Identification of Novel and Recurrent Variants in MYO15A in Ashkenazi Jewish Patients With Autosomal Recessive Nonsyndromic Hearing Loss

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Hearing loss is a genetically and phenotypically heterogeneous disorder. The purpose of this study was to determine the genetic cause underlying hearing loss in four Ashkenazi Jewish families. We screened probands from each family using a combination of targeted mutation screening and exome sequencing to identify the genetic cause of hearing loss in each family. We identified four variants in MYO15A, two novel variants never previously linked to deafness (c.7212+5G>A and p.Leu2532ArgfsTer37) and two recurrent variants (p.Tyr2684His and p.Gly3287Gly). One family showed locus heterogeneity, segregating two genetic forms of hearing loss. Mini-gene assays revealed the c.7212+5G>A variant results in abnormal splicing and is most likely a null allele. We show that families segregating the p.Gly3287Gly variant show both inter and intra-familial phenotypic differences. These results add to the list of MYO15A deafness-causing variants, further confirm the pathogenicity of the p.Gly3287Gly variant and shed further light on the genetic etiology of hearing loss in the Ashkenazi Jewish population.

Keywords: RNA-splicing, deafness, Ashkenazi Jewish, hearing loss, genotype-phenotype correlation, MYO15A

INTRODUCTION

Myosins are a superfamily of actin-based motor proteins that play an essential role in a wide variety of cellular activity ranging from intracellular transport and signaling, cell migration, and adhesion to muscle contractions (Manor and Kachar, 2008; Coluccio, 2020a; Coluccio, 2020b). Pathogenic variants in the genes that encode myosin proteins have been linked to many diseases, including hearing loss (Friedman et al., 2020). Currently, pathogenic variants in six myosin genes (MYO3A, MYO6, MYO7A, MYH14, MYH9, and MYO15A) have been linked to human deafness (Lalwani et al., 2000; Walsh et al., 2002; Donaudy et al., 2004; Friedman et al., 2020).

It is well established that pathogenic variants in MYO15A underlie autosomal recessive nonsyndromic hearing loss at the DFNB3 locus (Rehman et al., 2016; Hirsch et al., 2021). To date, more than 370 variants in MYO15A have been linked to DFNB3-related hearing loss (https://deafnessvariationdatabase.org/) (Azaiez et al., 2018). This allelic diversity is strongly mimicked at the phenotypic level, with variable hearing loss thresholds (ranging from mild to profound), onset...
(congenital-to-postlingual), and stability (progressive vs. non-progressive) (Friedman et al., 2002; Rehman et al., 2016).

Here we add to the pathogenic allelic heterogeneity of MYO15A by implicating two new pathogenic variants in MYO15A, one splice-altering and one frameshift, that cosegregate with deafness in Ashkenazi Jewish families. We also shed light on the phenotypic variability associated with a common pathogenic synonymous variant in the Ashkenazi Jewish community.

METHODS

Subjects

Four families of Ashkenazi Jewish ancestry, segregating autosomal recessive sensorineural hearing loss (ARSNHL), were ascertained for this study. Affected individuals underwent clinical examination and pure tone audiometry to measure hearing thresholds. After written informed consent to participate in this study was given, blood samples were obtained from all affected and unaffected family members, and genomic DNA was extracted.

Variant Identification and Segregation Analysis

One proband from each family underwent genetic screening using a panel of 60 hearing-loss-causing mutations common in the Ashkenazi Jewish community, performed at Dor Yeshorim (http://doryeshorim.org), as described (Hirsch et al., 2021). Probands in which only one pathogenic allele was identified underwent direct sequencing of all exons and flanking introns of MYO15A. Probands negative for targeted mutation screening subsequently underwent Exome Sequencing (ES), bioinformatic analysis and variant prioritization, as described (Hirsch et al., 2021). Candidate variants were screened using gene-specific primers in all available family members to confirm segregation. All variants have been submitted to the Deafness Variation Database (https://deafnessvariationdatabase.org/) (Azaiez et al., 2018) for curation and incorporation.

In Silico and In Vitro Splicing Analysis

Variants c.7212+5G>A and c.9861C>T impact on splicing were computationally predicted using MaxEnt (Wang et al., 2004) and Human Splicing Finder (HSF) (Desmet et al., 2009). In vitro minigene assays for splicing were carried out as described (Tompson and Young, 2017; Booth et al., 2018; Booth et al., 2019; Booth et al., 2020; Hirsch et al., 2021). Wildtype (WT) MYO15A (NM_016239.4) exons 34 and 35 were PCR amplified with gene-specific primers and ligated into the pre-constructed pSPL3 Exontrap vector. The c.7212+5G>A variant was introduced into the wildtype sequences using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA), using the manufacturer’s protocol. Colonies were selected and grown, and plasmid DNA was harvested using the ZymoPure Plasmid II Miniprep and Midiprep Kits (ZYMO Research, Irvine, CA). Following sequence confirmation, WT and mutant minigenes were transfected in triplicate into HEK293 and COS7 cells, and total RNA was extracted 48 h post-transfection using the Quick-RNA Miniprep Plus kit (ZYMO Research, Irvine, CA). Using a random primer mix (ThermoFisher Scientific, Waltham, MA), cDNA was synthesized using AMV Reverse Transcriptase (New England Biolabs, Ipswich, MA). After PCR amplification, products were visualized on a 2% agarose gel, extracted, cloned, and then sequenced.

RESULTS

Subjects, Variant Identification, Prioritization, and Segregation Analysis

Affected individuals of Family 1 were born to non-consanguineous Ashkenazi Jewish parents. The proband (II.1) and his affected brother (II.4) have bilateral severe NSHL. The proband was diagnosed at 13 months and underwent cochlear implantation at 25 years old. His affected brother was diagnosed at 7 months and only uses hearing aids. The proband now 48 years old, reports a good outcome of his CI, and has impaired speech. Besides hearing loss, clinical evaluations were unremarkable. The proband underwent genetic testing using a high-throughput NGS panel for 60 known pathogenic variants that cause hearing loss, which revealed a heterozygous missense variant in MYO15A (c.8050T>C; p.Tyr2684His) (Table 1; Figure 1A). Direct sequencing of all coding exons of MYO15A and flanking introns revealed an ultra-rare second variant (c.7212+5G>A) in intron 35 (Table 1). Segregation analysis confirmed these two variants are in trans and segregate with the deafness in the family (Figure 1A).

In Family 2, a homozygous synonymous variant in MYO15A (c.9861C>T; p.Gly3287Gly) was identified in the proband (II.1) and was found to segregate with the mild-to-moderate hearing loss in the family (Table 1; Figure 1B). The proband was initially diagnosed with mild, downsloping hearing loss at 12 years old and has reportedly progressed to moderate. His affected siblings are reported to have a similar hearing loss and progression. Age of diagnosis ranged considerably amongst the siblings, from 12 years old to 35 years old. Besides hearing loss, clinical evaluations in all affected individuals were unremarkable. Several affected individuals benefit from the use of hearing aids.

Family 3 is a large consanguineous family of Ashkenazi Jewish descent. The proband (II.1) was diagnosed at birth with bilateral, moderate, sensorineural hearing loss and had normal prenatal and postnatal clinical courses and neurodevelopment. At the time of testing, none of the seven siblings reported hearing loss. High-throughput NGS panel testing revealed the proband to be homozygous for a known pathogenic frameshift variant (c.948delG; p.Leu316PhefsTer5) in OTOGL and heterozygous for a known pathogenic synonymous variant (c.9861C>T; p.Gly3287Gly) in MYO15A. Segregation analysis revealed both unaffected parents are heterozygous for the OTOGL variant and the MYO15A variant (Figure 1D). Segregation in the extended family revealed four siblings with reported normal hearing to be homozygous for the p.Gly3287Gly variant in MYO15A (Figure 1C).
### TABLE 1 | Variant table.

| Gene | Chr.pos | Variant | cDNA | Protein | gnomAD | Conservation | Deleteriousness | Splicing | DVD | ClinVar | ACMG criterion | Final classification | References |
|------|---------|---------|------|---------|--------|-------------|----------------|----------|-----|---------|-----------------|----------------------|------------|
|       |         |         |      |         |        |             |                |          |     |         |                 |                      |            |
| MYO15A| 17:18052690G>A | c.7212+5G>A (1.7212.7213ins7212+1) | p.Ala2402SerTer29 | 0.0008 | 0.02 | Aut. | C | C | 22.9 | 4.9 | 1.36 | Broken WT Donor Site | VUS | — | P | VPS1, PS3 | PM2, PM3, PM1, PP1, PP3 | P | This Study |
| MYO15A| 17:18054544_18054545insGGGA | c.7594_7595insGGGA | p.Leu2532ArgfsTer37 | 0 | 0 | — | — | — | — | — | — | — | P | Con | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

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Nucleotide numbering starting at the +1 position of transcripts NM_016239.4 and NM_001368062.1 for MYO15A and OTOGL, respectively. Minor allele frequency (MAF) from Genome Aggregation Database (gnomAD) version 2 (Havilla et al., 2017). Deleteriousness was assessed using CADD, Combined Annotation Dependent Depletion (Kircher, 2014) and REVEL (Ioannidis et al., 2016). Variant impact on splicing was assessed using MaxEnt (Wang et al., 2004) and HSF, Human Splicing Finder (Desmet et al., 2009).ESE: Exonic Splicing Enhancer; ESS: Exonic Splicing Silencer. ACMG Criteria and Final Classification scoring based on Hearing loss specific ACMG guidelines (Ziga et al., 2018). C, conserved; _, score not given or absent; Con, Conflicting entries; P, Pathogenic; LP, Likely Pathogenic; VUS, Variant of Uncertain Significance; _M, moderate; _P, supporting; _S, strong.
Affected individuals in Family 4 are reported to have prelingual nonsyndromic moderate-profound SNHL. The proband (II.1) underwent genetic testing using a high throughput NGS panel for 60 known pathogenic variants that cause hearing loss, which revealed a heterozygous synonymous variant in \textit{MYO15A} (c.9861C>T; p.Gly3287Gly), but no second \textit{MYO15A} pathogenic allele. Subsequently, the proband underwent ES, revealing a novel frameshift insertion (c.7594_7595insGGGA; p.Leu2532ArgfsTer37) in \textit{MYO15A}. Segregation analysis confirmed these two variants are in trans and segregate with the deafness in the family (Figure 1D).

### Computational and in Vitro Splicing Analysis

The c.7212+5G>A variant is computationally predicted to alter the WT splicing and impact splicing (Table 1). To test improper splicing, we carried out \textit{in vitro} mini-gene assays for splicing using the pSPL3 Exontrap vector. Visualization of the splicing products for WT and mutant mini-genes revealed a 263 bp band for the empty vector corresponding to the 5’ and 3’ native exons; a 519 bp product with WT sequence showing; and a 535 bp band for the mutant (Figure 2).

### DISCUSSION

In this study, we used a combination of ethnicity-specific mutation screening and Exome Sequencing to implicate \textit{MYO15A} and \textit{OTOGL} as the causal genes underlying ARNSHL in four families of Ashkenazi Jewish descent. Of the variants identified, two affect RNA splicing, two are frameshifts, and one is a
previously described pathogenic missense variant (Table 1). All affected individuals from families with reported consanguinity are homozygotes for the variants.

In Family 1, we initially identified a heterozygous previously described pathogenic variant (c.8050C>T; p.Tyr2684His) in MYO15A using a targeted variant panel. Subsequent ES revealed an ultra-rare variant (c.7212+5G>A) in intron 35 of MYO15A. The G to A transition alters the highly conserved canonical +5 guanine fundamental for the spliceosome protein U1 to bind to the 3’ end of an exon (Baralle and Baralle, 2005; Park and Cartegni, 2017). Using a mini-gene splicing assay we showed the G to A transition alters wildtype splicing via the loss of the canonical donor site and the activation of a new cryptic donor site. The use of this donor site adds 16 nucleotides to the reading frame of MYO15A, resulting in a frameshift and a new termination codon in exon 36 out of 66. Since this new termination codon is more than 50 base pairs from the last exon-exon junction, it is expected that this mutant transcript is subjected to nonsense-mediated decay (NMD) (Hentze and Kulozik, 1999; He and Jacobson, 2015) and is a null allele. It is possible that other mutant transcripts in vivo are created and were not detected due to the design of the mini-gene. In our analysis of the spliced products of the mutant mini-gene, three colonies (10%) out of the 30 colonies analyzed showed a wildtype splicing pattern. While this is only an approximation for correctly spliced transcripts, other methods such as RT-qPCR, would provide a more accurate quantification of the correctly spliced transcripts.

The pathogenic synonymous variant c.9861C>T (p.Gly3287Gly) in MYO15A was identified in three of the four
families in this study. Affected individuals in Family 2 were homozygous for the variant. These individuals report a progressive post-lingual hearing loss, with a reported age of onset between 12 and 35 years old. Whereas affected individuals in Family 4 are compound heterozygous for p.Gly3287Gly and the novel frameshift c.7594_7595insGGGA, they are reported to have prelingual moderate-to-profound HL. Given its location in the gene, we expect the c.7594_7595insGGGA mutation to cause NMD.

In summary, we have identified two novel loss of function variants (one splice-altering and one frameshift) in MYO15A that cause ARNSHL in the Ashkenazi Jewish population. We provide more genetic support for the pathogenicity of the p.Gly3287Gly variant and illustrate that this variant exhibits both inter- and intra-familial phenotypic differences. Finally, our report highlights the importance of carrier screening and segregation analysis as it can identify individuals that will develop or have milder forms of disease.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://deafnessvariationdatabase.org/, DVD_001_KTB

**ETHICS STATEMENT**

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

**AUTHOR CONTRIBUTIONS**

Conception and Study design: KTB, YH, and DPC. Data generation, data collection and data analysis: KTB, YH, ACV, JE, DY, AQ, and TW. Drafted manuscript: KTB. Critically read and revised manuscript: YH and DPC. Reviewed the manuscript: ACV, JE, DY, AQ, and TW. All authors have approved the finalized manuscript. KTB and YH share firstAUTHOR CONTRIBUTIONSship.

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