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# Molecular epidemiological analysis of mitochondrial DNA12SrRNA A1555G, *GJB2*, and *SLC26A4* mutations in sporadic outpatients with nonsyndromic sensorineural hearing loss in China

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## Abstract

**Conclusion—***GJB2* mutation was frequent in sporadic outpatients and its mutation frequency was significant higher in the prelingual group than in the postlingual group, whereas the mutation of mtDNA A1555G and *SLC26A4* was very rare in Chinese sporadic outpatients with nonsyndromic sensorineural hearing loss (NSHL). Standard and comprehensive inclusion and grouping criteria are necessary for epidemiological studies of deafness-related gene mutations.

**Objectives—**This study aimed to examine the mutations of the three common deafness genes *GJB2, SLC26A4*, and mtDNA A1555G in Chinese sporadic outpatients withNSHLandtodiscussthefactorsthatinfluencethedetection accuracyofmutationfrequencies.

**Methods—**A total of 473 sporadic NSHL patients without any type of inner ear malformation, including both prelingual and postlingual groups were enrolled in this study. Three genes of mtDNA A1555G, *GJB2*, and *SLC26A4* were screened for mutation in our study cohort. A chi-square test was performed to compare mutation frequencies between prelingual and postlingual groups.

**Results—**The mutation frequencies of MtDNA A1555G, *GJB2*, and *SLC26A4* were 1.63%, 13.63%, and 0%, respectively, in our study cohort. The mutational hot spot of *GJB2* was *c. 235delC*, whose allele frequency was 12.68% in sporadic outpatients. Mutation frequency of *GJB2* in the prelingual group was significantly higher than in the postlingual group ($p < 0.05$).

### Keywords

Genetic testing; deafness genes

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## Introduction

With the rapid development of molecular genetics of human deafness, more and more research supports the proposition that genetic factors are the major etiological factors of human deafness and contribute to more than 50% of all cases. Among 46 well known nonsyndromic sensorineural hearing loss (NSHL) risk genes, *GJB2* and *SLC26A4* are the most common chromosome genes, and mitochondrial DNA12SrRNA A1555G (referred to as the MtDNA A1555G or *m.A1555G*) is the most common mitochondrial gene mutation. In the past 10 years, researchers have found that the mutation hot spot in a particular deafness-related gene varies among populations with different regions or ethnic origins. For example, the mutation hot spot of *GJB2* is *c.35delG* in Caucasians from Europe, America, and South Asia [1,2], while it is *c.235delC* in Mongolians from East Asia regions [3], and *c.71G>A* (*p.Trp24X*) in Spanish Romani [4]. It was proposed that the differences in these mutation hot spots were caused by different racial or regional genetic backgrounds.

However, our further studies revealed there were large differences and some inconsistent data from many studies. The differences were mainly found in mutation frequencies, including disease-causing mutation frequency (homozygous or compound heterozygous mutation), and risk allele carrying frequency (homozygous, compound heterozygous and heterozygous mutation). For example, the reported *m.A1555G* mutation frequency in Chinese NSHL patients varied from 1.67% to 15.5% in several independent studies [5,6]. The difference among reported mutation frequencies of *GJB2* gene was very large. The risk allele carrying frequency of *GJB2* mutation ranged from 14.8% to 41% in China [7,8], 14.7% to 50% in Europe [9,10], and 3.01% to 26.3% in America [11,12]. Kimberling first raised the question as to why there was such a large difference among mutation frequencies in the same region or ethnic origin [13]. He thought that inconsistent selection criteria for recruiting patients could lead to substantial differences in estimating the frequencies of genetic mutations and thus result in different mutation frequencies among studies (selection bias). While Wu et al. [14] thought that there was a significant difference in epidemiological characteristics of deafness gene mutations among patients from different sources, such as rehabilitation centers or hospitals.

According to Kimberling's idea, to effectively avoid such differences and accurately examine the genetic epidemiological characteristics of deafness gene mutations, it is necessary to conduct a group study considering different characteristics or sources of patients. Thus, we established standard selection and grouping criteria in this study. In the present study, only sporadic outpatients with a clear clinical diagnosis of NSHL were recruited, and all patients with any inner ear malformations (including enlarged vestibular aqueduct) were excluded. The aim of standardizing the selection criteria was that we could avoid any bias in examining the genetic epidemiological risks in these deafness patients without clear disease causes and thus provide data for molecular etiological diagnosis.

## Material and methods

### Subject recruitment

From 2003 to 2009, 473 sporadic deafness patients were recruited from the outpatient department in the Clinical Hearing Diagnostic Center, Department of Otolaryngology, Head and Neck Surgery, People's Liberation Army General Hospital (Beijing, China). By analysis of medical history, routine physical examination, laboratory examinations and tests, audiological testing, and temporal bone imaging examination, these patients were all diagnosed as having NSHL, and all types of inner ear malformations were excluded. Peripheral venous blood samples were collected from patients and their parents. The clinical audiological data were evaluated based on the Grades of Hearing Impairment recommended

by the World Health Organization in 1997 (http://www.who.int/pbd/deafness/hearing_impairment_grades/en/index.html). Informed consent, blood samples, and clinical evaluations were collected from all the participants according to the protocols approved by the Chinese People's Liberation Army General Hospital Institution Review Board of the Ethics Committee.

### Mutation screening

**Genomic DNA extraction—**Genomic DNA was extracted from peripheral venous blood using phenol extraction. MtDNA12SrRNA, the second exon of *GJB2* and exon 8,10,15,18 of *SLC26A4* including the flanking sequences were amplified by polymerase chain reaction (PCR) using the genomic DNA as template. The primer pairs were designed referring to previous reports by Guo et al. [3] and Wang et al. [15], and synthesized by Invitrogen Corporation. All PCR reactions were conducted in an ABI 9700 PCR Thermal Cycler. The PCR products were separated by 1.5% agarose gel electrophoresis and showed a single and specific band with correct size.

**Restriction fragment length polymorphism (RFLP) analysis of MtDNA12SrRNA mutation—**The MtDNA12SrRNA sequence contains a single restriction site between 1552 and 1556 bp that can be specifically recognized by Alw26I (BsmAI) endonuclease. PCR was performed as described previously followed by Alw26I digestion. If no MtDNA A1555G point mutation exists, the PCR product can be cut by the Alw26I and results in two bands of 326 bp and 441 bp, while the PCR product shows a 767 bp band if it carries the point mutation.

**Sequencing—**The PCR products of *GJB2* and *SLC26A4* were directly sequenced and the PCR products of MtDNA12SrRNA with single bands were also sequenced to further confirm MtDNA A1555G mutation. All PCR products were purified with Millipore plates and then sequenced using an ABI 3730 Sequencer (Applied Biosystems). Sequencing data were analyzed and aligned with NCBI GenBank reference sequences of MtDNA12SrRNA (AC_000021), *GJB2* (NC_000013), and *SLC26A4* (NC_000007.13) using Sequencher 4.8 Demo software.

### Statistical analysis

Comparisons of mutation frequencies of the *m.A1555G* and *GJB2* genes between the prelingual and postlingual group were conducted by chi-square test using SPSS 18.0 software. A $p$ value $< 0.05$ was considered as a statistically significant difference.

## Results

### Demographic characteristics of the subjects

The 473 recruited sporadic NSHL outpatients included 275 males and 198 females and their ages ranged from 0.3 to 67 years (average age 9.97, median age 5.00). Patients were from more than 10 Chinese ethnic groups and the highest proportion was Han Chinese, 93.02% (440/473). The majority of subjects had severe or profound hearing loss, 79.91% (378/473). Among the 68 patients without clear onset age in our study cohort, there were 30 patients aged 3 years or younger. The age at onset of the other 405 patients ranged from 0 to 65 years (average age 5.69, median onset age 2.00). Patients with an onset age younger or equal to 3 years were defined as prelingual patients (298), and those older than 3 years were postlingual patients (137).



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### m.A1555G mutation screening

A total of 434 patients including 270 prelingual, 129 postlingual, and 35 without clear onset age were screened for the *m.A1555G* mutation. Seven patients (7/434, 1.63%) were found to carry the *m.A1555G* mutation, including three prelingual patients (3/270, 1.11%) and four postlingual patients (4/129, 3.10%). There was no significant difference in *m.A1555G* mutation frequencies between the prelingual and postlingual groups ($\chi^2$ test, $p = 0.157$) (Table I). A survey of the medical histories indicated that all seven patients carrying *m.A1555G* had a history of exposure to aminoglycoside drugs.

### GJB2 mutation screening

A total of 411 patients including 264 prelingual, 117 postlingual, and 30 patients without clear onset age were subjected to *GJB2* mutation screening. In all, 56/411 patients (13.63%) were found to carry the *GJB2* disease-causing mutation. Thirty-four (34/411, 8.27%) patients were found to have monoallelic mutation. Among them, further analysis showed that 51 patients (51/264, 19.32%) were prelingual, 2 (2/117, 1.71%) were postlingual, and the other 3 patients had no clear onset age. Thus, the mutation carrying frequency of *GJB2* (including unknown mutations and novel variants, but excluding the polypeptide variant) was 21.90% (90/411). The 90 patients with the *GJB2* mutation included 74 (74/264, 20.03%) prelingual and 8 (8/117, 6.84%) postlingual patients. A $\chi^2$ test showed that both disease-causing mutation frequency and carrying frequency in the prelingual group were significantly higher than in the postlingual group ($\chi^2$ test, $p = 0.000$) (Table I).

In this study, we screened a total of 16 different genotypes of *GJB2* mutations including 5 known disease-causing mutation genotypes (2 homozygous genotypes and 3 compound heterozygous genotypes). The most common disease-causing mutation geno-type was *c. [235delC]+[235delC]*, which was found in 32 patients, followed by *c. [235delC] +[299_300delAT]* in 16 patients. Two compound heterozygous mutation genotypes, *c. [235delC]+[257C>G]* and *c.[235delC]+[263C>A]*, were first reported in this study. *c. 257C>G* and *c.263C>A* were novel variants; we could not confirm whether these two genotypes cause deafness and further studies are needed. We also found another nine heterozygous genotypes; among them the most common genotype was *c.[235del]C+[=]* found in 16 patients, followed by *c.[299_300delAT]+[=]* in 4 patients (Table II).

We detected 15 *GJB2* allelic variations in our study cohort. Among these variants, seven types were previously reported as disease-causing mutations, including six frameshift mutations and one missense mutation. Allelic mutation *c.235delC* was the most common, with 12.68% allele frequency, followed by *c.299_300delAT*, whose allele frequency was 3.02%. We also found four novel variants (*c.257C>G, c.263C>A, c.478G>A, c.11G>A*), one unknown mutation (*c.368C>A*), and five polymorphisms (*c.79G>A, c.341A>G, c. 109G>A, c.608T>C, c.558G>A*) (Table III).

### SLC26A4 mutation screening

A total 129 patients were screened for *SLC26A4* mutations. None was found to carry any *SLC26A4* mutation or variant.

## Discussion

A hospital outpatient department and a special school for deaf-mute subjects were the two main sources of patients with deafness. Patients with NSHL were common in the outpatient department. At present, due to a lack of clear pathological mechanisms and etiological factors, the treatments for NSHL are not very effective, except for the use of hearing aids and cochlear prosthesis implantation. Thus the etiological study of NSHL was very

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important and urgent. In this study, we analyzed the molecular etiology of NSHL in outpatients by examining mutations of three common deafness genes, *m.A1555G, GJB2*, and *SLC26A4* in Chinese sporadic NSHL outpatients. As Kimberling suggested, to avoid differences caused by inconsistent selection criteria, all selected patients followed our inclusion and grouping criteria, as follows. (1) Diagnosis: NSHL. Syndromic hearing loss and inner ear malformations were excluded. (2) Family history: sporadic, not familial. (3) Source: outpatient department, not special school or others. (4) Cause of deafness: unknown. (5) Subgroups: according to onset age, patients were classified into prelingual and postlingual subgroups. The aforementioned criteria could unify the selection criteria of patients – ensuring that these patients had similar research background, avoiding differences caused by dissimilar characteristics or sources, and providing correct data for molecular etiological diagnosis of NSHL patients.

We showed that the mutation frequency of *m.A1555G* in Chinese sporadic outpatients with NSHL was 1.63%, similar to previous reported data (1.6%) in Japanese outpatients [16]. However, we found that the frequency of *m.A1555G* was relatively lower in our study than in other reports on the Chinese NSHL population. For example, previous findings showed that the frequency was 2.9% in patients with NSHL [14]. Guo et al. [3] reported that the frequency was 8.75% in NSHL patients in special education schools in northwest China, and 15.5% was reported by Liu et al. [6], even up to 40% in 10 Taiwan families with deafness [17]. Noguchi et al. [16] reported that the mutation frequency was higher in familial patients than in sporadic patients. The aforementioned data suggested two aspects, as follows. (1) The previous reported *m.A1555G* frequencies of the deaf Chinese population were quite different. (2) The mutation frequency of *m.A1555G* should be higher in familial patients than that in sporadic cases. Thus, it is possible that the existence of familial patients in previous studies might be one of the causes of bias. We excluded all familial patients, and this might be the reason why the mutation frequency of sporadic patients in the present study was less than that in previous reports. We did not find significant differences in the *m.A1555G* mutation frequencies between the prelingual and postlingual groups; thus this mutation might not affect the onset age of NSHL patients. Interestingly, we noted that all seven patients with *m.A1555G* had a history of exposure to aminoglycoside drugs, which were known as the most important cause leading to hearing loss in *mA1555G* carriers. Therefore, the correlation of *m.A1555G* mutation frequency and onset age might be affected by using aminoglycoside drugs at a random time.

In the present study, the disease-causing *GJB2* mutation frequency and carrying frequency were 13.63% and 21.90% in sporadic outpatients with NSHL, respectively. There are no significant differences when comparing the two frequencies in our study with previous data in deaf Chinese patients. In 2009, Chen et al. [7] reported that *GJB2* mutation carrying frequencies in cochlear implant patients and NSHL patients were 36.5% and 41%, respectively, which were higher than our data in this study. Guo et al. [3] reported that *GJB2* mutation disease-causing frequency and carrying frequency in northwest China's special education schools were 9.14% and 19.26%, respectively, which were lower than our data. Liu et al. [18] found that the *GJB2* mutation disease-causing frequency in sporadic patients with NSHL was 26.7%, while it was only 5.2% in the familial patients. However, opposite findings were reported by other researchers, i.e. that *GJB2* mutation frequency in familial patients was significantly higher than that in sporadic patients [19]. These inconsistent data and controversial conclusions are confusing. It was believed that various factors might affect the accuracy of the data. In the present study, we found a significant difference in *GJB2* mutation frequency between prelingual and postlingual groups. It is suggested that onset age might be one factor that influenced the accuracy of *GJB2* mutation data. Deafness in the majority of patients with the *GJB2* mutation usually occurred at birth or in the neonatal period. Therefore, the mutation frequency of *GJB2* was higher in the prelingual group than

in the postlingual group. We further confirmed that *c.[235delC]+[235delC]* was a major genotype of *GJB2* mutations, and *c.235delC* was a major mutation hot spot in Chinese patients with NSHL. In addition, we identified four novel *GJB2* variants (*c.257C> G, c. 263C> A, c.478G> A, c.11G> A*) and further studies are needed to determine whether they are disease-causing mutations.

It is well known that *SLC26A4* gene mutation is one of the major causes of enlarged vestibular aqueduct syndrome (EVA). In Chinese EVA patients, the disease-causing mutations frequency of *SLC26A4* was near 90%, and the risk mutation carrying frequency is more than 90% or even 100% [15]. In this study, the *SLC26A4* mutation was not found in sporadic NSHL outpatients, suggesting that *SLC26A4* gene mutation might not be a deafness risk in NSHL patients without EVA. In the present study, we excluded all EVA patients because all these EVA patients already had clear pathogenic etiology and clinical diagnosis. It was reported that *SLC26A4* gene mutations had a very high mutation frequency, not only in Chinese EVA patients, but also in familial and NSHL patients [3,20], and there was a significant difference in SLC26A4 mutation frequency between rehabilitation centers and outpatient departments [14]. Taken together, it is indicated that *SLC26A4* gene mutation frequencies might be quite different in patients from various sources or with different clinical characteristics, and it should be necessary to classify patients into different subgroups when performing disease-associated genetic mutation analysis.

To sum up the above discussion, we propose that it is necessary to select pertinent factors for consideration during the formation of inclusion criteria when conducting molecular epidemiological studies of deafness-related genes. The main factors include sample size, country, region, ethnic group, hospital or special school, familial or sporadic, prelingual or postlingual. Thus, we should also consider the history of exposure to aminoglycoside drugs in a study of mtDNA A1555G and consider EVA or Mondini dysplasia in a study of *SLC26A4* mutation. If samples in one study include patients with different characteristics and sources, we should classify these samples into several subgroups to ensure that the patients in the same subgroup have the same characteristics and sources.

## Conclusion

*GJB2* mutation was frequent in sporadic outpatients and its mutation frequency was significantly higher in the prelingual group than in the postlingual group, whereas the mutation of mitochondrial DNA12SrRNA A1555G, and SLC26A4 was very rare in Chinese sporadic NSHL patients without inner ear malformations. Many factors may influence the accuracy of mutation frequency data. Thus, it is necessary to formulate standard and comprehensive inclusion and grouping criteria in conducting epidemiological studies of deafness gene mutation in the future.

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**Table I**

Comparison of mutation frequencies in prelingual and postlingual deafness groups.

| **Parameter** | ***m.A1555G*** | ***GJB2* disease-causing mutation** | ***GJB2* mutation carrying** |
|---|---|---|---|
| Prelingual deafness | 1.11% (3/270) | 19.32% (51/264) | 20.03% (74/264) |
| Postlingual deafness | 3.10% (4/129) | 1.71% (2/117) | 6.84% (8/117) |
| *p* value | > 0.05 | < 0.05* | < 0.05* |

*The mutation frequencies including disease-causing frequency and carrying frequency between prelingual and postlingual groups were significantly different ($\chi^2$ test, $p < 0.05$).

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**Table II**

Number of patients with different mutation genotypes of *GJB2* gene.

| Main genotype of *GJB2* | No. of patients |
|---|---|
| *c.[235delC]+[235delC]* | 32 |
| *c.[235delC]+[299_300delAT]* | 16 |
| *c.[235delC]+[176_191del16]* | 5 |
| *c.[299_300delAT]+[299_300delAT]* | 2 |
| *c.[299-300delAT]+[605ins46]* | 1 |
| *c.[235delC]+[257C>G]* | 3 |
| *c.[235delC]+[263C>A]* | 1 |
| *c.[235delC]+[=]* | 16 |
| *c.[299_300delAT]+[=]* | 4 |
| *c.[176_191del16]+[=]* | 3 |
| *c.[30_35delG]+[=]* | 2 |
| *c.[508_511dupAAACG]+[=]* | 1 |
| *c.[416G>A]+[=]* | 1 |
| *c.[368C>A]+[=]* | 1 |
| *c.[478G>A]+[=]* | 1 |
| *c.[11G>A]+[=]* | 1 |
| Total | 90 |

previously reported as disease-causing mutations, including six frameshift mutations and one missense mutation. Allelic mutation *c.235delC* was the most common, with 12.68% allele frequency, followed by *c.299_300delAT*, whose allele frequency was 3.02%. We also found four novel variants (*c.257C>G, c.263C>A, c.478G>A, c.11G>A*), one unknown mutation (*c.368C>A*), and five polymorphisms (*c.79G>A, c.341A>G, c.109G>A, c.608T>C, c.558G>A*) (Table III).

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**Table III**

Mutation spectrums and mutated allele frequency of *GJB2* gene.

| Nucleotide change | Codon change | Mutated alleles | Mutated allele frequency |
|---|---|---|---|
| *GJB2* (822 alleles) | | | |
| *c.235delC* | Frameshift | 105 | 12.73% |
| *c.299_300delAT* | Frameshift | 25 | 3.04% |
| *c.176_191del16* | Frameshift | 8 | 0.97% |
| *c.30_35delG* | Frameshift | 2 | 0.24% |
| *c.605ins46* | Frameshift | 1 | 0.12% |
| *c.416G>A* | *p.Ser139Asn* | 1 | 0.12% |
| *c.508_511dupAACG* | Frameshift | 1 | 0.12% |
| *c.368C>A* | *p.Thr123Asn* | 1 | 0.12% |
| *c.257C>G* * | *p.Thr87Arg* | 3 | 0.35% |
| *c.263C>A* * | *p.Ala88Glu* | 1 | 0.12% |
| *c.478G>A* * | *p.Gly160Ser* | 1 | 0.12% |
| *c.11G>A* * | *p.Gly4Asp* | 1 | 0.12% |
| *GJB2* polymorphisms | | | |
| *c.79G>A* | *p.Val27Ile* | 198 | |
| *c.341A>G* | *p.Glu114Gly* | 153 | |
| *c.109G>A* | *p.Val37Ile* | 29 | |
| *c.608T>C* | *p. Ile203Thr* | 7 | |
| *c.558G>A* | *p.Thr186Thr* | 1 | |

*Novel mutations.