Excess of rare missense variants in hearing loss genes in sporadic Meniere disease

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Abstract

Meniere's disease (MD) is a clinical spectrum of rare disorders with an estimated prevalence of 75/ 100.000 individuals, characterized by vertigo attacks, associated with sensorineural hearing loss (SNHL) and tinnitus involving low to medium frequencies. Although it shows familial aggregation with variable expressivity with incomplete phenotypic forms,, most cases are considered sporadic. The aim of this study was to investigate the burden for rare variation in SNHL genes in patients with sporadic MD.

We conducted a targeted-sequencing study by a custom gene panel including SNHL and familial MD genes in 890 sporadic MD patients. For this case-control study, we compared the frequency of rare variants in SNHL-related genes in sporadic MD cases, using three independent public datasets as controls.

Patients with sporadic MD showed a significant enrichment of missense variants in SNHL genes that were not found in the reference population. The list of genes includes *GJB2, USH1G, SLC26A4, ESRRB* and *CLDN14*. A rare synonymous variant with unknown significance was found in the *MARVELD2* gene in several unrelated patients with MD.

There is a burden of rare variation in certain SNHL genes in sporadic MD. Examination of the role of specific gene variants can assist to decipher the complex genetic architecture of MD and it will contribute to design a gene panel for the genetic diagnosis of MD.

Author summary

Meniere disease is a syndrome defined by sensorineural hearing loss, tinnitus and episodes of vertigo. The condition shows familial aggregation with autosomal dominant inheritance, but most of cases are considered sporadic. . We have chosen a set of genes to design an exome-sequencing panel to search for rare variants in more than 800 sporadic cases of Meniere disease diagnosed in Spain. The large number of variants found in certain genes provides a better understanding of the rare variants contribution in Meniere disease. Our results show an excess of missense variants in hearing loss genes such as GJB2, USH1G, SLC26A4, ESRRB and CLDN14 and they will facilitate the design of a gene panel for the diagnosis of Meniere disease.

Introduction

Meniere's disease (MD, MIM 156000) is a chronic disorder of the inner ear characterized by episodes of vertigo, associated with low to middle frequency sensorineural hearing loss (SNHL), tinnitus and aural fullness [1]. The disorder produces an accumulation of endolymph in the membranous labyrinth, and it may affect both ears in 25% - 40% of patients (termed bilateral MD) and most cases are considered sporadic [2]. However, heterogeneity in the phenotype is observed and some patients have co-morbid conditions such as migraine or systemic autoimmune disorders [3,4]. This phenotypic spectrum can make the clinical diagnosis challenging considering that some of the clinical symptoms overlap with other vestibular disorders such vestibular migraine (VM) or autoimmune inner ear disease (AIED) [5–8].

Epidemiological evidence showing a genetic contribution in MD is based on familial aggregation studies with a high siblings recurrence risk ratio (λ_s= 16-48) [9] and the description of multiple familial cases in European and Asian descendant populations [10,11]. Exome sequencing has identified private variants in the *FAM136A, DTNA, PRKCB, SEMA3D* and *DPT* genes in 4 families with autosomal dominant MD, showing incomplete penetrance and variable expressivity [12–14]. Moreover, some relatives in familial MD show partial syndromes, either with SNHL or episodic vertigo, increasing the granularity in the phenotype in a given family. However, the genetic contribution of familial genes in sporadic cases has not been investigated and the occurrence of recessive and de novo variation (DNV) is not known. More than 110 genes and 6000 variants have been related to hereditary non-syndromic hearing loss, making gene sequencing panels important for genetic diagnosis of hearing loss [15,16]. Those genes include, 25 associated to autosomal dominant SNHL while 70 genes were related to recessive hearing loss [17].

Targeted gene sequencing panels have been demonstrated to be an excellent tool for molecular diagnosis of rare variants in known genes with allelic heterogeneity [18,19], as well as in sporadic cases of hearing impairment in specific populations [20,21]. So, we selected SNHL genes and designed a custom gene panel to search for rare and DNV in sporadic MD.

In the present study, we describe the genetic variation found in a custom exon-sequencing panel of 69 SNHL genes in a large cohort of MD sporadic cases. We report that certain genes such as *GJB2*, *USH1G*, and *SLC26A4* seems to show an excess of missense variants in sporadic MD cases when compared to controls in the Iberian population, suggesting that several rare variants in these genes may contribute to the SNHL phenotype in sporadic MD.

Results

Single rare variant analysis

A total of 2770 SNV were selected from the raw merge data (18961 SNVs) after filtering by quality controls. For rare variants analysis, SNV that only appeared in more than one pool were selected, remaining 1239 variants. After that, we filtered by variants present in the control pools, leaving only 392 exonic SNV in case pools, of which 278 were missense, 111 were synonymous, 2 stopgain variants and 1 stoploss variant.

A final set of 162 SNV with MAF <0.001 were retrieved (143 missense, 18 synonymous, 1 stoploss, 1 stopgain). All the exonic variants were annotated and scored using different priorization tools. Of them, 136 SNVs were not previously described in any population database. We considered all of them as possible de novo variants (DNVs).

After prioritizing exonic variants 31 rare variants remained. Of them, 6 variants were validated by Sanger sequencing in more than 2 individuals in the following genes: *GJB2*,

ESRRB, *USH1G*, *SLC26A4*, which were selected according to the results found in the gene burden analysis (Table S3). The rest of the variants were considered benign or likely benign since they did not reach the pathogenicity threshold. However, a synonymous variant in *MARVELD2* was suspected to form acceptor splice site and was validated consequently.

The minor allelic frequencies in SNV in mitochondrial genes included in the panel were compared with the reference data obtained from MITOmap through its automated mtDNA sequence analysis system Mitomaster[22]. However, we did not found any SNV associated with MD (data not shown).

Gene burden analysis

To analyse the interaction of multiple variants, we considered SNV with a MAF<0.1 threshold for gene burden analysis. A total of 957 exonic variants were retrieved and their frequencies were compared with global and NFE frequencies from ExAC and with Spanish population frequencies from CSVS. ExAC datasets including 60706 individuals (33370 NFE) were used as population controls in order to increase the statistical power of the analysis.

A gene burden analysis using our gene set was performed using these three reference datasets. After Bonferroni correction, some genes showed a significant enrichment of rare variants in the three comparisons, making them candidate genes to be selected for a diagnosis panel for MD (Table 1). The genes showing an excess of variants are highlighted in Table 1. Moreover, 6 genes (*FAM136A, ADD1, SLC12A2, POU4F3, RDX* and *PRKCB*) presented novel variants that were not described in global ExAC or CSVS datasets. Although these variants could not be sequenced in the parents of these patients, we considered them as DNV.

Table 1. **Gene burden analysis 1**. List of 29 genes showing a significant excess of missense exonic variants in patients with sporadic MD, according to MAF observed in global ExAC population, non-Finnish European ExAC population (NFE) and Spanish population from CSVS.

Gene	# variants	Odds ratio ExAC[a]	P value	P corrected[b]	Odds ratio ExAC NFE[a]	P value	P corrected[b]	Odds ratio CSVS[a]	P value	P corrected[b]
MYH14	50	13,54 (5,85-31,37)	1.20E-09	6.01E-08	56,94 (25,13-128,98)	<1.00E-15	<1.00E-15	38,52 (16,95-87,56)	<1.00E-15	<1.00E-15
MYO7A	43	3,11 (1,73-5,57)	1.41E-04	6.08E-03	4,64 (2,65-8,13)	7.72E-08	3.32E-06	3,85 (2,18-6,81)	3.60E-06	1.55E-04
ADD1	29	249,89 (96,17-649,29)	<1.00E-15	<1.00E-15	318,03 (122,45-826)	<1.00E-15	<1.00E-15	747,23 (287,95-1939,08)	<1.00E-15	<1.00E-15
WHRN	28	24,63 (7,96-76,17)	2.68E-08	7.51E-07	39,97 (13,03-122,58)	1.12E-10	3.13E-09	62,91 (20,62-191,98)	3.44E-13	9.64E-12
TPRN	26	36,63 (10,61-126,49)	1.24E-08	3.22E-07	46,93 (13,64-161,49)	1.03E-09	2.68E-08	81,9 (23,93-280,26)	2.24E-12	5.81E-11
USH1G	24	4,04 (1,91-8,51)	2.46E-04	5.90E-03	18,38 (9,26-36,48)	<1.00E-15	<1.00E-15	8,12 (4-16,49)	6.57E-09	1.58E-07
USH1C	22	5,12 (2,58-10,17)	3.13E-06	6.88E-05	10,68 (5,54-20,59)	1.54E-12	3.38E-11	5,12 (2,58-10,18)	3.06E-06	6.73E-05
P2RX2	22	20,34 (9,75-42,44)	<1.00E-15	<1.00E-15	23,74 (11,41-49,41)	<1.00E-15	<1.00E-15	23,51 (11,29-48,94)	<1.00E-15	<1.00E-15
ESPN	19	27,88 (17,06-45,55)	<1.00E-15	<1.00E-15	24,35 (14,88-39,84)	<1.00E-15	<1.00E-15	17,39 (10,59-28,56)	<1.00E-15	<1.00E-15
RDX	17	94,37 (36,06-247)	<1.00E-15	<1.00E-15	57,38 (21,85-150,66)	<1.00E-15	<1.00E-15	75,26 (28,72-197,23)	<1.00E-15	<1.00E-15
SLC26A4	15	1,79 (1,02-3,15)	4.31E-02	6.46E-01	4,14 (2,5-6,86)	3.37E-08	5.06E-07	3,65 (2,19-6,09)	6.62E-07	9.93E-06
ESRRB	14	12,54 (7,26-21,63)	<1.00E-15	<1.00E-15	10,67 (6,16-18,48)	<1.00E-15	<1.00E-15	7,54 (4,31-13,18)	1.41E-12	1.98E-11
DTNA	13	6,07 (4,68-7,87)	<1.00E-15	<1.00E-15	4,87 (3,74-6,35)	<1.00E-15	<1.00E-15	4,78 (3,67-6,23)	<1.00E-15	<1.00E-15
PRKCB	13	27,62 (18,65-40,91)	<1.00E-15	<1.00E-15	17,6 (11,84-26,16)	<1.00E-15	<1.00E-15	40,79 (27,61-60,28)	<1.00E-15	<1.00E-15
SLC12A2	12	21,91 (15,95-30,1)	<1.00E-15	<1.00E-15	55,14 (40,31-75,43)	<1.00E-15	<1.00E-15	79,32 (58,04-108,41)	<1.00E-15	<1.00E-15
KCNQ1	11	7,93 (5,92-10,63)	<1.00E-15	<1.00E-15	13,75 (10,33-18,29)	<1.00E-15	<1.00E-15	6,96 (5,18-9,34)	<1.00E-15	<1.00E-15

NFKB1	10	1,87 (1,3-2,71)	7.81E-04	7.81E-03	1,92 (1,33-2,77)	4.69E-04	4.69E-03	3,79 (2,72-5,29)	4.44E-15	4.44E-14
DPT	10	1,88 (1,2-2,92)	5.36E-03	5.36E-02	8,03 (5,5-11,74)	<1.00E-15	<1.00E-15	4,38 (2,94-6,51)	3.01E-13	3.01E-12
CEACAM16	9	1,25 (1,04-1,49)	1.56E-02	1.40E-01	1,33 (1,12-1,59)	1.48E-03	1.33E-02	2,45 (2,09-2,87)	<1.00E-15	<1.00E-15
FAM136A	8	31,18 (27,22-35,71)	<1.00E-15	<1.00E-15	38,36 (33,5-43,92)	<1.00E-15	<1.00E-15	14,71 (12,81-16,89)	<1.00E-15	<1.00E-15
GRHL2	8	11,72 (9,35-14,7)	<1.00E-15	<1.00E-15	9,28 (7,39-11,66)	<1.00E-15	<1.00E-15	6,45 (5,11-8,15)	<1.00E-15	<1.00E-15
EYA4	7	36,65 (28,83-46,59)	<1.00E-15	<1.00E-15	25,31 (19,88-32,22)	<1.00E-15	<1.00E-15	7,68 (5,97-9,88)	<1.00E-15	<1.00E-15
COCH	7	3,41 (2,54-4,58)	<1.00E-15	<1.00E-15	3,39 (2,52-4,56)	<1.00E-15	<1.00E-15	3,95 (2,95-5,28)	<1.00E-15	<1.00E-15
CCDC50	5	1,25 (0,99-1,59)	6.22E-02	3.11E-01	1,62 (1,3-2,03)	2.51E-05	1.25E-04	3,22 (2,63-3,94)	<1.00E-15	<1.00E-15
KCNJ10	5	4,38 (3,9-4,93)	<1.00E-15	<1.00E-15	3,14 (2,78-3,55)	<1.00E-15	<1.00E-15	2,3 (2,02-2,61)	<1.00E-15	<1.00E-15
SEMA3D	4	1,56 (1,03-2,38)	3.60E-02	1.44E-01	1,13 (0,72-1,77)	5.84E-01	2.34E+00	3,79 (2,63-5,47)	1.09E-12	4.35E-12
CLDN14	4	4,51 (2,56-7,94)	1.74E-07	6.97E-07	22,86 (13,56-38,55)	<1.00E-15	<1.00E-15	4,72 (2,69-8,29)	6.61E-08	2.65E-07
POU4F3	4	7,31 (6,53-8,18)	<1.00E-15	<1.00E-15	4,92 (4,38-5,52)	<1.00E-15	<1.00E-15	9,2 (8,23-10,28)	<1.00E-15	<1.00E-15
MSRB3	4	13,35 (11,34-15,71)	<1.00E-15	<1.00E-15	10,85 (9,21-12,79)	<1.00E-15	<1.00E-15	4,06 (3,4-4,83)	<1.00E-15	<1.00E-15

[a]Odds ratios were calculated in the 95% confidence interval.

[b]P values were corrected with Bonferroni method approach.

A second variant filtering using only missense exonic variants described in CSVS Spanish population database was made (Table 2). Eighteen genes showed an excess of missense variants (46 in total). Of note, 5 genes causing autosomal recessive SNHL showed the highest accumulation of missense variants remained in the European population and Spanish population datasets: *SLC26A4*, *GJB2, CLDN14, ESRRB* and *USH1G*. These five genes were selected for validation through Sanger sequencing as Spanish population enriched genes.

Table 2. **Gene burden analysis 2**. List of 18 genes showing a significant excess of missense exonic variants in patients with sporadic MD, according to MAF observed and described in CSVS Spanish database, compared with global ExAC population and non-Finnish European ExAC population.

Gene	Number variants	Percentage variants retained	Odds ratio ExAC[a]	P value	P corrected[b]	Odds ratio ExAC NFE[a]	P value	P corrected[b]	Odds ratio CSVS[a]	P value	P corrected[b]
GJB2	6	80	0,5 (0,27-0,93)	2.91E-02	1.75E-01	**3,2 (2,12-4,83)**	**2.75E-08**	**1.65E-07**	**2,06 (1,33-3,19)**	**1.14E-03**	**6.85E-03**
SEMA3D	2	50	1,1 (0,76-1,61)	6.13E-01	1.00E+00	0,8 (0,53-1,21)	2.85E-01	5.70E-01	**2,67 (1,94-3,68)**	**2.03E-09**	**4.06E-09**
CLDN14	2	50	4,47 (2,55-7,83)	1.67E-07	3.35E-07	**23,18 (13,81-38,9)**	**<1.00E-15**	**<1.00E-15**	**4,64 (2,65-8,11)**	**7.47E-08**	**1.49E-07**
SLC26A4	6	40	1,18 (0,72-1,93)	5.04E-01	1.00E+00	**2,88 (1,89-4,38)**	**8.13E-07**	**4.88E-06**	**2,33 (1,51-3,59)**	**1.23E-04**	**7.37E-04**
NFKB1	3	30	1,37 (0,99-1,91)	5.92E-02	1.78E-01	1,43 (1,03-1,98)	3.37E-02	1.01E-01	**2,73 (2,03-3,66)**	**2.21E-11**	**6.62E-11**
POU4F3	1	25	1,56 (1,47-1,66)	<1.00E-15	<1.00E-15	1,06 (1-1,14)	6.16E-02	6.16E-02	**1,84 (1,73-1,95)**	**<1.00E-15**	**<1.00E-15**
ESRRB	3	21	4,41 (3,31-5,89)	<1.00E-15	<1.00E-15	**3,39 (2,52-4,55)**	**<1.00E-15**	**<1.00E-15**	**1,84 (1,33-2,54)**	**2.04E-04**	**6.12E-04**
USH1G	5	21	2,51 (1,38-4,56)	2.66E-03	1.33E-02	**20,27 (12,06-34,06)**	**<1.00E-15**	**<1.00E-15**	**4,67 (2,68-8,17)**	**6.11E-08**	**3.05E-07**
CCDC50	1	20	0,54 (0,44-0,66)	7.23E-10	7.23E-10	0,7 (0,59-0,84)	1.41E-04	1.41E-04	1,38 (1,19-1,61)	3.38E-05	3.38E-05
P2RX2	3	14	2,57 (1,93-3,42)	8.71E-11	2.61E-10	3,14 (2,38-4,14)	<1.00E-15	<1.00E-15	2,67 (2,01-3,55)	1.12E-11	3.36E-11
FAM136A	1	13	140,36 (131,25-150,11)	<1.00E-15	<1.00E-15	76,75 (71,75-82,09)	<1.00E-15	<1.00E-15	3,68 (3,41-3,97)	<1.00E-15	<1.00E-15

RDX	2	12	4,49 (3,59-5,61)	<1.00E-15	<1.00E-15	2,69 (2,12-3,4)	<1.00E-15	<1.00E-15	3,33 (2,65-4,2)	<1.00E-15	<1.00E-15
TPRN	3	12	2,24 (1,7-2,96)	1.46E-08	4.37E-08	6,55 (5,1-8,4)	<1.00E-15	<1.00E-15	2,95 (2,26-3,86)	<1.00E-15	<1.00E-15
ESPN	2	11	10,69 (9-12,69)	0.00E+00	<1.00E-15	10,26 (8,64-12,19)	<1.00E-15	<1.00E-15	2,01 (1,64-2,46)	9.24E-12	1.85E-11
SLC12A2	1	8	281,71 (246,32-322,17)	<1.00E-15	<1.00E-15	308,08 (269,39-352,33)	<1.00E-15	<1.00E-15	14,77 (12,86-16,96)	<1.00E-15	<1.00E-15
PRKCB	1	8	15,1 (13,03-17,48)	<1.00E-15	<1.00E-15	10,5 (9,05-12,19)	<1.00E-15	<1.00E-15	5,54 (4,75-6,46)	<1.00E-15	<1.00E-15
MYH14	3	6	14,44 (10,42-20,03)	<1.00E-15	<1.00E-15	28,12 (20,38-38,79)	<1.00E-15	<1.00E-15	5,86 (4,16-8,25)	<1.00E-15	<1.00E-15
ADD1	1	3	563,42 (492,71-644,27)	<1.00E-15	<1.00E-15	308,08 (269,39-352,33)	<1.00E-15	<1.00E-15	14,77 (12,86-16,96)	<1.00E-15	<1.00E-15

[a]Odds ratios were calculated in the 95% confidence interval.

[b]P values were corrected with Bonferroni method approach

Excess of rare variants in hearing loss genes in familial cases

We used exome sequencing datasets from familial MD previously reported in order to search for rare variants identified in our panel in the sporadic cases. Although no single missense variant was found segregated in all the cases in the same family, we found several rare missense variants in at least one case per family. These MD families show phenotypic differences in terms of age of onset, hearing profile and disease progression and the presence of rare variants can be addressed as potential modulators of the phenotype in each familial case (Table 3).

Table 3. **Missense variants found in familial MD cases**. Variants were retrieved from familial cases segregating a partial phenotype in different families.

Cases	Family	SNV	Gene	Described in	Pathogenicity	MAF (gnomAD)	MAF (ExAC)
All	1	chr2:70527974 G>A	FAM136A	Requena et al, 2015	Pathogenic		
1	1	chr4:6303197 G>A	WFS1	No	Conflicting interpretations of pathogenicity	0.0041	0.0045
2	1	chr13:20763264 C>T	GJB2	No	Benign/Likely benign	0.0094	0.0106
All	1	chr18:32462094 G>T	DTNA	Requena et al, 2015	Likely pathogenic	3.66E-05	2.47E-05
2	2	chr1:41296865 G>A	KCNQ4	No	Unknown significance	2.13E-05	3.49E-05
All	2	chr1:168665849 G>A	DPT	Martin-Sierra et al, 2017	Likely pathogenic	2.03E-05	2.5E-05
2	2	chr4:2900221 A>G	ADD1	No	Unknown significance	8.12E-06	8.24E-06
All	3	chr7:84642128 G>A	SEMA3D	Martin-Sierra et al, 2017	Pathogenic		
All	3	chr8:102555482 G>T	GRHL2	No	Unknown significance		
All	4	chr16:23999898 G>T	PRKCB	Martin-Sierra et al, 2016	Pathogenic		
1	4	chr22:38119405 C>T	TRIOBP	No	Likely benign	4.06E-05	2.49E-05
1	4	chr3:191098660 A>G	CCDC50	No	Benign	0.0061	0.0065
1	4	chr11:110134833 T>C	RDX	No	Unknown significance		

Effect of rare variant interaction

We selected exonic variants from the gene burden analysis to analyze their potential additive effect at protein-protein interaction interfaces by the tool INSIDER for our selected five genes. However, protein interfaces for *ESRRB*, *CLDN14* and *SLC26A4* genes could not be loaded and processed on the database (lacking predicted interfaces on ÉCLAIR database or crystalized protein structures on Protein Data Bank (PDB) database). Of note, most relevant affected interaction affected the self-interaction *GJB2-GJB2* by the known variants observed in the burden analysis (significant spatial clustering with 4 SNV, p=0.0009) (Fig 1A).

Other interactions of interest were founded between the *USH1G – USH1C* genes, but the involved variants were not located in the known interaction surface of *USH1G* (Fig 1B).

Discussion

This study shows that patients with sporadic MD have an enrichment of few rare variants in certain hearing loss genes such as *GJB2*, *SLC26A* or *USH1G*. This excess of missense variants in some genes may increase the risk to develop hearing loss in MD and may contribute to explain the heterogeneity observed in the phenotype [23]. To understand the relevance of population frequencies in our cohort, we performed the association analysis between variants observed in MD cases against their respective frequencies on a healthy population for each gene of the panel. From the total amount of variants, we selected exonic variants for all the targeted genes. We applied a stronger filter for the selection of missense variants by choosing previously described variants for each gene significantly overrepresented in MD cases.

Since many missense variants were not found in the Spanish control population from CSVS, a third comparison limited to those variants described in CSVS database was carried

out. We followed this conservative approach to reduce false positive findings in the readings. This third restrictive gene analysis was limited to 132 variants observed at least once in the Spanish reference population.

From the final analysis, we found that some genes such as *SLC26A4*, *ESRRB*, *CLDN14*, *GJB2* and *USH1G* retained the higher number of missense variants among Spanish MD patients. We also found one synonymous variant was representative in *MARVELD2* gene. Besides from its functional implications, it may also generate a cryptic splice site. However, more testing is needed to confirm this hypothesis.

Gene panel for familial MD

The Genomics England project (https://www.genomicsengland.co.uk/) has designed gene panels for the diagnosis of many genetic disorders including familial MD (https://panelapp.genomicsengland.co.uk/panels/394/). This panel is in an early stage of development because it only considers 130 genes with limited evidence to few families. The results of this study can be used to improve the design of panels for the diagnosis of MD.

For the design of our panel, we chose a total of 69 genes. Most of the genes were selected according to the hearing loss profile (low frequency or pantonal hearing loss). However, more than 90 genes have been described as related to hearing loss, so more hearing loss genes could be involved in the phenotype (fully accessible from Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org/). Genetic evidence of hearing loss has been obtained from linkage analyses until the emergence of NGS techniques [17], that have facilitated the clinical development of genetic diagnosis in hearing loss. Custom panels and microarrays have been the flags of a new age of discovery of novel and rare variants for genetic diagnostic of hearing loss [19,24].

Our panel was designed considering hearing loss as the main symptom shared by all patients with MD, since the vestibular phenotype and other associated co-morbidities such as migraine or autoimmune disorders are more variable. To improve the diagnostic yield of MD and to decrease this granularity in the phenotype, it will be recommendable to select sporadic patients with an early age of onset for future studies.

Rare variants in hearing loss genes in sporadic MD

The frequency of hearing loss related genes is population-specific [15]. Herein, we present a study for MD patients in the Spanish population. As a part of the study, we consider a panel of genes related to hearing loss and other symptoms. Besides from the validated variants in singletons, only a few rare variants such as *ESRRB* rs201448899, *MARVELD2* rs369265136, *SLC26A4* rs200511789 and *USH1G* rs151242039 have been validated in more than one sporadic case in the entire cohort. All these genes had been previously considered as pathogenic for hearing loss but never involved with MD.

ESRRB is the estrogen-related receptor beta, also known as nuclear receptor subfamily 3, group B, member 2 or NR3B2. This gene encodes for a protein like the estrogen receptor but with a different and unknown role. Mutations in the mouse orthologue have been described relevant in placental development and autosomal recessive SNHL [25,26].

MARVELD2 encodes a protein found in the tight junctions, between epithelial cells. The encoded protein seems to forge barriers between epithelial cells such the ones in the organ of Corti, where, in case of lack of these barriers, normal hearing is affected. Defects in this gene are a cause of deafness autosomal recessive type 49 (DFNB49) [27].

SLC26A4 gene encodes pendrin, a protein extensively studied in hearing loss. Its alteration is one of the most common causes of syndromic deafness, an autosomal recessive SNHL. It is also associated with enlarged vestibular aqueduct syndrome (EVAS) [28].

USH1G is a gene translating to a protein that contains three ankyrin domains, a class I PDZ-binding motif and a sterile alpha motif. This protein is well-known to interact with harmonin (*USH1C*) in the stereocilia of hair cells, a protein associated with Usher syndrome type 1C [29]. This protein plays a role in the development and maintenance of the auditory and visual systems and functions in the cohesion of hair bundles formed by inner ear sensory cells. Alterations in the integrity of the protein seem to be the cause of Usher syndrome type 1G [29,30].

However, *ESRRB* rs201448899 has been observed in more Spanish controls than expected. This increased frequency on the Iberian population when compared with other known largest frequencies as non-finnish Europeans, suggests that this is a population specific variant rather than a MD disease variant. Only the *MARVELD2* rs369265136 variant remains as a proper DNV related to MD cases. However, the functional effect of a synonymous variant is unknown and functional studies will be required to decipher the relevancy of this variant in MD cases in the future.

Burden analysis of rare missense variants in sporadic MD

Our results demonstrate a burden of rare missense variants in few SNHL genes, including *GJB2, ESRRB, CLDN14, SLC26A4* and *USH1G*. We speculate that the additive effect of several missense variants in the same gene could interact with the same or other genes at the protein level resulting in the hearing loss phenotype.

Population analysis was addressed in order to obtain a better image of our cohort. Despite the limitation that represents the small number of genes considered in our panel, we have found a significant increase of missense variants on several hearing loss genes in the Iberian population (Table 2). These findings demonstrate the involvement of multiple missense variants in the same gene and may explain several clinical findings in MD. So, incomplete phenotype found in relatives of patients with familial MD or even the variable expressivity observed could be explained on the differences found in multiple rare variants among individuals of the same family [12–14]. In addition, some sporadic cases where a single rare variant with unknown significance cannot explain the phenotype could be singletons individuals with several low frequency variants probably following a compound heterozygous recessive pattern of inheritance. Our results start to decipher the complex interaction between rare and ultrarare variations (MAF< 0.0001) in the same or different genes in sporadic MD, adding more evidence to the genetic architecture of MD.

First, the phenotype of MD could be the result of additive effect of low frequency or rare variants in the same gene. A good number of common or low frequency variants in the same gene can be a rare situation, as rare as the disease. As much changes are added to the protein, its integrity could be affected, reflecting a pathological output. In our case, GJB2, that forms a hexamer with a transmembrane channel function, has been determined as possible affected by these changes in their interactions. Previous studies have determined how certain changes in the monomer can affect to the develop of the hexamer [31,32]. Here, bioinformatics models show how the interaction of low frequency variants found in MD patients can impact the interaction between connexins monomers.

A second hypothesis points to the interaction of several genes in the disease phenotype, following its complex disease definition [33,34]. In this case, high significant genes in our panel could be added to the pool of possible targets of the disease, although a single candidate

variant could not be enough to explain the disease. So, the interaction between some of our selected genes and other, a priori, not related SNHL genes could be relevant in the expressivity. *USH1G* interacts with *USH1C*, a known gene involved in Usher syndrome. *USH1G* has been observed to have a minor role in Usher syndrome in Spanish population (35), but not in MD, even though they share similar hearing loss profile. Although no one of the missense variants in *USH1G* were in an interaction domain, this could be of interest when considering interaction between different proteins as a main factor to develop a mild phenotype. This hypothesis may be reinforced through the data found in sequenced familial cases. For instance, the variant rs748718975 in *DPT* gene was only associated with the SNHL phenotype in the family where it was described, but these cases showed different characteristics in the age of onset or hearing loss outcome. These differences between the cases can be explained with other variants found in *KCNQ4* (rs574794136) and *ADD1* (rs372777117) genes, though being described previously as variants of unknown significant. So, the variant rs574794136 was found in two sisters, but not in the third one, that was carrier of rs372777117. This excess of rare variants in certain genes observed in familial cases could explain the differences in expressivity in a given family.

This panel was made as an early screening diagnostic panel. Here we can show how certain SNHL gene variants can be related to MD in the Iberian population and show that multiple rare variation in the same gene should be consider as likely pathogenic. Our results will contribute to design a better gene panel for the genetic diagnosis of MD.

Materials and methods

Sample selection

A total of 890 Spanish and Portuguese patients with MD were recruited. This study protocol was approved by the Institutional Review Board for Clinical Research (MS/2014/02), and a written informed consent to donate biological samples was obtained from all subjects. All patients were diagnosed by neurotology experienced clinicians from the Meniere's Disease Consortium (MeDiC), according to the diagnostic criteria for MD formulated by the International Classification Committee for Vestibular Disorders of the Barany Society in 2015 [1]. Among them, 830 were considered sporadic MD cases while 60 were familial MD cases. Details from the selected cases are described in Table 4. As controls, 40 healthy individuals were selected from the same population.

Table 4. **Participant individuals in this study.** Number of individuals and distribution of the selected cases and controls for targeted-gene sequencing. SMD, sporadic Meniere disease; FMD, familial Meniere disease

Diagnosed	*N*	**Sex**	%	**Location**	*N*
SMD	830	**Male**	40	**North**	500
				South	320
FMD	60	**Female**	60	**Centre**	90
Control	40			**Portugal**	20

Selection of target genes

Target genes were selected from a literature search attending to human phenotype (hearing profile, comorbid vestibular symptoms) and phenotype observations in mouse and

zebrafish models. Most of them were selected from HearingLoss.org website gene list for monogenic SNHL. Additional genes were added because they have been previously found in familial MD [12–14], or allelic variations associated with hearing outcome in MD had been described, such as NFKB1 or TLR10 genes [36,37]. Mitochondrial genes were added since maternal inheritance is suspected in several families with MD. Relevant information about the location, size, bibliography and other characteristics about each gene included in the panel is presented in Table S1 and Table S2.

The custom panel (Panel ID: 39351-1430751809) were designed by the Suredesign webtool (Agilent) to cover the exons and 50 bp in the flanking regions (5' and 3' UTR). This allowed the sequencing around 533.380kb with more than 98.46% coverage.

Sample pooling

Enrichment technology allows the selective amplification of targeted sequences and DNA sample pooling, reducing the costs of reagents and increasing sample size. We decided to pool patient samples according to their geographical origin. Each pool consisted of 10 DNA samples from the same hospital for a total of 93 pools (930 samples).

DNA concentration and quality were measured on each sample using two methods: Qubit dsDNA BR Assay kit (TermoFisher Scientific) and Nanodrop 2000C (ThermoFisher Scientific). All samples had quality ratios ranging 1.8 and 2.0 in 280/260 and 1.6 to 2.0 in 260/230.

Libraries preparation protocol

The HaloPlex Target Enrichment System (Agilent, Santa Clara, CA) was used to prepare the DNA libraries, according to the manufacturer protocol. Validation of the protocol and library performance was analyzed with a 2100 Bioanalyzer High Sensitivity DNA Assay

kit. Expected concentrations were between 1 and 10 ng/ul. Higher concentrations than 10 ng/ul were diluted 1:10 in 10mM TRIS, 1mM EDTA. Targeted-sequencing was performed in an Illumina HiSeq 2000 platform.

Data generation pipelines

Raw data downloaded and sequencing adapters were trimmed following manufacturer indications. The minimum coverage considered was 30X mean depth, however mitochondrial sequences reached higher coverages due to their shorter sizes. Bioinformatic analyses were performed according to the Good Practices recommended by Genome Analysis ToolKit (https://software.broadinstitute.org/gatk/).

Two methods were used to find differences in how UnifiedGenotyper and HaplotypeCaller (the old and the most recent tools for variant calling in GATK suite) address sequenced pools. Both custom pipelines use BWA-mem aligner and GATK suite tools following the GATK protocol for Variant Calling against GRCh37/hg19 human reference genome. Left normalization for multi-allelic variants were addressed by separated. Calling was made in the first pipeline with UnifiedGenotyper modifying number of chromosomes per sample (per pool, there are 20 chromosomes). The second pipeline used HaplotypeCaller, which cannot allow the same approach, but can automatically address high number of calls with a different approach. Variants with read depth (RD) <10 and genotype quality (GQ)<20 were excluded in all the calling pipelines following recommended hard filtering steps by GATK suite.

A third caller tool, VarScan, was used to filter and annotate quality strand data per variant to compare its output with GATK-based callers. VarScan allows the variant filtering using the information obtained according to each strand polarity. The method retrieves those variants that were only called in one strand, but not in the reverse strand, leading to false

positive calls. This step was used as internal quality control to avoid strand bias usually generated in Haloplex data, as it has been reported in other studies [38].

Positive control SNV validation

Positive control testing was addressed using samples from patients with familial MD with known variants on certain genes. These individuals come from previous familial studies with independently validated variants by Sanger sequencing. Known variants were also sequenced and validated by Sanger (Table S3). Coverage and mapping quality after each pipeline were annotated and measured. Representative chromatographs from validated SNVs are detailed in Figure S1.

Selection and prioritization of pathogenic SNV

In order to obtain more information of each SNV, we annotated the merged files using the ANNOVAR tool. Minor allele frequencies (MAF) were retrieved for each candidate variant from gnomAD and ExAC database (total individuals and non-Finnish European (NFE) individuals). Since the estimated prevalence of sporadic MD in Spain is 0.75/1000 individuals [39], we selected variants with MAF <0.001 for single rare variant analysis. For burden analysis of common and rare variants, we chose a higher MAF value <0.1. The Collaborative Spanish Variant Server (CVCS) database including 1644 unrelated individuals was also used for annotation of exonic rare variants and to retrieve MAF in Spanish population (dataset fully accessible from http://csvs.babelomics.org/) [40].

KGGseq suite (grass.cgs.hku.hk/limx/kggseq) was used for the selection of rare and DNV to prioritize the most pathogenic variants according to the integrated model trained algorithm with known pathogenic variants and neutral control variants.

Enrichment analysis for each gene was made with all the exonic variants found with a MAF <0.1. This analysis required to divide the total amount of variants into three groups: those described in global gnomAD population, those found in NFE population, and finally those included in CVCS Spanish population. These three reference datasets were used for enrichment analysis comparison.

Validation of candidate pathogenic SNV

Candidate SNV were visually revised in the BAM files using Integrative Genomics Viewer (https://software.broadinstitute.org/software/igv/) and validated in the different pools where they were called using Sanger sequencing.

Population statistics

Statistical analysis was performed with IBM SPSS v.20 program, Microsoft Excel suite tools, and diverse python and java encoded public scripts. Due to the overrepresentation of Spanish population in our dataset, most of the selected variants were filtered through exome sequencing data from Spanish controls of CSVS database. The MAF was calculated for each variant in our dataset and rare and DNV on MD patients were identified in our gene panel. Odd ratios with 95% confidence interval were calculated for each variant using MAF obtained from Spanish and NFE populations as controls. All p values were corrected for multiple testing.

Gene burden analysis was addressed using 2x2 contingency tables counting total exonic alternate allele counts per gene in our cases against total and NFE controls in ExAC and CSVS controls. Odds ratios with 95% confidence intervals were calculated using Fisher's

exact test and obtaining one-sided p-values. P-values were also corrected for multiple testing by the total amount of variants found for each gene following Bonferroni approach.

Position of variants in significant enriched genes

Several models were generated for rare variant-enriched domains in significant enriched genes by using the INSIDER modelling tool [41]. The selected variants per gene are detailed in results. Prediction values were annotated with their calculated p values.

Acknowledgements

We acknowledge to all members of the Meniere disease Consortium (MeDiC), a network of clinical and research centres contributing to the study of Meniere disease. List of participants in MeDiC: Juan Carlos Amor-Dorado (Hospital Can Misses Ibiza, Spain), Ismael Aran (Complexo Hospitalario de Pontevedra, Spain), Angel Batuecas-Caletrío (Hospital Universitario Salamanca, Spain), Jesus Benitez (Hospital Universitario de Gran Canaria Dr. Negrin, Las Palmas de Gran Canaria, Spain), Jesus Fraile (Hospital Miguel Servet, Zaragoza, Spain), Ana Garcia-Arumí (Hospital Universitario Vall d'Hebron, Barcelona, Spain), Rocio Gonzalez-A (Hospital Universitario Marqués de Valdecilla, Santander, Spain), Juan M Espinosa-Sanchez (Hospital Virgen de las Nieves, Granada, Spain), Raquel Manrique Huarte, Nicolas Perez-Fernandez (Clinica Universidad de Navarra, Spain), Pedro Marques (Centro Hospitalar de São João, Porto, Portugal), EM-S, Ricardo Sanz (Hospital Universitario de Getafe, Madrid, Spain), Manuel Oliva Dominguez (Hospital Costa del Sol Marbella, Spain), PP (Hospital Cabueñes, Asturias, Spain), HP-G, VP-G (Hospital La Fe, Valencia, Spain), SS-P, AS-V (Complexo Hospitalario Universitario, Santiago de Compostela, Spain), MT (Instituto Antolí Candela, Madrid, Spain), Roberto Teggi (San Raffaelle Scientific Institute,

Milan, Italy), and GT (Complejo Hospitalario Badajoz, Spain). We also acknowledge Joaquin Dopazo (Clinical Bioinformatics Area, Hospital Virgen del Rocio, Sevilla, Spain) to facilitate us the access to CSVS datasets. AGM is a PhD student in the Biomedicine program at University of Granada and this work is part of his doctoral thesis.

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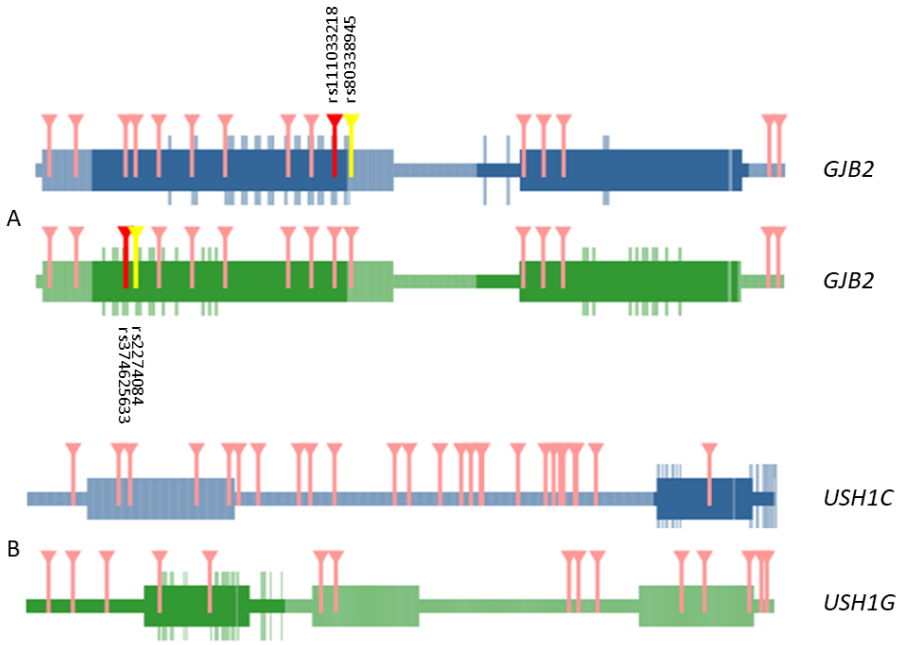
Figures

Figure 1. **Representation of domains and interactive interface in GJB2 – GJB2 (A) and USH1G – USH1C (B) interaction**. Marked in darker color boxes, functional regions of the protein. Aminoacids in the interactive surface of the protein are highlighted in the same color. Marked in red and yellow aminoacids that affect interaction regions between both protein s. Only in GJB2 – GJB2 self-interaction missense mutations are relevant in the interaction (dbSNP ids detailed in black). The rest of the variants affecting aminoacids tested in both interactions, and not found in the interactive surface region, are marked in pink.



Supporting information

Figure S1. Chromatographs for validated SNV through Sanger sequencing

Table S1. Design of panel. Regions included contain 75 bp upstream and 75 bp downstream of the entire genes selected. Mitochondrial genes were also included in this panel. However, their analysis were separated and not included in the present study.

Table S2. References used to support the gene panel selection for MD panel. DFNA, DFNB and others genes are separated in three tables.

Table S3. Validated variants. List of rare variants validated through Sanger sequencing in MD patients. DbSNP accession numbers are detailed for each tested variant. Number of individuals porting the variant are described between parenthesis.