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 χ2 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: χ2=37.17, p=2.45×10−5; DXS984: χ2=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-
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

### Table 1. Primers used to amplify DNA fragments encompassing the twelve microsatellite markers and the four single nucleotide polymorphisms.

| Name       | Primer sequence (5'→3')                                                                 | Length of product (bp) | Annealing temperature |
|------------|-----------------------------------------------------------------------------------------|------------------------|----------------------|
| DXS8090    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 154–172                | 55 °C                |
| rs11266282 | F-cgaagatgcgtgaggg F-ttgcaatctcgcgtaga                                               | 666                    | 60 °C                |
| rs11771    | F-ccacagt gacgggg F-ttgcaatctcgcgtaga                                               | 350                    | 56 °C                |
| DXS1069    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 254–268                | 55 °C                |
| DXS1068    | F-cctctaaacaggtggtgtaga R-ccaccttcgagaacgctg                                         | 245–259                | 55 °C                |
| DXS8109    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 229–239                | 55 °C                |
| rs6623918  | F-ccacagt gacgggg F-ttgcaatctcgcgtaga                                               | 436                    | 58 °C                |
| rs5923859  | F-ccacagt gacgggg F-ttgcaatctcgcgtaga                                               | 379                    | 56 °C                |
| DXS6803    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 110–126                | 55 °C                |
| DXS1196    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 209–227                | 55 °C                |
| DXS1222    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 234–240                | 55 °C                |
| DXS8074    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 221–231                | 55 °C                |
| DXS1211    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 159–175                | 55 °C                |
| DXS984     | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 154–184                | 55 °C                |
| DXS1205    | M13 tailed-F-CGTTGTAACAGCGGCGAGTT gccgt gaaatctca-tcgcgtaga                        | 184–202                | 55 °C                |
| DXS1227    | F-agaggtccgtgctttctccac R-ataagggtttactccccaa                                          | 77–99                  | 55 °C                |
| M13 probe  | CGTTGTAACAGCGGCGAGTT                                                                 | 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.
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
| Genotype   | Length (bp) | LHON (n=175) | Normal controls (n=100) | $\chi^2$ 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                      |                |         |
| Genotype   | Length (bp) | LHON (n=175) | Normal controls (n=100) | $\chi^2$ value | p value |
|------------|-------------|--------------|-------------------------|---------------|--------|
| DXS1222    |             |              |                         |               |        |
| 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            |                         |               |        |
| 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           |                         |               |        |
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).

**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 | LHON (n=175) | Controls (n=100) | χ² | P     |
|---------|----------|--------------|------------------|----|-------|
| 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 ddH₂O 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 test.
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 ($\chi^2=37.17, \ p=2.45 \times 10^{-5}$) and DXS984 ($\chi^2=33.88, \ p=1.66 \times 10^{-6}$). 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.

### 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 $\chi^2$ value of Fisher's exact test was 12.468 and p value was 0.931.

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: $\chi^2=37.17, \ p=2.45 \times 10^{-5}$) 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 ($\chi^2=33.88, p=1.66 \times 10^{-6}$) 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 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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