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GENETIC ANALYSIS OF JAPANESE PEDIGREES WITH LEBER'S HEREDITARY OPTIC NEUROPATHY

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INDEXING WORDS

Leber's hereditary optic neuropathy; mitochondrial DNA mutation; two locus control; lyonization

SYNOPSIS

Leber's hereditary optic neuropathy (LHON) is a maternally transmitted disease, characterized by bilateral optic atrophy mainly in young men. The strict maternal inheritance pattern of LHON can be explained by specific primary mitochondrial (mt) DNA mutations. However, intrafamilial phenotypic variation requires additional pathogenetic factors. Homo- or heteroplasmy of the primary mtDNA mutation, synergistic or antagonistic effects of secondary mtDNA mutations co-existing with the primary mutation, and involvement of an X-linked recessive gene have been postulated to be such factors. In an attempt to determine whether one of the three factors affects LHON expression in Japanese pedigrees, mtDNA analyses by means of RFLP and Southern blot hybridization of PCR products were performed for 22 members in 10 LHON pedigrees, and segregation analysis were conducted for 82 published LHON pedigrees. All of the LHON maternal relatives tested possessed only a homoplasmic mtDNA mutation at position 11778 with none of the secondary mtDNA mutations. Taking into account an extremely high prevalence of the 11778 mutation in Japanese LHON pedigrees (~ 90 %), the results indicated

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homogeneity of the mitochondrial mutation in Japanese LHON pedigrees. On the other hand, a goodness-of-fit test revealed that the two-locus mitochondrial and X-linked gene control theory accorded well with the characteristic inheritance trait of predominance in males and reduced penetrance with late onset in females. However, the penetrance in heterozygous LHON maternal line females of Japanese pedigrees was about twice as high as that in foreign pedigrees, indicating an ethnic difference in LHON genetics.

INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease, characterized by bilateral acute onset optic atrophy mainly in young men.[8] To date, 5 LHON-specific mitochondrial (mt) DNA point mutations have been identified at nucleotide position (np) 3460, 4160, 11778, 14484, and 15257 (Table 1), which can well account for the strict maternal transmission of this disorder.[8] Out of these 5 mutations, a guanine-to-adenine mutation at np 11778 in the NADH dehydrogenase subunit (ND) 4 gene,[11], which changes the 340th arginine to histidine of ND4, is most common, especially in Japan, since about 90 % and 50 % of Japanese and foreign LHON pedigrees, respectively, are known to have this mutation.[3,5] However, the presence of one of these mutations is not sufficient for LHON expression, because a maternal relative with one of these mutations in a LHON pedigree does not always manifest symptoms and men are preferentially affected. Three additional genetic factors are presumed to affect LHON expression.[1,6-8] The first is a proportion of mutant relative to wild-type mtDNA molecules. Each cell possesses thousands of mtDNA genomes, and one of the above mutations first occurs in only a small fraction of those, which are unequally distributed to daughter cells and propagated during every mitosis. Thus a proportion of mutant relative to wild-type mtDNA molecules varies among offsprings from an obligate mother. Only individuals with sufficient numbers of mutant mtDNA molecules may be affected. According to this hypothesis, abnormally homoplasmic individuals should always express LHON phenotype. The second is co-existence of secondary

mtDNA mutations. Additional mtDNA mutations or polymorphisms, which are pathogenetically silent by themselves, may exert synergistic or antagonistic effects on LHON expression by the primary mutation. Currently, 7 secondary mutations have been reported at np 3394, 4136, 4216, 4917, 5244, 7444, 13708, and 15812[8] (Table 1). Of these, the mutations at 3394, 4216, 4917, and 13708 co-exist in the 11778 mutated LHON pedigrees. The third possibility is that both abnormalities of X-linked recessive gene and mtDNA are necessary for the development of optic atrophy in LHON (the two locus model).[1,7] The above three issues have been tested for white populations, but not substantially for Japanese LHON pedigrees.

In this study, I aimed to elucidate whether one or more of the above three genetic factors regulate the LHON phenotype in Japanese and to clarify differences in genetic characteristics between Japanese and foreign LHON pedigrees.

Table 1. Primary and secondary mtDNA mutations reported

	Mutation Site	Amino Acid Change	Frequency in All LHON(%)	Frequency in 11778 mutated LHON(%)	Frequency in Control(%)
Group A	ND1 3460	A → T	8-25	0	0
	ND1 4160	L → P	1 pedigree	0	0
	ND4 11778	R → H	40-90	100	0
	ND6 14484	M → V	10	0	0
	CytB 15257	D → N	7-9	0	0.3
Group B	ND1 3394	Y → H	4	7	1-2
	ND1 4136	Y → C	1 pedigree	0	0
	ND1 4216	Y → H	38	21	7-13
	ND2 4917	D → N	17	3	3-4
	ND2 5244	G → S	1 pedigree	0	0
	COI 7444	ter → K	2 pedigrees	0	1
	ND5 13708	A → T	31	23	5-6
	CytB 15812	V → M	4	0	0.1

Group A: Primary mtDNA mutation. Group B: Secondary mtDNA mutation.
ND: Reduced NADH dehydrogenase subunit. CytB: cytochrome b. COI: cytochrome c oxidase.

MATERIALS AND METHODS

mtDNA analyses

The primary mtDNA mutations tested in the present study were those at np 3460, 4160, and 11778, since the other two were reported during the preparation of this manuscript. The secondary mtDNA mutations tested were those at np 3394, 4136, 4216, 4917, and 13708, because all of the present pedigrees possessed the 11778 mutation.

Blood samples were obtained with informed consent from 22 family members of 10 Japanese LHON pedigrees, which consisted of 13 male patients (10 probands and their 3 affected brothers), one subjectively asymptomatic brother with peripapillary microangiopathy, 7 asymptomatic maternal female relatives, and one internal control male (a son of one male patient). Ten unrelated normal controls were also included. DNA extraction, PCR, RFLP, and conventional or sequence-specific oligonucleotide (SSO) probe Southern blot hybridization were performed as described elsewhere.[6]

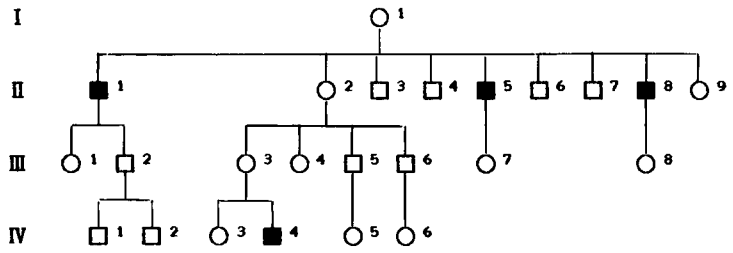

Figure 1. A representative LHON pedigree.

Segregation analysis for the presumed X-linked recessive gene

Based on the maternal line sibships in the published and present Japanese LHON pedigrees, two data sets were constructed. Data set I consisted of 27 pedigrees who were ascertained to have the 11778 mutation, while data set II of 55 pedigrees without molecular testing. Definition of LHON and criterion in enrolling pedigrees into the data sets were adapted from the previous study.[7] A heterozygous mother was defined as an affected or unaffected mother who had at least one unaffected or

affected son, respectively. Since the expected number of affected male offsprings from mating of a presumed heterozygous mother with a random male follows a truncate binominal distribution with a parameter = 1/2, a goodness-of-fit test was performed to determine the eligibility of the two-locus mitochondrial and X chromosomal gene model.[1,7] When the two locus model is valid, a female LHON patient should be not only an mtDNA mutant but also either a homozygote at the X-linked locus or a heterozygote affected by unfortunate inactivation (lyonization) of a normal allele on an X-chromosome. Segregation ratio (*S*), heterozygote penetrance (*Pe*), and the frequency of an abnormal X-linked gene (*q*) were estimated as described previously.[7] I then determined genetic differences between Japanese and foreign LHON pedigrees by the statistic comparison of the present *S* and *Pe* values and the previous.

RESULTS

Analyses for mtDNA genotype

1) Homoplasmy of the 11778 mutation

In an attempt to determine whether LHON expression is regulated by the ratio of 11778-mutant to normal mtDNA molecules, 312 bp DNA region encompassing np 11778 was amplified by PCR and the PCR products were hybridized with either wild or mutant-type 19 base SSO probe radiolabeled with [γ -^{32}P]-ATP.[6] Figure 2 shows results of the PCR-SSO hybridization for a representative large LHON pedigree (Fig. 1). Except for an internal reference individual III -2, a son of a male LHON patient II -1, and a normal control (N), all of the other lanes exhibited positive bands for only the mutant SSO probe with no traces of bands for the wild-type probe (Fig. 2 A and B). III -2 and N demonstrate the reciprocal results (Fig. 2 A and B). These indicate that all of the maternal lines of this pedigree, regardless of clinical phenotypes, possess the homoplasmic 11778 mutation. To discriminate between the above homoplasmy and PCR amplification bias, conventional Southern blot hybridization of *Sfa* NI-digested DNA to the probe of the above 312 bp PCR products was also employed. All lanes other than III -2 and N in Figure 2 C showed a 1595 bp band with no traces of two bands of 916 and 679 bp

length, reflecting the loss of the *Sfa* NI recognition site due to the 11778 G-to-A homoplasmic mutation. Similar results were obtained in other LHON members tested, thus showing further evidence of 11778 homoplasmy in all maternal relatives of the present LHON pedigrees, whether affected or asymptomatic.[6]

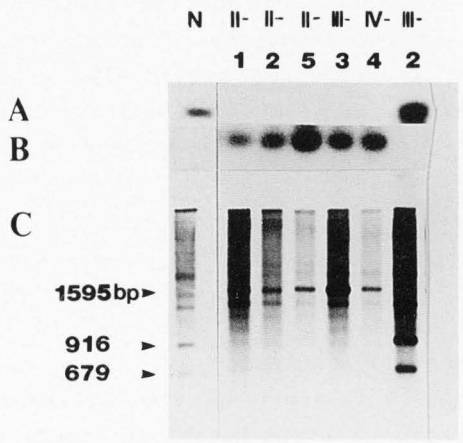

Figure 2. Homoplasmy of the mitochondrial DNA mutation at position 11778. (A) and (B). Southern blot hybridizations using normal and mutant probe, respectively, specific for np 11778. Lane numbers correspond to family members of Fig. 1. N indicates a normal control. Note that all of the tested members other than III -2 exhibited positive bands for mutant probes but not at all for normal-type probe. (C). Conventional Southern blot hybridization using probe of PCR products encompassing np 11778. Note that two bands of 879 and 916 bp lengths were produced by an *Sfa* NI cut and a single 1595 bp band was by the *Sfa* NI site loss.

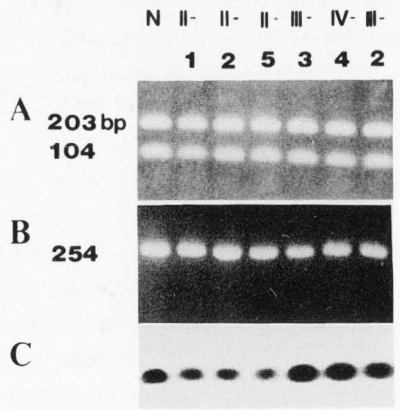

Figure 3. Exclusion of the secondary mtDNA mutations. (A). *Bsa* HI digestion of 307 bp PCR products. All cases showed two bands of 104 and 203 bp lengths due to preservation of the normal sequence at np 3460 in all cases. (B). *Sph* I or *Nsp7524* I digestion of 254 bp PCR products bracketing np 4136, 4160, and 4216. Note that all lanes including N exhibited a single 254 bp band, indicating the normal sequence at np 4136 and 4216 in all cases. (C). Southern blot hybridization using wild-type 19 base oligonucleotide probe specific for np 4160.

2) mitochondrial homogeneity

In order to examine whether the secondary mtDNA mutations occasionally observed in foreign LHON patients with the 11778 mutation affect the LHON phenotypes, PCR-RFLP analyses using appropriate restriction endonucleases and PCR-SSO hybridization were employed. Figure 3 A shows that 307 bp PCR products bracketing np 3460 were cut into 104 and 203 bp fragments by *Bsa* HI for all tested members, indicating the normal sequence at this specific site. Digestions of 254 bp PCR products bracketing np 4136 and 4216 with *Sph* I and *Nsp7524* I, respectively, resulted in no cleavage for all family members of the representative pedigree as well as the normal control because of preservation of the wild-type sequences at the two tested sites (Fig. 3 B). Figure 3 C presents evidence that 254 bp PCR products encompassing np 4160

from all of the tested members of the pedigree were hybridized to the wild-type SSO probe. The same results were obtained in the other LHON members tested. In addition, all of the 22 family members tested in 10 LHON pedigrees possessed normal sequences at both np 3394 and 13708 (data not shown).

Segregation analysis for the presumed X-linked recessive gene

1) Eligibility of the two-locus control

A goodness-of-fit test performed on the individual male sibship data revealed an elegant fit for the 1:1 segregation of the putative X-linked recessive gene in both data sets (Table 2), supporting the two-locus mitochondrial and X-linked model that we have recently reported for Japanese LHON pedigrees.[7]

Table 2. Goodness-of-fit tests on individual male sibship data for two locus model

			No. affected					Segregation
Data set	s[a]	n_s[b]	Obs.	Exp.	Var.	χ^2[c]	P	ratio[d]
I	1	13	13	13.0	0.00			0.500
	2	19	25	25.3	4.22			0.494
	3	9	11	15.4	4.41			0.357
	4	5	10	10.7	3.91			0.469
	5	2	5	5.2	2.16			0.484
Total		48	64	69.6	14.70	2.13	>0.15	0.460
II	1	23	23	23.0	0.00			0.500
	2	28	36	37.3	6.21			0.482
	3	17	28	29.1	8.33			0.480
	4	8	14	17.1	6.26			0.410
	5	2	4	5.2	2.16			0.387
	6	1	3	3.0	1.38			0.492
Total		79	108	114.7	24.34	1.86	>0.15	0.471

[a] Sibship size.

[b] The number of sibships.

[c] $\chi^2 = (\Sigma \text{Obs.} - \Sigma \text{Exp.})^2 / \Sigma \text{Var.}$

[d] Segregation ratio = Obs. / (Exp. × 2).

2) Estimation of S, Pe, and q, and comparison with the previous values

Table 3 indicates the pooled female sibship data, showing no significant differences between *S* of the two data sets nor between *Pe* of the two groups. The combined *S* for the two data sets was 0.150 ± 0.028 with a 95 % confidence interval of 0.095-0.205. The combined *Pe* was 0.190 ± 0.038 with a 95 % confidence interval of 0.115-0.265. When the fixed points of *S* and *Pe* values were applied to an equation, $S = q/2$ (a probability that the female offspring is homozygous affected) + $Pe/2$ (a probability that the female offspring is heterozygous times the penetrance value), *q* was estimated to be 0.11. Approximately 37 % of the affected females will be X-homozygotes, while about 63 % of those will be X-heterozygotes who become LHON patients by lyonization.

Table 3. Pooled female data for estimates of segregation ratio and penetrance in maternal females

	Numbers		
	Affected	Unaffected	Total
Data set I			
Offsprings	5	46	51
Heterozygous mother	9	37	46
Data set II			
Offsprings	19	90	109
Heterozygous mother	11	48	59

The present *S* did not differ statistically from the previous segregation ratio (= 0.093).[1] On the other hand, the present *Pe* was significantly higher than the previous penetrance value (= 0.111)[1] with a 5 % level of significance. These results further indicates a higher proportion of affected heterozygotes caused by an X-inactivation in LHON maternal line females in Japan compared with foreign nations.

DISCUSSION

The present study further supports the unique characteristics of LHON genetics in Japanese described earlier.[5-7] The first is the mitochondrial homogeneity characterized by homoplasmy of 11778 mutation and no locus heterogeneity in the mtDNA genome in LHON family members, whether they are affected or not. In foreign nations, heteroplasmy at np 11778 can well account for intrafamilial phenotypic variations in some LHON pedigrees, in which LHON patients had a higher proportion of mutant mtDNA molecules than did their asymptomatic maternal relatives.[4,8] On the other hand, about 21 % and 23 % white LHON patients with the 11778 mutation have been reported to simultaneously possess secondary mtDNA mutation at np 4216 and 13708, respectively (Table 1).[8] Other minor secondary mutations have been reported (Table 1).[8] All of these other than the 4136 mutation may synergize LHON expression resulting from the 11778 or other LHON-specific primary mutations. Conversely, the 4136 base change may be an "intragenic suppressor" mutation which ameliorates the biochemical defect of mitochondrial oxidative phosphorylation enzyme complex I and neurological abnormalities caused by the 4160 mutation.[3] The previous[6] and present studies, however, have presented evidence against both of the above scenarios. Neither heteroplasmy at np 11778 of mtDNA nor synergistic effects by additional secondary mtDNA mutations can explain the intrafamilial phenotypic variations at least in the 10 Japanese pedigrees currently available. There must be extramitochondrial factors necessary for the LHON expression.

In this regard, segregation analysis on the reported LHON pedigrees has clarified the second characteristics of LHON genetics as follows. (1) LHON expression is regulated by both mtDNA mutation and X-linked recessive gene[1,7] (Table 2). (2) Japanese heterozygous female offsprings of LHON maternal lines may be genetically more susceptible to LHON compared with foreign counterparts. The high penetrance for a heterozygous female may arise from a lower threshold of LHON in them. The overwhelming majority of Japanese LHON pedigrees harbor the 11778 mutation as previously documented,[5] and a patient with the 11778 mutation has been reported to have a worse visual prognosis than a patient

with other primary mtDNA mutations.[2] Whether the extraordinarily high prevelance of the 11778 mutation in Japanese affects the disease threshold in heterozygous females remains to be elucidated. Although the previous X-linkage analyses are still controversial,[9,10] the previous[7] and present studies have presented evidence for the two-locus mitochondrial and X-linked gene control theory for LHON expression. Further investigations remain to isolate the X-linked gene for studying the precise pathogenesis of this particular disease.

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