Comparison of the Mutation Spectrum of Common Deafness–Causing Genes in 509 Patients With Nonsyndromic Hearing Loss in 4 Different Areas of China by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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BACKGROUND: The aim of this study is to compare the spectrum and frequency of GJB2, SLC26A4, GJB3, and MT-RNR1 mutations in 4 different areas of China.

METHODS: A total of 509 patients from Linyi, Xinxiang, Yichang, and Baise were enrolled in this study. Twenty of the most common mutation sites were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

RESULTS: Patients from Yichang (in central China; 22.50%) and Linyi (in eastern China; 23.55%) carried a greater proportion of the 5 most common GJB2 mutations compared with patients from the other 2 areas surveyed, namely Xinxiang (in the North China Plain) and Baise (in south-central China). In comparison, patients from Yichang (10.00%) and Xinxiang (12.94%) had a higher prevalence of the 11 most common SLC26A4 mutations. Interestingly, only 1 patient (1.47%) from Baise was confirmed to carry the c.1229C>T mutation of SLC26A4. Among the 20 mutation sites analyzed across these 4 genes, c.235delC in GJB2 and c.919-2A>G in SLC26A4 were the most common mutations across all 4 geographical regions. The c.235delC mutation in GJB2 was significantly more prevalent in Yichang (18.13%) and Linyi (18.31%) than in the other 2 areas, whereas the c.919-2A>G allele of SLC26A4 was present at much higher frequencies in Yichang (8.44%) and Xinxiang (8.74%) compared with the other areas.

CONCLUSIONS: This study increases the available data on hearing loss-associated mutations and provides evidence of the need for risk assessment and genetic counseling of populations from these 4 areas of China.

KEYWORDS: Gene arrays, hereditary, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), mutation spectrum, nonsyndromic hearing loss (NSHL),

INTRODUCTION

Globally, hearing loss is the most common sensory disorder, with the incidence of hearing loss among children thought to be approximately 1/1000.1 Hearing loss is also the most common disability in China, with 28 million individuals suffering from hearing loss accounting for one-third of the total disabled population. According to the Chinese Birth Defects Prevention Report issued in 2012,2 35,000 new cases of congenital hearing impairment are reported each year, including hearing loss caused by genetic factors (accounting for 60% of the total), late-onset hearing loss, and drug-induced hearing loss.
Hereditary hearing loss is divided into syndromic hearing loss and nonsyndromic hearing loss (NSHL). NSHL involves the partial or total loss of hearing that is not associated with other signs and symptoms. NSHL accounts for 60%-70% of inherited hearing impairments. More than 110 genes (>170 loci) have been causally implicated in NSHL (http://hereditaryhearingloss.org/). Approximately 77%-93% of NSHL cases involve autosomal recessive loci, whereas 10%-20% of cases involve autosomal dominant loci. The remaining cases are related to X-linked genes or mitochondrial inheritance. However, many of the genes involved in the pathogenicity of hearing loss have not yet been identified. The high heterogeneity of hearing loss-related genes presents a major challenge to the clinical genetic diagnosis of hereditary hearing loss. Further, the genetic spectrum of hearing loss in 1 geographic region can differ from that in other areas.

To date, studies have shown that the genes most closely related to genetic hearing loss in China are GJB2, SLC26A4, MT-RNR1, and GJB3. China is the most populous country in the world, and people from different regions often have different genetic backgrounds because of geographical separation. The aim of this study is to elucidate the genetic factors associated with hearing loss in 4 regions of China: Linyi, Xinxiang, Yichang, and Baise. Linyi is located in southern Shandong Province, eastern China. Xinxiang is a city located in the north of Henan Province, located in the North China Plain, north of the Yellow River. Yichang is located in southwestern Hubei Province, which is located in central China. Baise is the westernmost city of the Guangxi Zhuang Autonomous Region, which is in south-central China. All 4 cities are relatively conservative areas with low migration rates and more limited communication compared with other regions of China because of their remote geographical locations. Therefore, the mutation spectra of hearing loss genes in these regions display unique characteristics. To the best of our knowledge, no previous studies have been performed in these areas to compare their mutation spectra of hearing loss-causing genes.

A total of 509 patients with NSHL were recruited from the 4 areas studied in China. After analyzing 20 mutation sites across 4 genes (GJB2, SLC26A4, MT-RNR1, and GJB3) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), we compared the gene mutation spectra of the four different populations. Comprehensive genetic analysis of patients with NSHL in different areas of China would help to clarify important NSHL-associated genetic factors in China and would provide additional epidemiological information to aid in effective genetic testing.

**MATERIALS AND METHODS**

**Patients and Collection of Blood Samples**

All 509 patients were diagnosed with NSHL and were recruited from four regions of China: Linyi, Xinxiang, Yichang, and Baise. The patient cohort consisted of 265 males and 244 females, with an age range of 3-25 years. No related systemic findings were identified by careful medical examination or medical history for any of the 509 patients with NSHL. Patients with obvious external auditory or middle-ear malformations, or other medical conditions related to syndromic hearing loss such as retinal dystrophy, were excluded from the study. Basic demographic and medical information were obtained for each patient, including their name, age, address, and family history of hearing loss, as well as clinical information regarding their hearing loss, such as the age of onset and the use of aminoglycoside antibiotics. Pure-tone audiometry (PTA), otoscope examination, and tympanometry were performed for audiological evaluation. Hearing was measured in decibels (dB). The severity of hearing loss was graded as mild (26-40 dB), moderate (41-55 dB), moderately severe (56-70 dB), severe (71-90 dB), or profound (>90 dB).

Informed consent to undergo analysis of hearing loss-causing genes was obtained from each participant. In the case of minors, written consent was obtained from their parents/guardians on their behalf. The study was performed with the approval of the Ethics Committees (No. 2016-067-1, November 2016-November 2018). The study conformed to the principles of the Declaration of Helsinki.

Whole-blood samples were obtained from patients with NSHL and treated with the anticoagulant ethylenediaminetetraacetic acid disodium salt. Collected blood samples were immediately placed on ice and stored at −80°C until further analysis.

**Genomic DNA Extraction and Multiplex Polymerase Chain Reaction (PCR) Analysis**

Genomic DNA was extracted from the leukocytes of 2 mL of peripheral blood using a Blood DNA Extraction Kit (Baio, Shanghai, China). Multiplex PCR was performed in 5-µL reaction volumes containing 0.5 µL of 10x PCR buffer with 20 mM MgCl₂, 0.4 µL of 25 mM MgCl₂, 0.1 µL of 25 mM dNTP mix, 1 µL of 0.5 µM primer mix, 0.2 µL of 5 U/µL PCR enzyme, and 2.8 µL of extracted DNA sample. PCR primers are summarized in Supplementary Table S1. The PCR protocol was as follows: initial denaturation at 95°C (2 min), followed by 45 amplification cycles under the following conditions: denaturation at 95°C (30 s), annealing at 56°C (30 s), and elongation at 72°C (60 s), with a final elongation at 72°C for 5 min.

The resulting PCR products were then treated with shrimp alkaline phosphatase (SAP) to remove excess dNTPs. A 2-µL volume of SAP reaction buffer (1.53 µL of HPCL-grade water, 0.17 µL of 10× SAP buffer, and 0.30 µL of 1.7 U/L SAP enzyme) was added to each multiplex PCR sample in the reaction plate and gently mixed. Sample plates were then centrifuged at 1000 rpm prior to incubation. The SAP reaction was performed at 37°C (40 min) and then at 85°C (5 min).

**iPLEX Genotyping Reaction**

A 2-µL volume of iPLEX reaction cocktail (0.619 µL of HPCL-grade water, 0.2 µL of 0.222× iPLEX buffer, 0.2 µL of 1× iPLEX termination mix, 0.94 µL of 0.84/1.04/1.57 µM extend primer mix, and 0.041 µL of 1× iPLEX enzyme) was added to each well of the multiplex PCR assay plate. The iPLEX reaction was performed as follows: initial denaturation at 94°C (30 min), followed by 40 amplification cycles of denaturation at 94°C (5 s), and five cycles each of annealing at 52°C (5 s) and elongation at 80°C (5 s), followed by a final elongation at 72°C (3 min). The mixture was then purified by treatment with a cationic resin to remove salts that accumulated during the multiplex PCR and iPLEX reactions.

**Mutation Analysis by MALDI-TOF MS**

For MALDI-TOF MS, 0.75 µL of each multiplex PCR product was spotted onto a Spectrochip and ionized on a Sequenom MassARRAY device (Bioyong Technologies Inc., Beijing, China). To analyze real-time
mass spectra, and for primary processing and documentation of the experimental results, MassARRAY TYPER 4.0 (Agena Bioscience, Inc., San Diego, Calif, USA) was used. Several samples were sequenced using the Sanger chain-termination method with fluorescently labeled terminators of each single nucleotide polymorphism (SNP), followed by capillary gel electrophoresis using ABI PRISM 3730 (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, Mass, USA) for genotype verification. Overall, 134 samples were verified by Sanger sequencing. No differences in the genotype characteristics obtained by MALDI-TOF MS and direct sequencing were detected, and the results were highly consistent. In total, we screened 20 mutation sites across the 4 genes (GJB2, SLC26A4, GJB3, and MT-RNR1; Table 1).

Table 1. Hearing Loss-Causing Mutations in GJB2, SLC26A4, MT-RNR1, and GJB3

| Gene    | Mutation Sites                                                                 |
|---------|-------------------------------------------------------------------------------|
| GJB2    | c.235delC, c.299_300delAT, c.176_191del16, c.167delT, c.35delG                |
| SLC26A4 | c.919-2A>G, c.2168A>G, c.1229C>T, c.281C>T, c.1174A>T, c.1226G>A, c.1975G>C, c.589G>A, A, c.1707+5G>A, A, c.2027T>A, A, c.2162C>T |
| MT-RNR1 | m.194C>T, m.1555A>G                                                          |
| GJB3    | c.538C>T, c.547G>A                                                            |

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Statistical Analysis
All data were analyzed using GraphPad Prism 6 (GraphPad Software Company, La Jolla, Calif, USA) and are presented as mean ± standard deviation of the mean for all experiments. A contingency table chi-square test was used to compare the frequencies of mutations among different areas. Statistical significance was assumed at P < .05.

RESULTS

Patients and general characteristics
The average age of the 509 NSHL patients was 13.74 ± 5.74 years. The cohort comprised 244 females (47.94%) and 265 (52.06%) males. Of these patients, 168/509 had hearing loss that was present at birth. The remaining patients had hearing loss that was present before the age of 6 years. Hearing tests demonstrated that all 509 patients presented severe to profound bilateral NSHL (Table 2). There was no significant difference in the sex ratio among the 4 patient groups or in the ethnic distribution of patients from Yichang, Linyi, and Xinxiang. Most of the examined patients from these 3 regions were Han Chinese, whereas all the patients from Baise were Zhuang Chinese.

Mutation Analysis of GJB2
In this study, 12 different allelic combinations were detected for GJB2. None of the patients from Baise were found to have any mutations in GJB2. Patients from Yichang (22.50%, 72/320) and Linyi (23.55%, 81/344) had the greatest prevalence of the most common mutations of GJB2 (5 mutations considered). In Yichang, 21 patients were homozygous for the c.235delC allele, with 2 of these patients also carrying a monoallelic mutation in one other NSHL-associated genes (c.547G>A of GJB3 or c.919-2A>G of SLC26A4) (Table 3). Eleven patients were compound heterozygotes, with 2 different allelic combinations observed: c.235delC and c.299_300delAT (n = 10) and c.176_191del16 and c.235delC (n = 1). Eight patients had a monoallelic mutation in GJB2. In Linyi, we identified 18 homozygotes displaying 2 different allelic combinations: c.235delC (n = 17) and c.299_300delAT (n = 1). Another 11 patients were found to be compound heterozygotes of GJB2, while 23 patients had a monoallelic mutation of this gene (this was combined with a monoallelic mutation of one other NSHL-associated gene in 2 patients). In Xinxiang, 9 patients were homozygous for the c.235delC allele, whereas 5 patients were compound heterozygotes. Thirteen patients were heterozygotes with a single mutant GJB2 allele (this was combined with a monoallelic mutation of c.2168A>G in 1 patient).

The most common mutant GJB2 allele in patients from Linyi, Yichang, and Xinxiang was c.235delC (Table 4). The allele frequencies for c.235delC in Yichang (18.13%, 58/320) and Linyi (18.31%, 63/344) were higher than that calculated for patients from Xinxiang (11.54%, 33/286) (P = .037). The allele frequencies for the c.299_300delAT and c.176_191del16 mutations were similar.

Mutation Analysis of SLC26A4
Patients from Yichang (10.00%, 32/320) and Xinxiang (12.94%, 37/286) had a higher prevalence of the most common mutations of SLC26A4 (11 mutations considered).

In Yichang, 7 patients were homozygous for the c.919-2A>G allele (Table 5). Four patients were compound heterozygotes, with 4 different allelic combinations identified. Another 10 patients had a monoallelic mutation of SLC26A4. In Linyi, 4 patients were homozygous for the mutant allele c.919-2A>G, and 4 were compound heterozygotes of SLC26A4 (1 of these patients had the mutant allele c.235delC of GJB2 combined with c.589G>A and c.1229C>T of SLC26A4). In total, 6 patients had a monoallelic mutation of SLC26A4, with 1 patient being homozygous for the c.235delC allele of GJB2 and carrying the monoallelic mutation, c.919-2A>G. In Xinxiang, there were 6 homozygotes (c.919-2A>G) and 8 compound heterozygotes who contributed 5 different allelic combinations. Another 9 patients had a monoallelic mutation of SLC26A4, with 1 patient carrying both of
the monoallelic mutations c.2168A>G and c.299_300delAT. Finally, in Baise, we only identified 1 heterozygote (1.47%, 1/68) with a mutation in SLC26A4 (c.1229C>T).

The most common SLC26A4 mutant allele in these regions was c.919-2A>G (Table 6). The allele frequencies of c.919-2A>G in Xinxiang (8.74%, 25/286) and Yichang (8.44%, 27/320) were significantly higher than that calculated for patients from Linyi (3.78%, 13/344) (P = .019).

Mutation Analysis of MT-RNR1
Among 509 patients, 11 carried the mutation m.1555A>G, whereas 2 carried m.1494C>T. Only 1 of the patients had the heteroplasmic m.1555A>G mutation. A similar mutation frequency at the MT-RNR1 locus was observed among the Yichang (2.50%, 4/160), Linyi (4.65%, 8/172), and Xinxiang (0.7%, 1/143) patient groups (χ² = 4.632, P = .099). None of the patients from Baise carried mutations in MT-RNR1.

Mutation Analysis of GJB3
One patient from Linyi had a monoallelic mutation of c.538C>T and 2 patients from Yichang had a monoallelic mutation of c.547G>A (1 of these 2 patients had a combined homozygous mutation of c.235delC). A similar allele frequency in GJB3 was observed in patients from Linyi and Yichang (χ² = 0.412, P = .521). No c.538C>T and c.547G>A mutations were detected in patients from Xinxiang or Baise.

**DISCUSSION**

**MALDI-TOF MS-Based SNP Detection**
Many of the genotyping methods currently used to detect SNPs, including direct sequencing, PCR–restriction fragment length polymorphism analysis, and microarray analysis, are not suitable for the genetic screening of a large number of samples because of assay complexity and lengthy times to result. Instead, in this study, we analyzed mutations in NSHL using MALDI-TOF MS analysis. MALDI-TOF MS, with its ability to perform multiplexed assays in a single reaction, is a high-throughput, cost-saving approach to SNP screening. Notably, we found that the MALDI-TOF MS results were highly consistent with the Sanger sequencing results. Many other studies have also demonstrated the advantages of MALDI-TOF MS-based SNP detection.

**GJB2**
In 1997, GJB2 became the first gene reported to be associated with hearing loss, exhibiting an autosomal recessive inheritance pattern. GJB2 encodes connexin 26 (Cx26), an important protein in gap junctions in the developing cochlea. Mutations in GJB2 are responsible for up to 50% of cases of recessive hearing loss. The clinical manifestations of mutations in this gene involve severe congenital sensorineural hearing loss. In this study, the prevalence rates of GJB2 mutations in Linyi (30.23%) and Yichang (25.00%) were higher than those reported previously in most other areas of China, even though only 5 of the most common mutations of GJB2 were considered in this study.

The c.235delC mutation is reportedly the most common GJB2 mutation in Asians, with much lower frequencies of this mutation...
observed in Europe, the United States, and Oceania. Here, consistent with the findings of these previous studies, c.235delC was found to be the most common pathogenic mutation of GJB2. The rates of this mutation in patients from Yichang (18.13%) and Linyi (18.31%) were higher than those reported in most other cities in China, including Tengzhou (12.5%) and Wenzhou (14.6%). Various investigators have reported that the c.35delG mutation is the predominant mutation in GJB2 in many ethnic groups worldwide, accounting for 85% of all mutations in GJB2. Mean carrier frequencies of the c.35delG mutation were shown to be 1.89, 1.52, 0.64, 1, and 0.64 for European, American, Asian, Oceanian, and African populations, respectively. Interestingly, none of the patients included in this study carried the mutations c.35delG or c.167delT. Thus, our study revealed the differences in GJB2 mutant alleles in different areas of China, and these findings are consistent with those of a previous study.

SLC26A4
SLC26A4 encodes a transmembrane anion exchanger. Mutations in SLC26A4 are the most common detectable causes of enlarged vestibular aqueduct (EVA) and Pendred syndrome, with the restoration of SLC26A4 expression and function reducing or preventing fluctuations in hearing in EVA patients. According to previous reports, the prevalence of SLC26A4 mutations varies among different ethnic groups, with highly prevalent mutations differing between groups. For example, c.919-2A>G is the predominant mutation in Taiwan and mainland China, whereas p.H723R is more common in Japan.
However, these mutations are rare among populations with European ancestry and among deaf patients living in the United States.23 In contrast, various SLC26A4 mutations have been found in Chinese patients, among which c.919-2A>G and 2168A>G are the most prevalent.24,25 We found that the most common SLC26A4 mutant allele in our cohort was c.919-2A>G. Allele frequencies in Xinxiang (8.74%) and Yichang (8.44%) were close to that observed in a large Chinese deaf population,26 whereas a lower frequency was observed in Linyi.

The frequency of the c.2168A>G mutation among patients in our cohort differed from frequencies recorded in Tengzhou (2.88%), northern China (3.7%), Japan (4.10%), and Korea (10.34%).27 These differences in Asian countries and elsewhere might be explained by ethnic and environmental factors.

MT-RNR1

Mutations in MT-RNR1 change the structure of the mtDNA, resulting in the formation of new aminoglycoside antibiotic-binding sites that lead to drug-induced hearing loss. The m.1555A>G and m.1494C>T mutations are the most common in MT-RNR1. In our study, MT-RNR1 mutations occurred at frequencies of 4.65% in Linyi and 2.5% in Yichang, the latter being similar to that reported for Tengzhou (2.60%) (Ma et al. 2016). The frequency of 17.40% previously reported for Wenzhou was significantly higher than that recorded in our cohort.8

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Table 6. Variations in the Frequencies of SLC26A4 Mutations in Patients from Yichang, Linyi, and Xinxiang

| Nucleotide | Yichang (320) | Linyi (344) | Xinxiang (286) | Statistical Analysis |
|------------|---------------|-------------|----------------|----------------------|
| Change     | Number(n)     | Frequency (%) | Number(n)     | Frequency (%) | Number(n)     | Frequency (%) | χ²  | P     |
| c.919-2A>G | 27            | 8.44         | 13            | 3.78       | 25            | 8.74         | 7.960 | .019  |
| c.2168A>G  | 0             | 0.00         | 5             | 1.45       | 5             | 1.75         | 5.264 | .072  |
| c.1229C>T  | 1             | 0.31         | 1             | 0.29       | 0             | 0.00         | 0.878 | .645  |
| c.281C>T   | 0             | 0.00         | 0             | 0.00       | 1             | 0.35         | -     | -     |
| c.1174A>T  | 1             | 0.31         | 0             | 0.00       | 2             | 0.70         | -     | -     |
| c.1226G>A  | 0             | 0.00         | 1             | 0.29       | 1             | 0.35         | -     | -     |
| c.1975G>C  | 0             | 0.00         | 1             | 0.29       | 1             | 0.35         | -     | -     |
| c.589G>A   | 1             | 0.31         | 1             | 0.29       | 0             | 0.00         | -     | -     |
| c.1707+5G>A| 1             | 0.31         | 0             | 0.00       | 0             | 0.00         | -     | -     |
| c.2027T>A  | 0             | 0.00         | 0             | 0.00       | 2             | 0.70         | -     | -     |
| c.2162C>T  | 1             | 0.31         | 0             | 0.00       | 0             | 0.00         | -     | -     |
| Total      | 32            | 10.00        | 22            | 6.40       | 37            | 12.94        | 7.816 | .020  |

*Statistical significance was assumed at P < .05.

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Figure 1. Geographic distribution and proportions of the NSHL-associated mutant alleles studied in four regions of China.
The m.1555A>G mutation was previously detected in 0.7% of German patients, 1.8% of Hungarian patients, 2.4% of Polish patients, and 4.5% of Syrian patients. Studies from different regions of China have reported m.1555A>G mutation frequencies in NSHL populations ranging from 0.26% to 17.00%. In the current study, the observed frequencies of 0.70%-3.48% were lower than those previously reported for patients from northern China and Wenzhou.

GJB3
Gap junction channels are made up of connexin protein subunits, which are encoded by a multigene family that includes GJB3. GJB3 encodes gap junction protein 31 (Cx31), and mutations in GJB3 may be responsible for bilateral high-frequency hearing impairment. Mutations in GJB3 were also shown to underlie an autosomal dominant form of NSHL in Chinese patients. Common mutations in GJB3 include c.538C>T and c.547G>A. Our study showed a low mutation rate in GJB3 consistent with that reported in previous studies.

Overall Analysis of Our Cohort
We noted significant differences in the frequency of hearing loss-causing mutations between the population from Baise and those from the other 3 areas of China. The patients from Baise were generally Zhuang Chinese, whereas the patients from the other areas were Han Chinese. We, therefore, hypothesize that ethnic factors might have been an important factor in the higher prevalence of certain mutations in patients with NSHL from Baise. However, enrolled NSHL patients in Baise were relatively smaller (n = 34) compared with other regions. This is a limitation in this study, and more patients should be enrolled to confirm the above conclusions.

However, one major limitation of this study was that while the method used can generally identify the genetic cause of NSHL at a reasonable cost, it limits the identification of uncommon variants or variants of uncertain significance. Thus, it may not be appropriate for the identification of hearing loss-causing gene variants in certain NSHL populations that are genetically distinct, such as the patients from Baise. In the future, we plan to screen other hearing loss-causing gene mutation panels using other analytical methods to identify hearing loss-causing gene mutations in Baise.

Further, some patients with single heterozygous mutations of GJB2, SLC26A4, and GJB3 were identified in this study. Among patients with monoallelic mutations, we identified 5 variable mutation combinations: c.235delC of GJB2 combined with c.589G>A and c.1229C>T of SLC26A4, c.235delC of GJB2 combined with c.919-2A>G of SLC26A4, c.919-2A>G of SLC26A4 combined with the homozygous c.235delC mutation of GJB2, c.547G>A of GJB3 combined with the homozygous c.235delC of GJB2 mutations, and c.2168A>G of SLC26A4 combined with c.299-300delAT of GJB2. In many previous genetic screening studies, some deaf patients with 1 causative recessive mutation were revealed using methods such as Sanger sequencing and the SNPscan technique, among others. Therefore, other gene mutations may have caused hearing loss in patients with monoallelic mutations.

This study showed different frequencies of mutation spectra among the 4 areas studied in China (Figure 1). We also noted different mutation spectra within each gene among the different areas, which warrants further study. Given the variation in geographical environments, ethnic factors, and genetic factors, other hearing loss-causing gene mutation panels may be useful for studies using populations from different regions.

CONCLUSION
Our study revealed regional differences in the most common alleles of GJB2, SLC26A4, MT-RNR1, and GJB3 in different parts of China. These findings increase the amount of available data on hearing loss-causing mutations and provide additional data for risk assessment and genetic counseling for populations from these areas of China.

Ethics Committee Approval: The Ethics Committees of the China Rehabilitation Research Center (No. 2016-067-1, 11.2016-11.2018).

Informed Consent: Informed consent to undergo analysis of hearing loss-causing genes was obtained from each participant. In the case of minors, written consent was obtained from their parents/guardians on their behalf.

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Conflict of Interest: The authors have no conflict of interest to declare.

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