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Rapid identification of aminoglycoside-induced deafness gene mutations using multiplex real-time polymerase chain reaction

Shasha Huang [a,1], Guangxin Xiang [b,c,d,1], Dongyang Kang [a], Chen Wang [b,c], Yanling Kong [b,c], Xun Zhang [b,c], Shujian Liang [b,c], Keith Mitchelson [b,c,e], Wanli Xing [b,c,d,e,*], Pu Dai [a,*]

[a] Department of Otolaryngology, PLA General Hospital, Do. 28 Fuxing Rode, Beijing 100853, People's Republic of China
[b] Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310003, P. R. China
[c] National Engineering Research Center for Beijing Biochip Technology, Beijing 102206, P. R. China
[d] CapitaBio Corporation, Beijing 102206, P. R. China
[e] Medical Systems Biology Research Center, Department of Biomedical Engineering, Tsinghua University School of Medicine, Beijing 100084, P. R. China

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ABSTRACT

Background: Exposure to aminoglycoside antibiotics can induce ototoxicity in genetically susceptible individuals carrying certain mitochondrial DNA (mtDNA) mutations (C1494T and A1555G), resulting in hearing loss. So, a rapid diagnostic approach is needed to accurately identify subjects carrying such gene mutations.

Methods: In the present study, we describe a rapid and reliable four-color, real-time quantitative polymerase chain reaction (qPCR) assay for simultaneously detecting two mtDNA 12S rRNA gene variants, A1555G and C1494T, which are prevalent in the Han Chinese population. This multiplex assay incorporates three allele-specific TaqMan probes labeled with different fluorophores in a single reaction, providing high genotyping accuracy for clinical blood samples.

Results: Tests with C1494T, A1555G and wild-type DNA exhibited high sensitivity, specificity, reproducibility and accuracy of discriminating mutations from wild-type.

Conclusions: This study shows that this simple and inexpensive method can be used for routine molecular diagnostics and potentially for large-scale genetic screening.

1. Introduction

Hearing loss is the most common sensory disease affecting 1 in 1000 newborns worldwide; an equal number of people lose their hearing by adulthood, and half of the population experience significant hearing impairment by the age of 65 [1]. In China, there are more than 27 million people with hearing loss, ranking it the top disability in the Chinese population [2]. Hearing loss can be congenital or caused by environmental factors, including infection, trauma or ototoxic drugs [3]. Aminoglycosides such as streptomycin, gentamycin and tobramycin are clinically important antibiotics that exert antibacterial effects by directly targeting bacterial 16S ribosomal RNA (rRNA), causing miscoding or premature termination of protein synthesis [4]. The use of aminoglycoside antibiotics in genetically susceptible individuals can frequently lead to ototoxicity, resulting in hearing impairment and deafness. Mutations in mitochondrial DNA (mtDNA) have been found to be associated with a wide range of human diseases. At least 5% of Caucasian individuals with post-lingual, nonsyndromic hearing impairment carry mtDNA mutations, and the frequency may be higher in oriental populations [5]. Particularly, two base substitutions in mitochondrial 12S rRNA, namely C1494T and A1555G, have been found to be associated with aminoglycoside-induced and nonsyndromic hearing loss in families with different ethnic backgrounds [6]. While the wild-type (WT) C1494 and A1555 do not form a base pair, the C1494T or A1555G mutation creates a new U–A or C–G base pair, altering the secondary structure of human mitochondrial 12S rRNA to resemble bacterial 16S rRNA more closely, thus becoming a primary target for aminoglycosides [3,7].

In China, the C1494T and A1555G mutations in mitochondrial 12S rRNA are the common cause of nonsyndromic sensorineural hearing loss (3.43%). Regarding maternal inheritance, all matrilineal

* Corresponding authors at: Department of Otolaryngology, PLA General Hospital, Do. 28 Fuxing Rode, Beijing 100853, PR China. Tel.: +86 1066937623; fax: +86 1068156974.
E-mail addresses: wlxing@tsinghua.edu.cn (W. Xing), daipu301@vip.sina.com (P. Dai).
[1] Equal contributors.

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descendants will carry mitochondrial DNA A1555G or C1494T mutation in a Chinese family(1:10 ratio of one patient to matrilineal descendants: if there was one person with A1555G or C 1494T mutation, there would be at least 10 matrilineal descendants carrying the same mutation in this family) [8]. Thus, to avoid hearing loss induction or worsening by the administration of aminoglycosides, a rapid diagnostic approach is needed to accurately identify subjects carrying such gene mutations.

DNA-based mutation detection technologies offer a precise method for the identification of at-risk individuals. Several deafness analysis platforms are currently available. Because of its simple operation and low cost, real-time quantitative polymerase chain reaction (qPCR) has become one of the most commonly used techniques to measure gene expression sensitively and quantify nucleic acids [9]. Previous methods have utilized monoplex real-time PCR to assay single-nucleotide mutations related to aminoglycoside-induced deafness [9–12]; however, multiplex assays should be more attractive to a clinical hearing screening program for the detection of multiple mutations in a single reaction [13]. Here, we report the development and optimization of a simultaneous four-color triplex real-time PCR assay for the detection of mitochondrial 12S rRNA C1494T and A1555G mutations. We propose that the triplex real-time PCR method is a highly accurate, rapid and economical clinical assay, with potential for use in neonatal hearing screening and for personalized medicine.

2. Subjects and methods

2.1. Subjects

A total of 143 Chinese subjects with hearing loss were recruited from the Department of Otolaryngology, Chinese PLA General Hospital. All the patients have been detected mt DNA 12S rRNA A1555G and C1494T mutation by Sanger sequence, including the samples with A1555G and C1494T mutation, and some negative cases. Only 2 authors knew the results of the 143 samples, other authors inculding the investigators of the new method did not know the outcome. After obtaining informed consent from the parents of each participant, whole blood, dried blood spots (DBS) and/or buccal swab samples were collected following standard methods. The study was performed according to protocols approved by the ethics committee of Chinese PLA General Hospital.

2.2. Plasmid construction

Plasmids containing the C1494T or A1555G mutation regions were constructed from PCR-amplified mtDNA fragments [position 1093 to 2643] obtained from a characterized patient's total DNA. The following primers were used for amplification: forward primer: 5′-CTTAGCCCTAAACCTCAACAG-3′; reverse primer: 5′-CGTGGA GCCATTCATACAG-3′. PCR products were then purified using the TaKaRa MiniBEST DNA Fragment Purification Kit Ver.3.0 (TaKaRa, Otsu, Japan) and transformed into *Escherichia coli* (TIANGEN, Beijing, China) using the pGEM-T Easy Vector System I (Promega, Madison, WI, USA) according to the manufacturer's instructions. Plasmids were isolated using the PurePlasmid Mini Kit (CoWin Biotech, Beijing, China) and were sequence confirmed. Wild-type (WT) plasmids carrying the mitochondrial region [position 16318 to 16427] were constructed following the same procedure with the forward primer 5′-AGCCATTTACCGTACATAG-CACATT-3′ and the reverse primer 5′-GGGATATTGATTTCACGGAG-GAT-3′.

2.3. Primers and probes for real-time PCR

The primers and probes specific for the detection of C1494T and A1555G mutations were designed using Primer Express Software for Real-Time PCR v3.0 (Applied Biosystems, Grand Island, NY, USA), and synthesized by Invitrogen (Oslo, Norway) and Applied Biosystems, respectively. Each probe consisted of an oligonucleotide with a fluorescent reporter dye at the 5′ end, a non-fluorescent quencher (NFQ) and a minor groove binder (MGB) at the 3′ end. Specifically, in the present study, the C1494T probe was labeled with a FAM fluorophore, and the A1555G probe was labeled with a VIC fluorophore at the 5′ end. The following sequences were used: forward primer: 5′-CCCTGAAGCGCGTA-CACA-3′; reverse primer: 5′-GCTACACTCTGGTTCGTCCAAGT-3′; C1494T probe: 5′-CCGTCAC TCTCCTCAA-3′; A1555G probe: 5′-ACGACTTGCCTCCT-3′.

Additionally, a separate set of primers and probe were synthesized to assay a conserved region in human mtDNA for quality control. Specifically, the forward control primer was 5′-AGCCATTTACCGTACATAGCACATT-3′, and the reverse control primer was 5′-GGGATATTGATTTCACGGAGGAT-3′. The control probe was 5′-CCATGGATGACCC-3′, which was labeled with an NED fluorophore at the 5′ end together with an NFQ and an MGB at the 3′ end.

2.4. Mutation analysis by real-time PCR

Plasmid DNA was isolated using the PurePlasmid Mini Kit (CoWin Biotech, Beijing, China). Genomic DNA was isolated from whole blood samples using the FlexiGene DNA Kit (Qiagen, Hilden, Germany), from DBS using the TIANamp Blood Spots DNA Kit (TIANGEN, Beijing, China) and from buccal swabs using the TIANamp Swab DNA Kit (TIANGEN). Quantification of the C1494T and A1555G mutations was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems). A PCR master mix was prepared and divided into aliquots at the volume of 20 μl per reaction, which contained 0.5 μl of Rox (50×), 0.4 μl of Taq polymerase, 0.03 μl of UNG (1 U/μl), 1 μl each of the forward and reverse primers (10 μM), 2 μl each of the forward and reverse control primers (10 μM), 1 μl each of the C1494T and A1555G probes (10 μM), 2 μl of control probe (10 μM) and PCR Buffer. Five microliters of DNA samples (2 ng/μl) were then added to each reaction to a final volume of 25 μl. Thermal cycling conditions were 37 °C for 5 min, 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 1 min. PCR data were analyzed using ABI 7500 Software v2.0 (Applied Biosystems) for plasmid DNA samples (This software was need to be calibrated through ROX fluorescence, and the fluorescence cannot be reflected in the figures because of being added to the solution, so there are only three colors in the figures.), and by the DeafTest_PCR Software (Capitalbio, Beijing, China) for genomic DNA samples.

2.5. Mutation confirmation by Sanger sequencing

Real-time PCR-based genotyping results were confirmed by Sanger sequencing in an ABI 3730xl DNA Analyzer using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. The primers used for sequencing were the pGEMT-vector common primers SP6 and T7, the forward primer 5′-CCCTGATGAAGGCTACAAAG-3′ and the reverse primer 5′-TGGCTAAGGTTGTCTGGTAG-3′. The resultant sequence data were compared with the complete genome of *Homo sapiens* mitochondria (GenBank accession number: NC_012920.1) for the presence of mutations. Control samples were also sequenced.