Research Paper

Rare coding variants involving *MYO7A* and other genes encoding stereocilia link proteins in familial meniere disease

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A R T I C L E   I N F O

A B S T R A C T

The *MYO7A* gene encodes a motor protein with a key role in the organization of stereocilia in auditory and vestibular hair cells. Rare variants in the *MYO7A* (myosin VIIA) gene may cause autosomal dominant (AD) or autosomal recessive (AR) sensorineural hearing loss (SNHL) accompanied by vestibular dysfunction or retinitis pigmentosa (Usher syndrome type 1B). Familial Meniere's disease (MD) is a rare inner ear syndrome mainly characterized by low-frequency sensorineural hearing loss and episodic vertigo associated with tinnitus. Familial aggregation has been found in 6–8% of sporadic cases, and most of the reported genes were involved in single families. Thus, this study aimed to search for relevant genes not previously linked to familial MD. Through exome sequencing and segregation analysis in 62 MD families, we have found a total of 1 novel and 8 rare heterozygous variants in the *MYO7A* gene in 9 non-related families. Carriers of rare variants in *MYO7A* showed autosomal dominant or autosomal recessive SNHL in familial MD. Additionally, some novel and rare variants in other genes involved in the organization of the stereocilia links such as *CDH23, PCDH15* or *ADGRV1* co-segregated in the same patients. Our findings reveal a co-segregation of rare variants in the *MYO7A* gene and other structural myosin VIIA binding proteins involved in the tip and ankle links of the hair cell stereocilia. We suggest that recessive digenic inheritance involving these genes could affect the ultrastructure of the stereocilia links in familial MD.

1. Introduction

Monogenic sensorineural hearing loss (SNHL) is sometimes associated with vestibular dysfunction, leading to a complex clinical syndrome which may range from episodic vertigo to a progressive vestibular dysfunction (Roman-Naranjo et al., 2017). Mutations in the *MYO7A* gene are involved in several types of SNHL with variable vestibular dysfunction, including autosomal recessive (DFNB2), autosomal dominant (DFNA11) SNHL, and Usher syndrome type 1B (USH1B) (Friedman et al., 2020). DFNB2 is caused by homozygous or compound heterozygous variants in *MYO7A*, and it was firstly described in a large consanguineous family in which 22 individuals presented profound bilateral deafness affecting all frequencies. Additionally, 4 of these individuals presented vestibular symptoms (Weil et al., 1997). Later, this family was revaluated, reporting mild retinal degeneration in 5 patients and an abnormal vestibular function in 7 patients. On the other hand, DFNA11 has been reported in different families with progressive mild-to-profound hearing loss and an age of onset ranging from 1 to 47 years. No evidence of retinal degeneration has been described for DFNA11 (Yamamoto et al., 2020). USH1B disease is an autosomal recessive disorder characterized by profound deafness affecting all frequencies, variable vestibular dysfunction, and early onset retinitis pigmentosa (RP) (Friedman et al., 2020).

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The *MYO7A* gene encodes an unconventional myosin whose expression in the inner ear is restricted to the stereocilia of the hair cells of the vestibular and cochlear organs. The functional effect of rare variants in this gene has been demonstrated in several animal models such as mice or zebrafish. Homozygous mutations in *MYO7A* in mice led to a phenotype known as shaker-1, characterized by deafness and balance impairment (Gibson et al., 1995), showing hair bundles anomalies (Batiste Boëda et al., 2002). Zebrafish carrying mutations in *myo7a*, known as *mariner* mutants, showed a phenotype very similar to shaker-1 mice, resulting in hair bundle disorganization in the inner ear and lateral line, leading to hearing loss and balance dysfunction (S Ernest et al., 2000). To date, a total of 683 rare variants affecting the *MYO7A* gene have been reported in humans and classified as likely pathogenic or pathogenic (http://deafnessvariationdatabase.org/; Release: 4 Jan 2021; Accessed: 24 Jun 2021), causing mostly USH1B.

Meniere disease's (MD [OMIM 156,000]) is a rare inner ear disorder with an estimated prevalence 75/100.000 in Southern European population (Morales Angulo et al., 2003). It is characterized by SNHL affecting initially low and medium frequencies, episodes of vertigo, tinnitus, and aural fullness (Lopez-Escamez et al., 2015).

Familial aggregation has been described in 9% of European descendant patients with MD (Requena et al., 2014). Most families with MD show an autosomal dominant inheritance pattern and rare missense variants in genes such as *DTNA, PRKCB, DPT* or *SEMA3D* have been described in four unrelated MD families (Escalera-Balsera et al., 2020; Gallego-Martinez et al., 2020). However, none of these variants have been reported in other families with MD, supporting genetic heterogeneity in familial MD. More recently, applying multiallelic inheritance models in SNHL genes in 46 unrelated families, we found an enrichment of rare missense variants in the *OTOG* gene in 15 families with MD (Roman-Naranjo et al., 2020). Homozygous and compound heterozygous mutations in *OTOG* cause DFNB18, an autosomal recessive monogenic SNHL with vestibular dysfunction (Schraders et al., 2012).

Here, we have performed exome sequencing in a total of 62 MD families and 9 of them showed rare heterozygous variants in the *MYO7A* gene associated with the MD phenotype.

2. Material and methods

2.1. Diagnosis of patients & clinical evaluation

Patients included in this study were diagnosed according to the diagnostic criteria defined by the International Classification Committee for Vestibular Disorders of the Barany Society in 2015 (Lopez-Escamez et al., 2015). Vestibular and hearing functions were assessed in all cases, discarding other vestibular diseases that could explain the phenotype. Pure-tone audiograms were retrieved to assess hearing loss since the onset of the disease. A total of 94 Spanish and Swiss patients with familial MD over 18 years old from 62 different families were selected for exome sequencing.

2.2. Whole exome sequencing

Saliva or blood samples were collected to obtain DNA as previously described (Szczepek et al., 2019). DNA samples were extracted with prepIT-L2P (DNA Genotek, Ottawa, Canada) and QI-Aamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) following manufacturer's protocols, according to the quality controls required for exome sequencing. Coding regions were selected using SureSelect Human All Exon V6 capture kit (Agilent Technologies, Santa Clara, CA, USA) and paired-end sequenced on the Illumina HiSeq 4000 platform with a mean coverage of 100X.

2.3. Bioinformatics

2.3.1. Processing and dataset generation

The raw unmapped paired-end reads of each sample, contained in FASTQ files, were aligned to the GRCh37/hg19 reference genome using the Burrows-Wheeler Aligner with maximal exact matches algorithm. After a post-alignment processing, where duplicated reads were located and removed and the quality of the alignment itself was assessed (McKenna et al., 2010), genetic variants were called using the Haplotypecaller function from GATK. Thus, a Variant Call Format (VCF) file for each individual was generated, retrieving single nucleotide variants (SNVs), insertion and deletions (indels) from the sequenced exomes. Annovar was used for prediction and annotation purposes (Wang et al., 2010).

2.3.2. Data analysis and prioritization strategy

Based on the data from a multi-ethnic study that assessed the pathogenicity of reported non-syndromic hearing loss variants (Shearer et al., 2014), we selected MAF thresholds of 0.005 and 0.0005 to identify, autosomal-recessive and autosomal-dominant rare variants in the familial MD cohort, respectively. Allelic frequencies were retrieved for non-Finish European (NFE) population from the gnomAD ($N = 33,365$) and ExAC (32,299) databases, and for the Spanish population ($N = 1942$) from the Collaborative Spanish Variant Server (CSVS) database (Peña-Chilet et al., 2020).

Candidate variants were classified according to the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (Richards et al., 2015), considering the specific guidelines for variant interpretation in genetic hearing loss (Oza et al., 2018). Furthermore, *in silico* tools, such as SIFT or CADD were used to prioritize and classify each variant according to its predicted pathogenicity.

As a method of variant prioritization, candidate genes harbouring rare variants were associated to mammalian phenotypes using the Mouse Genome Database (MGD; http://www.informatics.jax.org). Likewise, the Human Phenotype Ontology Project (HPO; https://hpo.jax.org/app/) and the Online Mendelian Inheritance in Man (OMIM; https://omim.org/) databases were used to determine associations between candidate genes and phenotypes in humans.

2.3.3. Variant validation and representation

Regions with candidate rare variants were visually inspected using the Integrative Genome Viewer software. Novel variants were validated using Sanger sequencing. Primers for PCR were designed in the regions flanking the variants using Primer v4.1 (http://bioinfo.ut.ee/primer3/), Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and Oligoanalyzer (https://eu.idtdna.com/calc/analyzer/). The primers used to validate candidate variants are showed in Supplemental Table 1. Candidate genes and variants were represented using Illustrator for Biological Sequences Version 1.0 (Wenzhong et al., 2015).

2.3.4. Statistical analysis

To assess the statistical significance of the candidate variants, European and Spanish databases (i.e., ExAC, gnomAD and CSVS) were used as reference to compare the observed MAF in FMD. Thus, odds ratios (OR) with 95% confidence interval (CI) were calculated for each single or set of variants. One-sided p-values were corrected for multiple testing following the Bonferroni approach. The same approach was followed to assess whether a single variant or a combination of variants drive the association.

2.3.5. Protein modeling

The effect of candidate variants on the myosin VIIA protein structure was evaluated by protein modeling. When it was possible, wild-type and mutated functional domains were modelled
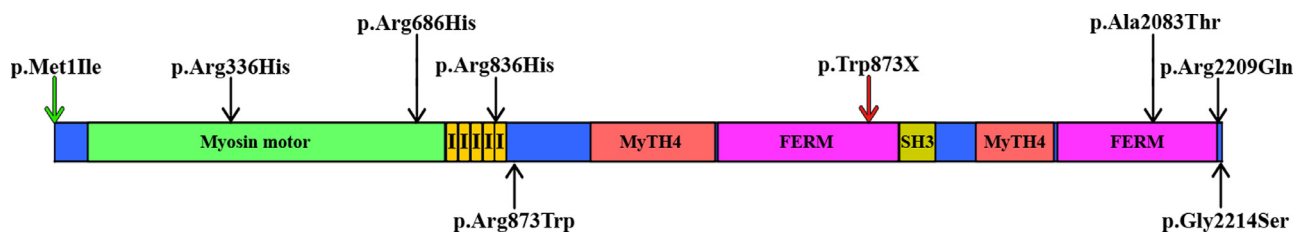
Fig. 1. Variants distribution across the MYO7A gene sequence in the studied FMD cohort. Nine rare variants were identified in the *MYO7A* gene, 6 of them with a frequency lower than 0.0005 in at least a reference population. Arrows indicate the position of a given variant within the gene. The green arrow indicates a start loss variant. The red arrow indicates a stop gain variant. Functional domains are indicated by colored boxes. I: IQ motif; MyTH4: Myosin Tail Homology 4 domain; SH3: SRC homology domain 3.

using SWISS-MODEL, using as templates crystal structures already published. Thus, crystal structures of the human myosin VIIA MyTH4-FERM domains in complex with harmonin-a (PDB ID: 5MV9) and the mouse myosin VIIA IQ5 domain in complex with apocalmodulin (apo-CaM, PDB ID: 5WSU) were used to assess the effects of R836H and A2083T variants.

2.4. Hearing assessment and analysis

Standard audiometric evaluations for air and bone conduction elicited by pure tones from 125 to 8000 Hz were retrieved from the clinical records to analyze the time course of the hearing profile in FMD cases with candidate variants. Regression analysis was performed to estimate the outcome of hearing loss for each frequency.

2.5. Variant data submission

All candidate variants in the *MYO7A* gene have been submitted to Clinvar database (http://www.ncbi.nlm.nih.gov/clinvar/). The accession numbers for these variants are SCV001451438, SCV001451439, SCV001451440, SCV001451441, SCV001451442, SCV001451443, SCV001451444, SCV001451445 and SCV001451446.

3. Results

3.1. Rare missense variants in MYO7A and other genes in the stereocilia links suggest digenic inheritance in familial MD

A total of 18,998 and 12,164 nonsynonymous or splice site variants were considered for FMD after applying quality controls for recessive and dominant inheritance models, by using MAF thresholds of 0.005 and 0.0005, respectively. Using the most restrictive MAF threshold, we found variants in a total of 7389 genes, whereas variants in 9612 genes were retained for MAF threshold $\leq$ 0.005. From them, 355 genes (393 for MAF threshold 0.005) were already related to hearing/vestibular/ear phenotype (MP:0,005,377) in different mouse models (data not shown).

The gene with the majority of rare variants in our dataset for both MAF thresholds was the *MYO7A* gene. Nine variants in 9 individuals were identified fulfilling frequencies criteria in at least one of the reference populations used in this study (Table 1).

The 9 variants found were scattered across the gene sequence, affecting different functional domains in Myosin VIIA protein (Fig. 1). Variants were found in heterozygous state in 9 over 62 families, accounting for the 14.5% of the studied families. Of note, we found two Loss of Function (LoF) variants: a start loss variant p.Met1Ile found in F1 segregating the MD in the three affected individuals of this family (Supplemental Table 2), and a novel stop gain variant p.Trp1545Ter found in F6. There were no mutations in the 5′ untranslated region (UTR) that generated a novel transcription start site to compensate the start loss variant identified in F1. The predicted pathogenicity of these two LoF variants

was high, being classified as likely pathogenic variants (Supplemental Table 3). Frameshift indels in the *MYO7A* gene were analyzed to search for additional LoF variants, however only a common deletion (rs111033223) found in 11,084/21,494 Non-Finish European from gnomAD and classified as benign was identified in F1, F2, F3, F7 and F9.

Our segregation analyses in familial MD revealed several rare variants in genes encoding proteins involved in the formation of the mechanotransduction complex in the stereocilia of hair cells. We assessed digenic inheritance in families carrying rare variants in the *MYO7A* gene and genes codifying for proteins involved in the stereocilia links. Interestingly, two families (F4 and F5) carried rare missense variants in *MYO7A* and *ADGRV1* genes, other two families (F2 and F6) carried rare variants in *MYO7A* and *CDH23* genes, and three families carried variants in *SHROOM2* (F1), *USH1C* (F7) and *PCDH15* (F8) genes (Supplemental Table 4). Thus, 7 of the 9 families carrying rare variants in the *MYO7A* gene also carried rare variants in genes with a structural function in the hair cell stereocilia.

Statistically, some of these variants in the *MYO7A* gene could explain the phenotype of certain families by themselves (Supplemental Table 5), such as M1I (F1), R836 (F4), W1545X (F6), A2083T (F7) and R2209Q (F8). On the other hand, a second variant in a gene involved in the stereocilia of hair cells is needed in some families to show statistical significance (Supplemental Table 6). In families such as F2 and F5 the phenotype could be explained by the combination of rare variants in *MYO7A* and *CDH23* genes, and *MYO7A* and *ADGRV1* genes, respectively. The co-segregation of these variants was confirmed by Sanger sequencing in F2 (II-1 and II-6) and F5 (I-1 and II-1).

3.2. Carriers of rare variants in MYO7A show autosomal dominant or autosomal recessive inheritance in familial MD

The segregation of rare variants in the *MYO7A* gene was assessed in each family. Among the 9 families, 3 followed an autosomal dominant inheritance pattern (F1, F5 and F7), whereas in the other 6 families was found an autosomal recessive inheritance pattern (Fig. 2). Digenic or compound heterozygous inheritance patterns could likewise explain these latter families. In total, 29 individuals with MD were diagnosed in these 9 families, and in 3 families (F1, F7 and F8) were observed partial syndromes in 3 individuals (i.e., only hearing loss or vertigo), suggesting a variable expressivity. The LoF variant p.Met1? found in F1 causes a start loss in the *MYO7A* gene, codon 12 being the next in-frame start codon. Although this variant could explain the MD phenotype in F1 by itself, it was found in conjunction with a variant in the *SHROOM2* gene (p.Gly211Ser). Two variants in genes involved in the architecture of the mechanotransduction complex in the hair cell stereocilia (*MYO7A* and *CDH23*) were found in F2, which seems to follow a recessive digenic inheritance pattern. Despite its frequency (0.22% of NFE population), the variant p.Arg336His in the *MYO7A* gene was classified as variant of unknown significance (VUS), since
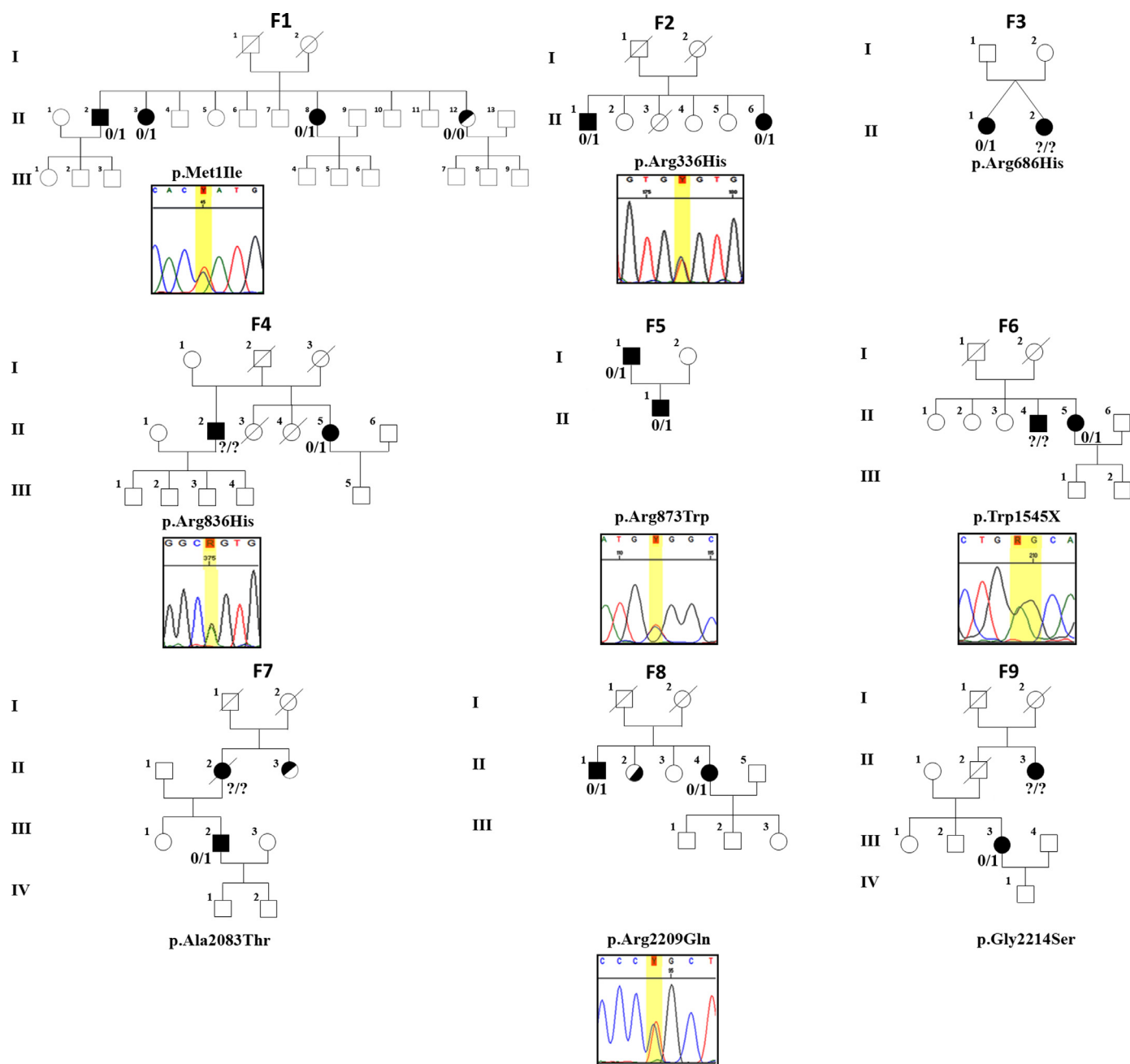
Fig. 2. Nine MD families carrying rare variants in the *MYO7A* gene. Variant segregation was assessed when at least two samples of the same family were available. Variants in *MYO7A* found in this study are indicated under each family by protein nomenclature. Similarly, Sanger sequencing chromatograms are displayed for those variants with the lowest minor allele frequency in the Spanish population in our cohort. Solid squares (male) and circles (female) indicate patients with definite MD. Those patients with only vertigo or hearing loss are indicated, respectively, with the upper or the lower half of their symbols filled. "0/1″: heterozygous variant. "0/0″: homozygous for the reference allele. "?/?": Sample not available.

it is predicted to impact the protein (CADD=24.1; M-CAP=0.139). The third family (F3) appears to be following a recessive inheritance pattern, likely caused by digenic inheritance. Only a heterozygous variant in the *MYO7A* gene, affecting a highly conserved arginine (p.Arg686His), classified as VUS was found in this family. *MYO7A* rare variant in the functional domain IQ together with a rare variant in the *ADGRV1* gene were found in F4 (p.Arg836His & p.Pro2528Ser) and F5 (p.Arg873Trp & p.Arg5547His). The pedigree of F4 suggests a recessive/digenic inheritance pattern, whereas F5 is suggestive of a dominant/digenic inheritance pattern. In the last four families (F6; F7; F8 and F9) we found variants affecting

the FERM functional domain of the *MYO7A* gene following a recessive/digenic inheritance pattern. The variant found in F6 cause a novel stop codon (p.Trp1545Ter) in exon 35 of the canonical and longest isoform of the *MYO7A* gene (NM_000260.4). In addition, a rare variant classified as a VUS in the *CDH23* gene (p.Arg2171His), which is part of the tip link complexes, was found in this family. Variants in genes of these complexes were also found in F7 and F8, who presented variants in the *USH1C* gene (p.Pro608Arg) and the *PCDH15* gene (p.Pro1748Thr), respectively. Supplemental Table 4 shows the most relevant associations in these families.

Table 1
Rare variants in the *MYO7A* gene (N = 9) found in the studied familial MD cohort (N = 62).

Genomic location	Nucleotide change	Protein change	Exon	Info	rs	MD family	MAF NFE	MAF CSVS	CADD	Domain	ACMG
11:76841683G>A	c.3G>A	p.Met1?	2	StartLoss	rs782787126	F1	8.9×10^{-5}	-	24.0	-	LP
11:76870496G>A	c.1007G>A	p.Arg336His	10	Nonsyn	rs45629132	F2	2.2×10^{-3}	4.9×10^{-4}	24.1	Myosin Motor	VUS
11:76885923G>A	c.2057G>A	p.Arg686His	17	Nonsyn	rs781991817	F3	7.8×10^{-5}	4×10^{-3}	29.8	Myosin Motor	VUS
11:76890920G>A	c.2507G>A	p.Arg836His	21	Nonsyn	rs782179888	F4	1.8×10^{-5}	-	24.5	IQ	VUS
11:76891450C>T	c.2617C>T	p.Arg873Trp	22	Nonsyn	rs200454015	F5	1.6×10^{-3}	2.5×10^{-4}	24.8	IQ	VUS
11:76910646G>A	c.4635G>A	p.Trp1545Ter	35	StopGain	-	F6	-	-	43.0	FERM	LP
11:76922875G>A	c.6247G>A	p.Ala2083Thr	46	Nonsyn	rs41298759	F7	3.1×10^{-4}	2.5×10^{-4}	21.8	FERM	VUS
11:76925719G>A	c.6626G>A	p.Arg2209Gln	49	Nonsyn	rs776881443	F8	1.1×10^{-5}	-	22.8	FERM	VUS
11:76925733G>A	c.6640G>A	p.Gly2214Ser	49	Nonsyn	rs111033231	F9	3.6×10^{-4}	1×10^{-3}	10.4	FERM	LB

Reference sequence: NM_000260.4 (*MYO7A*); MAF: Minor allele frequency; NFE: Non-Finish European; CADD: Combined Annotation Dependent Depletion; ACMG: American College of Medical Genetics and Genomics; LP: Likely pathogenic; VUS: Variant of unknown significance; LB: Likely benign.

3.3. Hearing assessment in MYO7A patients

Standard audiometric evaluations were obtained and hearing profiles for the patients (2 males, 7 females) with rare variants in the *MYO7A* gene were studied. Six patients showed bilateral hearing loss, whereas the other three individuals presented unilateral hearing loss (2 left-sided and 1 right-sided SNHL). So, 3 ears had a normal pure-tone audiogram (16.6%), 6 ears from 5 patients showed a flat shaped audiogram (33%), 4 ears from 3 patients showed a ski-slope shaped audiogram (22.2%), 3 ears from 2 patients showed a reverse ski-sloped audiogram (16.6%) and only an ear from a patient showed a tent shaped audiogram (5.5%). The age of onset of the first symptom considered to be caused by MD was 38.3 ± 11.5 (Supplemental Table 7). The estimated hearing threshold at onset was 72.1 ± 1.2 dB for low frequencies (125–250–500 Hz) and 58.9 ± 2.1 dB for high frequencies (1000–2000–4000 Hz) (Supplemental Figure 1).

3.4. Protein modeling

The identified p.Arg836His and p.Arg873Trp variants were in (or near of) IQ5 domain of myosin VIIA, and the p.Arg2083Thr was in the FERM domain (Fig. 3). Complexes of apo-CaM with p.Arg836His mutant IQ5 domains were modelled (Fig. 3A). In the IQ5 wild-type (IQ5-WT) model, no bonds were observed between the highly conserved arginine 836 (R836) of MYO7A and the phenylalanine 92 (F92) of apo-CaM. On the other hand, the IQ5 histidine 836 mutant (H836-Mutant) model shows the formation of a new NH/π interaction between this histidine and the F92 of apo-CaM. The variant affecting arginine 873 (R873) was also assessed with the IQ5-WT model (Fig. 3B), observing three hydrogen bonds between R873 and the glutamic acid 877 (E877) of MYO7A. In the tryptophan 873 mutant (W873-mutant) model, these three hydrogen bonds were lost. This loss is predicted to affect the stability/folding of the protein. The opposite occurs with the variant involving the alanine 2083 (A2083) in the FERM domain (FERM-WT) model of MYO7A (Fig. 3C). In the threonine 2083 mutant (T2083-mutant) model, a hydrogen bond not showed between T2083 and serine 2080 (R2079) in FERM-WT appears between T2083 and R2079.

4. Discussion

Rare coding variants in the *MYO7A* gene are known to underlie different cochleovestibular syndromes, such as DFNB2, DFNA11 and USH1B (Friedman et al., 2020). Hearing loss is a common trait for these three syndromes, however the onset and affected thresholds vary widely between them. Vestibular dysfunction and RP are other manifestations that can appear in the carriers of rare variants in *MYO7A*. In this study, 9 rare variants in the *MYO7A* gene were identified in 9 unrelated families with MD by exome sequencing. Interestingly, three of these variants have been reported before in Usher syndrome patients. The start loss variant (MYO7A:c.3G>A;p.Met1?) that segregates the MD phenotype in F1 has been previously found along with the p.Leu1839Pro *MYO7A* variant in a patient causing Usher Syndrome type 2 (Fuster-García et al., 2018). Moreover, the p.Arg873Trp variant in the *MYO7A* gene found in F5 has been previously observed in two patients with Usher Syndrome type 2 (Västinsalo et al., 2013); and the *MYO7A* variant found in F2 (p.Arg336His) has been already observed in patients with Usher Syndrome type 1, being classified as a disease causing variant (Jaijo et al., 2006). We suggest that rare missense variations in this gene could also affect the stability of myosin VIIA in the hair cell stereocilia in familial MD.

Familial MD typically shows an autosomal dominant inheritance pattern with incomplete penetrance (Escalera-Balsera et al., 2020; Requena et al., 2014), however compound recessive inheritance has
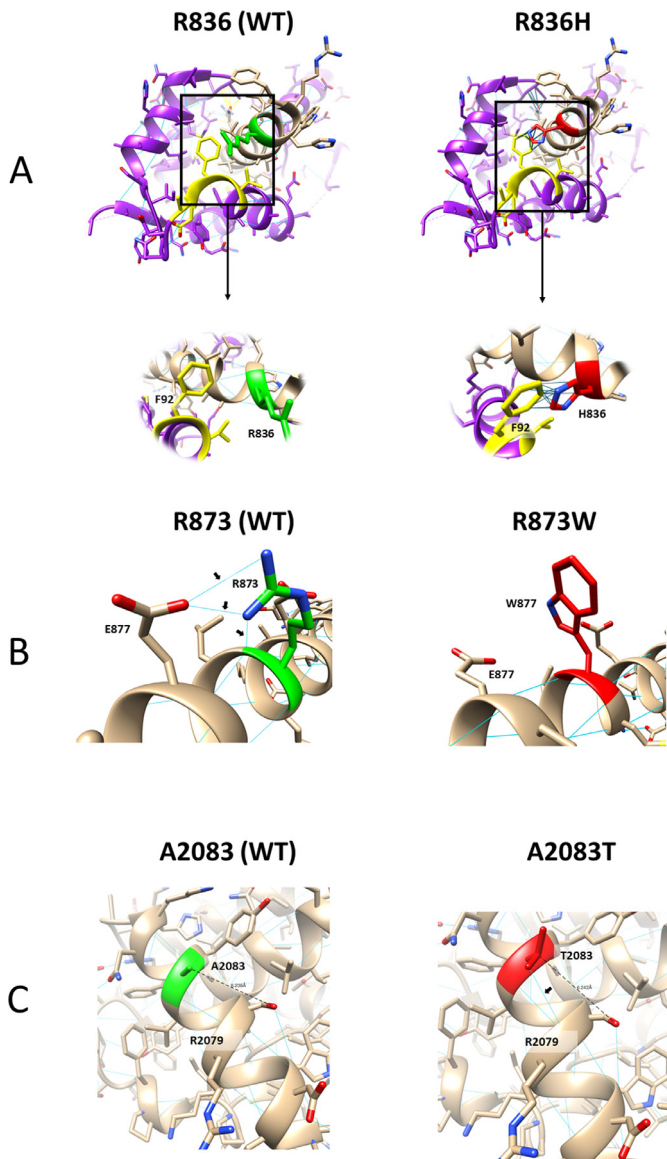
Fig. 3. Predicted effect of variants in MYO7A structure. **A**, wild-type (WT) and R836H mutant MYO7A IQ5 domain (brown) interacting with Apo-CaM (purple). Below each model, a close-up of the region of interest. **B**, WT and R873W mutant MYO7A IQ5 domains. **C**, WT and A2083T mutant MYO7A FERM domains. In green, the WT amino acid. In red, the mutant amino acid. In yellow, the surrounding amino acids of Apo-CaM. Arrows show new or lost hydrogen bonds. Apo-CaM: Apocalmodulin.

Fig. 4. Inner ear hair cell and the tip link (TL) and ankle link (AL) complexes. This diagram represents the proteins thought to participate between the stereocilia. Myosin VIIa, and several proteins, are involved in these structures. MET: Mechanotransduction; LTDL: Lower tip link density; UTLD: Upper Tip link density.

been recently reported in several families with MD, revealing *OTOG* as a key gene in familial MD (Roman-Naranjo et al., 2020). The *OTOG* gene encodes otogelin, an extracellular protein that participates in, among others, the otolith tethering in the otolithic membrane, the tectorial membrane attachment crowns, and the horizontal top connectors between stereocilia (Avan et al., 2019). Stereocilia are connected to each other through different links involving a large number of proteins, one of them being myosin VIIA (Goodyear et al., 2005).

Myosin VIIA, encoded by the *MYO7A* gene, is a motor protein highly expressed in the cochlea involved in the formation of tip links and ankle links in the stereocilia of hair cells (Morgan et al., 2016). Its canonical isoform encodes for a 2215 amino acid large protein (ENST00000409709) containing, from N-terminal to C-terminal, several functional domains: a motor domain, five IQ motifs and two repeated regions consisting of a MyTH4 and a
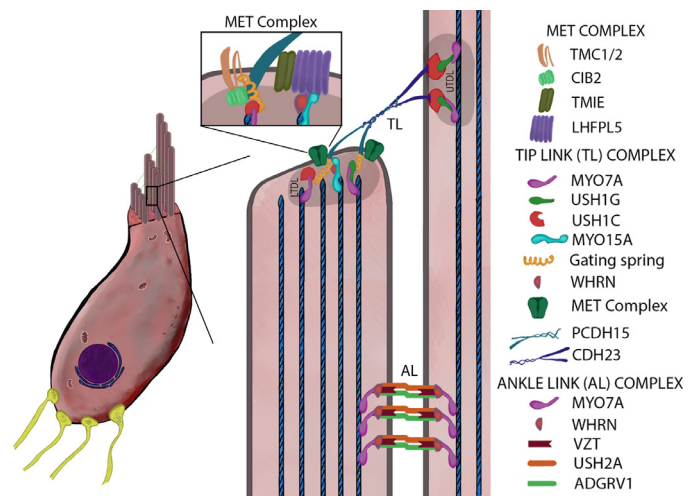
FERM domain separated by a SH3 domain. Through these domains, myosin VIIA interacts with other proteins involved in the tip and ankle links of stereocilia, encoded by genes such as *USH1C, CDH23* (Batiste Boëda et al., 2002), *ADGRV1* (Michalski et al., 2007) or *USH1G* (Weil et al., 2003) (Fig. 4).

Because of its location and function in the transduction complex of hair cells, Myosin VIIA has been proposed to function as a motor that tensions the hair cell mechanotransduction (MET) complex in the tip links (Li et al., 2020). We suggest that rare variations in the *MYO7A* gene, alone or combined with rare variants in genes encoding myosin VIIA binding proteins, will lead to fragile tip and ankle links, loss of cohesiveness among stereocilia, abnormal opening of the MET complex with sustained depolarization of hair cells, and ultimately hearing loss and/or vestibular dysfunction. Thus, we found rare variants in the *MYO7A* gene in 9 MD families, associated with missense variants in genes encoding myosin VIIA binding proteins, including *ADGRV1, CDH23, PCDH15, USH1C* and *SHROOM2* genes. Seven of these 9 families showed, at least, a rare variant in *MYO7A* and a variant in one of the genes mentioned above, suggesting a potential digenic inheritance in MD.

The contribution of the *MYO7A* gene to digenic inheritance in hearing loss has been studied in the *Myo7a^{sh1−8J}* mouse model, characterized by carrying a complex deletion affecting exons 38–40 and 42–46 (Zheng et al., 2012). The combination of recessive mutations was assessed making use of double heterozygotes of *Myo7a* and other Usher genes involved in the organization of the hair cell stereocilia. Thus, hearing loss was assessed in double heterozygotes of *Myo7a* and *Ush1g, Cdh23* or *Pcdh15* genes. *Myo7a, Ush1g, Cdh23* and *Pcdh15* single heterozygotes mice showed normal phenotype, whereas double heterozygotes had progressive hearing loss. This study reinforces the key role of the *MYO7A* gene in the architecture of hair bundles and raises the hypothesis of recessive digenic inheritance in familial MD by the combination of rare variants in *MYO7A* with variants in genes involved in the cohesiveness of the stereocilia.

In this study, two rare missense heterozygous variants were found in the *ADGRV1* gene in two families (F4 & F5) carrying *MYO7A* gene variants. The *ADGRV1* gene, an ankle-link component which encodes for the adhesion G-coupled receptor V1, has been associated in different studies with Usher syndrome type 2C (USH2C) (Weston et al., 2004). USH2C is an autosomal recessive disorder mainly characterized by prelingual sensorineural hear-

ing loss and progressive RP. To date, two different mouse models have been generated, revealing defects in hair bundle formation at P2, progressively decreasing their cohesion and becoming disorganized. This is likely caused because of the role of the Adhesion G-protein coupled receptor V1 protein in the ankle link complex (Michalski et al., 2007). Although it has not been replicated, digenic inheritance was reported in USH2C patients with variations in *ADGRV1* and *PDZD7* genes (Bonnet et al., 2016; Ebermann et al., 2010), however there are no reports of any phenotype caused by the combination of variants in *MYO7A* and *ADGRV1* genes in humans. Nevertheless, some studies suggest a direct MYO7A-ADGRV1 interaction and a key role of functional *MYO7A* in the localization of *ADGRV1* in the ankle link region (Michalski et al., 2007; Morgan et al., 2016). The additive effect of rare missense variants in *MYO7A* and *ADGRV1* could affect the stability of ankle link proteins leading to loss of cohesiveness among stereocilia.

Similarly, two rare variants were found in the *CDH23* gene in two unrelated MD families (F2 & F6) with rare variants in the *MYO7A* gene. The *CDH23* gene encodes cadherin-related 23, a structural protein of the tip links associated to autosomal recessive deafness 12 (DFNB12) and Usher syndrome type 1D (USH1D) (Bork et al., 2001). DFNB12, mainly diagnosed in patients carrying homozygous or compound heterozygous variants in *CDH23* gene, is characterized by severe to profound SNHL affecting high or all frequencies and normal vestibular function. On the other hand, USH1D, characterized by SNHL, RP and abnormal vestibular function, is assumed to be caused by variants disrupting more severely the function of the cadherin-related 23 protein. At least four mouse models have been generated to study the function of *Cdh23* gene (i.e., *waltzer*[v2J], *salsa, Cdh23*[12J], *Cdh23*[Ahl] and *Cdh23*[CBA/CaJ]). In short, these mice were characterized by flat progressive hearing loss, mainly caused by a reduction of outer hair cells and progressive loss of tip links (Bolz et al., 2001; Schwander et al., 2009). The variant found in II-1 and II-6 of F2 in the *CDH23* gene, which is chr10:73491873A>*G* (NM_022124.6:c.3845A>*G*; p.Asn1282Ser), was considered likely benign since it is a neutral amino acid change. However, the variant chr10:73553197G>*A* (NM_022124.6:c.6512G>*C*; p.Arg2171His), found in F6 in the same gene, is affecting a highly conserved calcium-binding element (DXNDNR). These motifs are thought to be essential in the function of cadherins (Nagar et al., 1996), and variants affecting them have been described causing hearing loss (De Brouwer et al., 2003). Digenic inheritance has not been postulated in humans for *MYO7A* and *CDH23* genes. However, although myosin VIIA does not bind to cadherin-related 23, they participate and cooperate together with the adaptor protein harmonin (encoded by the *USH1C* gene), providing the scaffold by which the F-actin-bound motor connects to the upper end of the tip link in the sensory hair cell bundles (Batiste Boëda et al., 2002).

A variant in *USH1C* gene, that encodes harmonin, was found in our familial MD cohort. Variants in this gene are phenotypically related to Usher syndrome type 1C and autosomal recessive deafness 18A (DFNB18A) (Ahmed et al., 2002). We found a variant in F7, chr11:17531093G>*C* (NM_153,676.4:c.1823C>*G*; p.Pro608Arg), previously described in a Chinese family causing Usher syndrome 1C, when present in homozygous state (Ouyang et al., 2002). Although it was found as a heterozygous variant in our cohort, the presence of a variant in the *MYO7A* gene in F7 raises the possibility of digenic inheritance.

In addition, variants in *PCDH15* and *SHROOM2* genes were found in F8 and F1, respectively. The *PCDH15* gene encodes protocadherin related 15, a component of the tip links, and it is associated to autosomal recessive deafness 23 (DFNB23) and Usher syndrome 1F (Ahmed et al., 2003). Notably, Yoshimura et al. (2014) reported the first case of *MYO7A/PCDH15* digenic inheritance (Yoshimura et al., 2014), two genes that encode proteins that can directly inter-

act with each other in the tip links in hair cell stereocilia (Senften et al., 2006). The co-segregation of *MYO7A*:p.Arg2209Gln and *PCDH15*:p.Pro1706Thr variants was confirmed in both relatives from F8 (II-1 and II-IV). On the other hand, although the *SHROOM2* gene is expressed in the hair cells, and an interaction with the tail of myosin VIIA has been suggested, its function is not well-known (Etournay et al., 2007; Hickox et al., 2017).

The audiograms of FMD patients carrying rare variants in the *MYO7A* gene showed moderate-to-severe hearing loss (73.2 ± 1.9 dB) at low frequencies and moderate hearing loss (58.8 ± 1.9 dB) at high frequencies since the first years of the disease.

The phenotypes and traits associated with the variants in *MYO7A* and related genes showed in this study are expected to be milder compared to phenotypes showed in the different knockout mouse models mentioned before. We can speculate that the variants in myosin VIIA, or in other proteins of its network, may lead to hearing loss because of a disorganization in the stereocilia, suggesting a key role in MD of the proteins involved in the tip links and ankle links that connect the stereocilia among them.

The results of this study suggest that some of these families may follow a composite digenism inheritance pattern, where a variant in a main gene (the *MYO7A* gene) is enough to produce the phenotype, but an additional variant in a second candidate gene (e.g., *CDH23, PCDH15, ADRV1…*) can modify the phenotype (e.g., modifying the age of onset). Composite digenism could explain the variable expressivity typically found in MD. Examples of this type of inheritance could explain the phenotype in F1 (*MYO7A/SHROOM2*), F4 (*MYO7A/ADGRV1*), F6 (*MYO7A/CDH23*) or F8 (*MYO7A/PCDH15*). However, we cannot discard other inheritance patterns involving *MYO7A* variants in MD, such as true digenism (two variants needed to show the phenotype) or dominant inheritance.

5. Conclusions

We have found co-segregation in several novel and rare variants in the *MYO7A* gene with other genes such as *CDH23, PCDH15* or *ADGRV1* involved in the mechanotransduction complex and the intercilliary links of the hair cells in families with MD. These findings suggest that digenic inheritance could be a novel mechanism in familial MD.

Declarations of Competing Interest

None.

Data availability

The datasets during and/or analyses during the current study are available from the corresponding author upon reasonable request.

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Declaration of Interest and Source of Funding

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Author contributions

Conceptualization: J.A.L.E.; Data curation: P.R-N., J.A.L.E.; Formal analysis: P.R-N., A.E.B.; Funding acquisition: J.A.L.E., A.H.E.; Methodology: P.R-N.; Resources: I.A., M.D.C.M., A.S.V., D.B., M.G.F., A.H.E., J.A.L.E.; Supervision: J.A.L.E.; Validation: P.R-N., Writing – original draft: P.R-N., J.A.L.E.; Writing – review & editing: P.R-N., J.A.L.E.

Ethics declaration

This study protocol was approved by the local ethics committees (MS/2014/02, Institutional Review Board for Clinical Research, Universidad de Granada, Spain; KEK-ZH-Nr. 2019-01006, Kantonale Ethikkommission, Zurich, Switzerland) and a written informed consent to donate biological samples was obtained from all subjects.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.heares.2021.108329.

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