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# X-Inactivation patterns in females harboring mtDNA mutations that cause Leber hereditary optic neuropathy

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**Purpose:** Leber hereditary optic neuropathy (LHON) is a common cause of genetically determined blindness in young adults. LHON preferentially affects males and is primarily due to a mutation affecting complex I genes of mitochondrial DNA (mtDNA). While LHON primarily affects men, a number of women are affected. Segregation analysis has implicated an interacting recessive X-chromosomal locus, with skewed X-inactivation as an explanation for visual failure in affected women. Small studies have failed to detect dramatic skewed X-inactivation in women transmitting LHON mutations. However, segregation analyses predicted skewing only in a proportion of women, which would not have been detected in these studies.

**Methods:** The aim of the present study was to determine whether affected or unaffected women with LHON have subtle skewed X-inactivation patterns as a whole, or whether extreme skewing was more common in affected women than in unaffected women.

**Results:** We studied X-inactivation by measuring methylation status of the androgen receptor (*AR*)-$(CAG)_n$ repeat in 192 women homoplasmic for established LHON mtDNA mutations and 96 healthy female controls.

**Conclusions:** We found no evidence of subtle skewed X-inactivation or an excess of skewed inactivation in affected or unaffected women with LHON mtDNA mutations. The frequency of *AR* homozygotes was greater in affected LHON females than unaffected women or healthy controls, implicating the androgen receptor in the pathophysiology of LHON either directly, or through linkage disequilibrium with a different visual loss susceptibility gene.

Leber hereditary optic neuropathy (LHON; MIM 535000) is a cause of subacute bilateral visual failure, which affects about 1 in 14,000 adult men due to retinal ganglion cell loss predominantly affecting the papillomacular bundle [1]. Over 95% of LHON cases have a mitochondrial DNA (mtDNA) mutation affecting one of three genes which code for subunits of complex I of the respiratory chain (3460G>A in *MTND1*, 11778G>A in *MTND4*, and 14484T>C in *MTND6*) [2]. However, only about 40% of males and about 10% of females who inherit the LHON mtDNA mutation develop visual failure, implicating additional environmental as well as genetic factors in the pathophysiology of the disorder [3]. Anecdotal reports have pointed to tobacco and alcohol consumption as precipitants of the visual loss in some cases [4], but there has been no conclusive evidence of an environmental trigger [5]. The clinical penetrance of LHON mtDNA mutations is influenced by polymorphic variants of mtDNA that fall within specific mtDNA haplogroups [6]. Approximately 1/3 of LHON mutation carriers have both mutated and wild-type mtDNA (heteroplasmy) which reduces the risk of blindness [7,8], but these factors cannot explain the gender bias that characterizes the disorder.

Segregation analyses have implicated a recessive X-chromosomal locus interacting with the primary LHON mtDNA mutations in some families [9], although this has not been a universal finding [10]. Early linkage studies have failed to identify a region linked to visual failure on the X-chromosome [11,12], but recent evidence has implicated Xp21 [13]. However, the underlying gene responsible has yet to be identified. Males harboring a high-risk recessive haplotype are thought to be at increased risk of visual failure because they do not carry a protective allele. Only a minority of females are homozygous for the susceptibility locus, which may explain why they are less likely to develop blindness. This cannot, however, be the whole explanation. Under these circumstances, all the sons of affected women would inherit the susceptibility allele and thus develop visual failure-something that is far from the case. Skewed X-inactivation may be a possible reason for the lack of visual failure in the offspring of affected women [9].

In mammals, X-inactivation provides a mechanism of dosage compensation to overcome the autosome/X-chromosome ratio in females [14-16]. This is achieved by the random methylation of one of the X-chromosomes during early embryonic development under the control of the X-inactivation

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center (Xic), centered around *XIST* [17]. *XIST* codes for a 15 kb RNA which is responsible for the *cis*-limited "coating" of the inactivated chromosome blocking translation [18]. Silencing of a protective allele in women harboring a LHON mtDNA mutation would increase their risk of visual failure, reaching similar levels to men. However, women who were blind because their protective allele was silenced would go on to have about 50% unaffected male offspring because the methylation pattern is effectively erased during germ line development, thus providing an explanation for the normal males born to women with LHON.

The choice of an X-chromosome for inactivation within an individual embryonic cell is thought to be random, leading to an equal number of daughter cells, where one or the other cell is active, and an X-inactivation ratio of 50:50 [17]. However, for many X-linked diseases, the inactivation ratio is skewed because of secondary cell selection during development. This is thought to explain why X-linked disorders are rare in females, due to the silencing of the X-chromosome carrying the mutant allele [19-21]. Skewed X-inactivation can therefore indicate carrier status of a deleterious allele on the X-chromosome which, as in LHON, may only become manifest in females harboring a pathogenic mtDNA mutation.

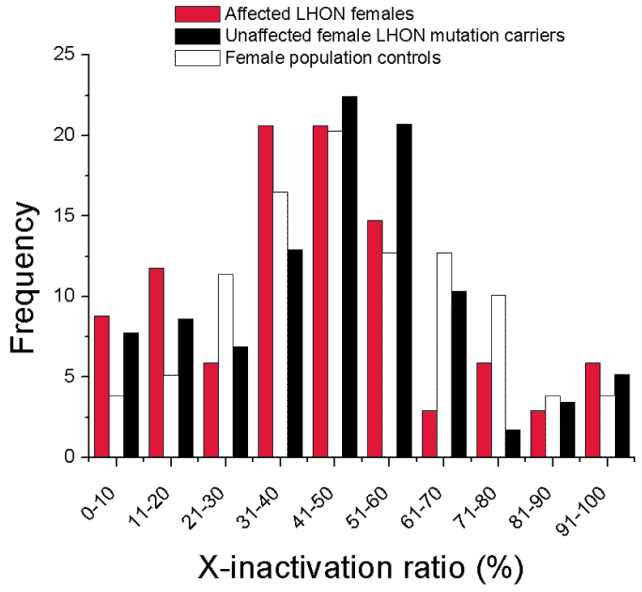


Figure 1. X-activation ratios in Leber hereditary optic neuropathy females and controls. There was no difference in the frequency of different X-activation ratios in affected and unaffected LHON mutation carriers and controls.

Three previous studies looked for evidence of non-random X-inactivation in females harboring LHON mtDNA mutations (16 females in one [22], and 35 females in another [23]), including one study of multiple tissues that included the optic nerve and retina (two females [24]). However, pedigree data and segregation ratios have indicated that X-inactivation only plays a part in a number of women [9], who could potentially be missed in studies of this size. Moreover, the biochemical phenotype in LHON is subtle, and unlikely to dramatically compromise the X-inactivation pattern in otherwise healthy women. An alternative possibility is that subtle skewing of X-inactivation is manifest across all affected women-again, this was not excluded by the previous studies. Through a multi-national study, we identified 192 women harboring a primary LHON mutation. This allowed us to detect subtle skewing of the X-inactivation ratio, possibly only manifest in some women, as expected in LHON.

## METHODS

We studied 192 females homoplasmic for the following primary LHON mtDNA mutations: 3460A>G (n=36), 11778G>A (n=147), 14484 (n=8), and 14495 (n=1) determined by PCR/RFLP analysis or primer extension assay [13]. Unlike the larger of the previous studies, we only included females who were homoplasmic for the LHON mtDNA mutation, thus eliminating a heteroplasmy as a confounding variable from this study. The clinical phenotype was determined by a local ophthalmologist [13]. Unaffected individuals had no vision symptoms and were older than the median age of onset for LHON (24 years). Based on these criteria, 41 were affected and 151 were unaffected mutation carriers. These were compared to 96 ethnically-matched healthy female control subjects. We described about the LHON female selections in the earlier paragraph. The control subjects were healthy population fe-

Table 1. Comparison of X-inactivation ratios in affected and unaffected women carrying Leber hereditary optic neuropathy mtDNA mutations and 96 control subjects

| | LHON mtDNA mutation | | | | Comparison of LHON mutation carriers with controls | | |
|---|---|---|---|---|---|---|---|
| X-inactivation ratio | Affected | Unaffected | p [a] | Control subjects | p [b] | p [c] | p [d] |
| <30:70 or >70:30 | 14 | 38 | 0.41 | 28 | 0.67 | 0.76 | 0.29 |
| <20:80 or >80:20 | 9 | 27 | 0.82 | 12 | 0.19 | 0.20 | 0.13 |
| <10:90 or >0:90 | 4 | 13 | 1.0 | 6 | 0.48 | 0.47 | 0.49 |
| <5:95 or >9:95 | 0 | 3 | 1.0 | 3 | 0.55 | 0.68 | 0.42 |
| Total | 34 | 116 | | 79 | | | |

Frequency of different degrees of X-skewing in affected and unaffected women harbouring Leber hereditary optic neuropathy mtDNA mutations and healthy controls. There was no significant difference between the different groups.

male controls and they were selected as being all >30 years of age for a different study. The youngest LHON case was 31 years old and the oldest was 71 years old.

X-inactivation was determined by restriction enzyme digestion and subsequent PCR amplification of the of the Androgen receptor gene repeat (*AR*-$(CAG)_n$) [22]. Total genomic DNA (100 ng), extracted from peripheral blood lymphocytes, was dually digested with 10 U of *HpaII* and 10 U of *Hha1* at 37 °C for 16 h, restriction enzymes were then heat inactivated at 85 °C for 30 min [25]. Digest products, as well as an undigested correlate sample, were then PCR amplified using *AR*-$(CAG)_n$ specific fluorescent primers (Forward, D3-TCC AGA ATC TTC CAG AGC GTG CGC, Reverse GGC TGT GAA GGT TGC TGT TCC TCA T). *AR*-$(CAG)_n$ length was determined by PCR fragment comparison using a Beckman Coulter CEQ8000 fluorescent DNA analyzer, according to the manufacturer's standard protocol (Beckman Coulter, Fullerton, CA). To take into account preferential amplification of smaller length alleles, we calculated the dosage quotient; normalizing the digested sample against the uncut sample (expressed as Dosage quotient (DQ=area short D allele/area longD allele/area shortU allele/area long U allele), where D is digested and U is undigested). X-inactivation patterns were recorded as the proportion of PCR product from one allele relative to the total amount of PCR product from both alleles. (Expressed as percentage X-inactivation=DQ/DQ+1x100). Statistical analysis was performed using SPSS V14.0 (Lead Technologies Inc., Chicago, IL).

## RESULTS

Seventeen (41%) affected and 25 (18%) unaffected LHON mutation carriers were homozygous for the *AR* $(CAG)_n$ repeat (n=42; 21% in total), as were 17 (18%) of the healthy controls. These were thus excluded from further analysis. For the remaining 150 heterozygotes, there was no significant difference in the distribution of X-inactivation ratios between clinically affected women and unaffected mtDNA mutation carriers (Figure 1, p=0.308). There was also no significant difference in the frequency of different degrees of skewed X-inactivation between affected and unaffected women within LHON families with 70:30, 80:20, and 95:5 X-inactivation ratios (Table 1). Sub-classification into LHON pedigrees with a high clinical penetrance (14484T>C and 11778G>A pedigrees belonging to mtDNA haplogroup J) versus those with a low clinical penetrance (all other 14484T>C and 11778G>A families) also did not reveal a significant difference in the frequency of skewed X-inactivation between affected women and unaffected mutation carriers (Table 2). There was no significant difference in the distribution or frequency of different X-inactivation ratios between different in LHON mutation carriers and controls (Figure 1; p=0.261 for affected women versus controls; p=0.825 for unaffecteds mutation carriers versus controls; p=0.419 for affected and unaffected LHON mutation carriers versus controls).

## DISCUSSION

We found no evidence to support skewed X-inactivation in women harboring mtDNA mutations that causes LHON, whether clinically affected or unaffected by the disease. Given that the visual phenotype is strongly linked to the X-chromosome in LHON pedigrees, could this result be a technical artifact? One possible explanation is incomplete digestion with *HpaII* or *Hha1*. We think this is unlikely for two reasons: (1) we included a known skewed sample and found close corre-

**Table 3. Frequency of different degrees of X-skewing in 96 control subjects in this study and 415 published phenotypically normal women**

| X-inactivation ratio | Control subjects from this study | Published data [26] | p |
|---|---|---|---|
| <20:80 or >80:20 | 12 | 60 | 0.73 |
| <10:90 or >10:90 | 6 | 15 | 0.13 |
| <5:95 or >9:95 | 3 | 7 | 0.06 |
| Total | 79 | 415 | |

There was no significant difference between the different groups.

**Table 2. Comparison of X-inactivation ratios in high and low penetrance Leber hereditary optic neuropathy families harboring the 14484T>C and 11778G>A Leber hereditary optic neuropathy mtDNA mutations.**

| | High penetrance | | | Low penetrance | | |
|---|---|---|---|---|---|---|
| | 14484T>C and 1778G>A belonging to mtDNA haplogroup J | | | 14484T>C and 1778G>A not belonging to mtDNA haplogroup J | | |
| X-inactivation ratio | Affected | Unaffected | p | Affected | Unaffected | p |
| <30:70 or >70:30 | 2 | 11 | 0.71 | 12 | 28 | 0.24 |
| <20:80 or >80:20 | 2 | 10 | 1.0 | 7 | 17 | 0.31 |
| <10:90 or >10:90 | 0 | 7 | 0.32 | 4 | 6 | 0.18 |
| <5:95 or >9:95 | 0 | 2 | 1.0 | 0 | 4 | 0.57 |
| Total | 10 | 38 | | 24 | 78 | |

Frequency of different degrees of X-skewing in high and low penetrance Leber hereditary optic neuropathy (LHON) families harboring the 14484T>C and 11778G>A LHON mtDNA mutations. There was no significant difference between the different groups.

spondence (within 5%) to data obtained in another laboratory; and (2) our control data is similar to other published series, including 415 healthy controls (Table 3) [26].

Although the results of the methylation assay do correlate with gene expression levels [16]. X-inactivation ratios can vary from tissue to tissue, raising the possibility that some affected women with LHON have skewed X-inactivation in the retinal ganglion cells but not in peripheral blood leukocytes. However, major variation is uncommon, and was not the case in two women harboring different LHON mtDNA mutations described in the literature [24]. Finally, the range of X-inactivation ratios is greater on older subjects [26], raising a further confounding variable. However, there were no significant differences in the mean age of affected (mean=56.26 years SD 15.60) and unaffected (mean=56.53, SD=19.50) LHON mutation carriers in this study, excluding age as an explanation for the absence of skewing in this study. Although our study does not exclude the possibility of subtle skewed X-inactivation, particularly given that segregation analysis implicates only skewing in 60% of females [9], the strikingly similar frequency distributions between affected women, unaffected women and controls (Figure 1), makes this unlikely.

The absence of skewed X-inactivation cannot be taken as evidence against the role of the X-chromosome in LHON because skewed X-inactivation in blood is not seen in a number of well-established X-linked diseases [27-29], and disease severity does not always correlate with the degree of skewing [30]. It is also intriguing that the number of *AR*-$(CAG)_n$ homozygotes in this study was greater in affected than unaffected LHON mutation carriers (p=0.001). This provides further independent evidence to support linkage of visual failure in LHON families to a recessive X-chromosomal locus [13], which includes the *AR* gene, and probably explains why the frequency of homozygotes in this study was greater than previously published values (cf. about 8% in 1284 healthy females in one study [26]). It remains to be established whether the androgen receptor itself is important in LHON, or it is simply in linkage disequilibrium with the visual loss susceptibility gene.

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