Mitochondrial DNA Mutation in Leber's Hereditary Optic Neuropathy

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Leber's hereditary optic neuropathy (LHON) causes acute or subacute central visual loss in healthy young males. Recently, it has been thought to be caused by a single nucleotide change in the ND4 gene in the mitochondrial genome. Mitochondrial DNA (mtDNA) of leukocytes and hair follicle cells from five patients in four families with LHON and nine relatives were analyzed by Sfa NI and Mae III enzyme digestion and DNA sequencing. Loss of Sfa NI site was found in all patients and maternal lineages but not in nonmaternal lineages and normal controls. Mae III digested all the mtDNAs that lost the Sfa NI site. The restriction fragment pattern of polymerase chain reaction (PCR) products exhibited mtDNA heteroplasmy in the hair follicle cells but not in blood cells of the proband in one family. Direct sequencing of PCR-amplified mtDNA fragments encompassing the ND4 gene of the patients disclosed a transition from guanine to adenine at nucleotide position 11778. These results confirm previous reports that a G to A point mutation is associated with LHON and that tissue variability and heteroplasmy of mtDNA exist in some, but not all, LHON patients. Invest Ophthalmol Vis Sci 33:2561-2566, 1992

Leber's hereditary optic neuropathy (LHON) is characterized by sudden loss of central vision in the second and third decades of a patient's life. Males are most affected. Initially, the optic discs are hyperemic and the nerve fiber layer is swollen and opacified. There is peripapillary telangiectasis without leakage of fluorescein dye. Loss of visual acuity generally is severe (6/60 or less). Optic atrophy is apparent within 2 mo.

The disease is familial, but the transmission does not follow the classic mendelian law. It is transmitted exclusively by women to offspring of both sexes.1 Parental transmission has never been described. Because mitochondrial DNA (mtDNA) is the only maternally inherited component of human genetic materials, mitochondrial inheritance has been implicated in the etiology of this disease.12 Wallace et al3 reported in 1988 a point mutation at nucleotide position 11778 of mtDNA isolated from patients with LHON. The mutation causes a change from arginine to histidine at the 340th amino acid position in subunit 4 of NADH dehydrogenase. The change from adenine to guanine at position 11778 of the mtDNA causes a concurrent loss of a restriction site for the restriction endonuclease Sfa NI. Stone and coworkers4 described in 1990 the advantage using the restriction enzyme Mae III to avoid a false-positive diagnosis. Mae III digests mtDNA near nucleotide position 11778 only when the Wallace mutation is present. The mechanism of the pathogenesis of this disease is still unknown, but these findings provide simple molecular diagnostic tests, especially for patients who lack a family history of a similar illness.

Using these methods, we investigated the mtDNAs of members of four families with Leber's disease in Taiwan.

Materials and Methods

Patients and Pedigrees

Five patients from four families and seven unaffected maternal linked relatives and two unaffected nonmaternal linked relatives were studied. All of the unaffected relatives were visually normal. They had uncorrected or corrected vision equal to or better...
than 20/20. All could read 15 of 15 Ishihara color plates and all had normal fundi without microangiopathy. Seven normal persons, one person with traumatic optic atrophy, and one person with Ethambutol-induced optic neuropathy were studied as controls.

The pedigree of family A is shown in Figure 1. Patients III and III2 are mother and son. The mother had the visual attack right after she delivered the affected son, and the son was found to have optic atrophy at age 10 without knowledge of the exact time of attack. In the other three families, the patient was the only affected person. Figure 2 shows the pedigree of family B.

Preparation of Mitochondrial DNA

Informed consent for molecular diagnosis was obtained from all the examinees. One milliliter of whole blood was obtained from each individual and was kept in a glass tube containing ethylenediaminetetraacetic acid (EDTA). Root ends of 0.5 cm from five hairs were obtained and kept in an eppendorf tube.

We extracted the mtDNA from the whole blood and hair follicle cells of all examinees. Two hundred microliters of the blood sample were washed twice with 500 μl of the TE buffer (20 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0) and centrifuged for 1 min at 13,000 × g. The supernatant was discarded. The washed blood cells or hair samples were mixed with lysis buffer (TE buffer, 100 μg/ml proteinase K, and 0.5% sodium dodecyl sulfate) to a final volume of 400 μl. The samples then were incubated at 56°C for 1 hr, mixed with 1.0 mol/l NaCl, and stored at 4°C for 2 hr. The mixture was centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected and added with one volume of phenol, and the mixture was centrifuged at 12,000 × g for 2 min. The aqueous phase was collected and added with one volume of phenol/chloroform (1:1). The mixture was centrifuged at 12,000 × g for 2 min. The aqueous phase was collected and added with one volume of chloroform, and the mixture was centrifuged at 12,000 × g for 2 min. The aqueous phase was collected and added with one volume of chloroform, and the mixture was centrifuged at 12,000 × g for 2 min. The aqueous phase was collected and added with one volume of isopropanol. After it stood at −20°C for 30 min, the mixture was centrifuged at 4°C for 15 min. The precipitate was collected and washed with 800 μl of 70% cold ethanol. The mixture was centrifuged at 4°C for 3 min. The precipitate containing mtDNA was air dried and dissolved in 40 μl of the TE buffer.
Amplification and Analysis of Mitochondrial DNA

Five microliters of the mtDNA solution was used as the template for amplifying the desired mtDNA segment by polymerase chain reaction (PCR). Two pairs of primers (sense 19 mer between 11673 and 11691 and antisense 20 mer between 12169 and 12188; sense 20 mer between 10744 and 10763 and antisense 20 mer between 12169 and 12188) were prepared with a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The primers were used to amplify a 515 base pair (bp) and a 1444 bp mtDNA segment, respectively, encompassing ND4 gene by the PCR technique in a DNA amplification system (Perkin-Elmer/Cetus [Norwalk, CT] Thermal Cycler). Thermal profiles consisted of 30 cycles of denaturing for 2.5 min at 94°C, annealing for 1 min at 56°C, and extension for 1.5 min at 72°C. After amplification of the mtDNA, a small fraction of the PCR product was electrophoresed in 1.2% TBE-agarose gel to check for the purity of the amplified fragment as a single band of expected size. Twenty one microliters of each PCR product was digested with 1.5 U of Sfa NI (New England Biolabs Inc., Beverly, MA) at 37°C for 16 hr, and 15.5 µl of the PCR product was digested with 2.5 U of Mae III (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 55°C for 2 hr. The samples then were subjected to electrophoresis in 1.5% TBE-agarose gel at 80 V for 4 hr. The gel was stained with ethidium bromide for 5 min, visualized with an ultraviolet transilluminator, and photographed.

DNA Sequencing

The 515 bp fragment was directly sequenced with a synthetic primer (from 11673 to 11691) using asymmetrically amplified mtDNA fragment as the template, following the procedure described previously.5

Results

Sfa NI digestion of the 1444 bp fragment generated from the wild type mtDNA yielded a 915 bp fragment (nucleotide positions 10872 to 11787), a 401 bp fragment (nucleotide positions 11787 to 12188), and a 128 bp fragment (nucleotide positions 10744 to 10872). Sfa NI digestion of the DNA fragment bearing the Leber's hereditary optic neuropathy mutation generated a 1316 bp fragment (nucleotide positions 10872 to 12188) and a 128 bp fragment (nucleotide positions 10744 to 10872). All the controls and non-maternal lineages exhibited 401 bp and 915 bp fragments after Sfa NI digestion of the 1444 bp PCR product, whereas all the patients and their maternal lineages showed that these fragments fused into a 1316 bp fragment. Figures 3 and 4 show the results of the restriction analysis of the 1444 bp PCR product from leukocyte mtDNA of patients and relatives of family A and family C. Homoplasmy was found in all individuals with Sfa NI site loss.

Sfa NI digestion of the 515 bp fragment amplified from the wild type mtDNA yielded a 401 bp fragment and a 114 bp fragment, whereas the fragment from mutant mtDNA lost the restriction site. In family B, mtDNA heteroplasmy (mixture of mutant and normal mtDNA) was observed in the unaffected maternal lineages and in the patient’s hair follicle mtDNA, although his leukocyte mtDNA showed homoplasmy (Fig. 5). Homoplasmy was observed in the other three families. The results from family A are shown in Figure 6.

Mae III digestion of the 515 bp fragment from the wild type mtDNA yielded fragments of 232 bp, 226 bp, and 57 bp, whereas digestion of the 515 bp fragment from mutant mtDNA lost the restriction site. In family B, mtDNA heteroplasmy (mixture of mutant and normal mtDNA) was observed in the unaffected maternal lineages and in the patient’s hair follicle mtDNA, although his leukocyte mtDNA showed homoplasmy (Fig. 5). Homoplasmy was observed in the other three families. The results from family A are shown in Figure 6.

Mae III digestion of the 515 bp fragment from the wild type mtDNA yielded fragments of 232 bp, 226 bp, and 57 bp, whereas digestion of the 515 bp fragment from mutant mtDNA yielded fragments of 226 bp, 131 bp, 101 bp, and 57 bp, respectively. Figure 7 shows the results obtained from Mae III digestion of the 515 bp mtDNA fragment from family B. Direct sequencing of the PCR-amplified mtDNA fragment of all the patients and their maternal lin-
kaged relatives also disclosed a substitution of an adenine (Leber's hereditary optic neuropathy) for a guanine (wild type) at nucleotide position 11778 (Fig. 8).

Discussion

Human mitochondria are unique among cellular organelles in that they contain their own genetic material of about 2–10 copies of a double-stranded, circular DNA molecule that is approximately 16.5 kilobases long. It is transmitted exclusively by mothers. It codes for 2 rRNA, 22 tRNA, and 13 polypeptides that are involved in the mitochondrial respiration and oxidative phosphorylation.

Since Wallace et al.3 reported in 1988 a point mutation at position 11778 of the mtDNA of a LHON patient, the mutation has been found in LHON pedigrees from the United States (both black and white), Canada, Finland, Greece, England, Ireland, and Japan.4 The present study confirmed that the same mutation occurs in Chinese pedigrees with LHON.

Because China has been divided into Mainland China and Taiwan without communication for more than 40 yr, family history is difficult to trace. Clinically, our three LHON patients are sporadic cases of the disease. Without catching the acute phase, the diagnosis is difficult. Identification of the loss of Sfa...
NI site, including the 11778 position of point mutation, provides a simple diagnostic test. Presence of Mae III restriction site further confirmed the mutation.

Heteroplasmy was found in the mtDNA of LHON patients by Holt et al6 and Wallace et al.8 The mutation was present in only four out of eight families studied by Holt et al. They concluded that a poor prognosis for visual recovery was associated with this mutation. The more abundant the mutant mtDNA, the poorer the visual prognosis. Newman et al9 studied the phenotypic characteristics of 49 pedigrees with 11778 mutation; 28 pedigrees were singleton cases. Seven families, including six singleton pedigrees, had maternal family members with heteroplasmy. However, Vikki et al10 found homoplasmy in all individuals with Sfa NI site loss. Our results show that the mutation was seen in all of the patients and their maternal lineages examined in this study. However, three out of four families showed homoplasmia. Heteroplasmy was noted only in one family of the maternal lineages and the patient's hair follicle mtDNA. However, his leukocyte mtDNA was homoplasmic.

The molecular mechanism of the pathogenesis of this disease is still unknown. Why are the patients healthy except loss of vision? Why do the relatives who carry mutant mtDNA in their blood and hair follicle cells not lose their vision? Why do the patients born with this mutation not lose their vision until later in life. Environmental factors such as smoking and nutritional deficiency have been proposed by some investigators.10 Heteroplasmy or tissue variability8 provide another explanation. Because the presence of wild type mtDNA molecules in LHON patients would reduce the extent of the respiratory Complex I deficiency, individuals whose optic nerve cells contain heteroplasmic mtDNA would be less likely to lose their vision. However, the strong male bias, the strict tissue specificity, and the delay expression of the disease cannot be readily explained by a single mitochondrial gene defect alone.

Wallace12 proposed a hypothesis that the disease could be the result of an interaction between an X-linked gene and the mtDNA defect. By linkage analysis, Chen13 excluded the involvement of an X-linked gene in Leber's families, but Vikki14 found evidence of linkage of Leber's optic atrophy to the locus DXS7 on the proximal Xp of X-chromosomal gene. Interestingly, the same region has been assigned by linkage studies for three other hereditary eye diseases—Norrie's disease,15 X-chromosomal congenital stationary night blindness,16 and X-linked retinitis pigmentosa.17

It is unclear why the disease is male predominant and manifests in adolescence. Worth mentioning is that our A11 patient lost her vision right after she delivered her affected son. Is there a hormonal factor that prohibits females from being affected and that changes in the male during adolescence to trigger the visual attack? Was the factor broken down in our A11 patient during her delivery of the affected son?
The results from this study and other reports can only suggest that Leber's patients and their maternal lineages exhibited mutant mtDNA with a G to A point mutation at the 11778 nucleotide position. However, the relationship between the mutation and the mechanism of the visual loss still warrants further investigation.

Key words: Leber's hereditary optic neuropathy, mitochondrial deoxyribonucleic acid, point mutation, heteroplasmy, tissue variability

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References