Mutations of SLC26A4 can cause hearing loss with enlargement of the vestibular aqueduct (EVA) either as an isolated auditory trait (nonsyndromic EVA) or in association with a thyroid iodination defect that can lead to goiter in Pendred syndrome (PS). SLC26A4 encodes the polytopic transmembrane protein pendrin (SLC26A4) that can exchange a variety of bases and anions across the plasma membrane of epithelial cells. Most subjects with PS do not have a goiter at the initial examination because it is not a completely penetrant feature and is often unapparent until the second decade of life. Computed tomography (CT) or magnetic resonance imaging (MRI) of the temporal bones reveals EVA or enlargement of its soft-tissue contents, the endolymphatic sac and duct, as a completely penetrant feature of PS.

About 25% of all subjects with EVA have 2 detectable mutant alleles (termed M2) of SLC26A4, 25% have 1 mutant allele (M1), and 50% seem to have no mutant SLC26A4 alleles (M0). Bilateral EVA has been observed in subjects with M0, M1, and M2 genotypes. Unilateral EVA, unilateral hearing loss, or both have been reported in a few subjects carrying 2 SLC26A4 sequence variants. However, the identities of the specifically associated variants have not been reported, some are co-
Incidental benign variants,\(^8\,^12\) and trans configuration was not demonstrated. In other studies, unilateral EVA has correlated with only 1 or 0 mutant alleles of SLC26A4.\(^5\,^9\,^{13}\)

The degree of hearing loss is significantly greater in M2 ears than in M1 or M0 ears with EVA.\(^14\) The number of mutant alleles of SLC26A4 is the strongest correlate of the severity of hearing loss in ears with EVA.\(^14\) When the number of mutant alleles of SLC26A4 is accounted for, there is no independent association of cochlear malformations with the severity of hearing loss in ears with EVA.\(^14\) Furthermore, the M2 genotype is correlated with abnormal thyroid iodination and gland volume.\(^9\,^{15}\)

Therefore, periodic thyroid surveillance is indicated in subjects with M2 genotype.\(^15\)

The recurrence risk for EVA in siblings of probands with M2 genotypes is statistically indistinguishable from the recurrence risk for siblings of probands with M1 genotypes.\(^7\) This likely reflects the existence of undetected mutations in another gene or in a noncoding region of SLC26A4 in subjects with M1 genotypes, although we have been unable to identify such mutations.\(^7\) Thus, EVA seems to be inherited as a recessive trait with a 1 in 4 recurrence risk in siblings of probands with M1 or M2 genotypes. In contrast, the recurrence risk is much lower, close to 0, in nonwinn siblings of probands with M0 genotypes.\(^7\) These recurrence risks are based on our definition of EVA as a midpoint diameter larger than 1.5 mm. It is possible the recurrence risk would be different if EVA were defined as a midpoint diameter of greater than or equal to 1.0 mm.\(^16\)

SLC26A4 test results can be informative for empirical counseling of subjects with EVA and their families regarding hearing prognosis, the need for thyroid surveillance, and recurrence risk in siblings (Table 1). Although the diagnostic yield and use of genetic testing for autosomal recessive, unilateral, nonsyndromic, sensorineural hearing loss is probably very low or 0 for other deafness genes, we hypothesized that SLC26A4 testing for unilateral EVA could yield diagnostic results with clinical use. In this study, we tested that hypothesis by evaluating the results of SLC26A4 testing in a cohort of subjects with unilateral EVA.

### Methods

This study was approved by the Combined Neuroscience Institutional Review Board, National Institutes of Health, Bethesda, Maryland. (The study objective was formulated before data were collected.) Written informed consent was obtained for all subjects. Self-reported race and ethnicity was classified according to our institutional review board reporting guidelines. We have accrued an additional 27 subjects with EVA since the report by Choi et al\(^8\) for a total of 116 subjects with EVA.

Subjects were evaluated at the National Institutes of Health Clinical Center as described.\(^8\,^9\,^{14}\,^{15}\) EVA was defined as a vestibular aqueduct (VA) diameter exceeding 1.5 mm at the midpoint between the posterior cranial fossa and the vestibule of the inner ear or a grossly malformed overall morphology of the VA.\(^3\) Evaluations included pure-tone and speech audiometry and CT and MRI of the temporal bones.\(^14\) There were 10 male and 14 female subjects with unilateral EVA: 21 white/Caucasian, 1 mixed, 1 Hispanic, and 1 black. Mean and median ages of the 24 subjects were 10.3 and 7 years, respectively (age range, 5–39 years). Six of the 24 subjects with unilateral EVA had bilateral hearing loss. We reviewed the radiologic images of the contralateral ear in these 6 subjects, and found 3 (subjects 1580, 1702, and 1949) with a normal-sized VA but a cochlear malformation (hypoplastic modiolus); 1 (subject 1979) with a VA midpoint diameter of 1.0 mm that would be considered enlarged by Madden et al\(^10\); and 2 (subjects 1623 and 1659) in which the contralateral ear was normal.

We extracted genomic DNA and sequenced the 21 exons and flanking intronic regions of SLC26A4 as described.\(^8\,^9\) We classified the pathogenic potential of SLC26A4 variants as described.\(^8\) p.R776C (c.2326C>T; rs111033255) was classified as a benign polymorphism and p.L597S (c.1790T>C; rs55638457) was classified as pathogenic in the context of trans configuration with a pathogenic variant.\(^8\) p.F335L (c.1003T>C; rs111033212) and p.M775T (c.2324T>C) were reclassified as pathogenic due to their respective control carrier frequencies of 0.186% and 0 in European Americans in the Exome Variant Server (http://evs.gs.washington.edu/EVS/; accessed December 21, 2012). The trans configuration of all compound heterozygous variants was confirmed by sequence analysis of parental DNA.

To assess the pathogenic potential of p.R185T, we generated an SLC26A4 complementary DNA expression construct fused at its C-terminus to green fluorescent protein, transfected COS-7 cells, and evaluated intracellular localization by confocal microscopy as described.\(^8\) We evaluated anion exchange activity of the p.R185T variant encoded by complementary RNA injected into Xenopus oocytes. Chlorine-36 (\(^36\text{Cl}^-\)) influx and efflux were measured and analyzed as reported previously.\(^8\,^{16}\,^{17}\)

### Table 1. Phenotypes and Recurrence Risks Associated With SLC26A4 Test Results in 87 National Institutes of Health Subjects With EVA

<table>
<thead>
<tr>
<th>No. of Mutant Alleles of SLC26A4</th>
<th>Identification of Etiology</th>
<th>Thyroid Phenotype(^a)</th>
<th>Proportion of Subjects With Unilateral EVA, No./Total No. (%)(^b)</th>
<th>Degree of Hearing Loss in Affected Ear(^c)</th>
<th>Recurrence Risk in Sibling(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Yes</td>
<td>Abnormal iodination; increased gland volume</td>
<td>2/27 (7)</td>
<td>More severe</td>
<td>1:4</td>
</tr>
<tr>
<td>1</td>
<td>Partial</td>
<td>Normal</td>
<td>3/17 (18)</td>
<td>Less severe</td>
<td>1:4</td>
</tr>
<tr>
<td>0</td>
<td>No</td>
<td>Normal</td>
<td>16/43 (37)</td>
<td>Less severe</td>
<td>&lt;&lt;1:4</td>
</tr>
</tbody>
</table>

Abbreviation: EVA, enlargement of the vestibular aqueduct.

\(^a\) Includes subjects with unilateral and bilateral EVA.

\(^b\) Nontwin siblings.

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Results

We reviewed our cohort of 116 subjects with EVA and identified 24 subjects (20.7%) with unilateral EVA. EVA was present in the right ear of 12 subjects and in the left ear of 12 subjects. SLC26A4 variants were detected in 10 (42%) of 24 subjects with unilateral EVA. Their genotypes and laterality of EVA are listed in Table 2. After classifying the variants as either benign variants or pathogenic mutations, a total of 18 subjects (75%) were M0, 4 subjects (16.7%) were M1, and 2 subjects (8.3%) were M2. Therefore, 25% of our cohort of subjects with unilateral EVA had SLC26A4 mutations. χ² Testing confirmed no association of SLC26A4 genotypes (M0, M1, or M2) with laterality (right vs left) of EVA (not shown).

Subjects 1627 and 2026 are exceptions to the collective observation that unilateral EVA is rarely, if ever, associated with 2 mutant alleles of SLC26A4. Thus, we explored their clinical and molecular genetic phenotypes in detail. Both subjects were described as white by their parents according to our institutional review board guidelines for race classification. Subject 1627 was a 5-year-old boy with unilateral (right) hearing loss (Figure 1A) and enlargement of the right VA and right endolymphatic sac and duct (Figure 2A) whose data are previously reported. His left ear was audiometrically and radiologically normal (Figure 1A and Figure 2B). Ultrasonography revealed normal thyroid volume and texture. He was compound heterozygous for the prevalent splice-site mutation c.1001 + 1G>A (IVS8 + 1G>A) and a rare hypofunctional missense variant c.1003T>C (p.F335L).

Table 2. EVA Laterality and Genotypes of 10 Subjects With SLC26A4 Variants and Unilateral EVA

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>EVA Laterality</th>
<th>SLC26A4 Genotype</th>
<th>Genotype Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1167*</td>
<td>RE</td>
<td>p.L597S / +</td>
<td>M0</td>
</tr>
<tr>
<td>1282*</td>
<td>RE</td>
<td>p.R776C / +</td>
<td>M0</td>
</tr>
<tr>
<td>1304</td>
<td>RE</td>
<td>p.L445W / +</td>
<td>M1</td>
</tr>
<tr>
<td>1580</td>
<td>RE</td>
<td>p.E384G / +</td>
<td>M1</td>
</tr>
<tr>
<td>1627</td>
<td>RE</td>
<td>p.F335L / IVS8 + 1G&gt;A</td>
<td>M2</td>
</tr>
<tr>
<td>1639*</td>
<td>LE</td>
<td>p.R776C / +</td>
<td>M0</td>
</tr>
<tr>
<td>1702</td>
<td>RE</td>
<td>p.M775T / +</td>
<td>M1</td>
</tr>
<tr>
<td>1981*</td>
<td>LE</td>
<td>p.L597S / +</td>
<td>M0</td>
</tr>
<tr>
<td>2026</td>
<td>LE</td>
<td>p.R185T / c.890delC</td>
<td>M2</td>
</tr>
<tr>
<td>2083</td>
<td>LE</td>
<td>c.365InsT / +</td>
<td>M1</td>
</tr>
</tbody>
</table>

Abbreviations: EVA, enlargement of the vestibular aqueduct; LE, left ear; RE, right ear.

* Subjects 1167, 1282, 1639, and 1981 had no mutations according to our study criteria (see Methods). The other 14 subjects in the study had no SLC26A4 variants.
Subject 2026 was a 6-year-old girl with unilateral (left) hearing loss at the time she was evaluated at the National Institutes of Health Clinical Center (Figure 1B). Her right ear continued to have normal pure-tone hearing thresholds when she was tested at age 10 years (Figure 1C). She had enlargement of the left endolymphatic sac and duct (Figure 2D) and left VA (Figure 2F). Her right ear was radiologically normal (Figure 2C and E). She had a normal thyroid phenotype and was compound heterozygous for the frameshift mutation c.890delC and the c.554G>C (p.R185T) missense allele of SLC26A4. We assume that c.890delC is a functional null allele based on its predicted effect on SLC26A4 expression and function. p.R185T has been reported as pathogenic. We observed that SLC26A4 R185T is retained in the endoplasmic reticulum of COS-7 cells (Figure 3F) and fails to traffic to the plasma membrane of Xenopus oocytes (Figure 3E). It exhibits a complete loss of Cl⁻/Cl⁻, Cl⁻/I⁻ (iodine ion), and Cl⁻/HCO₃⁻ (bicarbonate ion) exchange activities compared with wild-type pendrin (Figure 3A-D). These results confirm the SLC26A4-deficient, M2 genotype status of subject 2026.

**Discussion**

Because SLC26A4 mutant alleles are recessive, SLC26A4 test results are fully diagnostic only for subjects with M2 genotypes. In our cohort of subjects with unilateral EVA, 8.3% of subjects had a fully diagnostic result that guides recommendations to monitor the thyroid gland and provides precise information for genetic counseling. Although M0 and M1 geno-
type results cannot completely explain the cause of EVA, they can be used to empirically counsel subjects and their families regarding prognosis and recurrence risk.\(^7\)

The diagnostic yield of SLC26A4 mutation testing seems to be lower when the definition of EVA is less stringent (ie, smaller diameter). Madden et al\(^{10}\) identified SLC26A4 vari-

![Figure 3. Anion Transport Function](image-url)

A, Chlorine-36 (\(^{36}\)Cl\(^{-}\)) influx into uninjected Xenopus oocytes or oocytes expressing wild-type (WT) or mutant pendrin (R185T). B, \(^{36}\)Cl\(^{-}\) efflux from WT or R185T mutant pendrin-expressing oocytes exposed sequentially to salt bath compositions are shown. C and D, \(^{36}\)Cl\(^{-}\) efflux rate constants. E, Pendrin-GFP variants and normalized fluorescence intensity (FI) at surface of oocytes. F, WT or R185T mutant pendrin (green) and concanavalin-A staining (red) of plasma membranes\(^8\) of COS-7 cells. Co-localization of WT pendrin with concanavalin-A at the plasma membrane is seen as yellow (green arrow), while R185T mutant pendrin does not localize to the plasma membrane (red arrow). Scale bars indicate 20 μm; mM, millimolar; ln, natural logarithm; I\(^{-}\), iodine ion; and HCO\(_3\)^\(^{-}\), bicarbonate ion.
variants in 19 (27%) of 71 North American children with unilateral or bilateral EVA when EVA was defined according to the criteria of Vijayasekar et al.19: midpoint width larger than 0.9 mm, operculum width of larger than 1.9 mm, or both. In comparison, we previously identified SLC26A4 variants in 56 (65%) of 86 North American subjects with unilateral or bilateral EVA defined as a midpoint diameter larger than 1.5 mm.8,9 Even when we used stricter criteria to classify an SLC26A4 variant as a pathogenic mutation,8 39 (45%) of 86 National Institutes of Health subjects with EVA still had SLC26A4 mutations. The difference in SLC26A4 variant prevalence between the National Institutes of Health and Madden et al.10 cohorts is significant (Fisher exact test, 2-tailed, \( P < .001 \)) and could reflect a difference in cohort demographics, the difference in EVA size criterion, or both.

The EVA cohort reported by Madden et al.10 also included 2 subjects with unilateral EVA with 2 variants of SLC26A4. However, trans configuration was not confirmed and the variants were not specified. They may have included the nonpathogenic variants p.V609G or p.G740S.8,12,20 The pathogenic potential of 2 of the other variants, p.A189S and p.N324Y, is indeterminate. They have high allele frequencies of 52 of 4354 (1.19%) and 118 of 4406 (2.68%), respectively, among African American controls (Exome Variant Server; http://evs.gs.washington.edu/EVS/; accessed December 21, 2012), indicating they are common polymorphisms in that population. Although the ethnicity of the subjects with unilateral EVA carrying those variants was not specified, 13 of the subjects (19%) in the Madden et al.10 cohort were African American, raising the possibility that they were coincidentally detected benign variants. The uncertain pathogenic potential of rare hypofunctional SLC26A4 variants must be considered when interpreting SLC26A4 test results for individuals as well as cohorts of subjects. For the classification of variants in this study, we adapted the criteria from Choi et al.7 If we further increase the stringency of criteria for pathogenicity, and reclassify p.F335L and p.M775T as benign variants, 20 of our subjects (83%) were M0, 3 subjects (13%) were M1, and 1 subject (4%) was M2. Even with these rigorous criteria, there was still at least one SLC26A4 mutation in 17% of subjects with unilateral EVA. Nevertheless, SLC26A4 testing loses its utility when the pathogenic potential of the variants cannot be definitively interpreted and the subject cannot be conclusively classified as M0, M1, or M2. This is a problem for a small percentage of subjects and should be discussed with them as a potential risk during pretest counseling.

A perchlorate discharge test, which detects the thyroid iodination abnormality of PS, can add causative diagnostic information when SLC26A4 genotype results are inconclusive.15 However, inconclusive SLC26A4 genotypes are increasingly rare because of the rapidly deepening databases of human genomic variation that enable reliable interpretation of almost all sequence variants of SLC26A4. Ultrasonography is a practical initial evaluation of the thyroid in subjects with M2 genotypes and unilateral or bilateral EVA.15

In summary, we observed unilateral EVA associated with the full range of possible SLC26A4 genotypes: M0, M1, and M2. Because each of these genotypes is correlated with a different combination of natural history and recurrence probability, we conclude that SLC26A4 mutation testing can be clinically useful in subjects with unilateral EVA for which the midpoint diameter is larger than 1.5 mm. SLC26A4 mutation testing should always be preceded and followed by genetic counseling, irrespective of the results.

References


