Journal of Human Genetics (2013) 58, 102–108

www.nature.com/jhg

ORIGINAL ARTICLE

Phenotypic variability of *CLDN14* mutations causing DFNB29 hearing loss in the Pakistani population

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Human hereditary deafness at the *DFNB29* locus on chromosome 21q22.1 is caused by recessive mutations of *CLDN14*, encoding claudin 14. This tight junction protein is tetramembrane spanning that localizes to the apical tight junctions of organ of Corti hair cells and in many other tissues. Typically, the DFNB29 phenotype is characterized by prelingual, bilateral, sensorineural hearing loss. The goal of this study was to define the identity and frequency of *CLDN14* mutations and associated inner ear phenotypes in a cohort of 800 Pakistani families segregating deafness. Hearing loss in 15 multi-generational families was found to co-segregate with *CLDN14*-linked STR markers. The sequence of the six exons and regions flanking the introns of *CLDN14* in these 15 families revealed five likely pathogenic alleles. Two are novel missense substitutions (p.Ser87Ile and p.Ala94Val), whereas p.Arg81His, p.Val85Asp and p.Met133ArgfsX23 have been reported previously. Haplotype analyses indicate that p.Val85Asp and p.Met133ArgfsX23 are founder mutations. The p.Val85Asp accounts for ~67% of the mutant alleles of *CLDN14* in our cohort. Combined with the previously reported data, *CLDN14* mutations were identified in 18 of 800 Pakistani families (2.25; 95% CI, 1.4–3.5). Hearing loss in the affected individuals homozygous for *CLDN14* mutations varied from moderate to profound. This phenotypic variability may be due to environmental factors (for example drug and noise exposure) and/or genetic modifiers.
Journal of Human Genetics (2013) **58,** 102–108; doi:10.1038/jhg.2012.143; published online 13 December 2012

Keywords: *CLDN14*; claudin 14; *DFNB29*; mild hearing loss; Pakistan; profound deafness

INTRODUCTION

Mutations of *CLDN14* cause autosomal recessive nonsyndromic deafness at the *DFNB29* locus. To date, six different pathogenic variants of human *CLDN14* have been identified in families segregating severe to profound hearing loss, but no obvious vestibular phenotype.[1–4] Similarly, a *Cldn14* knockout mouse is also deaf.[5] Although claudin 14 is expressed in the mouse vestibular sensory epithelium, *Cldn14* knockout mice appear to have no obvious vestibular disorder such as circling behavior or head bobbing.[5]

The mammalian claudin family of 27 genes encodes tight junction proteins that function to maintain integrity of the apical and basolateral membrane domains and prevent diffusion of solutes and solvent molecules through intercellular spaces within epithelial sheets.[6–9] The claudin proteins are predicted to have four transmembrane domains and short cytosolic amino and carboxy termini.[10,11] Although the first and the fourth transmembrane regions as well as the extracellular loops are highly conserved among the different claudin species, the second and the third transmembrane regions are variable.[12] The first extracellular loop of these proteins has an important role in homophilic interactions (Figure 1a).[13,14] To date, most of the known mutations of claudin 14 are within or close to the second or third transmembrane domains (Figure 1a) and some of them have been shown to affect membrane localization. For example, p.Val85Asp impairs the ability of claudin 14 to form tight junction strands.[4]

The structure of most intercellular tight junctions in the inner ear is similar to that reported in other epithelia.[15–18] However, the structure of the bicellular junctions between hair cells and supporting cells, especially between an outer hair cell and adjacent Deiter's cell are more elaborate and highly specialized to maintain the ionic barrier between endolymph and perilymph.[16–18] These tight junctions contain a high amount of claudin 14 and are prominently stained with anticlaudin 14 antibody (Figures 1b and c). The apical junctional complexes between the cells of the organ of Corti lack desmosomes and gap junctions and have a combination of tight junction and adherens junction features, and

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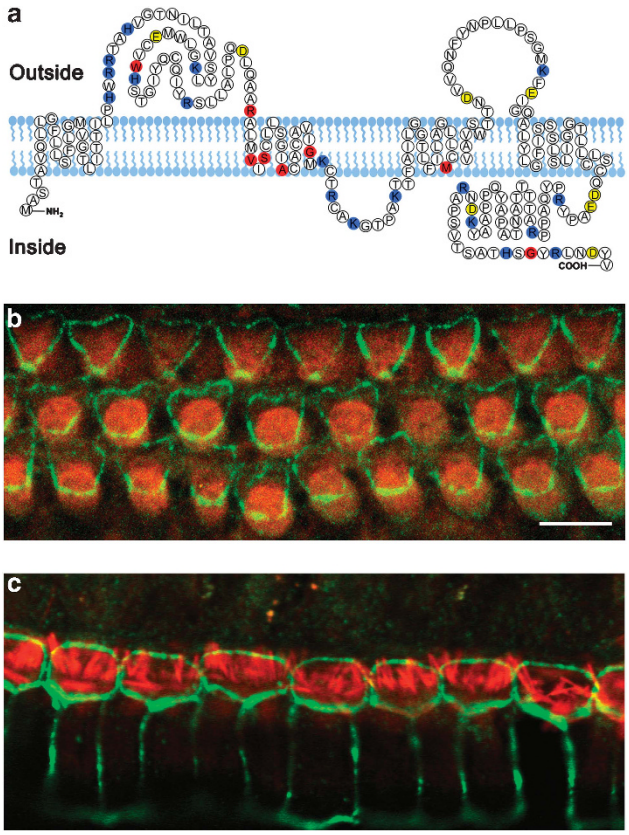
Received 28 August 2012; revised 5 November 2012; accepted 19 November 2012; published online 13 December 2012



extend down the depth of the reticular lamina, a region spanning 3–5 μm.[18]

The goal of this study was to determine the spectrum of mutant alleles, and the frequencies of these alleles in 800 Pakistani families segregating nonsyndromic deafness, and to measure variability in the clinical phenotype of *CLDN14* pathogenic variants. We found that pathogenic alleles of *CLDN14* are associated with hearing loss that ranges from moderate to profound, and that mutant alleles of this gene appear to be a common cause of heritable hearing loss among Pakistanis.

METHODS

Family participation and clinical evaluation

This study was approved by IRBs at the National Centre of Excellence in Molecular Biology (NCEMB), Lahore, Pakistan (FWA00001758), at the National Institutes of Health, USA (Combined Neuroscience IRB; OH-93-N-016), and at the Cincinnati Children's Hospital Research Foundation, USA (2009-0684;

Figure 1 Eight different pathogenic alleles of *CLDN14* are associated with hearing loss in human. (**a**) Schematic of human claudin 14. Topology of claudin 14 was predicted by TMpred software. Yellow and blue amino acids indicate negatively and positively charged residues, respectively. The positions of eight residues mutated in all reported DFNB29 families are red. (**b**) Localization of claudin 14 in the apical bicellular tight junctions between the outer hair cells and Deiters' cells (green). (**c**) Localization of claudin 14 (green) in the tight junctions between inner hair cells and pillar cells and between two adjacent pillar cells. Filamentous actin is highlighted by rhodamine–phalloidin (red). Scale bar = 5 μm.

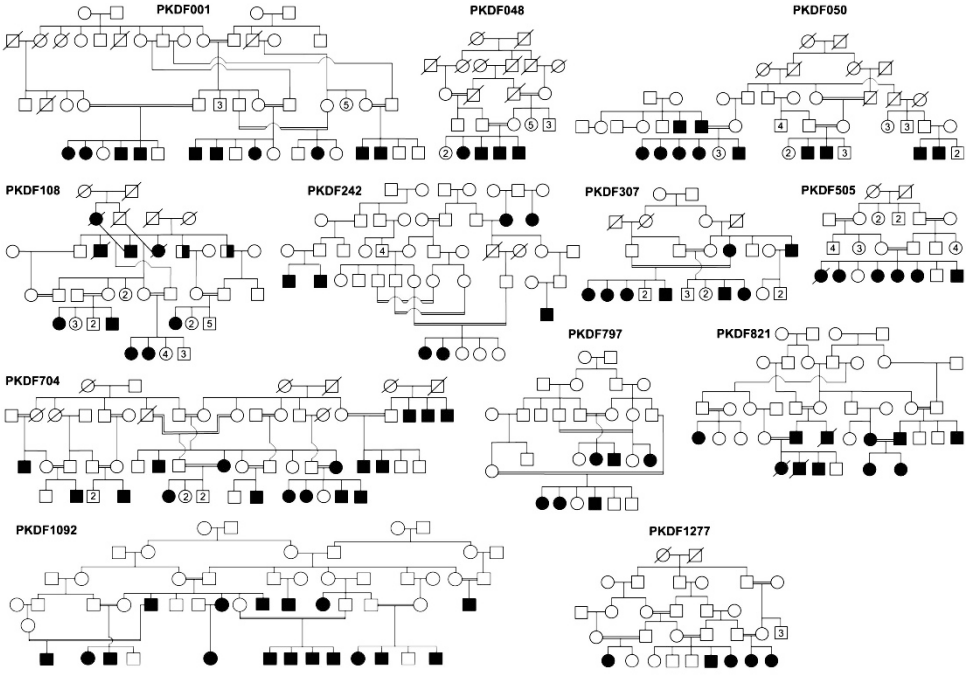


Figure 2 Pakistani DFNB29 families. Filled symbols represent individuals with prelingual, sensorineural hearing loss and double horizontal lines indicate a consanguineous marriage. Half filled symbols in family PKDF108 are individuals (ages 72 and 75 years) that have age-related hearing loss. Numbers represent unaffected siblings.

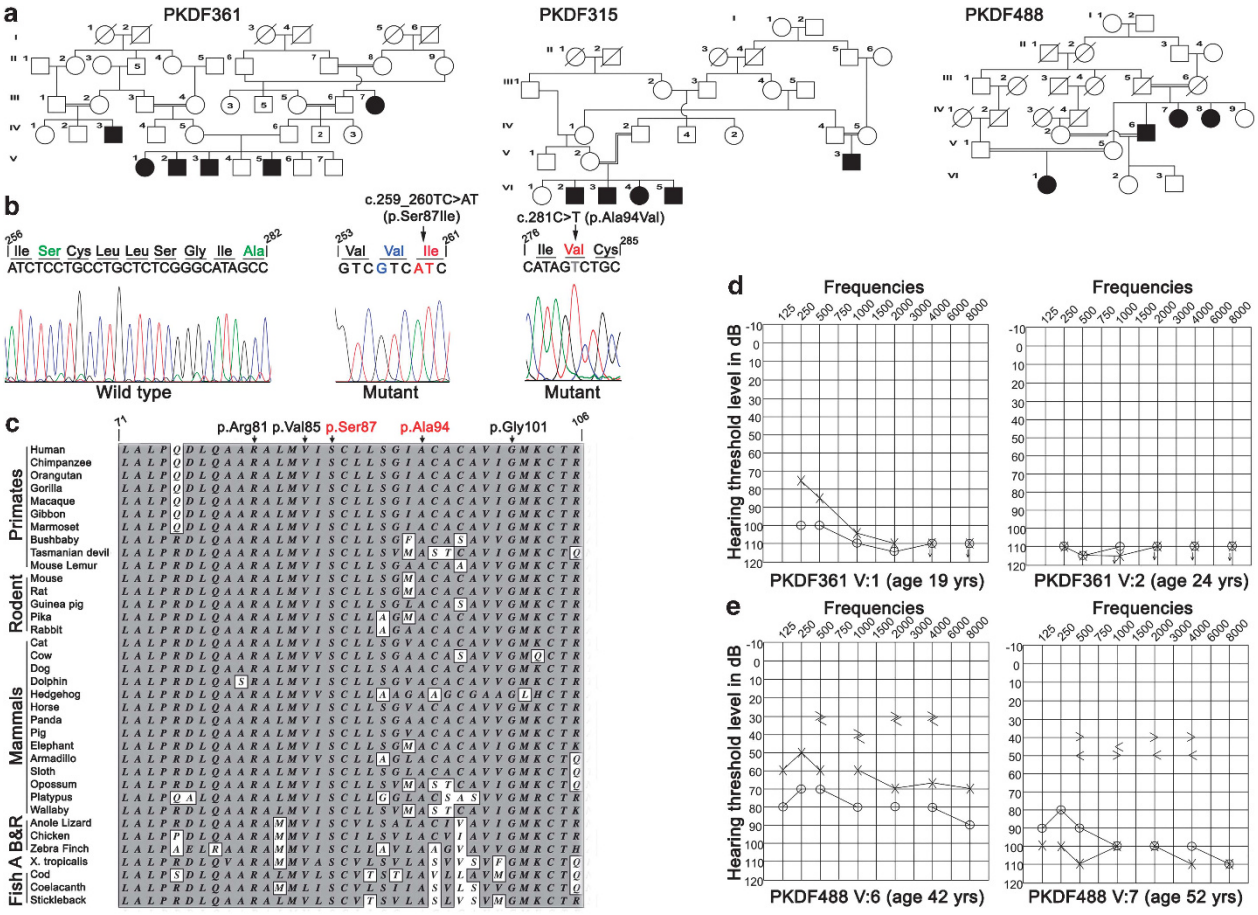


Figure 3 Pedigree of three DFNB29 families, sequencing chromatograms, pure tone audiograms and ClustalW alignment of 36 claudin 14 orthologs. (**a**) Pedigrees of families PKDF361, PKDF315 and PKDF488. Filled symbols represent affected individuals. (**b**) Wild-type and homozygous mutant nucleotide sequence chromatograms of exon 3 of *CLDN14* illustrating homozygosity for the c.259_260TC>AT (p.Ser87Ile) and c.281C>T (p.Ala94Val) mutations (arrows). Shown in green are the amino acids that are mutated (red) in the DFNB29 families, while blue color represent the non-deleterious change found in family PKDF361. (**c**) ClustalW multiple sequence alignment of the 36 amino acids of claudin 14 shows that p.Ser87 and p.Ala94 residues are conserved across species (shaded background). For comparison, three previously reported mutated residues, p.Arg81, p.Val85 and p.Gly101 are also shown. Amino acids are numbered with reference to GenBank Accession number NP_036262. B&R: birds & reptiles; A: amphibians. (**d**) Pure tone air and bone conduction thresholds for family PKDF361 individuals V:1 (19 yo female), V:2 (24 yo male). Individual V:1 has severe to profound hearing loss in her left ear, whereas right ear showed profound deafness. Right ear air conduction: O; Left ear air conduction: X; Right ear bone conduction: >; Left ear bone conduction: <;↓indicates the threshold level beyond the measurable range. (**e**) Pure tone air and bone conduction thresholds for family PKDF488 individuals V:6 (42 yo male) revealed moderate sensorineural hearing loss in his left ear, whereas the right ear showed a severe degree of hearing loss. In contrast, individual V:7 (52 yo female) had severe to profound, bilateral, sensorineural hearing impairment.

2010-0291). Written informed consent was obtained from adult subjects and parents of minor subjects. Hearing was evaluated in audiology clinics by pure tone audiometry at octave frequencies with intensities up to 110 dBHL. Vestibular function was evaluated by tandem gait and Romberg testing.[19]

Genotype and mutational analysis

Genomic DNA was extracted from 10 ml of peripheral venous blood as described.[20,21] Three fluorescently labeled microsatellite markers (*D21S2078*, *D21S1252* and *D21S2080*) linked to *CLDN14* were PCR-genotyped as described.[20] Primers for PCR amplification and *CLDN14* sequencing were designed using Primer3 (http://frodo.wi.mit.edu/).

Co-segregation of the mutations with hearing loss in each family was demonstrated for all subjects participating in this study. Control DNA samples from ethnically matched Pakistanis were sequenced to ascertain novel variants of *CLDN14*. Three prediction programs, SIFT,[22] Polyphen-2 (Adzhubei *et al*.[23]) and MutationTaster[24] were used to evaluate the potential effect of each novel missense mutation.

Immunolocalization of claudin 14 in the mouse organ of Corti

Immunolocalization of claudin 14 using tissue from C57BL/6 mouse organ of Corti was performed as described previously using a custom rabbit polyclonal PB108 anti-claudin 14 antibody with validated specificity. There was no immunolocalization signal observed when tissue from a *Cldn14* knockout mouse was used.[5]

RESULTS

Pathogenic variants of CLDN14

We reported that mutations of *CLDN14* cause DFNB29 deafness.[1] Subsequently, deafness segregating in 15 additional families (Figures 2 and 3) was found to be linked to STR markers for *CLDN14* (Table 1). Sequence analysis of *CLDN14* revealed four likely pathogenic variants. Among the five variants of *CLDN14*, two were novel missense substitutions p.Ser87Ile (c.259_260TC>AT) and p.Ala94Val (c.281C>T). In family PKDF361, we detected three nearly adjacent nucleotides changes (c.256A>G and c.259_260TC>AT; Figure 3b),

Table 1 *CLDN14* variants in Pakistani families segregating DFNB29 hearing loss[a]

		Haplotype								
Family	*Ethnicity*	*D21S2078*	*D21S1252*	*D21S2080*	*Nucleotide variation*	*Predicted effect*	*SIFT*	*Polyphen-2*	*MutationTaster*	*Reference*
PKSR9a	Punjabi	152	244	174	c.254T>A	p.Val85Asp	Deleterious	Damaging	Disease causing	1
PKDF001	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF009	Punjabi	152	244	174	c.254T>A	p.Val85Asp				25
PKDF048	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF050	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF108	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF242	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF307	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF505	Punjabi	156	244	166	c.254T>A	p.Val85Asp				This study
PKDF704	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF797	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF821	Punjabi	152	244	174	c.254T>A	p.Val85Asp				This study
PKDF1277	Balochi	154	234	166	c.242G>A	p.Arg81His				2,This study
PKDF1092	Punjabi	154	246	166	c.398delT	p.Met133ArgfsX23	Deleterious	Damaging	Disease causing	This study
PKSN6	Punjabi	154	246	166	c.398delT	p.Met133ArgfsX23				1
PKDF361	Sindhi	156	246	166	c.256A>G	**p.Ile86Val**	Tolerated	Benign	Polymorphism	This study
					c.259_260TC>AT	**p.Ser87Ile**	Deleterious	Damaging	Disease causing	This study
PKDF315	Punjabi	154	288	172	c.281C>T	**p.Ala94Val**	Deleterious	Damaging	Disease causing	This study
PKDF488	Punjabi	154	228	172	c.281C>T	**p.Ala94Val**				This study

[a]All variants were found in the homozygous state, and previously unreported variants are shown in bold.

which are predicted to result in two substitutions (p.Ile86Val and p.Ser87Ile; Table 1). SIFT, Polyphen-2 and MutationTaster predicted that p.Ile86Val is a benign polymorphism. Furthermore, claudin 14 orthologs in Armadillo, cow and hedgehog have a valine residue at position 86. Therefore, we considered p.Ile86Val as a non-pathogenic substitution although no carriers of c.256A>G were found in our 184 control subjects or in 1000 Genome and NHLBI-ESP databases. These data indicate that c.256A>G is a rare and benign variant whereas p.Ser87Ile is predicted to be deleterious (Table 1).

In addition, we detected p.Ala94Val in families PKDF315 and PKDF488 (Figures 3a and b). Both p.Ser87Ile and p.Ala94Val mutations affect amino acid residues that are conserved among 36 claudin 14 orthologs (Figure 3c). No carriers of c.259_260TC>AT and c.281C>T were found among 384 ethnically matched control chromosomes that we Sanger-sequenced, in the 1000 Genome database, or in 5400 individuals listed in the NHLBI-ESP variant database. Our data indicate that these variants are not common polymorphisms, and in each family homozygosity for the mutant allele of *CLDN14* co-segregated with deafness, whereas carriers had normal hearing.

We observed the previously reported variants c.254T>A (p.Val85Asp) in 12 families, c.242G>A (p.Arg81His) in one family and c.398delT (p.Met133ArgfsX23) in one family. All of these mutations co-segregated with deafness. STR markers linked to *CLDN14* were genotyped in unrelated affected individuals homozygous for the c.254T>A and c.398delT mutations and for both alleles the flanking haplotypes were consistent with a founder effect (Table 1).

DFNB29 hearing loss phenotype

Pure tone air and bone conduction audiometry revealed inter- and intra-familial variability in the severity of hearing loss (Figures 3d, e and 4) in these families. The affected individuals of family PKDF361 had prelingual severe to profound hearing loss across all the tested frequencies (Figure 3d). The 42-year-old affected individual (V:6) of family PKDF488 had bilateral moderate to severe, sensorineural hearing loss, whereas his sibling (V:7 age 52 years) had profound hearing loss across almost all the frequencies (Figure 3e). Phenotypic variability was associated with the known p.Val85Asp mutation (Figure 4), an allele we reported in two large multi-generation Pakistani families segregating prelingual, severe to profound hearing loss.[1,25] In this study, we identified 11 additional families segregating the p.Val85Asp allele of *CLDN14*. Hearing loss in members of these families ranged from moderate to profound with greater severity at higher frequencies (Figure 4), which is in agreement with degeneration of sensory hair cells from base to apex seen in the *Cldn14* knockout mice.[5]

DISCUSSION

CLDN14 mutations are a common cause of recessive hearing loss in the Pakistani population as mutations of this gene account for 2.25% (18 of 800 families; 95% CI, 1.4–3.5) of deafness in the NCEMB Pakistani study cohort (Table 2).[1–3,25] The probands of the NCEMB deafness cohort are usually students in schools for the hearing impaired and are usually profoundly deaf from birth. We may have underestimated the contribution of mutations of *CLDN14* to hearing loss by overlooking individuals with mild or delayed-onset hearing loss due to mutations of *CLDN14*.

Severity of hearing thresholds in our DFNB29 families does not seem to be directly correlated with age of the subject (Figure 4). For example, although similar in age, a 23-year-old affected woman of family PKDF505 has significantly better hearing, especially at low frequencies, than her 21-year-old sister (Figure 4). Similarly, in our previous study, audiograms from multiple affected individuals of family PKDF009 did not show any correlation between hearing thresholds and age of the affected individuals.[25] It is possible that an environmental factor (for example, drugs, noise and so on) may be the cause of this inter- and intra-familial phenotypic variability in hearing thresholds. We hypothesized that a genetic modifier is the

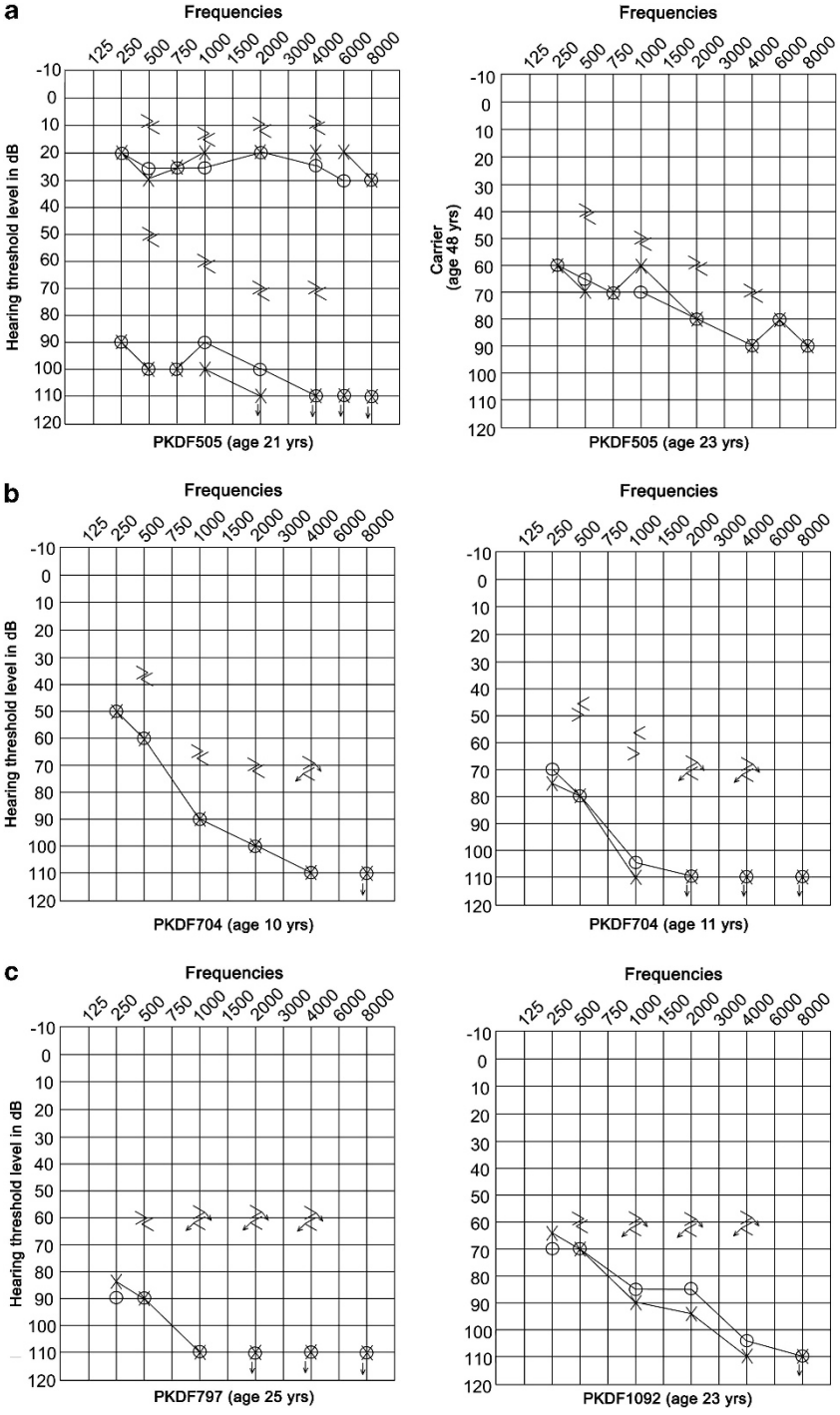


Figure 4 Pure tone air and bone conduction measurements from DFNB29 families segregating p.Val85Asp revealed intra- and inter-familial variability in thresholds. (**a**) Twenty-one-year-old affected individual of family PKDF505 has profound, bilateral, sensorineural hearing loss, whereas his 23-year-old sibling has moderate to severe, bilateral sensorineural hearing impairment. Shown also is their 48-year-old mother, a carrier of p.Val85Asp allele, with normal hearing thresholds across all frequencies. (**b**) Ten-year-old affected individual of family PKDF704 has moderate to profound, bilateral, sensorineural hearing loss, whereas her 11-year-old sibling has severe to profound, bilateral sensorineural hearing impairment. (**c**) Twenty-five-year-old affected individual of family PKDF797 has severe to profound hearing loss, whereas a 23-year-old affected individual of family PKDF1092 has moderate to profound, sensorineural hearing loss.

cause of this inter- and intra-familial phenotypic variability in hearing thresholds, especially in individual harboring the same *CLDN14* mutation. Similar phenotypic variability has been documented for many other deafness-causing mutations in humans.[26–28] The sensory epithelium of mouse inner ear expresses claudin family members 1, 2, 3, 9, 10, 12, 14 and 18,[29] and variation in expression of these other claudins in the auditory system may be modulating the severity of the hearing loss phenotype.

Table 2 Contribution of different genes to hearing loss in Pakistani families

Gene	Locus	Percentage (fraction) of families	95% CI	Reference
CLDN14	*DFNB29*	2.25 (18/800)	1.4–3.5	1,25,This study
SLC26A4	*DFNB4/PDS*	7.23 (56/775)	5.6–9.2	35,36
GJB2	*DFNB1*	6.12 (12/196)	3.5–10.4	37
HGF	*DFNB39*	5.12 (41/800)	3.8–6.8	38
TMC1	*DFNB7/11*	3.41 (19/557)	2.2–5.3	39,40
MYO15A	*DFNB3*	3.33 (20/600)	2.2–5.1	41,42
OTOF	*DFNB9*	2.33 (13/557)	1.4–4.0	43
TRIC	*DFNB49*	1.30 (11/841)	0.7–2.3	28,44
TRIOBP	*DFNB28*	1.29 (10/775)	0.7–2.3	45,46
ILDR1	*DFNB42*	1.29 (11/850)	0.7–2.3	47
MYO6	*DFNB37*	1.20 (3/250)	0.4–3.4	48
GIPC3	*DFNB72*	0.75 (6/800)	0.3–1.6	49
TPRN	*DFNB79*	0.50 (4/800)	0.2–1.2	50
RDX	*DFNB24*	0.36 (2/557)	0.1–1.2	51

Abbreviation: CI, confidence interval.

Mutations in other tight junction proteins are also known to cause deafness in humans and mice.[28,30,31] Claudin 11-deficient mice are deaf, demonstrating that this tight junction protein is also necessary for maintenance of the intra-strial compartment and generation of the endocochlear potential.[30] Both claudin 14 and claudin 9 mutant mice also display deafness with no vestibular defects.[5,31] Claudin 14 is expressed specifically by the cells forming the reticular lamina (Figures 1b and c) and the vestibular sensory epithelia, whereas claudin 9 is present in nearly all of the epithelia lining the scala media and the vestibular organs.[5,31,32] Mouse mutants of *Cldn14* and *Cldn9* both display cochlear hair cell loss by the second week of life, which progresses rapidly to include the entire cochlea within the next few weeks.[5,31] Loss of either of these claudins results in increased paracellular permeability of K^+ in the reticular lamina and an elevation in the K^+ concentration around the basolateral regions of hair cells, which is toxic.[5,31,33,34] Thus, both claudins are required to form a permeability barrier against cations.[5,31] The two novel mutations identified in this study, p.Ser87Ile and p.Ala94Val, are within the second transmembrane domain and in the vicinity of p.Val85, which has been shown to affect the membrane localization of claudin 14.[4] Therefore, these two new mutations might also impair the trafficking of claudin 14 to the plasma membrane.

Two of the mutations (p.Arg81His; p.Ser87Ile) of *CLDN14* identified in this study were found only once. However, unlike these two rare mutations, three other mutations (p.Val85Asp; p.Met133ArgfsX23; p.Ala94Val) account for ~89% of the *CLDN14* alleles we found in our cohort of Pakistani families segregating deafness (Table 1). In conclusion, there is considerable genetic and allelic heterogeneity that accounts for recessively inherited deafness in Pakistan (Table 2). *CLDN14* mutations are a frequent cause of genetic deafness in this population and are associated with marked inter- and intra-familial variability in hearing thresholds.

ELECTRONIC DATABASE INFORMATION

NHLBI-ESP variant database, http://evs.gs.washington.edu/EVS/
Primer3, http://frodo.wi.mit.edu/
1000 Genome, http://browser.1000genomes.org/
TMpred, http://www.ch.embnet.org/software/TMPRED_form.html
SIFT, http://sift.jcvi.org/
Polyphen-2, http://genetics.bwh.harvard.edu/pph2/
MutationTaster, http://www.mutationtaster.org/index.html

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the families for the participation and cooperation, and R Bhatti and T Kausar for technical assistance and Drs D Drayna, G Nayak and K Kurima for critiques of the manuscript. This work was also supported by an Action on Hearing Loss grant and National Institute on Deafness and Other Communication Disorders (NIDCD/NIH) research grants R01 DC011803 and R01 DC011748 to SR and intramural funds from NIDCD DC000039-15 to TBF.

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