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A newly identified mutation (c.2029 C > T) in SLC26A4 gene is associated with enlarged vestibular aqueducts in a Chinese family

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Abstract

Background: The enlarged vestibular aqueduct (EVA), associated with mutations in the SLC26A4 gene, characterized by non-syndromic hearing loss, is an autosomal recessive disorder. Here, we intended to investigate genetic causes of hearing loss in a Han Chinese man.

Method: First, whole-exome sequencing was performed to identify the gene mutations responsible for hearing loss in the proband. Sanger sequencing was used to verify the candidate mutations detected in the family. Next, we collected blood samples and clinical data from the three-generation pedigree. Finally, SLC26A4 mRNA and protein expression levels were detected by qPCR and western blotting.

Result: The proband suffered from bilateral progressive sensorineural hearing loss with EVA. The sequence analysis of SLC26A4 revealed that the proband and his sister both harbored a compound heterozygous mutation of c.2168A > G/c.2029C > T, inherited from their father and mother respectively. c.2029C > T mutation has not been recorded in the relevant literature previously. Relative mRNA levels of the SLC26A4 gene in individuals carrying a compound heterozygous mutation were significantly lower compared to a heterozygous mutation. SLC26A4 protein levels of 293t cells which transfected with recombinant plasmids [GV219-SLC26A4-mut (c.2029C > T) and GV219-SLC26A4-mut (c.2168A > G/c.2029C > T)] were significantly lower than normal control recombinant plasmids (GV219-SLC26A4-wt).

Conclusion: This study found a novel heterozygous mutation c.2029 (exon17) C > T compound with c.2168 (exon19) A > G in the SLC26A4 gene in a patient with EVA. The c.2029 (exon17) C > T mutation is proved to be pathogenic. This finding broadens the spectrum of variants in SLC26A4 gene.

Keywords: Hearing loss, SLC26A4, Mutation, c.2168A > G, c.2029C > T, Enlarged vestibular aqueduct

Background

Hearing loss is one of the most prevalent sensory dysfunctions, with a significant influence on the quality of life and physical and mental well-being of the affected individuals in some degree [1, 2]. In China, it is estimated that 30,000 babies are born with congenital hearing loss every year [3]. As far as we know, at least 60% of early-onset hearing loss or hypoacusis cases are correlated with a genetic cause [4]. Most hereditary hearing loss is

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sensorineural (SNHL) and inherited as a simple Mendelian trait. The hereditary SNHL is classified as autosomal recessive (80%), autosomal dominant (15–20%), x-linked (1%) or mitochondrial inheritance (1–5%) [5].

A mutation in the SLC26A4 gene is the second most common contributor to genetic hearing loss after GJB2 mutations [6]. Mutations in the SLC26A4 gene are involved in syndromic deafness featured on congenital sensorineural hearing impairment and goiter (Pendred syndrome, OMIM 274600) [7] as well as in congenital isolated deafness (DFNB4; OMIM 600791), both of which are relevant to an enlarged vestibular aqueduct (EVA) [8]. EVA, a common type of autosomal recessive hearing loss, is the most common radiological deformity of the inner ear relevant to hereditary SNHL [9].

The SLC26A4 gene is located on chromosome 7q22-31, contains 21 exons, and encodes a highly hydrophobic membrane protein named pendrin, which is a member of the SLC26 family of anion transporters [10]. Pendrin is primarily presented in the thyroid and the inner ear [11]. The complete loss of pendrin-induced anion transport is deemed as cause Pendred syndrome, while function-reducing mutations cause DFNB4, goiter being absent or having a late onset [12]. The SLC26A4 gene has an extensive mutation spectrum spreading across all exons and their flanking sequences. Missense mutations account for a large percentage of SLC26A4 mutations, the rest of which is frameshift mutations, splice site mutations, insertions or deletions that create a stop codon. The entire spectrum of known mutations in the SLC26A4 gene can be viewed at <https://deafnessvariationdatabase.org/gene/SLC26A4>.

In this paper, we identified a compound heterozygous mutation of c.2168A>G/c.2029C>T in SLC26A4 which was associated with EVA. This will prolongate the scope of knowledge of the spectrum of SLC26A4 mutations in the Chinese population, and it is beneficial for us to explore the relationship between phenotype and genotype in the mutations.

Methods

Subjects

The study investigated non-syndromic autosomal dominant sensorineural deafness in a Chinese pedigree. This work and study protocol were approved by the Medical Ethics Committee of the Yantai Yu Huang ding Hospital, Qingdao University, Yantai, China. The genealogical proband was a 20-year-old man recruited from the Department of Otolaryngology Head and Neck Surgery of the Yantai Yu Huang ding Hospital in December 2018. Written informed consents were gained from the pedigree. Furthermore, all of the participants consented to

the publication of their results on clinical characteristics and genetic data on the premise of privacy protection.

Audiological and imaging evaluation

A physical examination was conducted in the pedigree, including a CT of the temporal bone, functional thyroid tests and thyroid sonography. A specific medical history was acquired, including instances of family history of hearing loss and consanguineous marriages, history of ototoxic drugs and noise exposure, and years of onset of deafness, age, progression, Comprehensive audiometric evaluations are composed by distortion product otoacoustic emission (DPOAE), auditory brainstem response (ABR), tympanometry, pure tone audiometry (PTA). Normal hearing was classified as $PTA \leq 25$ dB HL (Hearing Level), mild hearing loss as $25 < PTA \leq 40$ dB HL, moderate hearing loss as $40 < PTA \leq 60$ dB HL, severe hearing loss as $60 < PTA \leq 80$ dB HL, and profound hearing loss as $PTA > 80$ dB HL [13]. A patient was diagnosed with EVA if the midpoint between the common crus and the external aperture was > 1.5 mm by temporal bone CT [14] or MRI.

SLC26A4 mutation screening

Whole-exome sequencing (WES)

The genome variants of the proband (III-1) were identified by whole-exome sequencing by Yin Feng Biotechnology Company (Jinan, China). Potential pathogenic variants were identified in the DNA of the other subjects (I-1, I-4, II-1, II-2, II-3, II-4, II-5, II-6, and III-2). DNA was extracted from the sample, and the main DNA quality testing procedures were as follows: (1) the DNA concentration was tested by Nanodrop lift (thermo scientific, CA, USA); (2) the purity was confirmed using a Nanophotometer (IMPLEN, CA, USA); and (3) the integrity was detected by 1% agarose gel electrophoresis.

The libraries were tested for: (1) exon enrichment using a Sure Select XT Target Enrichment System (G7530–90000); (2) for size distribution using an Agilent 2100 Bioanalyzer system; and (3) for concentration using a Bio-Rad CFX 96 quantitative fluorescence PCR instrument and Bio-Rad KIT iQ SYBR GRN. The samples were then sequenced on a NovaSeq 6000 platform. Variant analysis software (GATK) was used to extract the potential sites of SNP and InDel in the whole genome and compared to the reference database Human_GRCh38_dbSNP141.

Sanger sequencing

SLC26A4 gene variants identified by WES in the pedigree were confirmed by Sanger sequencing. Genomic DNA of all family members was prepared from 3 mL of peripheral blood with a QIAGEN Universal DNA Purification kit. The primer pairs are present in Table 1. PCR was carried

Table 1 The primer pairs used in the present study

| Gene | Forward sequence (5'–3') | Reverse sequence (5'–3') |
|-----------------|--------------------------|--------------------------|
| SLC26A4(c.2029) | GCTGAGGTGAAACCCATCCT | AGCCCATGTATTGCCCTGT |
| SLC26A4(c.2168) | CCACAAGGTTGACTACGACCA | CCAGATGCGCTACTGTTGTG |
| SLC26A4 qPCR | GTGGGATCTGTTGTTCTGAGCAT | GGCACTGGCAATCAGGACTC |
| Actin qPCR | CACCATTGGCAATGAGCGGTC | AGGTCITTTGCGGATGTCCACGT |

on according to these steps: an initial denaturation at 96 °C for 1 min, 96 °C for 10 s (denaturation), 50 °C for 5 s (annealing), 60 °C for 4 min (extension) and a final 25 cycles. In the end, the PCR products were sequenced with an ABI 3730 Sequencer (Applied Biosystems).

Detection of relative mRNA expression levels of SLC26A4

Peripheral blood samples were collected from all family members. RNA was isolated using Trizol reagent and reverse transcribed into cDNA according to the manufacturers' protocols (Accurate biology, Hunan, China). Relative mRNA levels of the SLC26A4 target gene were detected by quantitative PCR performed on an Applied Biosystems StepOnePlus system (Foster City, CA). Primer pairs were showed in Table 1. Data were collected and the $2^{-\Delta\Delta C_t}$ method was used to determine the relative mRNA expression level.

Detection of protein levels of SLC26A4

Three groups of recombinant overexpression plasmids containing a SLC26A4 single mutation site (GV219-SLC26A4-mut (c.2029C>T)), double mutation sites (GV219-SLC26A4-mut (c.2168A>G/c.2029C>T)) and wild-type (GV219-SLC26A4-wt) fragments were constructed by Shanghai Genechem Co., Ltd. The recombinant vectors were transiently transfected into 293 T cells for overexpression. Transfected cells were cultured for 48 h and then collected for western blotting analysis. The SLC26A4 antibody (NBP1-60106) is from Novus Biologicals.

Results

Family investigation and clinical evaluation

Here we identified a three-generation Han Chinese family with 10 members. The proband (III-1) had normal hearing at birth. However, when he was 18, he developed sensorineural deafness in the right ear and mild hearing loss in the left ear after a bad cold. The results presented in Fig. 1a and b showed a progressive aggravation of hearing loss at 19 and 20 years old. On tympanogram, the proband was classed as type A; CT scans of the inner ear showed EVA bilaterally without cochlea malformations (Fig. 1c, d) (Additional file 1: Supplementary figure 1.a, b); and MRI showed lymphatic enlargement

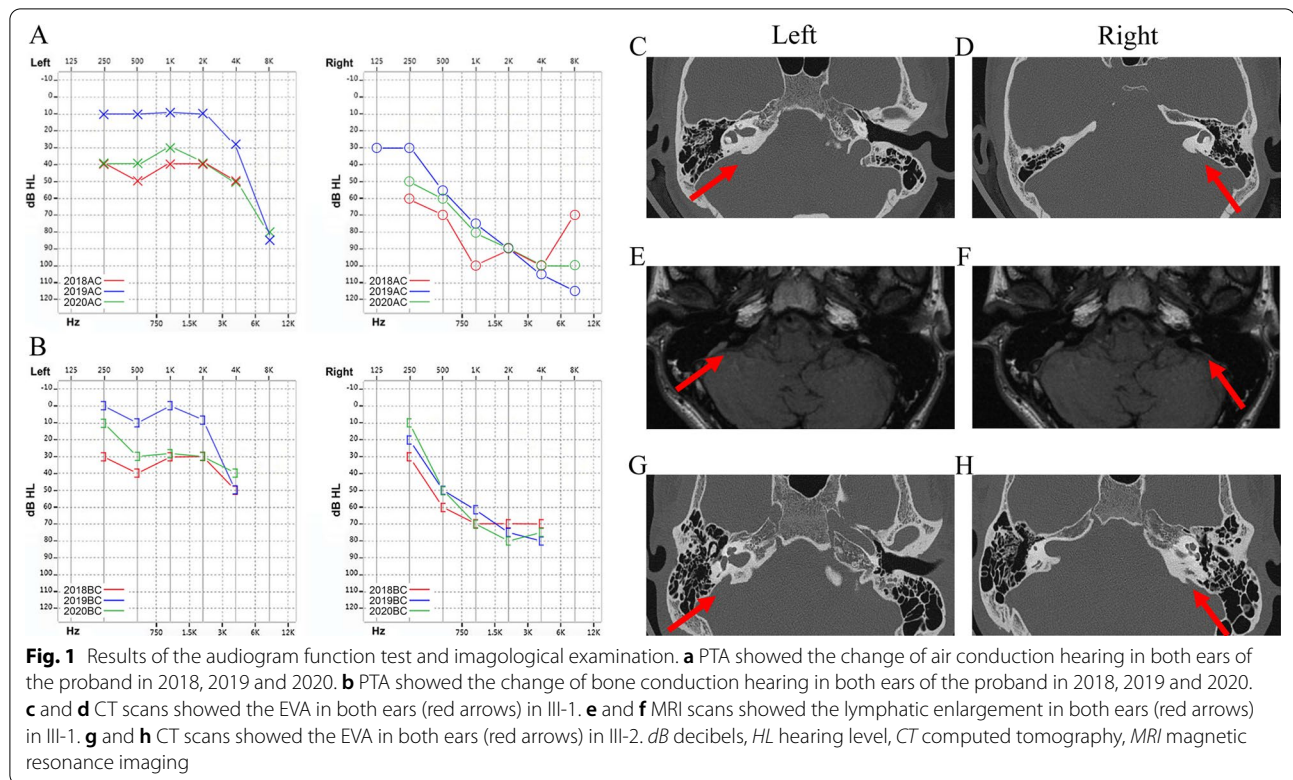
and no vestibular endolymphatic hydrops (Fig. 1e, f) (Additional file 1: Supplementary figure 2.a, b). Thyroid goiter was excluded based on normal thyroid function tests and ultrasonogram. Overall, the proband (III-1) was correlated with non-syndromic hearing loss with EVA. Another member of this family (III-2) also had EVA and mild hearing loss on PTA with no clinical symptoms (Fig. 1g, h) (Additional file 1: Supplementary figure 3.a, b). None of the family members had a history of ototoxic drug exposure. The clinical phenotype presentations of the pedigree are shown in Table 2.

Identification of SLC26A4 gene mutations

A compound heterozygous mutation of SLC26A4 (c.2168A>G/c.2029C>T) was detected in proband III-1 by WES. The results of Sanger sequencing showed that both III-1 and III-2 carried the c.2029C>T and c.2168A>G mutations, however I-4, II-3, II-4, II-5 and II-6 were heterozygous carriers of the c.2029C>T mutation. I-1, II-1 and II-2 were heterozygous carriers of the c.2168A>G mutation. Of note, we were able to determine details of the mutations and the pedigree map. Genotypes of the family members are listed in Fig. 2. The c.2029 (exon17) C>T mutation led to a substitution of Trp with Arg at position 677, the c.2168 (exon19) A>G mutation led to a substitution of Arg with His at position 723 (Fig. 3). The important information of both variants was displayed in Table 3.

Bioinformatics analysis

The SLC26A4 c.2168 A>G variant has been reported to be pathogenic previously [20, 21], but c.2029 C>T is a novel missense mutation, so far whether the mutation is pathogenic was unknown. In this case, we further dissect the pathogenicity of the new SLC26A4 c.2029C>T, p.Trp677Arg mutation by using several bioinformatics prediction software. PolyPhen-2 (version 2, <http://genetics.bwh.harvard.edu/pph2/>) was used to predict function of the peptide and the possible impact of an amino acid substitution on the structure. It is specifically designed to predict missense mutations and mainly performs function prediction on SNP and point mutation. It suggested that the SLC26A4 c.2029C>T mutation is probably destructive,



with a score of 0.967 (sensitivity, 0.61; specificity, 0.93) (Fig. 4a). In addition, SWISS-MODEL software was used to generate the 3D protein structures in specific R677 position of wildtype and mutant SLC26A4 protein, which were displayed in Fig. 4b. We also use DNASTAR Megalign software to analyze the residue conservation of Trp at position 677. The result of multiple protein sequence alignment indicated that the Trp at position 677 of SLC26A4 is evolutionally highly conserved among various species (Fig. 4c). All these bioinformatics analyses strongly suggested that SLC26A4 c.2029C>T is a pathogenic mutation.

SLC26A4 mRNA and protein expression

To further investigate the impact of compound heterozygous mutation c.2168A>G and c.2029C>T on SLC26A4, we analyzed SLC26A4 mRNA expression levels in some individuals of pedigree by qPCR. The results showed that SLC26A4 mRNA levels in III-1 and III-2 with a compound heterozygous mutation (c.2168A>G/c.2029C>T) were significantly lower compared to II-2 and II-3, especially lower than II-2, which only carried a heterozygous c.2168A>G mutation (Fig. 5a). The SLC26A4 protein level in 293 T cells transfected by recombinant plasmids (GV219-SLC26A4-mut (c.2029C>T) and GV219-SLC26A4-mut

(c.2168A>G/c.2029C>T) was significantly lower than the wildtype control recombinant plasmids (GV219-SLC26A4-wt, Fig. 5b) (Additional file 1: Supplementary figure 4.a, b).

Discussion

SLC26A4 mutation was the main cause of EVA, and no other mutations associated with these clinical phenotypes have been found. The incidence of SLC26A4 gene mutations is 20.35%, ranking second only to GJB2 mutations (25.65%) [15]. Some studies believe that the phenotype of EVA has a strong correlation with number of mutant alleles of SLC26A4 in Western populations [16]. Wang et al. [17] investigated 95 Chinese simplex families correlated with EVA maldevelopment, available data suggested that 88.4% of patients carried biallelic variants, monoallelic mutation account for 9.5%, while the other 2.1% did not have a mutant SLC26A4 allele.

We summarized the missense mutations located in exon 19 of the SLC26A4 gene accompanied with pathogenic clinical phenotype in Table 4 based on ClinVar database. We found that the reported SLC26A4 mutations were mainly located between positions 508–708, with clinical manifestations of Pendred syndrome or sensorineural hearing loss. In this study, we discovered that the proband carried a compound heterozygous mutation (c.2168A>G/c.2029C>T) in the SLC26A4 gene and his

Table 2 Clinical characteristics of the family individuals

| Subject | Age (years) | Sex | Pure tone test | Degree of hearing loss | DPOAE | Binaural ABR threshold (dB HL) | Tympanometry |
|---------|-------------|--------|---|-------------------------------|--|-----------------------------------|-------------------|
| I-1 | 72 | Male | 4000–8000 HZ frequency sensorineural deafness in left ear | Mild on left side | 1000 HZ, 1500 HZ frequency extraction in normal range in left ear 700–1500 HZ frequency extraction in normal range in right ear | 40 in right ear 50 in left ear | Binaura "A" curve |
| I-4 | 71 | Female | Binaural high-frequency sensorineural deafness | Mild on both side | Binaural full frequency extraction a bin normal range | 50 in right ear 60 in left ear | Binaura "A" curve |
| II-1 | 48 | Male | Binaural 2000–4000 HZ frequency sensorineural deafness | Mild on both side | 1000–8000 HZ frequency extraction in abnormal range in left ear | 40 in both ears | Binaura "A" curve |
| II-2 | 45 | Male | Binaural normal hearing | Normal hearing | 1000–2000 HZ frequency extraction in normal range in left ear 1500 HZ frequency extraction in normal range in right ear | 40 in right ear 50 in left ear | Binaura "A" curve |
| II-3 | 45 | Female | Low frequency sensorineural deafness in left ear | Mild on left side | Binaural down 1000 HZ frequency Extraction in normal range | 40 in both ears | Binaura "A" curve |
| II-4 | 41 | Female | Binaural high-frequency sensorineural deafness | Mild on both side | Binaural 500–2000 HZ frequency extraction in normal range | 60 in both ears | Binaura "A" curve |
| II-5 | 35 | Female | Binaural normal hearing | Normal hearing | Binaural 6000 HZ 8000 HZ frequency extraction in abnormal range | 30 in both ears | Binaura "A" curve |
| II-6 | 31 | Male | Binaural high-frequency sensorineural deafness | Mild on both side | Binaural down 2000 HZ frequency extraction in normal range | 40 in right ear 60 in left ear | Binaura "A" curve |
| III-1 | 20 | Male | Sensorineural deafness in right ear Sensorineural deafness with high-frequency in left ear | Severe profound on both sides | Full frequency extraction in abnormal range in right ear 1500–3000 HZ frequency extraction in normal range in left ear | 90 in right ear 50 in left ear | Binaura "A" curve |
| III-2 | 16 | Female | Binaural low frequency sensorineural deafness | Mild on both side | Binaural full frequency extraction in normal range | 20 in both ears | Binaura "A" curve |

DPOAE distortion product otoacoustic emission, ABR auditory brainstem response, dB decibels, HL hearing level

clinical presentation was profound high frequency hearing loss.

In general, SLC26A4 mutations give rise to hearing loss, which is more pronounced at high frequency. Yoshida et al. [18] reported a patient, had low-frequency SNHL and endolymphatic hydrops, carrying SLC26A4 gene c.1105A>G mutation, In acute low-frequency SNHL, mild or more pronounced endolymphatic hydrops was reported in the cochlea in 88% of vestibula and 82% of ears [19]. EVA patients with bi-allelic mutations had more severe deafness, earlier age of onset, and more fluctuating hearing levels compared to patients with no

pathogenic mutations. The proband in this study had an asymptotic high-frequency hearing loss, which was consistent with the bi-allelic mutation phenotype.

The c.2168 A>G variant is considered to be pathogenic [20, 21]. What attracted our attention was that the c.2029 (exon17) C>T mutation in SLC26A4 has already previously been deposited in the ClinVar database, but so far it is not clearly whether the mutation is pathogenic. It has been classified as a variant of unknown significance (VUS). The mutation led to a substitution of Trp with Arg at position 677 of the amino acid. While Trp is a neutral polar amino acid, Arg is an alkaline

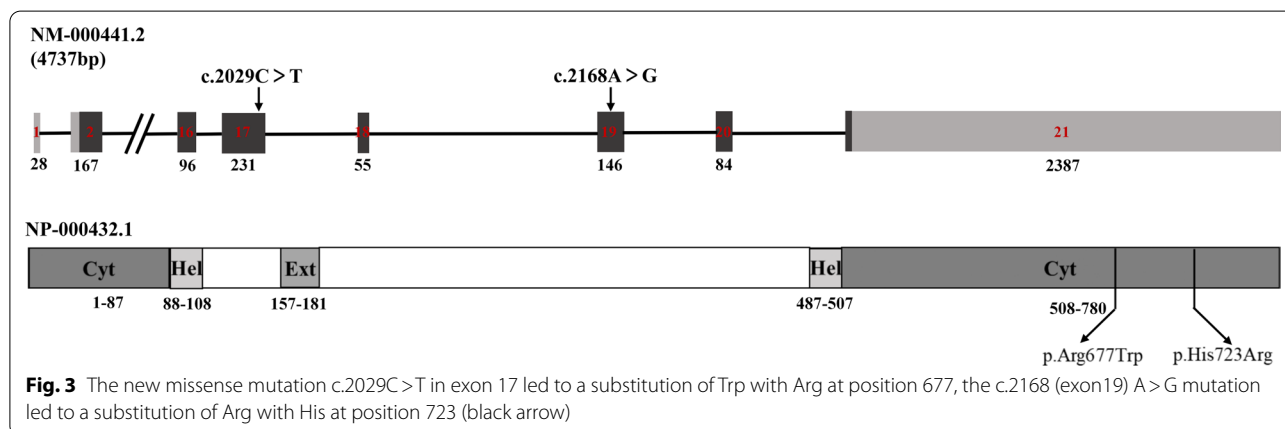
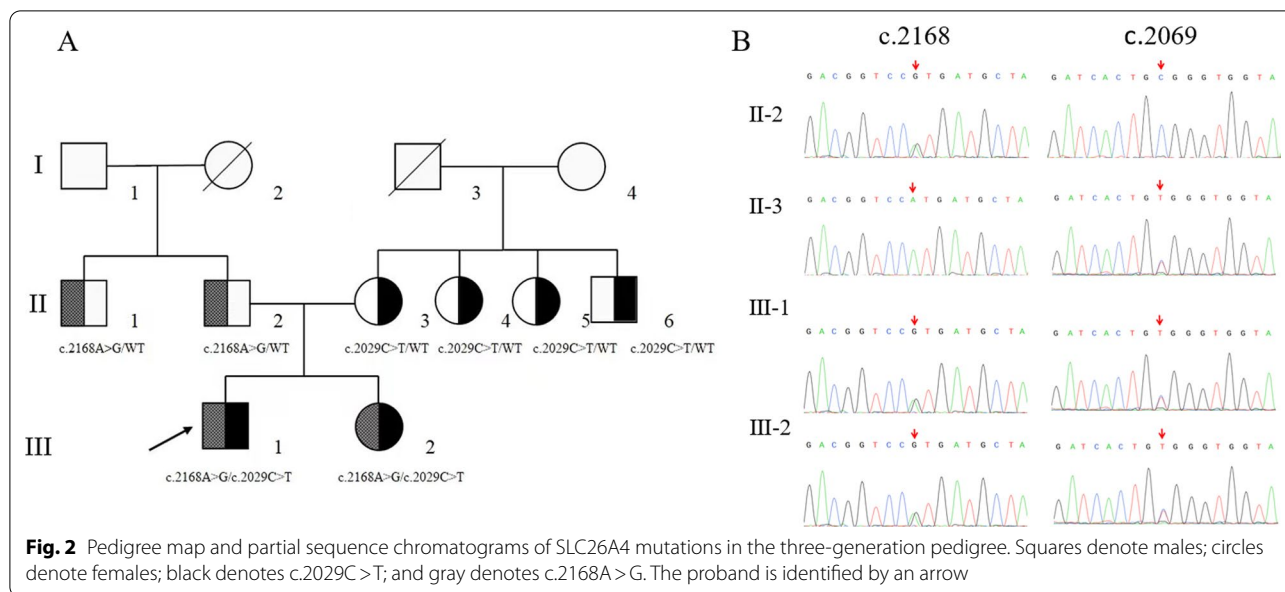


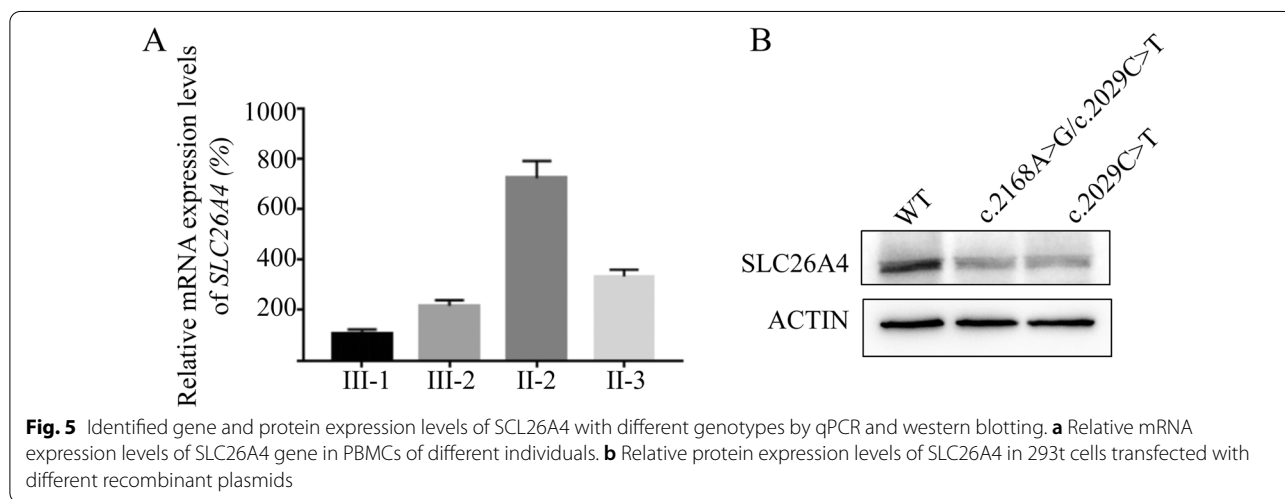
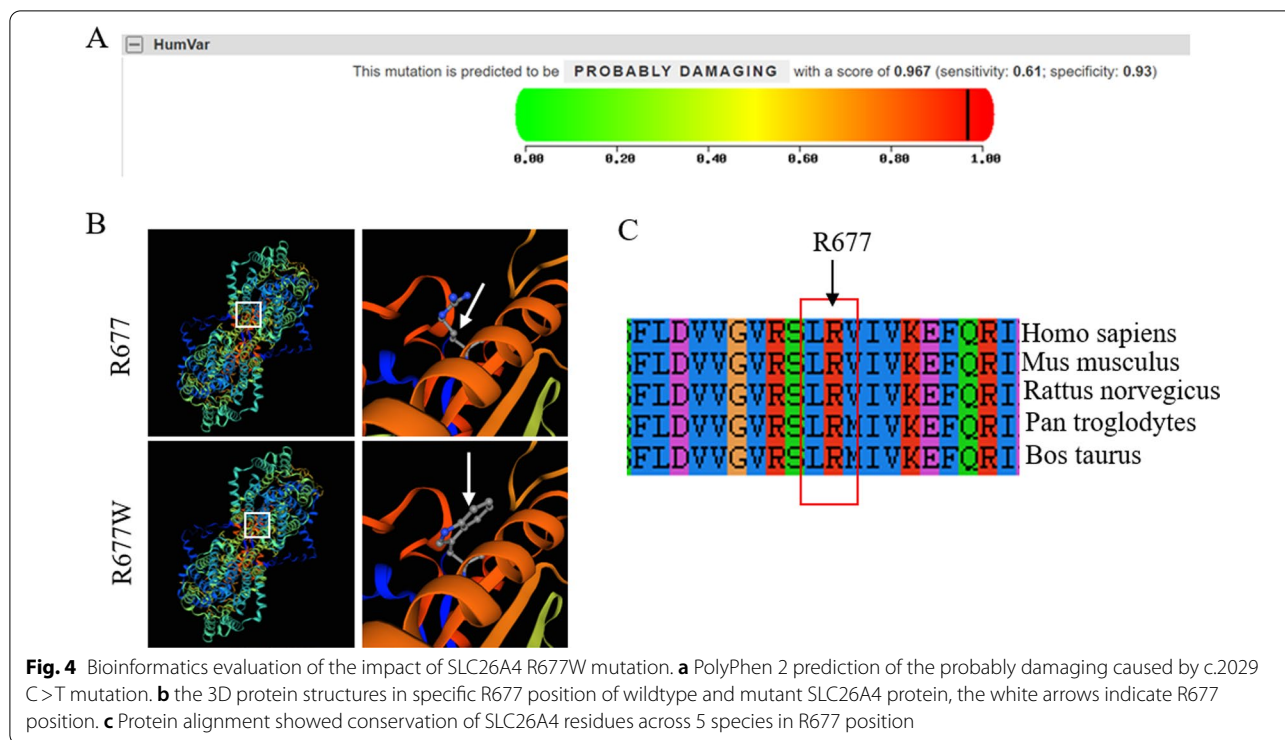
Table 3 The variant information of SLC26A4 c.2168A>G and c.2029C>T

| Nucleotide change | Amino acid change | chromosomal location | rs-numbers | Population frequency (GnomAD) (‰) |
|-------------------|-------------------|----------------------|-------------|-----------------------------------|
| c.2168 A>G | p. His723Arg | Chr7:107350577 | rs121908362 | 0.113 |
| c.2029 C>T | p. Arg677Trp | Chr7:107342497 | rs397516426 | 0.0460 |

polar amino acid. We suggest that Arg might be crucial for Pendrin function by influencing the Cl⁻/I⁻ transporter or Cl⁻/HCO₃⁻ exchange at the level of protein expression and function (Additional file 1).

We discovered that mRNA and protein expression of SLC26A4 with a c.2029 (exon17) C>T mutation is comparatively low. We speculated it may be related to the location of c.2029 C>T. The c.2029 C>T missense

mutation located closely to the edge of splice site of exon 17. As splicing site is very important for gene transcription, it may be a possible explanation on the lower mRNA/protein expression levels. Using SWISS-MODEL software, we detected that the substitution of Trp with Arg is able to destabilize the protein conformation, however c.2029 C>T mutation has no significant effect on SLC26A4 3D protein structure. We found a PolyPhen-2



prediction score of 0.967 (sensitivity, 0.61; specificity, 0.93). Generally, the closer the score is to 1, the damage is likely to be. Collectively, these results strongly indicated that the c.2029C>T mutation was likely to be deleterious to the protein. Therefore, we can presume that the c.2029 (exon17) C>T mutation in the SLC26A4 gene may be a specific mutation closely associated with hearing loss in Chinese patients related to EVA.

SLC26A4 mutations do not change the polarity sensitivity of the auditory nerve fibers to electrical stimulation,

which mainly lead to inner ear dysplasia and cochlear dysfunction [22]. Wu Ko et al. [23] indicated that cochlear implants should be valid in patients which carried SLC26A4 gene mutations. The proband in this work may receive single-sided cochlear implantation. Patients with same SLC26A4 genotype may have different phenotypes, whether they are from the same family or not. Chen et al. [24] reported two Chinese patients with a compound heterozygous mutation (c.919A>G and c.1548insC) in SLC26A4. Although they came from the same family,

Table 4 The missense mutation of exon 19 of the SLC26A4 gene with clinical phenotype as pathogenicity

| Nucleotide change | Protein change | Variant classification | Phenotype | Protein position | Consequence |
|-------------------|----------------|------------------------|--|------------------|------------------|
| c.2141G>A | p.Arg714Lys | Pathogenic | Hearing loss | 714 | missense_variant |
| c.2145G>T | p.Lys715Asn | Pathogenic | Hearing loss | 715 | missense_variant |
| c.2153T>C | p.Phe718Ser | Pathogenic | Enlarged vestibular aqueduct | 718 | missense_variant |
| c.2162C>T | p.Thr721Met | Pathogenic | Deafness, non-syndromic, autosomal recessive | 721 | missense_variant |
| c.2167C>G | p.His723Asp | Pathogenic | Deafness, non-syndromic, autosomal recessive | 723 | missense_variant |
| c.2167C>T | p.His723Tyr | Pathogenic | Enlarged vestibular aqueduct | 723 | missense_variant |
| c.2168A>G | p.His723Arg | Pathogenic | Pendred syndrome | 723 | missense_variant |
| c.2170G>A | p.Asp724Asn | Pathogenic | Pendred syndrome | 724 | missense_variant |
| c.2171A>G | p.Asp724Gly | Pathogenic | Deafness, non-syndromic, autosomal recessive | 724 | missense_variant |
| c.2173G>C | p.Ala725Pro | Pathogenic | Pendred syndrome/DFNB4 | 725 | missense_variant |
| c.2179C>T | p.Leu727Phe | Pathogenic | Enlarged vestibular aqueduct | 727 | missense_variant |
| c.2182T>C | p.Tyr728His | Pathogenic | Deafness, non-syndromic, autosomal recessive | 728 | missense_variant |
| c.2219G>T | p.Gly740Val | Pathogenic | Enlarged vestibular aqueduct | 740 | missense_variant |

they showed different phenotypes. The younger sister of this proband has the same genotype: temporal bone CT showed that she also had EVA and PTA, indicating a slight hearing loss in both ears. Such a patient may suffer fluctuation of hearing loss which may eventually develop into severe or total deafness after external factors such as colds, coughs, sneezes, or trauma to the head. To prevent those situations, she should try to avoid trauma and other predisposing factors in her daily life.

Conclusions

In summary, using whole-exome sequencing we identified a new compound heterozygous mutation (c.2168A>G/c.2029C>T) in a young Chinese man which will further strengthen the association between SLC26A4 mutation and EVA. We also demonstrated that the novel mutation (c.2029 C>T) is pathogenic. Further functional studies could assist in clarifying the relationship between the pathogenic mechanism and the novel mutation. In addition, there is still much room to explore the precise molecular mechanism of each mutation and how they affect phenotypic expression.

Abbreviations

EVA: Enlarged vestibular aqueduct; SNHL: Sensorineural hearing loss; ABR: Auditory brainstem response; PTA: Pure tone audiometry; DPOAE: Distortion product otoacoustic emission; dB: Decibels; HL: Hearing level; CT: Computed tomography; MRI: Magnetic resonance imaging; WES: Whole-exome sequencing.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12920-022-01200-4>.

Additional file 1. The original data of imaging examination and Western blotting examination.

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Authors' contributions

TW, LC and YM conceived and designed the project, drafted the manuscript. YS and FH coordinated the project. Participants were recruited and by WG and DL. JQ, CX and JZ prepared Figs. 1, 2, 3, 4 and 5 and Tables 1, 2, 3 and 4. All authors have read and approved the final manuscript.

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Availability of data and material

All data supporting the findings of this study are included in this published article. Meanwhile, data supporting the manuscript is available from any of the two corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by Medical Ethics Committee of the Yantai Yuhuangding Hospital, and all methods were carried out in accordance with its guidelines and regulations. Written informed consent for study participation was obtained from all subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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