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X chromosome-linked and mitochondrial gene control of Leber hereditary optic neuropathy: Evidence from segregation analysis for dependence on X chromosome inactivation

(two-locus inheritance/cytoplasmic inheritance/reduced penetrance)

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ABSTRACT **Leber hereditary optic neuropathy (LHON) has been shown to involve mutation(s) of mitochondrial DNA, yet there remain several confusing aspects of its inheritance not explained by mitochondrial inheritance alone, including male predominance, reduced penetrance, and a later age of onset in females. By extending segregation analysis methods to disorders that involve both a mitochondrial and a nuclear gene locus, we show that the available pedigree data for LHON are most consistent with a two-locus disorder, with one responsible gene being mitochondrial and the other nuclear and X chromosome-linked. Furthermore, we have been able to extend the two-locus analytic method and demonstrate that a proportion of affected females are likely heterozygous at the X chromosome-linked locus and are affected due to unfortunate X chromosome inactivation, thus providing an explanation for the later age of onset in females. The estimated penetrance for a heterozygous female is 0.11 ± 0.02. The calculated frequency of the X chromosome-linked gene for LHON is 0.08. Among affected females, 60% are expected to be heterozygous, and the remainder are expected to be homozygous at the responsible X chromosome-linked locus.**

Leber hereditary optic neuropathy (LHON), first described by Leber in 1871, is maternally transmitted and associated with acute and subacute visual loss in both young adult males and females. The age of onset is the early 20s, and it is an average 2–15 years later for females than for males (refs. 1 and 2; M. L. Savontaus, personal communication). The visual loss, usually permanent and bilateral, is caused by optic atrophy and large centrocecal scotomata. LHON has been shown to commonly involve a mitochondrial DNA (mtDNA) point mutation (3, 4), which explained the maternal transmission pattern. Yet there remain several confusing aspects of its inheritance, which cannot be explained by mitochondrial inheritance alone. These include male predominance (a mitochondrial disorder should have 1:1 sex ratio), reduced penetrance (i.e., clearly unaffected females transmitting the disease), later age of onset for females, and expression limited to optic tissue. In this paper, we propose a two-locus mitochondrial and X chromosome-linked nuclear gene model that, taking X chromosome inactivation into account, provides an explanation for these intriguing features of LHON inheritance.

About 85% of LHON patients are male (1). In the early stage of research on the segregation pattern of LHON, an X-linked mode of inheritance was proposed on the basis of this male predominance. However, this mode of inheritance failed to explain the lack of transmission of the disease through males (i.e., to their daughters). On the basis of linkage analysis, Chen *et al.* (5) excluded an X-linked gene alone as the cause for LHON. Earlier, Imai and Moriwaki (6) advanced the theory of cytoplasmic inheritance. The identification of a mtDNA point mutation for LHON by Wallace *et al.* (3) and Singh *et al.* (4) subsequently proved the decades-old hypothesis regarding the cytoplasmic inheritance of LHON (6). As mentioned above, however, a mitochondrial mutation alone still cannot explain many of the features of the transmission pattern of LHON, including the strong male bias and the reduced penetrance of LHON in the maternal line pedigree. These features, together with the conspicuous tissue-specific expression of LHON, suggest the involvement of nuclear gene(s), presumably an X-linked gene. Wallace (7) has proposed a nuclear–cytoplasmic interaction hypothesis. However, even the proposal of both X-linked and mitochondrial gene mutations does not fully explain the inheritance of LHON. Under an X-linked recessive and mitochondrial model, all male offspring of an affected mother should be affected. If the nuclear gene is X-linked dominant, there should be more affected females than affected males. Both of these conflict with the actual observations. It has been further proposed by Wallace in 1987 (7) that differential expression in males could reflect the difference in X chromosome number in males and females if this nuclear gene escapes X inactivation. We propose here the hypothesis that it is X inactivation of a small number of embryonic precursor cells for the optic tissue that can resolve the transmission pattern for LHON, and we support this hypothesis by a formal analysis of a large number of LHON pedigrees.

By the theory of X chromosome inactivation, a heterozygous female's clinical phenotype will be dependent on the combined effect of each individual cell of the involved tissue. The phenotype of each individual cell is determined by whether the normal or abnormal X chromosome is active. Thus one heterozygous genotype corresponds to two different phenotypes—affected or unaffected. This leads to potential difficulties in linkage studies as regards the correct classification of a heterozygous female. However, even with these difficulties, Vilkki *et al.* (8) have recently reported a linkage study indicating that a nuclear gene determining the clinical manifestation of LHON is localized to the region of proximal Xp.

A particularly interesting aspect of mitochondrial genetics is that some peptide subunits are encoded by mtDNA and other proteins are encoded by nuclear DNA. Given the fact of nucleus–mitochondria interaction (9), it could be inferred that some mitochondrial disorders are due to deleterious interactions between the two genomes. Herein we have

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

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modified our two-locus mitochondrial and nuclear gene models (10) by incorporating the random inactivation of the X chromosome. We show here that extending this analysis to the LHON case can fully account for the unique transmission pattern observed in this disease.

METHODS

Family Data. Our goal was to test the hypothesis that the clinical aggregation of disease in LHON families is due to simultaneous involvement of two loci, one mitochondrial and one X-linked. Three published pedigree data sets were used in this analysis. An individual with optic atrophy was defined as affected in all three data sets. We included in our segregation analysis only the maternal line members whose age at examination or age at death was beyond the mean age of onset of the disease, or who were not part of the youngest generation in the pedigree if information on age was not available. In this manner, we minimized the sampling error that would otherwise result from including those clinically unaffected individuals who might become affected at a later age.

Data set one contained four Finnish pedigrees (8, 11). All four pedigrees shared the same mitochondrial mutation (causing LHON disease) and showed no evidence of heteroplasmy for this mutation. The details of the pedigree structures were further clarified by direct communication with the authors of refs. 8 and 11.

Data set two included Seedorff's 20 pedigrees from Denmark (2, 12–14). Among them, pedigree A comprised nine generations and 254 maternal line individuals, and pedigree C comprised eight generations and 124 members.

Data set three contained an amalgamation of seven pedigrees from the literature (3, 5, 15–17). In all, our analysis included 31 pedigrees totaling more than 1200 individuals.

Two-Locus X-Linked and Mitochondrial Gene Model. The data were analyzed under our proposed two-locus X-linked and mitochondrial gene model (10). In the case of mitochondrial disorders, we know that the offspring inherit their mtDNA only from their mothers. Therefore, we limited the analysis to the sibships descended from genetically related females, a group we term the maternal line pedigree. All such maternal line family members will share the mtDNA mutation. Any disease will reflect only the segregation at the nuclear locus. Thus, restricting the analysis to the maternal line family members allows us to simplify the two-locus problem into a one-locus model, in this case X-linked. Let A and a represent, respectively, the normal X-linked allele and the disease X-linked allele. Thus, the large pedigrees were first simplified to maternal line pedigrees and then broken down into nuclear families.

We first tested this X-linked hypothesis in the male population of these data sets, since this is the more straightforward analytic situation. For the mating between a heterozygous female and a random male, on the average 50% of their male offspring will be affected. We ascertained the heterozygous females in these families by the following criteria: either an unaffected mother with at least one affected male offspring or, conversely, an affected mother with at least one unaffected male offspring. Therefore, we have two conditionally independent groups of families that we could use to independently test the same hypothesis. For each group of families, we can perform the goodness-of-fit test on either pooled male sibship data or individual sibship data. Under this ascertainment scheme, the study population (LHON pedigrees) will be exhaustively examined. A nuclear family with a large number of affected male children is no more likely to be included than one with a small number if they both meet the criteria above. The distribution of affected male children among selected nuclear families is approximately binomial except for omission of the class with no affected males, and thus is a truncated distribution. This selection method is thus equivalent to truncate selection. To account for the ascertainment bias introduced here, a correction was made in estimating the segregation ratio and the expected number of the affected and unaffected male offspring (18–21).

To examine this hypothesis of X inactivation at the nuclear locus, we then analyzed the aggregation of LHON in the female population of these data sets. Our model stated that there are two ways in which a female can be affected—(*i*) that she is homozygous at the X-linked locus and (*ii*) that she is heterozygous but has undergone unfortunate X inactivation with a predominant number of cells with the abnormal X chromosome active in the optic tissue.

Let q be the frequency of the abnormal X-linked gene (a) for LHON, and K the probability of a heterozygous female being affected—i.e., the penetrance. The probability of a female offspring being affected with LHON from the mating between a heterozygous female and a random male will be defined as the segregation ratio T. T therefore is the sum of the probability that the female offspring is homozygous affected at the X-linked locus ($q/2$), plus the probability that a female offspring is heterozygous (i.e., ½) times the probability for a heterozygous female to be affected (K). Thus

$$T = q/2 + K/2. \quad [1]$$

A heterozygous female will be affected with LHON when the proportion of affected cells in her optic tissues exceeds a certain limit. We define this limit as the disease threshold for the heterozygous female, which is dependent on the parameter K. The higher that K is, the lower the disease threshold.

Goodness-of-Fit Test for Males. For the male sibships in the families with a presumed heterozygous mother, the ascertainment bias was adjusted by the Haldane method (18, 19). The goodness-of-fit test on pooled male sibship data is a straightforward χ^2 test.

As for the test on the individual sibship data, the expected number of affected male offspring from a family with an obligate heterozygous mother at the X-linked locus follows a binomial distribution with only the parameter $T = ½$, which is the probability of a male offspring being affected. For a family with male sibship size s and r observed affected males, the expected number of affected offspring is (20, 21)

$$E_s(x) = \sum_{r=1}^{s} \frac{r\binom{s}{r}(½)^s}{1-(½)^s} \quad [2]$$

and the variance is

$$V_s(x) = E_s(x)^2 - [E_s(x)]^2 = \sum_{r=1}^{s} \frac{r^2\binom{s}{r}(½)^s}{1-(½)^s} - \left[\sum_{r=1}^{s} \frac{r\binom{s}{r}(½)^s}{1-(½)^s}\right]^2. \quad [3]$$

Therefore, a more critical goodness-of-fit test can be performed on the individual male sibship data.

Statistical Test and Inference for Females. Since the number of nuclear families ascertained through an unaffected mother and at least one affected male offspring is far more than that of the other group, we based our estimation on this former group. The expected segregation ratio T was estimated from the observed segregation ratio—i.e., the proportion of affected female offspring among the total female offspring of unaffected heterozygous mothers. Since the ascertainment bias for selecting these families comes from eliminating those families with no affected male offspring, the observed segregation ratio for female offspring will be an unbiased esti-

mate. A χ^2 test was used to test the heterogeneity among the observed segregation ratios of the three data sets. Fisher's exact test was used whenever appropriate (22). Since there was no significant difference among the data sets, they were combined to estimate the parameter more efficiently. An analogous test for the obligate heterozygous mothers, both affected and clinically normal, was performed. After the adjustment for selection bias, the ratio of the number of affected heterozygous mothers to total heterozygous mothers is an unbiased estimate of the heterozygote penetrance K. With the two parameters known in Eq. 1, $T = q/2 + K/2$, the gene frequency q was calculated.

RESULTS

Goodness-of-Fit Test for Males. The adjusted segregation ratios (±SE) for the three data sets with unaffected heterozygous mothers are 0.47 ± 0.10, 0.48 ± 0.04, and 0.44 ± 0.06. A goodness-of-fit test was performed on the pooled male sibships from each of the three data sets. Remarkably close agreement of the observed number of affected males with the expected number of affected males under the proposed two-locus X-linked and mitochondrial gene model was found (Table 1).

Besides an analysis of the pooled sibship data, we performed an even more critical analysis of the distribution of the individual sibship data. The expected number of affected male offspring from a heterozygous mother follows a binomial distribution with the sole parameter $T = ½$, which is independent of gene frequency. The expected values are given in Table 2 for each of the three data sets. There was again remarkably close agreement between the proposed two-locus X-linked and mitochondrial gene model and each of the observed family data sets (Table 2), and also for the summation of the three family data sets ($\chi^2 = 0.99, P > 0.30$).

We next analyzed the families ascertained through an affected mother and at least one normal male offspring. Because of their small sample sizes, the three data sets were combined. We again observed close agreement between these data and the proposed model (observed = 23, expected = 20.9, $\chi^2 = 1.67$, $P > 0.19$), supporting the X-linked and mitochondrial model.

Statistical Tests and Estimations of Penetrance for Heterozygous Female and Gene Frequency. Because we ascertained the families through the status of male offspring, we needed to test the equality of the offspring's sex ratio after correcting for the ascertainment bias. The adjusted offspring's sex ratio for the families ascertained through an unaffected mother and at least one affected male offspring was 1.061 ($\chi^2 = 0.67$, $P > 0.40$). For the families with an affected mother and at least one normal male offspring, after correcting for the ascertainment bias, a balanced distribution of male and female offspring was also found (sex ratio = 1.004, $\chi^2 = 0.0003$, and $P > 0.98$).

We first obtained the segregation ratio T. A χ^2 test of heterogeneity for the female offspring from the mating between an unaffected mother and a random father was performed. We found no evidence for heterogeneity among the three data sets—i.e., there was no significant difference among the segregation ratios of the three data sets ($\chi^2 = 0.74$, $P = 0.69$). Similar results were found by likelihood ratio χ^2 test ($G = 0.69$, $P > 0.70$) and Fisher's exact test ($P = 0.63$). The combined segregation ratio T for the three data sets was 0.093 ± 0.016 (30 divided by 321) with 95% confidence interval of 0.062 to 0.125.

We then proceeded to calculate the heterozygote penetrance K. A heterogeneity test of the ratio of adjusted number of affected heterozygous mothers to total adjusted number of heterozygous mothers for the three data sets was performed. There was no significant difference among these three groups ($P > 0.10$). A similar result was found by the likelihood ratio χ^2 test ($P > 0.10$). The estimate of the heterozygous penetrance K was 0.111 ± 0.020 (28.7 divided by 258.1) with a 95% confidence interval of 0.072 to 0.150.

Since we had obtained T and K, by using Eq. 1, we could calculate q, the gene frequency for the mutant X-linked nuclear gene. The point estimate of q was 0.076, with a range of 0.013 to 0.139 when the point estimate of K was used, or with a point estimate of 0.075 and a range of 0.036 to 0.114 when the point estimate of T was used. Comparing the estimates of $q/2$ (affected homozygotes) and $K/2$ (affected heterozygotes), we calculated a value for the proportion of affected females who were heterozygous at the X-linked locus. Approximately 40% of the affected females were calculated to be homozygous at the X-linked locus, and the rest of the affected females (60%) were estimated to be due to the consequence of unfortunate X inactivation.

Table 1. Goodness-of-fit tests for two-locus X-linked and mitochondrial gene model: Pooled male sibship data with unaffected heterozygous (*Aa*) mother

Data set	No. affected*		χ^2	P
	Obs.	Exp.		
One	17.3	18.5	0.16	>0.65
Two	96.5	100.5	0.32	>0.55
Three	48.8	55.0	1.38	>0.20

*The observed and expected numbers of affected are calculated from the observed and expected segregation ratios, respectively.

Table 2. Goodness-of-fit tests for two-locus X-linked and mitochondrial gene model: Individual male sibship data with unaffected heterozygous (*Aa*) mother

Data set	s	n_s	No. affected		χ^2	P
			Obs.	Exp.		
One	1	8	8	8.0		
	2	7	10	9.3		
	3	3	3	5.1		
	4	1	3	2.1		
	5	2	5	5.2		
Total		21	29	29.7	0.10	>0.75
Two	1	23	23	23.0		
	2	22	31	29.3		
	3	22	33	37.7		
	4	13	31	27.7		
	5	4	8	10.3		
	6	2	7	6.1		
	7	1	2	3.5		
Total		87	135	137.6	0.22	>0.60
Three	1	17	17	17.0		
	2	16	18	21.3		
	3	9	17	15.4		
	4	4	7	8.5		
	5	3	6	7.7		
	6	2	7	6.1		
	8	1	4	3.5		
Total		52	76	79.5	0.72	>0.35

s is sibship size and n_s is the number of families.

DISCUSSION

The object of this work was to determine whether the extension of methods of segregation analysis for two-locus nuclear and mitochondrial disorders could clarify the mode of inheritance of LHON. We demonstrated herein that (*i*) the aggregation of LHON in males is consistent with simulta-

neous X-linked and mitochondrial inheritance; (*ii*) the aggregation in females is also consistent with this model, provided that a female can be affected either by being homozygous at the X-linked locus (40%) or by being heterozygous with unfortunate X inactivation (60%); (*iii*) the X-linked gene frequency is 0.08; and (*iv*) the estimated penetrance for a heterozygous female is 0.11. These calculations also provide an explanation for later age of onset in females, suggesting that it is due to the mixture of homozygous and heterozygous affected females. Presumably, the proportion of optic cells with the normal X chromosome active in the latter group delays their clinical onset of disease.

One of the assumptions for two-locus mitochondrial and nuclear gene models is that the cell is homoplasmic, or close to homoplasmic, for the involved mitochondrial gene. Since each cell has hundreds to thousands of mitochondria, both homoplasmic and heteroplasmic hypotheses are theoretically possible. The available data suggest that homoplasmy is the more frequent phenomenon. Thus a study of the restriction fragment patterns of mtDNA in 70 affected or unaffected maternal line individuals from 17 families with LHON showed no evidence of heteroplasmy (23). Heteroplasmy of mtDNA for the restriction site *Sfa*NI (the site of the mutation for LHON) was found in only 1 probable LHON pedigree out of 10 pedigrees studied (24). mtDNA heteroplasmy was also found in some other LHON patients (25). Of interest, a rapid meiotic segregation rate of the heteroplasmic family members with LHON was reported (26, 27). This observation—i.e., a shift from heteroplasmy to homoplasmy in one or two generations—suggests that heteroplasmy may not be maintained for many generations. Large LHON pedigrees with many affected individuals would thus tend toward a homoplasmic mutant state (3). Comparable observations of rapid development from heteroplasmic cells to homoplasmic cells in bovine maternal lineages and in *Drosophila* were reported by several groups (28–31). These data support the interpretation that homoplasmy or near homoplasmy of mtDNA should be expected when the mitochondrial mutation already exists in a maternal line pedigree for several generations. Shoubridge *et al.* (32) reported that deletion mutants are functionally dominant over wild-type mitochondrial genomes in skeletal muscle fiber segments in mitochondrial disease. If this is also the case for the mitochondrial gene of LHON, theoretically the two-locus mitochondrial and X-linked nuclear gene model would hold even in circumstances of heteroplasmy of mtDNA.

The heterogeneity of LHON has been long debated. Genetic heterogeneity was confirmed by the identification of one of the mitochondrial mutations for LHON. Several LHON families in which the *Sfa*NI restriction enzyme site is not lost have been described, establishing heterogeneity of the mitochondrial gene defect (3, 24, 25). Nevertheless, as long as the involved nuclear gene(s) for LHON is X-linked, the segregation analysis in this study should be valid for male offspring. If there is further heterogeneity (i.e., a different X-linked locus), this will complicate the analysis in female offspring, whose aggregation is a function of disease frequency.

In all our analyses, there was no significant difference among the segregation ratios of the three independent data sets. For these three data sets, genetic homogeneity of the mode of inheritance of LHON is thus supported. Since data set one was homogeneous for the mitochondrial mutation, we would infer that all or most of the families from the other two data sets share a mitochondrial mutation as well. It may well be the same mutation, or it may consist of a different mitochondrial mutation but occur in the same locus as those in data set one, or it may even be a different mitochondrial locus. Use of segregation analysis can only establish a two-locus mitochondrial and X-linked nuclear gene mode of inheritance. However, the mitochondrial and X-linked mutations could vary from pedigree to pedigree and still be consistent with the segregation analysis model presented here.

Our analyses strongly suggest that the clinical manifestations of LHON in females appear to be dependent on X chromosome inactivation of a small number of responsible embryonic precursor cells. At the cellular level of the optic tissue, a female LHON patient could be homozygously affected (i.e., all the involved optic cells uniformly affected) with a homozygous or a heterozygous X-linked genotype (the latter due to uniform X inactivation of the normal X allele) or heterozygously affected (i.e., a certain percent of involved optic cells affected) with a heterozygous X-linked genotype. Mosaic expression of the X-linked gene would be expected in most of the heterozygous females, based on random X chromosome inactivation. Discordant female monozygotic twins and affected heterozygous females have been reported for several other X-linked disorders, such as Duchenne muscular dystrophy, Fabry disease, hemophilia, and glucose-6-phosphate dehydrogenase deficiency (33–38).

The reported data indicate that females have an average age of onset 2–15 years later than males (refs. 1 and 2; M. L. Savontaus, personal communication). One explanation would be that a proportion of the affected females are not homozygous at the X-linked locus, but heterozygous, and thus mosaic for the affected tissues. Compared with her homozygous affected counterparts, a heterozygous affected female would thus tend to have a later age of onset because the proportion of optic cells with the normal X-linked gene active might delay the onset of LHON. Even prior to identification of the X-linked gene, this question can be examined in the following manner. By appropriate inspection of the pedigree, one can distinguish a group of affected females who are obligate heterozygotes (an affected mother with one or more normal male offspring) versus a remaining group consisting of a mixture of indistinguishable affected homozygotes and heterozygotes. The respective ages of onset between these two groups could be compared. This will require the identification of age of onset of an adequate number of affected females with the details of the pedigree structure, data that are not yet available.

Parker *et al.* (39) have reported a marked decrease in the activity of complex I of the mitochondrial respiratory chain from one LHON family. If there are affected heterozygotes, they should, in principle, be a mixture of two distinct cellular phenotypes (i.e., regarding activity of complex I), each phenotype corresponding to one of the two different X chromosomes. By assaying the relevant enzymes in cloned populations of cells (i.e., descended from a single ancestral cell), the existence of two populations of cells can then be tested by obtaining the appropriate tissues (e.g., fibroblasts, neurons, and lymphoblasts) from obligate heterozygous females (affected or unaffected) identified by methods described in our pedigree analysis.

We utilized a method of segregation analysis particularly suited for mitochondrial disorders, in which restricting our analysis to the maternal line pedigree allows us to simplify the two-locus model into a "one nuclear X-linked locus model." By this simplification, the number of parameters to be estimated is minimized. The excellent fit between the observed and expected strongly suggests that the straightforward segregation analysis methods utilized here are adequate and appropriate. Other existing methods of segregation analysis (40, 41), in which reduced penetrance and heterogeneity are allowed, cannot test the maternal transmission *per se*. Therefore they do not appear to be suitable for the diseases with a maternal transmission pattern, such as LHON. Our maternal line pedigree method could cope with this difficulty satisfactorily. In addition, while these other existing methods

are extremely sophisticated in a variety of other situations, they may create certain potential difficulties regarding the analysis of LHON, such as misclassification of the affection status of the marrying-in males. These latter individuals may have the nuclear mutation, but they are not affected because they lack the mitochondrial mutation.

In summary, by extending methods of segregation analysis to the possibility of two-locus mitochondrial disorders, we found strong evidence for a two-locus nuclear X-linked and mitochondrial gene mode of inheritance for LHON, in which the heterozygous female's clinical manifestations are dependent on X inactivation. The estimates of the segregation ratio for female offspring and the heterozygous penetrance for females, therefore, predict the existence of appreciable number of affected female heterozygotes.

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