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# MGB probe assay for rapid detection of mtDNA11778 mutation in the Chinese LHON patients by real-time PCR*

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**Abstract:** Objective: Leber's hereditary optic neuropathy (LHON) is a maternally inherited degeneration of the optic nerve caused by point mutations of mitochondrial DNA (mtDNA). Many unsolved questions regarding the penetrance and pathophysiological mechanism of LHON demand efficient and reliable mutation testing. This study aims to develop a minor groove binder (MGB) probe assay for rapid detection of mtDNA11778 mutation and heteroplasmy in Chinese LHON patients by real-time polymerase chain reaction (PCR). Methods: Forty-eight patients suspected of having LHON and their maternal relatives underwent a molecular genetic evaluation, with 20 normal individuals as a control group at the same time. A real-time PCR involving two MGB probes was used to detect the mtDNA11778 mutation and heteroplasmy. A linear standard curve was obtained by pUCmLHONG and pUCmLHONA clones. Results: All 48 LHON patients and their maternal relatives were positive for mtDNA11778 mutation in our assay, 27 heteroplasmic and 21 homoplasmic. Eighteen cases did not show an occurrence of the disease, while 9 developed the disease among the 27 heteroplasmic mutation cases. Eleven did not show an occurrence of the disease, while 10 cases developed the disease among 21 homoplasmic mutation cases. There was a significant difference in the incidence between the heteroplasmic and the homoplasmic mutation types. The time needed for running a real-time PCR assay was only 80 min. Conclusion: This real-time PCR assay is a rapid, reliable method for mtDNA mutation detection as well as heteroplasmy quantification. Detecting this ratio is very important for predicting phenotypic expression of unaffected carriers.

**Key words:** Leber's hereditary optic neuropathy (LHON), Mitochondrial DNA (mtDNA), MtDNA11778 mutation, Minor groove binder (MGB) probe, Real-time polymerase chain reaction (PCR)

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## INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is a common genetic disease, mainly affecting the retina and the papillomacular bundle at the front part of the optic foramen of sclera, and causing neurodegeneration. The clinical manifestation is the acute or subacute loss of the central visual field in both eyes, which is not consistent with Mendelian inheritance (Mashima, 2002; Howell, 2003). Since the discovery of mitochondrial DNA G11778A (mtDNA11778) mutation with this disease by Wallace *et al*.(1988), molecular genetic study of the relation between LHON and mtDNA mutations has become the research focus of the disease. Recently, with the development of molecular diagnosis, sporadic cases of LHON were found among patients with optic neuritis of unclear causes (Yen *et al*., 2003; Man *et al*., 2003; Houshmand *et al*., 2004). Among these patients, some

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were found to have pathogenic mtDNA mutations, especially mtDNA11778 mutation, which has been shown to be prevalent in Japanese and Chinese LHON patients (Sudoyo *et al*., 2002; Jia *et al*., 2006). Various methods have been published to detect the mtDNA mutation, such as conventional polymerase chain reaction (PCR)-sequencing, allele-specific oligonucleotide (ASO) PCR analysis and PCR-restriction fragment length polymorphism (RFLP) (Zhang *et al*., 2005; Wang *et al*., 2007). It has been suggested that heteroplasmy might influence the expression and inheritance pattern of LHON, but there have been no rigorous prospective studies to address this possibility. Preliminary data suggest that heteroplasmy might contribute to incomplete penetrance with the risk of blindness being minimal if the mutational load is less than 60% (Chinnery *et al*., 2001).

We present here another technical approach for detecting mtDNA11778 mutation using real-time PCR with TaqMan minor groove binder (MGB) probe, and attempt to provide diagnostic laboratory detection with basic information regarding the mtDNA11778 mutation. This new molecular testing method will be available to dissect the peculiarities of mitochondrial genetics that complicate both disease manifestation and clinical molecular diagnostics. Real-time PCR is widely used for absolute or relative quantification of human mtDNA (Chabi *et al*., 2003; Gourlain *et al*., 2003; Bai *et al*., 2004). In addition to quantifying gene expression, potential applications for real-time PCR include mtDNA deletion, depletion and over replication. It might be able to replace the current techniques such as sequencing, single strand conformation polymorphism and restriction enzyme digestion. MGB probe is a new type of probe developed from the TaqMan probe. It can be inserted into the sulcule of the double-coiled spiral structure of DNA to form a non-covalent bonding, which is effective for raising the reannealing temperature of the probe. A small amount of nucleic acid monomer is attached to the ordinary probe of oligonucleotide with the same melting temperature ($T_m$) value (Yao *et al.*, 2006), which can make the single basic group sensitively interfere with the renaturation between the wild type and the mutant of mtDNA11778, thus effectively detecting the heterogenicity of mtDNA mutation.

In this study, a real-time PCR using MGB probe technology was performed to detect the mtDNA11778 mutation on 48 subjects (19 patients with LHON and their 29 family members) and a normal control group of 20 subjects. It aimed to develop a quick and effective gene diagnosis method to rapidly detect the mtDNA11778 mutation and to evaluate the heterogenicity of mtDNA for a further understanding of this disease.

## MATERIALS AND METHODS

### Patients and subjects

Six Chinese families (including 19 patients with LHON and their 29 participating family members) were selected through the First Affiliated Hospital, School of Medicine, Zhejiang University, China. Informed consent, blood samples and clinical evaluations were obtained under the protocols approved by the Human Subjects Ethics Committee of Zhejiang University. The ophthalmologic examinations of probands and other members of these families were conducted, including visual acuity, visual field examination, visual evoked potentials and fundus photography. All six families demonstrated clinical characteristics consistent with the diagnosis of LHON (Newman *et al*., 1991). No other ophthalmologic cause of visual loss was apparent. None of them had any systemic disease such as diabetes mellitus or other clinical abnormalities. DNA samples of the twenty controls used for screening for the presence of mtDNA11778 mutations were obtained from a panel of unaffected and unrelated subjects of Chinese ancestry. All volunteers gave informed consent before measurements commenced. MtDNA11778 mutation was confirmed in the probands and some of the family members by using allele-specific PCR amplification and direct sequencing (Zhang *et al*., 2005; Wang *et al*., 2007).

### Equipment and reagent

The multi-fluorescence-passage PCR equipment ABI7000 was purchased from Applied Biosystems Inc. (Austin, USA), the common PCR equipment GeneAmp PCR system 2400 from PerkinElmer (Foster, USA), DNA sequencer (ABI3730) from Applied Biosystems Inc. (Austin, USA), UV spec-

trophotometer (Pharmacia LKB, Biochrom 4060) from Amersham Biosciences (Chalfont, UK), hypothermy high speed centrifuge (Avanti 30 Centrifuge) from Beckmam (Foster, USA), heat start Tag enzyme from Roche Diagnostics Corporation (Indianapolis, USA). Primers of dNTPs (P1: 5′-CAT CCT CAT TAC TAT TCT GCC TAG CA-3′; P2: 5′-GGA GTA GAG TTT GAA GTC CTT GAG A-3′) and probe sequences (MT-1: 5′-FAM-CAC TCA CAG TCA CAT CA-MGB-3′; MT-2: 5′-VIC-AGG ATT ATG ATG CGA CTG T-MGB-3′) were provided by Invitrogen Corporation (Carlsbad, California, USA).

**Detection method**

1. Sample preparation

Three millilitres of peripheral vein blood was collected from each of the 19 patients with LHON and their 29 family members, and the normal control group (20 cases). Anticoagulated with natrium citricum and twice diluted with 0.9% (w/v) NaCl solution, the whole blood was then put into a centrifuge tube having the same volume of Ficoll 100 and centrifuged at 2500 r/min for 10 min. The middle layer of mononuclear cells was sucked out, put into a tube of 1.5 ml Eppendorf, and then centrifuged at 5000 r/min for 5 min. After the supernatant was removed, the peripheral blood mononuclear cell was left as sediment. DNA was extracted from 3 ml of peripheral vein blood from patients with LHON and their family members (48 cases) and the normal control group (20 cases) using a commercial kit (QI Aamp DNA minikit, Qiagen, Hilden, Germany) as per the manufacturer's protocol. The DNA concentration was determined following spectrophotometric measurement using a biophotometer (Eppendorf BioPhotometer 6131, Hamburg, Germany). The 100 μl DNA extract was taken out, preset for 10 min, and centrifuged at 13000 r/min for 10 min. The supernatant was used as the framework for PCR amplification.

2. Plasmid construction of the wild type and the mutation type of mtDNA11778

PCR primer sequences were P1: 5′-AGC CCT CGT AGT AAC AGC CA-3′; P2: 5′-GGA GTA TAG GGC TGT GAC CA-3′. The total volume of the reaction system was 40 μl, with the above primer 0.5 μmol/L, 10× PCR reaction mixture 4 μl, $MgCl_2$ 2.0 mmol/L and dNTP 200 nmol/L, Taq DNA polymerase 1.5 U and DNA extract 4 μl. PCR started with denaturation at 94 °C for 5 min, with the following cycle 35 times (i.e., denaturation at 94 °C for 30 s, renaturation at 57 °C for 30 s, and elongation at 72 °C for 45 s), and a final elongation at 72 °C for 5 min. The PCR product of the target DNA was reclaimed using the gel-reclaiming kit and cloned on the bearer ended with T. The plasmids were screened for the wild type and the mutation type of mtDNA by nucleotide sequencing.

3. Detection of the wild type and the mutant in the plasmid of mtDNA11778

PCR reaction system included 20 μl of 2× TaqMan PCR reaction buffer, 0.75 μmol/L of primer, 0.25 μmol/L of the probe, 1.5 U Taq DNA polymerase, 10 ng of each plasmid sample, then adding deionized water to 40 μl. The procedure of PCR included denaturation beforehand at 95 °C for 2 min, 40 amplification circulations (93 °C for 10 s, 60 °C for 40 s), and detection of the fluorescence increment at the FAM passage and at the VIC passage with FAM and VIC serving as the double passage for the fluorescence collection passage to obtain the $C_t$ value in the two passages.

4. Standard curve of the ratio of mtDNA in the mutation type to that in the wild type (*MT*/*WT*) and detection of mutation on mtDNA11778 in blood samples

Mix the standard samples of plasmid of the mutation type and the wild type with the cloned mtDNA11778 site according to the following proportions: 1:1, 2:1, 4:1, 8:1, 16:1, 1:2, 1:4, 1:8, 1:16. Take the above mixed liquors as the framework. Following the operational procedures abovementioned, detect the fluorescence increment at the FAM passage and at the VIC passage. Obtain the ratio of the $C_t$ value in the FAM passage to the one in the VIC passage ($C_{t,FAM}/C_{t,VIC}$), and draw the standard curve based on this ratio and the ratio of *MT*/*WT*, $C_{t,FAM}/C_{t,VIC}$=2.0 (*MT*/*WT*=16:1) and $C_{t,FAM}/C_{t,VIC}$= 0.085 (*MT*/*WT*=1:16) serving as the quantitation limits. Thus, when $C_{t,FAM}/C_{t,VIC}>2.0$, it is the mutation type; when $C_{t,FAM}/C_{t,VIC}<0.085$, it is the wild type; others are the heteroplasmy. The results are shown in Table 1. Based on the standard curve, the ratio of *MT*/*WT* was determined through the $C_t$ value in the two passages detected. The FAM fluorescent signal represented the mutation type, and the VIC fluorescent signal represented the wild type.

**Table 1 The ratio of $C_t$ value in the FAM passage to the one in the VIC passage based on the ratio of mtDNA in the mutation type to that in the wild type (*MT*/*WT*)**

| *MT*/*WT* | $C_{t,FAM}$ | $C_{t,VIC}$ | $C_{t,FAM}/C_{t,VIC}$ |
|---|---|---|---|
| 1:1 | 85 | 245 | 0.346 |
| 2:1 | 115 | 200 | 0.575 |
| 4:1 | 150 | 200 | 0.750 |
| 8:1 | 140 | 100 | 1.400 |
| 16:1 | 140 | 70 | 2.000 |
| 1:2 | 115 | 350 | 0.328 |
| 1:4 | 70 | 320 | 0.218 |
| 1:8 | 50 | 280 | 0.178 |
| 1:16 | 30 | 350 | 0.085 |

## RESULTS

### Analysis of the correlation between mutation proportion and $C_{t,FAM}/C_{t,VIC}$

The logarithm of the original value of $C_{t,FAM}/C_{t,VIC}$ was analyzed for the correlation between the mutation proportion ($Y$) and the quantization value. The dependability reached 0.8658 ($P$=0.99). The linear equation is $Y$=0.4575$C_{t,FAM}/C_{t,VIC}$+0.20089. The method used in this study can be employed to analyze the mutation proportion among the maternal family members of the patients with LHON.

### Correlation between morbidity and the blood samples of homoplasmic mutation and heteroplasmy

Eighteen cases did not show an occurrence of the disease, while 9 developed the disease among 27 heteroplasmic cases. Eleven did not show an occurrence of the disease, while 10 cases developed the disease among 21 homoplasmic mutation cases. The type of either homoplasmic mutation or heteroplasmy of a blood samples can be shown directly by computer (Fig.1). These results show that there was a significant difference in the familial incidence between the heteroplasmic type and the homoplasmic type.

### Analysis of the $C_{t,FAM}/C_{t,VIC}$ of the LHON patients

When analyzing the correlation between the incidence and $C_{t,FAM}/C_{t,VIC}$ among 19 patients with mtDNA11778 mutation, the ratio of *MT*/*WT* of 16 patients was more than 8:1. As shown in Fig.2, the more mtDNA mutation was found in peripheral blood, the higher the incidence was. When *MT*/*WT* was higher than 16:1, the incidence significantly increased.

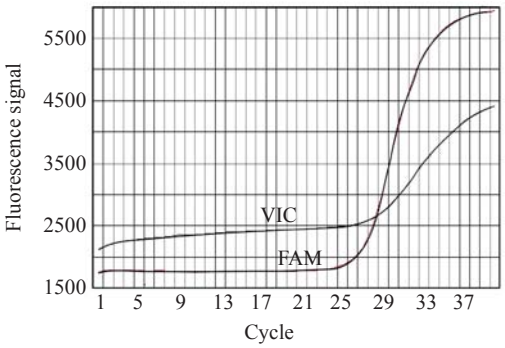


**Fig.1 Detection of the mutation type or wild type of mtDNA11778**
This mtDNA genotyping is heteroplasmy. The FAM fluorescent signal represented the mutation type and the VIC fluorescent signal represented the wild type. Two fluorescent signals were measured simultaneously

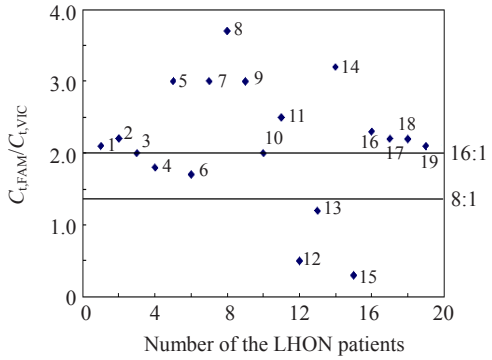


**Fig.2 Analysis of the $C_{t,FAM}/C_{t,VIC}$ of the LHON patients**

### Time consumed

Detection of mtDNA11778 mutation using real-time PCR only took 80 min, much less than the time used for sequencing.

## DISCUSSION

LHON is a common clinical optical nerve disease, which can cause blindness. It is frequently found in young adults. The symptom is the acute or subacute damage to the visual function in both eyes, simultaneously or respectively. So far no effective treatment has been found. In 1988, Wallace *et al*.(1988) firstly

found that it was caused by mtDNA mutation. Since then sporadic cases of optic neuropathy without clear explanation have been found and clinically diagnosed as LHON, with the test of molecular genetics. The occurrence rate of sporadic cases is 50%~80%, 50% and 40% in America, Europe and Japan, respectively. It was reported that 90% of LHON cases in Asia were caused by mtDNA11778 mutation and that in China 40% of the cases of optic neuropathy without clear explanation actually belonged to LHON (Sudoyo *et al*., 2002; Marotta *et al*., 2004; Jia *et al*., 2006; Volod′ko *et al*., 2006; Zhang and Qi, 2008). Because of the heteroplasmy of mtDNA mutation, it is difficult to make a quick and convenient detection of mtDNA mutation.

Our other studies published previously had used the conventional PCR sequencing, the methylation-specific PCR detection method (allele-specific PCR) and the PCR-RFLP to detect the mtDNA11778 mutation (Zhang *et al*., 2005; Wang *et al*., 2007). These approaches can in some instances result in significant false-negative results, with the following disadvantages: (1) they are not sensitive enough for detecting the low level of heteroplasmy if ethidium bromide is used for detection; (2) much of the time needed is devoted to post amplification steps, verifying the presence of PCR products by gel; (3) there is a possibility of incomplete digestion by restriction enzyme or the inability to do multiplexing.

We present here a technical approach using real-time PCR with a TaqMan MGB probe, which is more amenable to high throughput screening, as it does not require extensive post amplification manipulation. Using this approach, the mtDNA genotyping results can be obtained within less than 2 h. Discrimination between the wild type and mutant type is based on the difference in melting temperature ($\Delta T_m$) between the differently-labeled probes. In this method TaqMan probes conjugated to an MGB group form hyper stabilized duplexes with complementary mtDNA by folding the MGB probe into the duplex, thus giving a higher $T_m$ with an increased attributable specificity to increase the discrimination in detecting the mtDNA11778 mutation. In addition, each result was also verified by examining the PCR amplification curve generated from the software to eliminate false positives due to aberrant light emission. The simple, rapid and reproducible real-time PCR genotyping method presented here constitutes a significant improvement over current techniques.

By using this method the blood samples have been successfully analyzed. All the blood samples of the 20 normal subjects were mutation negative, which is in accordance with the result in the clinics. Among LHON patients and their family members, 18 cases did not show an occurrence of the disease, while 9 developed the disease among 27 heteroplasmic cases. Eleven did not show the occurrence of the disease, while 10 cases showed the onset of the disease among 21 homoplasmic mutation cases. It was indicated that this real-time PCR technology provides us with a convenient, fast and accurate detection method for mtDNA11778 mutation. It is suitable for clinical laboratory diagnostics. At the same time, this method can inform the ratio of *MT*/*WT*, which improves the accuracy of evaluating the heteroplasmy of mtDNA. It is of great importance for further exploration of the mutation threshold of mtDNA in LHON. Moreover, the results of this study also show that there was a significant difference between the incidence of the heteroplasmic family constellation and that of the homoplasmic mutation family constellation. The mutation ratio of mtDNA in the peripheral blood of patients with LHON has a direct correlation with the occurrence of LHON. The amount of mtDNA11778 mutant has a direct correlation with the severity of the optic nerve impairment in patients with LHON. Fig.2 shows that when the ratio of *MT*/*WT* reached 16:1 (the mutation mtDNA accounting for 94.12%), the incidence increases significantly.

As a matter of fact, LHON, a major disease in neuro-ophthalmology and an important disease model in mitochondriopathy, can be caused by oxidative stress, potential nuclear genetic or environmental factors, as well as mtDNA mutation. Thus further follow-up studies should be done on its pathogenesis.

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