The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/138088

Please be advised that this information was generated on 2017-11-26 and may be subject to change.
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 Mazhar3, 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 Otolaryngology, 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 Colombia, 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 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 MSRB3 (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.


Editor: Francesc Palau, Instituto de Ciencia de Materiales de Madrid - Instituto de Biomedicina de Valencia, Spain

Received January 1, 2014; Accepted May 22, 2014; Published June 20, 2014

Copyright: © 2014 Shafique et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Higher Education Commission (HEC) grant no 2155 to RQ under the National Research Program for Universities, by The Oticon Foundation (grant 09-3742 to HK), and by ZorgOnderzoek Nederland/Medische Wetenschappen (grant 40-00812-98-09047 to HK). The authors also acknowledge the Higher Education Commission of Pakistan for providing funds to S. Shafique and S. Siddiqi to work in The Netherlands. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* 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 # 120111), myosin XVA (MYO15A, MIM # 602666), transmembrane channel-like 1 (TMCL1, MIM # 606706), solute carrier family 26 (anion exchanger) member 4 (SLC26A4, MIM # 605646), ototerin (OTOF, MIM # 603681) and cadherin-related 23 (CDHR3, 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 have been shown to cause deafness in the remaining cases. In the Pakistani population mutations in MYO15A account for 5% of the recessive deafness [10].

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 spectrum of mutations in the disease in the current panel.

Materials and Methods

Ethics statement

The current study conformed to the tenets of the Helsinki 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 Schraders et al. [13]. Primers were designed using Primer3 software (URL: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/; [14]). Primer sequences are available upon request. PCR was performed using standard conditions.

Prior to whole genome single nucleotide polymorphism (SNP) mapping all families were pre-screened for mutations in GJB2 by Sanger sequence analysis. For the MYO15A locus, microsatellite markers (D17S843, D17S2196, D17S783 and D17S1824) were genotyped and in families in which haplotype analysis showed compatibility with genetic linkage, Sanger sequence analysis was performed for MYO15A. For family 11DF, microsatellite markers (D9S1866, D9S1806 and D9S1876) were used for exclusion of the TMCL1 region.

Families with no identified mutation in GJB2 and MYO15A were further genotyped using the Illumina HumanOmniExpress whole genome single nucleotide polymorphism (SNP) microarray (>700 K SNPs) or the Illumina Human Linkage-12 panel according to the manufacturer’s protocols. Homozygosity mapping was performed using an online tool Homozygosity Mapper (URL: http://www.homozygositymapper.org; [15]). The logarithm 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].

Exons and exon-intron boundaries of the known candidate genes [BSND (NM_003782), GJB2 (NM_000405), MSRB3 (NM_001081162), SLC26A4 (NM_000441), TMPRSS3 (NM_147196), TMC1 (NM_138691), TMPRSS9 (NM_024922) and TMB1 (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 TstI, 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.8866+1G>A), (c.8767C>T), (c.8222 T>C); TMCL1 (c.362+18A>G) and BSND (c.97G>C), variants was done by sanger sequencing. While for TMPRSS3 (c.726C>G) and MSRB3 (c.207T>G) variants AciI and TstI 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 TMCL1 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 pCI-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 TMCL1 exon 8, along with flanking intronic sequences were generated with the following primers, 5’-GGGGAGAAGTTTGTGATACAAAAAGAGAGCGTTT-Cggggcctaatgttgactgc-3′ and 5’-GGGGACCACCTTGTGATACAAAAAGGAGGCGTTGcggggctaatgttgactgc-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’-GGGGAGAAGTTTGTGATACAAAAAGAGGCGTTT-Cggggcctaatgttgactgc-3′ and 5’-GGGG-
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*83)</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.
doi:10.1371/journal.pone.0100146.t001

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.390G>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.3866+1G>A and c.3866+1G>A, one was a nonsense (c.8767C>T) and one a missense mutation (c.8222 T>G). The c.390G>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.390G>A mutation on splicing, a minigene approach was used. This showed correct splicing of the wildtype MYO15A exon 61, while the c.390G>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 nonsense 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 rs7976686, 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 S1F). The mutation abolishes a Tsd 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 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. 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>GJB2 (NM_004004.5)</td>
<td>Ex-2: c.598G&gt;A (p.Gly200Arg)</td>
<td>3.43; Deleterious;</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>DFR23</td>
<td>ND</td>
<td>17</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>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>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>MYO15A (NM_016239.3)</td>
<td>Ex-45: c.8222T&gt;C (p.Phe2741Ser)</td>
<td>4.97; Deleterious;</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>11DF</td>
<td>ND</td>
<td>9</td>
<td>ND</td>
<td>TMCI (NM_138691.2)</td>
<td>In-8: c.362+18A&gt;G (p.Glu122Trfs*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; rs7521242</td>
<td>BSND (NM_057176.2)</td>
<td>Ex-1: c.97G&gt;C (p.Val33Leu)</td>
<td>1.09 Deleterious;</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>DFR24</td>
<td>3.49</td>
<td>21</td>
<td>rs2835063; rs881969</td>
<td>TMPRSS3 (NM_024022.2)</td>
<td>Ex-8: c.726C&gt;G (p.Cys242Trp)</td>
<td>~0.28 Deleterious;</td>
<td>NA</td>
<td>Absent</td>
</tr>
<tr>
<td>DFR18</td>
<td>2.92</td>
<td>12</td>
<td>rs6581511; rs11176432</td>
<td>MSRB3 (NM_001031679.2)</td>
<td>Ex-4: c.207G&gt;T (p.Leu7Arg)</td>
<td>3.76; Tolerated;</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).
Pakistan and India have a shared genetic ancestry, p.Trp24* has a high prevalence in the Indian population as well. The variant (n = 70). Using these data, the frequency was found to be reported studies of Santos et al. (n = 430) and Bukhari et al. was obtained from the current (n = 125) as well as previously and c.231G
of the two most common nonsense variants c.71G
Pakistani deaf patients. 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.Gly12Valfs*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>C (p.Arg34*) is
Table 3. Recurrent mutations in known autosomal recessive non-syndromic hearing loss (ARNSHL) genes in 6 Pakistani families.

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Size of Region (Mb)</th>
<th>Chr. Position (in hg19)</th>
<th>Candidate Gene</th>
<th>Mutation (Predicted protein change)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFR22</td>
<td>9.23</td>
<td>rs4275319; rs2295861</td>
<td>TMC1, (NM_138691.2)</td>
<td>Ex-15: c.1114G&gt;A (p.Val372Met)</td>
<td>[23]</td>
</tr>
<tr>
<td>19DFS</td>
<td>11.96</td>
<td>rs10867845;rs10867778</td>
<td>HGF</td>
<td>Ex-7: c.100C&gt;T, (p.Arg34*) Absent</td>
<td>[40]</td>
</tr>
<tr>
<td>HGF+</td>
<td>26.06</td>
<td>rs10485886;rs2073791</td>
<td>TMC1, (NM_138691.2)</td>
<td>In-4: c.482_1986_1988delTGA (p.?) Absent</td>
<td>[25]</td>
</tr>
<tr>
<td>DFR39</td>
<td>29.83</td>
<td>rs2285507;rs1025936</td>
<td>SLC26A4, (NM_000441.1)</td>
<td>Ex-11: c.362+18A&gt;G (p.Leu7Arg)</td>
<td>[41]</td>
</tr>
<tr>
<td>26DF</td>
<td>3.85</td>
<td>rs304838;rs536036</td>
<td>MSRB3</td>
<td>Ex-3: c.20T&gt;G (p.Leu7Arg)</td>
<td>[27]</td>
</tr>
</tbody>
</table>

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>G (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 (3.4%) 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. Haplotype 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
Hearing Loss Gene Mutations in Pakistani Families

Supporting Information

Figure S1 Multiple-alignment of the corresponding stretches of protein sequences across different species. The blue color shading represents the intensity of conservation, where the dark blue shading represents highly conserved stretch of protein sequences across different species. A) Amino acid sequence conservation of p.Leu7 across 10 species. B) Amino acid sequence conservation of p.Cys24 across 10 species. E) Non-conserved residue of p.Leu7 across 10 species. (TIF)

References


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

Acknowledgments

We thank all the families for their cooperation in the current study. We thank Saskia van der Velde-Viser for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: S. Shafigue S. Siddiqi MO TMS M. Azam HK. RQ. Performed the experiments: S. Shafigue S. Siddiqi MS MO HA AB M. Azam. Analyzed the data: S. Shafigue S. Siddiqi MS MO HA AB M. Azam. CzS TMS AM KM STAS AH M. Ajmal. Contributed reagents/materials/analysis tools: MS TMS HK RQ. Wrote the paper: S. Shafigue S. Siddiqi M. Azam MO HA AB M. Azam. Contributed to the writing of the manuscript: MO TMS HK RQ. Performed the experiments: S. Shafique TMS. M. Azam HK RQ.

Author Contributions

Conceived and designed the experiments: S. Shafigue S. Siddiqi MO TMS M. Azam HK. RQ. Performed the experiments: S. Shafigue S. Siddiqi MS MO HA AB M. Azam. Analyzed the data: S. Shafigue S. Siddiqi MS MO HA AB M. Azam. CzS TMS AM KM STAS AH M. Ajmal. Contributed reagents/materials/analysis tools: MS TMS HK RQ. Wrote the paper: S. Shafigue S. Siddiqi M. Azam MO HA AB M. Azam. Contributed to the writing of the manuscript: MO TMS HK RQ.


