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ORIGINAL ARTICLE

A novel variant c.902C>A (p. A301D) in *KCNQ4* associated with non-syndromic deafness 2A in a Chinese family

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Abstract

Background: Deafness autosomal dominant 2A (DFNA2A) is related to non-syndromic genetic hearing impairment. The *KCNQ*4 (Potassium Voltage-Gated Channel Subfamily Q Member 4) can lead to DFNA2A. In this study, we report a case of autosomal dominant non-syndromic hearing loss with six family members as caused by a novel variant in the *KCNQ4* gene.

Methods: The whole-exome sequencing (WES) and pure tone audiometry were performed on the proband of the family. Sanger sequencing was conducted on family members to determine if the novel variant in the *KCNQ*4 gene was present. Evolutionary conservation analysis and computational tertiary structure protein prediction of the wild-type KCNQ4 protein and its variant were then performed. In addition, voltage-gated channel activity of the wild-type KCNQ4 protein and its variant were tested using whole-cell patch clamp.

Results: It was observed that the proband had inherited autosomal dominant, non-syndromic sensorineural hearing loss as a trait. A novel co-segregating heterozygous missense variant (c.902C>A, p.Ala301Asp) of the *KCNQ4* gene was identified in the proband and other five affected family members. This variant was predicted to cause an alanine-to-aspartic acid substitution at position 301 in the KCNQ4 protein. The alanine at position 301 is well conserved across different species. Whole-cell patch clamp showed that there was a significant difference between the WT protein currents and the mutant protein currents in the voltage-gated channel activity.

Conclusion: In the present study, performing WES in conjunction with Sanger sequencing enhanced the detection of a novel, potentially causative variant (c301 A>G; p.Ala301Asp) in exon 6 of the *KCNQ4* gene. Therefore, our findings contributed to the mutation spectrum of the *KCNQ4* gene and may be useful in the diagnosis and gene therapy of deafness autosomal dominant 2A.

KEYWORDS
deafness autosomal dominant 2A, hearing loss, *KCNQ*4:c.902C>A

1 | INTRODUCTION

Deafness autosomal dominant 2A (DFNA2A) is a form of post-lingual non-syndromic progressive sensorineural hearing loss that begins with impairment at high frequencies and progresses to include mid- to low frequencies (Kamada et al., 2006). Hearing loss is a common disability in humans, with at least 60% being attributed to genetic factors (Cui et al., 2022). Hearing loss that is inherited can be divided into two groups depending on the mode of inheritance and the specific clinical features, which can be either syndromic or non-syndromic (Huang et al., 2017). Based on the mode of inheritance, non-syndromic hearing loss (NSHL) includes autosomal dominant, autosomal recessive, X-linked, and mitochondrial types (Vallian Broojeni et al., 2023). Approximately 20% of all hereditary hearing loss cases are due to autosomal dominant non-syndromic hearing loss (ADNSHL) (Li et al., 2021). The mutations in *WFS1*, *KCNQ4*, *COCH*, and *GJB2* are frequently identified as the causes of ADNSHL. Among these, *KCNQ4* is responsible for approximately 9% of ADNSHL cases (Sloan-Heggen et al., 2016). DFNA2A associated with the *KCNQ4* gene is characterized by a progressive hearing loss pattern (Yen et al., 2021). *KCNQ4* is a member of the voltage-gated potassium channel gene family. This gene-mapped chromosome 1p34.2 forms a homologous tetrameric structure with 14 exons, and it encodes a polypeptide with 695 amino acids and a mass of approximately 77 kDa (Yen et al., 2021). A typical KCNQ channel consists of four subunits, each of which consists of six transmembrane segments (S1–S6) and the distribution of N and C terminus in the membrane. The S4 segment contains a voltage channel sensing area. The S5 and S6, together with the P-loop between them, form the pore area. Four P-loops form ion channel selectors. All KCNQ channels have long C segments, which can integrate multiple signal channels (Rim et al., 2021).

In our study, a novel co-segregating heterozygous missense variant (c.902C>A; p.Ala301Asp) of the *KCNQ4* gene was identified in a family that exhibited non-syndromic sensorineural hearing loss. This variant was predicted to cause an alanine-to-aspartic acid substitution at position 301 in the KCNQ4 protein. Our findings broadened the mutation spectrum of the *KCNQ*4 gene and may further contribute to the diagnosis and gene therapy of DFNA2A.

2 | MATERIALS AND METHODS

2.1 | Family recruitment and clinical evaluations

The proband was a 35-year-old man, who visited the Department of Center of Hearing, Guizhou Provincial People's Hospital in 2021, as a result of hearing loss. A history of hearing loss was reported in the patient's family line. This patient was a part of a Chinese family with three generations and exhibited a progressive, postverbal, non-syndromic type of sensorineural hearing loss, which is a common feature of ADNSHL. Prior to the commencement of the study, informed consent was obtained from participants involved in the study. The study was conducted in accordance with the relevant regulations of Guizhou Provincial People's Hospital.

2.2 | DNA sample collection and extraction

Genomic DNA was extracted by the QIAamp DNA Blood Mini Kit (Qiagen, Germany) from the peripheral blood lymphocytes of the participants. DNA quality was estimated using agarose gel electrophoresis and was quantified by the Qubit 2.0 fluorimeter (ThermoFisher Scientific).

2.3 | Whole-exome sequencing and data analysis

The SureSelect Human All Exon V6 kit (Agilent Technologies) was used to prepare a library that was enriched with sequences for exomes. The library was sequenced on a NovaSeq 6000 platform (Illumina) using a paired-end 150 bp strategy for an average of 100-fold depth. The sequencing reads were aligned to the human reference genome (hg38/GRCh38) using Burrows–Wheeler Aligner tool, and PCR duplicates were removed by using Picard v1.57 (http://picard.sourceforge.net/). Variant annotation and interpretation were conducted by ANNOVAR (Wang et al., 2010) and the Enliven® Variants Annotation Interpretation System authorized by Berry Genomics. Annotation databases mainly included gnomAD (http://gnomad.broadinstitute.org), SIFT (http://sift.jcvi.org), MutationAssessor (http://mutationassessor.org), CADD (http://cadd.gs.washington.edu), SPIDEX , (Xiong et al., Science 2015) OMIM (http://www.omim.org), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), HGMD (http://www.hgmd.org), HPO (https://hpo.jax.org/app/), etc. According to the guidelines established by the American College of Medical Genetics and Genomics (ACMG), the variants were categorized into five groups: “pathogenic,” “likely pathogenic,” “uncertain significance,” “likely benign,” and “benign” (Richards et al., 2015). Variants with minor allele frequencies (MAF) less than 1% in the exonic region or those having a splicing impact were carefully analyzed for their significance. This analysis

encompassed the evaluation of the ACMG category, evidence of pathogenicity, and the clinical synopsis and inheritance model linked to the disease.

2.4 | Sanger sequencing

The specific PCR primers for the *KCNQ*4 variant were designed by the Primer 5.0 software and synthesized by Sangon Biotech. The primers' sequences for NM_004700.4: c. 902C>A were F:5′–TTCCCTCATGATCAGGCT–3′; R:5′– ATCTTGTACCTGGATGAGGTT–3′. Sequencing of the amplified products was conducted using an ABI 3730 Genetic Analyzer (Foster City, CA, USA). The sequence chromatograms were compared and visualized by the TBtools software.

2.5 | Evolutionary conservation analysis

The sequence for alignment comprised an Ala301 residue, in addition to the amino acid residues preceding and following it. A multiple sequence alignment of six species was conducted on the UCSC Genome Browser.

2.6 | Three-dimensional (3D) structural modeling

The human KCNQ1 complex coordinates (PDB: 6UZZ) were aligned with the KCNQ4 and complex maps in CHIMERA. The KCNQ1 sequence was mutated to match the corresponding residues of KCNQ4 in COOT, and each residue was manually verified. The chemical properties of amino acids were taken into account during the model-building process. The structures of HA, HB helices, and CaM (PDB: 6B8L) were fitted into the respective densities using CHIMERA. Initially, the crystal structure was manually adjusted to a relatively accurate position. Then, the structure was docked into the density using the "Fit in Map" tool in the software (Rashid, 2021), which forms the structural model of KCNQ4 PDB ID 7byn. The PDB ID 7byn was used to forecast the three-dimensional structures of both the wild-type (WT) KCNQ4 and its variant. PyMOL (Version 1.6; Schrödinger, LLC) was used to display the three-dimensional structures of both the WT KCNQ4 protein and its variant form.

2.7 | Cell culture and transfection

PCR amplification of the KCNQ4 gene CDS (Coding DNA Sequence) using XbaI and AgeI as restriction enzyme sites, followed by recovery of the digested fragments. PCR amplification of the mutant KCNQ4 CDS sequence fragments, followed by recovery of the digested fragments. Digestion and recovery of the empty vector pcDNA3.4 using XbaI and AgeI, and subsequent ligation of the mutant and wild-type fragments to the vector separately. Validation of sequence correctness through first-generation sequencing. Culturing cells and transfecting them with KCNQ4 plasmids was performed for the purpose of electrophysiology. HEK293T cells were kept in a culture medium of DMEM, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at a temperature of 37°C and a CO_2 concentration of 5%. Before transient transfection, the cell density reached 70% to 90% confluency. To determine the whole-cell ion currents of KCNQ4 channels, KCNQ4 wild-type and p.Ala301Asp were cloned into the pcDNA3.4 vector. The cells were transfected with the plasmids that expressed wild-type and mutant KCNQ4 using Lipofectamine 2000 (Invitrogen, USA), prior to incubation at 37°C for 24 h.

2.8 | Whole-cell patch clamp

Conventional whole-cell patch clamp technique was used to record KCNQ4 channel currents. Patch pipettes were fabricated from borosilicate glass tubing (WPI, Sarasota, FL, USA), and the pipette tip was fire-polished using a microforge (MF-83; Narishige, Japan). The pipette tip resistance, when filled with pipette solution, ranged from 1.5 to 3 MΩ. An Axopatch-1D amplifier (Axon Instrument, USA) was employed for recording the whole-cell K+ currents. When the whole-cell seal was formed, the membrane voltage clamp was −80 mV. The clamping voltage was divided step by step from −80 mV to +100 mV, all maintained for 5 s. It was then quickly maintained at −80 mV, and a current of the Kv7 channel was detected. The data were repeated at 20 s intervals, and the densities were calculated.

2.9 | Statistical analysis

Data was expressed as mean ± SEM and performed by one-way analysis of variance and Dunnett's multiple comparison tests. For all conducted experiments in this study, $p < 0.05$ stood for statistical significance.

3 | RESULTS

3.1 | Clinical features of the patient

The family that was selected for the study had nine individuals with hearing loss. One of the family members was

deceased (I:2), and others (II:2, II:10) could not participate in the study due to private reasons. The proband had begun to gradually decline when he was around ten years old, and by the time he was older, he was suffering from severe hearing loss. The remaining seven family members (II:2, II:6, II:8, II:10, II:12, III:6, III:7) exhibited hearing impairment. Specifically, the onset ages were 10, 30, 20, 45, 3, 18, and 30 years old, respectively. The proband and his father have tinnitus sometimes. The other affected family members also exhibit different levels of tinnitus. The remainder of the family members reported without hearing loss, of which two members (II:4, III:2, III:5) (Figure 1a).

The audiological condition and clinical history of the proband with impaired hearing abilities showed progressive, post-lingual, symmetrical, bilateral, and non-syndromic sensorineural hearing loss (Figure 1b). Other family members with a hearing impairment did not accept hearing test because of private reasons.

3.2 | Identification of a novel mutation in *KCNQ4*

To identify the genetic cause of hearing loss, WES was performed on the proband. WES detected a novel potential causative variant (c.902 C>A; p.Ala301Asp) in exon 6 of the *KCNQ4* gene. This variant was also detected in individuals II:6, II:8, II:12, III:4, III:6, and III:7, by Sanger sequencing (Figure 2a), who showed as being impaired with regard to hearing. II:10 with a hearing impairment did not accept gene test because of private reasons. However, the variant was not found in individuals II:4 and II:5 who had normal hearing (Figure 2b).

3.3 | Amino acid conservation analysis

The location of the *KCNQ4* c.902 C>A variant, the associated protein region, and the residue conservation analysis among different species are shown (Figure 3). Apparently, the variant resulted in an alanine-to-aspartic acid substitution at position 301 in the KCNQ4 protein. The alanine at position 301 was conserved throughout evolution.

3.4 | Prediction of secondary structure and tertiary protein structure p.Ala301Asp

The *KCNQ4* c.902 C>A variant resulted in the conversion of alanine-to-aspartic acid, where alanine is a non-polar amino acid, and aspartic acid is an acidic (polar) amino acid. A computational secondary protein structure prediction of the wild-type and p.Ala301Asp variant of the KCNQ4 protein was performed (Figure 4a). We hypothesized that this variant affected the helical structure of the *KCNQ4* gene. A computational analysis of the tertiary structures of the wild-type and p.Ala301Asp variant of the KCNQ4 protein was conducted. Our study mapped the mutation position onto the crystal structure

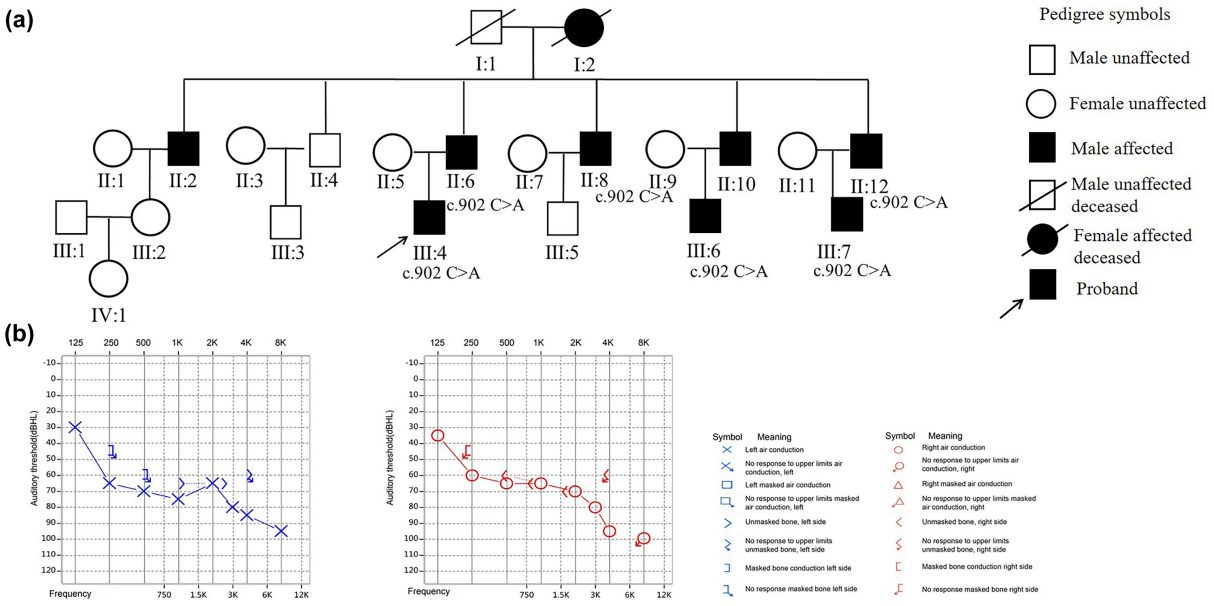

FIGURE 1 Family pedigree and audiograms of the proband. (a) The pedigree of Family A. The arrows indicate the probands; (b) the audiograms of the proband.

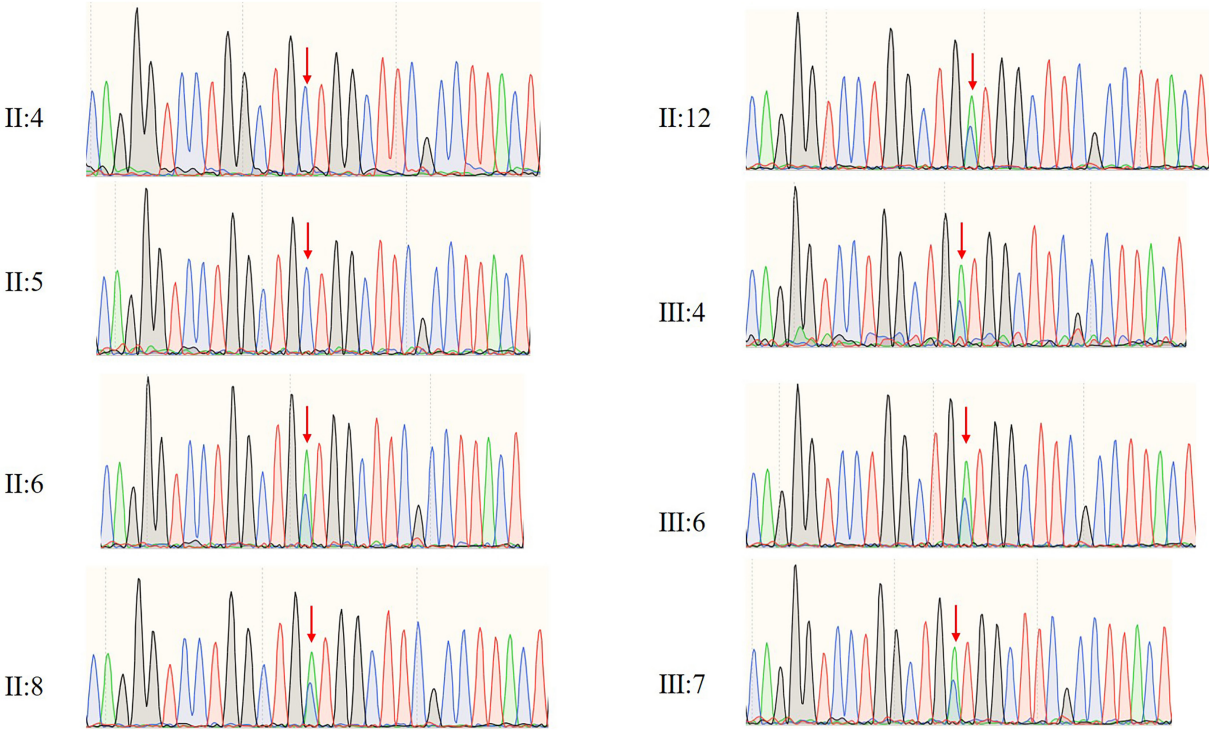

FIGURE 2 Sanger sequencing of the family. The mutant type was detected in II:6, II:8, II:12, III:4, III:6, III:7 by Sanger sequencing and was not found in individuals II:4 and II:5.

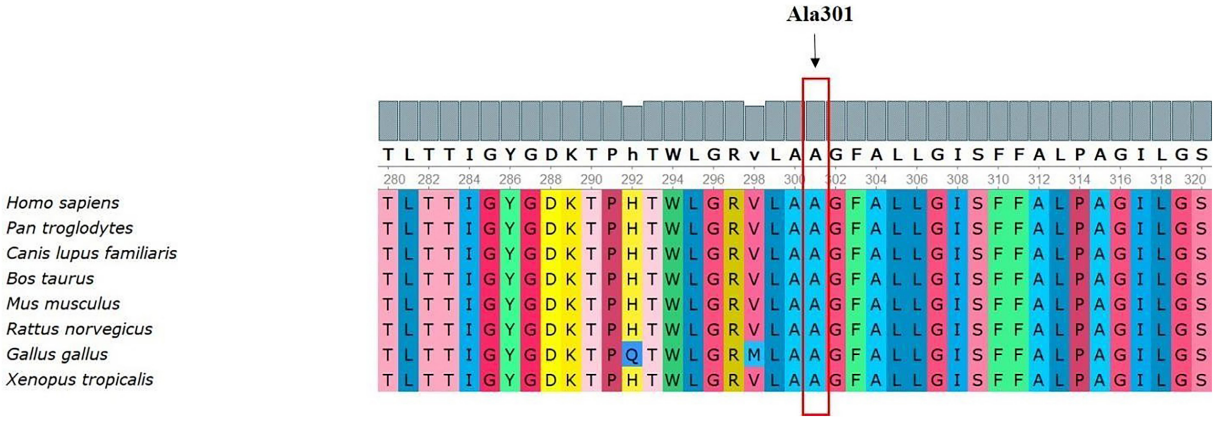

FIGURE 3 Amino acid conservation analysis. High conservation for the mutated amino acid position 301 was shown in the KCNQ4 protein among different species, with the red box marking the position where the amino acid residue changes occurred in our study.

of KCNQ4 (PDB ID:7byn). Three-dimensional structural model showed that the affected residues were located in the core region of KCNQ4, which may affect the global conformation and activity of protein (Figure 4b). When alanine was replaced by aspartate, all hydrogen bonds above were kept and two hydrogen bonds were built with Thr278. By modeling proteins, it was observed that the KCNQ4 variants experienced changes in their conformational space, leading to potential functional deficits (Figure 4c,d).

3.5 | Effects of novel variants on KCNQ4 channel function

To assess the effects of the KCNQ4 variants on voltage-gated channel activity, we recorded whole-cell currents in HEK293T cells that were transiently transfected with plasmids that expressed the wild type (WT), mutant type (MT), and mixture of Kv7.4wt and Kv7.4mut (WT&MT) via patch-clamp recording. HEK293T cells that expressed the WT protein exhibited voltage-dependent potassium

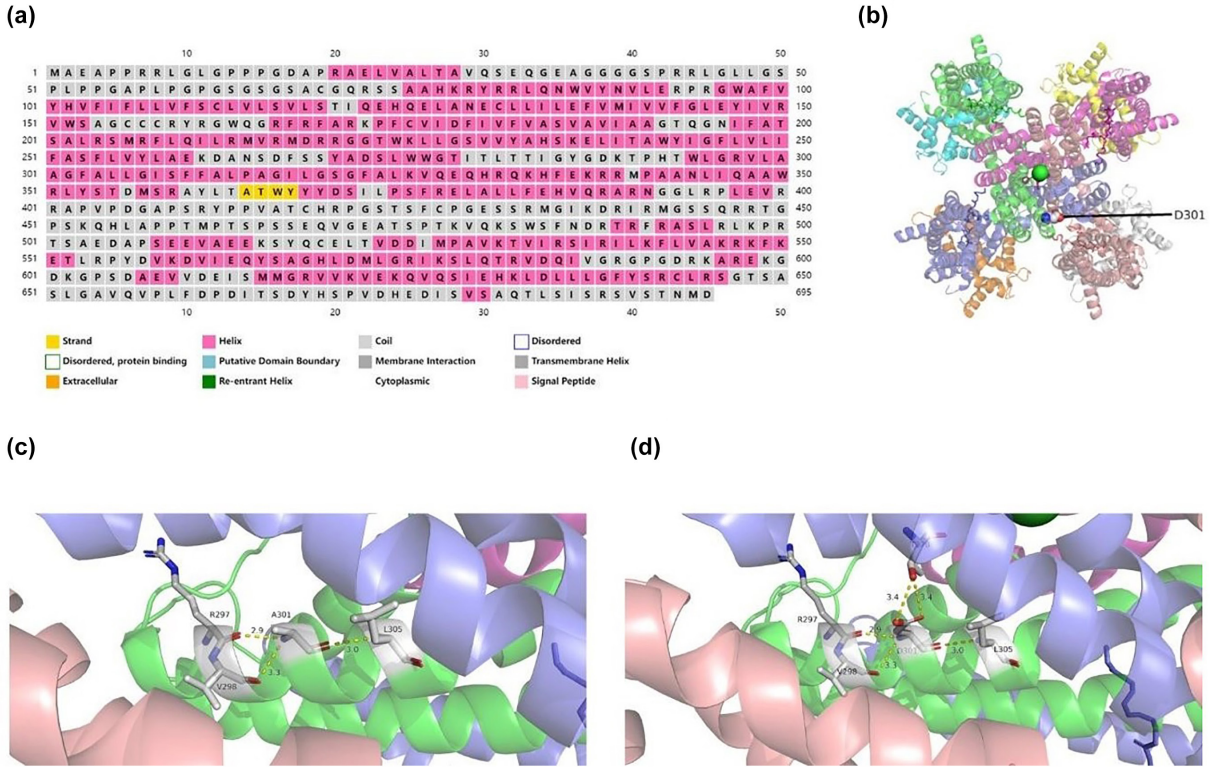

FIGURE 4 Prediction of secondary structure and tertiary protein structure p. Ala301Asp. (a) A computational secondary protein structure prediction of the wild-type and p.Ala301Asp variant of the KCNQ4 protein. It was speculated that the variant affected the helical structure of the KCNQ4 gene. (b) A computational tertiary protein structure prediction of the variant. (c) A computational tertiary protein structure prediction of the wild-type Ala301. (d) A computational tertiary protein structure prediction of the mutant type Asp301.

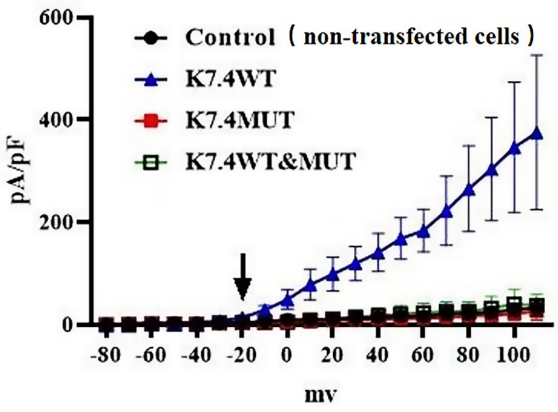

FIGURE 5 Effects of novel variants on KCNQ4 channel function. HEK293T cell with the WT protein showed voltage-dependent potassium currents with a peak current density of 375.3 ± 134.5 pA/pF at +110 mV. The outward potassium currents with mutant channels were barely detectable, similar to the control. Starting from 20mv, a significant difference in the WT protein currents and the mutant protein currents was noted ($p < 0.05$).

currents with a peak current density of 375.3 ± 134.5 pA/pF at +110 mV ($n = 10$). In contrast, the outward potassium currents that were generated by mutant channels were barely detectable, similar to the control (non-transfected cells). Starting from 20mv, a significant difference in the WT protein currents and the mutant protein currents was noted ($p < 0.05$) (Figure 5). Therefore, we speculate the newly identified KCNQ4 variant could reduce voltage-gated channel activity.

4 | DISCUSSION

In this study, a novel KCNQ4 variant, c.902 C>A (p.Ala301Asp), was found in all six affected individuals of a Chinese family with ADNSHL.NGS, and Sanger sequencing was used to emphasize this detection. It was observed that the alanine at position 301 was well conserved across species. No record of the *KCNQ*4 variant was found in the clinical disease-related databases ClinVar and HGMD, nor in the East Asian population of ExAC, 1000 Genomes, and the GnomAD database. REVEL score was 0.963 and CADD value was 26.5, which revealed that the variation triggered harmful effects on the gene product. Furthermore, the variant was found to co-segregate with the progressive hearing loss phenotype because six affected individuals carried the variant (II:6, II:8, II:12, III:4,

III:6, III:7) in the family. The *KCNQ4* c.902 C>A variant resulted in the substitution of alanine with aspartic acid, altering the polarity of the amino acid. Computational analysis of the protein's secondary and tertiary structures indicated that this variant may affect the helical structure and global conformation of the KCNQ4 protein, potentially impairing its function. Whole-cell patch clamp showed that the *KCNQ*4 variant reduced voltage-gated channel activity. Therefore, it was extrapolated that *KCNQ4* c.902 C>A is more likely to be a pathogenic mutation, in accordance with the ACMG/AMP guideline based on the evidence of PM2_supporting+PP3_strong +PP1 + PS3 moderate.

KCNQ4 is also referred to as voltage-gated potassium channel subunit Kv7.4. The protein is thought to be responsible for the formation of a potassium channel, which is believed to be necessary for controlling the electrical activity of neurons, particularly in the auditory cells of the cochlea. The protein encoded can potentially form either a homomultimeric potassium channel or a heteromultimeric potassium channel in conjunction with the protein encoded by the *KCNQ*3 gene (Gutman et al., 2005; Homma, 2022). DFNA2A, which is caused by a mutation in the *KCNQ4* gene, follows an autosomal dominant inheritance pattern (Rashid, 2021). In this study, affected individuals were observed in every generation, and at least one of the parents of an affected individual was also affected, which follows an autosomal dominant inheritance mode.

So far, 53 likely pathogenic or pathogenic variants of the *KCNQ4* gene have been reported to be associated with hearing loss in the ClinVar database, including 26 missense variants, 11 deletion variants, 8 duplication variants, 3 nonsense variants, 3 splicing variants, and 2 microsatellite variants. Missense variants are believed to disrupt the normal physiological function of potassium channels, leading to dominant-negative effects. Patients carrying a *KCNQ*4 missense variant typically exhibit early-onset, severe hearing loss that affects all frequencies. On the other hand, deletion variants result in frameshift changes, causing most of the expressed truncated proteins to be unable to fully carry out their physiological function. Patients with deletion mutations generally experience milder symptoms, characterized by delayed onset and involvement of high frequencies. Most of missense variants are located in exons 1, 3–8, and 14 of the *KCNQ*4 gene (Li et al., 2021). These variants, most of which are clustered around the pore area, play a role in loss of hearing. The pore area is critical for ion selectivity of potassium channels. The GYG (Gly-Tyr-Gly) characteristic sequence is located in the narrowest area of pores, which is essential to maintain the pore structure and function (Doyle et al., 1998). The missense variants on the K+ ion-selective filter have been proved to destroy the highly conservative GYG characteristic sequence, thereby resulting in severely damaged non-conductive channels and eventually serious loss of the ability to hear (Kubisch et al., 1999). The variant altered the first glycine of the GYG characteristic sequence. Several variations in this region have also been discovered in succession. Amino acid changes in other domains can also contribute to hearing loss. These include the missense variant *KCNQ*4: c.891G>T, p.R297S that is located in the S6 trans domain and the amino acid deletion *KCNQ*4: c. 689T>A, p.V230E that is located on the S4-S5 linker, both of which were identified in Japan (Naito et al., 2013). The majority of *KCNQ*4 variants, which are responsible for DFNA2A, are clustered in the S5−S6 region surrounding the ion-permeating pore region (amino acids 271–292). These variants disrupt ion permeation, regardless of channel gating, and are believed to have dominant-negative inhibitory effects, which may be the underlying cause of DFNA2A (Kubisch et al., 1999). In our study, the novel variant *KCNQ4*: c. 902 C>A, p.Ala301Asp is located in exon 6 and the S6 trans domain. In order to assess the effects of the *KCNQ*4 variants on voltage-gated channel activity, we transfected the wild type, mutant type, and wild-mutant type into HEK293T cells. Our findings showed that the mutant produced almost no current and the wild-mutant type produced very little current, compared with the wild type. It is speculated that the variant would affect the function of the ion permeation and exhibit dominant negative effects, thereby causing hearing loss. But the underlying mechanism needs to be further studied. Patients with the *KCNQ*4 gene mutations may exhibit varying phenotypes due to different penetrance and genetic heterogeneities.

In this study, affected family members (II:2, II:6, II:8, II:10, II:12, III:6, III:7) exhibited hearing impairment, with ages of onset were 10, 30, 20, 45, 3, 18, and 30 years old, respectively, which is consistent with the characteristic of late-onset in most cases of ADNSHL. The timing of the onset and degree of hearing loss in patients with *KCNQ*4 gene mutations varied across studies (Zhang et al., 2023). DFNA2A with *KCNQ*4 gene mutations is usually characterized by a symmetrical, gradual, high-frequency hearing loss that appears in the later stages of life, followed by a full-frequency hearing loss (Oh et al., 2023). Mutations in the KCNQ4 gene result in hereditary hearing loss, characterized by an average annual decrease in hearing thresholds of 0.72 dB. The most significant decline in hearing occurs between the ages of 45 and 50, with an average annual loss of 0.89 dB (Thorpe et al., 2022). So far there is no known specific treatment for hereditary hearing loss caused by mutations in the *KCNQ4* gene. Nevertheless, hearing aids and cochlear implants can provide some level of improvement in the hearing condition of affected individuals.

5 | CONCLUSION

In a word, our study demonstrated that the co-segregating heterozygous missense variant (c.902 C>A; Ala301Asp) in the S6 trans domain of the KCNQ4 channel was the probable pathogenic variant in this ADNSHL family. Our findings contributed to expanding the mutation spectrum of the *KCNQ4* gene and may be useful in the diagnosis and gene therapy of ADNSHL.

AUTHOR CONTRIBUTIONS

ZW-C and SW-H conceptualized the study; LY-R, JF-W, and YK contributed to methodology; K C and MM-J performed experiments; ZZ-Z and K C analyzed data; ZZ-Z contributed reagents or other essential material; LY-R wrote the paper. All authors have read and agreed to the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS STATEMENT

Written informed consent was obtained from all participants. The present study was approved by the Ethics Committee of Guizhou Provincial People's Hospital.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Guizhou Provincial People's Hospital.

INFORMED CONSENT STATEMENT

Prior to the commencement of the study, informed consent was obtained from participants involved in the study. No minors or illiterates were involved in this study.

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