GAP (GCG). The allele frequency of G11696A mutation in ND4 gene was determined by PCR amplification of fragments spanning the corresponding regions, using the genomic DNA derived from Chinese controls as templates and performing subsequent sequence analysis of PCR products, as described above.

Results

Clinical presentation

In family WZ7, the proband (IV-11) complained of painless, progressive deterioration of bilateral visual impairment and came to the Ophthalmology Clinic at Beijing Dongfang Hospital at the age of 17. Ophthalmological

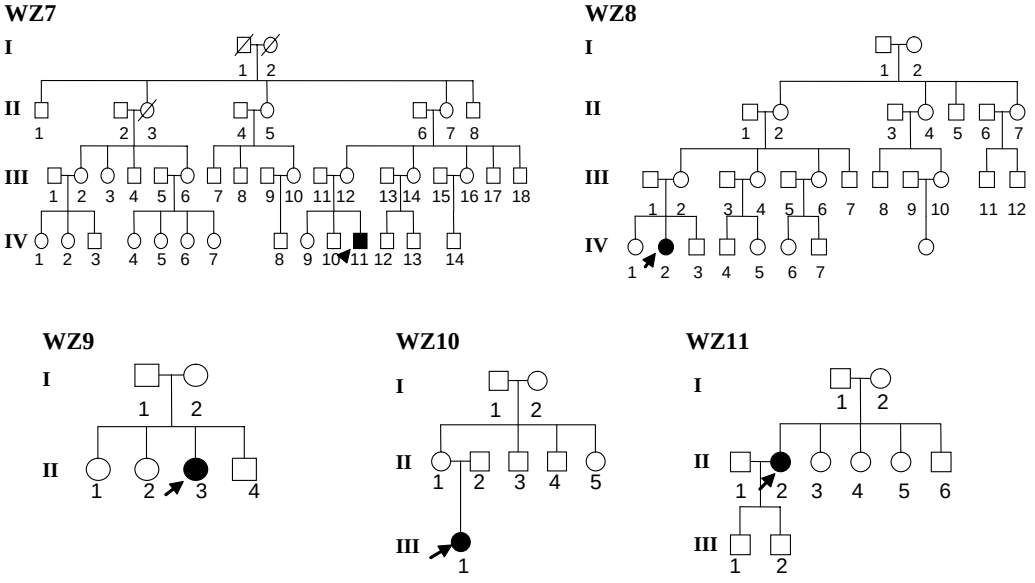

Fig. 1. Five Chinese pedigrees with Leber's hereditary optic neuropathy. Vision impaired individuals are indicated by filled symbols.

evaluation showed that his visual acuity was 0.05 in the right eye and 0.03 in the left eye. Fundus examination showed that both his temporal optic disks were pale and reflex on fovea centralis was normal. Intraocular pressure (IOP) is 14 mmHg in the right eye and 12 mmHg in the left eye. Visual field testing demonstrated large centrocecal scotomata in both his eyes. Therefore, he exhibited a typical clinical feature of LHON. No other abnormality was found on radiological and neurological examination. Furthermore, he had no other significant medical history. The family originated from Shandong Province in Eastern China. However, none of other 32 matrilineal relatives in this family exhibited visual impairment.

In WZ8 pedigree, the proband (IV-2) came to the Ophthalmology clinic at the Beijing Dongfang Hospital at the age of 15. She suffered from painless, progressive deterioration of bilateral visual impairment at the age of 14. Her visual dysfunction occurred within a month, first in the left eye and then four weeks later in the right eye. She saw a dark cloud in the center of vision and had tinnitus. Her visual acuity was 0.05 in the right eye and 0.1 in the left eye. Furthermore, she had normal color vision and movement in both eyes. Fundus examination showed that both her optic disks were abnormal: vascular tortuosity of the central retinal vessels, a circumpapillary telangiectatic microangiopathy, and no reflex on fovea centralis. IOP was 17 mmHg in the right eye and 16 mmHg in the left eye. Encephalon CT showed sphenoiditis. Visual field testing revealed large centrocecal scotomata in both her eyes. The flash VEP showed bilaterally decreased amplitudes with delayed latencies. Thus, she showed a typical clinical feature of LHON. She had no other significant medical history. The family originated from the Shanxi Province in Northwestern China. Further familiar history and clinical evaluation revealed that none of other 20 matrilineal relatives in this family exhibited a visual deficit.

In family WZ9, the proband III-3 came to the Ophthalmology clinic at the Wenzhou Medical College at the age of 19. She exhibited bilateral visual impairment at the age of 18. Her visual acuity was 0.1 in the right eye and 0.08 in the left eye. Clinical evaluation revealed that she had a typical clinical feature of LHON. The family, as shown in Fig. 1, originated from Shandong Province in Eastern China. Further familial history and clinical evaluation revealed that all other matrilineal relatives in this family exhibited normal vision.

In family WZ10, the proband (III-1) was diagnosed as LHON by Ophthalmology clinic at the Wenzhou Medical College at the age of 8. She began suffering bilateral visual impairment at the age of 7. Ophthalmological examination showed that her visual acuity was 0.12 in the right eye and 0.4 in the left eye. Visual field testing demonstrated large centrocecal scotomata in both her eyes. None of other matrilineal relatives in this family living in Shandong Province in Eastern China had visual deficit.

In family WZ11, the proband (II-2) was female of 38 years. She exhibited bilateral visual impairment at the age of 38. She was diagnosed as LHON by the Ophthalmology clinic at the Wenzhou Medical College. Her visual acuity was 0.1 in both eyes, respectively. Visual field testing showed large centrocecal scotomata in both her eyes. The flash VEP showed bilaterally decreased amplitudes with delayed latencies. However, other matrilineal relatives in this family living in the Shandong Province in Eastern China have normal vision.

Furthermore, there is no evidence that any member of those families had any other known cause to account for visual impairment. Comprehensive family medical histories of these individuals showed no other clinical abnormalities, including diabetes, muscular diseases, hearing impairment, and neurological disorders.

Mitochondrial DNA analysis

To elucidate the molecular basis of visual impairment, we have performed a mutational analysis of the mitochondrial genome in these families. First, we examined three commonly known LHON-associated mtDNA mutations (G3460A, G11778A, and T14484C) by PCR amplification and subsequent restriction enzyme digestion analysis of PCR fragments derived from each proband of those families. We failed to detect the presence of G3460A, G11778A, and T14484C mutations. We then performed a PCR-amplification of fragments spanning entire mitochondrial genome and subsequent DNA sequence analysis in these probands. Interestingly, the G-to-A transition at position 11696 (G11696A) in the ND4 gene resulting in the substitution of an isoleucine for valine at amino acid position 312 has been found in those subjects. Indeed, this mutation has been associated with LHON and hereditary spastic dystonia in a large Dutch family [17]. Further sequence analysis, as shown in Fig. 2, confirmed the presence of the homoplasmic G11696A mutation in other matrilineal relatives of these families but not other members of these families. The allele frequency analysis of G11696A mutation showed that one (25-year-old male) of 167 unrelated Chinese control subjects carried the G11696A mutation.

In addition to the identical G11696A mutation, as shown in Table 1, these subjects exhibited distinct sets of mtDNA polymorphism. Of other nucleotide changes in these mitochondrial genomes, there are 22 variants in the D-loop, 25 silent mutations in the protein encoding genes, five variants in 12S rRNA gene, and two variants in the 16S rRNA gene [8]. Five probands shared 17 known variants including C5178A (L237M) in ND2, A8701G (T59A), and A8860G (T112A) in the A6 gene, and A10398G (T114A) and the A15326G (T164A) in the Cyt *b* gene. Furthermore, four porbands (WZ7, WZ9, WZ10, and WZ11) shared the C8414T (L17F) variant in the A8 gene. Interestingly, WZ8 pedigree carried two known missense variants A5301G (I278V) in the ND2 gene and A12026G (I423V) in the ND4 gene, and two novel variants G11330C (A191F) in the ND4 gene and C15891G in the $tRNA^{Thr}$ gene. In addition, WZ10 pedigree harbored two novel mis-

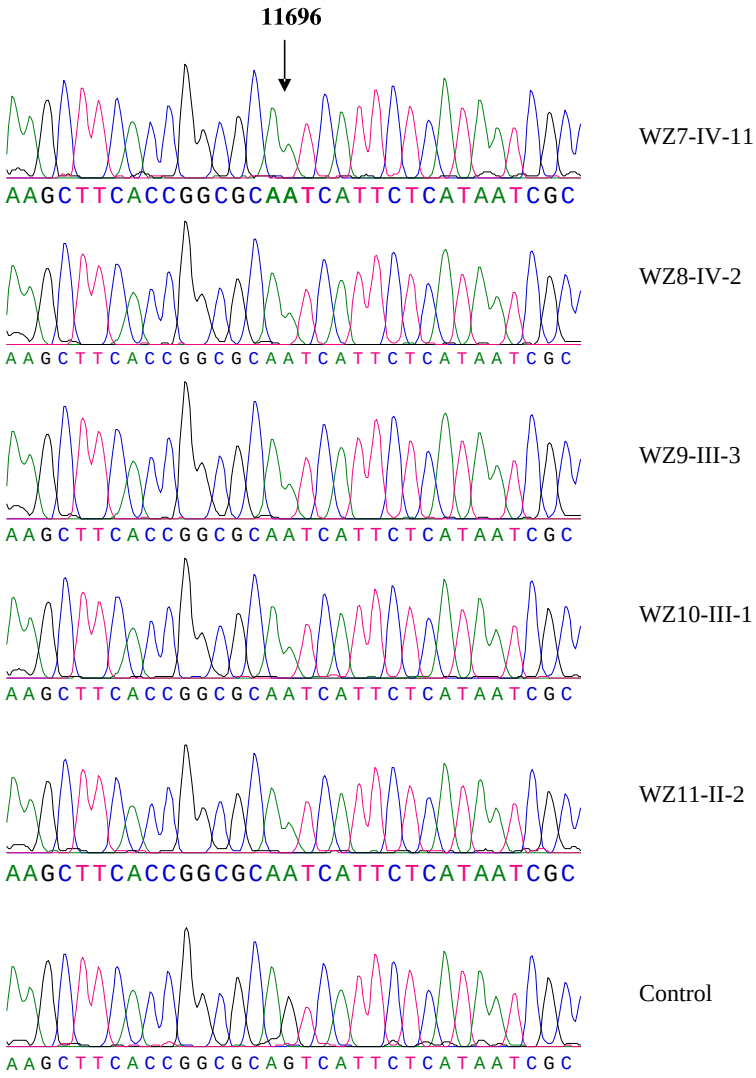

Fig. 2. Identification of the G11696A mutation in the ND4 gene. Partial sequence chromatograms of ND4 gene from five affected individuals and one Chinese control. An arrow indicates the location of the base changes at position 11696.

sense variants: A13549G (N405D) in the ND5 gene and G5820A in the $tRNA^{Cys}$ gene. Furthermore, WZ9 proband contained the novel variant A15656G (I204V) in the Cyt *b* gene.

These RNA and protein variants were further evaluated by phylogenetic analysis of these variants and nucleotides or residues from other organisms. In fact, the N405D in the ND5 and the G5820A in the $tRNA^{Cys}$ in the WZ10 pedigree are localized at sites which are highly conserved from human [16], mouse [18], bovine [19], and *Xenopus laevis* [20], whereas the sites of other missense variants in protein encoding genes, C15891G variant in $tRNA^{Thr}$, the five variants in the 12S rRNA, and two variants in 16S rRNA, are not evolutionarily conserved.

Discussion

In the present study, we have performed the clinical, genetic, and molecular characterization of five Chinese families with Leber's hereditary optic neuropathy. The bilateral visual impairment as a sole clinical phenotype was only present in the maternal lineage of those pedigrees, suggesting that the mtDNA mutation is the molecular basis for this disorder. Sequence analysis of the complete mitochondrial genomes in these pedigrees showed the distinct sets of mtDNA polymorphism, in addition to the identical G11696A (V312I) mutation in ND4. Indeed, this mutation is present in homoplasmy only in the maternal lineage of those pedigrees but not other members of these families. The valine at position 312 in the ND protein is located in the predicted transmembrane region, 28 amino acids aminoterminal to the R340H LHON mutation [21]. This LHON-associated mtDNA mutation was first identified to be heteroplasmy in a large Dutch family [17]. In fact, the occurrence of the G11696A mutation in these several genetically unrelated subjects affected by visual impairment strongly indicates that this mutation is involved in the pathogenesis of visual impairment.

Table 1
mtDNA mutations in five chinese families with LHON

Gene	Position	Replacement	Conservation (H/B/M/X)[a]	CRS[b]	WZ7	WZ8	WZ9	WZ10	WZ11	Previously reported[c]
D-Loop	73	A to G		A	G	G	G	G	G	Yes
	150	C to T		C		T				Yes
	152	T to C		T		C				Yes
	200	A-to-G		A		G				Yes
	263	A to G		A	G	G		G	G	Yes
	310	CTC or TCC		T	CTC	TCC		TCC	CTC	Yes
	489	T-to-C		T	C	C	C	C	C	Yes
	514	del C		C		del C				Yes
	515	del A		A		del A				Yes
	16164	A to G		A		G				Yes
	16172	T-to-C		T		C				Yes
	16182	A-to-C		A		C				Yes
	16183	A-to-C		A		C				Yes
	16184	C to T		C	T					Yes
	16188	C-to-T		C			T			Yes
	16189	T-to-C		T		C				Yes
	16214	C to T		C			T			Yes
	16223	C to T		C	T	T	T	T		Yes
	16266	C to T		C		T				Yes
	16311	T-to-C		T	T			C		Yes
	16362	T to C		T	C		C	C		Yes
	16519	T to C		T			C	C		Yes
12S rRNA	689	T to G	T/T/T/-	T	G					No
	722	C to T	C/T/A/-	C	T					No
	752	C to T	A/A/A/G	C		T				Yes
	1107	T to C	T/C/T/T	T		C				Yes
	1438	A to G	A/A/A/G	A	G		G	G	G	Yes
16S rRNA	2706	A to G	A/G/A/A	A	G	G	G	G	G	Yes
	3010	G to A	G/G/A/A	G	A		A	A	A	Yes
ND2	4769	A to G		A	G	G	G	G	G	Yes
	4883	C to T		C	T	T	T	T	T	Yes
	5178	C to A (Leu to Met)	L/T/T/T	C	A	A	A	A	A	Yes
	5301	A to G (Ile to Val)	I/I/M/L	A		G				Yes
$tRNA^{Cys}$	5820	G to A	G/G/G/G	G				A		No
CO1	7028	C to T		C	T	T	T	T	T	Yes
CO2	7768	A to G		A			G			Yes
	7897	G to A		G			A			No
	8020	G to A		G	A					Yes
A8	8414	C to T (Leu to Phe)	L/F/M/W	C	T		T	T	T	Yes
	8512	A to G		A		G				Yes
A6	8701	A to G (Thr to Ala)	T/S/L/Q	A	G	G	G	G	G	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	A	G	G	G	G	G	Yes
	9180	A to G		A		G				Yes
CO3	9540	T to C		T		C	C	C		Yes
ND3	10397	A to G		A		G				Yes
	10398	A to G (Thr to Ala)	T/T/T/A	A	G	G	G	G	G	Yes
	10400	C to T		C	T	T	T	T	T	Yes
ND4	10873	T to C		T	C	C		C	C	Yes
	11239	T to C		A			G			No
	11330	G to C (Ala to Pro)	A/S/S/A	G		C				No
	11696	G to A (Val to Ile)	V/T/T/M	G	A	A	A	A	A	Yes
	11719	G to A		G	A	A	A	A	A	Yes
	11914	G to A		G					A	Yes
	11944	T to C		T		C				Yes
	12026	A to G (Ile to Val)	I/I/M/L	A		G				Yes
ND5	12705	C to T		C	T	T	T	T	T	Yes
	13020	T to C		T			C			Yes
	13549	A to G (Asn to Asp)	N/N/N/N	A				G		No

(*continued on next page*)

Table 1 (*continued*)

Gene	Position	Replacement	Conservation (H/B/M/X)[a]	CRS[b]	WZ7	WZ8	WZ9	WZ10	WZ11	Previously reported[c]
	13608	T to C		T				C		No
ND6	14668	C to T		C	T		T	T	T	Yes
Cyt *b*	14783	T to C		T	C	C	C	C	C	Yes
	15043	G to A		G	A	A	A	A	A	Yes
	15301	G to A		G	A	A	A	A	A	Yes
	15326	A to G (Thr to Ala)	T/M/I/I	A	G	G	G	G	G	Yes
	15529	C to A		C			A			No
	15656	A to G (Ile to Val)	I/I/M/M	A			G			No
	15670	T to C		T			C			Yes
$tRNA^{Thr}$	15891	C to G	C/T/T/C	C		G				No

[a] Conservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X).
[b] CRS, Cambridge reference sequence [15].
[c] See the online mitochondrial genome database http://www.mitomap.org.

In contrast to our previous data that there is a high penetrance of visual loss in Chinese pedigrees carrying the G11778A mutation [12–14], the penetrances of visual impairment in these five Chinese families carrying the G1696G mutation were extremely low. In particular, only one matrilineal relative of each Chinese pedigree exhibited visual impairment. In addition, one of 167 Chinese subjects carried this mutation. Furthermore, as shown in Table 2, the age-at-onset for vision loss in those subjects varied from 7 to 36 years, with an average of 18.6. These data are comparable with our previous observation that the age-at-onset for visual loss in four Chinese families carrying the G11778A mutation is 17, 20, 18, and 22 years, respectively [12–14]. In addition, the severity of visual impairment in those affected subjects varied from mild to moderate to severe. Furthermore, biochemical characterization showed that there were reduced activities of the respiratory-chain complexes, especially in the complex I activity in a muscle biopsy derived from one affected subject of the Dutch family [17]. These biochemical data seem to support the genetic evidence that the G11696A mutation is a primary mutation underlying the development of LHON.

The extremely low penetrance of visual loss, the mild biochemical defect and the presence of one/167 controls indicated that the G11696A mutation itself is not sufficient to produce the clinical phenotype. Thus, the modifier factors including nuclear backgrounds, other environmental factors, and mitochondrial haplotypes are necessary for the phenotypic manifestation of the G11696A mutation. In particular, the mitochondrial haplotypes have been shown to influence the penetrance and expressivity of visional loss associated with primary mtDNA mutations. mtDNA mutations at positions 4216 and 13708 labeled as second LHON mutations were implicated to increase the penetrance of the LHON-associated ND4 G11778A mutation and ND6 T14484C mutation [22]. Furthermore, the mitochondrial haplogroup J can influence the phenotypic manifestation of the primary LHON G11778A and T14484C mutations in a very large cohort of families of European ancestry [23,24]. Strikingly, these five Chinese pedigrees shared the identical seventeen mtDNA variants. Indeed, these mitochondrial genomes of WZ7, WZ9, WZ10, and WZ11 pedigrees belong to Eastern Asian haplogroup D4, while mtDNA of WZ8 pedigree belong to haplogroup D5b [25]. This implied that the G11696A mutation in those Chinese families may originate from a common ancestry, in contrast with the fact that the G11778A mutation occurred sporadically and multiplied through evolution of the mtDNA [13,14,22,24]. The N405D in the ND5 and G5820A in the $tRNA^{Cys}$, showing high evolutional conservation, may contribute to the phenotypic expression of the G11696A mutation in the WZ10 pedigree. However, there was the absence of functionally significant mutations in tRNA and rRNAs or secondary LHON mutations in Chinese pedigrees WZ7, WZ8, WZ9, and WZ11. Thus, these variants may not have a potential modifying role in the development of visual

Table 2
Summary of clinical and molecular data for affected matrilineal relatives of five Chinese families with LHON

Subjects	Sex	Age of test (years)	Age of onset (years)	Visual acuity right	Visual acuity left	Level of visual impairment	Number of matrilineal relatives	mtDNA haplogroup
WZ7-IV-11	M	17	17	0.05	0.03	Severe	33	D4
WZ8-IV-2	F	15	14	0.05	0.1	Moderate	21	D5a
WZ9-III-3	F	19	18	0.1	0.08	Moderate	5	D4
WZ10-III-1	F	8	7	0.12	0.4	Mild	6	D4
WZ11-II-2	F	38	36	0.1	0.1	Moderate	8	D4

impairment associated with G11696A mutation in those families. Therefore, nuclear modifier gene(s) or environmental factor(s) may play a role in the phenotypic expression of the LHON-associated G11696A mutation in these Chinese pedigrees.

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