Int J Clin Exp Med 2017;10(9):13361-13370
www.ijcem.com /ISSN:1940-5901/IJCEM0055588

Original Article

Mutation of genes associated with hearing loss levels in the southern Fujian area of China

Qingyuan Lin[1*], Pengfei Mu[1*], Kun Liu[1*], Qian Tao[2*], Dan Liu[1], Wei Shao[1], Wensheng Yang[1], Tianhai Ji[1]

[1]Department of Pathology, Affiliated Chenggong Hospital, Xiamen University, Xiamen 361003, China; [2]PLA No.174 Clinical College, Anhui Medical University, Xiamen 361003, China. [*]Equal contributors.

Received April 17, 2017; Accepted August 17, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: Objective: The objective of this study is to examine loci's mutation frequencies for the four widespread deafness-associated genes in Han Chinese with hearing loss in the southern Fujian region and to establish the correlation between hearing loss levels and nucleotide modifications. Methods: Microarray-based mutation was detected nine hot spot mutations of the most prevalent deafness-related genes, including GJB2, GJB3, SLC26A and mitochondrial 12S rRNA. The hearing loss individuals also underwent hearing tests and medical examinations. Results: The prevalence of mutations in GJB2 (13.90% vs. 0.44%, P<0.001), SLC26A (11.05% vs. 0.44%, P<0.001) and mitochondrial 12S rRNA (12.19% vs. 0.44%, P<0.001) in the 1085 hearing loss individuals was significantly higher than those in the 452 healthy individuals with no hearing loss. There was no difference between hearing loss individuals and controls with GJB3 mutations (P > 0.05). Furthermore, the frequency of the c. 235 del C mutation in GJB2 was 12.44% (135/1085), slightly higher than that of c. 299_300 del AT (1.20%) and others. The most common mutation in SLC26A4 was c. IVS7-2A > G (9.77%, 106/1085). The mutation rate of mtDNA 12S rRNA was 12.19% (132/1085), most of these being the m. 1555 A > G mutation (11.15%, 121/1085). Significantly, c. 235 del C or c. IVS7-2 > G mutations were grander in patients with severe and profound hearing loss levels, just the reverse of m. 1555 A > G mutation (P<0.05), the percentage of the mitochondrial 12S rRNA mutations group who were taking aminoglycoside was 42.42%, significantly higher than that of other groups (P<0.01). Conclusions: Our data indicated that c. 235 del C, c. IVS7-2A > G and m. 1555 A > G were the most frequent mutations of southern Fujian populations, and that there was a strong association between specific hotspot mutations (GJB2, SLC26A and mitochondrial 12S rRNA) with hearing loss levels. The degree of hearing loss associated with c. 235 del C and c. IVS7-2A > G was mainly mild to severe and profound, but for m. 1555 A > G the associated hearing loss was mainly mild to moderate.

Keywords: GJB2, GJB3, SLC26A, mitochondrial 12S rRNA, mutation, hearing loss, ranks

Introduction

Hearing loss (HL) is relatively common among human population speech disorders and can result from environmental, genetic, or combined etiologies that prevent the normal function of the cochlea, the peripheral sensory organ. Severe hearing losses can have negative effects on a person's quality of life [1, 2]. Prevailing studies have demonstrated that in developed countries, the most frequent sensory defect leading to HL, with an incidence of approximately 1% in the newborn population [3], is attributed to genetic reasons. Approximately 70% of prelingual hearing loss is non-syndromic, and the remaining 30% is accompanied by additional clinical findings and is considered syndromic [1, 2]. In view of hearing's complexity, a lot of genes are involved in this function. Although a great number of mutations in a lot of genes may contribute to hearing loss, epidemiological research shows that the mutations of GJB2, SLC26A4, and mitochondrial 12S rRNA genes play important roles in HL. Knowledge of the patterns of high frequency mutations in certain populations and their population-wide distributions is essential for identifying hotspot mutations for carrier screening [4, 5]. Mutations in the GJB2 gene, which encodes the connexin 26 (Cx26), are considered to be a major reason of non-syndromic hearing loss in plenty of populations worldwide, and the spec-

tra of GJB2 mutations vary among different ethnic groups [1, 6-8]. Of these variants, c. 35 del G is the most common GJB2-deafness-causing mutation in European populations, while c. 235 del C and c. 167 del T are the most frequent variants in Eastern Asian populations [9-12]. In Southeast Asia and East, c. 235 del C has been reported as the most common variant, acting as a frameshift mutation causing premature protein protein termination in hearing-impaired patients, while lower frequencies have been observed in Oceania and Europe [13-15]. The SLC26A4 gene accounts for approximately 1%~12% of the causes of sensorineural HL in kids, with over 200 mutations currently identified in this gene. In different ethnicities or nations, specific variants of the gene were discovered in diverse studies [16]. Mutations in SLC26A4 are responsible for roughly 5-7% of congenital recessive hearing loss' all instances in the Caucasian and East Asian populations [17, 18]. The mutations of MT-RNR1, which are inherited from the mother, may increase the susceptibility to aminoglycoside ototoxicity [19].

A microarray developed by CapitalBio (CapitalBio, Beijing, China) was devised to detect nine hotspot mutations in the Chinese population, based on large-scale epidemiological studies. The results demonstrated that the majority (> 80%) of inherited hearing loss in Chinese patients is associated with those nine hotspot mutations [20-22]. The parallelism offered by the microarray platform makes it suitable for genotyping of genetically heterogenous conditions of hearing loss [23, 24].

The study described in this report aimed to detect nine hotspot mutations in four common deafness-associated genes, including GJB2 (35delG, 176_191del16, 235delC, 299_300delAT), GJB3 (538C > T), SLC26A4 (IVS7-2A > G, 2168A > G), and 12S rRNA (1555A > G, 1494C > G) in the southern part of Fujian province. The goals were to find the correlation between the age of onset and the related genes, as well as the nucleotide change associated with hearing loss rank. Elucidation of the genetic basis of an individual's deafness may assist in early diagnosis. This information will also be helpful in the early detection and proper management of neonatal deafness, as well as in genetic counseling for prenatal and postnatal diagnosis of HL.

Materials and methods

Patients

In our study, a total of 1085 unrelated sensor neural hearing loss individuals (582 females, 503 males; median age 29 years, range 1 month to 69 years) from southern Fujian were recruited through the Disabled Persons Federation. The hearing loss ranks (levels) were determined by the Department of Otolaryngology, of the affiliated Chenggong Hospital. A total of 452 normal hearing individuals (233 females, 219 males; median age 32 years, range 3 months to 61 years) were enrolled from the same region to act as controls. The majority of the participants were from Xiamen city, Longyan city, Putian city and the Zhangzhou city. The study cohort was of a significant feature of South Fujian province origins. A comprehensive history and physical examination record were obtained for each hearing loss patient, including name, age, address, family history, clinical history (age of onset), infection, use of aminoglycoside antibiotics (including streptomycin, gentamicin, neomycin, tobramycin, amikacin), as well as hereditary factors that be related to the hearing loss. Informed permission was acquired from each patient. The research was in compliance with the Ethics Committee of the Chinese People's Liberation Army 174 Hospital (Affiliated Chenggong Hospital, Xiamen University).

DNA samples

Genomic DNA was separated from 1085 hearing-loss patients' entire blood and from 452 salubrious unrelated persons with normal hearing in southern Fujian. I Informed assent was acquired from all the themes before blood sampling and genetic testing. Genomic DNA was separated from the entire blood through standard procedures utilizing a FlexGen Blood DNA Kit.

According to the manufacturer's protocol, as ymmetrical PCR was used to acquire adequate single-strand DNA for hybridization. After the heat denaturation procedure, the hybridization mixture was utilized to the array. The chip was incubated at 60°C for 1 hour, and washed twice at 42°C in 0.3% SSC (Sodium Citrate Buffer Solution)/0.1% SDS (Sodium Dodecyl Sulfate) for 120s and in 0.03% SSC for 60s, and centri-

Table 1. Hearing loss ranks detected according to the WHO standard

Ranks	The mean of the audiometric thresholds (dB HL)	Expression
Normal	≤ 25	No or only very light listening, can hear a whisper
Mild	26-40	Can hear or repeat the normal voice at 1m distance
Moderate	41-60	Can hear and repeat loud speech at 1m distance
Severe	61-80	Can hear shouting of some words at 1m distance
Profound	≥ 81	Can't hear or understand shouting

Table 2. Genetic screening for 1085 patients with hearing loss and 452 controls in southern Fujian

Gene	Nucleotide change	HL Carrier number (%)	Controls (%)	P
GJB2 mutations	c. 35 del G	0 (0%)	0 (0%)	> 0.05
	c. 176_191 del 16	3 (0.28%)	0 (0%)	> 0.05
	c. 235 del C	135 (12.44%)	2 (0.44%)	<0.001
	c. 299_300 del AT	12 (1.12%)	0	> 0.05
GJB3 mutations	c. 538 C > T	5 (0.46%)	1 (0.22%)	> 0.05
SLC26A4 mutations	c. 2168 A > G	14 (1.29%)	1 (0.22%)	<0.01
	c. IVS7-2 > G	106 (9.77%)	1 (0.22%)	<0.001
Mitochondrial 12S rRNA mutations	m. 1494 C > T	11 (1.01%)	1 (0.22%)	<0.001
	m. 1555 A > G	121 (11.15%)	1 (0.22%)	<0.001

fuged to separate the substances. Eventually, the chip was scanned with a LuxScan™ 10 K-B Microarray Scanner. The Detection Array Kit, purchased from CapitalBio Corporation (Beijing, China) was used to detect the most widespread deafness-associated mutations of the four genes (GJB2, GJB3, SLC26A4 and 12S rRNA), which were screened simultaneously based on DNA microarray technology. The target genes and mutations were GJB2 (35delG, 176_191del16, 235delC, 299_300delAT), SLC26A4 (IVS7-2A > G, 2168A > G), GJB3 (538C > T), and Mitochondrial 12S rRNA (1555A > G, 1494C > G). Acting in accordance with directions of the producer, nine loci from these four genes were multiplex PCR amplified with allele-specific PCR primers utilizing arrayed primer extension technology.

Hearing test

Hearing examinations illustrated that the levels of hearing loss was severe to fundamental in all instances. Most of patients, the hearing thresholds were decided through pure-tone audiometry, utilizing a clinical tonal audiometer GSI 60 in a soundproof room pursuant to current clinical standards. According to the world health organization (WHO) criterion in 1997, the severity of hearing loss is classified into five ranks [W, Reardon. Genetic deafness [J]. J Med Genet, 1992, 29: 521]: normal <26 decibel (dB); mild =26-40 dB; moderate =41-70 dB; severe =71-90 dB; and profound > 90 dB (**Table 1**). Systemic qualitative gender pathological varies or HL that was combined with other hereditary diseases were eliminated in accordance with the hospital ethics committee's regulations signed informed permission.

Statistical analysis

Distinctions of quantitative parameters between categories were evaluated utilizing the Mann Whitney U test. Differences of qualitative data were compared utilizing the chi-squared test. The Spearman Rank Correlation was used to analyze correlation and the Bonferroni Correction was used to counteract for the problem of multiple comparisons. Analysis was performed with statistical software spss19.0. A *P* value of <0.05 was regarded to be significant.

Results

Table 2 summarizes the percentage of nine hot spot mutations in HL and Control (normal hearing) patients. Of the 1085 cases of deafness, 13.90% (150/1085) were GJB2 mutations. As in most areas of China, there were four types of GJB2 polymorphisms. The most common mutation of GJB2 in southern Fujian was c. 235

Table 3. The percentage of the age of onset of hearing loss in four genes mutations

Age of onset (years)	GJB2 Mutations (%)	GJB3 mutations (%)	SLC26A4 mutations (%)	mitochondrial 12S rRNA mutations (%)
≤ 1	88 (58.67%)	1 (16.67%)	55 (45.83%)	4 (3.03%)
≤ 3	24 (16.00%)	2 (33.33%)	27 (22.50%)	10 (7.58%)
≤ 7	18 (12.00%)	2 (33.33%)	19 (15.83%)	35 (26.52%)
8-16	8 (5.33%)	1 (16.67%)	5 (4.17%)	38 (28.79%)
> 16	7 (4.67%)	0 (0%)	10 (8.33%)	37 (28.03%)
No clear	5 (3.33%)	0 (0%)	4 (3.33%)	8 (6.06%)
Total	150 (100%)	6 (100%)	120 (100%)	132 (100%)

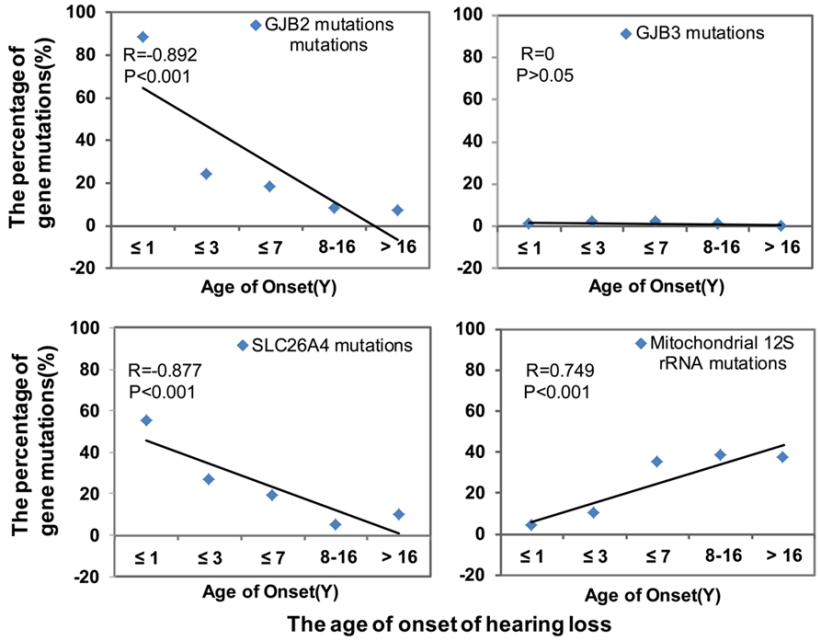

Figure 1. Correlation between the age of onset of hearing loss and the four gene mutations.

del C, 12.44% (135/1085). Five were GJB3 mutations 0.46% (5/1085), 11.06% (120/1085) were SLC26A4 mutations, and 12.17% (132/1085) were mitochondrial 12S rRNA mutations. The most common mutation of SLC26A4 and mitochondrial 12S RNA were c. IVS7-2 > G and m. 1555 A > G. The GJB2, SLC26A4 and mitochondrial 12S RNA mutation carrier rates were significantly higher than the rates for controls and GJB3 mutations (P<0.05). Rates for GJB2, SLC26A4 and mitochondrial 12S RNA mutations were similar.

Table 3 summarizes the age of onset in hearing loss patients in four common HL-related gene mutations. Depending on the age of onset and severity, HL is classified as congenital (≤ 1), prelingual (≤ 3), and postlingual (Preschool in the following sentences) (> 3). Of the 150 patients with GJB2 mutations, 58.67% (88/150) were congenital (≤ 1), 16.00% (24/150) were prelingual (≤ 3), and 12.00% (18/150) were preschool (≤ 7). Of the 6 patients with GJB3 mutations, the ages on onset were almost all prior to the teenage years, The onset ages of the 120 patients with SLC26A4 mutations were similar to those of the GJB2 mutations: 45.83% (55/120) were congenital, 22.50% (22/120) were prelingual, and 15.83% (19/120) were preschool. The deafness of patients with mitochondrial 12S rRNA mutations was primarily postlingual (> 3), with a carrier rate of 83%. Significantly, there was a strong negative correlation between the presence of GJB2 and SLC26A4 mutations and the age of onset of hearing loss (r=-0.892, P<0.001 and r=-0.877, P<0.001), but the opposite was true for mitochondrial 12S rRNA mutations (r=0.749, P<0.001). There was no correlation of GJB3 mutations with onset age (**Figure 1**).

Hearing loss rank was based on the World Health Organization (WHO) standard in 1997 [W, Reardon. Genetic deafness [J]. J Med Genet, 1992, 29: 521] in the 1085 hearing loss patients, whose tests for hearing loss levels were available, as indicated in the section "Patients and methods". Significantly, rates of patients with GJB2 mutations of severe and profound were 41.33% (62/150) and 44.00% (69/150), respectively, SLC26A mutation rates of severe and profound were 40.00% (48/120) and 41.67% (50/120), respectively (**Table 4**). Furthermore, prevalence of severe or profound

Table 4. The correlation between the hearing loss rank and four gene mutations and others

Ranks gene \ Hearing	Normal	Mild	Moderate	Severe	Profound
GJB2 mutations	1	5	13	62 (41.33%)	69 (46.00%)
GJB3 mutations	0	1	3	2	0
SLC26A4 mutations	1	7	14	48 (40.00%)	50 (41.67%)
Mitochondrial 12S rRNA mutations	10	48	45	13	16
Others	21	76	99	102	87

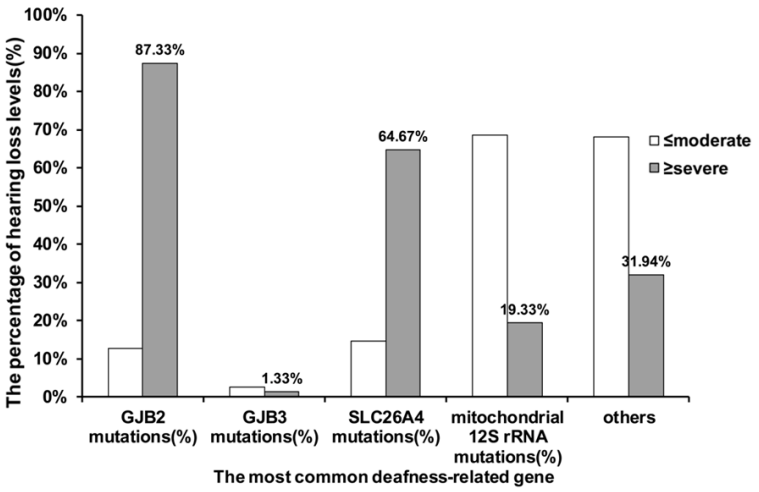

Figure 2. The percentage of hearing loss level in the most common deafness-related gene groups and others.

in patients with GJB2 mutations and SLC26A mutations was significantly higher than that of patients with GJB3 mutations, mitochondrial 12S rRNA mutations and others (not considered as common gene mutations or no mutation) (P<0.05, **Figure 2**).

Correlation analysis of 8 hot spot mutations in three of the most widespread deafness-associated genes and hearing loss ranks showed that the rates of patients with c. 235 del C exhibited a higher frequency of severe and profound in patients with GJB2 mutations. Also the rates of patients with c. IVS7-2 > G exhibited a higher frequency of severe and profound in patients with SLC26A mutations, and the m. 1555 A > G nucleotide change showed the highest frequency in mitochondrial 12S rRNA mutations. Moreover, there was a hard positive correlation between c. 235 del C and hearing loss rank (R=0.835, P<0.001). Similarly, there was a hard positive correlation between c. IVS7-2 > G and hearing loss rank (R=0.959, P<0.001), but there was a weak negative correlation between m. 1555 A > G and hearing loss rank (R=-0.470, P<0.01) (**Table 5** and **Figure 3**).

However, significant differences were observed between the mitochondrial 12S rRNA mutations group and other groups (including GJB2, GJB3, SLC26A4 mutations and all others) who were taking an aminoglycoside (including streptomycin, gentamicin, neomycin, tobramycin and amikacin) (P<0.05, **Table 6**). The percentage of the mitochondrial 12S rRNA mutations group taking an aminoglycoside was 42.42% and was significantly higher than that others (P<0.05, **Figure 4**).

Discussion

This study indicated that the mutation frequency of the nine loci for the four widespread deafness-related genes is 37.60% in Han Chinese with hearing loss in southern Fujian, including Xiamen city, Longyan city, Putian city and the Zhangzhou city. The mutation frequencies are meaningfully higher in hearing loss patients than in control subjects, uniform with the results of Xin F et al [20, 25, 26]. Mutations in the GJB2 gene have been reported to be the most widespread molecular defects in the Chinese deaf population [27, 28], with c. 235 del C being the most common. As previous reports indicated, ariants of the GJB2 gene could occupy the molecular etiology of 8 to 72 percent of non-syndromic hearing damages [29-31]. The most prevalent mutation in Caucasians, c. 35 del G, accounted for exclusively 0.33% of mutant alleles in the South China

Table 5. The correlation between the hearing loss ranks and nucleotide change

Nucleotide change \ Hearing ranks	Normal	Mild	Moderate	Severe	Profound
c. 35 del G (0)	0	0	0	0	0
c. 176_191 del 16 bp (4)	0	1	2	1	0
c. 235 del C (135)	1	5	10	58	61
c. 299_300 del AT (13)	0	2	3	4	4
c. 2168 A > G (23)	1	3	6	6	7
c. IVS7-2 > G (106)	0	4	11	41	52
m. 1494 C > T (13)	3	4	2	3	1
m. 1555 A > G (121)	7	45	44	10	15
Total	12	64	80	124	142

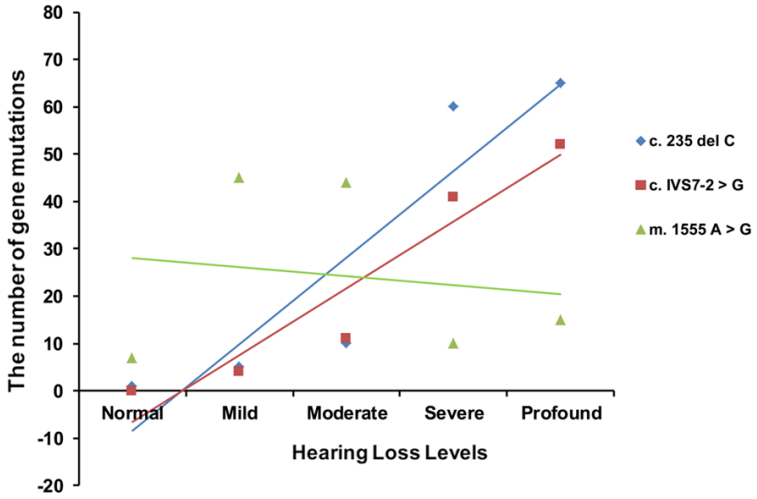

Figure 3. Correlation between hearing loss ranks and c. 235 del C, c. IVS7-2 > G, m. 1555 A > G mutations.

hearing population [32, 33]. In an earlier nationwide study, Dai analyzed GJB2 mutation of 2063 unrelated NSHL (Non-Syndromic Hearing Loss) students' GJB2 mutation from 23 different areas of China, and the c. 235 del C mutation allele frequency was 12.34% (509/4126), not involving southern Fujian province [27]. The frequency of c. 235 del C found in the southern Fujian deaf population was 12.17% (132/1085), similar to the percentage observed by Dai in a majority of Chinese deaf population [27]. Mutations of GJB3 lead to the growth of hearing loss in a minor percentage of sporadic hearing loss instances [34]. Deafness-related variation c. 538 C > T in the GJB3 gene was uncommon in our study cohort. Furthermore, two pathogenic mutations, c. IVS7-2 > G and c. 2168 A > G, in the SLC26A4 gene were discovered in 11.89% of the mutants in our study cohort, with c. IVS7-2 > G being the most common mutant develop in the SLC26A4 gene (9.77%, 106/1085). The most widespread mutation of SLC26A that is seen in the Chinese deaf population is c. IVS7-2A > G, with a detection rate as high as 12.5% [21]. According to previous reports, mutations in SLC26A4 are responsible for approximately 5% to 7.5% of all instances of non-syndromic that hears loss in the Easterly Asian and Caucasian populations. [17, 18] In this study, the 9.77% (106/1085) frequency of c. IVS7-2A > G was lower to that observed by Dai (12.5%) but higher than that observed by Park HJ [17]. Mitochondrial mtDNA mutations are among sensorineural hearing loss' most significant reasons in some nations [35, 36], with m. 1555 A > G having the greatest frequency in mitochondrial mtDNA mutations [37]. The incidence of the well-studied m. 1555 A > G mutation is between 3.0% and 7.0% in hearing-impaired individuals of Chinese descent and 0.7-3.8% in the European population [22, 38-40]. In our study, the 11.15% (121/1085) frequency of m. A1555G A > G was higher than that noticed by Lu et al (3.96%) [36]. A number of preceding investigations demonstrated that the A1555G mutation was a main factor underlying the development of deafness, but was not sufficient to develop a deafness phenotype [41]. The m. A1555G mutation newly is another identified mutation in mitochondrial 12S rRNA, with a predisposition to aminoglycoside ototoxicity and NSHL. According to a report, the 1494C > G mutation occupied 0.45% of hearing-impaired pediatric instances [38]. Furthermore, the percentage that took an aminoglycoside was 42.42% in the mitochondrial 12S mtDNA mutation patients, including streptomycin, gentamicin, neomycin, etc. Our data demonstrated that aminoglycoside ototoxicity is a

Table 6. The percentage of patients taking aminoglycoside in different gene mutation populations

Gene / Aminoglycoside	GJB2 mutations	GJB3 mutations	SLC26A4 mutations	Mitochondrial 12S rRNA mutations	Others
Streptomycin	8	0	3	25	35
Gentamicin	5	0	6	17	20
Neomycin	3	1	2	8	7
Tobramycin	3	0	3	7	10
Amikacin	2	0	1	8	11
No clear	8	0	3	25	242

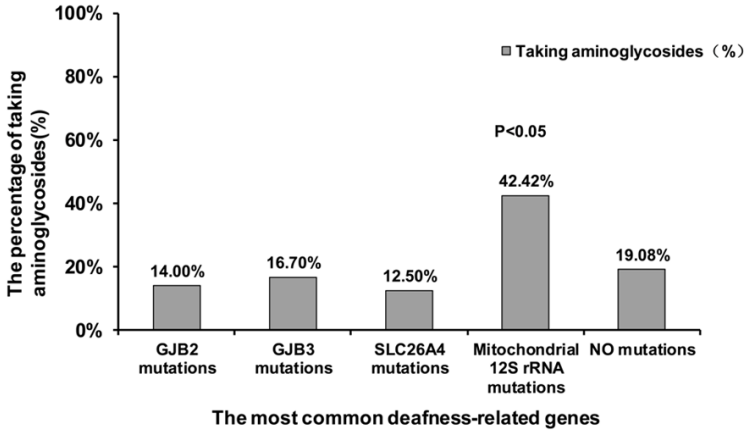

Figure 4. The percentage of patients taking aminoglycosides in different gene mutation populations.

general health issue in China, and mitochondrial 12S rRNA gene mutations were considered important reasons of aminoglycoside-induced hearing loss, due to aminoglycoside ototoxicity [36, 42].

Nevertheless, as indicated in our study, the age of onset of hearing loss patients with GJB2 and SLC26A gene mutations was primarily prelingual, and the hearing loss ranks tended to be severe and profound. This can be attributed to GJB2 and SLC26A gene mutations being the most common autosomal recessive deafness-related genes in most non-syndromic deafness patients. However, the percentage of severe and profound was relatively small in mitochondrial mtDNA mutation populations, and the percentage of postlingual deafness patients was greater than the percentage with prelingual HL. Furthermore, our data confirmed that there is a strong correlation between mitochondrial 12S mtDNA mutations and the number of hearing loss patients taking aminoglycosides. Preceding reports discovered that the 1555A > G mutation conferred sensitivity and mild mitochondrial dysfunction to aminoglycosides [43].

Chinese people from different regions may have different genetic backgrounds [44]. The most ancient Fujian resident migrated from the Chinese Central Plains area and the close-relative marriage principle can be followed back to ancient China, where intermarriages among persons with the same family name or among clansmen were prohibited [45]. Wide ranges of penetrance, severity and age-at-onset of hearing loss have been detected among southern Fujian populations. Therefore, the screening of deafness-related gene mutations can help us understand or account for some of the hearing losses. The GJB2, SLC26A4, and mitochondrial 12S rRNA genes should be the most common autosomal recessive deafness-related genes and may be applied in prenatal diagnosis of deafness and in routine newborn genetic screening.

Conclusions

In summary, we suggested that the c. 235 del C and c. IVS7-2A > G mutations that are reliable for inborn deafness are the most widespread among instances in southern Fujian, and the degree of hearing loss associated with c. 235 del C and c. IVS7-2A > G was mainly mild to severe and profound, but for m. 1555 A > G the associated hearing loss was mainly mild to moderate. This information will be helpful for developing optimum testing strategies with deaf patients in this region.

Acknowledgements

We are very grateful to the Department of Otolaryngology at the Chenggong Hospital affiliated with Xiamen University, and the Disabled Federation for collecting the hearing loss patients and funding the study. This work was also supported by the National Natural Science Foundation of China (81402064) and the Science and Technology Project of Xiamen (3502Z20154039 and 3502Z20164043).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Tianhai Ji, Department of Pathology, Affiliated Chenggong Hospital, Xiamen University, Xiamen 361003, China. Tel: +86-59-2633-5744; Fax: +86-59-2633-5744; E-mail: skysea_ji@sina.com

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