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# Mutations of *KCNJ10* Together with Mutations of *SLC26A4* Cause Digenic Nonsyndromic Hearing Loss Associated with Enlarged Vestibular Aqueduct Syndrome

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Mutations in *SLC26A4* cause nonsyndromic hearing loss associated with an enlarged vestibular aqueduct (EVA, also known as DFNB4) and Pendred syndrome (PS), the most common type of autosomal-recessive syndromic deafness. In many patients with an EVA/PS phenotype, mutation screening of *SLC26A4* fails to identify two disease-causing allele variants. That a sizable fraction of patients carry only one *SLC26A4* mutation suggests that EVA/PS is a complex disease involving other genetic factors. Here, we show that mutations in the inwardly rectifying $K^+$ channel gene *KCNJ10* are associated with nonsyndromic hearing loss in carriers of *SLC26A4* mutations with an EVA/PS phenotype. In probands from two families, we identified double heterozygosity in affected individuals. These persons carried single mutations in both *SLC26A4* and *KCNJ10*. The identified *SLC26A4* mutations have been previously implicated in EVA/PS, and the *KCNJ10* mutations reduce $K^+$ conductance activity, which is critical for generating and maintaining the endocochlear potential. In addition, we show that haploinsufficiency of *Slc26a4* in the *Slc26a4*$^{+/-}$ mouse mutant results in reduced protein expression of Kcnj10 in the stria vascularis of the inner ear. Our results link *KCNJ10* mutations with EVA/PS and provide further support for the model of EVA/PS as a multigenic complex disease.

Enlargement of the vestibular aqueduct (EVA) is the most common bony inner-ear malformation resolved by computed tomography. It is associated with nonsyndromic hearing loss (DFNB4 [MIM 600791]) and with Pendred syndrome (PS [MIM 274600]), a common type of syndromic hearing loss that includes thyroid dysfunction as part of the disease phenotype. EVA/PS has been causally linked to mutations in the anion transporter gene *SLC26A4*, which encodes the protein pendrin (MIM 605646).[1,2] In the inner ear, pendrin is expressed in cells of the external sulcus, epithelium cells of the endolymphatic duct and sac, and nonsensory cells at the margin of the maculae of the utricle and saccule.[3,4] In all of these cell types, the apical surface is exposed to endolymph, consistent with pendrin's role as an anion transporter. The mouse mutant homozygous for the targeted deletion of *Slc26a4* recapitulates the human phenotype—it is profoundly deaf and has EVA.[5]

Although pathogenic mutations in *SLC26A4* have been shown to abolish membrane targeting or abrogate ion transport,[6,7] the exact link between the loss of functional pendrin and hearing impairment is not well understood. There is evidence to support a role for pendrin in mediating secretion of $HCO_3^-$ from epithelial cells of the spiral prominence into the cochlear endolymph, where loss of pendrin in *Slc26a4*$^{-/-}$ mice causes endolymphatic acidification and $Ca^{2+}$ overloading. These ionic changes could potentially inhibit mechanosensory transduction and lead to hair cell degeneration.[8] However, it has also been postulated that cochlear development in *Slc26a4*$^{-/-}$ mice is compromised by local hypothyroidism (S. Billings et al., 2008, Assoc. Res. Otolaryngol., abstract).

Wangemann and colleagues have raised another intriguing possibility based on protein expression studies in the *Slc26a4*$^{-/-}$ mouse, which show that loss of pendrin leads to reduced protein levels of the $K^+$ channel Kcnj10.[9,10] KCNJ10 (MIM 602208) is an inwardly rectifying $K^+$ channel subunit abundantly expressed in the plasma membrane of intermediate cells of the stria vascularis. Temporal expression of murine Kcnj10 correlates with the onset of the endocochlear potential, a potential essential for auditory function. This potential is absent in *Kcnj10*$^{-/-}$ mice and is abolished when $K^+$ channel blockers are used to inhibit Kcnj10 channel activity, suggesting that deafness in the *Slc26a4*$^{-/-}$ mutant mouse is secondary to loss of Kcnj10 function.[11–14]

The onset of hearing loss varies considerably in EVA/PS. Although many patients are congenitally deaf, in others the onset of hearing loss occurs during childhood, suggesting that EVA/PS is compatible with hearing and that environmental or other genetic factors contribute to the loss of hearing. On the basis of the close link between protein expression levels of KCNJ10 and pendrin and the functional importance of KCNJ10 in the generation of the endocochlear potential, we hypothesized that mutations in *KCNJ10* might play a role in the pathogenesis of EVA/PS.

## Subjects and Methods

### Subjects

Persons with EVA/PS were ascertained through hearing loss referrals based on results of temporal bone computed tomography or magnetic resonance imaging. Their evaluation also included

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a complete history and physical examination. For classification of EVA, enlargement of the vestibular aqueduct had to be greater than 1.5 mm at a point midway between the endolymphatic sac and vestibule. For classification of Mondini dysplasia, the cochlea also had to be abnormal, with incomplete partition and a scala communis. All procedures were approved by the institution review board at the University of Iowa, and all subjects or the parents of minors gave written informed consent for genetic testing.

### Mutational Analysis of *SLC26A4* and *KCNJ10*

DNA was extracted from whole blood via standard procedures.[15] Mutation screening of *SLC26A4* was completed by denaturing high-performance liquid chromatography (DHPLC) and bidirectional sequencing, as previously described.[15,16] For EVA/PS patients who carry only one *SLC26A4* coding-sequence mutation, *KCNJ10* was screened by bidirectional sequencing of amplicons generated by PCR amplification of all exons and splice sites. Primer sequences are listed in Table S1 (available online).

### Expression of KCNJ10 in *Xenopus* Oocytes

The *KCNJ10* coding region was PCR amplified from cDNA and cloned into the pSP64 Poly(A) vector (Promega, Madison, WI). The p.P194H and p.R348C mutations were introduced into *KCNJ10* expression constructs by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). In vitro transcription of *KCNJ10* was performed with the mMESSAGE mMACHINE kit (Applied Biosystems/Ambion, Austin, TX), and the generated transcript (50 nl of a 1 ug/ul solution) was injected into defolliculated *Xenopus* oocytes. After injection, oocytes were incubated in MES-buffered saline (MBS) at 18°C. Electrophysiological studies were performed 24 hr later.

### Electrophysiology

Whole-cell electrophysiological studies were performed via two-electrode voltage clamping. In brief, currents were amplified with an Oocyte Clamp OC-725C (Warner Instruments, Hamden, CT), digitized with a MacLab/200 interface (ADInstruments, Colorado Springs, CO), and recorded and analyzed with Chart software (ADInstruments). The microelectrodes were filled with 3 M KCl, and during experiments, oocytes were bathed in a solution containing 90 mM KCl, 3 mM $MgCl_2$, 5 mM HEPES, and 150 μM niflumic acid at pH 7.4. Oocytes were held at a potential of 0 mV by voltage clamping, and voltage steps of 2 s were applied in 20 mV increments. Differences between currents of wild-type (WT) and mutant KCNJ10 ($n = 6$) were compared with the Student's unpaired t test. Slopes of the current-voltage relationship in the linear range (−120 mV to 0 mV) were calculated by linear regression analysis.

### Tissue Preparation and Protein Extraction of Mouse Stria Vascularis

Cochleae were removed from P18 *Slc26a4*$^{+/+}$, *Slc26a4*$^{+/-}$, and *Slc26a4*$^{-/-}$ mice ($n > 4$ for each group) via procedures approved by the Institutional Animal Care and Use Committee of the University of Iowa. Stria vascularis fractions were obtained by microdissection under the surgical microscope and were then immediately transferred to 40 μl Tris-Triton buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X) and homogenized on ice for 30 s by ultrasonic homogenization (Model 150VT, BioLogics, Manassas, VA). Extracted protein was stored at −80°C until use.

**Table 1. *KCNJ10* and *SLC26A4* Mutations Identified in Two Probands with EVA/PS**

| Patient No. | Inner-Ear Malformation | *KCNJ10* Mutation | *SLC26A4* Mutation |
|---|---|---|---|
| 82120-1 | EVA | p.R348C / + | c.919-2A→G / + |
| 7740-1 | EVA, Mondini dysplasia | p.P194H / + | p.F335L / + |

### Quantitative Immunoblot Analysis

Immunoblotting was performed as previously described,[9] with slight modification, and all procedures were performed at room temperature unless otherwise noted. In brief, equal volumes of protein extract and Laemelli buffer containing 5% β-mercaptoethanol were mixed and heated at 70°C for 5 min, followed by SDS-PAGE gel electrophoresis (10% Ready-gel, BioRad Laboratories, Hercules, CA). Separated proteins were electrophoretically transferred to a nitrocellulose membrane (ProTran BA85, 0.45 μm pore size, Whatman, Florham Park, NJ), blocked at 4°C overnight in 5% nonfat dry milk in TBS with 0.1% Tween-20, then incubated with primary antibodies (rabbit anti-Kcnj10, 1:1000, Cat# APC-035, Alomone Labs, Jerusalem, Israel; rabbit anti-tubulin, 1:500, Cat# ab6046, Abcam, Cambridge, MA) in blocking buffer for 1 hr. Unbound primary antibody was removed by three 10 min washes with Tween TBS, and the membrane was then incubated with HRP-conjugated secondary antibody (Peroxidase AffiniPure Goat anti-Rabbit IgG, 1:4000, Cat# 111-035-045, Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 min. After three washes with Tween TBS, the membrane was developed by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagents, GE Healthcare, Florham Park, NJ), read on a low-light digital camera (LAS-1000, Fujifilm Medical Systems, Stamford, CT), and quantified with Image Gauge software (Fujifilm Medical Systems, Stamford, CT). All experiments were repeated in triplicate. Kcnj10 protein expression differences in *Slc26a4*$^{+/+}$, *Slc26a4*$^{+/-}$, and *Slc26a4*$^{-/-}$ mice were compared by one-way analysis of variance (ANOVA) and the Dunn's post hoc test.

## Results

### Genetic Analysis of *KCNJ10* Mutations

We completed *KCNJ10* mutation screening in 89 patients who had a clinical diagnosis of EVA/PS and were known carriers of only one *SLC26A4* coding sequence mutation; promoter mutations and deletions of *SCL26A4* were also excluded in this patient cohort. In two patients, we identified missense mutations in *KCNJ10*—a p.P194H (c.581C→A) mutation in patient 7740-1 and a p.R348C (c.1042C→T) mutation in patient 82120-1. Patient 7740-1 also carries a p.F335L (c.1003T→C) mutation in *SLC26A4*, and in Patient 82120-1, we identified a c.919-2A→G mutation (Table 1). In Family 82120, we were able to reconstruct haplotypes, which showed that the mother carries *KCNJ10* p.R348C and the father carries *SLC26A4* c.919-2A→G. An unaffected sibling carries only the *KCNJ10* p.R348C variant (Figure 1). Both amino acid changes in *KCNJ10* are conserved across most mammalian species (Figure S1), and neither change was found in ethnically matched normal-hearing controls of European ($n = 200$, 400 chromosomes) and Chinese ($n = 200$, 400 chromosomes) descent.

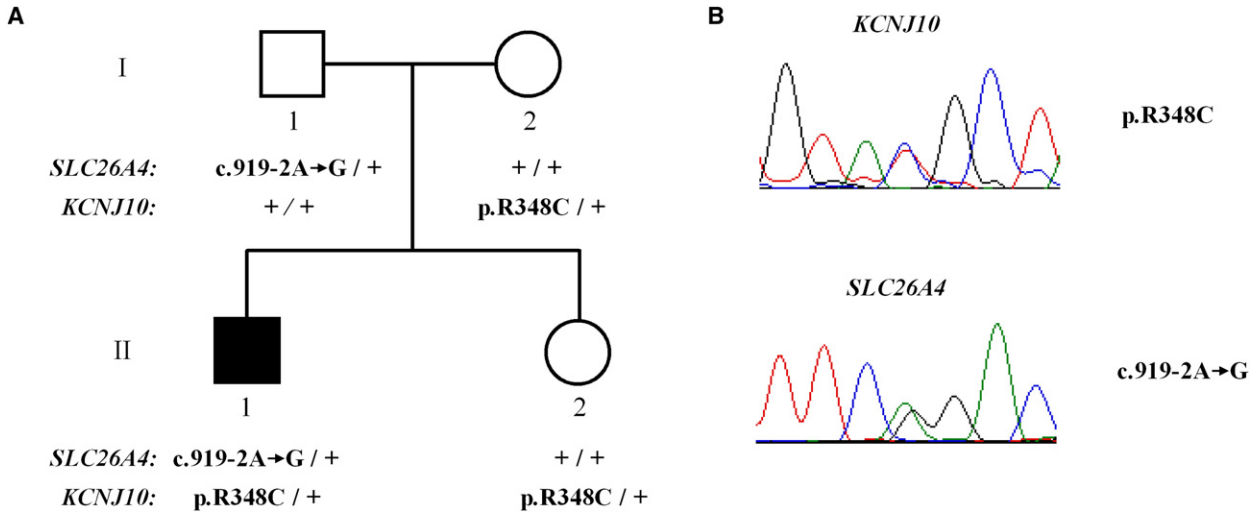


**Figure 1. Double Heterozygosity for Mutations in *KCNJ10* and *SLC26A4* in Family 82120 with EVA/PS**

(A) Pedigree and genotypes of the family, showing that the parents and their unaffected child carry a single mutation in either *SLC26A4* or *KCNJ10* and that the affected child carries both mutations.

(B) Representative chromatograms of the *SLC26A4* c.919-2A→G and *KCNJ10* p.R348C mutations identified in this family.

### Electrophysiology

To determine whether the identified *KCNJ10* mutations change $K^+$ channel currents, we expressed WT and mutant KCNJ10 in *Xenopus* oocytes by mRNA injection and recorded evoked $K^+$ currents in a two-electrode voltage clamp experiment. Figure 2A shows a representative current recording in an oocyte expressing WT KCNJ10. Control oocytes injected with equal volumes of water showed little current under evoking voltage commands (data not shown). So that the specificity of the measured currents was ensured, 150 μM niflumic acid was added in the bathing solution, blocking endogenous $Cl^-$ currents. In addition, the evoked current could be abolished when 1 mM $Ba^{2+}$ was applied.

Expression of WT KCNJ10 in *Xenopus* oocytes resulted in a weak inwardly rectifying $K^+$ current similar to previously reported data.[17] In oocytes expressing either H194 or C348

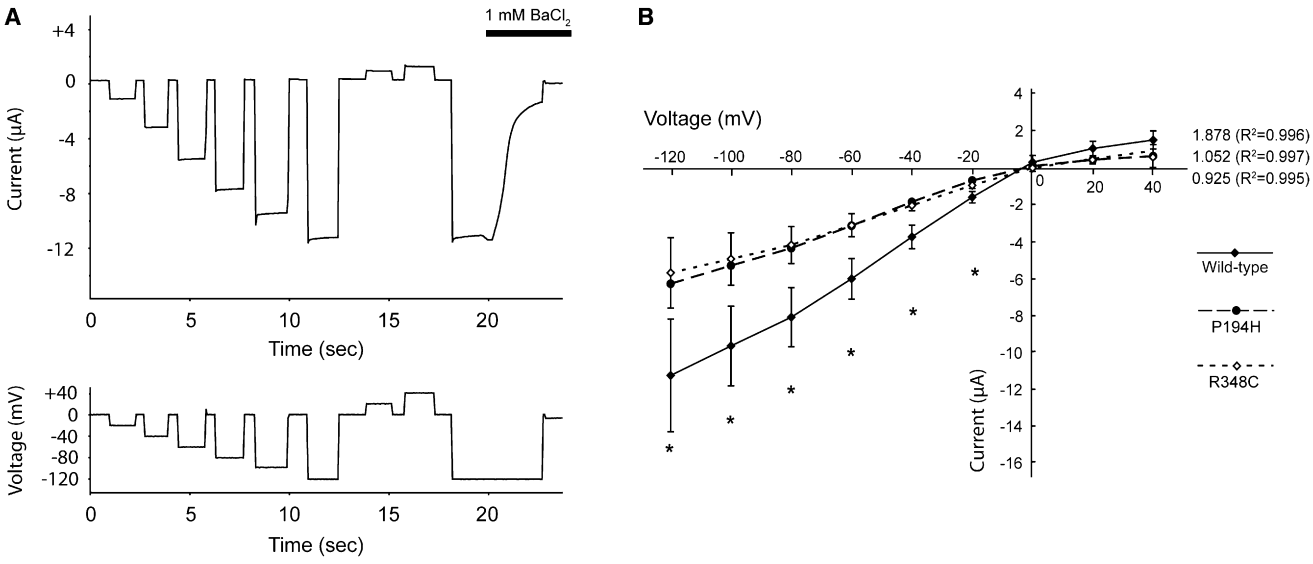


**Figure 2. Voltage-Clamp Analysis of $K^+$ Conductance of WT and Mutant KCNJ10**

(A) Representative trace of current (top) and voltage commands (bottom) versus time in *Xenopus* oocyte expressing WT KCNJ10. A 1 mM final concentration of channel blocker $BaCl_2$ was added into the extracellular bath at the time point near 20 s, as indicated (black bar).

(B) Steady-state current-voltage relationship for WT and mutant KCNJ10 (mean ± SE, n = 6). For clarity, error bars are shown only for WT and R348C mutant KCNJ10. The differences between the currents of WT and mutant KCNJ10 are statistically significant at voltages between −120 mV and −20 mV ($p < 0.05$), as indicated by asterisks. The slopes of the current-voltage relationship in the linear range between −120 mV and 0 mV were calculated by linear regression analysis and are shown at the right of each curve (from top: WT, R348C, and P194H; $R^2$ values are shown in parentheses).

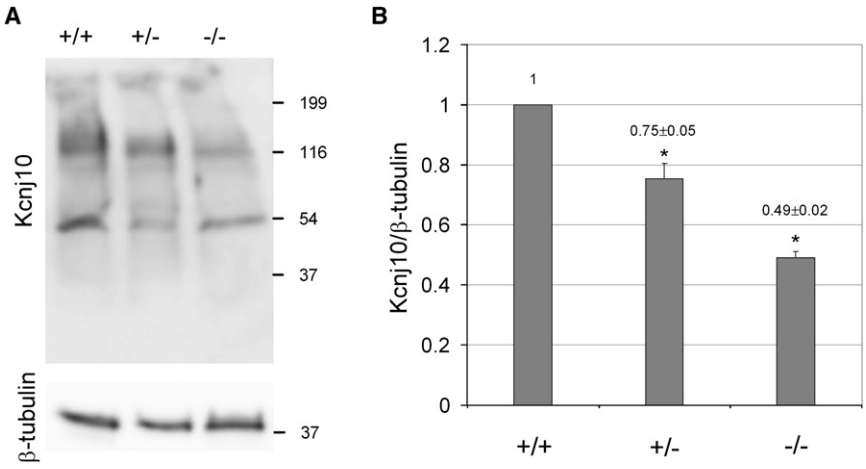


**Figure 3. Quantitative Immunoblot Analysis of Kcnj10 Expression in Mouse Stria Vascularis**

(A) Representative immunoblot showing protein expression of Kcnj10 and β-tubulin in equal sample volumes from *Slc26a4*$^{+/+}$, *Slc26a4*$^{+/-}$, and *Slc26a4*$^{-/-}$ mice. Molecular weights (kDa) of protein standards are marked on the right side. The two major bands of 120 kDa and 50 kDa and the minor smears of Kcnj10 reflect variable protein glycosylation, stoichiometry, and ubiquitination, as previously reported,[9] and all are included in the quantitative analysis.

(B) Quantification of Kcnj10 expression normalized against β-tubulin control expression. Reduced expression of Kcnj10 in the stria vascularis of *Slc26a4*$^{+/-}$ and *Slc26a4*$^{-/-}$ mice as compared to WT mice (mean percentage ± SE, n = 3) is shown as scaled bars. The differences in Kcnj10 expression between *Slc26a4*$^{+/-}$ and *Slc26a4*$^{+/+}$ mice and between *Slc26a4*$^{-/-}$ and *Slc26a4*$^{+/+}$ mice are statistically significant ($p < 0.05$), as indicated by asterisks.

mutant KCNJ10, $K^+$ channel conductance markedly decreased (Figure 2B). Compared to WT, the $K^+$ currents of the mutant KCNJ10 were significantly reduced ($p < 0.05$) in the linear range (−120 mV to −20 mV), and the mean slopes (ΔI/ΔV) of the mutant KCNJ10 current-voltage relationships were reduced by 44% and 51% for C348 and H194, respectively ($R^2 > 0.99$). These results suggest that $K^+$ conductance is severely impaired by the mutations that we identified in these two patients.

### Kcnj10 Expression in Slc26a4$^{+/-}$ Mice

In family 82120, the c.919-2A→G splice-site mutation in *SLC26A4* is predicted to cause skipping of exon 8, leading to premature protein truncation and haploinsufficiency. In the mouse mutant with a targeted deletion of *Slc26a4*, exon 8 is replaced by a *neo*$^R$-containing cassette that similarly results in loss of functional protein.[5] Given that deficiency of pendrin in *Slc26a4*$^{-/-}$ mice leads to loss of Kcnj10 protein in stria vascularis, we investigated whether haploinsufficiency would affect the Kcnj10 protein level in the stria vascularis by studying *Slc26a4*$^{+/-}$ animals.

The stria vascularis of P18 mouse cochleae from *Slc26a4*$^{-/-}$, *Slc26a4*$^{+/-}$, and *Slc26a4*$^{+/+}$ mice was isolated by microdissection, and total protein extracted from these tissue fractions was used for quantitative immunoblot analysis (Figure 3). In *Slc26a4*$^{+/-}$ mice, we observed reduced protein expression of Kcnj10 as compared to *Slc26a4*$^{+/+}$ WT controls (75%, $p < 0.05$). In *Slc26a4*$^{-/-}$ mice, Kcnj10 protein expression was further reduced (49%, $p < 0.05$). These results indicate that haploinsufficiency of pendrin leads to reduced KCNJ10 protein expression.

## Discussion

Many studies have suggested that EVA/PS is a complex disease.[18–20] However, in the ten-year span following discovery of *SLC26A4* as the primary gene responsible for EVA/PS, identification of factors that promote the onset of hearing loss in patients with EVA/PS has been limited to infections and head injuries and to genetic factors such as the expression of *FOXI1*, which controls the expression of *SLC26A4*.[21,22] The goal of identifying genetic factors has been limited by the availablity of a sufficient number of families showing clear digenic inheritance that would make conventional positional cloning approaches feasible. As an alternative, we have adopted a candidate gene approach, selecting for in-depth study genes that have a strong functional link to *SLC26A4*. In addition, we have identified a cohort of patients in which to screen these candidates.

Using this strategy in our earlier work, we selected *FOXI1* for study on the basis of the observation that endolymphatic sac expression of *Slc26a4* is absent in the *Foxi1*$^{-/-}$ mouse. We were able to show that heterozygosity for mutations in this transcription factor and *SLC26A4* are causally related to the EVA/PS phenotype in humans.[22] In this study, on the basis of work demonstrating that loss of Kcnj10 protein expression is a key event in the etiology of deafness in *Slc26a4*$^{-/-}$ mice, we hypothesized that genetic factors affecting expression of functional KCNJ10 channels might also contribute to penetrance of hearing loss in EVA/PS patients.

We identified two patients who have been diagnosed with EVA/PS and who segregate mutations in both *SLC26A4* and *KCNJ10*. The p.P194H and p.R348C mutations in *KCNJ10* change two well-conserved aminio acids and were not observed in 800 control chromosomes. Our electrophysiologic study showed that these mutations are detrimental to channel activity and reduce $K^+$ conductance by 44%–51% (Figure 2). Notably, given that KCNJ10 may form homomeric tetramers,[17] heterozygosity for these mutations is predicted to affect over 90% of multimeric KCNJ10 channels.

The p.F335L *SLC26A4* mutation carried by patient 7740-1 has been reported in 14 of 668 EVA/PS patients but in none of 358 normal-hearing controls ($p < 0.01$, Fisher's exact test).[19,22–25] Functional studies of this variant have shown decreased $Cl^-/HCO_3^-$ exchange, suggesting that leucine at this position results in a hypofunctional protein.[24] Family 82120 is of Chinese origin, and the affected child carries the *SLC26A4* c.919-2A→G mutation, which is highly prevalent in the Chinese population, where it accounts for more than half of all reported *SLC26A4* mutant alleles.[26] This mutation is within the 3′ splicing site of exon 8 and is predicted to lead to premature protein truncation and haploinsufficiency.

Our quantitative immunoblot analysis in *Slc26a4*$^{-/-}$, *Slc26a4*$^{+/-}$, and *Slc26a4*$^{+/+}$ mice indicates that haploinsufficiency for Slc26a4 leads to decreased expression of Kcnj10 in the stria vascularis, providing a pathogenic link between mutations in these two genes. Haploinsufficiency of only Slc26a4 in *Slc26a4*$^{+/-}$ mice or Kcnj10 in *Kcnj10*$^{+/-}$ mice, however, does not affect the magnitude of the endocochlear potential; in contrast, homozygous loss of Slc26a4 in *Slc26a4*$^{-/-}$ mice or of Kcnj10 in *Kcnj10*$^{-/-}$ mice leads to a complete loss of this potential.[8,12] Confirmation of a digenic disease mechanism in the patients that we have described will require generation of a mouse model carrying orthologous mutations in *Slc26a4* and *Kcnj10*.

The mechanism for the reduction of Kcnj10 protein expression in *Slc26a4*$^{+/-}$ mice is not understood. Previous studies have suggested that maintenance of normal endolymphatic $K^+$ concentration in *Slc26a4*$^{-/-}$ mice leads to free-radical stress in the stria vascularis and that this free-radical stress causes loss of strial Kcnj10 and thereby loss of the endocochlear potential.[8,9] It is noteworthy that *SLC26A4* and *KCNJ10* are expressed in distinct cell types in the cochlear lateral wall, the former found in the external sulcus cells and the latter in the intermediate cells of the stria vascularis. These distinct localizations suggest that the existence of a functional link such as oxidative stress is plausible. The oxidative stress hypothesis is supported by protein expression studies of cultured mouse stria vascularis. When surrounded by a uniform culture medium, striae vascularis harvested from *Slc26a4*$^{+/-}$ and *Slc26a*$^{-/-}$ mice show similar expression levels of *Kcnj10*.[9]

Recent studies have suggested that most rare missense alleles in humans are deleterious and make a substantial contribution to multifactorial diseases.[27,28] The detection of numerous rare variants in the same candidate gene, unlikely in conventional association studies based on common polymorphisms, has proven to be a useful strategy for discovery of disease-causing genes in complex diseases such as colorectal cancers and disorders of lipid metabolism.[29,30] The effectiveness of this strategy is clearly demonstrated by our finding of single *KCNJ10* mutations in two patients who also carry single mutations in *SLC26A4* and have an EVA/PS phenotype.

While this article was being written, Scholl and colleagues reported homozygous or compound heterozygous mutations in *KCNJ10* in members of four kindreds with a recessive complex syndrome that includes seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance.[31] This complex phenotype is similar to that seen in *Kcnj10*$^{-/-}$ mouse mutants that exhibit severe ataxia, stress-induced seizures, spongiform vacuolation, axonal swellings, and degeneration, in addition to hearing loss.[32,33] In contrast, the EVA/PS phenotype associated with double heterozygosity for mutations in *KCNJ10* and *SLC26A4* represents a distinct disease entity in which only hearing loss is observed.

Our functional characterization of the pathogenic interaction between *SLC26A4* and *KCNJ10* mutations suggests that this digenic combination of mutations is specifically associated with inner-ear dysfunction. On the basis of our data, we propose that *KCNJ10* is an important genetic factor in the pathogenesis of EVA/PS. The mutations in *SLC26A4* lead to hypofunction or haploinsufficiency. In association with EVA/PS, haploinsufficiency of SLC26A4 and oxidative stress converge to reduce strial expression of KCNJ10 protein. The consequence of decreased KCNJ10 expression is a reduced supply of $K^+$ to marginal cells in the stria vascularis. In turn, these cells reduce their rate of $K^+$ secretion. This self-limiting mechanism might account for the intermittent hearing-threshold recovery that is well documented in EVA/PS patients and leads to fluctuating and progressive hearing loss.[21,34,35] This paradigm provides new insight into the pathogenesis of EVA/PS and, as a corollary, suggests that if strial expression of KCNJ10 can be maintained, perhaps through controlling endolymph pH or limiting oxidative stress through medical therapy, hearing loss might be preventable in some persons with an EVA/PS phenotype.

## Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.ajhg.org/.

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## Web Resources

The URLs for data presented herein are as follows:

GenBank, http://ncbi.nlm.nih.gov/Genbank/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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