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Heteroplasmy analysis in the Polish patients with 11778A mutation responsible for Leber hereditary optic neuropathy✪

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We have analysed the heteroplasmy level in 11 individuals from 3 families harbouring the mitochondrial 11778A mutation responsible for Leber hereditary optic neuropathy using last cycle hot PCR. The mutation level exceeded 90% both in affected and in unaffected individuals. We also checked whether any of the families belonged to the J haplogroup of mitochondrial DNA and obtained a negative result.

Human mitochondrial DNA (mtDNA) is a small circular molecule coding for 13 respiratory chain proteins, 22 tRNAs and 2 rRNAs used in mitochondrial translation and is maternally inherited. In one cell a few hundred to a few thousand mtDNA molecules are present – from 1 to about 10 in one mitochondrion. Divisions of these molecules are not correlated with the cell cycle and during mitosis or meiosis mitochondria with mtDNA are randomly distributed to daughter cells. The existence of multiple DNA molecules, all equally susceptible to mutation can lead to the phenomenon called heteroplasmy when in one cell two or more different mtDNA molecules exist (mutated and not mutated). In consequence random distribution and drift can lead to differences in mutation load between tissues in the same individual or between individuals in the same family. Mutations in mitochondrial

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Abbreviations: LHON, Leber hereditary optic neuropathy; mtDNA, mitochondrial DNA.

DNA can lead to diseases most often affecting the neural and muscle system. Several dozen of such pathogenic mutations are known, with many of them being heteroplasmic and showing a strong threshold effect (symptoms of the disease appear when the mutation level exceeds a certain value, frequently even 85–90%) [1].

Leber hereditary optic neuropathy (LHON) was recognised for the first time at the end of the 19th century as a familial neuro-ophtalmological disease [2]. It is characterised by acute or subacute, painless visual loss. Eyes can be affected simultaneously or sequentially. The mean age of onset is about 27–34 years but cases from 1 to 70 years were reported.

In most of the cases loss of vision is the only clinical presentation but LHON occasionally can be associated with additional neurological symptoms and in rare cases even with severe neurodegenerative disease [3]. The existence of mutations specific for Leber hereditary optic neuropathy has also been suggested in multiple sclerosis but was excluded in multiple studies [4, 5].

Till now the disease has been associated with 18 missense mutations in mitochondrial DNA. All reported mutations mapped in protein coding genes, but the exact influence and role of each mutation in the disease is not clear. However, it was possible to distinguish three so-called primary mutations at base pair positions 11778, 3460 and 14484 (all nucleotide positions are given according to the Cambridge sequence [6]) which are present in at least 90% of LHON families, with 11778 being the most frequent one. The role of secondary mutations (additional mutations in mtDNA initially believed to contribute to disease symptoms) in the disease is now being questioned and it seems that they are rather polymorphisms associated with two Caucasian mtDNA forms (haplogroups J and T) [7]. For 11778 and 14484 a strong association with haplogroup J was observed suggesting that a combination of mutations specific for one of the J haplotypes increases the penetrance and disease occurrence risk [7, 8].

Genotype–phenotype correlation in the other diseases caused by mtDNA mutations is not straightforward and simple but some mutations seem to cause a more severe form of the disease than the others. For example 11778 mutation in most severe patients can lead to complete loss of vision, while 3460 severe patients retain light perception. The presence of one of the primary mutations is a necessary but not sufficient condition for disease development. Although maternally inherited, LHON does not show the typical pedigree pattern, where all children of an affected mother will be affected as male bias is observed in European patients harbouring common mutations. This observation led to the idea of an additional X-linked recessive mutation participating in disease development; later excluded by linkage analysis in LHON families [9]. LHON cases can be homoplasmic or heteroplasmic for mtDNA mutation with more severe mutations like 14459A found in one of the Hispanic families or 11778A common mutation being rather hetero- than homoplasmic [10]. Nevertheless even for a given mutation (11778A) both homo- and heteroplasmic affected individuals have been reported. In general few data are available on tissue distribution in heteroplasmic cases, but in general a uniform distribution is described [1].

Almost unique limitation of the symptoms to the optic nerve is puzzling and no explanation has been found yet.

11778A mutation causes an arginine to histidine change in amino acid 340 of NADH dehydrogenase 4 (ND4), a member of the respiratory chain complex I. Studies of the influence of this mutation on respiration capacity, enzyme activity and expression level were carried out on lymphoblasts and cybrids (cells resulting from fusion of rho0 cells lacking mitochondrial DNA and enucleated cells possessing mtDNA). They showed a significant decrease in respiration of the mutated cells and slight re-

duction in complex I activity. Reduction in growth rate on galactose was also observed. The expression pattern of mitochondrial proteins appeared to be unchanged [11, 12].

LHON had not been diagnosed at the DNA level in Poland till 2000. We describe the results of heteroplasmy analysis for three families harbouring the 11778A mutation performed in order to check the possibility of correlation between mutation level and disease expression.

MATERIALS AND METHODS

DNA samples were from LHON patients and members of families with G11778A mutation, altogether 11 subjects. Family A was primarily molecularly diagnosed in the Wills Eye Hospital (Philadelphia, PA, U.S.A.), family C was primarily diagnosed in the Tubingen University Clinic using similar molecular methods, but the heteroplasmy level was not checked and family B was diagnosed in the Department of Genetics, University of Warsaw (Fig. 1). DNA was isolated from leukocytes from blood using standard methods.

Existence of the mutation was confirmed by amplifying in 30 PCR cycles in standard conditions 230 bp fragment using primers:

F: CAGCCACATAGCCCTCGTAG (11632–11651)

R: GCGAGGTTAGCGAGGCTTGC (11843–11862)

followed by digestion with restriction enzyme *Mae*III (Roche) sensitive for G11778A mutation. Results were analysed on 3% agarose gels.

For quantitative analysis last cycle hot PCR was used. The PCR fragment was labelled with [α ^{32}P]dATP (approximately 0.01 mCi per sample; 3000 Ci/mmol) in one cycle of PCR and digested with *Mae*III according to manufacturer's instructions. Digested and undigested samples were run on 10% native polyacrylamide gels and exposed in PhosphorImager (Molecular Dynamics) cassettes and analysed by the ImageQuant programme (Molecular Dynamics).

To check if the individuals belong to haplogroup J region 15879–16545 of mtDNA was PCR amplified and the PCR product was digested with *Hin*fI (NEB, New England Biolabs) restriction enzyme in conditions suggested by manufacturer.

The T test was performed to check statistical differences in heteroplasmy level between affected and unaffected individuals and between males and females.

RESULTS AND DISCUSSION

For 5 LHON patients and 6 healthy family members, all carrying the 11778A mutation heteroplasmy analysis was performed using last cycle hot PCR (Fig. 2). Existence of an additional restriction site for *Mae*III just after

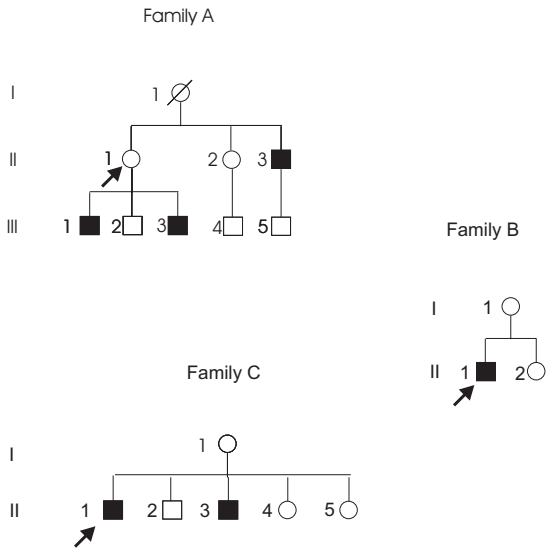
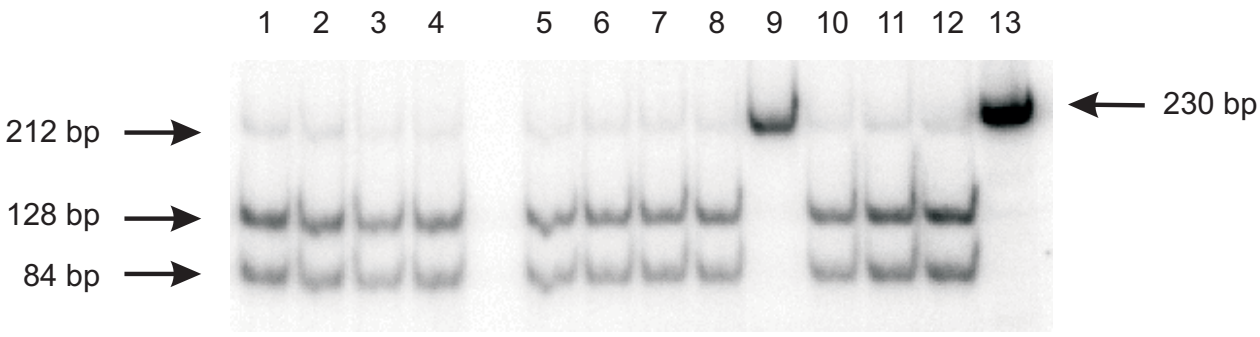


Figure 1. Pedigrees of families with G11778A mutation. Probands marked with an arrow.



1	family C, patient II-1	7	family A, patient II-2
2	family B, patient II-1	8	family A, patient III-4
3	family A, patient II-1	9	family A, patient III-5, control
4	family A, patient III-1	10	family A, patient II-3
5	family A, patient III-3	11	family B, patient II-2
6	family A, patient III-2	12	family C, patient II-2
		13	control, not digested

Figure 2 Quantitative analysis of mutation level by last cycle hot PCR and digestion with *Mae*III

Figure 2. Quantitative analysis of mutation level by last cycle hot PCR and digestion with *Mae*III.

the primer allowed distinguishing between truly heteroplasmic cases and not fully digested samples. In all 11 cases heteroplasmy was observed, for the negative control (individual III-5) no digestion in position 11778 was present. Mutation level was quantified in all heteroplasmic cases and data obtained are shown in Table 1. The use of last cycle hot PCR allows a quantitative estimation of the percentage of mutated mtDNA.

In all patients the mutation level was high, exceeding 90%. This is not very surprising bearing in mind that even in the individuals homoplasmic for the mutation no signs of disease may be present. No difference in heteroplasmy can be observed between affected and

Table 1. Percent of G11778A mutation in three families.

Results are the average from three repetitions.

Family	Patient	Mutation level in leukocytes (%)	Standard deviation (percentage points)
A	II-1	94.3	1.0
	II-2	92.1	2.5
	II-3	91.2	1.7
	III-1	95.5	0.7
	III-2	94.3	0.9
	III-3	95.2	1.7
	III-4	93.8	1.4
	III-5 – control	0	
B	II-1	92.7	0.5
	II-2	93.4	0.2
C	II-1	92.3	1.6
	II-2	93.5	0.7

non-affected cases (p = 0.77). This phenomenon can be explained in several different ways. First, the tissue used for the analysis was blood leukocytes while the pathology is present in the optic nerve. If the possibility of studying the optic nerve existed, the results might have been different. The other explanation may be the involvement of unknown nuclear factors modifying the disease process. Although all affected individuals were males no difference in heteroplasmy between males and females can be seen (p = 0.76) so that cannot explain the observed male bias.

Affiliation to haplogroup J also was checked by screening for disappearance of the restriction site for *Hin*fI in position 16 065 being the marker of J haplogroup. In all families the above restriction site was present, meaning that they do not belong to haplogroup J. Thus in these cases of Leber hereditary optic neuropathy the secondary mutations found in the J haplogroup mtDNA are not necessary for disease development.

All data suggest that the differences in heteroplasmy cannot be responsible for the differences in expression of the Leber hereditary optic neuropathy and the mechanism is probably much more complicated and needs further studies.

The presented data for the first time show the molecular analysis of the Polish patients with 11778A LHON mutation dealing with the heteroplasmy problem and haplogroup affiliation of mtDNA in this disease.

REFERENCES

1. Howell, N. (1999) Human mitochondrial diseases: Answering questions and questioning answers. *Int. Rev. Cytol.* **186**, 49–111.

2. Leber, T. (1871) Ueber hereditaere und congenital angelegte Sehnervenleiden. *Graefes Arch. Ophthal.* **17,** 249–291.

3. Wallace, D.C. (1970) A new manifestation of Leber's disease and a new explanation for the agency responsible for its unusual pattern of inheritance. *Brain* **93**, 121–132.

4. Nishimura, M., Obayashi, H., Ohta, M., Uchiyama, T., Hao, Q. & Saida, T. (1995) No association of the 11778 mitochondrial DNA mutation and multiple sclerosis in Japan. *Neurology* **45,** 1333–1334.

5. Leuzzi, V., Carducci, C., Lenza, M., Salvetti, M., Ristori, G., Di Giovanni, S. & Torroni, A. (1997) LHON mutations in Italian patients affected by multiple sclerosis. *Acta Neurol. Scand.* **96**, 145–148.

6. Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. & Young, I.G. (1981) Sequence and organization of the human mitochondrial genome. *Nature* **290,** 457–465.

7. Torroni, A., Petrozzi, M., D'Urbano, L., Sellitto, D., Zeviani, M., Carrara, F., Carducci, C., Leuzzi, V., Carelli, V., Barboni, P., De Negri, A. & Scozzari, R. (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. *Am. J. Hum. Genet.* **60,** 1107–11021.

8. Brown, M.D., Sun, F. & Wallace, D.C. (1997) Clustering of Caucasian Leber hereditary optic neuropathy patients containing the 11778 or 14484 mutations on an mtDNA lineage. *Am. J. Hum. Genet.* **60**, 381–387.

9. Chalmers, R.M., Davis, M.B., Sweeney, M.G., Wood, N.W. & Harding, A.E. (1996) Evidence against an X-linked visual loss susceptibility locus in Leber hereditary optic neuropathy. *Am. J. Hum. Genet.* **59,**103–108.

10. Novotny, E.J., Singh, G., Wallace, D.C., Dorfman, L.J., Louis, A., Sogg, R.L. & Steinman, L. (1986) Leber's disease and dystonia: A

mitochondrial disease. *Neurology* **36,** 1053–1060.

11. Brown, M.D., Trounce, I.A., Jun, A.S., Allen, J.C. & Wallace, D.C. (2000) Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation. *J. Biol. Chem.* **275**, 39831–39836.

12. Vergani, L., Martinuzzi, A., Carelli, V., Cortelli, P., Montagna, P., Schievano, G., Carrozzo, R., Angelini, C. & Lugaresi, E. (1995) mtDNA mutations associated with Leber's hereditary optic neuropathy: Studies on cytoplasmic hybrid (cybrid) cells. *Biochem. Biophys. Res. Commun.* **210**, 880–888.