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# Exonic mutations and exon skipping: lessons learned from *DFNA5*

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## Abstract

Dysregulation of splicing is a common factor underlying many inherited diseases including deafness. For one deafness-associated gene, *DFNA5*, perturbation of exon 8 splicing results in a constitutively active truncated protein. To date only intronic mutations have been reported to cause exon 8 skipping in patients with DFNA5-related deafness. In five families with postlingual progressive autosomal dominant non-syndromic hearing loss, we employed two next generation sequencing platforms – OtoSCOPE and whole exome sequencing – followed by variant filtering and prioritization based on both minor allele frequency and functional consequence using customized bioinformatics pipeline to identify three novel and two recurrent mutations in *DFNA5* that segregated with hearing loss in these families. The three novel mutations are all missense variants within exon 8 that are predicted computationally to decrease splicing efficiency or abolish it completely. We confirmed their functional impact *in vitro* using mini-genes carrying each mutant *DFNA5* exon 8. In so doing, we present the first exonic mutations in *DFNA5* to cause deafness, expand the mutational spectrum of DFNA5-related hearing loss, and highlight the importance of assessing the effect of coding variants on splicing.

### Keywords

Non-syndromic hearing loss; DFNA5; RNA-Splicing; Deafness; Exon-skipping

## Introduction

Like most cellular processes, the formation of mature messenger RNA (mRNA) from transcribed RNA involves a process known as RNA splicing, which is highly conserved and

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tightly regulated by five small nuclear ribonucleoproteins (snRNP) and many associated protein cofactors (Jurica & Moore, 2003; Will & Lührmann, 2011). Working in synergy, these proteins form the highly dynamic spliceosome and facilitate both constitutive and alternative splicing (Matera & Wang, 2014), the latter being fundamental to time- and tissue-specific gene expression. Dysregulation of splicing, which underlies many inherited and sporadic diseases (Paronetto, Passacantilli, & Sette, 2016), can be caused by mutations in spliceosome subunits (Boon et al., 2007; Utz, Beight, Marino, Hagstrom, & Traboulsi, 2013) or the target DNA sequence essential for spliceosome recognition and binding. The functional consequence includes exon skipping, intron retention and the formation of pseudo-exons (Baralle & Baralle, 2005; Dhir & Buratti, 2010).

A diverse group of genes encode the proteins required for development, function and maintenance of the auditory system. Defects in approximately 30 of these genes (http://hereditaryhearingloss.org/) carrying in aggregate over 200 causal variants (http://deafnessvariationdatabase.org/) cause autosomal dominant non-syndromic hearing loss (ADNSHL) (Morton, 1991), which typically manifests as post-lingual, high frequency, progressive hearing loss (Angeli, Lin, & Liu, 2012; Petersen, 2002). DFNA5-related hearing loss is also post-lingual, high frequency and progressive (Cheng et al., 2007; De Leenheer et al., 2002; Van Camp et al., 2000; Van Laer et al., 2004), but in contrast to the robust mutational heterogeneity shown by all other ADNSHL-associated genes, in *DFNA5* (MIM# 608798), all hearing loss results from a single unique RNA event (De Leenheer et al., 2002; Van Camp et al., 2000; Van Laer et al., 2004). The six genomic mutations linked to DFNA5-realted deafness all cause omission of exon 8 to yield the same truncated protein (Bischoff et al., 2004a; Chai, Chen, Wang, Wu, & Yang, 2014; Cheng et al., 2007; Li-Yang et al., 2015; Rogers et al., 2017; Van Laer et al., 1998; Yu et al., 2003). Functional studies on the truncated protein have shown that DFNA5 is a component of the apoptotic pathway (Op De Beeck, Van Laer, & Van Camp, 2012; Rogers et al., 2017).

Until now, only intronic mutations have been identified in DFNA5 families. Herein we present three novel *exonic* mutations in exon 8 of *DFNA5*, which segregate in three families with post-lingual progressive ADNSHL. We also further confirm the pathogenicity of two reported intronic mutations segregating in two families with post-lingual progressive ADNSHL.

## Materials and Methods

### Subjects

Five families segregating post-lingual progressive hearing loss were ascertained for this study, and based on clinical examination, which excluded syndromic features, a diagnosis of ADNSHL was made. Pure tone audiometry was performed to determine air conduction thresholds at 0.25, 0.5, 1, 2, 4, and 8 kHz. After obtaining written informed consent to participate in this study, blood samples were obtained from all family members. All procedures were approved by the human research Institutional Review Boards at the Welfare Science and Rehabilitation University and the Iran University of Medical Sciences, Tehran (Iran), and the University of Iowa, Iowa City, Iowa (USA).

### Next Generation Sequencing and Bioinformatic Analysis

We completed targeted genomic enrichment and massively parallel sequencing (TGE + MPS) using either the OtoSCOPE® v6 platform (families CDS-6824 and CDS-7393) or Agilent SureSelect Human All Exon v5 whole exome capture (families L-8700115, 11330 and 10490), followed by bioinformatic analysis using the same genomic alignment and variant calling methods as we have described (Azaiez et al., 2014, 2015; Booth et al., 2015; Sloan-Heggen et al., 2016). Variant annotation, filtering and prioritization was carried out using the Qiagen Ingenuity Variant Calling (IVA) software (www.qiagen.com/ingenuity) based on quality (QD>5), minor allele frequency (<0.5%) and the predicted conservation and consequence of the variant. Predicted deleteriousness was assessed using SIFT, PolyPhen2, MutationTaster, LRT and Combined Annotation Dependent Depletion (CADD) (Azaiez et al., 2014, 2015; Booth et al., 2015; Sloan-Heggen et al., 2016). Variant predicted effects on splicing were assessed using Human Splicing Finder (v.3.0) (http://www.umd.be/HSF3/). Genes with candidate variants identified by whole exome sequencing were evaluated for inner ear expression using the gene Expression Analysis Resource (gEAR) web portal (http://gear.igs.umaryland.edu/). Copy number variation was assessed using a sliding window approach to compare read-depth ratios (Nord, Lee, King, & Walsh, 2011). All causative variants were submitted to the Deafness Variation Database (http://deafnessvariationdatabase.org/) for expert review, interpretation and integration. Variant nomenclature follows the recommended HGVS naming convention (den Dunnen et al., 2016).

### Segregation analysis Sanger Sequencing

Segregation analysis of candidate variants was completed by Sanger sequencing on an ABI 3730 Sequencer (Perkin Elmer, Waltham, MA). All sequencing chromatograms were compared to published cDNA sequence for *DFNA5* (NM_004403.2); *USH2A* (MIM# 608400) (NM_206933.2) nucleotide changes were detected using Sequencer v5 (Gene Code Corporation, Ann Arbor, MI).

### In vitro splicing analysis

*In vitro* splicing assays were conducted using the pre-constructed pET01 Exontrap vector (MoBiTec) encoding a 5′ and 3′ exon separated by a multiple cloning site. Wild-type *DFNA5* exon 8 (193 base pairs) plus 88 and 83 nucleotides from the 3′ and 5′ flanking sequences, respectively, was PCR amplified with gene-specific primers that contained either Sall or SacII restriction enzyme sites. After restriction enzyme digestion, the PCR fragment was ligated into the pET01 vector and sequenced confirmed. Mutations were then introduced into the wild-type sequence using QuikChange Lightning Site-Directed Mutagenesis (Agilent) according to the manufacture's protocols. All mutant minigenes were sequenced to confirm the correct mutation.

Wild-type or mutant mini-genes were transfected in triplicates into COS7 cells using TransIT-LT1 Transfection Reagent (Mirus). Cells were harvested 36h after transfection and total RNA was extracted using Quick-RNA MiniPrep Plus kit (ZYMO Research). cDNA was transcribed using 750ng of isolated RNA SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific) using a primer specific to the 3′ native exon of the pET01 vector

according to manufacture protocol. PCR amplification followed using primers specific to the 5′ and 3′ native exons of the pET01 vector and products were visualized on a 1.5% agarose gel. As positive control and negative controls, the previously described c.991-2A>G mutation and the snp rs138980048:G>A was used to test and confirm the functionality of the designed mini-gene, respectively.

## Results

### Families

We identified five families segregating progressive ADNSHL (Figures 1 and 2) representing 3 ethnic backgrounds. Families CDS-6824 (Figure 1A) and 11330 (Figure 2B) are of European ancestry, families CDS-7393 (Figure 1B) and 10490 (Figure 2B) are of East Asian ancestry, and family L-8700115 (Figure 1C) originates from Iran (Table 1). In all five families the deafness was reported to start in after the first decade as mild high frequency loss, which then progressed to a severe loss in the high frequencies and a mild loss in the mid-frequencies (Figure 1A–C and Figure 2A–B). Physical examination in all affected individuals in all families was otherwise unremarkable.

### Variant Identification

Probands from families CDS-6824 and CDS-7393 were analyzed using OtoSCOPE® v6, identifying 1627 and 1619 variants, respectively; after filtering, 6 and 9 variants remained. In CDS-6824, two variants were considered possible based on mode of inheritance and minor allele frequency - 1 in *DFNA5* and 1 in *SLC17A8* (MIM# 607557) – but only the highly conserved *DFNA5* c.1102C>G variant in exon 8 segregated with the deafness phenotype (Figure 1A). Similarly, in family CDS-7393, only one variant, a highly conserved and novel variant (c.1183G>A) in exon 8 of *DFNA5*, segregated with the hearing loss in the extended family (Figure 1B).

Families L-8700115, 11330 and 10490 underwent whole exome capture. Sequenced samples had an average depth of coverage of 10X at >98% and 30X at 95%. In family L-8700115, three affected individuals were analyzed: III.5, IV.6 and V.4 (Figure 1C). After confidence and MAF filtering, the three individuals shared 59 heterozygous variants. Further filtering for exonic and splice-site variants narrowed the list of candidates to 14 variants, six of which were in genes that are expressed in the inner ear; segregation analysis eliminated all of these variants. Re-filtering variants to those shared only between individuals revealed 166 heterozygous variants in common to IV.6 and V.4. Of these variants, one variant (c.1154C>T) was identified in *DFNA5*; Sanger sequencing confirmed segregation of this variant in the outer loop of the pedigree (Figure 1C). Variant filtering using a recessive model was utilized for individual III.5. After removing low quality variants and variants seen at >0.5%, 128 genes were identified carrying 162 compound heterozygous or homozygous variants. One variant, an ultra-rare highly conserved homozygous variant (c.5144A>G, p.E1715G) in *USH2A*, was present in individual III.5 and confirmed by segregation analysis in III.10 (Figure 1C and Table 1). Both variants were absent in >2000 ethically matched chromosomes.

In family 11330, three affected individuals and one unaffected family member were analyzed (Figure 2A). Intersecting the final filtered variant list of all affected individuals after removing all variants shared with the unaffected family member yielded 54 variants. This list included a previously described ultra-rare pathogenic variant in *DFNA5* (c. 991-2A>G). Sanger sequencing confirmed its segregation with the hearing loss phenotype.

In family 10490, whole exome sequencing was completed on two affected individuals and one unaffected individual. Comparing the overlapping variants shared by affected individuals that were not present in the unaffected left 134 variants in 94 genes, including a previously described 3-basepair deletion, c.991-15_991-13delTTC in the pyrimidine tract of intron 7 in *DFNA5*. Segregation analysis showed that the deletion segregates in affected members (Figure 2B).

### Splicing Analysis

To characterize the effects on splicing of both the novel exonic variants and a previously described mutation, we first assessed splicing when a 364bp genomic fragment, which included wild-type *DFNA5* exon 8, was inserted into the pET01 exon trap vector (Figure 3A). Visual analysis of the splicing products showed a 242bp band, which corresponded to splicing of the pET01 native 5′ and 3′ exons, and a 435bp product, which was generated by inclusion of the 193bp exon 8 of *DFNA5* (Figure 3A). Mutant *DFNA5* constructs containing c.1102C>G, c.1183G>A, c.1154C>T or c.919-2, yielded only the 242bp product similar to the empty pET01 vector (Figure 3A). The snp rs138980048:G>A yielded similar results to the wild-type mini-gene (Supp Figure 1).

## Discussion

RNA splicing is a fundamental cellular process coordinated by both internal sequence signals and external splicing factors (Baralle, 2005; Matera & Wang, 2014; Pagani & Baralle, 2004; Will & Lührmann, 2011). Internal sequence signals provide genomic motifs for the binding of splicing factors and the coordinated removal of intron sequences to form functional mRNA for translation. The recognized motifs are dispersed between introns and exons and act to either enhance or silence a splicing signal (Matera & Wang, 2014; Will & Lührmann, 2011). While these signals are present in all transcripts, cell specific expression of the splicing factors which bind to these motifs can provide exquisite control of gene isoform expression (Lee & Rio, 2015). Here we show that the disruption of predicted exonic splicing enhancers (ESEs) or the creation of predicted exonic splicing silencers (ESSs) in exon 8 of the *DFNA5* gene results in skipping of this exon the phenotypic consequence of which is non-syndromic hearing loss.

The *DFNA5* gene (also known as *GSDME*) encodes the DFNA5 protein, which is comprised of 496 amino acids and belongs to a family of proteins involved apoptosis and necrotic activity (Rogers et al., 2017). Its N- and C-terminal domains are separated by a linker region and upon Caspase-3 mediated cleavage of the C-terminus, a DFNA5 protein is generated with necrotic activity. DFNA5-related deafness is reflective of this activity, which arises as a result of RNA mis-splicing of the *DFNA5* exon 8 and leads to cochlear hair cell loss through apoptosis. The loss of exon 8 in the *DFNA5* mRNA transcript translates into a truncated

protein with constitutive necrotic activity due to loss of the inhibitory C-terminal domain (Groves et al., 2015; Rogers et al., 2017; Van Laer et al., 1998, 2004; Van Rossom et al., 2012). To date, all reported DFNA5 mutations that lead to ADNSHL, of which there are six, lie in the introns flanking exon 8. In this study, we add to the mutational spectrum coding variants that affect splicing. Two different genetic causes of deafness segregate in family L-8700115. In the larger pedigree, which spans multiple generations, we identified a novel and predicted deleterious missense mutation (c.1154C>T, p.Thr385Ile) in *DFNA5* (Figure 1C). Occurring 29 nucleotides upstream from the 3′ end of exon 8, the C>T transition abolishes the motif for the ESE SRp40 and SF2/ASF and create a new ESS site (Figure 3C). Similar to the other exonic mutations, the c.1154C>T mutation causes skipping of exon 8 in vitro (Figure 3A). In the two hearing-impaired individuals (III.5 and III.10) who do not carry the *DFNA5* c.1154C>T variation, we identified homozygosity for an ultra-rare predicted deleterious missense variant in *USH2A* (c.5144A>G, p.Glu1715Gly) (Figure 1C). This variant is in located in the extracellular laminin G-like-2 domain of the USH2A protein, a region where multiple missense variants have been reported further supporting this variant's pathogenicity (http://deafnessvariationdatabase.org/). This family highlights the mutational, phenotypic and genetic complexities of hereditary hearing loss.

After identifying c.1154C>T in family L-8700115 and characterizing its effects on splicing, we sought to identify other missense variants in exon 8 of *DFNA5*, which potentially alter proper RNA-splicing. We identified two more *DFNA5* variants, c.1102C>G and c. 1183G>A, one in each of two families that co-segregate with the ADNSHL phenotype. In family CDS-6824, we considered two possible variants as the genetic cause of ADNSHL. One, an ultra-rare and predicted deleterious missense variant (c.161C>T, p.Pro54Leu) in the *SLC17A8* gene, did not segregate with the deafness phenotype; the other, a novel missense variant in *DFNA5* (c.1102C>G, Gln368Glu) co-segregated with hearing loss.

Although highly conserved at both the nucleotide and amino acid level, the Gln368Glu amino acid change itself is otherwise unremarkable. It is not predicted deleterious by PolyPhen2, SIFT, LRT or Mutation Taster and has a CADD score of only 2.33. Given that all pathogenic mutations in *DFNA5* result in skipping of exon 8, we assessed the effect of c. 1102C>G on splicing in-silico using Human Splicing Finder (HSF). HSF predicted altered splicing secondary to the creation of both a cryptic donor site and cryptic acceptor site. Although the new cryptic splicing signals were not as strong as the native signals for the wild-type exon 8, the c.1102C>G mutation did alter sequence motif resulting to increase the splicing signal by 22.35% and 68.75% for the new cryptic donor and acceptor sites, respectively (Figure 3C). The c.1102C>G mutation also creates a predicted Intron Identity Element (IIE) motif (Figure 3C). IIEs are sequence motifs that recruit two classes of splicing proteins: Intron Splicing Enhancers (ISE) and Exonic Splicing Silencers (ESS). This specific mutation increases the binding signal ~30% for the ESS hnRNP A1 (Figure 3C). While the direct mechanism by which mis-splicing occurs is not clear, in vitro analysis using a mini-gene with exon 8 of *DFNA5* shows that the c.1102C>G mutation causes skipping of exon 8 (Figure 3A).

In the other family, CDS-7393, the proband carried a novel c.1183G>A mutation in *DFNA5*, which segregated with the hearing loss. This residue is not only conserved but the transition

(G→A) is predicted to be deleterious and has a CADD score of 24.7 (Table 1). This mutation occurs at the last nucleotide in exon 8 and is predicted to break the wild-type donor site resulting in mis-splicing. Consistent with this prediction, in our mini-gene carrying the c.1183G>A mutation, exon 8 is skipped.

Finally, we identified two previously reported pathogenic variants in the intronic regions of *DFNA5* in families 11330 and 10490. The c.991-2A>G mutation in family 11330 has been reported in a large Chinese pedigree with late onset progressive ADNSHL (Chai et al., 2014), and the c.991-15_991-13delTTC mutation in family 10490 has been reported in families of East Asian descent (Nishio et al., 2014; Park et al., 2010; Yu et al., 2003). This mutation occurs in the polypyrimidine tract of intron 7 and may be a founder mutation as family 10490 is also of East Asian descent.

Most of the emphasis on missense variant interpretation focuses on the impact the amino acid change has at the protein level. Rarely, if the missense variant is located outside the traditional splicing window of +/−5 base pairs from the start and end of the exon, is alteration of splicing considered in variant interpretation. Recent studies, however, suggest that missense variants alter splicing more frequently than recognized (Cartegni, Chew, & Krainer, 2002; Pagani & Baralle, 2004; Savisaar & Hurst, 2017; Soemedi et al., 2017; Xiong et al., 2015) or predicted by in silico tools (Soukarieh et al., 2016). Our data emphasize the importance of comprehensive variant interpretation irrespective of its predicted translational impact.

In summary, we have increased the mutational spectrum of DFNA5-related deafness to include missense variants. Importantly, the three novel variants we describe are exonic but have an identical mode of action. We anticipate that traditionally overlooked silent variants in addition to other missense within exon 8 will be identified that alter splicing. Families segregating high frequency progressive ADNSHL that links to *DFNA5* should be carefully reviewed for genomic alterations not only in the conventional splice-region of exon 8 but also for variants in its flanking introns and within the exon itself. Highly conserved exonic variants in exon 8 should be considered and investigated for potential effects on splicing. Expanding our understanding of the mutational spectrum of *DFNA5* and magnifying the role coding variants play in splicing is an important step in providing the correct molecular interpretation and diagnosis for these families.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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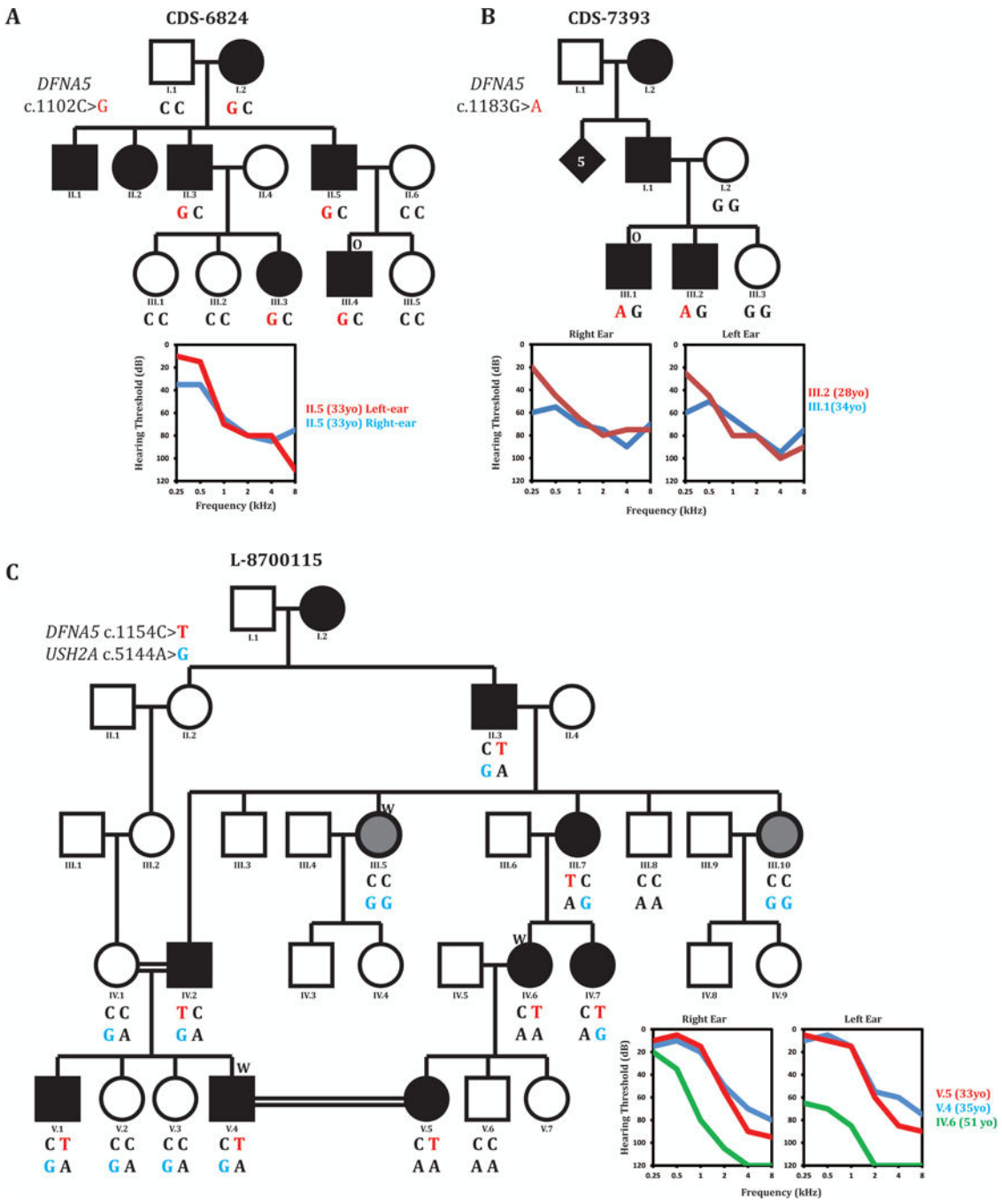


**Figure 1.**

ADNSHL pedigrees showing segregation of novel mutations in *DFNA5* with the deafness phenotype. Filled symbols denote affected individuals; red and bold and blue and bold represent the *DFNA5* and *USH2A* mutant alleles, respectively; grey filled symbols indicate individuals with USH2A. Audiograms were obtained using pure tone audiometry with air conduction from frequencies from 250 Hz to 8,000 Hz. ("O", OtoSCOPE; "W", Whole Exome Sequencing; A-C: Pedigrees for family CDS-6824, CDS-7393 and L-8700115, respectively)

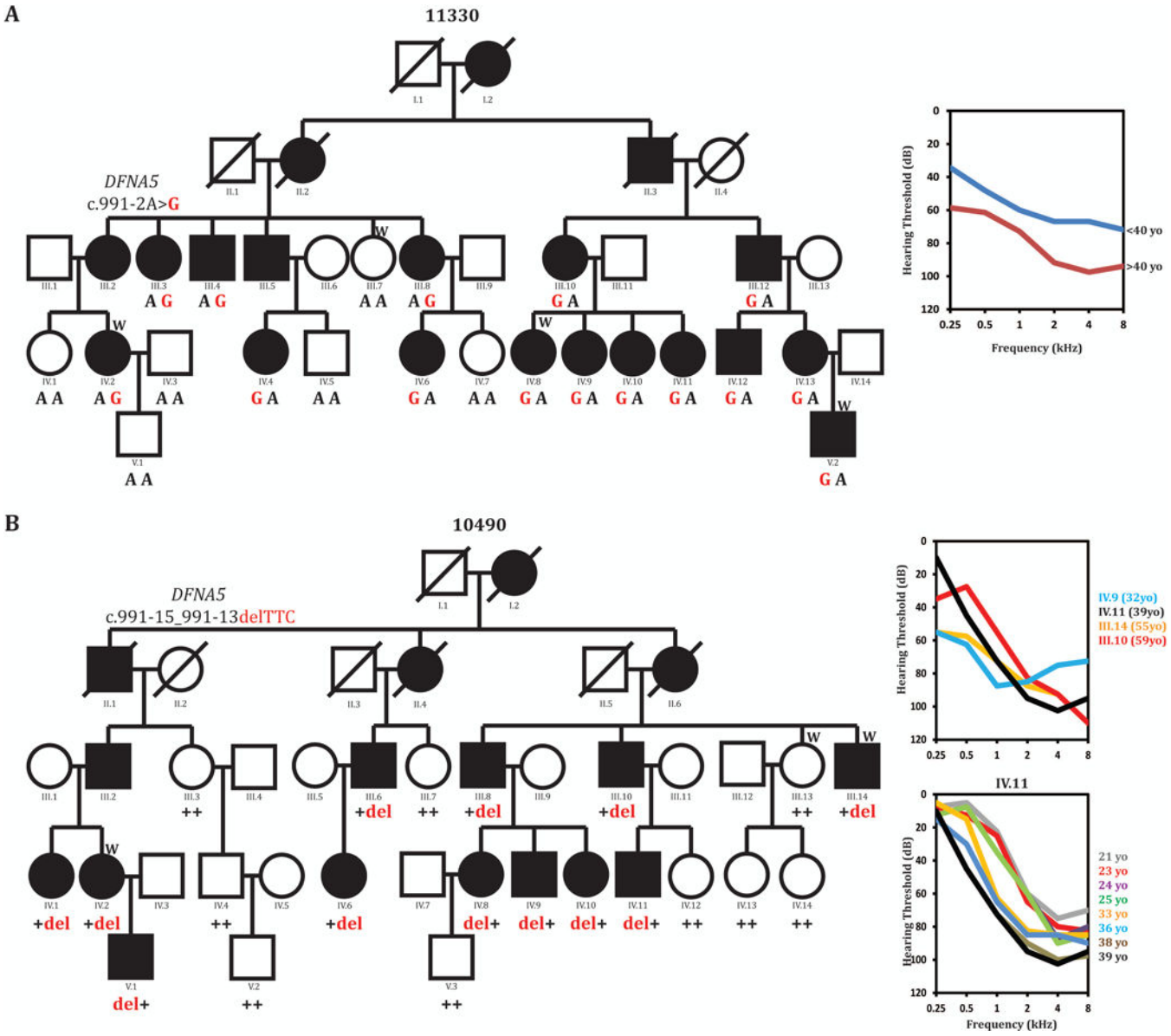


**Figure 2.**

ADNSHL pedigrees segregating recurrent mutations in *DFNA5*. Filled symbols denote affected individuals; red and bold represent the *DFNA5* mutant allele segregating with the HL. Audiograms were obtained using pure tone audiometry with air conduction from frequencies from 250 Hz to 8,000 Hz. ("W", Whole Exome Sequencing; A: family 11330, B: family 10490 with serial audiograms for individual IV.11)

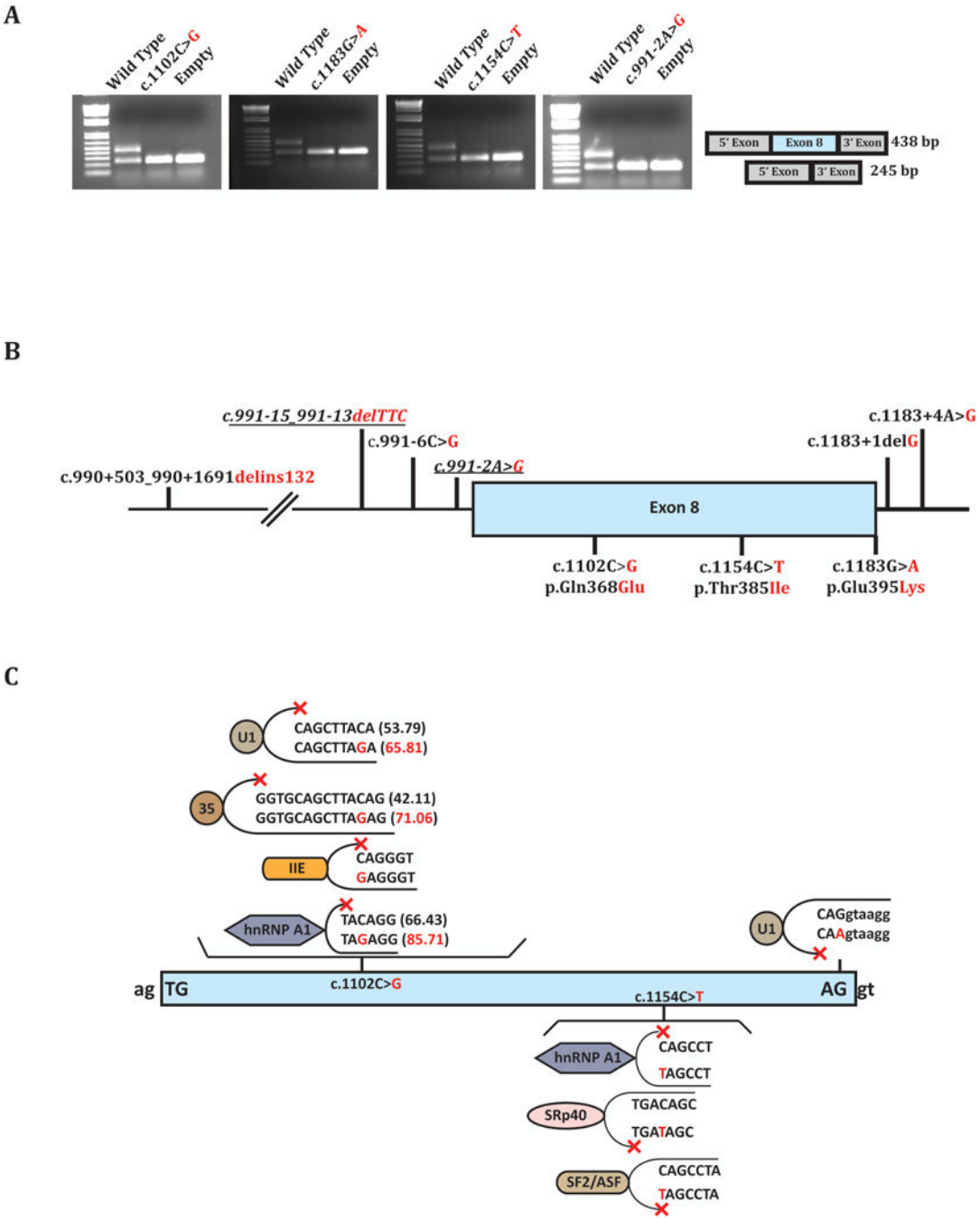


**Figure 3.**

Mini-gene splicing analysis and schematic representation of *DFNA5* exon 8 variants and splicing factor binding. A: Gel electrophoresis of wild-type *DFNA5* exon 8 and the c.1102C>G, c.1183G>A, c.1154C>T and c.991-2A>G variants and empty pET01 vector. The inclusion of exon 8 results in a 438bp product and its exclusion result in a 245bp band. B: Novel and previously described variant alignment to *DFNA5* exon 8 and its flanking introns. Novel variants are on bottom and previously described variants are on top. Italics and underline denote previously identified variants also identified in this study. C: Predicted

binding sites of splicing factors to the novel variants in this study. Values between parentheses represent predicted splicing signal, wild-type black and mutant red.

**Table 1**

*DFNA5* Mutations

| Family ID | Ethnicity | g.DNA | cDNA | GERP | PhyloP | CADD | ExAC (%) | gnomAD (%) | Ref |
|---|---|---|---|---|---|---|---|---|---|
| CDS-6824 | European | chr7:24745884G>C | c.1102C>G | C | C | 2.3 | 0 | 0 | This Study |
| CDS-7393 | East Asian | chr7:24745803C>T | c.1183G>A | C | C | 24.7 | 0 | 0 | |
| L-8700115 | Iranian | chr7:24745832G>A | c.1154C>T | C | C | 20.9 | 0 | 0 | |
| | | chr1:216258063T>C | c.5144A>G | C | C | 22.5 | 0 | 0.0004 | |
| 11330 | European | chr7:24745997T>C | c.991-2A>G* | C | C | 23.1 | 0 | 0 | This study+[17] |
| 10490 | E. Asian | chr7:24746008delGAA | c.991-15_991-13delTTC* | – | – | 0.543 | 0 | 0 | This study+[38,39] |
| | Dutch | chr7:24746055_24747243delins | c.990+503_990+1691del1189ins132 | – | – | – | 0 | 0 | [16] |
| | Dutch | chr7:24746001G>C | c.991-6C>G | | | | 0 | 0 | [18] |
| | Chinese | chr7: 24745802delC | c.1183+1delG | | | | 0 | 0 | [20] |
| | Chinese | chr7: 24745799T>C | c.1183+4A>G | | | | 0 | 0 | [15] |

Nucleotide numbering: the A of the ATG translation initiation site is noted as +1 using transcript NM_004403.2 of DFNA5 and NM_206933.2 of USH2A. Italics indicate the USH2A variant identified in two individuals in family L-8700115. Genomic Evolutionary Rate Profilin (GERP); Exome Aggregation Consortium (ExAC v.0.3); genome Aggregation Database (gnomAD v.2.0.2); C, predicted Conserved; dashes denote mutations outside functional domains. An asterisk "*" denotes previously described variants also identified in this study. Scores >0.0 and >0.95 are predicted conserved by GERP and PhyloP, respectively.