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Leber's Hereditary Optic Neuropathy—The Spectrum of Mitochondrial DNA Mutations in Chinese Patients

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Purpose: To investigate the spectrum of mitochondrial DNA (mtDNA) mutations in Chinese patients with Leber's hereditary optic neuropathy (LHON), optic atrophy of unknown etiology, and optic neuropathy of known etiology.

Methods: Twenty-seven patients from 25 LHON pedigrees, 22 patients with bilateral optic atrophy of unknown etiology, 21 patients with optic neuropathy of known etiology, and 25 normal healthy controls were included in this study. Twelve pairs of primers that covered the 21 reported mtDNA mutations were utilized. Single-strand conformation polymorphism analysis and DNA sequencing were used to detect base substitutions in mtDNA.

Results: Twenty-three LHON pedigrees (92%) had the 11778 mtDNA primary mutation. Two pedigrees (8%) had the 14484 mutation. No 3460 mutations were detected in this group. Thirteen other sequence changes were found in these patients, but only the 4216 mutation had been reported previously. Thirteen pedigrees had multi-mutation patterns consisting of one primary mutation together with other sequence changes. No primary mutations were found in patients with optic atrophy of unknown etiology or in patients with optic neuropathy of known etiology.

Conclusions: High frequency of 11778 mtDNA mutation was found in Chinese patients with LHON. No specific multi-mutation pattern such as the European mtDNA haplogroup J was found. **Jpn J Ophthalmol 2002;46:45–51** © 2002 Japanese Ophthalmological Society

Key Words: Haplogroup, Leber's hereditary optic neuropathy, mitochondrial DNA, mutation spectrum.

Introduction

Leber's hereditary optic neuropathy (LHON) is an inherited form of bilateral optic atrophy in which the primary etiologic factor is a mutation in the mitochondrial genome. It is characterized by acute bilateral blindness predominantly in healthy young men. In 1988, Wallace et al[1] first identified a point mutation of mitochondrial DNA (mtDNA). They found that Leber's patients carried a G to A point mutation at nucleotide position 11778 that converts the highly conserved 340th amino acid from arginine to histidine in subunit 4 of Complex I of the mitochondrial electron transport chain.

Since the 11778 mutation was reported, more than 17 point mutations in mtDNA have been described in LHON patients.[2–3] Based on genetic, clinical, and biochemical parameters, mutations at nucleotide positions (np) 3460, 11778, and 14484 are regarded as primary mutations in that each of these mutations alone can cause LHON. These primary mutations are not found in normal controls. These mutations result in amino acid substitutions in the ND1, ND4, and ND6 mitochondrial genes, respectively, which are three of the seven genes that encode subunits of Complex I of the respiratory chain. The other mutations are regarded as secondary mutations. The secondary mutations are found in normal controls, but

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at a much lower frequency than in LHON patients. The pathogenic or etiological role of the secondary mutation has not yet been established.

The three primary LHON mutations are estimated to account for 8–25%, 50–70%, and 10–15% of all Caucasian LHON patients described, respectively.[4–5] However, the np 3460 and np 14484 mutations have rarely been reported in Asian LHON patients. In Japan, the prevalence of the np 11778 mutation among families with LHON is significantly higher (91.7%) than in Europeans,[6] although a Japanese group of investigators found three patients with the np 3460 mutation (4%) and six patients with the np 14484 mutation (9%) among 68 LHON patients.[7] However, no mutation other than the 11778 mtDNA mutation has ever been reported in Chinese LHON patients.[8–10]

The aim of this study was to investigate the spectrum of mtDNA mutations in Chinese patients with LHON using single-strand conformation polymorphism (SSCP) analysis[11] and direct DNA sequencing. In addition, screening was performed for Leber's mtDNA mutations on patients with bilateral optic atrophy of unknown etiology and patients with optic neuropathy of known etiology.

Materials and Methods

Methods, including proper consent and approval for this study from the National Science Council in Taiwan, were in compliance with the Declaration of Helsinki.

Subjects

Twenty-five Chinese LHON pedigrees, including 27 patients and 16 maternally unaffected relatives were studied as group 1. Of the LHON patients, 26 were men and one was a woman. The age of onset ranged between 10 and 40 years with a mean age of onset of 22 years. All had acute or subacute bilateral sequential visual loss. Their visual acuity ranged from only light perception to 6/20. All patients had optic neuropathy only, and no associated neurological or cardiac abnormalities. Eleven pedigrees had familial optic atrophy and 14 were singleton cases. They had tested positive for mtDNA 11778 or 14484 mutation. Four patients were not clinically typical for LHON. The diagnosis was made after molecular testing. All of the unaffected maternal relatives (3 men and 13 women) were visually normal, being able to see 6/6, read 15 out of 15 Ishihara color plates, and had normal fundi without microangiopathy.

The clinical symptoms and signs of LHON can vary greatly.[12–16] To assure that no case of LHON was included as optic atrophy of unknown etiology, 22 cases of the latter were included as group 2. Eighteen patients were men and four were women. The age of onset ranged between 7 and 52 years with a mean age of onset of 31 years. All had subacute or chronic progressive visual loss in one or both eyes. Optic neuropathy was the only clinical deficit. Vision ranged from 1/60 to 6/10. Their fundi did not show telangiectatic microangiopathy. They had no maternal relatives within three generations with visual problems. Clinically, they did not fit the diagnosis of LHON, although LHON should be suspected. They subsequently tested negative for mtDNA 3460, 11778, or 14484 mutations.

Twenty-one patients with optic neuropathy of known etiology were included as group 3. Sixteen had acute optic neuritis, 1 had anterior ischemic optic neuropathy, 4 had dominant optic atrophy. Four of the 16 patients with acute optic neuritis were diag-

Table 1. Primers and Sizes of Polymerase Chain Reaction Products Used in This Study

Number	Gene	5′ Primer	3′ Primer	Size (bp)	Previously Reported Mutations That Are Covered in This Study
1	ND1	3368–3388	3609–3630	263	3394, 3460
2	ND1	4057–4076	4245–4264	208	4136, 4160, 4216
3	ND2	4791–4810	4955–4974	184	4917
4	ND2	5184–5206	5336–5356	173	5244
5	COI	7357–7377	7514–7536	180	7444
6	COIII	9377–9396	9535–9554	178	9438
7	COIII	9744–9764	9893–9914	171	9804
8	ND4	11690–11709	11849–11869	180	11696, 11778
9	ND5	13655–13679	13810–13830	176	13708, 13730
10	ND6	14406–14425	14560–14580	175	14459, 14482, 14484, 14498, 14568
11	Cyt b	15197–15216	15347–15367	171	15257
12	Cyt b	15752–15771	15904–15926	175	15812

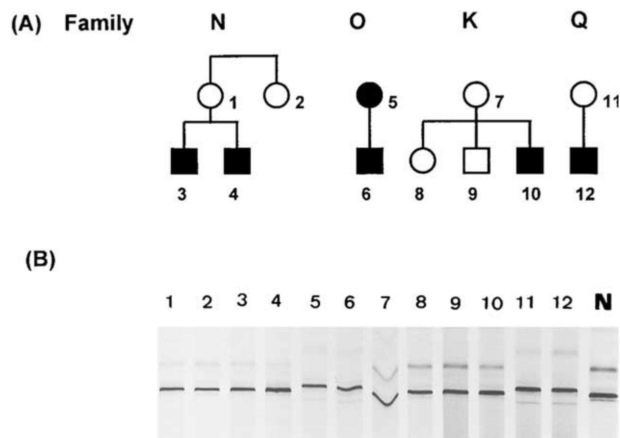

Figure 1. (**A**) N, O, K, and Q are pedigrees of Leber's hereditary optic neuropathy (LHON) subjects from group 1. □: unaffected male, ○: unaffected female, ■: affected male, ● affected female. Numbers 4, 6, 10, and 12 are probands of LHON. Subjects with numbered symbols had clinical and molecular examinations. (**B**) Single-strand conformation polymorphism analysis of polymerase chain reaction products amplified with primer pair 1. Electrophoretic condition: 500 V, 5°C, 2 hours. Analysis showed band shift in lanes 5, 6, 11, and 12. N: normal control.

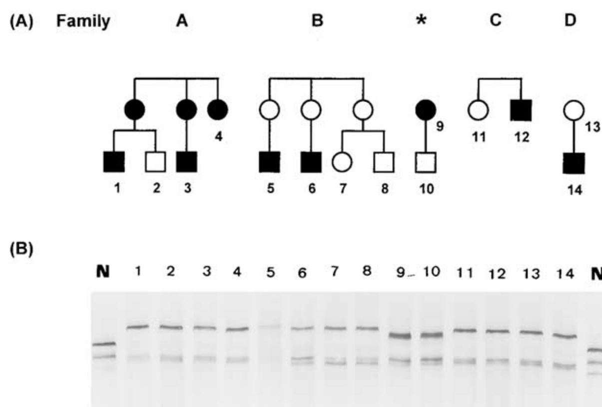

Figure 2. (**A**) A, B, C, and D are pedigrees of the Leber's hereditary optic neuropathy (LHON) subjects from group 1. *One pedigree of optic neuropathy of known etiology from group 3. Number 9 is a case of anterior ischemic optic neuropathy. □: unaffected male, ○: unaffected female, ■: affected male, ● affected female. Numbers 1, 6, 12, and 14 are probands of LHON. (**B**) Single-strand conformation polymorphism analysis of polymerase chain reaction products amplified with primer pair 8. Electrophoretic conditions: 500 V, 5°C, 3 hours. N: normal control. All lanes show band shift compared to normal control.

nosed with multiple sclerosis. The age of patients with optic neuritis ranged from 8 to 69 years with a mean age of 34.

Twenty-five normal healthy people were recruited as group 4, a control group. Nineteen were men and six were women. Their ages ranged from 2 to 86 years with a mean age of 44.

Blood Sampling

With informed consent, blood samples were obtained from LHON patients, their unaffected maternal relatives, patients with optic atrophy of unknown cause, patients with optic neuropathy of known cause, and healthy controls. Five milliliters of whole blood was withdrawn and collected in a glass tube containing EDTA.

DNA Isolation and PCR-RFLP Analysis

Total DNA was extracted from the blood cells and purified using a DNA purification kit (Puregene; Gentra Systems, Minneapolis, MN, USA). Twelve pairs of primers (Table 1) covering the 21 reported mtDNA mutations were prepared with a DNA synthesizer (Applied Biosystems, Foster City, CA, USA). The primers were used to amplify DNA segments ranging from 171 base pairs (bp) to 265 bp in length with the polymerase chain reaction (PCR) technique using a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT, USA). In total, 2236 base pairs of mtDNA were screened. The thermal profile consisted of 30 cycles of denaturation for 20 seconds at 94°C, annealing for 30 seconds at 56°C, and extension for 30 seconds at 72°C.

Single-Strand Conformation Polymorphism Analysis

Five milliliters of the PCR product plus 5 μL of the denaturing dye (25 mL containing 23.75 mL of 99% formamide, 1.25 mL of 1% xylene cyanol, and 10 mg of bromophenol blue) were denatured by heating at 95°C for 5 minutes. The samples were electrophoresed using a GenePhor Electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) at 5°C, 500 V for 3 hours. The gel was stained with a DNA silver staining kit using an automatic gel stainer (Pharmacia Biotech).

DNA Sequencing

The bands with a shift on SSCP analysis were chosen for DNA sequencing. The fragments were purified with a purification kit (Boehringer Mannheim, Mannheim, FRG), mixed with a dye terminator cycle sequencing kit (Perkin-Elmer), and sequenced using an autosequencer (Model 310, Perkin-Elmer).

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Table 2. Mutations of Mitochondrial DNA Found in Each Group

Mutation	Gene	Amino Acid Change	LHON* (n = 25)	OAUE† (n = 22)	ONKE‡ (n = 21)	Normal (n = 25)
G3460A	ND1	A52T	0	0	0	0
G11778A	ND4	R340H	23	0	0	0
T14484C	ND6	M64V	2	0	0	0
T3394C	ND1	Y30H	2	0	0	0
A3434G	ND1	Y43C	0	1	0	0
C3497T	ND1	A64V	1	0	2	0
T3548C	ND1	I81T	1	0	0	0
C3571T	ND1	L89F	1	0	1	0
T4115C	ND1	F270S	1	0	1	0
A4129G	ND1	T275A	0	0	1	1
T4216C	ND1	Y304H	2	0	0	0
A4833G	ND2	T122A	0	0	0	1
G4924T	ND2	S152I	1	0	0	0
G4959A	ND2	A164T	1	0	1	0
A5301G	ND2	I278V	0	1	0	0
C5363T	ND2	A298V	0	1	1	0
C13702G§	ND5	R456G	1	0	0	2
C13712T	ND5	A459V	1	0	0	0
A13748G	ND5	N471S	0	0	0	1
C13754G	ND5	S473C	0	1	0	0
G13759A	ND5	A475T	0	3	5	1
A15236G	Cyt b	I164V	1	1	0	0
C15324G	Cyt b	A193G	1	0	0	0
A15326G§	Cyt b	T195A	13	19	16	12
G15884A	Cyt b	A480T	0	1	0	0

*LHON: Leber's hereditary optic neuropathy.
†OAUE: optic neuropathy of unknown etiology.
‡ONKE: optic neuropathy of known etiology.
§Previous analyses have shown that mutations at np 13702 and 15326 are Cambridge Reference Sequence errors or rare polymorphisms.

Results

Figure 1 shows the SSCP analysis of PCR products amplified with primer pair 1. Band shift is shown in lanes 5, 6, 11, and 12. DNA fluorescent autosequencing revealed an np 3394 T to C point mutation.

Figure 2 shows the SSCP analysis of PCR products amplified with primer pair 8. All lanes show band shift when compared to the control. DNA fluorescent autosequencing of lanes 1–8 and lanes 11–13 all revealed an np 11778 A to G mutation. Patient 9 was a 70-year-old female patient with ischemic optic neuropathy in whom *Sfa* NI analysis gave a positive result for mtDNA 11778 mutation. SSCP analysis showed band shift. However, DNA fluorescent autosequencing revealed np 11782 C to T silent mutation.

After excluding the silent mutations, the spectrum and the frequency of mutations of the four groups are listed in Table 2. In the LHON group, 23 pedigrees (92%) had the np 11778 and 2 pedigrees (8%) had the np 14484 primary mutation. No np 3460 primary mutation of mtDNA was found. In addition to the primary mutations, 13 mutations were found in this group. Six occurred in the ND1 gene, and 10 involved the mitochondrial encoded Complex I subunits. Two of the 13 were also found in controls. These mutations comprised 13 different mutation patterns (Table 3). A single-mutation pattern consisting of the 11778 primary mutation was found in 11 pedigrees. Fourteen pedigrees had multi-mutation patterns consisting of one primary mutation together with one or more secondary mutations. Although three pedigrees had 11778+15326 mutation, the 15326 mutation occurred at a high frequency in the other optic neuropathy and control groups. Other patterns of multi-mutation occurred individually in only one pedigree each (Table 3).

No np 3460, np 11778, or np 14484 primary mutations were found in the group of patients with optic atrophy of unknown etiology. However, four mutations, 3434, 5301, 13754, and 15884, were each found in one patient in this group. They involved the ND1, ND2, ND5, and CYTB genes, respectively. They

Table 3. Frequency of Mitochondrial DNA Mutations Associated with Leber's Hereditary Optic Neuropathy

	Primary Mutations			Secondary Mutations												
Number of Pedigrees	3460	11778	14484	3394	3497	3548	3571	4115	4216	4924	4959	13702	13712	15236	15324	15326
11	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
1	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	+
1	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+
1	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+
1	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+
1	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
1	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+
1	−	+	−	+	−	−	−	−	+	+	+	−	−	−	−	−
1	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+
1	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	+
1	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+
1	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	+
25 control	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	12/25

were not found in LHON, or optic neuropathy of known etiology patients, or controls.

No primary mutation was found in patients with optic neuropathy of known etiology or in controls.

Discussion

Our results showed a false-positive *Sfa* NI analysis (patient 9 in Figure 2). The most common pathogenic mutation of mtDNA for LHON is at np 11778 and is usually detected by loss of an *Sfa* NI restriction site. The *Sfa* NI restriction site includes five base pairs 5′-GCATC-3′. Substitution of any of the nucleotides from np 11778 to 11782 will cause the loss of the *Sfa* NI cutting site. The false-positive rate of *Sfa* NI analysis was found to be 2.3–2.8%.[17–18] It requires double confirmation either by Mae III restriction[19] or by SSCP and direct sequencing.

Multiple mtDNA mutations have been reported in LHON patients.[20] The significance of secondary mutations is unclear. The accumulation of mtDNA mutations was thought to have a synergistic effect on the clinical expression of LHON. Howell et al[21] described a large Queensland family with the np 4160 mutation that presented with neurological abnormalities and infantile encephalopathy in addition to characteristic ophthalmological changes. One branch of this family carried both the np 4136 and np 4160 mutations. The affected members in this branch had only ophthalmological abnormalities. The np 4136 mutation was thought to be an intragenic suppressor mutation, that may ameliorate the biochemical defect of Complex I and the neurological abnormalities. However, no other report supported this synergism.

Haplotype analysis of mtDNA found that >70% of 14484-positive Caucasian LHON patients or Caucasian patients from North America had significant clustering and 11778-positive patients had moderate clustering on Caucasian mtDNA haplogroup J.[22,23] Haplogroup J is one of the European-specific mtDNA haplogroups that is characterized by two secondary mutations at np 4216 and 13708. It was found that this combination in haplogroup J increased both the penetrance of the two primary mutations 11778 and 14484, and the risk of disease expression. However, in vivo study did not support synergism of the 4216 and 13708 secondary mutations with the 11778 primary mutation in determining the deficit of energy metabolism in LHON.[24] In this study, neither pedigree showed the mutation pattern of European-specific haplogroup J and no specific multi-mutation pattern was found because every pattern occurred in only one pedigree. Patients who had simultaneous multiple mutations were not clinically different from those with a single mutation.

The three primary mtDNA mutations account for >95% of the LHON pedigrees of northern European descent.[5] The frequency of the np 11778 mtDNA mutation is about 50% in Caucasians.[25–29] The frequency of the np 11778 mutation reported by Mackey et al[5] included 75% (12/16) of Australian/New Zealand patients, 72% (48/67) of United Kingdom/Northern Ireland patients, 60% (25/42) of Dutch patients, 86% (19/22) of Danish patients, and 50% (6/12) of Finnish patients. The np 11778 mutation is significantly more prevalent in Japanese LHON patients. About 87% of Japanese LHON pedigrees possess this mutation.[6,7,30] Mutations at np

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3460 and np 14484 were found in 4% and 9%, respectively, of Japanese LHON pedigrees.[7] In this study, mutation at np 11778 and np 14484 were found in 92% and 8% of pedigrees, respectively. No np 3460 mtDNA mutation was found. The np 11778 mutation is also significantly more prevalent in Chinese LHON patients.

No primary mutations at np 3460, np 11778, or np 14484 were found in the group of patients with optic atrophy of unknown etiology. However, four mutations, 3434, 5301, 13754, and 15884, were each found in 1 patient in this group. They involved the ND1, ND2, ND5, and Cyt. b genes, respectively. The mutations were not found in the LHON, optic neuropathy of known etiology, and control groups, and have not been described previously. The pathological roles of these mtDNA mutations warrant further investigation.

In conclusion, the np 11778 mutation is significantly more prevalent in Chinese LHON patients. Although no 3460 mutations were found, we cannot exclude its existence in Chinese patients with LHON. Fourteen pedigrees had multi-mutation patterns consisting of one primary mutation together with other sequence changes. No specific multi-mutation pattern was found, and none was associated with the European-specific haplogroup J.

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