Original Article

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A novel compound heterozygous mutation in the *GJB2* gene is associated with non-syndromic hearing loss in a Chinese family

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Summary **Autosomal recessive (AR) non-syndromic hearing loss (NSHL) is the most common form of hereditary deafness. Mutations in the gap junction protein beta 2 (*GJB2*) gene encoding connexin 26 (Cx26) account for about 50% of cases of ARNSHL. In the current study, a combination of exome sequencing and Sanger sequencing in a Chinese Dong family with ARNSHL allowed identification of a novel compound heterozygous mutation c.240G>C(p.Q80H)/C.109G>A(p.V37I) in exon 2 of the *GJB2* gene, which co-segregated with the disease phenotype in this family and was not evident in 100 healthy controls. Bioinformatic analysis revealed that the two mutations in the *GJB2* gene were probably pathogenic. Results indicated that the compound heterozygous variants, p.Q80H and p.V37I, in the *GJB2* gene are associated with ARNSHL. The Q80H variant was initially identified in patients of Dong Chinese origin with NSHL. The current results broaden the spectrum of *GJB2* mutations responsible for NSHL and have important implications for molecular diagnosis, treatment, and genetic counseling for this family.**

Keywords: Exome sequencing, hearing loss, *GJB2* gene, mutation

1. Introduction

Hearing loss is the most frequent human sensory disorder, affecting 1 in 1,000 newborns (*1*). More than 50% of the cases of hearing loss are attributable to genetic causes that may be non-syndromic or syndromic (*2*). Non-syndromic hearing loss (NSHL) generally refers to deafness without other clinical symptoms, and accounts for 70% of inherited deafness. Approximately 80% of NSHL is autosomal recessive (AR), about 20% is autosomal dominant (AD), and less than 1% is inherited in an X chromosome-linked or a mitochondrial manner (*3*). To the extent known, at least 59 genes associated with ARNSHL have been identified (*http://hereditaryhearingloss.org*). For many populations, the most common cause of ARNSHL is a mutation in the gap junction protein beta 2 (*GJB2*) gene that encodes connexin 26 (Cx26) (*4-6*). Cx26 is highly expressed in epithelial supporting cells of the mammalian cochlea and is believed to play an important role in the recycling of the K^+ ion from the hair cells to the endolymph (*7-9*). So far, over 100 pathogenic mutations in the *GJB2* gene have been reported (The Connexin-deafness Homepage: *http://davinci.crg.es/deafness*).The p.V37I variant is highly prevalent in East Asian deafness, but the pathogenic role of p.V37I is debated. p.V37I was previously reported to be a polymorphism without pathogenicity in some studies; nevertheless, results have increasingly revealed that a homozygous p.V37I mutation or a compound p.V37I mutation with some other *GJB2* pathogenic variation is associated with mild to moderate hearing loss (*10-12*).

Using conventional Sanger sequencing to identify variants causing ARNSHL is extremely time-consuming and expensive due to the condition's high level of genetic heterogeneity; in contrast, exome sequencing is a powerful and cost-effective tool with which to reveal the genetic basis of Mendelian diseases (*13*). The current study used a combination of exome sequencing and Sanger sequencing, and results revealed a novel compound heterozygous mutation c.240G>C(p.Q80H)/

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c.109G>A(p.V37I) of the *GJB2* gene in a Chinese Dong family with ARNSHL.

2. Materials and Methods

2.1. *Subjects*

Subjects were a nonconsanguineous Chinese Dong family with ARNSHL from an ethnic minority region of western Hunan Province, China. Five members of the family, including three affected siblings (II:1, II:2, and II:3, Figure 1A) and two unaffected parents (I:1, I:2, Figure 1A), were included in this study. Clinical evaluations were conducted and pure tone audiometry (PTA) was performed at the First People's Hospital of Huaihua, China. Auditory function was divided into normal hearing (< 20 dBHL), mild deafness (20-40 dBHL), moderate deafness (41-70 dBHL), severe deafness (71-95 dBHL), and profound deafness (> 95 dBHL) (*14*). Three patients (II:1, aged 12 years; twins,II:2 andII:3 , aged 8 years) had congenital hearing loss, and PTA indicated moderate bilateral sensorineural deafness (Figure 2). Audiometric data are summarized in Table 1. The parents (I:1, aged 38 years; I:2, aged 35 years) and 100 ethnicity-matched unrelated normal controls had bilateral normal hearing according to PTA. The study was approved by the Ethics Committee of Hunan University of Medicine, Huaihua, China. Written informed consent were obtained from all participants or guardians in accordance with the principles of the Declaration of Helsinki.

2.2. *Exome sequencing*

A genomic extraction kit (Tiangen Biotech Co. Ltd, Beijing, China) was used to isolate genomic DNA from leukocytes in peripheral venous blood from all participants. The proband (II:1) in the family was subjected exome sequencing. According to the manufacturer's protocol, no less than 1.5 μg of genomic DNA was used to construct the exome library. Genomic DNA of the proband was fragmented using Covaris sonication, and the DNA library was pooled and hybridized for enrichment of exons using Agilent SureSelect Human All Exon V5. Enriched exome fragments were sequenced on the HiSeq 2000 platform. A mean sequencing depth of 128.16× was obtained to

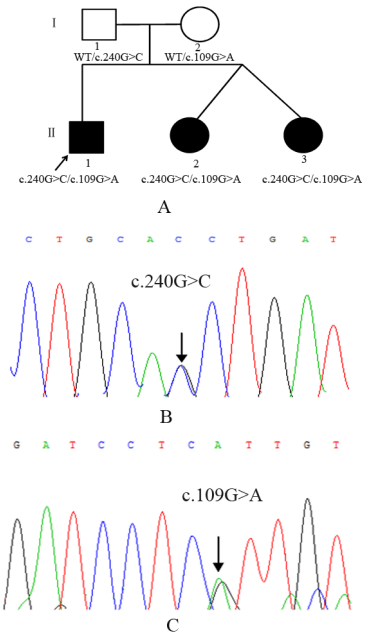

Figure 1. Pedigree and mutation analysis. (A) A Dong family with ARNSHL. The black arrow indicates the proband (II:1) of the family, and the filled symbols represent affected members. The patients (II:1,II:2, II:3) have compound heterozygote mutations in the *GJB2* gene. **(B)** A heterozygous *GJB2* c.240G>C mutation of the proband. **(C)** A heterozygous *GJB2* c.109G>A mutation of the proband.

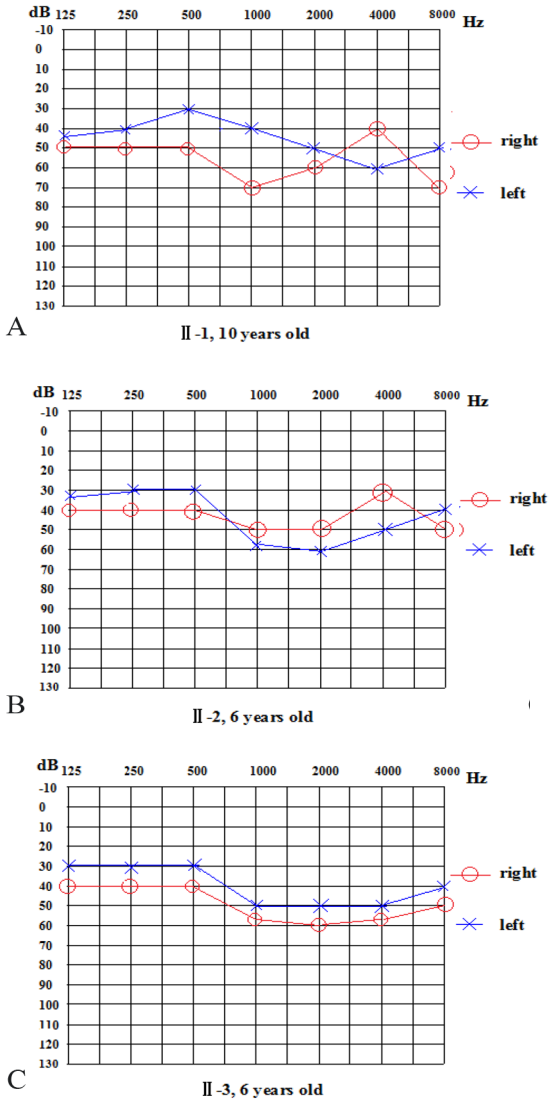

Figure 2. Pure tone audiograms of three affected family members. Hearing thresholds are the hearing level in decibels. The patients display moderate bilateral deafness. The patient age at diagnosis is indicated at the bottom of the audiogram. The "o" and "x" symbols represent air conduction pure-tone thresholds at different frequencies in the right and left ear. dB, decibels; Hz, Hertz.

Table 1. Characterization of the audiometric data for three affected family members

Individual	Gender	Current age	Newborn hearing screening	Age of onset	Age at initial visit	Age at audiometric testing	Degree of hearing loss	Other symptoms
II1	M	12	moderate	birth	10	10	moderate	N
II2	F	8	moderate	birth	6	6	moderate	N
II3	F	8	moderate	birth	6	6	moderate	N

F, female; M, male; N, without other symptoms

accurately determine variants at 99.20% of the targeted exome.

The sequence reads were aligned to a human genome reference obtained from the UCSC database version hg19 (*http://genome.ucsc.edu*) using the Burrows-Wheeler Alignment tool. SAMtools was used to detect single nucleotide polymorphisms (SNPs) and insertions/deletions, and Picard was used to delete duplicate reads (produced mainly during PCR). Possible variants were filtered against databases including SNP database version 137 (dbSNP137), 1000 Genomes data (April 2012 version), HapMap8, the YanHuang1 (YH1) project, and synonymous mutations. For bioinformatic analysis, four in-silico tools were used to predict potential deleterious effects of missense mutations, including PolyPhen-2 (*http://genetics.bwh.harvard.edu/pph2/*), SIFT (*http://sift.jcvi.org*), Mutation Taster (*http://www.mutationtaster.org/*) and the American College of Medical Genetics and Genomics (ACMG). The software ANNOVAR (Annotate Variation) was used to annotate possible variants. In order to confirm the conservation of amino acid substitutions during species evolution, typical protein sequences from multiple species were aligned using the online tool Clustal W to compare mutation sites with conserved domains.

2.3. *Verification with Sanger sequencing*

After exome sequencing, Sanger sequencing was used to verify genetic defects. Sequences of primers for potential causative mutations in the *GJB2* gene (NM_004004.5) were designed and synthesized as follows: 5'-ACACGTTCAAGAGGGTTTGG-3' and 5'-GGGAAATGCTAGCGACTGAG-3'. The size of the PCR product was 1,003 bp, and the product contained the coding sequence of the *GJB2* gene. PCR was performed with 30-μL reaction mixtures containing 40 ng of genomic DNA, 1.0 μM of the forward and reverse primers, and 15 μL of 2X Taq Master Mix (Huiling Biotech Co. Ltd, Shanghai, China). Thermocycling was performed using the following program: initial denaturation at 95℃ for 2 min, followed by 35 cycles of 94℃ for 10 s, 60℃ for 30 s, and 72℃ for 1 min, and final extension at 72℃ for 5 min. PCR products were purified with the Cycle-Pure Kit (OMEGA; Bio-Tek, Doraville, GA) and sequenced using an ABI PRISM 3730 automated sequencer (Applied Biosystems). Co-segregation analysis was subsequently performed with available DNA samples from family members. A mutation was considered to be novel if it was not in the National Center for Biotechnology Information dbSNP database (*http://www.ncbi.nlm.nih.gov/projects/SNP/index.html*), the Human Mutation Database (HGMD) (*http://www.hgmd.cf.ac.uk/ac*), or the Exome Variant Server (*evs.gs.washington.edu*) and had not been published.

2.4. *Structure-based model building and analysis*

Human wild-type and mutant proteins were modeled using SWISS-MODEL online (*https://swissmodel.expasy.org*), and PyMOL Viewer was used to visualize the effects of mutated residues on the structure of proteins.

3. Results

3.1. *Exome sequencing*

The proband generated 60,456,963 raw reads with a mean read length of 150 bp according to exome sequencing; 98.14% (59332463) of these raw reads were aligned to the human reference genome. Synonymous variants and known common variants described in dbSNP137, 1000 Genomes data, HapMap8, and the YH1 project were excluded. Non-synonymous variants were predicted using SIFT, PolyPhen-2, and Mutation Taster to eliminate benign variants or tolerated variants. In the known genes for hearing loss, a novel compound heterozygous mutation, c.240G>C(p.Q80H) /c.109G>A(p.V37I), in the *GJB2* gene was identified in the proband.

3.2. *Identification of causative mutations*

The compound heterozygous variants c.240G>C(p.Q80H) and c.109G>A(p.V37I) in the *GJB2* gene (Figure 1B and 1C) were found in all three affected family members according to Sanger sequencing, and the heterozygous variants c.240G>C(p.Q80H) and c.109G>A(p.V37I) were found in the unaffected father and mother, respectively, but were absent in the 100 ethnically matched normal controls. The compound heterozygous variants in the *GJB2* gene co-segregated with the ARNSHL phenotype in this family, suggesting

Table 2. Prediction of the functionality of compound heterozygous mutations

Variants	AA change	PolyPhen2	SIFT	Mutation Taster	ACMG
c.240G>C	p.Q80H	Probably damaging (1.00)	Damaging (0.00)	Disease-causing	Likely pathogenic
c.109G>A	p.V37I	Probably damaging (1.00)	Tolerated (0.66)	Disease-causing	pathogenic

Figure 3. A multiple-sequence alignment of the amino acid sequence in Cx26 from different species. Results indicate that both V37 and Q80 in Cx26 protein are highly conserved among many species.

that the c.240G>C(p.Q80H) and c.109G>A(p.V37I) variants were likely responsible for ARNSHL in this family.

The p.Q80H variant of the *GJB2* gene was predicted to be probably damaging according to PolyPhen-2, SIFT, MutationTaster, and ACMG (Table 2), and this mutation was novel since it had not been previously reported nor was it present in dbSNP, HGMD, or Exome Variant Server. The p.V37I variant in the *GJB2* gene was predicted to affect the features of the protein and it was predicted to be disease-causing according to MutationTaster, PolyPhen-2, and ACMG (Table 2). Both p.Q80 and p.V37 in Cx26 were highly conserved amino acid residues among different species (Figure 3), indicating their importance structurally and functionally.

3.3. *Structural modeling*

A 3D model was constructed for structural analysis of WT/Mut Cx26 proteins to determine the pathogenicity of mutant Cx26 according to SWISS-MODEL. When amino acid 37 is changed to isoleucine, the side chains of Cx26 also change as a result and tend to be longer than those of a structure in which amino acid 37 is valine (Figure 4A). The variant H80 protein has a different side chain than the wild-type protein as a result of a heterocyclic histidine being substituted for an aliphatic glutamine (Figure 4B). Therefore, the compound heterozygous mutations are predicted to affect the amino acid side chain. This might disrupt Cx26 function and interactions with other molecules and residues.

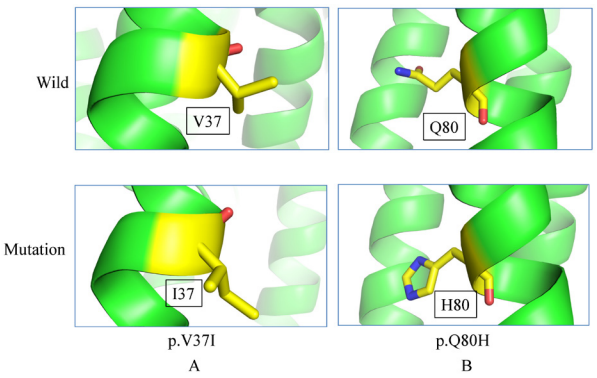

Figure 4. Protein molecular models of wild types and GJB2 mutations. (A) The mutated I37 protein has a longer side chain than the wild-type V37 protein. **(B)** The mutated H80 protein has a different side chain than the wild-type Q80 protein.

4. Discussion

The novel compound heterozygous mutations c.240G>C(p.Q80H) and c.109G>A(p.V37I) in the *GJB2* gene were likely responsible for ARNSHL in a Chinese family because the variants co-segregated with the disease phenotype in this family and because these variants were predicted to be disease-causing mutations. Moreover, the current study indicated that the compound mutations may give rise to the moderate hearing loss present in this family.

GJB2 mutations were first identified in three consanguineous pedigrees from Pakistan with ARNSHL in 1997 (*8*). Following dominant or recessive deafness, mutations in *GJB2* are considered to be the most common cause of nonsyndromic deafness (*14,15*). Over 50% of cases of ARNSHL are associated with *GJB2* mutations (*3*). The *GJB2* gene encodes the gap-junction protein Cx26, which is highly expressed in the cochlear cells (*16*). The sequences of Cx26 are extremely conserved with four transmembrane domains (TM1-4), two extra-cellular loops (E1 and E2), a cytoplasmic

loop (CL), and the N- and C-terminal cytoplasmic ends (*17*). Cx26 belongs to a large family of proteins that constitute intercellular channels and that contribute to the rapid exchange of small molecules between adjacent cells (*18,19*). Cx26 is essential for auditory transduction by recycling endolymphatic potassium ions and also for cell survival within the cochlea. Cx26-deficient mice have marked hearing impairment and cell death in the cochlea (*20*).

GJB2 mutations may affect the function of the Cx26 protein in various ways and may depend on the position and type of amino acid substituted. Previous studies suggested that hemichannels (hexamers) played an important role in the smooth functioning of ion channels in the WT-Cx26 protein. The novel c.240G>C mutation in the *GJB2* gene led to a positively charged histidine (His) being substituted for an uncharged glutamine (Gln) at amino acid 80 (p.Q80H), which is located in the TM2 segment of Cx26 protein. Therefore, the rare mutation may generate a functional null protein by disrupting the normal formation of hexamers (*21*). Three missense mutations (Q80K, Q80P and Q80R) and one nonsense mutation (Q80X) at the same position (The Connexin-deafness Homepage: *http://davinci.crg.es/deafness*) were reported to be responsible for hearing loss. For example, the variants c.238C>A (Q80K) and 35delG were identified as compound heterozygous mutations in a patient with severe hearing loss (*22*). In the current study, the novel p.Q80H variant of the *GJB2* gene was predicted to be deleterious according to SIFT, PolyPhen-2, MutationTaster, and ACMG. The c.109G>A variant, resulting in substitution of isoleucine for valine at position 37 (p.V37I), was initially reported as a polymorphic change in one study (*23*). However, greater awareness of *GJB2* has led to several studies describing p.V37I as a variant causing diverse hearing phenotypes, ranging from severe-to-profound hearing loss to normal hearing and from congenital onset to delayed-onset (*24-28*). In contrast, the homozygous p.V37I variant and the compound p.V37I variant with some other *GJB2* disease-causing mutation are rare (*25*). A study by Huang *et al*. (*28*) indicated that the p.V37I variant of the *GJB2* gene is mainly associated with mild or moderate hearing impairment in the Chinese population. The *GJB2* p.V37I variant was also predicted to be a pathogenic mutation according to PolyPhen-2, MutationTaster, and ACMG. Both the p.V37I and p.Q80H variants of the *GJB2* gene, located in the TM1 and TM2 domains, respectively, might interfere with the interaction of transmembrane regions or the proper folding and/or oligomerization of connexins or they might generate defective channels and thus cause hearing loss (*17*). In addition, both p.Q80 and p.V37 in Cx26 are highly conserved throughout all members of the connexin family, indicating their importance structurally and functionally.

In summary, the current results indicated that novel compound heterozygous variants (p.Q80H and p.V37I) in the *GJB2* gene were associated with congenital moderate hearing loss in a Chinese family. The p.Q80H variant was initially identified in patients of Dong Chinese origin with ARNSHL. The current study further supports the hypothesis that the p.V37I variant is devoid of functional activity and causes hearing loss (*29*). Exome sequencing is a rapid, exact, and cost-effective method with which to identify genes causing hearing impairment. The current findings broaden the spectrum of *GJB2* mutations associated with NSHL and may also shed new light on genetic counseling for individuals with hearing loss.

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