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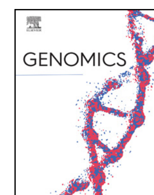

# A novel pathogenic variant in the *MARVELD2* gene causes autosomal recessive non-syndromic hearing loss in an Iranian family

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ABSTRACT

*Background and aims:* Hearing loss (HL) is the most common sensorineural disorder and one of the most common human defects. HL can be classified according to main criteria, including: the site (conductive, sensorineural and mixed), onset (pre-lingual and post-lingual), accompanying signs and symptoms (syndromic and non-syndromic), severity (mild, moderate, severe and profound) and mode of inheritance (Autosomal recessive, autosomal dominant, X-linked and mitochondrial). Autosomal recessive non-syndromic HL (ARNSHL) forms constitute a major share of the HL cases. In the present study, next-generation sequencing (NGS) was applied to investigate the underlying etiology of HL in a multiplex ARNSHL family from Khuzestan province, southwest Iran.
*Methods:* In this descriptive study, 20 multiplex ARNSHL families from Khuzestan province, southwest of Iran were recruited. After DNA extraction, genetic linkage analysis (GLA) was applied to screen for a panel of more prevalent loci. One family, which was not linked to these loci, was subjected to Otogenetics deafness Next Generation Sequencing (NGS) panel.
*Results:* NGS results showed a novel deletion-insertion variant (c.1555delinsAA) in the *MARVELD2* gene. The variant which is a frameshift in the seventh exon of the *MARVELD2* gene fulfills the criteria of being categorized as pathogenic according to the American College of Medical Genetics and Genomics (ACMG) guideline.
*Conclusion:* NGS is very promising to identify the molecular etiology of highly heterogeneous diseases such as HL. *MARVELD2* might be important in the etiology of HL in this region of Iran.

## 1. Introduction

Hearing loss (HL) is one of the most common congenital impairments, occurring in approximately 1–2 in 1000 newborns worldwide [1,2]. Half of the cases of this highly heterogeneous disease are attributed to genetic causes. Different criteria are routinely used for classification of HL. Genetic HL is divided into syndromic and non-syndromic forms considering other accompanying signs and symptoms [3]. Based on the site of the defect, HL is categorized as conductive, sensorineural and mixed. According to the time of onset, it is either pre-lingual or post-lingual. In terms of severity, HL is classified as mild, moderate, severe and profound. Finally, HL can have different modes of inheritance, including autosomal recessive, autosomal dominant, X-linked or mitochondrial [4,5].

HL is a main health problem in developing countries. Obviously, early onset HL prevents proper speech acquisition and negatively affects the quality of life and the socioeconomic position of the child [4] while early diagnosis can easily result in speech development and

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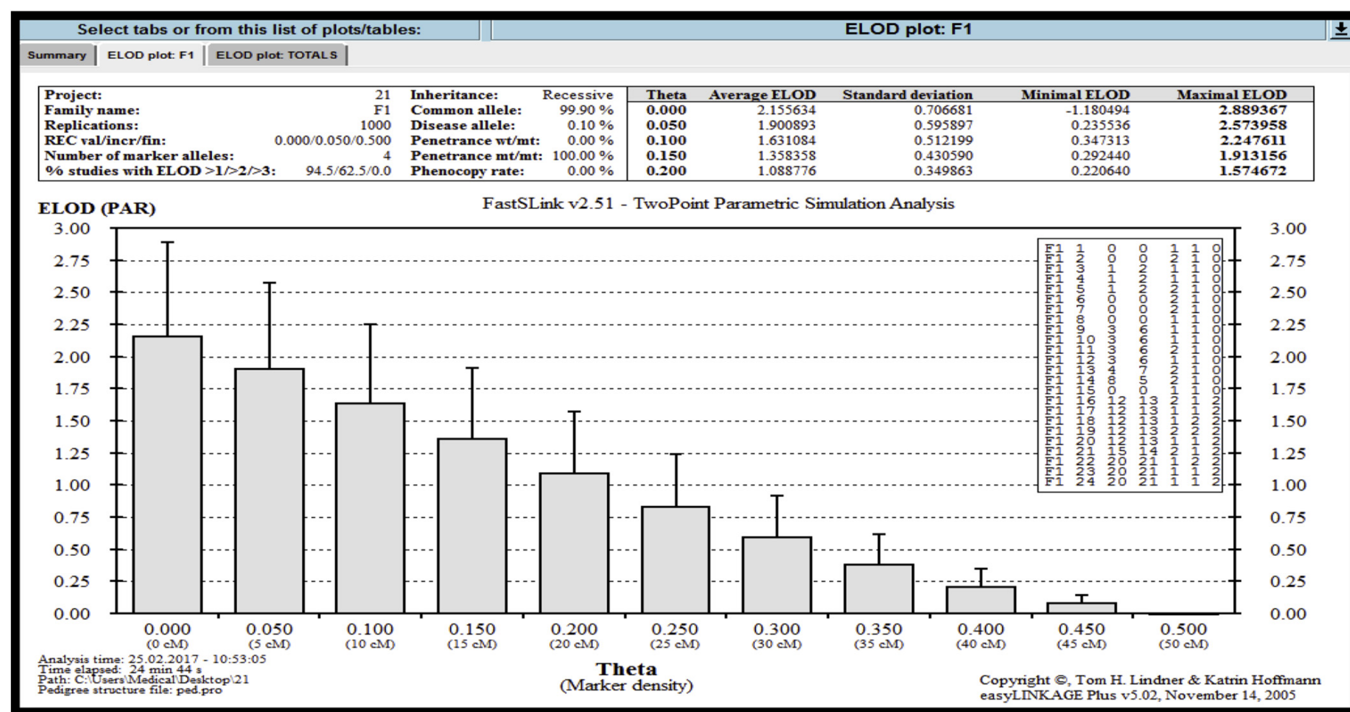


**Fig. 1.** Ahv-12 family SLINK value. The power to detect linkage depends on a variety of factors, such as the structure of the pedigree, the number of affected individuals and the informativeness of the markers. This image is the output of FastSlink v2.51 (easyLINKAGE program package). The maximum Slink value for Ahv-12 family is 2.8.

prevents further problems [6]. Non-syndromic forms (NSHL) constitute over 70% of HL cases and pattern of inheritance of nearly 80% of NSHL is autosomal recessive (ARNSHL) [7,8]. Previous studies have established involvement of about %1 of human genes in hearing development, defects in which cause HL. So far, 88 out of 148 known loci for NSHL are known to contribute to ARNSHL forms, which are usually more severe than other forms. To date, 64 genes have been identified to contribute to ARNSHL and 30 genes to autosomal dominant NSHL [9]. NSHL loci are numbered according to the order of discovery and classified bad on pattern of inheritance. DFNB and DFNA represent Loci with recessive and dominant inheritance, respectively. Likewise, loci with X-linked inheritance are shown as DFNX [10]. Although there are numerous genes contributing to HL, a few constitute a significant portion of the etiology [11]. Mutations in the *GJB2* gene are the main cause of ARNSHL all over the world, and, on average, cause about 18.5% of HL etiology in the Iranian population [12–14]. These findings emphasize the various mutational loads in NSHL genes in different populations. Genetic linkage analysis (GLA) has been used to detect the genetic cause of HL in different populations uncovering a considerable number of recessive HL genes for decades [15]. However, the approach has largely been superseded by the novel Next generation sequencing (NGS) technologies which offer a breakthrough in molecular research, facilitating more rapid diagnosis of different genetic diseases [16]. Although exome sequencing covers thousands annotated exons, the sequence coverage of regions of interest may be decreased in targeted regions. In comparison, targeted enrichment sequencing covers the target regions more efficiently and could reduce the analysis duration and cost [17–19]. NGS platforms have made their way into clinical laboratories, especially in diagnostic testing for hereditary disorders like HL [20,21]. NGS methods produce huge amount of data which need to be filtered and correctly classified. There are appropriate guidelines for NGS data interpretation [22,23].

The Iranian population with over 80 million people, and with many ethnic groups and high frequency of consanguineous marriages offers a valuable genetic resource to study ARNSHL [14]. Some of NSHL genes were initially discovered in the Iranian population through genetic linkage analysis (GLA) or have revealed many novel mutations [24–27].

*MARVELD2* (DBNB49) contributes to ARNSHL. *MARVELD2* encodes a membrane protein named tricellulin, a member of the tight junction-associated Marvel protein family which is found at the tight junctions between epithelial cells. Thus far, 6 mutations have been reported for *MARVELD2* gene to cause ARNSHL in different studies. Mutations in *MARVELD2* were the cause of HL in different studies from Pakistan, Iran and Central European Roma [28–31].

The present study is part of a larger study, which aimed to complete the genetic map of HL in Iran by investigating the pedigrees of families with hearing impaired members in each province. The result of this study would have implications in improved genetic counseling and prevention of HL using pre-implantation genetic diagnosis (PGD). In this study, 20 *GJB2* negative multiplex families from Khuzestan province, southwest Iran, were subjected to GLA to screen for more prevalent DFNB loci. A family (Named Ahv-12, Ahv stands for Ahvaz the birthplace of this family) with high Slink value not linked to the studied loci was subjected to NGS in order to clarify the etiology of HL and a novel pathogenic variant was found in *MARVELD2* gene (DFNB49).

## 2. Materials and methods

### 2.1. Family ascertainment and clinical diagnosis

Twenty consanguineous multiplex families (190 individuals) with at least 3 hearing impaired individuals in each family were recruited in this study. Families were from Khuzestan province with a high rate of consanguinity among the people. These families were selected from a collection of 50 families with hearing impaired individuals who had been visited by a specialist and the type of HL was confirmed as ARNSHL and had been subjected to Sanger sequencing for *GJB2* and to GLA for a panel of 11 common loci and were not linked to any of them. Affected members showed bilateral sensorineural moderate-to-profound HL. Pure tone audiometry and otoscopy were performed for all

the individuals by standard procedures. Simulation of linkage (SLINK) value was calculated for each pedigree as previously mentioned (Fig. 1 shows Ahv-12 family SLINK) [11]. Peripheral blood samples (5 ml) of all the 190 individuals were collected in EDTA-containing CBC tubes.

A written informed consent was filled out by all participants. The project was approved by the Review Boards of three different universities (Ahvaz Jundishapur University of Medical sciences, Shahrekord University of Medical Sciences and Isfahan University of Medical Sciences).

### 2.2. DNA extraction

DNA was extracted from 0.5 ml of blood sample for each individual by a standard phenol-chloroform procedure [32]. DNA purity and concentration was determined using a Nanodrop 2000 spectrophotometer (Nonodrop 2000 Thermo Scientific, USA) and its quality was checked on 1% agarose gel.

### 2.3. Genotyping STR markers and linkage analysis

In the previous study DNA sequencing of the coding region of *GJB2* was performed in one affected member of each family, using Sanger sequencing [11] and a panel of 11 loci was checked including: DFNB3, DFNB4, DFNB7/11, DFNB8/10, DFNB9, DFNB21, DFNB59, DFNB63, DFNB24, DFNB39, DFNB53.

In this study, we further examined the families using a panel of 4 other DFNB loci, including: DFNB35, DFNB42, DFNB48 and DFNB98 before performing NGS. Linkage analysis was performed using at least two informative Short Tandem Repeat (STR) markers located at or tightly linked to each locus. Each specific STR marker can have variable number of repeats among indviduals. NCBI MapViewer was used to choose the best STR markers. Primer sequences were obtained from the Probe database. Primer sequences are shown in Table 1. Touchdown PCR was performed in a thermal cycler machine (ASTEC PC-818, Fukuka, Japan) to amplify STR markers. For all the subjects, PCR products were genotyped via polyacrylamide gel electrophoresis. Different STRs had different sizes, heterozygosity and patterns but we used 10% polyacrylamide (19:1) to detect STR bands. As some STRs were uninformative in some of families we had to test more STRs to find at least 2 informative markers for each locus among families to confirm or reject linkage (Fig. 2). Haplotyping and logarithm of the odds (LOD) score calculations were done either to confirm or reject linkage.

Two-point and multi-point parametric LOD scores under a recessive model were, respectively, calculated by Superlink version 1.6 and Simwalk version 2.91(assuming a risk allele frequency of 0.001 and complete penetrance) (easyLINKAGE program package) [33,34]. Simwalk and Haplopainter software version 029.5 were used to reconstruct and visualize haplotypes, respectively [35].

### 2.4. NGS, data analysis and in silico prediction

NGS was performed using the Otogenetics Deafness Gene Panel (Otogenetics, USA), which uses custom oligonucleotide-based target capture of the coding regions of the 129 genes followed by Illumina HiSeq 2000 sequencing with > 100 fold coverage at every target base pair [36]. In summary, genomic DNA was fragmented to 200–300 base pairs, Illumina library was made and DNA fragments were captured using a customized array designed to target all exons, splicing sites and flanking intronic sequences of 129 deafness genes. The paired-end reads of 2 × 100 base pairs were then sequenced.

The released raw data were converted to the FASTQ file. Bioinformatics analysis included using BWA for read mapping to the reference genome (hg19, NCBI Build 38), Picard for removal of duplicate reads and GATK for variant calling. Variants were annotated with the Annovar software.

Homozygous missense, start codon change, splice site, nonsense, stop loss and indel variants with MAF < 1%, were filtered in dbSNP version 137, 1000 genomes database, NHLBI GO exome sequencing project (ESP) and exome aggregation consortium (ExAC). Several computational prediction tools were used to evaluate the pathogenic effect of the variants. We also used several online software tools including: FATHMM, PANTHER, SIFT, PROVEAN, MutationAssessor, I-Mutant2.0, PHD-SNP, PolyPhen-2 and Mutation taster for the investigation of the missense variant (Table 2). Next, the variants were investigated in the Human Gene Mutation Database (HGMD) and the literature to seek the novelty of the variant or its association with HL.

### 2.5. Sanger sequencing and NGS data validation

Only 3 out of all reported variants showed homozygote pattern, of which one was not found in the above-mentioned databases. Candidate variants were validated by Sanger sequencing in the proband, his parents and his affected uncle and aunt. The SeqMan software version 5.00 © (DNASTAR, Madison, WI, USA) was used to analyze the sequencing results. The American College of Medical Genetics and Genomics (ACMG) guidelines were used to interpret variants and classify them [23].

### 2.6. Co-segregation analysis

The following primers were designed to amplify the last exon of *MARVELD2* gene F: 5'-GCTGTAGAGACTTTTGCTATG-3′ R: 5′- CCTTG TACATCCCAATTC -3′. Co-segregation analysis was done for Ahv-12 family using Sanger sequencing. In addition, the variant in the *MARVELD2* gene was checked in a cohort of 50 healthy ethnically-matched controls using Sanger sequencing.

## 3. Results

### 3.1. Clinical evaluation and Slink result

The type of HL was detected as ARNSHL and its phenotype was bilateral moderate-to-profound HL. Pedigree of Ahv-12 family is shown in Fig. 3. The audiogram of the proband (Ahv-12) is shown in Fig. 4.

### 3.2. Linkage analysis

A panel of 4 loci was screened in this study. Polyacrylamide gel genotyping was done for all families and no linkage was found for the interested loci.

### 3.3. NGS data analysis

Out of all the variants found in this study, one met the criteria for further analyses. Table 3 summarizes the most probable candidates with in silico prediction.

A deletion of nucleotide c.1555G, replaced by nucleotides AA, changing ..AGAATGATCCTA.. to ..AGAATAAATCCTA.. (NM_001038603.2:c.1555delinsAA). It causes frameshift (NP_001033692.2: p.Asp519Lysfs*12) in the seventh exon of the *MARVELD2* gene. It creates alternation of 11 amino acids downstream of the insertion and leads to an early stop codon resulting in a truncated protein with 529 residues (versus 558 residues in the intact protein). This practically means the complete change of the last exon and deletion of about 30% of the occludin-ELL domain, which is essential for the protein interactions and tight junction function. This deletion-insertion mutation was assessed as being deleterious by mutation taster as well as several other prediction tools.

The variant was found to be co-segregating with the disease in the family: heterozygous in parents but homozygous in the three patients. The electropherogram of the last exon of *MARVELD2* gene is shown in Fig. 5.

**Table 1**
4 DFNB loci information. Details of screening markers are shown.

| Locus (gene) | MARKER | Forward and Reverse Primer sequences | PCR product range |
|---|---|---|---|
| DFNB98 )*TSPEAR)* | D21S1261 | F:GCAGCAGCACATCAACATTC<br>R:GAAGAGATGTTGAACTTCACTG | 194–204 |
| | D21S171 | F: GATCAAGTTAAGAGGAGGCT<br>R: GTGGGCTGCTGCTATCTTTG | 200–204 |
| | D21S1890 | F:TGACCACAGATTTCCCAATC<br>R:AAAAACACTCTGAACGATTAAGG | 143–173 |
| | D21S1260 | F: TCCAAGGGGTTCATCC<br>R: CCCAAGGCACTGTTCC | 200–214 |
| | D21S1446 | F:ATGTACGATACGTAATACTTGACAA<br>R: GTCCCAAAGGACCTGCTC | 209–223 |
| | D21S1912 | F: GAGCCCACCCTGGTAAC<br>R: CCCTCATACAGATTTAAAACACAC | 173–205 |
| DFNB48 (*CIB2*) | D15S1027 | F: CTGAAAACCAGCCCACTC<br>R: GAGTCCCTGGAGAGCCC | 196–210 |
| | D15S984 | F: GAGGCTCCGAGGGCAG<br>R: GCAGACACGCTCGCAT | 200–210 |
| | D15S1001 | F: TGGGCCTTGTGATTTTAG<br>R: CATCTGTGTGTCTGTGACTG | 259–270 |
| | D15S114 | F:AGAATGAGCAGCACTGTTTG<br>R:TTGTCACTGCTTTTCTCTGC | 177–191 |
| | D15S973 | F:ATCCACCTGACTCAAGGA<br>R:TTCTCCATCAGTAAATTGCG | 250–265 |
| | D15S991 | F: AGCTTGGTGACTCTATCAGGGTG<br>R: AACTGGCTGGTCTCTATTATCTGCG | 198–220 |
| | D15S1023 | F: GGTATTGTTTTGGACCACATCTTAG<br>R: GGGAGGCTGAGACAGTTTC | 272–293 |
| DFNB35 (*ESRRB*) | D14S53 | F:CTTTCCCCAGTGAAGTTGC<br>R:ACACTGCACTCCTACTCTG | 135–155 |
| | D14S61 | F: CCTGCTAAAAGTCAAGTGGG<br>R: AATGGCGTATCAGAGAGGAA | 197–227 |
| | D14S1036 | F:AAGCCCCTTTGTGACTGAC<br>R:GCCCATGTGATATTTGGTG | 203–217 |
| | D14S279 | F: GTGACCTCAGCATGTATTACTC<br>R: CAGGTATTCCCAATATGCAGC | 196–212 |
| | D14S270 | F:CCACTAATGATAACATTGTCGC<br>R: AGAGGCAGGTGAATGACT | 214–224 |
| | D14S983 | F: TGGACTGGTTAGCCTCAGTG<br>R: GCATCAACTGGCTTCCAATC | 222–270 |
| DFNB42 (*ILDR*1) | D3S3720 | F: CAAGCAATCCTCCAGCCTC<br>R: GCTGTATCTGAATCTAAAGCCT | 216–236 |
| | D3S3576 | F: GATCTAGTTGGCAAGGCG<br>R: TTGGACTCGAGCAATCC | 202–214 |
| | D3S3674 | F: GCCTTGGAAAACCTTGAA<br>R: TTGCCTGCAACAATGATTAC | 181–205 |
| | D3S3709 | F:TGCATGTGCGTGTATATTTG<br>R:ACAGTTGTGAGACACCACCTATT | 145–183 |
| | D3S3552 | F:GCCACTCCCAAATGTCTG<br>R:GACTGGAACTTGATTGCC | 161–177 |
| | D3S1278 | F: GGACACATGCTCCTGGAA<br>R: TGCACTAVAGGGCAGTTG | 203–231 |
| | D3S3645 | F: GCAGTGCTGAAAGATGGC<br>R: ACCTGGACTTGGGATGG | 164–212 |

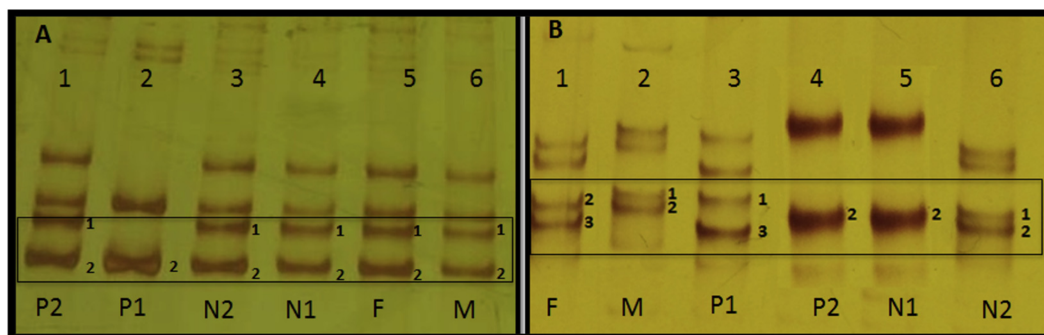


**Fig. 2.** Allele segregation in Ahv-12 family on 10% polyacrylamide (19:1) gel. No linkage was found. Key: M (Mother), F (Father), P (Patient with HL) and N (Normal). D15S1027 (DFNB48 (*CIB2* gene)) genotyping (A). F, M, N1, N2 and P2 are heterozygous while P1 is homozygous (A). D21S1446 (DFNB98) *TSPEAR* gene)) genotyping (B). F, M, P1 and N2 are heterozygous; while P2 and N1 are homozygous (B). P2 and N1 have the same pattern and the other patient shows heterozygosity. Patterns of allele segregation rejected linkage.

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**Table 2-**
The list of databases and prediction tools used in this study.

| | Tools/Databases | Electronic Address |
|---|---|---|
| 1 | SIFT | http://sift.jcvi.org |
| 2 | FATHMM | http://fathmm.biocompute.org.uk |
| 3 | PANTHER | http://www.pantherdb.org/tools/csnpScoreForm.jsp |
| 4 | MutationAssessor | http://mutationassessor.org/r3 |
| 7 | PolyPhen-2 | http://genetics.bwh.harvard.edu/pph2 |
| 8 | PROVEAN | http://provean.jcvi.org/index.php |
| 9 | Mutation taster | http://www.mutationtaster.org |
| 10 | I-mutant | http://folding.biofold.org/i-mutant/i-mutant2.0.html |
| 11 | String | http://string-db.org |
| 12 | Extasy | http://extasy.esat.kuleuven.be |
| 13 | Swiss-model | https://swissmodel.expasy.org/interactive |
| 14 | 1000 Genomes Project | http://www.1000genomes.org/ |
| 15 | DbSNP | http://www.ncbi.nlm.nih.gov/SNP/ |
| 16 | InterPro | https://www.ebi.ac.uk/interpro/ |
| 17 | Oligoanalyzer | https://eu.idtdna.com/calc/analyzer |
| 18 | MFEprimer-2.0 | http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/ |
| 19 | NCBIprimer blast | http://www.ncbi.nlm.nih.gov/tools/primer-blast/ |

## 4. Discussion

> 148 loci are known to cause hearing loss (HL) among which, 88 loci are attributed to ARNSHL [37]. DFNB1 (*GJB2*) is the most common known locus involved in HL worldwide, accounting for about 50% of ARNSHL cases in Western countries [11,38]. Even though mutations of *GJB2* are the most common cause of ARNSHL in Iran, they constitute about 18.5% of HL cases which highlights the role of other genes in Iran [12,13].

In this study, a consanguineous multiplex ARNSHL family negative for a panel of loci (investigated by GLA to find the cause of ARNSHL) was selected for further study using NGS by the Otogenetic HL gene panel and a novel deletion-insertion variant in the last exon of *MARVELD2* gene was found in this family.

The human *MARVELD2* gene (the *DFNB49* locus) is located on the chromosome 5q13.2 (Chromosome 5: 68,710,939–68,740,157) and encodes the MARVELD2/tricellulin protein [39]. The gene contains 7 exons and has 2 validated transcript variants [40,41]. MARVELD2, a transmembrane protein is composed of 558 amino acids and is mainly concentrated in tricellular tight junctions in the epithelial cells, including hair cells, cochlear supporting cells and marginal cells of stria vascularis [31,39]. Tight junction proteins (TJPs) have a definite role in regulating ionic and other molecular traffic. The inner ear epithelium is the main site for several tight junction proteins [42]. The MARVELD2 protein loss of function especially Occludin-ELL domain has been reported to be responsible for HL in several Pakistani families [30,31] and one Iranian family [28]. The locus DFNB49 was initially mapped to the long arm of chromosome 5 by Ramezan et al. who reported 2 Pakistani families linked to this region [40,43]. Riazuddin et al. identified the *MARVELD2* gene in this locus to cause HL in Pakistani families [31].

Riazuddin reported 4 different mutations in this gene in Pakistani families. IVS3-1G > A which resulted in the deletion of the first 17 nucleotides of exon 4 caused HL in a Pakistani family. Another reported variant was IVS4 + 2delTGAG that resulted in a premature stop codon found in 2 Pakistani HL families. IVS4 + 2 T > C was the cause of HL in 4 families resulting in premature termination of mRNA translation. c.1498C > T, a nonsense mutation in exon 5, caused HL in one Pakistani family [31]. Chishti et al. found 2 Pakistani families linked to DFNB49. Two different mutations were the causes of HL in these families. One was IVS4 + 2 T > C which had been previously reported by Riazuddin et al. [31] but the other, IVS4 + 1G > A, was a new mutation generating a truncated protein [44]. A single-base pair deletion (c.1543delA) in exon 6 of the *MARVELD2* gene which produces a premature stop codon (p.Lys517ArgfsX16) was reported as the cause of ARNSHL in an Iranian family by Babanejad et al. [28]. To date, 6 different pathogenic variants of the human *MARVELD2* gene have been identified in families with moderate-to-profound HL [28,30,31,44] (Table 5 and Fig. 6). Variants in the majority of TJPs have been associated with HL [30,39]. Riazuddin et al. accentuated the important role of occluding domian in the proper function of the tricellulin protein, and confirmed that the defect in this domain is associated with ARNSHL. Although it was confirmed that mutations in *MARVELD2* gene cause HL in humans and mice according to the study accomplished by Riazuddin, *Tric*−/− mice were generated by Kamitani et al. that suffered from an early-onset progressive HL associated with the destruction of cochlear hair cells probably owing to the disturbance in the epithelial barrier function in the organ of Corti. As the only pathological phenotype in their study was HL and loss of hair cells in *Tric*−/− mice, thus, contribution of tricellulin to the tight junctions especially in the organ of Corti was concluded [45].

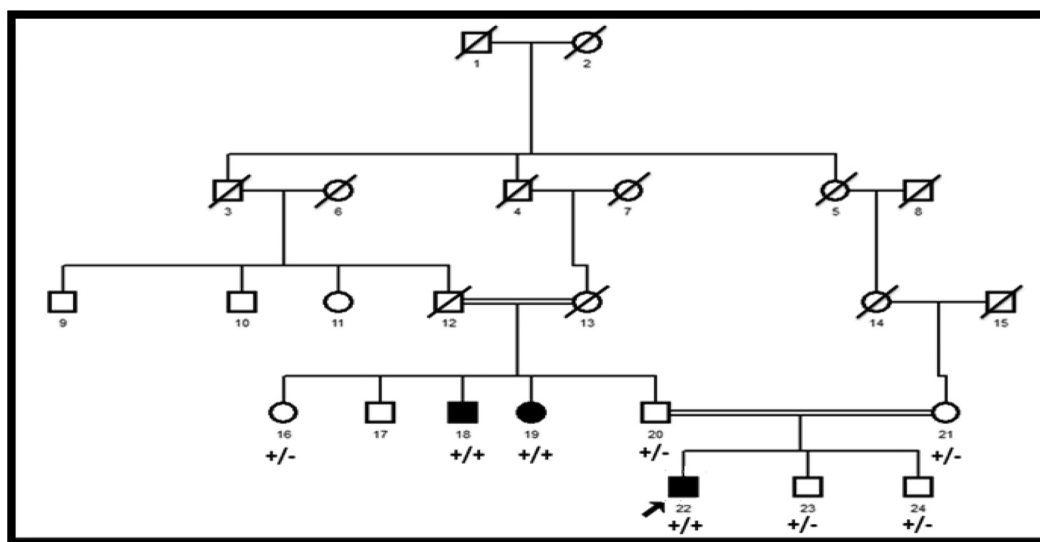


**Fig. 3.** The pedigree of Ahv-12 pedigree showing consanguinity among the nuclear families. Co-segregation of the novel deletion-insertion variant (c.1555delinsAA) with ARNSHL is shown in the pedigree. Individual #22 is the proband. Hearing impaired Individuals are shown with Filled symbols. (+) shows the new variant and (−) shows the wild-type allele.

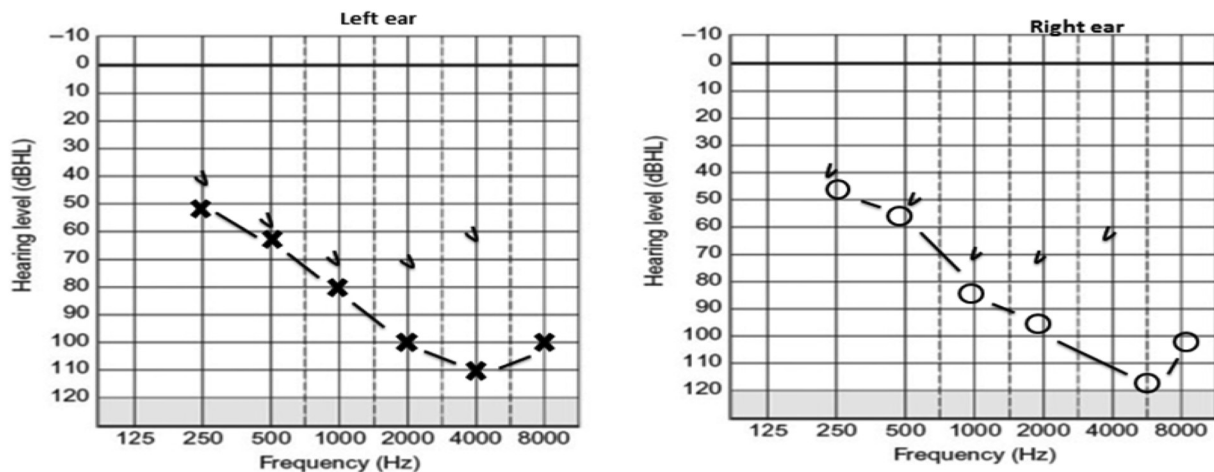


**Fig. 4.** Ahv-12 proband's audiogram confirming moderate-to-profound sensorineural defect in both ears. In air conduction testing, the symbol O stands for the right ear and the symbol X represents the left ear. A > symbol is used for the left ear and a < symbol is used for the right ear in bone-conduction vibration. The frequency and the loudness/intensity of sound are measured by Hertz (Hz) and decibels (db HL), respectively. The area above the symbols represents the sounds that the proband cannot hear and the area under the symbols indicates all of the sounds that the proband can hear.

**Table 3**
Bioinformatics analyses of the potential candidate variants found in this study.

| | *WHRN* | *MYO15A* | *OTOF* | *LHFPL5* |
|---|---|---|---|---|
| Zygosity | Homozygote | Homozygote | Heterozygote | Heterozygote |
| Variant type | Missense | Missense | Missense | Missense |
| Variant | P562A | A595T | R773C | H15Y |
| 1000 Genome Population Allele Frequency | 0.07 | 0.49 | 0.01 | 0 |
| Sift | Tolerated/1 | Damaging/0.05 | Damaging/0.02 | Damaging/0.04 |
| Polyphen | Possibly damaging/0.532 | Possibly damaging/0.68 | Possibly damaging/0.99 | Possibly damaging/0.98 |
| Provean | Neutral/−2.18 | Neutral/−0.68 | Deleterious/−4 | Deleterious/−3.15 |
| Mutation assessor | Medium/2.36 | Low/1.44 | Medium/2.29 | Medium/2.48 |
| Panther | Probably benign | Probably benign | Probably damaging | Probably damaging |
| Fathmm | Tolerated/3.71 | Damaging/−2.47 | Tolerated/−1.47 | Tolerated/−1.14 |
| I-mutant | Decrease stability | Decrease stability | Decrease stability | Decrease stability |

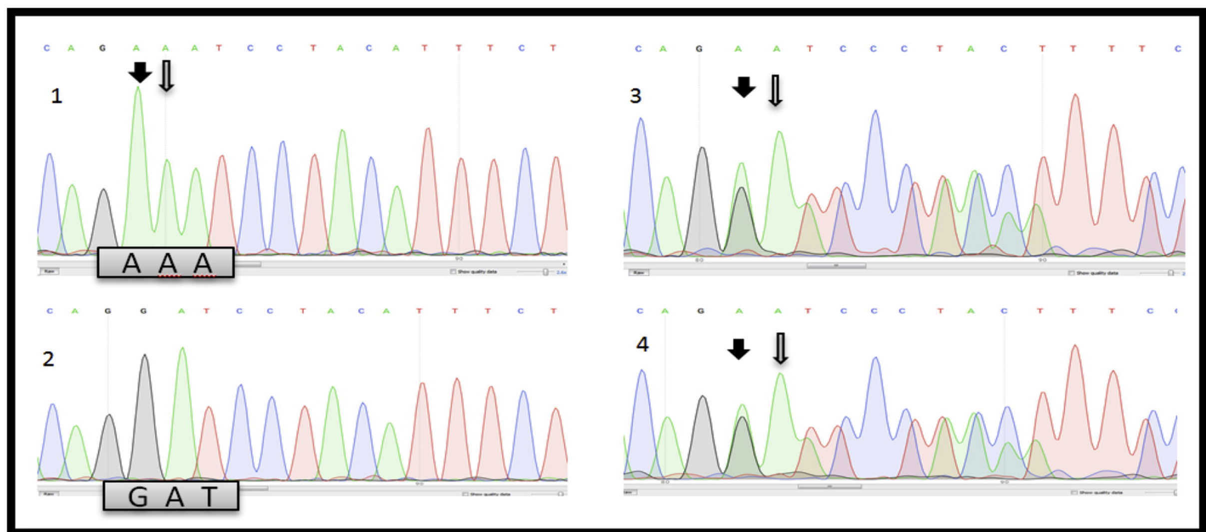


**Fig. 5.** Electropherogram of the last exon of the *MARVELD2* gene. Number 1, 2, 3 and 4 are the electropherogram of the patient, control individual and patient's father and mother, respectively. Heterozygosity of the deletion-insertion variant (c.1555delinsAA) in parents (heterozygote carrier), homozygous in the proband and homozygous wild-type alleles in a control individual are shown. Arrows show the position of deleted and inserted nucleotides.

In the present study, a novel deletion-insertion mutation (c.1555delinsAA) in *MARVELD2* was found to be the cause of HL in Ahv-12 family. The deletion-insertion variant was suggested to be deleterious by mutation prediction tools such as Mutation Taster. Even though this variant was not a missense mutation, we used different in silico prediction tools to determine the impact of one single amino acid alternation on the MARVELD2 protein functions and structure (Table 4). Surprisingly, it was reported as deleterious.

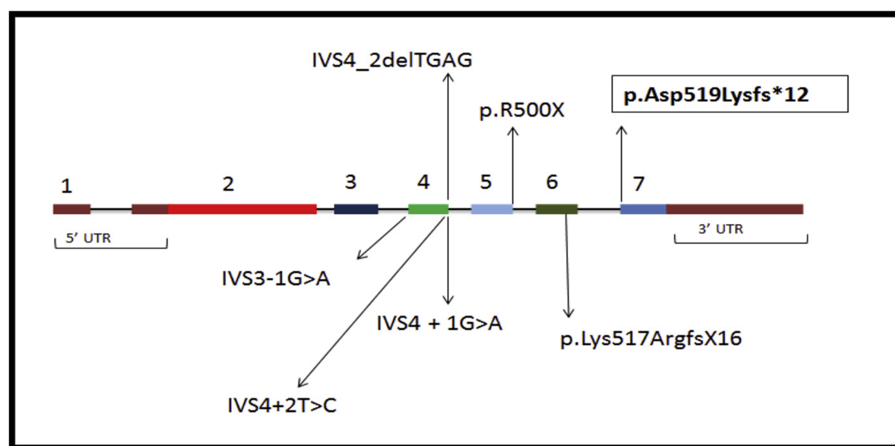


**Fig. 6.** Sites of known variants of the *MARVELD2* gene. The deletion-insertion variant c.1555delinsAA (p.Asp519Lysfs*12) which is located in exon 7, and 6 previously known variants are shown. Exons 5, 6 and 7 make the occludin domain.

**Table 4**
The impact of c.1555delinsAA, only assessed for D519K substitution, on the MARVELD2 protein predicted by in silico tools.

| PANTHER | SIFT | POLYPHEN | PROVEAN | MUTATION TASTER |
|---|---|---|---|---|
| Possibly damaging | Damaging/ 0.006 | Probably Damaging/1 | Deleterious/ −5.43 | Disease causing |

**Table 5**
The known mutations of the *MARVELD2* gene.

| Variant | Type | Study |
|---|---|---|
| IVS3-1G > A | Cryptic splice-acceptor site within exon 4 of *TRIC* | Riazuddin et al. (2006) |
| IVS4 + 2T > C | Exon4 skipping | |
| c.1498C > T (p.R500X) | Transition in exon 5 | |
| IVS4_2delTGAG | Premature stop codon | |
| IVS4 + 1G > A | Splice site mutation | Chishti et al. (2007) |
| p.Lys517Argfs*16 | Frameshift | Babanejad et al. (2012) |
| p.Asp519Lysfs*12 | Frameshift | Current study |

As the variant was in the last exon of the gene, the conservation of the exon as well as whole Occludin-ELL domain was checked more closely. The last exon of *MARVELD2* gene is highly conserved among species (Table 6) and has a key role in interaction between MARVELD2 and TJP1 (ZO-1) protein and also for the adequate function of tricellular tight junctions [46]. Defect in MARVELD2 and TJPs lead to moderate-to-profound forms of ARNSHL [45]. The C-terminal sequence of tricellulin and occludin which consists of 130 aa is completely conserved and contains a domain necessary for its interaction with ZO-1 a TJ plaque protein (Fig. 7) [47]. Even the last 35 amino acids of the MARVELD2 protein are reported as highly conserved by Interpro and Pfam [48]. Full-length tricellulin is necessary for proper tightness of TJPs of Corti. Truncated proteins are unable to associate with ZO-1, ZO-2 and ZO-3 [31,47]. The MARVELD2 protein interacts with TJP1 via the occludin domain, as described before. Length reduction of TRIC protein, which was the result of this frameshift variant, deteriorates the effective association between tricellulin and other TJPs.

The alteration we have found in this study is located in the cytosolic domain of the protein, which is highly vital for protein interactions and based on the previous findings we expect it to cause HL. The variant (p.Lys517ArgfsX16), reported by Babanejad et al., resulted in a premature stop codon [28], is close to this novel variant and both have similar effect on shortening the occludin domain.

The ACMG guidelines were used for variant interpretation [23] The deletion-insertion variant is a null allele leading to a truncated protein (PVS1), the variant was seen in all affected family members and was not detected in the controls (PS4), the variant is co-segregating with the disease in all affected family members (PP1) and bioinformatics analyses support a deleterious effect on MARVELD2 protein (PP3). It is located in a validated domain (PM1), this variant reduces the length of

**Table 6**
Occludin-ELL domain alignment among different species shows conservation of the last exon of the *MARVELD2* gene.

| Species | match | Gene | Change point (residue) | Alignment |
|---|---|---|---|---|
| Human | | ENSG00000152939 | 519 | IHEEFKKKKN**D**PTFLEKKERCDYLKNKLSHIKQRI QEYDKVMNWDVQGYS* |
| Mutated | Partly conserved | ENSG00000152939 | 519 | IH EEFKKKKN**K**SYISGKKRTL* |
| P.troglodytes | all conserved | ENSPTRG00000016951 | 520 | IHEEFKKKKN**D**PTFLEKKERCDYLKNKLSHIKQRI QEYDKVMNWDVQGYS |
| M.mulatta | all conserved | ENSMMUG00000009158 | 518 | IHEEFKKKKN**D**PTFLEKKERCDYLKNKLSHIKQRI QEYDKVMNWDVQGYS |
| F.catus | all conserved | ENSFCAG00000002488 | 318 | IHEEFKKKKN**D**PTFLEKKERCDYLKNKLSHIKQRI QEYDKVMNWNVQGYS |
| M.musculus | Partly conserved | ENSMUSG00000021636 | 516 | IHEEFKKKKN**D**PSFLEKKERCDYLKNKLSHIKQRI QEYDKVMNWDTQGY |
| T.rubripes | Partly conserved | ENSTRUG00000018563 | 520 | IHEEFKKKKN**D**PTFLEKKERCEYLKSKLSHIKQKI QEYDKVMEWN-DGY |

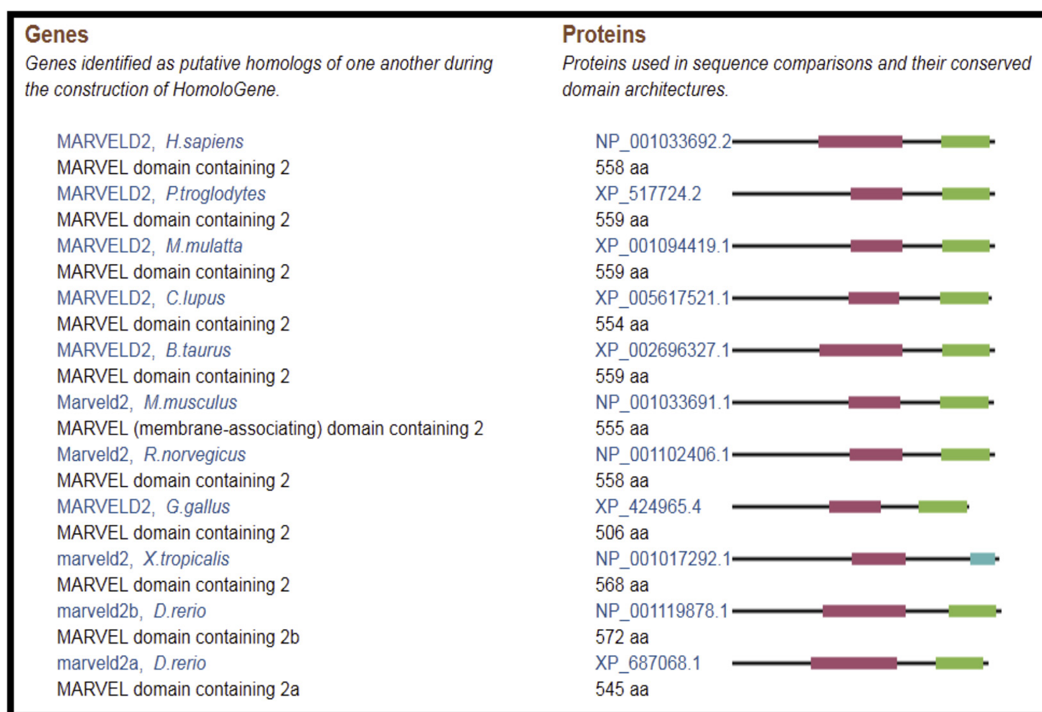


**Fig. 7.** Marveld2 conservation among different species obtained from NCBI. Pink rectangles (left) show marvel domain and green boxes (right) show occludin domain of protein and the occludin domain is highly conserved. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the protein (PM4) and was not reported by dbSNP database and 1000 genome project (PM2) [23]. Thus, the variant is categorized as being pathogenic.

Mašindová et al. studied the central European Roma population to determine the prevalence of DFNB49 mutations [29]. Nayak et al. estimated the frequency of *MARVELD2* mutations 1.5% in a cohort of 800 Pakistani families with ARNSHL [30]. Our study is the second study which shows the DFNB49 HL in Iran. We obtained the frequency of 5% (1 family) among the 20 families negative for *GJB2* mutations in this study. This might suggest that *MARVELD2* mutations should be considered in the diagnosis of HL in Iran.

In summary, we identified a novel variant in the *MARVELD2* gene (DFNB49). The gene should be studied in larger series of families in Iran for a more thorough understanding of its role in causing HL. While GLA is a cost effective and reliable method to study genetic diseases, using GLA in studying heterogeneous disorders like HL with numerous known loci; may be time consuming and expensive. However, in situations where there is limited access to NGS technologies, it seems logical to apply GLA for common loci. This study highly recommends the application of the NGS methods in the study of heterogeneous disorders such as HL.

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