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Point mutations associated with Leber hereditary optic neuropathy in a Latvian population

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Purpose: To study mutations associated with Leber hereditary optic neuropathy (LHON) in patients suspected of having this mitochondrial disorder in a Latvian population. Additional aims were to determine the heteroplasmy status of all non-synonymous polymorphisms identified in the current study and to identify the mitochondrial haplogroups of the studied participants because these factors may contribute to the manifestation of LHON.

Methods: Twelve patients, including patients in two families, were enrolled in the current study. LHON was suspected based on the findings of ophthalmologic examinations. In clinically affected individuals, the presence of all previously reported LHON-associated mutations was assessed with sequencing analysis. Additionally, the SURVEYOR endonuclease assay was used to detect heteroplasmy. The mitochondrial haplogroups were identified with restriction analysis and the sequencing of hypervariable segment 1.

Results: In one family (mother and son), there was one primary LHON-associated mutation, G11778A. In addition, one rare previously reported LHON-associated polymorphism, A13637G, was detected in two unrelated patients. A non-synonymous polymorphism at T6253C was found in one individual. This mutation was reported in the background of the 3460 mutation among LHON patients in a Chinese population. No non-synonymous point mutations in mitochondrial DNA were found in five of the study participants.

Conclusions: Molecular analysis of 12 patients with suspected LHON confirmed the diagnosis in four patients and allowed the use of appropriate prophylactic measures and treatment. Further investigations and additional studies of different populations are necessary to confirm the role of the non-synonymous polymorphisms A13637G and T6253C in the manifestation of LHON and the associations of these polymorphisms with mitochondrial haplogroups and heteroplasmy.

Leber hereditary optic neuropathy (LHON) is a mitochondrial disorder characterized by bilateral or, rarely, unilateral painless acute or subacute visual failure without a clear etiology. The incidence of LHON according to various authors varies from 1:50,000 to 1:31,000 [1,2]. The provisional diagnosis of this disease is based on ophthalmologic examinations that reveal swelling of the optic nerve head and vascular changes such as peripapillary telangiectasia, microangiopathy, and vascular tortuosity [3]. The clinical manifestations of LHON and the age of onset are highly variable. Both sexes can be affected, but the clinical symptoms of this disease most often appear in 20- to 30-year-old men [4]. Visual failure usually develops with visual blurring and impairment of the central visual field in one eye, and some months later, the same symptoms are found in the second eye. However, there have been rare cases of LHON in which the second eye remained unaffected for years [5]. This state can progress to atrophy of the optic nerve, causing blindness [6].

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Approximately 90% of individuals affected by LHON have one of three point mutations in mitochondrial DNA (mtDNA): G3460A, G11778A, or T14484C [7]. Molecular analysis of individuals who do not harbor these mutations but exhibit clinical manifestations of LHON has revealed other point mutations in mtDNA. Currently, 69 non-synonymous polymorphisms in the MITOMAP database may contribute to this disease when the three strictly LHON-associated mutations are not present.

To cause phenotypic pathology, the amount of mutant mtDNA should exceed a critical threshold level, which may differ for different positions in the mitochondrial genome [8]. It is thought that heteroplasmic LHON-associated mutations in mtDNA can cause significant effects if the proportion of the mutant variant exceeds 60% [6].

When a mitochondrial disease as complex as LHON is studied, it should be kept in mind that the penetrance, phenotypic expression, and prognosis of this disorder may depend on many factors, e.g., the mtDNA haplogroup-defining polymorphisms (selectively neutral), the mutation type (e.g., patients with the 14,484 mutation have a better prognosis and a greater chance of partial vision recovery than patients

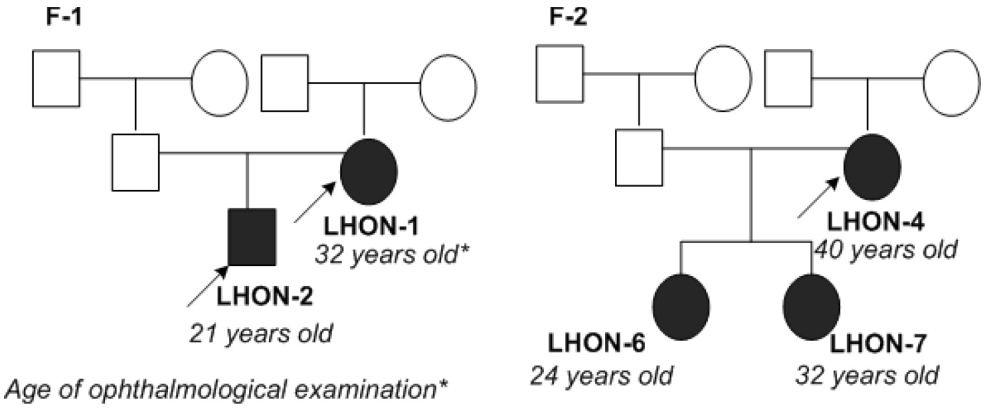

Figure 1. Two families (F1 and F2) involved in the current study.

with the 11,778 mutation), hormonal factors, environmental factors, and other factors. Moreover, the possible effects of the nuclear DNA background on the severity and penetrance of LHON have been widely discussed [2,9,10].

The epidemiological study of this rare mitochondrial disease is based on molecular analysis of the mitochondrial genome of patients suspected of having LHON based on ophthalmologic examinations. Only three common LHON-associated mutations are usually analyzed, and the rare LHON-associated polymorphisms have been comparatively poorly studied [11].

It may be of critical importance to analyze all non-synonymous polymorphisms in the mtDNA of patients with clinical manifestations of LHON in different populations to evaluate the role of these polymorphisms in the development of this mitochondrial disorder. The primary aim of this study was to detect LHON-associated mutations in patients suspected of having LHON in a Latvian population. A secondary aim was to report all new non-synonymous polymorphisms in the mitochondrial genome found in the study population. Additionally, the heteroplasmy status of each non-synonymous polymorphism was determined, and the mitochondrial haplogroups of the study participants were identified because these factors may also contribute to the manifestation of LHON.

METHODS

Patients: Twelve patients, including patients in two families, F1 and F2 (Figure 1), were enrolled in this study. In the first family, the mother and son were affected, and in the second family, visual failure was observed in only one family member (LHON-4) and not in her offspring (LHON-6 and LHON-7). The current study was approved by the Ethics Committee and complied with the Declaration of Helsinki [12]; all participants provided written informed consent. Two samples (LHON-1 and LHON-2) were obtained in 2007, and other samples were obtained in 2011–2012. Information on the health state and family history of the studied participants was collected using health and hereditary questionnaires.

Ophthalmologic examination: The ophthalmologic examinations were performed in the Ophthalmology Clinic of Pauls Stradins Clinical University Hospital, Riga. The following tests were used: visual acuity and visual field examinations, fundus photography, and optical coherence tomography (OCT). Other possible causes of visual failure were excluded, such as metabolic diseases (e.g., diabetes mellitus), toxicity (alcohol), trauma, and inflammation.

Molecular analysis of the mitochondrial genome: Whole DNA was extracted from blood leukocytes using the standard phenol chloroform method [13]. A total of 72 point mutations in mtDNA that had been previously reported to be associated with LHON according to the MITOMAP database (last update in August 2012) were analyzed. The polymorphism phenotyping (PolyPhen) and Sorting Tolerant From Intolerant (SIFT) databases were used to evaluate the interspecies conservation of these mutations and the possible or probable damaging status of each mutation (PolyPhen, SIFT). This information was considered when the results were interpreted. Twenty-two mtDNA fragments were amplified using oligonucleotide primers as described previously [14-17] (Appendix 1). PCR was performed in a final volume of 12.5 µl. The master mix contained (per tube) 1.25 µl of 10x *Taq* Buffer containing $(NH_4)_2SO_4$ (Thermo Scientific, Vilnius, Lithuania), 1.25 µl of 25 mM $MgCl_2$ (Thermo Scientific), 0.125 µl of dNTPs (10 mM each; Thermo Scientific), 0.5 µl of 10 µM forward primer (Metabion, Martinsried, Germany), 0.5 µl of 10 µM reverse primer (Metabion), 0.25 µl of *Taq* DNA Polymerase (5 U/µl; Thermo Scientific), and 7.63 µl of nuclease-free water (Thermo Scientific). To each tube was added 1 µl of 20 ng/µl DNA. The conditions of the

Table 1. Clinical data and results of ophthalmologic examination.

Code of the sample	Gender	Family history of visual failure	Age of onset	Age of enrolment into the genetic study	Visual acuity		Visual fields	
					OD	OS	OD	OS
LHON-1	F	No	35	50	0.06	0.06	CS	CS
LHON-2	M	Yes (LHON-1; mother)	21	27	0.03	0.04	CS	CS
LHON-3	M	NA	39	43	0.01	0.01	CS	CS
LHON-4	F	No	40	55	0.05	0.06	CCS	CCS
LHON-5	M	No	53	54	0.05	0.75	CCS	N
LHON-6	F	Yes (LHON-4; mother)	-	24	75	0.75	N	N
LHON-7	F	Yes (LHON-4; mother)	-	32	1	1	N	N
LHON-8	F	No	30	49	0.1	0.1	CS	CS
LHON-9	F	No	51	53	0.4	0.4	CCS	CCS
LHON-10	M	No	58	58	0.1	0.05	CCS	CCS
LHON-11	F	No	41	45	0.3	0.4	CCS	CCS
LHON-12	M	No	26	36	0.2	0.5	CCS	N

Abbreviations: CS – central scotoma; CCS – cecocentral scotoma; N – normal; NA – not available; OD – oculus dexter (right eye); OS – oculus sinister (left eye).

PCR were as follows: one cycle at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, primer annealing for 30 s (temperatures are given in Appendix 1) and 72 °C for 30 s (for long fragments, 13 and 15, the elongation time was 2 min); and a final elongation at 72 °C for 5 min. Each fragment was evaluated using an agarose gel stained with 10 mg/ml ethidium bromide and then was purified using the *Exo*I and *FastAP* enzymes (Thermo Scientific). The fragments were sequenced in both directions using the forward and reverse primers and a 3130×l Genetic Analyzer (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. For PCR fragments 13 and 15, long reads were used, and the capillary length was changed from 50 cm to 80 cm.

Analysis of mitochondrial DNA haplogroups: The mtDNA haplogroups of the participants were determined by sequencing hypervariable segment I (HVS-I) and with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis [15].

Detection of mtDNA heteroplasmy using the SURVEYOR assay: Because sequencing is not sufficiently sensitive to detect heteroplasmy (the limit of detection is approximately 20% [18,19]), the SURVEYOR mismatch endonuclease assay was used. The limit of detection for heteroplasmy by this method is 3% [14]. The mismatch endonuclease is able to digest a DNA heteroduplex on the 3′-side of the mismatch site formed by heating and slow cooling if heteroplasmy is present in the studied mtDNA fragment. All PCR products were treated using the SURVEYOR mismatch endonuclease and analyzed with 4% polyacrylamide gel electrophoresis with staining with ethidium bromide. The SURVEYOR Mutation Detection Kit (Transgenomic, San Jose, CA) was used in this study. The reactions were performed according to the manufacturer's protocols and Bannwarth's method of detection of heteroplasmic mutations in a whole mtDNA using SURVEYOR mismatch endonuclease [14].

RESULTS

Five affected men, five affected women, and two unaffected women participated in the study. The average age of onset was 38.5 years. The results of the ophthalmologic examinations and molecular analysis are summarized in Table 1 and Table 2, respectively.

Analysis of individual cases:

First family: Mother (LHON-1) and son (LHON-2)— In the first family (F1), mother and son had significant visual impairment. The clinical manifestation was more severe in the son (LHON-2) than in his mother (LHON-1). A significant decrease in visual acuity and central scotoma occurred in LHON-2 at the age of 21 years, but 1 year later, an ophthalmological examination revealed a decrease in the size of the central scotoma. Both individuals harbored a primary LHON-associated homoplasmic mutation in the MT-*ND4* gene: G11778A (p.Arg340His).

Patient LHON-3—The family history was not available for this patient. At the age of 39 years, this patient experienced significant visual loss in both eyes (visual acuity, 0.01) and

Table 2. Summary of molecular data on analyzed samples.

Code of the sample	Detected polymorphisms*	Change of amino acid	Prediction of pathogenicity		Other mtDNA coding region polymorphisms	HVS-I haplotype	Polymorphisms that determine haplogroup and RFLP restriction site	HG
			Poly Phen-2	SIFT				
LHON-1	G11778A (MT-*ND*4)	Arg340His	Damaging	Predict Not Tolerated	Not checked due to discovery of the primary with LHON-associated mutation	rCRS	7028C (−7025 *Alu*I)	H
LHON-2	G11778A (MT-*ND*4)	Arg340His	Damaging	Predict Not Tolerated	Not checked due to discovery of the primary with LHON-associated mutation	rCRS	7028C (−7025 *Alu*I)	H
LHON-3	T6253C (MT-*COX*I)	Met117Thr	Benign	Predict Tolerated	A4769G, T5267C, G8860A, G11914A, T14953C, A15326G	16,126-16184	7028C (−7025 *Alu*I)	H
LHON-4	A13637G (MT-*ND*5)	Gln434Arg	Benign	Predict Tolerated	T3197C, A7768G, C9477T, A11467G, A12308G, G12372A, T13617C, T14182C	16,126-16189–16325	7028T (+7025 *Alu*I); A12308G (+12308 *Hinf*I)	U5b
LHON-5	A13637G (MT-*ND*5)	Gln434Arg	Benign	Predict Tolerated	A3861G, A4769G, C8441T,G8860A, T9457C, C9477T, A11653G, G11719A, A12308G, G12372A, A12634G, T13617C, A13630G, T14182C, C14766T, G15497A	16,093-16258–16270–16292–16362	7028T (+7025 *Alu*I); A12308G (+12308 *Hinf*I)	U5b
LHON-6	A13637G (MT-*ND*5)	Gln434Arg	Benign	Predict Tolerated	T3197C, A7768G, C9477T, A11467G, A12308G, G12372A, T13617C, T14182C	16,126-16189–16325	7028T (+7025 *Alu*I); A12308G (+12308 *Hinf*I)	U5b
LHON-7	A13637G (MT-*ND*5)	Gln434Arg	Benign	Predict Tolerated	T3197C, A7768G, C9477T, A11467G, A12308G, G12372A, T13617C, T14182C	16,126-16189–16325	7028T (+7025 *Alu*I); A12308G (+12308 *Hinf*I)	U5b
LHON-8	Not revealed				A4793G, G8860A, A13434G, A15326G	rCRS	7028C (−7025 *Alu*I)	H
LHON-9	Not revealed				A8860G, G9477A, G9622A, A10283G, A12612G, T14182C	16,189-16270	7028T (+7025 *Alu*I); A12308G (+12308 *Hinf*I)	U5a
LHON-10	Not revealed				A15326G	16304	7028C (−7025 *Alu*I)	H
LHON-11	Not revealed				G4580A, A4793G, G8860A, T11899C, A15326G	16,153-16233–16298	A4580G (−4577 *Nla*III)	V
LHON-12	Not revealed				T3552C, A4793G, G4924C, A11467G, A11530G, G11719A, T14110C, A15326G	16,192-16256–16270–16304–16399	7028T (+7025 *Alu*I); A12308G (+12308 *Hinf*I)	U5a

*Non-synonymous polymorphisms that are already reported to be associated with LHON disease or novel mutations that are not associated with patient mitochondrial haplogroup; Abbreviations: rCRS – revised Cambridge Reference Sequence [34]; HG – mitochondrial haplogroup; HVS-I – Hypervariable Segment I; F – Female; M –Male; NA – not available; RFLP – Restriction Fragment Length Polymorphism.

atrophy of the optic nerve. Analysis of the mtDNA revealed one non-synonymous mutation, T6253C (p.Met117Thr), in the MT-*COX*I gene, which is not noted as associated with LHON in the MITOMAP database. To assess an interspecies conservation and the possible pathogenicity of the T6253C mutation, the PolyPhen and SIFT databases were used. These databases indicated that this mutation may be benign and tolerated, respectively.

Second family: Affected mother (LHON-4) and two unaffected daughters (LHON-6 and LHON-7): In this family (F2), only the mother (LHON-4) exhibited clinical features of LHON, which appeared at the age of 40 years. The rare LHON-associated mutation A13637G was found in the MT-*ND*5 gene of this patient. Then the two daughters (LHON-6 and LHON-7) of this patient were examined ophthalmologically to assess the state of their vision. At the examination, they were 24 and 32 years old, respectively. In addition, fragment number 14 of each daughter was sequenced to determine if there was a mutation at position 13,637. The same mutation was found in both daughters even though they were phenotypically healthy at the time of the study. In the PolyPhen database, the A13637G mutation is listed as benign, and in the SIFT database, it is listed as "predict tolerated."

Patient LHON-5: This male patient, who was 53 years old, had progressive visual impairment; over a period of 1 month, his visual acuity decreased from 0.3 to 0.05 in his right eye and from 1.0 to 0.75 in his left eye. The ophthalmological examination revealed edema, atrophy of the optic nerve, and cecocentral scotoma only in the right eye. Sequencing of the MT-*ND*5 gene revealed the presence of a secondary LHON-associated point mutation, A13637G, the same as that found in LHON-4.

Patients LHON-8, LHON-9, LHON-10, and LHON-11: These unrelated patients were suspected of having LHON due to significant visual impairment with an unclear etiology, but the molecular analysis of mtDNA did not confirm this diagnosis, as no LHON-associated mutations were found.

Patient LHON-12: The sex and age of onset of this study participant were characteristic of LHON. The patient was male and experienced significant visual impairment at the age of 26 years. This patient's decrease in visual acuity was not as severe as that for the patients discussed above. Visual field impairment (cecocentral scotoma) occurred only in the right eye. Molecular analysis of the mtDNA did not reveal any LHON-associated mutations.

Analysis of the mtDNA haplogroups of the studied patients: The majority of the participants belonged to haplogroup H or U, which are abundant mtDNA genotypes in the Latvian population [20] (Table 2).

Analysis of mtDNA heteroplasmy: As described previously, all non-synonymous point mutations in the mtDNA found in the current study were present only in the homoplasmic state. The homoplasmic state of these polymorphisms was suggested by the analysis of the sequencing chromatograms and was confirmed using the SURVEYOR mismatch endonuclease assay (Figure 2).

DISCUSSION

The results of studies on the prevalence of LHON and reports of individual cases of this disease are available for some European and Asian populations [21,22]. The current study provides additional information about the distribution of LHON-associated mutations in a Latvian population.

Prevalence of Leber hereditary optic neuropathy in European populations: As described previously, there is the great probability of identifying of one of the most common with LHON-associated mutations (G11778A, G3460A, or

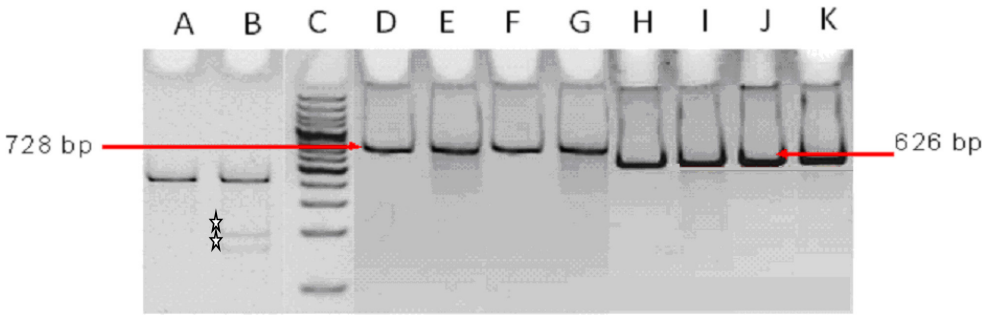

Figure 2. Detection of mitochondrial deoxyribonucleic acid heteroplasmy using SURVEYOR mismatch endonuclease on 4% polyacrylamide gel. Abbreviations: Lanes A, D, F, H, J, undigested PCR products; lanes B, E, G, I, K, digested PCR products with SURVEYOR mismatch endonuclease (Transgenomic); lane C, size marker (100 bp Ladder plus, Thermo Scientific). Lane A, a negative control for heteroplasmy (homoplasmic sample); lane B, a positive control for heteroplasmy (heteroplasmic sample). Stars indicate the location of cleavage fragments. Lanes D, E: LHON-1, 15 amplified fragment; lanes F, G: LHON-2, 15 amplified fragment; lanes H, I: LHON-3, 4 amplified fragment; lanes J, K: LHON-4, 14 amplified fragment.

T14484C) among individuals suspected of having LHON. In the current study, the members of one family (two affected members) with distinct clinical features of LHON harbored the G11778A mutation. None of the studied individuals possessed the G3460A or T14484C mutation. However, the low prevalence of this disease in European populations should be considered. The absence of other primary LHON-associated mutations among the studied participants may be due to the small number of patients enrolled in the current study. Furthermore, the current project started recently and represents data collected over a comparatively short period of time. These data may be insufficient for evaluating the prevalence of such a rare disease. However, the project is ongoing, and all collected data may be used to develop a LHON registry in Latvia. In Finland, which is geographically close to Latvia, 36 families with LHON were identified over a period of 34 years. The most common LHON-associated mutation was G11778A (19 families with LHON). The A3460G and C14484T mutations in the mtDNA were found less frequently (four families and one family, respectively). In the remaining 12 families with clinical manifestations of LHON, none of the three primary LHON-associated mutations were found [1]. Sixteen families with LHON were identified in an epidemiological study performed in northeast England. One of the three primary LHON-associated mutations was found in each family by sequencing of the entire mtDNA. The most common LHON-associated mutation in this study was G11778A (60%), as observed in the Finnish population. The G3460A mutation was found in 33% of families with LHON, and the T14484C mutation was found in 7% [23]. Another epidemiological study was performed in the Netherlands, and among the 63 analyzed Dutch families suspected of having LHON, 56 carried one of the primary LHON-associated mutations. In 33 cases, it was the G11778A mutation, in 11 cases, it was G3460A, and in 12 cases, it was T14484C. Seven patients did not harbor any primary LHON-associated mutations [24].

Haplogroup association with LHON: In some studies of European populations, an association between LHON and haplogroup J has been found [1,15]. Moreover, in some cases, the clinical manifestations and penetrance of LHON have been found to be milder in families belonging to haplogroup H than among families belonging to haplogroup J [1,25]. Additionally, one clinical study showed that rare LHON-associated point mutations may be deleterious or, conversely, beneficial in the context of different mitochondrial haplogroup backgrounds [26]. It is important to consider the distribution of the mitochondrial haplogroups in the region under study to evaluate the mitochondrial genetic background and its possible influence on the penetrance of LHON. Haplogroup J is more common in the Italian population than in the Latvian population (14.3% versus 6.4%, respectively) [20,27]. Haplogroup J is found in the Finnish population at the same proportion as in the Latvian population (6.3% and 6.4%, respectively) [20,28]. Although haplogroup J is not common in these populations, it could be overrepresented among individuals with LHON due to the possible influence on the clinical manifestation of this disease. Based on phylogenetic analysis, in regions where haplogroup J is not common, there is an association between LHON and other definitive geographic region-specific haplogroups, e.g., M7b for the Chinese population [29]. This result may indicate that various combinations of selectively neutral polymorphisms in mtDNA may be associated with the penetrance of this disease. However, the more abundant haplogroups in the region under study should also be considered because of the possibility of masking the association of LHON with comparatively rare haplogroups, especially if the sample size is not large enough. For example, haplogroups H and U are more frequent in the Latvian population, and therefore, there is a high probability that individuals with LHON in this region may belong to these haplogroups instead of haplogroup J, which is rare [20]. Published data on the association of LHON with specific haplogroups are more abundant for the three common LHON-associated mutations, especially for G11778A [15]. Currently, there are insufficient data on the effect of the mitochondrial haplogroup background on the severity and age of onset of LHON in patients harboring the only rare LHON-associated mutation in European populations. In the current study, the majority of affected individuals belonged to haplogroups H and U, and none of subjects belonged to haplogroup J; therefore, it is not possible to compare the penetrance and severity of LHON between the two mtDNA phylogenetic branches: haplogroup H and haplogroup J.

In epidemiology studies of LHON in European populations, there were many sporadic cases of this disease and families without primary LHON-associated mutations; these patients harbored other non-synonymous polymorphisms in the mitochondrial genome [1,24]. Many of these newly identified polymorphisms are included in the MITOMAP database as predictably associated with the development of LHON. The majority of these polymorphisms are localized in MT-*ND* genes (49 (71%) out of 69 mutations), which encode the subunits of NADH dehydrogenase, which is part of OXPHOS (oxidative phosphorylation) respiratory chain complex I.

The previously reported rare LHON-associated mutation in the MT-*ND*5 gene A13637G was found in two unrelated individuals with LHON in the current study. These individuals belonged to the mitochondrial haplogroup U5b,

which is listed as associated with this polymorphism in the PhyloTree database. According to the PhyloTree database, the A13637G polymorphism is also associated with two Asian haplogroups: M1a3 and N1c. This association may indicate that this polymorphism arose in different populations due to independent mutational events [30]. In addition, the A13637G polymorphism was found in one Chinese family that harbored the primary LHON-associated mutation G3460A. This family belonged to the haplogroup M10a1, which has not been shown to be associated with the A13637G polymorphism according to the PhyloTree database [31].

In the study of LHON in a Chinese population, the non-synonymous polymorphism T6253C was also observed [29]. This same polymorphism was found in patient LHON-3 in the current study. According to the MITOMAP database, this point mutation was previously reported to be associated only with prostate cancer [32,33]. According to the PhyloTree database, the T6253C polymorphism is also associated with various mitochondrial haplogroups: L1c2, M13'46'61, D5b1, and H15.

The PolyPhen and SIFT databases, which are widely used to predict probable deleterious effects of non-synonymous polymorphisms, did not confirm the pathogenicity of the A13637G and T6253C mutations. In light of all this information, these non-synonymous polymorphisms in the mtDNA may not be sufficient for the development of LHON. However, we could not exclude the possibility that these polymorphisms may be possible risk factors for LHON that could contribute to the clinical manifestation of this disease under certain conditions (e.g., stress, trauma) or when accompanied by other risk factors (e.g., a definitive nuclear background, excessive alcohol consumption). Further investigations and additional reports on the association between the molecular and clinical characteristics are necessary to evaluate the significance of these mutations in the development of this complex mitochondrial disease.

Conclusions: In a Latvian population, one family affected with LHON with a primary LHON-associated mutation, G11778A, was identified. Additionally, the A13637G mutation was identified in the mtDNA of two affected unrelated participants in this study, and the T6253C mutation was found in one patient. The analysis of the A13637G and T6253C mutation using the SIFT, PolyPhen data analysis software, and PhyloTree databases revealed that these polymorphisms are not conserved among species and are widely distributed among different mitochondrial haplogroups. Further investigations and additional reports are necessary to confirm the roles of these polymorphisms in the manifestation of LHON.

APPENDIX 1. PRIMER PAIRS USED FOR AMPLIFICATION AND SEQUENCING ANALYSIS IN THE CURRENT STUDY.

To access the data, click or select the words "Appendix 1."

ACKNOWLEDGMENTS

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