Brief Report

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Analysis of mutations in the *FOXI1* and *KCNJ10* genes in infants with a single-allele *SLC26A4* mutation

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Summary **The current study investigated how the *FOXI1* and *KCNJ10* genes were affected in infants with a single-allele mutation in the *SLC26A4* gene, and it determined the audiological phenotypes of infants with double heterozygous mutations (DHMs) in the three genes. Subjects were 562 infants with a single-allele *SLC26A4* mutation detected during neonatal deafness genetic screening; the infants were seen as outpatients by Otology at Beijing Tongren Hospital. All subjects underwent *SLC26A4* sequencing. Twenty infants had a second-allele variant while the remaining 542 had an *SLC26A4* single-allele mutation. Infants also underwent *FOXI1* and *KCNJ10* sequencing. All patients with double heterozygous mutations in the aforementioned genes underwent an audiological evaluation and a limited imaging study; variants and audiological phenotypes were analyzed. Of 562 patients, 20 had *SLC26A4* bi-allelic mutations; 8 carried single mutations in both *SLC26A4* and *KCNJ10*. No pathogenic mutations in the *FOXI1* gene were found. Four missense mutations in *KCNJ10* were detected, including c.812G>A, c.800A>G, c.53G>A, and c.1042C>T. Eight individuals with a DHMs all passed universal newborn hearing screening, and all were found to have normal hearing. These data suggest that individuals with an *SLC26A4* single-allele mutation, combined with *FOXI1* or *KCNJ10* gene mutations, do not suffer hearing loss during infancy, though this finding is worthy of further follow-up and in-depth discussion.**

Keywords: Infants, *SLC26A4* gene, *FOXI1* gene, *KCNJ10* gene, audiological evaluation

1. Introduction

SLC26A4 gene mutations are associated with deafness. In China, the rate of mutation in the *SLC26A4* gene in patients with an enlarged vestibular aqueduct (EVA) is approximately 97%. The most common variant is c.919-2A>G (*1*). According to Chinese studies, 11.6-38% of patients with EVA were unable to be identified based on pathogenic factors. Of those, 7.4-24% had an *SLC26A4* mono-allelic mutation (*2-4*). Foreign studies found that among patients with EVA the rate of *SLC26A4* bi-allelic and mono-allelic mutations was 16-83.9% and 16-36%, respectively (*5-8*).

A large number of individuals with a single-allele *SLC26A4* mutation have been identified as genetic screening for neonatal deafness has advanced in China. Clinicians are increasingly emphasizing the diagnosis of this condition and genetic counseling for patients. In China, Zhao *et al*. reported that the frequency of a second-allele variant in infants with a known single-allele mutation in the *SLC26A4* gene was 3.50% (13/371) for any type of variant and 2.96% (11/371) for pathogenic mutations (9). In other words, around 97% of these infants could not be genotyped using *SLC26A4* gene sequencing.

Several recent studies have indicated that mutations

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in the *FOXI1* and *KCNJ10* genes might also be associated with PDS/DFNB4 and that these mutations are inherited with heterozygous mutations in *SLC26A4* (*10,11*). Most of the subjects in studies that analyzed mutations in *FOXI1* or *KCNJ10* were patients with sensorineural hearing loss (SNHL) or EVA.

One aim of the current study was to analyze mutations in the *FOXI1* and *KCNJ10* genes in infants with a single-allele *SLC26A4* mutation. Another aim of this study was to identify the audiological phenotypes of infants with DHMs in those three genes.

2. Methods

2.1. *Subject recruitment*

Subjects were 562 Chinese newborns with a single-allele *SLC26A4* mutation that was detected during neonatal deafness genetic screening between April 2015 and March 2019. Exonic and flanking splice site regions of the *SLC26A4* gene were sequenced in all subjects. Subjects were screened for 4 genes and 15 pathogenic variants. Sequencing of the *FOXI1* and *KCNJ10* genes was conducted for patients without an *SLC26A4* second-allele variant. All patients with DHMs in the aforementioned genes underwent an audiological evaluation and a limited imaging study.

2.2. *DNA analysis*

Genomic DNA was extracted from 2 mL of whole blood from each patient using the Blood DNA kit (Tiangen Biotech, Beijing, China). Exons (coding areas) and the flanking splice sites of the *SLC26A4*, *FOXI1*, and *KCNJ10* genes were screened for mutations *via* amplification with PCR and bidirectional sequencing. The American College of Medical Genetics (ACMG) guidelines were used for variant interpretation (*12*).

2.3. *Bioinformatics and validation of the variants*

Sequence data were analyzed by aligning sequences with NCBI reference sequences of *SLC26A4* (NG_008489.1), *KCNJ10* (NG_016411.1) and *FOXI1* (NG_012068.2) using the software DNA Star 5.0. The 1000 Genomes Project database, ClinVar and dbSNP databases of the NCBI, and the Deafness Variation Database (DVD) were used as references to assess the novelty of the mutations found. Online tools including Mutation Taster, SIFT, CADD, and PolyPhen-2 were used to predict functional outcome of variants. GERP and Phylop were used to determine predicted conservation scores for variants.

2.4. *Audiological evaluation*

A comprehensive audiological evaluation including ABR, DPOAE, auditory steady-state response (ASSR), and acoustic immittance (AI) was performed. AI (226 Hz) was classified as A, B or C. AI (1,000 Hz) was classified as unimodal, bimodal, or flat (*13,14*).

Audiological evaluations were performed in accordance with the description of HSHL by Mazzoli *et al*. (*15*). The hearing threshold was calculated as the average hearing level at 0.5, 1.0, 2.0, and 4.0 kHz according to WHO standards (1997). Given the subjects' young age, the ABR threshold and ASSR were recorded, and mean thresholds at frequencies in the 0.5 to 4 kHz range were averaged to obtain an approximation for the behavioral hearing threshold (*16,17*).

2.5. *Imaging study*

Computed tomography of the temporal bone or magnetic resonance imaging of the inner ear was performed.

3. Results and Discussion

3.1. *Demographic data*

Of 562 subjects (359 males and 203 females), 20 had an *SLC26A4* second-allele variant while 542 had only an *SLC26A4* single-allele mutation. Subjects ranged in age from 3 to 34 months. The average age at first visit was 5.16 ± 3.21 months. A flowchart of research procedures for patients undergoing neonatal deafness genetic screening and *SLC26A4*, *FOXI1*, and *KCNJ10* sequencing is shown in Figure 1. Eight subjects carried single mutations in both *SLC26A4* and *KCNJ10*. No pathogenic mutations in *FOXI1* were found.

3.2. *Information on variants of the KCNJ10 gene*

KCNJ10 sequencing identified 4 variants (Table 1), including a novel variant, c.800A>G (p.Asp267Gly), that was not found in the ClinVar, DVD, dbSNP, and HGMD databases and that had never been described in clinical reports. The proband (BJNS-6) was found to have normal hearing at 8 months; the His genotype was c.919-2A>G/c.800A>G DHMs. The c.1042C>T (rs137853074) mutation was found in the ClinVar, PubMed, and HGMD databases and was labeled ‘pathogenic' in DVD. In the current study, however, BJNS-1 and 2 with a DHMs (twin sisters whose father and mother had a c.919-2A>G and c.1042C>T heterozygous mutation, respectively) were both found to have normal hearing. The genotype of the proband (BJNS-3) was also c.919-2A>G/c.1042C>T DHMs, and her hearing phenotype was normal as well. Two other variants, c.812G>A (rs3795339) and c.53G>A (rs115466046), were labeled as ‘Benign' in ClinVar and DVD. Sequence electropherograms of abnormal sequences in the *SLC26A4* and *KCNJ10* genes from BJNS-1,2 and 3 are shown at Figure 2.

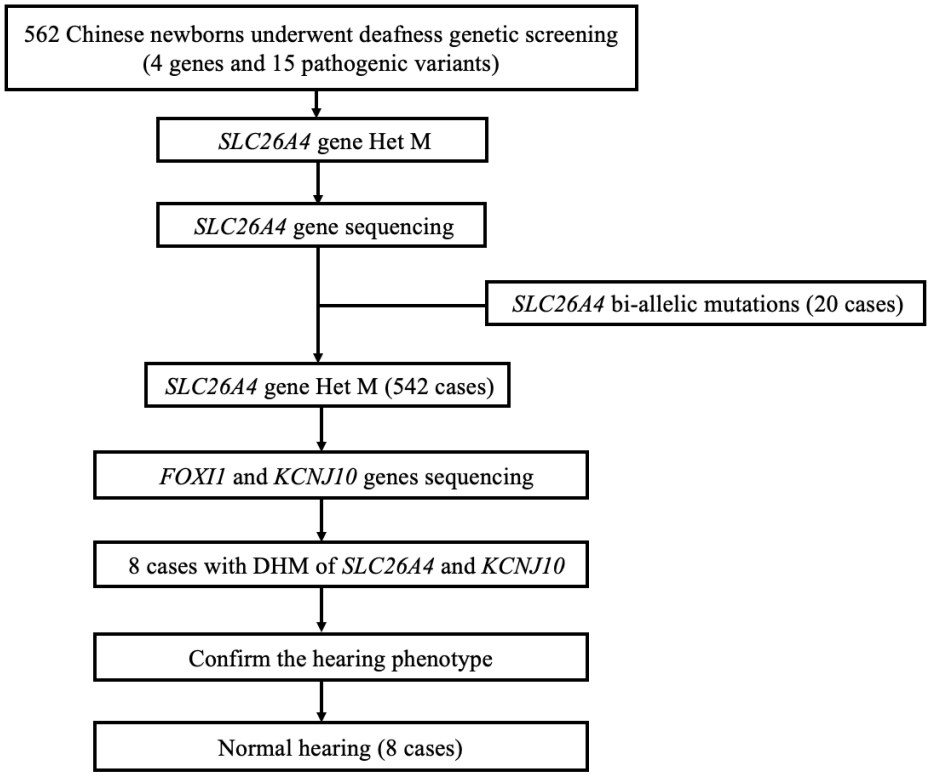

Figure 1. Flowchart of the research procedures for subjects undergoing neonatal deafness gene screening and *SLC26A4*, *FOXI1*, and *KCNJ10* sequencing. *Abbreviations*: Het M, heterozygous mutation; DHM, double heterozygous mutation.

Table 1. Information on *KCNJ10* gene variants

Variant	Amino acid	Location	Chromosomal position	Type of mutation	Pathogenicity*	CADD score	Mutant allele frequency
c.1042C>T	p.Arg348Cys	Exon 2	g.160011281:G>A	Missense	Pathogenic	23.6	18.75% (3/16)
c.812G>A	p.Arg271His	Exon 2	g.160011511:C>T	Missense	Benign	23.1	18.75% (3/16)
c.800A>G	p.Asp267Gly	Exon 2	g.160011523:T>C	Missense	No data	12.12	6.25% (1/16)
c.53G>A	p.Arg18Gln	Exon 2	g.160012270:C>T	Missense	Benign	22.6	6.25% (1/16)

*As reported in the Deafness Variation Database (DVD)

3.3. *Hearing phenotypes of the 8 subjects with DHMs of the SLC26A4 and KCNJ10 genes*

Table 2 summarizes genotypes and phenotypes of subjects with DHMs of the *SLC26A4* and *KCNJ10* genes. Eight individuals all passed universal newborn hearing screening (UNHS) and they were all found to have normal hearing at different ages. Three of the individuals agreed to undergo a CT scan of the temporal bone, and results were normal.

3.4. *Prediction of the functional outcome of variants*

Prediction of the variant effect was performed using SIFT, Mutation Taster, Polyphen-2, GERP, and Phylop. c.1042C>T was predicted to be "probably damaging" according to Polyphen-2 and SIFT, "disease causing" according to Mutation Taster, and "conserved" according to GERP. c.800A>G was predicted to be "tolerated" according to SIFT, "benign" according to Polyphen-2, and "conserved" according to GERP and Phylop.

The Cochrane Library, Pubmed, Embase and other databases were searched, and no studies were found to have analyzed mutations in the *FOXI1* and *KCNJ10* genes in infants with a single-allele *SLC26A4* mutation. Therefore, this may be the first study in China to analyze mutations in the two aforementioned genes in infants with a single-allele *SLC26A4* mutation.

3.5. *Genetic testing*

KCNJ10 sequencing identified 4 variants, including a novel variant, c.800A>G(p.Asp267Gly). This is a missense variant, located in exon 2, that causes an amino acid substitution at position 267 from aspartic

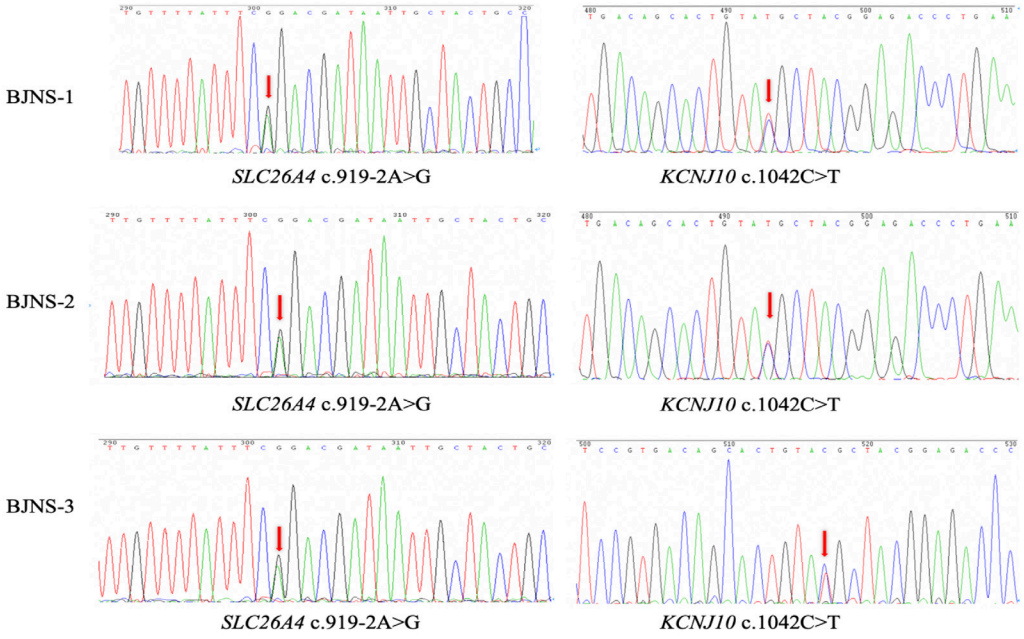

Figure 2. Sequence electropherograms of abnormal sequences in the *SLC26A4* and *KCNJ10* genes from three probands.

Table 2. Genotype and phenotype of subjects with DHMs

Patient No.	Age	Genotype	Phenotype	Imaging evaluation	UNHS results	
					R	L
BJNS-1	34 m	c.919-2A>G/c.1042C>T DHM	Normal	Normal	Pass	Pass
BJNS-2	34 m	c.919-2A>G/c.1042C>T DHM	Normal	Normal	Pass	Pass
BJNS-3	19 m	c.919-2A>G/c.1042C>T DHM	Normal	No data	Pass	Pass
BJNS-4	17 m	c.919-2A>G/c.53G>A DHM	Normal	Normal	Pass	Pass
BJNS-5	13 m	c.919-2A>G/c.812G>A DHM	Normal	No data	Pass	Pass
BJNS-6	10 m	c.919-2A>G/c.800A>G DHM	Normal	No data	Pass	Pass
BJNS-7	14 m	c.2168A>G/c.812G>A DHM	Normal	No data	Pass	Pass
BJNS-8	10 m	c.919-2A>G/c.812G>A DHM	Normal	No data	Pass	Pass

Abbreviations: DHMs, double heterozygous mutation; UNHS, universal newborn hearing screening.

acid to glycine. Online tools predicted this variant to be tolerated or benign. The proband (BJNS-6) was found to have normal hearing at 8 months and was advised to undergo a regular follow-up. Based on ACMG guidelines, c.800A>G is "likely benign." No pathogenic mutations in *FOXI1* gene were found. Only two known synonymous polymorphisms were identified in *FOXI1*; both were found in dbSNP (c.279G>A, rs2277944 and c.726C>T, rs35678180).

c.812G>A was previously reported by Chai *et al*., who found it in 5% of controls with normal hearing, suggesting that this variant maybe a polymorphism in the Chinese population (*18*). Zhao *et al*. analyzed genotypes of *SLC26A4* and *KCNJ10* in 1,056 Chinese patients with NSEVA and found that the most frequently detected *KCNJ10* mutation was c.812G>A. The incidence of c.812G>A in patients with NSEVA does not differ significantly from that in control subjects with normal hearing (*19*). Subjects in the two aforementioned studies had EVA or hearing loss, while subjects in the current study were all infants with an *SLC26A4* single-allele mutation. Three subjects (BJNS-5, 7 and 8) with c.919-2A>G/c.812G>A DHMs were identified. These subjects had normal hearing, which agrees with the findings of previous studies.

The mutation c.1042C>T was first reported by Yang, and he proposed that single mutations in both *SLC26A4* and *KCNJ10* (an inwardly rectifying potassium channel) lead to digenic NSEVA. c.1042C>T was found to reduce K+ conductance activity and is regarded as a pathogenic SNV according to 1000 Genomes (*11*). However, the study by Zhao *et al*. (*19*) did not find c.1042C>T in patients with NSEVA with zero or one *SLC26A4* mutation, but c.1042C>T was found in a control subject with normal hearing and both *KCNJ10* c.1042C>T and *SLC26A4* pathogenic mutations were carried by parents with normal hearing who had children with NSEVA. These facts suggest that c.1042C>T might be a benign variant in the Chinese population. The current study also found three subjects

(BJNS1, 2, and 3) with c.919-2A>G/c.1042C>T DHMs, all of whom currently have normal hearing. Therefore, the pathogenicity of c.1042C>T still warrants further study.

3.6. *Phenotypes and genotypes of individuals with DHMs*

A meta-analysis indicated that, overall, 1.3% and 3.1% of patients suspected of having PDS/DFNB4 had variants in *FOXI1* and *KCNJ10*, respectively (*20*). In the current study, eight subjects with DHMs all passed UNHS and were found to have normal hearing. Three of those individuals agreed to undergo a CT scan of the temporal bone, and results were all normal. Together, these results suggest that individuals with *SLC26A4* single-allele mutation, combined with *FOXI1* or *KCNJ10* gene mutations, do not suffer hearing loss during infancy, though this finding is worthy of further follow-up and in-depth discussion. The actual contribution of *FOXI1* and *KCNJ10* mutations to SNHL may be more limited, *i.e*. maybe they affect only a relatively small number of patients with EVA, as suggested by a study that found no *FOXI1* or *KCNJ10* variants (*21*).

4. Conclusion

This may be the first study in China to analyze mutations in the *FOXI1* and *KCNJ10* genes in infants with a single-allele *SLC26A4* mutation. These data suggest that individuals with an *SLC26A4* single-allele mutation, combined with *FOXI1* or *KCNJ10* gene mutations, do not suffer hearing loss during infancy, though this finding is worthy of further follow-up and in-depth discussion.

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References

1. Wang Q, Zhao Y, Rao S, Guo Y, Yuan H, Zong L, Guan J, Xu B, Wang D, Han M, Lan L, Zhai S, Shen Y. A distinct spectrum of *SLC26A4* mutations in patients with enlarged vestibular aqueduct in China. Clin. Genet. 2007; 72:245-254.
2. Wu C, Lu Y, Chen P, Yeh P, Su Y, Hwu W, Hsu C. Phenotypic analyses and mutation screening of the *SLC26A4* and *FOXI1* genes in 101 Taiwanese families with bilateral nonsyndromic enlarged vestibular aqueduct (DFNB4) or Pendred syndrome. Audiol Neurootol. 2010; 15:57-66.
3. Zhao J, Yuan Y, Chen J, Huang S, Wang G, Han D, Dai P. *SLC26A4* gene copy number variations in Chinese patients with non-syndromic enlarged vestibular aqueduct. J Transl Med. 2012; 10:82.
4. Pang XH, Chai YC, Chen P. The incidence and pathogenic correlation of SLC26A4 mono-allelic mutations in deaf patients with enlarged vestibular aqueduct. Chinese Scientific Journal of Hearing and Speech Rehabilitation. 2015; 3:190-194. (in Chinese)
5. Rah YC, Kim AR, Koo JW, Lee JH, Oh SH, Choi BY. Audiologic presentation of enlargement of the vestibular aqueduct according to the *SLC26A4* genotypes. Laryngoscope. 2015; 125:216-222.
6. Tsukamoto K, Suzuki HD, Namba A, Namba A, Abe S, Usami S. Distribution and frequencies of PDS (SLC26A4) mutations in Pendred syndrome and nonsyndromic hearing loss associated with enlarged vestibular aqueduct: A unique spectrum of mutations in Japanese. Eur J Hum Genet. 2004; 11:916-922.
7. Pryor SP, Madeo AC, Reynolds JC, Sarlis NJ, Arnos KS, Nance WE, Yang Y, Zalewski CK, Brewer CC, Butman JA, Griffith AJ. SLC26A4/PDS genotype-phenotype correlation in hearing loss with enlargement of the vestibular aqueduct (EVA): Evidence that Pendred syndrome and non-syndromic EVA are distinct clinical and genetic entities. J Med Genet. 2005; 42:159-165.
8. Albert S, Blons H, Jonard L, *et al. SLC26A4* gene is frequently involved in nonsyndromic hearing impairment with enlarged vestibular aqueduct in Caucasian populations. Eur J Hum Genet. 2006; 14:773-779.
9. Zhao X, Huang L, Wang X, Wang X, Zhao L, Cheng X, Ruan Y. Genotyping and audiological characteristics of infants with a single-allele *SLC26A4* mutation. Int J Pediatr Otorhinolaryngol. 2019; 116:153-158.
10. Yang T, Vidarsson H, Blomqvist SR, Rosengren SS, Enerback S, Smith RJ. Transcriptional control of SLC26A4 is involved in Pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (DFNB4). Am J Hum Genet. 2007; 80:1055-1063.
11. Yang T, Gurrola JG, Wu H, Chiu SM, Wangemann P, Snyder PM, Richard JH. Mutations of KCNJ10 together with mutations of SLC26A4 cause digenic nonsyndromic hearing loss associated with enlarged vestibular aqueduct syndrome. Am J Hum Genet. 2009; 84:651-657.
12. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier FS, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet. Med. 2015; 17:405-424.
13. Lidén G. The scope and application of current audiometric tests. J Laryngol Otol. 1969; 83:507-520.
14. Jerger J. Clinical experience with impedance audiometry. Arch Otolaryngol. 1970; 92:311-324.
15. Mazzoli M, Camp GV, Newton V, Giarbini N, Declau F, Parving A. Recommendations for the description of genetic and audiological data for families with nonsyndromic hereditary hearing impairment. Audiol. Med. 2009; 1:148-150.
16. Kim SY, Park G, Han KH, Kim A, Koo JW, Chang

SO, Seung HO, Park WY, Choi BY. Prevalence of p.V37I variant of GJB2 in mild or moderate hearing loss in a pediatric population and the interpretation of its pathogenicity. PLoS One. 2013; 8:e61592.
17. Liu J, Huang L, Fu X, Liu H, Yang Y, Cheng X, Ni T. The audiological characteristics of large vestibular aqueduct syndrome in infants and young children. J Clin Otorhinolaryngol Head Neck Surg (China). 2016; 30:1702-1709. (in Chinese)
18. Chai Y, Huang Z, Tao Z, Li X, Li L, Li Y, Wu H, Yang T. Molecular etiology of hearing impairment associated with nonsyndromic enlarged vestibular aqueduct in East China. Am J Med Genet. 2013; 161:2226-2233.
19. Zhao J, Yuan Y, Huang S, Huang B, Cheng Y, Kang D, Wang G, Han D, Dai P. *KCNJ10* may not be a contributor to n Chinese Subjects. PLoS One. 2014; 9: e108134.
20. Pique LM, Brennan ML, Davidson CJ, Schaefer F, Greinwald J, Schrijver I. Mutation analysis of the *SLC26A4*, *FOXI1* and *KCNJ10* genes in individuals with congenital hearing loss. Peer J. 2014; 2:e384.
21. Chen K, Wang X, Sun L, Jiang H. Screening of *SLC26A4*, *FOXI1*, *KCNJ10*, and *GJB2* in bilateral deafness patients with inner ear malformation. Otolaryngol Head Neck Surg. 2012; 146:972-978.

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