

# Clinical and Genetic Characterization of Gap Junction Protein $\beta$ -6 Variants in Non-Syndromic Hearing Loss: A Case Report with Familial Evaluation and *In Silico* Analyses

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## What's Known

- Gap junction protein  $\beta$ -6 (*GJB6*) encodes connexin 30, a gap junction protein essential for cochlear ion homeostasis and normal hearing.
- Large deletions in *GJB6* are well-established causes of non-syndromic hearing loss, but single-nucleotide variants in this gene are rare, and their pathogenic significance remains unclear.

## What's New

- A novel *GJB6* gene variant (c.446C>T) was identified by whole-exome sequencing (WES) and confirmed by Sanger sequencing in the family.
- The integration of clinical and *in silico* analyses suggests a potential impact on protein structure.
- Literature review confirming the rarity of *GJB6* single-nucleotide variants, particularly in Iranian populations.

## Abstract

Non-syndromic sensorineural hearing loss (NSHL) exhibits substantial genetic heterogeneity, and the identification of its causative variants remains an important challenge. The gap junction protein  $\beta$ -6 (*GJB6*), expressed in multiple tissues including the sensory epithelium of the inner ear, is essential for auditory function. Although large *GJB6* deletions are well characterized, single-nucleotide variants (SNVs) have been reported infrequently, and their pathogenic significance remains insufficiently defined. In this study, we identified a novel homozygous missense variant in *GJB6*, NM\_001110219.3:c.446C>T (p.Ala149Val), through whole-exome sequencing and confirmed it by Sanger sequencing in a 32-year-old Iranian female from a consanguineous family, presenting with prelingual bilateral sensorineural hearing loss without any additional phenotypes or syndromic features. She was referred to the Medical Genetics Laboratory in Tehran, Iran, in March 2025. *In silico* predictions, mammalian conservation, and structural modeling suggest that this substitution may disrupt normal *GJB6* function. To contextualize this finding, we reviewed all reported SNVs in *GJB6*, identifying 20 distinct variants in 47 patients, with most mutations clustering in the extracellular loop 1, cytoplasmic loop, transmembrane domain 3, and extracellular loop 2. These results expand the mutational spectrum of *GJB6* and highlight its critical role in NSHL pathogenesis. Collectively, this study presents the first comprehensive report of SNVs in *GJB6* and supports their further investigation in the genetic architecture of hereditary hearing loss.

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**Keywords** • Hearing loss • Sensorineural • Whole exome sequencing • Point mutation

## Introduction

Hearing, which involves the conversion of physical sound waves into electrochemical signals, is a highly coordinated and dynamic event involving intricate biological mechanisms.<sup>1, 2</sup> The cochlea, a fluid-filled sensory organ of the inner ear, plays a

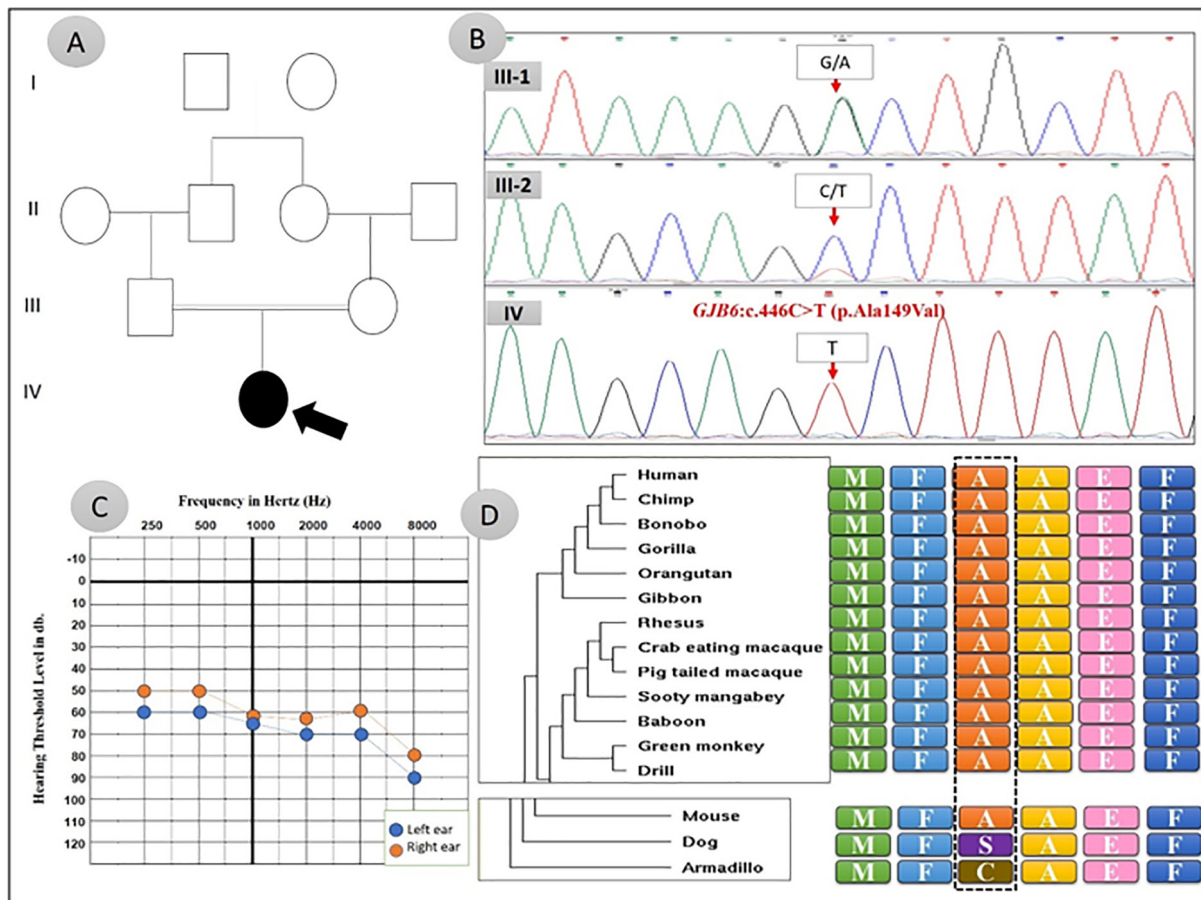
central role in auditory transduction and depends on the precise regulation of ionic gradients, particularly potassium ( $K^+$ ) homeostasis. Gap junction channels composed of connexin proteins, such as gap junction protein  $\beta$ -6 (*GJB6*), in cochlear supporting cells facilitate  $K^+$  recycling and enable direct intercellular communication necessary for maintaining the endocochlear potential. Moreover, *GJB6* has been implicated in the repair and maintenance of cochlear epithelial integrity following sensory cell damage, highlighting its essential role in inner ear homeostasis and normal hearing.<sup>1-3</sup> While large *GJB6* deletions are well characterized, single-nucleotide variants (SNVs) are rarely reported, and their pathogenic significance remains unclear.<sup>1-3</sup>

In this study, we report a novel homozygous variant of the *GJB6* gene in a woman with moderate to severe bilateral sensorineural hearing loss (SNHL) and no ectodermal dysplasia or palmoplantar keratoderma. Using *in silico* analyses, we evaluated the structural and functional impacts of the identified variant and compared them with those of other previously

reported SNVs in *GJB6*.

### Case Presentation

A 32-year-old woman, born to healthy consanguineous parents, was referred to the Medical Genetics Laboratory in Tehran, Iran, in March 2025. She was born at full term via an uneventful vaginal delivery with no perinatal complications, neonatal hospitalization, or significant medical issues during childhood. Developmental milestones were reportedly normal, except for an early-onset hearing impairment diagnosed before 6 months of age. Pure-tone audiometry and the Weber test revealed moderate to severe bilateral SNHL. Speech test scores indicated reduced speech clarity compared to normal, even with amplification of the sound. The patient has been using hearing amplification devices since childhood. At the most recent evaluation, no progression of hearing loss was observed. Comprehensive clinical and paraclinical evaluations ruled out external ear



**Figure 1:** Multiple molecular analyses that were conducted in the family to further understand the etiology of the hearing loss are shown. a) The family's pedigree indicates autosomal recessive inheritance. b) Sanger sequencing confirmed the presence of homozygous and heterozygous variants in the proband and her parents, respectively. c) Pure-tone audiogram of the proband indicates moderate to severe bilateral sensorineural hearing loss. d) Alanine and its surrounding amino acids in gap junction protein  $\beta$ -6 are highly conserved in mammals, as shown by evolutionary conservation studies.

malformations, dermatological findings (e.g., eczema or psoriasis), visual abnormalities, and signs suggestive of metabolic or syndromic involvement. No other systemic abnormalities were observed. Based on the clinical findings and family pedigree analysis, the case was classified as non-syndromic sensorineural hearing loss (NSHL) with an autosomal recessive inheritance pattern (figure 1). Informed written consent was acquired from the proband and her parents for the release of clinical and genetic data. The study was approved by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences (IR.USWR.REC.1403.199).

#### *Molecular Evaluation: Whole-Exome Sequencing and Copy Number Variant Analysis*

Genomic DNA was extracted from whole blood samples of the proband and her parents using the standard salting-out method.<sup>4</sup> Since no pathogenic variants were identified in *GJB2*, whole-exome sequencing (WES) was performed to detect additional candidate genes associated with hereditary hearing loss. The patient's DNA was fragmented and enriched using the Agilent SureSelect All Exon V7 kit (Illumina genome sequencing service in Macrogen, Seoul, South Korea). Variant calling was carried out by Genome Analysis Toolkit (GATK), and the resulting VCF file was annotated using wANNOVAR. Variant filtering was based on the assumed autosomal-recessive inheritance pattern, clinical significance databases, and population allele frequencies from the Genome Aggregation Database (gnomAD) and Iranome (minor allele frequency <0.01). All candidate variants were manually inspected in the Integrative Genomics Viewer to eliminate false positives. Copy number variations (CNVs) were analyzed using GATK (v.4.0.10.1).

#### *The Result of Molecular and Bioinformatics Analyses in the Family*

Analysis of WES data identified a novel homozygous variant, NM\_001110219.3:c.446C>T (p.A149V), located in exon 5 of *GJB6* (figure 1). No CNVs were detected in the proband across the known hearing loss-associated genes. According to the American College of Medical Genetics guidelines,<sup>5</sup> the detected variant was classified as a variant of uncertain significance (VUS). This classification was based on PM1 (moderate), as the missense variant is found in a recognized protein functional domain, and PM2 (moderate), due to its very low allele frequency across large population databases. No additional strong or supporting criteria were fulfilled; therefore, the currently available evidence remains insufficient to establish pathogenicity (figure 1, table 1). The variant at this position had a combined annotation dependent depletion (CADD [v1.7]) score of 13.13, placing it within the top 5-10% of potentially deleterious variants but below the commonly used pathogenicity threshold (CADD≥20). Furthermore, the A149V substitution in *GJB6* was modeled and compared with the wild-type structure using the SWISS-MODEL server. The resulting models were subsequently loaded and visualized in PyMOL (v2.5.5). Global backbone alignment showed no measurable deviation (Cα RMSD≈0.02 Å), indicating the preservation of the overall fold. The major effect was local and side-chain specific: the solvent-accessible surface area (SASA) at residue 149 nearly doubled (WT side chain≈36 Å<sup>2</sup> vs A149V≈70 Å<sup>2</sup>), with a moderate increase in the local segment (residues 146–152, +22 Å<sup>2</sup>). These changes reflect the bulkier and more hydrophobic side chain of valine, which protrudes further into the surrounding environment (figure 2).

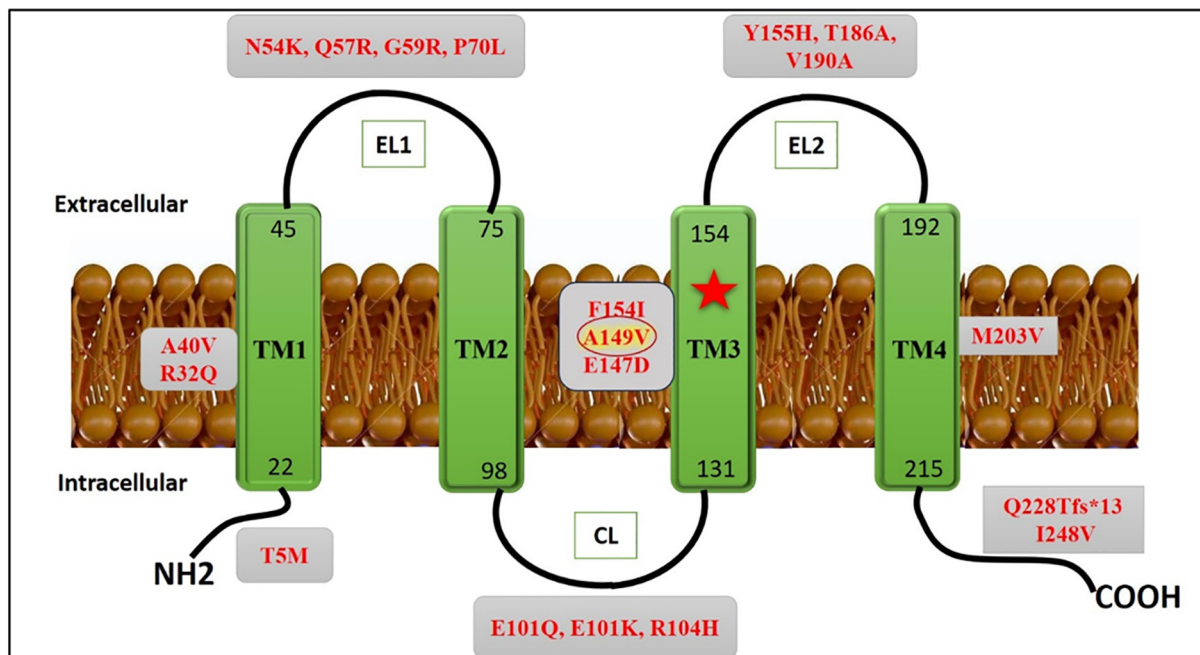
**Table 1:** *In silico* predictions and population frequencies of *GJB6*:c.446C>T (p.Ala149Val)

| Bioinformatics software prediction * |          |                     |                                  |          |                                  |                      |                         |               |
|--------------------------------------|----------|---------------------|----------------------------------|----------|----------------------------------|----------------------|-------------------------|---------------|
| SIFT                                 | PolyPhen | MutationT@ster 2025 | MetaSVM                          | MetaLR   | PROVEAN                          | M-CAP                | DEOGEN2                 | Fathmm XF     |
| Tolerated                            | Benign   | Damaging            | Tolerated                        | Damaging | Neutral                          | Damaging             | Damaging                | Neutral       |
| Conservation Scores (dbNSFP v5.2)    |          |                     |                                  |          |                                  |                      |                         |               |
| Phastcons470way_Mammalian            |          |                     | 1                                |          | Strong conservation in Mammals   |                      |                         |               |
| Phastcons17way_Primate               |          |                     | 0.602                            |          | Partial conservation in Primates |                      |                         |               |
| Protein Stability                    |          |                     |                                  |          |                                  |                      |                         |               |
| DynaMut2                             |          |                     | Protein stability                |          |                                  | ΔΔG: -0.26 kcal/mol  |                         | Destabilizing |
| mCSM                                 |          |                     | Protein stability                |          |                                  | ΔΔG: -0.016 kcal/mol |                         | Destabilizing |
|                                      |          |                     | Protein-protein complex affinity |          |                                  | ΔΔG: -0.994 kcal/mol |                         | Destabilizing |
| Population Frequencies               |          |                     |                                  |          |                                  |                      |                         |               |
| GnomAD v2.1.1                        |          |                     | 0.000003976                      |          |                                  | Iranome              | 0.000416                |               |
|                                      |          |                     | Number of homozygotes: 0         |          |                                  |                      | Number of homozygotes:0 |               |

\* The data presented in the table were obtained using multiple *in silico* resources, including Ensembl Variant Effect Predictor (VEP), PubVar, UCSC, and dbNSFP v5.2.(Web Query).



**Figure 2:** Bioinformatics analyses by several tools were performed to further understand the function of NM\_001110219.3:c.446C>T (p.Ala149Val). a) A multiple sequence alignment of *GJB6* and *GJB2* was conducted using CLUSTAL O (1.2.4) through the EMBL-EBI platform. The study indicated that alanine at position 149 is conserved in both proteins. b) The hydrophobicity and side-chain volume of valine are stronger than those of alanine, indicating its tendency to be buried in the protein structure.



**Figure 3:** According to UniProt, *GJB6* protein (O95452 CXB6\_HUMAN) comprises five topological domains and four transmembrane segments: TM1 (residues 23-45), TM2 (residues 76-98), TM3 (residues 132-154), and TM4 (residues 193-215). These transmembrane segments (TM) are interspersed with an N-terminal cytoplasmic domain, one cytoplasmic loop (CL), two extracellular loops (EL1 and EL2), and a C-terminal cytoplasmic domain (<https://www.uniprot.org/>). The asterisk marked the position of the variant (TM3) found in the proband.

While these changes suggest potential local effects, their functional consequences remain speculative and require experimental validation.

#### Review of Literature and Genotype-Phenotype Correlation

A total of 47 patients with *GJB6* have been reported across 14 papers, representing 20 variants (including 17 missense, 2 synonymous, and 1 insertion variant) from different ethnicities (table 2).<sup>1,2,6-17</sup> Syndromic hearing loss was reported in 13 patients (27.6%). The remaining cases exhibited isolated hearing loss. The identified variants were distributed across multiple domains of the *GJB6* protein. Most mutations were located within the extracellular loop 1 (EL1), cytoplasmic

loop (CL), transmembrane domain 3 (TM3), and extracellular loop 2 (EL2) (figure 3). Variants located within the transmembrane domains included seven distinct substitutions, collectively reported in 12 individuals (25.5%).<sup>7, 9-11, 13, 16</sup> Functional studies indicate that the T5M mutation exerts a dominant-negative effect, causing profound hearing loss in heterozygous humans, whereas in mice, heterozygotes remain unaffected and homozygotes exhibit only mild hearing impairment (table 2).<sup>18-21</sup> While reported *GJB6* variants span multiple protein domains and are associated with both syndromic and non-syndromic phenotypes, clear genotype-phenotype correlations remain limited, particularly for rare missense variants.

Table 2: Clinical evidence and functional studies of GJB6 single-nucleotide variants.

| Study                                     | Variant                      | Phenotype <sup>2</sup>                                                                    | Inh <sup>3</sup> | CADD <sup>4</sup> | AF <sup>5</sup> | C.S.             | Dom.              | No. of cases                         | Method                                                                                                                 | Eth-note                                                                                                                                                                                                                                           |
|-------------------------------------------|------------------------------|-------------------------------------------------------------------------------------------|------------------|-------------------|-----------------|------------------|-------------------|--------------------------------------|------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Clinical evidence <sup>1</sup>            |                              |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
| Grifa and colleagues (1999) <sup>8</sup>  | GJB6: T5M/WT                 | Bilateral middle high frequency HL <sup>*</sup>                                           | AD               | 16.43             | 2.489e-5        | VUS <sup>*</sup> | NH2 <sup>*</sup>  | 3 cases (f)                          | SSCP <sup>*</sup> analysis, Secondary structure prediction, and electrophysiological studies in <i>Xenopus</i> oocytes | Italian-p.T5M: c.14C>T, causes HI via dominant inhibition of wild-type CX30 channels                                                                                                                                                               |
| Yang et al., (2007) <sup>7</sup>          | GJB6: A40V/WT                | NSHL <sup>*</sup>                                                                         | AD               | 24.7              | 5.567e-5        | VUS              | TM1 <sup>*</sup>  | 1 case                               | PCR, Direct sequencing of the coding regions                                                                           | Taiwanese -c.119C>T, p.A40V. Novel missense heterozygous mutation; required for CX30 transport to plasma membrane; TM1 mutants retained in ER <sup>*</sup> or Golgi.                                                                               |
|                                           | GJB6:P87P/WT <sup>**</sup> & |                                                                                           | AD               | 0.016             | 1.998e-5        | VUS              | TM2               | 2 cases                              |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB6:P87P/P87P               |                                                                                           | AR?              |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB6:L132L/WT &              |                                                                                           | AD               | 9.442             | 1.626e-4        | B                | TM3               | 3 cases (1 Het, 2 C.H <sup>6</sup> ) |                                                                                                                        |                                                                                                                                                                                                                                                    |
| Nemoto-Hasebe et al., (2009) <sup>8</sup> | GJB6:L132L/WT                | Mild PPK <sup>*</sup> , severe SNHL <sup>*</sup> , knuckle pads and pseudo-ainhum of toes | AD               | 24.6              | Absent          | LP               | EL1 <sup>*</sup>  | 1 case                               | PCR, Direct sequencing, restriction enzyme digestion, histological evaluation, electron microscopy                     | Japanese -c.175G>C; p.Gly59Arg, First PPK-deafness case caused by mutation in CX30 E1 domain. E1 loop is essential for connexon-connexon interaction to form intercellular channels                                                                |
|                                           | GJB6:G59R/WT                 |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
| Asma et al., (2011) <sup>9</sup>          | GJB6: R32Q/WT                | Bilateral severe to profound SNHL                                                         | AD <sup>6</sup>  | 28.8              | 1.768e-5        | LP               | TM1               | 4 cases                              | PCR, sequencing                                                                                                        | Malay, Indian- Four novel GJB6 mutations E147D, R32Q, E101K and Y155H                                                                                                                                                                              |
|                                           | GJB2:V37I/WT                 |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB6: E101K/WT               |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB2:V37I/WT                 |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB6: E147D/WT               |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
| Battelino et al., (2012) <sup>10</sup>    | GJB2:V37I/WT                 |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB6: Y155H/WT               |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           | GJB2:W24X                    |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
| Oh et al., (2013) <sup>11</sup>           | GJB6:M203V/WT                | Mild progressive HL                                                                       | AD               | 12.11             | 8.663e-4        | LB               | TM4               | 1 case                               | PCR, Sanger Sequencing                                                                                                 | Caucasian -The patient showed onset at 26 years, with a duration of 7 years and a mean Pure-tone audiometry change of 15 dB over this period, GJB2: Normal                                                                                         |
|                                           | GJB6:P87P/WT                 |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
| Oh et al., (2013) <sup>11</sup>           | GJB6:P87P/WT                 | SNHL                                                                                      | AD               | 0.016             | 1.998e-5        | VUS              | TM2               | 1 case                               | PCR, sequencing, cloning and transfection, biochemical and ionic measurement assays                                    | Korean -2 variants (I248V, A40V) selected for functional studies. Cx30-p.I248V is nonpathogenic; it has normal ionic coupling and a minor effect on biochemical coupling; differences are likely due to molecule size; further studies are needed. |
|                                           | GJB6:I248V/WT                |                                                                                           |                  |                   |                 |                  |                   |                                      |                                                                                                                        |                                                                                                                                                                                                                                                    |
|                                           |                              |                                                                                           |                  | 17.27             | Absent          | VUS              | COOH <sup>*</sup> | 1 case                               |                                                                                                                        | Cx30-p.A40V was nonpathogenic in this study; others show Golgi accumulation, possibly due to different experimental methods.                                                                                                                       |

| Study                                   | Variant                                | Phenotype <sup>2</sup>                                    | Inh <sup>3</sup> | CADD <sup>4</sup> | AF <sup>5</sup> | C.S. | Dom. | No. of cases | Method                                                                                                                            | Eth.-note                                                                                                                                                                                                                                                     |
|-----------------------------------------|----------------------------------------|-----------------------------------------------------------|------------------|-------------------|-----------------|------|------|--------------|-----------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Miyagawa et al., (2013) <sup>12</sup>   | GJB6:T186A/WT                          | Bilateral SNH                                             | AD               | 22.9              | 3.977e-6        | VUS  | EL2* | 1 case       | MPS*, Sanger sequencing                                                                                                           | Japanese-Found in the Early Onset group                                                                                                                                                                                                                       |
| Beck et al., (2015) <sup>13</sup>       | GJB6:V190A/WT                          | NSHL                                                      | AD               | 23.8              | Absent          | LP   | EL2  | 3 cases      | direct sequencing, PCR                                                                                                            | German -Three novel variants reported; no second mutation found to establish a causative effect.                                                                                                                                                              |
|                                         | GJB6:M203V/WT                          |                                                           |                  | 12.11             | 8.663e-4        | LB   | TM4  |              |                                                                                                                                   |                                                                                                                                                                                                                                                               |
|                                         | GJB6: 682insA /WT                      |                                                           |                  | 24.3              | Absent          | LP   | COOH |              |                                                                                                                                   |                                                                                                                                                                                                                                                               |
| Pandey et al., (2016) <sup>14</sup>     | GJB6:N54K/WT                           | HL and skin abnormalities                                 | AD               | 23.3              | Absent          | VUS  | EL1  | 12 cases     | Linkage analysis, Sequencing, PCR, Cell expression, immunostaining, confocal imaging, dye transfer assays                         | Indian-GJB6:N54K mis localized to cytoplasm; fails neurobiotin transfer, showing loss of gap junction function. Confirmed by linkage analysis in 12 members of a family.<br>GJB2: p.R127H (c.380G>A) in cis with GJB6: p.N54K;<br>p.R127H may modulate p.N54K |
|                                         |                                        |                                                           |                  |                   |                 |      |      |              |                                                                                                                                   |                                                                                                                                                                                                                                                               |
| Alkowari et al., (2017) <sup>15</sup>   | GJB6:P70L/P70L                         | SNHL                                                      | AR?              | 28.2              | 1.415e-5        | LP   | EL1  | 1 case       | Targeted Re-Sequencing, Sanger sequencing                                                                                         | Qatari-First GJB6 allele detected in Qatari and Gulf populations; located in conserved domain crucial for protein structure. The proband has no family history of HL.                                                                                         |
| Nonose et al., (2018) <sup>16</sup>     | GJB6:F154I/WT<br>SLC26A4:c.918+2T>C/WT | Postlingual progressive HL; mixed loss in right ear; EVA* | AD <sup>6</sup>  | 25.1              | Absent          | VUS  | TM3  | 1case        | Microsatellite STR* genotyping, haplotype analysis, PCR, Sanger sequencing, MLPA*, WES*                                           | Brazilian-WES performed to identify second variant in two monoallelic SLC26A4 cases; c.460A>T found in pt.83, alongside other variants—None definitively causative.                                                                                           |
| Amritkumar et al., (2018) <sup>17</sup> | GJB6:R104H/WT                          | Moderate to profound prelingual HI                        | AD <sup>6</sup>  | 22.1              | 2.053e-4        | VUS  | CL   | 1 Het, 2 C.H | PCR, Sequencing                                                                                                                   | South Indian-Reported three novel variants p.Q57R, p.E101Q, p.R104H in the coding region of GJB6 gene                                                                                                                                                         |
|                                         | GJB2:E42D/WT                           |                                                           |                  |                   |                 |      |      |              |                                                                                                                                   |                                                                                                                                                                                                                                                               |
|                                         | GJB6:Q57R/WT                           |                                                           |                  | 24.1              | Absent          | LP   | EL1  | 3 cases (f)  |                                                                                                                                   |                                                                                                                                                                                                                                                               |
|                                         | GJB6:E101Q/WT                          |                                                           |                  | 15.67             | 5.978e-5        | VUS  | CL   | 4 cases (f)  |                                                                                                                                   |                                                                                                                                                                                                                                                               |
| Morgan et al., (2018) <sup>2</sup>      | GJB6:P70L/WT                           | NSHL                                                      | AD               | 28.2              | 1.415e-5        | LP   | EL1  | 1case        | Targeted re-sequencing, high-density SNP* arrays                                                                                  | Italian-The variant reported sporadic in one case as a likely damaging allele                                                                                                                                                                                 |
| Alkhdhir et al., (2024) <sup>11</sup>   | GJB6:P70L/WT                           | Moderate HL                                               | AD               | 28.2              | 1.415e-5        | LP   | EL1  | 1case        | GJB2 gene sequencing, chromosomal microarray, targeted familial variant testing, HL gene panel, WES, mitochondrial genome testing | Qatari-Reported as having uncertain association to the NSHL phenotype.                                                                                                                                                                                        |

| Study                               | Variant | Phenotype <sup>2</sup>                                               | Inh <sup>3</sup> | CADD <sup>4</sup> | AF <sup>5</sup> | C.S. | Dom. | No. of cases | Method                                                                                                                                                        | Eth.-note                                                                                                                                                           |
|-------------------------------------|---------|----------------------------------------------------------------------|------------------|-------------------|-----------------|------|------|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Functional evidence                 |         |                                                                      |                  |                   |                 |      |      |              |                                                                                                                                                               |                                                                                                                                                                     |
| Schütz et al., (2010) <sup>18</sup> | T5M     | Middle/high-frequency HL                                             | HL               | 16.43             | 2.489e-5        | VUS  | NH2  |              | Homologous recombination; ABR <sup>*</sup> ; immunolabelling; Western blot; dual patch-clamp; calcein dye transfer; Ca <sup>2+</sup> imaging via hemichannels | T5M is dominant-negative in humans, causing profound HL in heterozygotes; in mice, heterozygotes are unaffected, and homozygotes show mild HI <sup>*</sup>          |
| Wang et al., (2011) <sup>19</sup>   | A40V    | ADNSHL <sup>*</sup>                                                  |                  | 24.7              | 5.567e-5        | VUS  | TM1  |              | Cloning, HeLa/tet-on expression, fluorescent tagging, immunostaining, homology modeling                                                                       | p.A40V missense mutation causes Cx30 accumulation in Golgi, with dominant negative effect on both Cx30 and Cx26                                                     |
| Zhang et al., (2013) <sup>20</sup>  | G45E    | Keratitis-ichthyosis-Deafness (KID) syndrome in a <i>GJB2</i> mutant |                  | 27.5              | 7.971e-6        | P    | EL1  |              | Site-directed mutagenesis, HEK293 transfection, dye/Ca <sup>2+</sup> transfer assays                                                                          | The role of the G45E mutation was investigated in cochlear connexins 30, 32, and 43, revealing that G45E causes increased hemichannel activity (leaky hemichannels) |
| Berger et al., (2014) <sup>21</sup> | T5M     | NSHL                                                                 |                  | 16.43             | 2.489e-5        | VUS  | NH2  |              | Molecular cloning, Cell culture transfection, immunostaining, gap junction and hemichannel assays,                                                            | Mechanisms of four autosomal dominant Cx30 mutations investigated.                                                                                                  |
|                                     | V37E    | Clouston syndrome or keratitis-ichthyosis-deafness syndrome          |                  | 24.6              | 3.976e-6        | VUS  | TM1  |              | apoptosis (TUNEL), ER stress (XBP1 splicing), Western blotting                                                                                                | T5M: Functional channels; may alter permeability; reduces Cx26 levels                                                                                               |
|                                     | G59R    | Vohwinkel and Bart-Pumphrey syndromes                                |                  | 24.6              | Absent          | LP   | EL1  |              |                                                                                                                                                               | V37E: ER-retained; loss-of-function; dominant-negative on Cx30, Cx26, Cx43; induces apoptosis                                                                       |
|                                     | A88V    | Clouston syndrome                                                    |                  | 28.9              | Absent          | P    | TM2  |              |                                                                                                                                                               | G59R: Golgi-retained; loss-of-function; defective oligomerization and hemichannel formation                                                                         |
|                                     |         |                                                                      |                  |                   |                 |      |      |              |                                                                                                                                                               | A88V: Intracellular-retained; leaky hemichannels; dominant-negative effect on Cx26/Cx30/Cx43; induces apoptosis                                                     |

1: Studies including both clinical and functional data were classified as clinical.2: For cases without an individual phenotype report, the cohort phenotype was assigned.3: The mode of inheritance (Inh) was described based on the data presented in the article. 4: The CADD\_PHRED score (GRCh37-v1.7) was used for variant pathogenicity assessment. 5: Allele frequencies (AF) were obtained from gnomAD v2.1.1. 6: Both variants were detected in the heterozygous state in a single individual, suggesting a possible autosomal dominant inheritance, although a digenic effect cannot be excluded.

\*Abbreviations: Eth: Ethnicity; HL: Hearing loss; C.S: Clinical significance; CADD: Combined annotation-dependent depletion; VUS: Variant of uncertain significance; NH: N-terminus amino-terminal; SSCP: Single-strand conformation polymorphism; NSHL: Non-syndromic sensorineural hearing loss; B: Benign; LB: Likely benign; LP: Likely pathogenic; P: Pathogenic; TM1: Transmembrane domain 1; ER: Endoplasmic reticulum; PPK: Palmoplantar keratoderma; SNHL: Sensorineural hearing loss; EL1: Extracellular loop 1; CL: Cytoplasmic loop; COOH: Carboxyl-terminal c-terminus; Cx: Connexin; EL2: Extracellular loop 2; MPS: Massively parallel sequencing; STR: Short tandem repeat; MLPA: Multiplex ligation-dependent probe amplification; WES: Whole-exome sequencing; SNP: Single nucleotide polymorphism; ABR: Auditory brainstem response; HI: Hearing impairment; ADNSHL: Autosomal dominant non-syndromic hearing loss; F: Family members; Het: Heterozygous; C.H: Compound heterozygote.

\*\*Synonymous variants are included in the table but are not shown in the figure.

Note: Variants *GJB6*:c.366delT (reported by Asma et al., 2011, appears inconsistent with current HGVS annotation) and *GJB6*:p.Gly21Arg (reported by Sommen et al., 2016, lacking sufficient clinical data) were not included in the table.

## Discussion

Gap junctions in humans and other mammals are composed of two hemichannels (connexons), each formed by six connexin subunits. Among the connexin family, *GJB2* and *GJB6* are the most abundant in the cochlea and work together to maintain inner ear homeostasis. These two genes share high sequence homology (approximately 76%) and contain several conserved structural domains essential for normal auditory function.<sup>19</sup> Studies suggest that, due to the established functional interplay between *GJB6* and *GJB2*, digenic or modifier effects should be considered even when no pathogenic *GJB2* variants are detected.<sup>3, 9, 10</sup> Reports of incomplete penetrance have been observed, and while *GJB6* deletions in combination with *GJB2* variants can exacerbate disease severity, the resulting phenotype largely depends on the deletion location and its effect on *GJB2* expression.<sup>3, 9, 10</sup>

In contrast to *GJB2*, point mutations and deletions in *GJB6* are uncommon.<sup>22</sup> Variants such as G21R, T5M, and A40V are associated with non-syndromic hearing loss,<sup>6, 19, 23</sup> whereas N54K, G11R, and N14S are linked to specific forms of ectodermal dysplasia, with or without deafness.<sup>14, 24, 25</sup> In *GJB6*, skin-related pathogenic variants are proposed to act by increasing hemichannel open probability, causing excessive efflux of vital metabolites; for example, A88V in TM2 leads to hidrotic ectodermal dysplasia without affecting hearing.<sup>21</sup> However, altered permeability to signaling molecules, such as inositol 1,4,5-trisphosphate (IP<sub>3</sub>), together with impaired Ca<sup>2+</sup> signaling propagation, represents a potential pathogenic mechanism by which *GJB6* mutations contribute to the development of NSHL.<sup>22</sup>

Some variants impair auditory function by reducing the number of functional gap junction channels; notably, N54K is retained in the cytoplasm, thereby blocking neurobiotin transfer, resulting in aberrant intracellular localization with subsequent loss of channel activity.<sup>14</sup> The N54K mutation in either *GJB6* or *GJB2* results in a similar phenotype comprising hearing impairment and cutaneous abnormalities, highlighting the essential role of connexin genes in both epidermal and auditory systems.<sup>14, 22</sup> The *GJB6*:p.T5M, located within the N-terminal domain,<sup>6</sup> disrupts both Ca<sup>2+</sup> signaling and IP<sub>3</sub> permeability, whereas *GJB6*:p.A40V,<sup>19</sup> Alanine in this region, together with other amino acids, contributes to the formation of a hydrophobic core, which is essential for establishing a functional and stable intramolecular structure of *GJB6*.<sup>19, 22</sup>

In another study, *GJB6*: p.P70L was detected in the homozygous state in a patient from a consanguineous family, in which relatives were either unaffected wild-type carriers or heterozygous for this variant. The patient presented with bilateral prelingual deafness and no family history of hearing loss.<sup>15</sup>

In our study, WES identified a homozygous NM\_001110219.3:c.446C>T (p.A149V) variant in the proband, which was confirmed by Sanger sequencing in her family. Residue 149 is located within TM3 (figure 3). Previous studies have indicated that TM3 is located at the periphery of the hemichannel and is in contact with the lipid environment.<sup>26, 27</sup> In connexins, TM3 primarily contributes to helix packing, inter-subunit interactions, and structural stability rather than lining the channel pore.<sup>26, 27</sup> In the p.A149V variant, valine exhibits stronger hydrophobicity and side-chain volume than alanine, which is associated with its heightened propensity to be buried in the protein structure.<sup>26, 27</sup> Thus, the observed increase in side-chain exposure is more consistent with a potential disturbance in helix packing, membrane embedding, or connexon assembly/trafficking than with a direct alteration in pore diameter.<sup>26</sup> Overall, the modeling suggests that A149V does not destabilize the backbone structure but introduces a local sidechain perturbation in TM3 that may affect protein folding, subunit packing, or oligomerization. These findings alone are insufficient to establish pathogenicity; functional studies (trafficking assays, gap-junction coupling, etc.) are required to clarify the biological impact.

The variant's low frequency in Iranome, gnomAD, and other databases, including GenomeAsia (with no reported homozygotes) supports its rarity. However, its relatively higher frequency in a population-matched database (Turkish variome) may reflect reduced penetrance or population-specific variation, potentially requiring additional genetic or environmental factors for NSHL to manifest. Moreover, this study has inherent limitations. Segregation analysis was restricted by the proband being an only child, and longitudinal follow-up data were limited, although no progression was reported. The rarity of *GJB6* point mutations and poorly defined genotype-phenotype correlations for missense variants limit broader generalization. Furthermore, while extensive *in silico* analyses were performed, these computational predictions cannot replace functional validation. Nevertheless, the findings provide valuable insight into this rare variant and contribute to expanding the current

understanding of *GJB6*-associated hearing loss.

## Conclusion

Next-generation sequencing plays a crucial role in NSHL diagnosis. In this study, we report a novel *GJB6* missense variant (NM\_001110219.3:c.446C>T [p.Ala149Val]) associated with NSHL, thereby expanding the known genetic spectrum of this gene.

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## Authors' Contribution

F.ZA: Conceptualization, study design, experimental procedures, genetic testing, and drafting; M.M: Data interpretation and processing, and reviewing the manuscript; E.E: Data gathering and reviewing the manuscript; H.Gh: Data gathering and reviewing the manuscript, HR.KhKh: Data interpretation and reviewing the manuscript; R.N: Conceptualization, Study design, experimental procedures, genetic testing, and drafting; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Declaration of AI

No AI tools were used for data analysis or manuscript preparation.

**Conflict of Interest:** None declared.

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