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Expanding the Mutation Spectrum of Autosomal Recessive Non-Syndromic Hearing Loss in the Iranian Families

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ABSTRACT

Hearing loss is known as the most common sensory disorder in humans, with an incidence of 466 million people worldwide. This disorder is genetically highly heterogeneous, so among the 180 genes responsible for hearing loss, a disproportionate share of genes is involved in different ethnicities. Here, we report the underlying genetic cause of non-syndromic hearing loss segregating in four unrelated Iranian families. In the first step, patients were examined for mutations in the common genes *GJB2* and *GJB6*. After confirming the negativity of mutations in these genes, the affected patients were subjected to targeted-exome sequencing. Subsequently, Sanger sequencing was used to confirm the mutations found in the patients and their family members. *In-silico* analyses were used to consider the possible deleterious effect of the identified variants on encoded proteins. Targeted-exome sequencing revealed a novel intronic mutation c.490-8C>A in the *CABP2* gene, a novel ~154 kb deletion mutation including the *OTOA* gene involved in hearing loss, and two previously reported mutations: a pathogenic/likely pathogenic variant c.413C>A in the *TMPRSS3* gene and a c.966dupC variant with conflicting classifications of pathogenicity in the *COL11A2* gene. However, the audiological evaluations, segregation analysis, and *in-silico* approaches confirmed the disease-causing nature of all mutations found. Our findings could extend the pathogenic mutation spectrum of non-syndromic hearing loss, highlight the high genetic heterogeneity of hearing loss, and also aid in conducting genetic counseling, prenatal diagnosis, and clinical management of hearing loss in the Iranian population.

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Introduction

Hearing loss (HL) is a highly heterogeneous disorder and is recognized as the fourth most common disability worldwide. According to a recent global estimate, the number of people with hearing impairment will reach 2.45 billion by 2050, mainly due to age-related hearing impairments (ARHI) (Trpchevska *et al.*, 2022). ARHI is the most common type of sensorineural hearing loss (SNHL), and some of the factors contributing to its occurrence include the complex interplay between cochlear aging, genetic predisposition, and exposure to loud noise. In most cases, SNHL is caused by the degeneration of inner ear hair cells, and studies have shown a positive genetic correlation between SNHL and diabetes, obesity, and smoking (Kalra, 2021). Although more than 500 genes are predicted to be responsible for the normal functioning of the auditory system, only 180 genes that cause HL have been identified to date (Ajam-Hosseini *et al.*, 2023a). The diagnostic rate of genetic testing for HL is approximately 35-50%, depending on factors such as clinical phenotype, age of onset,

inheritance pattern, and ethnic background. This variability reflects the complexity of obtaining a definitive genetic diagnosis and the contribution of genes associated with HL that have not yet been identified (Bazazzadegan *et al.*, 2025). Genetic factors account for almost half of the reports related to hearing impairment, and more than two-thirds of these cases appear as a single symptom and without any other defect under the name of non-syndromic hearing loss or NSHL (Li *et al.*, 2025). About 80% of NSHL cases are inherited in an autosomal recessive manner, while approximately one-fifth are inherited in an autosomal dominant pattern. It is interesting to note that with increasing age, the frequency of autosomal dominant and autosomal recessive inheritance patterns increases and decreases, respectively (Sloan-Heggen *et al*., 2015). On the other hand, consanguineous marriages increase the chance of having genetic disorders with autosomal recessive inheritance by about 0.25 to 20% (Zafar *et al.*, 2020), and Iran is no exception to this rule, with nearly 40% consanguinity (Parvini *et al.*, 2022). Hereditary deafness is considered the second disability in Iran after intellectual disability. The *GJB2* gene accounts for a proportion of mutations compared to other common NSHL-causing genes such as *SLC26A4*, *MYO15A*, *MYO7A*, *CDH23*, and *TMC1*, with an average frequency that is 16% in the HL population in Iran (Ajam-Hosseini *et al.*, 2023b; Parvini *et al*., 2022). Here, we report two novel mutations in the *CABP2* and *OTOA* genes, as well as two previously reported mutations in the *TMPRSS3* and *COL11A2* genes, identified in four unrelated Iranian patients. These findings further expand the gene mutation spectrum and emphasize the high genetic heterogeneity of HL in the Iranian population. Beyond any doubt, such studies show rapid progress in the field of next-generation sequencing technologies, enabling more accurate and cost-effective diagnosis of a wide range of hereditary disorders (Noavar *et al.*, 2019; Fahimi *et al.*, 2021; Ajam-Hosseini *et al.*, 2023b).

Materials and Methods

Patients and clinical evaluations

In this research, we investigated the genetic cause of the NSHL in four Iranian unrelated families (HL01, HL02, HL03, and HL04). The clinical phenotype of the participants was evaluated through careful medical history and physical examination. Pure tone audiometry with air conduction at frequencies ranging from 250-8000 Hz was completed according to standard protocols. After obtaining informed consent, peripheral blood samples from all patients and their family members were collected. Genomic DNA was extracted using QIAamp DNA Blood Mini Kit Cat No. 51104 (QIAGEN, Germany) according to the manufacturer's instructions. The experimental procedures were approved by the ethics committee of the Pharmaceutical Sciences Branch of Islamic Azad University, Tehran, Iran (ethics approval code no. IR.IAU.PS.REC.1396.91).

Screening of *GJB2* and *GJB6* genes mutations

In the first step, the presence or absence of the 35delG mutant allele of the *GJB2* gene was detected using an allele-specific polymerase chain reaction (AS-PCR) assay based on a previously established method (Fahimi *et al.*, 2021). This assay was applied to screen all four HL-affected patients. Since all four studied patients were negative for the 35delG mutant allele, the entire non-coding (exon 1), coding (exon 2), and flanking intronic regions of the *GJB2* gene were amplified and directly sequenced using the following primers: CXF' 5' GAAGGCGTTCGTTCGGATTG 3'/CXR' 5' CCAAGGACGTGTGTTGGTC 3' (974 bp) for amplification of exon 1 and CXF 5' CTCCCTGTTCTGTCCTAGCT 3'/CXR 5' CTCATCCCTCTCATGCTGTC 3' (809 bp) for amplification of exon 2.

The PCR reactions were subjected to an initial denaturation step at 95°C for 10 seconds, followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds. Subsequently, all four patients who tested negative for 35delG and the other mutations of the *GJB2* gene were also screened for the known deletion mutations del(*GJB6*- D13S1830) and del(*GJB6*-D13S1854) by multiplex PCR, as previously described by Del Castillo *et al.* (2005)

TES and bioinformatics analysis

After ruling out *GJB2* and *GJB6* gene mutations, all four patients were investigated by targeted

exome sequencing (TES) to enrich all exons of 180 protein-coding genes (the annotated genes causing hearing impairment in the OMIM database) as well as some other important genomic regions involved in hearing. The TES was performed using an Agilent V.6 kit (Agilent, Santa Clara, USA) on an Illumina platform using the Illumina NextSeq500 instrument and a sequencing depth of 100X. Data filtering was based on frequency. Then, the data were filtered by removing all variants in non-coding, upstream, downstream, 3′-UTR, 5′-UTR, and intergenic regions, as well as synonymous variants in exonic regions. Generally, the testing platform covered more than 95% of the targeted regions with a sensitivity of greater than 99%. The TES results were analyzed using the open-access bioinformatics tools BWA (Burrows-Wheeler aligner) (Li and Durbin, 2010), Annovar (ANNOtate VARiation) (Wang *et al.*, 2010), and GATK (Genome Analysis Toolkit) (McKenna *et al.*, 2010), as well as public databases, including ClinVar, Kaviar, GME (Greater Middle East Variome), and gnomAD. The online tool VarSome (https://varsome.com) was used to classify variants according to the American College of Medical Genetics and Genomics (ACMG) guidelines. In addition, the local population database BayanGene, which includes data from over 4100 unrelated individuals, was utilized to assess the mutation frequency in the Iranian population. As a control, 300 healthy individuals of the same ethnic background as the studied patients were also screened for the identified mutations.

To predict the potential functional impact of the identified mutations, several *in-silico* bioinformatics tools were employed, including MutationTaster (http://www.mutationtaster.org/), Mutation Assessor (http://mutationassessor.org/r3/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (https://sift.bii.a-star.edu.sg/), FATHMM (http://fathmm.biocompute.org.uk/), and Combined Annotation Dependent Depletion (CADD) score (https://cadd.gs.washington.edu/score) (Table 1). Additionally, to assess the evolutionary conservation of the affected regions, multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Fig. 3).

Table 1. Bioinformatics data of the suspected variants identified in the proband.

Family ID	Genes	cDNA Change	Protein Change	Zygosity	CADD	FATHMM	Mut Tast*	Mut Ass*	Polyphen 2	SIFT	ACMG Classification	Ref.
HL01	*CABP2*	c.490-8C>A	-	Homo	0.598	N/A*	Disease causing	N/A	N/A	N/A	Likely pathogenic (PP3, PM2)	This study
HL02	*OTOA*	Whole gene deletion	No protein	Homo	N/A	N/A	Disease causing	N/A	N/A	N/A	N/A	This study
HL03	*TMPRSS3*	c.413C>A	p.Ala138Glu	Homo	23.7	Damaging (0.86614)	Disease causing	M*	Probably damaging (0.958)	D*	pathogenic (PP5, PM2)	Weegerink *et al.*, 2011
HL04	*COL11A2*	c.966dupC	p.Thr323fs	Homo	N/A	N/A	Disease causing	N/A	N/A	N/A	pathogenic PVS1, PP5, PM5)	Vona *et al.*, 2017

*N/A= Not applicable; M= Medium; D= Damaging; Mut Ass= Mutation Assessor; Mutation Taster= Mut Tast. *= Criteria used.

Sanger sequencing and segregation analysis

Sanger sequencing was performed for the patients and their family members to confirm the presence of the identified mutations and their segregation. The primers used were as follows: F-5′ GTGGCGGAAAGGTGGACTT 3′ and R-5′ CTCTTGCCTATGGGGATAACAATTC 3′ (PCR product: 544 bp) for *CABP2* gene, F-5′ ATCATAGCTCACCACAGTCTCCTGG 3′ and R-5′ ATTAGTGAGCAGCACAGGCCTGTAG 3′ (PCR product: 572 bp) for the *OTOA* gene, F-5′-AGGCTGCCGTGGACAAGAAG-3′ and R-5′-AAGCTGAGGAGCTGGAGGGTT-3′ (PCR product: 584 bp) for *TMPRSS3* gene and F-5′-ATCCACCACTTCTTCCCACTG-3′ and R-5′-TTCACTTACGGCTCCTGAGTG-3′ (PCR product: 426 bp) for *COL11A2* gene. Finally, the sequencing data were analyzed using Chromas software.

Results

Four unrelated families-HL01, HL02, HL03, and HL04-with a history of severe to profound HL were included in this study (Table 1). Pedigree analysis revealed autosomal recessive inheritance patterns and parental consanguinity in all cases (Fig. 1- 4). None of the affected individuals exhibited symptoms beyond hearing impairment. Based on the initial screening, all four patients tested negative for pathogenic variants in the *GJB2* gene and the two common large deletions

in *GJB6*. Subsequently, TES analyses revealed the following variants: a novel intronic mutation (NM_016366.3:c.490-8C>A) in the *CABP2* gene in family HL01; a novel ~154 kb deletion encompassing the *OTOA* gene in family HL02; and two previously reported variants, a missense mutation (NM_024022.2; c.413C>A; p.Ala138Glu) in exon 5 of the *TMPRSS3* gene in family HL03, and a frameshift mutation (NM_080680.3; c.966dupC; p.Thr323fs) in exon 8 of the *COL11A2* gene in family HL04 (Table 1). In family HL01, the proband was a 22-year-old boy who suffered from moderate to severe bilateral hearing impairment. He was born to consanguineous parents and has an affected brother with a similar phenotype (Fig. 1).

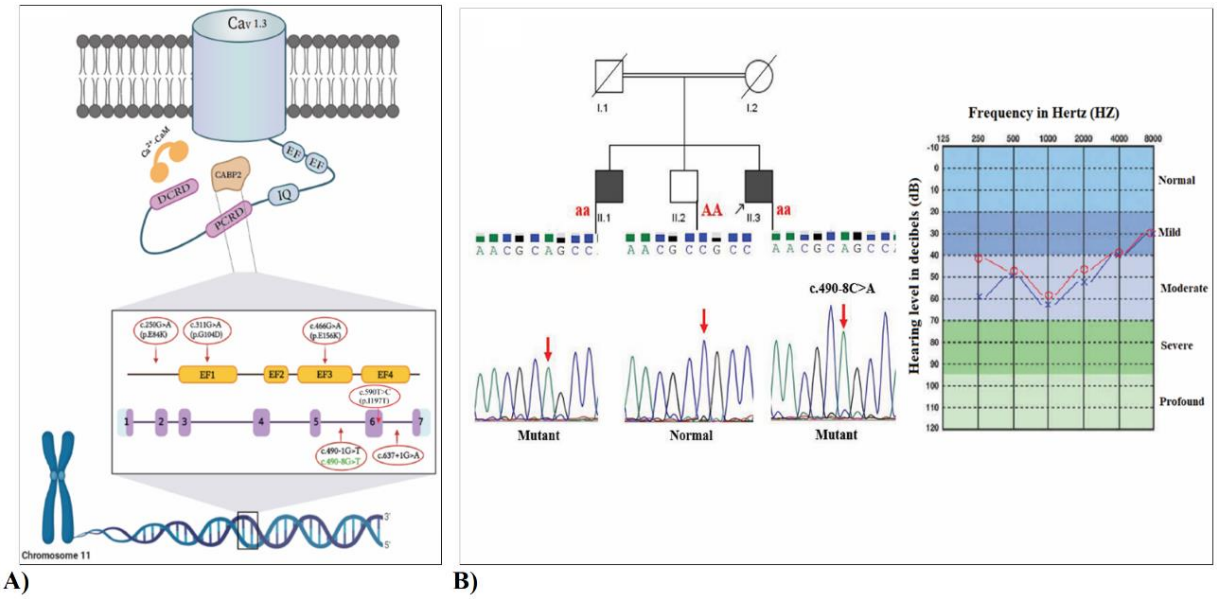

Fig. 1. Schematic representation of the CABP2 gene/protein structure, segregation analysis, and audiology output of the proband (II-3): A) CaV1.3 channel has proximal and distal COOH-terminal regulatory domains (PCRD and DCRD). The EF-hand motif together with the IQ motif forms the $Ca2^{+}$-dependent inactivation complex. The CABP2 protein prevents the binding of $Ca2^{+}$-CaM to the channel, leading to a decrease in calcium-dependent inactivation. The positions of 6 *CABP2* mutations are shown both at the protein (top) and at the gene (bottom) levels. The exons are numbered with the coding sequence shaded purple and the untranslated regions unshaded. The novel mutation identified in this study is marked with a green color; B) Family pedigree, along with the results of Sanger sequencing for the studied individuals of family HL01. The proband (II-3, aa) and his affected brother (II-1, aa) were homozygote for the mutation found, whereas their only healthy brother (II-2, AA) was unaffected. The proband's audiogram shows moderate to severe deafness.

Analysis of TES data disclosed a homozygous intronic variant (NM_016366.3); chr11:67519948G>T, c.490-8C>A at the 3'-end of intron 5 of the *CABP2*. This variant is associated with autosomal recessive non-syndromic hearing loss (ARNSHL), specifically DFNB93 (OMIM 614899). Functional and computational bioinformatics data support a deleterious effect of the identified variant on CABP2 (ACMG criterion: PP3). According to the ACMG guidelines, the c.490-8C>A variant was predicted to be likely pathogenic. Our review of public databases, including the local population database BayanGene, and relevant literature confirmed the novelty of this mutation. In addition, none of the 300 healthy controls showed the identified mutation, supporting its novelty. This mutation was confirmed by Sanger sequencing and segregated with the other family members (Fig. 1). Point mutations in splice consensus sequences can lead to the production of aberrant transcripts from the mutated gene due to the misrecognition of exon-intron boundaries. Typically, such

mutations cause errors in the splicing process and disrupt splicing regulatory sequences, such as extrinsic and intrinsic enhancers and repressors, ultimately altering the open reading frame (Anna and Monika, 2018). It should be noted that splicing variants commonly act as loss-of-function changes, accounting for 19.4% of all predicted loss-of-function variants (including nonsense and frameshift variants) in gnomAD. Furthermore, loss-of-function intolerance scores in gnomAD suggest a high likelihood that a gene will become intolerant to these mutations (Chaleshtori *et al.*, 2014). With respect to family HL02, the proband was a 36-year-old male with prelingual severe bilateral hearing impairment who had a brother with a similar phenotype. They are the offspring of a consanguineous marriage (Fig. 2b). The TES results revealed a novel ~154 kb large deletion mutation including three genes *OTOA*, *METTL9*, and *IGSF6*. The *IGSF6* gene overlaps with *METTL9* but is transcribed in the opposite direction (Fig. 2).

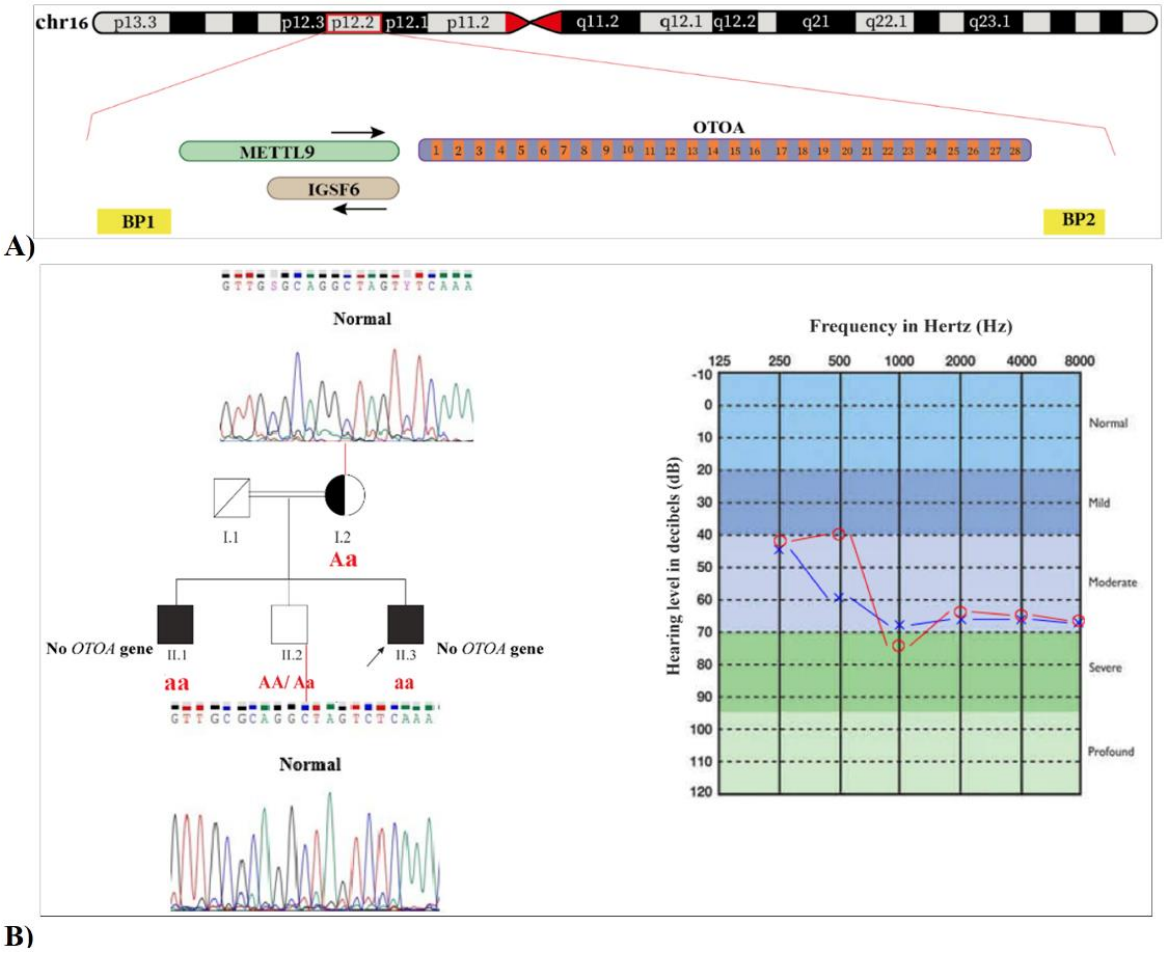

Fig. 2. Schematic representation of the *OTOA* gene/protein structure, segregation analysis and audiology output of the proband (II-3): A) Position of identified ~154 kb deletion mutation (band 16P12.2) including three genes *OTOA*, *METTL9*, and *IGSF6* which are surrounded by two fragments BP1 and BP2; B) Family pedigree along with the results of Sanger sequencing for the studied individuals of family HL02. The proband (II-3, aa) and his affected brother (II-1, Aa) were revealed to be homozygote for the detected mutation, whereas their healthy mother (I-2, AA) and brother (II-2, AA/Aa) were unaffected. The audiogram of the affected proband (II-3) shows severe hearing loss.

The *OTOA* gene is located on chromosome 16p12.2 and encodes otoancorin, a glycoprotein of the acellular gels of the inner ear (Souissi *et al.*, 2021). This protein is essential for connecting the tectorial membrane (TM) to the spiral limbus (Sugiyama *et al.*, 2019). Loss-of-function variants in *OTOA* cause ARNSHL type DFNB22 (OMIM 607039). Interestingly, there are a few cases of hearing loss associated with loss of function of *OTOA*; however, homozygous whole gene deletions are the most common molecular defect (Table 2). PCR and Sanger sequencing confirmed the identified deletion mutation. As expected, the affected brother of the proband (II-1) was homozygous for the reported mutation, whereas his healthy mother and brother were unaffected (Fig. 2).

Table 2. Pathogenic variants and size variation of the *OTOA* gene deletion mutations in NSHL patients to date.

cDNA/ (aa change)	Refseq	Origin	E/ I	Ref.	cDNA/ (aa change)	Refseq	Origin	E/ I	Ref.
c.120+1G>A	144672	Chinese	I3	Xiang *et al.*, 2020	c.2359G>T (p.Glu787*)	144672.4	NA	E18	Laurent *et al*., 2021
c.151+1G>A	144672.4	Iran	I3	Sloan-Heggen *et al*., 2015	IVS12+2T>C	144672	Palestine	E1-19	Fontana *et al.*, 2017
c.442C>T (p.R148*)	144672	Japan	E3	Sugiyama *et al.*, 2019	c.2960-2961sdelAT (p.H987Rfs)	144672.4	Western-European	E22	Sugiyama *et al.*, 2019
c.827delT (p.S277Vfs)	144672.4	Western-European	E10	Sugiyama *et al.*, 2019	Whole gene deletion (~500 kb)	NA	Palestine	E1-29	Sugiyama *et al.*, 2019
c.1320+5G>C	144672.4	Korean	I13	Lee *et al.*, 2013	Whole gene deletion	144672.	Spanish	NA E1-29	Sugiyama *et al.*, 2019
c.1352G>A (p.G451D)	144672.4	Pakistan	E14	Lee *et al.*, 2013	Whole gene deletion	NA 144672.4	Iran	NA E1-29	Sloan-Heggen *et al*., 2015
c.1426A>C (p.S476R)	144672	Chinese	E11	Xiang *et al.*, 2020	Whole gene deletion	144672	Japan	NA E1-29	Sugiyama *et al.*, 2019
c.1642C>T (p.P548S)	001161683.2	Iran	E13	Sloan-Heggen *et al*., 2015	Whole gene deletion	NA	Caucasus	---------	Sugiyama *et al.*, 2019
c.1765delC (p.Gln589Rfs)	144672.4	Korean	E17	Kim *et al.*, 2019	Large deletion (110 kb)	144672.4	NA	E2 of METTL9 to E22 of OTOA	Laurent *et al.*, 2021
c.1807G>T (p.V603F)	144672.4	Algeria	E18	Ammar-Khodja *et al.*, 2015	Large deletion (>190 kb)	NA 144672.4	Turkey	NA	Kim *et al.*, 2019
c.1865T>A (p.L622H)	144672.4	Italy	E18	Fontana *et al*., 2017	Large deletion (154 kb)	NA 144672.4	Iran	METTL9, IGS6 and OTOA	This study
c.1879C>T (p.P627S)	144672.4	Pakistan	E18	Lee *et al.*, 2013	Large deletion (228.5 kb)	144672.4	Italy	NA	Fontana *et al.*, 2017
c.1971-1G>A	001161683.2	Iran	I15	Sloan-Heggen *et al*., 2015	Microdeletion (~165 kb)	NA	NA	METTL9, IGSF6 and OTOA	Tassano *et al.*, 2019
c.2301+1G>T	144672	Philippines	E18	Truong *et al.*, 2019	Microdeletion	144672.4	Qatar	NA	Kim *et al.*, 2019

*Refseq NM= Reference sequence NM; E/I= Exon/ Intron; NA, Not available.

Concerning family HL03, the proband was a 43-year-old male with severe bilateral hearing impairment who also had an HL-affected brother and two healthy siblings. They are the offspring of a consanguineous marriage. The TES results revealed a previously homozygous missense mutation (NM_024022.2); chr21:43808545 G>T, c.413C>A (p.Ala138Glu) in the *TMPRSS3* gene, causing ARNSHL type DFNB8/10 (OMIM 601072). The *TMPRSS3* gene (OMIM 605511) consists of 13 exons and encodes an enzymatic protein of the serine protease family. It includes several functional domains: a transmembrane region, a low-density lipoprotein receptor A (LDLRA) domain, a scavenger receptor cysteine-rich (SRCR) domain, and a trypsin-like serine protease domain, as shown in Fig. 3A (Gao *et al.*, 2017). Furthermore, Sanger sequencing confirmed the presence and homozygosity of the mutation in the proband and segregated with the autosomal recessive inheritance pattern of HL in all tested members of family HL03 (Fig. 3B). The identified variant c.413C>A leads to an amino acid change p.Ala138Glu located in the SRCR domain of the TMPRSS3 protein. Multiple amino acid sequence alignments using Clustal Omega showed the high evolutionary conservation of mutated residue Ala138 across different species, except zebrafish (Fig. 3C and 3D).

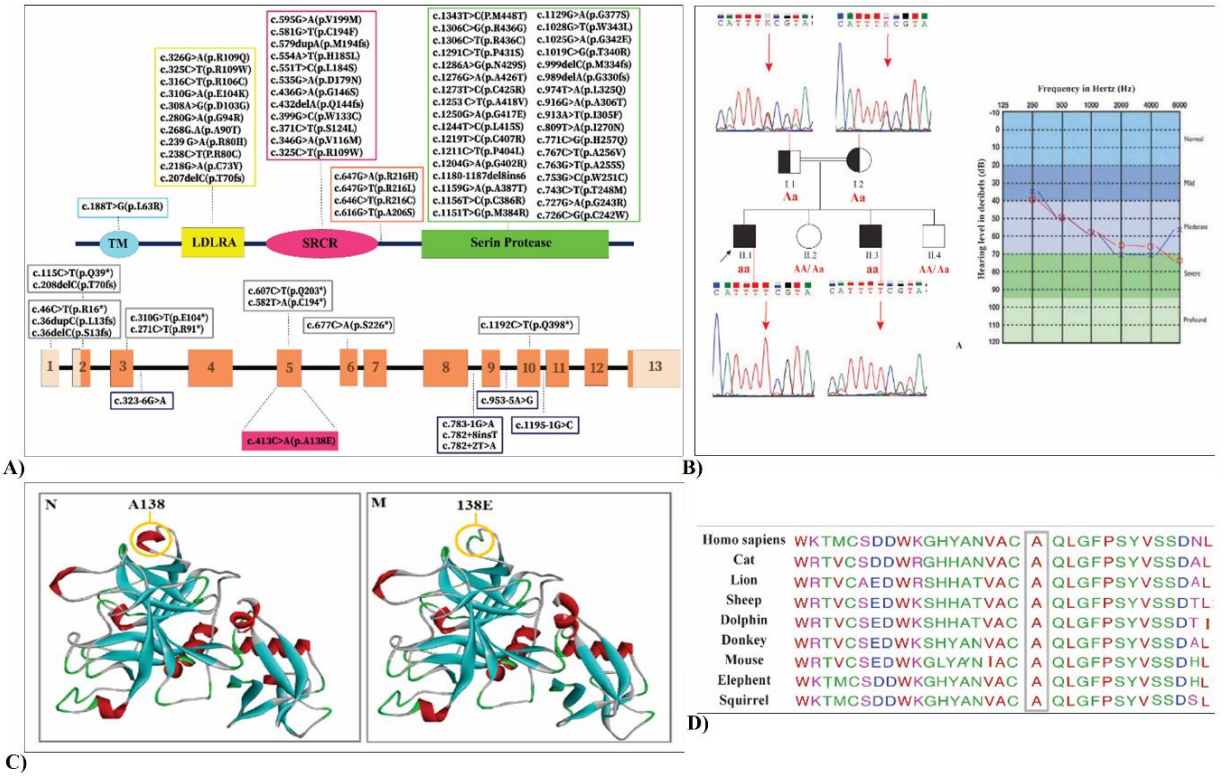

Fig. 3. Schematic representation of the *TMPRSS3* gene/protein structure, segregation analysis, and audiology output of the proband (II-1): A) The positions of 80 previously reported *TMPRSS3* mutations are shown both at the protein level (top) and at the gene (bottom). The exons are numbered with the coding sequence shaded orange and the untranslated regions unshaded. The identified mutation of this study (c.413C>A [p.A138E]) is marked with a different color; B) Family pedigree and the results of Sanger sequencing for the studied individuals of family HL03. As expected, the proband (II-1, aa) and his affected brother (II-3, aa) were homozygote for the detected mutation, and their healthy parents (I-1 and I-2, Aa) were heterozygote carriers. The proband's audiogram shows severe deafness; C) Phyre2 3D representation of the normal (N) and mutant (M) TMPRSS3 protein. The mutation resulted in a structural change of the SRCR domain; D) Alignments of the partial sequence of the TMPRSS3 protein, including the mutated position p.Ala138Glu, of nine different organisms.

The identified mutation was predicted to be damaging by various *in-silico* tools and ACMG guidelines (Table 1). Regarding family HL04, the proband was a 20-year-old female with prelingual severe bilateral hearing impairment. She has a healthy brother, and the family is the offspring of a consanguineous marriage (Fig. 4A). The TES analysis identified a previously reported homozygous frameshift mutation (NM_080680); chr6:33184297, c.966dupC (p.Thr323Hisfs) in exon 8 of the *COL11A2* gene, causing ARNSHL type DFNB53 (OMIM 609706). The c.966dupC mutation in the *COL11A2* gene causes a frameshift that introduces a premature stop codon shortly downstream. This change is predicted to result in either nonsense-mediated mRNA decay (NMD), resulting in complete loss of protein, or the production of a truncated, nonfunctional protein lacking normal biological activity (Fig. 4B).

Although according to Clinvar, the *COL11A2* gene variant (c.966dup; p.Thr323His fs) has conflicting interpretations of pathogenicity, previous studies have reported this variant as the causative agent of ARNSHL in three unrelated Iranian families of Turkic and Persian ethnicities. The insertion of cytosine in the 966 position leads to a frameshift and the creation of a premature stop codon in exon 8 of the *COL11A2* gene (Vona *et al.*, 2017). The recurrence of this variant in at least three ethnically diverse Iranian families with identical clinical presentation strongly supports its pathogenicity through genotype-phenotype correlation. Furthermore, based on different *in-silico* analyses, this variant is classified as pathogenic (Table 1 and Fig. 4B). The Sanger

sequencing confirmed the identified mutation and co-segregated with the autosomal recessive inheritance pattern of HL in family HL04 (Fig. 4a).

Discussion

In the current study, we report two novel mutations in the *CABP2* (c.490-8C>A) and the *OTOA* (a ~154 kb deletion containing *OTOA*) genes, as well as two previously reported mutations in *TMPRSS3* (c.413C>A) and *COL11A2* (c.966dupC), identified in four unrelated Iranian patients with ARNSHL. To the best of our knowledge, this is the first report worldwide of a patient with an intronic mutation c.490-8C>A in the *CABP2* gene, and the third report of a *CABP2*-related mutation in the Iranian population. CABP2 is a member of the Ca^{2+}-binding proteins (CABPs) subfamily with high similarity to calmodulin. This protein is expressed in the cochlea and modulates presynaptic calcium influx in inner hair cells through voltage-gated calcium channels to regulate auditory sensitivity (Sheyanth *et al.*, 2021). The frequency and spectrum of *CABP2* mutations in most ethnic populations are mainly unknown. A mutation in this gene leads to ARNSHL type DFNB93. To date, only six pathogenic variants in the *CABP2* gene have been identified in Iran (Chaleshtori *et al.*, 2014), Italy (Picher *et al.*, 2017), Turkey (Bademci *et al.*, 2016), Danish Caucasian (Sheyanth *et al.*, 2021), Pakistan (Park *et al.*, 2020), and Egypt (Nawaz *et al.*, 2024).

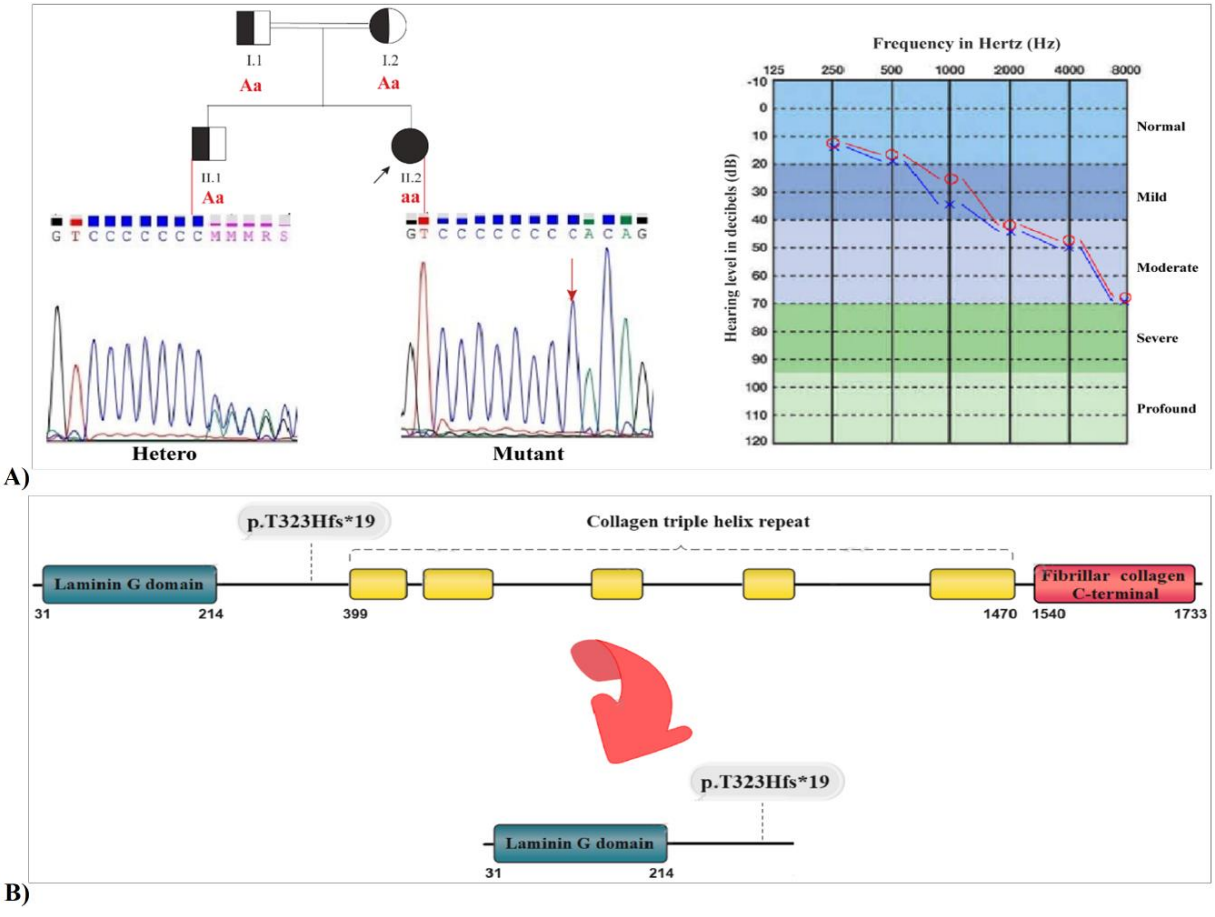

Fig. 4. Schematic representation of the COL11A2 protein structure, segregation analysis, and audiology output of the proband (II-2): A) Family pedigree and the results of Sanger sequencing for the proband and her healthy brother. The proband (II-2, aa) and her healthy brother (II-1, Aa) were homozygous mutant and heterozygous carriers, respectively. The proband's audiogram shows moderate to severe deafness; B) Production of truncated COL11A2 protein resulting from the frameshift mutation p.T323Hfs, which leads to the loss of a significant part of the protein.

Previous studies have shown that the protein encoded by *CAPB2* is also present in the retina. Therefore, ophthalmological examinations are necessary for individuals with *CABP2*-related HL. However, no ocular abnormalities have been reported to date (Sheyanth *et al.*, 2021). Regarding our patient, no ophthalmologic complication has been found. Mutations in the *OTOA* gene lead to moderate to profound ARNSHL type DFNB22 due to the disturbance in the stimulation of inner ear hair cells (Lee *et al.*, 2013). Interestingly, three large segmental duplications (BP1, BP2, and BP3) are located at chromosome band 16p12.2, a region associated with frequent recombination and chromosomal rearrangements. Moreover, these segmental repeats can act as a hotspot for copy number variants. There is a highly homologous sequence between the segmental repeats BP1 and BP2 that includes the *OTOA*, *METTL9*, and *IGSF6* genes (Tassano *et al.*, 2019). As a result, the variation in deletion size in these regions can be seen even in different ethnic groups (Table 2). Accordingly, the present study reports a novel ~154 kb deletion mutation and further supports the evidence of the pathogenic role of *OTOA* in ARNSHL.

Mutations in the *TMPRSS3* gene can lead to either prelingual (DFNB10) or post-lingual (DFNB8) ARNSHL. However, the occurrence of ARNSHL with post-lingual onset is rare. The phenotype of *TMPRSS3* mutations depends on the type and position of mutations that occurred, indicating the critical role of the *TMPRSS3* gene in the auditory system (Gao *et al.*, 2017). The c.413C>A (p.Ala138Glu) missense pathogenic variant in exon 5 of the *TMPRSS3* gene has been previously reported in the literature as homozygous in two siblings affected by ARNSHL. Similarly, researchers in one study identified the c.413C>A pathogenic variant as the causative agent of ARNSHL in two families from the United Kingdom and Korea. Furthermore, this variant has been observed in trans with another pathogenic variant (Ala306Thr) and has also co-segregated with the disorder among multiple affected family members in several families (Weegerink *et al.*, 2011). Regarding previous studies, mutations in the SRCR and LDLRA domains of TMPRSS3 can cause misfolding in these regions, which impairs the ability of its target protein, ENaC, to recognize the TMPRSS3 binding site. ENaC is a sodium channel that is expressed in many Na^+ reabsorbing tissues, such as the inner ear, and plays a role in regulating sodium concentration in the endolymph. The ENaC sodium channel activation, along with catalytic activity, is one of the important features of TMPRSS3, which is performed by the serine protease domain (Wong *et al.*, 2020). Although the exact function of the *TMPRSS3* gene in the auditory system has not yet been fully clarified, its expression has been reported in inner hair cells, supporting cells, and stria vascularis of the cochlear canal, and especially spiral ganglion neurons (Gao *et al.*, 2017). To date, 87 pathogenic and likely pathogenic variants have been reported in TMPRSS3. Of these, 80 variants are presented below, along with copy number variants as follows: 8bp deletion and insertion of 18 monomeric β-satellite repeat units, deletion of E1-5 and E13, 5 exons deletion, E6-10 deletion, 4 exons duplication, duplication of E7-10, and complex genomic rearrangement (Fig. 3a). Beyond hearing loss, *TMPRSS3* is known to be a tumor-associated gene, and several studies have shown its overexpression in pancreatic, ovarian, and breast tumors. Through its proteolytic activity, *TMPRSS3* contributes to the proliferation, migration, and survival of tumor cells in cancer development (Akhavanfard *et al.*, 2020).

The *COL11A2* gene encodes one of the two alpha chains of type XI collagen and is expressed in the developing cochlea. *COL11A2* variants are related to several disorders that include autosomal dominant (DFNA13) or recessive (DFNB53) NSHL, as well as Stickler syndrome, otospondylomegaepiphyseal dysplasia, fibrochondrogenesis, and Weissenbacher-Zweymuller syndrome (Vona *et al.*, 2017). Up to now, 61 pathogenic or likely pathogenic variants in *COL11A2* have been reported, 12 of which, including the c.966dupC mutation found in this study, lead to NSHL (http://www.hgmd.cf.ac.uk/ac/), which was shown in Table 3.

Notably, this homozygous variant has previously been identified in two Iranian families of Persian and Turkish ethnicities, with individuals exhibiting prelingual and profound ARNSHL. In addition, the same variant has been introduced as the cause of hearing loss in an Iranian child with

Ellis-van Creveld syndrome. So far, homozygosity of this allele has been observed only in Iranians, suggesting a possible founder effect (Vona *et al.*, 2017). The structural integrity of TM is crucial for the hearing process. Accordingly, *COL11A2* pathogenic variants lead to hearing impairment due to the abnormal distribution of collagen XI in the tissue membrane and the change in the structure of the TM (Chakchouk *et al.*, 2015). In conclusion, we have identified two novel homozygous mutations in the *CABP2* and *OTOA* genes, as well as two previously reported mutations in the *TMPRSS3* and *COL11A2* genes in four unrelated Iranian patients with moderate to profound HL. It seems that the functional study on these mutations may provide deeper insight into their pathogenic mechanisms at the molecular level. Altogether, current research, while confirming the high genetic heterogeneity of ARNSHL in Iran, emphasizes the importance and revolutionary impact of NGS methods in diagnosing the genetic cause of hearing impairment in *GJB2* and *GJB6-negative* patients. Thus, such an approach can play a critical role in genetic counseling, prenatal diagnosis, and subsequently clinical management of hearing impairment for families at high risk of this disorder.

Table 3. Pathogenic and likely pathogenic mutations in *COL11A2* (NM_080680) causing non-syndromic hearing loss.

cDNA /AA*	Phenotype	Origin	Exon	Ref	cDNA /AA*	Phenotype	Origin	Exon	Ref
c.109G>T (p.A37S)	DFNB53	Tunisia	1	Chakchouk *et al.*, 2015	c.2207G>T (p.G736V)	DFNA13	NA	29	Vona *et al.*, 2017
c.966dupC (p.T323Hfs)	DFNB53	Iran	8	Vona *et al.*, 2017	c.2423G>A (P.G808E)	DFNA13	NA	28	Chakchouk *et al.*, 2015
c.970G>A (p.G323E)	DFNA13	Dutch	31	Sloan-Heggen *et al.*, 2015	c.2662C>A (p.P888T)	DFNB53	Turkey	32	Chakchouk *et al.*, 2015
c.1638C>T (p.R549C)	DFNA13	America	42	Sloan-Heggen *et al.*, 2015	c.3100C>T (p.R1034C)	DFNA13	NA	39	Chakchouk *et al.*, 2015
c.1861C>A (p.P621T)	DFNB53	Iran	17	Chakchouk *et al.*, 2015	c.3392G>A (p.R1131Q)	DFNA13	NA	46	Vona *et al.*, 2017
c.2002C>T (p.P668S)	DFNB53	Japan	20	Vona *et al.*, 2017	c.3743C>T (p.P1248L)	DFNA13	NA	51	Vona *et al.*, 2017

*cDNA /AA= cDNA /AA change; NA: Not available

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Conflict of interest

All authors declare there is no conflict of interest.

Data availability statement

The identified novel variant in this study is accessible on the ClinVar repository under accession number "SCV002553195" for the *CABP2* gene. In addition, the original sequence results and other experimental results are accessible as supplementary file 1 via following link:https://drive.google.com/file/d/1A1qgY5XKej9jnYaq9IcI-3OLa1WhdyN2/view?usp=sharing

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