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Consensus interpretation of the p.Met34Thr and p.Val37Ile variants in *GJB2* by the ClinGen Hearing Loss Expert Panel

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Competing Interests Statement

JS, AMO, HD, ML, KZ, SSA, HLR, ANAT worked for pay for service diagnostic laboratories providing genetic testing. PK, SG, BM-H, KM, NN, AW worked for commercial laboratories providing genetic testing.

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Abstract

PURPOSE—Pathogenic variants in *GJB2* are the most common cause of autosomal recessive sensorineural hearing loss. The classification of c.101T>C/p.Met34Thr and c.109G>A/p.Val37Ile in *GJB2* are controversial. Therefore, an expert consensus is required for the interpretation of these two variants.

METHODS—The ClinGen Hearing Loss Expert Panel collected published data and shared unpublished information from contributing laboratories and clinics regarding the two variants.

Functional, computational, allelic, and segregation data were also obtained. Case-control statistical analyses were performed.

RESULTS—The panel reviewed the synthesized information, and classified the p.Met34Thr and p.Val37Ile variants utilizing professional variant interpretation guidelines and professional judgment. We found that p.Met34Thr and p.Val37Ile are significantly overrepresented in hearing loss patients, compared to population controls. Individuals homozygous or compound heterozygous for p.Met34Thr or p.Val37Ile typically manifest mild to moderate hearing loss. Several other types of evidence also support pathogenic roles for these two variants.

CONCLUSION—Resolving controversies in variant classification requires coordinated effort among a panel of international multi-institutional experts to share data, standardize classification guidelines, review evidence, and reach a consensus. We concluded that p.Met34Thr and p.Val37Ile variants in *GJB2* are pathogenic for autosomal recessive nonsyndromic hearing loss with variable expressivity and incomplete penetrance.

Keywords

ClinGen; hearing loss; incomplete penetrance; variant classification; variant interpretation

INTRODUCTION

Variants with incomplete penetrance for Mendelian conditions pose significant challenges for clinical interpretation because they are relatively common in the population and present in healthy individuals[1]. No such variants have been rigorously reviewed and classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines[2]. To demonstrate the best practice to interpret such variants, the ClinGen Hearing Loss Expert Panel (HL-EP) applied hearing-loss-gene-specific ACMG/AMP guidelines[3] to interpret two controversial variants in *GJB2*.

GJB2 encodes connexin 26, a member of the gap junction protein family. Gap junctions are intercellular channels allowing the coupling of adjacent cells to share molecules, ions, and electrical signals. Each gap junction channel is composed of two connected hemichannels called connexons, one on either membrane of neighboring cells. Each connexon is a hexamer of the same or different connexin units. Biallelic pathogenic variants in *GJB2* (NM_004004.5) are the most frequently identified cause of autosomal recessive sensorineural hearing loss[4,5]. Hundreds of *GJB2* variants have been reported in patients with hearing loss. Premature termination codons (PTC), such as c.35delG, c.167delT, and c.235delC common in European, Ashkenazi Jewish, and Asian populations respectively, are established pathogenic variants. However, classifications of two notable missense variants c.101T>C/p.Met34Thr and c.109G>A/p.Val37Ile have been controversial. P.Met34Thr was first reported as being associated with dominant hearing loss[4], but its pathogenicity for dominant hearing loss was later challenged because of subsequent identification of its occurrence in individuals with normal hearing[5,6], and an autosomal recessive mode of inheritance was suggested[7–9]. P.Val37Ile was first identified as a polymorphism in a heterozygous control[10], and later found to be homozygous or *in trans* with known pathogenic *GJB2* variants in affected individuals[7,11]. Both variants were found relatively

frequently in the general population. Some homozygotes for these variants appeared to have normal hearing[12,13]. Reduced penetrance was proposed to explain the inconsistency[14]. We surveyed clinical laboratories in the United States and Canada regarding the classification of these variants and found significant variability across different laboratories (Supplementary Information). Therefore, we considered it a priority to resolve the controversy and reach a consensus.

Herein, we report the ClinGen and ClinVar effort to resolve controversies regarding p.Met34Thr and p.Val37Ile classification assertions and demonstrate the best practice to interpret variants with incomplete penetrance.

MATERIALS AND METHODS

ClinGen Hearing Loss Expert Panel

The ClinGen HL-EP includes otolaryngologists caring for patients with hereditary hearing loss, medical geneticists, clinical laboratory diagnosticians, molecular pathologists, genetic counselors, and investigators specialized in auditory research[3]. This group was convened to develop specifications of the ACMG/AMP guidelines for interpreting sequence variants in genes associated to hearing loss[3].

Data sources

Fifteen genetic testing laboratories and clinics contributed data to this study (Table 1). Individuals tested for p.Met34Thr or p.Val37Ile were designated as cases if hearing loss was the indication or as population controls if carrier screening was the indication. Counsyl data were solely based on carrier screening, thus representing the general population. We also considered information from the Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org/) as population controls. Testing methods included specific variant testing, *GJB2* sequencing, large multigene panels, and exome sequencing. Individuals from sites with unspecified total numbers tested and relatives of probands were excluded from statistical analyses. Data collected included total number of individuals tested for p.Met34Thr or p.Val37Ile in *GJB2*, ethnicities, allele states, and phenotypic information (age at onset of hearing loss, age at testing, type of hearing loss, severity, laterality, frequency range affected, family history, and other clinical features). The study was approved by respective Institutional Review Boards, Helsinki Committees, or equivalent ethics committees of participating institutions involving research on human subjects.

Variant interpretation

Data were analyzed and interpreted according to ACMG/AMP guidelines[2] and hearing-loss-gene-specific criteria by the ClinGen HL-EP[3]. Original evidence codes included a first letter P (“pathogenic”) or B (“benign”), followed by VS (“very strong”), S (“strong”), M (“moderate”), P (“supporting”), or A (“standard alone”) to indicate the strength level and a category number[2]. Modified codes included a suffix of an underscore and the adjusted strength level[3].

Statistical analysis

For 2×2 contingency tables, odds ratios (OR), 95% confidence intervals (CI), Z statistics, and p values were calculated using MEDCALC (https://www.medcalc.org/calc/odds_ratio.php). Four types of case-population comparisons were performed: (1) homozygotes in overall populations, (2) homozygotes in ethnicity-stratified populations, (3) alleles in ethnicity-stratified populations, and (4) homozygotes and compound heterozygotes in ethnicity-stratified populations. GnomAD data were included except for analyses involving compound heterozygotes, where only Counsyl data were used as population controls, because individual data were unavailable in gnomAD. Compound heterozygosity was presumed in individuals with two variants in *GJB2* never reported to have occurred *in cis*.

For comparisons of the severity among different genotype groups, an ordinal logistic regression model was used. No covariates such as age and sex were included, because the information was not available for all individuals. The proportions of the sample sizes of the genotype groups were used as weights for the analysis. Type 1 test was used to test the overall significant difference among all groups and ad-hoc analysis was performed to compare the estimated odds ratio between each pair of specific gene types. The statistical significance was defined as p value <0.05. The analysis was performed with SAS software (version 9.4).

RESULTS

Case-control Evidence

P.Met34Thr and p.Val37Ile have been reported in patients with hearing loss in various populations (Supplementary Information). To perform accurate case-control comparisons, the HL-EP decided not to rely on published cases in the literature due to concerns of the publication bias. Instead, we obtained data from 15 contributing sites (Table 1). The level of details provided by each site varied. Three sites (BCH, DY, and GDWC) reported only cases with biallelic *GJB2* variants involving p.Met34Thr or p.Val37Ile. Eleven sites reported results from 17,635 probands with hearing loss tested for these two variants. Ethnicity information was available from eight sites on 7,962 European probands and 2,066 Asian probands. Five sites (Counsyl, CUHK, NTMC, TAU, and UNC) provided population screening data from 664,114 individuals, including 306,982 Europeans and 66,423 Asians (Tables 2 and 3).

We compared frequencies of individuals with these two variants between cases in our multicenter cohort and population controls. P.Met34Thr and p.Val37Ile homozygotes are significantly enriched in cases with ORs of 16 (95%CI 11–25, Z=13, p<0.0001) (Table 2) and 20 (95%CI 17–24, Z=31, p<0.0001) (Table 3) respectively.

Because the allele frequencies of p.Met34Thr and p.Val37Ile were observed highest in European and East Asian populations, respectively, we performed statistical analyses on stratified subpopulations by ethnicity to remove the confounding factor of different ethnic compositions between cases and controls. The significance still held when we performed ethnicity-specific analysis. P.Met34Thr homozygotes were significantly enriched in cases

over population controls in Europeans (OR=9.8, 95%CI=5.5–17, Z=7.8, p<0.0001) (Table 2) and p.Val37Ile homozygotes in Asians (OR=12, 95%CI=9.1–15, Z=19, p<0.0001) (Table 3), respectively. When we considered all biallelic cases involving p.Met34Thr or p.Val37Ile, including homozygotes and compound heterozygotes with another pathogenic variant in *GJB2*, the enrichments were more significant with ORs of 29 (95%CI 22–37, Z=25, p<0.0001) for p.Met34Thr in Europeans (Table 2) and of 19 (95%CI 15–24, Z=26, p<0.0001) for p.Val37Ile in Asians (Table 3) in cases with hearing loss in our multicenter cohort over those who underwent carrier screening at Counsyl. Therefore, both variants meet PS4 (prevalence in affecteds statistically increased over controls) according to ACMG/AMP guidelines[2].

Although alleles were statistically enriched in cases in our multicenter cohort over the general population with ORs of 1.3 (95%CI 1.2–1.5, Z=4.2, p<0.0001) for p.Met34Thr in Europeans (Table 2) and of 1.7 (95%CI 1.5–1.9, Z=8.1, p<0.0001) for p.Val37Ile in Asians (Table 3), the ORs did not exceed 5 to satisfy PS4. We concluded that comparing genotype frequencies is more appropriate than comparing allele frequencies in case-control analyses to interpret variants associated with an autosomal recessive condition, because the unaffected heterozygous carriers contributed the majority of alleles.

Computational predictions

The REVEL scores were 0.702 and 0.657 for p.Met34Thr and p.Val37Ile, respectively. PP3 (computational predictions support pathogenicity) could be applied to p.Met34Thr (REVEL>=0.7) but not to p.Val37Ile because its REVEL score did not reach the threshold (0.7) to support pathogenicity but exceeded the ceiling (0.15) to support a benign role as specified by the ClinGen HL-EP[3].

Different amino acid changes at positions p.Met34 and p.Val37 have been reported in hearing loss patients (Supplementary Information). While they do not constitute sufficient evidence for PM5 (different change at the same residue as a known pathogenic variant), multiple variants of uncertain significance leaning towards pathogenicity affecting the same amino acid residues corroborate with each other.

Segregation Evidence

Literature review showed that both variants segregated with hearing loss in families. Furthermore, we reported at least 35 affected siblings with the same homozygous or compound heterozygous genotypes as probands, including 16 with p.Met34Thr and 21 with p.Val37Ile (Table S2). Therefore, both variants meet PP1_Strong (co-segregation in families, modified to strong)[2,3].

P.Met34Thr and p.Val37Ile homozygotes were present in large population databases and identified by population carrier screening. However, clinical information was unavailable. Some individuals may be underdiagnosed. Furthermore, it has been reported that p.Val37Ile homozygotes lose hearing at ~1 dB/year[15], implicating an age-dependent penetrance. The penetrance by young adulthood was estimated to be 17%[16]. Therefore, we did not consider hearing individuals with biallelic p.Met34Thr or p.Val37Ile as observations in controls or non-segregation.

Allelic Evidence

Both variants have been identified in affected homozygotes or compound heterozygotes. In our multicenter cohort, we observed 138 p.Met34Thr and 141 p.Val37Ile compound heterozygotes (Table S2). However, because of high population allele frequencies and large sample sizes, we applied PM3 (*in trans* with a pathogenic variant in an affected individual) without modifying to strong despite the large number of biallelic cases identified.

Functional Evidence

P.Met34Thr altered gap junction function in *Xenopus* oocytes[17–19] and mammalian cells[20–22]. It had a similar subcellular localization pattern as wild-type connexin 26 in human sweat glands, in transfected HeLa cells, and in transfected COS-7 cells[18,20,21]. Paired *Xenopus* oocytes showed robust conductance when injected with *in vitro* transcribed human wild-type *GJB2* mRNA, reduced conductance when co-injected with wild-type and variant mRNA, and no coupling conductance above background when injected with variant mRNA only[17–19]. Co-injection of *GJB2* c.101T>C with wild-type connexin 30 (encoded by *GJB6*) or connexin 31 (encoded by *GJB3*) also showed reduced conductance[19]. Single channel conductance in c.101T>C transfected HeLa cells was reduced[22], as supported by molecular dynamics simulations[23]. Furthermore, c.101T>C transfected HeLa cells did not transfer Lucifer yellow (a fluorescent dye) to neighbors across gap junctions as wild-type connexin 26 transfected cells[20,22,24] and the ability to transfer neurobiotin was also reduced.[21] However, c.101T>C transfected HeLa cells was able to load the dye in response to non-physiological zero extracellular Ca^{2+} stimulus[25], suggesting that p.Met34Thr variant connexin hemichannels may retain some residual function under unusual circumstances. The atomic structure of human connexin 26 gap junction channels revealed that the methyl group in p.Met34 interacts with p.Trp3 in the amino-terminal helix of an adjacent protomer to stabilize the hexameric channel[26], and alteration of the residue was predicted to impact gap junction coupling[27].

Similarly, Xenopus oocytes injected with mRNA encoding p.Val37Ile showed no conductance above background, and co-injection of p.Val37Ile variant connexin 26 with wild-type connexin 26, connexin 30, or connexin 31 showed reduced conductance[19,28]. Propidium iodide dye transfer was impaired in *GJB2* c.109G>A transfected HEK293T cells[29]. Homozygous knock-in mouse model of c.109G>A in *Gjb2* was reported to have progressive mild hearing loss, more pronounced at higher sound frequencies[30]. However, because the codon for p.Val37 in mouse (GTG) was different from that in human (GTT), c.109G>A in mouse would translate into p.Val37Met, it was not counted towards strong functional evidence. Nonetheless, this *in vivo* animal study was consistent with *in vitro* findings suggesting alteration of p.Val37 impacts GJB2 function.

These functional studies suggest p.Met34Thr and p.Val37Ile impact connexin 26 function; however, they may not truly reflect the biologic process in human cochlea.

Genotype-Phenotype Correlation

We obtained clinical information for 472 cases with biallelic *GJB2* genotypes involving p.Met34Thr or p.Val37Ile (Table S2). P.Met34Thr was with a PTC in 86 cases, including 73

with c.35delG. P.Val37Ile was with a PTC in 92 cases, including 32 with c.235delC (Table 4). We characterized hearing loss for different genotypes, although information was incomplete and non-uniform. Most cases had hearing loss before 18 years old. It was typically bilateral, mild to moderate, and affecting mid- to high- sound frequencies. Progression was reported in all genotype categories (Table 4). In some individuals, profound hearing loss could be due to age-dependent progression or other etiologies. For example, one had meningitis at 1.5 years old, one was 76 years old when tested, and one infant had asymmetric hearing loss with moderate loss in the left ear and severe-to-profound loss in the right ear (Table S2).

Ordered logistic regression models showed no statistically significant difference in severity among p.Met34Thr or p.Val37Ile homozygotes, [p.Met34Thr];[p.Val37Ile], and their corresponding compound heterozygotes with a PTC ($p=0.93$), but p.Met34Thr or p.Val37Ile homozygotes or compound heterozygotes with a PTC presented significantly milder hearing loss than a cohort of 63 c.35delG homozygotes ($p<0.05$), the majority of whom had profound hearing loss. [p.Met34Thr];[p.Val37Ile] compound heterozygotes also had milder hearing loss than c.35delG homozygotes, but the small sample size precluded statistical significance (Table 4).

One infant with [p.Val37Ile];[c.235delC] passed newborn hearing screening but audiology evaluation detected unilateral mild high-frequency hearing loss at five weeks, which progressed to bilateral mild high-frequency hearing loss at 20 weeks. One self-reported unaffected parent was found to be [p.Met34Thr];[c.167delT] via familial testing, and follow-up audiology evaluation revealed mild high-frequency hearing loss at 47 years of age.

Taken together, phenotypic manifestation of *GJB2* p.Met34Thr and p.Val37Ile related hearing loss varied from apparently normal to profound. However, these two variants were typically associated with bilateral mild to moderate sensorineural hearing loss, affecting high- to mid-sound frequencies. Progression has been reported. There was no significant difference in severity among p.Met34Thr or p.Val37Ile homozygotes and compound heterozygotes with another PTC (Table 4). The penetrance could not be calculated. However, among all individuals with biallelic *GJB2* variants involving p.Met34Thr or p.Val37Ile, more compound heterozygotes than homozygotes were observed in our multicenter case cohort compared to the carrier screening population by Counsyl for both p.Met34Thr (OR 2.03, 95%CI 1.22–3.37, $Z=2.7$, $p<0.0064$) and p.Val37Ile (OR 1.90, 95% 1.35–2.66, $Z=3.7$, $p=0.0002$), suggesting a higher penetrance for compound heterozygous genotypes than p.Met34Thr or p.Val37Ile homozygosity.

Summary Interpretation

In summary, p.Met34Thr and p.Val37Ile were classified as pathogenic based on PS4 (homozygotes and compound heterozygotes are significantly enriched in cases over population controls), PP1_Strong (segregated with hearing loss in many affected family members), and PM3 (found *in trans* with many different pathogenic *GJB2* variants in patients with hearing loss).

DISCUSSION

Interpreting variants using case-control statistics

Maximum population allele frequencies for p.Met34Thr and p.Val37Ile are 2% in Finnish and 8% in East Asian in gnomAD, respectively, and in 3.5% (7/198) of Finnish and 17% (32/186) of Chinese Dai in Xishuangbanna in 1000 Genomes Project, respectively. The BA1 (stand-alone for benign, population allele frequency >5%) criterion is not applicable here to p.Val37Ile, because of conflicting evidence suggesting pathogenicity. Based on professional judgement, the ClinGen HL-EP ruled that PS4 overrides BA1.

When applying PS4, we need to choose appropriate cases and controls. Because mild hearing loss may be underdiagnosed without a formal audiology evaluation, we opted to compare cases vs. population controls. Should results be significant in cases over population controls, they would be even more so in cases over unaffected controls.

We chose to use laboratory contributed data for cases instead of relying on published reports to avoid publication biases. Negative results are difficult to get published. Some patients may be included in multiple studies. Large cohort studies do not generally provide detailed case level data, and cases studies do not specify the total number of cases tested for the variant of interest.

In this study, we performed different types of case-control analyses. Although alleles were significantly enriched in cases with ORs of 1.3 (95%CI 1.2–1.5, Z=4.2, p<0.0001) for p.Met34Thr in Europeans (Table 2) and of 1.7 (95%CI 1.5–1.9, Z=8.1, p<0.0001) for p.Val37Ile in Asians (Table 3), the ORs did not exceed 5 to satisfy PS4 according to ACMG/AMP guidelines[2], consistent with a previous meta-analysis[31]. However, ORs of homozygote frequencies met PS4. Hence, comparing individual frequencies is more appropriate than comparing allele frequencies in case-control analyses to interpret variants associated with an autosomal recessive condition, because unaffected heterozygous carriers contributed the majority of alleles.

We observed a larger effect when including compound heterozygotes, which implied a higher penetrance of other pathogenic variants in *GJB2* than p.Met34Thr and p.Val37Ile. Our data indicate that it is preferable to include compound heterozygotes in analyses. Because individual data are unavailable from population databases such as gnomAD, population carrier screening data are invaluable.

Ethnicity information is crucial in case-control studies, because different ethnicity compositions could confound results. In this study, we found significances were inflated when ethnicity information was disregarded. Proportions of p.Met34Thr and p.Val37Ile homozygotes varied significantly among laboratories due to different population compositions tested. Combining data from different laboratories while ignoring ethnicity information would bias towards the ethnic composition of the laboratory that contributed the most data. Stratifying patients by ethnicity significantly reduces the bias but decreases the sample size. Combining data from laboratories around the world allowed us to perform unbiased analysis with sufficient statistical power. Unfortunately, ethnicity information is not

always submitted to testing laboratories, and some laboratories do not collect such information. Furthermore, ethnicity in gnomAD was inferred based on principal component analysis, which may differ from self-reported ethnicity; gnomAD tends to over-estimate major populations as people with a self-reported mixed ethnic background would likely be counted towards one of the major populations. Therefore, we recommend laboratories collect ethnic information on the test requisition form and request patients and physicians to provide such information when ordering genetic tests.

Genotype-phenotype correlation

Variable expressivity of p.Met34Thr and p.Val37Ile has been reported[16,32]. The hearing loss may be unilateral or bilateral, from mild to profound, affecting different frequency ranges, even in individuals with the same genotype. P.Val37Ile has also been associated with pathopoieia[33] and sudden hearing loss[34]. There have been over 100 publications reporting individuals with these variants. However, case-level genotype and phenotype data were limited. This multicenter study found over 96% of biallelic p.Met34Thr or p.Val37Ile cases with bilateral hearing loss including a small percentage with asymmetric presentations, 84% with mild to moderate hearing loss, and a majority with high-frequency loss, consistent with previous reports[35–38]. Individuals with profound hearing loss may have an alternate etiology such as infection or old age. Unilateral or asymmetrical hearing loss may progress to bilateral uniform.

The majority of diagnosed cases in our multicenter cohort had a pediatric onset of hearing loss. The findings could be due to ascertainment bias, because most individuals undergoing genetic diagnosis are younger than 18 years old. Adults with mild hearing loss may not seek audiology evaluation or genetic testing. Progression of hearing loss was only reported in a small percentage of cases, because the progression was slow[15] and long-term follow-up information was unavailable.

We confirmed incomplete penetrance of these two variants. Three Asian p.Val37Ile homozygotes and two Ashkenazi Jewish p.Met34Thr compound heterozygotes were confirmed unaffected by audiology evaluation with the oldest known age at testing of 30 years (Table 5). We also identified 411 individuals with biallelic p.Met34Thr (189) or p.Val37Ile (238) via carrier testing. However, the penetrance could not be quantified based on our data, because they might be truly unaffected or underdiagnosed. It has been estimated that the penetrance of p.Val37Ile homozygotes was 17% in children in China[16], but the number could vary by age, ethnicity, and the allele *in trans* in compound heterozygotes. Our data suggest that the penetrance is higher in p.Met34Thr or p.Val37Ile compound heterozygotes than in corresponding homozygotes.

The mechanism for incomplete penetrance and variable expressivity remains elusive. Environmental and genetic modifiers may play a role. It is unclear how many different haplotypes were involved and whether the phenotype is haplotype-dependent. The haplotype with c.−684_−675del in 5’UTR of NM_004004.5 (rs139514105, referred to as c.−493del10) *in cis* with p.Met34Thr was confirmed in an affected sibpair from the UK[8] and seven affected German individuals[39], but its frequencies in all affected and unaffected biallelic p.Met34Thr populations are unknown. This haplotype did not abolish *GJB2* expression in

cultured keratinocytes, which argues against a regulatory role of the noncoding cis variant[8]. Identification of the modifiers will not only help interpret the genetic findings but also point to effective therapeutic strategies for *GJB2*-related hearing loss, the most common form of hereditary hearing loss. Given the high allele frequency of these two variants in the population, further studies to identify modifiers through population genetic screening and phenotypic evaluation of biallelic individuals are warranted and feasible.

Barriers to accurate variant interpretation

Our experience in analyzing p.Met34Thr and p.Val37Ile in *GJB2* revealed barriers to accurate variant interpretation. First, no phenotypic information was available in large population studies or population screening. Although we were able to achieve statistical significance with a large sample size, should hearing status in the general population be known, a much smaller sample size would be sufficient to provide the same level of statistical power. Second, incomplete ethnicity, family history and clinical information from laboratories and publications diminished the usefulness of many cases. We would urge ordering physicians and testing laboratories to collect and share such information and editors and peer-reviewers to encourage publication of detailed case-level information. Third, case information was biased towards published case reports usually by clinicians and genetic service centers. Although they provided detailed clinical information, matched control information was mostly unavailable. A systematic patient registry to document harmonized clinical and genetic information will overcome this. Finally, given the slow progressiveness and variable expressivity, we still could not accurately determine the penetrance of these variants in manifesting hearing loss and pinpoint the factors that influence the penetrance. Although age seems to be a factor, it could not explain everything. Long-term follow-up of individuals with these variants in prospective studies will illuminate in this area.

CONCLUSION

We conclude that the ACMG/AMP variant interpretation framework can be applied to variants with incomplete penetrance. Large and diverse sample sizes are required to overcome limitations and draw valid conclusions. The ClinGen HL-EP established a collaborative model of operation to collect case-level data, which allowed unbiased analysis of p.Met34Thr and p.Val37Ile, two controversial variants in *GJB2*. Based on compelling statistical and supporting functional evidence, we conclude that the two *GJB2* variants meet criteria to be classified as pathogenic for autosomal recessive sensorineural hearing loss, typically bilateral mild to moderate and slowly progressive over time[15]. When these variants are identified in homozygosity or compound heterozygosity in *GJB2* in cases with profound hearing loss, an alternate etiology should be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Contributing sites

Symbol	Name	Country/Region	Cohort	Type	All[1]	Ethnicity[2]	Biallelic[3]
BCH	Boston Children's Hospital	USA	Individuals with hearing loss	Diagnostic	0	0	35
CCHMC	Cincinnati Children's Hospital Medical Center	USA	Individuals with hearing loss	Diagnostic	1491	692	63
CHOP	Children's Hospital in Philadelphia	USA	Individuals with hearing loss	Diagnostic	163	121	13
Counsyl	Counsyl	USA	Reproductive adults	Screening	654426	364788	0
CUHK	Chinese University of Hong Kong	Hong Kong	Individuals with hearing loss; general population	Diagnostic and screening	5839	5839	6
DY	Dor Yeshorim	USA, Israel	Individuals with hearing loss and family members	Diagnostic and screening	0	0	18
EGL	EGL Genetics	USA	Individuals with hearing loss	Diagnostic	1113	0	32
GDWC	Guangdong Women and Children Hospital	China	Individuals with hearing loss; reproductive adults	Diagnostic and screening	0	0	27
HRyC	Hospital Ramon y Cajal	Spain	Individuals with hearing loss	Diagnostic	3881	3777	65
IG	Integrated Genetics, LabCorp	USA	Individuals with hearing loss	Diagnostic	2883	0	0
LMM	Laboratory for Molecular Medicine, Partners Personalized Medicine	USA	Individuals with hearing loss	Diagnostic	3472	1697	135 (1)
NTMC	National Hospital Organization Tokyo Medical Center	Japan	Individuals with hearing loss	Diagnostic and screening	1884	1884	56 (2)
TAU	Tel Aviv University (including those tested at Rabin and Sheba Medical Centers)	Israel	Individuals with hearing loss; reproductive adults	Diagnostic and screening	719	0	3 (2)
UNC	University of North Carolina	USA	Individuals with hearing loss; general population	Diagnostic and screening	5833	4590	8
VGH	Taipei Veterans General Hospital	Taiwan	Individuals with hearing loss	Diagnostic	45	45	11
				Total	**680985**	**383388**	**472 (5)**

Symbols, names, geographical locations, tested populations, and the type of tests performed are listed.

1. The total number of affected probands and screened populations contributed to statistical analyses of overall populations

2. The total number of probands with relevant ethnicity information contributed to the statistical analyses on ethnicity-stratified populations

3. The total number of p.Met34Thr or p.Val37Ile homozygotes and compound heterozygotes with clinical information contributed to the genotype-phenotype correlation analysis. The numbers of unaffected individuals confirmed by audiology evaluation are in parentheses.

Table 2.

Summary statistics for p.Met34Thr

All[1]	*Cases*[2]	*Population*[3]	*Counsyl*	*OR*	*95% CI*	*Z*	P value
Total number of individuals tested for GJB2	17635	802339	654426				
Total number of alleles tested for GJB2	35270	1604678	1308852				
Total number of individuals with p.Met34Thr	391	10835	8336				
Total number of p.Met34Thr heterozygotes	362	10754	8282				
Total number of p.Met34Thr homozygotes	29	81	54	**16**	**11–25**	**13**	**<0.0001**
Total number of p.Met34Thr compound heterozygotes[4]	147	NA[5]	135				
Total number of p.Met34Thr alleles	420	10916	8390				
Overall p.Met34Thr allele frequency	0.0119	0.0068	0.0064				
Number of European individuals tested for GJB2	7962	382842	304433				
Number of European alleles tested for GJB2	15924	765684	608866				
Number of European individuals with p.Met34Thr	207	7915	5769				
Number of European p.Met34Thr heterozygotes	193	7846	5725				
Number of European p.Met34Thr homozygotes	14	69	44	**9.8**	**5.5–17**	**7.8**	**<0.0001**
Number of European p.Met34Thr compound heterozygotes[4] *with another GJB2 pathogenic allele*	84	N/A[5]	88[6]	**29**[6]	**22–37**[6]	**25**[6]	**<0.0001**[6]
Number of European p.Met34Thr alleles	221	7984	5813	1.3	1.2–1.5	4.2	<0.0001
p.Met34Thr allele frequency in Europeans	0.0139	0.0104	0.0095				

1. Only probands (unrelated individuals) were counted. However, we could not rule out the possibility of related cases from different sites because cases were de-identified before being shared. Nevertheless, the likelihood of such occurrence would be low and would not significantly impact the conclusion.

2. The total number of cases included in statistical analyses did not include BCH, DY, and GDWC where the total number of individuals tested at these sites were not available.

3. The total population data were from Counsyl, CUHK, TAU, UNC, and gnomAD.

4. Compound heterozygosity was presumed in individuals with a second pathogenic or likely pathogenic variant in *GJB2* that had never been reported to have occurred *in cis*.

5. NA: Not available, because individual allele state information is not available from gnomAD.

6. Analyses involving compound heterozygotes were performed using Counsyl data as the population control (see Methods).

Table 3.

Summary statistics for p.Val37Ile

All[1]	*Cases*[2]	*Population*[3]	*Counsyl*	*OR*	*95% CI*	*Z*	P value
Total number of individuals tested for GJB2	17635	802339	654426				
Total number of alleles tested for GJB2	35270	1604678	1308852				
Total number of individuals with p.Val37Ile	464	10233	7609				
Total number of p.Val37Ile heterozygotes	313	9887	7370				
Total number of p.Val37Ile homozygotes	151	346	239	**20**	**17–24**	**31**	**<0.0001**
Total number of p.Val37Ile compound heterozygotes[4]	115	N/A[5]	96				
Total number of p.Val37Ile alleles	615	10488	7848				
Overall p.Val37Ile allele frequency	0.0174	0.0065	0.0060				
Number of Asian individuals tested for GJB2	2066	75857	60355				
Number of Asian alleles tested for GJB2	4132	151714	120710				
Number of Asian individuals with p.Val37Ile	197	6173	4023				
Number of Asian p.Val37Ile heterozygotes	113	5898	3852				
Number of Asian p.Val37Ile homozygotes	84	275	171	**12**	**9.1–15**	**19**	**<0.0001**
Number of Asian p.Val37Ile compound heterozygotes[4] *with another GJB2 pathogenic allele*	49	N/A[5]	46[6]	**19**[6]	**15–24**[6]	**26**[6]	**<0.0001**[6]
Number of Asian p.Val37Ile alleles	281	6360	4194	1.7	1.5–1.9	8.1	<0.0001
p.Val37Ile allele frequency in Asians	0.0680	0.0419	0.0347				

1. Only probands (unrelated individuals) were counted. However, we could not rule out the possibility of related cases from different sites because cases were de-identified before being shared. Nevertheless, the likelihood of such occurrence would be low and would not significantly impact the conclusion.

2. The total number of cases included in statistical analyses did not include BCH, DY, and GDWC where the total number of individuals tested at these sites were not available.

3. The total population data were from Counsyl, CUHK, TAU, UNC, and gnomAD.

4. Compound heterozygosity was presumed in individuals with a second pathogenic or likely pathogenic variant in *GJB2* that had never been reported to have occurred *in cis*.

5. NA: Not available, because individual allele state information is not available from gnomAD.

6. Analyses involving compound heterozygotes were performed using Counsyl data as the population control (see Methods).

Table 4.

Characteristics of hearing cases with biallelic *GJB2* variants involving p.Met34Thr or p.Val37Ilele, including homozygotes and compound heterozygotes with another pathogenic or likely pathogenic variant.

Genotype	[Met34Thr]; [P/LP]	[Met34Thr]; [Met34Thr]	[Met34Thr]; [Val37Ile]	[Met34Thr]; [PTC]	[Val37Ile]; [P/LP]	[Val37Ile]; [Val37Ile]	[Val37Ile]; [PTC]	c.[35delG]; [35delG][1]
Total probands	138	27	17	78	131	139	78	86
Total with characterization[2]	183	28	17	86	127	150	91	86
Age of Onset[3]	177	28	17	83	139	144	78	86
Childhood (<18 years)	155 (88%)	23 (82%)	16 (94%)	72 (87%)	113 (81%)	120 (83%)	50 (64%)	84 (98%)
Laterality	133	21	11	62	109	111	66	86
Unilateral	4 (3%)	0	1 (9%)	2 (3%)	5 (5%)	4 (4%)	3 (5%)	0 (0%)
Bilateral	129 (97%)	21 (100%)	10 (99%)	60 (97%)	104 (95%)	107 (96%)	63 (95%)	86 (100%)
Asymmetrical	15 (12%)	3 (27%)	2 (20%)	9 (15%)	6 (6%)	7 (7%)	1 (2%)	0 (0%)
Frequency ranges[4]	66	14	6	30	59	59	34	1
High	41 (62%)	9 (64%)	5 (83%)	13 (43%)	35 (59%)	41 (69%)	21 (62%)	1 (100%)
Mid	17 (26%)	4 (29%)	1 (17%)	11 (37%)	3 (5%)	10 (17%)	2 (6%)	0 (0%)
Low	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (12%)	0 (0%)	6 (18%)	0 (0%)
Progression	20 (11%)	2 (7%)	4 (24%)	10 (12%)	20 (16%)	10 (7%)	13 (14%)	4 (4%)
Severity	146	24	14	63	121	133	77	63
Mild	55 (38%)	10 (42%)	5 (36%)	26 (41%)	29 (24%)	48 (36%)	23 (30%)	0 (0%)
Moderate	67 (46%)	8 (33%)	7 (50%)	29 (46%)	55 (45%)	52 (39%)	34 (44%)	7 (11%)
Mild+Moderate	*122 (84%)*	*18 (75%)*	*12 (86%)*	*55 (87%)*	*84 (69%)*	*90 (75%)*	*57 (74%)*	*7 (11%)*
Moderately severe	12 (8%)	3 (13%)	2 (14%)	3 (5%)	20 (17%)	12 (9%)	9 (12%)	9 (14%)
Severe	4 (3%)	0 (0%)	0 (0%)	3 (5%)	16 (13%)	20 (15%)	10 (13%)	9 (14%)
Profound	8 (5%)	3 (13%)	0 (0%)	2 (3%)	1 (1%)	1 (1%)	1 (1%)	38 (60%)
Statistics (OR[95%CI], p)[5]								
[Met34Thr];[Val37Ile]		0.86 [0.0–673], 0.97						
[Met34Thr];[PTC]		0.76 [0.02–34], 0.89	0.88 [0.0–281], 0.97					

Genotype	[Met34Thr]; [P/LP]	[Met34Thr]; [Met34Thr]	[Met34Thr]; [Val37Ile]	[Met34Thr]; [PTC]	[Val37Ile]; [P/LP]	[Val37Ile]; [Val37Ile]	[Val37Ile]; [PTC]	c.[35delG]; [35delG][1]
[Val37Ile];[Val37Ile]		1.35 [0.04–49], 0.87	1.56 [0.0–432], 0.88	1.77 [0.47–7.7], 0.40				
[Val37Ile];[PTC]		1.36 [0.04–57], 0.87	1.00 [0.34–3.00], 0.88	1.78 [0.33–9.4], 0.50		1.00 [0.34–3.00], 0.99		
c.[35delG];[35delG]		**79 [1.5–4210], 0.0315**	91 [0.3–32548], 0.13	**103 [11–902], <0.0001**		**58 [10–336], <0.0001**	**58 [7.7–438], <0.0001**	

Abbreviations: [P/LP], a pathogenic or likely pathogenic allele in *GJB2*; [PTC], an allele with a premature termination codon in *GJB2*; OR, odds ratio; CI, confidence interval; p, p value.

1. Data on c.35delG cases were from the Laboratory for Molecular Medicine only.

2. All information is not available for all individuals.

3. When age of onset was not available, age of testing was used as a surrogate.

4. Flat audiogram shapes that affect all frequency ranges are under-reported.

5. Odds ratio refers to the genotype in the column header more likely to be milder than that in the row header. Statistically significant differences in severity are in bold.

Table 5.

Unaffected p.Met34Thr or p.Val37Ile homozygotes or compound heterozygotes confirmed by audiology evaluation.

Site	ID	Var 1 DNA	Var 1 AA	Var 2 DNA	Var 2 AA	Age tested	Family history	Ethnicity
LMM	56s	c.109G>A	p.Val37Ile	c.109G>A	p.Val37Ile	7 years old	sibling	Asian
NTMC	101	c.109G>A	p.Val37Ile	c.109G>A	p.Val37Ile	28 years old	no	Asian
NTMC	102	c.109G>A	p.Val37Ile	c.109G>A	p.Val37Ile	30 years old	no	Asian
TAU	13	c.101T>C	p.Met34Thr	c.269T>C	p.Leu90Pro	Adult	unknown	Ashkenazi/Iraqi Jewish
TAU	14	c.101T>C	p.Met34Thr	c.167delT	p.Leu56Argfs	unknown	unknown	Ashkenazi Jewish

Individuals without phenotypic information or audiology evaluation are not listed.