Letter to editor

A novel method of detecting mitochondrial m.1494C>T and m.1555A>G mutations in a single PCR reaction using base-quenched probe

Dear Editor,

Aminoglycoside antibiotics have widely been used for treating patients suffering from multidrug-resistant tuberculosis (MDR-TB) and other bacterial infections in China[1]. However, aminoglycoside antibiotics are a double-edged sword, despite its efficacy and cost effectiveness they have known severe toxic side–effects, especially ototoxicity that may lead a permanent hearing loss in certain individuals who are hypersensitive to the drugs [2]. It has been reported that several mutations in the mitochondrial DNA (mtDNA), including m.1555A>G[3], m.1494C>T[4], m.1095T>C[5], m.837A>G[6], and m.1291T>C[7] may possibly associate with the sensorineural hearing loss, and mutations of m.1555A>G and m.1494C>T at the highly conserved decoding site of 12S rRNA are well documented being associated to either aminoglycoside antibiotics-induced or non-syndromic hearing loss in families with different ethnic backgrounds [3, 4, 8, 9].

It has been demonstrated that mutation m.1494C>T might associate with aminoglycoside-induced hearing loss in a large Chinese family[8, 10, 11]., while the mutation m.1555A>G may contribute to both aminoglycoside-induced and nonsyndromic hearing loss in families worldwide[12, 13]. Both m.1555A>G and m.1494C>T mutations locate in a highly conserved region of 12SrRNA that is implicated in aminoglycoside binding in bacteria. Screening these mutations in newborns or children may warn clinicians to avoid subscribing aminoglycoside antibiotics, which may minimize the occurrence of drug-induced deafness. In the present study we describe a simple, quick

and economic method to detect these mutations by using the base-quenched probe technique that we established previously [14]. The key point of this method is based on the phenomenon that certain fluorescent dyes can be influenced by the DNA bases. During hybridization to its complementary sequence, fluorescence intensity of the probe was quenched by the adjacent DNA base. Subsequently, fluorescence would be largely increased during strand dissociation of the melting curve process. For a typical homozygous wild-type sample of human chromosome DNA, a single melting valley could be observed; for mixed alleles, two valleys could be observed; and for a homozygous mutated sample, only a single valley at a melting temperature different from that of wild-type allele could be observed.

In the present study, four 360-bp fragments were synthesized as the positive controls. These four amplicons represent the four possible haplotype combinations such as 1494C/1555A, 1494C/1555G, 1494T/1555A and 1494T/1555G (Figure 1). Fragments were amplified and purified, and then ligated to pUC57 vector. The ligated products were transformed into *E. coli.* JM109 competent cells. Extracted plasmid DNAs were used to generate four standard melting curves for four different haplotypes.

Primers and probes for detecting m.1494C>T and m.1555A>G were designed according to the sequence in the NCBI (NC_001807) (Table 1). Primers and probes were synthesized and fluorescence modified by the Sangon. The probe should be blocked with phosphate at the 3'end if FAM (Carboxyfluorescein) was labeled at the 5' end. In brief, PCR was performed as follows: 40-80 ng of DNA template, 2.5 μl of 10× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM 4×dNTPs, 1.25U Taq DNA polymerase, 10 pmol of each primer and 2 pmol of probe in a final reaction volume of 25 μl. Thermal cycling was performed

in a LightCycler (Roche) under the following conditions: the cycling program consisted of 2 min of initial denaturation at 95°C, followed by 40 cycles at 95 °C for 15 s (temperature transition rate 20°C /s), 58°C for 5 s, and 72 °C for 25 s (temperature transition rate 20°C /s). The analytical melting program involved heating the amplicon/probe heteroduplex at 95°C for 30 s, 30°C for 4 min, and increased to 80 °C at a temperature transition rate of 0.1 °C /s, with continuous acquisition of fluorescence data in fluorescence channel 1 (F1). Then the probe melts off when the temperature is slowly increased. At the same time, the fluorescence is largely increased. When mutated haplotype exists, it will occur at different temperature compared with the wild-type haplotype as we described previously[14]. Fig. 2 shows melting curves for genotyping analysis. A sudden increase in fluorescence indicates sensor probe melting as shown in Fig. 2(A). A mathematical transformation of the data-the negative first derivative of fluorescence with respect to temperature (-dF/dT) vs. T[15] allows for an easier interpretation of the results where the maximum value represents the TM as shown in Fig. 2. The A to G transition at position 1555 caused TMs shifted from 42°C to 51°C. The mutant in 1494 from C to T could induce TMs shifted from 61°C to 51°C. In other words, the TMs of 1494C, 1494T, 1555A and 1555G were 61°C, 51°C, 42°C and 51°C, respectively. For example, when TMs were obtained as 42°C and 61°C, it could interpret A and C in 1555 and 1494 site, respectively. When the single melting valleys were visible there were T and G genotype in 1494 and 1555 site, because 1494T and 1555G has the same TMs. Thus the genotype could easily be distinguished from the melting curves.

In order to verify this base-quenched probe method for detecting hearing loss mutations,

we have performed a mutational analysis of the mitochondrial 12S rRNA gene in 117 deaf students of Changzhou Deaf School. All participants were informed by written consent. 5 ml-whole blood was obtained from each volunteer. Genomic DNA was isolated from whole blood by the UNIQ-10 column (Shanghai, China) according to the manufacturer's instructions. Among them, 3 subjects harbored the homoplasmic mitochondrial DNA m.1555A>G mutation in the 12S rRNA gene. The frequency of the m.1555A>G mutation is 2.56%. And no m.1494C>T mutation was detected in this sample population. Although it is previously reported that the prevalence of 12S rRNA m.1494C>T mutation is about 0.41% (13/3133) in the Chinese patients suffered from non syndromic hearing loss[11]. Small experimental population may limit the detection of m.1494C>T mutation in the present study. And in the other hand the ethnicity or regions are also important factors in prevalence of a number of genetic diseases.

Furthermore this base-quenched probe method for detecting mutations was further validated by the direct DNA sequencing analyses. The PCR products of m.1555A>G mutation (3 samples) and non-mutation (12 samples) detected by base-quenched probe were selected randomly from the 117 volunteers and sequenced directly on an automatic sequencer from the Applied Biosystems (model 3730, Invitrogen, Shanghai, China). It results a complete concordance between DNA sequencing and the base-quenched probe method.

It has well been documented that mutations of 12S rRNA gene are the hot spots related to the aminoglycoside antibiotics-induced and/or non-syndromic hearing loss[16] and several deafness-associated mtDNA mutations have been identified in this gene. m.1555A>G mutation in a highly conserved decoding site of the 12S rRNA could

associate with both aminoglycoside-induced and non-syndromic hearing loss in families with different ethnic backgrounds,[3, 9, 17], and the m.1494C>T mutation is also structurally equivalent to the m.1555A>G mutation in the 12SrRNA gene [4, 10, 11, 18].

In the present study we have described a simple, quick and economic method for detecting m.1555A>G and m.1494C>T mutations of mtDNA simultaneously. During recent years, certain techniques, including restriction fragment length polymorphism (RFLP) genotyping, direct DNA sequence, and the resequencing Mitochip array have been described and are even commercially available for detecting mtDNA mutations [3, 9, 17, 19], however, potential risk of contamination makes it difficult to avoid using electrophoresis detection method. Although fluorescence resonance energy transfer (FRET) by using two fluorescent dyes in close proximity is one of the most powerful and promising methods for SNP genotyping[20], the expensive costs of fluorescent modification and requirement of special equipment may limit its application widely in the clinical testing.

Our method requires only one pair of primers and two probes and can detect m.1555A>G and m.1494C>T mutations of mtDNA simultaneously in one real-time PCR running. The precision of the method has been approved by the direct DNA sequence. During the melting of the final PCR product, the sequence alteration can be detected as a change in the TM of the base-quenched probe on Light-Cycler (version 3.5, Roche). In the whole testing process there is no need to open the tubes, which could avoid secondary contamination effectively.

In conclusion, comparing to existing methods, the base-quenched probe technique can detect m.1494C>T and m.1555A>G mutations simultaneously in one PCR reaction,

which is a quick, economic and convenient method and it can also minimize the second contaminant.

Reference

[1] Matteelli A, Migliori GB, Cirillo D, Centis R, Girard E, Raviglion M. Multidrug-resistant and extensively drug-resistant Mycobacterium tuberculosis: epidemiology and control. Expert Rev Anti Infect Ther 2007; 5:857-871.

[2] Guan MX, Fischel-Ghodsian N, Attardi G. A biochemical basis for the inherited susceptibility to aminoglycoside ototoxicity. Hum Mol Genet 2000; 9:1787-1793.

[3] Human H, Lombard D, de Jong G, Bardien S. A South African family with the mitochondrial A1555G mutation on haplogroup L0d. Biochem Biophys Res Commun 2009; 382:390-394.

[4] Zhao H, Li R, Wang Q, et al. Maternally inherited aminoglycoside-induced and nonsyndromic deafness is associated with the novel C1494T mutation in the mitochondrial 12S rRNA gene in a large Chinese family. Am J Hum Genet 2004; 74:139-152.

[5] Wang Q, Li R, Zhao H, et al. Clinical and molecular characterization of a Chinese patient with auditory neuropathy associated with mitochondrial 12S rRNA T1095C mutation. Am J Med Genet A 2005; 133A:27-30.

[6] Xing G, Chen Z, Wei Q, et al. Mitochondrial 12S rRNA A827G mutation is involved in the genetic susceptibility to aminoglycoside ototoxicity. Biochem Biophys Res Commun 2006; 346:1131-1135.

[7] Ballana E, Morales E, Rabionet R, et al. Mitochondrial 12S rRNA gene mutations affect RNA secondary structure and lead to variable penetrance in hearing impairment. Biochem Biophys Res Commun 2006; 341:950-957.

[8] Zhao L, Wang Q, Qian Y, et al. Clinical evaluation and mitochondrial DNA sequence analysis in two Chinese families with aminoglycoside-induced and non-syndromic hearing loss. Biochem Biophys Res Commun 2005; 336:967-973.

[9] Tang X, Yang L, Zhu Y, et al. Very low penetrance of hearing loss in seven Han Chinese pedigrees carrying the deafness-associated 12S rRNA A1555G mutation. Gene 2007; 393:11-19.

[10] Yuan H, Chen J, Liu X, et al. Coexistence of mitochondrial 12S rRNA C1494T and CO1/tRNA(Ser(UCN)) G7444A mutations in two Han Chinese pedigrees with aminoglycoside-induced and non-syndromic hearing loss. Biochem Biophys Res Commun 2007; 362:94-100.

[11] Zhu Y, Li Q, Chen Z, et al. Mitochondrial haplotype and phenotype of 13 Chinese families may suggest multi-original evolution of mitochondrial C1494T mutation. Mitochondrion 2009; 9:418-428.

[12] Jacobs HT, Hutchin TP, Kappi T, et al. Mitochondrial DNA mutations in patients with postlingual, nonsyndromic hearing impairment. Eur J Hum Genet 2005; 13:26-33.

[13] Yuan H, Qian Y, Xu Y, et al. Cosegregation of the G7444A mutation in the mitochondrial COI/tRNA(Ser(UCN)) genes with the 12S rRNA A1555G mutation in a Chinese family with aminoglycoside-induced and nonsyndromic hearing loss. Am J Med Genet A 2005; 138A:133-140.

[14] Luo G, Zheng L, Zhang X, Zhang J, Nilsson-Ehle P, Xu N. Genotyping of single nucleotide polymorphisms using base-quenched probe: a method does not invariably depend on the deoxyguanosine nucleotide. Anal Biochem 2009; 386:161-166.

[15] Frances F, Portoles O, Sorli JV, Guillen M, Gonzalez JI, Corella D. Single tube optimisation of APOE genotyping based on melting curve analysis. Clin Biochem 2008; 41:923-926.

[16] Fischel-Ghodsian N. Genetic factors in aminoglycoside toxicity. Pharmacogenomics 2005; 6:27-36.

[17] Ding Y, Li Y, You J, et al. Mitochondrial tRNA(Glu) A14693G variant may modulate the phenotypic manifestation of deafness-associated 12S rRNA A1555G mutation in a Han Chinese family. J Genet Genomics 2009; 36:241-250.

[18] Zhao H, Young WY, Yan Q, et al. Functional characterization of the mitochondrial 12S rRNA C1494T mutation associated with aminoglycoside-induced and non-syndromic hearing loss. Nucleic Acids Res 2005; 33:1132-1139.

[19] Leveque M, Marlin S, Jonard L, et al. Whole mitochondrial genome screening in maternally inherited non-syndromic hearing impairment using a microarray resequencing mitochondrial DNA chip. Eur J Hum Genet 2007; 15:1145-1155.

[20] Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 1995; 4:357-362.

Lu Zheng

Guanghua Luo*

Xiaoying Zhang

Jun Zhang

Comprehensive Laboratory, Third Affiliated Hospital,

Suzhou University,

Changzhou 213003, China

Ning Xu*

Section of Clinical Chemistry & Pharmacology, Institute of Laboratory Medicine,

Lunds University,

S-221 85 Lund, Sweden

***Corresponding author.** Guanghua Luo, MD.,

Tel: +86 519 68870619;

Fax: +86 519 86621235.

E-mail: shineroar@163.com.

Ning Xu, MD, PhD.

Tel: +46 –46 173487;

Fax: +46 –46 130064,

E-mail: ning.xu@med.lu.se.

Legend to figures

Fig. 1. The figure shows partial DNA sequences of four positive control amplicons for the different genotype combinations, which represents four possible genotype combinations, 1494C/1555A, 1494C/1555G, 1494T/1555A and 1494T/1555G, respectively.

Fig. 2. Melting curve analysis. Curves of fluorescence (F) versus temperature (T) for sequence-specific base-quenched probe complementary to the mitochondrial DNA sequence was shown in Fig. 2A. Fig. 2 (B,C,D,E) shows the derivative melting curves (-dF/dT vs. T) that depict the same data as in Fig. 2A. All of the derivative melting valleys are oriented in negative scale and afford easier visualization of TMs. Fig 2B shows the melting curve of 1494C/1555A haplotype. For the 1555A haplotype, TM is about 42 °C, and for 1494C haplotype, the TM is about 61 °C. Melting curve of 1494C/1555G was show in Fig. 2C. The TM of 1494C and 1555G are 61 °C and 51°C, respectively. Fig. 2D shows the melting curve of 1494T/1555A haplotype. Two melting valleys were obtained in 51°C and 42 °C. 1494T and 1555G haplotype has the same TM, 51°C, but the area of melting valleys were increased (Fig. 2E).

Table 1. Sequences of primers and probes of A1555G and C1494T

	Sequence (5′ to 3′)	annealing position
Sense primer	TCACCACCTCTTGCTCAGCCTAT	1235nt-1257nt
Antisense primer	TGCACTTTCCAGTACACTTACCATGTT	1564nt-1590nt
C1494T probe	GCCCGTCACCCTCCTCA -FAM	
A1555G probe	TAGAGGAGGCAAGTC-FAM	

Fig. 1.

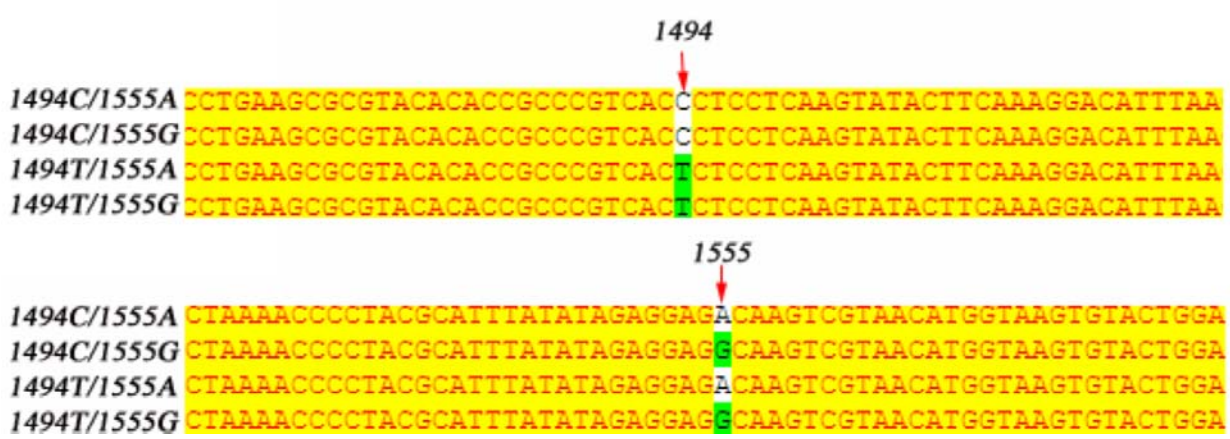
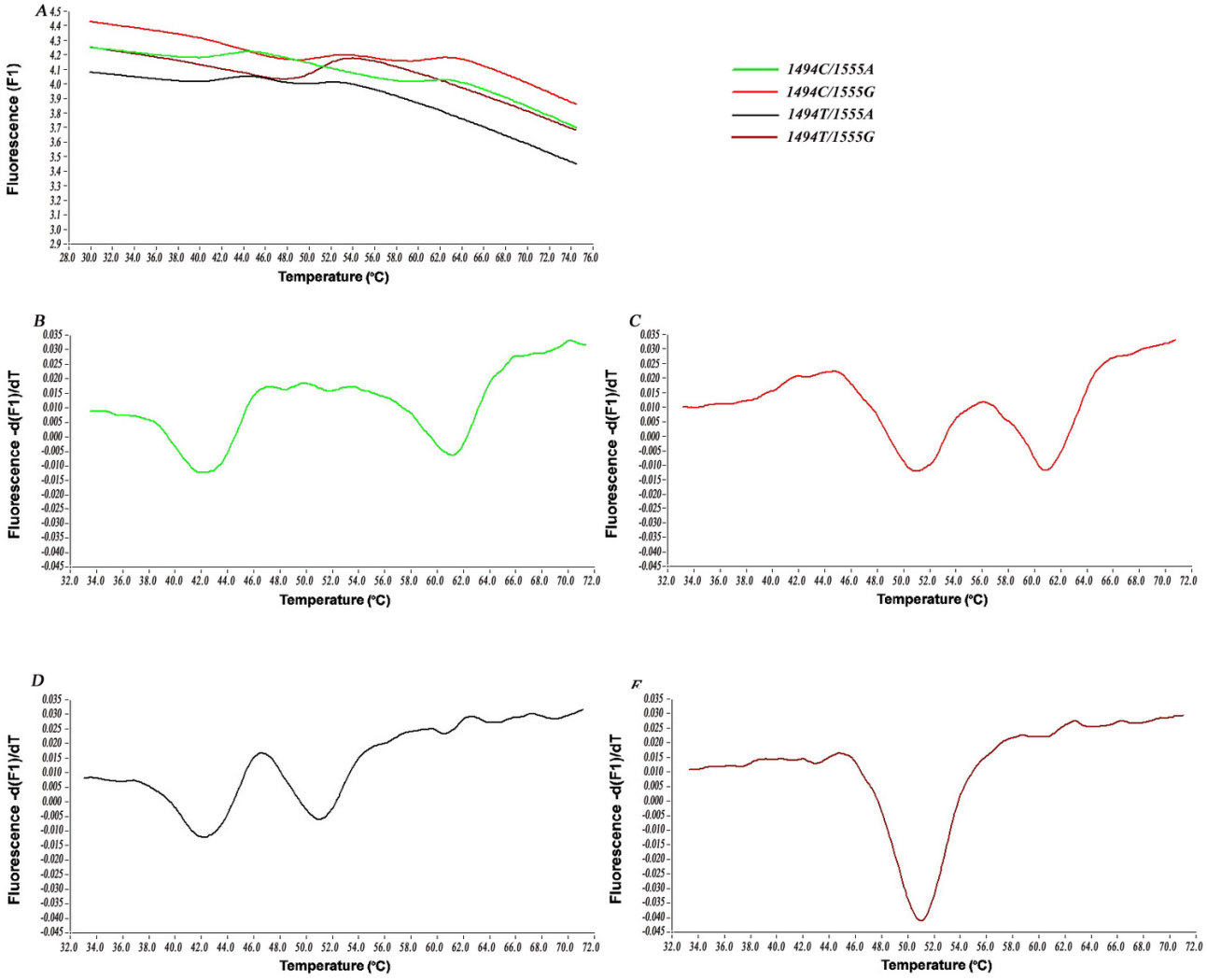

1494
1494C/1555A CCTGAAGCGCGTACACACCGCCCGTCACCCTCCTCAAGTATACTTCAAAGGACATTTAA
1494C/1555G CCTGAAGCGCGTACACACCGCCCGTCACCCTCCTCAAGTATACTTCAAAGGACATTTAA
1494T/1555A CCTGAAGCGCGTACACACCGCCCGTCACTCTCCTCAAGTATACTTCAAAGGACATTTAA
1494T/1555G CCTGAAGCGCGTACACACCGCCCGTCACTCTCCTCAAGTATACTTCAAAGGACATTTAA
1555
1494C/1555A CTAAAACCCCTACGCATTTATATAGAGGAGACAAGTCGTAACATGGTAAGTGTACTGGA
1494C/1555G CTAAAACCCCTACGCATTTATATAGAGGAGGCAAGTCGTAACATGGTAAGTGTACTGGA
1494T/1555A CTAAAACCCCTACGCATTTATATAGAGGAGACAAGTCGTAACATGGTAAGTGTACTGGA
1494T/1555G CTAAAACCCCTACGCATTTATATAGAGGAGGCAAGTCGTAACATGGTAAGTGTACTGGA

Fig. 2.


A
Fluorescence (F1)
Temperature (°C)
1494C/1555A
1494C/1555G
1494T/1555A
1494T/1555G
B
Fluorescence -d(F1)/dT
Temperature (°C)
C
Fluorescence -d(F1)/dT
Temperature (°C)
D
Fluorescence -d(F1)/dT
Temperature (°C)
F
Fluorescence -d(F1)/dT
Temperature (°C)