

***SLC26A4* mutation testing for hearing loss associated with enlargement of the vestibular aqueduct**

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or *SLC26A4* mutations. Two mutant alleles of *SLC26A4* are detected in 1/4 of North American or European EVA populations, one mutant allele is detected in another 1/4 of patient populations, and no mutations are detected in the other 1/2. The presence of two mutant alleles of *SLC26A4* is associated with abnormal iodide organification, increased thyroid gland volume, increased severity of hearing loss, and bilateral EVA. The presence of a single mutant allele of *SLC26A4* is associated with normal iodide organification, normal thyroid gland volume, less severe hearing loss and either bilateral or unilateral EVA. When other underlying correlations are accounted for, the presence of a cochlear malformation or the size of EVA does not have an effect on hearing thresholds. This is consistent with observations of an *Slc26a4* mutant mouse model of EVA in which hearing loss is independent of endolymphatic hydrops or inner ear malformations. Segregation analyses of EVA in families suggest that the patients carrying one mutant allele of *SLC26A4* have a second, undetected mutant allele of *SLC26A4*, and the probability of a sibling having EVA is consistent with its segregation as an autosomal recessive trait. Patients without any mutations are an etiologically heterogeneous group in which siblings have a lower probability of having EVA. *SLC26A4* mutation testing can provide prognostic information to guide clinical surveillance and management, as well as the probability of EVA affecting a sibling.

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Key words: *SLC26A4*; Pendred syndrome; Genetic testing; Goiter; Hearing loss; Vestibular aqueduct; Genotype-phenotype correlation

Abstract

Pendred syndrome (PS) is characterized by autosomal recessive inheritance of goiter associated with a defect of iodide organification, hearing loss, enlargement of the vestibular aqueduct (EVA), and mutations of the *SLC26A4* gene. However, not all EVA patients have PS

Core tip: Enlargement of the vestibular aqueduct (EVA) is a common inner ear anomaly. We review the correlation of phenotype with genotype of *SLC26A4*. *SLC26A4* mutations are the most prevalent known cause of hearing loss associated with EVA. The number of mutated alleles is correlated with the presence or absence of a

thyroid iodination defect, thyroid gland volume, severity of hearing loss, laterality (bilateral *vs* unilateral) of the inner ear anomaly, and probability of recurrence of EVA in a sibling. We discuss the risks and benefits of genetic testing and counseling for affected patients. These concepts may be of broad interest to otolaryngologists, audiologists and other clinicians.

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PENDRED SYNDROME AND NONSYNDROMIC HEARING LOSS WITH ENLARGEMENT OF THE VESTIBULAR AQUEDUCT

Pendred syndrome (PS) is characterized by autosomal recessive inheritance of goiter and hearing loss, first reported in two sisters by Pendred^[1] in 1896. Fraser^[2] estimated this syndrome accounted for 5.6% of congenital hearing loss in his series of 2355 children. The causative gene for PS was mapped to chromosome 7q in 1996^[3] and identified as *SLC26A4* in 1997^[4]. Molecular testing for *SLC26A4* mutations and temporal bone imaging have established that PS is always accompanied by inner ear deformities, with enlargement of the vestibular aqueduct (EVA) as the most penetrant feature^[5] (Figure 1). The identification of *SLC26A4* mutations associated with PS suggested a possible association of nonsyndromic hearing loss with EVA (NSEVA) with mutations of this gene. Usami *et al*^[6] identified *SLC26A4* mutations in sporadic and familial cases of NSEVA, showing that *SLC26A4* mutations are commonly associated with NSEVA. These observations were confirmed in numerous studies of large cohorts of PS and NSEVA patients from different ethnic populations^[7-11].

Most clinicians now rely upon molecular testing of *SLC26A4* for the etiologic diagnosis of PS and NSEVA. There are over 200 reported mutations in *SLC26A4* associated with sporadic and familial forms of PS and NSEVA. Furthermore, a large-scale study demonstrated mutations of *SLC26A4* in approximately 5%-10% of individuals with childhood deafness among several large global populations^[12]. This percentage is coincident with Fraser's phenotypic estimate of the prevalence of PS^[2]. However, in North American and European populations, *SLC26A4* mutations cannot be detected in up to one half of patients with hearing loss and EVA, while only one mutant *SLC26A4* allele is identified in one fourth of patients^[9-11,13]. EVA has also been detected in a subset of patients with branchio-oto-renal or branchio-oto syndrome^[14], Waarden-

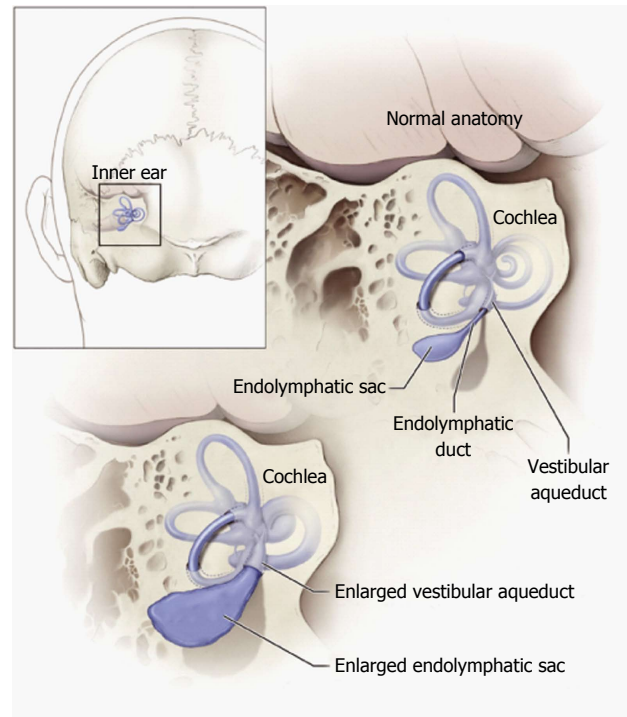


Figure 1 Schematic illustration of the relationship of the vestibular aqueduct with the endolymphatic sac and duct. Normal anatomy of the inner ear structures is shown above. Pathologic enlargement of the endolymphatic sac and abnormal enlargement of the vestibular aqueduct are shown below. Some ears with enlargement of the vestibular aqueduct also have a reduced number of cochlear turns. Reproduced from <http://www.nidcd.nih.gov/health/hearing/vestAque.htm>.

burg syndrome^[15], and deafness associated with the recessive form of distal renal tubular acidosis^[16]. However, there is no published evidence that mutations of the genes underlying these syndromes cause PS or NSEVA.

SLC26A4 encodes a transmembrane protein, called pendrin, comprised of 780 amino acids and 12 or more predicted membrane-spanning domains^[4,17-21]. Mouse *Slc26a4* is expressed in a restricted tissue distribution that includes the inner ear, thyroid, kidney, lung, and several other organs^[4]. Pendrin has been shown to exchange anions across the plasma membrane in several heterologous expression systems. Physiologically predominant functional modes are thought to include Cl⁻/I⁻ exchange in the thyroid^[22] and Cl⁻/HCO₃⁻ exchange in the inner ear^[23]. This anion exchange activity is critical during late embryonic and early postnatal development of the inner ear^[24]. A variety of cellular details of the pathogenic events have been described^[25-28]. Here we summarize the clinical phenotypes, genetics, and a novel mouse model of EVA.

CORRELATION OF *SLC26A4* GENOTYPE WITH THYROID PHENOTYPE

The pathogenesis of goiter in PS is thought to be a thyroidal iodine organification defect^[29]. The goiter tends to be diffuse at first, but later becomes nodular^[2]. The organification defect can be detected by measuring the discharge of inorganic radioiodide from the thyroid after adminis-

tration of potassium perchlorate. Potassium perchlorate is a competitive inhibitor of the sodium-iodide symporter, which transports iodide into thyroid folliculocytes across their basolateral membrane. An abnormally high discharge of iodide from the thyroid gland in response to perchlorate administration is a relatively specific finding for the clinical diagnosis of PS. For decades, it was the gold standard for the diagnosis of PS. Goiter, an abnormal perchlorate discharge, or both is identified in one third to one fourth of patients with hearing loss and EVA^[30,31]. Goiter is an incompletely penetrant feature of PS. Furthermore, an onset during adolescence is typical^[2,32]. The distinction between PS and NSEVA can therefore be difficult to make during childhood. This problem is exacerbated by the insensitivity of the physical examination for detection of goiter. While ultrasound examination with volume determinations may be helpful, normal gland size varies with age, and volume determinations have typically not been reported in a normalized fashion. In addition, goiter of other etiologies is common in some regions and populations, leading to phenocopies that increase the potential for misdiagnosis^[33].

SLC26A4 mutations are responsible for both PS and some cases of NSEVA, which suggested a possible correlation between particular types of mutations and the presence of the goiter^[8,34]. Scott *et al*^[7] concluded that normal thyroid function in NSEVA patients is the consequence of residual pendrin activity encoded by hypofunctional *SLC26A4* variants as compared to functional null alleles in PS patients. However, subsequent studies of cohorts with EVA and hearing loss failed to support this hypothesis^[8,35]. Alternatively, a correlation between clinical phenotype and the number of mutant alleles of *SLC26A4* has been suggested. With a definition of PS as > 15% discharge of iodide 2 to 3 h after administration of perchlorate, there was strong correlation between PS and the presence of two (M2) mutant *SLC26A4* alleles, while NSEVA was associated with either one (M1) or zero (M0) mutant alleles^[9,10]. Moreover, a multivariate analysis concluded that thyroid gland volume is primarily dependent on the presence of two mutant alleles of *SLC26A4*, at least in pediatric (< 10 years old) EVA patients^[30].

CORRELATION OF *SLC26A4* GENOTYPE WITH AUDITORY PHENOTYPE

Radiologically detectable inner ear deformities are often considered to be pathologic changes that contribute directly to congenital deafness. Inner ear deformities were first reported by Mondini^[36] in a temporal bone histopathological study in 1791. For centuries afterwards, the term “Mondini dysplasia” was often used for any inner ear malformation. Over many years, the classification and interpretation of inner ear anomalies, especially cochlear deformities, were based on a linear developmental model in which a developmental arrest occurred during embryogenesis^[37,38].

However, certain observations do not support the developmental arrest model for all inner ear malformations.

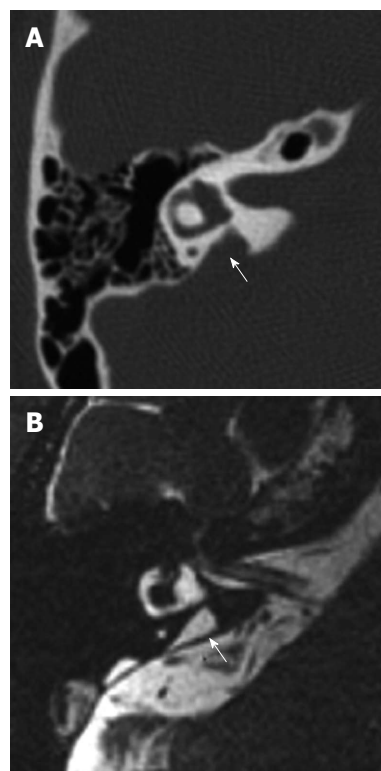


Figure 2 Right temporal bone of a patient with enlargement of the vestibular aqueduct. A: Axial computer tomography image of a right temporal bone with an enlarged vestibular aqueduct (arrow); B: Equivalent magnetic resonance image of the same temporal bone showing an enlarged endolymphatic duct (arrow). Reproduced from <http://www.nidcd.nih.gov/health/hearing/Pages/eva.aspx>.

The vestibular aqueduct (VA) is a narrow bony canal that opens onto the medial surface of the temporal bone and contains the endolymphatic sac and duct (Figure 1). The VA continues to grow throughout fetal life, but does not reach its full mature size before birth^[39]. Some temporal bone studies indicate that the VA continues to grow postnatally in size until 3 years of age^[40,41]. These observations were inconsistent with the hypothesis of arrested development^[38]. Kim *et al*^[42] reported that EVA and scala media expansion occurred at embryonic day 14 in the *Slc26a4*-null mouse model. Their model postulated that enlargement depends on disruption of the normal balance between endolymph secretion and absorption in the labyrinth and endolymphatic sac. They speculated that lumen enlargement might be a form of hydrops caused by increased endolymphatic osmotic pressure due to impaired resorptive ion transport. This observation suggested that a developmental distortion, as well as arrest, occurs during fetal embryogenesis, thus explaining the concomitance of EVA and Mondini dysplasia.

EVA in humans is conventionally defined as a VA diameter exceeding 1.5 mm, measured at the midpoint between the common crus and external aperture (Figure 2). This original radiologic criterion was proposed by Valvassori *et al*^[43] in 1978. A recent study demonstrated that 1.0 mm is a more sensitive criterion for EVA^[44]. EVA can occur as an isolated anomaly, as well as in combination with other

inner ear deformities^[45,46]. Inner ear deformities have been detected in 20%-30% of patients with congenital deafness^[46-49]. EVA is the most common inner ear deformity, recognized in approximately 5%-15% of ears of deaf children^[49-52].

No significant association has been reported between the type or number of mutant alleles of *SLC26A4* and the presence of cochlear anomalies^[10,45,53]. In contrast, two mutant alleles of *SLC26A4* (M2) are tightly correlated with bilateral EVA, while unilateral EVA is correlated with only one (M1) or zero (M0) mutant allele of *SLC26A4*^[9]. Unilateral EVA is found with and without other inner ear deformities and is two to six times less frequent than bilateral EVA in North American and European populations^[34,54-56].

The hearing loss associated with *SLC26A4* mutations is predominantly sensorineural or mixed, asymmetric, with an onset in the first few years of life. The degree of hearing loss can vary from mild to profound^[31,38,45,55]. The hearing loss often shows fluctuation and overall downward progression that can be precipitated by minor head trauma or barotrauma. Hearing loss progression has been observed in 36%-88% of ears and fluctuation has been observed in 30%-92% of ears associated with *SLC26A4* mutations^[10,31,45]. Almost one half of the ears with fluctuating hearing loss eventually showed overall progressive loss of hearing. Even in the ears with normal to moderate hearing loss, hearing loss could progress at the rate of about 1 dB/year, with no apparent effect of environmental factors^[45].

No significant relationship has been reported between the degree of hearing loss and the type of mutation or the presence of cochlear deformities, whereas the degree of hearing loss associates significantly with the number of mutant alleles of *SLC26A4*^[9,10,45,55]. The presence of two mutant alleles (M2) is associated with more severe hearing loss than only one (M1) or zero (M0) mutant alleles. Most reports have failed to reveal significant effects of number of mutant alleles of *SLC26A4* or the presence or absence of cochlear anomalies on longitudinal hearing^[10,45]. The degree of hearing loss does not correlate with the degree of enlargement of the VA or its contents, the endolymphatic duct^[45,57]. This strongly suggests that endolymphatic hydrops is not a direct cause of hearing loss. Although others have reported potential correlations of radiologic findings with hearing loss phenotypes^[58], these conclusions were based upon univariate analyses that did not account for underlying factors and correlations such as *SLC26A4* genotype, age, and other genetic diagnoses.

PATHOGENESIS OF HEARING LOSS ASSOCIATED WITH EVA

Although hearing loss is often sensorineural, bone conduction threshold testing can reveal a mixed (conductive plus sensorineural) hearing loss at low frequencies associated with normal tympanometry and middle ear findings^[59-62], and an abnormal vestibular evoked myo-

genic potential result^[63]. These findings are thought to be due to a "third window" effect upon sound transmission within the labyrinth^[64].

The pathogenesis of sensorineural component in hearing loss ears with EVA has been enigmatic. It was initially believed that trauma or barotrauma increases intracranial pressure with reflux of the contents of the endolymphatic sac and duct into the scala media through the enlarged endolymphatic duct. However, there is little evidence to support this theory, as obliteration of the endolymphatic sac and duct does not reverse or even prevent further hearing loss in patients with EVA^[38]. It has also been suggested that sudden drops of hearing might be caused by rupture of Reissner's membrane^[38], hemorrhage in the endolymphatic sac^[65] or a fistulous round window membrane^[66]. There may be occasional examples of these pathogenic mechanisms, but recent research indicates that the underlying mechanism is more often attributable to an intrinsic disruption of endolymphatic homeostasis.

Studies of an *Slc26a4*-null mouse model suggested scala media expansion and endolymphatic acidosis are early consequences of a lack of pendrin expression^[67,68]. Subsequently, oxidative stress, abnormal cell stretching, impaired cell-to-cell communication, and loss of KCNJ10 expression occur in the stria vascularis, associated with a reduced endocochlear potential (EP) and hearing loss^[23,69-71].

Slc26a4 is expressed in multiple non-sensory cell populations of the cochlea, vestibular labyrinth, and endolymphatic sac and duct^[70,72,73]. The *Foxi1* gene encodes a fork-head transcription factor^[74], which regulates transcription of *Slc26a4* in the endolymphatic sac and duct^[75-77], but not in the cochlea or vestibular labyrinth. The observation of EVA and deafness in a *Foxi1*-null mouse, in which pendrin is expressed in the cochlea and vestibular labyrinth but not in the endolymphatic sac, suggested that pendrin expression in the endolymphatic sac is essential for the acquisition of normal hearing^[75].

Slc26a4- and *Foxi1*-null mice are profoundly deaf with severe inner ear malformations and degenerative changes that do not model the less severe human phenotype. Choi *et al.*^[24] reported a binary transgenic mouse line with doxycycline-inducible pendrin expression, in which pendrin expression during embryonic day 16.5 to postnatal day 2 was necessary and sufficient to acquire normal hearing at 1 mo of age. Lack of pendrin during this period could lead to endolymphatic acidification, loss of the EP and mild to severe hearing loss, even without significant scala media expansion or EVA. The timing of pendrin expression could be manipulated to generate mice with unilateral or asymmetric hearing loss associated with minimal, if any, EVA and no other morphogenetic anomalies (Figure 3). Since this latter model more closely approximated the human phenotype, endolymphatic acidification appears to be more important than scala media expansion for the pathogenesis of hearing loss. Although there are no histopathological specimens from patients with isolated EVA to corroborate these observations in mouse models, it seems doubtful that endolymphatic hydrops plays a direct causative role in the hearing loss associated with EVA^[78].

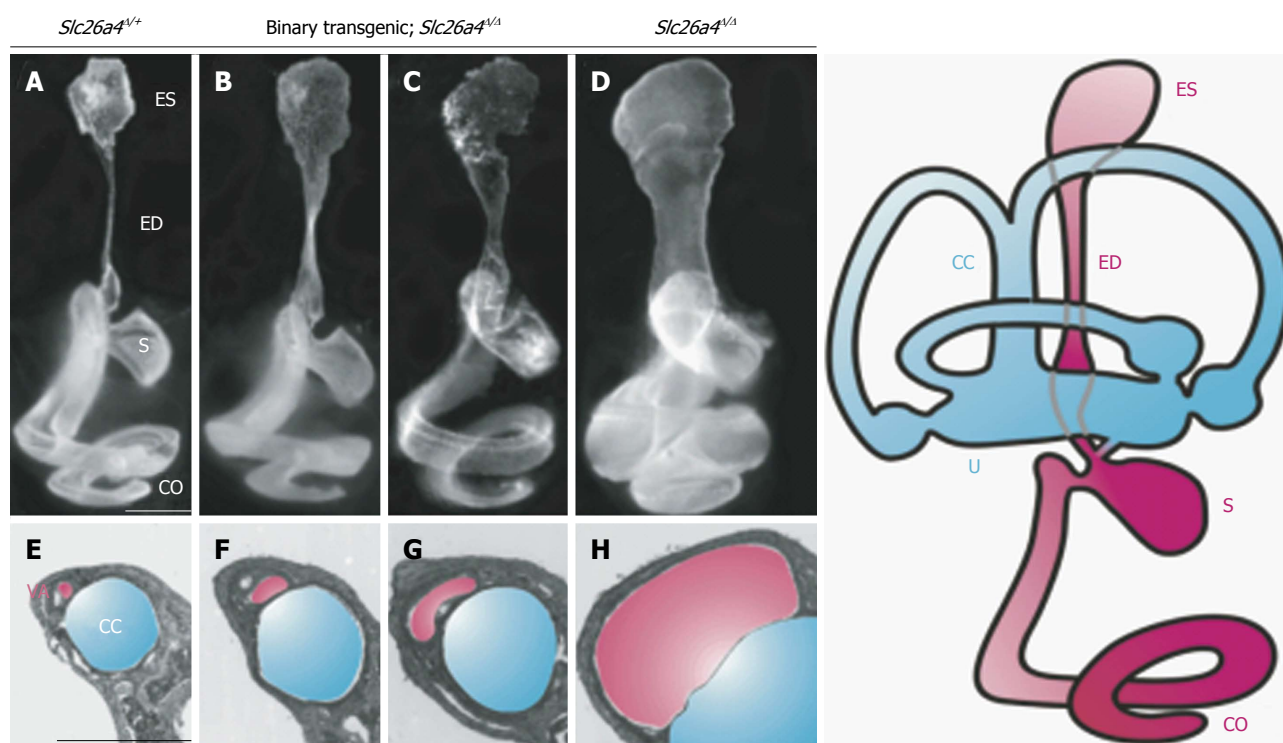


Figure 3 Morphology of the endolymphatic sac and duct and vestibular aqueduct in *Slc26a4* mutant mouse models of enlargement of the vestibular aqueduct. *Slc26a4*^{+/+} normal control (A and E), binary transgenic; *Slc26a4*^{ΔΔ} (B, C, F and G), or *Slc26a4*^{ΔΔ} mutant control mice (D and H) were sacrificed at P3 for paint-fill analysis (A-D) or between P28 and P109 for cross-sectional histopathology of the vestibular aqueduct (VA, shaded pink) adjacent to the common crus (CC, shaded blue; E-H). Scale bars: 500 μm (A, applies to A-D; E, applies to E-H). Manipulating pendrin expression in binary transgenic; *Slc26a4*^{ΔΔ} mice results in less enlargement of the endolymphatic duct and sac and vestibular aqueduct (B, C, F and G). ES: Endolymphatic sac; ED: Endolymphatic duct; S: Saccule; U: Utricle; CO: Cochlea. Reproduced with modification from Choi *et al.*^[24].

ETIOLOGY OF EVA IN PATIENTS WITH NON-DIAGNOSTIC *SLC26A4* GENOTYPES

A single mutant allele of *SLC26A4* is unlikely to be sufficient to cause hearing loss and EVA. There are no published reports of vertical co-segregation of EVA with a single mutant allele of *SLC26A4* or of sporadic cases associated with a single *de novo* mutant allele of *SLC26A4*^[9]. To elucidate the genetic causes and recurrence probability of EVA in families of probands with non-diagnostic *SLC26A4* genotypes (M1 or M0), Choi *et al.*^[79] compared segregation ratios of EVA in M1 and M0 families with M2 families. A segregation ratio is a measure of the frequency of the phenotype among a proband's siblings and, thus, provides an estimate of recurrence probability in siblings. The segregation ratio of EVA in M1 families was not significantly different from that in M2 families, consistent with the predicted ratio (25%) for an autosomal recessive trait with full penetrance and viability. The results suggested the existence of a second, undetected *SLC26A4* mutation in the M1 families^[79]. It is also possible that a single pathogenic mutation of *SLC26A4* might cause EVA in combination with a mutation in another gene^[9]. Yang *et al.*^[80] described digenic heterozygosity for mutations of *SLC26A4* and *FOXI1*^[77] or *KCNJ10* in EVA patients. However, these results have not been reproduced in other studies of EVA cohorts^[54,81-83] and the pathogenic potential of *FOXI1* and *KCNJ10* variants thus remains undeter-

mined^[84,85]. Furthermore, *SLC26A4*-linked polymorphic DNA markers co-segregated with EVA in M1 families. This result is consistent with the hypothesis that current mutation analyses are failing to detect mutations that affect *SLC26A4* or its expression on the apparently wild type allele of *SLC26A4* in M1 families. Taken together, the data suggest that there is a second, undetected mutation of *SLC26A4* that alters a promoter or enhancer or creates a cryptic splice site within an intron. Alternatively, epigenetic modifications of *SLC26A4* such as DNA methylation might repress transcription^[86] and account for the observed co-segregation of EVA and *SLC26A4* in M1 families. The correlation of the absence of goiter, and less severe inner ear deformities and hearing loss with M1 genotypes may reflect undetected mutant or epigenetically-modified alleles of *SLC26A4* that act as hypomorphic alleles with residual function^[79], in a tissue- or time-specific manner^[24], or a combination of these mechanisms.

In M0 families, the segregation ratio was significantly lower than in M2 families and there was discordant inheritance of *SLC26A4*-linked DNA markers with EVA. These results suggested etiologic heterogeneity that includes environmental causes, mutations in other genes, or a combination of these factors^[79]. Congenital cytomegalovirus (CMV) infection can produce a very similar auditory phenotype to that associated with EVA^[87]. However, congenital CMV infection was ruled out as a common or significant cause of EVA^[88].

GENETIC TESTING FOR EVA

Most patients want to know the cause of their hearing loss and have a positive attitude toward genetic testing^[89-91]. Genetic testing for *SLC26A4* mutations can provide useful information for EVA patients. In some families, it may alleviate parental anxiety or guilt about the cause of hearing loss in their children. Second, it can guide the decision to longitudinally monitor the thyroid gland for enlargement or dysfunction. Third, it can be used to estimate the severity of hearing loss^[10,45,55]. Fourth, it provides data for genetic counseling about recurrence probability, and the relative likelihood that EVA would be unilateral or bilateral if it does affect a sibling.

Assuming full viability and full penetrance of EVA in persons with two mutant alleles of *SLC26A4*, the probability of EVA in the sibling of an M2 EVA proband is 25%. Similarly, the probability of EVA in a sibling of a heterozygous (M1) proband with hearing loss and EVA is statistically indistinguishable from that for a sibling of an M2 proband^[79]. The probability of EVA in a sibling of an M0 proband is significantly less than that for a sibling of an M1 or M2 proband, although the probability (about 11%) is not zero^[79]. In the NIH cohort of EVA subjects, when EVA was observed in M0 sibling pairs, the siblings were often monozygotic or dizygotic twins. It is not clear if this reflects ascertainment bias or a relationship of twinning with the development of EVA.

We conclude that genetic testing for *SLC26A4* mutations can be beneficial for some patients with EVA. However, it should always follow pre-test counseling so that patients and parents understand what testing can and cannot reveal. Pre-test counseling should also include a discussion of potential risks, including the possibility that testing may reveal unexpected biological relationships, implied carrier status in relatives, or potential insurance or employment discrimination. It is rare for otolaryngologists to have the time and expertise to conduct pre- and post-test counseling for genetic testing. A genetic counselor can provide pre- and post-test counseling, as well as educate the patient and family about genetics and inheritance. Genetic counselors can also collect pedigree and medical information^[90,91].

FUTURE DIRECTIONS

The advent of massively parallel DNA sequencing (also known as “next-generation” DNA sequencing) provides clinicians and researchers with the ability to sequence entire genomes or entire coding regions of genomes (also known as “exomes”). This opportunity also presents a challenge: the interpretation of DNA sequence variants of unknown pathogenicity. In the absence of conclusive genetic evidence linking mutations of genes other than *SLC26A4* to EVA, direct Sanger di-deoxy sequencing of *SLC26A4* currently remains the most efficient and reliable routine diagnostic test for the etiology of EVA. In the future, research should be directed toward identifying or confirming other genetic causes of EVA. Another

avenue of research is to identify the etiologic, probably genetic, co-factors that cause EVA in patients with one detectable mutant allele of *SLC26A4*.

CONCLUSION

Genetic testing for *SLC26A4* mutations in patients with hearing loss associated with EVA can provide useful information for establishing the etiology of the hearing loss, prognosis, clinical surveillance and management of the thyroid gland, and counseling families about the probability of EVA in one or both ears and severity of hearing loss in siblings of patients with EVA. The most informative aspect of an *SLC26A4* genotype is the number of mutant alleles, since this shows the strongest correlation with the severity of hearing loss, laterality (unilateral *vs* bilateral) of EVA, thyroid gland volume, and recurrence probability. Patients with two mutant alleles of *SLC26A4* typically have bilateral EVA, more severe hearing loss, a thyroid iodide organification defect associated with increased thyroid gland volume, and a 25% recurrence probability of EVA for each sibling. Patients with one mutant allele have unilateral or bilateral EVA, less severe hearing loss, on average, in the ear(s) with EVA, a normal thyroid gland, and a recurrence probability that is similar to that of patients with two mutant alleles. Patients with no mutations of *SLC26A4* have thyroid and auditory phenotypes that are indistinguishable from those in patients with one mutant allele, but the probability of EVA in their siblings is much lower. Therefore even a “negative” *SLC26A4* mutation test result can provide useful diagnostic, prognostic, and familial recurrence information.

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