

***SLC26A4* Mutations in Patients with Moderate to Severe Hearing Loss**

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Received: 6 August 2012 / Accepted: 15 November 2012 / Published online: 17 March 2013
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Abstract Mutations in *SLC26A4* cause either syndromic or nonsyndromic hearing loss. We identified a link between hearing loss and *DFNB4* in 3 of the 50 families participating in this study. Sequencing analysis revealed two *SLC26A4* mutations, p.V239D and p.S57X, in affected members of the 3 families. These mutations have been previously reported in deaf individuals from the subcontinent, all of whom manifested profound deafness. The patients investigated in our study exhibited moderate to severe hearing loss. Our results show that inactivating *SLC26A4* mutations that cause profound deafness can also be involved in the etiology of moderate to severe hearing loss. The type of mutation cannot predict the severity of the hearing loss in all cases, and there may be additional epistatic interactions that could modify the phenotype.

Keywords Pendrin · Moderate to severe hearing loss · *DFNB4* · Deafness · Pakistan

Introduction

Hearing loss is one of the most prevalent sensory impairments in humans. Hundreds of syndromic and nonsyndromic forms of deafness have been described. Pendred syndrome (MIM 274600) involves sensorineural hearing loss along with enlarged vestibular aqueduct and also includes thyroid dysfunction, which can be manifested as a goiter (Pendred 1896). *DFNB4* (MIM 600791) is described as nonsyndromic deafness with enlarged vestibular aqueduct (Li et al. 1998). Recessively inherited mutations in *SLC26A4* cause both Pendred syndrome and *DFNB4* (Li et al. 1998; Everett et al. 1997).

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SLC26A4 encodes pendrin, a membrane protein of 85 kDa comprising 780 amino acids. It functions in mediating the exchange of chloride with bicarbonate, formate, and iodide (Scott et al. 1999). *SLC26A4* is expressed in the thyroid (Royaux et al. 2000), the inner ear (Everett et al. 1999), and the kidney (Soleimani et al. 2001). More than 300 mutations of the gene causing hearing loss have been described. These include missense, nonsense, splice site, and frameshift mutations.

To understand the role of *SLC26A4* in the pathogenesis of the inner ear, researchers have generated many mouse mutants that have engineered mutations or lack the gene. These include two knockout *Slc26a4*^{-/-} mice (Everett et al. 2001; Kim and Wangemann 2011) and two knock-in mice: *Slc26a4*^{loop/loop} with the engineered missense mutation p.S408F (Dror et al. 2010) and *Slc26a4*^{tm1Dontuh/mlDontuh} with the c.919-2 A→G mutation (Lu et al. 2011). The loss of hearing in these mouse models is profound in degree and offers no clues to the molecular basis of the observed differences in hearing thresholds due to a few particular *SLC26A4* mutations in humans. Experiments with transgenic mice Tg[E];Tg[R];*Slc26a4*^{Δ/Δ}, however, in which *Slc26a4* expression is inducible by doxycycline on a background of absent endogenous pendrin, have demonstrated that expression of *Slc26a4* at early embryonic stages of E0-E17.5 is required for acquisition of normal hearing in these mice (Choi et al. 2011). Ablating expression of *Slc26a4* at the early embryonic stages results in complete hearing loss in the mice, and its absence in early postnatal stages results in partial hearing loss. This variability in hearing loss recapitulates the phenotypes documented for many humans with mutations in *SLC26A4*.

It has been suggested that many missense mutations affect pendrin function severely and cause profound deafness, whereas some mutations (p.V480D, p.V653A) retain residual function of pendrin and cause a less severe phenotype (Campbell et al. 2001). Moreover, nonsense mutations usually result in profound deafness. We have found, however, that identical mutations in *SLC26A4*, previously known to cause profound deafness, can also result in moderate to severe hearing loss in humans.

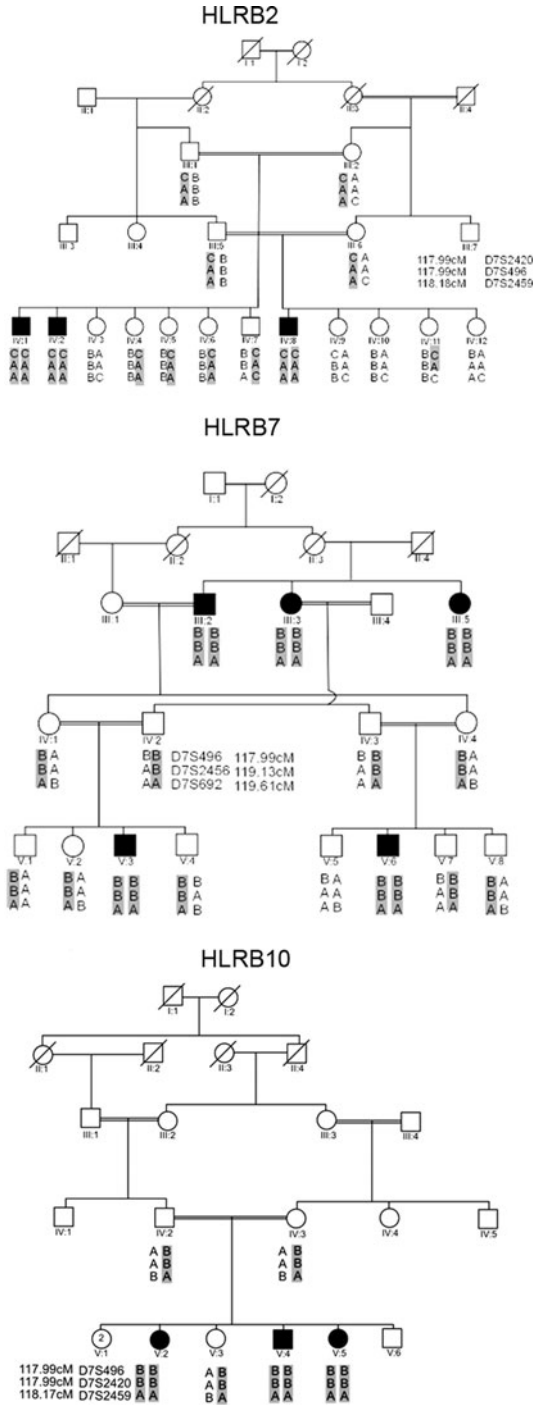
Materials and Methods

Three consanguineous families from Pakistan, HLRB2, HLRB7, and HLRB10, were recruited as part of a larger study on the genetics of moderate to severe hearing loss, which included 47 additional families from Pakistan with less than profound hearing loss. All families had multiple affected individuals. Approval was obtained from the institutional review board of the School of Biological Sciences, University of the Punjab, Lahore. Samples were obtained with written informed consent from all participants or from their parents in the case of children. DNA was extracted from blood samples from the participants (Grimberg et al. 1989; Miller et al. 1988).

Clinical Investigation

Detailed medical examinations and histories were completed for the participants. HLRB2 was a consanguineous family with three affected children aged 8–11 years.

Fig. 1 Pedigrees of families HLRB2 (top), HLRB7 (center), and HLRB10 (bottom) with phenotype data and genotypes of microsatellite markers flanking *SLC26A4*. Solid circles (females) and squares (males) denote affected individuals. Haplotypes associated with deafness are shaded gray (Color figure online)



HLRB7 had five affected individuals aged 14–70 years, and HLRB10 had three affected individuals, aged 9–14 years, in a single consanguineous loop (Fig. 1). Hearing status was evaluated by pure tone audiometry using a portable audiometer (Danplex DA65, Denmark). Hearing was evaluated at frequencies of 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz. The severity of hearing loss was graded according to hearing thresholds: mild (20–40 dB), moderate (41–55 dB), moderately severe (56–70 dB), severe (71–90 dB), and profound (95–120 dB) (Smith et al. 2005). Vestibular function was assessed by Romberg and tandem gait tests. Thyroid status was evaluated by palpation.

Genotyping and Sequencing

We sequenced the coding exon of *GJB2* and genotyped DNA samples from all 50 families with microsatellite markers specific for 16 deafness loci, including *DFNB4*. These loci were known to be either a common cause of deafness in Pakistan or responsible for less severe or progressive hearing loss in humans. Genotyping data were analyzed using GeneMapper ID version 3.2 (Applied Biosystems, Foster City, CA). Two-point likelihood of odds (LOD) scores for each marker were calculated using the MLink program of the Linkage package (Terwilliger and Ott 1994). Deafness was coded as a fully penetrant recessive disorder with a disease allele frequency of 0.001.

The 21 exons of *SLC26A4* and their flanking intronic regions greater than 60 bp were PCR-amplified with specifically designed primers. The PCR products were treated with exonuclease I and shrimp alkaline phosphatase (Fermentas, Glen Burnie, MD) to remove excess primer and dNTPs. Sequencing was completed with a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) as described previously (Malik et al. 2010).

Results

Subjects and Clinical Investigation

Clinical history, interviews, and physical examinations revealed no apparent phenotype except hearing loss in the participants. Palpable goiters were absent in the individuals with hearing loss. Pure tone audiometry revealed moderate to severe sensorineural hearing loss in all affected individuals in families HLRB2, HLRB7, and HLRB10 (Fig. 2). Romberg and tandem gait tests were normal, suggesting that vestibular function was not impaired.

Genotyping and Mutational Analysis

Genotyping with microsatellite markers and haplotype analysis of the affected members in the three families revealed linkage of hearing loss to the *DFNB4/PDS* locus on chromosome 7q31. Markers *D7S2459*, *D7S2420*, and *D7S496* were

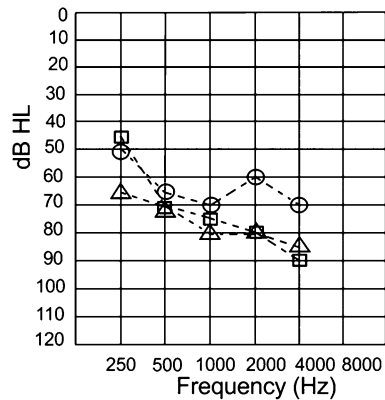
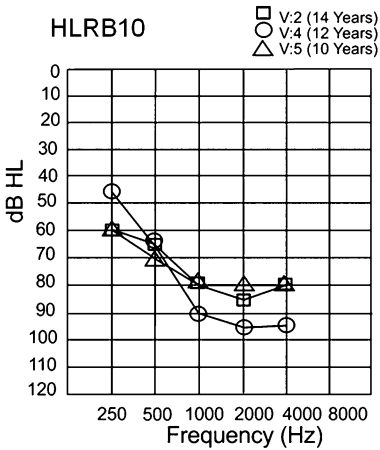
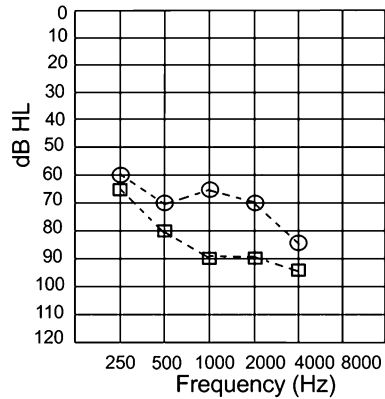
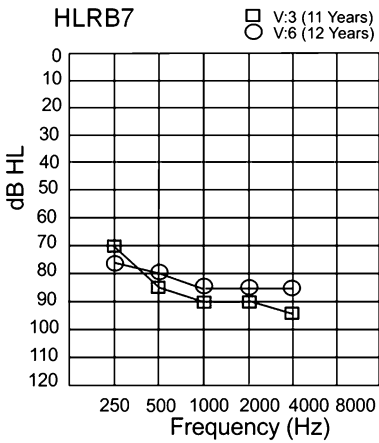
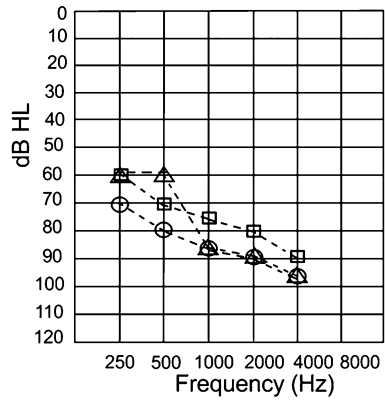
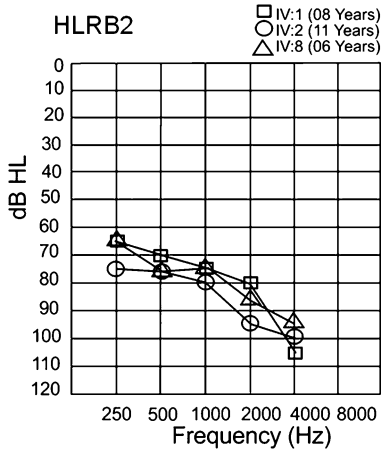
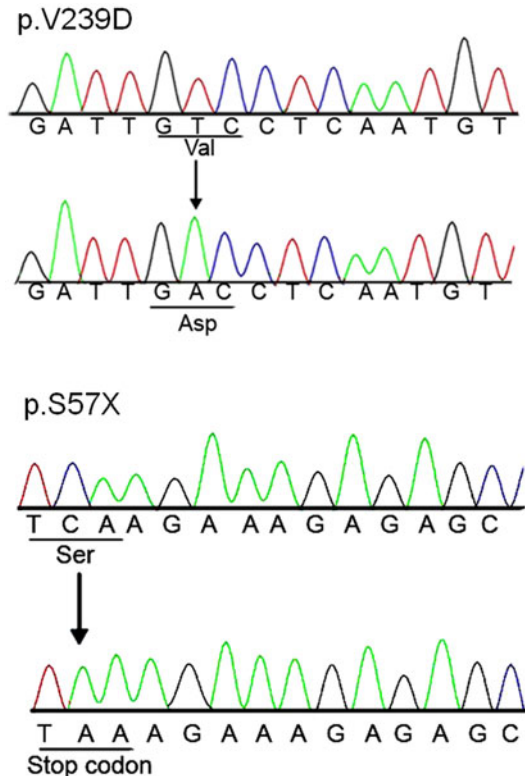


Fig. 2 Pure tone audiograms for three children of family HLRB2 (*top*), two children of family HLRB7 (*center*), and three children of family HLRB10 (*bottom*). All affected individuals (identified by codes in Fig. 1) have moderate to severe hearing loss. Hearing thresholds are depicted for the left ear (*solid lines*) and the right ear (*dotted lines*)

homozygous in affected individuals and heterozygous in the obligate carriers. Maximum two-point LOD scores of 2.5 were obtained with markers *D7S2459* and *D7S2420* at recombination fraction $\theta = 0$ for family HLRB2. For family HLRB7, marker *D7S496* yielded an LOD score of 3.5. Markers *D7S2420* and *D7S496* supported an LOD score of 1.6 for family HLRB10. Hearing loss did not segregate with markers specific for *DFNB4/PDS* in any of the other 47 participating families.

We sequenced the 21 exons of *SLC26A4* from one affected individual for each family demonstrating linkage to *DFNB4/PDS*. Two mutations were identified in *SLC26A4* that were subsequently determined to segregate with hearing loss in members of families HLRB2, HLRB7, and HLRB10 (Fig. 3). The transversion mutation c.716 T→A (p.V239D) was detected in exon 6, on both alleles of *SLC26A4*, in affected individuals of HLRB2 and HLRB7. This mutation is predicted to affect transmembrane domain 5 of *SLC26A4*. Another transversion mutation, c.170 C→A, was identified in exon 3 of *SLC26A4* as a homozygous mutation

Fig. 3 Representative sequence electropherograms of the mutations p.V239D (*top*) and p.S57X (*bottom*). Arrows indicate mutation sites. The normal and mutated codons are *underlined* in the respective traces (*Color figure online*)



among affected members of family HLRB10. This mutation causes termination of pendrin at amino acid 57 (p.S57X). The termination occurs at the amino terminus of *SLC26A4*, and the protein is predicted to lack most of the important domains.

Discussion

Mutations in *SLC26A4* are a common cause of hearing loss in humans, with no single prevalent mutation worldwide. A few mutations, however, are specifically found in particular ethnic groups in some populations (Park et al. 2003; Tsukamoto et al. 2003). Mutations in *SLC26A4* are one of the most common causes of recessively inherited profound deafness in Pakistan, accounting for 7.2% of the disorder (Anwar et al. 2009). Our results demonstrate that the ratio of *SLC26A4* mutations is similarly high in individuals with less than profound deafness in Pakistan and account for hearing loss in 6% of the families in our cohort.

Previously, a few splice site mutations in *SLC26A4* have been reported to cause intrafamilial variability in degree of hearing loss (Van Hauwe et al. 1998). These splice site mutations affect the C-terminus of *SLC26A4* and are hypothesized to produce truncated proteins, which may maintain the residual function of pendrin and therefore could explain the presence of less severe phenotype in the affected patients (Van Hauwe et al. 1998). Moreover, two mutations, IVS4+7 A→G (Lopez-Bigas et al. 1999) and IVS8+4 A→G (Park et al. 2003), have been described that affect nucleotides away from the canonical splice junction sequences and create new splice sites within *SLC26A4*. The use of these newly created splice sites leads to frameshifts of the open reading frame of *SLC26A4*. It is hypothesized that some correctly spliced *SLC26A4* may still be produced, since the actual splice sites are intact. The presence of residual wild-type *SLC26A4* may explain the less severe hearing loss observed in some patients in families with these mutations (Lopez-Bigas et al. 1999).

Hearing loss associated with Pendred syndrome is profound. Nonsyndromic deafness (*DFNB4*) may be profound or may vary from mild to moderate in degree. Mutations p.T416P, p.L236P, and p.E384G (Campbell et al. 2001; Van Hauwe et al. 1998), which cause Pendred syndrome, have been demonstrated to result in retention of pendrin within the endoplasmic reticulum in an in vitro functional study. Because three frequent mutations observed in Caucasians and associated with *DFNB4* (p.V653A and p.I490L/p.G497S) allow continued pendrin activity (Scott and Karniski 2000; Rotman-Pikielny et al. 2002), it has been hypothesized to constitute the difference between mutations that cause Pendred syndrome or *DFNB4*. Other *SLC26A4* mutations in patients with nonsyndromic hearing loss also cause the retention of protein in the endoplasmic reticulum (Taylor et al. 2002). The mutation p.V239D has been described in Pakistani, Indian (Park et al. 2003), Turkish, and Palestinian families (Fitoz et al. 2007; Tekin et al. 2003; Walsh et al. 2006). It is involved in the etiology of both Pendred syndrome and *DFNB4*. Trafficking of wild-type and intracellular localization of mutant (p.V239D) pendrin has also been evaluated, showing that p.V239D mutant protein is retained in the endoplasmic reticulum and wild-type protein is targeted to the plasma membrane

Table 1 *SLC26A4* mutations identified in this study

Family	Nucleotide change	Amino acid change	Country	Hearing loss phenotype	Source
–	c.716 T→A	p.V239D	India and Pakistan	Profound	Park et al. (2003)
–	c.716 T→A	p.V239D	Turkey	Profound	Tekin et al. (2003)
–	c.716 T→A	p.V239D	Palestine	Profound	Walsh et al. (2006)
HLRB2, HLRB7	c.716 T→A	p.V239D	Pakistan	Moderate to severe	This study
–	c.170 C→A	p.S57X	India and Pakistan	Profound	Park et al. (2003)
–	c.170 C→A	p.S57X	Pakistan	Profound	Anwar et al. (2009)
HLRB10	c.170 C→A	p.S57X	Pakistan	Moderate to severe	This study

(Walsh et al. 2006). A similar spectrum of mutations in Pendred syndrome and the *DFNB4* population contradicts the molecular study of pendrin. It has been hypothesized that since in vitro functional studies show outcomes for single mutations, other factors (including intervention of regulatory elements, modifier genes, or nutrition affecting iodine uptake) may determine the thyroid phenotype in vivo (Albert et al. 2006).

The mutation p.S57X has previously been reported only in deaf individuals from Pakistan and India (Anwar et al. 2009; Park et al. 2003) and may be confined to the subcontinent because of a founder effect. Previously, the nonsense mutations p.K440X and p.Q514X were reported to cause less severe hearing loss, each in one of two affected individuals (Wang et al. 2007). It was hypothesized that the p.K440X mutation affects transmembrane domain 10 and that p.Q514X affects the carboxy terminus of *SLC26A4*, conserving many of the functional domains of the protein. This may be sufficient for preserving some degree of hearing. All other reported truncating mutations (p.S23X, p.E37X, and p.S57X) cause profound deafness. In family HLRB10, the p.S57X mutation is associated with moderate to severe hearing loss, even though no functional pendrin is predicted to be present in the affected members, either because the most important protein domains are lacking or because of nonsense-mediated decay of the *SLC26A4* transcript.

Identification of identical mutations (p.V239D, p.S57X) in both those individuals with profound deafness and those with moderate to severe hearing loss (Table 1) suggests that the effects of some pathogenic mutations in *SLC26A4* may be modified by genetic and epigenetic factors. Research on individuals from other populations segregating moderate to severe hearing loss should reveal whether additional missense and nonsense mutations in *SLC26A4* can cause similar variability in hearing thresholds.

Acknowledgments We are grateful to the study participants for their cooperation. We thank Dr. Thomas Friedman for providing some of the primers used in this research. The work was funded by grant no. R01TW007608 from the Fogarty International Center and National Institute of Deafness and Other Communication Disorders, National Institutes of Health, USA.

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