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Novel *CLDN14* Mutations in Pakistani Families With Autosomal Recessive Non-Syndromic Hearing Loss

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Abstract

Mutations in the *CLDN14* gene are known to cause autosomal recessive (AR) non-sydromic hearing loss (NSHL) at the DFNB29 locus on chromosome 21q22.13. As part of an ongoing study to localize and identify NSHL genes, the ARNSHL segregating in four Pakistani consanguineous families were mapped to the 21q22.13 region with either established or suggestive linkage. Given the known involvement of *CLDN14* gene in NSHL, DNA samples from hearing-impaired members from the four families were sequenced to potentially identify causal variants within this gene. Three novel *CLDN14* mutations, c.167G>A (p.Trp56*), c.242G>A (p.Arg81His), and c. 694G>A (p.Gly232Arg), segregate with hearing loss (HL) in three of the families. The previously reported *CLDN14* mutation c.254T>A (p.Val85Asp) was observed in the fourth family. None of the mutations were detected in 400 Pakistani control chromosomes and all were deemed damaging based on bioinformatics analyses. The nonsense mutation c.167G>A (p.Trp56*) is the first stop codon mutation in *CLDN14* gene to be identified to cause NSHL. The c.242G>A (p.Arg81His) and c.694G>A (p.Gly232Arg) mutations were identified within the first extracellular loop and the carboxyl-tail of claudin-14, respectively, which highlights the importance of the extracellular domains and phosphorylation of cytoplasmic tail residues to claudin function within the inner ear. The HL due to novel *CLDN14* mutations is prelingual, severe-to-profound with greater loss in the high frequencies.

Keywords

autosomal recessive non-sydromic hearing loss; claudin-14; *CLDN14*; Pakistan

INTRODUCTION

Tight junctions are important to maintain compartmentalization in eukaryotic organ systems, and in the inner ear, they form a barrier between the endolymphatic and perilymphatic fluid compartments while maintaining membrane cell polarity and modulating inter-cellular permeability to solutes, ions and water. Of the proteins that comprise tight junctions, only

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ELECTRONIC DATABASE INFORMATION

The following URLs were accessed for data in this article: Hereditary Hearing Loss Homepage (http://hereditaryhearingloss.org) UCSC Genome Browser (http://genome.ucsc.edu) OMIM (http://www.omim.org)

Conflict of interest: All authors declare no competing interests.

the claudin proteins are known to polymerize into tight junction fibrils, and this ability is probably related to the function of selective paracellular permeability which is thought to be exclusive to claudin proteins [Angelow et al., 2008]. Knowledge of claudin function has been culled mainly from in vitro expression studies, mouse knockout models and human phenotypes from claudin mutations [Angelow et al., 2008]. Claudin proteins are widely expressed in the inner ear, with 10 types of claudin proteins of differential distribution or localization [Kitajiri et al., 2004]. Claudin-14 protein increases transepithelial resistance by decreasing cation permeability, particularly for potassium [Ben-Yosef et al., 2003], which has higher concentrations in endolymph than perilymph. Although mouse mutant models for *Cldn14* [Ben-Yosef et al., 2003], *Cldn11* [Gow et al., 2004], and *Cldn9* [Nakano et al., 2009] are deaf, so far from the *Claudin* family of genes only the *CLDN14* gene (OMIM 605608) has been shown to be involved in human hearing loss (HL). Previously 3 *CLDN14* mutations (1 frameshift and 2 missense) have been reported to be responsible for autosomal recessive (AR) non-sydromic hearing loss (NSHL) due to the DFNB29 locus on 21q22.13 [Wilcox et al., 2001; Wattenhofer et al., 2005]. In this report, four *CLDN14* mutations, of which 3 (1 non-sense and 2 missense) are novel, were identified in four consanguineous Pakistani families. From a collection of 353 consanguineous Pakistani families with ARNSHL, who have either undergone genome-wide linkage scans and/or for which the causal ARNSHL gene variant has been identified, these are the only families which were identified with NSHL due to mutations in the *CLDN14* gene.

MATERIALS AND METHODS

Study approval was granted by the Institutional Review Boards of the Quaid-I-Azam University and the Baylor College of Medicine and Affiliated Hospitals. Informed consent was obtained from all participating family members. The four consanguineous families in which the *CLDN14* mutations were identified (Fig. 1) come from different provinces of Pakistan with varied linguistic origins: Family 4158 from Sindh province speaks Sindhi; Family 4209 from Punjabi province speaks Sairiki; Family 4306 speaks Pashto, and is from Khyber Pakhtoonkhwa; and Family 4413 resides in the Kashmiri-speaking region of Azad Jammu and Kashmir province. From the clinical history, HL was prelingual and no environmental factors (e.g., maternal/perinatal, infectious, traumatic, ototoxic) were identified as possible causes of HL. Syndromic and vestibular features were ruled out through careful physical examination that included balance and gait testing. Hearing in selected individuals with HL was tested at 250–8,000 Hz using a portable audiometer (Fig. 2b).

Venous blood was obtained from 2 hearing and 3 HL members of Family 4158, 4 hearing and 6 HL members of Family 4209, and from 7 members of Family 4413, 3 of whom have HL (Fig. 1). For Family 4306, 3 of 6 family members who provided venous blood have HL. After standard extraction of genomic DNA, the coding region of the *GJB2* (OMIM 121011) gene was sequenced in HL individuals and was negative for *GJB2* variants. DNA samples from the four families underwent a whole genome linkage scan at the Center for Inherited Disease Research (CIDR) using the Infinium HumanLinkage-12 panel which contains ~6,000 SNP marker loci. Quality control of genotype data was performed using PedCheck [O'Connell and Weeks, 1998] to detect Mendelian inconsistencies and through MERLIN [Abecasis et al., 2002] to detect occurrences of double recombination events over short genetic distances, which are most likely due to genotyping error. MLINK of FASTLINK package [Cottingham et al., 1993] and Allegro 1.2c [Gudbjartsson et al., 2000] were used for 2-point and multipoint linkage analyses, respectively, while SimWalk2 [Sobel and Lange, 1996] was used for haplotype reconstruction. An AR mode of inheritance with complete penetrance and a disease allele frequency of 0.001 were assumed. Marker allele frequencies were estimated from observed and reconstructed genotypes of founders from the 4 families

and 50 additional families who underwent genome scan at the same time at CIDR. The Rutgers combined linkage-physical map of the human genome Build 36 version [Matise et al., 2007] was used to determine genetic map positions for the multipoint linkage analysis. For markers which are not on the Rutgers map, the genetic map positions were interpolated using the physical positions from the human reference sequence (Build36).

The coding region with in exon3 of the *CLDN14* (NM_001146077.1) gene was sequenced in 2 HL and 1 hearing relative per family, namely: HL individuals V-1 and V-2 and hearing individual IV-1 for family 4158; HL individuals VI-1 and VI-4 and hearing individual III-2 for family 4209; HL individuals IV-1 and IV-3 and hearing individual III-1 for family 4306; and HI individuals V-3 and V-5 and hearing individual IV-1 of Family 4413 (Fig. 1). After PCR-amplification and purification, sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA). When *CLDN14* mutations were identified using Sequencher software V4.9 (Gene Codes Corp., Ann Arbor, MI), the coding region was sequenced using DNA samples from additional family members and 200 unrelated hearing individuals from Pakistan. Bioinformatics analyses tools PolyPhen-2 [Adzhubei et al., 2010], SIFT [Ng and Henikoff, 2001], Pmut [Ferrer-Costa et al., 2005], and SNAP [Bromberg et al., 2008] were used to determine pathogenicity of identified mutations as well as previously reported ARNSHL *CLDN14* mutations. Homologous proteins were searched at the UniProt Knowledgebase (Swiss-Prot + TrEMBL) [Bairoch et al., 2004] using blastp [Altschul et al., 1990] and aligned with ClustalW2 [Thompson et al., 1994] to determine evolutionary conservation.

RESULTS

All four families were shown to have either established (LOD ≥ 3.3) or suggestive (2.0 ≤ LOD <3.3) linkage to chromosome 21q22.13, with maximum multipoint LOD scores ranging from 2.1 to 5.5 (Table I). The homozygous region for all four families includes the chromosomal region 21q22.13 which contains the known ARNSHL gene, *CLDN14* (Fig. 1).

Upon sequencing of the *CLDN14* gene, a novel non-sense mutation c.167G>A (p.Trp56★) was identified to segregate with ARNSHL in Family 4158. Two novel missense mutations c.242G>A (p.Arg81His) and c.694G>A (p.Gly232Arg) which segregate with HL were also identified in Families 4209 and 4413, respectively (Table II). Although an additional missense substitution c.11C>T (p.Thr4Met) was also identified in Family 4413, this variant was deemed to be non-causal because it was detected in 8 out of 400 control chromosomes and was also classified as non-pathogenic in other NSHL studies [Uyguner et al., 2003; Wattenhofer et al., 2005]. Additionally bioinformatic tools Polyphen-2, SIFT, Pmut, and SNAP all deemed the variant to be benign (data not shown). In Family 4306 a known pathogenic mutation, c.254T>A (p.Val85Asp), was also uncovered in the *CLDN14* gene. This mutation was previously observed in three Pakistani families [Wilcox et al., 2001; Bashir et al., 2010] (Table II).

All four mutations, c.167G>A(p.Trp56★),c.242G>A(p.Arg81His), c.254T>A (p.Val85Asp), and c.694G>A (p.Gly232Arg), as well as two previously described causal mutations in the *CLDN14* gene, were not detected in 400 Pakistani control chromosomes. The three missense mutations were also deemed damaging, pathological, or non-neutral by PolyPhen-2, SIFT, Pmut, and SNAP (Table II). The four residues Trp56, Arg81, Val85, and Gly232 are identical in homologous proteins from at least 9 non-human species, including 6 mammalian, 1 amphibian, 1 avian, and 1 fish. Among 25 human claudin proteins, the Arg81 residue is highly conserved in 20 claudin proteins, while the Val85 residue is conserved in 12 claudin proteins. On the other hand, the Gly232 residue is conserved only in claudin-3

and claudin-9, which have the same D-Y-V sequence for the PDZ domain-binding motif at the C-terminus.

DISCUSSION

Two novel mutations p.Trp56★ and p.Arg81His occur within the first extracellular loop (EL1), while the novel mutation p.Gly232Arg can be found at the carboxyl tail of claudin-14 (Fig. 2a). The positively charged Arg residue at the end of EL1 is highly conserved among claudin proteins, and a missense mutation p.Arg149Lys at this position in claudin-1 results in familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) [Weber et al., 2001] due to a complete loss of function, presumably from misfolding and/or lack of transport to the plasma membrane [Hou et al., 2005]. Based on the hypothesis that charged amino acids of EL1 have specific spatial location and facing in order to maintain the cation pore [Krause et al., 2009], it is possible that the charge reduction from the p.Arg81His mutation would also result in misfolded protein. On the other hand, the production of 183 amino acid residues, which contribute to the formation of three transmembrane domains and three topological domains, is predicted to be stopped by the non-sense mutation p.Trp56★. Stop mutations at the EL1 and C-terminus of claudin-16 also cause FHHNC with retention in the endoplasmic reticulum or endocytosis to lysosomes after weak surface expression [Müller et al., 2006]. Truncated claudin-14 protein may also be hypothesized to curtail transport to or lack expression in plasma membrane, therefore preventing proper formation of tight junctions.

The Gly232 residue is located between Ser231 and Tyr233, which are identified as phosphorylation sites by GPS 2.1 [Xue et al., 2008] (Fig. 2a). The Gly232 residue is identical in homologous proteins from 11 non-human species, but among 25 human claudin proteins, it is conserved only in claudin-3 and claudin-9. It should be noted that both claudin-3 and claudin-9 are expressed in mouse inner ear [Kitajiri et al., 2004], while deafness has been demonstrated in a *Cldn-9* mutant mouse [Nakano et al., 2009]. Although the carboxyl tail is highly variable in structure among the claudins, according to SIFT prediction, any change in residue for Gly232 and Tyr233 in claudin-14 will not be tolerated and will result in dysfunction. Since phosphorylation can occur at multiple sites of claudin protein simultaneously in order to ensure apical localization and modulate transepithelial resistance of the permeability barrier [D'Souza et al., 2007], it is plausible that the Gly232 residue facilitates protein kinase activity at adjacent residues, and that the increase in positive charge from the p.Gly232Arg mutation interferes with phosphorylation. Dephosphorylated claudin proteins are dissociated from tight junctions and translocated to lysosomes for degradation [Ikari et al., 2006]. Though the main phosphorylation site within the C-terminus is the PDZ domain-binding site at residues 237–239 of claudin-14 which attaches to other tight junction proteins, such as zonula occludens proteins [Itoh et al., 1999], the cytoplasmic tail residues prior to the PDZ domain-binding motif of claudin proteins have been shown to direct proper localization within the tight junction [Ruffer and Gerke, 2004] or increase stability through half-life regulation independent of the PDZ domain-binding site [Van Itallie et al., 2004].

The previously reported c.254T>A (p.Val85Asp) mutation, which is located in the second transmembrane domain (Fig. 2a), also results in mislocalized protein from failed export to the plasma membrane [Wattenhofer et al., 2005]. This mutation can cause variable severity in hearing thresholds with greatest affectation in the high frequencies, although average thresholds for three *CLDN14* families with this mutation mostly cluster at the severe-to-profound range and all audiograms display a downward slope [Ahmed et al., 2002; Bashir et al., 2010]. Likewise in three HL individuals reported here, each of whom belongs to a different family and carries a different *CLDN14* mutation, the HL is severe-to-profound

with the same pattern of greater loss at higher frequencies as previously reported for *CLDN14* mutations (Fig. 2b).

Although non-progression of hearing severity over time has not been well-documented for *CLDN14* [Bashir et al., 2010], it is unlikely that the variability in hearing thresholds is age-related given that in this report the variable hearing levels seen in three individuals with *CLDN14* mutations were documented at similar ages (Fig. 2b). Additionally, in previous reports, audiograms from multiple affected individuals from the same family and across different families with the same p.Val85Asp mutation did not support an increase in HL severity with age [Ahmed et al., 2002; Bashir et al., 2010]. Non-progressive, prelingual HL with profound high-frequency loss due to *CLDN14* mutations in humans is consistent with the *Cldn14*$^{-/-}$ mouse model which has been demonstrated to have degeneration of hair cells primarily of the cochlear base prior to the age of onset of hearing function [Ben-Yosef et al., 2003]. Weak or no surface expression of mutant claudin-14 protein might also be related to the early-onset and severe-to-profound HL. For *CLDN16*, mutations that result in failure to localize to the tight junction caused earlier-onset FHHNC with faster progression when compared to other mutations that are associated with partial loss of function [Konrad et al., 2008].

The finding of novel mutations in claudin proteins have allowed the identification of critical residues around the paracellular ion pore, thus permitting hypothesis formation on claudin and tight junction physiology [Krause et al., 2009]. Because claudin proteins have been used as cancer biomarkers (e.g., in gastrointestinal epithelium) [Semba et al., 2008], synthetic peptides that mimic-specific EL regions have been constructed and were shown to cause reversible changes to tight junction permeability in vivo, which suggests their potential utility for drug delivery across epithelia [Mrsny et al., 2008]. Similarly, increased knowledge of critical residues and domain functions of claudins that are expressed in the inner ear can provide new insights on pathophysiology and therapeutic possibilities for HL.

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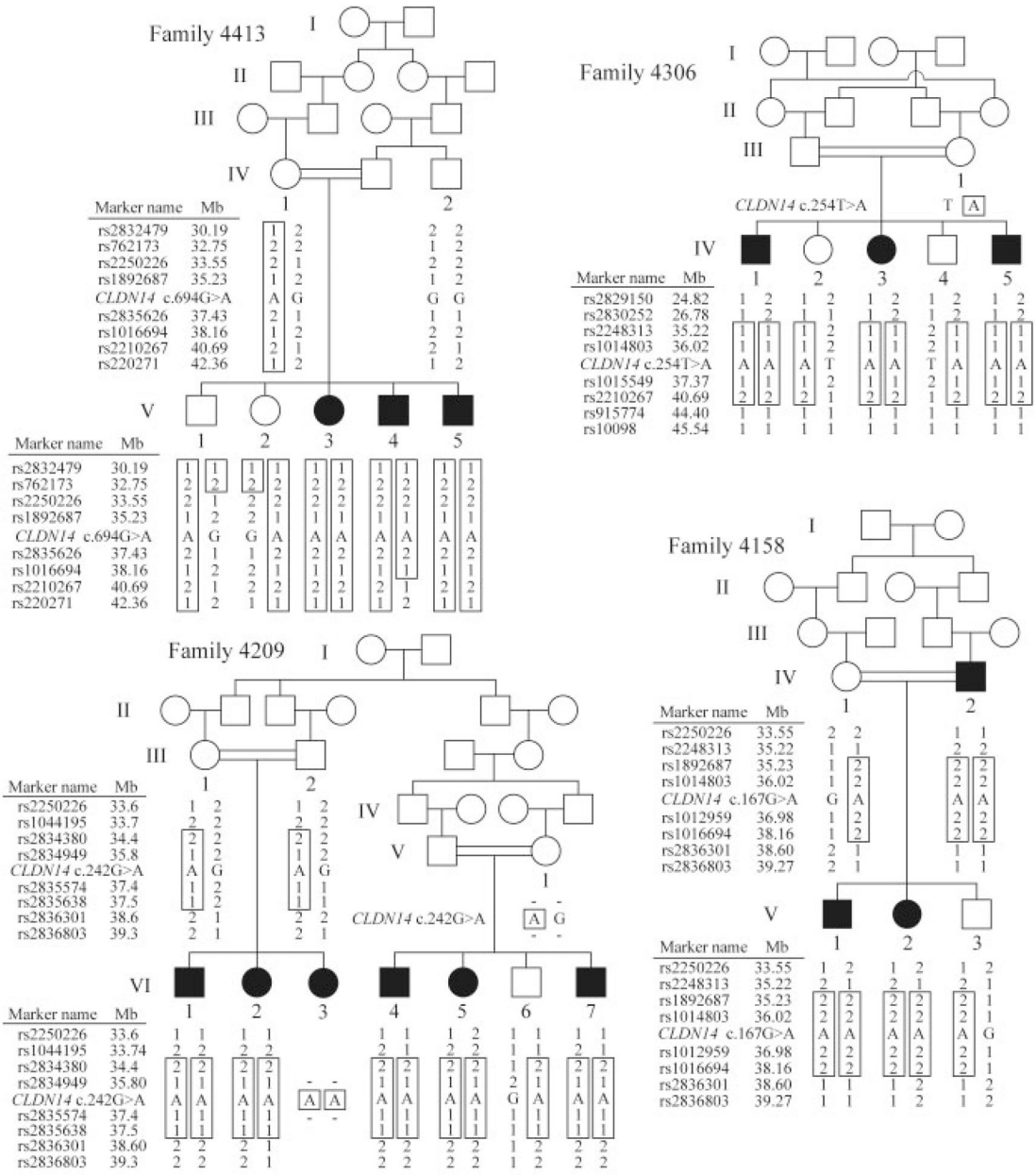

FIG. 1.

Pedigree drawings of three ARNSHL families with *CLDN14* mutations. *Filled* symbols represent hearing-impaired individuals, *clear* symbols denote hearing individuals. The haplotype segregating with ARNSHL is shown in a *box* and includes genotypes for SNP markers within the region and variant for the *CLDN14* mutation. Family 4158 segregates the novel mutation c.167G>A (p.Trp56*), Family 4209 the novel mutation c.242G>A (p.Arg81His), Family 4306 the known mutation c.254T>A (p.Val85Asp), and Family 4413 the novel mutation c.694G>A (p.Gly232Arg).

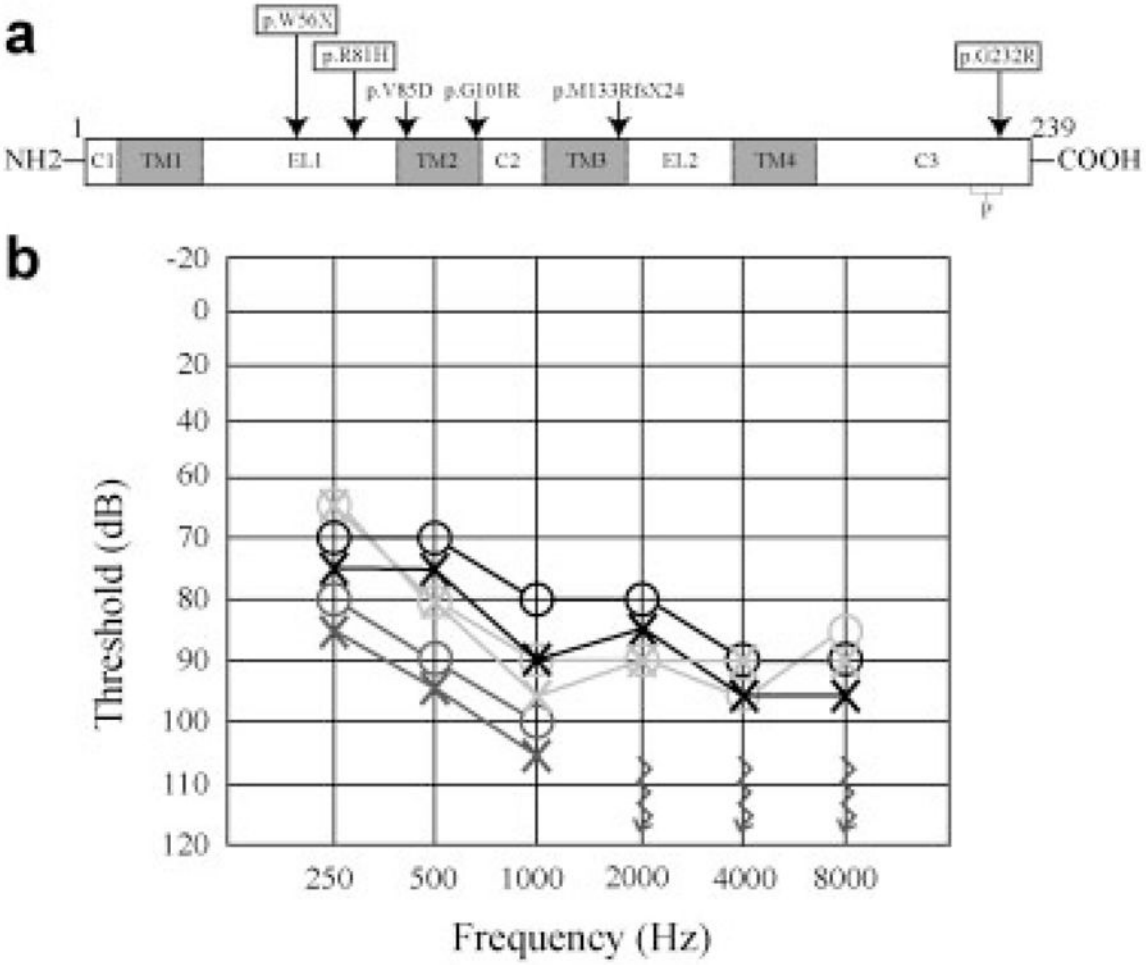

FIG. 2.

Panel a: Schematic representation of the coding region of *CLDN14* gene. The relative positions of causal variants are indicated with *arrow*. The *box* indicates newly identified causal variants from this study. EL, extracellular loop; TM, transmembrane domain; C, cytological domain; P, phosphorylation sites. Panel b: Air-conduction thresholds for individuals with *CLDN14* mutations. Right hearing is denoted by *circles*, left by *crosses*. *Black* markings indicate audiogram for individual V-4 (23 years of age) of Family 4413 who has the c.694G>A (p.Gly232Arg) mutation, while markings in *light gray* are from individual VI-1 (20 years of age) of Family 4209 with the c.242G>A (p.Arg81His) mutation. Both audiograms show bilateral severe-to-profound hearing loss at all frequencies. Audiogram in *dark gray* for individual IV-1 (25 years of age) of Family 4306 who is homozygous for the c. 254T>A (p.Val85Asp) mutation demonstrates severe-to-profound loss at 250–1,000 Hz and no hearing at higher frequencies.

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TABLE I

LOD Scores for Families 4158, 4209, 4306, and 4413 Within the 21q Region Which Includes the *CLDN14* Gene [*]

				2-Point LOD at recombination fraction θ =						
Marker loci[a]	**Physical map position[b]**	**Genetic map position[c]**	**Multipoint LOD**	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
Family 4158 (21q22.12–q22.13)										
rs2250226	33,554,186	37.77	−2.0	−2.6	−1.2	−0.6	−0.3	−0.1	0.0	0.0
rs2248313	**35,223,308**	**38.03**	−2.0	−2.6	−1.2	−0.6	−0.3	−0.1	0.0	0.0
rs1892687	35,233,892	40.73	2.1	0.8	0.7	0.7	0.6	0.4	0.2	0.1
rs1014803	36,015,910	42.59	2.1	0.9	0.9	0.8	0.6	0.4	0.2	0.1
rs1012959	36,983,492	45.16	2.1	0.7	0.7	0.6	0.5	0.4	0.2	0.1
rs1016694	38,156,688	46.76	2.1	1.0	0.9	0.8	0.7	0.5	0.2	0.1
rs2836301	**38,599,459**	**47.66**	−∞	−∞	−1.3	−0.6	−0.4	−0.2	−0.1	0.0
rs2836803	39,273,650	49.22	−∞	−∞	−1.2	−0.6	−0.3	−0.2	−0.1	0.0
Family 4209 (21q22.11–q22.2)										
rs2250226	33,554,186	37.77	−∞	−∞	0.0	0. 6	0.7	0.5	0.3	0.1
rs1044195	**33,743,677**	**38.03**	−∞	−∞	−2.3	−1.0	−0.5	−0.2	−0.1	−0.1
rs2834380	34,413,370	39.02	4.4	1.1	1.1	1.0	0.9	0.6	0.4	0.1
rs2834949	35,801,553	42.08	5.5	3.2	3.1	2.8	2.4	1.7	1.0	0.4
rs2835574	37,377,194	45.73	5.4	1.6	1.6	1.4	1.2	0.8	0.5	0.2
rs2835638	37,456,403	45.83	5.4	1.6	1.5	1.4	1.2	0.8	0.4	0.2
rs2836301	**38,599,459**	**47.66**	−∞	−∞	0.8	1.2	1.2	1.0	0.6	0.2
rs2836803	39,273,650	49.22	−∞	−∞	0.7	1.1	1.1	0.9	0.5	0.2
Family 4413 (21q21.3–q22.3)										
rs2832479	30,187,890	32.57	1.8	0.2	0.2	0.7	0.1	0.1	0.0	0.0
rs762173	**32,754,546**	**36.24**	2.6	0.4	0.4	0.3	0.2	0.1	0.1	0.0
rs2250226	33,554,186	37.77	3.0	0.9	0.9	0.8	0.7	0.5	0.2	0.1
rs1892687	35,233,892	40.73	3.2	1.2	1.2	1.0	0.9	0.6	0.3	0.1
rs2835626	37,434,568	45.81	3.2	1.6	1.5	1.3	1.1	0.7	0.4	0.1
rs1016694	38,156,688	46.76	3.3	1.4	1.4	1.2	1.0	0.6	0.3	0.1
rs2210267	**40,685,095**	**53.10**	−∞	−∞	−1.1	−0.3	−0.1	0.1	0.1	0.0
rs220271	42,363,322	60.52	−∞	−∞	−1.3	−0.6	−0.3	−0.1	−0.0	0.0

Marker loci[a]	Physical map position[b]	Genetic map position[c]	Multipoint LOD	2-Point LOD at recombination fraction θ = 0.00	0.01	0.05	0.10	0.20	0.30	0.40
Family 4306 (21q21.3–q22.3)										
rs2829150	24,816,606	25.03	−3.1	−3.3	−1.3	−0.6	−0.3	−0.1	−0.0	0.0
rs2830252	**26,776,572**	**27.31**	−3.1	−3.0	−0.8	−0.2	−0.0	0.1	0.1	0.0
rs2248313	35,223,308	40.70	2.2	1.2	1.2	1.1	0.9	0.6	0.3	0.1
rs1014803	36,015,910	42.59	2.3	1.3	1.3	1.2	1.0	0.7	0.4	0.1
rs1015549	37,370,750	45.72	2.3	1.2	1.2	1.1	0.9	0.6	0.3	0.1
rs2210267	40,685,095	53.10	2.4	1.8	1.7	1.6	1.3	0.9	0.5	0.1
rs915774	**44,400,145**	**64.78**	1.6	0.4	0.4	0.4	0.3	0.2	0.1	0.0
rs10098	45,539,973	66.92	1.6	0.4	0.4	0.3	0.3	0.2	0.1	0.0

*The *CLDN14* (NM_001146077.1) gene is located at 21q22.13 (chr21: 36,754,790–36,870,737).

[a]Markers in bold type delimit the homozygous region.

[b]Physical map positions in base pairs from the human reference sequence Build 36.

[c]Genetic map positions in cM from the Rutgers combined linkage-physical map of the human genome Build 36.

TABLE II

Identified Mutations and Previously Reported Causal Variants Nucleotide

Nucleotide change	rs number	Amino acid substitution	Family ID	Maximum LOD score	Alleles in controls	PolyPhen-2	SIFT	Pmut	SNAP
c.167G>A[a]	—	**p.Trp56***[a]	**4158**	**2.1**	**0/400**	—	—	—	—
c.242G>A[a]	—	**p.Arg81His**[a]	**4209**	**5.5**	**0/400**	**Probably Damaging**	**Damaging**	**Pathological**	**Non-neutral**
c.254T>A[b]	rs74315437	p.Val85Asp[b]	PKSR9a/HLBR5/4306	6.1/2.4	0/400	Probably Damaging	Damaging	Pathological	Non-neutral
c.301G>A[c]	rs74315438	p.Gly101Arg[c]	NA	NA	0/400	Probably Damaging	Damaging	Pathological	Non-neutral
c.398delT[b]	—	p.Met133Arg*fs*X24[b]	PKSN6	4.7	0/400	—	—	—	—
c.694G>A[a]	—	**p.Gly232Arg**[a]	**4413**	**3.3**	**0/400**	**Probably Damaging**	**Damaging**	**Pathological**	**Non-neutral**

[a]Mutations in bold type indicate newly identified functional variants from this study.

[b]These mutations were previously identified in consanguineous Pakistani families [Wilcox et al., 2001; Bashir et al., 2010]. The p.Val85Asp mutation was also observed in Family 4306 from this study.

[c]The mutation was identified in a single proband of Greek ancestry [Wattenhofer et al., 2005].