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Mutation analysis of the *SLC26A4*, *FOXI1* and *KCNJ10* genes in individuals with congenital hearing loss

Pendred syndrome (PDS) and DFNB4 comprise a phenotypic spectrum of sensorineural hearing loss disorders that typically result from biallelic mutations of the SLC26A4 gene. Although PDS and DFNB4 are recessively inherited, sequencing of the coding regions and splice sites of SLC26A4 in individuals suspected to be affected with these conditions often fails to identify two mutations. We investigated the potential contribution of large SLC26A4 deletions and duplications to sensorineural hearing loss (SNHL) by screening 107 probands with one known SLC26A4 mutation by Multiplex Ligation-dependent Probe Amplification (MLPA). A heterozygous deletion, spanning exons 4-6, was detected in only one individual, accounting for approximately 1% of the missing mutations in our cohort. This low frequency is consistent with previously published MLPA results. We also examined the potential involvement of digenic inheritance in PDS/DFNB4 by sequencing the coding regions of *FOXI1* and *KCNJ10*. Of the 29 probands who were sequenced, three carried nonsynonymous variants including one novel sequence change in *FOXI1* and two polymorphisms in *KCNJ10*. We performed a review of prior studies and, in conjunction with our current data, conclude that the frequency of *FOXI1* (1.4%) and *KCNJ10* (3.6%) variants in PDS/DFNB4 individuals is low. Our results, in combination with previously published reports, indicate that large SLC26A4 deletions and duplications as well as mutations of *FOXI1* and *KCNJ10* play limited roles in the pathogenesis of SNHL and suggest that other genetic factors likely contribute to the phenotype.
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Introduction

Pendred syndrome (PDS; OMIM #274600) and DFNB4 (OMIM #600791) are sensorineural hearing loss disorders that comprise a phenotypic spectrum resulting from, in most cases, mutations in the SLC26A4 gene (OMIM *605646) (Everett et al., 1997; Li et al., 1998). SLC26A4 is composed of 21 exons and encodes the 780 amino acid transmembrane anion transporter pendrin (Everett et al., 1997, Everett et al., 1999; Royaux et al., 2000; Royaux et al., 2001), which plays a key role in maintaining the endocochlear potential (Everett et al., 1999; Royaux et al., 2003).

PDS and DFNB4 are typically characterized by congenital, bilateral sensorineural hearing loss which can be progressive and is usually severe to profound. There is considerable variability of symptoms. Vestibular dysfunction as well as non-pathognomonic temporal bone abnormalities, in particular enlargement of the vestibular aqueduct (EVA), can also be present in these conditions. DFNB4, also known as nonsyndromic enlarged vestibular aqueduct (NS-EVA), is not associated with other clinical findings. PDS, in contrast, classically manifests additional symptoms such as the development of an incompletely penetrant euthyroid goiter, which can be present at birth but is more likely to develop in late childhood to early adulthood. PDS is also typically accompanied by Mondini dysplasia, a reduction of the number of turns of the cochlea combined with the characteristic bilateral EVA (Schrijver & Gardner, 2006). Although the Mondini malformation can be used as a criterion for diagnosis, it is thought to be clinically heterogeneous and it remains uncertain which proportion of Mondini malformations can be linked to Pendred syndrome (Reardon et al., 1997). Other, less well defined, temporal bone abnormalities can (and typically are) seen in those individuals lacking Mondini dysplasia. PDS was originally estimated to be responsible for 7.5% of cases of hereditary hearing loss (Fraser, 1965) but the actual incidence has not been determined due to difficulties inherent in diagnosing PDS, the degree of phenotypic variability (i.e. isolated hearing loss versus
multisystemic involvement), the frequently late onset and reduced penetrance of the goiter, and
the lack of pathognomonic findings (Blons et al., 2004). Nevertheless, PDS is thought to be one
of the most common forms of syndromic deafness and mutations of SLC26A4 were reported to be
the second most frequent cause of autosomal recessive nonsyndromic sensorineural hearing loss
worldwide (Hilgert, Smith & Van Camp, 2009).

More than 260 mutations in the SLC26A4 gene have been identified to date
(http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SLC26A4), including deletions spanning multiple
exons (Park et al., 2003; Hu et al., 2007; Pera et al., 2008a; Anwar et al., 2009; Siem et al., 2010).
Until recently, however, individuals with sensorineural hearing loss (SNHL) and possible PDS or
DFNB4 were not systematically analyzed for the presence of multiexon deletions and
duplications. Multiplex Ligation-dependent Probe Amplification (MLPA) analysis of 37 probands
in a Scandinavian cohort of 109 patients suspected to have PDS/DFNB4 identified a homozygous
SLC26A4 deletion of exons 4-6 in one individual, indicating that intragenic deletions and
duplications may contribute to the phenotype (Rendtorff et al., 2013). Mutations of the FOXII
and the KCNJ10 genes have also been associated with PDS/DFNB4 and were reported to be
digenically inherited with heterozygous mutations in SLC26A4 (Yang et al., 2007; Yang et al.,
2009). FOXII encodes a transcription factor that binds to the promoter region of SLC26A4 and is
responsible for upstream regulation of the gene. KCNJ10 encodes an inwardly rectifying
potassium (K⁺) channel that is involved in generating and maintaining the endocochlear potential
(Marcus et al., 2002). Intragenic deletions of SLC26A4 as well as digenic mutations with either
FOXII or KCNJ10 have all been implicated in PDS/DFNB4 pathogenesis but the extent of their
involvement as well as their clinical relevance for SNHL remains unclear.

We investigated the contribution of intragenic SLC26A4 copy number changes by
performing MLPA analysis of 107 probands with congenital SNHL who had only one identified
SLC26A4 mutation. Although it has been recommended to consider SLC26A4 mutation analysis if
there is progressive hearing loss, goiter, Mondini dysplasia, or EVA (Hilgert, Smith & Van Camp, 2009), the clinical testing strategy often adheres to the following algorithm: For individuals with congenital hearing loss that is congruent with autosomal recessive inheritance and that seems nonsyndromic, diagnostic testing typically starts with GJB2 sequence analysis. If that test does not identify two pathogenic mutations, then inner ear imaging studies by MRI or CT for temporal bone anomalies may be performed. However, clinical testing of SLC26A4 without MRI or CT is also considered acceptable because mutations in this gene are thought to be a frequent cause of SNHL (Alasti, Van Camp & Smith, 1998, updated 2012). This approach to the clinical work-up is quite common in the U.S., in part because the same DNA specimen can be used for both molecular tests sequentially and because imaging studies in the pediatric population can be challenging. As a result, our patients were selected based on the presence of congenital SNHL compatible with autosomal recessive inheritance, absence of two hearing loss-associated GJB2 mutations, and the presence of only one known mutation in SLC26A4. In these individuals, we performed MLPA of SLC26A4 and in a subset we also sequenced the coding regions and splice sites of FOXI1 and KCNJ10 to evaluate alternative etiologies.

Materials and Methods

Study subjects

A total of 107 individuals participated in this study. Participants provided written informed consent as appropriate. All were enrolled with IRB approval from the Stanford University Administrative Panels for the Protection of Human Subjects (IRB Approval Numbers IRB-14011 and IRB-8353); they included individuals from Stanford University Medical Center (n = 60, Stanford, CA), the Center for Genetic Testing at Saint Francis Hospital (n = 30, Tulsa, OK) and Cincinnati Children’s Hospital Medical Center (n = 17, Cincinnati, OH). All participants
were probands presenting with congenital SNHL who prior to enrollment, had all received GJB2 testing and been sequenced for mutations in the exons, splice sites and promoter region of the SLC26A4 gene (GenBank ID: NC_000007.13) as part of routine clinical care. Patients who did not have two pathogenic GJB2 mutations and who had only one identified SLC26A4 mutation were then eligible for additional genetic testing by MLPA in an effort to identify a second disease-causing mutation. FOXI1 (GenBank ID: NG_012068.1) and KCNJ10 (GenBank ID: NG_016411.1) were also sequenced in a subset of the Stanford patient group (n = 29/60 probands) for whom sample was available.

Because imaging analysis is not routinely ordered on children with phenotypically non-syndromic SNHL prior to SLC26A4 sequencing, imaging analysis results were available for only a portion of the subjects (n = 46/107; 43%) and indicated the presence of EVA in 56% (n = 26/46) and Mondini malformation in none of the probands. Vestibular dysfunction complaints were noted in only one of 54 participants for whom this information was reported. Thyroid manifestations were reported in none of the Stanford probands for whom this information was available (n = 0/37), including five individuals older than 13 years of age.

Detection and characterization of intragenic rearrangements

MLPA is a multiplex PCR technique for the detection of copy number variations that are typically not identified by conventional screening methodologies such as sequencing and Denaturing High Performance Liquid Chromatography (DHPLC) (Schouten et al., 2002). Genomic DNA samples extracted from peripheral blood by standard methods were analyzed for copy number mutations by the SALSA MLPA kit P280 Pendred-SLC26A4 (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer’s protocol. Capillary electrophoresis of PCR products was performed using either an ABI 310 or an ABI 3500 Genetic Analyzer (Life
Technologies, Grand Island, NY, USA) and the resulting data were analyzed using GeneMarker 1.51 software (SoftGenetics, LLC., State College, PA, USA).

For breakpoint analysis in the single proband with an identified SLC26A4 deletion, PCR products were electrophoresed on an agarose gel and the 494 bp fragment corresponding to the deletion was excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). The amplicon was then sequenced on an ABI 3730xl Genetic Analyzer (Life Technologies, Grand Island, NY, USA) to confirm the breakpoints of the deletion.

FOXII and KCNJ10 sequencing

Almost half (n = 29/60) of the Stanford University proband samples had direct DNA sequencing of the coding exons, including intron/exon boundaries ± 20 bp into the introns, of FOXII and KCNJ10. Primers were designed to amplify and sequence exons 1 and 2 of FOXII and exon 2 of KCNJ10 (Table 1). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD) and then sequenced on an ABI 3730xl Genetic Analyzer (Life Technologies, Grand Island, NY, USA). Sequences were compared against either the FOXII (GenBank ID: NG_012068.1) or the KCNJ10 (GenBank ID: NG_016411.1) reference sequence using Mutation Surveyor 2.51 software (SoftGenetics, LLC., State College, PA, USA).

Nonsynonymous sequence variants detected in the coding regions of the FOXII and KCNJ10 genes were further analyzed using the mutation interpretation tools SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/) and PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/), which predict the effect of an amino acid substitution on protein function.

Results
In order to assess the potential diagnostic benefits of deletion/duplication analysis of the *SLC26A4* gene, we performed MLPA on genomic DNA samples from 107 patients with congenital hearing loss in whom only one *SLC26A4* mutation had been found by sequencing. Only one individual, PDS41, (1/107 unknown alleles; 0.93%) had MLPA results indicative of such a copy number variation – a putative heterozygous deletion spanning exons 4-6 of the gene (Figure 1). Because deletions of these same *SLC26A4* exons were previously described in a Spanish patient (Pera et al., 2008a) and in two Norwegian patients of Lebanese descent (Siem et al., 2010; Rendtorff et al., 2013), we investigated the possibility that our MLPA assay had detected a known rearrangement in our proband. Flanking PCR primers were designed to analyze the breakpoints of the deletion (PDS IVS3 Forward: 5’- ACAATGTCCATGCCACAACC-3’ and PDS IVS6 Reverse: 5’- ACAGAGACCATTACATACATAC-3’). A duplex reaction including both the breakpoint primers and a set of control primers for the amplification of exon 4 (PDS Ex4 Forward: 5’- AGGCAAAGTCATAAGTGGAAC-3’ and PDS Ex4 Reverse: 5’- ACCTAATAGAGGTATAATGCAC-3’) resulted in two products: a 494 bp fragment corresponding to the deletion and a 289 bp fragment amplified from the control primers (data not shown). The presence of the 289 bp amplicon indicated that the deletion was indeed heterozygous. Subsequent sequencing of the 494 bp amplicon confirmed the presence of g.8091T-22145Cdel (Figure 1). The deletion removes 14,053 bp from the *SLC26A4* gene, disrupting the open reading frame and truncating the protein at amino acid residue 105 in the first transmembrane domain.

In addition to performing MLPA analysis on samples from our patient cohort, we investigated other genes potentially contributing to their SNHL by sequencing 29 probands for variants in the coding regions of the *FOXI1* and *KCNJ10* genes. We identified a heterozygous variant of *FOXI1*, c.677C>T (p.Thr226Ile), in one patient, PDS29 (1/29 unknown alleles; 3.4%). This *FOXI1* substitution has not been previously described in SNHL. It represents a rare variant.
(dbSNP:rs115399307; minor allele frequency of 0.3%,
significance. The SIFT and PolyPhen-2 programs predicted the substitution to be “tolerated” and
“benign”, respectively. Two heterozygous variants of KCNJ10 were also detected in this group
(2/29 unknown alleles; 6.9%), each in a separate proband. The substitution c.812G>A
(p.Arg271His) in PDS21 was predicted by SIFT and PolyPhen-2 to be “tolerated” and “benign”,
respectively. This substitution is also a rare sequence variant (dbSNP: rs3795339; minor allele
frequency of 0.6%, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=3795339) of
uncertain clinical significance that has been reported previously in a single heterozygous Chinese
proband with nonsyndromic EVA and zero mutations of SLC26A4 (Chai et al., 2013). In that
same study, however, the c.812G>A (p.Arg271His) variant was found in 10/200 or 5% of normal
hearing controls, suggesting that this substitution may be a polymorphism in the Chinese
population. The other KCNJ10 variant, c.811C>T (p.Arg271Cys), was discovered in patient
PDS23 and it also affects amino acid residue 271. Although this substitution is predicted to
“affect protein function” by SIFT and to be “possibly damaging” by PolyPhen-2, it is a reported
polymorphism of unknown clinical significance (dbSNP: rs1130183) with a minor allele
frequency of 2.3% (dbSNP: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?
type=rs&rs=1130183). This substitution has been previously reported in three Italian individuals
affected with SNHL, who also had bilateral inner ear malformations (Cirello et al., 2012) and has
been associated with seizure susceptibility (Buono et al., 2004; Lenzen et al., 2005).

Discussion

The autosomal recessive inheritance of PDS and DFNB4 is well established and yet, for
many affected individuals, analysis of the coding sequences and splice sites of the SLC26A4 gene
has failed to identify one or both of the mutations required to cause these disorders (Campbell et al., 2001; Tsukamoto et al., 2003; Pryor et al., 2005; Albert et al., 2006; Yang et al., 2007; Wu et al., 2010). In fact, an analysis of six studies with a total enrollment of 769 hearing impaired probands with EVA, a non-pathognomonic clinical finding that is a hallmark of PDS and DFNB4, reveals that only 25% have biallelic SLC26A4 mutations (Table 2). Of these same probands, 45% have at least one SLC26A4 mutation, a percentage slightly lower than the 50% reported by GeneReviews as the proportion of PDS/DFNB4 accounted for by mutations in SLC26A4 (http://www.ncbi.nlm.nih.gov/books/NBK1467/). Among the six included studies, there are considerable differences in the reported percentages of individuals segregating monoallelic or biallelic SLC26A4 mutations. The proportion of probands for whom at least one SLC26A4 mutation is detected ranges from as low as 30-40% (Campbell et al., 2001; Albert et al., 2006; Yang et al., 2007) to as high as 80-90% (Tsukamoto et al., 2003; Wu et al., 2010). The percentage of probands in whom biallelic mutations are found varies accordingly, from a low of 13% (Yang et al., 2007) to a high of 62% (Wu et al., 2010). This variation may be attributed to differences in the selection criteria of each study and/or to the patient population being tested; however, in all cases, there is a substantial proportion of individuals for whom the genotype is either incomplete or for whom no mutations in SLC26A4 were identified at all. This has implications for genetic counseling regarding recurrence risk, whether to pursue imaging, and, more broadly, understanding of disease pathogenesis. The inability to identify both mutations in individuals suspected of having a PDS/DFNB4 hearing loss etiology suggests the possible involvement of (1) mutations in unexamined regions of SLC26A4; (2) mutations in other, as yet to be implicated genes; or (3) other factors, such as those that may regulate gene expression.

The selection of study subjects is an important factor that is not consistent between studies. Our patient selection was based primarily on the genetic testing of GJB2 and SLC26A4 as is common in the clinical work-up for children with SNHL in the U.S., with imaging studies...
performed on only a subset of patients. Even in this relatively general SNHL patient population, however, we saw a considerable enrichment for probands with single \textit{SLC26A4} mutations, compared to unaffected individuals. In the experience of the Stanford Molecular Pathology Laboratory, the frequency of heterozygous \textit{SLC26A4} mutations in probands tested with a \textit{GJB2} and \textit{SLC26A4} algorithm and in whom two pathogenic mutations are not identified is 12.3%, which is significantly higher (p < 0.001) than the expected carrier frequency. Using a frequency of 1/500 for congenital bilateral hearing loss of \(\geq 40\) dB (Hilgert, Smith & Van Camp, 2009), and the estimate that \textit{SLC26A4} related SNHL would account for up to 7.5%, then the frequency of such hearing loss could approximate one in 7,000. Assuming Hardy-Weinberg equilibrium, the carrier frequency would be about 1:50 (2%). This is congruent with a study in which pathogenic or possibly pathogenic mutations were identified in 1.9% of normal-hearing controls (8/428 controls; p.E29Q, p.F354S, p.F667C, p.D724G, p.G740S) (Pera et al., 2008b). An “excess” of heterozygous mutations in individuals with SNHL compared to controls has also been observed for \textit{GJB2} (Putcha et al., 2007), a gene for which neighboring deletions can affect gene expression (Rodriguez-Paris & Schrijver, 2009).

Given that \textit{SLC26A4} related SNHL is autosomal recessive, we postulated that these ‘missing’ \textit{SLC26A4} mutations may be the result of intragenic deletions or duplications of one or more exons for which patients are not routinely tested. We selected 107 individuals with SNHL and monoallelic mutations of \textit{SLC26A4} for MLPA analysis of the \textit{SLC26A4} gene to explore this further. A handful of multiexon \textit{SLC26A4} deletions have been described in the literature (Park et al., 2003; Hu et al., 2007; Pera et al., 2008a; Anwar et al., 2009; Siem et al., 2010) but it is unclear how many of the probands in these cohorts were tested for intragenic deletions and duplications. Recently, however, in a study of 109 Scandinavian probands with suspected PDS/DFNB4, 37 individuals with only one or zero mutations of \textit{SLC26A4} were systematically screened for copy number variants by MLPA (Rendtorff et al., 2013). Only one harbored a
homozygous deletion, of exons 4-6 (n = 2/63 unknown alleles; 3.2%). In our study of hearing impaired individuals with one previously identified SLC26A4 mutation, we found an intragenic deletion in a single individual only (n = 1/107 unknown alleles; 0.93%). This deletion also spanned exons 4-6 of the SLC26A4 gene and was previously detected in a patient of Spanish descent (Pera et al., 2008a). An analysis of the deletion breakpoints confirmed that our subject carried a heterozygous copy of g.8091T-22145Cdel (Figure 1). To date, deletions and duplications seem to represent approximately 1.8% of missing SLC26A4 mutations overall (n = 3/170 unknown alleles) (Rendtorff et al., 2013; this study). Despite accounting for a low percentage of the ‘missing’ mutations, clinical testing for multiexon deletions and duplications of additional patients with potential PDS/DFNB4 etiology may remain warranted in order to more firmly establish frequencies and elucidate their relative contribution to the phenotype.

Alternatively, unrecognized mutations in unexamined, noncoding regions of the gene may be responsible for the ‘missing’ SLC26A4 mutations and contribute to the PDS/DFNB4 phenotype. For example, intronic mutations may create cryptic splice sites and mutations in the promoter region may disrupt the binding of regulatory elements. Indeed, a cis-regulatory element that binds transcription factor FOXI1 has been described in the SLC26A4 promoter region (Yang et al., 2007). The regulatory element consists of two head-to-head binding sites, FBS1 and FBS2, and a mutation within this cis-element, c.-103T>C, has been shown to disrupt transcriptional activation of the gene by FOXI1. However, none of the probands included in this study carried mutations in the promoter region.

Mutations of the FOXI1 gene itself have also been implicated in PDS and DFNB4. Monoallelic variants of FOXI1 were documented in six patients with either PDS or non-syndromic EVA and were shown to compromise the ability of FOXI1 to transcriptionally activate SLC26A4 (Yang et al., 2007). One of these six probands segregated the DFNB4 phenotype with one heterozygous mutation each of FOXI1 and SLC26A4. This finding was consistent with the
EVA phenotype observed in the \textit{Slc26a4}+/\textit{−}; \textit{Foxi1}+/\textit{−} double-heterozygous mouse model and suggests that the transcriptional regulatory machinery of \textit{SLC26A4} plays a role in PDS/DFNB4 pathogenesis. Mutations of \textit{KCNJ10}, a gene that encodes a K$^+$ channel protein, have also been associated with PDS/DFNB4 (Marcus et al., 2002). Protein expression studies in \textit{SLC26A4} knockout mice have indicated that the absence of pendrin expression reduces KCNJ10 protein levels, supporting the hypothesis that deafness in the mouse model is secondary to loss of KCNJ10 function (Wangemann et al., 2004). A similar reduction of \textit{KCNJ10} expression was observed in the stria vascularis of the inner ear in the haploinsufficient \textit{Slc26a4}+/\textit{−} mouse mutant (Yang et al., 2009). In that same study, two individuals with the PDS/DFNB4 phenotype were reported to be double heterozygous for mutations of the \textit{SLC26A4} and \textit{KCNJ10} genes, further supporting a digenic model of inheritance.

We examined to what extent digenic inheritance may contribute to the PDS/DFNB4 phenotype by sequencing the coding regions and splice sites of \textit{FOXI1} and \textit{KCNJ10} in about half (n = 29/60) of the Stanford University probands enrolled in the study for whom enough sample was available for the additional analysis. Our initial sequencing of \textit{FOXI1} and \textit{KCNJ10} in these subjects resulted in three nonsynonymous variants overall; all three of these heterozygous substitutions are listed in the dbSNP database as having unknown clinical significance. Nonetheless, functional studies must be conducted to investigate the effects of the \textit{FOXI1} variant on \textit{SLC26A4} transcriptional activation and the impact of the two \textit{KCNJ10} variants on K$^+$ channel conductance before a determination of the pathogenicity of these three variants and their effect on SNHL phenotypes can be more definitively made.

Although unlikely, if the \textit{FOXI1} variant and the two \textit{KCNJ10} variants detected in our probands are indeed digenic mutations acting in conjunction with mutations of \textit{SLC26A4}, then 3.4% and 6.9% of the missing mutant alleles in our SNHL patients would be attributable to variants in \textit{FOXI1} and \textit{KCNJ10}, respectively. However, a meta-analysis of published studies in
which *FOXI1* and *KCNJ10* were sequenced in SNHL patients with inner ear malformations does not support these frequencies (Table 3) and shows instead that, overall, 1.3% and 3.1% of suspected PDS/DFNB4 patients have variants in *FOXI1* and *KCNJ10*, respectively. In fact, considering that the *KCNJ10* variant c.812G>A (p.Arg271His) may be a polymorphism in the Chinese population and the *KCNJ10* variant c.811C>T (p.Arg271Cys) is a reported polymorphism in the dbSNP database, the frequency of *KCNJ10* variants in PDS/DFNB4 patients may be inflated by the inclusion of the Chinese and Italian probands carrying these substitutions and may actually be lower. The great majority of reported *FOXI1* and *KCNJ10* variants are from the initial studies that implicated the genes in the digenic inheritance of PDS/DFNB4 (Yang et al., 2007; Yang et al., 2009). However, the actual contribution of *FOXI1* and *KCNJ10* mutations to SNHL may be more limited, as illustrated by several subsequent studies in which either no *FOXI1* variants (Wu et al., 2010; Mercer, Mutton & Dahl, 2011; Lai et al., 2012; Chen et al., 2012; Chai et al., 2013), or no *KCNJ10* variants (Mercer, Mutton & Dahl, 2011; Chen et al., 2012) were identified.

The genetic basis of hearing loss is diagnostically challenging with over 100 genes implicated (http://hereditaryhearingloss.org). The phenotypic variability observed within the PDS/DFNB4 spectrum also complicates diagnosis with changes in the same gene, *SLC26A4*, responsible for syndromic as well as nonsyndromic hearing loss. Most clinical centers have historically utilized tiered testing in the assessment of hearing loss genetic etiology. However, with the advent of large-scale massively parallel sequencing (MPS), future approaches will likely employ testing platforms that are more comprehensive, cost effective and efficient (Shearer & Smith, 2012). Additional sources of genetic mutation, such as deletions and duplications, will need to be included in these new testing approaches.
References


(PDS) mutations in east and south Asians: global implications for the epidemiology of deafness.


Table 1 (on next page)

Primers used for the amplification of FOXI1 and KCNJ10.
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Figure 1

Identification of the heterozygous SLC26A4 deletion g.8091T-22145Cdel in subject PDS41.

Fig 1. Identification of the heterozygous SLC26A4 deletion g.8091T-22145Cdel in subject PDS41. (A) The SLC26A4 MLPA probe mix includes probes for the 21 SLC26A4 exons, 14 reference probes and three probes specific for the point mutations c.1001+1G>A (IVS8+1G>A), c.707T>C (p.Leu236Pro) and c.1246A>C (p.Thr416Pro). Subject PDS41 is heterozygous for a deletion (g.8091T-22145Cdel) spanning exons 4-6 in SLC26A4, as evidenced by the reproducible reduction in peak height for these three exon probes (peaks labeled 4, 5, 6) relative to the control. PDS41 also has a heterozygous c.1246A>C (p.Thr416Pro) mutation that was previously identified by sequencing and confirmed by these MLPA results (peak labeled p.T416P). (B) Sequencing chromatogram of the deletion breakpoints in SLC26A4 IVS3 and IVS6. The deletion removes a total of 14,053 bp. IVS = intervening sequence
Table 2 (on next page)

Percentages of PDS/DFNB4 probands with SLC26A4 mutations.
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<th>0 Mutations</th>
<th>Study Selection Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campbell, 2001</td>
<td>58</td>
<td>9 (16%)</td>
<td>14 (24%)</td>
<td>35 (60%)</td>
<td>Recessive HL with DVA or Mondini dysplasia</td>
</tr>
<tr>
<td>Tsukamoto, 2003</td>
<td>42</td>
<td>24 (57%)</td>
<td>10 (24%)</td>
<td>8 (19%)</td>
<td>Pendred (goiter) or bilateral HL with EVA</td>
</tr>
<tr>
<td>Pryor, 2005</td>
<td>39</td>
<td>14 (36%)</td>
<td>14 (36%)</td>
<td>11 (28%)</td>
<td>EVA in at least one ear</td>
</tr>
<tr>
<td>Albert, 2006</td>
<td>100</td>
<td>24 (24%)</td>
<td>16 (16%)</td>
<td>60 (60%)</td>
<td>Bilateral, recessive HL; EVA; no GJB2 mutations</td>
</tr>
<tr>
<td>Yang, 2007</td>
<td>429</td>
<td>57 (13%)</td>
<td>75 (17%)</td>
<td>297 (69%)</td>
<td>HL with EVA</td>
</tr>
<tr>
<td>Wu, 2010</td>
<td>101</td>
<td>63 (62%)</td>
<td>24 (24%)</td>
<td>14 (14%)</td>
<td>Bilateral EVA</td>
</tr>
</tbody>
</table>

Total = 769 | 191 (25%) | 153 (20%) | 425 (55%) |

HL=hearing loss; DVA=dilated vestibular aqueduct; EVA=enlarged vestibular aqueduct
Table 3 (on next page)

Meta-analysis of the frequency of FOXI1 and KCNJ10 variants in PDS/DFNB4 probands.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Population Origin</th>
<th>Reference</th>
<th>Controls</th>
<th>Inclusion Criteria for SLC26A4 Sequencing</th>
<th>Selection Criteria for FOXI1 Sequencing</th>
<th>Subjects with Nonsynonymous Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXI1</td>
<td>US/Sweden</td>
<td>Yang et al, 2007</td>
<td>250</td>
<td>EVA with or without Mondini dysplasia</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>6/372 (1.6%)</td>
</tr>
<tr>
<td>FOXI1</td>
<td>Taiwan</td>
<td>Wu et al, 2010</td>
<td>100</td>
<td>Bilateral EVA with or without other IEMs</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>0/38 (0.0%)</td>
</tr>
<tr>
<td>FOXI1</td>
<td>Australia</td>
<td>Mercer et al, 2011</td>
<td>96</td>
<td>EVA</td>
<td>Phenotype only</td>
<td>0/44 (0.0%)</td>
</tr>
<tr>
<td>FOXI1</td>
<td>Italy</td>
<td>Cirello et al, 2012</td>
<td>80</td>
<td>Bilateral IEMs/family history of PS/goiter</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>1/14 (7.1%)</td>
</tr>
<tr>
<td>FOXI1</td>
<td>China</td>
<td>Lai et al, 2012</td>
<td>100</td>
<td>EVA</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>0/8 (0.0%)</td>
</tr>
<tr>
<td>FOXI1</td>
<td>China</td>
<td>Chen et al, 2012</td>
<td></td>
<td>IEM's</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>0/15 (0.0%)</td>
</tr>
<tr>
<td>FOXI1</td>
<td>China</td>
<td>Chai et al, 2013</td>
<td>200</td>
<td>Nonsyndromic EVA</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>0/33 (0.0%)</td>
</tr>
<tr>
<td></td>
<td>US</td>
<td>This study</td>
<td></td>
<td>Sensorineural hearing loss</td>
<td>1 SLC26A4 mutation</td>
<td>1/29 (3.4%)</td>
</tr>
</tbody>
</table>

Reported FOXI1 Frequency = 7/524 (1.3%)  
Total Combined FOXI1 Frequency = 8/553 (1.4%)  

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNJ10</td>
<td>China/US/Canada</td>
<td>Yang et al, 2009</td>
<td>400</td>
<td>EVA with or without Mondini dysplasia</td>
<td>1 SLC26A4 mutation</td>
<td>2/89 (2.2%)</td>
</tr>
<tr>
<td>KCNJ10</td>
<td>Australia</td>
<td>Mercer et al, 2011</td>
<td>96</td>
<td>EVA</td>
<td>Phenotype only</td>
<td>0/44 (0.0%)</td>
</tr>
<tr>
<td>KCNJ10</td>
<td>Italy</td>
<td>Cirello et al, 2012</td>
<td>80</td>
<td>Bilateral IEMs/family history of PS/goiter</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>3/14 (21.4%)</td>
</tr>
<tr>
<td>KCNJ10</td>
<td>China</td>
<td>Chen et al, 2012</td>
<td></td>
<td>IEM's</td>
<td>0 or 1 SLC26A4 mutations</td>
<td>0/15 (0.0%)</td>
</tr>
<tr>
<td>KCNJ10</td>
<td>China</td>
<td>Chai et al, 2013</td>
<td>200</td>
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<td>0 or 1 SLC26A4 mutations</td>
<td>1/33 (3.0%)</td>
</tr>
<tr>
<td>KCNJ10</td>
<td>US</td>
<td>This study</td>
<td></td>
<td>Sensorineural hearing loss</td>
<td>1 SLC26A4 mutation</td>
<td>2/29 (6.9%)</td>
</tr>
</tbody>
</table>

Reported KCNJ10 Frequency = 6/195 (3.1%)  
Total Combined KCNJ10 Frequency = 8/224 (3.6%)  

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1. All controls had normal hearing and, unless otherwise noted, none harbored any of the novel variants.
2. Five variants (p.G258E, p.N161del, p.G258R, p.R267Q and p.G335V) were detected in six probands and were shown to compromise SLC26A4 transcriptional activation.
3. Functional analysis of the identified novel variant (p.P239L) showed no impairment of SLC26A4 transcriptional activation.
4. Two identified missense mutations (p.P194H and p.R348C) were shown to impair K+ channel conductance.
5. One previously reported variant (p.R271C) was identified in 3 probands.
6. One heterozygous variant (p.R271H) was detected in a single proband; this variant was also found in 10/200 normal hearing controls.

EVA=Enlarged vestibular aqueduct; IEM=Inner ear malformation; PS=Pendred syndrome