


ORIGINAL ARTICLE

Early truncation of the N-terminal variable region of *EYA4* gene causes dominant hearing loss without cardiac phenotype

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Award Number: SBGJ2018020152**Abstract****Background:** Autosomal dominant hearing loss (ADHL) accounts for about 20% of all hereditary non-syndromic HL. Truncating mutations of the *EYA4* gene can cause either non-syndromic ADHL or syndromic ADHL with cardiac abnormalities. It has been proposed that truncations of the C-terminal Eya domain lead to non-syndromic HL, whereas early truncations of the N-terminal variable region cause syndromic HL with cardiac phenotype.**Methods:** The proband and all the other hearing impaired members of the family underwent a thorough clinical and audiological evaluation. The cardiac phenotype was examined by ECG and echocardiography. Their DNA was subjected to target exome sequencing of 129 known deafness genes. The sequencing data were analyzed and the candidate variants were interpreted following the ACMG guidelines for clinical sequence interpretation. The effect of candidate variant on *EYA4* gene expression was assessed by quantitative PCR and western blot of gene production in blood.**Results:** We report a Chinese family cosegregating post-lingual onset, progressive ADHL with a novel nonsense mutation NM_004100.4:c.543C>G (p.Tyr181Ter) of *EYA4*. Two affected members show no cardiac abnormalities at least until now revealed by electrocardiography and echocardiography. The overall expression level of the *EYA4* gene in the proband was lower than that in his unaffected relative.**Conclusion:** This report expands the mutational spectrum of the *EYA4* gene and highlights the fact that more data are needed to elucidate the complex genotype–phenotype correlation of *EYA4* mutations.

Yanfang Mi and Danhua Liu are Joint first authors.

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KEYWORDS

autosomal dominant hearing loss, EYA4, next-generation sequencing

1 | INTRODUCTION

Hearing loss is the most common sensory defect in humans. The prevalence of permanent HL at birth is 1.07/1000 (Morton & Nance, 2006). HL is a heterogeneous disorder caused by genetic and environmental factors, yet half of the cases tend to be genetic (Nance, 2003). If the pathology is genetically related, it can be classified according to the pattern of inheritance (autosomal dominant, autosomal recessive, and X-linked), and the presence (syndromic) or absence (non-syndromic) of abnormalities in other parts of the body. It has been estimated that approximately 80% of genetic deafness is non-syndromic, where 80% of non-syndromic HL have been inherited in an autosomal recessive manner (Morton & Nance, 2006). In contrast to the autosomal recessive HL, which is congenital and pre-lingual, patients with autosomal dominant non-syndromic HL (ADNSHL) often have a family history of post-lingual HL.

So far, a total of 47 genes have been identified as causal genes for ADNSHL (<http://hereditaryhearingloss.org>), including *DIAPH1* (Lynch et al., 1997), *KCNQ4* (Kubisch et al., 1999), and *GJB3* (Xia et al., 1998). Autosomal dominant deafness 10 (DFNA10, OMIM #601316) locus was first identified and mapped on chromosome 10 in 1996 in a large American family as a causal gene for ADNSHL (O'Neill et al., 1996). In 2001, Wayne et al. identified nonsense mutations in the *EYA4* gene (OMIM: 603550) in two unrelated families segregating at DFNA10, and defined *EYA4* (Eyes Absent Homolog 4) as the causative gene for DFNA10 (Wayne et al., 2001). The *EYA4* gene is composed of 21 exons encoding a 640 amino acid protein, which contains a highly conserved region (*eya* homologous region, *eyaHR*) and a more divergent proline-serine-threonine (PST)-rich transactivation domain (*eya* variable region, *eyaVR*) (Borsani et al., 1999). *EYA4* is a member of the vertebrate *eya* gene family, a group of transcriptional activators that interact with other proteins to ensure the healthy development of multiple organs, including the eye, muscle, kidney, inner ear, and heart (Wang et al., 2008; Wayne et al., 2001). Although some mutations in *EYA4* cause syndromic HL with dilated cardiomyopathy (DCM) as described in a family (Schonberger et al., 2000, 2005) and a Japanese patient (Abe et al., 2018), the majority of mutations in this gene are associated with ADNSHL. Hearing impairment for *EYA4* mutations is characterized by post-lingual and progressive sensorineural HL. To date, 34 diseases causing *EYA4* mutations have been described, including four nonsense mutations, 12 missense mutations, nine small indels, six splicing mutations, and three gross deletions (Stenson et al., 2012).

In this study, we report on a case of a three-generation family with late-onset progressive sensorineural HL. The

genetic cause was investigated using targeted sequencing of 129 known deafness genes. A novel nonsense variant, NM_004100.4:c.543C>G (p.Tyr181Ter) in exon 8 of the *EYA4* gene, was identified in the family. The onset age and audiograms were compared with other studies, and the impact of reported mutations on cardiac phenotype has been discussed. This study expands the mutational spectrum of the *EYA4* gene and suggests that next-generation sequencing is effective in elucidating the etiology of familiar progressive HL.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was conducted according to the Helsinki Declaration and approved by the local institutional ethics committee in human research of Zhengzhou University (reference no. 2018008). Written informed consent for genetic testing and the submission of the study was obtained from all the studied individuals.

2.2 | Subjects and clinical evaluation

The proband was a 28-year-old male Chinese, who was referred to the Department of Otolaryngology at the Second Affiliated Hospital of Zhengzhou University in Zhengzhou. Detailed family history questioning revealed the other four affected members across three generations with AD inheritance patterns (Figure 1). All affected family members underwent a medical history interview that included questions on onset age and degree of HL, other clinical manifestations, and environmental factors (viral infection, noise, and ototoxic drugs). A complete clinical evaluation, audiological tests, and cardiac examination were performed in the affected family members who agreed to participate in this study. The level of HL was classified into four tiers in terms of pure tone average (averaged over 0.5, 1.0, 2.0, and 4.0 kHz): mild (21–40 dB), moderate (41–70 dB), severe (71–90 dB), and profound (>91 dB).

2.3 | Target exome sequencing of known deafness genes

Genomic DNA was isolated from peripheral blood leukocytes using the GenMagBio Genomic DNA Purification kit

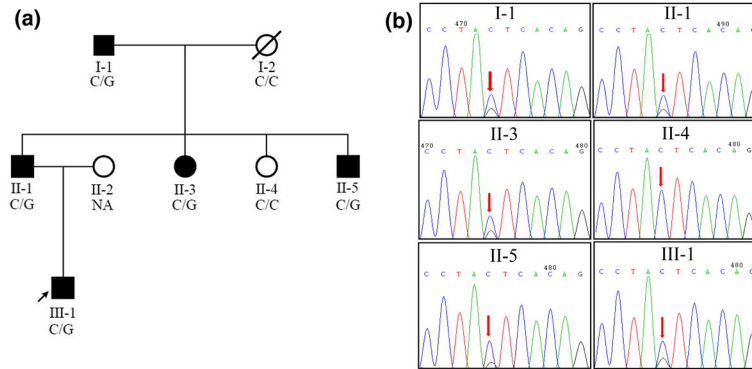


FIGURE 1 Pedigree of the family with non-syndromic autosomal dominant sensorineural hearing loss segregating *EYA4* c.543C>G (canonical transcript NM_172105.3). (a) Family pedigree. Open symbols unaffected; filled black symbols affected; diagonal line deceased. (b) Sanger sequencing. The red arrow indicates the *EYA4* c.543C>G mutation

(GenMagBio, Changzhou, China) following the manufacturer's standard procedures. Genomic DNA from affected and unaffected members was fragmented to an average size of 250 bp, and end repair, adapter ligation as well as PCR enrichment were performed following the protocol for VAHTS™ Universal DNA Library Prep Kit for Illumina V3 (Vazyme Biotech Co., Ltd). The enriched DNA libraries were captured with the Human Deafness Panel oto-DA3 (Otogenetics Corporation) with 129 known HL genes, following the manufacturer's protocol. The resulting libraries were sequenced on an Illumina HiSeq 4000 sequencer (Illumina Inc.) with the paired-end of 150 bp at the Precision Medicine Center of Zhengzhou University, Zhengzhou, China.

2.4 | Bioinformatics analysis and variant interpretation

Sequencing adapters and low-quality reads were trimmed from raw reads with Trimmomatic (Bolger et al., 2014). Clean reads were aligned to the human reference genome (version GRCh37) using Burrow-Wheeler Aligner (version 0.7.17-r1188) (Li, 2013). Duplicate reads were flagged by sambamba (version 0.6.6) (Tarasov et al., 2015). Single nucleotide variants (SNVs) and small indels were characterized using the Genome Analysis Toolkit version 4 (GATK4) HaplotypeCaller (DePristo et al., 2011). Variant annotation was performed using Vcfanno (Pedersen et al., 2016) with several variant frequency databases for healthy population, such as 1000 Genomes Project database (Genomes Project et al., 2015), dbSNP (Sherry et al., 2001), Exome Aggregation Consortium (ExAC) (Lek et al., 2016), Genome Aggregation Database (gnomAD) (Karczewski et al., 2019), ClinVar (Landrum et al., 2018), InterVar (Li & Wang, 2017), and dbNSFP (Liu et al., 2016), which compiled mutation prediction scores from many algorithms (Ioannidis et al., 2016; Kumar et al., 2009; Schwarz et al., 2014). All analysis

steps described above were performed in the framework of bcbio-nextgen (<https://github.com/bcbio/bcbio-nextgen>), which provides best practice pipelines for variant calling, annotation, and validation. We filtered out variants with minor allele frequency >0.05 in any general continental population in which at least 2000 alleles were observed in the gnomAD database, except the ones on ACMG benign stand-alone exception list or those linked to diseases in ClinVar database (Ghosh et al., 2018). Variant interpretation was done by an expert panel of an otorhinolaryngologist, a bioinformatician, and a molecular geneticist following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) guidelines for clinical sequence interpretation (Richards et al., 2015). Variant nomenclature was based on *EYA4* canonical transcript NM_172105.3. The phenotype and pathogenic variant were submitted to the LOVD database (<https://databases.lovd.nl/shared/individuals/00320009>).

2.5 | Sanger sequencing

To confirm candidate variants detected by next-generation sequencing, we performed PCR amplification and Sanger sequencing. The following primers were designed using Primer-BLAST software: forward, 5'-CATGGTGAGTGTGAATTTGCTCA-3'; reverse, 5'-GTGTAGCTACTTACCTGGCATT-3'. PCR products were purified by a PCR purification kit (Life Sciences) and sequenced using a SeqStudio Genetic Analyzer (Applied Biosystems/Life Technologies).

2.6 | Functional study of *EYA4* nonsense mutation

Whole peripheral blood from the proband (III-1) and his unaffected aunt (II-4) was collected in EDTA tubes,

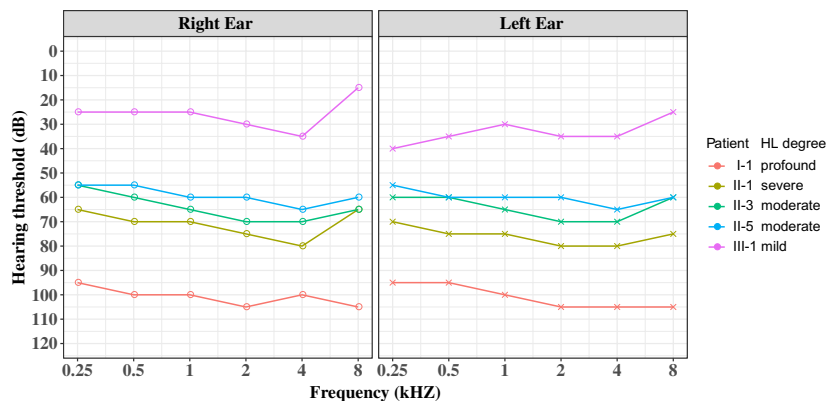


FIGURE 2 Audiometric phenotypes of five affected members in the family. Pure-tone air conduction thresholds are shown for right and left ears, and the HL severity is summarized for the ear with better hearing

and total RNA was extracted from blood mononuclear cells ~12 hours after collection using the TRIzol method (Cat. No. 15596026). RNA was reverse transcribed into cDNA using the HiScript III RT SuperMix for qPCR (Vazyme). The forward and reverse primers for *EYA4* were 5'-TCCCCACAGCTGTATCCTTC-3' and 5'-AACTGAGGCAGCCACTCTGT-3' as validated in a previous study (Zou et al., 2005). The primers for human *GAPDH* (internal control, PrimerBank accession number: 378404907c3) were 5'-CTGGGCTACACTGAGCACC-3' and 5'-AAGTGGTCGTTGAGGGCAATG-3'. The amplification length for *EYA4* and *GAPDH* was 250 bp and 101 bp, respectively. Relative expression levels of wild-type and mutant *EYA4* transcripts were measured by quantitative real-time PCR (qPCR) using AceQ qPCR SYBR Green Master Mix (Vazyme) and the ABI StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Each condition was replicated for three times and the $\Delta\Delta C_t$ method was used to quantify qPCR results.

The serum was removed from anticoagulated whole blood by centrifugation and the red blood cells were lysed using the red blood cell lysis buffer (Solarbio, cat no. R1010). Then, RIPA cell lysate (Solarbio, cat no. R0010) and protease inhibitor (MedChemExpress, cat no. HY-K0010) were used to rupture mononuclear cells to obtain total protein. The extracted protein was quantified by BCA protein assay kit (Beyotime, cat no. P0010S). The total protein was separated in a 10% gel with SDS-PAGE, and then transferred to PVDF membrane (Beyotime, cat no. FFP39). The PVDF membrane was blocked with 5% non-fat milk in TBST solution at room temperature and then incubated with the primary *EYA4* antibody (univ-bio, cat no. abs111928). Beta-actin served as a loading control. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Transgene, cat no. HS101-01).

3 | RESULTS

3.1 | Clinical features of the family

The three-generation family had five affected individuals, with age ranging from 28 (III-1) to 80 (I-1). The family exhibited a typical autosomal dominant inheritance pattern (Figure 1a). The self-reported onset age of HL ranged from the second to the fourth decade. The age of onset was 25 years old for the proband with mild sensorineural HL involving all frequencies; the average auditory threshold of air conduction measurements for the right and left ear was 26 dBHL and 33 dBHL, respectively (Figure 2). In the fifth decade, the severity of the HL generally became severe-to-profound across all frequencies. The affected individuals reported the use of hearing aids in the third or fourth decade, as II-1, II-3, and II-5. No family members received cochlear implants, and in most cases, the HL was not severe enough to warrant such intervention. The grandpa of the proband (I-1, 80 years old) manifested severe sensorineural HL, involving entire frequencies. No tinnitus or vestibular dysfunction (such as balance disorders, vertigo, or Meniere disease) was observed in any of the patients. All members denied any family history of cardiomyopathy, and the electrocardiogram and echocardiography conducted on two affected individuals (II-1 and III-1) showed no evidence of DCM or other abnormalities (Figure 3). The clinical examinations ruled out the probability of syndromic HL with heart problems.

3.2 | Target exome sequencing identifies a novel *EYA4* mutation

Target exome sequencing of 129 known deafness genes covered by the Otogenetics Human Deafness Panel



FIGURE 3 Normal cardiac phenotypes of the proband (a and c) and his father (b and d). (a and b) Electrocardiogram; (c and d) echocardiograph

oto-DA3 was performed for one unaffected and four affected individuals for the identification of potentially pathogenic variants. An average of one billion raw base pairs (1 Gbp) were generated for each sample, with more than 85% of bases having a Phred quality score $Q \geq 30$ (Q30). More than 99% of clean reads can map the human genome reference sequence (GRCh37 version), and the average sequencing depth of targeted regions was 171.5X (ranging from 158.3X to 187.9X), covering $\geq 95\%$ of target regions more than 20 times. After filtering out variants with allele frequency $>0.1\%$ in the database of the normal population (gnomAD and ExAC), a total of 14 variants were obtained, including variants in genes *MITF*, *GJB3*, and *EYA4*, which were previously associated with HL. The only variant in *EYA4* was cosegregated with HL in the family. Sanger sequencing of this variant in the family was verified, and the mutation was present in the affected members and absent in the unaffected ones (Figure 1b).

3.3 | Variant interpretation of the mutation as pathogenic

The *EYA4* (NM_172105.3) variant c.543C>G (p.Tyr181Ter) was found in exon 8; amino acid of 181 positions changed from tyrosine to termination codon, which led to early termination of protein translation. According to the standards and guidelines for interpreting genetic variants proposed by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) (Richards et al., 2015), this variant was classified as pathogenic. It was absent from all population databases, including gnomAD and ExAC (Pathogenic Moderate 2, PM2). The mutation is a null mutation that leads to a non-functional protein product (Pathogenic Very Strong 1, PVS1). The variant was cosegregated with the phenotype of DFNA10 in the affected family members (Pathogenic Supportive 1, PP1). This mutation has not yet been deposited in the database of HGMD professional edition and is a novel pathogenic variant.

3.4 | Functional study of *EYA4* nonsense mutation

To explore the effect of nonsense mutation on *EYA4* expression, we investigated the expression of *EYA4* gene at both transcriptional and protein levels in the proband and his unaffected relative. We extracted total RNA and total protein from the fresh peripheral blood from the proband and his healthy aunt. We observed that the mRNA levels of *EYA4* gene in the proband decreased by ~50% compared to the unaffected relative (Figure 4a). The western blot analysis revealed that compared with his aunt the protein level of *EYA4* gene was much lower in the proband (Figure 4b). These results suggest that the mutant transcript with nonsense mutation is subject to nonsense-mediated mRNA decay (NMD).

4 | DISCUSSIONS

In the present study, we reported on a three-generation Han Chinese family with post-lingual non-syndromic HL. All the affected individuals in the family had sensorineural deafness and onset age from 26 to 50 years old, affecting primarily low and middle frequencies. All members denied cardiac phenotype, and electrocardiogram and echocardiography conducted on two affected members who agreed to participate in the examinations showed no evidence of DCM and

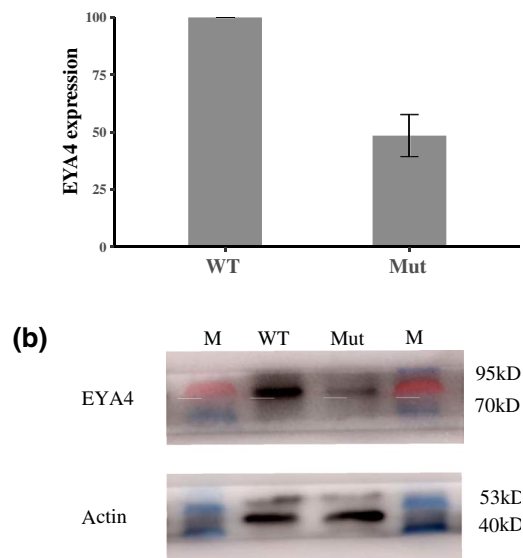


FIGURE 4 Reduced expression of *EYA4* in blood in the proband compared with his unaffected aunt. (a) Quantitative PCR analysis of *EYA4* expression (b) western blot analysis of *EYA4* protein expression in the proband (Mutant, Mut) and his aunt with normal hearing (wild-type, WT). M, protein makers. The qPCR experiment was repeated three times and bars represent standard deviation

other abnormality. Targeted sequencing of 129 HL genes showed that HL in the family segregated with a mutation in the *EYA4* gene. The mutation NM_004100.4: c.543C>G (p.Tyr181Ter) is in exon 8 and leads to the introduction of a premature termination codon of the coding protein lacking the entire *eyaHR* domain. The nonsense mutation was classified as pathogenic following the variant interpretation standards and guidelines of ACMG. To the best of our knowledge, this variant has not been reported before.

Patients harboring *EYA4* mutations often develop non-syndromic late-onset HL. However, so far, a few studies reported on patients with DCM and heart failure accompanied by HL. Schonberger et al. (2000) reported two families showing sensorineural HL and DCM transmitted by an autosomal dominant manner and mapped to 6q23–24 (Schonberger et al., 2000); by fine mapping and mutation analysis, they identified a 4846-bp deletion encompassed part of the *EYA4* gene as causal for the syndrome (Figure 5, E193) (Schonberger et al., 2005). In 2009, Dutrannoy et al. reported a de novo 9 Mbp deletion of 6q23.3-24.1 disrupting *EYA4* in a young patient who presented with HL, patent ductus arteriosus, and other mental problems (Dutrannoy et al., 2009). Moreover, Abe et al. (2018) reported progressive HL and mild cardiac abnormalities carrying a nonsense mutation in *EYA4* gene (NM_004100.5:c.1177C>T:p.Gln393Ter) in a Japanese patient (Abe et al., 2018); the mutation was previously characterized as a pathogenic variant in a Korean pedigree with non-syndromic HL (Figure 5) (Kim et al., 2015). Although the correlation between genotype (*EYA4* mutation) and phenotype (syndromic or non-syndromic HL) is not clear, it has been suggested that the mutation position of *EYA4* might determine whether HL is accompanied by cardiac abnormalities. A few scientists speculated that truncations of the C-terminal *Eya* domain cause non-syndromic HL, whereas deletions of the N-terminal variable region lead to HL with DCM (Makishima et al., 2007; Schonberger et al., 2005). This view was challenged by a study that reported on truncating *EYA4* mutation c.863C>A(p.Ser288Ter), leading to deletions of the N-terminal variable region in a Korean family with moderate hearing impairment and no DCM (Baek et al., 2012). Yet, the detailed investigation of the cardiac phenotype was not performed for the patient. Two recent studies further weaken this view by reporting more patients with non-syndromic HL and N-terminal truncating mutations, such as c.160G>T (p.Glu54Ter), c.223_224del (p.Val75PhefsTer32, Figure 5), and c.517C>T (p.Gln173Ter) (Morin et al., 2020; Shinagawa et al., 2020). The novel mutation we reported here c.543C>G, in exon 8 of *EYA4*, resulted in a shortened protein lacking part of the N-terminal variable region in a Chinese family with no apparent cardiac phenotypes, and further complicated the relationship between *EYA4* mutation and phenotype. Therefore, more studies are needed to investigate the correlation between *EYA4* mutation and syndromic phenotype.

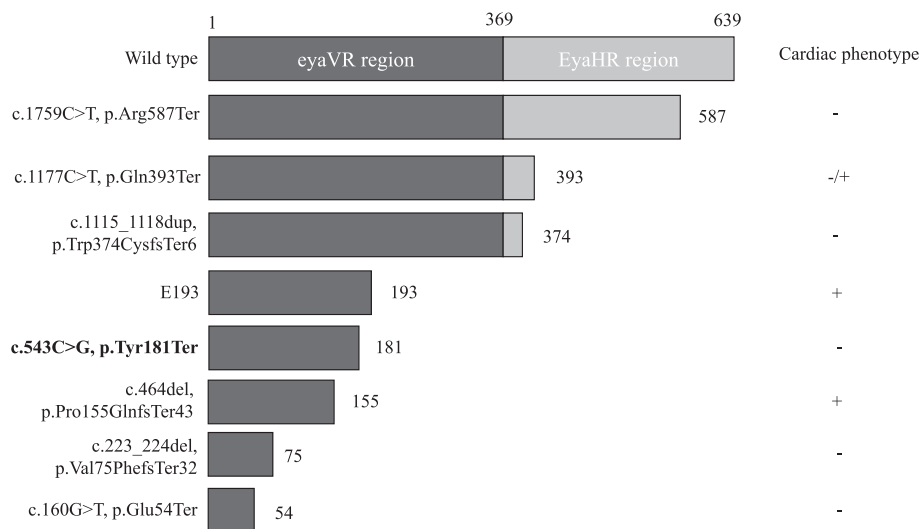


FIGURE 5 Effects of representative mutations on *EYA4* protein structure and the cardiac phenotype. The number of amino acids of each allele product is indicated. The mutation found in this study is in bold. Variant nomenclature was based on *EYA4* canonical transcript NM_172105.3

Until now, more than 50 pathogenic or likely pathogenic mutations in *EYA4* have been reported (Morin et al., 2020); among these, ~40% of mutations have been found in the East Asia population. The *EYA4* gene is not a common gene for ADNSHL, and thus far, only a limited number of mutations have been reported. Thus, the complete picture of audiological features associated with these variants needs to be further explored. The audiological features associated with *EYA4* mutations are highly heterogeneous. Specifically, the same or similar mutation can cause different clinical phenotypes, while similar clinical phenotypes can be caused by different mutations. The clinical phenotype of patients carrying *EYA4* mutations is the following: late-onset, post-lingual, progressive, and bilateral HL. For example, in relation to nonsense mutations, at least four nonsense mutations have been reported (Baek et al., 2012; Hu et al., 2018; Kim et al., 2015; Wayne et al., 2001). Most of the affected individuals with nonsense mutations suffered from moderate to severe bilateral, progressive, sensorineural HL in low- and mid-frequencies and all frequencies were affected by aging. The onset age of HL ranged from early childhood to adulthood. The hearing curve of patients at the beginning of onset is usually U-shaped but can also be seen in the descending and flat curves. This report expands the mutational spectrum of the *EYA4* gene and highlights the importance of genetic testing for patients with familial progressive HL.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

Study design: Yanfang Mi, Yulin Zhao, and Hongen Xu. Patient phenotypic analysis and genetic counseling: Yanfang Mi, Hui Zhang, and Bei Chen. Next-generation sequencing and Sanger sequencing: Yongan Tian, Juanli Zhang, and Wenxue Tang. Data analysis and variant interpretation: Danhua Liu, Bei Chen, Hong Xue, and Hongen Xu. qPCR and western blot: Beiping Zeng. Writing and review of original draft of the manuscript: Yanfang Mi, Danhua Liu, Yulin Zhao, and Hongen Xu. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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