Original Article

The genetic load for hereditary hearing impairment in Chinese population and its clinical implication

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Abstract Objective To understand the genetic load in the Chinese population for improvement in diagnosis, prevention and rehabilitation of deafness. **Methods** DNA samples, immortalized cell lines as well as detailed clinical and audiometric data were collected through a national genetic resources collecting network. Two conventional genetic approaches were used in the studies. Linkage analysis in X chromosome and autosomes with microsatellite markers were performed in large families for gene mapping and positional cloning of novel genes. Candidate gene approach was used for screening the *mtDNA 12SrRNA*, *GJB2* and *SLC26A4* mutations in population-based samples. **Results** A total of 2,572 Chinese hearing loss families or sporadic cases were characterized in the reported studies, including seven X-linked, one Y-linked, 28 large and multiplex autosomal dominant hearing loss families, 607 simplex autosomal recessive hereditary hearing loss families, 100 mitochondrial inheritance families, 147 *GJB2* induced hearing loss cases, 230 cases with enlarged vestibular aqueduct (EVA) syndrome, 169 sporadic cases with auditory neuropathy, and 1,283 sporadic sensorineural hearing loss cases. Through linkage analysis or sequence analysis, two X-linked families were found transmitting two novel mutations in the *POU3F4* gene, while another X-linked family was mapped onto a novel locus, nominated as *AUNX1* (auditory neuropathy, X-linked locus 1). The only Y-linked family was mapped onto the *DFNY1* locus (Y-linked locus 1, *DFNY1*). Eight of the 28 autosomal dominant families were linked to various autosomal loci. In population genetics studies, 2,567 familial cases and sporadic patients were subjected to mutation screening for three common hearing loss genes: *mtDNA 12S rRNA 1555G*, *GJB2* and *SLC26A4*. The auditory neuropathy cases in our samples were screened for *OTOF* gene mutations. **Conclusions** These data show that the Chinese population has a genetic load on hereditary hearing loss. Establishing personalized surveillance and prevention models for hearing loss based on genetic research will provide the

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opportunity to decrease the prevalence of deafness in the Chinese population.

Key words Hereditary hearing loss, linkage analysis, DFNY1, AUNX1, auditory neuropathy, enlarged vestibular aqueduct, senserineural hearing loss, genetic epidemiology

Introduction

The second national census in 2006 (http://www.cdpf.org.cn) showed that there were a total of 82.96 million disabled people in China, of which 27.8 million (34%) had a hearing disability. This number continues to grow, with 30,000 new deaf cases every year. Currently, the only effective intervention for congenital profound hearing loss or deaf patients is cochlear implantation. Currently in China, the minimal cost for cochlear implantation and following rehabilitation is 300,000 yuan (roughly $44, 118). A total treatment cost for the 27.8 million people would be 8,000 billion yuan ($1,176 billions). The annual incremental medical cost for the 30,000 new deaf babies could impose an up to 9 billion yuan ($1.3 billion) bill on China economy. Rapidly accumulating evidence in the last decades demonstrates that genetic factors contribute substantially and broadly to communication disorders in various races and geographic regions in the world. Therefore, there is a great demand for genetic studies to elucidate the underlying mechanisms for hereditary hearing loss, which can provide increased insights onto developing effective prevention and interventional measures.

Genetic factors contribute to 50% of deafness cases including syndromic (30%) and non-syndromic (70%) hearing impairment[1, 2]. Recent times have witnessed rapid progress in the field of genetic studies of hereditary hearing impairment, especially of non-syndromic hearing impairment. Transmission of non-syndromic hearing impairment can be autosomal recessive (77%), autosomal dominant (22%), X-linked (1%), matrili- neal(mitochondrial inheritance, about 1%), or Y-lin- ked [3-5]. More than 146 chromosomal loci and at least 78 genes (http://webhost.ua.ac.be/hhh) have been identified so far. Recent advancements in hearing loss genetic studies have provided a better understanding of the genetic mutations that cause prelingual deafness [6]. There have been reports revealing that prelingual severe or profound hearing loss is mainly due to recessive inheritance, and the mutations in *GJB2* (*DNFB1*, OMIM: 121011) and *SLC26A4* (*DFNB4*, OMIM: 600791) genes are thought to be the major causes of autosomal recessive nonsyndromic deafness[7]. *GJB2* gene mutation is currently recognized to be respon-sible for both nonsyndromic autosomal recessive and sporadic prelingual deafness[8, 9]. Mutations in the *SLC26A4* gene have been identified as a major cause for nonsyndromic hearing loss associated with enlarged vestibular aqueduct (EVA) and Pendred syndr - ome [10, 11]. The *1555A>G* mitochondrial mutation in the highly conserved coding region of the mitochondrial *12S rRNA* gene has been found to be the most prevalent mitochondrial mutation associated with both aminoglycoside-induced and nonsyndromic sensorineural hearing loss (OMIM: 561000) in many ethnic origins [12, 13].

Our questions are: What is the genetically attributed prevalence of hearing impairment in the Chinese population? What are the unique mutation profiles in hearing impairment in the Chinese population? And what can be done to decrease the prevalence of hearing impairment in the Chinese population?

Methods

1. Subject collection and audiological evaluation

Genetic resources are essential for finding novel loci/genes and for characterizing genetic epidemiological parameters of hereditary hearing loss. With the help from the Audiology Development Foundation of China (in provincial, municipal and county jurisdictions), we initialized a nation-wide campaign in 2000 for collecting genetic resources related to hearing loss and for constructing a large-scale database to document Chinese patients including case-enriched families or sporadic subjects. Subjects in the studies were either identified through the national network covering most regions of China or from patients who directly visited our clinic. Control subjects were either randomly sampled normal hearing individuals or patients of different disease categorizations than the phenotype under this study. Additional efforts were devoted in collecting pedigree-based samples once a proband was identified, including pedigree extension by procuring the demographic and audio-

metric data, and DNA samples for their relatives. The protocols of collecting the genetic resources were approved by the Chinese PLA General Hospital Institution Review Board of the Ethics Committee. All hearing loss patients and their relatives involved in the studies gave their informed consents. The data summarized here were collected from 2000 to 2007. Since 2007, we have begun newborn hearing concurrent gene screening and resources samples have accumulated quickly to more than 25, 000.

Clinical evaluation was conducted according to the protocols approved by the PLA General Hospital IRB. All participants were extensively interviewed by experienced otoloaryngologists to identify personal or family evidence of hearing impairment, tinnitus, vestibular symptoms, use of aminoglycosides, and other clinical abnormalities. These subjects were then given physical examinations of the hair, skin, sclera, iris, mouth, maxilla, mandible, eyes, interocular distance, spine, and extremities. Otolaryngological examinations focused on the auricle, external auditory meatus, and tympanic membrane. Audiometric evaluation included pure tone audiometry, tympanometry and acoustic reflexes, and auditory brainstem responses. The audiometric data were categorized based on the recommendations by the European HEAR project, as described by Stephens[14]. Sensorineural hearing impairment was defined as an air/bone gap ≤15 dB hearing loss averaged over 0.5, 1, and 2 kHz. The severity of hearing impairment was applied to the better hearing ear, averaged over 0.5, 1, 2, and 4 kHz and categorized as following: mild: 20 – 40 dB HL; moderate: 41 – 70 dB HL; severe: 71–95 dB HL; and profound: >95 dB HL. Frequency ranges were defined as following: low frequencies: ≤0.5 kHz; mid frequencies: between 0.5 and 2 kHz (including 2 kH z); high frequencies: between 2 and 8 kHz (including 8 kHz); and extended high frequencies: >8 kHz.

2. Molecular procedures for DNA isolation, genotyping and sequencing

Genomic DNA from peripheral blood leukocytes was obtained by the phenol/chloroform method. All PCR amplified products for the target fragments (microsatellites or gene fragments for genes *GJB2, SLC26A4 and mtDNA 12S rRNA A1555G*) were purified with the Millipore plate, and genotyped or sequenced with an ABI 3730 Sequencer (Applied Biosystems). Genotypes at >400 molecular markers, covering human genome every 10 cM each, were used for linkage analysis. Sequence data were analyzed by aligning with the reference sequences in NCBI (NC_000013 for *GJB2*, NT_007933 for *SLC26A* and AC_000021 for *mtDNA 12S rRNA A1555G*) using the DNAStar 5.0 and BioEdit software. Mutations or polymorphisms were identified per reference sequences.

GJB2 mutation screening The *GJB2* gene has two exons, and the coding region is in exon 2. With the use of the Primer 5.0 software package, the primer pair—forward primer (*GJB2*–F): TGCTTACCCAGACTCGAGAA and reverse primer (*GJB2*–R): CGACTGAGCCTTGACAGCTGA, were designed for the coding region of *GJB2*, and the PCR product was an 864bp fragment. In order to amplify the exon, touch–down PCR with the annealing temperatures of T1 = 68℃ (10 cycles) and T2 = 63℃ (25 cycles) was carried out in an ABI 9700 thermal cycler.

SLC26A4 mutation screening The *SLC26A4* gene contains an open reading frame of 2,343 bp, and encompasses 21 exons. Mutations in exons 8, 19, 10, 17 and 15 are often found in nonsyndromic hearing loss associated with EVA in the Chinese[15]. Hence, the following sequential procedures were used to systematically detect mutations in the *SLC26A4* gene. PCR amplifications of five exons (8, 19, 10, 17 and 15) were first performed. If a mutation was not detected in these five exons, the DNA sample was further screened for mutations in exons 3, 4, 5, 7, 11, 12 and 14, respectively. Exons 3, 5, 8 and 15 were amplified using the primer pairs described previously[16], which were designed using the online Primer 3.0 software. For exons 4, 7, 10, 11, 12, 14, 17 and 19, the primer pairs were designed based on those in Van Laer et al[17]. All the primer pairs were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd (Shanghai, China). Touch–down PCR with conditions as described by Guo Y–F et al[16] was conducted for amplifying exons 3, 5, 8 and 15 in an ABI 9700 thermal cycler. The other exons were amplified according to the reaction conditions described by Van Laer et al[17].

Mitochondrial *DNA A1555G* mutation screening With the use of the primer pair synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd–forward primer (M–F): TCAACCTCACCACCTCTT and reverse primer (M–R): TTTGTCGCCTCTACCTAT,

a 767 bp mtDNA fragment(nt1229~nt1995) was amplified using the annealing temperature of T=62℃ for 30 cycles. Alw26I digestion analysis was also used for detecting *mtDNA A1555G*. A volume of 6.0 μl of the PCR product was mixed with 2.0 μl of buffer and 0.2 μl of Alw26I restriction enzyme (Tango, Shanghai, China). ddH_2O was added to the mixture till the volume reached 25.0 μl. The reaction mixture was incubated at 37℃ for 150 minutes. The electrophoresis on the 2% agarose gel was run to examine the digested product. If the digested product showed the specific band for *mtDNA A1555G*, the PCR product was verified by direct sequencing.

3. Data analysis

Linkage analysis First, simple familial aggregation and transmission analysis was performed to identify the most likely inheritance models for the investigated families, after non-genetic environmental causes were excluded. Then, a genome-wide model-based linkage analysis was performed, and two point or multi-point logarithm of odds(LOD) scores between the disease locus and markers were calculated by LINKAGE software [18] or GENEHUNTER software [19]. For X-linked families, linkage analysis in X chromosome was implemented with X-linked microsatellite markers and linkage evidence was determined per chromosomewide significance. Following linkage analysis, candidate genes within the 95% confidence intervals of the mapped loci were investigated and genetic variants were screened by sequencing or other means.

Candidate gene approach Genetic epidemiological studies of several common hearing loss genes in the Chinese population were conducted using candidate gene approach to establish their influence on the Chinese at large. To obtain the population genetic parameters such as mutation rates and genotypic relative risks, an extensive screening of mutations in the candidate genes was conducted for different geographic regions or nationalities in this largest eastern country. In our studies, three common genes (*mtDNA 12S rRNA, GJB2 and SLC26A4*) were focused on. For sporadic patients or autosomal recessive pedigrees with profound or fluctuating hearing loss, or patients with a history of exposure to ototoxic medications, we used the candidate approach to characterize the genetic epidemiological profiles in Chinese deaf patients. Also, the three genes were used as the targets in our newborns screening.

Results

1. The national network of China for collecting the genetic resources related to hearing loss

The national network was constructed in 2000 and maintained since then by Institute of Otolaryngology, Chinese PLA General Hospital. The China Disabled Persons´ Federation and numerous local societies(in provincial, municipal and county jurisdictions) have provided consistent help in the largest campaign for collecting genetic resources(pedigrees and sporadic cases) related to hearing loss. Currently, this network covers more than 20 provincially administrated regions including. The largest database has documented a total of 1,120 simplex or multiplex pedigrees of different inheritances, and 1,452 sporadic cases (Table 1). In addition, this database restores the detailed clinical data and genetic testing for three common hearing loss genes (*mtDNA 12S rRNA, GJB2 and SLC26A4*) for 5,914 newborns coming from eight provincial jurisdictions (Table 1). These resources provide us rich materials for developing rigorous projects in both clinical and basic scientific research in the domain of hearing science, and consequently for defining better intervention measures delivered to hearing impaired patients in China.

2. Large-scale genetic studies for identifying the unique mutation spectrum and high-risk loci in Chinese population

There is a worldwide interest in studying the pathogen-

Table 1 List of genetic resources collected

Classification	N
Pedigrees, dominance	28
Pedigrees, recessive	607
Pedigrees, *GJB2*	147
Pedigrees, EVA	230
Pedigrees, *A1555G*	100
Pedigrees, X-linked	7
Pedigrees, Y-linked	1
Pedigrees, total	1,120
Cases, AN	169
Cases, SHL	1,283
Cases, total	1,452
Newborns screened	5,914

ic variants, especially in the three common hearing loss genes (*mtDNA 12S rRNA*, *GJB2* and *SLC26A4*) that confer high cross-race risks to multiple ethnic backgrounds and populations. Nevertheless, increasing evidence from previous studies indicates that not only the mutation rates are distinct, but also the genetic loci/variants can be quite different among different races. In several studies, we performed large-scale genetic studies of the three genes in order to develop reliable genetic testing for the Chinese population. In total, we have screened 2,567 (either familial or sporadic) patients for *12S rRNA 1555G* and *GJB2* or *SLC26A4* mutations. These large-scale efforts identified Chinese specific mutation hotspots and defined the unique spectrum [15]. In the study of 801 patients collected in the northwest regions (mainly Gansu province) [16], 26.2% were found to result from mutations in the three genes and the mutation rate was 8.4% for *mtDNA A1555G*, 9.0% for *GJB2* and 8.8% for *SLC26A4*, respectively. About 12% of the patients were *GJB2* or *SLC26A4* mutation-wild heterozygotes. As a result, the cumulative 38% of the patients were associated with genetic variations in the three common genes. This genetic epidemiological study also demonstrated that 26.2% of the prelingual deafness in northern China could be detected at young ages by genetic testing of the three common hearing loss genes, allowing early intervention to help them in language acquisition.

Among the 1,120 pedigrees collected, 147 were found to have hearing loss resulting from *GJB2* homozygotes or compound heterozygotes. Hearing loss in the 230 EVA pedigrees was due to *SLC26A4* homozygotes or compound heterozygotes. And in 100 families, hearing loss was caused by *12SrRNA A1555G* mutation. Hearing loss was from *12SrRNA C1494T* in 3 pedigrees. All the pedigrees had two or more affected members and 42.8% of the pedigrees were associated with mutations in the three genes, while the cause of deafness was unknown in the remaining 57.2% families .

In the study [15], 107 patients with EVA recruited from May 2003 to Dec. 2005 received comprehensive audiometric evaluation, imaging scanning of their ears and *SLC26A4* mutation screening. The audiometric tests included play audiometry or pure tone audiometry, tympanometry, middle ear muscle reflex thresholds and stapedius muscle reflex (acoustic and nonacoustic), otoacoustic emissions (OAEs), and auditory brain stem responses (ABRs). High-resolution computed tomography (CT) scan of the temporal bone and magnetic resonance imaging (MRI) were used to examine structures of the cochlea and endolymphatic sac. All 21 exons of the *SLC26A4* gene were sequenced. Of the EVA patients, 70% were found having a significant A-B gap at low frequencies (500 and 250 Hz), covering a span of 15-95 dB HL. In 75% of the patients, the short latency negative response (ASNR) was evoked during the routine ABR testing. The latency of ASNRs was 3.26 ± 0.57 ms. In this study, *SLC26A4* gene mutations were detected in 97.9% of the EVA patients. Based on these findings, we developed a systematic diagnosis procedure integrating audiometric tests, CT and MRI scans, and *SLC26A4* gene test [20, 21].

3. Positional cloning based on Chinese pedigrees and searching for novel loci candidate gene mutations

As shown in Table 2, the novel loci identified in our lab were nominated as *DFNC1*, *DFNA55* and *DFNA56* in the autosomal dominant inheritance pedigrees. Of the eight large pedigrees, the Z002 pedigree was mapped onto the *DFNA4* locus at 19q13.2-13.4, and the F013 pedigree onto 9q31.3-34.3 (nominated as *DFNA56*). The novel locus *DFNC1* at 12p11.23-13 for autosomal dominant conductive hearing loss with congenital ptosis was identified using a four-generation pedigree with nine patients affected by this disorder. The Z029 pedigree was mapped onto 11q13.4-22.1, the 686 pedigree onto 9p13.2-13.3 (nominated as *DFNA55*), and the 727 pedigree onto 1p31-35, a region partially overlapping with *DFNA47*. The linkage for the remaining two pedigrees (1301 and 1318) was yet to be characterized.

Besides the autosomal dominant gene mappings, two novel mutations in *POU3F4* causing congenital profound sensorineural hearing loss were found in two Chinese X-linked pedigrees [22-24]. The first pedigree represented an exceptional case of more than eight males affected in one family with the phenotype of profound hearing loss leading to deaf-mutism caused by a *POU3F4 de novo* mutation that had not been reported before. Mutation screening of *POU3F4* revealed a de novo missense

Table 2 Linkage results for eight Chinese pedigrees following inheritance of autosomal dominance

Pedigrees	Geographic origin	No subjects	DNA samples	No of patients	Age of onset (yrs)	Location
Z002	Jianxi	85 (6 generations)	37	14	20–35	19q13.2–13.4
F013 (DFNA56)	Shandong	40 (4 generations)	31	11	8–30	9q1.3–34.3
DFNC1	Liaoning	44 (4 generations)	19	9	congenital	12p12.3–13.2
Z029	Hebei	84 (6 generations)	49	19	13–40	11q13.4–22.1
686 (DFNA55)	Henan	63 (5 generations)	30	11	21–55	9p13.2–13.3
727	Liaoning	44 (4 generations)	16	8	8–20	1p31–1p35
1301	Shandong	61 (6 generations)	29	17	12–42	working on
1318	Henan	102 (5 generations)	39	15	4–18	working on

substitution (925T > C) in the well-known deaf gene. Very recently, another novel mutation in this gene was identified in another independent Chinese pedigree with CPSHI collected by our lab (unpublished data).

In the positional cloning study, we mapped the first X-linked locus for auditory neuropathy, *AUNX1*, in a Chinese family [25, 26]. We also mapped a Y-linked hearing impairment family on Y chromosome, nominated as *DFNY1* locus. Recent studies showed that a novel mechanism was responsible for the deafness phenotype [5, 27–29].

Discussion and perspective

In the last decade, genetic studies of hearing loss including positional cloning of the underlying genes, functional characterization of the identified candidate genes and large-scale population-based epidemiological investigation of mutations in common hearing loss genes in various races have provided us insights onto the molecular basis for the human auditory system, and thus new conceptions of diagnosis, prevention and ultimate cure for the most common communication disorder worldwide [30]. Further advances in the field to establish the molecular pathways or correlates to the observed clinical phenotypes would allow researchers and otology clinicians to view and interpret development of this disease from new standpoints. Since the first gene for nonsydromic hearing loss was identified in 1995, up to 146 genetic loci have been mapped and 46 disease genes have been cloned in 30% of the loci identified (http://webhost.ua.ac.be/hhh/). Some scientists reckon that the number of genes related to human auditory system takes up 1% (250–300) of the total number of human genes (25, 000–30, 000) [31]. However, we have to recognize that follow-up positional cloning effort takes much longer than identification of the susceptibility loci for hearing loss. The only exceptions are 1998 and 2001 when seven hearing loss genes were cloned in the respective year, giving an overoptimistic impression that the genetic basis for human auditory system could be fully decoded in the near future. However, with discoveries of a large number of new genetic loci for varied clinic audiological phenotypes, high heterogneities presented in several well-studied genes, multiple causal genes at a single Mendelian inheritance locus and modifier genes as well as evolutionary divergence of founder's mutation, it is agreed by more people that fully deciphering the molecular pathways of human hearing functionalities is still a very challenging task [6, 31].

In our otology clinic, when children with congenital profound hearing loss come, clinicians can reasonably believe that their symptoms may result from a *GJB2* mutation at *DFNB1* locus. This likelihood is 20%–50%. Likewise, when a patient is found sensitive to aminoglycoside ototoxicity and also has *12S rRNA 1555G*, our clinicians will suggest averting the use of this type of drugs. When a patient is found having a *POU3F4* mutation, the clinician will alert that the patient may be at risk of stapes gushers when an inner ear surgery is performed. Finally, early interventions may be given to an EVA patient with *SLC26A4* mutation to prevent his progressive hearing loss. In short, the recent advance in genetic studies of hearing loss is becoming an integrative

component in designing most effective diagnosis for hearing loss and in delivering solid and convincing advices to patients in otology clinic.

Nevertheless, before the genetic architecture underlying hereditary hearing phenotypes is fully understood, we have to face challenges in improving current clinic practice and research for hearing loss because of the partial or fragmented genetic knowledge accumulated so far. Large amount of work is to be completed, including deciphering the mutation-induced molecular mechanisms, biochemical or pathogenic pathways leading to varied phenotypes of hearing loss and phenotypic heterogeneities. For examples, among the *GJB2*-induced profound hearing loss, the patients have different forms of mutations at the same (mutant homozygote) or different loci (compound heterozygotes). In some cases, both the patient and his parent(s) have one mutant, but the parent's hearing is normal. Among *SLC26A4*-induced EVA patients, more than 164 mutation types have been identified [15], but the resulted clinical phenotypes are largely indistinguishable from each other. For late onset hearing loss of dominant inheritance, it is unclear why patients with in-born pathogenic mutation demonstrate progressive hearing loss in their first or second decade. It remains a myth how environmental factors, post-transcription DNA methylation and various modifiers interplay with the underlying genetic network in the human auditory system. Some scientists estimate that 250-300 genes are related to the system, but only slight more than 70 genes are cloned. Pathogenic genetic factors for varieties of hearing loss phenotypes, such as noise-induced, age-related, idiopathic and neuropathy-associated hearing loss, are yet to be identified. To further understand the underlying functional mechanisms, mouse models may prove promising. However, translation of findings in these models to humans remains a challenging and is a largely unresolved issue. Although we are now in the stage of developing a matured molecular diagnosis for various types of hearing loss, congenital profound sensorineural hearing loss in particular, the studies for post-diagnosis intervention are largely in infancy. Among others, regeneration of hair cells, gene therapy and development of personalized medication are likely future targets in the domain of otology clinic and science.

In otology clinics, early diagnosis and subsequently timely intervention are becoming the most effective strategy for improving hearing care in the largest population in the world. There are 800,000 deaf children under seven years of age in China. If we are able to fully implement a national newborn genetic screening program, based on the knowledge that approximately 30% of deaf children are a result of gene mutations, 240,000 children are expected to receive timely intervention to avoid complete loss of their communication ability. In the meantime, 72 billions yuan of medical cost can be saved. In China, a yearly increment is the births of approximately 30,000 deaf children. Based on these numbers, genetic testing can help one third of these children and save 3,000 millions yuan for China. Therefore, we advocate an integration of genetic testing into the existing newborn screening program. This conception will lead to identification of deaf mutation carriers in 3% of the populations. With planned birth in Chinese families, a substantial reduction in the genetic load and therefore the incidence rate of hearing suffers in the next generation of this population is possible.

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