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Original Article

Analysis of c.269T>C, c.35G>T and c.109G>A variant of GJB2 by high resolution melting approach combined with dried blood spot

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Abstract: Genetics accounts for approximately half of the cases of hearing impairment worldwide, it has previously been reported that c.269T>C, c.35G>T and c.109G>A variant of GJB2 are linked to non-syndromic hearing loss, therefore, an simplicity, high sensitivity and low cost detection is calling for clinic. Here, we established a High Resolution Melting (HRM) combined Dried Blood Spot (DBS) approach to screen for c.269T>C, c.35G>T and c.109G>A mutations of GJB2. In this study, we identified c.269T>C, c.35G>T and c.109G>A mutations in 400 non-syndromic hearing loss children by HRM using Bio-Rad CFX 96 melt curve analysis system, then all the samples were sent for direct DNA sequencing. The results of established method and DNA sequencing showed 100% concordance for c.269T>C and 99.75% concordance for c.35G>T and c.109G>A. Our results strongly prove that established HRM combined DBS approach is a reliable, sensitive simplicity and low-cost method for screening c.269T>C, c.35G>T and c.109G>A variant of GJB2 in diagnosis.

Keywords: c.269T>C, c.35G>T, c.109G>A, GJB2, HRM

Introduction

Hearing loss is the most common sensory deficit in developed countries, one in 1000 children is born profoundly deaf, and the prevalence of hearing impairment rises significantly with age to include 2.70/00 before age five and 3.50/00 before adolescence [1]. It is widely recognized that undetected hearing loss has profound implications for development of speech and language in infants and young children [2]. With the development of newborn hearing screening, more congenital hearing loss had been detected and intervened to avoid the developmental sequelae associated with late-diagnose hearing loss. Unlike congenital hearing impairment, early detection of the postnatal childhood impairment remains a challenge, especially late-onset and progressive hearing loss.

Recent advances provide ample evidence that genetics accounts for approximately half of the cases of hearing impairment worldwide [3]. More than 140 chromosomal loci is associated to Hereditary hearing loss (HHL), and approximately 60 genes so far identified as causative [4]. Diffusion of small molecules is a fundamental mechanism for the hearing loss process, gap junctions are the main system involved in ion homeostasis in the cochlea, and they restore the correct ion concentration in the endolymph after the activation of the signal transmission in response to acoustic stimuli. A lot of genes involved in ion homeostaisis have been shown to cause hearing loss (connexins, KCNQ4, SLC26A4, SLC26A5, TRIC, CLND14, CRYM, TMPRSS3) but a major role is due to GJB2, that explains more than the 80% of the recessive forms of HHL [5].

Until now many mutations in GJB2 gene have been identified, some of them are very common but some are rare. It has been documented that c.269T>C (L90P), c.35G>T (G12V) and c.109G>A (V37I) variant of GJB2 can be responsible for nonsyndromic hearing loss, and the three mutations are highly prevalent, c.109G>A

Table 1. Primers used to amplify c.269T>C, c.35G>T and c.109G>A

Gene		Sequence
c.269T>C	Forward primer 1	CTGCAAGAACGTGTGCTACGA
	Reverse primer 1	TCGATGCGGACCTTCTG
	Forward primer 2	TCCACGCCAGCGCTCC
	Reverse primer 2	CCACGTGCATGGCCACT
c.35G>T	Forward primer 1	GCTTGCTTACCCAGACTCAGA
	Reverse primer 1	GGATCATAATGCGAAAAATGAA
	Forward primer 2	CACGCTGCAGACGATCCTGGGGG
	Reverse primer 2	GCTGGTGGAGTGTTTGTTCACA
c.109G>A	Forward primer 1	GACACGAAGATCAGCTGCA
	Reverse primer 1	GTTCCTGTGTTGTGTGCATT
	Forward primer 2	CCTCCTTTGCAGCCACAA
	Reverse primer 2	TTTTTCGCATTAGGATCCTC

has a carrier rate of 11.6% in Taiwan, another two mutations c.269T>C and c.35G>T are common reported in hearing loss patients [6]. A recent study has shown that the c.109G>A, c.35G>T and c.269T>C were associated with mild-to-moderate hearing loss [5, 7], hence, screening these mutations in newborns may help clinicians to diagnosis HHL.

High-resolution melting analysis (HRM) is a highly powerful technical for SNP genotyping, mutation scanning and sequence scanning in DNA samples, it can characterize nucleic acid samples based on their disassociation behavior and detects small sequence differences in PCR amplified sequences, just by direct melting. The advantages of HRM include cost effective, simple, fast and low reagent consumption [8, 9]. Besides, dried blood spot (DBS) offers a number of advantages such as simple collection, less invasive, requires a smaller blood volume, easier transport and storage over whole blood collection techniques [10]. In this study, we established a reliable, sensitive simplicity and low-cost detection methods of c.269T>C, c.35G>T and c.109G>A for newborn hearing screening by HRM analysis combined with DBS technique, and 400 DBS samples were collected to evaluated the efficiency and the reliability of this method.

Materials and methods

Blood spotting and DBS DNA extraction

Blood samples were collected from 400 Chinese children (1-12y) with non-syndrome deafness from Children's Hospital of Fudan University, Shanghai, China. DBS samples were made by spotting 30 μL of whole blood standards onto the center of pre-printed circle on filter paper (Whatman 903, Dassel, Germany) using a calibrated pipette. The samples were left overnight to dry at room temperature. When required, DBS samples were stored in sealed plastic minibags until analysis.

For DNA extraction of DBS, 6 mm diameter spot was punched out using a generic hole puncher, and was put into a 2.0 mL polypropylene centrifuge tube, it was spike by adding 20 μl mixed formaldehyde and acetone (1:1, v/v) followed by 60°C water bath for 10-15 min, then the sample was perfomed with 50 μL TE buffer (10 mM Tris-Hcl 1 mM EDTA pH=8.0) at 95°C for 20 min, 60°C for 10 min, 95°C for 20 min again and 60°C for 10 min again. Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) was used to measure the OD 260/280 ratio of each DNA samples.

PCR amplification and HRM protocol

Assays were performed in 96 well plates. Amplification of c.269T>C, c.35G>T and c.109G>A were performed by two-round nested PCR using a Bio-Rad CFX 96 Real-Time PCR system (Bio-Rad, USA), primers as shown in **Table 1**. In this experiments, LC Green Plus and LightScanner system (Idaho Technology Inc, USA). The first round of the two-round nested PCR protocol is One cycle of 95°C for 2 min; 40 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s; One cycle of 72°C for 5 min. The first round PCR products were denatured at 94°C for 1 min and cooled to 15°C to form heteroduplexes. The second round of the two-round nested PCR is the HRM analysis. HRM protocol was set as follows: One cycle of 95°C for 2 min; 40 cycles of 95°C for 10 s, 60°C for 10 s; HRM analysis of all samples was undertaken postrun by incubation at 50°C for 20 s followed by ramping from 60 to 95°C, with fluorescence data acquisition at 0.2°C increments every cycle.

DNA sequencing

In attempt to validate the results of HRM, we analyzed 400 samples using both HRM analy-

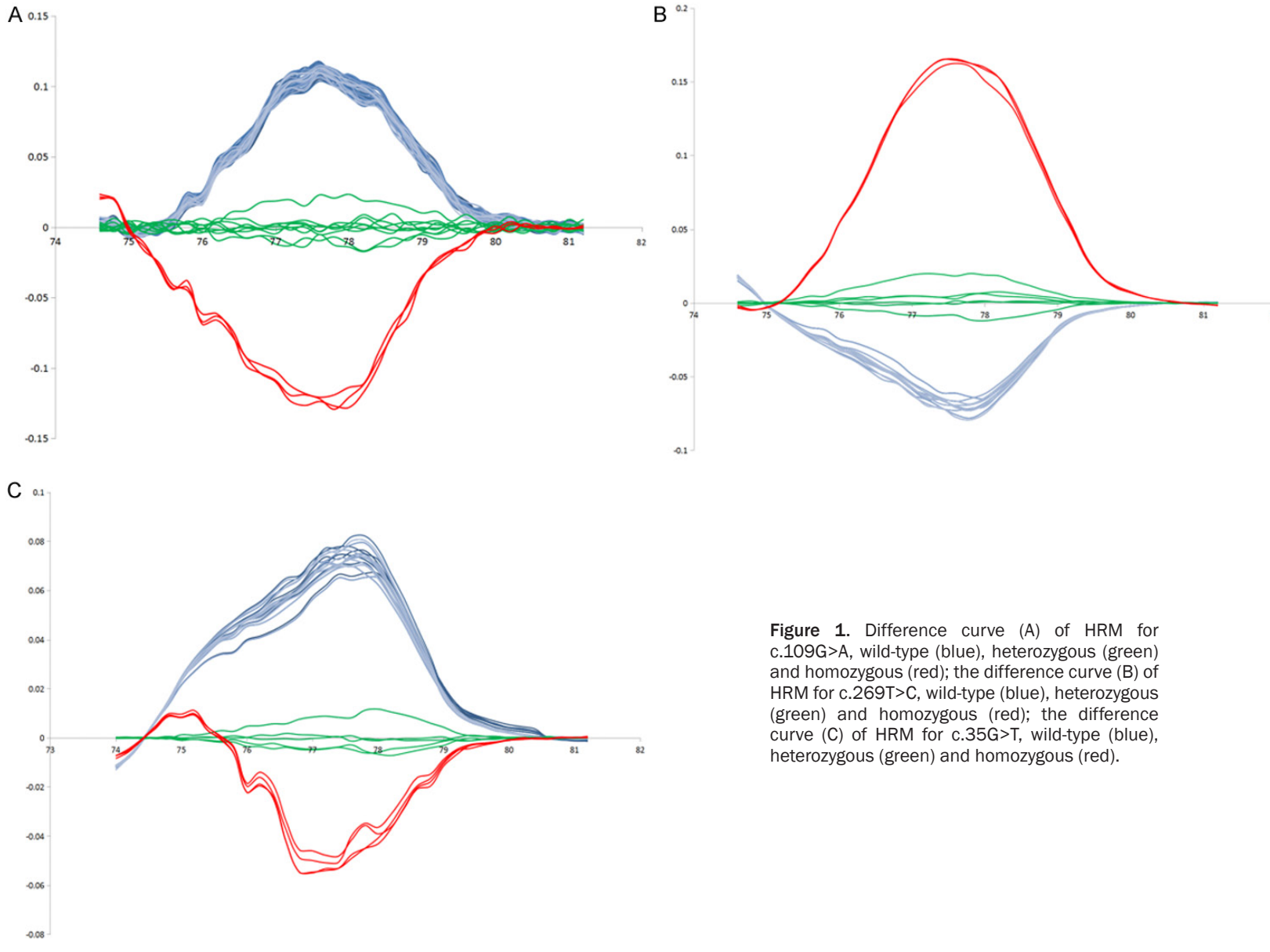

Figure 1. Difference curve (A) of HRM for c.109G>A, wild-type (blue), heterozygous (green) and homozygous (red); the difference curve (B) of HRM for c.269T>C, wild-type (blue), heterozygous (green) and homozygous (red); the difference curve (C) of HRM for c.35G>T, wild-type (blue), heterozygous (green) and homozygous (red).

sis and Sanger sequencing. The primer sequences of Sanger sequencing were GTAAAACGACGGCCAGTTCGTTGTGGCTGCAAAGG (M13 plus forward) and GTACATGAAGGCGGCTTCG (reverse) for c.269T>C, GTAAAACGACGGCCAGTGGTTCTGTCTTCACCTGTTTTG (M13 plus forward) and CCTGGCTGCAGGGTGTT (reverse) for c.35G>T, GTAAAACGACGGCCAGTTTTATCTCCCCCTTGATGAA (M13 plus forward) and GACAAACTAAGTTGGTTCTGTCTTC (reverse) for c.109G>A. PCR reaction system was similar to PCR-HRM without LC Green Plus and thermal cycling was set as follows: One cycle of 95°C for 5 min; 40 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 30 s; One cycle of 72°C for 5 min. ABI 3730 automatic sequencer was used to sequenced PCR products, the sequencing primer is GTAAAACGACGGCCAGT (M13 forward).

Results

Homogeneity of DNA samples were improved after nested PCR amplication

A rapid and simple DNA extraction procedure from DBS was developed, but the problem was that the quality of DNA extracted from the DBS samples were average poor (Mean OD260/280 ratio of 1.639, 95% CI 1.572-1.681) and cannot meet the demand of HRM detection. Instead of choosing commercialize dried blood spot DNA isolation Kit, we developed a two round nested PCR approach to obtain higher OD260/280 ratio of DNA samples for HRM, and the OD260/280 ratio improved a lot (mean 1.804, 95% CI 1.794-1.821).

Comparison of HRM and Sanger sequencing

We firstly screened c.269T>C, c.35G>T and c.109G>A in 400 non-system hearing loss children using HRM described in the Methods section. As shown in **Figure 1**, homozygous, heterozygous and wild-type of c.269T>C, c.35G>T and c.109G>A can be automatically detected due to distinctive melting curves. Among 400 patients, 30 and 7 were detected as heterozygous and homozygous for c.109G>A, respectively, 10 and 3 were detected as heterozygous and homozygous for c.269T>C, respectively, 6 and 4 were detected as heterozygous and homozygous for c.35G>T, respectively. To assess the sensitivity and the specificity of established HRM method, all the 400 samples were re-screened by direct sequencing, and the results indicated 100% concordance between DNA sequence analysis and HRM for c.269 T>C, and 99.75% concordance between DNA sequence analysis and HRM for c.35G>T and c.109G>A, there was one sample was detected as wild-type by Sanger sequencing but as heterozygous by HRM for c.35G>T and c.109G>A.

Discussion

A genetic basis accounts for approximately half of the cases of hearing impairment worldwide, some forms are due to single genetic mutations. With c.269T>C, c.35G>T and c.109G>A of GJB2 playing a important role in hearing loss, it is critical to establish a sensitive, simplicity and low-cost technology for genetic based testing, especially for neonatal screening. With the benefits of simplicity, speed, high sensitivity and low cost, HRM meets these demands rightly [11]. With HRM, these and other applications are done using low-cost generic dyes where previously custom labeled probes such as TaqMan® or fluorescence resonance energy transfer (FRET) probes were required. HRM is thus a simpler and much more cost-effective way to characterize samples [12]. Although many laboratories use HRM for genotyping of common DNA variants and have shown successful clinical use [13, 14], few studies with application on HRM genotyping of c.269T>C, c.35G>T and c.109G>A of GJB2 were reported. In this study, we first established a fast, reliable, high-thoughput and inexpensive HRM method to screen patients for c.269T>C, c.35G>T and c.109G>A mutations.

In this study, we choose DBS methods to screen the samples, which offer a number of advantages such as simple collection, less invasive, require a smaller blood volume, easier transport and storage over whole blood, plasma or serum collection techniques, it first used for detection of phenylke in newborns by Guthrie et al. It has potential applications in many other fields like DNA saving, preclinical, pharmacokinetics, toxicokinetics and therapeutic drug monitoring [10, 15, 16]. But DNA quality was poor after it directly extracted from the DBS samples; hence we used the two-round nested-PCR amplication to minimize the difference among the initial DNA samples. Our results showed that the quality of PCR product increased significantly with the additional round

of PCR amplification to ensure the accuracy of results.

Though it is not 100% concordance with DNA sequencing when genotyping c.35G>T and c.109G>A with HRM, there was one sample was observed in concordance, the reason maybe is poor quality of PCR product (OD-260/280=1.312), this reminds us to strictly control the DNA quality. But it also proved be sensitive in c.35G>T and c.109G>A genotyping. The 100% concordance was observed in genotyping c.269T>C with HRM when compared with DNA sequencing results. wild-type, heterozygous and homozygous of c.35G>T and c.109G>A and c.269T>C were all founded in our study, in fact, the frequency was higher in our study than that in other studies of China, it cannot be seemed as epidemiologic data for the blood samples were not randomly collected from population or patients.

In summary, our study indicated that the established HRM method for c.269T>C, c.35G>T and c.109G>A of GJB2 were simplicity, fast, high sensitivity and low cost, it is suitable for the large-scale genetic testing.

Disclosure of conflict of interest

None.

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