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Reevaluation of Enlarged Vestibular Aqueduct

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IMPORTANCE Enlarged vestibular aqueduct (EVA), the most prevalent inner ear malformation causing hearing loss (HL) in various populations, is predominantly genetically mediated. Despite advancements in genetic diagnostics, the comprehensive phenotypic and genotypic spectrum of EVA remains insufficiently characterized.

OBJECTIVES To characterize the natural history, clinical outcomes, phenotype, and genotype of EVA.

DESIGN, SETTING, AND PARTICIPANTS This single-center, longitudinal, retrospective cohort study was conducted from March 2003 to October 2022, with follow-up until July 1, 2024. Patients with EVA who were seeking medical advice at the Chinese PLA General Hospital were included.

MAIN OUTCOMES AND MEASURES This study presents a 21-year longitudinal analysis of Chinese patients with EVA, providing a systematic analysis of the natural history, phenotypic diversity, and molecular etiology of EVA.

RESULTS Of 2774 patients, 1453 (52.4%) were female individuals, and the median (range) age was 8 (4 months to 45 years) years. This study identified that 124 of 341 patients (36.36%) with EVA received passing newborn hearing screening results, while 375 of 597 (62.8%) received a diagnosis through combined audiological and radiological assessments. Recurrent vertigo (256 of 597 [42.9%]) and goiter (38 of 597 [6.4%]) were common comorbidities. Genetic analysis revealed that 2661 of 2774 patients (95.9%) carried biallelic *SLC26A4* variants, with 70 (2.5%) attributable to copy number variants and 13 (0.5%) to a deep-intronic variant (c.304 + 941C>T) that affected splicing. A de novo heterozygous *FOXI1* variant (c.483_485delCAA) was identified in an EVA family, indicating an autosomal dominant inheritance pattern. A stepped genomic analysis strategy was associated with an improved molecular diagnosis rate of 95.9%, highlighting the necessity of comprehensive genetic testing beyond traditional coding regions.

CONCLUSIONS AND RELEVANCE The results of this cohort study underscore the importance of periodic hearing surveillance and tailored genetic counseling for patients with EVA, offering substantial implications for prevention, management, and future gene therapy approaches. This study provides an extensive phenotypic and genotypic characterization of EVA, potentially advancing an understanding of its molecular underpinnings and clinical heterogeneity.

Supplemental content

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Enlarged vestibular aqueduct (EVA), also termed *DFNB4* (MIM 600791), is the most common inner ear malformation associated with congenital or childhood-onset hearing loss (HL).[1] It is frequently accompanied by an incomplete partition II (IP-II) of the cochlea,[2-4] with incidence as high as 19% among patients with HL.[5] EVA is a defining diagnostic feature of Pendred syndrome (PS; MIM 274600), a disorder characterized by HL and thyroid goiter. *DFNB4* and PS are inherited as autosomal-recessive traits, typically caused by biallelic variants (V2) in the *SLC26A4* gene. With the advent of high-throughput sequencing technologies and the accumulation of case data, genomic and clinical understanding of EVA has substantially advanced. Although studies across diverse cohorts have advanced the genomic and clinical characterization of EVA, the phenotypic and molecular spectrum of this condition (particularly in the Chinese population) to our knowledge still need to be fully elucidated. This study aimed to expand current knowledge of EVA by providing comprehensive clinical and genetic data from a large Chinese cohort.

The natural history of patients with EVA has been inconsistent and insufficiently understood, primarily due to small sample sizes and the lack of long-term follow-up studies. This gap in knowledge is critical, as understanding the natural progression of EVA is essential for designing effective protective and preventive strategies. EVA-related HL can vary widely in terms of severity, laterality, age of onset, and progression. While some studies suggest that HL in patients may be triggered by minor head trauma,[6,7] the many factors contributing to hearing loss remain poorly defined. Consequently, to our knowledge, no reproducible treatment has been established to prevent the onset or progression of HL in patients with EVA. Additionally, the accompanying manifestations of EVA and their associated treatments have not been systematically studied. For example, the lifetime prevalence of goiter in patients with EVA has been debated, with the penetrance rate ranging from 6% to 50%,[8-10] typically manifesting until adolescence.

SLC26A4 variants are among the most prevalent causes of hereditary HL worldwide.[11] Pendrin, a sodium-independent chloride/iodide transporter protein encoded by the *SLC26A4* gene (solute carrier family 26, member 4), plays a role in maintaining ion homeostasis in the endolymphatic fluid of the inner ear.[12] In China, *SLC26A4* variants account for 19.39% of all HL cases.[5] Despite the association between biallelic *SLC26A4* variants and EVA, some patients with EVA carry only 1 detectable variant allele (V1) or no variant allele (V0) in *SLC26A4*. The proportions of 2-variant (V2), V1, and V0 genotypes vary across different populations. The detection rates of *SLC26A4* variants in patients with EVA are higher among Asian individuals compared with other populations, with rates of 98.0% (92.0% V2) in Chinese patients, 82.0% (66.0% V2) in Japanese patients, 85.5% (59.3% V2) in Korean patients, and 93.4% (82.5% V2) in Taiwanese patients.[13-16] In contrast, the variant detection rate of *SLC26A4* in White patients with EVA is substantially lower; for example, a French study reported a detection rate of 40% (24% V2).[17] In a US study, *SLC26A4* variants were detected in 30.76% of patients with EVA, with only 13.29% being V2 status.[18] Although numerous studies have explored the association of *SLC26A4* variants with EVA, the data were largely confined to single-nucleotide variants (SNVs) and small indels in the coding and splicing regions of *SLC26A4*.

Key Points

Question What is the comprehensive landscape of the enlarged vestibular aqueduct (EVA)?

Findings In this cohort study and 21-year longitudinal analysis of 2774 Chinese patients with EVA, insights are provided on the natural history, phenotypic diversity, and molecular etiology of EVA.

Meaning This cohort study provides an extensive phenotypic and genotypic characterization of EVA and potentially advances an understanding of its molecular underpinnings and clinical heterogeneity.

In addition to *SLC26A4*, several other genes, including *FOXI1*, *KCNJ10*, and *EPHA2*, have been implicated in EVA pathogenesis. While these genes were initially thought to follow a strict autosomal recessive inheritance pattern, emerging evidence has suggested that their variants may interact with *SLC26A4* variants through a potential digenic mechanism, contributing to the phenotypic spectrum of EVA or PS.[8,18,19] Although *KCNJ10* variants exhibit relative high frequency in the Chinese population, to our knowledge their direct pathogenic role in EVA etiology remains unconfirmed.[20]

In this study, we systematically investigated a cohort of 2774 Chinese patients with EVA and aimed to elucidate the natural history, long-term clinical outcomes, phenotypic spectrum, and molecular characteristics of EVA. By demonstrating that EVA exhibits a broad phenotypic spectrum that ranges from mild HL without goiter to profound HL with goiter, we identified a core set of risk factors, novel variants, and effective detection strategies with substantially implications for management, surveillance, and genetic counselling. Finally, we adopted an integrated clinical, imaging, and molecular approach to provide biological insights into EVA, potentially advancing our understanding of this disorder.

Methods

Patient Cohort

A total of 2774 unrelated patients who received a diagnosis of EVA via high-resolution temporal bone computed tomography (CT) or magnetic resonance imaging (MRI) were enrolled from the Genetic Testing Center for Deafness at the Chinese PLA General Hospital between March 2003 and October 2022, with follow-up until July 1, 2024. The diagnostic criterion for EVA was a vestibular aqueduct diameter that was longer than 1.5 mm at the midpoint between the common crus and the external aperture of the vestibular aqueduct, as visualized on CT images.[21] We performed MRI assessments for patients with EVA with inconclusive CT findings.

This study was approved by the Research Ethics Committee of the Chinese PLA General Hospital, Beijing, China. Fully informed written consent was obtained from all participants

or their guardians for genetic testing and publication of clinical data.

Clinical Evaluation

Relevant family history, age of onset, and symptom progression were recorded for all patients. Comprehensive clinical evaluations included physical and otoscopic examinations, audiological assessments, and thyroid ultrasonography. HL was evaluated using pure-tone audiometry for patients 6 years or older. For children aged 2 to 5 years, hearing assessment relied primarily on age-appropriate behavioral methods (play/visual reinforcement audiometry). Auditory brainstem response and auditory steady state response were reserved for cases with unreliable behavioral results or when evaluating cochlear implant candidacy. According to the 2021 World Health Organization guidelines,[22] HL was categorized into 4 levels based on the average pure tone hearing threshold at 0.5, 1, 2, and 4 kHz: mild (26-40 dB HL), moderate (41-60 dB HL), severe (61-80 dB HL), and profound (≥81 dB HL).[23,24]

A subset of 597 patients (21.5%) was followed up via telephone interviews using a standardized questionnaire (eMaterial 1 in Supplement 1). Auditory and speech abilities were assessed using the Categories of Auditory Performance and Speech Intelligibility Rating scales.[25-27]

Variant Detection and Interpretation

Genomic DNA was extracted from peripheral blood using a commercial kit (Qiagen). For 2357 patients enrolled between March 2003 and December 2015, *SLC26A4* variants were analyzed using Sanger sequencing. Polymerase chain reaction (PCR) amplification targeted the coding exons and 50 to 100 base pairs of flanking intronic regions.[28]

A total of 555 patients (20.0%) underwent targeted deafness gene capture and next-generation sequencing (NGS), including138 patients with 1 or 0 variants detected by Sanger sequencing and 417 prospectively enrolled patients (December 2015 to October 2022). The custom-designed panel included 415 deafness genes, including 16 mitochondrial regions (eTable 1 in Supplement 1). For *SLC26A4* (chr7:107301080-chr7:107358252), exonic and intronic regions were sequenced. The details of deafness gene capture, sequencing, and bioinformatics analysis were described in our previous work.[29] Pathogenic or likely pathogenic SNVs identified by NGS were confirmed by Sanger sequencing. Copy number variants (CNVs) detected in *SLC26A4* through NGS testing were validated using multiplex ligation-dependent probe amplification with the SALSA MLPA Probemix P280-B3 *SLC26A4* kit (MRC Holland).

To assess the carrier frequency of *SLC26A4* variants in the general population, the coding and splicing regions of *SLC26A4* were sequenced in 1000 Chinese individuals with healthy hearing. An additional 498 individuals with health hearing underwent deafness gene panel testing. Variants were classified according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology guidelines,[30,31] with reference to 1500 ethnically matched controls with healthy hearing.

Reverse Transcriptase PCR Analysis of a Deep-Intronic Variant

To investigate the functional effect of the deep-intronic variant *SLC26A4* c.304 + 941C>T (chr7:107304821), RNA was extracted from peripheral blood using Phasemaker tubes using the TRIzol RNA kit (ThermoFisher). Complementary DNA was synthesized using random hexamer primers and the RT-PCR Transcriptor First Strand cDNA Synthesis kit (Roche). Primers spanning exon 2 (F: CTTTCCAGCAACAGCACGAG) and exon 5 (R: GGCACTGGCAATCAGGACTCTA) were used for amplification. The PCR products were subjected to NGS to assess splicing abnormalities. Six additional primer pairs were designed for Sanger sequencing to confirm the findings (eTable 2 in Supplement 1). The variant's distribution was assessed in 1000individuals with healthy hearing.

Statistical Analysis

The χ^2 test (R × C table), Friedman rank sum test (*M* test), and Cochran-Armitage test for trend were used to compare the distributions of *SLC26A4* variants across groups. Statistical analyses were performed using SPSS, version 26 (IBM), with 2-tailed $P \leq .05$ considered statistically significant.

Results

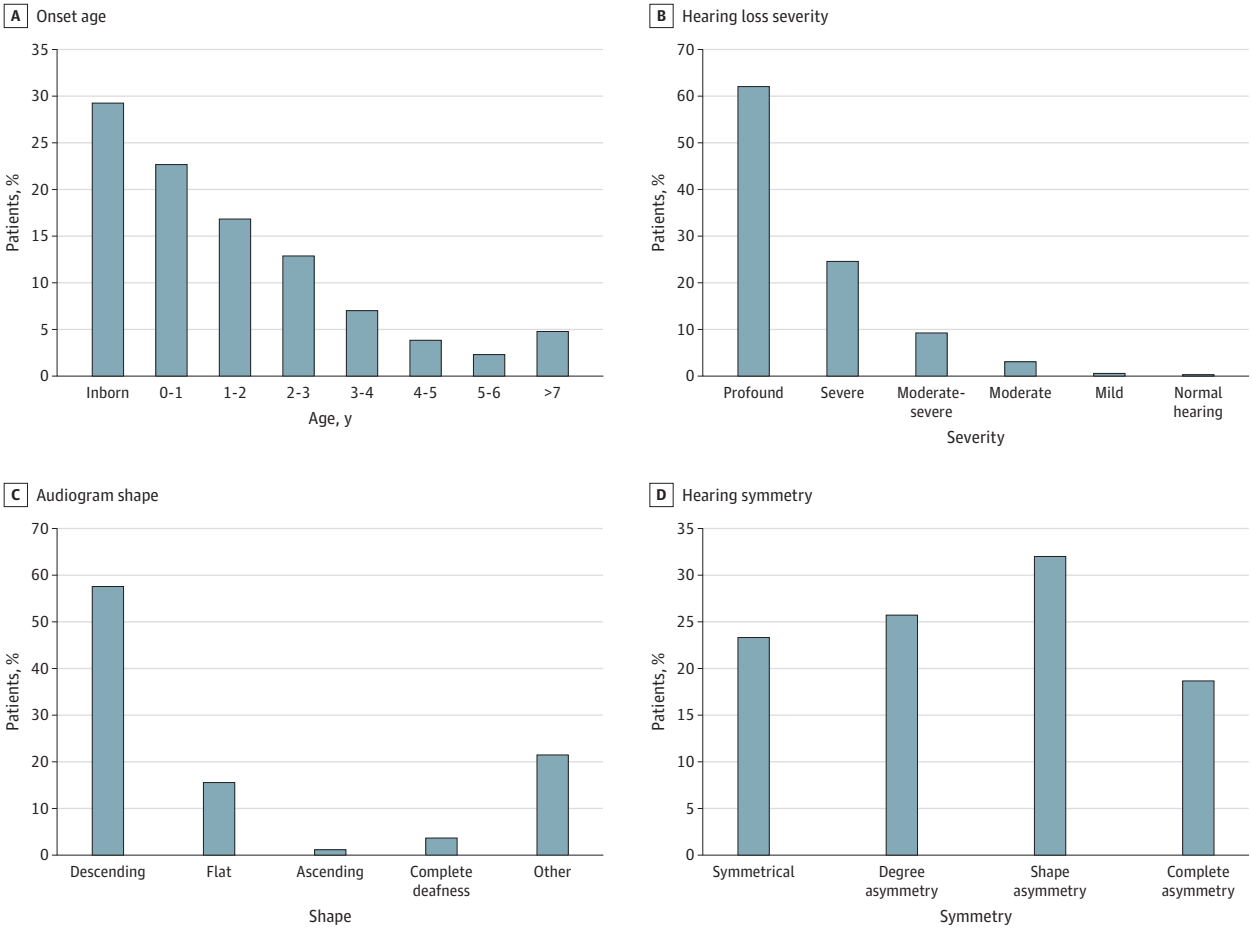
Natural History of EVA

The cohort comprised 1453 female patients (52.4%) and 1321 male patients (47.6%); the median age was 8 (range, 4 months to 45 years) years. The cohort included 2350 children (younger than 18 years; 84.7%) and 424 adults (18 years or older; 15.3%). Among 597 patients with EVA who were followed up via telephone interviews, 528 (88.4%) had no family history of HL. The primary reasons for referral to our institution fell into 2 categories: 375 (62.8%) were referred based solely on temporal bone CT results, while the other 185 (31.0%) were referred based solely on genetic testing that revealed pathogenic *SLC26A4* variants. A total of 124 patients (36.4%) passed newborn hearing screening tests, and hearing fluctuations were reported for 277 patients (46.4%). Vertigo was a common comorbidity that affected 256 patients (42.9%). Goiter was observed in 38 patients (6.4%), with 35 (92.1%) of these patients developing goiter after age 10 years (eTable 3 in Supplement 1).

Therapeutic Interventions for EVA

Of 597 patients, 574 (96.2%) underwent rehabilitation, including hearing aids or cochlear implantation. Specifically, 66 patients (11.50%) received bilateral cochlear implants, 162 patients (28.2%) received unilateral cochlear implants, 159 patients (27.7%) used a combination of hearing aids and unilateral cochlear implants (bimodal hearing), 177 (30.8%) used bilateral hearing aids, and 10 (1.7%) used a unilateral hearing aid. Postrehabilitation, 448 patients (78.1%) achieved Categories of Auditory Performance scores of 6 or greater, and 480 patients (83.5%) achieved Speech Intelligibility Rating scores of 4 or greater.

Figure 1. Audiometric and Phenotypic Characteristics of Hearing Loss (HL) in Patients With Enlarged Vestibular Aqueduct



A, Age of onset distribution: 81.9% of patients exhibited prelingual HL (onset 3 years or younger), while 18.2% developed postlingual HL. B, HL severity classification: profound (62.2%), severe (24.7%), moderate to severe (9.2%), moderate (3.0%), mild (0.6%), and normal hearing (0.3%; under surveillance). C, Audiogram configurations: descending (57.8%), flat (15.5%), ascending (1.3%), complete deafness (3.7%), and other patterns (21.6%). D, Symmetry analysis: 23.4% symmetric vs 25.79% asymmetric HL by degree; 32.09% asymmetric by configuration; 18.72% exhibited degree and configuration asymmetry.

Reproductive Counseling in High-Risk Families

Among 597 families, 314 families (52.6%) included children. Of these, 91 families (29.0%) did not pursue preventive measures, and there were 32 children (35.2%) with HL. In contrast, 205 families opted for a prenatal diagnosis (chorionic villus sampling or amniocentesis) after unassisted pregnancy, with only 4 children (2.0%) born with HL (the amniocentesis results showed all carrying biallelic *SLC26A4* variants and the parents chose to have their children be born). Additionally, 18 families underwent preimplantation genetic testing, resulting in unaffected children.

Phenotypic Characteristics of EVA

All 2774 patients underwent temporal bone CT or brain MRI. Unilateral EVA was observed in 19 patients (0.7%), while 2755 (99.3%) had bilateral EVA. Isolated EVA was presented in 213 patients (7.7%), whereas 2561 (92.3%) had EVA and IP-II. Unilateral HL was rare (5 [0.2%]), with 2769 patients (99.8%) exhibiting bilateral HL. Prelingual HL (defined as onset before age 3 years) was observed in 2271 patients (81.9%), while 503 (18.2%) developed HL after age 3 years (**Figure 1**).

Computed tomographic scans of the temporal bones were performed in the axial projection at 0.625-mm intervals. The vestibular aqueduct midpoint was measured in 867 patients (representing 1606 ears) who underwent temporal bone CT scans at our hospital to ensure accurate measurement. The range vestibular aqueduct size at the midpoint was 1.50 to 6.70 mm. The size of vestibular aqueduct midpoint for different subgroups can be found in eTable 4 in Supplement 1.

Hearing Phenotypes

Detailed hearing data were available for 1971 patients (71.0%). Profound HL was the most common phenotypes (1226 [62.2%]), followed by severe HL (486 [24.7%]), moderate to severe HL (181 [9.2%]), moderate HL (60 [3.0%]), mild HL (12 [0.6%]), and normal hearing (6 [0.3%]). Descending audiograms were predominant (1139 [57.8%]), while flat (307 [15.5%]), ascending (25 [1.3%]), and other patterns (425 [21.6%])

were also observed. Degree asymmetric HL was noted for 508 patients (25.8%), with 632 (32.1%) showing shape asymmetry (Figure 1).

Analyses using the Cochran-Armitage testing revealed significant differences in HL severity across age groups among patients with EVA, indicating a negative association between the degree of hearing loss and increasing age (eTable 5 in Supplement 1).

Genotypic Characteristics of EVA

Among the 2774 patients with EVA, 2661 (95.9%) carried biallelic *SLC26A4* variants (V2), 60 (2.2%) had monoallelic variants (V1), and 53 (1.9%) showed no detectable variants (V0). A total of 292 variants were detected (211 previously reported [72.3%], 81 novel [27.7%]), with 235 (80.5%) classified as pathogenic and 30 (10.3%) as likely pathogenic (**Figure 2**A). These variants comprised 6 categories: missense (150 [56.6%]), frameshift (50 [18.9%]), splicing (32 [12.1%]), nonsense (21 [7.9%]), CNVs (11 [4.2%]), and deep-intronic variants (1 [0.2%]) (Figure 2B). The 254 pathogenic/likely pathogenic SNVs are detailed in eTable 6 in Supplement 1. Eleven CNVs were detected in 70 patients (2.52%), with exons 1 to 3 deletions (36 of 70 [51.4%]) and exons 5 to 6 deletions (22 of 70 [31.4%]) representing the most frequent genomic rearrangements (Figure 2C). A topographic analysis of variant distribution across 2774 cases revealed variant hotspots that were concentrated in critical functional regions, particularly intron 7 and exon 19, 10, 17, and 5 (Figure 2D).

The novel deep-intronic variant c.304 + 941C>T was particularly interesting and was identified in 13 patients (0.5%). Functional characterization demonstrated that this variant induces multiple aberrant splicing events, including a 126-bp retention of intron 3 (chr7:107304694-107304819), generation of 2 novel intronic splice donor sites, and deletion of the first 82 bp of exon 5 (chr7: 107314609-107314690) (**Figure 3**; eTable 7 in Supplement 1).

Population screening in 1000 individuals with healthy hearing demonstrated a 4.8% pathogenic carrier frequency for pathogenic *SLC26A4* variants (eTables 8 and 9 in Supplement 1). A deafness gene panel analysis of an independent cohort (n = 500) identified no CNVs in *SLC26A4*.

Other Genes

Using targeted gene capture and NGS, this study detected *FOXI1* and *ATP6V1B* variants in 2 independent families. In the first family, a de novo heterozygous *FOXI1* variant (c.483_485delCAA; p.Asn161del) was identified in the affected proband and was absent in both unaffected parents (eFigure in Supplement 1). In the second family, the proband presented with distal kidney tubular acidosis, sensorineural HL, and EVA, and genetic testing identified the homozygous pathogenic variant c.1356delT (p.Phe452Leufs*35) in *ATP6V1B*. No pathogenic variants were detected in *EPHA2* among the patients with EVA.

Stepped Genomic Analyses Strategy

This study used a stratified genetic screening strategy for 2774 patients with EVA, with the diagnostic workflow schematically presented in **Figure 4**. *SLC26A4* was detected by Sanger sequencing in 2357 patients with EVA (84.9%), revealing biallelic variants in 2194 cases (93.1%), monoallelic variants in 113 cases (4.8%), and no variant in 50 cases (2.1%). Among these patients, 138 patients (88 with monoallelic SNVs and 50 with no variant detected by Sanger sequencing) were further analyzed using NGS. This follow-up analysis identified CNVs in 54 cases and the deep-intronic variant c.304 + 941C>T in 11 cases.

In a separate cohort of 417 patients with EVA, NGS was performed directly and identified biallelic variants in 402 cases (96.4%), monoallelic variants in 9 cases (2.2%), and no variant in 6 cases (1.4%). Among the 402 patients with biallelic *SLC26A4* variants, 16 (3.6%) carried CNVs and 2 (0.5%) harbored the c.304 + 941C>T variant in the deep intronic region.

A combined analysis revealed biallelic *SLC26A4* variants in 95.9% of patients with EVA, with 2.2% having monoallelic variants and 1.9% showing no variants. A comparison of detection methods revealed that Sanger sequencing identified 93.1% of patients with EVA with biallelic variants, traditional NGS (targeting coding exons and 50-100 bp flanking regions) detected 95.9%, and specific NGS (including exonic and intronic regions) achieved a detection rate of 96.4%.

Genotype-Phenotype Associations

Patients were stratified into 6 groups based on EVA phenotypes: (1) EVA with or without other inner ear malformations, (2) EVA with or without goiter, (3) unilateral or bilateral EVA, (4) EVA with varying degrees of HL, (5) vestibular aqueduct midpoint, and (6) hearing progression. A significant difference in *SLC26A4* variant status was observed in patients in group 3 and 5: patients with bilateral EVA had a higher rate of biallelic variants (98.6%) compared with patients with unilateral EVA (21.1%), and the vestibular aqueduct midpoint dimensions were significantly larger in the V2 group compared with the V0 and V1 groups. No significant associations were found between *SLC26A4*-specific variants (V2/V1/V0) and IP-II, goiter status, HL severity, or the hearing progression (**Table**).

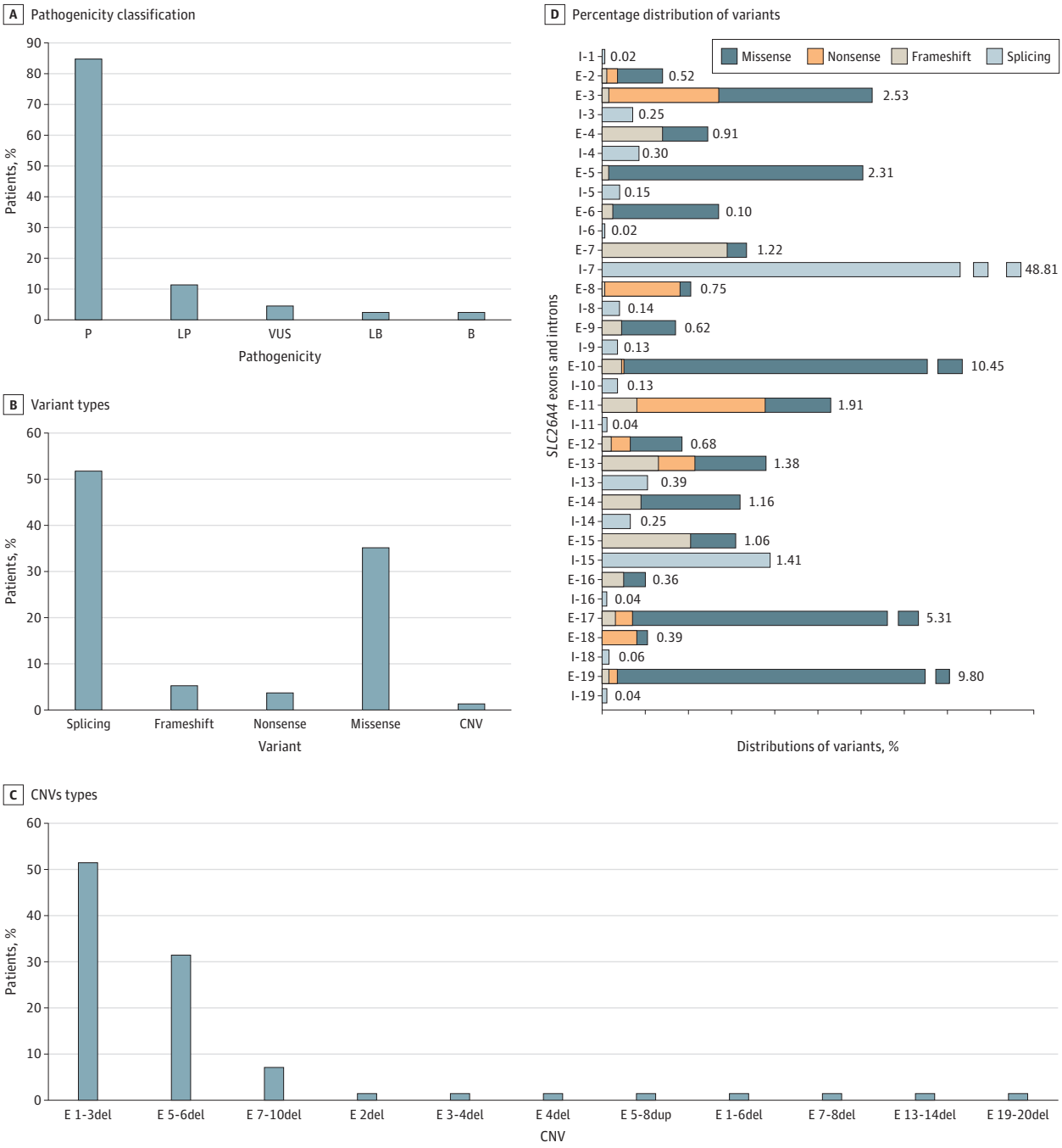
Discussion

Insight Into Disease Progression via Natural History of EVA

EVA is the most common inner ear malformation in otolaryngology and is primarily characterized by progressive and fluctuating HL due to the enlargement of the vestibular aqueduct. This condition is often associated with triggers, such as head trauma or barotrauma, and may be accompanied by vertigo. A subset of patients also exhibit goiter, a hallmark of PS. The clinical phenotypes and accompanying symptoms of EVA vary substantially among patients. Although previous studies have explored specific aspects of EVA,[6-10,32,33] a comprehensive understanding of its natural history remains limited.

To address this gap, we conducted a follow-up study of 597 patients with EVA via telephone questionnaires, capturing data on diagnosis, phenotypic characteristics (eg, newborn hearing screening, age of onset, hearing fluctuations, vertigo, goiter, and kidney status), and disease progression. Our findings suggest a general trend of progression HL in most patients with

Figure 2. Genomic Characteristics and Distribution Patterns of *SLC26A4* Variants

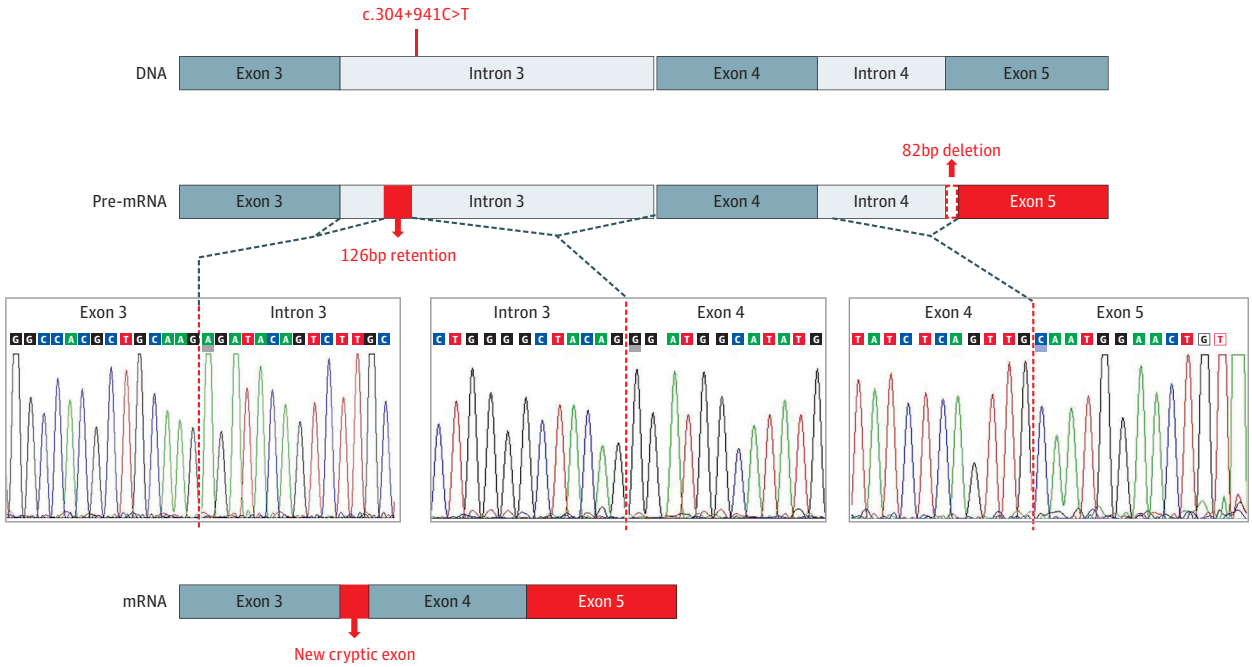


A, Pathogenicity classification of identified variants, demonstrating the proportion of pathogenic (P; 80.5%), likely pathogenic (LP; 10.3%), variants of uncertain significance (VUS; 4.5%), likely benign (LB; 2.4%), and benign (B; 2.4%). B, Types of P and LP variants: splicing variants (12.1%), frameshift variants (18.9%), nonsense variants (7.9%), missense variants (56.6%), copy number variants (CNVs; 4.2%), and deep-intron variants (0.4%). C, CNVs types: classification and frequency of CNVs detected in the *SLC26A4*, exon1-3del (51.4%), exon5-6del (31.4%), exon7-10del (5.7%), 1.4% for other CNVs, respectively. D, Percentage of distribution of variants across *SLC26A4* exons/introns, showing the proportion of unique variant alleles. Each variant is counted once, irrespective of patient recurrence, ad proportions are derived from eTable 3 in Supplement 1.

EVA, although individual disease courses vary. Long-term follow-up is essential to better understand the natural history of EVA, enabling physicians to provide more accurate patient counseling and management strategies.

Currently, hearing aids and cochlear implants are the most effective rehabilitation methods for patients with EVA.[34,35] Our data indicated that at least 78.1% of patients achieve effective rehabilitation. In China, EVA accounts for 19.0% of patients

Figure 3. Functional Association of the Deep-Intronic Variant in *SLC26A4* With Splicing



The variant c.304 + 941C>T induces 3 aberrant splicing events: (1) retention of a 126-bp segment of intron 3, (2) creation of 2 novel intronic splice donor sites, and (3) deletion of the first 82 bp of exon 5. mRNA indicates messenger RNA.

with HL,[5] and 52.6% of high-risk families express a need for reproductive counseling. Therefore, identifying pathogenic genes and variants is critical for clinical prevention. This study not only systematically investigated the natural history of EVA, but it also provided a detailed phenotypic and genotypic analysis of 2774 patients with EVA, offering insights for prevention and management in high-risk families.

Diverse Phenotypic Characteristics of EVA

Among the 2774 patients with EVA, 7.7% had isolated EVA, while 92.3% exhibited EVA and IP-II. Imaging results revealed that 99.3% of patients had bilateral EVA and only 0.7% (19 patients) had unilateral EVA. Of the latter, 5 (26.3%) exhibited unilateral HL, while 14 (73.7%) had bilateral HL. Reported prevalence rates of unilateral EVA have varied substantially across studies. Lower rates were consistently observed in East Asian populations: 0.68% (this study), 1.14%,[28] and 8.9%.[36] In contrast, Western populations exhibit markedly higher prevalence, with rates of 15.7% to 51.4%.[37-40] Despite this substantial geographical variation, a consistent pattern has emerged: the proportion of patients with unilateral EVA who carry biallelic pathogenic *SLC26A4* variants remains low (0%-15.8%).[28,36-40] Consequently, most researchers hypothesize that noninherited factors, such as developmental anomalies, likely contribute to unilateral EVA pathogenesis. However, the precise mechanisms require further elucidation. The complexity of its etiology, which potentially involves multifactorial interactions, may also underlie the observed population disparities in prevalence.

The hearing phenotypes of patients with EVA are highly diverse, ranging from normal to profound HL, often with fluctuation and progression.[41,42] This study revealed that severe to profound HL was the most common phenotype (86.9%), and 46.4% of patients reported hearing fluctuations, while there was a negative association between the degree of hearing loss and increasing age. Audiograms were predominantly descending (57.8%), with flat (15.5%), ascending (1.3%), complete deafness (3.7%), and other patterns (21.6%) also being observed. Asymmetric HL was noted in 44.5% of cases. Prelingual HL was observed in 81.9% of patients, which was consistent with earlier reports,[15,43-45] although some studies reported higher rates of postlingual HL.[9,46]

The prevalence of PS among patients with EVA varies by ethnicity, ranging 19.0% in Japanese patients[15] and 6% to 50% in White patients.[8-10] In our cohort, only 6.4% of patients had a diagnosis of PS. eTable 10 in Supplement 1 summarizes the phenotypic and genotypic differences in EVA across various populations.[9,10,15-18,47-49]

SLC26A4 as the Primary Pathogenic Factor in Chinese Patients With EVA

In this study, biallelic pathogenic variants in *SLC26A4* were identified in 95.9% of patients with EVA, confirming its role as the primary genetic determinant of EVA in the Chinese population. The genotypic spectrum of *SLC26A4* is highly diverse, with pathogenic variants distributed across coding and noncoding regions, including SNVs, CNVs, and deep-intronic variants.

Figure 4. Genetic Testing Results of 2774 Patients With Enlarged Vestibular Aqueduct (EVA)

Molecular diagnostic yield and variant distribution across cohort. The left side represents individuals who underwent Sanger sequencing first, and those for whom no biallelic variants were detected were subsequently tested with the *415HL* gene panel + next-generation sequencing (NGS), while the right side represents individuals who were directly tested with the hearing loss gene panel without prior Sanger sequencing. CNV indicates copy number variants; SNV, single-nucleotide variants.

Table. Variant *SLC26A4* Allelic Number in Different Groups

Groups	No. (%) V2	V1	V0	Total No. of patients	*P* value
1					
EVA	201 (94.4)	8 (3.8)	4 (1.9)	213	.45
EVA with IP-II	2460 (96.1)	52 (2.0)	49 (1.9)	2561	
2					
PS	18 (100)	0	0	18	.50
EVA with/without IP-II	2643 (95.9)	60 (2.2)	53 (1.9)	2756	
3					
Bilateral EVA	2658 (96.5)	59 (2.1)	38 (1.4)	2755	<.001
Unilateral EVA	3 (15.8)	1 (5.3)	15 (79.0)	19	
4					
Normal	4 (66.7)	0	2 (33.3)	6	.16
Mild	9 (75.0)	0	3 (25.0)	12	
Moderate	46 (76.7)	10 (16.7)	4 (0.7)	60	
Moderate to severe	167 92.3)	9 (5.0)	5 (2.7)	181	
Severe	458 (94.2)	26 (5.4)	2 (0.4)	486	
Profound	1145 (93.4)	69 (5.6)	12 (1.0)	1226	
5					
Midpoint, median (range) mm[a]	2.43 (1.52-3.36)	2.41 (1.56-4.04)	2.61 (1.50-6.70)	1606[b]	<.001
6					
Progressive	268 (96.8)	8 (2.9)	1 (0.4)	277	.19
Stable	302 (94.4)	16 (5.0)	2 (0.6)	320	

Abbreviations: EVA, enlarged vestibular aqueduct; IP-II, incomplete partition type II of the cochlea; PS, Pendred syndrome; V2, biallelic variants; V1, monoallelic variants; V0, no detectable variants (non-*SLC26A4*).

[a] The midpoint between the common crus and the external aperture of the vestibular aqueduct.

[b] 1606 Ears.

To date, more than 400 pathogenic *SLC26A4* variants have been recorded in the Deafness Variation Database.[50] Racial differences in the detection rates and mutational spectra of *SLC26A4* variants are well-documented.[13-18] While previous studies focused on SNVs in coding regions and splice sites, to our knowledge few explored CNVs and deep-intronic variants.[11-15,18] In this study, we systematically analyzed *SLC26A4* variants in 2774 Chinese patients with EVA, identifying 265 pathogenic and likely pathogenic variants, including 253 SNVs, 11 CNVs, and 1 variant in a deep-intronic region. Comprehensive reevaluation according to American College of Medical Genetics and Genomics/Association for Molecular Pathology guidelines for genetic hearing loss that incorporated phenotypic data and carrier frequencies across case and control cohorts led to the reclassification of 21 variants: 10 variants of uncertain significance were upgraded to pathogenic/likely pathogenic, while 11 variants (or uncertain significance or pathogenic) were downgraded to benign/likely benign. The molecular diagnostic rate reached 95.9%, higher than the 92.0% reported in our previous study.[14] However, 4.07% of patients with EVA do not have a molecular diagnosis, highlighting the need for future research.

Our results suggest that phenotypic differences in patients with EVA are not solely determined by the number of variant *SLC26A4* alleles but may also reflect the extent of vestibular aqueduct enlargement or environmental factors.[15,48] While some studies suggest that patients with V2 experience more profound HL earlier,[2,3] our findings aligned with a large body of evidence that has indicated no significant genotype-phenotype association.[15,46,49,51] This study not only confirmed that patients with bilateral EVA exhibit a significantly higher detection rate of biallelic pathogenic *SLC26A4* variants, but also revealed that patients who harbor biallelic pathogenic variants exhibit more pronounced vestibular aqueduct anatomical abnormalities, which was consistent with the results reported by Madden et al.[52]

Expanded Genetic Spectrum of EVA in Chinese Patients

Beyond *SLC26A4*, we investigated other EVA-associated genes. *FOXI1* is one of the earliest identified contributors to EVA pathogenesis. Murine models have demonstrated that *Foxi1* knockout mice exhibit inner ear malformations phenocopying human PS.[53,54] In 2007, Yang et al[18] proposed a digenic model for EVA pathogenesis: double heterozygosity (*SLC26A4*+/−; *FOXI1*+/−) in humans and mice that was associated with EVA phenotypes. Despite this hypothesis, subsequent studies failed to identify additional *FOXI1* variants in EVA cohorts, and its functional role remained unexplored. In our EVA cohort, we identified a de novo heterozygous *FOXI1* variant in 1 family. Combined with a report of another *FOXI1* de novo variant,[55] and the study by Smits et al[56] that reported heterozygous *FOXI1* variants in three sporadic EVA cases, these findings imply that heterozygous *FOXI1* variants may contribute to EVA. However, further clinical evidence and mechanistic studies are required to confirm this association. We also confirmed established syndromic associations through identifying a homozygous *ATP6V1B1* variant in a patient with concurrent EVA and distal kidney tubular acidosis while excluding *EPHA2* as a contributor in our cohort.

Diagnostic Strategies and Implications for Genetic Counseling

Our findings revealed that 95.5% of pathogenic/likely pathogenic *SLC26A4* variants are SNVs in coding/splicing regions, supporting Sanger sequencing as the primary detection method. While Sanger sequencing successfully identified biallelic variants in 93.1% of patients with EVA, confirming its diagnostic

efficacy, its application for this large gene presented throughput limitations. By comparison, NGS offers superior efficiency with comprehensive coverage of SNVs/CNVs. By optimizing our NGS protocol to encompass full exonic/intronic sequences, we achieved a substantially improved diagnostic rate of 96.4%, demonstrating enhanced detection capability for comprehensive molecular diagnosis of EVA. Thus, we recommend tailored detection strategies based on laboratory capacity, target population, and clinical needs to optimize molecular diagnosis and prevention efforts in high-risk families. While medical imaging remains the criterion standard for EVA, our findings suggest that genetic testing can take precedence.

This study confirmed a strong-effect *SLC26A4*-EVA association in a large homogeneous cohort. The 95.5% high detection rate in Chinese patients highlights their distinct genetic architecture, for whom *SLC26A4* variants constitute the primary etiology of EVA. These findings aligned with prior studies in East Asian populations (82%-98%),[13-16] whereas the diagnostic yield differs markedly in European and US populations (30%-40%),[17,18] indicating underlying genetic background as the primary determinant.

Limitations

A limitation of this study was the absence of EVA haplotype testing in White individuals, which significantly contributes to the molecular diagnosis of Pendred syndrome and *DFNB4*, particularly in White populations (50%-81% in V1 cohorts).[56,57]

Conclusions

This cohort study provided a comprehensive analysis of the natural history, phenotypic diversity, and genotypic landscapes of EVA in a large Chinese cohort. Our findings underscore the importance of long-term follow-up, stepped genomic analysis, and tailored genetic counseling for patients with EVA. *SLC26A4* is the primary genetic determinant of EVA in the Chinese population, and its diverse variant spectrum highlights the need for advanced diagnostic strategies. Furthermore, we hypothesized an autosomal dominant inheritance mechanism mediated by *FOXI1* pathogenic variants in the etiology of EVA. This study potentially deepens our understanding of its molecular underpinnings and clinical heterogeneity.

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