Prevalence of Mutations in *GJB2*, *SLC26A4*, and *mtDNA* in Children with Severe or Profound Sensorineural Hearing Loss in Southwestern China

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Aim: To study the distribution characteristics of common mutations in the *GJB2*, *SLC26A4*, and *mtDNA* genes in children with severe or profound sensorineural hearing loss (SNHL) in southwestern China. **Materials and Methods:** A total of 1,164 individuals were recruited to screen for the common *GJB2*, *SLC26A4*, and *mtDNA* mutations by microarrays. Subsequencing for the coding region of the *GJB2* gene in the samples without the *GJB2* hotspot mutations as well as subsequencing for the exon 1 of the *TRMU* gene in those samples with the *mtDNA* hotspot mutations was performed by Sanger sequencing. All mutations were analyzed in association with medical imaging. **Results:** In this study, 28.43% of all subjects carried mutations. The mutation frequencies in the *GJB2*, *SLC26A4*, and *mtDNA* genes were 17.27%, 7.04%, and 4.12%, respectively. No *TRMU* mutation was found in the study. The frequency of the *mtDNA* mutations in the multiethnic minorities was six times that in the Han (11.23% vs. 1.91%; p approaches 0.000) and in the urban group was one-third of that in the suburban group (1.49% vs. 4.47%; $p=0.047$). The frequency of the *GJB2* mutations in urban and suburban groups was 23.38% and 15.99%, respectively ($p=0.012$). The enlarged vestibular aqueduct (EVA) was the most common inner ear malformation and ~79.10% of EVA cases were associated with the *SLC26A4* mutations. **Conclusions:** More than one-fourth of children with severe or profound SNHL carried the common deafness mutations. The proportions of ethnic minorities and urban subjects could impact the frequency of the *GJB2* and *mtDNA* mutations. The *SLC26A4* hotspot mutations are prevalent and correlate strongly with EVA.

Introduction

HEARING LOSS IS ONE of the most common diseases worldwide. Permanent congenital bilateral hearing loss of moderate or greater degree has a prevalence of about 1 to 2 per 1000 births. Furthermore, the prevalence can reach up to 2.7 per 1000 in 5-year-olds and 3.5 per 1000 during adolescence (Morton and Nance, 2006). This disease is an etiologically heterogeneous trait with genetic and environmental factors, both of which could be self-existent or coexistent.

A genetic factor is estimated to cause about two-thirds of prelingual nonsyndromic hearing loss cases (Petit *et al.*, 2001; Smith *et al.*, 2005). The *GJB2*, *SLC26A4*, and *mtDNA* genes are the three most common deafness genes and account for 40% of the patients with hereditary hearing loss (Li *et al.*, 2010a). The c.35delG, c.167delT, c.235delC, and c.427C>T (p.R143W) in the *GJB2* gene are the most prevalent mutations among Caucasians, Ashkenazi Jews, Asian, and African populations, respectively (Lee *et al.*, 2008), which support

the ethnic differences in the genetic pathogenesis of deafness. A characteristic *SLC26A4* mutation spectrum is identifiable in highly prevalent mutations, such as c.IVS7-2A>G and p.H723R in East Asian subjects, but is rare in European lineages (Dai *et al.*, 2009). Exposure to aminoglycoside antibiotics can cause hypersensitivity and ototoxicity in patients carrying the 1555A>G or 1494C>T mutation in the *mtDNA* gene, leading to severe or profound sensorineural hearing loss (SNHL). Aminoglycoside antibiotics are widely used to treat tuberculosis and bacterial infections in developing countries, including China, India, and Africa.

There are previous studies reporting the frequency of common genes in congenital SNHL, including patients with various degrees of hearing loss. However, the frequency of the three common genes in children with >70 decibel (dB) hearing loss and specifically examining the Chinese multiethnic minority background has not been evaluated in detail. There is little information about the underlying risk factors associated with the distribution of the common deafness mutations, such as the

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multiethnic minorities, urban residential ratio of the deaf population, and modifying factor of the *mtDNA* mutations.

Materials and Methods

Subject recruitment and clinical evaluation

A total of 1,164 children (under 13 years old) from 1,072 families with severe or profound SNHL were recruited at the second Xiangya Hospital of Central South University between 2005 and 2013. Six hundred fifty-eight of the subjects were males. All of the patients were from 10 ethnic groups in southwestern China, including 888 from Han Chinese and 276 from the 9 ethnic minorities (90 Miao, 80 Tujia, 54 Dong, 41 Zhuang, 4 Yao, 2 Tibetan, 2 Hani, 2 Hui, and 1 Bai) (Fig. 1a). Informed consent was obtained from all participants before the study in accordance with the Institutional Review Board and Ethics Committee of the second Xiangya Hospital of Central South University.

A complete history and physical examination was obtained from all subjects by seven otologists. Audiometry was assessed by pure-tone audiometry, auditory brainstem response, auditory steady-state response, immittance testing, and distortion product otoacoustic emissions according to their age. High-resolution thin-section CT (HRTSCT) and magnetic resonance imaging (MRI) of temporal bone were performed in the 828 subjects who had undertaken or had prepared to undergo cochlear implantation. This study was approved by the Ethics Committee of Second Xiangya Hospital of Central South University.

Genetic analysis

Genomic DNA for deafness gene analysis was obtained from peripheral blood samples of the patients and their lineal relatives after an informed consent was obtained.

Microarray

Hereditary hearing loss allele-specific polymerase chain reaction (PCR) and universal array (ASPUA; CapitalBio) were used to simultaneously screen 9 mutations causing hereditary hearing loss (*GJB2*: c.35delG, c.176 del16, c.235delC, c.299-300delAT; *GJB3*: c.538C>T; *SLC26A4*: c.IVS7-2A>G, c.2168A>G; *mtDNA*: m.1555A>G, m.1494C>T). Multiplex allele-specific PCR was performed as described previously (Qu *et al.*, 2012). The microarrays were scanned in a LuxScan TMHT 24 Microarray Scanner (CapitalBio) and the data were analyzed.

Sanger sequencing

Subjects, who had been identified to carry none of the mutant alleles of the *GJB2* gene by microarray, were further screened for the coding regions of the *GJB2* gene and exon 1 of the *TRMU* gene by Sanger sequencing in the remaining samples without the *GJB2* mutations or with the *mtDNA* mutations, respectively. The primers for both genes were the same as previously reported (Bae *et al.*, 2012). Amplified DNA fragments were purified with a gel extraction kit (Qiagen) and then sequenced by the ABI 377 sequencer. All PCR products were sequenced on both strands and analyzed with DNASTAR software. The data were compared with GenBank, RefSeq (*GJB2*, NC_000013.11; *TRMU*, NC_000022.11).

Statistical analyses

Data were subjected to statistical analyses using SPSS software (version13.0; SPSS, Inc.). The χ^2 test was performed for statistical comparison of the difference in the mutation frequency between the Han and multiethnic minorities groups or between urban and suburban areas. A *p*-value ≤ 0.05 is considered significant.

FIG. 1. The population distribution and proportion of children with severe or profound sensorineural hearing loss in southwestern China. (a) The ethnic proportion (Han & multiethnic minorities). Other groups in the multiethnic minorities included the Yao, Tibetan, Hui, Bai, and Hani ethnic groups; (b) the urban proportion in total; (c) the urban residential proportion in the Han and multiethnic minorities groups.

Results

Clinical information

A total of 1,164 subjects with severe or profound hearing loss in southwestern China were studied. Males accounted for 61.98%. The average age was 4.5 ± 1.3 years. The degree of hearing loss was divided into five groups (70–79, 80–89, 90–99, 100–110, and ≥ 110 dB), and the data were the average values from 500, 1000, and 2000 Hz (Fig. 2). Because patients with more severe hearing loss could have a priority opportunity for free cochlear implantation surgery, 93.56% (1089/1164) of the patients we collected had a profound SNHL in the study. The urban residential proportions in all subjects, Han, and multiethnic minorities groups were 17.27% (201/1164), 19.36% (172/888), and 10.51% (29/276), and there was a significant difference between the Han and multiethnic minorities groups ($\chi^2=11.575$; $p=0.01$) (Fig. 1b, c).

Medical imaging

Overall, about 10.63% (88/828) of the images examined before cochlear implantation were found to have a malformation in the inner ear by HRTSCT and MRI. The vestibular aqueduct was considered to be enlarged (EVA) if the diameter was > 1.5 mm at the midpoint between the common crus and the external aperture of the vestibular aqueduct. Mondini dysplasia was defined as a complex malformation presenting a hypoplastic coil of one and a half turns. Internal auditory meatus malformation included absence, narrowing (diameter < 2.0 mm or an anteroposterior/ventrodorsal distance < 2.0 mm), or enlargement (diameter > 8 mm). The possibility of cochlear nerve abnormality is considered if the diameter of the cochlear nerve canal < 1.4 mm, as previously

reported (Li *et al.*, 2014). EVA was the most common abnormality identified in this study (7.85%, 67/828). Mondini dysplasia presented as an isolated form (seven cases) or compounded with the EVA (two cases). The second most common form of the inner ear malformations was internal auditory meatus narrowing (with or without cochlear nerve hypoplasia), which was 13.64% (12/88) in all cases with a malformation of the inner ear (Fig. 3a).

Mutation frequencies of common deafness genes

In the study, about 28.43% (331/1164) of subjects carried one or more mutant alleles in the three common genes (*GJB2*, *SLC26A4*, and *mtDNA*) through screening by microarrays. The frequency of common mutations in the three genes in the Han group (27.92%, 248/888) was a little less compared with the multiethnic minorities group (31.07%, 83/276), but without a significant difference ($\chi^2=0.476$, $p=0.49$). Furthermore, the frequencies of these common mutations in the urban and suburban populations were 31.84% (64/201) and 27.52% (265/963), respectively ($\chi^2=1.532$, $p=0.216$). Only 1 case was found to carry a heterozygous c.538C>T in the *GJB3* gene compounded with a heterozygous c.235delC in the *GJB2* gene.

The frequency of the *GJB2* mutations was 17.27% (201/1164) in total, as 18.47% (164/888) in the Han group and 13.41% (37/276) in the multiethnic minorities group ($\chi^2=3.778$, $p=0.052$) (Fig. 4), while it was 23.38% (47/201) in the urban area and 15.99% (154/963) in the suburban area ($\chi^2=6.359$, $p=0.012$) (Fig. 5). The screening of the coding region of the *GJB2* gene was used and the variants are listed as pathogenic mutation, probable pathogenic mutation, polymorphism, synonymous, or novel variants in Table 1 according to the categories of the connexin-deafness homepage website and the NCBI dbSNP. The c.235delC was still the main mutation either in the Han group or in the multiethnic minorities group. The frequency of mutation or mutant allele was similar in the two groups.

The frequency of *mtDNA* 1555A>G or 1494C>T was 4.12% (48/1164), 1.91% (17/888), and 11.23% (31/276) in the total subjects, the Han group, and multiethnic minorities group, respectively, and all mutations displayed a homoplasmic form. The frequency in the multiethnic minorities group was almost six times that in the Han group ($\chi^2=46.234$, p approached 0.000) (Fig. 4). There was a significant difference in the frequency between the urban and suburban area (1.49% and 4.47%; $\chi^2=3.871$, $p=0.047$) (Fig. 5).

The frequency of the *SLC26A4* mutations was 7.04% (82/1164), 7.54% (67/888), 5.43% (15/276), 7.06% (14/201), and 6.97% (68/963) among total subjects, the Han group, multiethnic minorities group ($\chi^2=1.432$, $p=0.231$), urban area, and suburban area ($\chi^2=0.002$, $p=0.961$), respectively. In addition, 53 (6.40%) of 828 subjects who underwent cochlear implantation were detected successfully to carry c.IVS7-2A>G or/and c.2168A>G in the *SLC26A4* gene (Fig. 3b). Interestingly, all cases carrying the two mutations had an EVA malformation. However, the remaining 14 cases with the EVA malformation were not found to carry either mutation.

Only one case with Mondini dysplasia carried a heterozygous *SLC26A4* c.IVS7-2A>G mutation; by imaging, this

FIG. 2. The deaf populations with different degrees of hearing loss in southwestern China. dB, decibel.

FIG. 3. The proportion of the inner ear malformations detected by high-resolution thin-section computed tomography and magnetic resonance imaging (a) and the correlation of the *SLC26A4* c.IVS7-2A>G/c.2168A>G mutation and inner ear malformation (b). EVA, enlarged vestibular aqueduct.

case had a Mondini dysplasia combined with an EVA malformation. None of the common mutations were found in our cases with isolated Mondini dysplasia or other forms of inner ear malformations.

Discussion

We sought to analyze mutations in a very young population with severe to profound SNHL. For inclusion in the present study, subjects were limited to the criteria discussed above, including audiometry demonstrating > 70 dB hearing loss. In fact, the proportion of enrolled subjects with profound hearing loss (≥ 90 dB) was 93.56%. In addition, the average age was only 4.5 ± 1.3 years. Because we employed multiaudiometry methods, we were able to exclude cases with uncertain or mild to moderate hearing loss. Furthermore, focusing on very young subjects with congenital hearing loss enabled us to minimize effects from age-related progressive hearing loss or other acquired risk

factors. Our new findings revealed that the frequency of the hotspot mutations in three common deafness genes was 28.43% among the total subjects, which is in accordance with the frequencies in previous reports (Guo *et al.*, 2008; Yuan *et al.*, 2009; Ji *et al.*, 2011; Qu *et al.*, 2012; Du *et al.*, 2014).

It is well known that mutations of the *GJB2* gene, encoding the protein connexin 26, may affect potassium recycling in the inner ear, and hotspot mutations of the *mtDNA 12S rRNA* can cause a range of phenotypes from mild to profound SNHL (Prezant *et al.*, 1993; Murgia *et al.*, 1999; Sobe *et al.*, 2000; Wilcox *et al.*, 2000; Zhao *et al.*, 2004; Wang *et al.*, 2006). In addition, the *SLC26A4* gene, encoding the pendred protein, can cause pre- or postlingual onset of sensorineural or mixed, fluctuating or progressive hearing loss with EVA (Li *et al.*, 1998; Albert *et al.*, 2006; Wang *et al.*, 2007). Therefore, the general level of the mutant frequency in the children with severe or profound SNHL could be interpreted by unfixed phenotypes of hearing loss in mutations of three genes.

FIG. 4. The frequency of the common mutations in *GJB2*, *SLC26A4*, and *mtDNA* in the Han group and multiethnic minorities group.

In our study, more subjects carried *mtDNA* 1555A>G or 1494A>G mutation in the multiethnic minorities group (11.23%), nearly six times the frequency of 1.91% in the Han group (p approaches to 0.000). Given the young age range studied in our cohort, our data are not confounded by possible

progressively acquired phenotypes. Thus, the ethnicities and other factors can be considered to be important in our subjects.

In general, the *mtDNA* mutations had a lower frequency compared with the frequency of other *GJB2* and *SLC26A4* gene mutations. However, different geographic areas and ethnicities had varying frequencies of *mtDNA* 1555A>G. Several large-scale screenings of the *mtDNA* 1555A>G mutation worldwide have emphasized the importance of this mutation in different racial populations with HL, with incidence ranging from 0% to 2.4% in European countries, 3% to 10% in Japan, 0.9% to 4.8% in Korea, 5.3% in Indonesia, and 6.7% in China (Bae *et al.*, 2008). There are over 56 ethnicities in China. Previous reports suggest that there is a difference in the prevalence of the 1555A>G mutation among different ethnicities. It was estimated at 8.56% in the northern region with a frequency of 0–3.92% among Uyghurs and 0–1.75% in Tibetans (Li *et al.*, 2010b; Yuan *et al.*, 2012). We speculate that there are several reasons for the racial diversity of hearing levels among the 1555A>G mutation frequencies, such as aminoglycoside antibiotics abuse, some modifying factors increasing the deafness penetrance of *mtDNA* mutations, and smaller population, but higher endogamy rate in the multiethnic minorities. The common usage of aminoglycoside antibiotics in developing countries and lack of awareness of the ototoxicity of this drug family in poor areas makes an accurate assessment of aminoglycoside drug exposure history difficult. A known modifying gene, *TRMU*, was analyzed in all the samples with *mtDNA* 1555A>G or 1494A>G, but no mutation existed in the exon 1 of this gene. So, the high frequency of *mtDNA* among the multiethnic minorities may be a major hearing level factor.

Interestingly, we found differences among urban residential proportions between the Han and multiethnic minorities groups. The urban residential group in the multiethnic minorities population was only half of that in the Han group ($p=0.01$). It demonstrated that the urban/suburban area would be another important factor to impact the distribution of mutations. To understand the impact of the urban/suburban area without the impact of the ethnic factor, we divided these subjects into two groups according to their residential address (Fig. 5). The frequency of *mtDNA* mutations in the urban group was one-third of that in the suburban group ($p=0.047$); in contrast, the frequency of the *GJB2* mutations in the urban group trended higher compared with the suburban group ($p=0.012$). The result for the *mtDNA* mutation indicated that urban subjects displayed a decrease in the frequency of the *mtDNA* mutations, which was consistent with the phenomenon of less urban population and more *mtDNA* mutations in the multiethnic minorities group.

There was no difference in the frequency of *SLC26A4* mutations, either between the Han and multiethnic minorities groups or between the urban and suburban geographic areas. Our study was consistent with previous reports suggesting that *SLC26A4* mutations account for 4–17% of inherited deafness cases (Guo *et al.*, 2008; Qu *et al.*, 2012; Xin *et al.*, 2013; Yang *et al.*, 2013). As expected, c.IVS7-2A>G was the most common *SLC26A4* gene mutation. The detection rate of two mutations, c.IVS7-2A>G and c.2168A>G, in the subjects with the EVA was 81.81%, and the frequency of the mutant alleles was 48.48% (64/132). Less than 20% of the EVA cases were associated with other mutations of the *SLC26A4* gene. One of two cases with Mondini dysplasia and

FIG. 5. The frequency of the common mutations in *GJB2*, *SLC26A4*, and *mtDNA* in the urban area and suburban area.

TABLE 1. THE PREVALENCE OF THE *GJB2* VARIANTS IN CHINESE CHILDREN WITH SEVERE OR PROFOUND SENSORINEURAL HEARING LOSS IN THE HAN AND MULTIETHNIC MINORITIES GROUPS

	Han (N=888, alleles=1776)		Multiethnic minorities (N=276, alleles=552)	
	N (mutations %)	n (alleles %)	N (mutations %)	n (alleles %)
Pathogenic mutation				
35delG	2 (0.23)	2 (0.11)	0	0
235delC	150 (16.89)	220 (12.39)	41 (14.85)	70 (12.68)
299-300delAT	39 (4.39)	45 (2.53)	8 (2.89)	10 (1.81)
176del16	5 (0.56)	5 (0.28)	0	0
R32H	0	0	1 (0.36)	1 (0.18)
E47K	5 (0.56)	5 (0.28)	0	0
R75W	1 (0.11)	1 (0.06)	0	0
R75Q	1 (0.11)	1 (0.06)	0	0
E147K	1 (0.11)	1 (0.06)	0	0
Probable pathogenic mutation				
V37I	130 (14.64)	157 (8.84)	47 (17.03)	53 (9.60)
Polymorphism				
V3V27I	265 (29.84)	297 (16.72)	67 (24.27)	74 (13.41)
E114G	193 (21.73)	214 (12.05)	62 (22.46)	66 (11.96)
T123N	5 (0.56)	5 (0.28)	4 (1.45)	4 (0.72)
L203T	35 (3.94)	37 (2.08)	6 (2.17)	6 (1.09)
Synonymous				
H94H	1 (0.11)	1 (0.06)	0	0
T186T	1 (0.11)	1 (0.06)	1 (0.36)	1 (0.18)
Novel variant				
T18A	1 (0.11)	1 (0.06)	0	0
A78I	1 (0.11)	1 (0.06)	0	0
A78V	0	0	1 (0.36)	1 (0.18)
T86R	2 (0.23)	3 (1.69)	0	0
W133S	1 (0.11)	1 (0.06)	0	0
Y155T	3 (0.34)	3 (1.69)	0	0
D159N	0	0	1 (0.36)	1 (0.18)
N215K	1 (0.11)	1 (0.06)	0	0

EVA was detected to harbor the c.IVS7-2A>G mutation, another with similar compound malformations, 7 cases with isolated Mondini dysplasia, and other cases with different inner ear malformations (12 cochlear nerve hypoplasia; 2 enlarged internal auditory canal) were not associated with any common mutations in the three deafness genes. Therefore, all common mutations in the *GJB2*, *SLC26A4*, and *mtDNA* genes do not appear to be associated with inner ear malformations, with the exception of the EVA malformation.

In conclusion, about 27–32% of the children with severe or profound SNHL were found to harbor eight common mutations in the *GJB2*, *SLC26A4*, and *mtDNA* genes among total population groups and various subsets of southwestern Chinese populations. The subjects belonging to the ethnic minorities were found to have an increased frequency of *mtDNA* mutations, while the subjects belonging to the urban residential groups were found to have decreased frequency. The frequency of *GJB2* mutations in urban geographic areas is higher than in suburban areas. The *SLC26A4* hotspot mutations are prevalent stably and correlate to EVA strongly, but not to other inner ear malformations.

Acknowledgments

The authors express their deepest thanks and gratitude to the families for participating in this study. This work was supported by the Cochlear Implant Program of Hunan, China,

and National Basic Research Program of China (973) (grants 2012CB967900 and 2012CB967904) to D.H.X. and by National Institutes of Health (NIH) (grant R01DC005575, R01DC012546, and R01DC012115) to X.Z.L.

Author Disclosure Statement

The authors declare that they have no competing interests.

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