

Mutation analysis of the *SLC26A4* gene in three Chinese families

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Summary

In order to investigate the genetic causes of hearing loss in a Chinese proband (in Family A) with enlarged vestibular aqueduct (EVA) and to investigate the genotype of two Chinese probands with *SLC26A4* single-allelic mutation and normal hearing (in Families B and C, respectively), the three probands and their parents were clinically and genetically evaluated. Twenty exons and flanking splice sites of the *SLC26A4* gene were screened for pathogenic mutations *via* amplification with PCR and bidirectional sequencing. As controls, a group of 400 healthy newborns from the same ethnic background underwent *SLC26A4* gene screening using the same method. The three probands all harbored two mutations in the *SLC26A4* gene in the form of compound heterozygosity. The genotypes of mutations in Families A, B, and C are c.1211C>A/c.919-2A>G, c.1729G>A/c.919-2A>G, and c.1286C>A/c.919-2A>G, respectively. The missense mutations c.1211C>A (p.T430Q) in exon 10 and c.1729G>A (p.V577I) in exon 16 are both reported for the first time and were absent in 400 healthy newborns. c.1211C>A has Glutamine (Gln) at amino acid 430 instead of Threonine (Thr), and c.1729G>A has Isoleucine (Ile) at amino acid 577 instead of Valine (Val). c.1286C>A, a mutation previously reported in DVD and HGMD, was associated with Mondini deformity, but a proband with the c.1286C>A mutation in this study was normal. This study has demonstrated that the novel missense mutation c.1211C>A in compound heterozygosity with c.919-2A>G in the *SLC26A4* gene is likely to be the cause of deafness in Family A. A novel variant, c.1729G>A, was identified and is likely benign. The pathogenicity of the c.1286C>A mutation warrants more in-depth study. These findings will broaden the spectrum of known *SLC26A4* mutations in the Chinese population, providing more information for genetic counseling and diagnosis of hearing loss with EVA.

Keywords: *SLC26A4*, novel mutation, enlarged vestibular aqueduct

1. Introduction

Deafness, which refers to various degrees of hearing loss, is one of the most common sensory disorders. The incidence of neonatal congenital deafness is

approximately 1-3‰ (1). *SLC26A4* (OMIM 605646, also called the PDS gene, NM_000441.1) maps on 7q22-31 (DFNB4 locus) (2) and encodes a 780-amino-acid protein called pendrin, a member of the solute carrier 26 protein family that functions as a chloride iodide transporter in cell expression systems (3). Mutations in the *SLC26A4* gene are known to vary by region and ethnicity. Domestic data have indicated that *SLC26A4* is the second most common gene that causes nonsyndromic hearing loss (NSHL), accounting for 14.5% (4). To date, about 539 mutations have been identified. (<http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SLC26A4>). Mutations in the *SLC26A4* gene result in two typical phenotypes: *i*) the syndromic form, called Pendred syndrome (PS) (OMIM 274600), that

Released online in J-STAGE as advance publication October 27, 2019.

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is characterized by hearing loss, goiter, and eventually hypothyroidism, with/without EVA or other inner ear malformations; *ii*) the nonsyndromic form, called DFNB4 or nonsyndromic EVA (OMIM 600791) (when EVA is present), that is characterized by hearing loss with/without EVA or other inner ear malformations (5-7).

Enlarged vestibular aqueduct (EVA) is an inner ear malformation of the temporal bone that predisposes patients to hearing loss from childhood as well as vestibular symptoms. Individuals with mutations in the *SLC26A4* may exhibit hearing loss, as well as EVA, at birth or during early childhood. Foreign data have revealed about 16-83.9% of patients with EVA have bi-allelic mutations in *SLC26A4* (including homozygous mutations and compound heterozygous mutations). Mono-allelic mutations in *SLC26A4* are found in about 16-36% of patients with EVA (8-11). NSHL with EVA is known to be typically characterized by congenital, bilateral sensorineural hearing loss (SNHL), which can be progressive and usually ranges from severe to profound (12).

Newborn genetic screening for deafness has been conducted in Beijing since 2012, and greater numbers of newborns with genes causing deafness have been detected. Dai and Huang reported that genetic screening of 180,469 infants revealed genes causing deafness in 8,136 (4.508%) (13). In 2017, the current authors' research team retrospectively analyzed 582 subjects with genetic mutations causing deafness and summarized the relationship between genotype and phenotype; results indicated that *SLC26A4* gene mutations were mainly associated with high-frequency hearing loss and profound-severe hearing loss (14). In addition, the *SLC26A4* gene is known to be closely associated with delayed-onset hearing loss and is often detected in deaf populations. Zhu *et al.* reported that the rate of homozygous and heterozygous mutations in the *SLC26A4* gene was 6.92% (22/318) and 18.55% (59/318), respectively, in 318 students with NSHL in Hebei Province, China (15). Later, the current authors' research group examined patients with a single-allele *SLC26A4* mutation revealed by newborn genetic screening for deafness. Zhao *et al.* found a variant of some type in 3.50% of these infants and a pathogenic mutation in 2.96% (16). The current authors examined patients with *SLC26A4* bi-allelic mutations and found a novel pathogenic frameshift mutation, c.574delC (p.Leu192Ter), in 2018 (17). Later on, the current authors' team investigated how the *FOXI1* and *KCNJ10* genes were affected in infants with a single-allele mutation in the *SLC26A4* gene. Results suggested that individuals with an *SLC26A4* single-allele mutation, combined with *FOXI1* or *KCNJ10* gene mutations, do not suffer hearing loss during infancy (18).

Based on the studies mentioned above, the current authors noticed that three families warrant study and

discussion. The current study investigated the *SLC26A4* gene in 4 members of one Chinese family with EVA and 6 members of two Chinese families with normal hearing. Two novel compound heterozygous mutations in *SLC26A4* were identified. The pathogenicity of the c.1286C>A (p.A429E) missense mutation warrants more in-depth study. The current findings will broaden the spectrum of *SLC26A4* mutations in the Chinese population.

2. Materials and Methods

Written informed consent was obtained from parents. The protocol was in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

2.1. Subjects and clinical evaluation

One Chinese family with EVA and two Chinese families with normal hearing were recruited by Otolaryngology and Head and Neck Surgery, Beijing Tongren Hospital (Beijing, China). The proband had a c.919-2A>G single-allele mutation according to genetic screening for deafness (15 variants in 4 genes, including *GJB2* c.235delC, c.299delAT, c.176del16, and c.35delG; *GJB3* c.538C>T; *SLC26A4* c.919-2A>G, c.2168A>G, c.1174A>T, C.1226G>A, c.1229C>T, c.1975G>C, c.2027T>A and c.IVS15+5G>A; and Mt 12SrRNA m.1555A>G and m.1494C>T). Clinical evaluation was performed and included family history, a detailed medical history, and a physical examination, including thyroid sonography and a high-resolution computed tomography (CT) scan of the temporal bone. Four hundred unrelated Chinese newborns with normal hearing were recruited as normal controls.

2.2. Mutation analysis

Genomic DNA was extracted from 2 mL of whole blood from each patient, using the Blood DNA kit (Tiangen Biotech, Beijing, China). Twenty exons and flanking splice sites of the *SLC26A4* gene were screened for mutations *via* amplification with PCR and bidirectional sequencing. Variants were interpreted in accordance with ACMG guidelines (19).

2.3. Bioinformatics and validation of the variants

Sequence data were analyzed by aligning them with the reference sequence of *SLC26A4* (NT_007933) from the National Center for Biotechnology Information (NCBI) using the software DNA Star 5.0. The 1000 Genomes Project database (<http://www.1000genomes.org/>), ClinVar (<https://www.st-va.ncbi.nlm.nih.gov/clinvar/>), the dbSNP database of NCBI (<http://www.ncbi.nlm.nih.gov/>).

gov/), and the Deafness Variation Database (<http://deafnessvariationdatabase.org/>) were used as references to assess the novelty of the mutations found in this study. Online tools including Mutation Taster, SIFT, CADD (v1.3.1) and PolyPhen-2 were used to predict the functional outcome of the variants. GERP and PhyloP were used to score the conservation of the variants.

2.4. Auditory evaluation

Subjects underwent universal newborn hearing screening and had specific results. A comprehensive audiological evaluation was performed and included pure tone audiometry (PTA), auditory brainstem response (ABR), 40-Hz auditory event-related potential, distortion product otoacoustic emission (DPOAE), auditory steady-state response (ASSR), acoustic immittance, and pediatric behavioral audiometry. The hearing threshold was calculated as the average hearing level at 0.5, 1.0, 2.0, and 4.0 k Hz according to the 1997 World Health Organization standard. The severity of hearing impairment was defined as mild (26-40 dB), moderate (41-60 dB), severe (61-80 dB), or profound (> 80 dB). Owing to the young age of subjects, the

ABR threshold and/or ASSR were recorded, and mean thresholds at frequencies in the 0.5-4 k Hz range were averaged to obtain an approximation for the directional conditioned reflex (20,21).

3. Results

3.1. Clinical, audiological, and imaging data

All members of the three families were negative for systemic and thyroid disease, and physical examination and otoscopy results were also normal. One subject 7 months of age in Family A had hearing loss, and pure-tone audiometry revealed normal hearing in the parents. This proband underwent universal neonatal hearing screening (UNHS) and was diagnosed with SNHL when first seen by a doctor at this Hospital at four months of age. The threshold of ABR air-conduction in the proband was 80 dB nHL on the right and 50 dB nHL on the left. The proband had a type "A" tympanogram, and the bilateral acoustic stapedial reflex was not elicited. DPOAE elicited no response from the patient in both ears. The results of objective audiometry and visual reinforcement audiometry (VRA) in the proband in Family A (4 months, 7 months, and

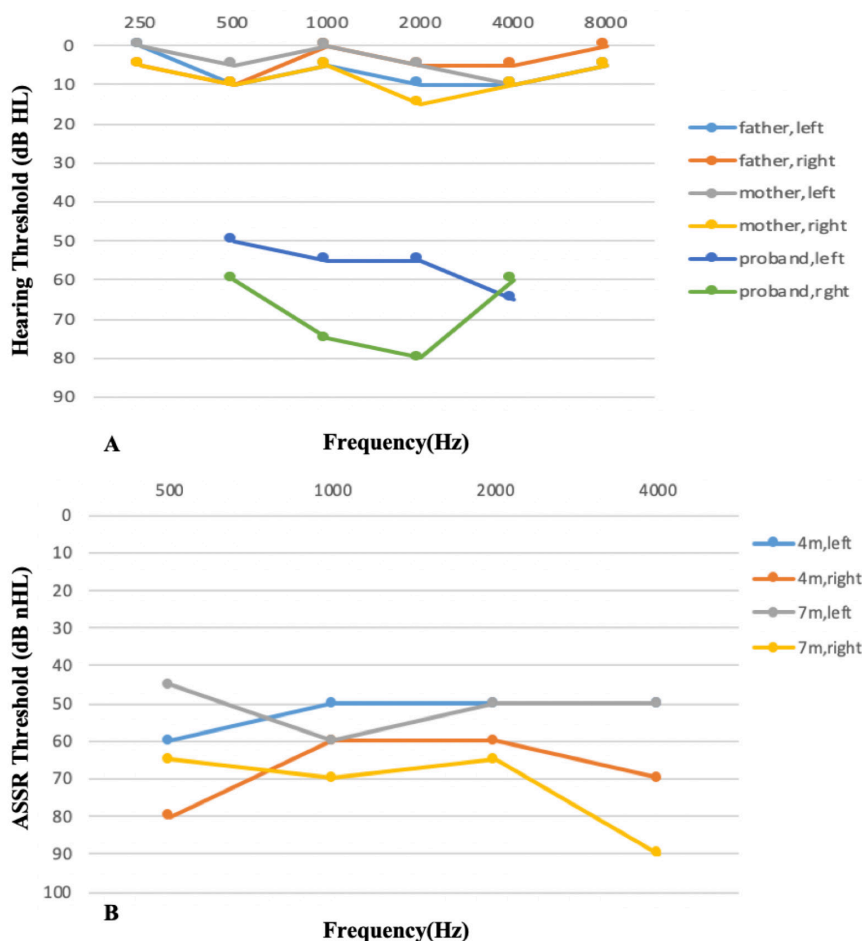


Figure 1. Audiograms of the proband in Family A and his parents. Frequency in Hertz (Hz) is plotted on the x-axis and the hearing level in decibels (dB HL and dB nHL) is plotted on the y-axis.

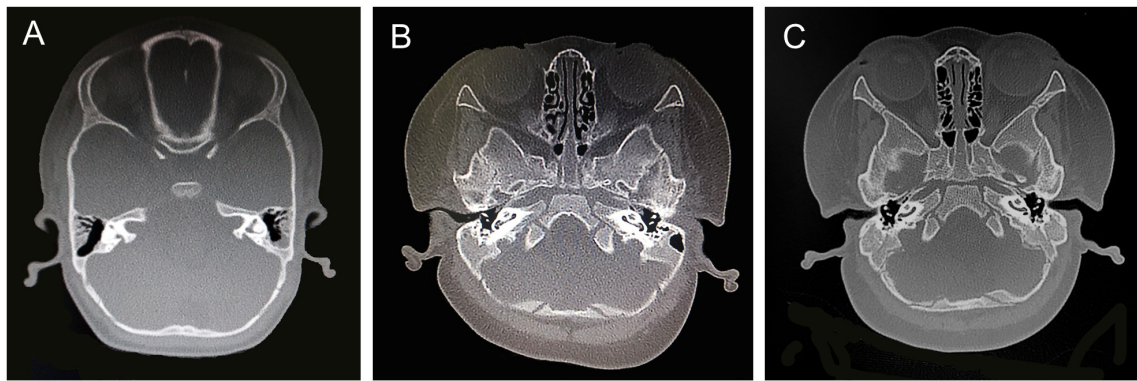


Figure 2. CT scan of the temporal bone of three probands. (A) A CT scan of the temporal bone of the proband in Family A (bilateral enlarged vestibular aqueducts); (B) A CT scan of the temporal bone of the proband in Family B; (C) A CT scan of the temporal bone of the proband in Family C.

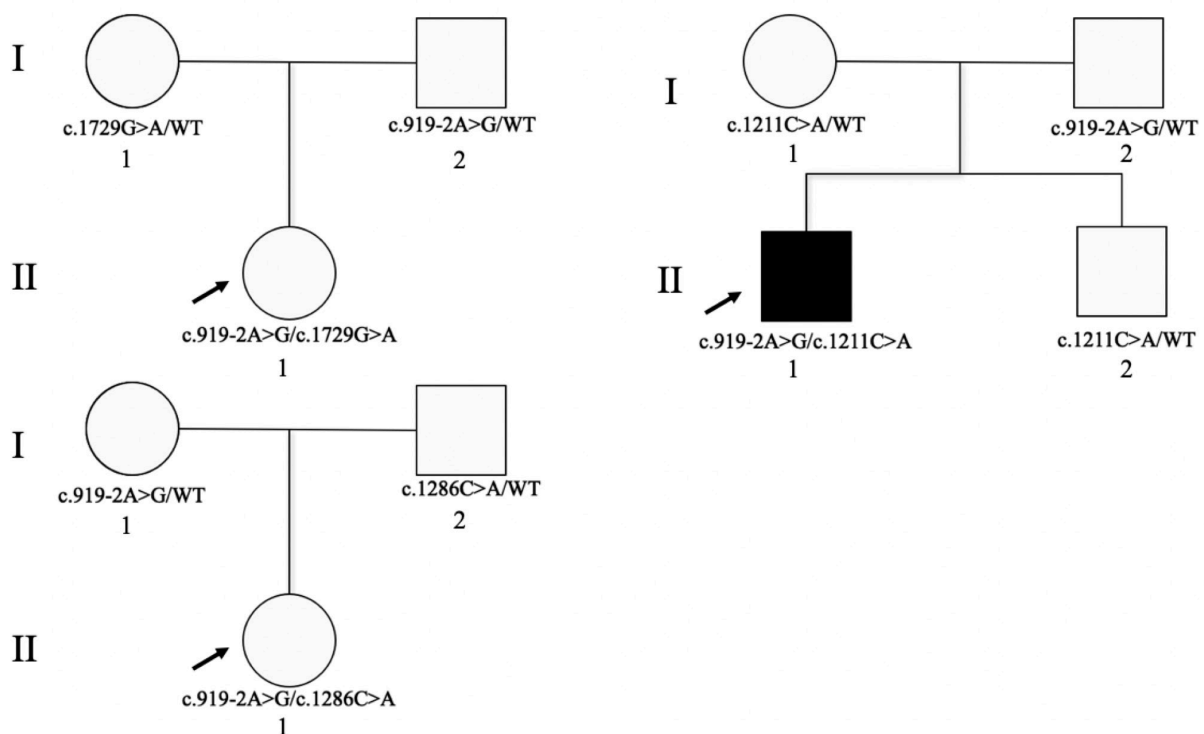


Figure 3. Pedigree map of the three families. Squares and circles denote male and female patients, respectively. WT, wild type.

11 months of age, respectively) and PTA in her parents are shown in Figure 1. A CT scan of the temporal bone of the proband revealed bilateral EVA with a vestibular aqueduct wider than 1.5 mm, pericochlear lucency, dilation around the bottom, parietal fusion, and paranasal sinusitis. She was diagnosed with bilateral large vestibular aqueduct syndrome at seven months of age according to the CT scan (Figure 2A). The probands in Families B and C underwent UNHS, and were both found to have normal hearing at 3 and 6 months of age, respectively. These two probands both a type "A" tympanogram. A CT scan of the temporal bone was normal at 8 and 6 months of age, respectively (Figure 2B and 2C).

3.2. Genetic analysis

Sequencing of *SLC26A4* indicated that the proband in Family A had compound heterozygosity of a c.919-2A>G (IVS7-2A>G) (rs111033313) mutation in intron 7 and a c.1211C>A (p.T430Q) missense mutation in exon 10. In addition, the father was a heterozygous carrier of the c.919-2A>G mutation, and the mother was a heterozygous carrier of the c.1211C>A mutation. The genotypes of the proband in Families B and C were c.1729G>A/c.919-2A>G compound heterozygous mutations and c.1286C>A/c.919-2A>G compound heterozygous mutations, respectively. Figures 3 and 4 show the pedigree map of the three

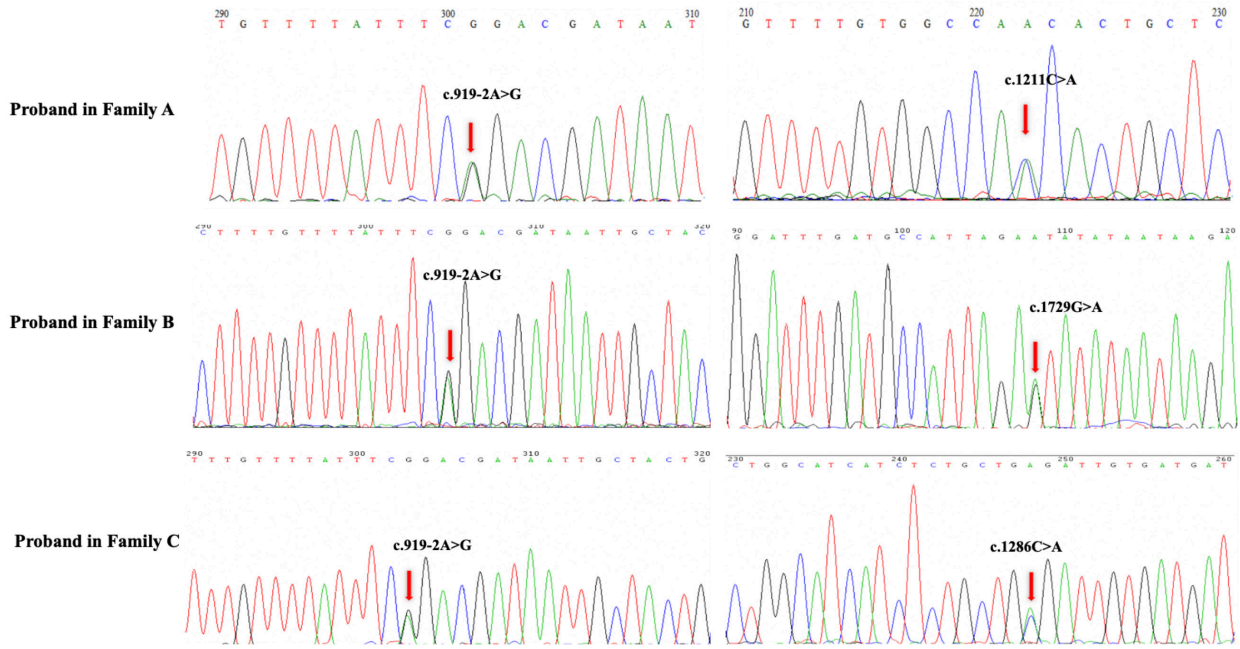


Figure 4. Sequence electropherograms of abnormal sequences from three probands in Families A, B and C.

Table 1. Prediction of variant effect using online tools

No	Variant	ACMG	SIFT score	Polyphen-2 score	Mutation Taster score	CADD score	GERP score	Phylop score
1	c.1211C>A	LP	0.003	0.949	0.998	28.4	5.1	3.261
2	c.1729G>A	LB	1.0	1.0	0.999	23.0	5.72	7.679
3	c.1286C>A	VUS	0.011	0.583	0.883	23.2	-1.65	-0.002

ACMG, American College of Medical Genetics; LP, likely pathogenic; LB, likely benign; VUS, variant of unknown significance.

families and sequence electropherograms of abnormal sequences from three probands in Families A, B, and C, respectively. The variants c.1211C>A (p.T430Q) and c.1729G>A (p.V577I) were not present in ClinVar, PubMed, Deafness Variation Database, dbSNP, the 1000 Genomes Project database, or HGMD and had never been described in clinical reports. These two novel mutations were not found in 400 healthy newborns. The variant c.1286C>A(rs753269996), which has previously been reported in DVD and HGMD, was associated with Mondini deformity. The variant c.1211C>A has Glutamine (Gln) at amino acid 430 instead of Threonine (Thr), and c.1729G>A has Isoleucine (Ile) at amino acid 577 instead of Valine (Val).

3.3. Prediction of the functional outcome of variants

The effect of variants was predicted using SIFT, Mutation Taster, Polyphen-2, CADD, GERP, and Phylop (Table 1). The mutation c.1211C>A was predicted to be "damaging" according to Polyphen-2 and SIFT, "disease causing" according to Mutation Taster, and "conserved" according to GERP and Phylop. The variant c.1729G>A was predicted to be "tolerated" according to SIFT and

"nonconserved" according to GERP and Phylop.

4. Discussion

EVA is a genetically autosomal recessive disorder. Subjects with bi-allelic mutations have earlier age of onset, more severe deafness, more fluctuating hearing loss, and a larger vestibular aqueduct than those without mutations (22,23). EVA is known to be closely linked to *SLC26A4* mutations; variants are highly heterogenous and differ among ethnic groups. p.V138F (c.412G>T) is the most common mutation in the Czech population (24). p.L236P (c.707T>C), p.T416P (c.1246A>C), and IVS8+1G>A (c.1001+1G>A) are mainly detected in Caucasians (25), and p.H723R (c.2168A>G) is mainly detected in Koreans (26). The p.V609G (c.1826T>G) mutation and the IVS8+1G>A (c.1001+1G>A) mutation are predominantly found in the deaf population in South America and North America, respectively (27). An increasing number of novel mutations have been found in Chinese patients as more genetic studies are reported. c.919-2A>G (IVS7-2A>G) and p.H723R (c.2168A>G) account for the majority of mutations in China (28).

The current study found that the proband's father

and mother (the heterozygous carrier of c.919-2A>G and c.1211C>A mutation, respectively) both had normal hearing, and the proband with *SLC26A4* compound heterozygous mutations (c.1211C>A/c.919-2A>G) had bilateral SNHL as well as bilateral EVA. Therefore, genetic mutations were transmitted from the parents to the offspring, and a distinction between the genotype and phenotype was apparent. The splice-site mutation of c.919-2A>G mentioned above is the most prevalent pathogenic mutation of *SLC26A4* in China. Another mutation, c.1211C>A, is not present in ClinVar, PubMed, or HGMD and has never been described in clinical reports. Therefore, this is the first study to report that the mutation is associated with EVA. This mutation has highly conserved residues and is predicted to be pathogenic. This mutation leads to a p.T430Q switch at amino acid 430 in the cytoplasmic topological domain. Therefore, the novel mutation discovered in this study may impair the anion-transporting activity of pendrin by altering the structure of the pendrin protein. The c.1211C>A (p.T430Q) mutation is located in the STAS domain which is included in members of the SLC26A family to regulate the stability, trafficking, and anion transport function of SLC26A family proteins. The structural significance of this domain has been substantiated by the disease-causing nature of mutations in SLC26A family proteins. Therefore, the novel mutation discovered in this study may be closely related to hearing loss (29,30). According to ACMG guidelines, c.1211C>A is likely to be pathogenic.

The patient with c.1729G>A/c.919-2A>G compound heterozygous mutations had normal hearing and a normal CT scan of the temporal bone, and her father and mother (heterozygous carriers of the c.919-2A>G and c.1729G>A (p.V577I) mutations, respectively) also had normal hearing. This mutation in exon 16 had not been previously reported and was absent in 400 healthy newborns. It results in Isoleucine (Ile) taking the place of Valine (Val) at amino acid 577. The variant c.1729G>A was predicted to be "tolerated" according to SIFT and "nonconserved" according to GERP and PhyloP. According to ACMG guidelines, c.1211C>A is likely to be benign.

The c.1286C>A (p.A429E) mutation is a documented SNP (rs753269996). It was first reported by Huang *et al.* in a study of extremely disparate mutations in *SLC26A4* among Chinese patients with an isolated Mondini deformity or EVA (31). They found that one of the patients with inner ear malformations carried the c.1286C>A mutation. The mutation affected residues that are conserved among *SLC26A4* orthologs [Mus (mouse)] and were not present in a screen of 50 patients without inner ear malformations and 200 normal Chinese controls in a study by Yuan *et al.*, suggesting that this mutation is likely to be pathogenic (32). In the current study, however, the proband with c.1286C>A/c.919-2A>G compound heterozygous mutations

currently has normal hearing and a normal CT scan of the temporal bone. His father and mother (heterozygous carriers of the c.1286C>A and c.919-2A>G mutations, respectively) also had normal hearing. Therefore, the pathogenicity of the c.1286C>A mutation warrants more in-depth study.

The current study analyzed the pathogenicity of three mutations in the *SLC26A4* gene in combination with clinical data. A possible follow-up would involve construction of a plasmid containing the corresponding *SLC26A4* mutants to verify protein expression at the cellular level.

5. Conclusion

This study has demonstrated that the novel missense mutation c.1211C>A in compound heterozygosity with c.919-2A>G in the *SLC26A4* gene is likely to be the cause of deafness in Family A. A novel variant, c.1729G>A, was identified and is likely benign. The pathogenicity of the c.1286C>A mutation warrants more in-depth study. The current findings have broadened the spectrum of known *SLC26A4* mutations in the Chinese population, providing more information for genetic counseling and diagnosis of hearing loss with EVA.

Acknowledgements

The authors wish to thank the patients and their family members for their participation in this study. This research was supported by the National Natural Science Foundation of China (grant number 81870730), the National Key R&D Program of China (grant number 2018YFC1002204), and the Beijing Natural Science Foundation (grant number 7172052).

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(Received October 4, 2019; Revised October 23; Accepted October 25, 2019)