

Title: A new sequence variant in mitochondrial DNA associated with high penetrance of Russian Leber hereditary optic neuropathy

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Abstract

We have analyzed mitochondrial DNA sequence in 15 Russian LHON patients and found the new mtDNA sequence variant in one family (2 patients) who showed 100% penetrance of the disease in men. This family has a T14484C primary mutation, and 4 secondary mutations (T4216C, G 13708A, G 15812A, G15257A), which belong to the European haplogroup J. The new sequence variant of A9016G in the ATPase 6 gene changed highly conserved amino acid of isoleucine to valine, has not been found in the rest of 13 LHON patients and controls. This novel sequence variant may contribute to the 100% penetration of LHON disorder in men of this family.

Key words: LHON, point mutation, penetrance, mitochondrial DNA

Introduction

Leber Hereditary Optic Neuropathy (LHON [MIM 535000]) is a maternally inherited genetic disorder associated with point mutations in the mitochondrial DNA (mtDNA) that cause blindness in young adults (Wallace et al., 1988; Howell. et al., 2003). It is characterized by acute or subacute, progressive, and bilateral loss of central vision due to focal degeneration of the retinal ganglion cell layer and optic nerve. Common primary LHON mutations are G3460A, G11778A, and T14484C not in association with each other, heteroplasmic and are absent among control are necessary for expression of the disease but not sufficient (Man et al., 2002). Secondary/intermediate LHON mutations showed different frequency pattern among the three classes of above common primary mutations in positive patients and controls, usually homoplasmic, and may contribute to the LHON by increasing the probability of expressing the phenotype (Torroni et al., 1997).

Patients and methods.

Patients.

Fifteen patients from 13 unrelated Russian families were fulfilled the clinical criteria of LHON (Wallace et al., 2001), however, patients 8, 9, 11, 12, 13, 14 and 15 have no family history. Three women and twelve men from 10 to 56 years old with mean age of onset about 22.3 years. The research followed the tenets of the Declaration of Helsinki. Informed consent

was obtained from the patients after explanation of the nature and possible consequences of the study. DNA was taken and studied for the various mtDNA mutations.

One family which has the highest penetrance among our 13 unrelated families is shown in Figure 1. The proband is a 35-year-old Caucasian man, who suffered from a rapid bilateral painless loss of central vision. The loss of vision began at the age of 18 years, and at the same period, the patient was admitted to the hospital with diabetic coma due to insufficient insuline production. He had a 6-month interval between the blindness in both eyes which was not caused by diabetic retinopathy. The visual acuity is 0.02-0.03 with the central scotomas. Bilateral loss of central vision was also detected in patient's mother, a 56-year-old woman, when she was 47 years old. She had two brothers and two uncles who were completely blind from their youth, showing 100% penetrance of LHON disease in men of maternal lineage. There is no family history of diabetes mellitus, deafness or neuromuscular disorders except blindness.

Sequencing analysis.

Total DNA was extracted from white blood cells using the standard method (King et al., 1992). The complete mitochondrial genome was amplified by long-PCR method in 7 overlapping fragments using 14 primers (Table1). The PCR conditions were: first, one cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 3 min, and finally, one cycle of 72 °C for 5 min followed by cooling to 4 °C. The quality and quantity of

template DNA were determined by 1% agarose gel electrophoresis. ExoSap-IT (USB Corporation, Ohio USA) utilizes two hydrolytic enzymes Exonuclease I and Shrimp Alkaline Phosphatase together in a specially formulated buffer, to remove unincorporated dNTPs and primers. The ExoSap-IT was added directly to the PCR product. After treatment, ExoSap-IT was inactivated simply by heating to 80 °C for 15 minutes. Using 40 forward primers (Table 2) and CEQ Dye Terminating Cycle Sequencing Kit (Beckman Coulter, Inc., Fullerton, CA), sequence reaction was performed as following: first, one cycle of 96 °C for 5 min, followed by 30 cycles of 96 °C for 20 sec, 50 °C for 20 sec, 60 °C for 3 min, and finally, one cycle of 60 °C for 5 min followed by cooling to 4 °C. The sequences were assembled in a contig using the program DNASIS Pro (Hitachi Software Engineering Co, Ltd, Tokyo, Japan) and the resulting contig was aligned to the Cambridge sequence (Anderson et al., 1981; Andrews et al., 1999).

PCR-RFLP analysis.

To confirm the heteroplasmic condition in primary and secondary mutation reported in LHON, or novel sequence variant, we performed the PCR-RFLP analysis using suitable sets of primers and restriction enzymes (Brown et al., 1995; Brown et al., 2001). Conditions for all PCR amplifications were as follows: first, one cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55.5 °C for 1 min, 72 °C for 45 sec, and finally, one cycle of 72 °C for 5 min followed by cooling to 4 °C. Diagnostic restriction endonuclease digestions were resolved by 12% polyacrylamide gel electrophoresis, and the DNA fragments were identified by ethidium

bromide.

For new A9016G sequence variant, we designed a set of primers as following: forward from np8833 to np8852 and reverse from np9140 to np9121 (5'-3'). This fragment contains a single restriction site for HpyCH4IV in the presence of mutation. To eliminate partial enzymatic digestion, all restriction enzyme reactions were carried out overnight, followed by the addition of 10 units of enzyme and 2 additional hours of incubation. Percentage of heteroplasmy was quantitated by one-dimensional densitometry using Image Quant software version 4.1 (Molecular Dynamics, Sunnyvale, CA, USA).

Results.

All nucleotide changes found in our study are shown in Table 3. Seven patients from 5 unrelated families have one of the primary mutations. Two unrelated patients (patients 1 and 7) have a G11778A mutation. One of them (patient 7: haplogroup J) has two secondary mutations of T4216C and G13708A, however, the other (patient 1: haplogroup W) has no secondary mutation. Three patients (2 families) have a G3460A mutation, one family (patients 4 and 5: haplogroup X) has no secondary mutations, and the other (patient 6: haplogroup T1) has a T4216C and A4917G mutations. Patients 2 and 3 (mother and son) belong to haplogroup J and have a primary mutation of T14484C and secondary mutations of a T4216C, G13708A, G15257A, and G15812A. Eight patients from 8 families (patients from 8 to 15) have no known

primary mutation in their mtDNA, however 3 patients (patients 8, 12, and 13) from this group have one of the secondary mutations of G13708A or A4917G. Remaining 5 patients have none of pathogenic mutations reported before. Moreover, we have found a novel sequence variant of A9016G in the ATPase 6 gene in mother and son (patients 2 and 3) who have a primary mutation of T14484C (Figure 2).

Among 13 unrelated families from Russian LHON, distribution of mtDNA haplogroups were as follows: 23% for haplogroup H (n=3); 23% for haplogroup J (n=3); 15.4% for haplogroup X (n=2); 15.4% for haplogroup T (n=2); 7.7% for haplogroup U2 (n=1); and 7.7% for haplogroup M (n=1).

Discussion.

LHON is characterized by incomplete penetrance and men are preferentially affected (~68%) when the primary mutation of T14484C exist (Wallace et al., 2001). Since our family (patients 2 and 3), who showed 100% penetrance of the disease in men in three generations, has the nucleotide changes at C5633T, A11251G, A12612G, and C15452A in addition to primary mutation of T14484C in the homoplasmic condition, (Herrnstadt et al., 2002) they are constituent of European mtDNA haplogroup J background which is present in European population at a frequency of 9%. The other factors such as smoking, alcohol excess, diet, psychological stress, exposure to toxins, head trauma have been among the epidemiologic

factors suspected of increasing the penetrance of LHON (Tsao et al., 1999). However in our index family, there are no obvious risk factors to increase the penetrance of this disorder. We found the novel A9016G sequence variant in both mother and son in this family. This sequence variant was heteroplasmic and results in a substitution of isoleucine for valine at a highly conserved residue in the ATPase 6 polypeptide (Figure 3). Hence, this is likely to be pathogenic mutation according to above information (Riordan-Eva and Harding, 1995; Brown et al., 2002). This sequence variant has not been reported in the literature, MITOMAP and mtSNP database, and has not been found in 13 Russian LHON patients and in 30 Japanese individuals. Though a number of studies have failed to make the important distinction between frank pathogenic mtDNA mutations and haplogroup-associated polymorphism (Chagnon et al., 1999; Lin et al., 1992), this A9016G sequence variant may increase the penetrance of the disease in LHON, when it is associated with the primary mutation of T14484C and secondary mutations of a T4216C, G13708A, G15257A, and G15812A. In addition, this family has the unique combination of the following polymorphisms of A9494G, G11718A, A15662G, and C16193T which are reported in the literature, however, they are not present in the rest of 12 families in this study.

We could not perform further biochemical analysis in this index family, because specimens were not available. Thirty-seven to fifty percent of LHON having T14484C primary mutation are reported to be recovered within 16 months interval (Wallace DC et al., 2001, Web

site of Mitochondrial Research Society). However we have not observed such recovery of blindness in the members of this family. Though the pedigree is so small and additional biochemical data is not available in this study, we suggest that an A9016G sequence variant is specific for this family and may increase the penetration of LHON with the combination of secondary mutations (Mackey, 1994). Above information will continue to provide us with new insights into the pathophysiology of mitochondrial disease.

Acknowledgements

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MITOMAP. The human mtDNA database: <http://www.mitomap.org>

GIFU International Institute of Biotechnology. GIIB-JST mitochondrial mtSNP database: <http://www.giib.or.jp>

Web site of Mitochondrial Research Society.

<http://www.neuro.wustl.edu/neuromuscular/mitosyn.html>

Table 1. PCR primers used for amplification of the entire mtDNA of Russian LHON patients.

Fragment	Forward primer	Reverse primer	Length(bp)
1	Gn549F:5'-caa cca aac ccc aaa gac ac-3'	Gn3424B:5'-aac gtt ggg gcc ttt gcg ta-3'	2876
2	Gn3205F:5'-cca cac cca ccc aag aac ag-3'	Gn5910B:5'-cga aca tca gtg ggg gtg ag-3'	2706
3	Gn5662F:5'-cca atg gga ctt aaa ccc ac-3'	Gn8028B:5'-gct tca atc ggg agt act ac-3'	2367
4	Gn7794F:5'-cca tca tcc tag tcc tca tc-3'	Gn10515B:5'-gtg aga tgg taa atg cta g-3'	2722
5	Gn10289F:5'-agc cct aca aac aac taa cc-3'	Gn12360B:5'-ggg tat agt agt gtg cat gg-3'	2072
6	Gn12144F:5'-aca tca tta ccg ggt ttt cc-3'	Gn14762B:5'-tgc gta ttg ggg tca ttg g-3'	2619
7	Gn14641F:5'-acc cac act caa cag aaa c-3'	Gn706B:5'-gga tgc ttg cat gtg taa tc-3'	2635

Table 2. Forward primers used for direct sequence.

Number	Primer	5'-position
1.	CQ549:5'-caa cca aac ccc aaa gac acc	549
2.	CQ611:5'-gaa aat gtt tag acg ggc tc	611
3.	CQ 1161:5'-aac tca aag gac ctg gcg gt	1161
4.	CQ1690:5'-cca ctc cac ctt act acc aga c	1690
5.	CQ2164:5'-ccc ata gta ggc cta aaa gca gcc	2164
6.	CQ2629:5'-atg aat ggc tcc acg agg gtt c	2629
7.	CQ3116:5'-cct ccc tgt acg aaa gga c	3116
8.	CQ3205:5'-cca cac cca ccc aag aac agg g	3205
9.	CQ3712:5'-gta gcc caa aca atc tca ta	3712
10.	CQ4240:5'-tcc agc att ccc cct caa ac	4240
11.	CQ4621:5'-gtt cca cag aag ctg cca tc	4621
12.	CQ5151:5'-cta cta cta tct cgc acc tg	5151
13.	CQ5662:5'-cca atg gga ctt aaa ccc ac	5662
14.	CQ6251:5'-tat agt gga ggc egg agc ag	6251

15.	CQ6801:5'-gac aca cga gca tat ttc ac	6801
16.	CQ7321:5'-gag aag cct tcg ctt cga ag	7321
17.	CQ7794:5'-cca tca tcc tag tcc tca tc	7794
18.	CQ8239:5'-ctt tga aat agg gcc cgt att tac c	8239
19.	CQ8634:5'-tct cat caa caa ccg act aa	8634
20.	CQ9091:5'-aca ctt atc atc ttc aca at	9091
21.	CQ9621:5'-gca tca gga gta tca atc ac	9621
22.	CQ10133:5'-aca act caa cgg cta cat ag	10133
23.	CQ10289:5'-agc cct aca aac aac taa cc	10289
24.	CQ10616:5'-caa cac cca ctc cct ctt ag	10616
25.	CQ11183:5'-cgc ctg aac gca ggc aca ta	11183
26.	CQ11671:5'-aac ccc ctg aag ctt cac cg	11671
27.	CQ11900:5'-gtg cta gta acc acg ttc tcc	11900
28.	CQ12144:5'-aca tca tta ccg ggt ttt cc	12144
30.	CQ12602:5'-tca tcc ctg tag cat tgt tc	12602
31.	CQ13101:5'-agg aat ctt ctt act cat cc	13101
32.	CQ13490:5'-cct cac agg ttt cta ctc c	13491
33.	CQ13949:5'-cct atc tag gcc ttc tta cga gcc	13949
34.	CQ14511:5'-cta tta aac cca tat aac ct	14511

35.	CQ14641:5'-acc cac act caa cag aaa c	14641
36.	CQ15121:5'-aac agc ctt cat agg cta tg	15121
37.	CQ15535:5'-cac ccc tcc cca cat caa gcc	15535
38.	CQ15838:5'-cct aat acc aac tat ctc cc	15838
39.	CQ16200:5'-aca agc aag tac agc aat ca	16200
40.	CQ132:5'-ctt tga ttc ctg cct cat cc °	132

Table 3. Sequence variation in the mtDNA of the 15 LHON patients.

Accession	1	20	24	400	402	6	7	8	9	10	11	12	13	14	15
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* and ** : showed the members of the same family.

Gray boxes showed nucleotide change in this study.

Black boxes showed primary mutation of LHON.

Green boxes showed secondary mutation of LHON.

Purple box showed novel polymorphism of LHON in our family.

Figure 1. Pedigree of the family.

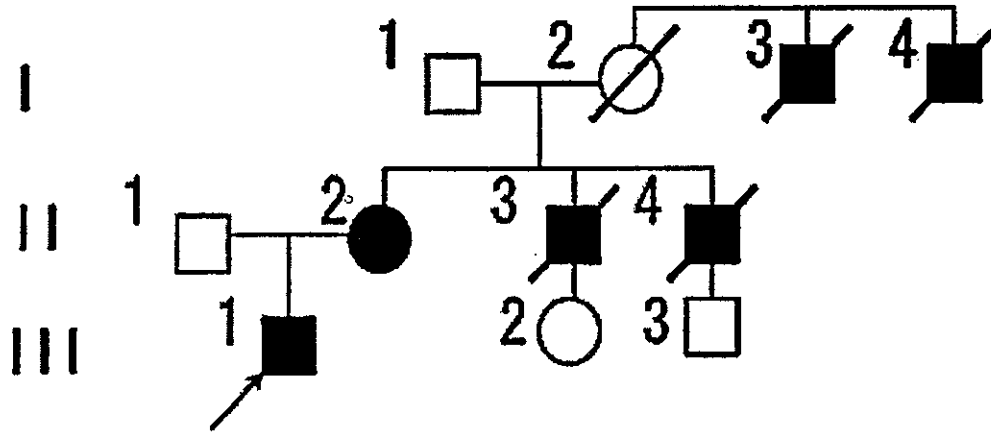


Figure 2. PCR-RFLP analysis of an A9016G mutation

