

A novel compound heterozygous mutation of *SLC26A4* in two Chinese families with nonsyndromic hearing loss and enlarged vestibular aqueducts

GUANG-JIE ZHU*, LU-SEN SHI*, HAN ZHOU, YE YANG, JIE CHEN and XIA GAO

Department of Otorhinolaryngology, Head and Neck Surgery, Drum Tower Hospital Affiliated to Nanjing University Medical School, Nanjing, Jiangsu 210008, P.R. China

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Abstract. Enlarged vestibular aqueduct (EVA)-associated hearing loss is frequently detected in individuals carrying the *SLC26A4* mutation in the Chinese population. The present study aimed to identify the causative *SLC26A4* coding mutations in a patient group with nonsyndromic hearing loss (NSHL) and EVA. Genomic DNA was extracted from blood samples obtained from 52 NSHL patients with EVA and from 60 normal controls. The mutation analysis for 20 coding exons of *SLC26A4* was performed by direct sequencing. The results of the mutational analysis showed that there were two probands from two separate families suffering from bilateral sensorineural hearing loss with EVA, carrying the same novel compound heterozygous mutation of *SLC26A4* (c.1644_1645insA and c.2168A>G). Other members of the two families had heterozygous mono-allelic mutations with normal hearing. However, neither of these mutations were detected in the 60 normal controls. These results are the first, to the best of our knowledge, to link the compound heterozygote mutation,

c.1644_1645insA and c.2168A>G, in the *SLC26A4* gene to NSHL patients with EVA. The two mutations identified in the present study were located in the anti-sigma factor antagonist domain, the core region for plasma membrane targeting of anion transporters, which suggested that the reduced or complete loss of *SLC26A4* function was the direct cause of hearing loss in the two patients. These results provide a foundation for further elucidating the genetic factors responsible for EVA-associated NSHL.

Introduction

Enlarged vestibular aqueduct (EVA) syndrome, most frequently found by radiological examination in patients with nonsyndromic hearing loss (NSHL), is typically characterized by congenital, bilateral profound sensorineural hearing loss (SHL) or progressive hearing loss (HL) (1,2). EVA in SHL is often accompanied with or without Pendred syndrome (PS; MIM: 274600), which is often caused by mutation of the pendrin protein encoded by the *SLC26A4* gene. Autosomal recessive NSHL with *SLC26A4* mutations has been frequently reported to present with inner ear malformations, including EVA, which can be associated with Mondini dysplasia (MD), and abnormality of cochlear spirals detected using computed tomography (CT) and magnetic resonance imaging (MRI) (3-5). *SLC26A4* comprises 21 exons and produces an 86 kDa protein containing 780 amino acids. The *SLC26A4* protein, a member of the solute carrier family 26A and also known as pendrin, is mapped on chromosome 7q31 (6). Pendrin, the gene product of *SLC26A4*, is important in the regulation of endolymphatic pH and the maintenance of endocochlear potential by mediating the exchange of fluid and ions, including formate, bicarbonate, chloride and iodide ions (7). *SLC26A4* mutation can cause PS and autosomal recessive nonsyndromic hearing impairment locus 4 (MIM: 600791)-type NSHL. To date, >200 *SLC26A4* mutations have been reported in patients with nonsyndromic EVA or PS (<http://www.healthcare.uiowa.edu/labs/pendredandbor/slcMutations.htm>). In addition, 98.9% of the *SLC26A4* mutants detected in patients with EVA are from the Chinese population (8). A previous study reported that children with PS and EVA showed improved performance in speech perception, compared with a reference group with an unknown

Correspondence to: Dr Jie Chen or Dr Xia Gao, Department of Otorhinolaryngology, Head and Neck Surgery, Drum Tower Hospital Affiliated to Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: njjiechen@163.com
E-mail: gaoxiadth@outlook.com

*Contributed equally

Abbreviations: EVA, enlarged vestibular aqueduct; NSHL, nonsyndromic hearing loss; STAS, anti-sigma factor antagonist; SHL, sensorineural hearing loss; PS, Pendred syndrome; MD, Mondini dysplasia; PTA, pure-tone audiometry; CT, computed tomography; MRI, magnetic resonance imaging; ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emissions; PCR, polymerase chain reaction; NCBI, National Center for Biotechnology Information; PKA, putative protein kinase A

Key words: nonsyndromic hearing loss, enlarged vestibular aqueduct, *SLC26A4*, novel compound heterozygous mutation

cause of hearing impairment following cochlear implantation (9). Expanding the *SLC26A4* mutation spectrum may be beneficial for molecular assessment, enabling early diagnosis and planning effective clinical strategies. Through the use of targeted sequence capture and direct sequencing, the present study investigated 20 coding exons of the *SLC26A4* gene in 52 NSHL patients diagnosed with EVA by CT and MRI. The results provided the first evidence, to the best of our knowledge, of the compound heterozygous mutation p.R549Kfs*15 and p.H723R in *SLC26A4*, which expands on the *SLC26A4* mutation spectrum in the Chinese population.

Patients and methods

Subjects and clinical investigations. All Chinese individuals enrolled in the present study were recruited from the Ear, Nose and Throat Department of Drum Tower Hospital Affiliated to Nanjing University (Nanjing, China). A total of 60 normal-hearing Chinese individuals underwent pure-tone audiometry (PTA) testing and otoscopy at the Drum Tower Hospital (male/female, 32/28; age, 7.86±4.71 years). Those individuals with normal tympanum morphology and an average threshold of PTA (250-8,000 Hz) <25 dB HL were considered as normal-hearing controls. A total of 52 NSHL patients with EVA (male/female, 30/22; age, 3.52±6.13 years) and their parents underwent systematic clinical examinations and audiometric evaluations prior to the patients receiving a cochlear implant at Drum Tower Hospital. The average age of the 52 NSHL patients with EVA was significantly lower, compared with that of the normal controls, determined using Student's t-test ($P < 0.001$). As the majority of the normal controls were inpatients suffering from adenoid hypertrophy and chronic tonsillitis, there were 37 pre-language HL patients in the group of 52 NSHL patients with EVA in the present study. In addition to PTA and otoscopy, the patients underwent tympanometry, auditory brainstem response (ABR), distortion products otoacoustic emissions (DPOAE) and imaging examinations. Imaging of the ear was performed by CT and MRI. The patients or their parents were also interviewed to obtain a detailed medical history and family history, details of the mother's health during pregnancy, the patient's clinical history, including history of head or brain injury, infection and the use of medicines, including aminoglycoside antibiotics. The data collected, including characteristics and age at diagnosis, are listed in Table I.

All participants and the parents of all minors recruited in the present study provided written informed consent, and the study protocol was performed in accordance with institutional bioethics guidelines approved by the Research and Ethics Committee of Drum Tower Hospital (201601502).

DNA isolation, polymerase chain reaction (PCR) and sequence data analyses. Blood samples were collected following the provision of written informed consent. Total genomic DNA was extracted from the peripheral blood using a gDNA Isolation Micro kit (Watson Biotechnologies, Shanghai, China). All 20 coding exons of the *SLC26A4* gene were amplified using PCR with primers designed using Primer3 software (<http://primer3.ut.ee/>; Table II). The PCR mixture contained 250 µm dNTP Mixture, 10X Ex Taq buffer and Ex Taq (5 U/µl)

Table I. Clinicopathological characteristics of 52 patients with NSHL and 60 normal controls.

Characteristic	Patients with NSHL and EVA n (%)	Normal controls n (%)	P-value
Age ^a (years)	3.52±6.13	7.86±4.71	<0.0001
Sex			
Male	30 (57.69)	32 (53.33)	0.644
Female	22 (42.31)	28 (46.67)	
Family history			
Yes	14 (26.92)	NA	
No	38 (73.08)	NA	
Hearing loss			
Pre-language	37 (71.15)	NA	
Post-language	15 (28.85)	NA	
Modini dysmorphia			
With	2 (3.85)	NA	
Without	50 (96.15)	NA	
c.2168A>G			
Positive	9 (17.31)	0	
Negative	43 (82.69)	60	
c.1644_1645insA			
Positive	2 (3.85)	0	
Negative	50 (96.15)	60	

^aAge is presented as the mean ± standard deviation. Difference between groups were determined using Student's t-test. Sex differences were analyzed using Pearson's χ^2 test. NSHL, nonsyndromic hearing loss; EVA, enlarged vestibular aqueduct; NA, not applicable.

polymerase (Takara Biotechnology Co., Ltd., Dalian, China), and underwent 30 cycles of amplification with denaturation at 96°C for 20 sec, annealing at 62°C for 20 sec, extension at 72°C for 60 sec, and extension at 72°C for 5 min in a PTC-200 PCR thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR amplified products were purified and then sequenced using Sanger sequencing with an ABI 3730XL genetic analyzer (Beijing Genomics Institute, Beijing, China). The sequence data were analyzed by comparing them with the sequence of *SLC26A4* (NM_000441.1) of the National Center for Biotechnology Information (NCBI) using DNASTar 5.0 software (DNASTar, Inc., Madison, WI, USA). In addition, the 60 normal controls were checked at the same locus.

Statistical analysis. Statistical analysis was performed using SPSS 22.0 for Windows (IBM SPSS, Armonk, NY, USA). The difference in age between the NSHL patients with EVA and the normal controls was determined using Student's t-test. The sex differences were analyzed using Pearson's χ^2 test. The statistical associations of the prevalence of the novel compound heterozygous mutation of *SLC26A4* were analyzed using Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference.

Table II. Specific primers used to amplify the coding exons of SLC26A4.

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
2	GGGGACTGGGTGGAAC	GCCCGAGACTGATGGAG	678
3	GCAAATTGGTTGTGACTGAG	GAAGGGTAAGCAACCATCTGTCAC	294
4	GTTGGGCAAATAATCTAACGCA	GCAGGCAAAACACTGAAATCC	575
5	GGTCCGGCTCAGCTTCTT	GCACCTGACCTAAAACAACGT	481
6	AGGAAGGGGAGTGATAGGGT	GTCTCAAACCTCTGGGCTCA	497
7-8	CATGGTTTTTTCATGTGGGAAGATTC	AGACTGACTTACTGACTTAATGT	502
9	GAGGACAAAGAAATCAGCCAGT	CCCCTTCTTTAGCTGACACC	456
10	ATCAGGTGCTATTTCTTG	TTTCAGGTGAGGGAGTG	526
11-12	TGTTTCAGTTTTGTGGCTTGAG	TCACATGGAAGACTTCATGGC	663
13	TGTTTGTGGATCATTGA	GCACAGCAGTAGAGGAC	527
14	ACCTTTCAGGGTTATGG	TTTCTCCCTTTGGCTAC	491
15	TTGAGTGCTGCTACCC	TTCTCATTGCCCTACA	346
16	CCCTTTGAGAAATAGCC	TTGCCAAGAAATACACT	587
17	CTACCCACCATAGAAGG	GCAATACTGGACAACCC	626
18	ATTTAGCACCTCCACG	CCACAGTCCCAGATAG	375
19	TTTCAAATCTGGGTCAC	CTACCAGGTAATTCCTAT	674
20	AGAAGCACCAGGAAAGC	AGGAAGGTCATAGGGTT	570
21	GGCAACAGTGAGTGAGATTCA	AGCTTACCCTGGACGCTG	462

Results

Clinical and audiological evaluation. The novel compound heterozygous mutation, p.R549Kfs*15 and p.H723R, in *SLC26A4* was detected in a 4-year-old boy (A3) from family A and a 5-year-old girl (B3) from family B in the present study (Fig. 1A-F). The two families were from different cities of the Jiangsu province of China, and there was no relationship between the families. There was no history of hearing loss in the families of the two the two individuals or any clinical evidence showing any syndrome or disease, for example hypothyroidism. There was no history of previous illness, including meningitis, or trauma, and pregnancies were normal. There were no occurrences of congenital NSHL or syndromic hearing infection, including PS, in either family. Audiological examinations were performed and the results of PTA test showed that the two probands (A3 and B3) exhibited bilateral profound SHL (Fig. 2). This type of hearing cannot be improved with ear hearing aids and they presented with deficiency in language development. The results of the ABR showed that no representative wave was initiated under the highest intensity (105 dB), and the DPOAE showed no representative response in the frequency range of 1-4 kHz in the two probands. The results of the otoscopic examinations revealed a normal external auditory canal and tympanic membranes, and tympanometric results were normal. The two probands showed no vestibular symptoms and had a walking age of 12 months. Their thyroids were of normal size, and the results of the laboratory investigations showed normal blood parameters and thyroid function. The other members of the two families had normal hearing (Fig. 2).

Radiological examination. EVA was defined as a diameter at the midpoint between the common crus and the external aperture

of >1.5 mm (10). The temporal bone CT examinations of the two probands showed no cochlear malformations, however, bilateral EVA was present and the width of the vestibular aqueduct was >1.5 mm (Fig. 3A and B). On MRI examination, enlargement of the endolymphatic sac and duct was observed in the left and right ears of the two probands (Fig. 3C-F).

Mutation analysis. Sequencing of the 20 *SLC26A4* coding exons in the 52 patients with EVA revealed the same compound heterozygous mutation in the two probands, the 4-year-old boy and a 5-year-old girl, from the two separate families. The two variants comprised an insertion of an adenine (c.1644_1645insA) in exon 15 (Fig. 1C) and an adenine to guanine substitution (c.2168A>G) in exon 19 (Fig. 1E). The first insertion was predicted to cause a frameshift and produce a truncated protein by a premature stop (p.R549Kfs*15). This variation has not been reported previously. Individuals A1 in family A, and B1 and B4 in family B were heterozygous for this variation. The second mutation led to the substitution of histidine by arginine (p.H723R). This variation was present in the NCBI dbSNP (rs121908362) and Deafness Variation Databases of the University of Iowa (<http://www.healthcare.uiowa.edu/labs/pendredandbor/slcMutations.htm>).

The missense mutation identified in *SLC26A4* has been previously reported to cause PS or NSHL with EVA (11,12). Patient A2 in family A and B2 in family B were heterozygous for this variation. The affected members (A3 and B3) of the two families were heterozygous for the p.R549Kfs*15 and p.H723R mutations. None of the compound heterozygous mutations were detected in the 60 ethnically-matched normal control subjects or in the 50 remaining NSHL patients with EVA. This finding indicated that the p.R549Kfs*15 variation was the NSHL-causing mutation in the two families, as a compound heterozygous mutation with p.H723R.

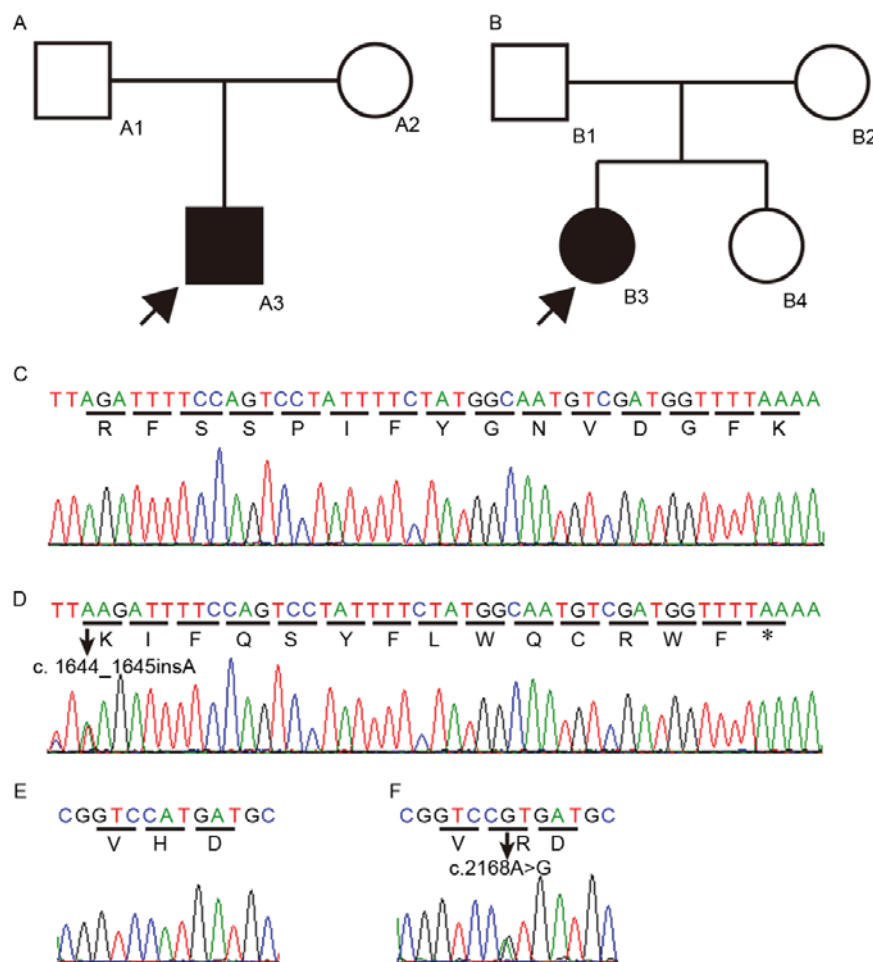


Figure 1. Autosomal recessive pedigrees of the two families. Pedigree showing the (A) 4-year-old boy (A3) from family A and (B) 5-year-old girl (B3) from family B with profound hearing loss examined in the present study (shaded and indicated with arrows) and the unaffected sister (2 years old) in family B. (C) Normal allele c.1642 and protein product of *SLC26A4*. (D) Heterozygous inheritance of the *SLC26A4* insertion c.1644_1645insA (p.R549Kfs*15) was detected using Sanger sequencing in the two probands. (E) Normal allele c.2162 and protein product of *SLC26A4*. (F) c.2168A>G missense mutation (p.H723R, rs121908362) detected using Sanger sequencing in the two patients.

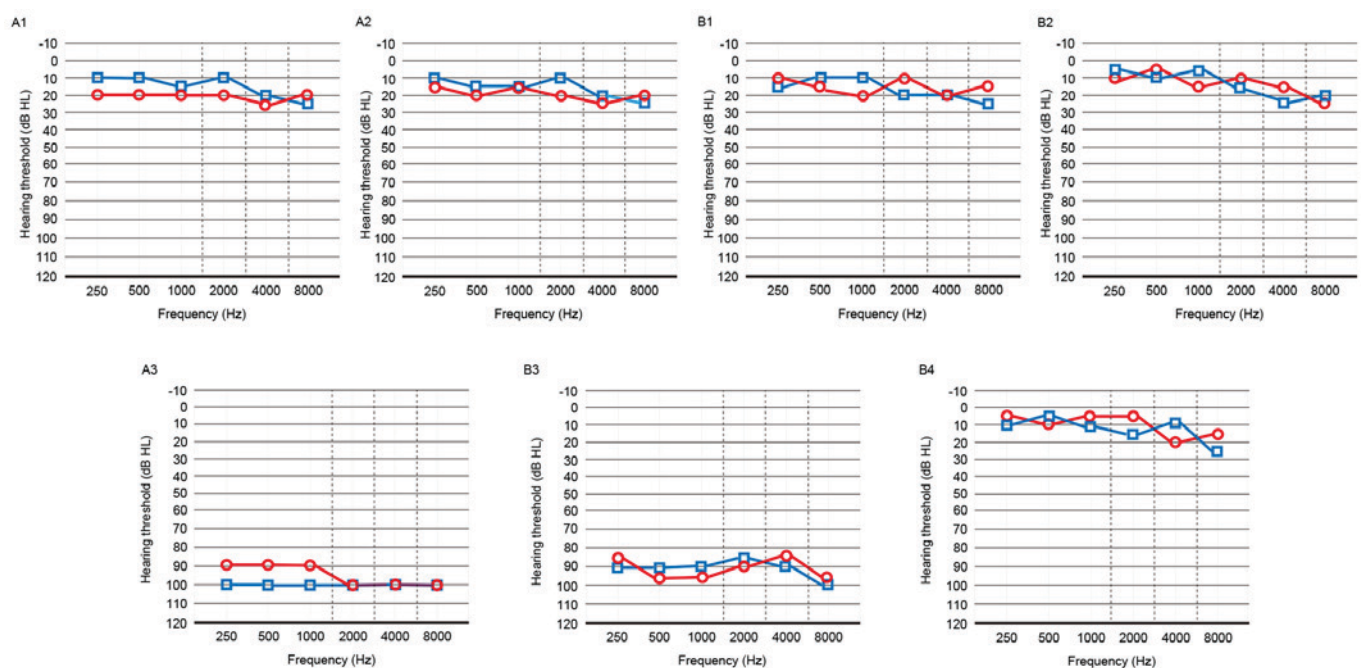


Figure 2. Bilateral pure tone audiograms from individuals in families A and B. Red circles in the audiograms represent right air conduction thresholds and blue squares represent left conduction thresholds. B4 was examined using behavior observation audiometry.

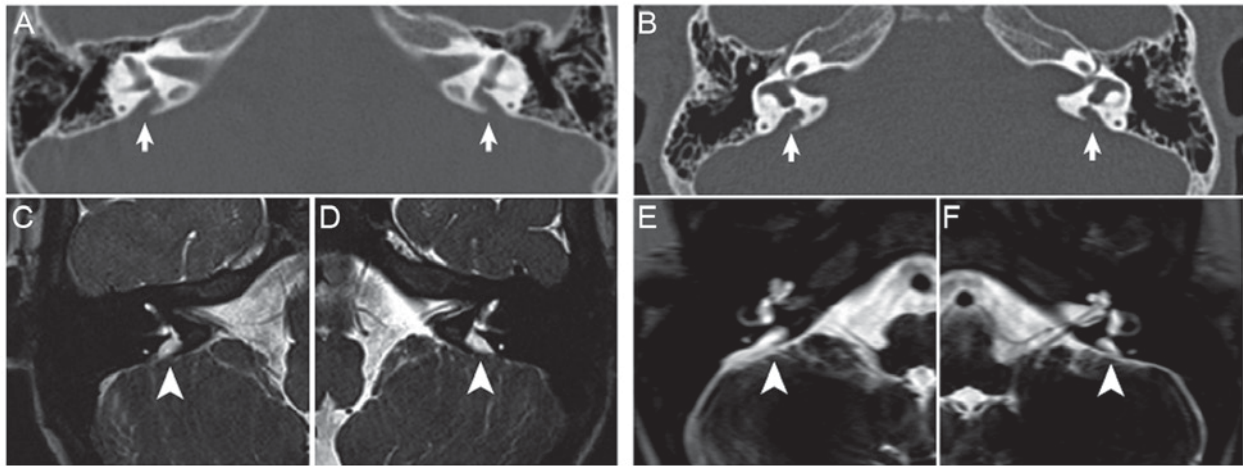


Figure 3. Radiological examinations. Axial temporal bone computed tomography images of patient (A) A3 and (B) B3 show enlarged vestibular aqueducts (white arrows). Inner ear magnetic resonance images of the (C) right and (D) left ear of patient A3, and (E) right and (F) left ear of patient B3 show the enlarged endolymph sac of the two probands (white arrows heads).

Discussion

In the present study, a novel compound heterozygous mutation of *SLC26A4* (c.1644_1645insA, c.2168A>G) was identified in two patients with profound bilateral SHL and EVA. The parents of the two patients and an unaffected member in family B (B4) carried a mutant allele separately with a normal allele and had normal hearing. The compound heterozygous mutation was inherited from the mutant alleles of the father and mother in the two probands. Therefore, it was concluded that the two affected families reported were consistent with an autosomal recessive disorder caused by bi-allelic function loss of the pendrin protein. Compound heterozygous patients with two *SLC26A4* mutant alleles have been reported in up to 50% of Chinese patients with HL and exhibit significant genetic heterogeneity in Asian populations (13-15). The mutant allele of c.2168A>G (p.H723R) has been reported to be a common pathogenic mutation among Chinese, Korean and Japanese patients with HL (14-17). The incidence of this mutation in patients with NSHL and EVA in the present study was 9/52 (17.31%; 95% CI, 8.23-30.33%). It has been reported that the mutant pendrin comprised p.H723R is localized predominantly in endoplasmic reticulum and was found to lack the activity of anion exchange (18). It was also found that patients with two bi-allelic mutations of p.H723R or a compound heterozygous mutation with the other mutation often suffered from bilateral SHL (19-21).

The other mutant allele c.1644_1645insA mutation of *SLC26A4* was identified as a novel compound heterozygous mutation with p.H723R in the present study. The incidence of this mutation in patients with NSHL and EVA in the present study was 2/52 (3.85%; 95% CI, 0.47-13.21%). The predicted result of the c.1644_1645insA mutation was a frameshift beginning at codon 549 (Arg to Lys) and a premature translation stop at position 562 in exon 15. The different conserved amino acid change (p.H723R) in exon 19 of pendrin caused by mutation of c.2168A>G and the early translational termination in exon 15 at the coding region of carboxyl terminus caused by c.1644_1645insA led to a deleterious effect on protein function, eventually resulting in a pathologic phenotype with profound HL and EVA.

Pendrin, as with other proteins in the SLC26 family, carries a sulfate transporter and anti-sigma factor antagonist (STAS) domain in its carboxy-terminus. Aravind and Koonin (22) reported that the STAS domain encompassed amino acids 535-573 and 654-729 of pendrin. The p.R549Kfs*15 and p.H723R mutations identified in the two probands in the present study were involved in the STAS domain. The STAS domain in proteins of the SLC26 family is reported to be involved in nucleotide binding and interactions with other proteins (23,24). The function of the STAS domain can be affected or lost by mutations reported in a number of patients with PS or EVA, which indicates that the STAS domain may be an important intracellular component of pendrin in the function of plasma membrane targeting and retention. The majority of the mutations in the STAS domain lead to its reduced or complete loss of function as an anion transporter, including iodide efflux (18,25-27).

It has also been confirmed that the c.2168A>G (p.H723R) mutant is a hot-spot region of the *SLC26A4* mutation in Chinese patients with PS and EVA (12,16), also affecting the putative protein kinase A (PKA) binding site. The PKA and STAS domains located in the carboxyl terminus region of pendrin are considered to be important components in the targeting of proteins to the plasma membrane (28).

In addition, the c.1644_1645insA (p.R549Kfs*15) mutation identified in the present study can cause an early stop codon and truncated protein. The early stop codon causes nonsense-mediated mRNA decay and these mRNAs are rapidly decayed (29). As the c.2168A>G (p.H723R) mutant is on another allele, the patients reported had no pendrin proteins with a normal function and exhibited NSHL with EVA. A number of reasons indicate the novel compound heterozygous mutation of the *SLC26A4* gene, including p.R549Kfs*15 and p.H723R, as the pathologic mutation. The 20 coding exon sequencing analysis of *SLC26A4* in the 52 NSHL patients with EVA revealed the compound heterozygous mutation (c.1644_1645insA, c.2168A>G) in two individuals (2/52; 3.85%). The high prevalence of this novel compound heterozygous mutation of *SLC26A4* was almost 4% among the patients with NSHL and EVA in the present study (P=0.03 with Fisher's

exact test). Therefore, the novel compound heterozygous mutation of *SLC26A4* identified in the present study expands on the wide mutational spectrum of *SLC26A4*. The results of the present study provide a foundation for future investigations of the molecular mechanisms of *SLC26A4* mutations associated with HL and EVA.

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