Mitochondrial DNA variation of Leber's Hereditary Optic Neuropathy (LHON) in Western Siberia

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Abstract

Leber's hereditary optic neuropathy (LHON) is a form of disorder caused by pathogenic mutations in a mitochondrial DNA. LHON is maternally inherited disease, which manifests mainly in young adults, affecting predominantly males. Clinically LHON has a manifestation as painless central vision loss, resulting in early onset of disability. Epidemiology of LHON has not been fully investigated yet. In this study, we report 44 genetically unrelated families with LHON manifestation. We performed whole mtDNA genome sequencing and provided genealogical and molecular genetic data on mutations and haplogroup background of LHON patients in the Western Siberia population. Known "primary" pathogenic mtDNA mutations (MITOMAP) were found in 32 families: m.11778G>A represents 53,10% (17/32), m.3460G>A – 21,90% (7/32), m.14484T>C – 18,75% (6/32), and rare m.10663T>C and m.3635G>A represent 6,25% (2/32). We describe potentially pathogenic m.4659G>A in one subject without known pathogenic mutations, and potentially pathogenic m.9444C>T, m.6261G>A, m.9921G>A, m.8551T>C, m.8412T>C, m.15077G>A in families with known pathogenic mutations confirmed. We suppose these mutations could contribute to the pathogenesis of optic neuropathy development. Our results indicate that haplogroup affiliation and mutational spectrum of the Western Siberian LHON cohort substantially deviate from those of European populations.

Introduction

Leber's hereditary optic neuropathy is a form of hereditary disorder caused by pathogenic mutations in a mitochondrial DNA. These mutations are non-synonymous and affect genes coding for different subunits of complex I of the mitochondrial respiratory chain. The occurrence of such kind of mutations in mtDNA subunits leads to dysfunction of the electron transport, increased reactive oxygen species production and defective ATP synthesis (Baracca, Solaini et al. 2005; Lin, Sharpley et al. 2012; Kim, Jurcute and Yu-Wai-Man 2018). Retinal ganglion cells are highly susceptible to death during LHON progression, because of their high sensibility to disrupted ATP production and oxidative stress (Meyerson, Van Stavern et al. 2015). Therefore, LHON is usually manifested as painless, acute or subacute, with central visual loss in both or one eye, results to early onset of disability. The onset of Leber hereditary optic neuropathy is relatively rare in childhood and has better visual prognosis. The peak age of onset of visual loss among LHON carriers is 20–30 years old (Majander, Bowman et al. 2016). In some cases, LHON patients have been reported as having additional neurologic, cardiac, and endocrine disorders (Finsterer and Zarrouk-Mahjoub 2016; Haas 2019, de Barcelos, Troxell and Graves 2019). Leber's disease is inherited maternally and manifests in young adults, predominantly affecting males – 50% of male and only 10% of female subjects experience vision loss. Interestingly, this sex predilection cannot be explained by the principles of mitochondrial inheritance (Meyerson, Van Stavern et al. 2015). At the present time a number of mtDNA point mutations have been described, but the most prevalent are m.3460G>A, m.11778G>A and m.14484T>C, accounting for about 90% of cases of LHON worldwide. The prevalence of each mutation varies among different populations, but the average is 69-92% for m.11778G>A, 3-19% for m.14484T>C and 1-13% for m.3460G>A (Mackey, Oostra et al. 1996; Mashima, Yamada et al. 1998; Kumar, Kaur et al. 2012, Romero, Fernandez et al. 2014; Jiang, Liang et al. 2015; Khan, Govindaraj et al. 2017). However, there are significant deviations from the average in some populations, for example, among French Canadians 87% of cases are due to m.14484T>C as a result of a founder effect (Laberge, Jomphe et al. 2005). Moreover, phenotypic expression of these primary mutations has been found to vary in different populations and different pedigrees. This incomplete penetrance suggests that other factors, such as mtDNA haplogroup background, nuclear genetic background and environmental factors, may influence the modulation of phenotypic expression and severity of the disease (Jacobi, Leo-Kottler et al. 2001; Kirkman, Yu-Wai-Man et al. 2009; Istikharah, Tun et al. 2013).

Consequently, the worldwide prevalence of LHON varies in different populations and is unknown for the majority of them. According to broad epidemiologic studies, it is estimated between 1:30000 and 1:50000 (Puomila, Hamalainen et al. 2007; Mascialino, Leinonen et al. 2012). Epidemiology of LHON has not been fully investigated in Russian Federation, and our

previous studies included the description of isolated cases (Brown, Zhadanov et al. 2001; Brown, Starikovskaya et al. 2002; Volod'ko, L'vova et al. 2006). Hence, in the present study, we reported 44 genetically unrelated LHON families, performed whole mtDNA sequencing and provided genealogic and molecular genetic data on mutations and haplogroup background of LHON patients in the Western Siberia population.

Materials and methods

Subjects

This study was approved by the Ethics Committee IRB 00001360 affiliated with Vector State Research Center of Virology and Biotechnology (SRC VB Vector), Novosibirsk, Russian Federation. The total number of subjects in the study is 178 individuals from 44 unrelated families (85 affected and 83 healthy carriers), including 17 cases from our previous studies (Brown, Zhadanov et al. 2001; Brown, Starikovskaya et al. 2002; Volod'ko, L'vova et al. 2006). The clinical follow-up of LHON patients has been carried out by the Novosibirsk Branch of Federal Eye Microsurgery Department since 1997, conducted by one of the authors. The clinical diagnosis was based on a combination of symptoms and signs: painless acute or subacute central vision loss, fundus changes and visual field abnormality such as pseudopapilledema, optic nerve atrophy, central or cecocentral scotoma. All the individuals made an informed decision to take part in the study and provided written consent. Family history was taken in each case to identify maternal inheritance of symptoms. The complete mtDNA genomes were sequenced for the family's probands, and for the other individuals the certain mutations were confirmed by sequencing of associated mtDNA regions.

mtDNA analysis

Whole peripheral blood samples were collected from the donors in 10-ml Vacutech EDTA tubes. Total DNA was extracted from buffy-coat layer using the SileksMagNA-G™ Blood DNA Isolation kit, according to the manufacturer's protocols. The complete sequencing procedure entailed PCR amplification of 22 overlapping mtDNA templates (Nochez, Arsene et al. 2009), which were sequenced in both directions with BigDye 3.1 terminator chemistry (PE Applied Biosystems, Foster City, CA, USA). The trace files were analyzed with Sequencher (version 4.5 GeneCode Corporation) software. To perform capillary electrophoresis on ABI Prism 3130XL DNA Analyzer we used core facilities of the "Genomika" Sequencing Center, SBRAS, Novosibirsk, Russian Federation. Variants were scored relatively to the Reconstructed Sapiens Reference Sequence, RSRS (Behar, van Oven et al. 2012). MtDNA haplotypes were identified following the nomenclature suggested by the PhyloTree Build 17 (van Oven and Kayser 2009).

42 mitochondrial genomes obtained through this study were deposited in GenBank with accession numbers XX000000-XX000000. Two genomes, EU807741.1 and EU807742.1, had been accessed earlier (Brown, Zhadanov et al. 2001).

Penetrance analysis

We determined penetrance as the proportion of affected individuals from all maternally related family members using family pedigrees (Puomila, Hamalainen et al. 2007). Values for both men and women were calculated separately. Pedigrees are available in supplementary materials.

Analysis of pathogenicity for non-synonymous mutations

To make sure that the revealed non-synonymous mtDNA mutations are not sequencing errors or hot points for the general population, and to find out the disease–associated polymorphisms among those published earlier, we used several databases: MITOMAP (Brandon, Lott et al. 2005), mtDB - Human Mitochondrial Genome Database, containing 2704 human mitochondrial genomes (Ingman and Gyllensten 2006) and HmtDB – Human Mitochondrial DataBase, containing 32922 human mitochondrial genomes (Attimonelli, Accetturo et al. 2005). To assess the possible pathogenicity of these mutations and to predict whether a protein sequence variation affects protein function, we used the following web applications: MutPred 1.2 (Li, Krishnan et al. 2009), MutPred 2 (Pejaver et al. 2017), PolyPhen – 2 (Adzhubei, Schmidt et al. 2010), PROVEAN (Protein Variation Effect Analyzer) (Choi, Sims et al. 2012) and SIFT (Sorting Intolerant from Tolerant) (Kumar, Henikoff et al. 2009) programs. All the sources are provided in the public domain.

Results

From 44 LHON families, 32 harbored a primary mutation; the results are shown in Table 1. Among families with a primary mutation, the m.11778G>A represents 53,10% (17/32), m.3460G>A – 21,90% (7/32), m.14484T>C – 18,75% (6/32). Rare m.10663T>C and m.3635G>A represent 6,25% (2/32) of the families.

According to the family pedigrees, only 50% (22/44) of cases had a family history of vision loss on maternal lineage in more than one generation, among which m.11778G>A represented 36% (8/22), the m.14484T>C – 14% (3/22), m.3460G>A – 23% (5/22) and without primary mutations (LHON-like cases) – 18% (4/22). Rare mutation cases (m.10663T>C and m.3635G>A) were family–inherited. 39% (17/44) of cases were sporadic, among which 13 cases were with only one affected person diagnosed and 4 cases with two affected persons in one generation. Among

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the sporadic cases m.11778G>A represents 41% (7/17), m.3460G>A – 12% (2/17), m.14484T>C – 6% (1/17), and the LHON-like cases – 41% (7/17).

Summary information on penetrance is shown in Table 2. The average penetrance among men was 32% (6-100%) and among women – 12% (0-58%). Our data correlate with data previously published by (Meyerson, Van Stavern et al. 2015). However, there are particular families with higher penetrance among females than among males: L24, L26, L28. The penetrance is highly variable between separate families, even with the same primary mutation as shown in pedigrees in supplementary materials.

In 12 families with a clear-cut LHON phenotype, no pathogenic mtDNA mutations were found. Analysis of the mtDNA revealed non-synonymous mutations: m.14002A>G, m.4766A>G, and m.13105A>G, which have not been noted as associated with LHON or other diseases in the MITOMAP database. All of pathogenicity prediction tools indicated low probability that these amino acid substitutions are disease-associated for these mutations. Mutation m.4659G>A has been previously reported as associated with Parkinson's disease (Khusnutdinova, Gilyazova et al. 2008), and in an Australian LHON pedigree that was heteroplasmic for the m.14484T>C (Mackey and Howell 1992). Polyphen-2 predicted the pathogenicity for this mutation as benign, MutPed 2 showed low probability score, but MutPred 1.2, PROVEAN and SIFT determined this mutation as deleterious. The results are shown in Table 3.

In several families with primary mutations (L01, L03, L12, L28, L30, L40, L43) we found out additional non-synonymous mutations, as shown in Table 4. Mutations m.8875T>C, m.14582A>G, m.8400T>C, m.4639T>C were neutral and mutation m.9444C>T had high probability to be pathogenic according to data of all the pathogenicity prediction tools; for other mutations we observed divergence of prediction results. Since prediction results for primary pathogenic mutations diverged too (see Table 3), novel non-synonymous nucleotide change was considered potentially pathogenic if it had extremely low frequency in the general population and it was predicted by at least three algorithms to have an effect on protein function. For mutations m.6261G>A and m.15468C>T only PolyPhen2 predicted pathogenicity as probably damaging and possible damaging respectively. However, mutation m.6261G>A has already been reported by Abu-Amero (Abu-Amero and Bosley 2006) in patient with optic neuropathy and also as somatic mutation associated with prostate cancer. Interestingly, that the family (L01) with m.6261G>A and m.11778G>A has the same haplogroup – T2, as case was reported by Abu-Amero. Other mutations (m.9921G>A, m.8551T>C, m.8412T>C, m.15077G>A) were predicted as pathogenic at least by three algorithms, but the first three of them have not been noted as associated with diseases in the MITOMAP database, and mutation m.15077G>A was reported as associated with maternally inherited isolated deafness (Gutierrez Cortes, Pertuiset et al. 2012).

Phylogenetic analysis clearly illustrates genetic origin of Siberian carriers of pathogenic LHON mutations and confirms that all families are unrelated and belong to different maternal phylogenetic lines. All classic and rare mutations occurred among European haplogroups J, H, V, and U. It is interesting that m.11778G>A were not occurred among haplogroups of branch M. Mutations m.3460G>A and m.14484T>C belong to diverse haplogroups – J, V, H of European origin, M8 and D of Asian origin. Phylogenetic trees are shown in Figures 1, 2 and 3.

Discussion

Primary LHON mutations

In Western Siberia, frequencies of primary mutations are different from frequencies reported for Europe and Asia. The most prevalent m.11778G>A (55%) is less common in Western Siberia than in Europe – 69% (Mackey, Oostra et al. 1996), China and Japan – 90% (Mashima, Yamada et al. 1998, Jiang, Liang et al. 2015). The prevalence of m.3460G>A (24%) is twice as much as in Europe, but m.14484T>C (13,8%) do not deviate from those of other European populations (Mackey, Oostra et al. 1996).

In twelve families with LHON manifestation no one known pathogenic mtDNA mutation was found. However, there are other elaborations in which the clinical diagnosis not confirmed by molecular-genetic analysis (Howell, Oostra et al. 2003, Abu-Amero and Bosley 2006, Aitullina, Baumane et al. 2013, Rosenberg, Norby et al. 2016). Apparently, it is difficult to distinguish the mtDNA mutations-caused LHON from the other forms of optic neuropathy, for example, from the dominant optic neuropathy (DOA), especially when it is the sporadic clinical case without any pattern of inheritance. Compared to LHON, DOA visual loss is detected between ages 4 and 6 in the majority of patients, and 58–84% of patients with DOA report visual impairment by age 11 (Fraser, Biousse et al. 2010). In our 11 cases ages of onset were between 13 – 36 years. We are going to study these cases for the mutation spectrum of common pathogenic genes for DOA in future.

Penetrance

We suppose that the reasons for low prevalence of LHON disease in Western Siberia are associated with incomplete penetrance and diagnostic difficulties of atypical (e.g. late onset) and combined (e.g. multiple sclerosis) forms of LHON. Patients with LHON could have also been wrongly diagnosed as suffering from toxic amblyopia, tobacco–alcohol amblyopia, or optic neuritis (Rosenberg, Norby et al. 2016). On the other hand, the problem is that patients do not now their family history. Molecular testing for LHON is not routinely performed in patients with optic atrophy in Russian Federation. Identification and registration of unaffected carriers plays an

important role for prevention of disease manifestation. For example, there is strong evidence now that smoking is associated with an increased risk of visual failure among LHON carriers – 93% penetrance of vision loss in male smokers versus a 66% penetrance in male non-smokers (Kirkman, Yu-Wai-Man et al. 2009). Our observations highlight the importance of molecular genetic examination for unaffected carriers. Since the proportion of sporadic cases is high, 39% according to our data, 40% according to published data (Meyerson, Van Stavern et al. 2015), the presence of pathogenic mutations should be tested not only for probands but for all relatives on the maternal line.

Potentially pathogenic mutation m.4659G>A

We found out m.4659G>A in one subject without any known primary mutations (L51). This sequence change is located at codon 120 in the functional domain of the CO1 gene and changes an alanine, a hydrophobic amino acid, into threonine - a neutral amino acid. Mutation m.4659G>A has been reported as associated with LHON in an Australian pedigree that also had heteroplasmic mutation m.14484T>C and m.5460G>A (Mackey and Howell 1992). This family had 10 maternally related descendants, five of whom with vision loss. Unfortunately, our patient does not know his family history and we couldn't confirm maternal inheritance for this mutation. However, m.4659G>A has very low frequency in the general population (0,0011) and high probability to be pathogenic according to data of different prediction tools (Table 3).

Additional non-synonymous mutations revealed in LHON cases

Phenomenon of co-existence of two pathogenic mutations in one family has already been described. In the first case, m.14484T>C, m.4659G>A and m.5460G>A in an Australian LHON pedigree, described above (Mackey and Howell 1992). In the second case, a family harboring two primary LHON mutations m.11778G>A and m.14484T>C, and both mutations had synergistic pathogenic effect on protein function, and a higher degree of heteroplasmy of the m.14484T>C correlated with an earlier age at onset (Catarino, Ahting et al. 2016). And the third example is a unique double-mutant ND4L with two concurrent mutations m.10609T>C and m.10663T>C in an Arab pedigree from Kuwait (Behbehani, Melhem et al. 2014).

We reported mutations m.9444C>T, m.6261G>A, m.9921G>A, m.8551T>C, m.8412T>C, m.15077G>A, which could be potentially pathogenic because of low frequency in the general population and high probability to be pathogenic according to data from different prediction tools. Two of them m.6261G>A and m.15077G>A have already been reported in subjects with optic neuropathy and maternally inherited isolated deafness respectively. However, we suppose that additional non-synonymous mutations could either have a synergistically pathogenic or a

protective effect. To demonstrate the full significance of novel mutations respiratory chain assay should be performed. For example, as in the study by Gutierrez Cortes, Pertuiset et al. 2012, where cybrids with m.15077G>A showed normal activities for mitochondrial electron chain enzymatic complexes.

Haplogroup analysis

Western Siberia region is represented predominantly by European haplogroups and includes aboriginal Asian populations. Asian haplogroups have been found in Altaian (L18) and Tuvinian (L24) as in Russian families (L10, L25).

Rare mutations m.10663T>C and m.3635G>A were found in Russian families from Kazakhstan (the first) and the Novosibirsk region (the second) associated with the European haplogroups J1c4 and J2b1c1, respectively (Brown et al. 2001, Brown et al. 2002). Mutation m.10663T>C was also reported in the background of the haplogroups J1c2c, L2a1, L3'4, L3f1b (Abu-Amero and Bosley 2006, Achilli, Iommarini et al. 2012, Behbehani, Melhem et al. 2014, Al-Kharashi, Al-Kharashi et al. 2016) and mutation m.3635G>A - R11a, D4g2b, M7b4, F1a, B5b, M7b (Yang, Zhu et al. 2009, Bi, Zhang et al. 2012). The presence of the same pathogenic mutations on the background of various mitochondrial haplogroups confirms that pathogenic LHON mutations arise de novo, independently of different mtDNA haplogroups and ethnic backgrounds. It is known that the clinical impact of mDNA mutations may be modulated by mitochondrial haplogroups. For example, Hudson et al. performed a multicenter study of 3,613 subjects from 159 different families and showed that the risk of visual failure is greater when m.11778G>A or m.14484T>C mutations are present in specific subgroups of haplogroup J, the m.3460G>A mutation is present in haplogroup K, and the risk of visual failure is significantly lower when m.11778G>A occurs in haplogroup H (Hudson, Carelli et al. 2007) Romero et al. supposed that haplogroup D has a protective effect in carriers of LHON mutations. His hypothesis was based on the fact that there was a markedly decreased frequency of haplogroup D in Chilean subjects with LHON, as haplogroup D is one of the most common in the Chilean population (Romero, Fernandez et al. 2014). Also, other experimental research serves as proof that cybrids and fibroblasts bearing LHON mutations have different response to neurotoxic agents depending on haplogroup background (Ghelli, Porcelli et al. 2009).

Association of mtDNA variation in different diseases could be a result of founder events. It is suggested, that at the end of the last glaciation phylogenetically more ancient mutations could have provided their carriers with the adaptive advantages upon the development of Central and Northern Europe, and positive selection could contribute to the fixation of weakly pathogenic LHON mutations, appearing at specific genetic background (Volod'ko, L'vova M et al. 2006).

Conclusion

These data may be insufficient for evaluating the prevalence of LHON. However, the project is still ongoing, and all the data collected might be used to develop a LHON registry in Russian Federation.

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Table 1. *Summary of the data for examined LHON families. Published previously by: *(Volod'ko, L'vova M et al. 2006); **(Brown, Starikovskaya et al. 2002; Volod'ko, L'vova et al. 2006); ***(Brown, Zhadanov et al. 2001; Volod'ko, L'vova et al. 2006).*

No.	Family name	Ethnicity on maternal line	Number of examined individuals (affected/ healthy)	Family history of visual loss	Primary mutation	mtDNA haplogroup
1	L1*	Russian	9 (1/8)	Yes	m.11778G>A	T2b
2	L2**	-/-	6 (2/4)	Yes	m.10663T>C	J1c4
3	L3*	-/-	4 (1/3)	Yes	m.11778G>A	T2d1b1
4	L5*	-/-	1 (1/0)	Yes	m.11778G>A	J1c2i
5	L6	-/-	2 (2/0)	No	-	U4a1d
6	L8	Unknown	1 (1/0)	No	-	U2e1
7	L9	Russian	2 (2/0)	Yes	-	U5a1b1c1
8	L10*	-/-	8 (3/5)	Yes	m.14484T>C	M9a1a1c1a
9	L12*	-/-	2 (1/1)	No	m.11778G>A	J2b1a1
10	L14*	-/-	5 (2/3)	No	m.11778G>A	T2b28
11	L17*	-/-	8 (4/4)	Yes	m.14484T>C	J1c2c1
12	L18*	Altaian	4 (2/2)	No	m.3460G>A	D4p
13	L20	-/-	3 (2/1)	Yes	-	U4b1b1
14	L23*	Azerbaijani	1 (1/0)	Unknown	m.11778G>A	J2b1
15	L24*	Tuvinian	19 (7/12)	Yes	m.3460G>A	C5d1
16	L25***	Russian	5 (3/2)	Yes	m.3460G>A	D5a2a2
17	L26*	-/-	13 (8/5)	Yes	m.11778G>A	J1c7a
18	L27***	-/-	11 (2/9)	Yes	m.11778G>A	H2a5b
19	L28***	-/-	9 (2/7)	Yes	m.11778G>A	T2b8
20	L30***	-/-	19 (11/8)	Yes	m.3635G>A	J2b1c1
21	L31***	-/-	3 (2\1)	Yes	-	U3b1b
22	L32	Unknown	1 (1/0)	Unknown	m.14484T>C	V
23	L38	Ukrainian	2 (2/0)	Yes	m.11778G>A	J1c2c2a
24	L39	Unknown	2 (1/1)	No	m.11778G>A	V
25	L40	Albanian	1 (1/0)	Yes	m.14484T>C	H
26	L41	German	2 (1/1)	No	m.3460G>A	H40a
27	L42	Belarusian	2 (1/1)	Yes	m.11778G>A	H1c
28	L43	Russian	3 (1/2)	No	m.11778G>A	H
29	L45	-/-	2 (1/1)	No	-	U5a2e
30	L46	-/-	2 (1/1)	No	-	H13a1d
31	L47	-/-	1 (1/0)	No	m.14484T>C	J1c5a1
32	L49	Unknown	1 (1/0)	No	m.11778G>A	K1c
33	L50	Unknown	1 (1/0)	Unknown	m.14484T>C	U5a2b1c
34	L51	Unknown	1 (1/0)	Unknown	-	U2c1b
35	L52	Russian	3 (1/2)	No	m.11778G>A	J1c2
36	L53	Unknown	3 (1/2)	Unknown	m.11778G>A	H1b2
37	L54	Russian	2 (1/1)	No	-	U4a2a
38	L56	-/-	2 (1/1)	No	-	J1c1b1
39	L57	-/-	1 (1/0)	Yes	m.3460G>A	V1a1
40	L58	-/-	2 (1/1)	Yes	m.3460G>A	J1c3
41	L59	Ukrainian	2 (1/1)	No	-	V7a
42	L60	Russian	2 (1/1)	No	m.11778G>A	H1b2
43	L61	-/-	4 (2/2)	Yes	m.3460G>A	H1b1
44	L62	-/-	1 (1/0)	Yes	-	H

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Table 2. *Summary information about penetrance. Pedigrees are available in supplementary materials.*

	m.11778G>A (N=15)	m.14484T>C (N=4)	m.3460G>A (N=7)	Total
Males	34%	46%	15%	31%
Females	12%	12%	10%	11%

Table 3. *Non-synonymous mutations revealed in LHON–like cases. Known primary mutations (m.11778G>A, m.3460G>A, m.14484T>C, m.10663T>C, m.3635G>A) were placed into this table to demonstrate differences between different prediction algorithms and frequencies in general population for pathogenic mutations. MutPred 1.2 score >0,75 referred to as confident hypotheses to be pathogenic. Mutpred 2 score >0.50 would suggest pathogenicity.*

Mutation	Protein-coding region of mtDNA	Amino acid substitution	PolyPhen – 2 score	MutPred 1.2/2 Score (cutoff 0,75/0,50)	PROVEAN/ SIFT prediction	Frequency in general population (mtDB)	Frequency in general population (HmtDB)	Family
m.14002A>G	ND5	T556A	0,002 (benign)	0,387/0,059	Neutral/Tolerated	0,0037	0,00289	L45
m.4766A>G	ND2	M99I	0,001 (benign)	0,571/0,225	Neutral/Tolerated	0	0,00009	L46
m.13105A>G	ND5	I257V	0,001 (benign)	0,198/0,032	Neutral/Tolerated	0,0612	0	L51
m.4659G>A	ND2	A64T	0,029 (benign)	0,790/0,256	Deleterious/ Damaging	0,0011	0,00161	
m.11778G>A	ND4	R340H	0,999 (probably damaging)	0,919/0,494	Deleterious/ Damaging	0,0097	0,00340	-
m.3460G>A	ND1	A52T	1,000 (probably damaging)	0,789/0,418	Neutral/ Damaging	0,0097	0,00058	-
m.14484T>C	ND6	M64V	0,993 (probably damaging)	0,618/0,787	Neutral/Damaging	0,0026	0,00146	-
m.10663T>C	ND4L	V65A	0,946 (probably damaging)	0,604/0,694	Deleterious/ Damaging	0	0,00003	-
m.3635G>A	ND1	S110N	0,999 (probably damaging)	0,873/0,493	Deleterious/ Damaging	0	0,00027	-

Table 4. Additional non-synonymous mutations revealed in LHON cases. MutPred 1.2 score >0,75 referred to as confident hypotheses to be pathogenic. Mutpred 2 score >0.50 would suggest pathogenicity.

Mutation	Protein-coding region of mtDNA	Amino acid substitution	PolyPhen – 2 score	MutPred 1.2/2 Score (cutoff 0,75/0,50)	PROVEAN /SIFT prediction	Frequency in general population (mtDB)	Frequency in general population (HmtDB)	Family
m.6261G>A	CO1	A120T	0,998 (probably damaging)	0,491/0,324	Neutral/ Tolerated	0,0048	0,00553	L01
m.8875T>C	ATP6	F117L	0 (benign)	0,251/0,429	Neutral/ Tolerated	0,0007	0,001276	L03
m.9921G>A	CO3	A239T	0,009 (benign)	0,543/0,624	Deleterious/ Damaging	0,0011	0,00082	L12
m.15468C>T	CYB	T241M	0,890 (possible damaging)	0,245/0,079	Neutral/ Tolerated	0,0004	0,00043	L28
m.8551T>C	ATP6	F9L	0,976 (probably damaging)	0,676/0,418	Deleterious/ Damaging	0,0007	0	L30
m.14582A>G	ND6	V31A	0,003 (benign)	0,245/0,181	Neutral/Tolerated	0,0086	0,00571	L40
m.8400T>C	ATP8	M12T	0 (benign)	0,504/0,118	Neutral/Tolerated	0,0011	0,00052	L43
m.9444C>T	CO3	R80W	0,999 (probably damaging)	0,875/0,586	Deleterious/ Damaging	0	0	
m.4639T>C	ND2	I57T	0,001 (benign)	0,297/0,047	Neutral/ Tolerated	0,0082	0,00395	L50
m.8412T>C	ATP8	M16T	0,711 (possible damaging)	0,677/0,542	Deleterious/ Tolerated	0	0,00039	
m.15077G>A	CYB	E111K	0,992 (probably damaging)	0,684/0,331	Deleterious/ Damaging (low confidence)	0,0007	0,00213	

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Figure 1, 2, 3. Phylogenetic trees based on complete mtDNA genome sequences of pedigree-probands with pathogenic mutations. The non-synonymous coding-region variants are denoted by "ns" (known pathogenic mutations designated "in bold"), the insertions and deletions are denoted by "ins" and "del," respectively, underlined mutations are recurrent.

R

JT

U

J

T

J1

J2

T2

J1c

J1c2

J1c3

J1c4

J1c5a1

J1c7a

J2b1

T2b

T2d

K1c1

U5a2b1c

J1c2c

J1c2i

J2b1a1

J2b1c1

T2b8

T2b28

T2d1b1

J1c2c1

J1c2c2a

513
4639ns
8412ns
13299
15077ns
16269
16270
14484

151
10256
16240
16311
11778

2442
16312
14484

195
5351
12172
3460

9132
10663

1824
2392
11887

6152
7891
9884
11778

152
7202
9921ns
11778

8551ns
10362
12070
16286
3635

195
6261ns
8266
11778

15468ns
15924
16147
16297
16390
11778

4917
8875ns
16296
11778

2706
11778

11778

11887

11778

L52 L17 L25 L05 L58 L02 L47 L26 L23 L12 L30 L01 L28 L14 L03 L49 L50