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Evaluation of the X-linked modifier loci for Leber hereditary optic neuropathy with the G11778A mutation in Chinese

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Purpose: To test the association of the X-chromosome regions (Xp21.1–q21.2 and Xq25–27.2) with Leber hereditary optic neuropathy (LHON) in Chinese patients.
Methods: One hundred and seventy-five male LHON patients with the G11778A mutation and 100 unrelated normal males participated. Twelve microsatellite markers and four single-nucleotide polymorphisms (SNPs) were genotyped for patients and controls. A χ^2 or Fisher's exact test was used to compare the frequencies of genotypes as well as haplotypes in the two groups.
Results: Significant differences between patients and controls were found in two isolated microsatellite markers (DXS6803: χ^2=37.17, p=2.45×10^{-5}; DXS984: χ^2=33.88, p=1.66×10^{-6}) based on genotype frequencies. However, no significant differences for genotype and haplotype frequencies were found in the other 14 markers located in the two reported regions of Xp21.1–q21.2 and Xq25–27.2.
Conclusions: Our results provide suggestive evidence of X-linked modifiers on the expression of LHON. Further studies are needed to identify the exact nuclear genes that might affect LHON expression.

Leber hereditary optic neuropathy (LHON, OMIM 535000) is one of the best studied mitochondrial genetic diseases. The prevalence of LHON is about 1 in 8,500 individuals in the general population of North East England [1]. The majority of LHON cases are caused by three common mitochondrial DNA (mtDNA) mutations, G11778A in the *ND4* gene [2], T14484C in the *ND6* gene [3,4], and G3460A in the *ND1* gene [5,6]. The distribution patterns of these three primary mutations differ remarkably among populations of Europe and East Asia [7,8] and about 90% of LHON cases among Chinese are associated with the G11778A mutation [8].

Only about one third of carriers of the three common mutations will develop LHON, and male carriers have a much higher risk of developing the disease than females. The incomplete penetrance and sex bias of LHON are not well explained by primary mtDNA mutations alone, suggesting that environmental [9-11] or additional genetic factors may contribute to the expression of LHON. Beyond primary mtDNA mutations, other genetic factors that might affect the clinical expression of LHON include additional mtDNA mutations [12], heteroplasmy [13,14], mtDNA haplogroup [7,15-19], and potential nuclear genes such as X-chromosome modified loci [20]. In European families, clear evidence demonstrates that the risk of visual failure is higher when G11778A or T14484C mutations are present in haplogroup J and when G3460A is present in haplogroup K, but is lower when G11778A exists in haplogroup H [7]. The effect of haplogroup J was narrowed to subclades J1c and J2b [19]. Our previous study showed that haplogroup M7b1'2 could increase the risk of visual failure and that M8a might have a protective effect in Chinese families with LHON, which (results of M7b12 and M8a) differ from those found among Europeans [21,22]. However, the effect of mtDNA haplogroups could only partly explain the different penetrance among different families. It could also not explain different penetrance within the same family where all maternal offspring have the same mutation under the same mtDNA background, yet some individuals develop the disease while others do not, and male family members are more likely than females to have the disease.

Previous segregation analysis found that some pedigrees are consistent with an X-linked susceptibility allele [23,24], leading to efforts to map and identify the suspected X-linked modified gene. However, linkage analysis of X-chromosome markers resulted in a series of inconsistent results [25-27]. Recently, Hudson et al. suggested that nuclear modifiers might be more common in the general population than the relatively rare primary mtDNA mutations [28]. Using a nonparametric complex-disease-mapping strategy, they identified an X-chromosomal haplotype DXS8090 (166)/ DXS1068 (258) in the Xp21.1–q21.2 region as a risk factor in Europeans, which is independent of the mtDNA background and could well explain the variable penetrance and sex bias in the studied pedigrees. In a recent study, X-

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TABLE 1. PRIMERS USED TO AMPLIFY DNA FRAGMENTS ENCOMPASSING THE TWELVE MICROSATELLITE MARKERS AND THE FOUR SINGLE NUCLEAOTIDE POLYMORPHISMS.

Name	Primer sequence (5′-3′)	Length of product (bp)	Annealing temperature
DXS8090	M13 tailed-F-CGTTGTAAAACGACGGCCAGTgggtgaaattccatcacaaa	154–172	55 °C
	R-acaaatgcagatgtacaaaaaata		
rs11266282	F-ccaaagatgaccgtgag	666	60 °C
	F-ctgccaatgttctggatgt		
rs11771	F-tggggttttaggtggtga	350	56 °C
	F-aaatgcaaagggtgatgc		
DXS1069	M13 tailed-F-CGTTGTAAAACGACGGCCAGTagcctaacccacataacagc	254–268	55 °C
	R-agctactatattnaccttggtcttg		
DXS1068	F-cctctaaagcatagggtcca	245–259	55 °C
	R-cccatctgagaacacgctg		
DXS8109	M13 tailed-F-CGTTGTAAAACGACGGCCAGTacaggctcggcttattaggg	229–239	55 °C
	R-5′-ctttcagtgccaggcatagg		
rs6623918	F-5′-tctatttccttactttcccaca	436	58 °C
	R-5′-ggaccctttccgcttgat		
rs5923859	F-5′-tattgttgtaaggtgggc	379	56 °C
	R-5′-cttggcttctgctgatat		
DXS6803	M13 tailed-F-CGTTGTAAAACGACGGCCAGTgaaatgtgctttgacaggaa	110–126	55 °C
	R-5′-caaaaagggacatatgctactt-3′		
DXS1196	M13 tailed-F-CGTTGTAAAACGACGGCCAGTctaaattctcctccaccgtg	209–227	55 °C
	R-tttccagagcagattttcagt		
DXS1222	M13 tailed-F-CGTTGTAAAACGACGGCCAGTgcaaaaatccccagcc	234–240	55 °C
	R-ttcattgccatccagattc		
DXS8074	M13 tailed-F-CGTTGTAAAACGACGGCCAGTataaattagccagaggtgttg	221–231	55 °C
	R-5′-ctaggtgtgtctgtaaaggtagg-3′		
DXS1211	M13 tailed-F-CGTTGTAAAACGACGGCCAGTccctccaatctggcagaa	159–175	55 °C
	R-aagacctgggtttggcct		
DXS984	M13 tailed-F-CGTTGTAAAACGACGGCCAGTttctgtctgccaagtgttt	154–184	55 °C
	R-tactgngccctactccattc		
DXS1205	M13 tailed-F-CGTTGTAAAACGACGGCCAGTcctacgcatgtggctc	184–202	55 °C
	R-attaatggcttagagtactttttca		
DXS1227	F-agaggtccgagtcttccac	77–99	55 °C
	R-ataagggtttactcccccaa		
M13 probe	CGTTGTAAAACGACGGCCAGT	21	x

Primers used to amplify DNA fragments encompassing the twelve microsatellite markers (DXS8090, DXS1069, DXS1068, DXS8109, DXS6803, DXS1196, DXS1222, DXS8074, DXS1211, DXS984, DXS1205, and DXS1227) and the four SNPs (rs11771, rs11266282 , rs5923859, and rs6623918).

TABLE 2. ENZYME AND DIGESTION FRAGMENTS FOR RFLP ANALYSIS OF TWO SNPS

SNP	Genotype	Enzyme	Digestion fragments (bp)
rs11771	C	HindIII	350
	T		251/99
rs11266282	T	HinfI	379/191/96
	A		271/191/108/96

Note: There are only two genotypes of each SNP because two makers lies in X chromosome and two groups of samples are all male.

chromosomal linkage analysis in a large Brazilian family with the G11778A mutation on a haplogroup J background revealed a novel LHON susceptibility locus on chromosome Xq25–27.2 [29]. Considering the extreme high rate of false-positive results in genetic association studies [30-35], replication is the first priority in a genetic association study of complex traits. In addition, it is necessary to test whether this X-chromosome locus also affects the clinical expression of LHON among Chinese, although we have seen differences in mtDNA haplogroups [7,21] as well as in sex bias (the male to female ratio was 2.2:1 to 2.4:1 among Chinese [8,21] but 3.7:1 to 12.4:1 in Caucasians [36-38]).

Here, we studied the distribution of the microsatellite and SNP markers on the two reported loci and the reported high-risk haplotype [DXS8090 (166)/DXS1068 (258)] in the

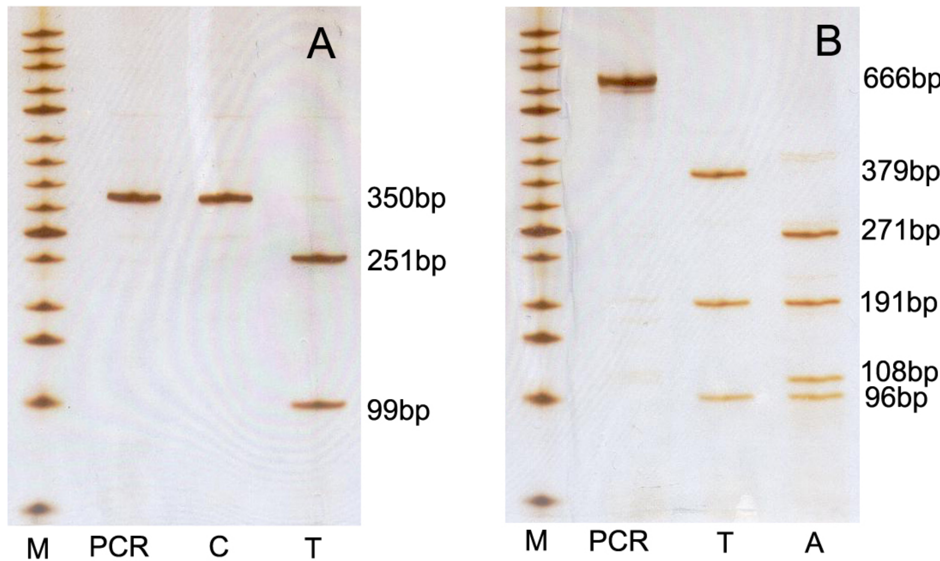

Figure 1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of rs11771 and rs11266282 in LHON patients and normal controls. **A**: The C/T genotype of rs11771 in the DYNLT3 gene was analyzed using HindIII digestion. **B**: The T/A genotype of rs11266282 in the LANCL3 gene was analyzed using HinfI digestion. M: Size marker of 50 bp DNA ladder (from bottom to top: 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 300 bp, 350 bp, 400 bp, 450 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, and 1000 bp).

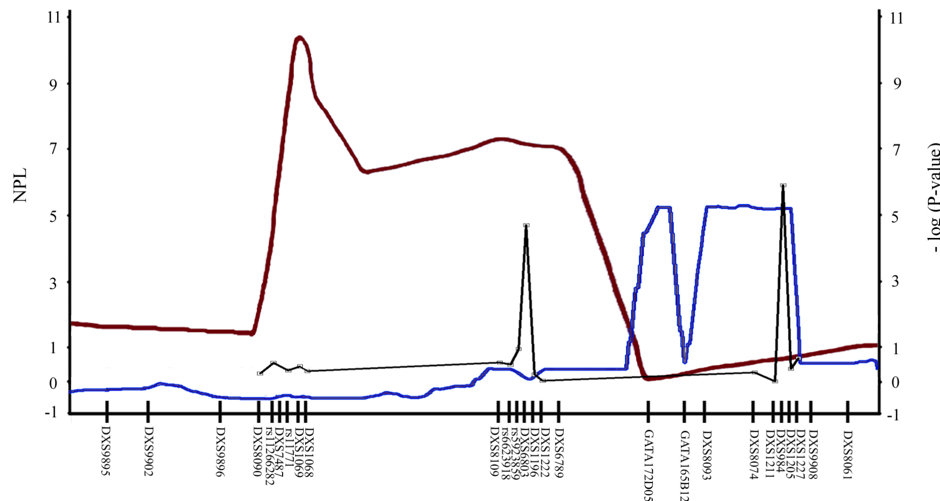

Figure 2. Ideogram of the modifier loci for LHON on the X-chromosome. Auburn and blue lines show the results of Hudson et al. [28] and Shankar et al. [29], respectively, where the nonparametric linkage score (NPL) is listed on the left vertical axis. Black line shows the results of our study, where the –log (p value) on the right vertical axis.

Xp21.1–q21.2 between Chinese with LHON and normal controls.

METHODS

Patients: One hundred and seventy-five unrelated male LHON probands with the G11778A mutation were identified from our clinic based on mutational detection of G11778A by allele-specific amplification and single-strand conformational polymorphism analysis as previously described [8,21]. In addition, one hundred unrelated normal males (age, gender, and birth-place matched) participated. Of the 175 LHON patients, 55 had a family history of LHON. All participating individuals were from the central and southeast region of China. Informed consent was obtained from participants before the study, conforming to the tenets of the Declaration of Helsinki and following the Guidance for Sample Collection of Human Genetic Disease (National 863-Plan) by the Ministry of Public Health of China. This study was approved by the Institute Review Board of the Zhongshan Ophthalmic Center. Genomic DNA was prepared from venous leukocytes.

Genotyping of microsatellite markers: We genotyped twelve microsatellite markers, including seven microsatellite markers (DXS8090, DXS1069, DXS1068, DXS6803, DXS8109, DXS1196, and DXS1222) in the Xp21.1–q21.2 region and five microsatellite markers (DXS8074, DXS1211, DXS984, DXS1205, and DXS1227) in the Xq25–27.2 region. Genotyping primers for DXS1068 and DXS1227 (Table 1) were from Panel 28 of the ABI Linkage Mapping Set v2.5 (Applied Biosystems, Foster City, CA). An M13-tailed primer PCR method [39] was used to genotype the other ten microsatellite markers where a 5′6-FAM labeled M13 probe was used (Table 1). The reaction mixture was composed of 0.5 µl reverse primer (10 µM), 0.125 µl M13-tailed forward primer (10 µM), 0.375 µl 5′6-FAM labeled M13 probe

Table 3. The genotypes distribution of twelve microsatellite markers between the LHON patients and normal controls.

Genotype	Length (bp)	LHON (n=175)	Normal controls (n=100)	χ2 value	p value
DXS8090	152	1	0	7.826	0.55
	154	5	4		
	156	1	1		
	158	10	2		
	160	21	8		
	162	43	32		
	164	74	47		
	166	14	5		
	168	5	1		
	170	1	0		
DXS1069	253	2	1	4.3	0.335
	255	30	24		
	257	137	73		
	257	4	0		
	263	2	2		
DXS1068	251	2	1	6.458	0.472
	253	64	28		
	255	8	3		
	257	21	14		
	259	63	47		
	261	10	4		
	263	0	1		
	265	1	0		
DXS8109	221	1	0	9.247	0.262
	225	0	1		
	227	3	3		
	229	3	0		
	231	13	15		
	233	133	68		
	235	15	9		
	237	5	4		
	241	2	0		
DXS6803	106	2	3	37.174	2.45×10–5
	108	1	0		
	110	28	12		
	112	11	9		
	114	31	11		
	116	44	48		
	118	47	5		
	120	1	0		
	122	10	10		
	126	0	1		
DXS1196	204	1	0	10.26	0.591
	206	2	0		
	208	53	23		

Table 3. Continued.

Genotype	Length (bp)	LHON (n=175)	Normal controls (n=100)	χ2 value	p value
	210	72	46		
	212	12	13		
	214	15	6		
	216	3	2		
	218	1	0		
	220	6	2		
	222	4	3		
	224	2	3		
	226	3	0		
	228	1	1		
DXS1222	227	0	1	3.733	0.897
	229	2	2		
	231	29	16		
	233	102	60		
	235	26	12		
	237	14	9		
	239	1	0		
	241	1	0		
DXS8074	220	143	88	3.393	0.51
	222	3	0		
	224	1	0		
	226	27	11		
	228	1	1		
DXS1211	157	48	32	3.662	0.925
	159	1	0		
	161	45	24		
	163	27	10		
	165	1	1		
	167	7	3		
	169	8	5		
	171	32	22		
	173	6	3		
DXS984	161	1	0	33.879	1.659×10^{-6}
	163	1	1		
	165	40	11		
	167	98	76		
	169	30	2		
	171	0	2		
	173	1	1		
	175	2	6		
	179	2	0		
DXS1205	179	3	1	12.365	0.402
	181	4	3		
	183	18	7		
	185	3	2		
	187	6	4		
	189	28	10		

Table 3. Continued.

Genotype	Length (bp)	LHON (n=175)	Normal controls (n=100)	χ2 value	p value
	185	3	2		
	187	6	4		
	189	28	10		
	191	60	48		
	193	26	7		
	195	2	4		
	197	5	3		
	199	12	7		
	201	2	2		
	203	3	2		
DXS1227	77	1	0	10.058	0.196
	79	4	1		
	83	108	59		
	85	33	15		
	87	2	2		
	89	2	0		
	91	19	21		
	95	6	1		
	97	0	1		

Note: The Fisher' s exact test was used to compare the frequency of the twelve microsatellites between the two groups.

Table 4. The genotype distribution of the four SNPs between the LHON patients and controls

SNP	Genotype				χ2	P
	LHON (n=175)		Controls (n=100)			
rs11266282	A (117)	T (53)	A (61)	T (37)	1.206	0.272
rs11771	C (133)	T (42)	C (80)	T (20)	0.583	0.445
rs6623918	G (150)	A (25)	G (81)	A (19)	1.052	0.305
rs5923859	A (142)	G (32)	A (89)	G (11)	2.622	0.105

(TaKaRa Biotechnology, Dalian, China; 10 µM), 2 µl Template DNA (40 ng/µl), 0.2 µl rTaq polymerase (5 U/µl), 0.8 µl dNTP (2.5 mM each), and ddH_2O to a total volume of 10 µl. PCR amplification was performed for the initial denaturation at 94 °C for 8 min, followed by 10 cycles of amplification at 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, an additional 20 cycles of amplification at 89 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min.

Fluorescence-labeled PCR products were separated by capillary electrophoresis using an ABI 3100 genetic analyzer. The lengths of the PCR products were calculated using GeneScan™ 400HD size standards and analyzed using Genemapper software (Applied Biosystems). For the ten microsatellite markers using the M13-tailed primer PCR method, the length of fragments was adjusted (the real length being 21 bp shorter due to the addition of a 21 bp M13-tailed probe on the forward primer).

Genotyping of single nucleotide polymorphisms: Four SNPs were genotyped. Of the four, rs11771 and rs11266282 in the Xp21.1–q21.2 region were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis, where the amplicons were digested by the restriction endonucleases HindIII and HinfI (TaKaRa Biotechnology), respectively (Table 2). The digested products were separated by 10% PAGE (PAGE; Figure 1). The other two SNPs (rs6623918 and rs5923859) in the Xp21.1–q21.2 region were genotyped by cycle sequencing. The primers used to amplify the fragments harboring these four SNPs are listed in Table 1.

Statistical analysis: Distributions of the genotype and haplogroup frequencies of the sixteen markers in the Xp21.1–q21.2 and Xq25–27.2 regions were compared between patients and controls using the chi-square or Fisher's exact

Table 5. The distribution of DXS8090-DXS1068 haplotype between the LHON patients and normal controls

Number	DXS8090-DXS1068	LHON (n=175)	Controls (n=100)
1	164 251	1 (1%)	1 (1%)
2	154 253	2 (1%)	1 (1%)
3	160 253	8 (5%)	2 (2%)
4	162 253	19 (11%)	14 (14%)
5	164 253	23 (13%)	10 (10%)
6	166 253	6 (3%)	1 (1%)
7	160 255	2 (1%)	1 (1%)
8	162 255	3 (2%)	1 (1%)
9	164 255	2 (1%)	1 (1%)
10	154 257	1 (1%)	1 (1%)
11	160 257	1 (1%)	1 (1%)
12	162 257	8 (5%)	4 (4%)
13	164 257	7 (4%)	7 (7%)
14	158 259	4 (2%)	1 (1%)
15	160 259	6 (3%)	4 (4%)
16	162 259	8 (5%)	10 (10%)
17	164 259	34 (19%)	25 (25%)
18	166 259	6 (3%)	4 (4%)
19	168 259	2 (1%)	1 (1%)
20	162 261	1 (1%)	2 (2%)
21	164 261	5 (3%)	1 (1%)
22	other haplotypes	26 (15%)	7 (7%)

Note: The $\chi2$ value of Fisher's exact test was 12.468 and p value was 0.931.

test (SPSS13.0, Chicago, IL). The haplotypes of the two reported markers (DXS8090 and DXS1068) were constructed using PHASE software. A p value of 0.05 or less was regarded as statistically significant, based on previous reports [28].

RESULTS

Twelve microsatellite markers and four SNPs were successfully genotyped except for a few samples (which failed to generate amplicons after several attempts). The locations of the analyzed markers on the X-chromosome are shown in Figure 2. The genotyping results for the twelve microsatellite markers are listed in Table 3 and for the four SNPs in Table 4. Two of the sixteen markers yielded significant differences between cases and controls, namely DXS6803 (χ^2=37.17, p=2.45×10^{-5}) and DXS984 (χ^2=33.88, p=1.66×10^{-6}). No statistically significant difference was found in the distribution of genotyping frequencies for the other fourteen markers between LHON patients and controls (Table 3, Figure 2).

Haplotypes of the reported markers DXS8090/DXS1068 were constructed using PHASE software (Table 5). There was no statistically significant difference in the distributions of these reported haplotypes between LHON patients and controls.

DISCUSSION

Several studies have shown that the incomplete penetrance and sex bias of LHON are associated with nuclear modifier genes on the X-chromosome. Recently, DXS8090 (166)/ DXS1068 (258) haplotypes in the Xp21.1–q21.2 region were shown to modulate the clinical expression of LHON in European patients [28]. This effect is independent of the mtDNA genetic background and could explain the variable penetrance and sex bias well in these pedigrees. Our results failed to confirm any DXS8090/DXS1068 haplotype with LHON expression among Chinese, but did find a significant difference in a nearby marker (DXS6803: χ^2=37.17, p=2.45×10^{-5}) in the Xp21.1–q21.2 region. This marker is located in the broader linkage region but not in the highly significant fine mapping region reported by Hudson et al. [28]. In addition, our study design of case–control series is different from that of Hudson et al. [28] whose controls were unaffected family members, which may partly explain our discrepant findings. However, a common locus may be detected by either strategy unless it is ethnic-specific.

In a recent study, X-chromosomal linkage analysis in a large Brazilian family with a G11778A mutation on a haplogroup J background revealed a novel LHON susceptibility locus on chromosome Xq25–27.2 [29]. We genotyped five microsatellite markers (DXS8074, DXS1211,

DXS984, DXS1205, and DXS1227) in the Xq25–27.2 region. Our results showed that DXS984 differed significantly (χ^2=33.88, p=1.66×10^{-6}) between LHON patients and controls, supporting a possible modifier locus in this region. These results need to be confirmed by additional studies, as two other nearby markers (DXS1211 and DXS1205) did not support the association.

Significant association for isolated markers is not uncommon and has been reported even in a genome-wide association study [40]. Replication and confirmation remains a challenge in association studies. Considering that most genetic risk factors (about 95%) reported for many other complex traits have been false positives [30-33], we must interpret our results with caution at this stage. Further linkage and genome-wide association studies on Chinese families with LHON are essential to provide additional information about the X-linked modifier gene in the Chinese population.

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