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ORIGINAL ARTICLE

Concurrent Genetic and Standard Screening for Hearing Impairment in 9317 Southern Chinese Newborns

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Objective: The goal of this study was to investigate the use of concurrent genetic screening together with standard newborn hearing screening (NHS) in an effort to provide a scientific basis for the beneficial use of concurrent genetic hearing screening in newborns. Our aim was to improve the neonatal detection rate of hearing impairment and the potential for hearing loss, allowing for increased early intervention and potentially allowing for prevention of later onset hearing loss. This information could also be used to increase the effectiveness of genetic counseling regarding hearing impairment. *Methods:* A total of 9317 neonates from Children's Hospital of Dongguan and Dongguan People's Hospital were included in this study between January 2015 and October 2015. Twenty hotspot hearing-associated mutations of four common deafness- susceptibility genes (*GJB2, GJB3, SLC26A4, and MTRNR1*) were analyzed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The results of genetic screening and NHS were concurrently analyzed. *Results:* A total of 129 infants (1.38%) exhibited hearing loss as determined by otoacoustic emission (OAE) testing. The genetic screening revealed that 348 (3.74%) individuals had at least one mutant allele. In total, 34 (0.36%) of the neonates carried a causal complement of mutations. The overwhelming majority of the genetically referred newborns passed the OAE hearing screening, but could be at risk for later hearing loss. *Conclusion:* This study furthers the understanding of the etiology of hearing loss and proves that it is beneficial to use genetic screening along with OAE screening of neonates to improve detection rates of at-risk infants. Our results show that this concurrent testing allows for better early identification of infants at risk for hearing loss, which may occur before speech and language development. Prevention of hearing loss can be achieved by avoiding the use of antibiotics containing amino glycosides in infants whose mutations make them extremely sensitive to these antibiotics. This information is also useful in genetic counseling, providing region-specific mutation information.

Introduction

Hearing loss is the most common sensory disorder, with an incidence rate of $\sim$1–3‰ among newborns (Morton and Nance, 2006; Cohen and Phillips, 2012). In terms of etiology, at least half of all cases of childhood hearing loss are estimated to have a genetic cause, and the majority ($\sim$70%) is nonsyndromic hearing loss (NSHL) (Morton, 1991; Bitner-Glindzicz, 2002). Previous epidemiological studies have shown that a majority of NSHL is caused by a limited amount of genes with recurrent mutations in the Chinese population, such as the gap junction beta-2 protein (*GJB2*), gap junction beta-3 protein (*GJB3*), solute carrier family 26 member 4 (*SLC26A4*), and mitochondrial 12S rRNA (*MTRNR1*) genes, which would facilitate clinical testing (Dai *et al.*, 2006; Sun *et al.*, 2008; Yuan *et al.*, 2009; Han and Shao, 2015).

Mutations in the *GJB2* gene encoding connexin 26 (Cx26) are the most frequent cause of nonsyndromic autosomal recessive sensorineural hearing loss in many populations. The mutation spectrum and prevalence of mutations vary significantly across different ethnic groups. Mutations in the *SLC26A4* gene are responsible for Pendred syndrome (PDS) and enlarged vestibular aqueduct syndrome (EVA), with

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hearing loss found at birth or in early childhood. The *GJB3* gene, which is related to hereditary NSHL, was first cloned in the Chinese population (Alexandrino *et al.*, 2004). Mutations in *GJB3* are associated with progressive hearing loss. The m.1555A>G and m.1494C>T mutations in *MTRNR1* are responsible for aminoglycoside antibiotic-induced deafness (Guan *et al.*, 2000; Hamasaki and Rando, 1997). The secondary structure of the 12S rRNA molecule is changed by these mutations, which can effect mitochondrial translation and protein synthesis, and is expected to create a binding site for aminoglycosides that facilitate interaction with the drugs.

Hearing loss currently cannot be completely cured, but early prevention, detection, and intervention are key mitigation strategies. Universal newborn hearing screening (NHS) has been widely performed in China, but many studies have identified limitations to traditional hearing screening; for example, neonatal nonsyndromic delayed deafness and drug-induced deafness cannot be detected by the common hearing screening tests (Han and Shao, 2015; Meng *et al.*, 2015). If deafness gene screening could be implemented, the population at high risk of genetic deafness could be identified through an analysis of the regional epidemiological characteristics of susceptible gene mutations (Dai *et al.*, 2006; Yuan *et al.*, 2009). Subsequently, effective prevention and intervention could be adopted. Therefore, screening would aid in reducing the incidence of deafness.

China is a large, multiethnic country with a population of more than 1.6 billion, which encompasses 9.6 million square kilometers. At present, genetic screening for deafness is not a routine procedure in China. Some molecular etiology reports are available for northern China (Zhang *et al.*, 2013) and eastern China (Shen *et al.*, 2015; Yu *et al.*, 2015), but very few reports are available for southern China. Genetic counseling for deafness should fully take into account the differences in regional backgrounds. Therefore, the establishment of a specific mutational database for the different regional populations would be indispensable. In this study, we report the first analysis of deafness-susceptibility genes in a large cohort of southern Chinese infants. According to the common hotspots of the gene mutation spectrum related to deafness in the Chinese population, 20 mutation sites in four selected genes (*GJB2*, *GJB3*, *SLC26A4*, and *MTRNR1*) indicating susceptibility to deafness were examined.

Materials and Methods

Patients

A total of 9317 newborns of Han Chinese from Children's Hospital of Dongguan or Dongguan People's Hospital were studied between January 2015 and October 2015. Infants with fetal abnormalities or potential risk factors for hearing loss, including low birth weight, premature delivery, congenital fetal malformation, and a family history of congenital deafness, were excluded from our study. The *GJB2*, *SLC26A4*, *GJB3*, and *MTRNR1* genes were tested simultaneously in 4744 males and 4573 females. This study was approved and conducted in accordance with the protocol of the Institutional Medical and Ethics Committee of Children's Hospital of Dongguan and Dongguan People's Hospital. Written informed consent was obtained from the parents or guardians of the infants.

Newborn hearing screening

A two-stage screening approach with an otoacoustic emissions (OAE) test was performed in the hearing screening. Every newborn was tested using OAE covering a spectrum of frequencies and volumes at ~48 to 72 h after birth in the maternity ward by an otolaryngologist. If the newborn failed the initial test, a repeated OAE test and an automatic auditory brainstem repose (AABR) assessment were performed at 42 days of age.

Molecular genetic analysis

The blood samples were collected by a heel prick within 72 h of birth. Three appropriate-sized blood spots were obtained using a customized dried blood collection card for screening, validation, and storage. This genetic screening involved 20 hotspot mutations in four primary NSHL genes: *GJB2*, *GJB3*, *SLC26A4*, and *MTRNR1*, which are frequently associated with deafness in the Chinese population (Table 1).

A multiple polymerase chain reaction (PCR)-based matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS) assay was used for genotyping by Shenzhen BGI co. The methods were validated by wild- and mutant-type DNA samples from patients with hereditary deafness previously confirmed by Sanger sequencing. All samples with positive results were further confirmed by sequencing. The sensitivity and specificity were >99.9%.

The Chelex 100 chelating resin (BioRad, Hercules, CA) was used for DNA extraction. A 0.5 cm^2 blood spot was obtained by a punch and added to a 1.5 mL Eppendorf tube. After 500 μL of purified water was added, the solution was mixed vigorously by vortexing. The tube was allowed to stand for 15 min at room temperature and was then centrifuged at 13,000 rpm for 3 min. Then, the supernatant was discarded. Well-mixed 5% Chelex 100 solution (150 μL) was added for precipitation. The solution was mixed by vigorous vortexing, incubated in a 56°C water bath for 30 min, and then boiled in a 100°C water bath for 8 min. After the solution had been centrifuged at 13,000 rpm for 5 min, it was stored at 4°C for further use.

The target sites in the 80- to 120-bp gene segments were amplified using 20 pairs of mixed primers. PCR assays were performed with 1 μL of extracted DNA template (1 μL H_2O was added to the negative control; 1 μL of the samples

Table 1. The 20 Mutations in Four Genes Examined in This Study

Gene	*Mutation sites*	*No. of sites*
GJB2	c.35delG, c.167delT, c.176_191del16, c.235delC, c.299_300delAT	5
GJB3	c.538C>T, c.547G>A	2
SLC26A4	c.281C>T, c.589G>A, IVS7-2A>G, c.1174A>T, c.1226G>A, c.1229C>T, IVS15+5G>A, c.1975G>C, c.2027T>A, c.2162C>T, c.2168A>G	11
MTRNR1	m.1494T>C, m.1555A>G	2
	Total	20

provided by BGI Tech were added to the positive controls). The reaction conditions were as follows: denaturing at 94°C for 15 min; 45 cycles of denaturing at 94°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and extension at 72°C for 3 min. Shrimp alkaline phosphatase-digested free dNTPs were added to the reaction system. The PCR products were purified with an iPLEX kit (Sequenom, Inc., San Diego, CA) according to the manufacturer's instructions, mixed well, and dispensed into a template tube before the test. The template was used for MALDI-TOF-MS (Sequenom, Inc.) in the final test. The data were processed using TYPER 4.0 software (Sequenom, Inc.).

The genetic screening results were designated as ''pass'' or ''refer,'' with ''pass'' indicating that the infant lacked mutations or genotypes associated with hearing loss and ''refer'' indicating that causative homozygous or compound heterozygous mutations were found in *GJB2*, *GJB3*, *SLC26A4*, or *MTRNR1*.

Statistical analyses

The statistical analyses were performed using the statistical package SPSS (version 17.0; SPSS, Inc., Chicago, IL). Chi-squared tests were performed to determine the statistical significance of differences in mutation carrier rates between the current and previous studies. All reported *p*-values were two-tailed, and the significance threshold was set at a $p < 0.05$.

Results

Results from hearing screening of 9317 newborns

A total of 9317 newborns with a gender ratio of 1.00 (female):1.04 (male) were examined in this study. The overall hearing screening results are summarized in Table 2. In the first step, OAE test for infants at 48–72 h after birth, a total of 8241 (88.45%) newborns passed the initial screening. Of those who failed the screening, 362 cases failed the left-ear hearing test and 257 cases failed the right-ear hearing test, for a total of 619 (6.64%) cases. A total of 457 (4.91%) cases failed both the right- and left-ear hearing tests. Overall a total of 1076 (11.55%) newborns failed the initial screening. For those who were referred after the initial test, repeated tests of OAE plus AABR were performed at 42 days after birth. The confirmatory test showed that a total of 129 referred infants (0.36% bi- and 1.02% unilateral referral) did not pass the rescreening.

Table 2. Hearing Screening Results of 9317 Newborns

Audiological test	*No. of cases (%)*
First step	
Otoacoustic emissions test	
Pass	8241 (88.45)
Bilateral referral	457 (4.91)
Unilateral referral	619 (6.64)
Second-step screening	
Passed	9188 (98.6)
Bilateral referral	34 (0.36)
Unilateral referral	95 (1.02)

Molecular screening spectrum of 20 NSHL mutations in 9317 newborns in the Dongguan region

The molecular screening data of the 20 NSHL mutations in this study of 9317 neonates are shown in Table 3. Three hundred forty-eight newborns were identified with at least one mutation in the pathogenic genes. Among the neonates examined, 310 newborns (3.33%) exhibited heterozygous mutations or heteroplasmic mtDNA mutations, 10 newborns (0.11%) exhibited compound heterozygous mutations, and 28 newborns (0.30%) exhibited homozygous or homoplasmic mutations. When each allele was counted individually (one infant with two mutation alleles was counted twice), the total carrier rate was 3.84% (358/9317). The highest mutation carrier rate was found for the *GJB2* gene at 1.86%, followed by the *SLC26A4* gene at 1.43%. The *GJB2* 235delC and *SLC26A4* IVS7-2A>G mutations were the most frequently detected in this study, with carrier rates of 1.48% and 0.86%, respectively. A detailed mutation spectrum is shown in Figure 1.

Analysis of hearing screening results according to the genotypes in the genetic screening

In total, 34 (0.36%) neonates carried a causal complement of mutations and therefore were genetically referred. Among these genetically referred cases, 30(0.32%) had an mtDNA 12S rRNA mutation, one was *GJB2* c. 235delC homozygous, and the remaining three were compound heterozygous in the *SLC26A4* gene, with *SLC26A4* c. [1229C>T]+[1975G>C], *SLC26A4* c.[1229C>T]+[IVS7-2A→G], and *SLC26A4* [IVS15+5G>A]+ [IVS7-2A→G], respectively. Among the 34 genetically referred cases, the overwhelming majority of genetically referred newborns passed the hearing screening (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/gtmb). A comprehensive analysis of the NHS and genetic screening is shown in Figure 2.

Comparison analysis of carrier rates for NSHL-associated mutations between different regions in China

We used the chi-squared test to compare the differences of the carrier rates of 20 deafness-associated mutations between this study and previous studies in other parts of China. Our study showed the total carrier rate of mutations was 3.84%, which was lower than the reported carrier rate in northern and eastern China ($p < 0.05$) (Zhang *et al.*, 2013; Meng *et al.*, 2015; Shen *et al.*, 2015; Wang *et al.*, 2015). However, the carrier rates of *MTRNR1* gene were higher than the carrier rates in northern and eastern China ($p < 0.05$). The detailed data are shown in Supplementary Table S2.

Discussion

In this study, the *GJB2*, *SLC26A4*, *GJB3,* and *MTRNR1* genes were simultaneously screened in 9317 newborns in the Dongguan region of southern China. In our study, the most frequent mutations were observed in *GJB2*. The carrier rate of the mutations in *GJB2* in the Dongguan region of southern China was 1.86%, which accounts for 48.32% (173/358) of all mutations that tested positive. The data showed a significantly lower carrier rate than that reported in northern China

Table 3. Mutation Spectrum of 20 Hotspot Deafness-Associated Alleles in 9317 Newborns in the Dongguan Region

Genetic screening	*Mode of inheritance*	*Homozygous*	*Heterozygous*	*Total*	*Carrier rate (%)*
GJB2	Autosomal recessive	1	172	173	1.86
c.235delC		1	137	138	1.48
299_300delAT		0	30	30	0.32
176_191del16		0	4	4	0.04
c.35delG		0	1	1	0.01
SLC26A4	Autosomal recessive	0	133	133	1.43
IVS7-2A>G		0	80	80	0.86
c.1229C>T		0	23	23	0.25
c.2168A>G		0	14	14	0.15
IVS15+5G>A		0	5	5	0.05
c.1975G>C		0	4	4	0.04
c.281C>T		0	3	3	0.03
c.2162C>T		0	1	1	0.01
c.1174A>T		0	1	1	0.01
c.1226G>A		0	1	1	0.01
c.2027T>A		0	1	1	0.01
GJB3	Autosomal recessive/	0	22	22	0.24
c.538C>T	autosomal dominant	0	15	15	0.16
c.547G>A		0	7	7	0.08
MTRNR1	Maternal inheritance	27	3	30	0.32
m.1555A>G		26	3	29	0.31
m.1494T>C		1	0	1	0.01
Total		28	330	358	3.84

(Tianjin 2.62%) and eastern China (Jiangsu 3.40%) (Zhang *et al.*, 2013; Meng *et al.*, 2015). The *GJB2* c.35delG mutation is common in Caucasian populations (Gasparini *et al.*, 2000; Van *et al.*, 2001), but we detected this mutation in only 1/9317 infants (0.01%) in the Dongguan population. No c.167delT mutation, which is a common mutation in Ashkenazi Jews, was detected in our study (Morell *et al.*, 1998; Sobe *et al.*, 1999, 2000; Lerer *et al.*, 2000). In this genetic screening, 172 newborns carried a monoallelic *GJB2* mutation; however, 147 (85.47%) of these newborns passed the hearing screening. One case exhibited a homozygous mutation of *GJB2* 235delC, and this child did not pass the NHS. For this newborn, who exhibited biallelic mutations, an audiologic diagnosis should be made as soon as possible, with cochlear implants being provided if necessary to allow the child to retain speech capacity. For other infants with monoallelic mutations, follow-up should be undertaken to prevent the occurrence of late-onset hearing loss, and the appropriate genetic counseling should be provided.

SLC26A4 gene mutations may cause NSHL associated with EVA and PDS, with hearing loss found at birth or in early childhood (Park *et al.*, 2005; Hilgert *et al.*, 2009). In China, 97.9% of the EVA patients in simplex families exhibited either biallelic or monoallelic mutations (Wang *et al.*, 2007). In this genetic screening, 133 infants with *SLC26A4* gene mutations were identified, including three compound heterozygous with biallelic mutations in the *SLC26A4* gene, indicating a higher carrier rate than that observed in northern China. The hearing screening results indicated that 88.7% newborns who carried a monoallelic mutation of SLC26A4

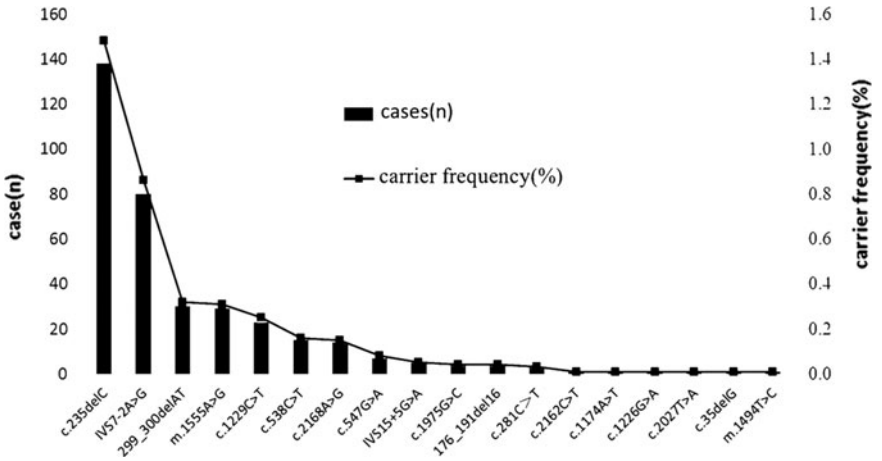

FIG. 1. Spectrum of 20 hotspot deafness-susceptibility mutations.

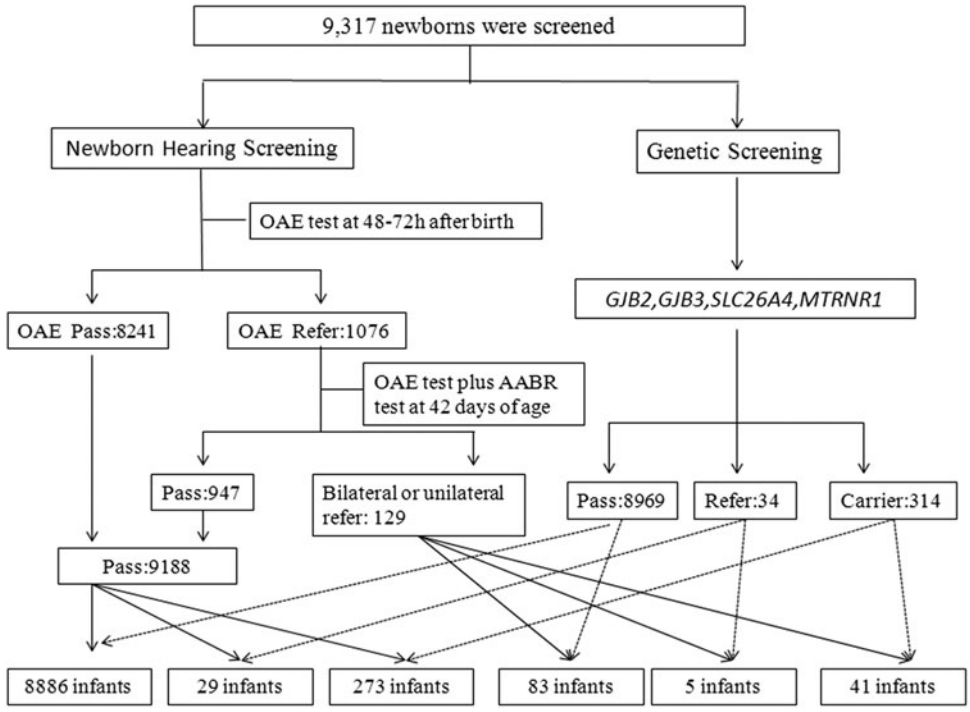

FIG. 2. Comprehensive analysis of the newborn hearing screening and genetic screening in this study. AABR, automatic auditory brainstem repose; OAE, otoacoustic emission.

passed the hearing screening. Auditory function may be normal at birth because of a monoallelic mutation of this gene, but within a few years of birth, auditory function may become abnormal due to some external factors, such as falls, head injury, and viral infection. For children with *SLC26A4* gene mutations, head injury should be avoided to reduce the risks of progressive hearing loss. Thus, the genetic screening of hearing loss may provide an important basis for the early prevention of deafness.

For *GJB3*, the carrier rate of mutations in infants was 0.24% in this study, whereas the reported carrier rates were 0.43% and 0.50% in northern and eastern China, respectively. The *GJB3* gene is mainly associated with acquired high-frequency hearing loss, and carriers of this gene mutation may experience delayed deafness (Sun *et al.*, 2008). Symptoms of deafness may not appear in neonates, but close follow-up of hearing function is necessary in these individuals.

The m.1494>T and m.1555A>G mutations in the *MTRNR1* mitochondrial gene are directly associated with aminoglycoside-induced deafness (Kupka *et al.*, 2002; Ramsebner *et al.*, 2007). Of the 9317 newborns in this study, 29 infants exhibited m.1555A>G mutations, and one infant exhibited an m.1494T>C mutation. However, 93.3% of these infants passed the NHS, yet they remain at risk for deafness in later life and are extremely sensitive to aminoglycoside antibiotics (including streptomycin, neomycin, kanamycin, and gentamicin); any exposure to these drugs is likely to lead to irreversible deafness (Stergaard *et al.*, 2002).Screening for drug-induced deafness gene mutations ensures that medication is used correctly in neonates after birth. Extending screening to family members and relatives has been recommended to avoid drug-induced deafness in identified carriers.

Congenital deafness accounts for the overwhelming majority among the prelingual deafness population. The timing of the identification of hearing impairment is a very important factor because it can affect language capacity. Therefore, early detection, diagnosis, and intervention are necessary for newborns who are susceptible to deafness. Deafness gene screening may identify the cause of deafness at the molecular level and allow the early detection of congenital inherited deafness. Young children can benefit from undergoing early treatment to prevent the occurrence and development of deafness, especially for mtDNA and SLC26A4 gene mutations. According to the results of deafness gene screening, guidelines for the safe use of medication and regular hearing care should be provided for children who carry deafness genes. Delayed hearing loss caused by heredity, drugs, and physical factors may be avoided or postponed. This study provides clinical references for regional deafness gene screening, which are beneficial for preventing deafness.

Conclusions

We have conducted the first analysis of deafness-susceptibility genes in south China. Our results show that genetic screening along with OAE screening of neonates allows for better early identification of infants at risk for hearing loss, which may occur before speech and language development. This study furthers the understanding of the etiology of hearing loss and provides useful and important information for effective genetic counseling and the early prevention and intervention of deafness.

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Author Disclosure Statement

No competing financial interests exist.

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