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Genetic causes of moderate to severe hearing loss point to modifiers

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Abstract

The genetic underpinnings of recessively inherited moderate to severe sensorineural hearing loss are not well understood, despite its higher prevalence in comparison to profound deafness. We recruited 92 consanguineous families segregating stable or progressive, recessively inherited moderate or severe hearing loss. We utilized homozygosity mapping, Sanger sequencing, targeted capture of known deafness genes with massively parallel sequencing and whole exome sequencing to identify the molecular basis of hearing loss in these families. Variants of the known deafness genes were found in 69% of the participating families with the *SLC26A4*, *GJB2*, *MYO15A*, *TMC1*, *TMPRSS3*, *OTOF*, *MYO7A* and *CLDN14* genes together accounting for hearing loss in 54% of the families. We identified 20 reported and 21 novel variants in 21 known deafness genes. Sixteen of the twenty reported variants, previously associated with stable, profound deafness were associated with moderate to severe or progressive hearing loss in our families. These data point to

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DISCLOSURE

The authors declare no conflict of interest

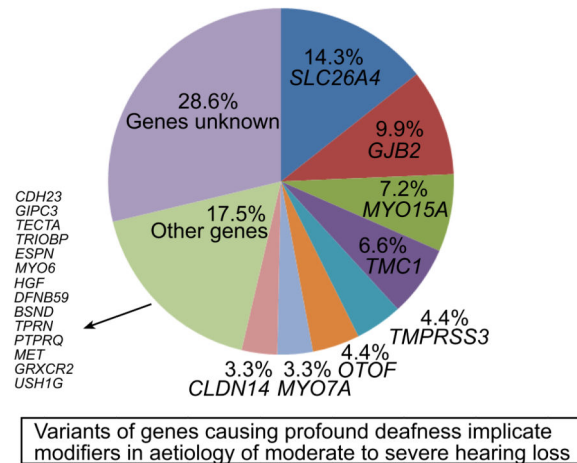
SUPPLEMENTARY MATERIAL

Supporting material is available for this article and includes a Supplementary file with Table S1, Table S2, Table S3, Table S4, Table S5, & Fig. S1 and Supplementary Information.

a prominent role for genetic background, environmental factors or both as modifiers of human hearing loss severity.

Graphical abstract

Contributions to aetiology of moderate to severe hearing loss



Keywords

Aetiology; Deafness; DFNB; Hearing loss; Modifiers; Mutation; Pakistan

INTRODUCTION

Hearing loss is a common sensory impairment that affects 278 million people worldwide (1). Dominant and recessive alleles account for approximately half of congenital or early onset childhood hearing loss that can have a broad range of severity (2). A majority of pathogenic recessive variants of genes associated with hearing loss cause congenital or prelingual, severe to profound, stable sensorineural deafness, which occurs in approximately 1 in 1,000 births (1). Autosomal dominant inheritance is typically associated with post-lingual, progressive hearing loss that can range from mild to profound at onset or over the course of many decades. In general, the hearing loss associated with dominant inheritance is less severe than that associated with recessive inheritance. A few mutant alleles of some profound deafness-associated genes such as *MYO7A* and *CDH23*, have been described to cause variable audiological phenotypes as well (3-5). The objective of this study was to determine if other genes previously associated with profound deafness also contribute to moderate or severe hearing loss. Our study implicates 21 different genes as well as modifiers in the etiology of recessively inherited moderate to severe hearing loss.

MATERIALS AND METHODS

Families

The study was approved by the Institutional Review Board at the School of Biological Sciences, University of the Punjab, Lahore and the Combined Neuroscience Institutional

Review Board (protocol OH-93-N-016) at the National Institutes of Health, USA. Samples were collected with written informed consent from each participant.

We ascertained 92 consanguineous Pakistani families; 90 residing in the province of Punjab and two families from Khyber Pakhtunkhwa. Probands were identified through special schools in Punjab where children with different disabilities are educated, from schools for the deaf and through referrals from audiologists. In order to identify probands from schools, we first inquired about the extent of a child's hearing ability from teachers, which were verified by a distraction test that involves recording a subject's response to sound stimuli coming from a hidden source. Pure tone thresholds of the probands and other family participants, who expressed willingness for audiometric testing, were measured at 0.25, 0.5, 1, 2, 4 and 8 kHz in a quiet room due to the lack of a sound proof room. The pure-tone average (PTA) was calculated by averaging the hearing thresholds at 0.5, 1, 2 and 4 kHz (6). Hearing loss was classified on the basis of PTA as moderate (41 to 70 dB HL), severe (71 to 95 dB HL), and profound (above 95 dB HL) (6). Individuals with hearing thresholds between 31-40 dB HL were classified with mild hearing loss while those with thresholds up to 30 dB HL were considered to have normal hearing since performing audiometry in ambient noise conditions may result in slightly elevated thresholds for those with normal hearing (7). Subjects were questioned about the age of the onset and progression in severity of their hearing loss. Vestibular function was evaluated by tandem-gait and Romberg tests. Otoscopic examinations and medical histories were completed in order to exclude affected individuals due to active external or middle ear disease, and syndromic or environmental causes, though families with Usher syndrome were included. Funduscopy and electroretinography examinations were conducted for the oldest affected individuals in a subset of families.

Molecular characterization

Blood samples were collected from the participants and genomic DNA was extracted by a standard method (8). The sequence of *GJB2* was analyzed for the affected participants from all families by Sanger sequencing (9). DNA samples from at least three affected individuals from 66 of 92 consanguineous families (Supplementary Table S1) were screened for linkage to genes associated with nonsyndromic recessively inherited deafness (*DFNB*) and Usher syndrome (*USH*), by homozygosity mapping as described (10, 11). In brief, samples from at least three affected individuals were chosen from each family, 2 to 5 tightly linked microsatellite markers for each deafness gene were PCR amplified using fluorescently tagged primers and the amplicons resolved by capillary electrophoresis. If affected individuals were homozygous for alleles of any of these markers, samples from all participants of the respective family were genotyped for the linked, as well as additional downstream or upstream markers to determine if there is co-segregation with the phenotype.

After homozygosity mapping, Sanger sequencing of the implicated genes was performed for 17 of the families (Supplementary Table S1). Samples from 53 families were screened with massively parallel sequencing (Supplementary Table S1). For targeted capture and massively parallel sequencing of 108 genes with a demonstrated role in hearing (Supplementary Table S2), custom probes were designed to capture the exons and approximately 50 bp of flanking

intronic sequence. Fragment library preparation and target enrichment were performed according to the manufacturers' instructions on one randomly selected sample from each family (Supplementary Table S3) and sequencing was performed on an ABI SOLiD 5500.

For whole-exome sequencing, one to three samples of affected individuals were randomly selected from the chosen families (Supplementary Table S3). Fragment libraries were prepared with a Nextera Rapid Capture library kit (REF#15034786) for paired end, dual index sequencing on an Illumina HiSeq 1000 platform at the NIDCD, USA, while some libraries were prepared and sequenced at Otogenetics, USA on an Illumina HiSeq 2000 platform. Variants were annotated using the web-based software wANNOVAR (<http://wannovar.usc.edu/>). Variants with overall population allele frequencies of >1% (0.01) in any of the public databases (1000 genome dataset, 6500 exome variant server, and Exome Aggregation Consortium, ExAC, database) were removed. Only variants in protein coding sequences or those affecting splice sites were considered further. For *HGF* (OMIM 142409), we also screened for the intronic variants that were shown to be associated with nonsyndromic deafness *DFNB39* (OMIM 608265) (12).

All suspected pathogenic variants were evaluated for co-segregation with the hearing loss phenotype in families by PCR amplification and Sanger sequencing using Big Dye Terminator V3.1 (Applied Biosystems, Foster City, USA). Sequences of PCR primers are available on request. Allele frequencies of the novel variants were assessed by Sanger sequencing using genomic DNA from 100 to 200 ethnically matched unrelated individuals from Pakistan with threshold for assigning the variant as a polymorphism set to its detection in at least 1% control chromosomes. The pathogenicity of the missense variants was evaluated using *in silico* tools PROVEAN, <http://provean.jcvi.org/>, SIFT, <http://sift.bii.a-star.edu.sg/>, and Mutation Taster, <http://www.mutationtaster.org/>. Possible splicing effects introduced by variants identified near exon/intron junctions were determined by Human Splicing Finder (<http://www.umd.be/HSF/>) and Skippy (<http://research.nhgri.nih.gov/skipper/>). Alignments of multiple protein sequences were performed with Clustal Omega <http://www.ebi.ac.uk/Tools/msa/clustalo/>. The sequences of orthologs for alignment of proteins were obtained from UCSC genome server (<https://genome.ucsc.edu/>).

Calculation of percentage contributions and confidence intervals

Individuals from 92 families participated in the study. Percentage contribution of deafness genes to hearing loss in this cohort were calculated based on 91 families which included seven families for which linkage of hearing loss was detected to a reported deafness locus but a pathogenic variant was not identified. The 95% Confidence Intervals for contribution of genes to deafness were calculated as described (<https://onlinecourses.science.psu.edu/stat200/node/48>). A family with an *USH1C* variant was excluded from these calculations as Usher syndrome was manifested by the affected participants at the time of enrollment, and therefore for this family only the reported genes for Usher syndrome were analyzed.

RESULTS

Families

Most of the families we studied had multiple affected individuals who were born to unaffected parents with consanguineous marriages. Exceptions occurred in five families, in which one (HLGM10, HLAI-10, HLAI-19, HLAI-20) or both parents (HLM1) were also affected, with clear evidence for autosomal recessive inheritance of hearing loss in other branches of the respective pedigrees. Hearing loss in 71 families was moderate or severe (Fig. 1a-d and Table 1). Another twenty families had a majority of individuals with moderate or severe hearing loss while one to three affected individuals presented profound deafness (Table 1). The remaining one family (HLM1) had five members affected with profound deafness at the time of enrollment, which was said to have developed in all individuals gradually from a mild hearing loss in childhood. In most cases, previous audiograms were not available and the age of onset of hearing loss could not be reliably ascertained, though the hearing loss was reported to be progressive in nature for 87 individuals in 22% of the participating families (Table 1). None of the affected individuals exhibited an imbalance on tandem gait or Romberg tests.

Genetic analyses

We identified 20 previously described and 21 novel pathogenic variants in reported deafness-associated genes for 57 of the 92 participating families (Table 1). For seven families, either a gene had not been identified for the implicated locus or no mutations in the reported exons of the corresponding genes were found although the phenotype was linked to markers of a deafness locus for which there are well-documented pathogenic alleles (Table 1). Of the twenty variants identified in this study that were previously reported to cause deafness (Supplementary Table S4), fifteen were present in the public nucleotide variant databases with overall allele frequencies ranging from 0.0000082 to 0.00024 (Supplementary Table S5). These variants were rare in the South Asian population as well, except for the p.W24X variant in *GJB2* with an allele frequency of 0.004 and the p.V239D variant of *SLC26A4* with an allele frequency of 0.0017. None of the 21 novel pathogenic mutations were detected in the DNA of ethnically matched controls (200 to 400 chromosomes) but five of these were identified in the ExAC database and had an overall allele frequency of 0.000008237 to 0.00005447 (Supplementary Table S5). The novel variants reported in our study have been deposited in the ClinVar database (<http://www.ncbi.nlm.nih.gov/clinvar/>).

Novel variants and involvement with moderate or severe hearing loss

We identified novel variants in thirteen known deafness-associated genes in 21 of the 92 participating families (Table 1). Fifteen of the 21 variants are described in this manuscript (Table 1, Supplementary Information) while the other six variants were reported earlier by us (Table 1, Supplementary Table S4). These variants (Table 1) did not differ substantially from those previously reported in the type of mutation or predicted pathogenicity (Table 2, Supplementary Information). The variants included frameshift, stop-gain, in-frame deletions and missense mutations affecting evolutionary conserved amino acid residues in *MYO7A* (OMIM 276903), *MYO15A* (OMIM 602666), *OTOF* (OMIM 603681), *CDH23* (OMIM

605516), *TRIOBP* (OMIM 609761), *TECTA* (OMIM 602574), *ESPN* (OMIM 606351), *MYO6* (OMIM 600970), *PTPRQ* (OMIM 603317) and *USH1C* (OMIM 605242), (Table 1, Fig. 2, Supplementary Information).

The variants of *TRIOBP*, *ESPN* and *MYO6* described here implicate for the first time these genes in recessively inherited moderate or severe hearing loss. Though specific mutations of *MYO15A* and *CDH23* are known to be involved with moderate or severe hearing loss (3, 13-15), some of the variants we identified do not correspond to the established genotype-phenotype correlations for these genes (Supplementary Information). For example, we found that homozygous mutations that affected both isoforms 1 and 2 of *MYO15A* (16) were associated with severe hearing loss (Table 1, Supplementary Information). This is in contrast to earlier findings in which it was hypothesized that mutations which affect both isoforms of *MYO15A* would result in profound deafness (13, 14).

We also found mutations in genes which are established causes of moderate to severe hearing loss (Supplementary Information) such as *TECTA* and *PTPRQ* (17, 18). It has been stated that *PTPRQ* mutations show weak evidence for association to deafness (19). However, different studies (18, 20-23) and the frameshift variant identified in three affected cousins in a participating family in our research (Table 1) together establish *PTPRQ* as a bona fide deafness gene.

Known variants as cause of moderate or severe hearing loss

SLC26A4 (OMIM 605646) variants were the most numerous in our cohort, associated with hearing loss in twelve families and in one of the eight affected individuals in family HLAM12B. We found the same five nonsense, missense and frameshift mutations (24) (Table 1) as cause of the moderate or severe hearing loss, which were previously reported to result in profound deafness in other studies (Supplementary Table S5), (Fig. 3). Audiometric thresholds among individuals homozygous for these *SLC26A4* variants were not significantly correlated with age (Ages, 6-45, Spearman's $r = 0.226$; $p = 0.11$) suggesting that other genetic or environmental factors can modify the phenotype associated with these mutations (Fig. 3). For example, an 18 year old individual in family HLAI-20 had profound deafness (PTA 104 dB HL) in contrast to the other older individuals in his family (ages 45 and 35 years; PTA 84 and 89 dB HL, respectively). Similarly, in families HLAI-15 and HLAM01B in which the affected individuals were homozygous for the p.Q446R variant of *SLC26A4*, the 25 and 26 years old individuals have better hearing (PTA 73 and 83 dB HL respectively) as compared to the younger affected individuals (ages 10 and 12 years, PTA 104 and 98 dB HL respectively). The type of mutation was also not correlated to the severity of the phenotype since three individuals homozygous for a nonsense (p.Ser57X) or a frameshift (c.965dupA) mutation in *SLC26A4* had moderate hearing loss (PTA 66, 63 and 69 dB HL) at the ages of 12, 25 and 28 years, respectively (Table 1, Fig. 3).

In addition to *SLC26A4*, some known variants of other genes including *GJB2* (OMIM 121011), *MYO15A* and *TMPRSS3* (OMIM 605511), were found segregating with moderate or severe hearing loss in this study but were previously reported as a cause of profound deafness (Table 1, Supplementary Information). Comparison of audiograms of affected

individuals with identical mutations in multiple families did not suggest that the observed hearing threshold differences were age related.

It has been hypothesized that homozygosity for *TMPRSS3* frameshift variants results in profound deafness (25). We identified a homozygous frameshift mutation as a cause of moderate or severe hearing loss in one participating family (Table 1, Supplementary Information). In addition, the age of onset of hearing loss in one family with a cryptic acceptor splice site variant in intron 4 of *TMPRSS3* (Table 1) was between 3-4 years and was moderate to severe at the age of 22 years in contrast to the deafness progressing to profound degree in two families with the identical mutation (26) (Supplementary Information).

Molecular heterogeneity

In families HLAI-05, HLAI-18, HLAI-20, HLAI-27, HLAM01A, HLAM12B and HLMR3, some of the affected individuals were not homozygous for the pathogenic mutation found in deaf siblings or close relatives (Supplementary Fig. S1), indicating that variants of other genes or environmental factors contribute to their hearing loss.

Overall contribution of deafness genes to the phenotype

Mutations of eight genes *SLC26A4*, *GJB2*, *MYO15A*, *TMC1*, *TMPRSS3*, *OTOF*, *MYO7A* and *CLDN14* together accounted for hearing loss in 54% of the families (Table 3). Variants of other genes were rare and collectively accounted for the phenotype in 15% of the families (Table 3).

DISCUSSION

Persons with hearing loss of moderate or severe degree may derive satisfactory benefit from hearing aids, while cochlear implantation is a recommended treatment to restore access to sound for individuals with profound deafness. Since profound deafness is clinically distinct from moderate and severe hearing loss, we hypothesized that there would be a spectrum of variants of unique deafness genes as well as distinct mutant alleles of the reported deafness genes as causes of moderate to severe hearing loss. Although we did discover mutations of *GRXCR2* (OMIM 615762) (27) and *MET* (OMIM 164860) (28) as novel causes of progressive or severe hearing loss, unexpectedly we found that exactly the same mutations previously reported to be associated with profound deafness can also cause moderate to severe hearing loss. In fact, the combined contribution of *SLC26A4* and *GJB2* to moderate or severe hearing loss in this cohort is as high as 24.2% in contrast to that of 13.35% due to the mutant alleles of these same two genes to profound deafness in Pakistan (29).

Phenotypic variability due to mutations in the same gene is usually attributed to a difference in severity of gene function disruption. Splice site variants not resulting in a complete loss of the protein and missense mutations can sometimes be less damaging than nonsense and frameshift mutations. However, of the 41 mutations that we identified in reported deafness genes, seven are nonsense (17%), eleven cause frameshifts in the canonical reading frames (27%), five are splice site mutations (12%), two are in-frame deletions (5%), one is a regulatory variant (2%) and the remaining fifteen are missense mutations (37%). The splice

site mutations of *MYO15A* and *USH1G* are predicted to result in the loss of the encoded proteins. Phenotypic variation for the same mutant allele suggests the presence of genetic modifiers, environmental factors or both. We did not find variants in the mitochondrial genome that might account for phenotypic variability observed in families in which affected individuals had identical mutations. In addition, we found no rare variants of *ATP2B2*, a known modifier gene of *CDH23* related deafness (15). The possibility remains to be explored that common variants of *ATP2B2* are modifiers.

Few studies have reported population wide involvement of specific genes in moderate or severe hearing loss. For example, copy number variations and point mutations of *STRC* (OMIM 606440) contributed to approximately 6% of mild to moderate hearing loss in the USA and Germany in a cohort of 763 individuals (30, 31). In Qatar, *GJB2* variants accounted for 15% of moderate to severe hearing loss in a cohort of 126 individuals (32). Our work has indicated the involvement of 23 genes in the genetics of moderate or severe hearing loss and implicates modifiers in reducing the severity of the phenotype. Future molecular genetic insight and epidemiological studies may be helpful in identifying specific modifier variants in the genetic background of our study subjects which, perhaps in combination with extrinsic factors, partially suppress profound deafness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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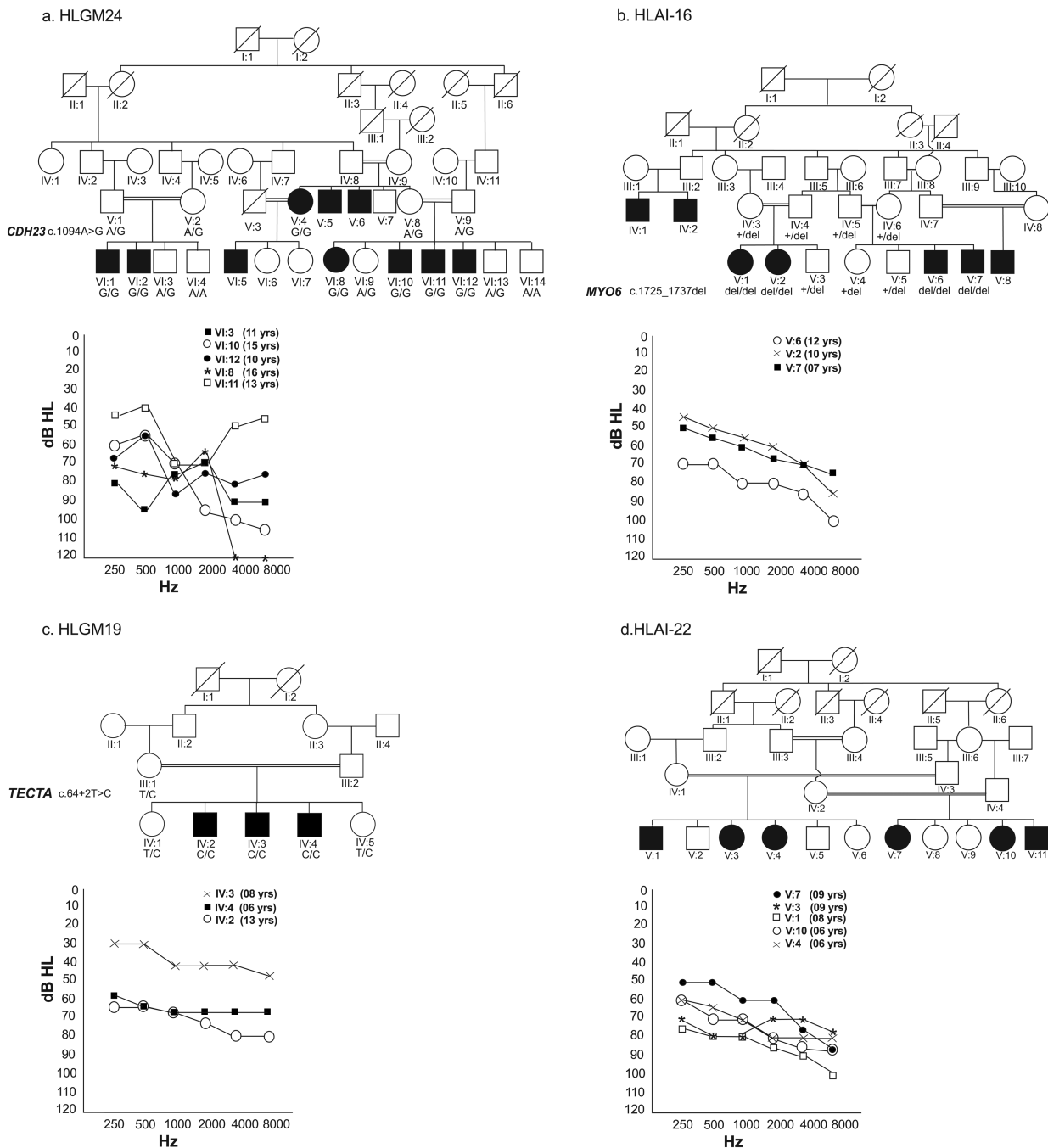


Fig. 1. Selected Pedigrees and Pure tone audiograms

Pedigree drawings are provided for four of the participating families with the Pure tone audiograms showing degree of hearing loss across the test frequency range. Hearing thresholds of unaffected age matched controls acquired in similar settings range between 20 dB HL and 30 dB HL. The ages of all affected individuals are noted on the right side of the audiogram. Results are plotted for the better hearing ear in each case. (a) Variable degree of mild-to-moderate, moderate-to-severe or moderate-to-profound hearing loss in family HLG M24 due to *CDH23* missense mutation (b) Moderate, moderate-to-severe or moderate-

to-profound hearing loss in family HLAI-16 due to *MYO6* frameshift mutation (c) Mild-to-moderate, moderate, and moderate-to-severe hearing loss as observed in family HLGMI9 which had a frameshift variant in *TECTA*. Note that one of the affected individuals has a significantly lesser degree of hearing loss (d) Moderate-to-severe or severe-to-profound hearing loss was present in family HLAI-22. The parents reported that the affected individuals had a progressive hearing loss.

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Fig. 2. CLUSTALO alignments among diverse vertebrate species
 Alignments show absolute evolutionary conservation of novel amino acid changes reported in this study. The sequences for MYO15A, OTOF, MYO7A and CDH23 correspond to the proteins encoded from transcripts NM_016239.3, NM_194248.2, NM_000260.3 and NM_022124.5, respectively.

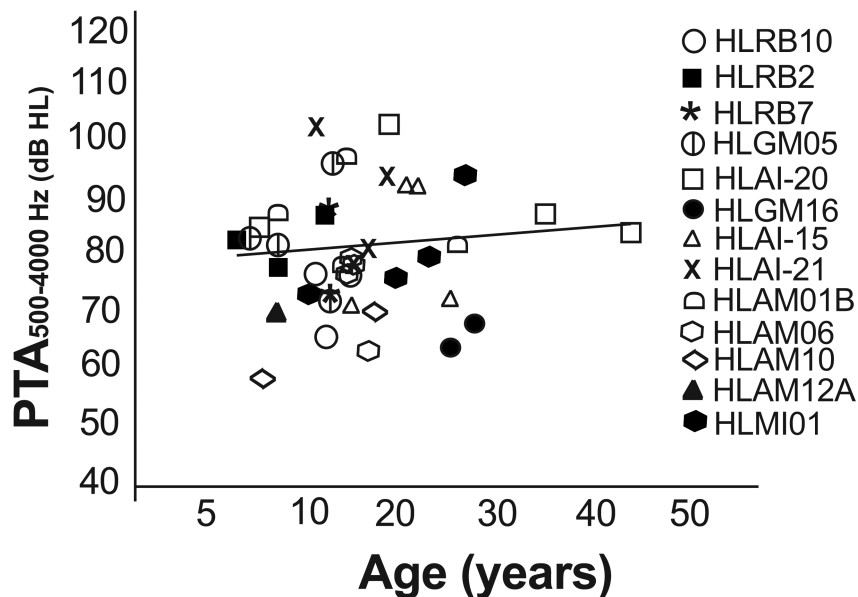


Fig.3. PTA of individuals with *SLC26A4* variants
 Pure tone averages (PTA) of hearing thresholds at 500, 1000, 2000 and 4000 Hz, for the better hearing ears in affected members with homozygous mutations in *SLC26A4* are plotted as a function of age of the participants. The degree of hearing loss is different across and within families with mutations in *SLC26A4* without correlation to age, an observation which is supported by the straight regression line in the graph.

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Table 1

Details on the participating families, their phenotypes and the identified mutations

Gene	Family ID	^a Affected individuals	Pure tone average (PTA _{500-4000 Hz}) db HL	Variant	# Comment
<i>SLC26A4</i> NM_000441.1	*HLRB10	3	66, 78, 78	c.170C>A:p.(Ser57X)	
	*HLRB2	3	79, 83, 88	c.716T>A:p.(Val239Asp)	
	*HLRB7	5	73, 89	c.716T>A:p.(Val239Asp)	
	HLGM05	4	75, 83, 84, 98	c.716T>A:p.(Val239Asp)	
	HLAI-20	6 [1]	84, 85, 89, 104 (Progressive reported)	c.716T>A:p.(Val239Asp)	
	HLGM16	3	63, 69	c.965dupA:p.(N322KfsX8)	
	HLAI-15	4	71, 73, 94, 94	c.1337A>G:p.(Gln446Arg)	
	HLAI-21	5	79, 81, 94, 104 (Progressive reported)	c.1337A>G:p.(Gln446Arg)	
	HLAM01B	4	78, 83, 89, 98	c.1337A>G:p.(Gln446Arg)	
	HLAM06	5	64, 78, 79, 79	c.1337A>G:p.(Gln446Arg)	
	HLAM10	3	59, 70	c.1337A>G:p.(Gln446Arg)	
	HLAM12A	2	70	c.1667A>G:p.(Tyr556Cys)	
	HLAM12B	1 [7]	70	c.1667A>G:p.(Tyr556Cys)	
	HLMI01	7	73, 76, 80, 95	-	
<i>GJB2</i> NM_004004.5	*HLAI-02	8	81, 86, 88, 98, 103, 104	c.71G>A:p.(Trp24X)	
	*HLAI-12	3	59, 71, 90	c.71G>A:p.(Trp24X)	
	*HLGM25	5	70, 70, 94	c.71G>A:p.(Trp24X)	
	*HLMS16	2	74, 79	c.71G>A:p.(Trp24X)	
	*HLMS34	2	75, 78	c.71G>A:p.(Trp24X)	
	*HLRB1	4	54, 65, 71, 103	c.231G>A:p.(Trp77X)	
	*HLRB8	6	70, 78, 78	c.71G>A/c.231G>A p.(Trp24X)/p.(Trp77X)	
	*HLAM09	3	61	c.71G>A/c.231G>A p.(Trp24X)/p.(Trp77X)	
HLMR2	3	88, 91	c.358_360 del: p.(Glu120del)		
<i>MYO15A</i> NM_016239.3	*HLRB3	3	78, 78, 86	c.1185dupC:p.(Glu396ArgfsX36)	
	HLAM05	3 [1]	70, 83, 86	c.1657delC:p.(Arg553GlyfsX76)	First report
	HLAI-06	3	68, 75	c.2456C>A:p.(Ser819X)	First report
	HLAM07	6	65, 81, 98	c.3866+1G>A	
	HLAI-10	4	83, 106	c.6589C>T:p.(Gln2197X)	
	HLAI-25	2	68, 70	c.8158G>A:p.(Asp2720Asn)	First report
HLAI-08	10	68, 73, 79, 103, 104	-		
<i>TMCI</i> NM_138691.2	*HLAM02	3	71, 75, 89	c.100C>T:p.(Arg34X)	

Gene	Family ID	^a Affected individuals	Pure tone average (PTA _{500-4000 Hz}) db HL	Variant	# Comment
	*HLAI-27	3	61, 80, 81 (Progressive reported)	c.596A>T:p.(Asn199Ile)	
	*PHLAI-01	4	78, 86, 93, 93 (Progressive reported)	c.1166G>A:p.Arg389Gln	
	*HLAI-04	4	65, 95, 101	c.1404+1G>T	
	*HLAI-14	3	70, 75, 88 (Progressive reported)	c.1788C>A:p.(Ser596Arg)	
	*HLAI-17	6	80, 84, 95	-	
<i>TMPRSS3</i> NM_024022.2	HLAI-13	5	66, 78, 84, 99 (Progressive reported)	c.208delC:p.(His70ThrfsX19)	
	HLAM08	3	80, 95	c.1219T>C:p.(Cys407Arg)	
	HLM1	5	110 (Progressive reported)	c.323-6 G>A	
	HLM102	4	70, 74, 85	c.323-6 G>A	
<i>OTOF</i> NM_194248.2	HLAI-09	4	73, 76	c.2965_2967del:p.(Phe989del)	First report
	HLGM14	3	76, 86, 89	c.3289-1G>T	First report
	HLAI-01	4	70, 84	c.4805G>T:p.(Gly1602Val)	First report
	HLGM06	6	73, 86, 88 100	-	
<i>MYO7A</i> NM_000260.3	HLAI-19	7	85, 85, 94 (Progressive reported)	c.1183C>T:p.(Arg395Cys)	First report
	HLGM12	3	80, 89, 90	c.6354G>C:p.(Lys2118Asn)	First report
	HLAI-07	3	73	-	
<i>CLDN14</i> NM_144492.2	*HLRB5	10	63, 74, 78, 79, 85, 88, 88, 88, 89, 91	c.254T>A:p.(Val85Asp)	
	HLAM04	4	76, 85, 94, 94	c.254T>A: p.(Val85Asp)	
	HLAM11	9	73, 83, 83, 84, 88, 91	c.254T>A: p.(Val85Asp)	
<i>CDH23</i> NM_022124.5	HLGM24	7	58, 74, 78, 83, 84	c.7814A>G:p.(Asn2605Ser)	First report
<i>GIPC3</i> NM_133261.2	HLAI-11	6	64, 83, 86, 96 (Progressive reported)	c.662C>T:p.(Thr221Ile)	
<i>TECTA</i> NM_005422.2	HLGM19	3	38, 69, 73	c.64+2T>C	First report
<i>TRIOBP</i> NM_001039141.2	HLAI-26	3	74, 86	c.2968C>T:p.(Arg990X)	First report
<i>ESPN</i> NM_031475.2	HLMR3	1 [2]	68	c.2019dupG:p.(Leu674AfsX72)	First report
<i>MYO6</i> NM_004999.3	HLAI-16	4	59, 63, 79 (Progressive reported)	c.1729_1741del:p.(Phe577IlefsX28)	First report
<i>HGF</i> NM_000601.4	HLAI-18	2 [2]	73 (Progressive reported)	c.482+1986_1988delTGA	
<i>DFNB59</i> NM_001042702.3	*HLGM15	6	79, 81, 89, 90, 90, 109 (Progressive reported)	c.1028G>C:p.(Cys343Ser)	

Gene	Family ID	^a Affected individuals	Pure tone average (PTA _{500-4000 Hz}) db HL	Variant	# Comment
<i>BSND</i> NM_057176.2	HLAI-23	5	70 (Progressive reported)	c.35 T>C:p.(Ile12Thr)	
<i>TPRN</i> NM_001128228.1	* HLRB6	3	79, 94 (Progressive reported)	c.42_52del11:p.(Gly15AlafsX150)	
<i>PTPRQ</i> NM_001145026.1	HLRB4	3	73	c.189delC:p.(Glu65LysfsX95)	First report
<i>GPSM2/DFNB32</i> NM_013296.4	HLRB11	5	75, 76, 90, 94	-	
	HLAI-24	4	61, 69 (Progressive reported)	-	
<i>MET</i> NM_000245.2	* HLG M17	9	74, 83, 89, 89, 89, 108	c.2521T>G:p.(Phe841Val)	
<i>GRXCR2</i> NM_001080516.1	* HLA I-05	3 [1]	54, 63, 73 (Progressive reported)	c.714dupT: p.(Gly239TrpfsX74)	
<i>USH1C</i> NM_005709.3	USH-1	3	64, 73	c.605dupC:p.(Gly203TrpfsX47)	First report
<i>USH1G</i> NM_173477.4	* HLRB12	3	69, 76, 81	c.163_164+13del	
Unknown	HLRB9	6	73, 73, 75, 80, 100		
	HLRB13	3	69, 83		
	HLGM02	3	78, 79, 88		
	HLGM03	3	78		
	HLGM04	3	85, 90, 94		
	HLGM07	4	79, 79, 90, 94		
	HLGM08	4	46, 49, 64, 84		
	HLGM09	3	71, 89		
	HLGM10	3	89, 93		
	HLGM11	3	84, 85, 85		
	HLGM13	3	83,88,111 (Progressive reported)		
	HLGM18	4	51, 56, 69 (Progressive reported)		
	HLGM20	4	60, 61, 63, 66		
	HLGM21	3	58, 66, 90		
	HLGM22	11	84, 85, 86, 90, 91, 95, 98, 101, 103		
	HLGM23	4	75, 78, 80, 81		
	HLGM26	7	45, 84, 99		
	HLAI-03	4	69, 90, 94 (Progressive reported)		
	HLAI-22	6	61, 74, 75, 76, 83 (Progressive reported)		
	HLAM01A	4	78, 81, 95		
HLAM03	5	76, 76, 78			

Gene	Family ID	^a Affected individuals	Pure tone average (PTA _{500-4000 Hz}) db HL	Variant	# Comment
	HLAM12B	7	80, 84, 85, 89		
	IHT01A	3	43, 46, 74 (Progressive reported)		
	IHT02	3	79, 85, 94		
	HLMR1	4	83, 88, 91, 99		
	HLMR4	3	76, 89, 96		

^aThe number of affected participants or individuals with pathogenic mutations in deafness genes is denoted by an integer while in case of families exhibiting genetic heterogeneity number of those affected individuals without the causative variants are also shown by a bracketed integer. The PTA_{500-4000Hz} of the latter group is not shown in the corresponding columns.

[#]See Supporting Table S4 for references to the first reports of these mutations.

^{*}Data for these families were previously reported by us.

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Table 2

Results of in silico predictions for novel missense variants

Gene	Variant	MT Score	MT Pred	Prov Score	Prov Pred	SIFT Score	SIFT Pred
<i>MYO15A</i> NM_016239.3	c.8158G>A:p.(Asp2720Asn)	23	D	-4.42	D	0.016	D
<i>OTOF</i> NM_194248.2	c.4805G>T:p.(Gly1602Val)	109	D	-8.87	D	0	D
<i>MYO7A</i> NM_000260.3	c.1183C>T:p.(Arg395Cys)	180	D	-7.75	D	0	D
	c.6354G>C:p.(Lys2118Asn)	94	D	-4.33	D	0.001	D
<i>CDH23</i> NM_022124.5	c.7814A>G:p.(Asn2605Ser)	46	D	-3.53	D	0	D

MT, Mutation Taster, Prov, Provean, SIFT, Sorting intolerant from tolerant, Pred, prediction, D, disease causing, deleterious and damaging predictions from Mutation Taster, Provean and SIFT, respectively.

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Table 3

Percentage contribution of genes to moderate or severe hearing loss in Pakistan

Gene/Locus	Number of families ^a	Percentage	95 % CI
<i>SLC26A4</i>	13	14.3	7.1-21.5
<i>GJB2</i>	9	9.9	3.8-16
<i>MYO15A</i>	7	7.7	2.2-13.2
<i>TMC1</i>	6	6.6	1.5-11.7
<i>TMPRSS3</i>	4	4.4	0.1-8.7
<i>OTOF</i>	4	4.4	0.1-8.6
<i>MYO7A</i>	3	3.3	0-7
<i>CLDN14</i>	3	3.3	0-7
Other 13 genes or loci	14	-	-
“New genes”, ^b	2	-	-
Unknown	26	28.6	-
Total	91	-	-

CI, Confidence Interval.

^aPercentages are calculated on the basis of 91 pedigrees without the data of an *USH1C* family and includes data for families for which linkage was detected to a locus with a LOD score of 3 and a pathogenic mutation was not identified after sequencing the corresponding gene (*MYO7A* for HLAI-07, *MYO15A* for HLAI-08, *SLC26A4* for HLMI01, *TMC1* for HLAI-17, *OTOF* for HLG06 and *GPSM2* for HLRB11).

^b*GRXCR2* and *MET*.