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## mtDNA/nDNA ratio in 14484 LHON mitochondrial mutation carriers

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**Abstract** Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease caused by mitochondrial DNA (mtDNA) mutations. In this study, the mtDNA/nuclear DNA ratio was evaluated in 11 LHON patients with the 14484 mutation, 13 asymptomatic carriers and 18 non-carrier relatives as controls, to reveal possible relationships between the disease and mtDNA content. DNAs from peripheral blood lymphocytes were subjected to quantitative PCR. Gender differences and age-dependent changes in the mtDNA content were not observed. Significant increase in the mtDNA content was observed only in the asymptomatic carriers ( $P < 0.05$ ). This indicated that individuals whose mtDNA content had increased and been maintained at certain levels were free from LHON development, whereas those whose levels had not, had developed LHON. Since the asymptomatic carriers are the stock of the future LHON patients, monitoring the mtDNA content in patients and their relatives may help to predict the prognosis of the disease.

**Keywords** Leber's hereditary optic neuropathy · 14484 mutation · mtDNA/nDNA ratio · Mitochondrial DNA · Quantitative PCR

### Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by acute or sub-acute bilateral visual loss occurring predominantly in young males. This disease was reported in detail more than 100 years ago by Leber (referred to in Newman et al. 1991; Riordan-Eva et al. 1995). More than 90% of LHON cases are due to one of the three mitochondrial DNA (mtDNA) point mutations at the nucleotide positions 3460, 11778, and 14484 (Wallace et al. 1988; Johns et al. 1992; Howell et al. 1991; Huoponen et al. 1991). These mutations are called primary (pathogenic) mutations because they have been found exclusively in LHON patients and their family members but never in control subjects.

These primary mutations are located on the genes of the complex I subunits in the mitochondrial respiratory chain (Howell 1994), and a decline in respiratory function in mitochondria with LHON mutations was reported (Smith et al. 1994; Brown et al. 2000). However, characteristics of this disease, such as incomplete penetrance and male predominance, cannot be explained by the decline in respiratory function alone. In the search for other genetic backgrounds that may exist as co-factors in the development of LHON, more than 20 secondary mutations were identified that proved to have weak or no association with LHON pathogenesis (Oostra et al. 1994; Mashima et al. 1998). Epidemiological investigations pointed to an association between environmental stresses, such as tobacco smoking and heavy alcohol drinking, and the onset of LHON (Smith et al. 1994; Riordan-Eva et al. 1995; Chalmers and Harding 1996). Despite many studies referring to a variety of aspects, the pathogenesis of LHON is still unclear.

Mitochondria carry their own DNA sets. The number of mitochondria and the number of mtDNA copies in a mitochondrion vary widely by cell type (Robin and Wong 1988). A recent study showed that the content of

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mtDNA in blood cells with the LHON 11778 mutation was greater than that of controls (Yen et al. 2002). This suggested that the content of mitochondrial DNA has certain relationships with the pathogenesis and incomplete penetrance of LHON; however, little is known about the relationships between LHON and mtDNA content. For this phenomenon to be generalized and ascertained, more data from other LHON primary mutations are awaited.

In this study, we investigated the mtDNA/nuclear DNA (nDNA) ratio using peripheral blood lymphocytes of LHON patients with the 14484 mutation, asymptomatic carriers, and controls to find relationships between LHON and the content of mtDNA.

## Subjects and methods

### Subjects

Heparinized blood specimens were collected from members of an Indonesian LHON family with the LHON 14484 mutation after informed consent had been obtained. All the individuals with the 14484 mutation were homoplasmic and did not have any other LHON secondary mutations (Nishioka et al. 2003). In total, 11 LHON patients (six male and five female) with the 14484 mutation, 13 asymptomatic 14484 mutation carriers (four male and nine female), and 18 non-carrier relatives as controls (12 male and six female) were included in this study (Table 1). We selected non-carrier relatives from controls in order to minimize diversity of nuclear DNA background between the LHON mutation carriers and the controls.

### DNA extraction and quantitative PCR

We centrifuged heparinized blood specimens at 1,000 rpm to separate platelets from the buffy coat layer. DNAs were extracted from peripheral blood lymphocytes in buffy coat by the NaI method (Wang et al. 1994).

Quantitative PCR was performed with a LightCycler (Roche Diagnostic, Mannheim, Germany). As a reference nuclear DNA, a part of the melanocortin one-receptor gene was selected and amplified with a primer set (forward: 5'-CGCCGTGGACCGCTACATCT-3'

and reverse: 5'-GCACGGCCATGAGCACCAG-3'). Primers for mitochondrial DNA at the bp14678–14930 region (forward: 5'-CTCGCACGGACTACAACC-3' and reverse: 5'-TGGGCGATTGATGAAAAG-3') were used. These PCR amplifications are well established with the current PCR conditions in our laboratory. The PCR was performed in a 20- $\mu$ l reaction mixture (2  $\mu$ l 10  $\times$  LightCycler DNA Master (LightCycler-FastStart DNA Master SYBR Green I; Roche Diagnostics), 3 mmol/l MgCl<sub>2</sub>(final concentration), 10 pmol forward primer, 10 pmol reverse primer, and 10 ng total DNA). For mitochondrial DNA amplification, 1 ng of total DNA was used. PCR cycling was initiated after a denature step at 95°C for 10 min. Each PCR step was at 95°C for 15 s, 65°C for 10 s, and 72°C for 12 s. Fluorescent signal was obtained at the end of the elongation step. Total PCR cycles were 50 cycles. We prepared 100, 10, 1, and 0.1 ng DNA templates of a healthy Japanese male for the standard curve. After performing quantitative PCRs, we calculated the concentration (equivalent to the standard DNA) of each DNA specimen from the standard curve using LightCycler Software (Roche Diagnostics). The relative amount of mtDNA (mtDNA/nDNA ratio) of each specimen was calculated as follows:

$$\begin{aligned} \text{mtDNA/nDNA ratio} \\ &= \text{concentration of mtDNA} / \text{concentration of nDNA} \\ &(\text{mtDNA/nDNA ratio} = 1 \text{ is equivalent to the mtDNA} \\ &\text{amount of the Japanese male used for the standard} \\ &\text{curve}) \end{aligned}$$

The measurement of mtDNA/nDNA ratio in each specimen was done in triplicate, and the mean value of three experiments was used for further analyses.

## Results

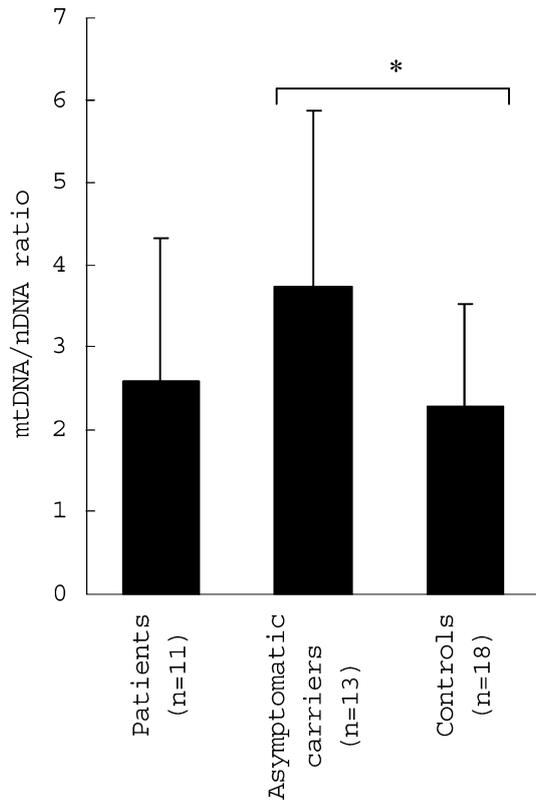
The mean mtDNA/nDNA ratios in the LHON patients, asymptomatic carriers, and controls are shown in Fig. 1. There was a significant difference in the mtDNA/nDNA ratio between the asymptomatic carriers and the controls ( $P < 0.05$ , Mann–Whitney test). However, the mtDNA/nDNA ratio in the LHON patients did not differ from that of the controls or that of the asymptomatic carriers.

We compared the mean mtDNA/nDNA ratio in males carrying the 14484 mutation with that of females carrying the 14484 mutation to clarify relationships between male predominance of LHON and mtDNA/nDNA ratio (Fig. 2a). There was no significant gender difference in the mtDNA/nDNA ratio in the three groups: the LHON patients, asymptomatic carriers and controls (Fig. 2b–d).

Relationships between age and mtDNA/nDNA ratio were also investigated (Fig. 3). In each group, LHON patients, asymptomatic carriers and controls, no corre-

**Table 1** Subjects studied for mtDNA/nDNA ratio. The mean age of the patient group was significantly higher than those of other two groups ( $P < 0.05$ , Fisher's LSD)

Characteristic	LHON patients	Asymptomatic carriers	Controls
Number (male/female)	11 (6/5)	13 (4/9)	18 (12/6)
Age range (years)	19–60	5–65	0.5–70
Mean age $\pm$ SD (years)	38.4 $\pm$ 12.9	21.0 $\pm$ 19.0	18.8 $\pm$ 15.4



**Fig. 1** mtDNA/nDNA ratio in LHON patients, asymptomatic carriers, and controls (mean + SD). \* $P < 0.05$ , Mann-Whitney test

lation between age and mtDNA/nDNA ratio was observed.

## Discussion

mtDNA content in the asymptomatic carriers was significantly greater than that in the controls, but that in the LHON patients was not. The increase in mtDNA in only the asymptomatic carriers might be attributed to several factors. Increase in the amount of mtDNA with aging is thought to be a compensatory response to the decline in the respiratory function of mitochondria (Barrientos et al. 1997; Lee et al. 1998). In this study the LHON patients' age was higher but their mean mtDNA/nDNA ratio was not higher than that of asymptomatic carriers. Moreover, in our study, there was no correlation between mtDNA/nDNA ratio and subjects' age (Fig. 3). Alternative explanations are required for the age-independent increase in the amount of mtDNA in the asymptomatic carriers.

As a decline in the respiratory function of mitochondria with an LHON mutation has been reported (Brown et al. 2000), the presence of a compensatory response that increases the amount of mtDNA in mitochondria with the LHON mutation is still presumed. The increase in mtDNA in the asymptomatic

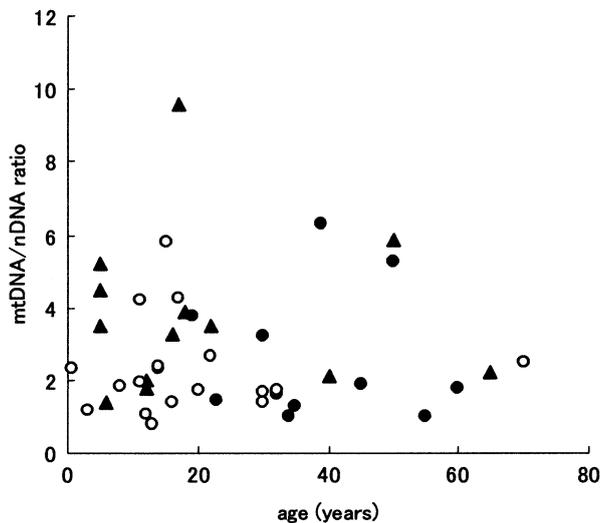
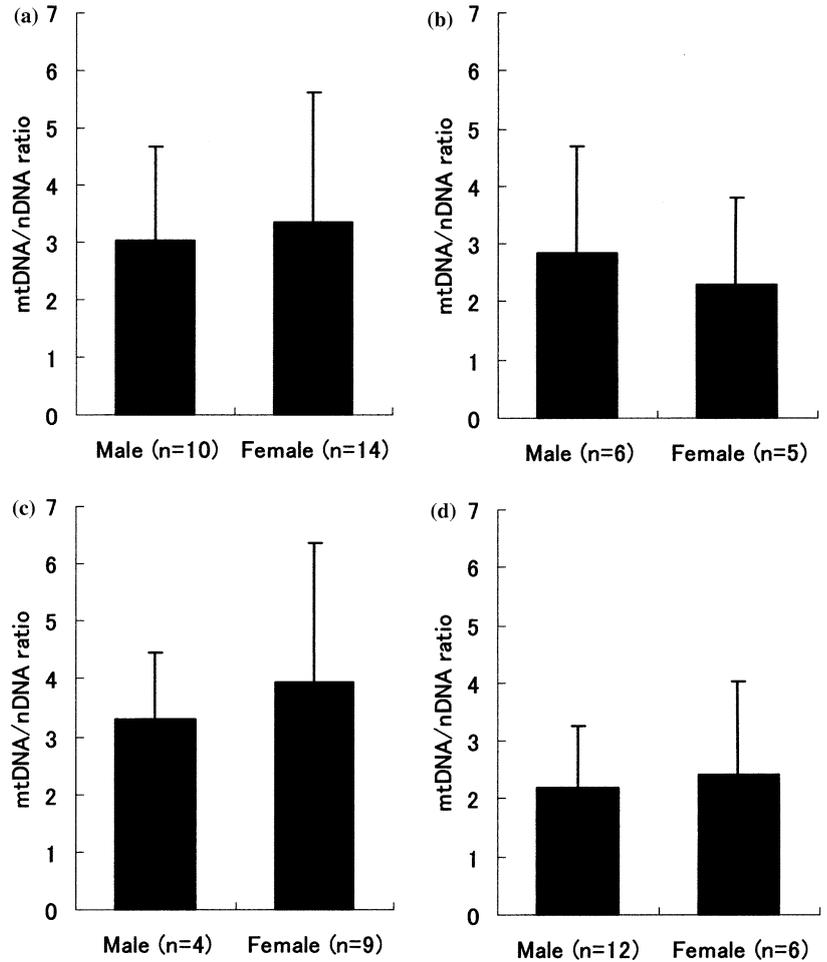
carriers might be attributable to a compensatory response to the reduced function of the mitochondria. The increase in mtDNA may vary in each individual; those individuals whose mtDNA content had increased up to certain levels would be free from LHON development, whereas those whose levels had not, as a result, would develop LHON; or, the mtDNA content in the 14484 mutation carriers had once been increased and then those whose elevated levels had been maintained could compensate for the functional levels, but those whose levels decreased would develop LHON.

In 11778 mutation carriers, the increase in the relative mtDNA content of both patients and their asymptomatic carriers was observed (Yen et al. 2002). Symptoms of LHON with the 14484 mutation are less severe than those of LHON with the 11778 mutation, while a better visual prognosis has been observed for LHON with the 14484 mutation (Riordan-Eva et al. 1995; Oostra et al. 1994; Johns et al. 1993; Mashima et al. 1998). Reduction in the complex I function in the 11778 mutant was larger than that in mitochondria with the 14484 mutation (Brown et al. 2000). We may find that the reduction in the mitochondrial respiratory function of the individuals with the 11778 mutation may be severe enough to cause the compensatory response in all individuals with the 11778 mutation but that the increase in mtDNA may not be sufficient for complete protection against LHON development.

In LHON, incomplete penetrance, male predominance and age-dependent onset are the unsolved issues with appropriate explanations. Our result showed no gender differences in the mtDNA/nDNA ratio in the controls, the 14484 mutation carriers or the carriers with or without LHON. Male predominance of LHON might be due to epigenetic factors such as physiological and/or functional difference between male and female. Variants of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, and gender-dependent predisposition to oxidative stress damage are nominated as causal factors; however, there was no gender difference in sensitivity to oxidative stress in vitro (unpublished data). As incomplete penetrance and age-dependent onset are related to each other in a sense, there might be factors other than mtDNA content that predispose these phenomena, since no age-dependent increases in mtDNA content were observed in the subjects.

Since the relationships between the increase in mtDNA and the development of LHON are still unclear, quantification as well as qualification of mtDNA with different LHON primary mutations is required. In neuronal cells, reduction in the amount of mtDNA is observed when precursor cells differentiate into neuronal form in vitro (Wong et al. 2002), and the mtDNA content varies widely in each cell type (Robin and Wong 1988). Changes in mtDNA content by cell type and condition should be taken into consideration when relationships between mtDNA content and LHON development are studied. Since the asymptomatic carriers are the stock of the future LHON patients, moni-

**Fig. 2a–d** mtDNA/nDNA ratios between **a** males and females carrying the 14484 mutation; **b** male patients and female patients; **c** male and female asymptomatic carriers; **d** male and female controls. Means and SD are represented



**Fig. 3** mtDNA/nDNA ratio and donor age. LHON patients (filled circles), asymptomatic carriers (filled triangles) and controls (open circles) are shown

toring the mtDNA content in the patients and their maternal relatives may help to predict the prognosis of the disease.

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