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Short Communication

Genetic Screening of Neonates for 20 Most Common Mutations in Deafness Associated Genes in Anhui Province of China

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Authors' Contribution
IB, JZ and JT conceived and designed the project. JT, GF and YS collected blood samples and performed experiments. JT, CW and MRK analyzed data and prepared first draft of the manuscript.

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ABSTRACT

Hearing loss is the most common human genetic disorder caused by mutations in various genes. Current study was initiated to analyze the 20 most common mutations by MALDI-TOF-MS method in deafness-associated genes (*GJB2*, *GJB3*, *SLC26A4* and mitochondrial 12SrRNA) in 3,331 newborns from Anhui Province of China. The analysis of data revealed that variations in *GJB2* has high frequency 2.82% while *SLC26A4*, *GJB3*, and mitochondrial 12SrRNA were found to have 2.49%, 0.42%, and 0.33% respectively. This study is conducted for the first time on newborns in Anhui province, China which confirms the significant role of mutations in etiology of deafness in this population.

Hearing loss is the most common sensorineural disorder limiting communication in humans, which is nearly found in 1-3 per 1,000 newborns. This could be caused by environmental or physical factors but the most commonly by genetic factors (Smith *et al*., 2005). These genetic factors normally inherited to offspring via autosomal dominant, autosomal recessive, X-linked and mitochondrial inheritance pattern (Xia *et al*., 2002; Finsterer and Fellinger, 2005; Duman and Tekin, 2012; Stanton *et al*., 2014). It is believed that the carrier frequencies of some mutational hot spots associated with NSHL vary from various racial and ethnic groups. It is difficult to analyze and determine the accurate genetic causes of heterogeneous traits of NSHL. However, it is evident that a large proportion of NSHL in Chinese population are due to a limited number of recurrent mutations resulting from common founders or a mutational hot spot (Ouyang *et al*., 2009). In China, previous epidemiological data showed that *GJB2*, *SLC26A4*, and mitochondrial 12SrRNA are the three most common deafness genes and account for 40% of the patients with hereditary hearing loss (Ouyang *et al*., 2009; Chen *et al*., 2011; Li *et al*., 2010; Wei *et al*., 2013; Lu *et al*., 2011; Du *et al*., 2014). However, the frequency of the four common genes in newborns has not yet been described in detail. Here, we screened 20 common mutations of these deafness-associated genes for the first time in newborns from Anhui province of China.

Materials and methods

Total 3331 unrelated Han Chinese neonates were recruited along with parents from 7 cities (HeFei, Lu'an, Bozhou, FiuYong, HuaiNan, Anqing, Chizhou) of Anhui Province of China.

Clinical and family history was obtained from the parents. Data regarding use of drugs during pregnancy was also collected. Among all parents, only one mother had aminoglycoside (antibiotics) associated deafness, the remaining parents had neither deafness nor family history. Written informed consents were taken from parents of the neonates and all the experimental procedures were approved by the Ethical Committee of Anhui Medical University. Three blood spots (about 150μm) from each neonate were collected by venipuncture of heel after 72 h of the birth. Genomic DNA was extracted from blood spots by DNA Beads Kit (Bioeasy Technology, Shenzhen, China) while the quantity and purity of the extracted DNA

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was tested using an ultraviolet spectrometry (ActGene Inc, Taipei, Taiwan).

Polymerase chain reaction (PCR), single base extension (SBE) and matrix assisted laser desorption ionization time of flight mass spectrometry MALDI-TOF-MS methods were used according to the manufacturer's protocols (BGI, Shenzhen, China) to simultaneously screen the 20 selected mutations causing hereditary hearing loss (Table I). To amplify the mutant sites of the genes different size of primers and unextended probes were used which previously designed and used by Zhang *et al*. (2013). Allele specific extending probes were used for every mutant allele having different molecular weight; these amplified primers were further separated by MALDI-TOF MS.

Table I.- Selected mutations in four deafness associated genes for genetic screening of Anhui's neonates.

Gene	Chromosomes location	Mutation
GJB2	13q11-q12	35delG, 167delT, 176_191del16GCTGCA AGAACGTGTG 235delC 299_300delAT
GJB3	1p34	538C>T, 547G>A.
SLC26A4	7q31	281C>T, 589G>A, 1174A>T, 1226G>A, 1229C>T, 1975G>C, 2027T>A, 2162C>T, 2168A>G, IVS7-2A>G, IVS15+5G>A.
Mitochondrial 12SrRNA		1494C>T, 1555A>G

The multiplex PCR was performed as previously reported (Zhang *et al.*, 2013; Imtiaz and Naz, 2012). The total volume of the reaction was 5μL consisting 1 μL of DNA template (10–25 ng/ μL), 1x PCR buffer (including 2 mmol/L magnesium chloride), 2 mmol/L magnesium chloride, 500 μmol/L deoxy-nucleoside triphosphate (dNTP) mix, 0.1 pmol/ μL of each preprimer, and 0.5 U of HotstarTaq. Following PCR condition was applied: denaturation at 94°C for 15 min, followed by 45 cycles of 20 s at 94°C, 30 s at 56°C, 1 min at 72°C, and a final extension was given for 3 min at 72°C. Then the PCR products were treated with shrimp alkaline phosphatase (SAP) to dephosphorylate remaining dNTPs.

The iPLEX primers extension reaction was performed according to manufacturer's guidelines (Sequenom, USA). Before the mass spectrometric analysis, 6mg of clean resin was added into the 384-well PCR plate to desalt the iPLEX extension products. After the purification, 3–10 nL products were dispensed into a 384-element SpectroCHIP bioarray (Sequenom,USA). TYPE 4.0 software (Sequenom, USA) was used to process and analyze iPLEX Spectro CHIP bioarrays. The analysis was performed according to the relative molecular mass of the single base extension products.

Results

Analysis of data revealed that overall 6.21% (207/3331) of subjects were carrying heterozygous or homozygous mutations in four selected genes (*GJB2*, *GJB3*, *SLC26A4*, and mitochondrial 12SrRNA) through screening by MALDI-TOF-MS. The frequency of the mutations in *GJB2* was higher (2.82%) than other genes which was detected in 94 cases, one case carried homozygous mutation, and one case carried compound heterozygous 235delC/299-300delAT and remaining 92/94 cases had heterozygous forms of the mutations (Supplementary Fig. 1A). Only 83 (2.49%) cases were carrying mutations in *SLC26A4*, 82 /83 cases were heterozygous carriers while one case had compound heterozygous 2168A>G/IVS7-2A>G mutation in *SLC26A4* (Supplementary Fig. 1B). There were 14 (0.42%) cases heterozygous carrier for 538C>T and 547G>A mutations in *GJB3* while 11 (0.33%) cases showed homozygous mutations 1555A>G in mitochondrial 12SrRNA (Table II, Supplementary Fig. 1C, D). In addition, three cases (0.09%) had heterozygous mutations 235delC and IVS7-2A>G in both *GJB2* and *SLC26A4* gene respectively. One case was found to have heterozygous 299-300delAT in *GJB2* as well as heterozygous 2168A>G in *SLC26A4* gene. Another individual had heterozygous 299-300delAT in *GJB2* gene compounded with a heterozygous IVS7-2A>G in *SLC26A4* (Table III).

Discussion

The current study was aimed to analyze the frequencies of 20 hot spot mutations in common deafness associated genes in newborns from Anhui province of China. Total five mutations in *GJB2*, 2 in *GJB3*, 11 in *SLC26A4* and 2 in mitochondrial 12SrRNA were studied. The finding of current study represents that mutation 235delC in *GJB2* is the most common in newborns of Anhui province, China, followed by IVS7-2A>G in *SLC26A4* gene.

GJB2 encodes the gap junction protein Connexin 26 (CX26), which is expressed in the cochlea and plays an important role in endolymphatic potassium recycling. It is well known that mutations in *GJB2* are the most common cause of inherited hearing loss in various populations of the world (Ouyang *et al.*, 2009). Approximately half of

Table II.- Genetic screening of 3331 neonates in Anhui Province of China.

Gene	Mutations	n		Carrier frequency (%)
		Homozygous	Heterozygous	
GJB2	235delC	1	71	2.16
	299-300delAT		15	0.45
	176-191del16		5	0.15
	35delG		1	0.03
	235delC/299-300delAT		1	0.03
SLC26A4	IVS7-2A>G		54	1.62
	IVS15+5G>A		6	0.18
	2168A>G		5	0.15
	1975G>C		4	0.12
	1174A>T		3	0.09
	1226G>A		3	0.09
	1229C>T		3	0.09
	2027T>A		3	0.09
	2162C>T		1	0.03
	IVS7-2A>G/2168A>G		1	0.03
GJB3	538C>T		7	0.21
	547G>A		7	0.21
Mitochondrial 12SrRNA	1555A>G	11		0.33

Table III.- Neonates carrying compound heterozygous mutations in two genes.

Gene	Mutations	n		Carrier frequency (%)
		Homozygous	Heterozygous	
GJB2/*SLC26A4*	235delC/IVS7-2A>G		3	0.09
	299-300delAT/2168A>G		1	0.03
	299-300delAT/IVS7-2A>G		1	0.03

the genetically inherited hearing loss cases in Caucasians and 1/3 of nonsyndromic hearing loss in the Greek population are associated with mutations in *GJB2* gene. Additionally, about 30% of autosomal nonsyndromic deafness in Turkey is attributed to mutations in *GJB2* (Kalay *et al*., 2005). In northern Pakistan *GJB2* mutations accounts for 4.28% (Bukhari *et al*., 2013) which is slightly lower than the reported prevalence of *GJB2* mutations in other Pakistani population (Santos *et al*., 2005; Salman *et al*., 2015). Dissemination of the *GJB2* mutations is significantly different among various populations. For example 35delG accounts for 70% European and American Caucasian deaf population with a carrier frequency ranging from 1.3 to 2.8%. The 167delT accounts for 40% of hearing loss in Ashkenazi Jews deaf population with 4% carrier frequency among Ashkenazi Jews (Ouyang *et al*., 2009). In the East Asian region, the 235delC of *GJB2* is the most frequent cause of inherited nonsyndromic deafness ranging from 5.10 to 34.0% (Abe *et al*., 2000; Wang *et al*., 2002; Ohtsuka *et al*., 2003; Hwa *et al*., 2003; Lee *et al*., 2008; Lu *et al*., 2011). Similarly in current study, 235delC (homozygous and heterozygous form) was found as the most prominent mutation in *GJB2* having 2.82% prevalence in neonates of Anhui province. Therefore, 235delC mutation can be considered as an important hot spot in genetic screening of hearing loss in Han Chinese population.

SLC26A4 gene is the second prominent (2.64% of carrier rate) in our cohort. *SLC26A4* encodes pendrin protein, is associated with Pendred syndrome and enlarged vestibular aqueduct (EVA). Pendred syndrome is a genetic disorder leading to congenital bilateral sensorineural hearing loss and goiter with occasional hypothyroidism, while EVA is described as inner ear malformation without goiter. The association of congenital sensorineural hearing loss with EVA can be diagnosed as enlargement of vestibular aqueduct by computed tomography (CT) scan and magnetic resonance imaging (MRI). Similar to *GJB2*, more than 100 mutations have been reported in *SLC26A4* with significant difference in the frequency and

distribution in different population (Ouyang *et al*., 2009). Khan *et al.* (2013) reported that mutations in *SLC26A4* exhibited moderate to severe familial hearing loss. In northern Europe deaf individuals, L236P, T416P, E384G and IVS8+1G>A are common mutations (Hilgert *et al*., 2009). While in China, the IVS7-2A>G mutation is the most common locus of *SLC26A4* in Han Chinese deaf population. Current study detected heterozygous form of the IVS7-2A>G mutation in 59/3331 cases.

In current study mutations in *GJB3* gene were found in 14 cases (0.42%) in Anhui's neonates. This gene is associated with bilateral profound hearing impairment (Li *et al*., 2014), and is initially cloned by (Xia *et al.*, 1998). In Jinan China 0.27% neonates had heterozygous mutation in *GJB3* (Xiang *et al*., 2015). Besides nuclear genes' mutations, the mutations of mitochondrial gene 12SrRNA are also associated with hearing loss, especially aminoglycoside-induced deafness (Hilgert *et al*., 2009). Individuals carrying 1555A >G or 1494C > T mutation in the mitochondrial 12SrRNA are susceptible to aminoglycoside antibiotics, which can cause hypersensitivity and ototoxicity (Li *et al*., 2005). Aminoglycoside antibiotics such as streptomycin, gentamycin and kanamycin are widely used to treat a wide spectrum of bacteria in China. Therefore, we selected 12SrRNA to screen in Anhui newly born babies and 0.33% cases were found to have 1494C > T mutation in this gene.

In summary, hearing loss is considered as common heredity sensorineural disorder. Genetic screening at neonatal stage can help them to give clue for further prevention therefore current study was undertaken. Although most of the cases were found heterozygous carrier for tested mutations but homozygous form of a mutation in *GJB2* (235delC) and mitochondrial 12SrRNA (1494C > T) was in some cases. It is therefore suggested that further inheritance and pathogenicity of carrier alleles can be prevented by marrying carriers with genetically normal counterparts.

Ethical approval number

The official reference number for ethical approval of this study is AMU26-080611.

Statement of conflict of interest

Authors have declared no conflict of interest.

Supplementary Figure 1 is available at website http://zsp.com.pk/pdf48/QPJZ-0459-2015-F%20(Supplementary%20Figure%201).pdf

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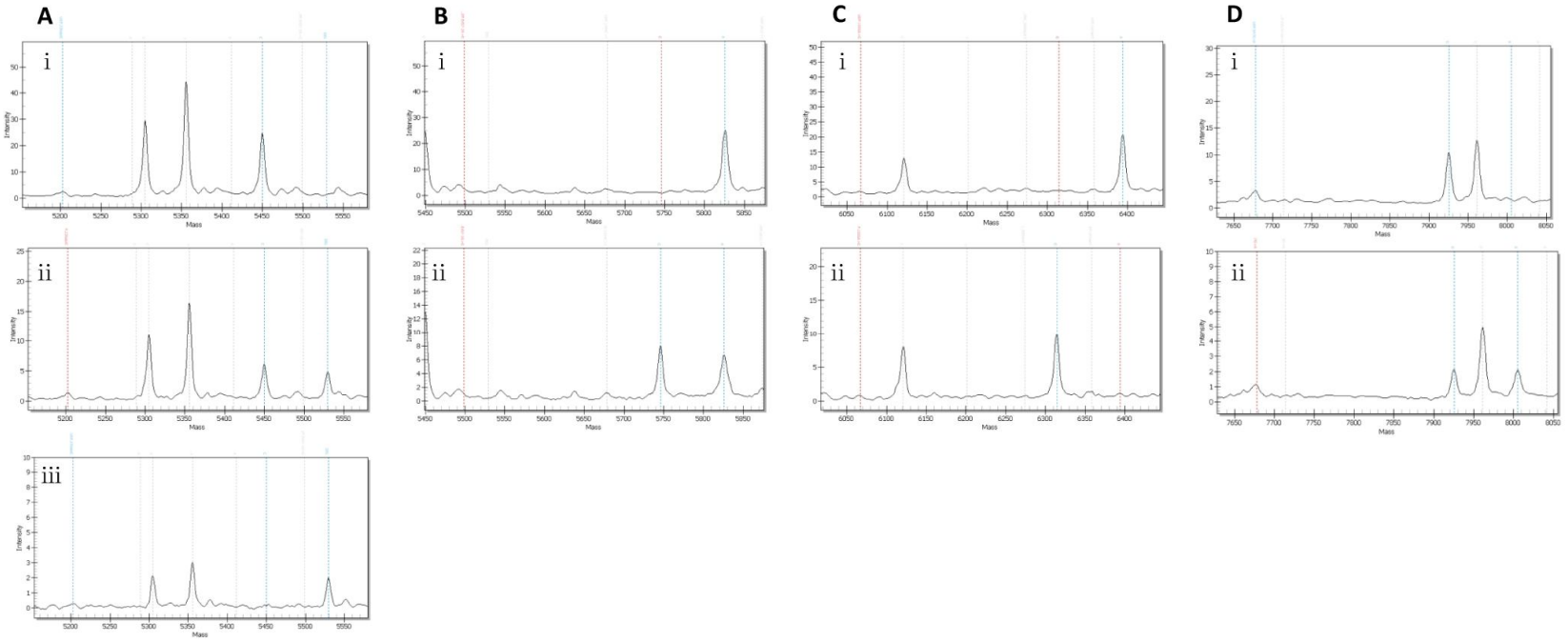

Supplementary Fig. 1. Mass chromatograms of mutations in deafness associated genes. A) shows Mass chromatograms of GJB2 c.235delC (i) Normal wild type (ii) Heterozygous and (iii) Homozygous form of the mutation. B) Mass chromatograms of SLC26A4 c. IVS7-2A>G (i) Normal wild type and (ii) Heterozygous form of the mutation C) Mass chromatograms of mitochondrial 12SrRNA c.1555A>G (i) Normal wild type and (ii) Homozygous form of the mutation D) Reveals Mass chromatograms of GJB3 c.547G>A (i) Normal wild type, and (ii) Heterozygous form of the mutation.