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Results of Mitochondrial DNA Sequence Analysis in Patients with Clinically Diagnosed Leber's Hereditary Optic Neuropathy

Hakan Gürkan[1], Sadık Altan Özal[2], Haluk Esgin[2]

[1]*Department of Medical Biology, İstanbul Faculty of Medicine, İstanbul University, İstanbul, Turkey*
[2]*Department of Ophtalmology, Faculty of Medicine, Trakya University, Edirne, Turkey*

ABSTRACT

Objective: To investigate possible mitochondrial DNA (mtDNA) mutations in patients with Leber's hereditary optic neuropathy (LHON) in order to provide a precise diagnosis and genetic counseling.

Material and Methods: Between 1982 and 2007, ten patients were clinically diagnosed with LHON and six of these patients agreed to be involved in this study. Six healthy individuals were also included as a control group. mtDNA was isolated from peripheral blood samples and polymerase chain reaction and mtDNA sequence analysis were performed.

Results: In one of the six patients, a homoplasmic mutant m.11778G>A mutation was detected. All of the clinically diagnosed LHON patients and the control groups had the m.14212C>T and m.14580G>A single nucleotide polymorphisms (SNPs). The m.11719A>G SNP was detected in three of six patients and four of the controls. Two of the six patients had the m.3197T>C SNP and, in addition, the m.14258G>A SNP was found in one of these two patients, while neither of these mutations were present in the control group.

Conclusion: The clinical diagnosis of LHON could be supported by molecular genetics only in one patient by the detection of one mutation. The m.3197T>C and m.14258G>A SNPs should be considered as potential mtDNA mutations due to the fact that they were detected in the patient group. These mutations should be investigated further in large case groups for suspected gene loci that could lead to optic neuropathy.

Key Words: Leber's hereditary optic neuropathy, familial optic atrophy, mitochondrial DNA, mitochondrial DNA mutations, single nucleotide polymorphism

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Introduction

Leber's hereditary optic neuropathy (LHON; OMIM 535000) is frequently seen in young adult males, resulting in bilateral central loss of vision due to the primary degeneration of retinal ganglion cells and optic atrophy, with an acute or subacute character and accompanying cardiac dysrhythmia. LHON is inherited in a mitochondrial or non-Mendelian pattern. In fact, LHON is the first mitochondrial disorder known to result from single point mutations. Wallace et al. demonstrated that the mutation resulting from the substitution of guanine (G) at nucleotide position 11778 of mitochondrial DNA (mtDNA) with adenine (A) is the most common mutation (69%) in LHON. This substitution causes a change in the gene encoding subunit 4 of the nicotinamide adenine dinucleotide (NADH) dehydrogenase (ND) 4 protein, which is one of seven subunits in NADH-ubiquinone oxidoreductase (NADH dehydrogenase) complex 1, which plays a role in energy production in mitochondria (1).

Studies conducted in recent years have shown that LHON is a heterogeneous disease, and that mutations in ND1, ND4 and ND6 might cause the disease independently. Up to now, about 35 mtDNA mutations in the subunits of the electron transport chain have been found in LHON (2). Some of these mutations are sufficient to cause the disease and are termed *primary mutations* without any presence in healthy individuals. Primary mutations are required for LHON but are not sufficient in the development of optic neuropathy. The primary mutations responsible for more than 95% of LHON cases are the m.3460G>A, m.11778G>A and m.14484T>C mtDNA point mutations (3). The most common one (50-76%) among these mutations is the m.11778G>A mutation. The m.3460G>A mutation has been reported to occur at a frequency of 7-30%, while the m.14484T>C mutation occurs at a frequency of 10-31% (4). The contributing environmental factors, however, remain unclear, and it is accepted that secondary factors influence the development of optic neuropathy (5).

The purpose of the present study was to investigate mtDNA mutations by DNA sequence analysis in patients with clinically diagnosed LHON in order to provide genetic support for the diagnosis.

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Address for Correspondence: *Dr. Hakan Gürkan, Department of Medical Genetic, Faculty of Medicine, Trakya University, Edirne, Turkey*
Phone: +90 284 235 76 42/1442 E-mail: dr_hakangurkan@yahoo.de

Material and Methods

We retrospectively reviewed the files of 329 patients with a diagnosis of optic neuropathy in our clinic from 1982-2007. Ten patients clinically diagnosed with LHON were invited to our clinic by mail. Six patients who responded our call in addition to six healthy individuals without any ophthalmological and systemic complaints were enrolled in the study (Table 1).

Six patients were diagnosed with LHON on the basis of painless, subacute, bilateral optic neuropathy by excluding the other causes of subacute optic neuropathy (Table 1).

Patient 1: Presence of a history of poor vision since childhood. Bilateral high hypermetropia (RE:+8.50, LE:+8.00), Bilateral optic atrophy.

Patient 2: Bilateral poor vision since the age of 8, no refractive error. Pale bilateral optic disc temporal branch, increase in vascular tilting more pronounced in the veins, presence of the optic disc with superficial telangiectatic vessels in the nasal branch. Prolongation of latency in bilateral VEP (P100 latency RE: 127.8 ms; LE: 169.4 ms).

Patient 3: Bilateral poor vision, bilateral hypermetropic astigmatism (RE:+0.50(+4.25/93), LE:+1.50(+2.75/86)). Optic disc hyperemia and superficial telangiectatic vessels more pronounced in the right optic disc.

Patient 4: Poor vision for 15 years, no refractive error. Bilateral flat optic atrophy.

Patient 5: Reduction in vision of the left eye and then the right eye with a four-month interval, pale right optic disk temporal branch in the fundus, optic atrophy in the left eye.

Table 1. Gender, age and visual acuity of the patients and the control groups

	Gender	Age	Visual Acuity in the Right Eye	Visual Acuity in the Left Eye
Patient 1	M	28	0.2	0.3
Patient 2	M	9	0.1	0.1
Patient 3	F	9	0.5p	0.5p
Patient 4	F	26	HM (+)	HM (+)
Patient 5	F	18	0.1	LP (-)
Patient 6	M	16	0.1	0.1
Control 1	E	40	1.0	1.0
Control 2	E	28	1.0	1.0
Control 3	E	28	1.0	1.0
Control 4	K	29	1.0	1.0
Control 5	K	27	1.0	1.0
Control 6	K	30	1.0	1.0

M: Male, F: Female, HM: Hand movement, LP: light perception

Patient 6: Poor vision since the age of 6, bilateral optic atrophy.

Peripheral blood samples were drawn from the individuals following a detailed ophthalmological examination and sent to Trakya University Medical Faculty, Department of Medical Biology for the investigation of possible mtDNA mutations by DNA sequence analysis.

Peripheral blood samples (5 ml) from each case were drawn into EDTA tubes and mtDNA was isolated by using the QIAMP DNA Blood Mini Kit (QIAGEN, Cat. No.:51104). Regions (16S rRNA, ND1, ND4 and ND6) including the m.11778G>A (R340H), m.3460G>A (A52T) and m.14484T>C (M64V) point mutations which have been reported to be responsible for 95% of the mtDNA mutations associated with LHON were amplified by polymerase chain reaction (PCR) using synthetic oligonucleotides (primer sequences) as described in Table 2.

We used the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) for amplification with the PCR program including one cycle at 94°C for 10 min, 40 cycles at 94°C for 30 sec, 52°C for 45 sec, 72°C for 1 min and 72°C for 5 min, then held at 4°C. The first PCR products were monitored with the help of a transilluminator to confirm the amplification by 2% agarose gel electrophoresis. The appropriate amplification products of the first PCR were purified using the QIAquick Post PCR purification kit (Catalog no: 28104) in order to remove residues according to the manufacturer's instructions. Following purification, regions on which mtDNA sequence analysis was to be be performed were amplified by DNA sequencing PCR, then the amplicons were run through spin columns containing Sephadex (Sigma-Aldrich, Catalog No: 064K1118) to remove residual oligonucleotides. Once prepared for mtDNA sequence analysis, samples were loaded into an Applied Biosystems (ABI) 3100 Avant automated DNA sequencer. The raw data obtained from the ABI 3100 Avant automated DNA sequencer regarding the forward and reverse nucleotide sequences were transferred to another computer and first matched using Proseq software. Common matched sequences were then transferred to the BioEdit software on the same computer and compared to the reference nucleotide sequence of the *Homo sapiens* mitochondrion complete genome obtained from NCBI Gene Bank (Accession No: NC_001807, Gene Bank Database); possible nucleotide changes (SNP/mutations) were then identified.

Results

Following mtDNA sequence analysis performed in all patients and controls, we could identify the homoplasmic m.11778G>A mutation only in one patient (patient 4) and as-

Table 2. Primer sequences used in PCR Amplification

Amplified Mutation Region	Forward Primer Sequence	Reverse Primer Sequence	Location
m.11778G>A (R340H)	gctccatctgcctacgac	ccacaacacaatggggct	ND4
m.3460G>A (A52T)	ggagtaatccaggtcggtttc	gctctcaccatcgctcttc	16S rRNA and ND1
m.14484T>C (M64V)	cctattcccccgagcaatc	gcacggactacaaccacg	ND6

sociated this mutation with LHON. We found the m.14212C>T and m.14580G>A homoplasmic nucleotide changes in all patients and controls. The m.11719A>G homoplasmic nucleotide change was detected in three of six patients and four of the controls. Two patients had the m.3197T>C homoplasmic nucleotide change, and in one of these two patients, we identified the m.14258G>A homoplasmic nucleotide change as well. Neither of these nucleotide changes were seen in the control group (Figure 1, Figure 2, Table 3).

Discussion

We investigated the three primary mutations (m.11778G>A, m.3460G>A and m.14484T>C) known to be the most common ones in the literature as a first priority in six patients being followed-up for clinically diagnosed LHON and in six healthy controls. The homoplasmic mutant m.11778G>A mutation was detected in only one patient (16%) (patient 4) and we have proven LHON as the precise diagnosis for this patient. We did not detect the m.3460G>A or m.14484T>C mutations either in the patients or in the control group.

LHON-associated mtDNA mutations have been identified in various ethnic populations. Mutations in the ND4 gene (m.11778G>A) account for 50-76% of cases, whereas mutations in the ND1 gene (m.3460G>A) are detected in 7-30% of cases and the ND6 gene (m.14484T>C) in10-31% of LHON cases in different ethnic backgrounds (6).

In a study by Harding et al. (7) investigating the mtDNA mutations of 89 patients diagnosed with LHON in the United Kingdom, they reported the incidence of the m.3460 mutation

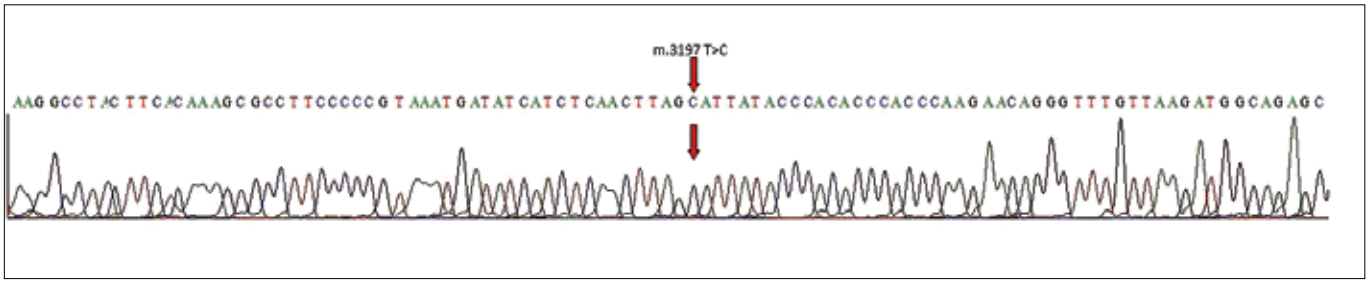

Figure 1. Nucleotide sequence and m.3197T>C SNP (homoplasmic, 16S rRNA location) of the samples obtained from the ABI 3100 Avant automated DNA sequencer

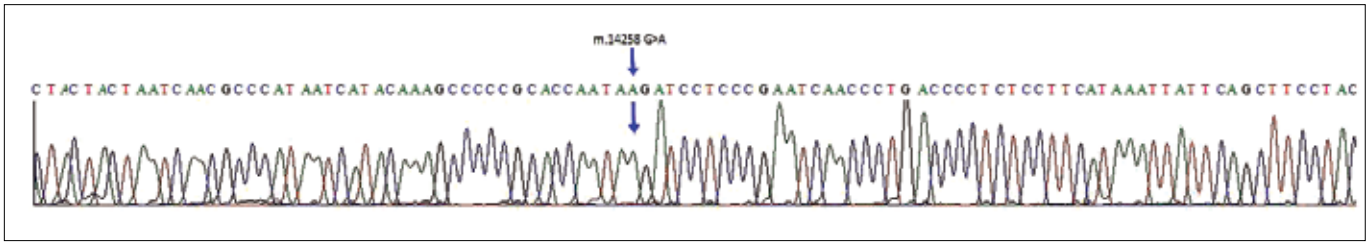

Figure 2. Nucleotide sequence and m.14258G>A SNP (homoplasmic, ND6 location, codon 139, second position, proline to leucine) of the samples obtained from the ABI 3100 Avant automated DNA sequencer

Table 3. Nucleotide changes detected in the patient (P) and control (C) groups

Name	m. 3197 T>C	m. 3460 G>A	m. 11696 G>A	m. 11719 A>G	m. 11778 G>A	m. 14212 C>T	m. 14218 T>C	m. 14258 A>G	m. 14323 G>A	m. 14580 G>A	m. 14620 C>T	m. 14668 C>T	m. 14484 T>C
P1	+	_	_	_	_	+	_	+	_	+	_	_	_
P2	+	_	_	+	_	+	_	_	_	+	_	_	_
P3	_	_	_	_	_	+	_	_	_	+	_	_	_
P4	_	_	_	_	+	+	_	_	_	+	_	_	_
P5	_	_	_	+	_	+	_	_	_	+	_	_	_
P6	_	_	_	+	_	+	_	_	_	+	_	_	_
C1	_	_	+	_	_	+	+	_	_	+	_	+	_
C2	_	_	_	+	_	+	_	_	_	+	_	_	_
C3	_	_	_	+	_	+	_	_	_	+	_	_	_
C4	_	_	_	+	_	+	_	_	_	+	_	_	_
C5	_	_	_	+	_	+	_	_	_	+	_	_	_
C6	_	_	_	_	_	+	_	_	_	+	+	_	_

in 9%, the m.14484 mutation in 13% and the m.11778 mutation in 78% of cases.

Zhou et al. (8) investigated 25 Chinese LHON patients who were clinically diagnosed; they concluded that the penetrance of the m.11778 mutation was very high.

Dogulu et al. (6) reported that three of 32 patients (9%) had the m.14484T>C mutation, one of 32 patients (3%) had the m.3460G>A mutation and one of the 32 patients (3%) had the m.11778G>A LHON mutation. According to their study, Dogulu et al. indicated that the incidence of these three primary LHON mutations in Turkish LHON patients is significantly lower than the previously studied populations.

According to our data, one of the six LHON diagnosed patients carried a primary LHON mutation, which supports the hypothesis of Dogulu et al. The effects of other mutations defined as secondary mutations on the disease remain unclear. These mutations were also detected in the control group, suggesting that they interact with other mutations and increase the risk of disease manifestation. These mutations are reported to be 3394 (Complex1/ND1 gene), 4160 (Complex1/ND1 gene), 4216 (Complex1/ND1 gene), 4917 (Complex1/ND2 gene), 5244 (Complex1/ND2 gene), 7444 (Complex4/COX1 gene), 7706 (Complex4/COX2 gene), 13708 (Complex1/ND5 gene), 13730 (Complex1/ND5 gene) and 15812 (Complex3/apocytochrome b gene). The most common secondary mutations are G13708A, A4160C, T3394C and G5244A (9-11).

When the secondary mutations were investigated in the five remaining patients which were clinically diagnosed as LHON and did not carry the primary m.11778G>A, m.3460G>A and m.14484T>C mutations, it appeared that they all had m.14212G>T and m.14580G>A nucleotide changes. However, we considered these nucleotide changes, which were also present in all control cases, as polymorphisms.

The m.3197T>C nucleotide change (16S rRNA location) that we found in our patients has been reported as an SNP in the related literature (T allele in 2608 sequences and C allele in 96 sequences of a reported total of 2704 sequences) (12-15). The m.14258G>A SNP (ND6 location, codon 139, second position, proline to leucine) that we determined in one of our patients has been reported in only one sequence of 2704 sequences in the related literature (12-16). The presence of the m.3197T>C and m.14258G>A SNPs in only one patient suggests that these SNPs might cause LHON or another optic neuropathy with a genetic background. According to our results, investigating primary and secondary mutations of clinically diagnosed LHON patients in the Turkish population is not adequate. The probability of identifying new, Turkish population-specific mtDNA mutations should be taken into account. On these grounds, analyzing the entire mitochondrial genome would be significant.

Conclusion

Consequently, LHON is a clinically diagnosed disorder. The diagnosis could be supported by molecular genetics in only one of six patients with clinically diagnosed LHON. The m.3197T>C and m.14258G>A SNPs should be considered as potential mtDNA mutations due to the fact that they were detected in a patient. Additional mutations should be investigated further in larger case groups for suspected gene loci that could lead to optic neuropathy.

Conflict of Interest

No conflict of interest was declared by the authors.

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