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Exclusive Homoplasmic 11778 Mutation in Mitochondrial DNA of Chinese Patients With Leber's Hereditary Optic Neuropathy

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Purpose: To investigate the degree of heteroplasmy of the 11778 mtDNA mutation in Chinese patients with Leber's hereditary optic neuropathy (LHON).

Methods: Seventeen Chinese Leber's pedigrees, including 24 patients, 17 unaffected maternal lineages, 4 internal controls, and 6 unrelated controls, were screened for the 11778 mtDNA mutation. This was carried out by analysis of the restriction fragment length polymorphism, single-strand conformation polymorphism, and DNA sequencing.

Results: All patients and unaffected maternal lineages, regardless of their symptoms, had homoplastic 11778 mtDNA mutation, which was revealed by restriction fragment length polymorphism analysis and single-strand conformation polymorphism analysis.

Conclusion: Exclusive homoplasmy of the 11778 mtDNA mutation in Chinese LHON patients was found in this study. Homoplasmy of the 11778 mtDNA mutation cannot account for the variation in the clinical phenotype of Chinese Leber's patients. **Jpn J Ophthalmol 1999;43:196–200** © 1999 Japanese Ophthalmological Society.

Key Words: Chinese Leber's hereditary optic neuropathy, homoplasmy, mitochondrial DNA, 11778 mutation.

Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by acute or subacute bilateral loss of central vision, predominantly in healthy young men.[1] In 1998, Wallace et al[2] reported a point mutation at nucleotide position (np) 11778 of mitochondrial DNA (mtDNA) in 9 of 11 LHON families. The mutation causes a change from arginine to histidine at the 340th amino acid position in subunit 4 of NADH dehydrogenase. Since the report of the 11778 point mutation, 17 other LHON-associated mtDNA point mutations have also been identified.[3] Among them, mutations at np 3460, 11778, and 14484 are regarded as primary mutations because they are the most deleterious and are not found in normal controls. These three mutations are estimated to account for 15%, 50%–70%, and 10% of LHON patients, respectively.[4]

The mechanism of the pathogenesis of Leber's disease is still unknown. The presence of a primary mtDNA mutation does not necessarily lead to visual loss. Heteroplasmy of the mtDNA mutation is considered a risk factor for Leber's disease because it has been demonstrated that some LHON patients have a higher proportion of mutant mtDNA, when compared with their asymptomatic maternal relatives.[5–15] Using single-strand conformation polymorphism (SSCP) analysis, Mashima et al[13] found that the degree of heteroplasmy ranged from 10%–94%

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in 14% of Japanese patients with LHON. The aim of this study was to investigate the degree of heteroplasmy in Chinese LHON patients by restriction fragment length polymorphism (RFLP) and SSCP analysis.

Materials and Methods

Subjects

Seventeen Chinese LHON pedigrees were studied, including 24 patients and 17 unaffected maternal lineages. In the patients with LHON, 21 were men and 3 were women. The age of onset ranged from 10–56 years. The visual acuity ranged from light perception to 6/20. Six pedigrees had a family history of LHON and 11 were sporadic cases. Clinical data of the study subjects are shown in Table 1. All the unaffected maternal relatives (3 men and 14 women) had normal vision, being able to see 6/6 and read 15/15 of the Ishihara color plates and having normal fundi without microaneurysm. Four internal controls (parental relatives) and six unrelated healthy controls were also examined.

Table 1. Clinical Data of Leber's Hereditary Optic Neuropathy Patients

Family	Family History	Family Relation	Sex	Age	Age of Onset	Vision OD	Vision OS
A	+	Proband	M	35	10	5/60	5/60
		Cousin	M	30	29	HM	HM
		Aunt	F	57	56	CF	CF
B	+	Proband	M	23	13	LP	LP
		Cousin	M	19	16	1/60	1/60
C	−	Proband	M	20	16	CF	2/60
D	−	Proband	M	15	14	CF	CF
E	−	Proband	M	28	28	CF	6/60
F	−	Proband	M	15	12	3/60	3/60
G	−	Proband	M	20	17	1/60	1/60
H	+	Proband	M	36	30	1/60	1/60
		Brother	M	33	32	2/60	3/60
I	−	Proband	M	30	29	CF	CF
J	−	Proband	M	25	16	6/60	6/60
K	−	Proband	M	40	39	3/60	2/60
L	−	Proband	M	11	11	3/60	3/60
M	−	Proband	M	23	19	6/60	CF
N	+	Proband	M	11	11	CF	3/60
		Mother	F	40	13	6/20	6/20
		Brother	M	19	6	6/30	1/60
O	+	Proband	M	19	17	CF	6/60
		Mother	F	40	CP	6/60	1/60
P	+	Proband	M	21	21	1/60	1/60
Q	−	Proband	M	17	17	3/60	1/60

CF: finger count, CP: chronic progressive without exact age of onset, F: female, HM: hand motion, LP: light perception, M: male, OD: right eye, OS: left eye.

Blood Sampling

Blood samples were obtained with consent from LHON patients, their unaffected maternal relatives, and healthy controls. One milliliter of whole blood was withdrawn from each subject and was kept 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). Three pairs of primers (sense 20 mer between np 11690 and 11709, antisense 20 mer between np 11869 and 11849; sense 20 mer between np 11690 and 11709, antisense 20 mer between np 11944 and 11923; sense 19 mer between np 11673 and 11691, and antisense 20 mer between np 12188 and 12169, respectively) were prepared with a DNA synthesizer (Applied Biosystems, Foster City, CA, USA). The primers were used to amplify a 180–base pair (bp), a 255-bp, and a 515-bp mtDNA segment by the polymerase chain reaction (PCR) technique using a DNA amplification system (DNA Thermal Cycler; Perkin-Elmer/Cetus, Norwalk, CT, USA). 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.

For the restriction fragment length polymorphism (RLFP) analysis, the 515-bp PCR product was digested with 1 unit of SfaNI (New England Biolabs, Beverly, MA, USA) at 37°C for 16 hours and electrophoresed in a 1.5% agarose gel at 80 V for 3 hours. The gels were stained with ethidium bromide and photographed under UV transillumination.

SSCP Analysis

Five microliters of the 180-bp or the 255-bp 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) was denatured by heating at 95°C for 5 minutes. The samples were then applied to the gels (GeneGel Excel 12.5/24 kit; Pharmacia Biotech AG, Uppsala, Sweden). The gels were electrophoresed using the GenPhor Electrophoresis Unit (Pharmacia Biotech AG) at 5°C, 500 V for 3 hours and were stained with a DNA silver staining kit (Pharmacia Biotech AG) using an automatic gel stainer (Pharmacia Biotech AG).

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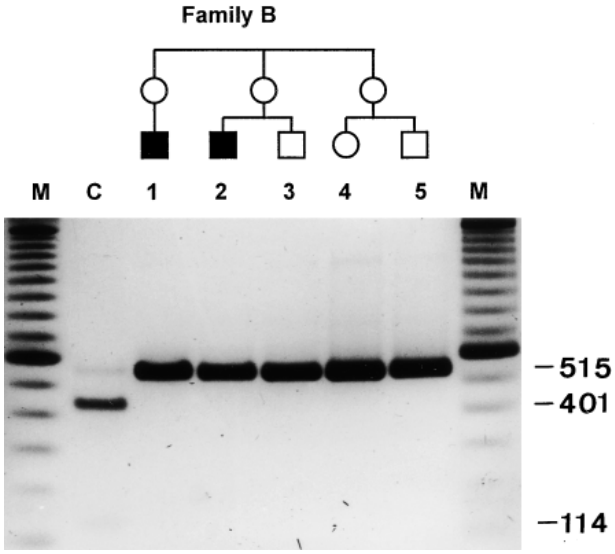

Figure 1. Agarose gel electrophoresis of PCR-amplified mtDNA fragments restricted by SfaNI. SfaNI digestion of the 515-bp fragment from normal mtDNA generated 401-bp and 114-bp fragments, but 515-bp fragments amplified from mtDNA with 11778 mutation were not cleaved. C: normal control, M: 100-bp ladder DNA size marker.

Quantification of Heteroplasmic Mutant mtDNA

The degree of heteroplasmy of the mutant mtDNA was calculated using an image analyzer system (IS-1000; Alpha Innotech, San Francisco, CA, USA).

DNA Sequencing

The 180-bp fragment was purified with a purification kit (Boehringer Mannheim, Mannheim, Germany), mixed with a dye terminator cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA), and was sequenced using an autosequencer (Model 373A; Perkin-Elmer/Applied Biosystems).

Results

The 515-bp fragment containing normal mtDNA sequence was digested by SfaNI into a 401-bp fragment and a 104-bp fragment, whereas the 515-bp fragment containing the 11778 mutation of mtDNA was undigested.

All the patients and unaffected maternal lineages, regardless of their symptoms, had homoplasmic 11778 mtDNA mutation, which was revealed by SfaNI RFLP analysis (Figure 1) and SSCP analysis (Figure 2).

DNA sequencing also confirmed that all the patients and maternal relatives had the 11778 G to A substitution. Besides the 11778 mtDNA point mutation, all members of one family (family B on Table 1) also had a 11782 C to T silent mutation. However, SSCP showed no difference between patients who had both 11778 and 11782 mutations and patients who had only 11778 mutation.

SfaNI restriction analysis and SSCP analysis was repeated with separate blood samples, using differ-

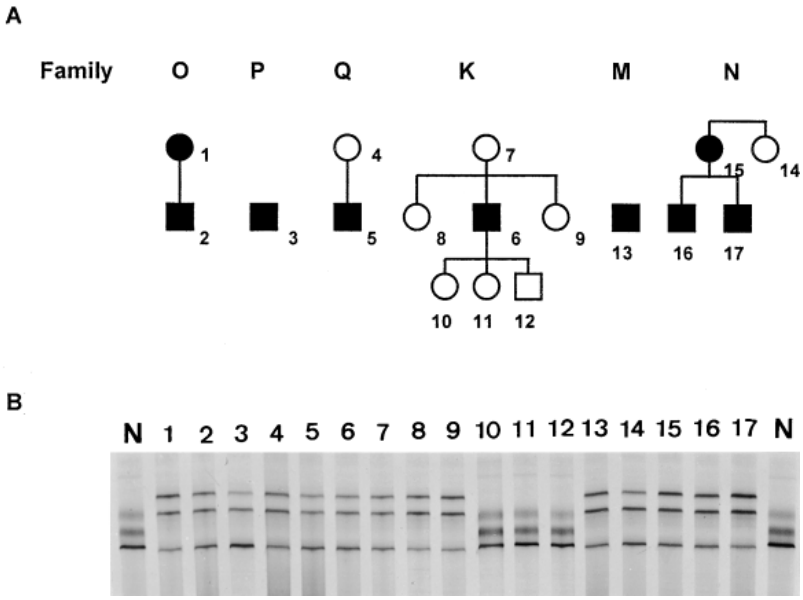

Figure 2. (**A**) Pedigrees of Leber's Hereditary Optic Neuropathy (LHON) patients. (**B**) Single-strand conformation polymorphism analysis of PCR-amplified 255-bp mtDNA fragments from LHON patients. Electrophoretic condition: 500 V, 5°C, 3 hours. Pattern showed no difference for all persons with 11778 mtDNA mutation. N: normal control

ent primers and different length PCR products (180 bp or 255 bp). Results of both repeated analyses were the same as in the first analysis.

Discussion

Heteroplasmy is the presence of a mixture of normal and mutant mtDNAs in tissue cells. Holt et al[5] first observed the heteroplasmic 11778 mutation of mtDNA in peripheral blood cells in three of four families with LHON. The percentage of the mutant mtDNA varied from >95% in an affected proband to 68% in one of his cousins. Holt et al[5] suggested that heteroplasmy of mutant mtDNA might have a clinical correlation. Later, Zhu et al[6] reported 76%–90% heteroplasmy in five affected LHON patients whereas Smith et al[7] reported 62%–90% heteroplasmy in six affected LHON patients. The latter also observed that heteroplasmic subjects were more likely to remain asymptomatic than those who harbor homoplasmic mutant mtDNA. However, the subjects with heteroplasmic 11778 mtDNA mutation who did become symptomatic did not seem to differ clinically from symptomatic subjects who carried homoplasmic mutant mtDNA.

Heteroplasmy was found in about 14% of the patients and unaffected relatives with the 11778 mtDNA mutation.[7,13,16] The proportion of the mutant mtDNA was found to shift markedly across generations[9–11] and within the tissues of an individual.[11,17] Heteroplasmy was also found in the LHON-associated 3460 mutation of mtDNA.[14,15] The actual threshold level required for the clinical expression of the disease in the optic nerve tissue is unknown. Although the proportion of the mutant mtDNA was thought to correlate with the severity of the disease, there was one affected subject with a very low mutant mtDNA level in peripheral blood cells (15%).[15]

Several methods, such as restriction enzyme assay with and without Southern blotting,[5–7,10–11] allele-specific oligonucleotide hybridization,[8,18] and solid-phase minisequencing[19] were used to quantify the proportion of heteroplasmic mtDNA mutation in patients with LHON. Using restriction enzyme assay and hybridization with allele-specific oligonucleotides, Nakamura et al[18] did not find heteroplasmy of the ND4 11778 mutation in seven Japanese LHON pedigrees. However, heteroplasmy was observed in 13 of 90 affected cases (14.4%) and 8 of 18 carrier cases (44.4%) in Japanese LHON in a report by Hotta et al.[20] Single-strand conformation polymorphism analysis is a simple, rapid, and accurate technique for detecting the substitution of even a single base in DNA.[21] Also, it can avoid the false-positive results of incomplete digestion of SfaNI in RFLP analysis. Using SSCP analysis, Mashima et al[13] found that the degree of heteroplasmy ranged from 10%–94% in 14% of Japanese LHON families. However, in this study, using the same method, all Chinese patients with LHON and their maternal lineages had homoplasmic 11778 mtDNA mutation. Heteroplasmy was not observed in this series. This study also demonstrated that SfaNI RFLP analysis was as sensitive as SSCP analysis for detecting heteroplasmic mtDNA mutation.

It is clear that most subjects who inherit homoplasmic 11778 mutation of mtDNA never develop visual symptoms during their lifetime. Although the percentage of mutant mtDNA in peripheral blood cells is not necessarily the same as that in optic nerve tissue, heteroplasmy or homoplasmy of the 11778 mtDNA mutation alone cannot explain the variation in the clinical phenotype of LHON patients.

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