Received: 5 May 2018 | Accepted: 29 May 2018

DOI: 10.1002/jcla.22592

RESEARCH ARTICLE

WILEY

GJB3/GJB6 screening in *GJB2* carriers with idiopathic hearing loss: Is it necessary?

Kaitian Chen[1,2] | **Xuan Wu**[1,2] | **Ling Zong**[3] | **Hongyan Jiang**[4]

[1]Department of Otorhinolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

[2]Institute of Otorhinolaryngology, Sun Yat-sen University, Guangzhou, China

[3]Department of Otorhinolaryngology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

[4]Department of Otorhinolaryngology, Hainan General Hospital, Haikou, China

Correspondence
Hongyan Jiang, Department of Otorhinolaryngology, Hainan General Hospital, Haikou, China.
Email: jhongy@mail.sysu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81500802 and 81700912; Natural Science Fund of Guangdong Province, Grant/Award Number: 2015A030310072 and 2016A030310286

Background: Genetic analysis detected excessive mono-allelic recessive *GJB2* mutations in individuals with idiopathic deafness; the remaining alleles in trans/cis are underdetermined. The aim of this study was to assess the contributions of variants in *GJB3* or *GJB6* to non-syndromic sensorineural hearing impairment (NSHI) in Chinese patients with mono-allelic *GJB2* mutations.

Methods: The entire coding sequences of *GJB3/GJB6*, as well as deletions in *GJB6*, in a cohort of NSHI patients (n = 100) carrying likely pathogenic heterozygous *GJB2* mutations, were tested. Targeted next generation sequencing was further performed in a multiplex family GDHY with moderate to profound NSHI.

Results: Putatively causative *GJB3* variant underlied 1% (1/100) in this cohort. In family GDHY, we identified a rare *GJB3* c.250G>A mutation, as double heterozygotes with *GJB2* c.109G>A and/or a novel *GJB2* mutation c.638T>C predicted to be damaging in a digenic inheritance after precluding other attributable mutations from 127 deafness genes. No *GJB6* mutation was found.

Conclusions: *GJB3/GJB6* variants account for a low proportion in autosomal recessive *GJB2* mutation carriers in our cohort. Environmental causes, or other NSHI relevant genes, revealed by targeted next generation sequencing or whole exome sequencing, may play major roles in triggering deafness in these patients.

KEYWORDS
GJB2, *GJB3*, *GJB6*, hearing impairment, targeted next generation sequencing

1 | INTRODUCTION

Different connexin (CX) proteins are co-expressed in the supporting cells in cochlea to execute cellular communication through the hemichannel or gap junctions.[1,2] It's generally estimated that *GJB2* (encoding connexin 26, CX26) causes about 50% of non-syndromic hearing impairment (NSHI) in many populations.[3-5] Other connexin genes mutations (eg, *GJB3/GJB6*) have also been linked to NSHI.[6-12] Particularly, the digenic inheritance model has been addressed. For example, c.235delC/c.497A>G, c.235delC/c.580G>A and c.299-300delAT/c.580G>A in *GJB2/GJB3* were found in three Chinese families respectively (probably in trans effect).[8] The coexistence of either common deletion *del(GJB6-D13S1830)* and *del(GJB6-D13S1854)* in *GJB6* with *GJB2* mutations, were demonstrated to disrupt *GJB2* expression at the transcriptional level, presumably by removing one or more cis-regulatory elements located within the deleted region (in cis effect).[7,9] Despite these suggestive findings, the estimated proportions of digenic mutations and the necessity of screening of either *GJB3* or *GJB6* in Chinese are still uncertain.

In our earlier large-scale cohort study,[3] general genetic analysis detected a heterozygous *GJB2* state in 14.0% of individuals with congenital hearing loss, suggesting a larger role for additional recessive genes in these cases. Since then, *GJB2* carriers are continuing to be found in our clinic. We hypothesized that there may be putatively causative *GJB3* or *GJB6* variants that interact with *GJB2* mutations to cause hearing loss in digenic heterozygotes in these carriers. In this study, we extended our study through examining the entire coding sequences of *GJB3/GJB6* in NSHI by Sanger sequencing. Targeted next generation sequencing (NGS) was further performed in a familial pedigree.

2 | PATIENTS AND METHODS

The institutional review board of the First Affiliated Hospital, Sun Yat-sen University approved our study. Informed consent had been obtained from parents or their guardians prior to this study.

2.1 | Study participants, PCR, Sanger sequencing

A total of 100 peripheral blood DNA samples with mono-allelic *GJB2* mutations were collected from our Chinese NSHI hereditary database (Table 1). Temporal imaging and auditory assessments with auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) in each participant had ruled out the risk of enlarged vestibular aqueduct or auditory neuropathy. No other known etiology was found, nor had these samples been studied in prior report.[13] Common mutations in *SLC26A4* or mitochondrial genes were previously excluded. Also, neither the splice site mutation c. IVS1 + 1G>A, nor defects in exon 1 and its basal promoter in *GJB2* was present.

DNA fragments spanning the *GJB2*, *GJB3* and *GJB6* coding exons were amplified by PCR methods (*GJB2*: Forward primer: TTGGTGTTTGCTCAGGAAGA; Reverse primer: GGCCTACAG GGGTTTCAAAT; *GJB3*: Forward primer: TACGATGGTTTTTCC TCTAATTCT; Reverse primer: TTGCATAACTTAGTGAACTCAGAG; *GJB6*: Forward primer: TATCACCGTGTCACTTTCC; Reverse primer: CAGGTTGGTATTGCCTTC). Two large deletions in *GJB6* were also tested as previously described.[7,9] PCR with a volume of 25 μL, containing 100 ng DNA template, 5pmol of each primer, 12.5 μL 2X PCR buffer mixtures and ddH_2O, were conducted with an annealing temperature 56°C. The products were purified and sequenced by Sanger sequencing using a 3730XL DNA analyzer (Applied Biosystems, Carlsbad, California, USA).

2.2 | Targeted NGS in GDHY-family

We extended our study by using targeted deafness genes capture and NGS on samples III:2 from the family-GDHY to explore other hidden modified alleles. The designed panel covers all coding exons, splice sites and flanking intron sequences of 127 known deafness genes including 20 autosomal dominant NSHI (DFNA) genes, 30 autosomal recessive NSHI (DFNB) genes, six DFNA/DFNB genes, three X-linked deafness genes, two maternally inherited deafness genes, 11 syndromic/non-syndromic deafness genes, and 55 syndromic deafness genes (Table S1). The captured regions were analyzed using illumina Hiseq2000 platform. Variants detected with >0.05 frequency in 1000 Genome, dbSNP, and Hapmap and >0.3 in YH database[14] were filtered. The identified mutations were further confirmed in the family members by Sanger sequencing as described above.

2.3 | In silico analysis

Multiple sequence alignments were completed with *ClustalX2*. We used *SIFT*, *Polyphen-2*, *MutationTaster* to evaluate possible pathogenicity. The crystalline structure of human CX31 (*GJB3*) was applied with its closest homologous protein family member, human CX26 (*GJB2*), which has been elucidated by crystallography (PDB entry 2ZW3) to a resolution of 3.5A°. Three-dimensional modeling of human wild-type CX26 and CX31, CX26-p.L213S, and CX31-p.V84I mutations was performed using *SWISS-MODEL*, an automated homology modeling program.[15]

3 | RESULTS

The basic information of this cohort was summarized in Table 1. Overall, one missense mutation c.250G>A (p.Val84Ile, 1/100) and one synonymous variant c.357T>C (rs41310442, p.Asn119=, 12/100) in *GJB3* exon 2 were finally detected in these unrelated families. The c.357T>C was classified as benign and its pathogenicity remains disputable, since synonymous variant may result in hearing loss as well.[16] Though the variant c.250G>A was classified as polymorphism in previous report,[17] deleterious effect in cellular level induced by this variant strongly debated its "Benign" significance.[18] This yielded a putative mutational rate of 1% in *GJB2*/*GJB3* genes

TABLE 1 Clinical data of the 100 unrelated NSHI patients

Demographic data		Degree of hearing loss (%)		Genotype			
				GJB2 variants	No.	MAF	Amino acid change
Gender (M: F)	61:39	Mild	0	c.109G>A	34	8.194%	p. Val37Ile
Age (y)	14.1 ± 8.5	Moderate	14	c.235delC	26	0.6412%	p. Leu79CysfsTer3
Prelingual deafness (No.)	89	Severe	34	c.299-300delAT	14	0.0901%	p. His100ArgfsTer14
Postlingual deafness (No.)	11	Profound	33	c.176-191del16	11	0.01739%	p. Gly59AlafsTer18
Sporadic cases (No.)	91	Anacusis	19	c.512insAACG	9	0.0265%	p. Ala171GlufsTer40
Familial cases (No.)	9			c.35delG	5	0.00%	p. Leu10TrpfsTer4
				c.638T>C	1	-	p. Leu213Ser

MAF, Minor Allele Frequency in gnomAD in East Asian populations.

responsible for NSHI in this cohort. Additionally, we failed to identify any potentially suspected allele in *GJB6* gene.

The *GJB3* c.250G>A was identified in the GDHY-family (Figure 1). The propositus (II:2) had congenital bilateral moderate hearing loss and was found to carry a novel *GJB2* missense mutation c.638T>C (p.L213S) in heterozygosity. The genetic trait of this variant was predicted to be possibly autosomal recessive by tracing the familial transmission and led us to perform *GJB3*/*GJB6* sequencing and NGS for this family. Both children (III:1 and III:2) were diagnosed as bilateral profound NSHI after birth and received cochlear implants at the age of six. Temporal computed tomography and magnetic resonance imaging of the two siblings were normal. The average depth of targeted region in the III:2 samples achieved a 322-fold coverage. In addition, 98.25% of the targeted sequences were covered. The sequencing depth was 96.65% of captured bases with over 30-fold coverage. Given the filtration criteria and the hereditary pattern, the mutation c.638T>C compound with c.109G>A (p.V37I, rs72474224) in *GJB2* exon 2, c.250G>A (rs145751680) in *GJB3* exon 2 were suspected as the disease-causing genes in this multiplex family exclusively by NGS. Only the father was heterozygous with normal audition. The contributions of other mutations from the NGS panel were excluded. The *GJB2* p.V37I/p.L213S/*GJB3* p.V84I mutations cosegregated with the phenotype of NSHI in this family. The tri-allelic mutations might result in increased penetrance of hearing loss (Figure 1D).

The change at position p.V84I in CX31 showed no gain or loss of hydrogen bonds (Figure S1). Nevertheless, the p.V84 is located in evolutionarily conserved position of the CX gene family across different species and the alteration of Val to Ile is suspected to have deleterious effect by *SIFT*, *Polyphen-2* and *MutationTaster*. Transfection of p.V84I plasmid into HEK293 and Hela cells showed defect in ionic and biochemical couplings, a result supporting the pathogenicity of p.V84I in vitro.[18]

The mutation c.638T>C was not found in the 1000 Genome, dbSNP, Hapmap or YH databases. It's also absent in 701 Chinese NSHI and 180 normal control.[3] The coding protein p.L213 is highly conserved in different species (Figure 2A). Bioinformatic analysis predicted the c.638T>C to be "damage causing". The modeling structure showed that p.L213 is present in the fourth transmembrane region of CX26. This alteration in the CX26 protein at p.L213S results in the gaining of a new hydrogen bond with p.E209, when compared to wild-type CX26 (Figure 2B).

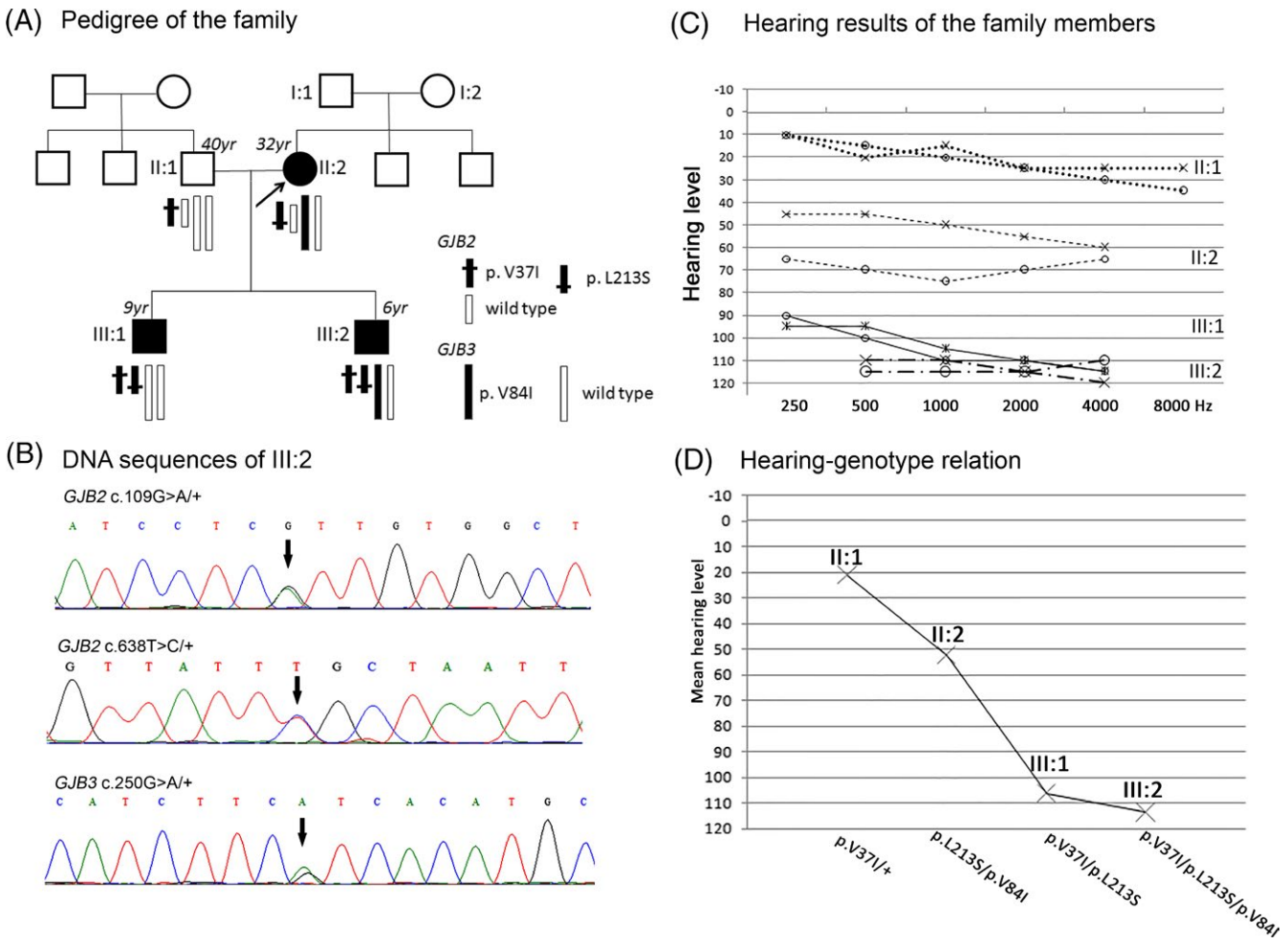

FIGURE 1 Illustration of the family with digenic tri-allelic *GJB2*/*GJB3* mutations. The pedigree showed mutations in *GJB2*/*GJB3* genes (A-B), with different hearing levels in each member (C). The genotype-phenotype relation was summarized in (D)

4 | DISCUSSION

In this cohort, *GJB3* mutants appear to account for a small proportion in double heterozygous state with autosomal recessive *GJB2* mutation in NSHI population (with an allelic frequency of 0.5%). Only one putatively pathogenic mutant existed in family GDHY, in which *GJB2* p.V37I/p.L213S/*GJB3* p.V84I digenic heterozygotes cosegregated with the hearing loss phenotype. It's inferred that screening for *GJB3* mutations may help illustrate the hereditary etiologies of deafness in limited cases bearing heterozygous *GJB2* mutations. Instead, the role of *GJB6* variants was extremely minor, implying the necessity for exploring other causative genes or environmental causes in our patients.

The allelic frequency of *GJB3* mutation p.V84I is estimated to be 0.25% among Chinese NSHI,[13] and 0.0037 in 1000 Genome. Despite the fact that the biallelic mutations in *GJB2* p.V37I/p.L213S were predicted to cause sufficient connexin dysfunction to affect audition, the additive effect of the homologous connexon gene *GJB3* p.V84I mutation cannot be disregarded. Based on previous functional studies and the complex interaction between homologous connexin genes (in trans or cis regulatory role),[2,6-9,18,19] we assumed that p.V84I in CX31 might interact with CX26 p.L213S to cause hearing loss with deleterious effect. The function and permeability of these gap junctions in the inner ear of the affected mother (II:2) were therefore impaired. The complexity of digenic tri-allelic mutations p.V37I/p.L213S/p.V84I in *GJB2*/*GJB3* increased significantly. According to the genotype-hearing correlation, these tri-allelic mutations might result in increased penetrance of hearing impairment. Functional evidence with ironic and biochemical studies on CX26/CX31 heteromeric hemichannels is needed.

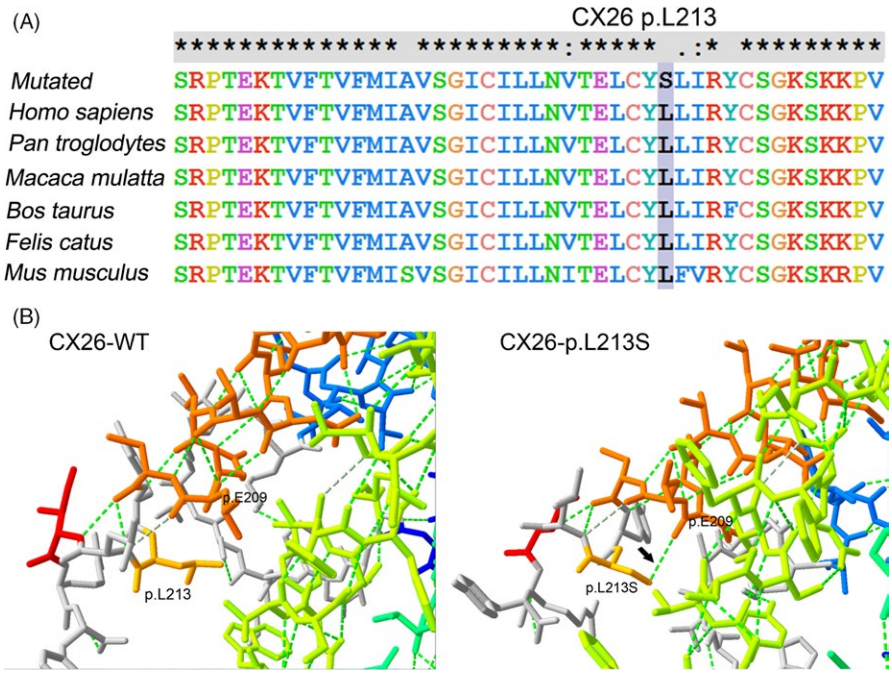

FIGURE 2 Analysis of the p.L213S mutation in *GJB2*. A, The novel mutation p.L213S in *GJB2* was highly conserved among different species. B, The modeled structure shows that p.L213S creates a new hydrogen bond (arrow) with p.E209 compared to wild-type

TABLE 2 Digenic *GJB2*/*GJB3* mutations in various populations

Patients	Phenotype	*GJB2*	*GJB3*	Hearing loss	Ethnicity	Reference
1	Palmoplantar keratoderma and deafness	p.M34T/p.D66H	p.R32W/+	N/A	UK	Kelsell et al
2	NSHI	p. Leu79CysfsTer3/+	p.N166S	Profound	Chinese	Liu et al
3	NSHI	p. Leu79CysfsTer3/+	p.A194T/+	Profound	Chinese	Liu et al
4	NSHI	p. His100ArgfsTer14/+	p.A194T/+	Profound	Chinese	Liu et al
5	NSHI	p.V37I /+	p.A194T/+	95/103 dB	Chinese	Chen et al
6	NSHI	p.L213S /+	p.V84I /+	52.5/70 dB	Chinese	Present study
7	NSHI	p.V37I/p.L213S	p.V84I/+	113.75/113.75 dB	Chinese	Present study
8	NSHI	p.V193E/+	p.A194T/+	87.5 dB	Korean	Kim et al

N/A, not available.

The digenic inheritance in autosomal recessive NSHI caused by mutations in *GJB2*/*GJB3* has been documented in different populations (Table 2). However, a digenic tri-allelic mutation inheritance for p.V37I/p.L213S/p.V84I in *GJB2*/*GJB3* was never seen, indicating the genetic heterogeneity of NSHI. Given this condition, targeted NGS panel is increasingly used as an alternative to reveal uncommon deafness-associated genes. Examples of this were the successful identifications of rare/novel mutations in *PCDH15*, *MYO7A*, *CDH23* genes in sporadic families by us and other authors.[6,20-24] Hence, it's likely to uncover the hidden genetic etiologies for *GJB2* carriers by NGS in future, which could not be achieved in this study owing to economic cost.

Great efforts have been made toward clarifying the contributions of *GJB3* and *GJB6* mutations to deafness in diverse populations during the past decades.[8,10,11,13,18,25-29] To address this issue, Yang et al screened 380 Chinese (260 with NSHI and 120 with normal hearing) for variants in eight connexin genes. Only three patients possessed heterozygote c.520G>A in *GJB3* (3/260; 1.15%) and another individual (1/260; 0.38%) was carrier of *GJB6* c.119C>T mutation.[27] Likewise, a genetic study of 129 Italian NSHI infants for *GJB6* and *GJB3* genes found no mutation.[28] Similar results were observed in American,[10] Moroccan,[25] and Indian[29] NSHI. The allele frequencies of variants in *GJB3* and *GJB6* were estimated to be "0-2.78%"[8,10,13,25-28] and "0%-1.42%"[13,25-29] respectively. Our present results, in combination with preceding studies, suggest that the effect of *GJB3* and *GJB6* in *GJB2* carriers may not be predominant. Environmental causes or unknown mutations in other genes are assumed to trigger hearing loss in these patients, who might otherwise be coincidental *GJB2* carriers. Advanced genetic tool, such as NGS or whole exome sequencing, may shed new light on the potential candidate genes, in them.

This study has several weaknesses. Apart from the limited numbers in this cohort, we were unable to collect the data of the I:1 and I:2 in this family, who did not prepare to participate in our study, since they "had normal hearing and language ability". Also, the *GJB3* p.V84I may be a potential modifier, but the mechanism was not understood yet. For such hypothesis to be verified, it is of utmost importance to verify the extent of gap junction damage at the cellular level by constructing digenic mutations model in vitro and in vivo.

5 | CONCLUSIONS

GJB3/*GJB6* variants account for a low proportion in autosomal recessive *GJB2* mutation carriers in our cohort. Environmental causes, or other NSHI relevant genes, revealed by targeted NGS or whole exome sequencing, may play a major role in triggering deafness in these patients.

ACKNOWLEDGEMENTS

We thank all participants in this study for their cooperation. The study was supported by grants from the National Natural Science Foundation of China (No. 81500802, 81700912) and the Natural Science Fund of Guangdong Province (No. 2015A030310072, 2016A030310286).

ORCID

Hongyan Jiang http://orcid.org/0000-0003-1308-5042

REFERENCES

1. Zhao HB, Kikuchi T, Ngezahayo A, White TW. Gap junctions and cochlear homeostasis. *J Membr Biol*. 2006;209:177-186.
2. Ahmad S, Chen S, Sun J, Lin X. Connexins 26 and 30 are co-assembled to form gap junctions in the cochlea of mice. *Biochem Biophys Res Commun*. 2003;307:362-368.
3. Chen K, Zong L, Liu M, et al. Developing regional genetic counseling for southern Chinese with nonsyndromic hearing impairment: a unique mutational spectrum. *J Transl Med*. 2014;12:64.
4. Dai P, Yu F, Han B, et al. GJB2 mutation spectrum in 2,063 Chinese patients with nonsyndromic hearing impairment. *J Transl Med*. 2009;7:26.
5. Rabionet R, Zelante L, Lopez-Bigas N, et al. Molecular basis of childhood deafness resulting from mutations in the GJB2 (connexin 26) gene. *Hum Genet*. 2000;106:40-44.
6. Kim SY, Kim AR, Kim NKD, et al. Unraveling of enigmatic hearing-impaired GJB2 single heterozygotes by massive parallel sequencing: DFNB1 or not? *Medicine (Baltimore)*. 2016;95:e302914.
7. Rodriguez-Paris J, Tamayo ML, Gelvez N, Schrijver I. Allele-specific impairment of GJB2 expression by GJB6 deletion del(GJB6-D13S1854). *PLoS ONE*. 2011;6:e21665.
8. Liu XZ, Yuan Y, Yan D, et al. Digenic inheritance of non-syndromic deafness caused by mutations at the gap junction proteins Cx26 and Cx31. *Hum Genet*. 2009;125:53-62.
9. Rodriguez-Paris J, Schrijver I. The digenic hypothesis unraveled: the GJB6 del(GJB6-D13S1830) mutation causes allele-specific loss of GJB2 expression in cis. *Biochem Biophys Res Commun*. 2009;389:354-359.
10. Mhatre AN, Weld E, Lalwani AK. Mutation analysis of Connexin 31 (GJB3) in sporadic non-syndromic hearing impairment. *Clin Genet*. 2003;63:154-159.
11. Liu XZ, Xia XJ, Xu LR, et al. Mutations in connexin31 underlie recessive as well as dominant non-syndromic hearing loss. *Hum Mol Genet*. 2000;9:63-67.
12. Kelsell DP, Wilgoss AL, Richard G, Stevens HP, Munro CS, Leigh IM. Connexin mutations associated with palmoplantar keratoderma and profound deafness in a single family. *Eur J Hum Genet*. 2000;8:141-144.
13. Chen K, Zong L, Zhou W, Liu M, Jiang H. GJB3 and GJB6 genes screening in 200 non-syndromic hearing impairment patients. *J Audiol Speech Pathol*. 2012;20:198-200. in Chinese.
14. Li G, Ma L, Song C, et al. The YH database: the first Asian diploid genome database. *Nucleic Acids Res*. 2009;37(Database issue):D1025-D1028.
15. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*. 2006;22:195-201.
16. Kim Y, Kim HR, Kim J, et al. A novel synonymous mutation causing complete skipping of exon 16 in the SLC26A4 gene in a Korean family with hearing loss. *Biochem Biophys Res Commun*. 2013;430:1147-1150.
17. Yuan Y, You Y, Huang D, et al. Comprehensive molecular etiology analysis of nonsyndromic hearing impairment from typical areas in China. *J Transl Med*. 2009;7:79.

18. Oh SK, Choi SY, Yu SH, et al. Evaluation of the pathogenicity of GJB3 and GJB6 variants associated with nonsyndromic hearing loss. *Biochim Biophys Acta*. 2013;1832:285-291.
19. Balciuniene J, Dahl N, Borg E, et al. Evidence for digenic inheritance of nonsyndromic hereditary hearing loss in a Swedish family. *Am J Hum Genet*. 1998;63:786-793.
20. Mutai H, Suzuki N, Shimizu A, et al. Diverse spectrum of rare deafness genes underlies early-childhood hearing loss in Japanese patients: a cross-sectional, multi-center next-generation sequencing study. *Orphanet J Rare Dis*. 2013;8:172.
21. Vona B, Muller T, Nanda I, et al. Targeted next-generation sequencing of deafness genes in hearing-impaired individuals uncovers informative mutations. *Genet Med*. 2014;16:945-953.
22. Yang T, Wei X, Chai Y, Li L, Wu H. Genetic etiology study of the non-syndromic deafness in Chinese Hans by targeted next-generation sequencing. *Orphanet J Rare Dis*. 2013;8:85.
23. Zhan Y, Liu M, Chen D, Chen K, Jiang H. Novel mutation located in EC7 domain of protocadherin-15 uncovered by targeted massively parallel sequencing in a family segregating non-syndromic deafness DFNB23. *Int J Pediatr Otorhinolaryngol*. 2015;79:983-986.
24. Zong L, Chen K, Wu X, Liu M, Jiang H. Compound heterozygous MYO7A mutations segregating Usher syndrome type 2 in a Han family. *Int J Pediatr Otorhinolaryngol*. 2016;90:150-155.
25. Nahili H, Ridal M, Boulouiz R, et al. Absence of GJB3 and GJB6 mutations in Moroccan familial and sporadic patients with autosomal recessive non-syndromic deafness. *Int J Pediatr Otorhinolaryngol*. 2008;72:1633-1636.
26. Beck C, Perez-Alvarez JC, Sigruener A, et al. Identification and genotype/phenotype correlation of mutations in a large German cohort with hearing loss. *Eur Arch Otorhinolaryngol*. 2015;272:2765-2776.
27. Yang JJ, Huang SH, Chou KH, Liao PJ, Su CC, Li SY. Identification of mutations in members of the connexin gene family as a cause of non-syndromic deafness in Taiwan. *Audiol Neurootol*. 2007;12:198-208.
28. Chinetti V, Iossa S, Auletta G, et al. Mutational analysis for GJB2, GJB6, and GJB3 genes in Campania within a universal neonatal hearing screening programme. *Int J Audiol*. 2011;50:866-870.
29. Adhikary B, Ghosh S, Paul S, et al. Spectrum and frequency of GJB2, GJB6 and SLC26A4 gene mutations among nonsyndromic hearing loss patients in eastern part of India. *Gene*. 2015;573:239-245.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Chen K, Wu X, Zong L, Jiang H. *GJB3/GJB6* screening in *GJB2* carriers with idiopathic hearing loss: Is it necessary? *J Clin Lab Anal*. 2018;32:e22592. https://doi.org/10.1002/jcla.22592