

Original Article

The mitochondrial *ND5* T12338C mutation may influence the phenotypic manifestation of Leber's hereditary optic neuropathy-associated *ND4* G11696A mutation

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Abstract: Mutations in mitochondrial genome are the important causes of Leber's Hereditary Optic Neuropathy (LHON). To investigate the pathophysiology of LHON, we recently initiated a systematic mutational screening for the candidate pathogenic mutations in mitochondrial genome. In this study, we described a Chinese family with LHON. Four of nine matrilineal relatives exhibited variable degree of vision loss, as well as different age at onset of LHON. Sequence analysis of the complete mitochondrial genome of the proband and the matrilineal relatives showed the presence of *ND4* G11696A (Val to Ile) and *ND5* T12338C (Met to Thr) mutations, as well as a set of polymorphisms belonging to human mitochondrial haplogroup F2. Of these, the G11696A was a primary mutation associated LHON and reported to modulate the clinical expression of deafness-associated A1555G mutation, in addition, the T12338C mutation was known to decrease the *ND5* mRNA level and to inhibit the processing of RNA precursors. Thus, the T12338C mutation, acted a modified factor, may increase the clinical expression of LHON-associated *ND4* G11696A mutation in this Chinese family.

Keywords: LHON, mtDNA mutations, G11696A, T12338C, modifier, Chinese family

Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited eye disease that generally affects children to young adults with the rapid, painless, bilateral loss of central vision [1, 2]. The maternal transmission of visual dysfunction in families with LHON indicates that mutations in mitochondrial DNA (mtDNA) are the molecular basis for this disorder. Of these, the *ND1* G3460A, *ND4* G11778A and *ND6* T14484C mutations, which involve genes encoding the subunits of respiratory chain complex I, account for approximately 90% of LHON pedigrees [3-5]. These LHON-associated mtDNA mutations often present near or at homoplasm. Typical features in LHON pedigrees are incomplete penetrance and male bias among the affected subjects, reflecting the complex etiology of this disease [6]. Matrilineal relatives within and among families, despite carrying the

identical LHON-associated mtDNA mutations, exhibited a wide range of severity, age-of-onset and penetrance of optic neuropathy. Therefore, other modifier factors including nuclear modifier genes, mitochondrial haplotypes, epigenetic factors and environmental factors should modulate the phenotypic manifestation of LHON-associated with those primary mtDNA mutations [7, 8]. In particular, a group of secondary LHON-associated mtDNA mutations such as T4216C, A4917G and G13708A, haplogroups J, M7b and M8a were implicated as influencing the phenotypic manifestation of the primary mtDNA mutations, including the G11778A and T14484C in Caucasian and Chinese families [9, 10].

With the aim of investigating the molecular basis of LHON in the Chinese population, a systematic and extended mutational screening of mtDNA has been initiated in Shenzhen People's

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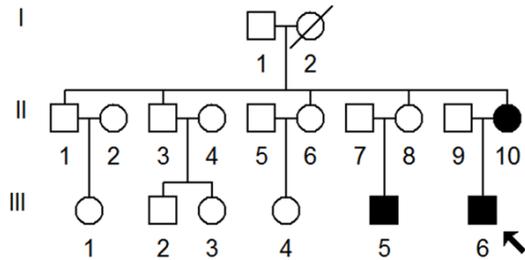


Figure 1. One Han Chinese family with LHON, vision impaired individuals are indicated by filled symbols, arrow indicates the proband.

Hospital. In the present study, we described a Han Chinese family with LHON, molecular analysis of the mitochondrial genome led us to identify the *ND4* G11696A and *ND5* T12338C mutations.

Materials and methods

Patients

As the part of the genetic screening program for visual impairment, one Chinese family (LS-201), as shown in **Figure 1**, was ascertained the Department of Ophthalmology, Shenzhen People's Hospital. Informed consent, blood samples, and clinical evaluations were obtained from all participating family members, under protocols approved by the Shenzhen People's Hospital. Members of this pedigree were interviewed at length to identify both personal and family medical histories of visual impairments and other clinical abnormalities. The 250 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 ophthalmic examinations of probands and other members of this family were conducted, including visual acuity, visual field examination (Humphrey Visual Field Analyzer II-i, SITA Standard; Carl Zeiss Meditec, Oberkochen, Germany), visual evoked potentials (VEP; RETI port gamma, flash VEP; Roland Consult, Brandenburg, Germany), and fundus photography (CR6-45NM fundus camera; Canon, Lake Success, NY). The degree of visual impairment was defined according to the visual acuity as follows: healthy greater than 0.3, mild equaled

0.3 to 0.1, moderate less than 0.1 to 0.05, severe less than 0.05 to 0.02, and profound less than 0.02.

Mutational analysis of mitochondrial genome

Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kits (Gentra Systems). The entire mitochondrial genome of the proband and matrilineal relatives were PCR amplified in 24 overlapping fragments using sets of the light (L) strand and the heavy (H) strand oligonucleotide primers as described previously [11]. Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. These sequence results were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_012920) [12].

Phylogenetic analysis

The mtDNA sequences of 17 different vertebrates were used in the interspecific analysis. The conservation index (CI) was calculated by comparing the human nucleotide variants with the other 16 vertebrates. The CI was then defined as the percentage of species from the list of 16 different vertebrates that have the wild-type nucleotide at that position. We regarded the $CI \geq 70\%$ as having functional potential [13].

Analysis of mtDNA haplogroup

The entire mtDNA sequence of the proband and the matrilineal relatives were assigned to the Asian mitochondrial haplogroups by using the nomenclature of mitochondrial haplogroups [14].

Results

Clinical characterization of the Han Chinese family with LHON

In this family (LS201), the proband (III-6) was a 20-year-old man who lived in Shenzhen area of Guangdong Province. He began to suffer to suffer bilateral vision loss when he was 17 years. He saw a dark cloud in the center of vision and had problems appreciating colors that all seemed a dark gray. As shown in **Table 1**, visual

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Table 1. Summary of clinical data for affected relatives in this family with LHON

Subjects	Gender	Age of test (year)	Age of onset (year)	Visual Acuity Right	Visual Acuity Left	Level of Visual impairment
II-10	Female	51	48	0.05	0.05	Moderate
III-5	Male	23	20	0.04	0.03	Severe
III-6	Male	20	17	0.02	0.03	Severe
II-9	Male	48	/	0.4	0.6	Normal

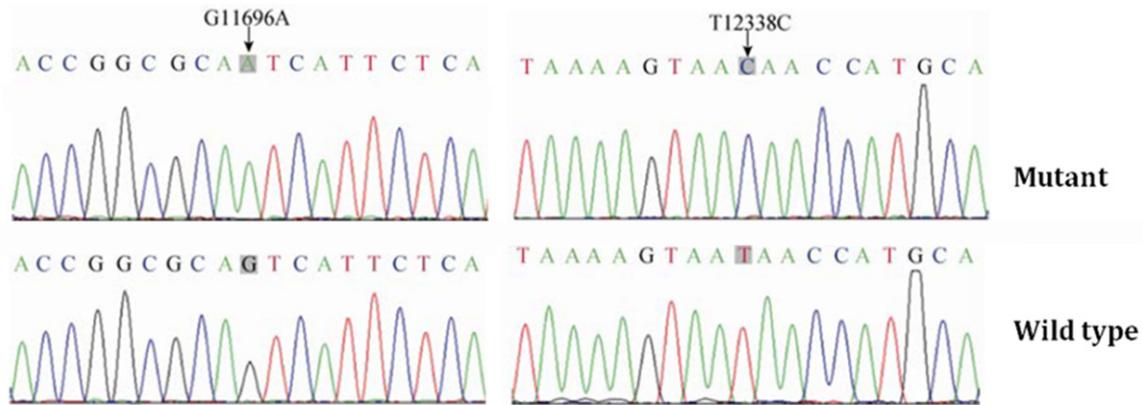


Figure 2. Identification of the *ND4* G11696A and *ND5* T12338C mutations. Partial sequences chromatograms of *ND4* and *ND5* genes were derived from the proband and the control subject. Arrows indicate the positions of 11696 and 12338.

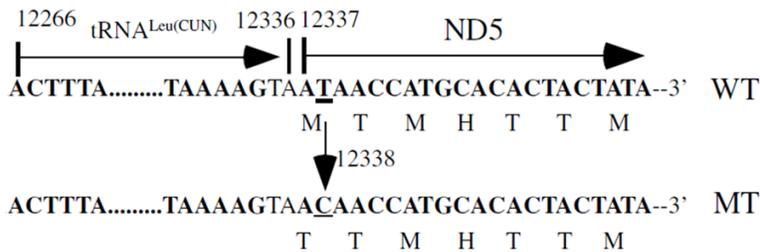


Figure 3. Scheme of the mitochondrial DNA sequence at position 12338 and the adjacent sequence of *ND5* and *tRNA^{Leu(CUN)}* in the wild and mutant types. Arrows indicate the position of the T12338C mutation. WT, wild-type; MT, mutant type.

acuity was 0.02 and 0.03 in his right and left eyes, respectively. 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. Among other matrilineal relatives, subject III-5 experienced loss of vision at the age of 20 years. Further familiar history and clinical evaluation revealed that none of other matrilineal relatives in this family exhibited a visual deficit.

MtDNA sequence analysis

To elucidate the molecular basis of visual impairment, we performed a mutational analysis of the mitochondrial genome in this family. We firstly examined the 3 common LHON-associated mtDNA mutations (G3460A, G1177-8A and T14484C) by PCR amplification and direct sequencing. However, we failed to detect the presence of these

primary mutations in the proband and matrilineal relatives in this family. We then performed PCR amplification of fragments spanning the entire mitochondrial genome and subsequently sequenced the PCR products. As shown in **Figure 2**, the homoplasmic *ND4* G11696A and *ND5* T12338C mutations were identified in these subjects. Notably, the known T12338C mutation in the *ND5* gene caused the replacement of the first amino acid, translation initiating methionine with a threonine in the *ND5* polypeptide (**Figure 3**). In addition, the G to A

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Table 2. MtDNA sequence variants in this family with LHON

Gene	Position	Replacement	Conservation (H/B/M/X) ^a	Previously reported ^b
D-loop	73	A to G		Yes
	103	G to A		Yes
	146	T to C		Yes
	263	A to G		Yes
	310	T to TC		Yes
	489	T to C		Yes
	16182	A to C		Yes
	16189	T to C		Yes
	16266	C to T		Yes
	16519	T to C		Yes
12S rRNA	1107	T to C		Yes
	1438	A to G		Yes
16S rRNA	2706	A to G		Yes
	3010	G to A		Yes
ND1	4071	C to T		Yes
ND2	4769	A to G		Yes
	5442	T to C (Phe to Leu)	F/F/M/L	Yes
CO1	6357	C to T		Yes
	7028	C to T		Yes
A6	8701	A to G (Thr to Ala)	T/S/L/Q	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	Yes
ND3	10398	A to G (Thr to Ala)	T/T/T/A	Yes
ND4	10861	T to C		Yes
	11696	G to A (Val to Ile)	V/T/T/M	Yes
	11719	G to A		Yes
ND5	12338	T to C (Met to Thr)	M/M/M/M	Yes
	13928	G to C (Ser to Thr)	S/T/S/T	Yes
ND6	14311	T to C		Yes
Cytb	14766	C to T (Thr to Ile)	T/S/T/S	Yes
	15301	G to A		Yes
	15326	A to G (Thr to Ala)	T/M/I/I	Yes

^aConservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M) and *Xenopus laevis* (X). ^bSee the online mitochondrial genome database (<http://www.mitomap.org>).

translation at position 11696 in *ND4* gene resulting in the substitution of an isoleucine for valine at amino acid position 313 had been found in these matrilineal relatives. In fact, this mutation had been associated with LHON and hereditary spastic dystonia in a large Dutch family [15]. The allele frequency analysis showed that T12338C and G11696A mutations were absent in 250 unrelated Chinese controls with normal vision.

To assess the contribution of mtDNA variants in the clinical expression of LHON, we performed

a PCR amplification of fragments spanning the entire mitochondrial genome and sequenced the PCR products. In addition to the identical T12338C and G11696A mutations, as shown in **Table 2**, the proband and the matrilineal relatives exhibited distinct set of polymorphisms belonging to human mitochondrial haplogroup F2 [14]. Among these, there were 10 variants in D-Loop region, 2 known variants in 12S rRNA gene and 2 variants in 16S rRNA gene, while other variants were mainly localized at protein-coding genes. In addition to the G11696A and T12338C mutations, there were 6 missense mutations, included the *ND2* T5442C (Phe to Leu), A6 A8701G (Thr to Ala) and A8860G (Thr to Ala), *ND3* A10398G (Thr to Ala), *Cytb* C14766T (Thr to Ile) and A15326G (Thr to Ala). These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis and sequences from other organisms including mouse [16], bovine [17] and *Xenopus laevis* [18]. Nevertheless, none of other variants showed evolutionary conservation, except for the T12338C and G11696A mutations.

Discussion

In the present study, we have performed the clinical, genetic, and molecular characterization of a Han Chinese family with LHON. Visual impairment as a sole clinical phenotype was only present in the maternal lineage of this pedigree, suggesting that mtDNA mutation may be the molecular basis for this disorder. The variable severity and age-of-onset in visual impairment were observed in the matrilineal relatives, although they exhibited the rapid, painless, bilateral loss of central vision. Sequence analysis of the complete mitochondrial genome showed a set of polymorphisms belonging to human mitochondrial haplogroup F2 [14], in addition to the identical *ND5* T12338C (Met to Thr) and *ND4* G11696A (Val to Ile) mutations. The following evidence suggested that the T12338C was a primary pathogenic mtDNA

Table 3. Summary of clinical and molecular data for 7 Chinese families carrying the *ND4* G11696A mutation

Pedigree	No. of matrilineal relatives	Ratio of affected male and female	Average age at onset (Year)	Penetrance	mtDNA haplogroup
LS201	9	2:1	28	33%	F2
WZ12	30	7:3	18	33%	D4
WZ7	33	1:0	17	3%	D4
WZ8	21	0:1	15	4%	D5a
WZ9	5	0:1	19	20%	D4
WZ10	6	0:1	8	16%	D4
WZ11	8	0:1	38	12%	D4

mutation that caused a genetic predisposition to optic neuropathy. Firstly, this mutation was present only in the matrilineal relatives in this pedigree, but was absent in 250 healthy controls. Secondly, the T12338C mutation resulted in the replacement of the first amino acid, translation-initiating methionine with a threonine in the *ND5* polypeptide (**Figure 3**). Notably, the first methionine in *ND5* polypeptide was an extraordinarily conserved residue in every sequenced *ND5* from bacteria to human mitochondria [19]. Therefore, the truncated *ND5* protein was expected to be shortened by 2 amino acids. Moreover, the *ND5* T12338C mutation was localized in 2 nucleotides adjacent to the 3' end of the tRNA^{Leu (CUN)} [12]. As result, the *ND5* T12338C mutation, which was similar to the *ND1* T3308C mutation, may cause a decrease in *ND5* mRNA level as well as altering the processing of RNA precursors [20], thereby leading to a reduction in tRNA^{Leu (CUN)} level. Recent experimental studies indicated that the T12338C mutation was associated with ovarian carcinomas [21], deafness [19], polycystic ovary syndrome and insulin resistance [22].

In addition to the T12338C mutation, the G11696A mutation was also observed in the matrilineal relatives in this LHON family. Indeed, the valine at position 313 in the *ND* protein was located in the predicted transmembrane region, 28 amino acids aminoterminal to the R340H LHON mutation [23]. The G11696A mutation was first identified in a large Dutch family and subsequently in 6 Chinese families [24, 25]. 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 [15]. These biochemical data seemed to support the genetic evidence that the G11696A mutation was a primary mutation underlying the development of LHON.

In this family, the average age at onset of vision impairment ranged from 17 to 48 years, with an average of 28 years old. The average age-at-onset for visual loss in matrilineal relatives of this family, as shown in **Table 3**, was compared with those in other families carrying the *ND4* G11696A mutation [24, 25]. The average-age-at-onset for visual impairment varied from 8 years to 38 years, with an average of 20 years. Furthermore, the penetrances of visual impairment were from 3% to 33%, with the average of 17% in these Chinese pedigrees with G11696A mutation [24, 25]. However, incompletely penetrance of visual loss, and the mild biochemical defect indicated that the G11696A mutation itself was not sufficient to produce the clinical phenotype, thus, other modifier factors including the nuclear genes, environmental factors and mitochondrial haplotypes may contribute to the clinical expression of LHON. Taken together, our data indicated that the mitochondrial haplogroup F2 specific T12338C mutation may increase the phenotypic manifestation of LHON-associated *ND4* G11696A mutation in this Chinese family.

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Disclosure of conflict of interest

None.

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