Genetic Spectrum of Autosomal Recessive Non-Syndromic Hearing Loss in Pakistani Families

Sobia Shafique1*, Saima Siddiqi2*, Margit Schraders3,4,5, Jaap Oostrik3,4,5, Humaira Ayub1, Ammad Bilal6, Muhammad Ajmal1, Celia Zazo Seco3,4,5, Tim M. Strom7, Atika Mansoor2, Kehkashan Mazhar2, Syed Tahir A. Shah1, Alamdar Hussain1, Maleeha Azam1, Hannie Kremer3,4,5,8*, Raheel Qamar1,9,10*

1 COMSATS Institute of Information Technology, Park Road, Islamabad, Pakistan, 2 Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan, 3 Department of Otornolaryngology, Hearing and Genes, Radboud University Medical Center, Nijmegen, The Netherlands, 4 Nijmegen Centre for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands, 5 Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands, 6 Simon Fraser University, Vancouver, British Columbia, Canada, 7 Institute of Human Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany, 8 Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands, 9 Al-Nafees Medical College & Hospital, Isra University, Islamabad, Pakistan

Abstract

The frequency of inherited bilateral autosomal recessive non-syndromic hearing loss (ARNSHL) in Pakistan is 1.6/1000 individuals. More than 50% of the families carry mutations in GJB2 while mutations in MYO15A account for about 5% of recessive deafness. In the present study a cohort of 30 ARNSHL families was initially screened for mutations in GJB2 and MYO15A. Homozygosity mapping was performed by employing whole genome single nucleotide polymorphism (SNP) genotyping in the families that did not carry mutations in GJB2 or MYO15A. Mutation analysis was performed for the known ARNSHL genes present in the homozygous regions to determine the causative mutations. This allowed the identification of a causative mutation in all the 30 families including 9 novel mutations, which were identified in 9 different families (GJB2 (c.598G>A, p.Gly200Arg); MYO15A (c.9948G>A, p.Gln3316Gln; c.3866+1G>A; c.8767C>T, p.Arg2923* and c.8222T>C, p.Phe2741Ser), TMC1 (c.362+18A>G), BSND (c.97G>C, p.Val33Leu), TMPRSS3 (c.726C>G, p.Cys242Trp) and MSR83 (c.20T>G, p.Leu7Arg)). Furthermore, 12 recurrent mutations were detected in 21 other families. The 21 identified mutations included 10 (48%) missense changes, 4 (19%) nonsense mutations, 3 (14%) intronic mutations, 2 (9%) splice site mutations and 2 (9%) frameshift mutations. GJB2 accounted for 53% of the families, while mutations in MYO15A were the second most frequent (13%) cause of ARNSHL in these 30 families. The identification of novel as well as recurrent mutations in the present study increases the spectrum of mutations in known deafness genes which could lead to the identification of novel founder mutations and population specific mutated deafness genes causative of ARNSHL. These results provide detailed genetic information that has potential diagnostic implication in the establishment of cost-efficient allele-specific analysis of frequently occurring variants in combination with other reported mutations in Pakistani populations.


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* Email: Hannie.Kremer@radboudumc.nl (HK); raheelqamar@hotmail.com (RQ)

† These authors contributed equally to this work.

‡ These authors also contributed equally to this work.

Introduction

Deafness or hearing loss is the most common congenital sensorineural disorder affecting about 1 in 1000 children. Genetic factors contribute to approximately half of the cases of hearing loss [1], which can be either syndromic or non-syndromic. The former is responsible for about 30% of prelingual deafness in combination with abnormalities of other organs. Non-syndromic deafness is usually due to abnormalities of the middle and/or the inner ear and is found in 70% of the hereditary cases [2]. The disease is genetically heterogeneous and currently 134 loci have been determined and 80 genes identified for the non-syndromic type (Hereditary Hearing Loss Homepage, URL: http://hereditaryhearingloss.org/). More than 400 syndromes are known with deafness as one of the symptoms and for many of these the causative genes have been identified (Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/omim).

Syndromic and non-syndromic hearing loss display an autosomal recessive, autosomal dominant, X-linked, Y-linked or mitochondrial pattern of inheritance [3]. Eighty percent of the early onset non-syndromic cases have an autosomal recessive inheritance pattern (ARNSHL), while autosomal dominant,
X-linked and mitochondrial non-syndromic deafness contribute to 18%, 1–3% and <1% of the cases, respectively [4]. In Pakistan, hearing impairment is severe and congenital in 70% of the cases and the increased occurrence of these conditions is due to a high rate of consanguineous marriages (60%); profound bilateral deafness occurs at 1.6 per 1000 individuals [5].

The genes most frequently involved in ARNSHL are those encoding gap junction protein beta 2 (GJB2, MIM# 120101), myosin XVA (MYO15A, MIM# 602666), transmembrane channel-like 1 (TMEM16, MIM# 608706), solute carrier family 26 (anion exchanger) member 4 (SLC26A4, MIM# 605646), otokeratin (OTOS, MIM# 606381) and cadherin-related 23 (CDHR2, MIM# 605516), each of which has been found to contain more than 20 different mutations, most of which have been reported in consanguineous families [6]. Mutations in GJB2 are the most common cause of ARNSHL and explain up to 50% cases in the Mediterranean regions [7,8,9] while mutations in multiple genes are the most common cause of ARNSHL and explain up to 50% cases in the consanguineous families [6].

In the present study a panel of 30 unrelated consanguineous Pakistani families was initially tested for involvement of GJB2 and MYO15A followed by whole genome homozygosity mapping and candidate gene sequencing. This approach resulted in defining the mutation spectrum of the disease in the current panel.

Materials and Methods

Ethics statement

Informed written consent of minors was obtained from their guardians. Written consent was taken before recruitment and sampling. Individuals were informed about the purpose of the study and hearing family members and 89 ethnically matched control individuals were informed about the purpose of the study and written consent was obtained. In Pakistan, the declaration and was approved by the Department of Biosciences Ethics Review Board of the COMSATS Institute of Information Technology, Islamabad, Pakistan. All patients, their normal hearing family members and 89 ethnically matched control individuals were informed about the purpose of the study and written consent was taken before recruitment and sampling. Informed written consent of minors was obtained from their guardians.

Genotyping

A total of 30 consanguineous families with ARNSHL were ascertained from different regions of Punjab, Pakistan. Audiometry was performed on a few members from each family to determine the level of hearing loss.

Blood samples were collected in EDTA containing vacutainers. DNA extraction from these samples was carried out using a standard phenol-chloroform/organic method [11]. Microsatellite markers were analyzed as described previously [12] and Sanger sequencing was performed according to the manufacturer's protocols. Homozygosity mapping was performed using an online tool Homozygosity Mapper (URL: http://www.homozygositymapper.org). [15]. The log-ratio of odds (LOD) score for the homozygous regions identified in family DFR18 was calculated using the Gene Hunter v2.1r5 program in the easyLINKAGE plus v5.08 software package [16].

The exons and exon-intron boundaries of the known candidate genes [BSND (NM_003716.2), GJB2 (NM_000603.5), GJB1 (NM_000601.5), MSRB3 (NM_00101816.12), SLC26A4 (NM_000441.1), TMEM16 (NM_138891.2), TMC1 (NM_029222.2) and TMPRSS3 (NM_147196) present in the homozygous regions were sequenced in the proband of the families. Segregation analyses for identified mutations in the corresponding families were performed by Sanger sequencing except for family DFR24 and DFR18. In these families, the segregation of TMPRSS3 and MSRB3 mutations was analyzed with restriction digestion with the enzymes AciI and TaqI, respectively (New England Biolabs Inc, UK).

Eighty nine ethnically matched controls were sequenced for the novel mutations identified in the current study. Control panel screening of GJB2 (c.598G>A); MYO15A (c.9948G>A), (c.3866+1G>A), (c.8767C>T); TMC1 (c.362+18A>G) and BSND (c.979G>C), variants was done by sanger sequencing. While for TMPRSS3 (c.726C>G) and MSRB3 (c.207T>G) variants AciI and TaqI PCR-RFLP were performed, respectively.

In silico prediction of the identified variants

In silico prediction of the identified variants was performed using online prediction tools. Sorting intolerant from tolerant (SIFT; http://sift.jcvi.org; [17]) and Polymorphism Phenotyping v2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2; [18]) were used for analyses of missense changes. Effects on splicing were evaluated with NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/; [19]). SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP-4.0/) was employed to predict the presence and location of the signal peptide/non-signal peptide cleavage sites [20]. In addition, the online tool, Have your Protein Explained (HOPE) (http://www.cmbi.ru.nl/hope/input) was used to predict the three dimensional structural changes at the protein level [21]. The exome variant server (EVS) and an in-house exome database (Human Genetics, Radboud University Medical Centre) were also searched for the presence of putative pathogenic variants.

Minigene Construction and Splicing Assay (for c.362+18A>G mutation in TMC1 and c.9948G>A mutation in MYO15A)

A plasmid containing the genomic region encompassing exons 3–5 of RHO inserted at the EcoRI/Sall sites in the pC1-NEO vector was used for in vivo splicing assays [22]. The plasmid was adapted from the Gateway cloning technology (Life technologies) according to the manufacturer’s protocol. PCR amplified fragments of wild-type and mutant TMC1 exon 8, along with flanking intronic sequences were generated with the following primers, 5’-GGGAGACAGTGTGGTACAGAAAAAGCGAGGTTT-Cggggctaaagtgtcgg-3’ and 5’-GGGAGACAGTGTGGTACAGAAAAAGCGAGGTTT-Cggggctaaagtgtcgg-3’, that contain the attB1 and attB2 sites necessary for Gateway cloning. Similarly, amplified fragments of wild-type and mutant MYO15A exon 61 were generated using primers, 5’-GGGAGACAGTGTGGTACAGAAAAAGCGAGGTTT-Cggggctaaagtgtcgg-3’ and 5’-GGGAGACAGTGTGGTACAGAAAAAGCGAGGTTT-Cggggctaaagtgtcgg-3’. Robinson et al. predicted the level of hearing loss.
Results

All families in this study were consanguineous (Table S1) and all patients in these families were diagnosed with severe to profound congenital hearing loss. Causal mutations were identified in all 30 families and the 9 novel mutations identified in the current study were not present in any of the 89 ethnically matched control individuals.

Sequence analysis of GJB2 in the current cohort identified 16 families with mutations in this gene (Table 1; Table 2) segregating with hearing loss. A recurrent nonsense mutation c.71G>A (p.Trp24*) was the most common and was found in 5 families (31%), followed by another nonsense variant, c.231G>A (p.Trp77*) that was identified in 4 families (25%; Table 1). In addition to the 6 recurrent GJB2 mutations, a novel homozygous change c.398G>A (p.Gly200Arg) was found in one consanguineous family DFR10 (Table 2); as predicted by the HOPE server the larger side chain of the mutant residue arginine might well affect the proper folding of the cysteine rich domain. In addition, the residue resides in the conserved region of the protein (Figure 1; Figure S1A).

Out of the 9 novel mutations (Table 2) identified in the current study, 4 were present in MYO15A, of which 2 were splice site mutations (c.9948G>A and c.3866+1G>A), one was a nonsense (c.8767C>T) and one a missense mutation (c.3822 T>G). The c.9948G>A variant changes the last nucleotide of exon 61 and is predicted to affect the splice donor site. The splice donor site in the reference sequence has a highly confident score of 94%, which is reduced to a score of 24% in the mutant. To determine the effect of the c.9948G>A mutation on splicing, a minigene approach was used. This showed correct splicing of the wildtype MYO15A exon 61, while the c.9948G>A mutation almost completely abolished the normal splicing (Figure 2). NetGene2 predicted the abolition of the splice donor site of exon 5 as a result of the c.3866+1G>A mutation in intron 5 of MYO15A. The nonsense mutation c.8767C>T (p.Arg2923*) was novel and predicted to lead to the synthesis of a truncated protein, while the missense mutation c.8222 T>G, leads to the substitution of serine for phenylalanine (p.Phe2741Ser) at amino acid position 2741 that resides in the conserved region of the protein (Figure 1; Figure S1B). This missense change was predicted to be deleterious by SIFT and Polyphen2 (Table 2).

Haplotype analysis of STR-markers flanking TMC1 showed compatibility with genetic linkage for family 11DF. Since sequence analysis of TMC1 revealed a novel intronic mutation, c.362+18A>G, this family was not further analyzed by SNP-array genotyping. The variant segregated with the hearing loss in the family (Figure 1) and was predicted to create a novel splice donor site with a similar confidence score as the original splice donor site. To determine the effect of the c.362+18A>G mutation on splicing, a minigene approach was used. This revealed correct splicing of the wild-type TMC1 exon 8, while the c.362+18A>G mutation resulted in a 17 bp extension of exon 8 (Figure 3). This leads to a frameshift and a premature stop codon (p.Glu122Tyrfs*10).

A novel homozygous missense mutation was identified in TMPRSS3 (MIM_605511), c.726C>G (p.Cys242Trp) that co-segregated with hearing impairment in family DFR24 (Figure 1) and was predicted to be probably damaging by Polyphen2 and deleterious by SIFT (Table 2). This mutation is located in the peptidase S1 domain and the HOPE server predicted the abolition of the catalytic activity of TMPRSS3. In addition the amino acid substitution is present in a region of the protein that is conserved across different species (Figure S1D) and therefore probably affects the core structure of the peptidase domain (Figure 4).

In family DFR18 SNP microarray data analysis revealed a 17.6 Mb homozygous region on chromosome 12 flanked by SNPs rs7973881 and rs7976868, a LOD score of 2.7 was calculated for this region. The known deafness gene, MSRB3 (MIM_613719), in the region was subsequently sequenced, revealing a novel homozygous nucleotide substitution c.20T>G (p.Leu7Arg) in exon 4 (Figure 1; Table 2). The leucine at position 7 is located in the signal peptide of the MSRB3 protein and as a result of the substitution by arginine this signal peptide loses its function as predicted by SignalP 4.0. Furthermore, the Leu7 residue is conserved across species (Figure S1E). The mutation abolishes a Tsf restriction site, which allowed the segregation of this variant in the family to be checked by restriction digestion. By using the same analysis, this variant was also found heterozygously in 3 out of 178 ethnically matched control alleles.

The novel BSND (MIM_606412) missense mutation c.97G>C (p.Val33Leu) in family 7DF (Figure 1) was predicted to be deleterious by SIFT and Polyphen2 (Table 2) and Val33 is conserved across species (Figure S1C).

Table 1. Spectrum of recurrent GJB2 mutations in Pakistani families with autosomal recessive non-syndromic hearing loss (ARNSHL).

<table>
<thead>
<tr>
<th>Mutation identified (Protein change)</th>
<th>Type of mutation</th>
<th>No. of families</th>
<th>No. of affected members</th>
<th>Frequency in EVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.71G&gt;A (p.Trp24*)</td>
<td>Nonsense (homozygous)</td>
<td>5</td>
<td>26</td>
<td>Absent</td>
</tr>
<tr>
<td>c.231G&gt;A (p.Trp77*)</td>
<td>Nonsense (homozygous)</td>
<td>4</td>
<td>14</td>
<td>AA = 0/AG = 1/GG = 4299</td>
</tr>
<tr>
<td>c.35delG (p.Gly12Valfs*2)</td>
<td>Frameshift (homozygous)</td>
<td>2</td>
<td>8</td>
<td>Absent</td>
</tr>
<tr>
<td>c.35delG (p.Gly12Valfs*2) + c.439G&gt;A (p.Glu147Lys)</td>
<td>Missense (compound heterozygous)</td>
<td>1</td>
<td>3</td>
<td>Absent</td>
</tr>
<tr>
<td>c.380G&gt;A (p.Arg127His)</td>
<td>Missense (homozygous)</td>
<td>2</td>
<td>4</td>
<td>AA = 0/AG = 26/GG = 4274</td>
</tr>
<tr>
<td>c.377_378insATGCGGA (p.Arg127Cysfs*85)</td>
<td>Frameshift (homozygous)</td>
<td>1</td>
<td>2</td>
<td>Absent</td>
</tr>
</tbody>
</table>

As reference sequence NM_004004.5 was employed. EVS, exome variant server; *The pathogenicity of this mutation is controversial.

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Gene expression was further investigated in HEK293T cells that were incubated at 37°C for 24 hours. RNA was then isolated from the transfected cells and reverse transcribed into cDNA and sequenced. Forward primer 5'-gcggaggtcaacaacgagtct-3' and reverse primer 5'-aggtagggagggagac-3' were used to amplify and sequence the amplified cDNA fragments along with flanking RHO sequences as present in the vector.

The splice donor site of exon 5 as a result of the c.3866+1G>T mutation in intron 5 of MYO15A was predicted by NetGene2 to lead to the synthesis of a truncated protein, while the missense mutation c.377_378insATGCGGA (p.Arg127Cysfs*85) was novel and predicted to lead to the synthesis of a truncated protein, while the missense mutation c.377_378insATGCGGA (p.Arg127Cysfs*85) was novel and predicted to lead to the synthesis of a truncated protein.
### Table 2. Novel mutations identified in known genes for autosomal recessive non-syndromic hearing loss (ARNSHL) in the current study.

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Size of homozygous regions (Mb)</th>
<th>Chr.</th>
<th>Flanking SNPs</th>
<th>Chr. position (in hg19)</th>
<th>Candidate gene (Acc. No.)</th>
<th>Mutation (Predicted protein change)</th>
<th>PhyloP; SIFT; Polyphen</th>
<th>NetGene2</th>
<th>Frequency in EVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFR10</td>
<td>ND</td>
<td>13</td>
<td>ND</td>
<td>ND</td>
<td>GJB2 (NM_004004.5)</td>
<td>Ex-2: c.598G&gt;A (p.Gly200Arg)</td>
<td>3.43 Deleterious; Damage</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>DFR23</td>
<td>ND</td>
<td>17</td>
<td>ND</td>
<td>ND</td>
<td>MYO15A (NM_016239.3)</td>
<td>Ex-61: c.9948G&gt;A (p.Gln3316Gln)</td>
<td>NA</td>
<td>Abolition of splice site</td>
<td>Absent</td>
</tr>
<tr>
<td>13DF</td>
<td>ND</td>
<td>17</td>
<td>ND</td>
<td>ND</td>
<td>MYO15A (NM_016239.3)</td>
<td>In-5: c.3866+1G&gt;A (p.??)</td>
<td>NA</td>
<td>Abolition of splice site</td>
<td>AA = 0, AG = 1, GG = 6051</td>
</tr>
<tr>
<td>DFR28</td>
<td>ND</td>
<td>17</td>
<td>ND</td>
<td>ND</td>
<td>MYO15A (NM_016239.3)</td>
<td>Ex-50: c.8767C&gt;T (p.Arg2923*)</td>
<td>NA</td>
<td>NA</td>
<td>TT = 0, TC = 1, CC = 6266</td>
</tr>
<tr>
<td>DFR3</td>
<td>ND</td>
<td>17</td>
<td>ND</td>
<td>ND</td>
<td>MYO15A (NM_016239.3)</td>
<td>Ex-45: c.8222T&gt;C (p.Phe2741Ser)</td>
<td>4.97 Deleterious; Damage</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>11DF</td>
<td>ND</td>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>TMC1 (NM_138691.2)</td>
<td>In-8: c.362+18A&gt;G (p.Glu122Trpfs*10)</td>
<td>NA</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>7DF</td>
<td>8.40</td>
<td>1</td>
<td>rs1242330;</td>
<td>53,396,842-61,803,889</td>
<td>BSND (NM_057176.2)</td>
<td>Ex-1: c.97G&gt;C (p.Val33Leu)</td>
<td>1.09 Deleterious; Damage</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>DFR24</td>
<td>3.49</td>
<td>21</td>
<td>rs2838063;</td>
<td>42,929,129-46,421,694</td>
<td>TMPRSS3 (NM_024022.2)</td>
<td>Ex-8: c.726C&gt;G (p.Cys242Trp)</td>
<td>-0.28 Deleterious; Damage</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>DFR18</td>
<td>2.92</td>
<td>12</td>
<td>rs6581511;</td>
<td>64,278,102-67,207,064</td>
<td>MSRB3 (NM_001031679.2)</td>
<td>Ex-4: c.207G&gt;T (p.Leu7Arg)</td>
<td>3.76 Tolerated; Possibly Damaging</td>
<td>NA</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Acc. No., accession number of reference sequence; Chr, chromosome; Ex, exon; EVS, exome variant server; hg19, human genome assembly 19; In, intron; NA, not applicable; ND, not determined; SNPs, single nucleotide polymorphisms; PhyloP, phylogenetic P-values; Polyphen, polymorphism phenotyping; SIFT, sorting intolerance from tolerance.

doi:10.1371/journal.pone.0100146.t002
Figure 1. Pedigrees and the segregation of novel mutations in known deafness genes. Unfilled circles indicate unaffected females, unfilled squares indicate unaffected males, filled circles indicate affected females, filled squares indicate affected males, double lines represent consanguineous marriages, slashed line across the symbols indicate deceased individual, + indicates wild type allele, M indicates mutant allele.

doi:10.1371/journal.pone.0100146.g001
Recurrent mutations in *TMC1* (c.1114G>A; p.Val372Met [23] and c.100C>T; p.Arg34* [24]), *HGF* (c.482+1991_2000delGAT-GATGAAA and c.482+1986_1988delTGA [25]), *SLC26A4* (c.1337A>G; p.Gln446Arg [26]) and *TMIE* (c.241C>T; p.Arg81Cys [27]) also segregated in the respective families and most likely are the disease causing mutations (Table 3).

**Figure 2.** Effect of MYO15A c.9948G>A using a minigene approach. An agarose gel containing RT-PCR products detected from HEK293T cells transfected with the wildtype and mutant minigene construct and a schematic representation of the identified splicing products. The RT-PCR products were verified by sequence analysis. The c.9948G>A mutation leads to skipping of exon 61.

doi:10.1371/journal.pone.0100146.g002

**Figure 3.** Effect of *TMC1* intronic mutation c.362+18A>G using a minigene approach. Electropherogram of the partial cDNA sequence of RNA derived from cells transfected with the pCI-NEO with either the mutant or wildtype *TMC1* exon 8. The mutation leads to the insertion of 17bp at the 3’ end of exon 8, which can be predicted to result in a premature stop codon in exon 9 (p.Glu122Tyrfs*10).

doi:10.1371/journal.pone.0100146.g003
In a cohort of 30 ARNSHL families the genetic defects were identified in 9 known deafness genes; GJB2, MYO15A, TMC1, BSND, TMPRSS3, MSRB3, HGF, SLC26A4 and TMIE. In the current panel recurrent as well as novel mutations were detected, the novel mutations were identified in GJB2, MYO15A, TMC1, BSND, TMPRSS3 and MSRB3. GJB2 was the most frequently mutated gene in these families. In this gene the novel mutation c.598G>A (p.Gly200Arg) affects a residue in the cysteine-rich domain that is involved in the formation of intramolecular disulphide bonds [28,29]. Although the effect of glycine on the intramolecular disulphide bond formation cannot be predicted, the mutant residue arginine may affect the proper folding of the cysteine-rich domain because of the larger size of the arginine side chain.

In two families the GJB2 variant c.380G>A (p.Arg127His) was found to segregate with the hearing loss, being present homozygously in 4 affected members. Other studies have also reported deafness patients carrying this mutation homozygously [29,30,31]. Based upon the higher carrier frequency in patients as well as controls, Padma et al. [30] suggested that it is unlikely that this variant plays a pathogenic role in deafness. However, Matos et al. [32] have proposed that this mutation is likely to be pathogenic when influenced by other unknown genetic or environmental factors [32]. In the EVS, however, no homozygous occurrence of the mutated allele has been reported to date (Table S1). Identification of 4 p.Arg127His homozygous patients out of 125 deafness patients in the current study and the previously reported cases of 9 heterozygous cases and 1 compound heterozygous case in 70 deaf patients by Bukhari et al. [31], demonstrates that this is the most frequently occurring mutation in Pakistani deafness families and therefore becomes important in genetic counseling of Pakistani deaf patients.

In the current study GJB2 mutations were found to be the most common, followed by MYO15A mutations. The overall frequency of the two most common nonsense variants c.71G>A (p.Trp24*) and c.231G>A (p.Trp77*) of GJB2 in Pakistani deafness patients was obtained from the current (n = 125) as well as previously reported studies of Santos et al. [33] (n = 430) and Bukhari et al. [31] (n = 70). Using these data, the frequency was found to be 5.9% (p.Trp24*) and 4.3% (p.Trp77*) [31,33]. The variant p.Trp24* has a high prevalence in the Indian population as well [34], Pakistan and India have a shared genetic ancestry [35], which could be the reason of the presence of this variant in both populations. The third mutation p.Gly12Val6*2 identified in the current study has previously been reported from Northern areas of Pakistan [31] and also in Caucasians and Turks [36], thus indicating a possible founder effect of this mutation. In the current cohort, 35% (60/173) individuals were found to be carriers of GJB2 mutations. Therefore, as a preliminary screening of deaf families the sequencing of this gene is suggested in Pakistan. In the current study MYO15A was found to be the second leading cause of deafness in the Pakistani population, but still no recurrent mutation was identified in this gene.

A total of 7 nonsense mutations were identified in GJB2, MYO15A and TMC1, these nonsense mutations are likely to cause nonsense mediated decay (NMD) because they are either present in the middle or near the 5’ end of the gene. In MYO15A two splice site, a nonsense and a missense mutations were identified. The c.9948G>A (p.Gln3316Gln) mutation affects the last nucleotide of exon 61 and changes the consensus splice site sequence. Based on the results of the minigene assay the c.9948G>A mutation is expected to lead to skipping of exon 61, which would result in a frameshift and NMD can be expected to occur at least for part of the mRNAs. The second splice site mutation c.3866+1G>A is a canonical splice site change and is predicted to remove the splice donor site of exon 5. An alternative splice site is predicted at position +99 in intron 5, if this alternative site is used it would lead to a frameshift and a premature stop codon (encoded by nucleotide 2–4 of intron 5). Both splice site mutations are thus predicted to lead to a frameshift and NMD could occur for at least part of the mRNA transcripts. The two other novel mutations, c.8222T>C (p.Phe2741Ser) and c.8767C>T (p.Arg2923*), found in two different families, are located in the region of the gene that encodes the tail region of MYO15A and are likely to cause a loss of function of this region. The p.Arg2923* mutation is present in the SH3 domain, which is involved in the protein-protein interactions [37]. Most of the previously reported mutations of MYO15A causing congenital severe to profound deafness were found in the motor head and the tail domains [6,38]. Collectively these results indicate that the motor head and tail regions of MYO15A are essential in the hearing process and any mutation in these regions is thus critical [39].

TMC1 encoding a transmembrane protein is expressed in the neurosensory hair cells of the mouse cochlea [40]. The recurrent nonsense mutation in exon 7 of TMC1, c.100G>T (p.Arg34*) is
the most common TMC1 mutation reported in Pakistan. It leads to a truncated protein and leads to congenital severe to profound hearing loss [40,41,42,43]. Ben Said et al. [42] have previously described that the p.Arg34* is an old founder mutation found in several populations including the Pakistani population. It is likely that the family described here carries the mutation on the founder haplotype. Also a novel intronic mutation in the TMC1 gene, c.362+18A>G was identified in the current study (Table 2), which creates a novel splice donor site and the insertion of 17 nucleotides as demonstrated in the minigene approach.

BSND encodes the barttin protein, which is a vital subunit of the chloride and voltage-sensitive Ka (CLCNKA) and Kb (CLCNKB) channels in the inner ear and the kidney. Chloride channels and the barttin protein form heteromers which function in the recycling of the K⁺ ions in the inner ear and salt reabsorption in the kidneys [44,45,46,47,48,49]. As predicted by in silico analysis, the currently identified BSND mutation p.Val33Leu residing in the transmembranal region might result in the malfunctioning of chloride channels CLCNKA and CLCNKB. However, in the current family there were no complaints of any renal problems.

Deafness caused by mutations in the TMPRSS3 gene is bilateral and severe to profound with no defects of the middle ear and the vestibular system [50]. Mutations in TMPRSS3 can also cause progressive hearing loss with a postlingual onset [51]. TMPRSS3 mutation c.726C>G (p.Cys242Trp) in exon 8 affects the serine protease domain as predicted by 3D modeling. Previously another mutation in the same exon of TMPRSS3, c. 647G>T (p.Arg216Leu) has been shown to result in a failure of the protein to undergo proteolytic cleavage resulting in the inactivation of the sodium channel [52].

The homozygous variant p.Leu7Arg (c.20T>G) in MSRB3 identified in the current study is located in the mitochondrial signal sequence and may result in mislocalization of the protein. Ahmed et al. [53] have shown the importance of the mitochondrial isoforms when they found a mutation c.55T>C (p.Arg19*) to underlie hearing impairment. This mutation also resides in the signal sequence for mitochondrial localization. However, the variant c.20T>G (p.Leu7Arg) was also found in heterozygous state in 3 out of 89 (1.7%) ethnically matched controls. The mutation might be a founder mutation in the corresponding population. However, currently it is uncertain whether this variant is the cause of hearing impairment in the family and further studies are necessary for a definite conclusion on the pathogenic effect of the variant.

**Conclusions**

In the present study 53% (16/30) of the families were found to carry causative mutations in GJB2 illustrating that the most frequently involved gene in deafness in the Pakistani population is GJB2 followed by MYO15A (13%, 4/30) and TMC1 (10%, 3/30). Based on these results it is therefore suggested that as an initial step for the genetic diagnosis of deafness, GJB2 should be analyzed in Pakistani patients and if this gene is excluded then microsatellite markers flanking MYO15A and TMC1 should be genotyped to exclude those genes or to indicate mutation analysis. Homozygosity mapping is an effective approach to determine the mutated genes in consanguineous families. Although a large number of deafness genes and mutations have already been identified, our study demonstrates that the full mutation spectrum in these genes is still not defined. Identification of novel mutations in these genes is important for genetic counseling and can provide handles for further studies on protein function. Genetic counseling of the families is important to better inform couples about the risk of their
offspring to be hearing impaired. In addition, as can be seen in the current study, recurrent as well as novel mutations in known genes define the disease in the families which seem to have some population specificity. This information can be important in developing screening strategies.

Supporting Information


Table S1 Characteristics of 30 Pakistani families diagnosed with autosomal recessive non-syndromic hearing loss (ARNSHL).

| References |


