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Molecular Analysis of Twelve Pakistani Families with Nonsyndromic or Syndromic Hearing Loss

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Aim: To investigate the causative genetic mutations in 12 Pakistani families with nonsyndromic or syndromic hearing loss.
Methods: Mutations in the most common causative gene for hearing loss, *GJB2*, were evaluated by Sanger sequencing. Targeted next-generation sequencing or whole-exome sequencing was used to analyze the genomic DNA samples from 11 probands with hearing loss. Sanger sequencing was performed to verify all identified variants.
Results: We found pathogenic, or likely to be pathogenic, mutations in all 12 families, including six known mutations in *GJB2*, *SLC26A4*, *LHFPL5*, and *USH2A* and eight novel mutations in *ESPN*, *MYO7A*, *LRTOMT*, *PCDH15*, *USH2A*, or *EPS8L2*. Notably, four compound heterozygous mutations in the *MYO7A* and *USH2A* genes were detected in two consanguineous families. In addition, the novel frameshift mutation in *EPS8L2* was first documented in Pakistan.
Conclusions: Our study increases the spectrum of mutations associated with hearing loss in the Pakistani population. In addition, our study highlights the fact that compound heterozygous mutations, although rare, can occur in consanguineous families.

Keywords: hearing loss, gene, mutation, targeted next-generation sequencing, whole-exome sequencing

Introduction

Hearing loss is the most common congenital sensorineural disorder and affects 1 in 500–1000 newborns (Morton and Nance, 2016). More than 50% of childhood prelingual hearing impairments are caused by genetic factors, while the remaining cases are mainly attributed to environmental factors such as antibiotic use, noise, and infection (Morton and Nance, 2016). Hearing loss can occur in syndromic or nonsyndromic (NSHL) forms. There are nearly 400 types of syndromic deafness, which are accompanied by other clinical abnormalities such as retinitis pigmentosa or goiter, and accounts for 30% of deafness cases. Meanwhile, NSHL or isolated deafness represents 70% of hearing loss cases. The prevalence of profound bilateral hearing loss is 1.6 per 1000 in the Pakistani population and 70% of deafness cases occur in consanguineous families (Elahi *et al.*, 1998).

NSHL and syndromic hearing loss are genetically heterogeneous, wherein most cases show autosomal recessive inheritance, although autosomal dominant, X-linked, or mitochondrial transmission modes also exist (Morton, 1991). To date, more than 100 genetic loci and 80 genes underlying NSHL have been identified (Hereditary Hearing Loss Homepage). The mutation spectrum of deafness genes may vary among different ethnic groups. For the Pakistani population, mutations in *GJB2*, *SLC26A4*, *MYO15A*, *TMC1*, *OTOF*, and *CDH23* were previously shown to be the major cause of hearing loss (Shafique *et al.*, 2014).

In this study, we used Sanger sequencing and next-generation sequencing (NGS) to analyze 12 consanguineous Pakistani families with hearing loss. We found mutations in eight known genes to confirm the extreme heterogeneity of this disorder.

Materials and Methods

Study subjects

A group of 12 consanguineous families with hearing loss were recruited from Pakistan. A clinical questionnaire was used to rule out any history of other diseases and environmental factors such as antibiotic use, noise, and infection that could cause hearing loss. Clinical information and blood samples were obtained from the probands and their family members after informed consent was provided. Genomic DNA was extracted from peripheral blood leukocytes using a

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QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's protocols. Genetic testing was performed in accordance with the Helsinki Declaration and approved by the Peking Union Medical College Institutional Review Board.

Screening for GJB2 mutations

The two coding exons and the flanking intronic sequences of *GJB2* were amplified by polymerase chain reaction (PCR) and then subjected to Sanger sequencing after purification. Primer sequences are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/gtmb).

Targeted NGS

Genomic DNA (20 ng) was amplified to prepare libraries using a designed panel containing 62 genes that are associated with hearing loss (Life Technologies). The amplifiable libraries were diluted to 100 pM and emulsion PCR was performed to obtain template-positive ion sphere particles (ISPs) according to the manufacturer's instructions (Life Technologies). ISPs were loaded and sequenced on an Ion 318V2 Chip using an Ion Torrent Personal Genome Machine (Life Technologies).

Whole-exome sequencing

For whole-exome sequencing, genomic DNA (100 ng) was amplified to prepare an exome library using the Ion AmpliSeq™ Exome Panel (Life Technologies). The amplifiable libraries were diluted to 100 pM. Then, we performed emulsion PCR using a OneTouch 2 instrument (Life Technologies) with an Ion PI Template OT2 200 Kit V3 (Life Technologies). ISP enrichment was achieved using the Ion OneTouch ES enrichment system (Life Technologies). An Ion Proton I chip (Life Technologies) was prepared and loaded according to the manufacturer's instructions.

Data analysis

Sequence data were aligned to the GRCh37/hg19 reference sequence with the Torrent Mapping Alignment Program. Genotype calling of multiallelic substitutions and indels was performed using a Torrent Variant Caller (version 4.4.3). After variant detection, ANNOVAR was used for annotation (Wang *et al.*, 2010). In addition, sequence data were visualized using the Integrative Genome Viewer (IGV) (Robinson *et al.*, 2011; Thorvaldsdóttir *et al.*, 2013). To assess missense mutation pathogenicity, three prediction programs (SIFT, Polyphen2, and Mutation Taster) (Kumar *et al.*, 2009; Adzhubei *et al.*, 2010; Schwarz *et al.*, 2014) and two conservation programs (PhyloP and GERP++) were used (Davydov *et al.*, 2010; Pollard *et al.*, 2010). Effects on splicing were evaluated with Human Splicing Finder (HSF) (Desmet *et al.*, 2009).

Mutation confirmation

To validate mutations detected by NGS, specific fragments were PCR amplified using site-specific primers (Supplementary Table S1) and analyzed by Sanger sequencing. Novel mutations were further analyzed in 200 ethnically matched control individuals.

Results

Clinical manifestations

This study enrolled 12 families that had 71 individuals, of whom 34 were affected by hearing loss. All families were derived from consanguineous mating, indicating a possible autosomal recessive inheritance, although other inheritance patterns, such as autosomal dominant, were also considered. The age of the patients ranged from 10 to 52 years. All of the probands had bilateral, prelingual, moderate to profound hearing impairment. The patients were otherwise healthy and developed normally, except those from F1 and F4. The proband F1 suffered from night blindness at the age of eight and Usher syndrome was diagnosed (Ahmed *et al.*, 2008). The affected individuals in F4 had goiter and presented with Pendred syndrome (Coyle *et al.*, 1998) (Table 1).

Mutation identification and verification

Details of the candidate variants are summarized in Figure 1 and Table 2. Screening for *GJB2* mutations identified a homozygous c. 35del G mutation (p. Gly12Valfs*2) in F8. Subsequently, the other 11 probands were selected for targeted NGS to identify pathogenic mutations. For each sample, 70 MB of data were obtained, and the mean depth reached to 216× coverage with 95.86% of the target region covered. These data showed the detection of ~300 variants. To identify plausible pathogenic mutations, a series of filter criteria were applied. First, only homozygous or compound heterozygous variants were selected as candidates arising from an autosomal recessive inheritance pattern. Second, all variants with a minor allele frequency <1% (dbSNP142 and Exome Aggregation Consortium [ExAC]) were retained. Third, only nonsynonymous variants in the coding region or splicing variants were selected. Finally, that status of the variants was confirmed visually using IGV (Robinson *et al.*, 2011; Thorvaldsdóttir *et al.*, 2013). The remaining variants were further verified using Sanger sequencing. If we did not find candidate variants with these filters, we focused on heterozygous *de novo* variants following an autosomal dominant inheritance pattern.

In total, we found eight homozygous and four compound heterozygous candidate variants in 10 different consanguineous deafness families using targeted NGS (Fig. 1; Table 2).

Table 1. Clinical Features of Probands in Twelve Pakistani Families with Hearing Loss

Family	*No. of probands*	*Gender*	*Age (years)*	*Severity*	*Other symptoms*
F1	IV-7	Male	10	Profound	Night blindness
F2	IV-1	Female	18	Profound	No
F3	IV-1	Male	16	Moderate	No
F4	IV-4	Male	38	Profound	Goiter
F5	V-5	Male	12	Profound	No
F6	IV-2	Female	12	Profound	No
F7	III-4	Male	52	Profound	No
F8	V-1	Female	16	Profound	No
F9	IV-1	Male	21	Profound	No
F10	V-5	Male	13	Severe	No
F11	IV-2	Male	25	Moderate	No
F12	IV-2	Male	12	Profound	No

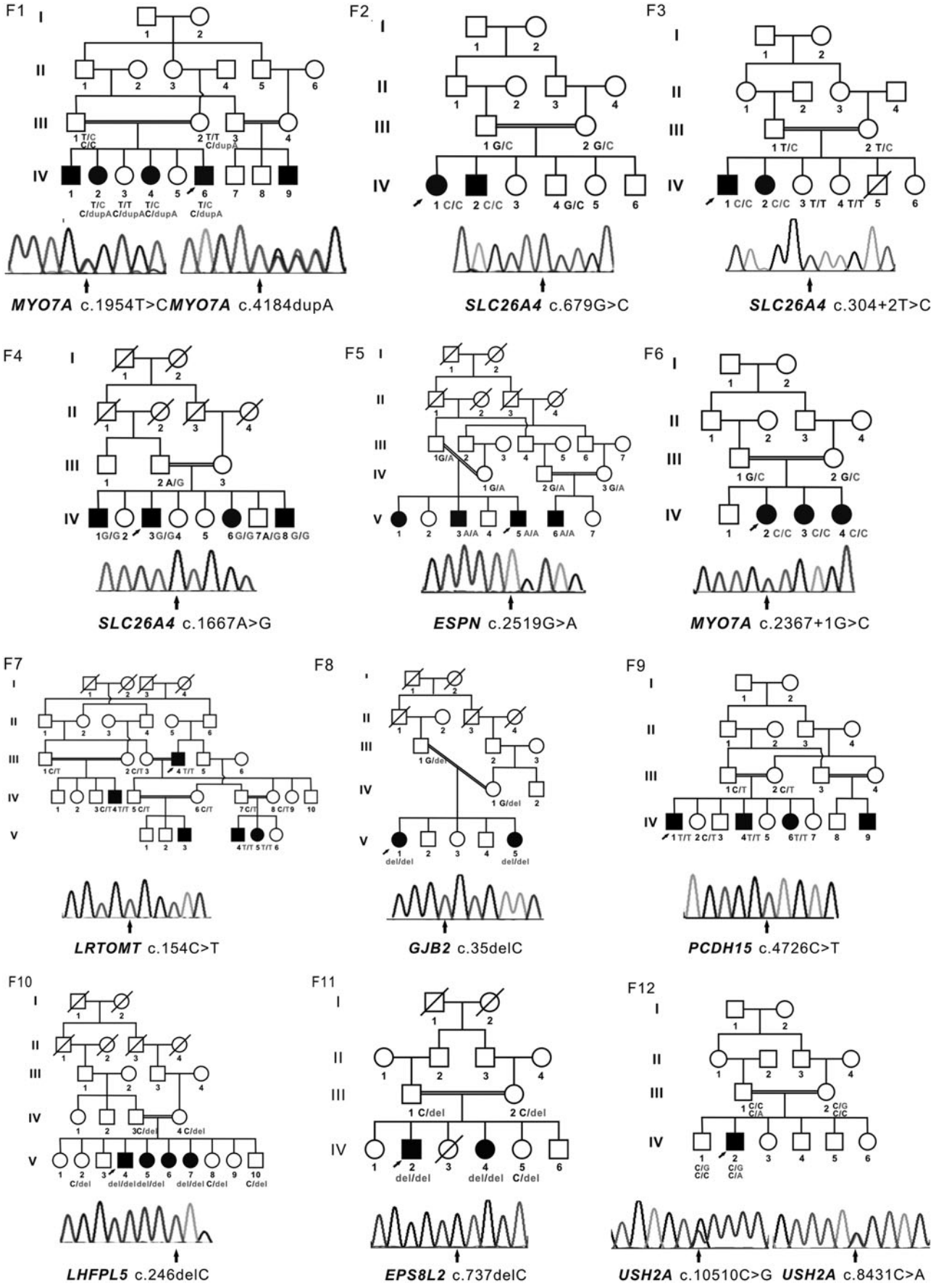

FIG. 1. Family pedigrees and the segregation of mutations in known genes. *Squares* and *circles* indicate males and females, respectively, whereas *solid* and *open symbols* represent affected and unaffected individuals, respectively. *Slashes* indicate deceased family members. *Arrows* indicate the probands or the mutant sites. Del, deletion; dup, duplication.

TABLE 2. MUTATIONS IDENTIFIED IN TWELVE PAKISTANI FAMILIES WITH HEARING LOSS

Family	*Gene*	*Transcript ID*	*cDNA*	*Predicted protein effect*	*ExAC(OAF)*	*PhyloP*	*GERP++*	*SIFT/Polyphen2/ mutation taster*	*References*
F1	*MYO7A*	NM_000260	c. 1954 T>C	p. Cys652Arg	0	1.972	5.21	D/D/D	Novel
			c. 4184dupA	p. Gln1395Thrfs*9	0	NA	NA	NA/NA/NA	Novel
F2	*SLC26A4*	NM_000441	c. 679 G>C	p. Ala227Pro	0	2.536	5.41	D/D/D	Jiang *et al.* (2010)
F3	*SLC26A4*	NM_000441	c. 304 + 2 T>C	p. Met103Lysfs*4	0.000008237	NA	NA	NA/NA/D	Anwar *et al.* (2009)
F4	*SLC26A4*	NM_000441	c. 1667 A > G	p. Tyr556Cys	0.00002487	2.117	5.51	D/D/D	Coyle *et al.* (1998)
F5	*ESPN*	NM_031475	c. 2519G > A	p. Trp840*	0	1.945	4.32	NA/NA/D	Novel
F6	*MYO7A*	NM_000260	c. 2367 + 1G > C	NA	0	2.272	4.87	NA/NA/D	Novel
F7	*LRTOMT*	NM_001145308	c. 154C > T	p. Arg52Trp	0	0.085	0.993	D/D/N	Novel
F8	*GJB2*	NM_004004	c. 35del G	p. Gly12Valfs*2	0.006040	NA	NA	NA/NA/NA	Zelante *et al.* (1997)
F9	*PCDH15*	NM_001142769	c. 4726 C>T	p. Gln1576*	0	0.188	0.931	NA/NA/D	Novel
F10	*LHFPL5*	NM_182548	c. 246del C	p. Gly82Glyfs*3	0	NA	NA	NA/NA/NA	Shabbir *et al.* (2006)
F11	*EPS8L2*	NM_022772	c.737delC	p. Ala246Alafs*6	0	NA	NA	NA/NA/NA	Novel
F12	*USH2A*	NM_206933	c.10510 C>G	p. Pro3504Ala	0.001964	0.562	3.3	T/B/D	van Huet *et al.* (2015)
			c.8431 C>A	p. Pro2811Thr	0.0003544	2.731	5.79	T/D/D	Novel

ExAC, exome aggregation consortium; OAF, overall allele frequency; NA, not available; D, deleterious; N, neutral; T, tolerated; B, benign.

All variants cosegregated with the phenotype within each family and none of the novel mutations was present in chromosomes from 200 ethnically matched control individuals. Five mutations were reported previously, including c.679G>C [p. Ala227Pro] and c.304+2 T>C [p. Met103Lysfs*4] in *SLC26A4*, c.246delC [p. G82Gfs*3] in *LHFPL5*, c.1667A>G [p. Tyr556Cys] in *SLC26A4*, and c.10510 C>G [p. Pro3504Ala] variants in *USH2A*. Among the remaining seven novel variants, two nonsense variants (c. 2519G>A [p. Trp840*] in *ESPN* and c. 4726 C>T [p. Gln1576*] in *PCDH15*) and the frameshift variant (c. 4184dupA [p. Gln1395Thrfs*9] in *MYO7A*) were predicted to lead to either synthesis of truncated protein products or to degradation by nonsense-mediated mRNA decay (NMD). HSF predicted a splicing variant (c. 2367+1G>C) in *MYO7A* (Desmet *et al.*, 2009) that would produce an aberrantly spliced transcript, whereas three missense variants (c. 1954 T>C [p. Cys652Arg] in *MYO7A*, c. 154C>T [p. Arg52Trp] in *LRTOMT*, and c.8431 C>A [p. Pro2811Thr] in *USH2A*) changed conserved amino acids. Two of the three prediction programs (SIFT, Polyphen2, or Mutation Taster) predicted these mutations to be possibly damaging (Kumar *et al.*, 2009; Adzhubei *et al.*, 2010; Schwarz *et al.*, 2014).

In addition, we performed whole-exome sequencing on the proband from F11 in which we failed to identify a causative mutation by Sanger sequencing and targeted NGS. We identified a homozygous frameshift variant (c.737delC [p. Ala246Alafs*6]) in *EPS8L2* in this individual (Fig. 1; Table 2). The homozygous variant cosegregated with deafness within the family, but was absent from public databases (dbSNP142 and ExAC) and the 200 control individuals. To our knowledge, this is the first report of mutations in *EPS8L2* in a Pakistani family with NSHL.

Discussion

In this study, we found 14 candidate mutations that accounted for deafness in all 12 families studied. Of these, three affected families harbored mutations in *SLC26A4* (3/12=25%) and two in *MYO7A* (2/12=16.67%). The other six families each harbored one or two pathogenic mutation in *GJB2*, *ESPN*, *LRTOMT*, *PCDH15*, *LHFPL5*, *USH2A*, or *EPS8L2*. This finding indicates that *SLC26A4* and *MYO7A* mutations make a notable contribution to populations with hearing loss in Pakistan, which is in line with previously reported studies, although the mutation frequency was higher (Shahzad *et al.*, 2013).

Among the 14 mutations, six were known mutations and eight were novel mutations. All of the mutations segregated with affected individuals in these families, but were not detectable in 200 control individuals, indicating that they were not merely common silent polymorphisms. Two mutations (c.679G>C [p. Ala227Pro] and c. 304+2 T>C [p. Met103Lysfs*4]) in *SLC26A4* as well as the c.246delC (p. G82Gfs*3) *LHFPL5* mutation were previously shown to be associated with NSHL (Shabbir *et al.*, 2006; Anwar *et al.*, 2009; Jiang *et al.*, 2010). In addition, the c.1667A>G (p. Tyr556Cys) *SLC26A4* mutation in F4 causes Pendred syndrome (Coyle *et al.*, 1998). Meanwhile, the potential significance of the c.10510 C>G [p. Pro3504Ala] variant in *USH2A* was unclear (van Huet *et al.*, 2015), yet the c.10510 C>A [p. Pro3504Thr] variant in the same position was

reported to be pathogenic (Dreyer *et al.*, 2008). Although mutations in *GJB2* have previously been reported to be the most common cause of NSHL in the Pakistani population (Bukhari *et al.*, 2013; Shafique *et al.*, 2014), only one known mutation (c. 35del G [p. Gly12Valfs*2]) (Zelante *et al.*, 1997) was detected in these 12 families. This result might be due to the small sample size in this study.

Because the frameshift mutations in *MYO7A* (c. 4184dupA [p. Gln1395Thrfs*9]) and *EPS8 L2* (c.737delC [p. Ala246Alafs*6]) produce stop codons in the middle of the two genes, they may activate NMD pathways and could conceivably underlie the pathogenesis of hearing loss through a loss-of-function mechanism (Dahmani *et al.*, 2015). With respect to the nonsense mutations in *ESPN* (c. 2519G > A [p. Trp840*]) and *PCDH15* (c. 4726 C > T [p. Gln1576*]) that are located in the last exons of both genes, these mutant mRNAs might escape degradation by the NMD pathway and are predicted to result in truncation of the encoded proteins. However, direct RNA analysis must be performed to verify this possibility. *ESPN* encodes the calcium-insensitive actin-bundling protein espin (Naz *et al.*, 2004). Amino acids 13 and 19 of the espin carboxy-terminus are known to be required for actin bundling and microvillar elongation activity, respectively (Bartles *et al.*, 1998; Loomis *et al.*, 2003). Hence, the nonsense c. 2519G > A mutation lacking the 15 amino acids of the espin C-terminus is presumed to be pathogenic. *PCDH15* encodes three alternative, evolutionarily conserved unique cytoplasmic domains (CD1, CD2, and CD3) (Ahmed *et al.*, 2006, 2008). Although ~74 different *PCDH15* mutations have been reported to cause NSHL, only the c.4542dupA ([p. P1515Tfs*4]) mutation and c.1103delT [p.Leu368Trpfs*58] mutation specifically affected the CD2 isoform (Pepermans *et al.*, 2014; Bonnet *et al.*, 2016). The c. 4726 C > T [p. Gln1576*] mutation found in this study adds a third example to illuminate the key function of the *PCDH15* CD2 isoform in mature auditory hair cells. In general, missense variants in *PCDH15* are associated with ''Deafness, autosomal recessive 23'' (DFNB23, OMIM 609533) (Ahmed *et al.*, 2008), whereas more severe pathogenic variants (e.g., splicing, frameshift, nonsense, and large deletions) cause Usher syndrome type I (OMIM 276900). The p. Gln1576* mutation that produces a PCDH15 protein lacking the C-terminal 214 amino acids might underlie NSHL because of its relatively mild effect on protocadherin-15. Both missense mutations (c. 154C>T [p. Arg52Trp] in *LRTOMT* and c. 1954 T > C [p. Cys652Arg] in *MYO7A*) are believed to be pathogenic because of their location and conservation of the affected amino acids, and because neither change has been observed in a series of normal controls. The c. 154C > T mutation is present within a mutational hot spot in the *LRTOMT*2 gene (Ichinose *et al.*, 2015), whereas the c. 1954 T > C mutation in *MYO7A* is located in the crucial region of the myosin head that is thought to be involved in transducing energy from the site of ATP hydrolysis to the regulatory domain (Mburu *et al.*, 1997). Thus, the pathogenicity of both mutations is highly probable. *USH2A* encodes a protein that contains laminin EGF motifs, a pentaxin domain, and several fibronectin type III motifs. Mutations within this gene are associated with Usher syndrome type IIa and retinitis pigmentosa (Eudy *et al.*, 1998; Rivolta *et al.*, 2000). In this study, the c.8431 C > A and c.10510 C > G mutations identified in *USH2A* were predicted to affect the fibronectin type III 14 and 20 motifs, respectively. Based on the low frequency of these mutations in population databases and the cosegregation with the phenotype, these mutations are likely to be pathogenic.

Notably, we identified compound heterozygous *MYO7A* and *USH2A* mutations in consanguineous families F1 and F12, indicating that the cause of deafness in this family was not due to inheritance of the same mutation from both parents, as is often the case for autosomal recessive disorders in consanguineous marriages.

In summary, we found 14 pathogenic mutations that accounted for deafness in all 12 Pakistani families studied. Because the methods used for sequence analysis offer high efficiency and accuracy, as well as cost-effectiveness, this study provides support for the clinical adoption of NGS to screen rare pathogenic genes associated with hearing loss.

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Author Disclosure Statement

No competing financial interests exist.

Web Resources

Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org
ANNOVAR: http://wannovar.usc.edu
IGV: http://www.broadinstitute.org/igv
SIFT: http://sift.jcvi.org
Polyphen2: http://genetics.bwh.harvard.edu/pph2
Mutation Taster: http://www.mutationtaster.org
HSF: http://www.umd.be/HSF3
dbSNP: http://www.ncbi.nlm.nih.gov/SNP
ExAC: http://exac.broadinstitute.org

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