Autosomal Dominant Non-Syndromic Hearing Loss Maps to DFNA33 (13q34) and Co-Segregates with Splice Site Variants in ATP11A, A Phospholipid Flippase Gene

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Abstract

Whole genome approaches are superior for identifying recessive genes, however discovery of dominant genes including deafness genes (DFNA) remains challenging. Herein we report a new DFNA gene, ATP11A, in a Newfoundland family with a variable form of bilateral sensorineural hearing loss (SNHL). Targeted screening of DFNA genes based on audioprofiles was unsuccessful. Genome-wide SNP genotyping linked SNHL to DFNA33 (Lod = 4.77), a locus on 13q34 previously mapped in a German family with variable SNHL in 2009. WGS identified 51 unremarkable positional variants on 13q34. Continuous clinical ascertainment identified several key recombination events and reduced the disease interval to 769 Kb, excluding all but one variant. ATP11A (NC_000013.11: g.190616G > A) is a novel point mutation predicted to be a cryptic donor splice site. RNA studies in patient-derived tissues verified *in silico* predictions, revealing the retention of 153bp of intron in the 3’ UTR of several ATP11A isoforms. A second, unresolved family from Israel with a similar, variable form of SNHL and a novel duplication in exon 28 of ATP11A that occurs within the splice donor sequence (intron 28). ATP11A is a type of P4-ATPase that transports (flip) phospholipids from the outer to inner leaflet of cell membranes to maintain asymmetry. Haploinsufficiency of ATP11A, the phospholipid flippase that specifically transports phosphatidylserine (PS) and phosphatidylethanolamine (PE), could leave cells with PS/PE at the extracellular side vulnerable to phagocytic degradation. Given that surface PS can be pharmaceutically targeted, hearing loss due to ATP11A could potentially be treated. It is also likely that ATP11A is the gene underlying DFNA33.

Introduction

Hereditary hearing loss is a common sensory disorder exhibiting extensive genetic and clinical heterogeneity (Morton and Nance 2006). Over 200 hearing loss genes have been identified (Van Camp G 2015); however, approximately one-third of the 60 mapped autosomal dominant loci (DFNA) evade discovery. Like other autosomal dominant traits, hearing loss is typically characterized by variable expressivity and reduced penetrance (Richard JH Smith 1999). Whole-genome sequencing (WGS) in patients with dominant conditions generates many heterozygous variants, which is particularly problematic when the underlying gene is novel. Well ascertained multiplex families and more traditional methods (e.g., linkage and haplotype analysis) can be critical to reduce the number of candidate variants segregating. In a similar way, well ascertained families provide truly unaffected members that can be used to filter out benign variants. Genetic isolates can also help where apparently unrelated families are members of a clan (Sherry et al. 2001), as well as the fact that sibships are usually large and genealogical connections with distant relatives are known. With the increased power to detect rare variants using WGS compared with whole-exome sequencing (WES), WGS analysis should include all known isoforms of candidate genes, and not be limited to validated isoforms (i.e., RefSeq) (Belkadi et al. 2015; Zhao and Zhang 2015).

A key feature of eukaryotic cell membranes is the non-random (asymmetrical) distribution of phospholipids, an essential feature to maintaining cell integrity (Segawa et al. 2014). Asymmetry is most evident at the plasma membrane (Zachowski 1993) and is maintained by the action of three classes of proteins: scramblases, floppases and flippases. Phospholipid flippases (or P4-ATPases) specifically transport or “flip” phospholipids from the outer to the inner leaflet (Paulusma and Elferink 2010). Mouse
studies have demonstrated the importance of the P4-ATPase class of flippases in phospholipid metabolism and maintaining normal auditory function (Coleman et al. 2014; Stapelbroek et al. 2009). For example, pathogenic variants in ATP8B1 (MIM: 605868) cause intrahepatic cholestasis type 1 (MIM: 211600), where patients sporadically develop hearing loss (Stapelbroek et al. 2009). Phospholipids have also been implicated in autoimmune conditions, such as antiphospholipid syndrome characterized by the presence of antiphospholipid antibodies that invoke an autoimmune response causing thrombosis, and complications during pregnancy and hearing loss (Mouadeb and Ruckenstein 2005; Wiles et al. 2006). We map SNHL in a family of Northern European descent to the DFNA33 locus (OMIM 614211) and identify two novel splicing mutations (one likely pathogenic and one unknown) in the 3’ region of the ATP11A gene (OMIM: 605868) in two unrelated families with variable SNHL.

### Materials And Methods

For the discovery phase, a six-generation family of Northern European descent was ascertained from the province of Newfoundland and Labrador (NL), Canada (Fig. 1). The proband (PID IV-7) presented at age 13 years with a progressive, sloping, bilateral sensorineural hearing loss and a family history consistent with autosomal dominant inheritance. Validation in a second pedigree, representing a four-generation family from Israel with roots in Afghanistan was studied at the University of Tel Aviv (Fig. 2). The proband (PID IV-1) is a 29 year old female with hearing loss who was seeking genetic counseling to genetically diagnose the cause of familial hearing loss. At age 17 years she had normal low-mid frequency hearing and a high frequency bilateral sensorineural hearing loss, sloping above 1 kHz. In both families, hearing loss was evaluated using standard pure tone measurements of air and bone conduction thresholds (Fig. 3a-e). Informed consent was obtained according to protocol #01.186 of the Human Research Ethics Board, St. John's, NL, Canada.

**Targeted Sequencing, Genome-Wide SNP Genotyping and Linkage Analysis**

For the discovery phase, genomic DNA was extracted from peripheral blood of the NL family using a modified salting out protocol (Miller et al. 1988) and screening was performed by bi-directional Sanger sequencing and analyzed using Mutation Surveyor Software (v5.0, SoftGenetics LLC State College, PA, USA). The NL proband was Sanger sequenced screened for all pathogenic variants causing hearing loss in this genetic isolated population, followed by select autosomal dominant genes matching the proband's (PID IV-7) audiometric data with that of reference audioproles of 34 autosomal dominant deafness loci (Audiogene (v4.0) (Hildebrand et al. 2009). We next performed genome-wide single nucleotide polymorphism (SNP) genotyping using the Illumina 610Quad genotyping chip (Illumina Inc., San Diego, CA, USA) on multiple family members. Starting with a set of > 500,000 high quality SNP markers, informative SNPs (n = 17,407) were imported into Superlink (v1.7) (Fishelson and Geiger 2004) and linkage analysis performed under an autosomal dominant model with 99% penetrance and a disease allele frequency of 0.0025.

**Whole-Genome Sequencing**
Genomic DNA libraries were prepared on four affected (Fig. 1: PID IV-4, IV-6, IV-7, and IV-12) and two unaffected members of the NL family (PID IV-1 and IV-17) using the Lucigen Shotgun NxSeq AmpFREE Low DNA Library Kit (Cat. #14000-1, Lucigen Inc., Madison, WI, USA). Prepared libraries were loaded on an Illumina paired end 150 base pair (bp) sequencing lane, and sequenced on the HiSeqX Sequencer (Illumina Inc., San Diego, CA, USA). Sequence reads were aligned to GRch37 in both RefSeq and Ensembl reference genomes, and single nucleotide variants (SNVs) and insertions and deletions (INDELs) were called using GATK (v4.0). Structural chromosomal variants were called using Lumpy (v0.2.13) and SVtyper (v0.5.2), while a Bioconductor package, cn.MOPS (v1.26.0), was used for copy number variation (CNV) analysis. Variants were functionally annotated with SNPeff (v4.3T). We filtered for rare variants (MAF < 1%) residing within linked regions with a minimum of 20X coverage.

Cascade Sequencing, Haplotype and In Silico Analysis

Candidate variants were amplified using a standard touchdown PCR protocol and sequenced in family members and compared to 202 SNHL probands and 326 ethnically matched controls from NL. Microsatellite markers and intergenic SNPs within linked regions were called with GeneMapper software (v4.0) (Pater et al. 2017) and phased manually. Candidate splicing variants within exon-intron boundaries were analyzed in silico using MaxEnt, Human Splicing Finder (v3.1), and NNSPPLICE (v0.9) to predict their effect on RNA splicing.

Experimental Validation with Patient-Derived Tissues

To validate variants predicted to alter RNA splicing, we extracted RNA from transformed B-cell lymphocytes from both affected and unaffected individuals (controls) using TRIzol-based methods (Thermo-fisher, Cat. #15596026). We prepared cDNA libraries with the Superscript VILO cDNA synthesis kit (Thermo-fisher, Cat. #11754050) followed by genomic DNA digestion (Turbo DNA-free kit, Invitrogen, Cat. #1907). Reverse transcription PCR (RT-PCR) was performed using a standard touchdown PCR protocol and primers that flanked splicing variants within two positional candidate genes (ATP11A: 5’ CCAGAGGGGTGTGAAGCA 3’ and 5’ CATCACACGAGCATTCCCAC 3’; COL4A1: 5’ GTTCACCTGGCTTACCTGGA 3’ and 5’ AAACCCACCTCACCCTTG 3’). RT-PCR products were electrophoresed through 1.5% agarose Tris-Borate-EDTA gel and stained with SYBR Safe (Invitrogen, Cat. #S33102). For positional candidate genes expressing multiple transcripts, distinct bands were excised from the gel and cloned using the TOPO TA-Cloning Kit with One Shot TOP10 Chemically Competent E. coli (Invitrogen, Cat. #K457540) according to the manufacturer’s protocol. Clones were amplified using colony PCR (Costa and Weiner 2006) and Sanger sequenced.

Results

Hearing Loss Phenotype

In the discovery family, sixteen members exhibit bilateral SNHL. Longitudinal audiograms on PIDs IV-4, IV-6, IV-7, IV-9, IV-12, IV-14, V-4, V-5, V-6, V-9, and V-11 reveal a progressive loss with variable onset and configuration, with auditory profiles ranging from high frequency sloping loss to low-mid frequency and flat
configurations (Fig. 3). For example, the proband (PID IV-7) has a sloping high frequency hearing loss whereas his son (PID V-6) has a relatively flat configuration. Longitudinal audiograms for a cousin (PID V-4) reveal a low-mid frequency rising configuration. The proband (PIDs IV-7) and his father (PID III-5) were identified with hearing loss in their first decade. In contrast, two members (PIDs V-6, VI-1) failed newborn screening. Other members reported hearing loss in the 2nd or 3rd decade (age 28 for PID V-5, Fig. 3d). The course of hearing deterioration is also variable. Hearing sensitivity declined into the severe to profound range by the 2nd decade for some members (PIDs V-4, V-6) and in the 3rd – 6th decade for others (PIDs IV-12, IV-7, IV-9, IV-14, IV-6).

SNHL in the Israel family is also progressive with variable onset and configuration. Twelve members have bilateral SNHL (PIDs II-2, III-4, III-6, III-8, III-10, IV-1, IV-2, IV-3, IV-4, IV-5, IV-12, and IV-13). Pure tone audiogram data for the proband (PID IV-1) (Fig. 3b), the proband's sibling (PID IV-2) and cousin (PID IV-3) show variability in the audiometric configuration and degree of hearing loss. Longitudinal audiograms for the proband PID IV-1 (Fig. 3e) show sloping high frequency hearing loss and progression over three years. Consecutive audiograms for PID IV-2 show all frequency loss and progression over six years (Fig. 3e). A single audiogram for PID IV-3 at age 40 shows moderate low and mid frequency loss, and a peak at 2kHz with severe high frequency loss (Fig. 3e). Hearing loss was present in the proband (PID IV-1) by the early 2nd decade; onset is reported during the first decade for a sibling (PID IV-2) and two other members (PID IV-3 and IV-12).

Linkage Analysis Links Hearing loss to DFNA33 (13q34)

The proband (PID IV-7) screened negative for all genetic variants we previously identified in this genetically isolated population (Table S1). Furthermore, although audiometric data analysis yielded positive gene matches, bidirectional Sanger sequencing of targeted genes based on audioprofiles yielded wild-type sequence (data not shown). Genome-wide SNP genotyping and two-point linkage analysis yielded statistically significant linkage (LOD = 4.77; Table 1) to a 3.6 Mb region on chromosome 13q34 overlapping DFNA33 (13q34-13qter) (Bonsch et al. 2009).
### Table 1

Genomic regions with maximum observed LOD > 1.5 in a two-point linkage analysis under a dominant model with 99% penetrance and a disease allele frequency of 0.0025.

| Chr | LOD | SNP     | cM     | Genomic Position | SNP     | cM     | Genomic Position | Region Size (Mb) |
|-----|-----|---------|--------|------------------|---------|--------|------------------|------------------|
| 1   | 2.54| rs591979| 85.54  | 61,368,955       | rs9629017 | 87.62  | 62,083,960       | 0.72             |
| 1   | 2.14| rs6593523| 101.44 | 76,486,908       | rs1360878 | 101.82 | 76,749,088       | 0.26             |
| 1   | 1.87| rs1325278| 109.17 | 85,400,182       | rs817485  | 109.42 | 85,573,095       | 0.17             |
| 5   | 1.56| rs253604 | 161.03 | 155,960,089      | rs6892282 | 163.77 | 159,360,485      | 3.40             |
| 5   | 1.55| rs11954477| 167.38 | 163,374,345      | rs253537  | 169.34 | 164,600,485      | 1.23             |
| 13  | 4.77| rs872484 | 117.51 | 110,708,368      | rs9324254 | 128.64 | 114,312,000      | 3.60             |
| 19  | 1.83| rs4527136| 25.72  | 8,186,519        | rs2042300 | 26.79  | 8,580,602        | 0.39             |
| 19  | 1.83| rs2060260| 38.32  | 15,704,783       | rs1558139 | 38.55  | 15,997,564       | 0.29             |

LOD: logarithm of the odds (to the base 10), Chr: chromosome, cM: centimorgan, SNP: single nucleotide polymorphism, Mb: megabase.

Haplotype Analysis Reduces the Number of Candidate Variants

Whole-genome sequencing yielded an average coverage of 44X with 94% of the genome covered at 25X (Table S2). No structural chromosomal rearrangements or CNVs were detected (data not shown). Of the 15,071 variants identified in affected family members, 51 resided within the 3.6 Mb disease interval on 13q34 (Table S3). Two candidate splicing variants, one novel: \( \text{ATP11A} \) (NC_000013.11: g.190616G > A; ENST00000415301.1: ATP11A-203: c.*11G > A; Fig. 4a) and one rare: \( \text{COL4A1} \) (NC_000013.11: g.110174539dupT; NG_011544.2:g.137617dup; NM_001845.6: c.3326-7dupT; Fig. 4b) were both absent in Newfoundland population controls. \( \text{In silico} \) tools predict that \( \text{COL4A1} \) c.3326-7dupT does not disrupt splicing, which we confirmed by RNA analysis (Fig. 4c). Furthermore, during the transcriptional analysis, we recruited two unaffected family members (PID III-4 and V-2) that harboured key cross over events and reduced the disease interval to 769 Kb, excluding the \( \text{COL4A1} \) c.3326-7dupT (Fig. 1). Subsequently, 202 Newfoundland probands with hearing loss were screened wild-type for \( \text{ATP11A} \) g.190616G > A.

RNA Analysis Reveals several \( \text{ATP11A} \) Products and an Extra 3’ Coding Exon

The \( \text{ATP11A} \) gene encodes a member of the family of P4-ATPase proteins and has 17 transcripts (Human GRCh37 Ensembl 93 build (Yates et al. 2016)), most of which are incompletely annotated. The \( \text{ATP11A} \) gene has three long isoforms. Ensembl reports \( \text{ATP11A-201} \) (ENST00000375630.6), \( \text{ATP11A-202} \) (ENST00000375645.7) and \( \text{ATP11A-212} \) (ENST00000487903.5), which also includes the two RefSeq transcripts: \( \text{ATP11A-202} \) (NM_015205: isoform a: 8,795 bp) and \( \text{ATP11A-201} \) (NM_032189: isoform b:...
In silico algorithms predict this novel ATP11A variant has the potential to disrupt a canonical donor splice site (Medium Impact; Table S4). According to NNSPLICE analysis, the ATP11A variant is predicted to activate a cryptic donor splice site 153 bp downstream of the canonical donor splice site. RT-PCR analysis of the 3' region of ATP11A (flanking the g.190616G > A variant) in unaffecteds and ATP11A g.190616G > A carriers (PID III-1, III-5 and III-7) revealed multiple products (Fig. 4d). PCR amplification and size fractionation of cloned ATP11A RT-PCR products revealed three products in unaffecteds and these three products plus three extra higher molecular weight products in ATP11A g.190616G > A carriers (Fig. 4e).

Sequencing of cloned RT-PCR products in unaffected and ATP11A g.190616G > A carriers show they all contained a 104 bp exon (i.e., NC_000013.11: g.190513–190616), which matches with exon 2 of the ATP11A-203 transcript (ENST00000415301.1; Fig. 4) and is consistent with the common lower-molecular weight bands (Fig. 4e). Downstream of the shared 104 bp exon, three unique sequences were observed, aligning to exon 3 of ATP11A-203 and exon 30 of ATP11A-202 (ENST00000375645.7, lower molecular weight), exon 29 of ATP11A-201 (ENST00000375630.6; NM_032189) and exon 30 of ATP11A-212 (ENST00000487903.5; NM_015205; higher molecular weight; Fig. 4e; Fig. 5). Whether or not the 104 bp exon represents the penultimate exon in ATP11A-201 (Fig. 5a) and exon 30 in the putative ATP11A-202/212 isoforms (Fig. 5b), or represents a novel transcript is unknown (Fig. 5). Interestingly, this 104 bp sequence is similar (61.5% conserved) to exon 29 (ATP11A-201) in the mouse (Figure S1), which is consistent with location of the 104 bp spliced region from our RNA analysis (Fig. 5). Full-length sequencing of these distinct RNA transcripts is required to confirm which of the many ATP11A isoforms are being expressed in blood.

RNA Validation of Aberrant Splicing in Patient-Derived Tissues

NNSPLICE analysis predicted that the ATP11A g.190616G > A variant may activate a cryptic splice site 153 bp downstream of the canonical donor splice site. Sequencing of cloned RT-PCR products in ATP11A g.190616G > A carriers show they retain 153 bp of intronic sequence at the 3'UTR of ATP11A and is consistent with the three aberrant higher-molecular weight products (Figs. 4e and 5). This intronic sequence was not observed in unaffected family members tested (Figs. 4e and 5). Whether or not the 153 bp extends exon 29 in the putative ATP11A-201 isoform (Fig. 5a) and exon 30 in the putative ATP11A-202/212 isoforms (Fig. 5b), or represents a novel transcript is unknown (Fig. 5). As the RT-PCR analysis and sequencing was restricted to the 3' region of ATP11A (flanking the g.190616G > A variant), full-length sequencing is required to confirm the variant effect on ATP11A isoforms.

Unrelated Family from Israel with Novel Splicing Variant in exon 28 of ATP11A

Genomic DNA from the proband (PID IV-1) and two cousins (PIDs IV-3, IV-12; Fig. 2) underwent clinical exomes (CEGAT Laboratory) and screened negative for DFNA genes but were heterozygous for a novel splice site duplication in exon 28 (ATP11A: NM_032189.3:c.3322_3327 + 2dupGTCCAGGT) in all three samples (data not shown). This variant of uncertain clinical significance has not been seen in 5,000 previous exome analyses at the CEGAT Laboratory or previously described in the gnomAD database.
Discussion

We report the first family with autosomal dominant SNHL to be linked to DFNA33 since the locus was mapped in 2009 (Bonsch et al. 2009) and identify two novel splice site variants in the 3’ region of ATP11A. Splice mutations in the 3’ region of ATP11A cause a bilateral, progressive SNHL with variable onset and configuration. The hearing loss in the German DFNA33 family is quite similar to ATP11A mutation carriers in both the NL and the Israel families. Although we cannot be certain that the ATP11A gene is DFNA33, it was noted to be a functional candidate based on mouse studies (Bonsch et al. 2009). Although a decade has passed since DFNA33 was mapped to chromosome 13q34-qter, no other families have been reportedly mapped to this locus. Although other phospholipid flippases (P4-ATPases) are associated with syndromic forms of hearing loss, this study documents the first association of ATP11A with a highly penetrant Mendelian phenotype.

Mutations in specific phospholipid flippases have been linked to familial intrahepatic cholestasis (Bull et al. 1998), severe neurological and motor disorders (Martin-Hernandez et al. 2016) and congenital hemolytic anemia (Arashiki et al. 2016). P4-ATPases comprise a subfamily of P-type ATPases that flip phospholipids from the exocytosplasmic to the cytoplasmic leaflet of cell membranes that both generates and maintains phospholipid asymmetry and are organized into five subclasses based on the sequence similarity of their catalytic subunits. These proteins contain a large catalytic or α-subunit composed of a nucleotide binding domain (N-domain), a phosphorylation domain (P-domain), and an actuator domain (A-domain) as well as a membrane domain (M-domain) comprised of 10 transmembrane segments (Andersen et al. 2016) and forms a heteromeric complex with CDC50 (Bryde et al. 2010). Furthermore, mammalian P4-ATPases can contain an extended C-terminal segment implicated in protein folding and regulation of its activity (Chalat et al. 2017).

ATP11A, which specifically transports phosphatidylserine (PS) and phosphatidylethanolamine (PE) across cell membranes, is ubiquitously expressed in various tissues and deletion of in atp11a in mice results in lethality during embryogenesis (Segawa et al. 2016; Wang et al. 2018). Of the 17 annotated ATP11A transcripts, the putative ATP11A g.190616G > A variant mapped to a short Ensembl isoform containing only three exons. RT-PCR and cloning analysis of the 3’ region of ATP11A (flanking the g.190616G > A variant) in unaffecteds and ATP11A g.190616G > A carriers revealed multiple products, three products in unaffecteds and these three products plus three extra higher molecular weight products in ATP11A g.190616G > A carriers. The predicted variant effect of the insertion of a 153 bp intronic sequence, which activates a cryptic donor splice site 153 bp downstream, extending the 3’UTR was confirmed with RNA analysis. However, the exact disease mechanism is unclear.

It is known that the 3’ end of the mammalian P4-ATPases are important for protein folding and regulation of its activity (Chalat et al. 2017). Given that the splicing variant is located in the 3’UTR, it is unlikely that it affects protein structure; however, it might affect protein function through modulating ATP11A gene expression. Furthermore, it is unknown if the likely pathogenic splicing variant acts in a dominant-negative effect or due to haploinsufficiency.
Evidence suggests that the genomic complexity of ATP11A expression is similar in mouse and humans. Several principal ATP11A transcripts are expressed in mouse auditory system, including sensory hair cells of the inner ear, otic progenitor cells and auditory and vestibular ganglion neurons (Shen et al. 2015). By combining single-cell and long-read RNA-seq technologies, Ranum et al (2019) has recently revealed clusters of genes that define inner hair cells, outer hair cells and deiter cells, and identified many heretofore unrecognized exons, alternative splicing diversity and isoform abundance in hearing loss genes (Ranum et al. 2019). According to the Molecular Otolaryngology and Renal Research Laboratories database, ATP11A-203 (exon 2) murine homolog, Atp11a (exon 29) was expressed in inner hair cells, outer hair cells and deiter cells. Most importantly, Atp11a was identified as a deiter cell defining gene. ATP11a exhibits moderate expression during mouse embryonic development, followed by a marked increase in expression after birth (Shen et al. 2015). That the mouse ATP11A-201 transcript contains an exon 29 that is similar to the new 104 bp exon that we identified between exons 28 and 29 in one RT-PCR product and that Atp11a (exon 29) was expressed in inner hair cells, outer hair cells and deiter cells, suggests that of 3’ end of the ATP11A product is likely ATP11A-201 in humans. A mouse model for ATP11A-induced hearing loss could help elucidate the molecular mechanisms.

Over the ten-years of study on the NL family, we have benefited from ongoing recruitment and comprehensive phenotyping which eventually identified key recombinations to the disease-associated haplotype. The use of a mixed approach including linkage and haplotype analysis reduced the number of candidate variants, which made the problem tractable. Given the complexity in genomes, a comprehensive bioinformatics pipeline targeting all known transcripts is essential as is the need to experimentally validate in silico predictions in patient-derived tissues.

Limitations of this study include a lack of insight as to protein structure and/or function, specifically with respect to hearing loss. In addition, given that the complete ATP11A 201/202/212 transcripts were not sequenced, it is uncertain if the penultimate exon is present in these ATP11A transcripts or represents the 3’ end of a novel transcript. Likewise, it is uncertain if the splicing variant in ATP11A carriers causes the retention of 153 bp in the penultimate exon of ATP11A 201/202/212 transcripts or represents the 3’ end of a novel transcript. The effect of the predicted splice site duplication in exon 28 (ATP11A: NM_032189.3: c.3322_3327+2dupGTCCAGGT) in the Israel family is unknown.

Future directions for this study include the quantitative analyses of ATP11A splicing variant, in addition to in vitro and in vivo functional characterization of the molecular mechanisms. Given that ATP11A specifically transports PS and PE across cell membranes, the deregulation of transport could redistribute PS to the extracellular side of plasma membrane flagging cells for their recognition, phagocytosis, and ultimate degradation by phagocytes (Schroit et al. 1985). Phagocytic signals such as PS at the cell surface are known pharmaceutical targets (Birge et al. 2016), so there is potential that hearing loss due to ATP11A could be pharmaceutically treated. It will be important, if possible, for the original German family used to map DFNA33 be sequenced in order to be certain that ATP11A is, in fact, DFNA33.

Web Resources
NNSPLICE: (https://www.fruitfly.org/seq_tools/splice.html)

Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org.

Audiogene (v4.0): https://audiogene.eng.uiowa.edu/

CEGAT Lab: https://www.cegat.de/en/diagnostics/exome-diagnostics/the-best-possible-exome/.

Genome Aggregation Database (gnomAD): https://gnomad.broadinstitute.org/

Ensembl: https://useast.ensembl.org/index.html

International mouse phenotyping consortium:
http://www.mousephenotype.org/data/genes/MGI:135473524

miRBase: the microRNA database: http://www.mirbase.org/

Declarations

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not Applicable

Authors' contributions

For the NL study, patients were recruited by AG; charts reviewed by AG, CP, SS, TB, SGS, JAP. Experiments performed by JAP, CP, JH, NA. Linkage analysis done by NMR, DV, PM. Genotype data analyzed by JAP, CP, LPD, JS, SW, GW, NA, JH, DDO, TLY. RNA analysis by JAP, CRF, DDO, JH, TLY. For the Israel family, the study was led by MS and samples sequenced by OB. Manuscript written by JAP, DDO, AG, SS, TLY. This study includes thesis work of JAP (PhD) and CP (MSc).

Conflicts of Interests

The authors declare they have no conflict of interest.

Ethics Approval

This study was approved by the institutional review board at Memorial University (#1.186).
Consent to participate

Informed consent was obtained from all individual participants included in this study.

Consent for Publication

Participants signed informed consent regarding publishing their data.

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**Figures**
Figure 1

Six generation Newfoundland pedigree with autosomal dominant bilateral SNHL segregating a DFNA33-linked disease haplotype on 13q34. Haplotypes (coloured vertical bars) represent a 3.6Mb region on chromosome 13q34 with polymorphic markers and SNPs and their relative genomic positions (left). The disease haplotype (yellow) is diminished by several key crossover events (red x). Black squares represent males and circles, females. Dashes above symbols indicate pure tone audiometry hearing testing was performed. Asterisks are the members that were selected for whole-genome sequencing.
Figure 2

Four generation Israel pedigree with bilateral sensorineural hearing loss segregating as an autosomal dominant trait. The proband (PID IV-1) presented for clinical whole exome sequencing (WES) due to concerns about hereditary hearing loss in her family. Sequencing was completed on her and her two cousins (PIDs IV-3 and IV-12). All screened negative for DFNA genes but were heterozygous for a novel duplication in exon 28 of ATP11A (NM_032189.3: c.3322_3327+2dupGTCCAGGT) that occurs within the splice donor sequence (intron 28).
Figure 3

Comparison of audiograms between two families with autosomal dominant, variable non-syndromic hearing loss and novel variants in the ATP11A gene. (a) Audiogram of Newfoundland (NL) proband. (b) Audiogram of Israel proband. (c) Audiograms of three NL family members with variable configuration and progression. (d) Comparison of audiograms between six NL family members (PID IV-7 age 27 years; IV-6 age 28 years; IV-9 age 29 years; IV-12 age 21 years; V-5 age 28 years; V-4 age 26 years) show variability of severity in the 3rd decade. (e) Audiograms of three Israel family members with variable configuration and progression of hearing loss. AC = air conduction; BC = bone conduction.

Figure 4
RNA analysis of candidate splicing variants derived from B-cells in unaffecteds (controls) and patients (ATP11A g.190616G>A carriers). (a) Electropherogram illustrating a heterozygous ATP11A (NC_000013.11: g.190616G>A) variant (Figure 1: PID: III-1). (b) Electropherogram illustrating a heterozygous COL4A1 (NC_000013.11: g.110174539dupT; NG_011544.2:g.137617dup; NM_001845.6: c.3326-7dupT) variant (Figure 1: PID: III-1). (c) RT-PCR analysis of the COL4A1 c.3326-7dupT variant revealed a single ~280bp amplicon in unaffecteds (controls) and COL4A1 c.3326-7dupT carriers (PID III-1, III-5, and III-7). (d) RT-PCR analysis of the 3′ region of ATP11A flanking the g.190616G>A variant revealed multiple bands in both unaffecteds (controls) and ATP11A g.190616G>A carriers. (e) Cloned RT-PCR products revealed three low-molecular weight bands common to unaffecteds (controls) and ATP11A g.190616G>A carriers and three higher-molecular weight bands only observed in carriers. NTC: non-template control. The 100 bp ladder is indicated on the left of the gels with the densely stained bands representing 600 bp and 1500 bp.

**Figure 5**

Sequencing of cloned ATP11A RT-PCR products from healthy controls and affected family members. The genomic position of the ATP11A variant (NC_000013.11: g.190616G>A) is indicated by a pink arrow and activates a cryptic splice site causing intron retention of 153 bp. For visualization purposes, 20 bp of the shared 104 bp exon and the first 20 bp of the next known 5′ exon are displayed. Cryptic spliced region is
represented by the first and last 10 bp of the 5’ and 3’ ends of the retained intronic sequence, respectively.  
(a) Upper electropherogram: sequence present in both controls and affected family members (Figure 3e, Lane 3 and Lane 9). The first 20 bp of this sequence aligns to ATP11A-203 (exon 2) and the following 20 bp aligns to both ATP11A-203 (exon 3) and ATP11A-202 (exon 30). Lower electropherogram: sequence harboring the ATP11A variant found in affected family members that activates a cryptic donor splice site 153 bp downstream (Figure 3e, Lane 6).  
(b) Upper electropherogram: sequence found in both control and affected family members (Figure 3e, Lane 2 and Lane 8) that aligns to ATP11A-203 (exon 2) and ATP11A-201 (exon 29). Lower electropherogram: sequence harboring the ATP11A variant found in affected family members that activates a cryptic donor splice site 153 bp downstream (Figure 3e, Lane 5).  
(c) Upper electropherogram: sequence present in both controls and affected family members (Figure 3e, Lane 1 and Lane 7) that aligns to ATP11A-203 (exon 2) and ATP11A-212 (exon 30). Lower electropherogram: sequence harboring the ATP11A variant found in affected family members that activates a cryptic donor splice site 153 bp downstream (Figure 3e, Lane 4). Control: wild-type ATP11A cDNA sequence, found in both control and affected family members. Carrier: ATP11A variant (g.190616G>A) cDNA sequence, which is exclusive to affected family members. Green box: DNA motif predicted by NNSPLICE as the most probable cryptic donor splice site, in the absence of the natural splice site (Table S5). Uppercase font denotes exonic sequence, and lowercase font indicates retained intronic cDNA.  

**Supplementary Files**

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- SupplementalDataFile.pdf