



Screening the three LHON primary mutations in the general Chinese population by using an optimized multiplex allele-specific PCR

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ABSTRACT

Background: Leber hereditary optic neuropathy (LHON) is one of the most common mitochondrial diseases, which is mainly caused by three mitochondrial DNA (mtDNA) mutations (*m.3460G>A*, *m.11778G>A* and *m.14484T>C*). Incomplete penetrance suggests that there might be asymptomatic carriers in general populations. These asymptomatic carriers are clinically important as they are potential future patients and the female carriers could transfer the pathogenic mutations to their offspring. Thus, screening the three LHON primary mutations in general populations is important for genetic counseling.

Methods: We optimized a multiplex allele-specific PCR method based on previous studies, and the sensitivity was evaluated. The three LHON primary mutations were screened by using this MAS-PCR method in 1571 subjects from general Chinese populations that are without symptoms or family history of optic neuropathy.

Results: The optimized MAS-PCR approach can detect a heteroplasmy level at 5%, 5%, and 20% for *m.3460G>A*, *m.11778G>A* and *m.14484T>C*, respectively. None of the three LHON primary mutations was detected in the 1571 subjects.

Conclusion: The three LHON primary mutations are rare in general Chinese populations. The optimized MAS-PCR assay provides an easier, faster and more cost-effective method for detection of the three LHON primary mutations, making it practical for clinical diagnosis.

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1. Introduction

Leber hereditary optic neuropathy (LHON; MIM 535000) is a typical mitochondrial genetic disease first described by the German ophthalmologist Theodore Leber in 1871 [1]. Acute or sub-acute central visual loss is the key clinical feature, and young adult males are the main susceptible population [1–3]. Epidemiological investigations show that LHON affects approximately 1 in 31,000 in the northeast English population [4,5] and 1 in 50,000 in the Finnish population [6]. Over 95% of LHON cases are caused by one of the three primary mitochondrial DNA (mtDNA) mutations, *m.3460G>A* in the *MT-ND1* gene, *m.11778G>A* in the *MT-ND4* gene, and *m.14484T>C* in the *MT-ND6* gene [7–10]. The frequency of the three LHON primary mutations varies greatly in worldwide populations. The frequencies of *m.3460G>A*, *m.11778G>A* and *m.14484T>C* are 1.1%, 90.2% and 8.7%, respectively, in East Asian patients, and are 22.6%, 56.6% and 20.8%, respectively, in European patients [11,12].

Incomplete penetrance (in which not all primary mutation carriers develop LHON) is one of the unsolved questions concerning LHON and

suggests that there might be asymptomatic carriers in the general population. Heteroplasmy of mtDNA mutations might influence the penetrance of LHON, and previous studies have estimated that mutated alleles with a frequency higher than 60% would lead to a higher risk of visual loss [13,14]. Female carriers with a heteroplasmic mutation would possibly transmit the mutation to their children at various levels of heteroplasmy, and the risk of visual loss of their offspring may increase (depending on the extent of the transmitted heteroplasmy of the pathogenic mutation and on the nuclear genetic background) [14–16]. Thus, screening the three primary mutations of LHON in the general population is important for genetic counseling and disease prevention. Recently, Elliott et al. [17] investigated the carrier frequencies of ten mtDNA pathogenic mutations in about 3000 random samples from England: the carrier frequencies were determined to be 0.11% for each of the three primary mutations, suggesting that mtDNA pathogenic mutations are not rare in general populations [17].

Point mutations can be detected through direct sequencing or other genotyping methods like allele-specific PCR (AS-PCR), which is rapid, convenient and inexpensive. Nørby et al. had previously designed a duplex AS-PCR which can detect *m.3460G>A* and *m.11778G>A* in a single assay [18,19]. Yang et al. had developed a multiplex AS-PCR (MAS-PCR) which can detect the three LHON primary mutations

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simultaneously [20]. In this study, we optimized a MAS-PCR method based on previous studies, which can simultaneously detect the three LHON primary mutations with reasonably high sensitivity. By using this optimized MAS-PCR assay, we further screened the three LHON primary mutations in 1571 subjects from the general Chinese population.

2. Materials and methods

2.1. Samples

Genomic DNA samples extracted from peripheral blood of 6 LHON patients (Le1027 and Le1037 with *m.3460G>A* [21], Le35 and Le924 with *m.11778G>A* [22], Le671 and Le1123 with *m.14484T>C* [these two patients have the typical clinical features of LHON and have a family history and will be reported in another manuscript]) were initially collected from the Genetic Clinic of the Eye Hospital, Zhongshan Ophthalmic Center. These LHON samples were used as the mutation-positive controls in this study, and their complete mtDNA genome sequences have been determined and confirmed to harbor a homoplasmic mutation [21,22]. The complete mtDNA sequence of another subject (II:3 in family ZJ170) was reported and confirmed to be without the three primary mutations [23] so it was used as a mutation-negative control in this study. Peripheral blood of 1571 donors who had no symptoms or family history of optic neuropathy was collected from Zhejiang Province (N = 554), Yunnan Province (N = 525) and Hunan Province (N = 492) in China. The genomic DNA was extracted using the standard phenol/chloroform method. Each subject gave informed consent, and the study was approved by the institutional review board of the Kunming Institute of Zoology.

2.2. MAS-PCR optimization for detecting the three LHON primary mutations

Primers designed for the MAS-PCR assay are listed in Table 1. The 3' end of the allele-specific primer was designed to be the mutated base; an additional mismatch was designed at the 3rd or 4th position upstream the 3' end to enhance the specificity of the MAS-PCR. All the primers were designed to have a similar annealing temperature. Primer pairs 3460-2L/H4227, L11760/H12118, 14484-2/H14906 are allele-specific for alleles 3460A, 11778A, and 14484C, respectively. The expected PCR products are 808 bp, 359 bp, and 462 bp, respectively. Primer pair L1156/H1782 can amplify a 664 bp fragment and was used as the inner control for PCR amplification.

The concentrations of the primers in the PCR mixture were adjusted to achieve a high yield of all target regions. The final optimized PCR reactions were conducted in a total volume of 20 μ l containing 1 \times PCR buffer (1.5 mmol/l MgCl₂), 0.5 units of TaKaRa rTaq (TaKaRa Bio Inc., Dalian, China), 175 μ mol/l of each dNTP, corresponding concentration of each primer (cf. Table 1), and 50 ng DNA template. The PCR was performed with the following procedures: a pre-denaturation cycle at 94 °C for 5 min; 30 amplification cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 35 s; and a final extension cycle at 72 °C for 7 min. Then,

2 μ l of PCR product was analyzed using 1.5% agarose gel electrophoresis at 130 V for 30 min.

2.3. Quality evaluation of the MAS-PCR assay

The specificity of the MAS-PCR was examined by comparing the PCR results with the predicted result based on previously determined sequences. The non-specifically amplified products were cloned into the PGM-T vector (Tiagen Bio Inc., Beijing, China) and were sequenced by using the universal primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTAGGTGACACTATAGAA-3'). The sequencing results were handled with the DNASTAR software package (DNASTAR Inc., Madison, WI, USA) and aligned with the revised Cambridge Reference Sequence (rCRS) [24].

To detect the sensitivity of the MAS-PCR assay in this study, DNA samples from healthy controls and LHON cases with a certain homoplasmic primary mutation were mixed to achieve final proportions of mutant DNA of 5%, 10%, 15%, and 20% (for *m.14484T>C* detection, we also included a proportion of 25% of mutant DNA). Then, a total of 50 ng mixed DNA was amplified by the optimized MAS-PCR method, the sensitivity of which is defined to be the smallest percentage of mutant DNA that can be detected by this method.

2.4. Screening of the three mutations in the general population

The three LHON primary mutations were screened in 1571 samples from the general Chinese population by using the above optimized MAS-PCR. For each sample, we used 50 ng DNA for amplification.

3. Results and discussion

Fig. 1 shows the MAS-PCR results of two subjects (ZJ171 and ZJ172), one mutation-negative control (II:3) and three mutation-positive controls (Le1037, Le924 and Le1123 who carry *m.3460G>A*, *m.11778G>A* and *m.14484T>C*, respectively). The PCR results were completely concordant with the sequencing results [21–23]. The 664 bp fragment, which is an inner control of the current MAS-PCR system to show the successful amplification condition, was detected in all subjects. The presence of fragments of 808 bp, 359 bp, and 462 bp indicate the existence of mutations *m.3460G>A*, *m.11778G>A* and *m.14484T>C*, respectively, and were only detected in the mutation-positive controls. Note that there is a 315 bp fragment for sample ZJ172 and Le1037 (Fig. 1), which is a non-specific band that was produced by primer pair L11760/H12118 when primer L11760 binds non-specifically at region 11803–11822, according to our sequencing result. This non-specific PCR product is easily discriminated from the other products by 1.5% agarose gel, and does not affect the MAS-PCR results.

Many methods like PCR-RFLP and SSCP have been used to genotype SNPs (single nucleotide polymorphisms) for decades [7,10,25]. However, these methods are time consuming, relatively complex and complicated by incomplete digestion so are not ideal in screening a

Table 1
Primer information for detecting the three LHON primary mutations.

Primer name	Sequence (5'-3') ^a	Product size	Mutant allele	Concentration in PCR
3460-2L H4227	TACTACAACCTTCGCTG <u>C</u> CA ATGCTGGAGATTGTAATGGG <u>T</u>	808 bp	3460A	0.15 μ mol/l
L11760 H12118	ACGAACGCACTCACA <u>T</u> CA GATGTCGGGGTTGAGGGG <u>A</u> T	359 bp	11778A	0.15 μ mol/l
14484-2 H14906	GTATATCCAAAGACAAC <u>G</u> AC TGAGCGCTCTGGTGA <u>G</u> TA <u>G</u> TG	462 bp	14484C	0.15 μ mol/l
L1156 H1782	GAACACTACGAGCCACAGC TCATCTTTCCTTGCGGTAC	664 bp	Inner control	0.05 μ mol/l

^a The 3' end of the mutation-specific primer is designed to be the mutant base and is underlined. An additional mismatch at the 3rd or 4th base from the 3' end of the mutation-specific primer is shown in lower-case and bold face.

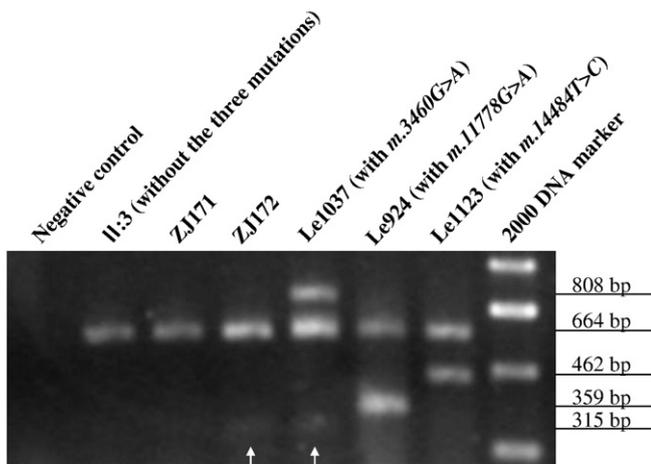


Fig. 1. Using the MAS-PCR to detect the three LHON primary mutations. PCR products were separated on 1.5% agarose gel. The fragment of 664 bp is the inner control of the MAS-PCR assay. Fragments of 808 bp, 359 bp and 462 bp are mutation-specific PCR products of *m.3460G>A*, *m.11778G>A* and *m.14484T>C*, respectively. Arrows refer to non-specific PCR products of 315 bp fragments. Negative control: PCR without DNA template. II:3: mutation-negative control, confirmed to be without the three LHON primary mutations in our previous study [23]. ZJ171 and ZJ172: two subjects from the general Chinese population in this study. Le1037, Le924, Le1123: mutation-positive controls and were confirmed to carry *m.3460G>A*, *m.11778G>A*, and *m.14484T>C* mutations, respectively [21,22].

large sample size. The AS-PCR has been widely used in genetic diagnosis of pathogenic mutations in different diseases because of its speed, simplicity, and affordability [26,27]. In their pioneer studies, Nørby et al. performed a duplex AS-PCR assay to detect *m.3460G>A* and *m.11778G>A* in a single tube [18,19]. They used a non-specific PCR product as the inner control for PCR assay, but the non-specific PCR product was found to be unstable in our experiments. Yang *et al.* developed a MAS-PCR method to detect the three LHON primary mutations simultaneously [20], however, they did not include an inner control to inspect the quality of PCR, so false negative results could not definitively be excluded.

We optimized the MAS-PCR method to overcome the shortcomings in those previously reported studies. First, we used one pair of primers (L1156/H1782), which completely match with rCRS [24], as an inner control to avoid false negative results. The amplification efficiency of the mutation-specific primer might be decreased by the additional mismatch, but the inner control should be consistently amplified unless the amplification condition was not optimal. In order to balance the amplification efficiency of the four pairs of primers that were used in the multiplex PCR, the concentration of the inner control primers was reduced to 1/3 of the concentration of each mutation-specific primer to obtain a balance between maximum sensitivity and maximum specificity. Because of the fixed position, primers L11760 and 14484-2 employed in this study are similar to the previously reported primers [18–20]: primer L11760 is one base shorter than primer WA-C17 reported by Nørby et al. [18,19], and primer 14484-2 is three bases shorter than primer 14484F reported by Yang et al. [20].

The MAS-PCR assay optimized in this study had a reasonably high sensitivity and could detect a heteroplasmy level at 5%, 5% and 20% for *m.3460G>A*, *m.11778G>A* and *m.14484T>C*, respectively (Fig. 2). Only 10%–15% of LHON patients are thought to suffer due to heteroplasmic mutations [13], and the threshold of the primary mutation heteroplasmy for the onset of LHON was estimated to be 75% to 80% [13], despite the fact that very few patients with mutation load less than 50% have developed the disease [15,28]. Moreover, the presence of *m.11778G>A* in Chinese patients is nearly all homoplasmic [11,22]. Therefore, we consider the optimized MAS-PCR to be sensitive enough for use in auxiliary diagnosis of LHON.

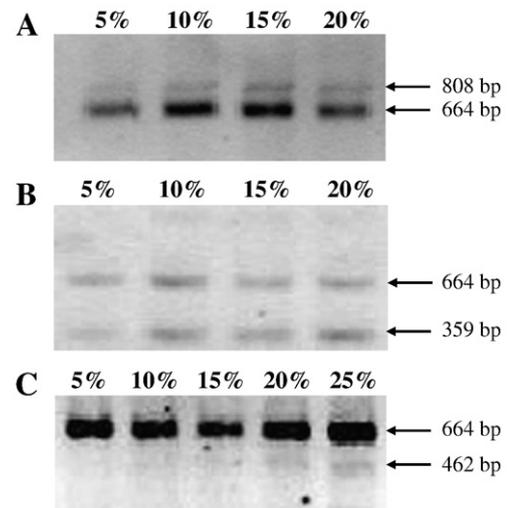


Fig. 2. Sensitivity of the optimized MAS-PCR assay. Mutant DNA was mixed with wild-type DNA to reach different concentrations of mutant DNA, stepwise from 5% to 20% (for *m.14484T>C* detection, to 25%) in intervals of 5%, to determine the smallest percentage of mutant DNA detectable by the optimized MAS-PCR method. PCR products were separated on 1.5% agarose gel. (A): Sensitivity for detection of *m.3460G>A*. (B): Sensitivity for detection of *m.11778G>A*. (C): Sensitivity for detection of *m.14484T>C*.

The incomplete penetrance of LHON indicates that there may be some asymptomatic carriers in the general population. A recent study shows that the presence of the mtDNA pathogenic mutations in the general population is not rare [17]. Among the samples that were analyzed, *m.3460G>A* had a frequency of 3/2807, whereas the frequencies of *m.11778G>A* and *m.14484T>C* reached 3/2770 and 3/2855, respectively [17]. In the current study, none of the 1571 subjects from the general Chinese population was found to harbor any of the three mutations. The relatively small sample size may account for the failure to identify LHON primary mutation carriers in this study. In addition, the heteroplasmy level of mtDNA mutation varies in different tissues [29,30], so whether other tissues, such as muscle tissues of the 1571 subjects, harbor any of the three LHON primary mutations remains unknown. It is also possible that some samples with very low levels of heteroplasmy of the mutation are below the sensitivity threshold of the current MAS-PCR method. Nevertheless, our results suggest that the three primary mutations are rare in the general Chinese population.

In conclusion, we have optimized a simple, fast and cost-effective MAS-PCR method, which can correctly and specifically discriminate the three LHON primary mutations simultaneously. No carrier of any of the three LHON primary mutations was found in 1571 subjects from the general Chinese population using this detection method. Compared with the previous investigation of carrier frequencies of the three LHON primary mutations in the general population from England [17], our results indicate a lower carrier frequency in the general Chinese population.

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