Report

Identification of an X-Chromosomal Locus and Haplotype Modulating the Phenotype of a Mitochondrial DNA Disorder

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Mitochondrial DNA (mtDNA) mutations are a major cause of human disease. A large number of different molecular defects ultimately compromise oxidative phosphorylation, but it is not clear why the same biochemical defect can cause diverse clinical phenotypes. There is emerging evidence that nuclear genes modulate the phenotype of primary mtDNA disorders. Here, we define an X-chromosomal haplotype that interacts with specific *MTND* mutations to cause visual failure in the most common mtDNA disease, Leber hereditary optic neuropathy. This effect is independent of the mtDNA genetic background and explains the variable penetrance and sex bias that characterizes this disorder.

Leber hereditary optic neuropathy (LHON [MIM 535000]) typically presents in young adult life with sequential bilateral visual failure due to focal degeneration of retinal ganglion cells within the optic nerve (Nikoskelainen et al. 1996; Howell 1997). In 95% of cases worldwide, LHON is due to one of three point mutations of mtDNA in genes that code for complex I of the respiratory chain: 3460G→A in *MTND1*, 11778G→A in *MTND4*, and 14484T→C in *MTND6* (Mackey et al. 1996). Only ~50% of male and ~10% of female mutation carriers develop symptoms, which indicates that additional genetic or environmental factors are required for the phenotypic expression of LHON (Newman 2002). Epidemiological studies have failed to substantiate anecdotal reports of a link with excess alcohol and tobacco (Kerrison et al. 2000), but segregation analyses

are consistent with a nuclear-encoded X-linked susceptibility allele in some pedigrees (Bu and Rotter 1991; Nakamura et al. 1993). Early attempts to map the locus were inconclusive (Chen et al. 1989; Vilkki et al. 1991; Carvalho et al. 1992), but these studies used widely spaced, often noninformative markers and failed to account for mtDNA heteroplasmy, which can influence the expression of LHON. Later studies were more comprehensive, but exclusion mapping was based on an X-linked recessive model, which cannot explain the segregation pattern in all pedigrees (Mackey 1993), and study size limited the power to exclude a substantial portion of the X chromosome (Juvonen et al. 1993; Chalmers et al. 1996).

LHON mutations are found in ~1 in 8,500 individuals in the general population and are inherited through the maternal line (Man et al. 2003). Large multigenerational pedigrees with LHON are well recognized, with affected individuals present in ≥10 generations. It is therefore unlikely that a nuclear modifier locus would be strictly coinherited with the primary mtDNA mutation throughout the whole pedigree. Given the relative rarity of the primary mtDNA mutation, it is far more likely that the nuclear modifier is common in the general popula-

tion and moves in and out of the maternal pedigree through random mating between mothers who transmit the LHON mtDNA mutation (who largely remain unaffected) and unrelated male partners not harboring the LHON mtDNA mutation. The nuclear modifier is therefore likely to be one or more ancient genetic variants that may be present at a high frequency in the population. We therefore used a nonparametric complex-disease-mapping strategy to identify the locus.

A total of 389 DNA samples were collected from affected and unaffected individuals from 100 families with LHON (6 families from Finland, 23 from the United Kingdom, 54 from Italy, 15 from Hungary, 1 from Spain, and 1 from Slovenia). The clinical phenotype was determined by local ophthalmologists. Affected individuals had the characteristic phenotype of LHON, presenting with acute or subacute painless sequential or bilateral visual failure in the presence of an established pathogenic mtDNA mutation, with no other inflammatory, metabolic, or structural cause identified. Unaffected individuals had no visual symptoms and were older than the median age at onset for LHON (24 years). The diagnosis in affected individuals was confirmed by direct sequencing of the *MTND* genes or by PCR-RFLP analysis. The percentage with heteroplasmy was determined by fluorescent primer-extension assay (Man et al. 2003). Only samples with >70% mutated mtDNA were included in the linkage study, because this level of mutated mtDNA is associated with the same penetrance as homoplasmic mutated mtDNA (Chinnery et al. 2001*a*). Subjects were homoplasmic for the following primary LHON mtDNA mutations: 11778G→A ($n = 290$ subjects; 51% male), 14484T→C ($n = 18$; 61% male), 3460A→G ($n = 75$; 49% male), and 14495A→G ($n = 6$; 50% male).

Linkage disequilibrium (LD) blocks in young, geographically isolated populations are generally larger than average, which facilitates low-density linkage mapping of complex traits (Wright et al. 1999). We therefore initially performed nonparametric linkage (NPL) analysis in six Finnish families. X-chromosome microsatellite analysis was performed using part of the 9-cM Weber human genome screening set (version 10aRG [Invitrogen]) in accordance with the manufacturer's conditions, and markers were sized using a Beckman Coulter CEQ 8000 fluorescent DNA analyzer. NPL analysis was performed using Genehunter, version 1.3. Markers between *DXS9896* and *DXS9893* had multipoint NPL scores >2, identifying a region with a maximal NPL score of 10.2 (*DXS1068* in fig. 1*a*). The region was mapped further with 12 additional markers identified from the UniSTS database, synthesized with a forward fluorescent tag (Proligo), and sized using the same method. Fine mapping confirmed our findings, with an NPL score >45 spanning 1 cM for markers *DXS8090, DXS8376,* and *DXS7487* (fig. 1*b*).

The X-linked reconstruction-combination transmission/disequilibrium test (XRC-TDT) (Horvath et al. 2000) with the use of informative markers confirmed that 2.4 Mb of the X chromosome flanked by *DXS8090* and *DXS8016* was being preferentially transmitted to affected male offspring in the Finnish families (fig. 1*c*). We then turned to two independent European cohorts with LHON. In 30 trios from the European mainland, two markers (*DXS1219* and *DXS8090*) showed significant transmission disequilibrium in the XRC-TDT, and, in 30 British trios, *DXS1069* showed significant transmission disequilibrium in the XRC-TDT, confirming linkage and association with the same region of the X chromosome (fig. 1*c*). Differences in the strength of the statistical association between adjacent markers in the different populations is likely a reflection of different marker allele frequencies in the different subgroups.
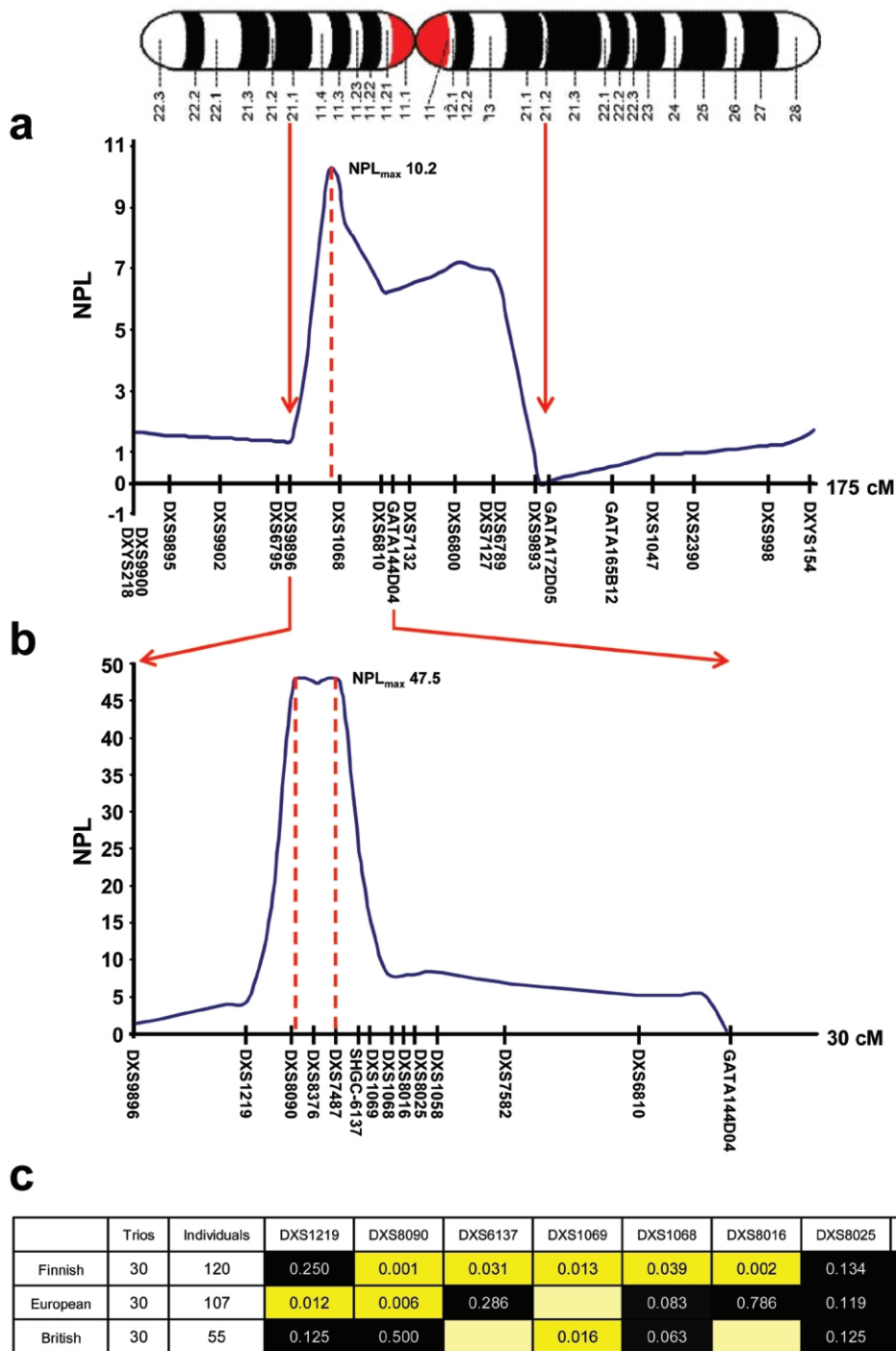
Table 1

Frequency of the *DXS8090*(166)-*DXS1068*(258) Haplotype in European Males with Different Primary LHON mtDNA Mutations (British and Mainland European Data Combined)

| LHON mtDNA Mutation and X-Chromosomal Haplotype | No. of Males | | P^a | OR (95% CI)[b] |
	Unaffected	Affected		
3460A→G:				
Non *DXS8090*(166)-*DXS1068*(258)	7	8	.66	1.75 (.24–12.64)
DXS8090(166)-*DXS1068*(258)	2	4		
Non 3460A→G:				
Non *DXS8090*(166)-*DXS1068*(258)	47	44	<.0001	35.25 (4.62–268.80)
DXS8090(166)-*DXS1068*(258)	1	33		

NOTE.—14495A→G has been described in two families with LHON, and, like 14484T→C, it affects the *MTND6* gene and is associated with a good prognosis (Chinnery et al. 2001*b*). The single 14495A→G family was pooled with the other non-3460A→G LHON pedigrees, because of the limited number of 14484T→C and 14495A→G pedigrees. Although it is possible that limited power explains the lack of association between *DXS8090*(166)-*DXS1068*(258) and visual failure in 3460A→G males, the increased penetrance of LHON in 3460A→G families (in this study, 62% of males and 29% of females are affected) suggests that a nuclear modifier locus is less influential in this subgroup.

[a] Fisher's exact *P*.
[b] Corresponding to the risk of visual failure.

Figure panels a and b (chromosome map and NPL score plots) and panel c table:

c

	Trios	Individuals	DXS1219	DXS8090	DXS6137	DXS1069	DXS1068	DXS8016	DXS8025	DXS1058
Finnish	30	120	0.250	0.001	0.031	0.013	0.039	0.002	0.134	1.000
European	30	107	0.012	0.006	0.286		0.083	0.786	0.119	1.000
British	30	55	0.125	0.500		0.016	0.063		0.125	1.000

Figure 1 Mapping of the nuclear modifier locus in Finnish pedigrees transmitting homoplasmic LHON mtDNA mutations. *a,* Human X chromosome with the corresponding NPL scores for the initial linkage screen (maximum NPL score = 10.2). *b,* Fine mapping of the linked locus, confirming earlier findings and revealing a maximum NPL score of 47.5. *c,* XRC-TDT for informative markers in the Finnish, mainland European, and British cohorts. As expected, the Finnish families show a large block of linkage and association corresponding to the linked region. There is significant transmission distortion for specific markers at the same locus for the British and mainland European cohorts, which confirms our initial findings and demonstrates the smaller block of LD present outside Finland. A black background indicates that the association is nonsignificant; a yellow background indicates that it is significant at the .05 level; and a yellow box without a value inside it indicates a marker that is uninformative when the number of alleles is <4.

Table 2

Logistic Regression Model of the Additive Effects of mtDNA Haplotype and the *DXS8090*(166)-*DXS1068*(258) Haplotype in Males Homoplasmic for 11778A→G, 14484T→C, and 14495A→G

Variables in the Equation	P	OR (95% CI)
Non mtDNA haplogroup J	.043	2.45 (1.03–5.82)
DXS8090(166)-*DXS1068*(258)	.001	33.84 (4.38–261.53)
Constant	<.01	.02

NOTE.—As expected, the frequency of non-3460A→G haplogroup J pedigrees (23%) was greater than the European average (found to be 9%–14% in the European populations studied here). The effect of each genetic variable was approximately the same when modeled independently as when both variables were included in the same regression model. Modeling did not identify a significant direct interaction between mtDNA haplotype and *DXS8090*(166)-*DXS1068*(258). χ^2 was greatest for the additive model ($\chi^2 = 35.68$; $P < .0001$). Together, these findings indicate that each genetic factor that exerts its effect is independent and additive. Logistic regression showed an increased risk of visual failure in non–haplogroup J pedigrees in this cohort. A formal study of the penetrance of LHON in haplogroup J pedigrees versus non-J pedigrees had not been performed previously.

By comparing microsatellite allele frequencies in 146 European men, we identified the major high-risk haplotype defined by *DXS8090* and *DXS1068*. Of the 89 men affected with LHON, 37 (42%) had the *DXS8090*(166)-*DXS1068*(258) haplotype, compared with only 3 (5%) of 57 unaffected men (Fisher's exact $P = .0001$; odds ratio [OR] 12.81; 95% CI 3.72–44.11).

The 3460A→G mutation is considered to be the most severe molecular defect, with a consistently reduced complex I activity (Howell et al. 1991), a less marked sex bias (Newman et al. 1991; Johns et al. 1993), and no association with the background mtDNA haplotype (Man et al. 2004). We therefore thought that a nuclear-mitochondrial interaction would be more prominent in non-3460A→G pedigrees. In keeping with this, there was no significant increase in the risk of visual impairment in 3460A→G males with the *DXS8090*(166)-*DXS1068*(258) haplotype, but non-3460A→G males were >35-fold more likely to develop visual failure if they had the *DXS8090*(166)-*DXS1068*(258) haplotype (table 1). In contrast to 3460A→G, both 11778G→A and 14484T→C are strongly associated with mtDNA haplogroup J (Man et al. 2004). We therefore used logistic regression to determine whether the *DXS8090*(166)-*DXS1068*(258) haplotype exerted its effect independent of haplogroup J status, modeling the effect of the nuclear or mtDNA haplotypes alone and through additive, synergistic, and antagonistic interactions. An additive interaction between *DXS8090*(166)-*DXS1068*(258) and mtDNA haplogroup was the most compelling model (tables 2 and 3). Non-3460A→G males who did not harbor *DXS8090*(166)-*DXS1068*(258) and who

belonged to mtDNA haplogroup J had a 35.1% risk of visual failure. The presence of *DXS8090*(166)-*DXS1068*(258) *or* not belonging to mtDNA haplogroup J was associated with a 58.9% risk of visual failure, but 100% of individuals who were *both* non-mtDNA haplogroup J *and* had *DXS8090*(166)-*DXS1068*(258) were visually impaired ($P < .0001$). The effect of the nuclear haplotype (OR 33.84; 95% CI 4.38–261.53) was >10-fold greater than that of the mtDNA haplotype (OR 2.45; 95% CI 1.03–5.82).

To confirm our observations in males, we turned our attention to non-3460A→G female subjects. Of 107 females, 20 (19.05%) were heterozygous for *DXS8090*(166)-*DXS1068*(258). Given that LHON was 97% penetrant in non-3460A→G males with LHON who were hemizygous for *DXS8090*(166)-*DXS1068*(258), we predicted that approximately four females would be homozygous for *DXS8090*(166)-*DXS1068*(258) and that most of them would also be visually impaired. Five women were homozygous, four of whom were also visually impaired, which supports a recessive haploinsufficiency model. Molecular testing of unaffected maternal relatives in known homoplasmic pedigrees with LHON has been of limited clinical use to date, confirming the expected inheritance of the mutation but not altering well-established recurrence risks. Here, we show that identification of *DXS8090*(166)-*DXS1068*(258) in hemizygous males or homozygous females greatly increases the chance of visual failure occuring during life. This may be of use in prenatal diagnosis and predictive testing.

We also identified a second, less-frequent predisposing haplotype in Europeans, *DXS1068*(258)-*DXS8016*(218), present in 14.7% of males in this study. All males harboring this haplotype were affected (Fisher's exact $P < .0001$), and the majority (74%) of these individuals also had *DXS8090*(166), indicating that this is a subgroup of the common disease haplotype. It is possible that diallelic mutation of microsatellites and recombination have generated other, less-frequent high-risk marker haplotypes that remain undetected in this study, contributing to marker heterogeneity and confounding attempts to map the locus. We therefore performed homozygosity mapping with the 10K SNP microarray (Affymetrix) for four unrelated women with visual failure due to the 11778A→G mutation who had more than three male

Table 3

Logistic Regression Models of the Effects of mtDNA Haplotype and the *DXS8090*(166)-*DXS1068*(258) Haplotype in Males Homoplasmic for 11778A→G, 14484T→C, and 14495A→G

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

offspring, all of whom were clinically affected. All four women were homozygous for SNPs adjacent to the critical region, which pointed toward a small, common shared haplotype block (fig. 2). On the other hand, the striking difference between the Finns and the other European subjects points to allelic heterogeneity at the nuclear visual-loss susceptibility locus, and not just at the flanking markers. Of the affected Finnish men, 63% had a high-risk haplotype defined by *DXS8090*(168)-*DXS1069*(262) ($P = .0259$; OR 7.71; 95% CI 1.28–46.37), found in only 3.7% of mainland Europeans in this study. Different nuclear alleles may behave in subtly different ways that are specific to particular LHON mtDNA mutations or geographic/ethnic regions.

Epigenetic mechanisms provide an alternative explanation for the reduced penetrance and sex bias in LHON. Although two studies failed to find skewed X inactivation in the blood (Pegoraro et al. 1996) and ocular tissue (Pegoraro et al. 2003) from women affected with LHON, many imprinted disease genes do not show X skewing (Renieri et al. 2003). In families with no known disease, the failure to fully correct a maternal imprint leads to the preferential transmission of grandpaternal chromosomes to male offspring (Naumova et al. 1998). The inheritance of a partially silenced grandpaternal gene could explain the characteristic penetrance of primary LHON mutations in males and could also explain the further reduced penetrance in women when it is superimposed on the normal female random inactivation pattern. X-chromosomal imprinting has been implicated in a number of common neurodevelopmental diseases (Falls et al. 1999), resulting in "parent of origin" effects. We therefore manually reconstructed the 3-generation X-chromosomal haplotypes in 52 males with homoplasmic LHON mtDNA mutations. Overall, we observed the same distortion of transmission of grandpaternal alleles that is well described in control populations (proportion of grandpaternal alleles = 0.60; 95% CI 0.45–0.73; $P = .87$, for comparison with control data of Naumova et al. [1998]). There was no difference in the frequency of grandpaternal alleles between affected and unaffected male offspring (table 4) nor between affected and unaffected female offspring, which suggests that imprinting is not the principal mechanism underpinning the phenotypic variability seen in LHON.

In conclusion, we have identified a region of the X

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Figure 2   Homozygosity mapping with the 10K SNP Affymetrix microarray for four unrelated women with visual failure due to a homoplasmic LHON mutation. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 4

Grandparental Origin of X-Chromosomal Markers in Males from 3-Generation Pedigrees with Homoplasmic LHON mtDNA Mutations

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The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

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chromosome containing a high-risk haplotype that explains the reduced penetrance and sex bias that characterizes LHON in the majority of pedigrees. Clarification of the underlying sequence variation is likely to have broader implications for our understanding of nuclear-mitochondrial interactions in health and disease, opening new avenues for therapeutic intervention.

Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for LHON)
UniSTS Database, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists

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