

CLINICAL SCIENCE

Increase of mitochondrial DNA in blood cells of patients with Leber's hereditary optic neuropathy with 11778 mutation

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Aims: To investigate the change of mitochondrial DNA (mtDNA) content in Leber's hereditary optic neuropathy (LHON) with 11778 mutation.

Methods: Mitochondrial DNA content in 27 LHON patients with 11778 mutation, 26 asymptomatic maternal relatives, and 23 normal controls was measured using a competitive polymerase chain reaction (PCR) method.

Results: The mean relative content of mtDNA (with respect to the β actin gene) in LHON patients, asymptomatic maternal relatives, and normal controls was 245.5 (162.3), 238.2 (118.4), and 156.5 (61.6), respectively. There was a statistically significant difference between patients and controls and between relatives and controls. However, no statistically significant difference between patients and unaffected relatives was found. There was no statistically significant difference in the relative content of mtDNA between all males and females carrying 11778 mtDNA mutation

Conclusion: The results suggest that the increase in mtDNA content in LHON patients with 11778 mtDNA mutation may be due to a compensatory effect for respiratory chain defects of mitochondria. However, the increase of mtDNA content is the result rather than the cause of defective mtDNA. It still cannot explain the pathogenesis of LHON.

Leber hereditary optic neuropathy (LHON) is a maternally transmitted disease and is characterised by acute or subacute bilateral loss of central vision in young adults.¹ It has been associated with 25 different mitochondrial DNA (mtDNA) mutations that were classified as primary and secondary mutations, according to their aetiological role in disease expression.²⁻⁴ Although the primary aetiological factor of LHON is a mutation in the mitochondrial genome, the presence of a primary mtDNA mutation does not necessarily lead to visual loss. The optic neuropathy in LHON shows incomplete penetrance, and there are additional genetic and environmental aetiological factors, which are rather poorly defined, that influence the onset of the disease.^{5,6}

Mitochondria are known to possess a second set of DNA that is structurally distinct from nuclear DNA. The mtDNA represents less than 1% of total cellular DNA.⁷ The number of mitochondria and the amount of mtDNA per cell are closely regulated within a given cell type but differ widely from cell type to cell type.⁸ The content of mtDNA has been found to increase with age.⁹⁻¹¹ It is thought that as the respiratory function of cells declines with age, the cells are able to compensate for reduced ATP synthesis by inducing the proliferation of mtDNA.¹¹

Mitochondrial respiratory function was reported to be compromised in LHON patients.¹²⁻¹⁷ The mitochondrial dysfunction is thought to be caused by one or more mutations of mtDNA. However, the content of mtDNA of LHON patients has not been studied. It was our purpose to investigate whether the content of mtDNA is altered in LHON patients.

MATERIALS AND METHODS

Twenty seven patients (25 male and two female) with LHON with 11778 mutation, 26 asymptomatic maternal relatives (seven male and 19 female), and 23 normal controls (16 male and seven female) were included in this study (Table 1). The mean age at onset of LHON patients was 19.8 years, with a range of 8-38 years. At the time of blood sampling, the patients had lost vision for 2 months to 20 years. The mean age of LHON patients when their blood was sampled was 24 years, with a range of 11.5-40 years. The mean age of asymptomatic maternal relatives was 33.8 years, with a range of 9-68 years. Both LHON patients and asymptomatic maternal relatives carry homoplasmic 11778 mutation of mtDNA. The mean age of normal controls was 35.3 years with a range of 5-83 years. They were tested negative for mtDNA 11778 mutation. They have no systemic disease as diabetes mellitus.

Table 1 Clinical data of LHON patients, asymptomatic maternal relatives, and normal controls

	LHON	Relatives	Controls
Number	27	26	23
Sex ratio (M/F)	25/2	7/19	16/7
Mean age (years) (SD)	24.0 (8.6)	33.8 (15.8)	35.3 (23.6)
Range	11.5-40	9-68	5-83
Mean age of onset (years) (SD)	19.8 (8.8)	-	-
Mean duration of visual loss (years)	5.1 (7.8)	-	-

Table 2 The nucleotide sequence of six primers used in this study

Primers	Sequence (5' to 3')	Target DNA
BA1	CATGTGCAAGGCCGGCTTCG	nDNA, β actin
BA2	CTGGGTCATCTTCTCGCGGT	nDNA, β actin
BA2-BA3	CTGGGTCATCTTCTCGCGGTGCAGCACGGGGTGCTCCTC	nDNA, β actin
L3540	TCTCACCATCGCTCTTCTAC	mtDNA, ND1
H3887	TTGGTCTCTGCTAGTGTGGA	mtDNA, ND1
H3887-H3836	TTGGTCTCTGCTAGTGTGGAGGCAGGAGTAATCAGAGGT	mtDNA, ND1

The study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects. With informed consent from each of the study subjects

according to a protocol approved by the institutional review board of Taipei Veterans General Hospital, blood samples were obtained from LHON patients with the mtDNA 11778

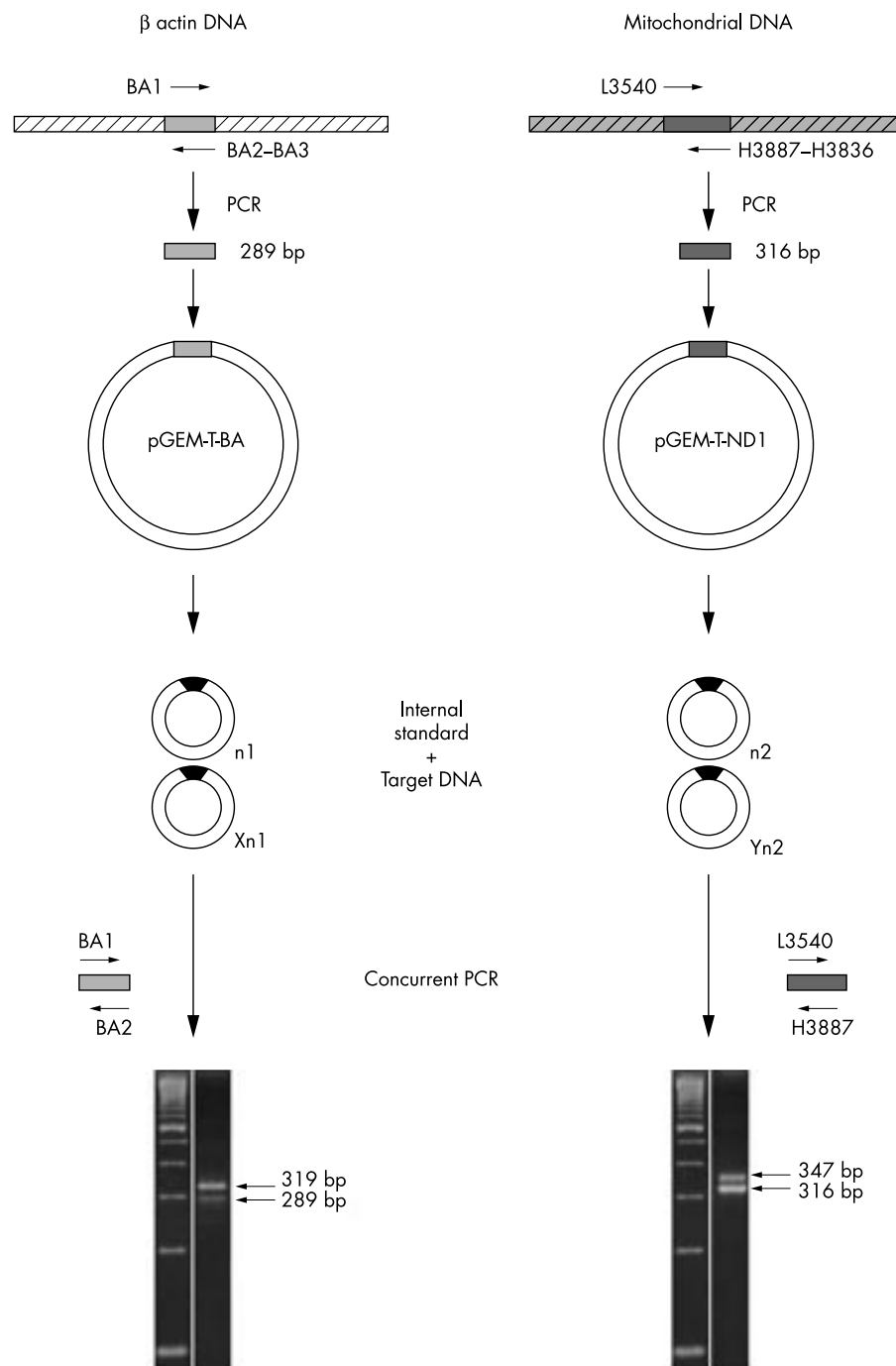


Table 3 Mean mtDNA content in LHON patients, asymptomatic relatives, and controls

Study subjects	Mean (SD)
LHON patients (n=27)	245.5 (162.3)*
Asymptomatic relatives (n=26)	238.2 (118.4)†
Controls (n=23)	156.5 (61.6)

*Patients v controls, $p < 0.05$, Mann-Whitney test.†Relatives v controls, $p < 0.05$, Mann-Whitney test.Patients v relatives, $p > 0.05$, Mann-Whitney test.

mutation, their unaffected maternal relatives, and normal controls. Five ml of whole blood was withdrawn and stored in an EDTA containing glass tube.

Total DNA was extracted from the blood cells and purified using a DNA purification kit (Puregene, Gentra System, Inc, Minneapolis, MN, USA).

Using β actin gene of nuclear DNA as an internal standard, the relative amount of mtDNA was determined by a competitive polymerase chain reaction (PCR) method modified from that developed by Gilliland *et al*¹⁸ and the competitor DNA was constructed by a method similar to that described by Celi *et al*.¹⁹ The nucleotide sequences of the primers used in this study are listed in Table 2. The whole procedure is summarised in Figure 1. To generate an internal DNA standard of β actin gene, a plasmid was constructed by insertion of the PCR product amplified with primers BA1 and BA2-BA3 to the pGEM-T vector (Promega Co, Madison, WI, USA). The internal DNA standard of β actin gene was designed having a 30 base pair (bp) deletion. A volume of 0.5 μ l of the internal DNA standard (5 μ g/ μ l) was introduced with DNA sample into the PCR mixture and was amplified with primers BA1 and BA2. Two products with sizes of 319 bp and 289 bp were generated from the endogenous template and from the internal DNA standard, respectively. With the same approach, we generated an internal mtDNA ND1 standard with another plasmid that was constructed by insertion of the PCR product amplified with primers L3540 and H3887-H3836 and cloned into the pGEM-T vector. The internal mtDNA ND1 standard was designed to have a 31 bp deletion. A volume of 0.5 μ l of the internal mtDNA standard (3 mg/ μ l) was introduced into the DNA sample and amplified with primers L3540 and H3887. Two products with sizes of 347 bp and 316 bp were then generated from the endogenous template and the internal ND1 standard, respectively.

The competitive PCR was carried out for 30 cycles in a DNA thermal cycler (Model 2400, Perkin-Elmer/Cetus). Each aliquot of 50 μ l reaction mixture contained 200 μ M of each dNTP, 20 pmol of each primer, 1 unit of Taq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT, USA), 50 mM KCl, 1.5 mM MgCl₂, and 10 mM TRIS-HCl (pH 8.3). The thermal profile was as follows: denaturation at 94°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 40 seconds. The PCR products were separated electrophoretically in 3% agarose gel and stained with 1 μ g/ml ethidium bromide at 25°C for 10 minutes. The intensities of the PCR products of the target and competitor DNA were analysed by digital scanning densitometry. A standard curve was constructed from a healthy person by plotting the competitor/target DNA intensity ratio against the logarithm of the content of the internal DNA standard added to the reaction mixture. Using the information derived from the best fit analysis of standard, the relative content of mtDNAs were normalised and calculated as an equivalent of one copy of β actin gene.

Relative mtDNA content with respect to one copy of β actin gene = ((intensity of 316 bp band/intensity of 347 bp band) \times (concentration of ND1 internal standard))/((intensity of 289 bp band/intensity of 319 bp band) \times (concentration of β actin internal standard)).

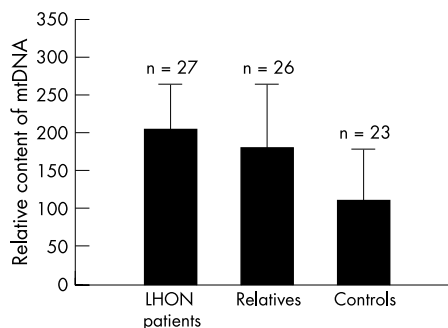


Figure 2 Comparison of the relative content of mtDNA in LHON patients, unaffected maternal relatives, and controls. The relative content of mtDNA in LHON patients (245.5 (162.3)) and unaffected maternal relatives (238.2 (118.4)) was higher than that of the controls (156.5 (61.6)) ($p < 0.05$, Mann-Whitney test). However, the relative content of mtDNA between LHON patients and unaffected maternal relatives has no statistically significant difference ($p > 0.05$, Mann-Whitney test).

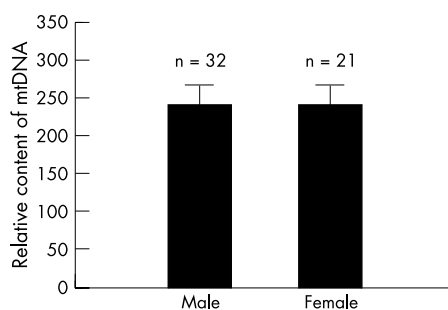


Figure 3 The relative content of mtDNA between all males (242.4 (26.7)) and female (241.2 (27.9)) carrying 11778 mtDNA mutation has no statistically significant difference ($p > 0.05$, Mann-Whitney test).

RESULTS

The mean relative content of mtDNA in blood cells of LHON patients with mtDNA 11778 mutation, in asymptomatic maternal relatives, and in normal controls was 245.5 (162.3), 238.2 (118.4), and 156.5 (61.6), respectively (Fig 2). There was a statistically significant difference in the relative content of mtDNA between the patients and normal controls and between relatives and normal controls (Mann-Whitney test, $p < 0.05$). However, the difference between LHON patients and the asymptomatic maternal relatives did not reach statistical significance (Mann-Whitney test, $p > 0.05$) (Table 3). The mean relative content of mtDNA in all males and females with 11778 mtDNA mutation no matter who was affected or unaffected was 242.4 (26.7) and 241.2 (27.9), respectively. The difference also did not reach statistical significance (Mann-Whitney test, $p > 0.05$). The content of mtDNA in LHON patients did not correlate with the age of onset or the duration from the onset of visual loss to the time of the blood test (Pearson correlation coefficient, $r < 0.5$, $p > 0.05$).

DISCUSSION

The increase in content of mtDNA with age has been considered to be a compensatory response for the decline in respiratory function.^{10 11 20} It is thought that as the respiratory function of tissue cells declines with age, the cells are mobilised to compensate for the reduced ATP synthesis by increasing the copy number of mtDNA. The energy deficit might be signalled to the nucleus, probably through a reactive oxygen species increase,^{21 22} which can activate the expression of nuclear DNA encoded transacting factors able to induce

mitochondrial proliferation.²³ Increased expression of mitochondrial transcription factor A and nuclear respiratory factor-1 were observed in aged human skeletal muscles.²⁴ Over-proliferation of mitochondria and increased synthesis of mitochondrial respiratory enzymes were observed in a patient with mitochondrial myopathy.²⁵ Increase in mitochondria and mtDNA was also found in human cells harbouring 4977 bp deleted mtDNA in response to oxidative stress.²⁶

Although LHON is known as a mitochondrial disease, the molecular mechanism of the disease is unknown. Variable functional defects of complex I in LHON have been reported.¹²⁻¹⁷ Brown *et al*²⁷ reported an extensive biochemical analysis of the mitochondrial defects in lymphoblasts and trans-mitochondrial cybrids harbouring LHON 3460, 11778 and 14484 mtDNA mutation. Respiration studies revealed that the maximal respiration rate reduced 20–28%, 30–36%, and 10–15% in the cybrids with 3460, 11778, and 14484 mtDNA mutation, respectively. From this study, we found the content of mtDNA is increased in LHON patients and their asymptomatic maternal relatives harbouring 11778 mutation of mtDNA. However, the content of mtDNA did not correlate with the age of onset of the disease (Pearson correlation coefficient, $p > 0.05$). We believe that the change is a compensatory response for respiratory chain defects of mitochondria harbouring the 11778 mutation.

However, mtDNA content does not increase in all pathological conditions. The content of mtDNA was found to be decreased in adult, but not fetal, pancreatic islets of diabetic rats compared with non-diabetic rats.²⁸ The content of mtDNA was found to be increased in light smokers, but decreased in heavy smokers.¹¹ These findings suggest that under some conditions, other factors, such as diabetes or smoking, may modulate the compensatory mechanism in the control of mtDNA replication. In this study, we did not find a modulatory factor that affects the compensatory effect in LHON patients. The compensation does not fade with time because the increased content did not correlate with the duration from the onset of visual loss to the time when the patient was examined (Pearson correlation coefficient, $p > 0.05$).

Typical LHON is often seen in young males. The male to female ratio of affected individuals is about 4.2 to 1.⁶ Why males are predominantly affected is unknown. Our study did not show a statistically significant difference of mtDNA content between all males and females carrying 11778 mtDNA mutation.

The content of mtDNA between LHON patients and asymptomatic maternal relatives did not show a statistically significant difference. Both LHON patients and asymptomatic maternal relatives carried the mtDNA 11778 mutation. Since the ages of asymptomatic maternal relatives overlap with the LHON patients, some of them might develop LHON later. The increased mtDNA content in both groups cannot explain the pathogenesis of the onset of LHON.

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