Identification of two novel mutations in FAM136A and DTNA genes in autosomal-dominant familial Meniere’s disease

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Meniere’s disease (MD) is a chronic disorder of the inner ear defined by sensorineural hearing loss, tinnitus and episodic vertigo, and familial MD is observed in 5–15% of sporadic cases. Although its pathophysiology is largely unknown, studies in human temporal bones have found an accumulation of endolymph in the scala media of the cochlea. By whole-exome sequencing, we have identified two novel heterozygous single-nucleotide variants in FAM136A and DTNA genes, both in a Spanish family with three affected cases in consecutive generations, highly suggestive of autosomal-dominant inheritance. The nonsense mutation in the FAM136A gene leads to a stop codon that disrupts the FAM136A protein product. Sequencing revealed two mRNA transcripts of FAM136A in lymphoblasts from patients, which were confirmed by immunoblotting. Carriers of the FAM136A mutation showed a significant decrease in the expression level of both transcripts in lymphoblastoid cell lines. The missense mutation in the DTNA gene produces a novel splice site which skips exon 21 and leads to a shorter alternative transcript. We also demonstrated that FAM136A and DTNA proteins are expressed in the neurosensorial epithelium of the crista ampullaris of the rat by immunohistochemistry. While FAM136A encodes a mitochondrial protein with unknown function, DTNA encodes a cytoskeleton-interacting membrane protein involved in the formation and stability of synapses with a crucial role in the permeability of the blood–brain barrier. Neither of these genes has been described in patients with hearing loss, FAM136A and DTNA being candidate gene for familiar MD.

INTRODUCTION

Meniere’s disease (MD, [MIM 156000]) is an inner ear disorder characterized by episodes of vertigo associated with sensorineural hearing loss (SNHL) and tinnitus (1). The prevalence of MD is ~0.5–1/1000 and it is more common in populations of European descent (2,3). There is a small female preponderance and the typical age of onset is 30–50 years (4,5). In 5–40% of cases, both ears are affected (bilateral disease) (6,7), leading to a severe hearing impairment and chronic imbalance, resulting in a huge burden for the patient and inability to work (8,9).

Histopathological studies in human temporal bones have found an accumulation of endolymph in the scala media of the cochlea, but the origin of the endolymphatic hydrops is unknown (10). The majority of patients with MD are considered sporadic cases, and MD is a multifactorial disorder in which genetic factors probably confer susceptibility to its development (11). The prevalence of MD anticipates that multiple genes would be associated with MD (12). However, the observed clinical heterogeneity in the phenotype among sporadic cases makes difficult the recruitment of a large homogeneous cohort of patients to perform a genome-wide association study, and it has prevented the identification of genes associated with MD. Of note, some patients report relatives with a history of vertigo or SNHL and the frequency of familial cases of MD is around 8–10% among sporadic cases in European population (11,13,14). Focus on multicase families with MD

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reduces clinical and genetic heterogeneity and the combination of
whole-exome sequencing (WES) followed by variant filtering by
using unaffected relatives sharing a common genetic background
is a reliable strategy to identify novel genes-causing disease
(15,16). Most of the families have an autosomal-dominant
pattern of inheritance with incomplete penetrance (14,17), but
families with recessive inheritance have also been described
(18), pointing to a genetic heterogeneity (19). Anticipation,
including both earlier onset and tendency to more severe symp-
toms in successive generations, has also been observed in MD
in Swedish (20,21), British (22) and German families (23).

Previous linkage studies in familial MD (FMD) have found
candidate loci at 12p12.3 in a large Swedish family (20) and
5q14–15 in another German family (23), but the genes were
not identified. However, next-generation sequencing (NGS)
techniques have led a new approach to identify new causal
genes and mutations in monogenic diseases as well as complex
diseases (24). Particularly, WES was used since, 85% of the
diseases causing variations are located in exonic regions in
Mendelian disorders (25).

We have performed a WES study in a family with three
affected cases in consecutive generations. Herein, we report
the identification of two novel mutations in FAM136A and
DTNA genes, which generate novel protein coding transcripts
which may explain the MD phenotype in the family.

RESULTS

FAM136A and DTNA genes

WES and bioinformatics analyses revealed two novel mutations
which segregated in all affected cases (Fig. 1A). The presence
of both mutations was assessed in different populations with the
use of three databases: 1000 Genomes, the National Heart,
Lung, and Blood Institute Exome Variant Server, and our
in-house exome database (1000 MD cases and 500 controls). In
total, data from >10,000 individuals from unrelated populations
were reviewed and none of them had the chr2:70527974C>T or
chr18:32462094G>T variants.

A single novel heterozygous nonsense variant of interest was
found in the gene, Family with sequence similarity 136,
member A gene (FAM136A [ENSG0000035141]) at position
chr2:70527974C>T (Fig. 1B). In humans, FAM136A has
four annotated protein coding transcripts: FAM136A_001
(ENST0000037869), FAM136A_002 (ENST00000450256),
FAM136A_004 (ENST00000430566) and FAM136A_005
(ENST00000438759; Supplementary Material, Fig. S1).
FAM136A_001 and FAM136A_002 isoforms encompass three
exons, but the alternative splice site in FAM136A_002 includes
part of the intronic sequence. FAM136A_004 and FAM136A_
005 isoforms have extra amino acids as a result of a differ-
ent splice site. FAM136A_003 (ENST00000460307), besides
FAM136A_006 (ENST00000498665), it has a retained intron
and no protein product is generated.

The novel mutation leads to a stop codon that disrupts three of
these four isoforms (isoforms 1, 4 and 5; Fig. 1C; Supplementary
Material, Fig. S1). The predicted pathogenicity of the latter
variant is very damaging since it truncates the FAM136A
protein, leading to shorter protein products of 76, 182 and 145
amino acids, respectively (Supplementary Material, Table S1).

The second single novel heterozygous missense variant was
observed in the Dystrobrevin α gene (DTNA [ENSG00000
134769]) at position chr18:32462094G>T (Fig. 1D). DTNA
has 38 annotated transcripts (Supplementary Material,
Table S2). The novel mutation produces an amino acid change
(VAL to PHE) that modified 15 of the 31 coding isoforms
(Fig. 1E) predicted in the Ensembl database. This DTNA
missense mutation generates a novel splice-site sequence predicted as a
constitutive acceptor (ttcgcggacCTGGGAAGACT).

FAM136A and DTNA expression

The Expression Atlas (EMBL-EBI) and BioGPS indicates that
FAM136A is highly expressed in lymphocytes. So, FAM136A
gene expression was analyzed in lymphoblastoid cell lines
derived from a patient (III.4) and a familial control (II.19).

Sequencing revealed two mRNA transcripts of 1810 and
936 bps lengths, but only the larger transcript harbored the
mutation. Of note, Reverse-transcriptase polymerase chain reaction
(RT–PCR) showed that the mutant mRNA (FAM136A_001
Transcript) has a significantly reduced expression ($P = 0.002
in lymphoblasts from patients with FMD when compared with
controls (Fig. 2). Immunoblotting confirmed the presence of
two wild-type protein isoforms (138 and 105 amino acids,
respectively) in patient lymphoblasts. However, we were not
able to detect the mutant-predicted protein resulting from the
novel mutation (76 amino acids, 8 kDa) in either patient (III.4)
or control (II.19) immunoblots (Fig. 3).

The Expression Atlas (EMBL-EBI) and BioGPS show that
DTNA is highly expressed in the brain, retina and inner ear.
No biopsy or autopsy material was available from the inner ear
or brain and lymphoblasts have a low DTNA expression, so
these cells are not the most appropriate cell type to evaluate
the impact of this mutation in DTNA expression. However, the
splice-site effect was tested in lymphoblasts and it was validated
(Fig. 4A). This splice-site skips exon 21 that carries the last five
amino acids of the DTNA protein (Fig. 4B and C).

Immunohistochemical studies

We carried out immunohistochemical studies in the rat inner ear
to define the location of FAM136A and DTNA within the vest-
tibular system.

Using confocal microscopy in inner ear rat tissue, we have
found that FAM136A co-localizes with the mitochondrial
marker COX IV in the basal part of hair cells in the crista ampul-
laris (Fig. 5A).

DISCUSSION

Our filtering strategy using an MAF threshold of 0.01 is rather
conservative, since it has been recently established an MAF
threshold of 0.005 for autosomal-recessive variants and 0.0005
for autosomal-dominant variants for genetic screening of non-
syndromic hearing loss (26). We have found novel single
nucleotide variants (SNVs) in FAM136A and DTNA genes in
this family and both variants may contribute by independent
mechanisms to develop the phenotype.
Although the novel variant of DTNA causes the skipping of the last five amino acids of the protein, it could still be a tolerated loss-of-function mutation (27); however, we cannot rule out this variant, since this splice-site skips a phosphorylation site in serine 740. Moreover, the splice site is close to another phosphorylation site in serine 716 and both could affect the function of the protein.

Both FAM136A and DTNA proteins have been reported to be expressed in the human inner ear (28,29), although orthologous proteins were also found in several species from \textit{Rattus norvegicus} to...
pared with controls (asterisk, significantly reduced Sybr Green expression in patient lymphoblasts when it was compared with controls (asterisk, \( P = 0.002 \)).

Figure 2. Gene expression of FAM136A in lymphoblastoid cells, as assayed by standard quantitative PCR on cDNA from individuals harboring chr2: 70527974C>T and healthy donors. The FAM136A_001 transcript has a significantly reduced Sybr Green expression in patient lymphoblasts when it was compared with controls (asterisk, \( P = 0.002 \)).

Figure 3. Immunoblotting of FAM136A and actin as a loading control from individuals harboring chr2:70527974C>T and healthy donors. The FAM136 band is located at an MW of 16 kDa. Each sample was replicated to minimize the error.

Danio rerio (Supplementary Material, Fig. S2). The Shared Harvard Inner-Ear Laboratory Database shows that FAM136A and DTNA proteins are differentially expressed in the mouse inner ear. During the development of the inner ear, FAM136 and DTNA are present in both the cochlea and vestibular organs (30,31), but no gene expression data are available in the adult mouse. The proteins are also present in cochlear and vestibular ganglia.

FAM136A gene appears to encode a protein present in the utricle and cochlea during prenatal development, but subsequently, only present in the utricle after birth. So, FAM136A could be an important protein for development of the inner ear at early stages.

The FAM136A_001 isoform is the most commonly expressed and the protein sequence encoded by this isoform is more similar to mouse and rat orthologs with a homology of 88% (Supplementary Material, Fig. S2). FAM136A_002, FAM136A_004 and FAM136A_005 have a lower homology with these species, ~70%. Our results show a decreased expression of both isoforms in patient lymphoblasts (FAM136A_001 and FAM136A_002) that could be explained by the loss of function of FAM136A protein and the regulation of FAM136A gene transcription. We predict that the mutation in patients from this family leads to a truncated protein with the formation of an abnormal inactive isoform, possibly processed in the proteasome, in the vestibular end organs that subsequently results in haploinsufficiency. Without autopsy material, or tissue from a FAM136A mutant or knockout model, it is not possible to determine whether the stop codon in FAM136A isoform 1 is aberrantly expressed in the vestibular system and thereby possibly leads to damage in the vestibular endorgans. The patients with this heterozygous mutation do not appear to carry other clinical abnormalities.

DTNA encodes \( \alpha \)-dystrobrevin, a structural protein of the dystrophin-associated protein complex, which has been associated with movement disorders such as Duchenne muscular dystrophy. The absence of glial \( \alpha \)-dystrobrevin causes abnormalities of the blood–brain barrier and progressive edema in the mouse model (32). In addition, DTNA shows differential expression in the vestibular system during maturation of the inner ear in mice, suggesting also a relevant role in the development of the vestibular system (28). Our immunohistochemical results confirm the presence of \( \alpha \)-dystrobrevin in supporting cells in the rat crista. \( \alpha \)-Dystrobrevin is associated with the dystrophin complex of proteins (such as \( \alpha \)- and \( \beta \)-dystroglycan, laminin and \( \alpha \)-syntrophin) that are found in inner ear endorgans (33,34) (Lysakowski’s unpublished data). \( \alpha \)-Dystrobrevin and the dystrophin-associated protein complex are an integral part of the cytoskeleton, closely connected to the plasma membrane. Since dystrophin is expressed in the cochlear hair cells in guinea-pig and mouse (28,29), it is expected that structural changes in this protein network can affect the motility of hair cells in the cochlea, and could explain the SNHL observed in this family.

DTNA transcripts are tissue specific and the inner ear transcripts will probably have more similarity with brain transcripts. Without autopsy material, or tissue from a DTNA mutant or knockout model, it is not possible to determine the effect of the new splice site in DTNA in the vestibular system. The distribution of both proteins in the vestibular neuroepithelium of the adult rat and the finding of novel mutations in FAM136A and DTNA genes support a role for both proteins in the pathophysiology of FMD.

In summary, our findings suggest that novel mutations in FAM136A and DTNA genes are probably causal variants in FMD. A decrease expression of FAM136A_001 coding transcript in patient lymphoblasts, leading to haploinsufficiency of FAM136A, and the generation of a novel splice site in DTNA gene, skipping exon 21, suggest a functional role for both mutations. Finally, the localization of FAM136A and \( \alpha \)-dystrobrevin in the neuroepithelium of the rat vestibular crista demonstrate that both genes are expressed in adult rats. Future work includes performing immunohistochemistry studies in the rat and mice cochlea to determine with cells types are involved at that level. Moreover, we plan to perform histological examinations in the heterozygous \( \alpha \)-dystrobrevin knockout mice to characterize its cochlear and vestibular phenotype to evaluate the development of endolymphatic hydrops.

MATERIALS AND METHODS

Patients and controls
A Spanish family including three affected women in consecutive generations with criteria for definite MD (35), highly suggestive
of an autosomal-dominant pattern of inheritance was diagnosed at the Hospital of Poniente, El Ejido, Almería (Fig. 1A). DNA was isolated from blood samples anticoagulant-treated peripheral blood mononuclear cells (PBMCs) using the GenoVision M-48 robot (Qiagen, Venlo, The Netherlands) and the MagAttract DNA Blood Mini M48 (192) kit from Qiagen from the three cases and an unaffected man. Two of the women presented MD with bilateral SNHL (I.6 and III.4) and the third one had MD in the left ear (II.18). The ages of onset were 33, 33 and 22, suggesting anticipation. None of the patients with MD had migraine, but another woman in the second generation (II.17) had episodic dizziness associated with migraine fulfilling the criteria for probable vestibular migraine (36). The unaffected man (II.19) was a sibling of two of the women in the second generation.

This study was approved by the Ethical Review Board for Clinical Research, and an informed consent was obtained from all subjects.

**Whole-exome sequencing**

Exons and flanking intron regions were selected and captured by Agilent’s All Exon 50 MB capture kit. The conditions and primer sets are available on commercial website. Library products were sequenced with SOLiD 5500xl platform and the sequences were analyzed with Lifescope(TM) (Applied Biosystems), SAMtools and MAQtools.

**Bioinformatics analysis**

The search for rare variants (MAF < 1%) that were exclusively found in the three affected women was carried out with different open access Webtools. Initially, 24 248 SNVs were found in common among the three cases. Finally, 167 SNVs, described in Supplementary Material, Table S3, remained after filtering by exome data from 2386 controls. Seventy-nine variants were coding and 88 located in non-coding regions (introns, utr-5, near gene-3 and near gene-5). Among the coding variants, 38 were synonymous variants and 41 were non-coding (1 nonsense, 40 missense). Functional annotation software (ANNOVAR) was used to prioritize non-synonymous SNVs according to: (i) the effect in protein structure and phylogenetic conservation by using a seven points scoring system to estimate the pathogenicity risk for each variant (SIFT, Sort Intolerant from Tolerant), PolyPhen2 (Polymorphism Phenotyping v2), Grantham's Matrix, GERP+(Genomic Evolutionary Rate Profiling), Mutation taster, PhastCons and PhyloP); (ii) cross-species phenotype comparison according to the inheritance pattern, and mouse as model organism phenotype by the Exomiser software (37) and (iii) genomic data fusion combining deleteriousness of the variant, haploinsufficiency prediction and similarity of the given gene to known genes associated with the phenotype by the eXtasy suite (38). The prediction and classification of new splice site was tested with three different tools: ASSP, Human Splicing Finder (Version 2.4.1) and Berkeley Drosophila Genome Project (39–41). The URLs for the software used are listed below in Supplementary Material, Table S4.

Small insertions and deletions (indels) shared by all affected cases were searched, but all of them were filtered by our controls. A copy number variation (CNV) analysis was performed to detect and to identify CNVs in WES data of cases segregating
with the familial phenotype by using Conifer software (42). Candidate CNVs were plotted with the Nexus Copy Number™ software, but we did not observe any CNV with significant Z-score in the family associated with the MD phenotype.

Finally, since all affected cases had a maternal inheritance, all SNVs in the mitochondrial DNA were considered and compared with Human Mitochondrial Genome Database (mtDB) and a human mitochondrial genome database (MITOMAP). After filtering by familial controls, no novel mutation was found.

**Accession number**

The clinical variant database accession numbers for the FAM136A and DTNA sequences reported in this paper are SCV000153677 and SCV000153678.

**Isolation and culture of lymphoblastoid cells**

PBMCs were isolated using a Ficoll gradient and incubated with Epstein-Barr virus to generate a lymphoblastoid cell line. PBMCs were seeded in a sterile Falcon® Cell Culture at a density of 1.5–2 × 10⁶ cells/ml in Gibco® RPMI 1640 containing 20% FBS. EBV crude stock at 1 : 1 ratio was added and placed in an incubator maintained at 37°C with 5% CO₂. After 24 h, medium containing viral supernatant was aspirated without disturbing the cells and fresh complete RPMI 1640 was added. After 3–4 weeks of incubation, rosette morphology of cells ascertained the transformed phenotype of PBLs. Lymphoblastoid cell lines were generated from patients and healthy donors. Both cell lines were cultured in Gibco® RPMI 1640 containing 10% FBS. Cells were harvested when confluence was 10⁷ cells/ml.

**Validation by Sanger sequencing and expression analyses**

Both novel variants were validated by Sanger sequencing of DNA samples in a 3130 Genetic Analyzer (Applied Biosystems). RNA was isolated by using the Qiagen kit (Qiagen) and cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen) both following the commercial instructions. GAPDH housekeeping gene was used to determine the quality and quantity of cDNA. RT–PCR was used to confirm which isoform of FAM136A was expressed in lymphoblast cell lines. A nested PCR was used to increase DTNA product and validate the splice-site predicted for DTNA gen in RNA obtained from a mutant DTNA lymphoblast cell line. PCR products were loaded on 4% agarose gels to identify which isoforms were expressed (Supplementary Material, Fig. S3). Bands were extracted and purified with the QIAquick Gel Extraction Kit (Qiagen) and the purified products were sequenced by the Sanger technique to validate the results.

Quantitative real-time PCR (Q-PCR) with SYBR® Green RT-PCR techniques (Life Technologies) were performed to determine the expression of FAM136A gene. The data were analyzed with ABI RQ Manager Software (Applied Biosystems). Values for each sample were expressed in ΔCt with their standard deviation.

The primers used to validate the variants of both genes and amplify FAM136A and DTNA isoforms as well as PCR conditions are given in Supplementary Material, Tables S5 and S6.
The amount of FAM136A protein in the lymphoblastoid cells was determined using the Bio-Rad Protein Assay and Infinite® PRO NanoQuant. The immunoblots were performed following the manufacturer’s description (Bio-Rad). The volumes loaded were adjusted so that the amount of protein was equal in all samples in a Mini-PROTEAN® TGX™ Precast Gels and subsequently transfer with Trans-Blot® Turbo™ Transfer System (Bio-Rad). The primary antibody (Santa Cruz, Cat. No. SC-246575) was used against an epitope mapping within an internal region of FAM136A homologous to human, rat and mouse FAM136A sequence. The primary antibody (Sigma, Cat. No. A1978) against housekeeping protein anti-β-actin was used to determine the quantity of FAM136A. The chemiluminescence of the blots was recorded in an ImageQuant LAS 4000 and the amount of FAM136 protein was analyzed by ImageJ (43).

**Animals and tissue preparation**

All the animal experiments were carried out in the facilities of Anatomy and Cell Biology of University Illinois Chicago (UIC). Rodents were housed in the Biological Research Laboratory at UIC. Animal experiments were approved by the local ethical review board and conformed to the Guide for the Care and Use of laboratory animals.

Rats (Rattus norvegicus) were sacrificed by an overdose of barbiturate anesthesia and transcardially perfused. The temporal bones containing the inner ear were dissected, fixed and cryosectioned as previously described (33,44).

**Immunofluorescence and antibodies**

For immunohistochemistry, 35 μm sections were stained and imaged as previously described (45). Briefly, sections were thawed and permeabilized with 4% Triton X-100 for 1 h at room temperature, blocked with 4% normal goat serum (NGS) in 1× PBS, and incubated with primary antibody in 1% NGS in 1× PBS overnight at 4°C. Goat polyclonal antibodies against FAM136A (Santa Cruz, Cat. No. SC-246575, 1:600), α-Dystrobrevin (Santa Cruz, Cat. No. SC-13812, 1:400), rabbit polyclonal COX IV (Cell Signaling Technology, Cat. No. #4844, 1:400) and mouse monoclonal antibody Anti-β-Spectrin II (BD Biosciences, Cat. No. 612562, 1:400).

Primary antibodies were visualized with Alexa-488-conjugated donkey anti-goat (Life Technologies, Cat. No A11055, 1:200) Alexa-595-conjugated donkey anti-rabbit (Life Technologies, Cat. No A21207, 1:200) Alexa-350-conjugated donkey anti-mouse (Life Technologies, Cat. No A10035, 1:200). Sections were rinsed and mounted in Mowiol mounting medium. For all immunoreactions, negative controls (only secondary antibody incubation) were also included. A laser scanning confocal microscope (LSM 710, Carl Zeiss, Oberkochen, Germany) was used for image collecting. Final image processing and labeling was done with Adobe Photoshop (San Jose, CA, USA).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.


