Genetic Analysis through OtoSeq of Pakistani Families Segregating Prelingual Hearing Loss

Mohsin Shahzad, PhD1*, Theru A. Sivakumaran, PhD2,3*, Tanveer A. Qaiser, PhD4, Julie M. Schultz, PhD5, Zawar Hussain, PhD4, Megan Flanagan2, Munir A. Bhinder, PhD4, Diane Kissell, MHSA2, John H. Greinwald Jr, MD2,6, Shaheen N. Khan, PhD4, Thomas B. Friedman, PhD5, Kejian Zhang, MD2,3, Saima Riazuddin, PhD1,2,4, Sheikh Riazuddin, PhD7,8, and Zubair M. Ahmed, PhD1,2,4

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. To identify the genetic cause of prelingual sensorineural hearing loss in Pakistani families using a next-generation sequencing (NGS)–based mutation screening test named OtoSeq.

Study Design. Prospective study.

Setting. Research laboratory.

Subjects and Methods. We used 3 fluorescently labeled short tandem repeat (STR) markers for each of the known autosomal recessive nonsyndromic (DFNB) and Usher syndrome (USH) locus to perform a linkage analysis of 243 multigenerational Pakistani families segregating prelingual hearing loss. After genotyping, we focused on 34 families with potential linkage to MYO7A, CDH23, and SLC26A4. We screened affected individuals from a subset of these families using the OtoSeq platform to identify underlying genetic variants. Sanger sequencing was performed to confirm and study the segregation of mutations in other family members. For novel mutations, normal hearing individuals from ethnically matched backgrounds were also tested.

Results. Hearing loss was found to co-segregate with locus-specific STR markers for MYO7A in 32 families, CDH23 in 1 family, and SLC26A4 in 1 family. Using the OtoSeq platform, a microdroplet PCR-based enrichment followed by NGS, we identified mutations in 28 of the 34 families including 11 novel mutations. Sanger sequencing of these mutations showed 100% concordance with NGS data and co-segregation of the mutant alleles with the hearing loss phenotype in the respective families.

Conclusion. Using NGS-based platforms like OtoSeq in families segregating hearing loss will contribute to the identification of common and population-specific mutations, early diagnosis, genetic counseling, and molecular epidemiology.

Keywords

hearing loss, Usher syndrome, microdroplet PCR, next-generation sequencing, clinical diagnosis, genetic etiology, PDS, MYO7A, CDH23.

Received March 19, 2013; revised May 1, 2013; accepted May 17, 2013.

Introduction

Hereditary deafness is phenotypically and genetically heterogeneous, affecting nearly 300 million people worldwide.1-3 Clinically, hearing loss is manifested by wide variation in associated symptoms, age of onset, the specific...
frequencies affected, and progressive loss of hearing thresholds.\textsuperscript{1-3} Genetically, it has been estimated that ~1% of all human genes may be implicated in the etiology of hereditary deafness.\textsuperscript{1,4} To date, the molecular genetic etiology for approximately 100 deafness genes have been reported.\textsuperscript{5-8} The major challenges and goals in the field have been to identify all the genes containing mutations leading to deafness and to decipher the functions of the encoded proteins in auditory and vestibular pathways. In the past 5 years, the emergence of genome enrichment arrays followed by massively parallel sequencing (next-generation sequencing, NGS) technologies has accelerated gene discovery for human disorders, including deafness.\textsuperscript{9,18} NGS has also had an important impact on research and clinical diagnosis in many diseases, including cancer,\textsuperscript{9,12,15} intellectual disabilities,\textsuperscript{14,18} mitochondrial disorders,\textsuperscript{19-21} polycystic kidney disease,\textsuperscript{22} and ciliary disorders\textsuperscript{23} because it has the capability to simultaneously interrogate a large set of genes. For example, OtoSCOPE (Otologic Sequence Capture Of Pathogenic Exons) was a platform developed to examine the exons of 54 known genes associated with human deafness.\textsuperscript{24} OtoSCOPE uses hybridization-based enrichment of targeted exons followed by NGS on Illumina and 454 sequencers.\textsuperscript{24} Additional platforms for the genetic diagnosis of hearing disorders were developed, which varied in the number of targeted exonsgenes and type of NGS analyzers.\textsuperscript{25,26}

Recently, OtoSeq, a microdroplet-based target enrichment\textsuperscript{27} followed by NGS to screen 24 reported genes associated with autosomal recessive nonsyndromic hearing loss, was developed at Cincinnati Children’s Hospital Research Foundation.\textsuperscript{28} Here, we report genetic screening of 34 consanguineous Pakistani families segregating prelingual hearing loss linked to short tandem repeat (STR) markers for MYO7A, CDH23, or SLC26A4 using OtoSeq. We found 24 mutations in 28 of the 34 families including 11 MYO7A alleles not previously reported. Among the 11 novel mutations, 7 were missense substitutions, 1 nonsense, 1 frameshift, and 2 splice site changes.

**Materials and Methods**

**Family Ascertainment**

The study of Pakistani families was approved by Institutional Review Boards at the National Centre of Excellence in Molecular Biology (NCEMB), Lahore, Pakistan (FWA00017939); at the National Institutes of Health, USA (Combined Neuroscience IRB; OH-93-N-016); and at the Cincinnati Children’s Hospital Research Foundation, USA (2009-0684; 2010-0291). Written informed consent was obtained from adult subjects and parents of minor subjects. We performed medical history interviews to identify possible clinical features of syndromic hearing loss and rule out potential environmental causes. In all families, hearing was evaluated in audiology clinics by pure tone audiometry at octave frequencies with intensities up to 110 dBHL, and vestibular function was evaluated by tandem gait and Romberg testing.\textsuperscript{29} For 11 available individuals with bi-allelic mutations of MYO7A, funduscopic examination was performed by an ophthalmologist to evaluate the individuals for retinitis pigmentosa.

**DNA Isolation and Genotyping in Pakistani Families**

Genomic DNA was extracted from peripheral blood samples using a standard protocol. Using genomic DNA from affected and unaffected members of 243 Pakistani families segregating autosomal recessive hearing loss, we screened for linkage of the deafness phenotype to STR markers for all of the reported DFNB/USH genes/loci using a previously described protocol.\textsuperscript{30} In 34 families, the deafness phenotype showed linkage to markers for the MYO7A, CDH23, or SLC26A4 genes. DNA samples from these families were screened for mutations using microdroplet PCR-based target enrichment and NGS.

**Target Enrichment and Next-Generation Sequencing**

Target enrichment and NGS were performed according to the protocol given in the article by Sivakumaran and coauthors.\textsuperscript{28} Briefly, the coding and flanking intronic regions of all 24 genes were enriched using microdroplet PCR (RainDanceTechnologies, Inc, Lexington, Massachusetts).\textsuperscript{28} The resulting enriched amplicons were gel purified, subjected to NGS library preparation using a Nextera DNA sample prep kit (Epiconcept Biotechnologies, Madison, Wisconsin), and sequenced using an Illumina HiSeq 2000 (Illumina, San Diego, California). The raw NGS sequence reads were filtered for quality and subjected to an NGS data analysis pipeline.\textsuperscript{28} The sequence variants observed in the patients were searched against the Human Gene Mutation Database (HGMD), dbSNP, and NHLBI Exome Variant Server (EVS). Variants listed as mutations in the HGMD and variants that were either absent or had low minor allele frequency in dbSNP or EVS were evaluated further. All novel missense variants were evaluated using in silico prediction programs, Polyphen-2, SNPs3D, Mutation Taster, and SIFT.\textsuperscript{31-34}

**Sanger Sequencing**

For confirmation and segregation analyses, the genomic DNA of each participating member of each family was PCR amplified with primers for exons harboring mutant alleles and sequenced as described.\textsuperscript{35} Briefly, 15 μl of PCR product was treated with 0.3 U of shrimp alkaline phosphatase and 3 U of exonuclease I at 37°C for 1 hr, followed by incubation at 80°C for 15 min. DNA sequencing reactions contained 3.2 pmol primer, 0.6 μl Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Foster City, California), and 2 μl 5× dilution buffer (400 mM Tris-HCl, pH 9 and 10 mM MgCl2) in a 10 μl reaction. The cycling conditions were 96°C for 2 min, followed by 45 cycles of 96°C for 10 s, 55°C for 10 s, and 60°C for 4 min. The sequencing reaction products were ethanol precipitated, and the pellets were suspended in 10 μl of
deionized formamide. Sequences were obtained on an ABI 3730 XL (Applied Biosystems).

**Results**

**Linkage Analysis**

The study participants comprised 243 families segregating severe-to-profound, prelingual, recessive deafness ascertained through NCEMB after the original 557 families reported by Riazzuddin et al. As part of our routine genetic screening protocol, we performed linkage analysis of the NCEMB cohort using STR markers flanking reported DFNB and USH locigenes. Through these linkage studies, we identified 34 families co-segregating recessive hearing loss and STR markers linked to MYO7A (32 families), CDH23 (1 family), or SLC26A4 (1 family) genes (Figures 1, 2). Further analysis using additional STR markers revealed shared MYO7A-linked haplotypes in some families (data not shown).

**Mutational Analysis**

For MYO7A, families PKDF896, PKDF1079, and PKDF1080 had the same haplotype for 3 markers closely linked to MYO7A (data not shown). Similarly, families PKDF624, PKDF937, PKDF948, and PKDF960 had the same MYO7A-linked haplotypes. Using a microdroplet PCR-based target enrichment followed by NGS (OtoSeq), we screened 1 affected individual from each of 29 Pakistani families with unique MYO7A, CDH23, and SLC26A4 linkage haplotypes (Figures 1, 2) for 24 known deafness-causing genes. Unbiased analysis (blinded to linkage data) of the NGS data revealed potential pathogenic mutations in 23 of 29 samples screened. We confirmed these mutations through Sanger sequencing (Figure 3). Finally, segregation analysis studies revealed potential pathogenic alleles in 28 of 34 Pakistani families (Table 1). None of the novel mutations were detected in 192 chromosomes from normal-hearing participants who were geographically and ethnically matched to study participants with hearing loss.

We identified 22 homozygous mutant alleles of MYO7A (Table 1) that segregated among 26 families (Figures 1, 2). Among the 11 novel mutations, 7 were missense substitutions of residues evolutionarily conserved in a variety of myosin VIIA orthologs (Figure 4); these are predicted to have deleterious effects on the function of this unconventional myosin (Table 1). In addition, we identified 1 new nonsense (p.Arg1967*), 1 frameshift (p.Asp1638Metfs*7), and 2 splice site (c.2367+1G>A, c.6354+1G>A) mutations. Assuming that these mutant mRNAs escape degradation by the nonsense-mediated decay pathway, these mutations are predicted to result in a truncation of the encoded protein.

We also identified 11 reported MYO7A alleles that segregated in 16 families, including 4 missense (p.Arg241Cys, p.Arg241His, p.Ala826Thr, p.Gly2163Ser), 2 nonsense (p.Lys420*, p.Trp838*), 3 frameshift (p.Glu166Argfs*3, p.Tyr1302Thrfs*96, p.Asp2613Valfs*31), and 2 splice site mutation (c.2695-9A>G, c.3504-1G>C) (Table 1). Surprisingly, 2 different homozygous, potentially pathogenic mutations of MYO7A (p.Gly434Arg, p.Ala826Thr) were segregating in family PKDF959 (Table 1). The p.Ala826Thr mutation was initially identified in USH1B subjects from Algeria and Morocco. We also previously identified the p.Ala826Thr mutation in a large consanguineous Pakistani family. Parents of these affected individuals were heterozygous for both alleles and had normal hearing, suggesting 2 possibilities: (1) Both alleles are present in the cis-configuration, and parents have 1 normal copy of MYO7A; (2) alternatively, p.Gly434Arg may be a rare polymorphism, which is in linkage-disequilibrium with the p.Ala826Thr pathogenic allele in family PKDF959. Currently, we do not have lymphoblast cell-derived cDNA samples available from these families, and at the genomic DNA level the 2 alleles are ~16 Kb apart, which precludes our attempt to determine the cis/trans configuration of these 2 alleles. Intriguingly, no potential pathogenic mutation in the protein coding exons or in the splice junctions of MYO7A was detected in 6 other families with linkage to chromosome 11q13.5 STR markers (data not shown).

In the USH1D family (PKDF475; Figure 1), we identified a homozygous transition mutation (c.8530C>T) that resulted in a substitution of serine for proline at position 2844 (p.Pro2844Ser) in the 27th ectodomain (Table 1). The p.Pro2844Ser mutation was recently reported to be associated with type 1 Usher syndrome segregating in another Pakistani family. In the PDS family (PKDF960; Figure 1), we identified a homozygous transversion mutation (c.716T>A) in exon 6 of SLC26A4 that results in a substitution of valine by aspartic acid (p.Val239Asp) in the encoded protein, Pendrin (Table 1).

Intriguingly, in 4 families (PKDF609, PKDF771, PKDF788, PKDF948), the pathogenic alleles identified through OtoSeq could not explain the deafness phenotype shared by all affected individuals in the family (Figures 1, 2). In these families, 1 or 2 affected individuals (indicated by grey filled symbols in Figures 1, 2) were either heterozygous for the identified mutation or were homozygous for the wild type allele. Segregation analysis using chromosome 11q13.5-linked STR markers also revealed non-segregation of the MYO7A mutation harboring haplotype in these individuals.

**Clinical Evaluation**

Hearing evaluation using pure tone audiometry revealed severe to profound or profound degree of hearing loss in the affected individuals of families segregating MYO7A, CDH23, or PDS mutations. Although not rigorously tested using posturography and a rotary chair, Romberg and tandem gait tests suggested impaired vestibular function among the affected individuals homozygous for MYO7A and CDH23 mutations. Only affected individuals from 5 (marked by in Table 1) families segregating MYO7A alleles were available for ophthalmologic examination. Funduscopic examination of the 2 oldest affected individuals from these families revealed signs of retinitis pigmentosa along with narrowing of retinal blood vessels, bone spicules, and waxy appearance of the optic disk. The severity of retinitis pigmentosa ranged from mild to severe loss of vision. Based on the clinical
presentation, these families were classified as segregating Usher syndrome type I. For family PKDF609, 2 affected individuals with homozygous PDS mutation were examined by a local physician and had palpable goiters.
Discussion

Screening of 243 Pakistani families with STR markers for known DFNB/USH loci revealed evidence of linkage to markers for USH1B/DFNB2, USH1D/DFNB12, and PDS/DFNB4 loci in 34 families. Because the genes mutated at the USH1B/DFNB2 (MYO7A), USH1D/DFNB12 (CDH23), and PDS/DFNB4 (SLC26A4) loci were included in OtoSeq, we used Otoseq to uncover mutations of these genes in these 34 families. Genetic screening using OtoSeq revealed 24 mutations (Table 1). As anticipated from linkage analysis studies, 22 of the identified alleles were present in the MYO7A gene. These include 11 missense, 3 nonsense, and 8 predicted frameshift alleles. The allelic heterogeneity for MYO7A in Pakistan is similar to that reported for MYO7A in the United States and United Kingdom. Our results indicate that MYO7A mutations are a significant cause of recessive hearing loss in Pakistan and in many other populations. Taken together with our earlier result, MYO7A mutations account for 6.25% (50 of 800 families; 95% CI, 4.77-8.15) of hearing loss in our NCEMB cohort of Pakistani families segregating prelingual hearing loss.

As of January 2013, 260 different mutant alleles of MYO7A have been reported and are listed in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php?gene=MYO7A). Although some of these are recurrent variants, most are private and distributed across the gene. In our study, only 2 mutations (c.721C>T and c.1258A>T) of MYO7A were found in multiple families (Table 1). Together with our earlier result, we observed a distinct spectrum of MYO7A mutations that did not include alleles prevalent in other populations, such as p.Arg666* (United Kingdom), p.Arg241Gly (Italy), p.Gln821* (Spain), and p.Cys31* (Denmark). The 3 most common alleles (c.496delG, c.721C>T, c.1258A>T) only accounted for ~24% of the cases of deafness in the NCEMB study.
population, and thus hierarchical mutation screening would not be a cost-effective approach for molecular diagnosis. By contrast, the p.Val239Asp allele of SLC26A4 segregating in family PKDF609 is a recurrent mutation and has

Figure 3. Sanger sequence chromatograms of novel MYO7A mutations in an affected individual and a wild type sequence in a control individual. Arrows in the homozygous mutant chromatograms indicate the location of the mutant nucleotide (red).
Table 1. Mutant alleles found in 28 Pakistani families with prelingual sensorineural hearing loss

<table>
<thead>
<tr>
<th>Family</th>
<th>Gene</th>
<th>Mutation</th>
<th>Effect on protein</th>
<th>Domain</th>
<th>Allele frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKDF475&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CDH23</td>
<td>c.8530C&gt;T</td>
<td>p.Pro2844Ser</td>
<td>EC27</td>
<td>0/188</td>
<td>This study</td>
</tr>
<tr>
<td>PKDF820</td>
<td>MYO7A</td>
<td>c.398A&gt;C</td>
<td>p.His133Pro</td>
<td>Motor</td>
<td>0/188</td>
<td>This study</td>
</tr>
<tr>
<td>PKDF764</td>
<td>MYO7A</td>
<td>c.496delG</td>
<td>p.Glu166Argfs*3</td>
<td>Motor</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>PKDF896&lt;sup&gt;b&lt;/sup&gt; PKDF1079</td>
<td>MYO7A</td>
<td>c.721C&gt;T</td>
<td>p.Arg241Cys</td>
<td>Motor</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>PKDF1080</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKDF514</td>
<td>MYO7A</td>
<td>c.722G&gt;A</td>
<td>P.Arg241His</td>
<td>Motor</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>PKDF930</td>
<td>MYO7A</td>
<td>c.1183C&gt;T</td>
<td>p.Arg395Cys</td>
<td>Motor</td>
<td>0/188</td>
<td>This study</td>
</tr>
<tr>
<td>PKDF624</td>
<td>MYO7A</td>
<td>c.1258A&gt;T</td>
<td>p.Lys420*</td>
<td>Motor</td>
<td>0/188</td>
<td>62</td>
</tr>
<tr>
<td>PKDF937 PKDF948 PKDF960</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CC, coiled coil region; EC, ectodomain.
<sup>a</sup>Novel mutations are in bold and were predicted pathogenic by Polyphen-2, SNPs3D, MutationTaster, and SIFT prediction algorithms.
<sup>b</sup>Vestibular, auditory, and funduscopic evaluations revealed Usher syndrome type I phenotype segregating in these families. Affected individuals of other families homozygous for MYO7A mutations had vestibular dysfunction, evaluated through Romberg and tandem gait tests, but no ophthalmology data are available from these families.
<sup>c</sup>Predicted by the Human Splicing Finder program (http://www.umd.be/HSF/).

been identified previously in 17 other Pakistani PDS families. We previously demonstrated that 3 alleles of SLC26A4 (p.Ser90Leu, p.Val239Asp, and p.Gln446Arg) account for ~66% of cases of DFNB4/PDS deafness in the NCEMB study population.<sup>54</sup> Thus, hierarchical mutation screening using a tetra-primer amplification refractory mutation system (ARMS) assay would have been a cost-effective approach for molecular diagnosis in the PKDF609 family.<sup>54</sup>

In 6 of the potential MYO7A (chromosome 11q13.5)-linked families, we did not observe mutations in the coding exons or splice junctions of MYO7A. There are at least 3 possible reasons for our failure to detect MYO7A mutations in these families. First, our assay is designed to only screen the coding exons and the flanking 20 bp that includes the donor and acceptor splice junctions. Hence, mutations in cis-acting regulatory or splice elements of MYO7A that are necessary for MYO7A expression in the inner ear and retina would not be detected. Second, the OtoSeq platform has limited resolution in detecting insertions (up to 22 nucleotides)<sup>28</sup> and may have missed complicated mutations like large insertions. Third, the hearing loss segregating in these 6 families may be spuriously linked to markers at 11q13.5. However, for 4 of these families the linkage scores for markers of chromosome 11q13.5 were statistically significant (>3.5). Fourth, there may be an additional gene on chromosome 11q13.5 in which mutant alleles cause hearing loss. There are examples of closely linked deafness genes and overlapping deafness loci in humans.<sup>55-57</sup>

**Conclusion**

Identification of 24 mutations, including 11 novel alleles, in 28 Pakistani families emphasizes the genetic heterogeneity of this disorder. Our findings, along with the results of previous studies,<sup>35,36,39,45,58-61</sup> suggest that besides HGF, CIB2, and PDS, mutant alleles of MYO7A and CDH23 are among the major causes of prelingual recessive hearing loss within the Pakistani population.
Acknowledgments

We would like to thank all the families for participating in the present study. We thank Thomas Jaworek, Cortney Washam, and Rashid Bhatti for their technical assistance and discussions of the results. We also thank Drs. Andrew Griffith and Kyoto Kurima for helpful discussions and critiques of the manuscript.

Author Contributions

Mohsin Shahzad, collection of study samples, acquisition and analysis of the data, paper preparation; Theru A. Sivakumaran, data analysis and interpretation, paper preparation; Tanveer A. Qaiser, collection of study samples and clinical data, perform linkage analysis studies and editing the manuscript; Julie M. Schultz, acquisition and analysis of sequencing data, paper preparation; Zawar Hussain, collection of study samples and clinical data, perform linkage analysis studies, and editing the manuscript; Megan Flanagan, acquisition and analysis of next-generation sequencing data, editing the manuscript; Kejian Zhang, acquisition and analysis of sequencing data, paper preparation; Saima Riazuddin, conception and study design, data analysis and interpretation, paper preparation; Zubair M. Ahmed, conception and study design, data analysis and interpretation, paper preparation.

Disclosures

Competing interests: Theru A. Sivakumaran, employee of the Molecular Genetics Laboratory at Cincinnati Children’s Hospital Medical Center, which offers OtoSeq as a commercial test; Diane Kissell, employee of the Molecular Genetics Laboratory at Cincinnati Children’s Hospital Medical Center, which offers OtoSeq as a commercial test; Kejian Zhang, employee of the Molecular Genetics Laboratory at Cincinnati Children’s Hospital Medical Center, which offers OtoSeq as a commercial test.

Sponsorships: None.

Funding source: Intramural funds NIDCD DC000039-15 to Thomas B. Friedman. NIDCD/NIH research grant R01 DC011803 to Saima Riazuddin. Career Development Award from RPB Foundation to Zubair M. Ahmed. NIDCD/NIH research grant R01 DC012564 to Zubair M. Ahmed.

References


