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 \textit{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...
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.
562 Chinese newborns underwent deafness genetic screening (4 genes and 15 pathogenic variants)

- **SLC26A4 gene Het M**
- **SLC26A4 gene sequencing**
- **SLC26A4 bi-allelic mutations (20 cases)**
- **SLC26A4 gene Het M (542 cases)**
- **FOXI1 and KCNJ10 genes sequencing**
- **8 cases with DHM of SLC26A4 and KCNJ10**
- **Confirm the hearing phenotype**
- **Normal hearing (8 cases)**

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 acid.
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 \textit{FOXI1} gene were found. Only two known synonymous polymorphisms were identified in \textit{FOXI1}; both were found in dbSNP (c.279G>A, rs2277944 and c.726C>T, rs35678180).

\textit{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 \textit{SLC26A4} and \textit{KCNJ10} in 1,056 Chinese patients with NSEVA and found that the most frequently detected \textit{KCNJ10} mutation was \textit{c.812G>A}. The incidence of \textit{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 \textit{SLC26A4} single-allele mutation. Three subjects (BJNS-5, 7 and 8) with \textit{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 \textit{c.1042C>T} was first reported by Yang, and he proposed that single mutations in both \textit{SLC26A4} and \textit{KCNJ10} (an inwardly rectifying potassium channel) lead to digenic NSEVA. \textit{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 \textit{c.1042C>T} in patients with NSEVA with zero or one \textit{SLC26A4} mutation, but \textit{c.1042C>T} was found in a control subject with normal hearing and both \textit{KCNJ10} \textit{c.1042C>T} and \textit{SLC26A4} pathogenic mutations were carried by parents with normal hearing who had children with NSEVA. These facts suggest that \textit{c.1042C>T} might be a benign variant in the Chinese population. The current study also found three subjects

Table 2. Genotype and phenotype of subjects with DHMs

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

\textit{Abbreviations: DHMs, double heterozygous mutation; UNHS, universal newborn hearing screening.}
(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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