HHS Public Access

Author manuscript

Clin Genet. Author manuscript; available in PMC 2021 July 01.

Published in final edited form as:

Clin Genet. 2021 July ; 100(1): 59–78. doi:10.1111/cge.13956.

Exome sequencing utility in defining the genetic landscape of hearing loss and novel-gene discovery in Iran

Marzieh Mohseni[*,1], **Mojgan Babanejad**[*,1], **Kevin T Booth**[2,3], **Payman Jamali**[4], **Khadijeh Jalalvand**[1], **Behzad Davarnia**[5], **Fariba Ardalani**[1], **Atefeh Khoshaeen**[6], **Sanaz Arzhangi**[1], **Fatemeh Ghodratpour**[1], **Maryam Beheshtian**[1], **Faezeh Jahanshad**[7], **Hasan Otukesh**[8], **Fatemeh Bahrami**[1], **Seyed Morteza Seifati**[9], **Niloofar Bazazzadegan**[1], **Farkhondeh Habibi**[10], **Hanieh Behravan**[1], **Sepide Mirzaei**[1], **Fatemeh Keshavarzi**[1], **Nooshin Nikzat**[1], **Zohreh Mehrjoo**[1], **Holger Thiele**[11], **Michael Nothnagel**[11,12], **Hela Azaiez**[2], **Richard J Smith**[2], **Kimia Kahrizi**[#1], **Hossein Najmabadi**[#1]

[1.]Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

[2.]Molecular Otolaryngology and Renal Research Laboratories, Department of Otolaryngology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

[3.]Harvard Medical School, Department of Neurobiology, Boston, Massachusetts, USA

[4.]Shahrood Genetic Counseling Center, Welfare Organization, Semnan, Iran

[5.]Department of Anatomy and Pathology, Ardabil University of Medical Sciences, Ardabil, Iran.

[6.]Mehrgan Genetic Clinic, Sari, Iran

[7.]Navid's Institute of Infertility, Tehran, Iran

[8.]Department of Pediatric Neurology, Iran University of Medical Sciences, Tehran, Iran

[9.]Medical Biotechnology Research Center, Islamic Azad University, Ashkezar, Yazd, Iran

[10.]Genetic Counseling Center of Welfare Organization, Rasht, Guilan, Iran

[11.]Cologne Center for Genomics (CCG), University of Cologne, Cologne, Germany

[12.]University Hospital Cologne, Cologne, Germany

[#] These authors contributed equally to this work.

Abstract

Correspondence: Hossein Najmabadi, Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Daneshjoo Blvd, Koodakyar St., Evin, Tehran, Iran. hnajm12@yahoo.com, Kimia Kahrizi, Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Daneshjoo Blvd, Koodakyar St., Evin, Tehran, Iran. kahrizi@yahoo.com.
[*]Marzieh Mohseni and Mojgan Babanejad are joint first authors

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability Statement

All variants identified in this study have been submitted to ClinVar (https://submit.ncbi.nlm.nih.gov/clinvar/, Submission ID: SUB8234804). The other data that support the findings of this study are available from the corresponding authors upon reasonable request.

Hearing loss (HL) is one of the most common sensory defects affecting more than 466 million individuals worldwide. It is clinically and genetically heterogeneous with over 120 genes causing non-syndromic HL identified to date. Here, we performed exome sequencing (ES) on a cohort of Iranian families with no disease-causing variants in known deafness-associated genes after screening with a targeted gene panel. We identified likely causal variants in 20 out of 71 families screened. Fifteen families segregated variants in known deafness-associated genes. Eight families segregated variants in novel candidate genes for HL: *DBH*, *TOP3A*, *COX18*, *USP31*, *TCF19*, *SCP2, TENM1*, and *CARMIL1.* In the three of these families, intrafamilial locus heterogeneity was observed with variants in both known and novel candidate genes. In aggregate, we were able to identify the underlying genetic cause of HL in nearly 30% of our study cohort using ES. This study corroborates the observation that high-throughput DNA sequencing in populations with high rates of consanguineous marriages represents a more appropriate strategy to elucidate the genetic etiology of heterogeneous conditions such as HL.

Introduction

Hearing, the process of translating physical sound waves into electrochemical signals is a highly dynamic and orchestrated process. The involvement of ~1% of the genes in the human genome makes hearing loss (HL) one of the most genetically heterogeneous conditions. Not surprisingly, this genetic heterogeneity is reflected at the phenotypic level with variability in type, onset, progression, symmetry, audioprofile, and severity.[1]

HL affects millions of people globally and could be due to genetic defects, environment, or both factors.[2] Congenital sensorineural HL affects 1 in every 500 newborns in developed countries and it is estimated that ~50% of individuals will experience HL in their lifetimes due to damage induced by noise or aging. The rate of congenital HL, like many genetic diseases, increases in populations with high rate of consanguineous marriages, such as Iran.[3] These populations have played an important role in the identification of novel genes, specifically those associated with autosomal recessive hearing loss (ARHL).

Traditional approaches to novel gene identification took years and required families large enough to perform linkage analysis. Through the years, this approach has led to the identification of more than 75% of genes known to be implicated in HL. However, many families that were too small to establish a significant log of the odds (LOD) score or families with intrafamilial locus heterogeneity remained unsolved.[4]

Advances in genomic high-throughput sequencing offered new opportunities in genetic studies, including discovering causative genes in Mendelian diseases regardless of family size.[5] Furthermore, they facilitated large-scale screening projects revealing an unprecedented genomic and variation diversity within the deafness-associated genes, and within distant ethnic groups.[6, 7] It has now become feasible to investigate complex families, small families, and sporadic cases.

Using both single-gene sequencing and high-throughput sequencing, we previously revealed that *GJB2* pathogenic variants are responsible for about 16% of autosomal-recessive non-syndromic HL (ARNSHL) in the Iranian population.[8] In a study of over 300 *GJB2*-negative

Iranian families, we identified the causative pathogenic variants in 67% of families using a custom Targeted Genomic Enrichment panel (OtoSCOPE V5&V6).[9] Since these studies, significant advances in identifying genetic causes of HL in the Iranian population has been made as ~80% of the studied families were offered a positive genetic diagnosis for their HL using the above-mentioned methods.

In recent years, there has been a shift away from targeted gene panels to exome and genome sequencing. Their advantage lies in their ability to facilitate the discovery of novel genes underlying Mendelian diseases in individuals where known disease-causing genes have already been ruled out. This approach has been shown to be fruitful in diseases that are both genetically and phenotypically heterogeneous such as recessive cognitive disorders.

Here, we used a similar approach utilized by Najmabadi et al,[10] to investigate the genetic causes of HL in 71 families. We identified pathogenic variants in known genes associated with HL, revealed an unexpected amount of intrafamilial locus heterogeneity, and identified eight novel candidate genes for HL. Overall, this study illustrates the power of using exome sequencing (ES) to unravel the genetics of HL and improves our understanding of the biology of hearing and deafness.

Materials and Methods

Patients included in this study were sequentially referred to the Genetics Research Center (GRC) at the University of Social Welfare and Rehabilitation Sciences (USWR) during the past 20 years for genetic testing. Prior to the start of the study, ethical approval was obtained from the Ethical Board of USWR. Each participant signed consent forms for their participation in this study. This study was conducted according to the ethical standards as defined by the Helsinki Declaration.

A detailed clinical examination was completed. Hearing thresholds were measured by pure-tone audiometry at 250, 500, 1000, 2000, 4000, and 8000 Hz following standard protocols.

Prior to this study, all patients underwent genetic screening for pathogenic variants in *GJB2* using Sanger sequencing. Patients negative for variants in *GJB2* were screened using OtoSCOPE panel (V5 & V6); a targeted gene panel covering deafness-associated genes as previously described.[9] Considering that OtoSCOPE-negative families are a valuable cohort to identify novel deafness-associated genes, we studied 70 OtoSCOPE-negative families by ES. We also performed ES on one additional family without any prior screening with OtoSCOPE (Figure 1). In each family, only one affected member underwent ES, except three families (L-665, L-1119, and L-8600574). This study was conducted between 2015 and 2019.

Exome enrichment was performed using Agilent SureSelectXT Human All Exon V6. All enriched libraries were sequenced on the Illumina NextSeq500 using 100 bp paired-end reads. After quality control assessment using the FastQC toolkit, 100–150 bp reads were aligned to the human reference genome hg19 using the Burrows-Wheeler Aligner (BWA).[11] Picard tool applied to perform BAM processing and then realignment of indel regions, recalibration of base qualities, and variant detection and calling were performed using the

Genome Analysis Toolkit (GATK)[12] to produce variant call format files, which were then annotated using ANNOVAR.[13] Variant filtering was based on quality/coverage depth (≥3) and minor allele frequency (MAF <0.5%) as reported in the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/).[14] We also considered MAF of all these variants in the Iranome Database (http://www.iranome.com/).[15] Variants meeting these criteria were further filtered based on inheritance mode, coding effect, and annotation for conservation and pathogenicity. All potentially causal variants detected by ES were validated by Sanger sequencing and co-segregation studies were performed, including all available and informative family members.

We prioritized candidate genes obtained through ES based on their expression pattern in the auditory system using the gEAR[16] (https://umgear.org/) and SHIELD (https://shield.hms.harvard.edu/).

The analysis of run of homozygosity (ROH) was performed with the Varbank v.3.4 exome pipeline of the Cologne Centre for Genomics (CCG), including alignment against the human GRCh38 reference genome using BWA-mem[11] and variant calling with the GATK haplotype caller.[12] Varbank uses a linkage-based approach with a recessive consanguineous inheritance model (https://varbank.ccg.uni-koeln.de/varbank2/). We constructed a pedigree with a first cousins' relationship and assumed a disease allele frequency of 0.0001. Known high-quality single-nucleotide variants (SNVs) (GQ > 98; VQSLOD>2; QD > 6; MQ > 60; FS < 20; MQRankSum> − 2.5) with a maximum population allele frequency less than 80% according to gnomAD v2.01[14] were chosen and run with Allegro v1.2c[17] in overlapping windows of 2000 SNVs each. Regions with a LOD-score < 0.8 (maxLOD = 1.2) were filtered out. ROHs were considered for families exhibiting consanguinity.

Variants in known deafness-associated genes were checked in the Deafness Variation Database (https://deafnessvariationdatabase.org/) and classified according to the deafness-specific ACMG/AMP guidelines and our expert curation.[7, 18]

The impact of amino acid substitutions and 3D modeling of wild-type and mutant amino acid in novel candidate genes were investigated using the Project Hope server, I-Tasser,[19] and PyMOL. Thermodynamic predictions were calculated using STRUM.[20]

Results

This study was conducted from 2015 to 2019 and some results have already been reported.[21, 22] In 20 out of 71 families investigated (~ 30%), plausible causal defects were identified in known deafness-associated genes as well as novel candidate genes, all co-segregating with HL. The phenotypic data of families are summarized in Table 1.

We identified 15 families with variations in known deafness-associated genes. Three families segregated pathogenic variants in *CDC14A* which are as follows: L-692 and L-1096: NM_003672.4:c.1126C > T:p.(Arg376*) and L-1347: NM_003672.4:c.1351_1352del: p.(Ala451Thrfs*43).[21] Two families (L-350 and L-8900107) segregated the same pathogenic variant in *NARS2* (NM_001243251.1: c.658A > G: p.[Met220Val]). Families L-1119 and L-8700223 segregated pathogenic variants in

TMIE, NM_147196.2:c.250C > T:p.(Arg84Trp) and c.122_125dup:p.(Pro43Alafs*73), respectively. In family L-665 a frameshift variant (NM_017433.5:c.1370_1371del:p.[Arg457Asnfs*25]) in *MYO3A* was identified. Affected individuals in family L-1108 were homozygous for the previously described pathogenic variant (NM_001302455.2:c. 293C > T:p.[Thr98Met]) in *EDN3*. A novel pathogenic frameshift variant (NM_078485.4:c.1915del:p.[Val639Trpfs*51]) in *COL9A1* was found co-segregating with Stickler syndrome (MIM:614134) in family L-1326. Pathogenic or likely pathogenic variants in *CLDN14* (NM_012130.4:c.40_41insTGGTGCACGGCCGTGCA:p.[Ser14Metfs*15]), *ATP6V0A4* (NM_130840.3:c.710_712del:p.[Lys237del]), *CLCNKA* (NM_001042704.2:c.55C > T:p.[Gln19*]), *MYO7A* (NM_000260.4:c.5442 T > G: *p.[Tyr1814*]*) and *SLC52A2* (NM_001253815.2:c.973 T > G:p.[Cys325Gly]) were found to be co-segregating with deafness in families L-3100, L-8600034, L-8600220, L-8600574, and L-9501254 respectively (Figure 2). Audiograms for these families are shown in Supplementary Figure 1.

In summary, we identified 13 likely pathogenic or pathogenic deafness-causing variants in 11 genes previously associated with non-syndromic or syndromic HL in our cohort. These variants comprise homozygous missense (n = 4), nonsense (n = 3), frameshift (n = 5), and in-frame deletion variants (n = 1). Five out of the 13 variants are novel and have not been previously linked to deafness (Table 2). In seven families, the causative genes are linked to syndromic forms of HL (*EDN3*, *COL9A1*, *ATP6V0A4*, *CLCNKA*, *NARS2, MYO7A*, and *SLC52A2*) (Table 1).

Family L-347 is a consanguineous family with Azeri ethnicity and profound ARNSHL (Figure 3A). We identified a missense variant (NM_000787.4:c.1486C > T:p.[Pro496Ser]) in *DBH* gene located in a ROH spanning 8 Mb. The p.(Pro496Ser) variant is highly conserved, predicted to be deleterious by in silico tools, and is absent from all population databases (Table 3).

Family L-665 with Fars ethnicity consists of multiple affected branches. Initially, DNA from individual V:5 was sent for ES, which revealed a homozygous frameshift variant in *MYO3A* (NM_017433.5:c.1370_1371del:p.[Arg457Asnfs*25]). This variant segregated with HL in generation V, but not in deaf individuals in generation VII (Figure 3(B)). The audioprofiles were different between the two branches (Table 1, Figure 3(B)). Subsequently, individual VII.1 was sent for ES. After variant filtering and prioritization, a missense variant (NM_004618.5:c.1651G > A; p.Val551Met) in *TOP3A* gene was identified. This novel variant alters a highly conserved residue, and it is predicted deleterious by in silico tools (Table 3).

Family L-1119 with Azeri ethnicity segregates HL in two branches (Figure 3(C)). Initially, we screened affected individual VI:4. After variant filtering, we identified an ultra-rare homozygous missense variant (NM_001300729.1:c.914G > A; p.[Arg305His]) in the *COX18*. This variant is located in a region of homozygosity of ~40 Mb. It is conserved and predicted to be damaging by in silico tools. This variant also segregated in a homozygous state in the affected sibling but not in the second branch of the family (Figure 3(C)). We then

performed ES on affected individual V:3 and identified the recurrent mutation (NM_147196.2:c.250C > T; p.Arg84Trp) in *TMIE*.

Two patients from a consanguineous family with Azeri ethnicity exhibited post-lingual moderate-to-severe NSHL (L-1208; Figure 3(D)). We identified a missense variant (NM_020718.3:c.2533A > G; p.[Ser845Gly]) in *USP31* gene. This variant, located in a ~ 35 Mb ROH, is ultra-rare (MAF < 0.0088% in gnomAD), impacts a highly conserved residue and is predicted damaging.

L-3033 is a non-consanguineous family of Fars ethnicity with three affected individuals (a mother and her two sons) with severe HL (Figure 3(E)). After exploring all inheritance modes; we identified a heterozygote missense variation (NM_001077511.2:c.482G > A; p.[Arg161Gln]) in the *TCF19* gene that segregated in this family. This variant is conserved and predicted to be deleterious by some in silico tools. It is rare (gnomAD MAF: 0.0011%) and absent from the Iranome database (Table 3).

In four affected individuals from a consanguineous marriage with Gilaki ethnicity and profound ARNSHL (L-8600456; Figure 3(F)), we identified a missense variant (c. 554A > G;p.[Asp185Gly]) in the *SCP2* gene. The variant is ultra-rare, highly conserved, and located in 17 Mb ROH.

In L-8600574 family, three out of the five affected individuals had retinitis pigmentosa (RP) and deafness whereas the other two exhibited non-syndromic deafness only (Figure 3(G)). ES performed on one affected individual with deafness and RP, identified a truncating pathogenic variant (c.5442 T > G; p.Tyr1814*) in *MYO7A*. However, this variant did not segregate in the two affected individuals with isolated deafness. Subsequent ES of an individual with isolated deafness identified a deleterious and conserved variant (c.3273G > T; p.Gln1091His) in *TENM1*. This variant co-segregated with the non-syndromic HL. Since these two affected siblings were male, *TENM1* gene represents a good novel X-linked candidate gene.

Family L-8900097 is of Azeri ethnicity with three affected individuals and profound deafness (Figure 3(H)). We identified a missense variant (NM_001173977.2:c.3245G > A;p.[Arg1082Gln]) in the *CARMIL1* gene that segregated with deafness. This variant is located inside a ROH of ~15 Mb. It is rare (MAF: 0.004416% in gnomAD), highly conserved, and predicted to be damaging.

Discussion

HL is the most common congenital sensory impairment, affecting around 466 million people worldwide. The HHL homepage currently lists more than 120 genes for NSHL; and it is estimated that HL is part of more than 400 genetic syndromes. The multitude of genes and variants and different inheritance patterns involved in HL pathogenesis along with the clinical heterogeneity, make the establishment of proper diagnosis difficult.[7]

This study involved 71 multiplex families from Iran. Using ES, we identified plausible causal genetic defects in 20 families (~30%). Pathogenic and likely pathogenic variants were

identified in 11 known deafness-associated genes. Five out of the 13 (38.4%) identified variants in known genes are novel and not yet linked to deafness. Variants in eight novel candidate genes were also identified (Tables 2 and 3).

In two families, pathogenic variants in *COL9A1* and *ATP6V0A4* made it possible to diagnose two families with syndromic HL; Stickler syndrome type IV and distal renal tubular acidosis (dRTA) 3, respectively. We identified a missense variant in *SLC52A2* gene in a consanguineous family with Brown Vialetta van Laere syndrome. Early diagnosis of this syndrome may be lifesaving as high-dose oral riboflavin therapy has been reported to result in clinical improvement.[23] Pathogenic variants in *EDN3* associated with Waardenburg syndrome type 4B and *CLCNKA* associated with Bartter Syndrome, Type 4B were found each in one family.

Variants in *NARS2* are involved in both syndromic (Combined oxidative phosphorylation deficiency 24 MIM: 616239) and NSHL (Deafness, autosomal recessive 94 MIM:618434). Families L-350 and L-8900107 with the same mutation in *NARS2* are not from the same village or geographical location or ethnicity. Affected individuals in family L-8900107 underwent clinical reevaluation and showed syndromic form of *NARS2*. We could not collect more detailed clinical data for family L-350, so it is unclear whether this family has syndromic HL or NSHL.

In three families, intrafamilial locus heterogeneity was detected. Family L-8600574 presented both syndromic and NSHL within the core family. In L-665 and L-1119, two different genes have been identified in different branches. These cases highlight the importance of comprehensive clinical and molecular characterization of patients within the same pedigree to recognize possible distinct phenotypes and underlying genetic defects. This is valuable in establishing a correct diagnosis and thereby providing appropriate genetic counseling.

The c.1486C > T variant in the *DBH* gene changes a highly conserved proline into serine at position 496. The wild-type residue and newly introduced mutant residue differ in size, charge, and hydrophobicity. Prolines are very rigid conferring a special backbone conformation; the alteration to serine is predicted to disturb this conformation (Figure 4(A)). This is supported by a large change in free energy folding between the wide-type and mutant residues ($\Delta\Delta G$:−1.65). The Dopamine beta-hydroxylase protein is predicted to interact with the LRTOMT protein, which is responsible for DFNB63-related deafness. The DBH is a biosynthetic enzyme catalyzing the formation of norepinephrine (NE) in the noradrenergic nerve endings of the central and peripheral nervous systems. In 2010, Maison et al. found that the $Dbh^{-/-}$ mice are significantly more vulnerable to middle-ear infection than littermate controls.[24] Shepard et al. suggested that the NE is required for critical period plasticity in the auditory cortex, while intrinsic tonotopic patterning of auditory cortical circuitry occurs independently from NE. In 2018, Lee et al. reported that the catecholamine NE acts in the auditory cortex to shape local processing of complex sound stimuli. Moreover, it also enhances the coding accuracy of neurons in the auditory cortex as well as the downstream sensorimotor cortex.[25]

Topoisomerase 3a is required for segregation of human mtDNA. Pathogenic variants in *TOP3A* have been linked to two different conditions: progressive external ophthalmoplegia with mitochondrial DNA deletions, autosomal recessive 5 (MIM: 618098), and microcephaly, growth restriction, and increased sister chromatid exchange 2 (MIM: 618097). The two affected individuals in L-665 family (branch 2) presented post-lingual NSHL with no other symptoms. The variant c.1651G > A:p.(Val551Met) in *TOP3A* gene changes a highly conserved residue and occurs in the active domain of TOP3A that may disturb contact with other molecules and might affect the signal transduction between the domains (Figure 4(B)). This gene is highly expressed in the inner ear in mice. The three identified DNA topoisomerases in mammalian mitochondria (I, IIβ, IIIα) could solve any topological problems in replication, transcription, and cellular transactions.[26–28] Wenjun Xia et al. recently identified pathogenic variants in *TOP2B* gene as a cause of ADHL in a Chinese family with 48 members. Affected individuals showed progressive mild post-lingual HL. They found significant abnormalities in the inner ear in *top2b* knockdown zebrafish. Their work showed that *top2b* has a critical role in the PI3K-Akt pathway and its alteration will reduce the number of supporting cells and hair cells.[29]

The c.914G > A variant in the *COX18* gene changes arginine into histidine at position 305. Molecular modeling showed that Arg305 interacts with three other amino acids: Pro302, Gln306, and Phe328. Changing residue 305 to His disturbs interactions with Gln306 and Phe328 (Figure 4(C)). This variant is in the repeated domain named mitochondrial matrix and is predicted to disturb its function. The COX18 protein is required for the insertion of integral membrane proteins into mitochondrial inner membrane. It is essential for the activity and assembly of cytochrome c oxidase and plays a central role in the translocation and export of the C-terminal part of the COX2 protein into the mitochondrial intermembrane space. A previous study in mice showed an association between the *Cox2* gene and sensorineural HL. There is an interaction between *COX18* and *COX2* for promoting translocation in the inner membrane.[30]

USP31 is an ubiquitin-related gene that recognizes and hydrolyzes the peptide bond at the C-terminal of ubiquitin and is involved in the processing of poly-ubiquitin precursors. In Border Collies, SNPs in *USP31* were strongly associated with adult-onset deafness, possibly by a dysregulation of NF-kB activity; which is associated with increased levels of cochlear apoptosis and HL.[31] The c.2533A > G variant in the *USP31* gene identified in L-1208 family changes serine into glycine at position 845. The mutant residue is smaller and more flexible than the wild-type residue, which might cause loss of interactions or disrupts protein conformation. 3D modeling showed alteration of the interaction of mutant residue with the other residues (Figure 4(D)). More recently, Kazmierczak et al. implicated another member of the ubiquitin protein family (USP53) as a novel component of tight junction-associated proteins in the cochlear epithelial cells that is essential for the survival of auditory hair cells and normal hearing in mice.[32]

The c.482G > A variant in *TCF19* gene changes arginine into glutamine at position 161. The wild-type residue is positively charged whereas the mutant residue is neutral. 3D modeling showed this change results in the loss of one hydrogen bond (Figure 4(E)). The TCF19 protein is a trans-activating factor that has a critical role in cell cycle progression.[33] In one

study, it has been shown that SPARC and TCF19 have critical roles in the development and function of cochlea in the rat. The *TCF19* gene is highly expressed in connective tissues of the cochlea.[34] It has also been shown that TCF19 is regulated by vezatin, a protein associated with actin filaments and is essential for the sound plasticity of cochlear hair cells.[35] It is possible that the p.Arg161Glu perturbs or alters this interaction resulting in dysregulation of TCF19. More recently, while exploring the role of ATOH1 in vestibular hair cell regeneration, Taylor et al. showed adenoviral transduction of human vestibular epithelium with *ATOH1* upregulated several important transcription factors, including TCF19. The addition of ATOH1 and the upregulation of these transcription factors supported the differentiation of supporting cells into hair cell-like cells.[36]

The c. 554A > G variant in the *SCP2* gene changes aspartic acid into a glycine at position 185. 3D modeling predicted that the wild-type residue interacts with Val25 and Ser81 but in the presence of Gly, the interaction with Ser81 is lost (Figure 4(F)). Furthermore, this variation impairs the thiolase_N domain, which is an important domain for the protein function. The SCP2 protein is a peroxisomal enzyme with thiolase activity that is required for the breakdown of branched-chain fatty acids. Variants in *SCP2* have been linked to a spectrum of phenotypes linked to abnormal fatty-acid acyl-CoA metabolism and Zellweger syndrome.[37, 38] A computational analysis of interacting partners of SCP2 revealed interaction with other members of the peroxisomal pathway including ACOX1, PEX5, PEX14, and HSD17B4.[39, 40] The latter is a known gene associated with syndromic deafness. It is worth noting that the affected individuals in family L-8600456 do not present any of the previously reported symptoms associated with SCP2 protein defect.

The c.3273G > T variant in the *TENM1* gene changes glutamine into histidine at position 1091. In 3D modeling, His1091, loses interaction with Tyr1089, and interacts with Lys1092 as compared to the wild-type residue (Figure 4(G)). The wild-type amino acid is highly conserved, and its alteration is predicted to be deleterious. The TENM1 protein belongs to the teneurins family genes, such as cell adhesion molecules and plays a role in regulating the establishment of proper connectivity within the nervous system. In mice, *TENM1* is expressed in the embryonic nervous system, sensory organs, and widely in the auditory system. In *Drosophila melanogaster*, this protein has a synaptic-partner-matching role between axons in the olfactory sensory neurons and target neurons. It also has a critical role in synapse organization in the olfactory system.[41] In chick, expression of teneurin-1 is detected in different brain regions involved in various processes including the processing of auditory information, general sensation, olfaction, and the coordination of complex motor behavior.[42]

The c. 3245G > A variant in the *CARMIL1* gene changes arginine into glutamine at residue 1082. The wild-type arginine has a positive charge, unlike the mutant glutamine that has no charged side chain. The wild-type arginine interacts with residues Ser1080 and Glu1081, but with the mutant residue, the interaction with Glu1081 is lost (Figure 4(H)). The *CARMIL1* gene encodes a F-actin uncapping protein comprised of 11 leucine-rich repeats, a coil–coil domain and a CAPZA2 inhibitory region. The p.Arg1082Glu variant is located in the CAPZA2 inhibitory region and could perturb this interaction, which may result in dysregulation of F-actin capping. The CARMIL1 multidomain protein has critical roles in

the cell such as cell development, differentiation, and disease. CARMIL1 regulates actin assembly, which is important to actin motility. For actin motility, CARMIL1 has interaction with Myosin 1E, Myosin 1C, and Twinfilin.[43] Pathogenic variants in myosin proteins have been linked to hearing problems, because of the contributions of this protein to intracellular functions such as transportation, actin-binding, and signaling. Studies showed that Twinfilin and CARMIL interaction regulates actin assembly in mechanosensory stereocilia in the inner ear.[44]

We identified 13 pathogenic variants in known genes. Missed diagnoses using a targeted gene panel were due to several reasons. In one case, the indel variant in *CLDN14* gene was identified but the genotype was inaccurate (heterozygote instead of homozygote) due to mapping error for indels in early versions of the analytical pipeline. In nine families, the identified causal genes were not covered on early versions of OtoSCOPE (V5 & V6) and thus were not screened. In two cases (L-665 and L-1119), intrafamilial genetic heterogeneity prevented the identification of the second cause of HL in the other branches of the family for which affected individuals were not screened with OtoSCOPE. Although the diagnosis for these families was initially missed; given evolving panel designs coupled with the high throughput, accuracy in variant calling and the ability to do high-resolution CNV calling, a tiered approach such as described here is still the most practical approach to diagnostics and novel gene discovery.

We identified eight potential novel candidate genes in Iranian patients (*DBH, TOP3A, COX18, USP31, TCF19, SCP2, TENM1*, and *CARMIL1*). We cannot conclusively assert their implication in HL due to the lack of additional families and functional studies. However, we believe publishing these findings and sharing them with the larger auditory community will give others the chance to look for them in their cohorts and potentially further validate them. Functional studies are required to confirm and evaluate the role of these genes in deafness.

For some families, our genetic study did not identify any genetic cause. Several reasons could explain these findings: aetiologic heterogeneity within families; poor ES depth of coverage in some regions; causative variant located in intronic or intergenic regions, and missed CNVs using ES data. Investigating these families using genome sequencing may resolve some of these challenges.

In conclusion, using technologies such as ES in genetically and clinically heterogeneous disorders and in populations with a high rate of consanguinity greatly accelerates the identification of the many novel genes that are hitherto unknown. The discovery of novel genes responsible for HL improves our knowledge of the mechanisms and pathways involved in this condition. It also creates opportunities for carrier screening among relatives and offer opportunities for informed choice in family planning, thus providing a direct benefit to families, communities, and to public health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank all patients and families who participated in this study.

This study was supported by the National Institute for Medical Research Development “NIMAD” (grant no. 982338) to HN, the Iran National Science Foundation (INSF) (grant no. 96011200) to KK, NIH/NIGMS grant support on T32 GM007748 to KTB, and NIDCDs R01’s DC002842, DC012049 and DC017955 to RJS.

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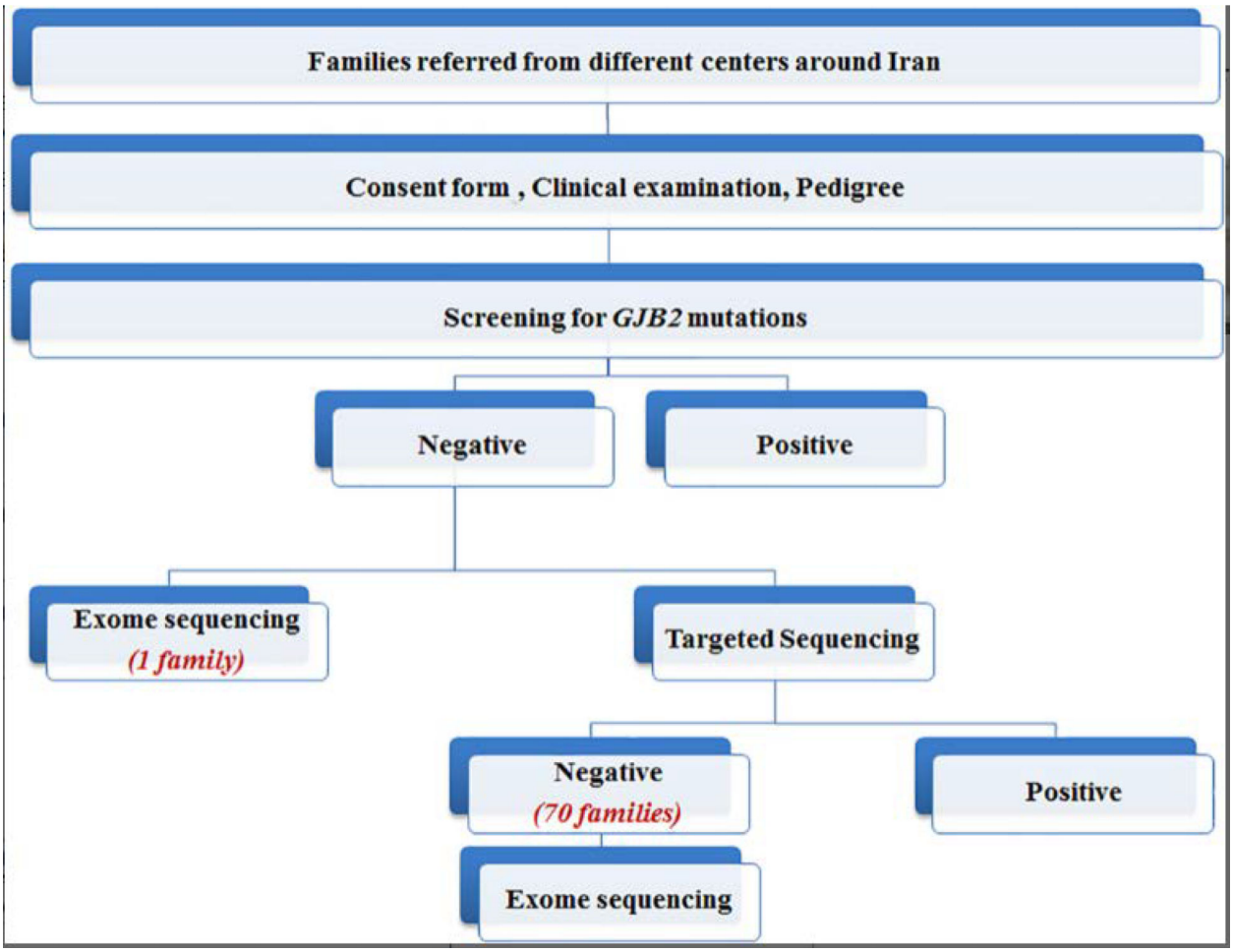
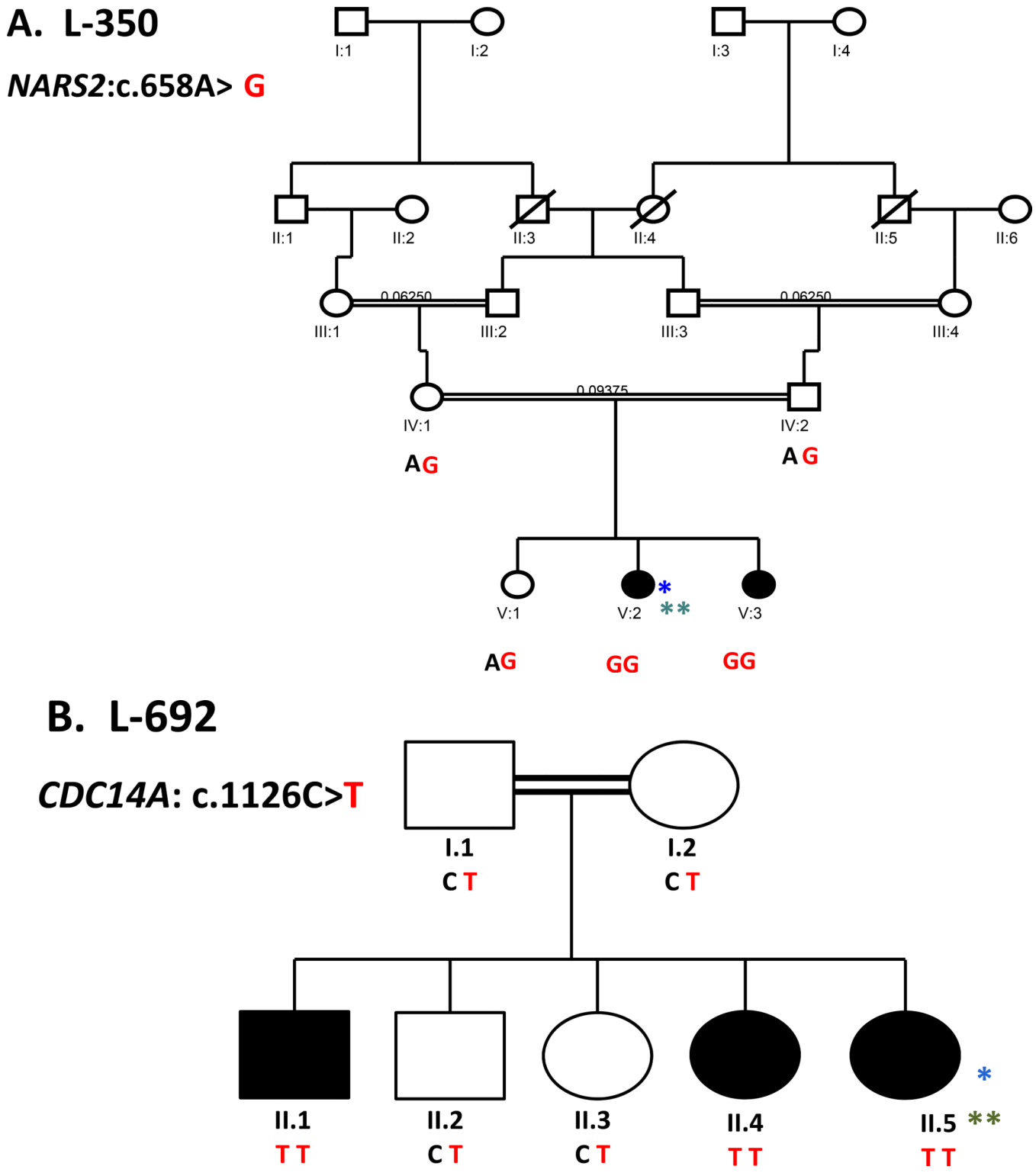
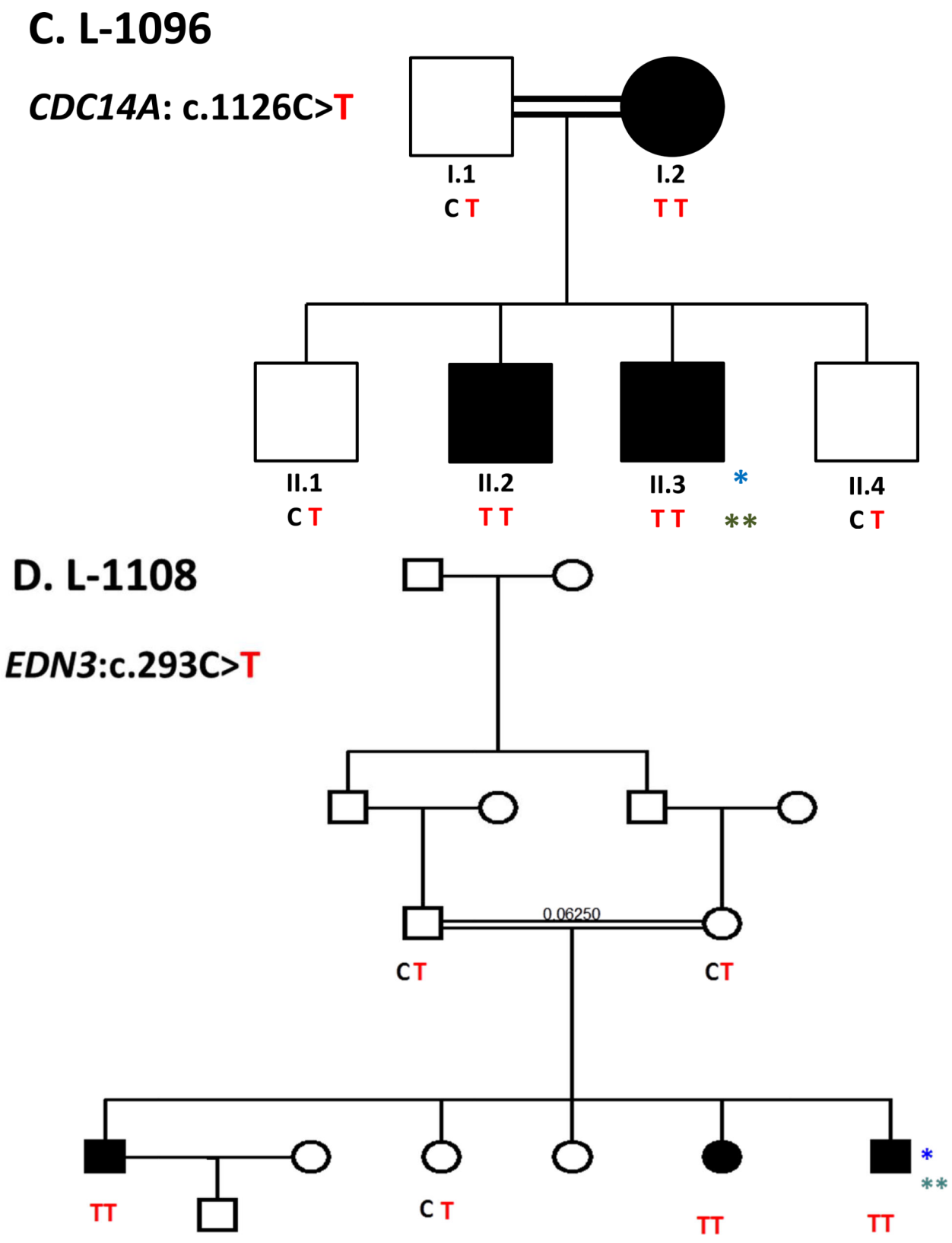
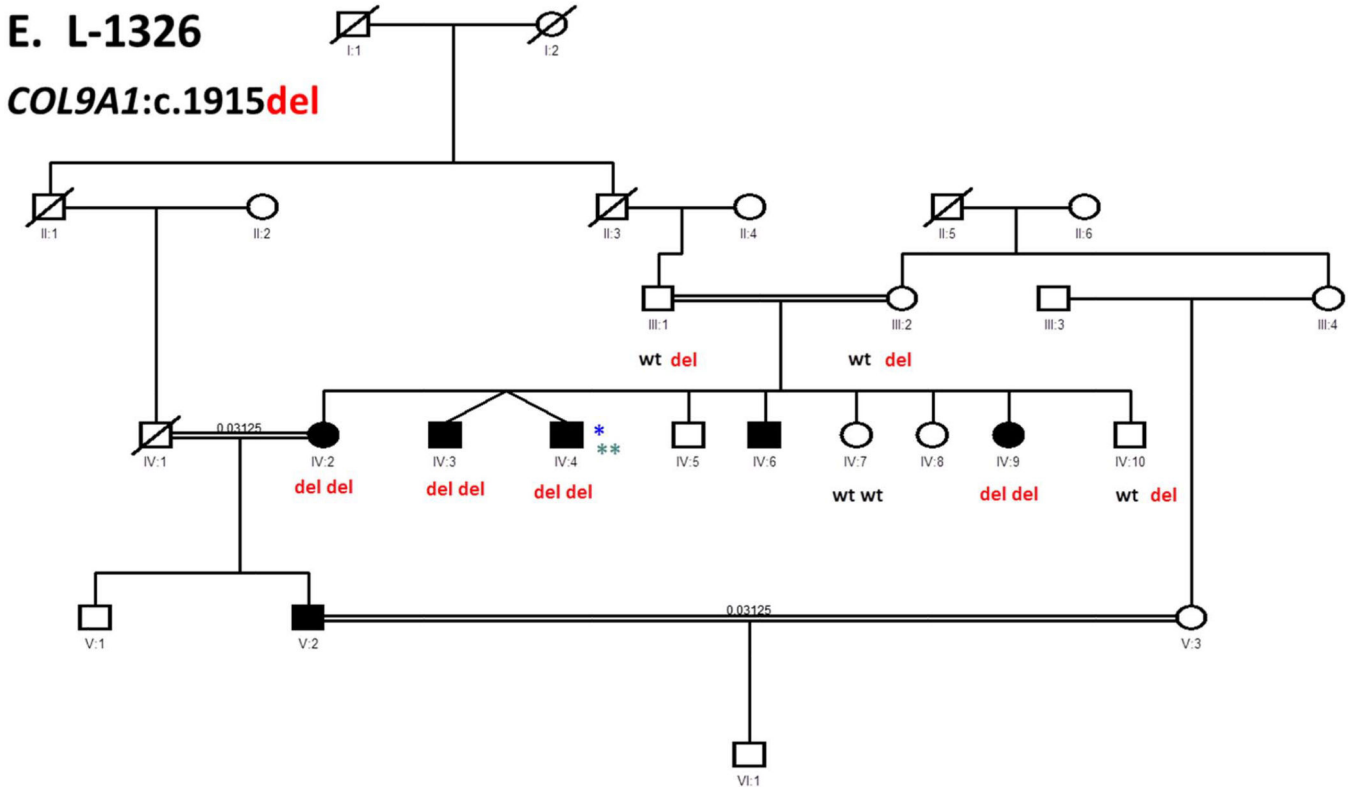
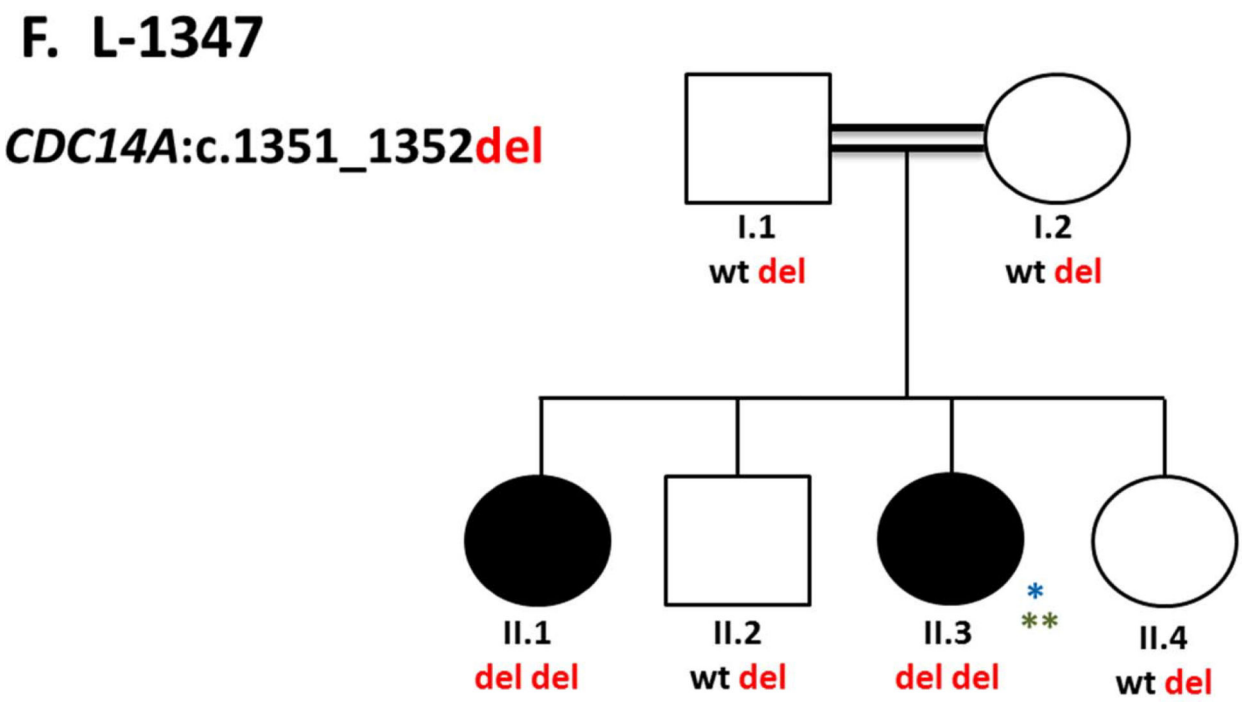


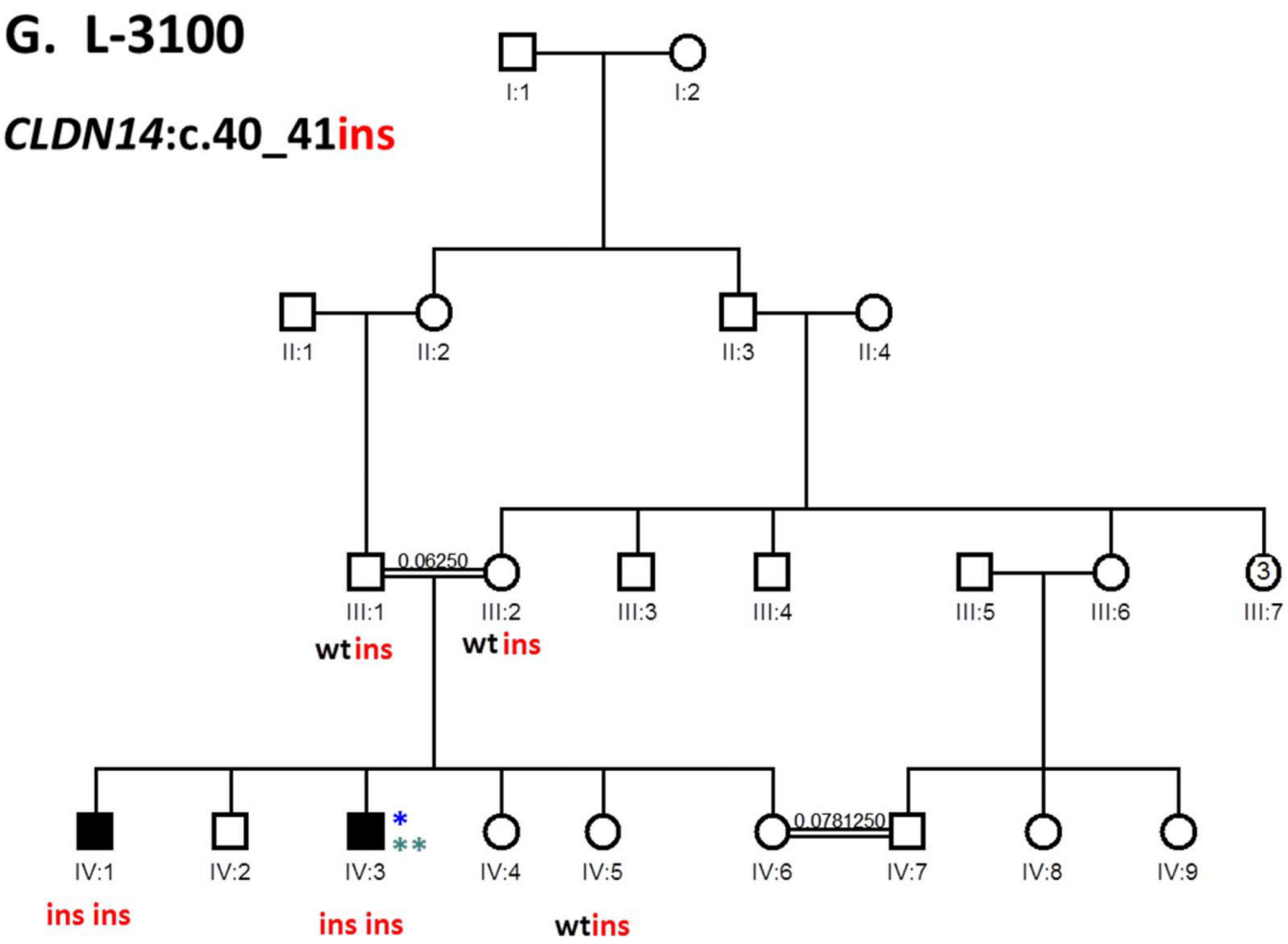
Figure 1:
Flow diagram demonstrates the process of identifying the genetic cause of hereditary hearing loss in affected families in this study

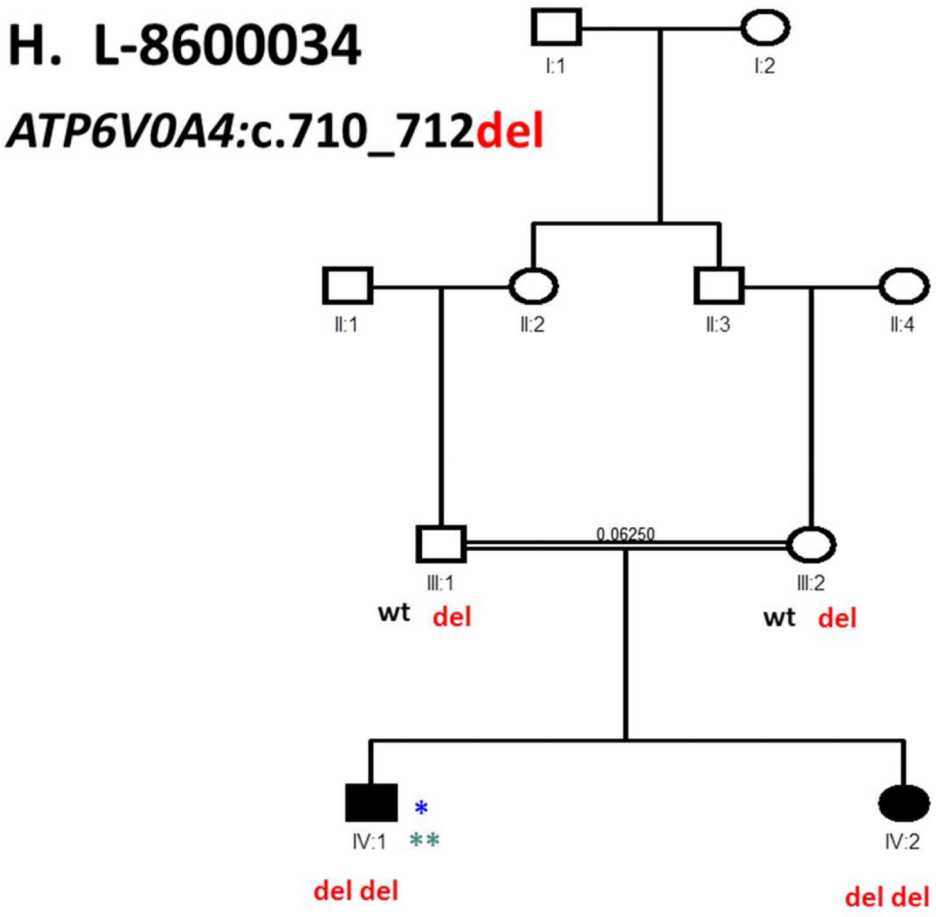


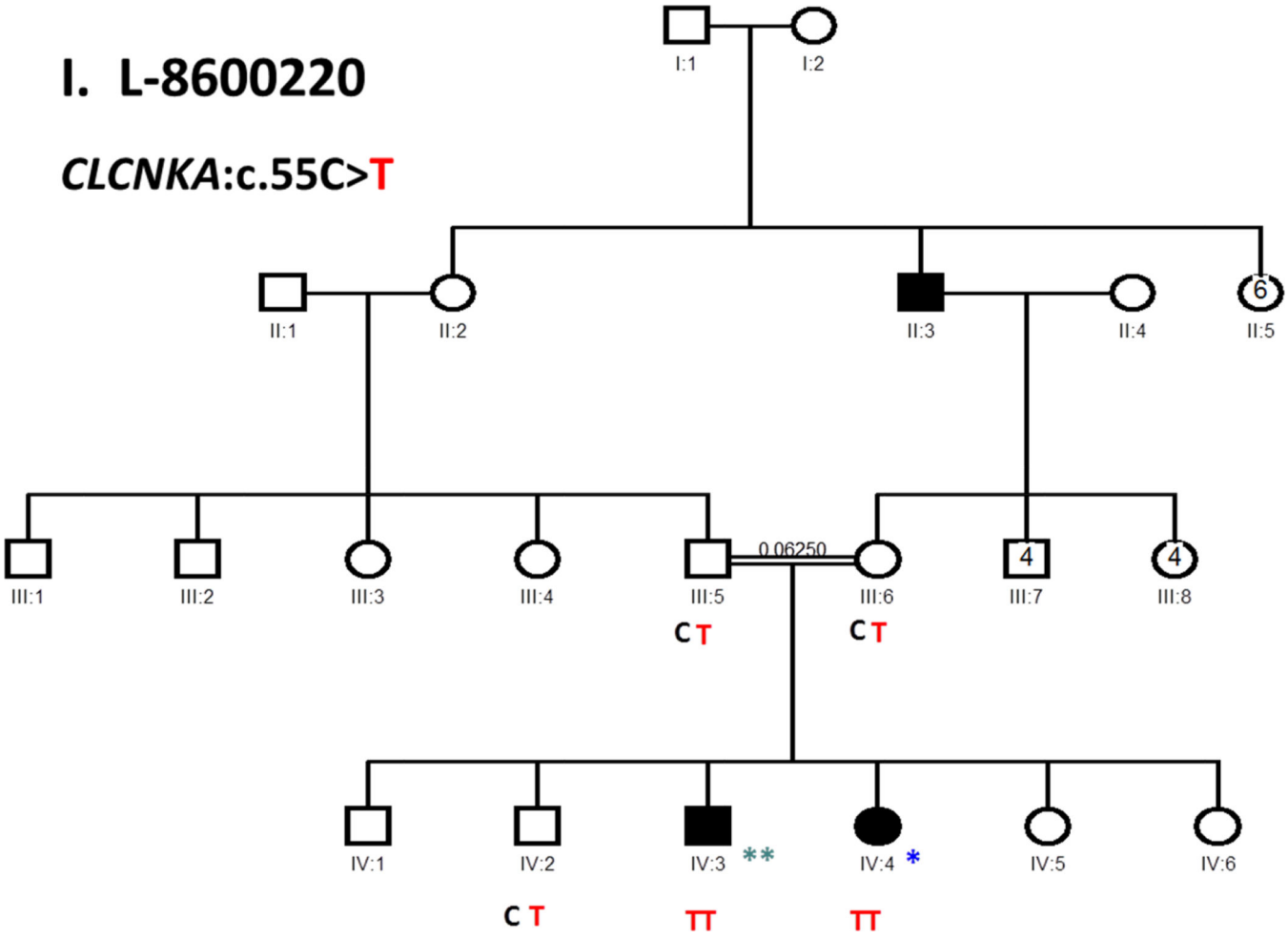


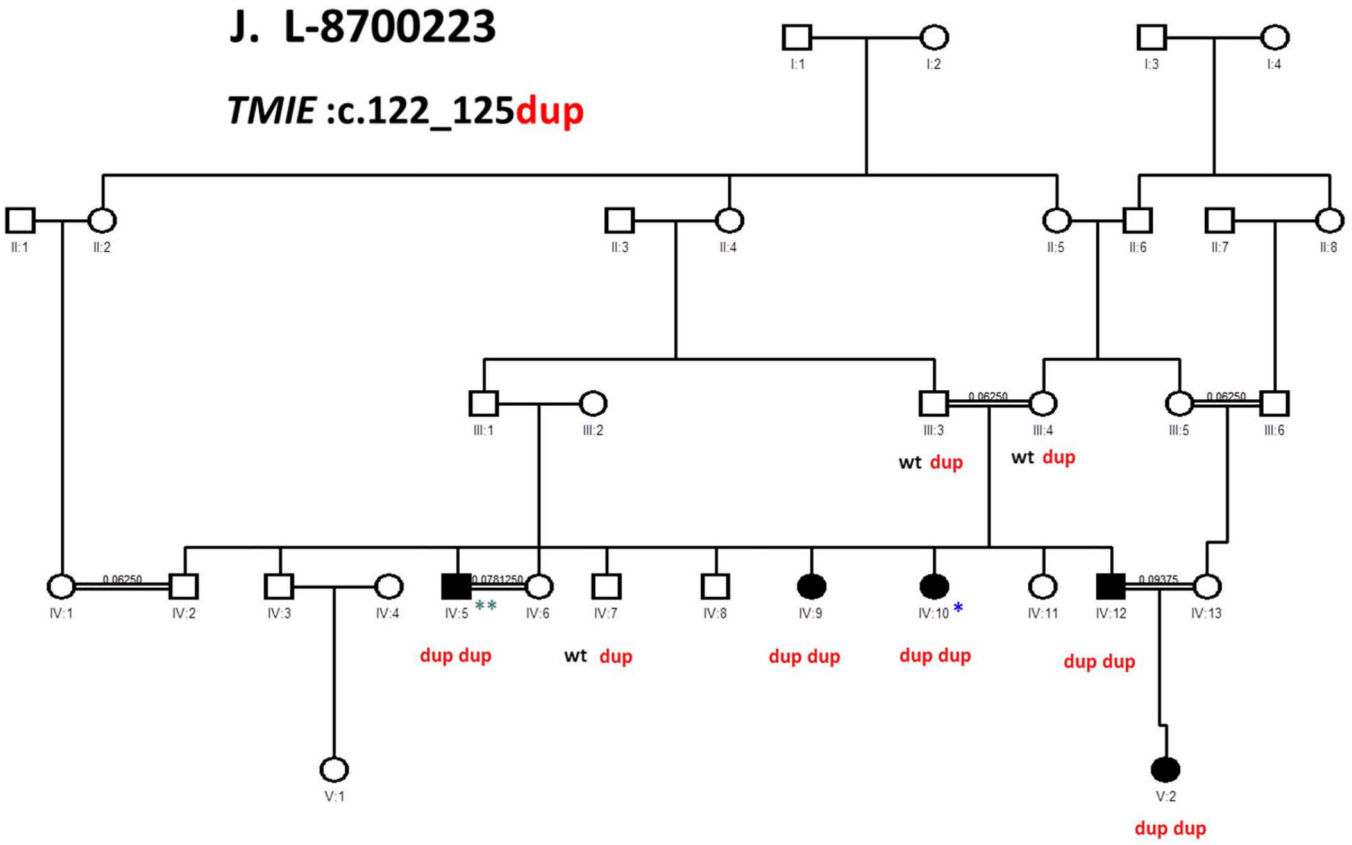


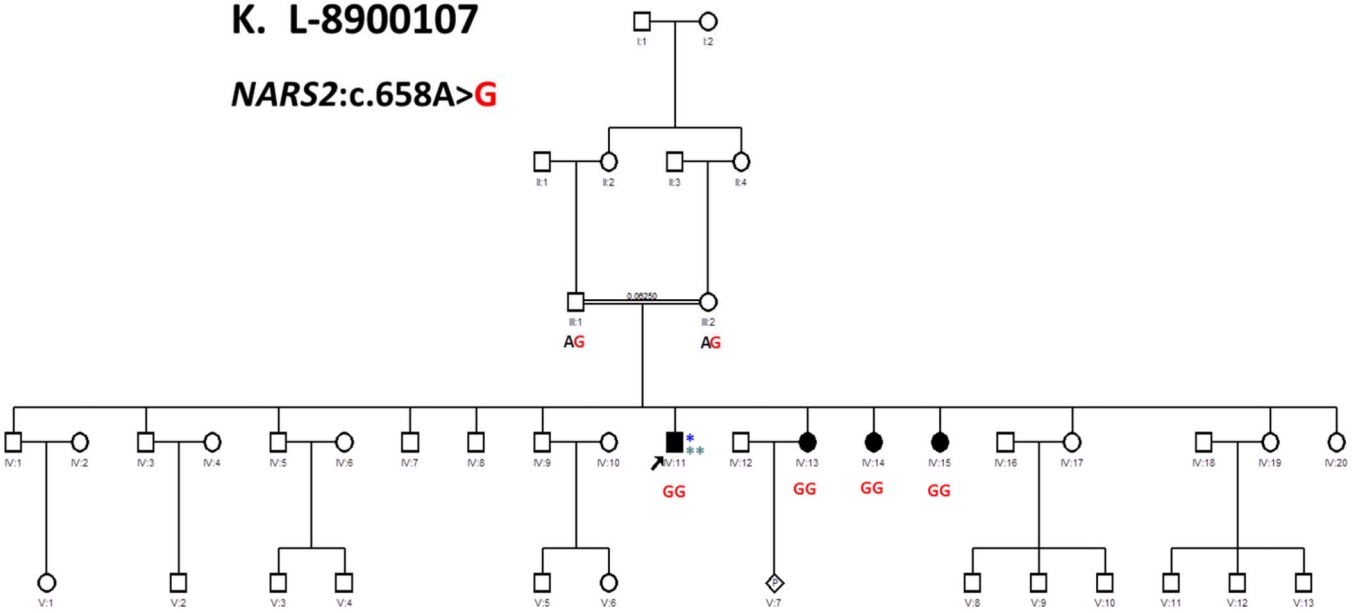












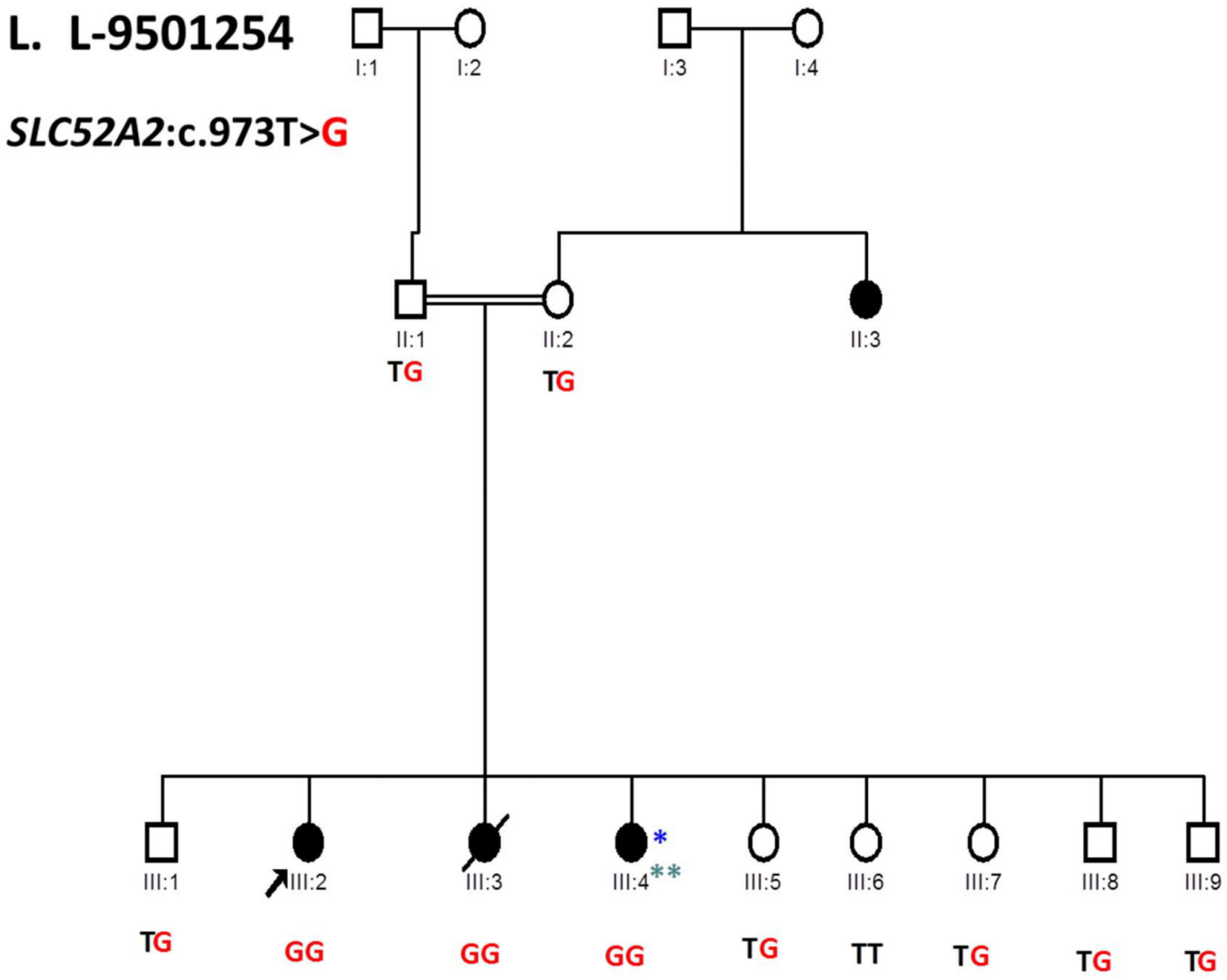
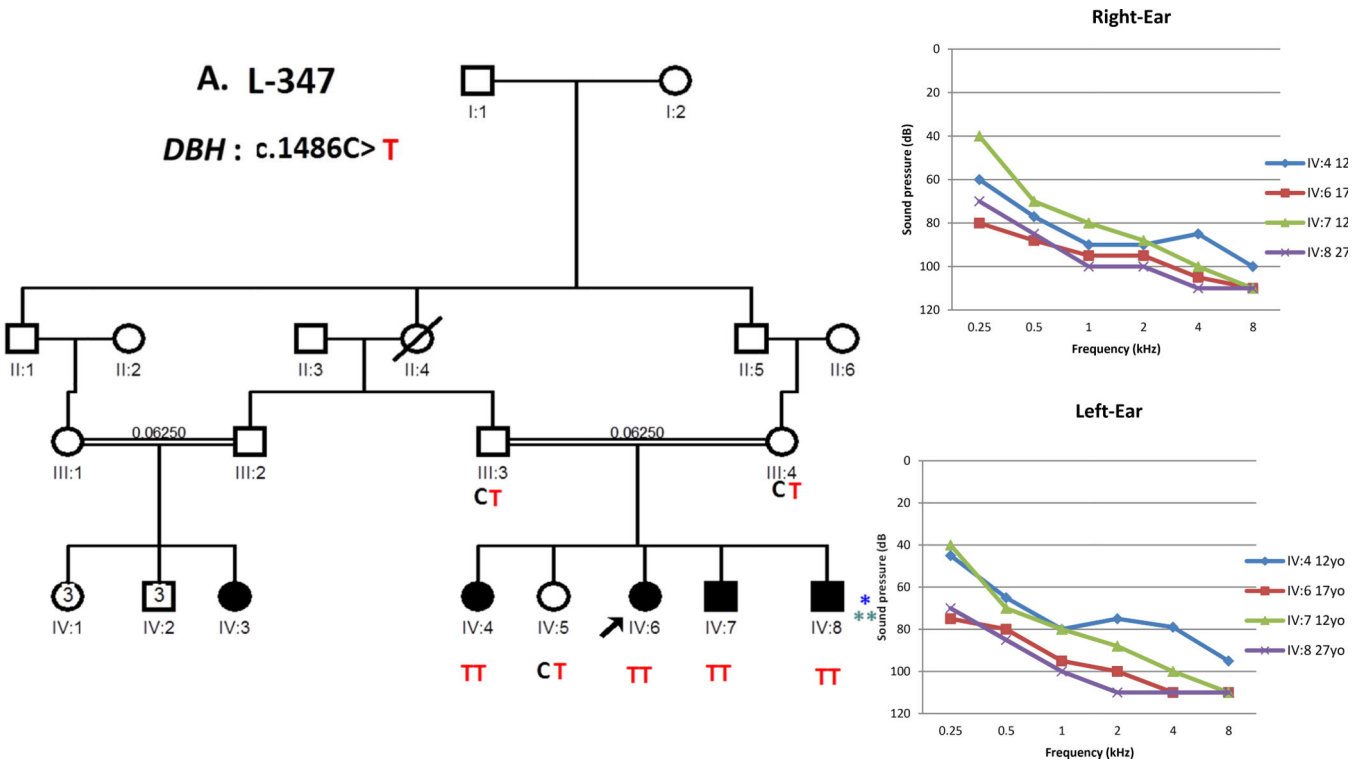
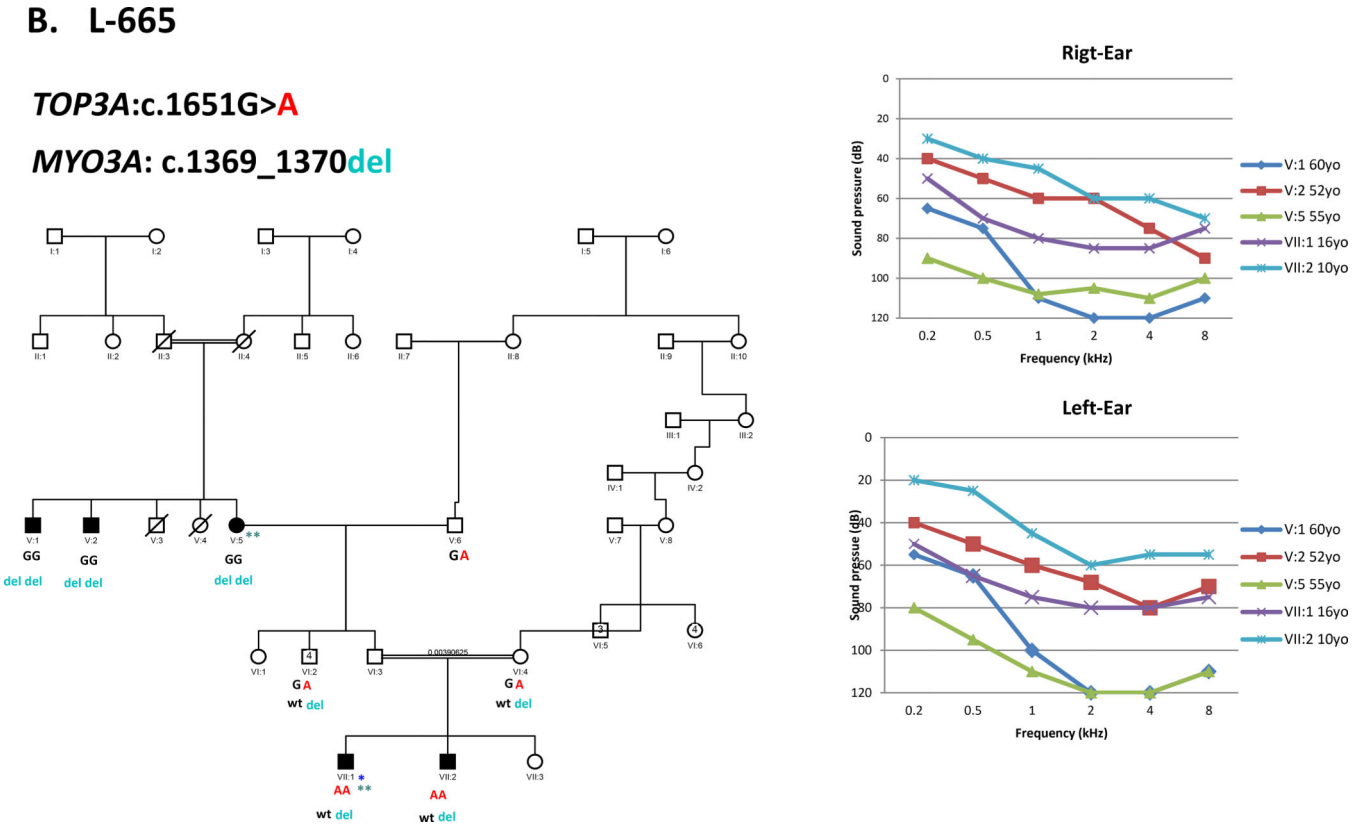
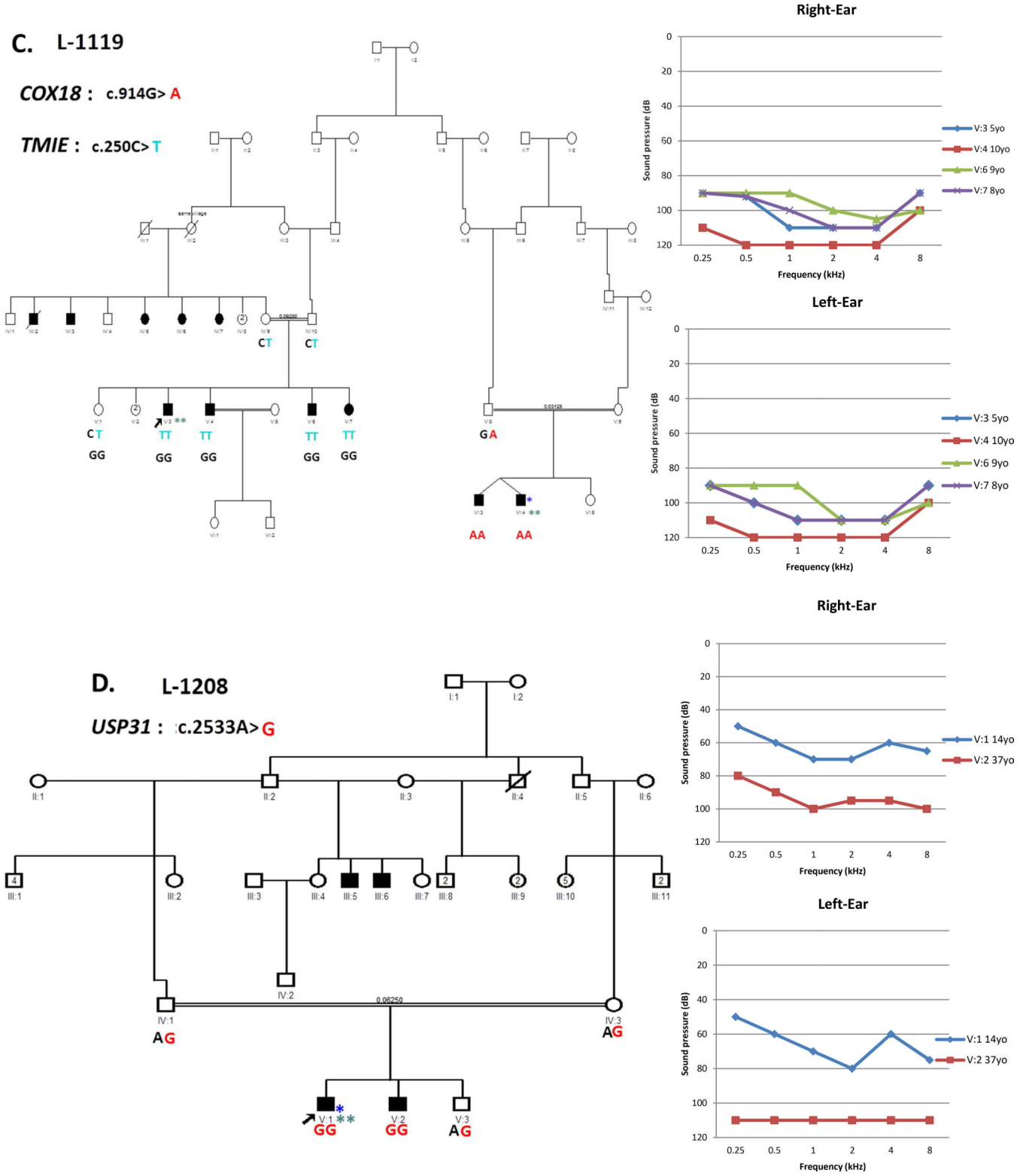


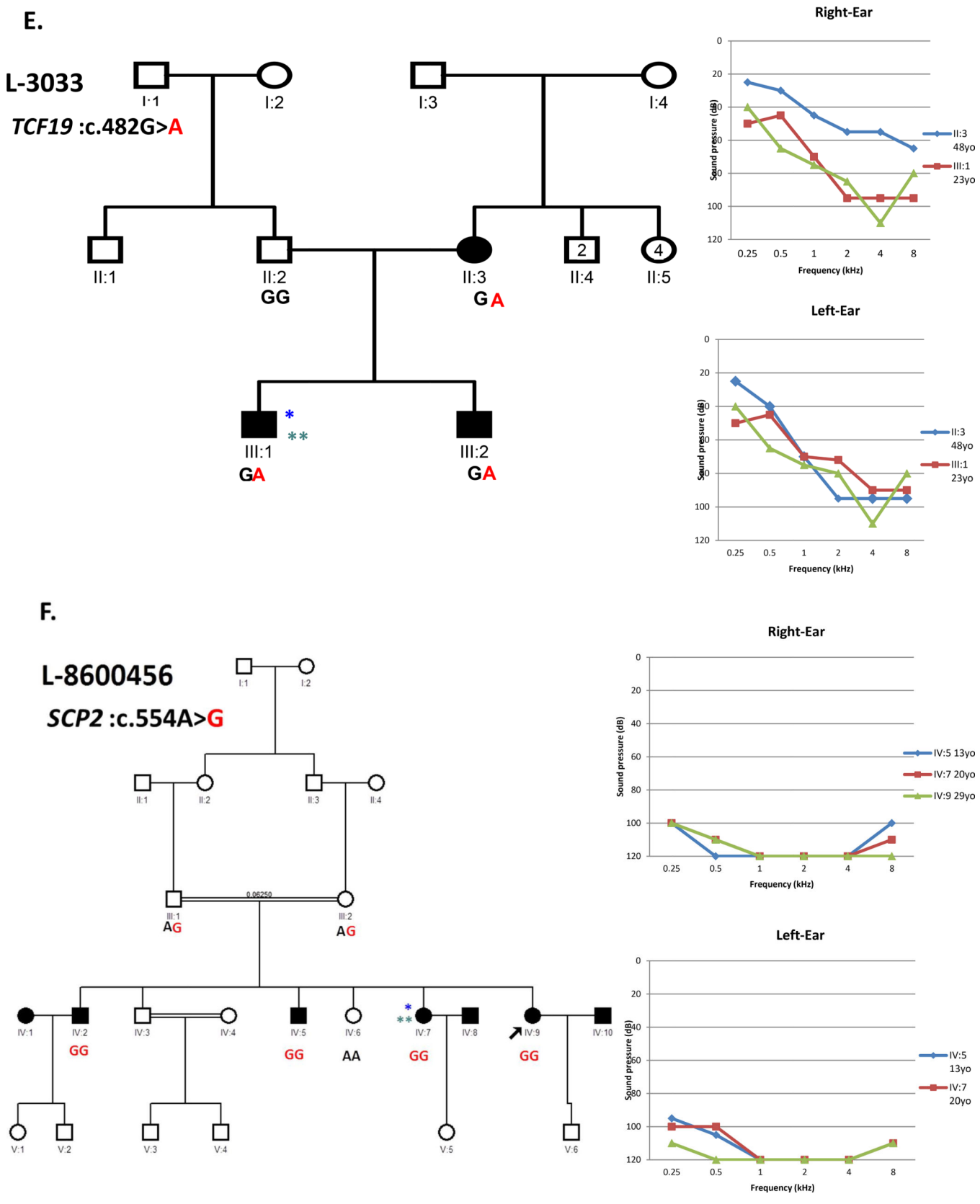
Figure 2:

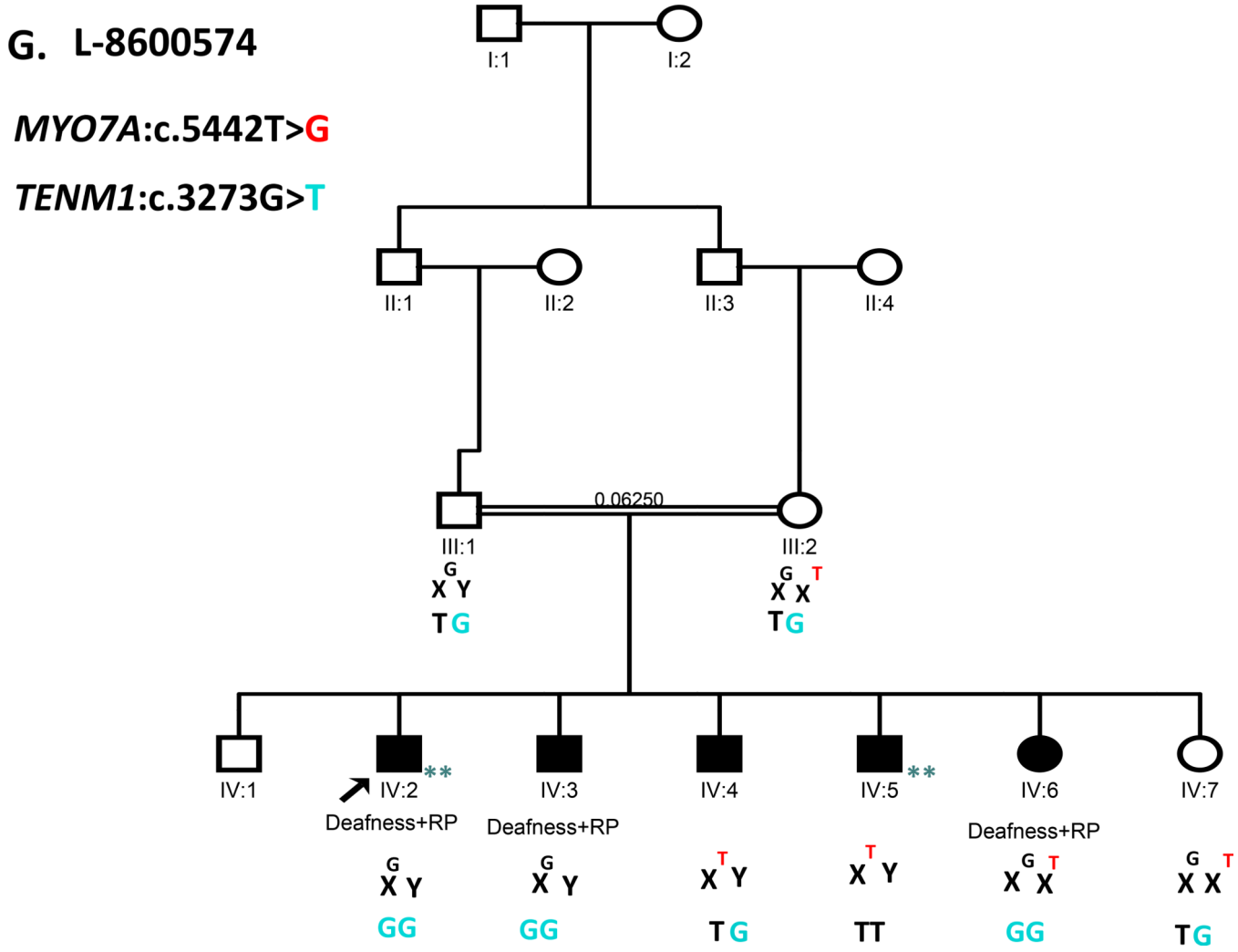
Pedigrees and genotypes of families with variants in known genes (*individuals run on OtoSCOPE, **individuals run on ES). ES, exome sequencing











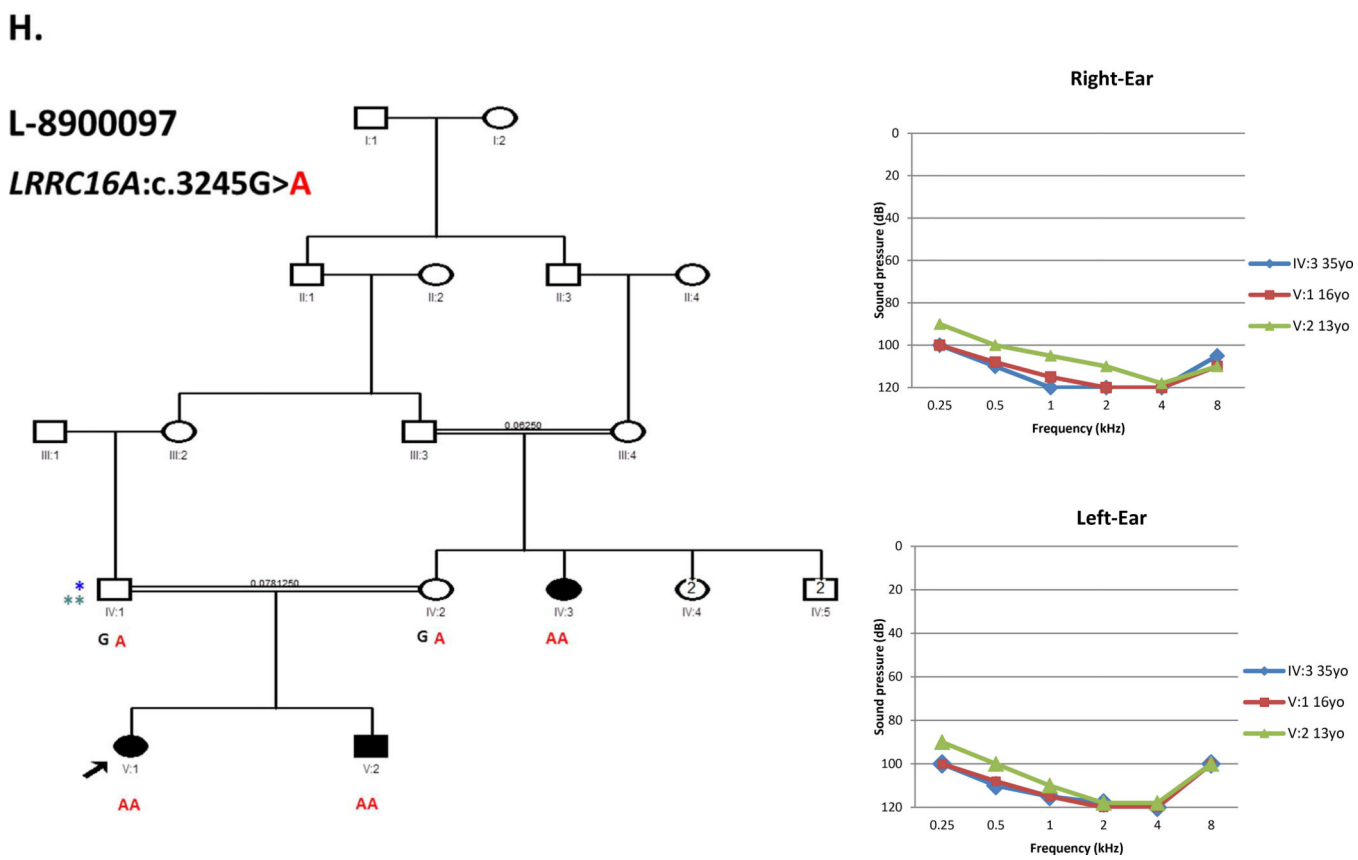


Figure 3:

Pedigrees, genotypes and audiograms of families with novel candidate genes (*individuals run on OtoSCOPE, **individuals run on ES). ES, exome sequencing

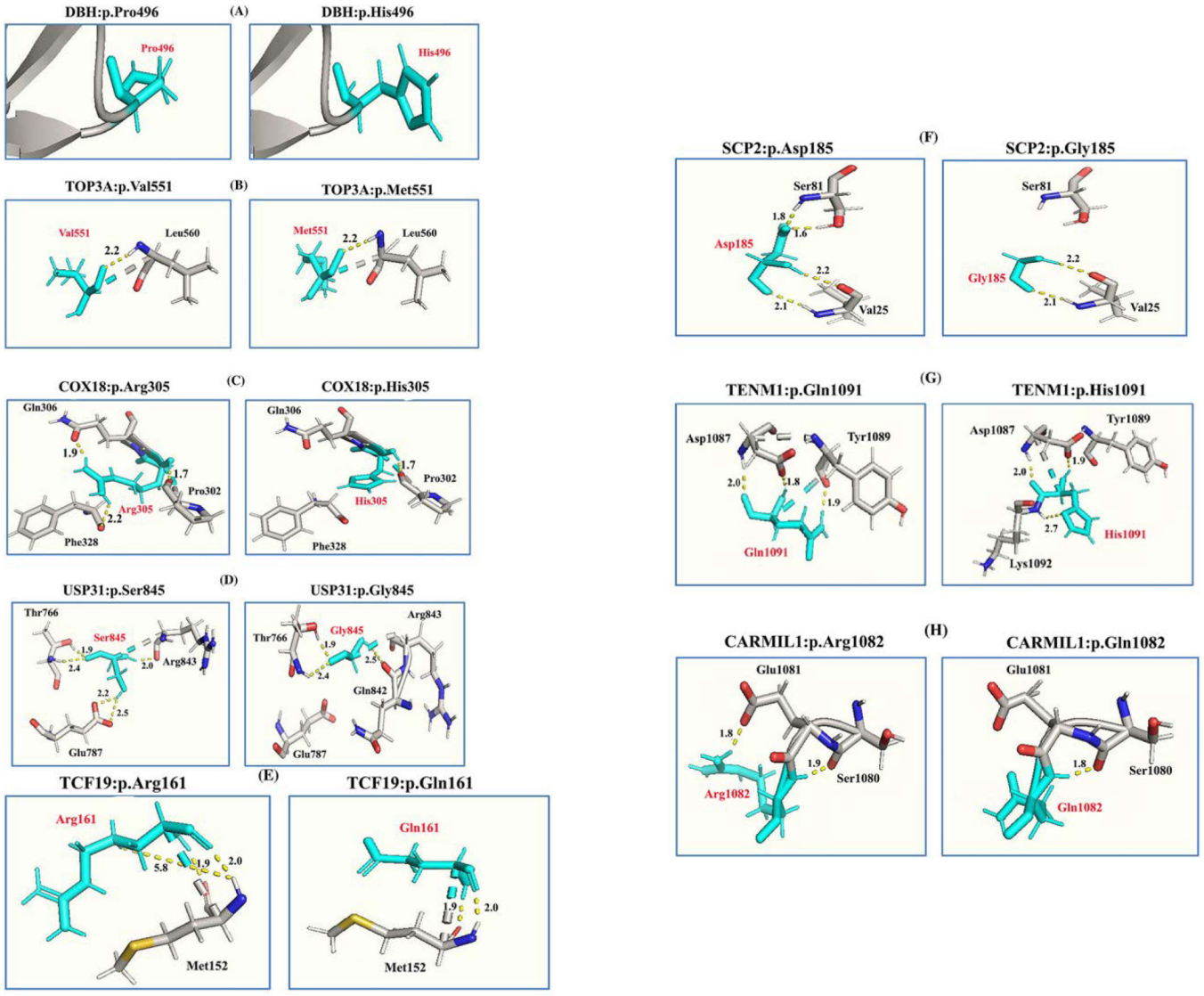


Figure 4:
3D modeling of wild-type and mutant residue in protein structure of novel candidate genes.

TABLE 1.

Phenotype of deaf families with variants in known and novel candidate genes (N: novel candidate gene)

Family ID			Age of onset	Kind of HL	Age at examination	Inheritance	Number of affected in core family	Number of affected in pedigree	Ethnicity	Parental relationship	Observed phenotype	OMIM Phenotype, Number
L-347			12 M	Prelingual	18 Y	AR	4	5	Turk	Consanguineous	Profound nonsyndromic hearing loss	N
L-665	Branch1	VII: 1	7Y	Postlingual (progressive)	10 Y	AR	2	5	Persian	Consanguineous	Moderate to severe progressive nonsyndromic hearing loss	Progressive external ophthalmoplegia with mitochondrial DNA deletions, autosomal recessive 5, phenotype MIM:618098
	Branch2	V:2	NA	Adult onset (decade 2–5)	55 Y		3	5			Profound progressive nonsyndromic hearing loss	Deafness, autosomal recessive 30, phenotype MIM: 607101
L-1119			2 Y	Prelingual	6 Y	AR	4	11	Kurd	Consanguineous	Profound nonsyndromic hearing loss	Deafness, autosomal recessive 6, phenotype MIM: 600971
L-1208			3 Y	Postlingual	14 Y	AR	2	2	Turk	Consanguineous	Moderate to Severe nonsyndromic hearing loss	N
L-3033			2.5 Y	Postlingual	24 Y	AD	3	3	Persian	Non-Consanguineous	Severe nonsyndromic hearing loss	N
L-8600456			2 Y	Prelingual	24 Y	AR	4	4	Gilak	Consanguineous	Profound nonsyndromic hearing loss	Leukoencephalopathy with dystonia and motor neuropathy, phenotype MIM:613724
L-8900097			18 M	Prelingual	16 Y	AR	2	3	Turk	Consanguineous	Profound nonsyndromic hearing loss	N
L-350			3 Y	Postlingual	3 Y	AR	2	2	Persian	Consanguineous	Profound nonsyndromic hearing loss (N/A for clinical reevaluation)	Deafness, autosomal recessive 94, phenotype MIM: 618434
L-692			12 M	Prelingual	31 Y	AR	3	3	Lur	Consanguineous	Severe to profound hearing loss (N/A for spermogram)	Deafness, autosomal recessive 32, with or without immotile sperm, phenotype MIM: 608653

Family ID		Age of onset	Kind of HL	Age at examination	Inheritance	Number of affected in core family	Number of affected in pedigree	Ethnicity	Parental relationship	Observed phenotype	OMIM Phenotype, Number
L-1096		2 Y	Prelingual	16 Y	AR	3	4	Lur	Consanguineous	Moderate to severe hearing loss, sperm dysmotility	Deafness, autosomal recessive 32, with or without immotile sperm, phenotype MIM: 608653
L-1108		6 M	Prelingual	24 Y	AR	3	3	Persian	Consanguineous	Profound hearing loss, skin hypopigmentation	Waardenburg syndrome type 4B, phenotype MIM: 613265
L-1326		8 Y	Postlingual	24 Y	AR	2	6	Persian	Consanguineous	Profound hearing loss, retinopathy, skeletal dysplasia	Stickler syndrome type IV, phenotype MIM: 614134
L-1347		1.5 Y	Prelingual	22 Y	AR	2	2	Persian	Consanguineous	Severe nonsyndromic hearing loss	Deafness, autosomal recessive 32, with or without immotile sperm, phenotype MIM: 608653
L-3100		2 Y	Prelingual	22 Y	AR	2	2	Persian	Consanguineous	Profound nonsyndromic hearing loss	Deafness, autosomal recessive 29, phenotype MIM: 614035
L-8600034		5 M	Prelingual	13 Y	AR	2	2	Lur	Consanguineous	Mild to moderate hearing loss, hypokalemic metabolic acidosis, primary distal renal tubular acidosis	Distal renal tubular acidosis 3, with or without sensorineural hearing loss, phenotype MIM: 602722
L-8600220		2 Y	Prelingual	13 Y	AR	2	3	Lur	Consanguineous	Severe hearing loss, polyuria, growth retardation	Bartter syndrome type 4b, phenotype MIM: 613090
L-8600574	IV:2	2 Y	Prelingual	16 Y	AR	5	5	Arab	Consanguineous	Severe hearing loss, retinitis pigmentosa, night blindness	Usher syndrome, type 1B, phenotype MIM: 276900
	IV:5	2 Y	Prelingual	14 Y	XLR					Sever hearing loss	N
L-8700223		2 Y	Prelingual	28 Y	AR	4	5	Persian	Consanguineous	Profound nonsyndromic hearing loss	Deafness, autosomal recessive 6, phenotype MIM: 600971
L-8900107		2 Y	Prelingual	24 Y	AR	4	4	Persian	Consanguineous	Profound hearing loss, optic atrophy	Combined oxidative phosphorylation deficiency 24, phenotype MIM: 616239
L-9501254		9 Y	Postlingual	35 Y	AR	3	4	Lur	Consanguineous	Severe hearing loss, ataxia, optic atrophy, visual loss	Brown-Vialetto-Van Laere syndrome 2, phenotype MIM:614707

Clin Genet. Author manuscript; available in PMC 2021 July 01.

PP: polyphen, MT: mutation taster, mSVM: metaSVM, mLR:

	cDNA Change	Protein Change	Known/ Novel variants	Minor Allele Frequency (%)			Conservation		Deleteriousness						Classification	ACMG scores	Reference
				gnomAD (max)	gnomAD (global)	Iranome	GERP	PhyloP	PP	SI	MT	mSVM	mLD	CADD			
1	c.658A>G	p.(Met220Val)	Novel	NA	NA	NA	5.84	3.2	P	D	D	D	D	24.9	Likely pathogenic	PM2, PM3_Supp, PP1, PP3,PP4	–
	c.1370_1371del	p.(Arg457Asnfs*25)	Known	0.002653	0.001203	NA	.	.	.	.	D	.	.	.	Pathogenic	PVS1, PM2, PP1_Moderate	–
	c.1126C>T	p.(Arg376*)	Known	0.01801	0.000431	0.06289	1.85	1.1	.	.	D	.	.	36	Pathogenic	PVS1, PM2_supp, PM3, PP1_Moderate	PMID: 32231217
	c.1351_1352del	p.(Ala451Thrfs*43)	Known	NA	NA	NA	.	.	.	.	D	.	.	28.6	Pathogenic	PVS1, PM2, PP1	PMID: 32231217
2	c. 293C >T	p.(Thr98Met)	Known	NA	NA	NA	4.8	5.9	D	D	D	D	D	34	Likely pathogenic	PM1, PM2, PM3_Supp, PP1_Moderate, PP3, PP4	PMID: 22876130
	c.250C>T	p.(Arg84Trp)	Known	0.004418	0.002405	0.001658	4.69	1.3	D	D	D	D	D	35	Pathogenic	PM2, PM3_Very Strong, PM5, PP1_Strong, PP3,	PMID: 12145746 PMID: 19438934
	c.1915del	p.(Val639Trpfs*51)	Novel	NA	NA	NA	.	.	.	.	D	.	.	32	Pathogenic	PVS1, PM2, PP1_Strong,	–
	c.40_41insTGGTGCACGGCCGTGCA	p.(Ser14Metfs*15)	Novel	NA	NA	NA	.	.	.	.	D	.	.	26.1	Pathogenic	PVS1, PM2, PP1,	–
	c.710_712del	p.(Lys237del)	Novel	0.003266	0.000398	NA	.	.	.	.	.	D	.	22.2	Likely Pathogenic	PM2, PM4, PP1, PP3, PP4	–
2	c.55C>T	p.(Gln19*)	Known	0.04658	0.01009	NA	4.12	1.3	.	.	D	.	.	35	Pathogenic	PVS1, PM2_supp, PP1, PP4	–
	c.122_125dup	p.(Pro43Alafs*73)	Known	0.009803	0.001203	NA	.	.	.	.	D	.	.	33	Pathogenic	PVS1, PM2_supp, PM3_Supp, PP1_Strong,	PMID: 12145746

cDNA Change	Protein Change	Known/ Novel variants	Minor Allele Frequency (%)			Conservation		Deleteriousness					Classification	ACMG scores	Reference
			gnomAD (max)	gnomAD (global)	Iranome	GERP	PhyloP	PP	SI	MT	mSVM	mLD	CADD			
c.5442T>G	p.(Tyr1814*)	Novel	NA	NA	NA	−9.2		.	.	D	.	.	29.7	Pathogenic	PVS1, PM2, PP1 strong,, PP4	–
c.973T>G	p.(Cys325Gly)	Known	NA	NA	NA	3.54	6.1	B	D	D	D	D	25.3	Pathogenic	PM1, PM2, PP1_Strong, PP3, PP4	PMID: 29287867

TABLE 3.

andidate hearing loss genes identified in 8 Iranian families with HHL (SI: SIFT, PP: polyphen, MT: mutation taster, mSVM: metaSVM, : PROVEAN, Fa Fathmm)

	g.DNA Change	RefSeq ID	cDNA Change	Protein Change	Minor Allele Frequency (%)			Located in ROH region	Conservation		Deleteriousness						ΔΔG STRUM	gEAR portal	HSF The Human Splicing Finder system
					gnomAD (max)	gnomAD (global)	Iranome		GERP	PhyloP	PP	SI	MT	mSVM	mLD	CADD			
	Chr9:g.136521696C>T	NM_000787.4	c.1486C>T	p.(Pro496Ser)	NA	NA	NA	Y	4.63	7.49	D	D	D	D	D	31	−1.65	Expressed in the cochlear duct	ESE Site Broken Alteration of an exonic ESE site. Potential alteration of splicing.
	Chr17:g.18188781C>T	NM_004618.5	c.1651G>A	p.(Val551Met)	NA	NA	NA	Y	5.61	7.81	D	D	D	T	T	34	4.54	Strong expression in the cochlear and vestibular sensory epithelium at E16 but weak expression in cochlea duct and HC at P0	New ESS Site Creation of an exonic ESS site. Potential alteration of splicing.
	Chr4:g.73923928C>T	NM_001300729.1	c.914G>A	p.(Arg305His)	0.005786	0.001195	NA	Y	4.52	3.49	D	D	D	T	T	27.5	−1.99	Weak expressed in cochlear at E16 and weak expressed in HC at P0	New ESS Site Creation of an exonic ESS site. Potential alteration of splicing. ESE Site Broken Alteration of an exonic ESE site. Potential alteration

	g.DNA Change	RefSeq ID	cDNA Change	Protein Change	Minor Allele Frequency (%)			Located in ROH region	Conservation		Deleteriousness						ΔΔG STRUM	gEAR portal	HSF The Human Splicing Finder system
					gnomAD (max)	gnomAD (global)	Iranome		GERP	PhyloP	PP	SI	MT	mSVM	mLD	CADD			
																			of splicing.
	Chr16:g.23080893T>C	NM_020718.3	c.2533A>G	p.(Ser845Gly)	0.000880	0.000398	NA	Y	6.16	4.68	D	D	D	T	T	24.2	1.38	Strong expression in cochlear at E16 and expressed in the cochlear duct	Cryptic donor site. Potential alteration of splicing New ESS Site Creation of an exonic ESS site. Potential alteration of splicing. ESE Site Broken Alteration of an exonic ESE site. Potential alteration of splicing.
	Chr6:g.31129467G>A	NM_001077511.2	c.482G>A	p.(Arg161Gln)	0.006033	0.001094	NA	Y	4.83	1.55	D	T	N	T	T	24.6	−2.07	Strong expression in cochlear at E16 and expressed in the cochlear duct but weak expression in HC at P0 and P1	New Acceptor Site Activation of an exonic cryptic acceptor site, with presence of one or more cryptic branch point(s). Potential alteration of splicing. ESE Site

	g.DNA Change	RefSeq ID	cDNA Change	Protein Change	Minor Allele Frequency (%)			Located in ROH region	Conservation		Deleteriousness						ΔΔG STRUM	gEAR portal	HSF The Human Splicing Finder system
					gnomAD (max)	gnomAD (global)	Iranome		GERP	PhyloP	PP	SI	MT	mSVM	mLD	CADD			
																			Broken Alteration of an exonic ESE site. Potential alteration of splicing.
	Chr1:g.53443900A>G	NM_001007098.2	c.554A>G	p.(Asp185Gly)	0.002596	0.001194	NA	Y	5.29	6.407	D	D	D	D	D	25.4	−0.29	Strong expression in cochlear at E16 and expressed in the cochlear duct but weak expression in HC at P0 and P1	New Donor Site Activation of an exonic cryptic donor site. Potential alteration of splicing. New ESS Site Creation of an exonic ESS site. Potential alteration of splicing. ESE Site Broken Alteration of an exonic ESE site. Potential alteration of splicing.
	ChrX:g.123654395C>A	NM_001163278.1	c.3273G>T	p.(Gln1091His)	0.03669	0.003418	NA	Y	3.65	2.24	D	D	D	D	D	26	0.67	Expressed in the cochlear and vestibular sensory	ESE Site Broken Alteration of an exonic ESE site. Potential

	g.DNA Change	RefSeq ID	cDNA Change	Protein Change	Minor Allele Frequency (%)			Located in ROH region	Conservation		Deleteriousness					ΔΔG STRUM	gEAR portal	HSF The Human Splicing Finder system
					gnomAD (max)	gnomAD (global)	Iranome		GERP	PhyloP	PP	SI	MT	mSVM	mLD	CADD		
																	epithelium at E16	alteration of splicing.
TMPRSS3	Chr6:g.25600667G>A	NM_001173977.2	c.3245G>A	p.(Arg1082Gln)	0.03269	0.004416	NA	Y	5.2	4.29	D	D	D	T	T	24.8	0.33	Strong expression in the cochlear and vestibular sensory epithelium at E16, cochlea duct and HC at P0.	ESE Site Broken Alteration of an exonic ESE site. Potential alteration of splicing.