

# A novel *KCNQ4* gene variant (c.857A>G; p.Tyr286Cys) in an extended family with non-syndromic deafness 2A

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**Abstract.** Deafness is one of the most common sensory disorders found in humans; notably, >60% of all cases of deafness have been attributed to genetic factors. Variants in potassium voltage-gated channel subfamily Q member 4 (*KCNQ4*) are etiologically linked to a type of progressive hearing loss, deafness non-syndromic autosomal dominant 2A (DFNA2A). In the present study, whole-exome sequencing (WES) was performed on three members of a five-generation Chinese family with 46 members with hearing loss. Pure tone audiometry and Sanger sequencing were performed for 11 family members to determine whether the novel variant in the *KCNQ4* gene was segregated with the affected family members. In addition, evolutionary conservation analysis and computational tertiary structure protein prediction of the wild-type *KCNQ4* protein and its variant were performed. The family exhibited autosomal dominant, progressive, post-lingual, non-syndromic sensorineural hearing loss. A novel co-segregating heterozygous missense variant (c.857A>G; p.Tyr286Cys) in the glycine-tyrosine-glycine signature sequence in the pore region of the *KCNQ4* channel was identified. This variant was predicted to result in a tyrosine-to-cysteine substitution at position 286 in the *KCNQ4* protein. The tyrosine at position 286 is well conserved across different species. The substitution of tyrosine with cysteine would affect the structure of the pore region, resulting in the loss of channel function. The *KCNQ4* gene is one of the most common mutated genes observed in patients with autosomal dominant, non-syndromic hearing

loss. Taken together, for the family analyzed in the present study, performing WES in conjunction with Sanger sequencing has led to the detection of a novel, potentially causative variant (c.857 A>G; p.Tyr286Cys) in exon 6 of the *KCNQ4* gene. The present study has added to the number of pathogenic variants observed in the *KCNQ4* gene, and the findings may prove to be useful for both the diagnosis of DFNA2A and in the design of early interventional therapies.

## Introduction

Hearing loss is one of the most common sensory defects in humans, and is often caused by the death of sensory hair cells (HCs) in the inner ear. HCs function in transducing sound waves into electric signals in the inner ear (1,2). Damage induced by a variety of intrinsic and extrinsic sources will affect cochlear HC function. The factors involved include genetic factors, ototoxic drugs, aging, chronic cochlear infections and noise overexposure (3-7); notably, >60% of all cases of deafness have been attributed to genetic factors (8-10). Based on inheritance patterns and the presence or absence of distinctive clinical features, hereditary deafness can be divided into two categories: Syndromic hearing loss and non-syndromic hearing loss. The inheritance patterns of non-syndromic hearing loss include autosomal dominant, autosomal recessive, X-linked and mitochondrial inheritance. Autosomal dominant non-syndromic hearing loss (ADNSHL) accounts for ~20% of cases of hereditary hearing loss (11,12).

In general, patients with autosomal recessive non-syndromic hearing loss have an early age of onset and more pronounced hearing loss. However, the principal manifestation of ADNSHL is post-lingual progressive sensorineural hearing loss, which begins with impairment in hearing high frequencies. Hearing loss is genetically heterogeneous. To date, >60 loci for ADNSHL have been mapped, and ~40 genes for ADNSHL have been identified (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. <https://hereditaryhearingloss.org>; accessed March 11, 2021). ADNSHL contains a number of different disease subtypes, DFNA2A being one of them.

The gene encoding potassium voltage-gated channel subfamily Q member 4 (*KCNQ4*) (Gene MIM number 603537; Online Mendelian Inheritance in Man. <https://omim.org/entry/603537>; accessed March 9, 2021). was discovered and

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cloned by Kubisch *et al* (13) in 1999. *KCNQ4* is mapped to 1p34, within a region that encompasses the deafness non-syndromic autosomal dominant 2A (DFNA2A; Phenotype MIM number 600101) locus (Online Mendelian Inheritance in Man. <https://omim.org/entry/600101>; accessed March 9, 2021) (14). The cDNA of the *KCNQ4* gene, which includes 14 exons, encodes a polypeptide of 695 amino acids with a mass of ~77 kDa. Pathogenic variants of the *KCNQ4* gene have been shown to cause progressive hearing loss, and the gene is inherited in an autosomal dominant manner. This gene is linked to the DFNA2A locus on chromosome 1p34, encoding the KCNQ4 protein. It is only expressed on sensory outer HCs, and not in the inner HCs or the stria vascularis in the inner ear (15). The major function of KCNQ4 protein is to contribute to potassium ion circulation in stimulated HCs (16,17). The major process of pathogenesis associated with *KCNQ4* affects potassium recycling in the endolymph, which subsequently changes the nature of its electrolyte milieu and reduces the endocochlear potential (18-20).

Four subunits associate with each other to form the voltage-gated channel. This arrangement allows potassium ions to selectively cross the cell membrane (19). Each subunit consists of six transmembrane domains, intracellular N- and C-termini, and a pore region (21). Six exons (exons 2-7) encode the six transmembrane segments (S1-S6). The S4 segment contains the voltage sensor of the channel, whereas the S5 and S6 segments are linked through the P-loop domain to form the pore region.

The *KCNQ4* gene is one of the most commonly mutated genes found in patients with ADNSHL, and this gene has been shown to lead to DFNA2A. To date, ~30 pathogenic variants in this gene have been reported as the cause of DFNA2A (The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff; <http://www.hgmd.cf.ac.uk>; accessed March 11, 2021). Variant hotspots in the *KCNQ4* gene that are linked to hearing loss have been shown to cluster around the pore region (22); these variants result in decreased membrane expression of the channel protein, or loss of channel function.

The present study reported on the genetic basis of progressive hearing loss in a Chinese family, in which a novel missense variant c.857A>G (p.Tyr286Cys) was identified in the pore region of the *KCNQ4* channel via whole-exome sequencing (WES) and Sanger sequencing.

## Patients and methods

**Family recruitment and clinical evaluations.** The proband was a 25-year-old woman. Due to hearing loss, she visited the Department of Otolaryngology, Head and Neck Surgery, Xijing Hospital in 2018. The patient had a family history of hearing loss. The patient was part of a five-generation Chinese family with 46 members. The family had autosomal dominant, progressive, post-lingual, non-syndromic sensorineural hearing loss and these features are consistent with ADNSHL (Fig. 1). A total of 11 members of this family participated in the present study, which included seven affected and four unaffected relatives. Written informed consent was obtained from the study participants and the study was performed in accordance with relevant regulations of Xijing Hospital. Other members of the family were unwilling to participate in the present study, and since it was not possible to obtain their consent, their information has not been used.

The following information was obtained from each study participant, which included basic information, age of onset, disease progression, mother's pregnancy and the study participant's delivery, noise exposure, ototoxic drug use, head trauma, infectious diseases, family history and other related information. Physical examinations confirmed a family history of non-syndromic hearing loss. Audiometric evaluations and otological examinations performed for the proband included electronic otoscope, pure tone audiometry (PTA), acoustic impedance, distortion production otoacoustic emission, auditory brainstem response, speech discrimination score, electronystagmography, vestibular evoked myogenic potential, tinnitus detection, temporal bone computed tomography and magnetic resonance imaging. The other family members were evaluated by PTA. Average value of the thresholds of air conduction were determined at 500, 1,000, 2,000 and 4,000 Hz to determine the degree of hearing loss in the family. The condition of an individual's hearing was assessed through measuring their hearing loss, and this was classified as mild (26-40 dB HL), moderate (41-60 dB HL), severe (60-80 dB HL) or profound hearing loss ( $\geq 81$  dB HL) (23). Detailed medical records were established for each family member. The majority of the patients in this family experienced moderate hearing loss: They were required to wear hearing aids to improve their hearing, although they did not require treatment with drugs; nor had they received surgery. For members of the family who were unwilling to participate in the present study, and for whom it was not possible to obtain their consent, their data have not been used.

**DNA extraction.** Genomic DNA from the 11 study participants was extracted from peripheral blood leukocytes using a blood DNA extraction kit (cat. no. CW0544M; Kangwei Century Biotech Co., Ltd.; <https://www.cwbiootech.com/goods/index/id/10199>). DNA concentration and purity were measured using an ultraviolet NanoQ Spectrophotometer (CapitalBio Technology, Inc.).

**Screening for variants in *SLC26A4*, *GJB2*, *GJB3* and mitochondrial 12S rRNA.** *SLC26A4*, *GJB2*, *GJB3* and mitochondrial 12S rRNA are the most common cause of hearing loss in the Chinese population (24). Sanger sequencing for *SLC26A4*, *GJB2*, *GJB3* and mitochondrial 12S rRNA was performed using PCR amplification and direct sequencing of exons. The primers referred to the studies of Dai *et al* (25) and Yuan *et al* (26). The PCR conditions and Sanger sequencing are described in the Segregation analysis sanger sequencing section of the text.

**WES.** Whole-exome capture was performed using the Agilent SureSelect V5 enrichment capture kit (Agilent Technologies, Inc.). The qualified genomic DNA was randomly fragmented to an average size of 180-280 bp by Covaris S220 sonicator (Covaris Inc.). Next, the DNA fragments were end-repaired and phosphorylated, followed by A-tailing and ligation at the 3'-ends with paired-end adaptors (Illumina, Inc.) with a single 'T' base overhang; the purification was carried out using Agencourt SPRIselect (cat. no. B23317 Beckman Coulter Inc.). Then, the size distribution and concentration of the libraries (2 nM) were determined by Agilent 2100 Bioanalyzer and quantified using PCR. Finally, the DNA library was sequenced

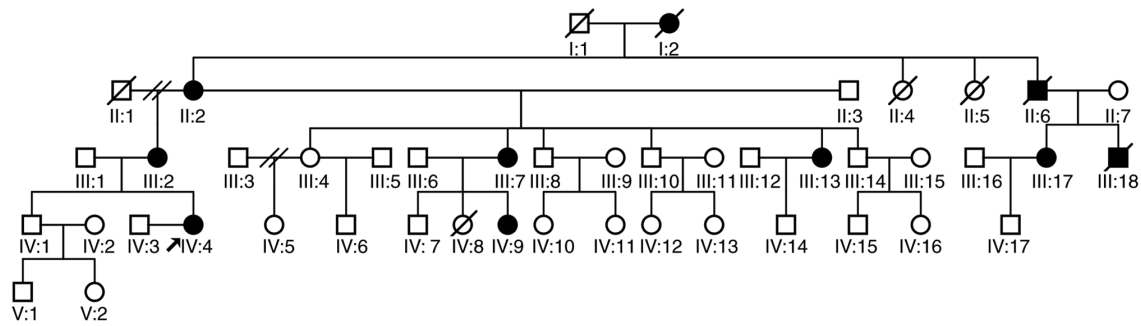


Figure 1. Family pedigree with autosomal dominant hearing loss. Hearing-impaired individuals are denoted by the black shapes; the arrow indicates the proband (IV:4).

on Illumina HiSeq 4000 (Illumina Inc.) for paired-end 150-bp reads. The sequence reads were aligned to the human reference genome (UCSC hg19/hg38) (UCSC Genome Browser; <https://genome.ucsc.edu>; accessed March 15, 2021) (27) using the Burrows-Wheeler Alignment (28) to get BAM file. SAMtools (29) and Sambamba (30) were used to sort BAM files and perform duplicate marking, local realignment, and base quality recalibration for coverage and depth analysis. Duplicate reads were removed for variants calling. ANNOVAR (31) software was used to annotate single nucleotide polymorphism (SNP), insertion and deletion (Indel) and copy number variation (CNV). The variants were filtered based on the monogenic autosomal dominant trait. First, variants with minor allele frequencies >1% in the 1000 Genomes database (32), the NHLBI Exome Sequencing Project (ESP6500) [Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP); accessed March 11, 2021] or the Genome Aggregation Database (gnomAD; <https://gnomad.broadinstitute.org>; accessed March 13, 2021) were excluded. Second, variants present in the homozygous or hemizygous state in individuals without hearing loss were excluded. Third, synonymous variants and intronic variants not located within the splice site regions were excluded. Finally, variants of all genes known to be monogenic factors for hearing loss were systematically evaluated.

**Segregation analysis sanger sequencing.** Following exome sequencing, segregation analysis of candidate variants was completed by Sanger sequencing. Sequences of primers for potential causative variants in the *KCNQ4* gene were designed and synthesized as follows: 5'-TTCCCTCATGATCAG GCT-3' and 5'-ATCTTGATACCTGGATGAGGTT-3'. PCR was performed with 25  $\mu$ l reaction mixtures containing 100 ng of genomic DNA, 1  $\mu$ l of the forward and reverse primers, and 22  $\mu$ l of 1.1X Golden Star T6 Super PCR Mix (cat. no. TSE101, TsingKe Biological Technology). Thermocycling was performed using the following program: Initial denaturation at 98°C for 2 min, followed by 30 cycles of 98°C for 10 sec, 62°C for 10 sec, and 72°C for 10 sec and final extension at 72°C for 1 min. PCR products were purified with the Cycle Pure kit (cat. no. D6492 OMEGA Bio-Tek) and sequenced using Applied Biosystems 3730 DNA Analyzer (Thermo Fisher Scientific, Inc.).

**Evolutionary conservation analysis.** The target sequence for alignment contained Tyr286 residue as well as its upstream and downstream amino acid residues. Multiple sequence

alignment was performed across 13 species using BLAT (33) on the UCSC Genome Browser (<https://genome.ucsc.edu>).

**Three-dimensional (3D) structural modeling.** The BLAST search protocol in BIOVIA Discovery Studio (DS) (v. 19.1.0x64; 3D EXPERIENCE Company, Dassault Systèmes) (2019) was used to find template structures that had similar regions to the domain of *KCNQ4* protein. This was accomplished by searching against the PDB\_nr95 sequence database. The residue sequence of the mammalian Shaker Kv1.2 potassium channel (from Ser289 to Phe413) bore a 31% similarity to that of *KCNQ4* protein (from Ala325 to Phe449), which included the ion transport domain. The structure was subsequently retrieved from the Protein Data Bank (PDBID:2A79) (34,35). The Prepare Protein protocol in DS was then used to restore missing atoms and remove water molecules. The tertiary structure of the *KCNQ4* domain was constructed based on the template using the Build Homology Models protocol in DS. Subsequently, the structure of the mutant (Tyr286→Cys) was constructed using the Build Mutants Protocol, and then the model with the highest Discrete Optimized Protein Energy score was selected. Finally, molecular visualization and 3D images were processed using PyMol (v2.1; Schrödinger Inc.; <https://pymol.org/2/#download>).

## Results

**Clinical description.** The family selected for the study had 10 individuals with hearing loss, three of whom (I:2, II:6, III:18) were deceased and were unable to participate in the study. Their hearing had begun to decline gradually at about age 20, and by the time they are older, they had suffered severe hearing loss. The remaining seven individuals (II:2, III:2, III:7, III:13, III:17, IV:4 and IV:9) were all diagnosed with hearing impairment. The remainder of the family members had good hearing, of which four members (III:4, III:8, III:14 and IV:1), underwent PTA and their hearing was deemed normal (Table I). The audiological condition and clinical history of the hearing-impaired family members showed progressive, post-lingual, symmetrical, bilateral, non-syndromic sensorineural hearing loss. The age at onset of hearing impairment ranged from 20-30 years, and the degree of hearing loss was positively associated with age.

**WES identifies a causative variant in *KCNQ4*.** To identify the genetic cause of hearing loss, WES was performed on this family. Variants in *GJB2*, *GJB3*, *SLC26A4* and mtDNA

Table I . Phenotype and genotype of individual family members.

Family member	Age, years	Nucleotide change	PTA-right, dB HL	PTA-left, dB HL	Age of onset, years	Noise exposure	Ototoxic drugs	Head trauma
II:2	79	c.857A>G	86.25	77.5	25	No	No	No
III:2	56	c.857A>G	71.25	73.75	30	No	No	No
III:4	53	Wild-type	17.5	16.25	/	No	No	No
III:7	50	c.857A>G	63.75	63.75	20	No	No	No
III:8	48	Wild-type	8.75	7.5	/	No	No	No
III:13	44	c.857A>G	68.75	68.75	20	No	No	No
III:14	42	Wild-type	15	18.75	/	No	No	No
III:17	37	c.857A>G	75	70	25	No	No	No
IV:1	34	Wild-type	10	8.75	/	No	No	No
IV:4	25	c.857A>G	52.5	55	25	No	No	No
IV:9	24	c.857A>G	35	38.75	30	No	No	No

PTA, pure tone audiometry; HL, hearing level.

were excluded. Autosomal dominant inheritance was suspected on the basis of the family pedigree, and WES was performed on individuals III:1, III:17 and IV:4. WES detected a novel potentially causative variant (c.857 A>G; p.Tyr286Cys) in exon 6 of the *KCNQ4* gene. This variant resulted in a tyrosine-to-cysteine substitution at position 286 in *KCNQ4* protein. The tyrosine at position 286 is conserved throughout evolution, as shown in Fig. 2. Subsequently, Sanger sequencing was performed to determine whether the c.857 A>G variant in the *KCNQ4* gene segregated with the affected status in this family (Fig. 3). This variant was detected in individuals II:2, III:2, III:7, III:13, III:17, IV:4 and IV:9, and all these family members were diagnosed as being hearing-impaired (Fig. 4A). The variant was not detected in individuals III:4, III:8, III:14 and IV:1, who had good hearing (Fig. 4B). The variant was found to co-segregate with the progressive hearing loss phenotype. Furthermore, this variant was not present in the 1000 Genomes database or in the NHLBI Exome Sequencing Project (ESP6500). The c.857 A>G (p.Tyr286Cys) variant in the *KCNQ4* gene was identified in ClinVar (Variation ID: 228776; (ClinVar; <https://www.ncbi.nlm.nih.gov/clinvar/variation/228776>; accessed March 11, 2021) (36), although the clinical significance was listed as ‘uncertain significance’, and the condition was ‘not specified’ (20).

**Structural modeling of p.Y286C (p.Tyr286Cys).** A computational tertiary protein structure prediction of the wild-type and p.Y286C variant of *KCNQ4* protein was performed (Fig. 5). The model covered the target sequence of *KCNQ4* protein. The tyrosine residue at position 286 of the *KCNQ4* protein was identified in the pore region of *KCNQ4* protein. DS software predicted that this variant affected the amino acid side chain due to the replacement of tyrosine with cysteine. This variant led to the hydroxyphenyl group in the amino acid side chain being changed to a sulfhydryl group. This substitution led to a mismatch in the disulfide bond, which was predicted to affect the structure of the

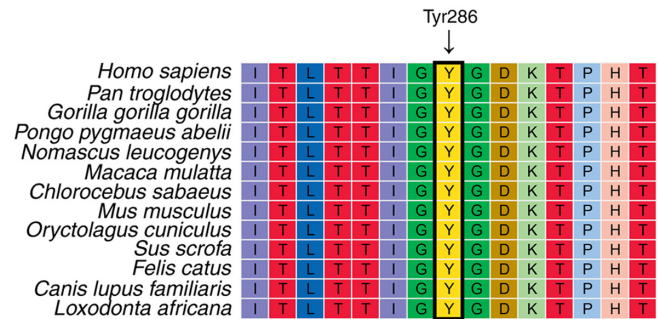


Figure 2. Evolutionary conservation of the Tyr286 residue of the potassium voltage-gated channel subfamily Q member 4 gene across a range of species. Protein alignment demonstrates high conservation for the mutated amino acid position 286 in the *KCNQ4* protein among different species. The amino acid residues shown in the figure are all highly conserved in different species, with the black arrow marking the position where the amino acid residue changes occurred in this study.

pore region, and hence changed the glycine-tyrosine-glycine (GYG) signature sequence of the K<sup>+</sup> channel pore. These three amino acids (GYG) are found in the narrowest part of the pore (19), and variants in these three amino acids affect its selectivity and abolish channel function in the majority of patients.

## Discussion

In the present study, a novel variant (c.857 A>G; p.Tyr286Cys) in the *KCNQ4* gene was identified in a Chinese family with ADNSHL. The variant gene was co-segregated with progressive hearing loss. The tyrosine at position 286 has been shown to be well conserved across species. The replacement of tyrosine with cysteine was suggested to affect the structure of the pore region and abolish channel function.

The protein encoded by the *KCNQ4* gene belongs to the Kv7 channel family. The Kv7 channel family is a distinct branch of the superfamily of voltage-gated potassium channels



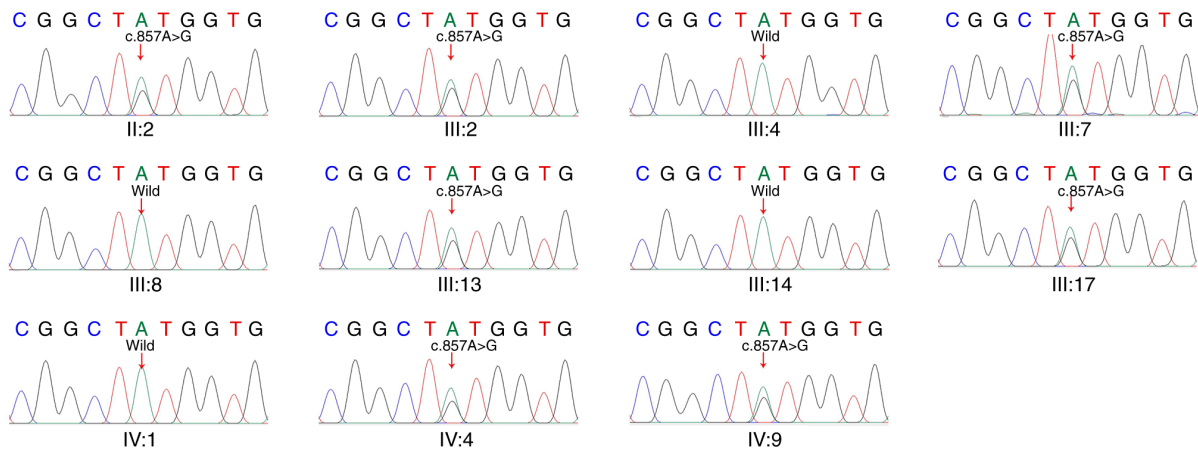


Figure 3. Sanger sequencing results of individual family members are presented. Sanger sequencing results of the variant c.857A>G in all family members. Arrows indicate the position of the nucleotide changes identified in this study. II:2, III:2, III:7, III:13, III:17, IV:4 and IV:9 carried the variant. The variant was not detected in individuals III:4, III:8, III:14 and IV:1.

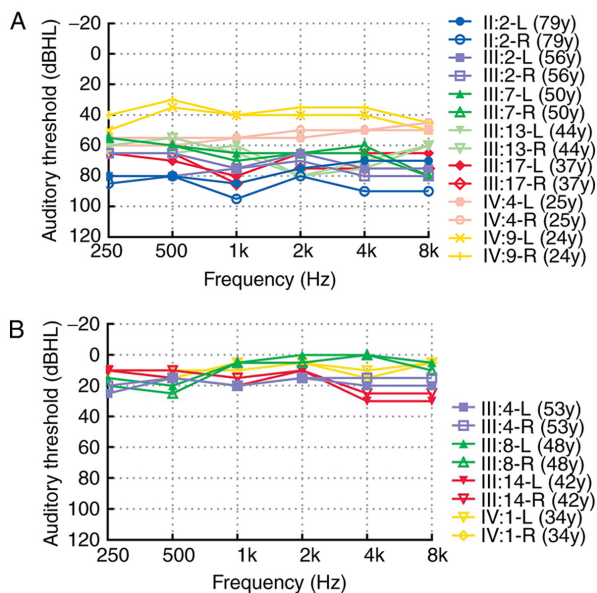


Figure 4. Pure-tone audiograms from individual family members. (A) Individuals II:2, III:2, III:7, III:13, III:17, IV:4 and IV:9, and (B) individuals III:4, III:8, III:14 and IV:1. y, years.

encoded by *KCNQ* genes. There are five members in this family (Kv7.1-Kv7.5), and the channel proteins are encoded by the *KCNQ1-KCNQ5* genes, respectively. They fulfill important roles in the brain, heart, kidney and inner ear (37). Variants of *KCNQ1* (for example, KvLQT1) have been shown to cause heart diseases, long QT syndrome and Jervell-Lange-Nielsen syndrome (38,39). Variants of *KCNQ2* or *KCNQ3* have been causally associated with benign familial neonatal seizures, whereas variants in the *KCNQ4* gene have been shown to cause DFNA2A (20,40). Variants of both the *KCNQ1* gene and the *KCNQ4* gene may cause hearing loss. However, deafness associated with *KCNQ1* gene is syndromic, severe, congenital and autosomal recessive, whereas that associated with the *KCNQ4* gene is non-syndromic, progressive autosomal dominant inheritance (41,42).

To date, 40 pathogenic variants in the *KCNQ4* gene have been reported to be associated with hearing loss (Deafness

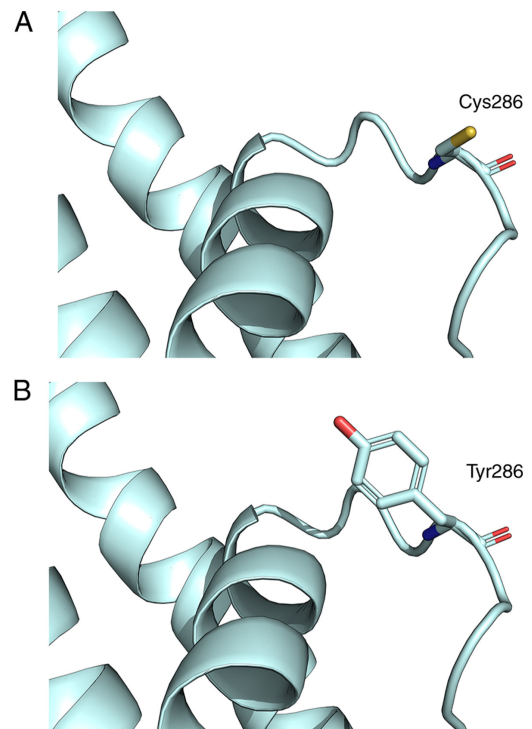


Figure 5. Computational tertiary structure prediction of the potassium voltage-gated channel subfamily Q member 4 wild-type and p.Tyr286Cys proteins. The (A) mutant Cys286 and (B) wild-type Tyr286 proteins are shown.

Variation Database. <http://deafnessvariationdatabase.org>; accessed March 11, 2021) (43). All these variants are located in exons 1, 3-8 and 14 of the *KCNQ4* gene. The majority of these variants have been linked to hearing loss, and are clustered around the pore region. The pore region is responsible for the ion-selectivity of the potassium channel. The GYG signature sequence located in the narrowest region of the pore is critical for maintaining pore structure and function (19). Missense variants at the K<sup>+</sup> ion selectivity filter have been shown to disrupt the highly conserved GYG signature sequence, resulting in severely impaired non-conducting channels to induce severe hearing loss. The hearing loss induced

by variants at the first (p.Gly285Cys; p.Gly285Ser) and third (p.Gly287Arg) amino acids in the GYG signature sequence has been demonstrated to be causal (13,44,45). Furthermore, the variants in the second (p.Tyr286Cys) amino acid in the GYG signature sequence have been documented in ClinVar by previous investigators (20). However, the clinical significance was denoted as ‘uncertain significance’, and the condition as ‘not specified’. To the best of our knowledge, the pathogenicity of the c.857 A>G (p.Tyr286Cys) variant has not been demonstrated in previous studies (46). The novel missense variant p.Y286C in the GYG signature sequence was co-segregated with progressive hearing loss in the family investigated in the present study.

The reduction in Kv7 channel function induced by *KCNQ* gene variants has been shown to lead to a variety of diseases, such as epilepsy, arrhythmia and deafness (13,41,47–51). Therefore, the use of *KCNQ* activators may be helpful for the treatment of these diseases. Retigabine, a small molecule that activates *KCNQ2-5* channels, but not the *KCNQ1* channel, was approved by the US Food and Drug Administration in 2011 (52,53). It was the first *KCNQ* activator to be used for the treatment of epilepsy. However, retigabine has a broad regulatory effect on almost all Kv7 channels and other ion channels. Systemic side effects, such as dizziness, drowsiness, memory loss, urinary retention, vertigo and slurred speech, have been observed in patients treated with retigabine (54). Non-selective *KCNQ* activators have also been shown to cause several side effects; therefore, the development of selective *KCNQ* activators is necessary to overcome these limitations. To date, several new small-molecule modulators (activators) of *KCNQ* have been developed that may have potential benefits, while reducing adverse effects. It may ultimately be possible to rescue Kv7 channel functionality using *KCNQ* activators, thereby preventing hearing loss in patients with DFNA2A.

Collectively, our study demonstrated that the co-segregating heterozygous missense variant (c.857A>G; p.Tyr286Cys) in the glycine tyrosine glycine signature sequence in the pore region of the *KCNQ4* channel was the pathogenic variants in this ADNSHL family. This finding supports the pathogenicity of the missense variant (c.857A>G; p.Tyr286Cys) in the *KCNQ4* gene. Data presented here extend the pathogenic variant spectrum of the *KCNQ4* gene. The finding has implications in genetic counseling for hereditary deafness and enable otolaryngologists to select appropriate clinical interventions for patients with ADNSHL.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request. The sequencing dataset has been deposited in NCBI Sequence Read Archive, and the BioProject ID is PRJNA689907 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA689907>).

## Authors' contributions

DZ and JC conceptualized and designed the study. PL, SW, JW and WL researched the family's history and recruited the family members. JW performed pure tone audiometry testing, disease diagnosis and DNA extraction. PL and SW completed PCR amplification and Sanger sequencing. QL, WL, YY and XA were responsible for analyzing the WES data; and QL performed the molecular genetic studies and the sequence alignment analysis. QL drafted the manuscript. QL and PL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of the Air Force Medical University (approval number KY20212002-C-1), and informed consent was obtained from all the study participants.

## Patient consent for publication

Enrolled study participants and the patient provided written informed consent for the publication of this manuscript.

## Competing interests

The authors declare that they have no competing interests.

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