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No genetic differences between affected and unaffected members of a German family with Leber's hereditary optic neuropathy (LHON) with respect to ten mtDNA point mutations associated with LHON

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In order to investigate possible synergistic influences of different mtDNA mutations on penetrance and severity of Leber's hereditary optic neuropathy (LHON), a large German LHON pedigree is characterized with respect to 10 different mutations associated with LHON. All members of the family carry three different mtDNA mutations (at nucleotide 4,216, 11,778 and 13,708) in a homoplasmic form, regardless of whether or not they are clinically affected. Testing for another 7 mutations reveals negative results in all family members. Hence, the variable disease expression of the family members cannot be explained by varying combinations of LHON-associated mtDNA mutations.

Mitochondrial DNA; Leber's hereditary optic neuropathy; Mutation; Restriction endonuclease; Mismatch primer

1. INTRODUCTION

The maternally transmitted Leber's hereditary optic neuropathy (LHON) leads to bilateral loss of central vision as a result of optic nerve death [1]. The disease is associated with defined mutations of the mitochondrial DNA (mtDNA) [2]. About 50–80% of LHON patients can be attributed to a G-to-A transition mutation at position 11,778 in the ND4 subunit gene of complex I of the respiratory chain [3]. For the remainder of the LHON patients not carrying this mutation several other mutations have been described [2]. In LHON families, usually both clinically affected and unaffected family members carry the mt genetical defect. Therefore the question arises, what regulates the penetrance of the disease? Since in most sporadic LHON cases, as well as in LHON pedigrees, various but distinct mtDNA mutations [4] have been described, it was suggested that no particular mutation, but rather a cluster of synergistically interacting mutations may define the apparently variable expression of the disease. This assumption was confirmed recently by observations in a large LHON pedigree from Queensland [5]. While all members of this family carry a ND1 gene mutation, one clinically less severely affected branch of this pedigree exhibits an additional second mutation. To investigate the possibility of synergistically interacting mtDNA mutations we have characterized a large German LHON family with respect to 10 different mutational sites reported as being associated with LHON (Fig. 1). This was done by restriction fragment analysis and by use of allele-specific point mutation primers.

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Abbreviations: bp, base pairs; nt, nucleotide; LHON, Leber's hereditary optic neuropathy; ND, complex I = mitochondrial NADH dehydrogenase (EC 1.6.99.3).

2. MATERIALS AND METHODS

2.1. *Patients*

The pedigree of the German LHON family is given in Fig. 2.

2.2. *Analysis of mtDNA*

Total DNA was extracted from 10 ml EDTA-blood as described [6] using standard proteinase K procedures. Point mutation priming and restriction analysis for the different mtDNA positions to be investigated were performed as follows: PCR amplification was done with allele-specific mismatch primers (Table I). These primers produce amplified fragments only in the presence of mutated mtDNA. To confirm the results, fragments amplified by PCR using the primer combinations given in Table II were cut by the respective restriction endonucleases. Samples prepared by both methods were visualized in 1% agarose gels containing ethidium bromide.

3. RESULTS

All 10 members of the LHON family under study carry three different mtDNA mutations (Fig. 3). First, the most common mutation, G-to-A, at position 11,778 in the ND4 gene, which eliminates a *Sfa*NI restriction site and creates a new one for the endonuclease, *Mae*III. The mutation replaces a highly conserved arginine by a histidine at position 340 in the ND4 subunit of com-

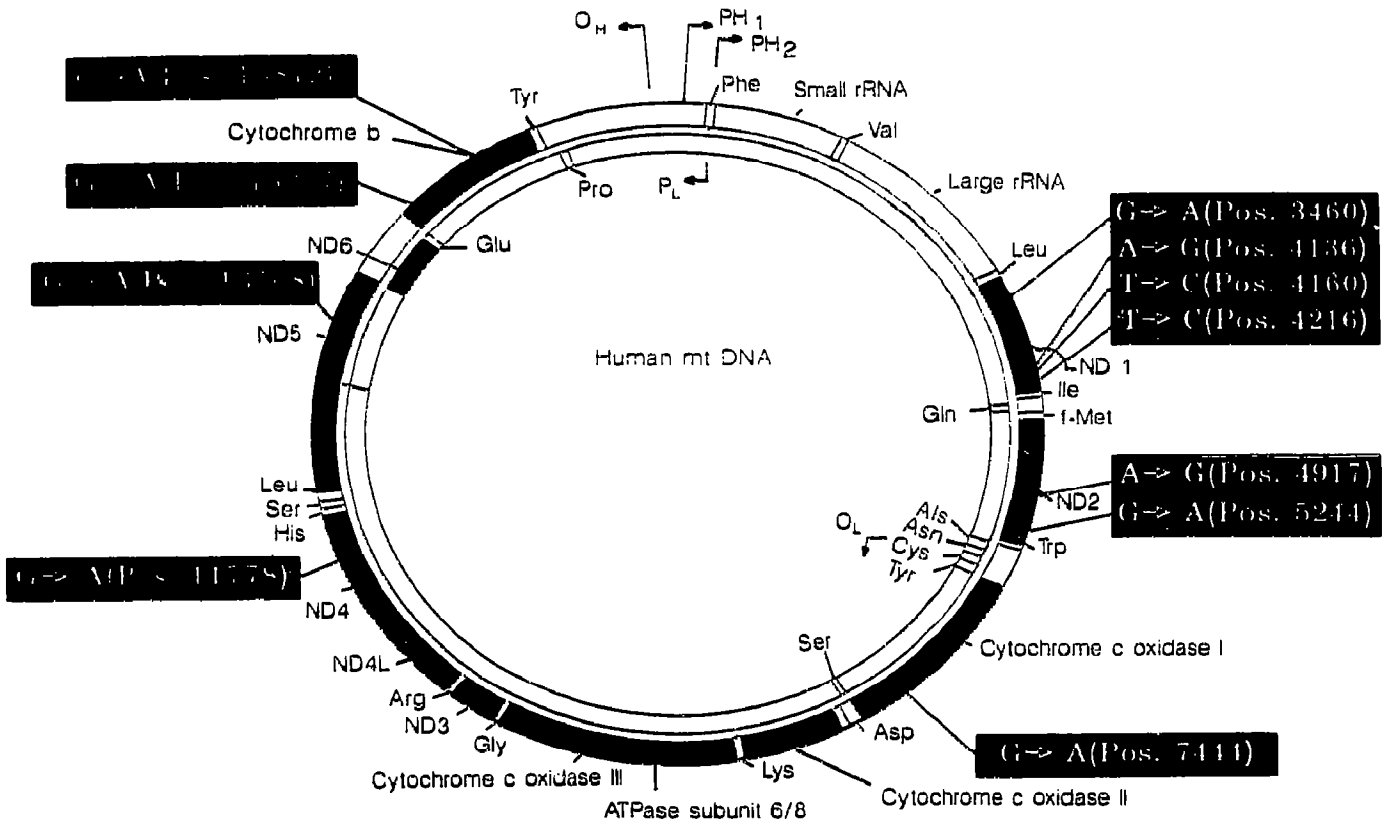

Fig. 1. Point mutations of the mtDNA associated with LHON. Black regions indicate genes on the heavy (outer circle) and the light (inner circle) strand; tRNA genes are marked by solid lines with the respective amino acid abbreviations. O_H, O_L, P_H, P_L are the respective origins of replication and the promotors for the heavy and light strands. LHON mutation base substitutions are shown outside the double stranded mt genome with the respective nucleotide positions.

plex I of the respiratory chain. The second mutation was found at position 13,708 (G-to-A) in the ND5 subunit gene. As the restriction sites of the two endonucleases, *Bst*NI and *Fnu*4HI, overlap at position 13,708, the mutation can be detected by failure of both enzymes to cleave the 450 nucleotide (nt) product. The mutation

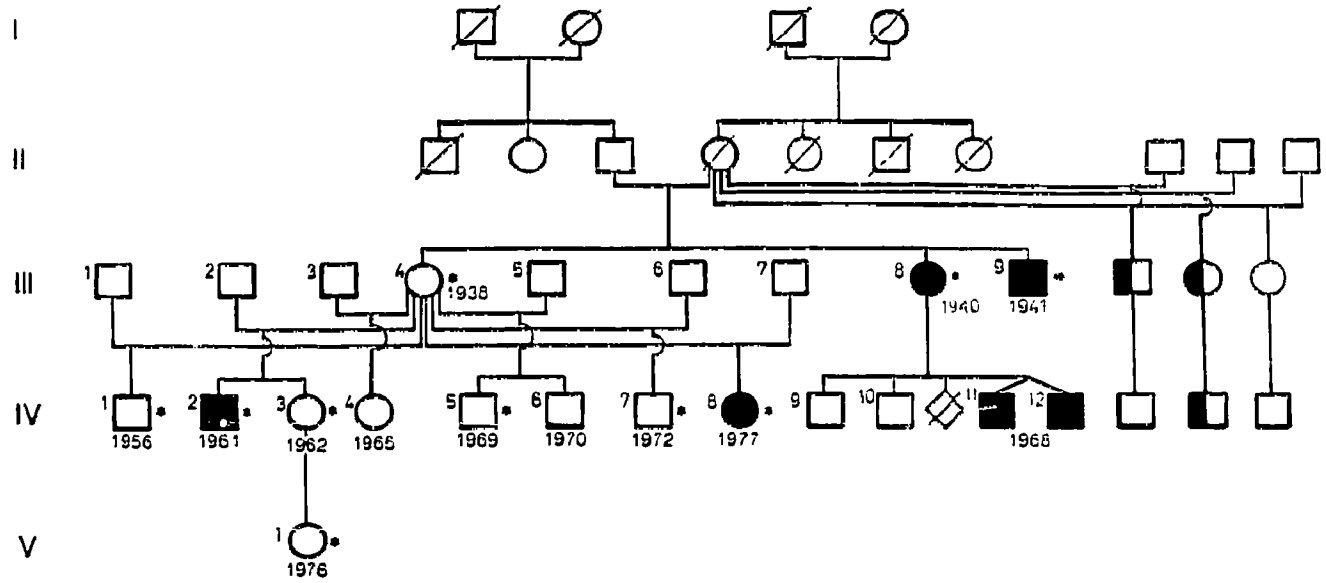

Fig. 2. Pedigree of the LHON family. Numbers represent the years of birth; *cases investigated in this study.

Table I

PCR primers and restriction endonucleases

Mutation (gene)	5′ primer (fragment length)	3′ primer	Restriction endonuclease	W	M
3,460 (ND1)	3,275– 3,304 (671 nt)	3,945– 3,916	*Bsa*HI	+	–
4,136 (ND1)	3,275– 3,304 (2598 nt)	5,872– 5,844	*Nla*III	–	+
4,216 (ND1)	3,275– 3,304 (2598 nt)	5,872– 5,844	*Nla*III	–	+
4,917 (ND2)	4,704– 4,730 (429 nt)	5,132– 5,103	*Mae*I	–	+
5,244 (ND2)	4,808– 4,835 (654 nt)	5,461– 5,434	*Hpa*II (5,242)	+	–
7,444 (COX I)	7,172– 7,199 (646 nt)	7,817– 7,788	*Xba*I	+	–
11,778 (ND4)	11,576–11,602 (561 nt)	12,136–12,110	*Sfa*NI *Mae*III	+ –	– +
13,708 (ND5)	13,570–13,595 (450 nt)	14,019–13,990	*Bst*NI *Fnu*4HI	+ +	– –
15,257 (Cyt. *b*)	15,015–15,042 (938 nt)	15,952–15,925	*Acc*I (15,255)	+	–
15,812 (Cyt. *b*)	15,260–15,287 (899 nt)	16,158–16,131	*Rsa*I	+	–

W, wild type; M, mutation; +, restriction site present; –, restriction site lost.

changes a non-polar hydrophobic alanine to a hydrophilic threonine at position 458 in the ND5 subunit. The third mutation found in all LHON family members was a T-to-C transition at position 4,216 in the ND1 subunit gene. It creates a new restriction site for the endonuclease, *Nla*III, and results in a tyrosine/histidine exchange in the ND1 gene product.

All except one of the tested members of our LHON family carry these three mutations in a homoplasmic form, i.e. restriction digests of the amplified fragments only showed the mutation patterns. The only exception is patient III/9 who shows two bands after *Sfa*N1 digestion, indicating a heteroplasmic 11,778 mutation (Fig. 3a). Testing for another 7 mutations listed in Tables I and II produced negative results with both methods in all family members.

Table II

LHON allele-specific mismatch primers

Positions	Primer sequence
3,438– 3,460	5′-G CTA CTA CAA CCC TTC GCT AAC A-3′ G G
4,182– 4,216	5′-CTT CCT ACC ACT CAC CCT AGC ATT ACT TAT AAG AC-3′ T T
4,888– 4,917	5′-CA ATC ATA TAC CAA ATC TCT CCC TCA ATA G-3′ C A
5,223– 5,244	5′-GGA GGC CTG CCC CCG CTA GCC A-3′ A G
7,411– 7,444	5′-ACA CAT TCG AAG AAC CCG TAT ACA TAA AAT ATA A-3′ C G
11,745–11,778	5′-C TCA AAC TAC GAA CGC ACT CAC ACT CA-3′ G G
13,680–13,708	5′-C ACC CTA CTA AAC CCC ATT AAA GGC GTG A-3′ C G
15,231–15,257	5′-AA TGA ATC TGA GGA GGC TAC TCA ATA A-3′ G G
15,785–15,812	5′-TTT ACC ATC ATT GGA CAA GTA GCA GCC A-3′ T G

The mutation site is underlined. Wild-type nucleotides are given under the primer sequence.

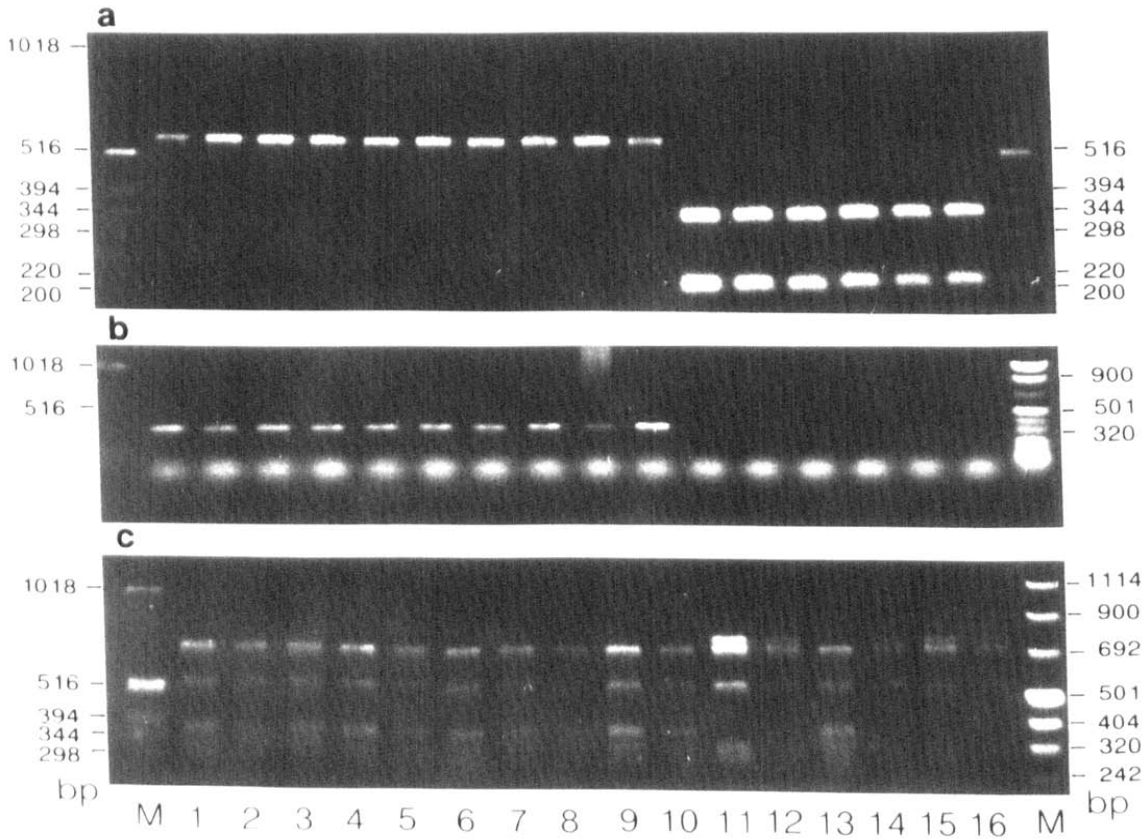

Fig. 3. Detection of LHON mutations by electrophoresis of PCR fragments amplified from blood. LHON family (lanes 1–10) and controls (lanes 11–16). M, marker ladder. (a) Detection of mutation G→A at nt 11,778: the amplification products resulting from the use of the primers given in Table I were digested with *Sfa*NI. All tested LHON family members show the undigested 535 bp fragment, indicating the absence of the restriction site and the presence of the mutation. Control samples are cut into a 321 bp and 214 bp fragment, respectively, indicating the presence of the restriction site and the absence of the mutation. (b) Detection of the mutation G→A at nt 13,708 by use of the allele-specific mismatch primer given in Table I. All LHON family members show the 311 bp band, indicating the presence of the mutation. No band is detectable in the controls. (c) Detection of mutation T→C at nt 4,216. The amplification products resulting from the use of the primers given in Table II were digested with *Nla*III. All controls, except control 13, show the wild-type pattern consisting of the 738, 687, 527, 318, 287 and 40 bp fragments. The mutation produces an additional cleavage site within the 738 bp fragment which is consequently cut into a double band of 374/364 bp. This pattern is found in all LHON family members as well as in control 13, indicating the presence of the mutation, while all other controls are negative for the mutation. As there is no further band detectable, we can also exclude the presence of the A→G mutation at nt 4,136 in all samples.

4. DISCUSSION

Variable penetrance is observed in almost all LHON pedigrees. The reason for this fact, however, remains unknown. Furthermore, the time of onset of LHON differs intrafamilially. To follow the idea that interacting LHON mtDNA point mutations [4,5] would perhaps explain some of these variations, we have investigated a German LHON family with respect to the 10 LHON-associated mutations reported so far [2]. All 10 family members under study carry the same LHON point mutation pattern independently of whether or not they are clinically affected. They are positive for the mutations at nt 4,216, 11,778 and 13,708, as well as negative for the mutations at nt 3,460, 4,917, 5,244, 7,444, 15,257 and 15,812, thus resembling single cases reported by Johns and Berman [9]. The intrafamilial variation of disease onset in our family is, for instance, demonstrated by patients III/4 and IV/8. While the first does not show any symptoms of the disease at the age of 54, the latter had already developed partial optic atrophy at the age of 15. Thus there is no evidence at present that a varying pattern of known LHON-associated point mutations is responsible for the variable manifestation of the disease, at least in this family. There is also no evidence for a correlation between the age of onset and varying proportions of mutated mtDNA, since all examined family members, except the affected member III/9, bear the mutations in homoplasmic form, i.e. only mutated mtDNA is present. It still remains to be discovered what additional mitochondrial, nuclear or environmental factors regulate the expression of the genetic defect as a clinical disorder.

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