Identification of a novel CNV at the EYA4 gene in a Chinese family with autosomal dominant nonsyndromic hearing loss

Weixin Zhang1,2,3,4,5†, Jing Song1,2,3,4†, Busheng Tong6, Mengye Ma1,2,3,4, Luo Guo1,2,3, Yasheng Yuan1,2,3,4,5,7* and Juanmei Yang1,2,3,4,7*

Abstract

**Background:** Hereditary hearing loss is a heterogeneous class of disorders that exhibits various patterns of inheritance and involves many genes. Variants in the EYA4 gene in DFNA10 are known to lead to postlingual, progressive, autosomal dominant nonsyndromic hereditary hearing loss.

**Patients and methods:** We collected a four-generation Chinese family with autosomal-dominant nonsyndromic hearing loss (ADNSHL). We applied targeted next-generation sequencing (TNGS) in three patients of this pedigree and whole-genome sequencing (WGS) in the proband. The intrafamilial cosegregation of the variant and the deafness phenotype were confirmed by PCR, gap-PCR and Sanger sequencing.

**Results:** A novel CNV deletion at 6q23 in exons 8–11 of the EYA4 gene with a 10 bp insertion was identified by TNGS and WGS and segregated with the ADNSHL phenotypes.

**Conclusions:** Our results expanded the variant spectrum and genotype–phenotype correlation of the EYA4 gene and autosomal dominant nonsyndromic hereditary hearing loss in Chinese Han individuals. WGS is an accurate and effective method for verifying the genomic features of CNVs.

**Keywords:** EYA4, DFNA10, Copy number variation (CNV), Deafness, Whole genome sequencing (WGS)

Introduction

Hearing loss is the most common sensory deficit in modern society and affects approximately 466 million people worldwide, 34 million of whom are children [1, 2]. Hereditary hearing loss patterns vary among autosomal dominant, autosomal recessive, X-linked, and mitochondrial patterns of inheritance. Unlike syndromic hearing losses, non-syndromic hearing losses are not associated with other clinical abnormalities [3]. In 75–80% of cases, NSHL is inherited autosomally recessively, while 20% are inherited autosomally dominantly. X-linked (2–5%) or mitochondrial patterns of inheritance (1%) are rare in NSHL [3, 4]. The main characteristics of autosomal dominant hearing loss are high genetic and clinical heterogeneity and a delayed onset (postlingual), which are easily overlooked during hearing screening of newborns. At present, 51 different genes and 67 different loci have been linked to autosomal-dominant NSHL [5].

The EYA4 gene, which is located on chromosome 6q22.3-q23.2, was first identified as a causal gene of DFNA10 in a large American family in 1996 [6]. The EYA4 gene encodes eye absent 4 protein and is considered necessary for the proper development of multiple human organs, including the eye, inner ear, and heart.

1 Weixin Zhang and Jing Song contributed equally to this work.
*Correspondence: yuan_yasheng@163.com; yangjuanmei1982@126.com
1 Department of Otolaryngology and Skull Base Surgery, Eye Ear Nose and Throat Hospital, Fudan University, Shanghai 200031, China Full list of author information is available at the end of the article
The EYA4 protein comprises two functional domains that contain a 271-amino-acid highly conserved C-terminal EYA domain (eyaHR) and an N-terminal variable region with a proline-serine-threonine (PST)-rich transactivation domain (eyaVR) [7]. By mediating interactions with the sine oculis family of proteins (Six1–Six6), mammalian EYA proteins function as transcriptional coactivators [8].

Researchers from different countries have found novel variants of the EYA4 gene and deletions of the EYA4 allele in different families with hearing loss based on sequencing analysis [9–14]. Late-onset, progressive, sensorineural hearing loss and age of onset from 6 to 50 years are the common characteristics among the tested families. Mid-frequency hearing is affected first, and all frequencies gradually become affected with increasing age. The degree of hearing loss also ranges from mild to moderate or severe with spontaneous evolution [15].

In recent years, next-generation sequencing (NGS) technology, including both targeted and whole-genome sequencing (WGS), has been considered an efficient and swift method to detect potential variants [16, 17]. This method provides a guiding role in the diagnosis and treatment of hereditary hearing loss [18]. Copy number variations (CNVs) are genomic variants within species that reflect differences in copy numbers, including deletions, duplications, and amplifications of DNA sequences. By using cyto genomics techniques such as comparative genomic hybridization (CGH), SNP arrays, WES and WGS, many novel CNVs associated with NSHL phenotypes have been identified [19–23]. With the development of diagnostic WGS, the accessibility, robustness and accuracy of CNVs throughout the genome have dramatically improved. Compared to genomic microarrays, the investigation of genomic features such as copy number, content and positional information has become more precise through the WGS method and algorithm [24, 25].

In the present study, we present a four-generation Chinese pedigree with autosomal dominant nonsyndromic hearing loss. A novel CNV deletion at 6q23 was identified in the affected individuals by targeted next-generation sequencing (TNGS) and WGS, and this information sheds new light on the pathogenic mechanism of EYA4 variants.

**Patients, materials and methods**

**Family and clinical evaluation.**

A Chinese family (FY-140) classified as of Han origin presented with late-onset, progressive hearing loss. Approval for the study was obtained from the Ethics Committee of the Eye & ENT Hospital, Fudan University for Human Studies. Written informed consent was obtained from the participants or the parents of minors. The assessment of all the individuals was based on audiological methods, including pure-tone audiometry, auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE) and otological examination. Clinical information, such as age of onset, degree of hearing loss, progression of hearing loss, noise exposure and history of using aminoglycosides, was collected from family members if available. Information on deceased family members was obtained from relatives. The proband (II-2) was subjected to a high-resolution CT scan of the temporal bone. All the individuals accepted electrocardiography for the reason that some references indicated that EYA4 gene variants may cause heart disease in patients.

**Targeted genomic capturing and next-generation sequencing**

Genomic DNA was isolated using the TIANamp Blood DNA Midi Kit (TIANGEN Biotech, Beijing China) and fragmented to 150 bp using an ultrasonoscope (Covaris S220, Massachusetts, USA). End repair, adenylation and adapter ligation were performed for library preparation using a standard library construction kit (MyGenostics Inc., Beijing, China). Targeted DNA fragments were captured by a sequence capture array (MyGenostics Inc., Beijing, China). High-throughput sequencing and processing and bioinformatic data analysis were performed using the DNBSEQ-T7 sequencing platform (MGI Tech Co, Shenzhen China). The raw sequence reads were filtered using the BWA MultiVision software package and then aligned to GRCh38/hg38 (University of California Santa Cruz version). SNPs and indels were identified using the GATK Indel Genotyper and ANNOVA software. A CNV analysis was performed using the log2 ratio of the read depth on each exon.

**Whole-genome sequencing**

The genomic DNA samples were fragmented by sonication to a size of 300–500 bp. Sequence analysis was performed using the TruSeq Nano DNAHT Sample Prep Kit (Illumina Inc., Massachusetts, USA) following the manufacturer’s instructions. The total effective data yield of the sample was approximately 430 million reads, and the data showed a coverage of >99.62% at 20X. After the raw sequence reads were mapped to the human genome reference sequence (USSC) (GRCh38/hg38), SpeedSeq software was used. SNPs, indels, CNVs and SVs were captured using GATK HaplotypeCaller, ANNOVAR and SpeedSeq software.

**PCR, gap-PCR and Sanger sequencing**

PCR, gap-PCR and Sanger sequencing were performed to analyze the cosegregation of variants with NSHL in this family. All primers were designed with Primer3 software.
Gap-PCR was designed to detect certain CNVs of the EYA4 gene. The following seven PCR primers were used for analysis of the suspected variants: EYA4 gene (Forward1, 5'-ATGAAGCCCAACACATATTCAAA-3'; Forward2, 5'-TAGTGGCTACAGCCGAGATCA-3'; and Reverse, 5'-AGACTTTGTGATGACGTTTCAATG-3') and CDH23 gene (Forward1, 5'-CACCCAGGAGGTGATCCAGT-3'; Reverse1, 5'-GGAGGAGGAGATCGCTGATGTTGC-3'; Forward2, 5'-CAGTACCTGCTGACAGTC-3'; and Reverse2, AGCAGGCGATAGTGTCATCTAC-3'). The cycling program was as follows: 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 64–0.5 °C per cycle for 40 s, and 72 °C for 1 min; 24 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 1 min; 72 °C for 2 min; and 4 °C for the rest of the time. The standard protocols for Sanger sequencing were performed using an ABI 3730XL Dx Genetic Analyzer (Applied Biosystems) and PolyPhred software to confirm the detected variants in patients (II-2, II-3, II-6 and III-1) and healthy family members (I-2, III-2, III-3, III-5, IV-1 and IV-2).

Results
Clinical evaluation
The pedigree of the family includes 15 members (eight men and seven women) over four generations (Fig. 1A). Five individuals were diagnosed with NSHL based on their medical history and audiological function examination results. The self-reported age of onset of hearing impairment ranged from 26 to 43 years. Assessments of the four affected living members showed mild to severe bilaterally symmetric NSHL across all frequencies, and the disease affected both sexes (Table 1). In addition to hearing loss, the patients had no other clinical symptoms or signs. The temporal bone scans and cardiac examinations of the proband yielded normal results. The initial hearing loss showed an audiogram pattern called a “cookie bite”, which was usually mild and only affected mid-frequencies. Progressive hearing loss expanded to other frequencies at later stages (Fig. 1B). Other audiometric tests’ results in affected individuals including ABR, DPOAE, acoustic impedance and otological examination were also consistent with the diagnosis of NSHL.

Identification of novel CNV by TNGS
Patients II-2, II-3, and III-5 were subjected to targeted NGS of 147 deafness-related genes, and 8 SNPs and 1 CNV were detected. A novel CNV in exons 8–11 of the EYA4 gene and two previously identified variants (c.7630T > G, p. Leu2544Val and c.8257G > A p. Ala2753Thr) in the CDH23 gene were found in all three patients (Fig. 2A, B). The intrafamilial cosegregation of the variants and the hearing loss phenotype were confirmed by long-range PCR and Sanger sequencing in all family members.

We found that both variants of the CDH23 gene (NM_022124), c.7630T > G (p. Leu2544Val) in exon 52 and c.8257G > A (p. Ala2753Thr) in exon 54, were been carried by four patients (II-2, II-3, II-6 and III-1) and five healthy family members (I-2, III-2, III-3, III-5, IV-1 and IV-2) by Sanger sequencing. These four patients and five healthy family members are all heterozygous for both variants of the CDH23 gene. According to the pedigree diagram, the inheritance pattern of the hearing loss in this family was dominant. Both variants of the CDH23 gene did not co-segregate with the phenotype (BS4-ACMG). The genotype for each variant in the pedigree and the Sanger sequencing results of these family members are described in the supplemental material (Additional file 1: Fig. S1, Additional file 2: Fig. S2, Additional file 3: Fig. S3). We believe they are in cis-mutation and not in...
compound heterozygosity in each subject. Although they have been reported in a previous paper that detected both variants in a deafness patient from China, these variants did not appear in the general populations databases [26]. The minor allele frequency (MAF) of p. Leu2544Val variant is 0.000051 (East Asian) in GnomAD, while the p. Ala2753Thr variant is novel (PM2-ACMG). These variants were predicted as benign by silico pathogenicity prediction tools REVEL, MutationTaster, SIFT and Polyphen 2 (BP4-ACMG). According to ACMG standards and guidelines, both variants in the \textit{CDH23} gene are classified as PM2, BP4 and BS4, and the criteria for both alleles will be of uncertain significance (Table 2). In general, \textit{CDH23} gene mutations cause autosomal recessive non-syndromic hearing loss [4, 27]. Based on the ACMG classification and intrafamilial cosegregation analysis of these variants, we inferred that the c.7630T > G and c.8257G > A variants in the \textit{CDH23} gene may not be the cause of the disease in this family. In contrast, a CNV in the \textit{EYA4} gene was found only in the patients, and none of the healthy family members were carriers of this deletion (Fig. 2C). Therefore, this variant in the \textit{EYA4} gene could be considered the cause of the disease. Because patient I-1 died, we were unable to collect a sample of his DNA. We inferred that patients II-2, II-3 and II-6 inherited the heterozygotic variant from their father and that patient III-1 inherited the variant from patient II-2.

### Verification of CNV by WGS

We performed WGS using genomic DNA from patient III-1 to further identify potential variants. We identified on average 3,585,580 SNPs and 732,397 indels in coding regions or introns. Then, Single-nucleotide variants (SNVs) and InDel from WGS data were filtered as follows: (1) variants with MAF below 0.01 in 1000Genomes, ExAC03 Asian population and gnomAD Asian population (2) coding/splicing variants (3) variants that are predicted to be likely pathogenic/pathogenic by any of the following software such as SIFT, POLYPHEn V2, MutationTaster, Cadd, Dann, and dbscSNV, were considered as likely causal variants. CNV analysis was performed using the log2-ratio of read depth on each exon. Priority was given to variants found in deafness genes (annotated as deafness genes in one or more of the following databases: OMIM, HPO, HGMD, InterVar, HPO, MGI, ClinVar, ISCA and MalaCards). There were 64 variants predicted as candidates, including 47 SNVs, 3 indels, and 2 CNVs. With WGS, TNGS, Sanger sequencing, and

| Subjects | Gender | Age (years) | Pure-tone average (dBHL) | Audiogram shape | Degree of hearing loss |
|----------|--------|-------------|--------------------------|-----------------|-----------------------|
|          |        |             | At testing | Left | Right | At onset | Left | Right |        |
| I-1      | Male   | 76          | –          | 82   | 90   | –        | Severe |
| I-2      | Female | 72          | –          | Normal | Normal | –        | Normal hearing |
| II-1     | Male   | 62          | –          | Normal | Normal | –        | Normal hearing |
| II-2     | Female | 57          | 42         | 77   | 98   | Flat     | Moderate–severe |
| II-3     | Male   | 54          | 43         | 83   | 94   | Flat     | Severe |
| II-4     | Female | 51          | –          | Normal | Normal | –        | Normal hearing |
| II-5     | Male   | 53          | –          | Normal | Normal | –        | Normal hearing |
| II-6     | Female | 44          | 37         | 75   | 71.25 | Flat–sloping | Moderate–severe |
| III-1    | Male   | 32          | 26         | 55   | 45   | Cookie–bite | Moderate |
| III-2    | Male   | 30          | –          | Normal | Normal | –        | Normal hearing |
| III-3    | Male   | 27          | –          | Normal | Normal | –        | Normal hearing |
| III-4    | Female | 26          | –          | Normal | Normal | –        | Normal hearing |
| III-5    | Female | 26          | –          | Normal | Normal | –        | Normal hearing |
| IV-1     | Male   | 4           | –          | Normal | Normal | –        | Normal hearing |
| IV-2     | Female | 2           | –          | Normal | Normal | –        | Normal hearing |

Table 1 Summary of the phenotypic information of the family members.

(See figure on next page.)

Fig. 2 Identification of a novel copy number variation in the \textit{EYA4} gene in a Chinese family. a Schematic diagram showing the position of the \textit{EYA4} gene deletion on chromosome 6. The red bar indicates the alignment position of the deletion. b Copy number of each exon calculated from the fluorescence peak ratios identified from the CNV analysis. c Scheme of the normal and inverted alleles. d Gap-PCR product of the distal breakpoint junction showing segregation with the phenotype in the family. e Sanger sequencing of the inverted allele by \textit{EYA4}_Ex7_F and \textit{EYA4}_In11_R covering the two breakpoints and a 10-bp insertion.
Fig. 2 (See legend on previous page.)
Table 2  Identified CDH23 variant and in silico molecular genetic analysis

| Gene  | Transcription accession number | cDNA change | Protein change | Position (GRCh38/hg18) | REVEL | MutationTaster | SIFT | Polyphen 2 | ACMG Classification |
|-------|--------------------------------|-------------|----------------|-------------------------|--------|----------------|------|------------|---------------------|
| CDH23 | NM_022124;exon52               | c.7630T>G   | p.Leu254Val    | chr10:71803045          | Benign | Disease causing | Benign          | Benign | Uncertain (PM2 + BP4 + BS4) |
| CDH23 | NM_022124;exon56               | c.8257G>A   | p.Ala2753Thr   | chr10:71807355          | Benign | Disease causing | Benign          | Benign | Uncertain (PM2 + BP4 + BS4) |

Discussion

CNVs are a common cause of hereditary hearing loss and are thought to play a role in nearly 20% of non-syndromic HL diagnoses [19]. In this study, we performed a comprehensive genetic analysis that included TNGS, WGS, gap-PCR and Sanger sequencing in a four-generation Chinese Han family with autosomal dominant NSHL. All the affected individuals in the family exhibited sensorineural deafness, which primarily affected low and mid-frequencies and had onset ages in the range of 26 to 43 years.

We identified a novel CNV deletion in exons 8–11 of the EYA4 gene with a 10 bp insertion. First, this variant cosegregated with NSHL symptoms in patients and was not detected in normal family members. Then, this CNV is predicted to affect the eyaHR domain. By interacting with members of the SIX and DACH protein families in a conserved network, the highly conserved C-terminal region of EYA4 (eyaHR) regulates embryonic development and follow-up functions after development of the mature organ of Corti. It regulates Na+/K+-ATPases and the development of mechanosensory cells of the inner ear [12]. Finally, we attempted to construct a three-dimensional structure of the CNV using the SWISS-MODEL software but failed because the structure was severely affected. These findings may also suggest that this novel CNV deletion is pathogenic in auditory function.

The hearing loss phenotype in the present family is similar to that reported for patients carrying EYA4 variants, i.e., late-onset, postlingual, progressive, and bilateral HL. In previous reports, flat-type hearing loss was observed in patients with truncating EYA4 variants. At onset, hearing impairment was usually mild and detected at mid-frequencies, resulting in an audiometric profile commonly referred to as a “cookie-bite” pattern. During its progression, hearing loss began to involve other frequencies. The progression rate of hearing loss caused by EYA4 was approximately 5.75 dB/year (95% CI 4.50–7.00 dB/year), which is relatively severe compared to POUL4F3 and MYO6 gene mutation in ADNSHL patients [28, 29].

To date, variants in the EYA4 gene have been associated with HL in more than 50 ethnic groups worldwide. It was believed that EYA4 variants led to syndromic and non-syndromic NSHL, but EYA4 is not a frequently mutated gene in ADNSHL compared with other reported genes. The characteristics of all known EYA4 variants are summarized in Table 3. According to these variants, the severity of hearing loss was not significantly related to the types or locations of variants.

Several CNVs in EYA4 have been linked to deafness (Fig. 3). One CNV disrupts the EYA4 gene andspares only exons 1–3 from the deletion [30]. The deleted sequence of the promoter and the first two exons was previously identified in a Japanese boy [31]. A deletion of four exons and a deletion spanning exons 4–20 have been reported to cause severe ADNSHL in Japanese individuals [9]. In addition, the EYA4 variant reportedly causes dilated cardiomyopathy accompanying NSHL in a single large family. In this family, a 4846-bp genomic deletion...
### Table 3  Summary of all known EYA4 variants and their hearing loss phenotypes

| Variant type | Nucleotide Change | Exon/Intron | Amino Acid Change | Origin | Age at HL onset | HL degree | Audiogram profile | References |
|--------------|------------------|-------------|-------------------|--------|----------------|-----------|-------------------|------------|
| Splicing     | c.84-2A > G      | Intron 3    |                   | Chinese Indian | N/A           | N/A       | N/A               | Chen et al. 2016 [36] Panigrahi et al. [37] |
| Missense     | c.152C > T       | Exon 4      | p.Ser51Phe        | America  | N/A            | N/A       | N/A               | Sloan-Heggen et al. [38] |
| Nonsense     | c.160G > T       | Exon 4      | p.Glu54*          | Spanish  | 42 years       | Mild      | MF/LF             | Morin et al. [13] Shinagawa et al. [9] |
| Frameshift   | c.222_223del     | Exon 5      | p.Val75Phefs*32   | Japanese | 61 years       | Mild to moderate | HF/LF     | Van Beelen et al. [39] |
| Frameshift   | c.464delC        | Exon 8      | p.Pro155Glnfs*43  | Swedish Dutch | N.A Childhood | N.A Moderate | N.A MF/HF      | Neveling et al. [39] |
| Frameshift   | c.498del         | Exon 8      | p.Thr167Leufs*31  | Japanese | 13 years       | Mild       | LF                | Shinagawa et al. [9] |
| Missense     | c.511G > C       | Exon 8      | p.Gly171Arg       | Chinese  | 6–50 years     | Mild to severe | HF/Flat    | Liu [15] Shinagawa et al. [9] |
| Nononsense   | c.517C > T       | Exon 8      | p.Gln173*         | Japanese | 48 years       | Moderate    | Flat              | Morin et al. [13] |
| Missense     | c.543G > G       | Exon 8      | p.Tyr181Ter       | Chinese  | Second to the fourth decade | Severe to profound | Flat | Mi et al. [32] |
| Frameshift   | c.579_580insTACC | Exon 8      | p.Asp194Tyrfs*52  | Swedish | 4–40 years     | Mild to profound | N/A        | Frykholm et al. [41] |
| Frameshift   | c.580+1G > A     | Intron 8    |                   | Japanese | 45 years       | Moderate    | Flat              | Shinagawa et al. [9] |
| Frameshift   | c.614dupA        | Exon 9      | p.Glu205Argfs*40  | Chinese  | 20–40 years    | Moderate to profound | HF/Flat | Huang et al. [42] Morin et al. [13] |
| Frameshift   | c.781del         | Exon 10     | p.Thr261Argfs*34  | Spanish  | 26–44 years    | Mild to moderate | Gently downsloping | Varga et al. [43] |
| Missense     | c.804G > C       | Exon 10     | p.Gln268His       | Slovak   | 10–40 years    | Moderate    | Gently downsloping | |
| Nonsense     | c.863C > A       | Exon 11     | p.Ser288*         | Korean   | Korean          | N.A Moderate to severe | Reverse U-shaped Flat | Baek et al. [44] Kim et al. [10] |
| Missense     | c.866C > T       | Exon 11     | p.Thr289Met       | American | N.A            | N.A        | N.A               | Miszalski-Jamka et al. [45] |
| Frameshift   | c.910del         | Exon 11     | p.Ser305Leufs*15  | Japanese | 30 years       | Severe      | Flat              | Shinagawa et al. [9] |
| Missense     | c.978C > G       | Exon 12     | p.Phe326Leu       | Korean   | N.A            | Moderate    | Down sloping     | Choi et al. [46] |
| Nonsense     | c.988C > T       | Exon 12     | p.Gln330*         | Japanese | 16 years       | Moderate    | Flat              | Shinagawa et al. [9] |
| Frameshift   | c.1026_1027dupAA | Exon 12     | p.Thr343Lysfs*62  | American | N.A            | Moderate to profound | Flat/Gently sloping | Wayne et al. [12] |
| Frameshift   | c.1048_1049dupAA | Exon 12     | p.Arg352Profs*53  | American | N.A            | Moderate to severe | MF/HF     | Makishima et al. [47] |
| Missense     | c.1078C > A      | Exon 12     | p.Pro360Thr       | Spanish  | 44 years       | Mild to moderate | Gently downsloping | Morín et al. [13] |
| Missense     | c.1107G > T      | Exon 12     | p.Glu369Asp       | Spanish  | 10–11 years    | Moderate to severe | Gently downsloping | Morín et al. [13] |
| Missense     | c.1109G > A      | Exon 13     | p.Glu370His       | Philippines | N.A            | N.A        | N.A               | Truong et al. 2019 [48] |
| Missense     | c.1109G > C      | Exon 13     | p.Arg370Pro       | Japanese | 30 years       | Mild to moderate | MF         | Shinagawa et al. [9] |
| Missense     | c.1109G > C      | Exon 13     | p.Val371Met       | Belgium  | N.A            | N.A        | N.A               | Sommen et al. [49] |
| Variant type | Nucleotide Change | Exon/Intron | Amino Acid Change | Origin       | Age at HL on set | HL degree       | Audiogram profile | References |
|--------------|-------------------|-------------|-------------------|--------------|------------------|----------------|-------------------|------------|
| Frameshift   | c.1115_1118dup    | Exon 13     | p.Trp374Cysfs*6   | Hungarian    | N.A              | N.A            | N.A               | Pfister et al. [50] |
| Missense     | c.1122G>T         | Exon 13     | p.Trp374Cys       | Australian   | 10–25 years      | Mild to severe | Gently downsloping | Morin et al. [13] |
| Missense     | c.1154C>T         | Exon 13     | p.Ser385Leu       | Italian      | N.A              | Mild to profound | MF               | Cesca et al. [51]  |
| Nonsense     | c.1177C>T         | Exon 13     | p.Gln393*         | Korean       | 26 years         | Moderate        | HF               | Kim [10] Shinagawa et al. [9] |
| Frameshift   | c.1194del         | Exon 14     | p.Met401Trpfs*3   | Korean       | N.A              | Moderate        | Down sloping     | Choi et al. [46]  |
| Missense     | c.1216G>C         | Exon 14     | p.Gly406Arg       | Japanese     | 5 years          | Moderate        | Flat              | Shinagawa et al. [9] |
| Missense     | c.1223G>A         | Exon 14     | p.Arg408His       | America      | N.A              | N.A            | N.A               | Miszalski-Jamka et al. [45] |
| Missense     | c.1281G>A         | Exon 14     | p.Glu427Glu       | Spanish      | 26 years         | Moderate to profound | Flat            | Morin et al. [13] |
| Splicing     | c.1282-12T>A      | Intron 14   |                   | Australian   | N.A              | Mild to profound | Flat              | Hildebrand et al. [52] |
| Splicing     | c.1282-1G>A       | Intron 14   |                   | Spanish      | 12 years         | Mild to moderate | MF/Flat           | Morin et al. [13] |
| Missense     | c.1301T>A         | Exon 15     | p.Ile434Lys       | Chinese      | 8–38 years       | Mild to severe  | MF/flat           | Tan et al. [53] Vona et al. [54] |
| Splicing     | c.1341-19T>A      | Intron 15   |                   | German       | N.A              | N.A            | N.A               | Morin et al. [13] |
| Nonsense     | c.1601C>G         | Exon 17     | p.Ser534*         | Spanish      | 3–16 years       | Moderate to severe | Flat            | Iwasa et al. [56] Neveling et al. [39] Van Beelen et al. [40] |
| Missense     | c.1643C>G         | Exon 18     | p.Thr548Arg       | Chinese      | 17–40 years      | Mild to profound | Flat              | Sun et al. [11]    |
| Missense     | c.1663G>C         | Exon 18     | p.Ala555Pro       | Japanese     | 25 years         | Moderate        | N.A               | Shinagawa et al. [9] |
| Splicing     | c.1739-1G>A       | Intron 18   |                   | America      | 50 years         | N.A            | N.A               | Cirino et al. [55] Wayne et al. [12] |
| Nonsense     | c.1759C>T         | Exon 19     | p.Arg587*         | Belgian      | 6–40 years       | Mild to moderate | N.A              | Xin et al. [57] Shinagawa et al. [9] |
| Frameshift   | c.1790del         | Exon 19     | p.Val597Glyfs*4   | Japanese     | 35 years         | Moderate        | Flat              | Iwasa et al. [56] Neveling et al. [39] Van Beelen et al. [40] |
| Missense     | c.1810G>T         | Exon 19     | p.Gly604Cys       | Swedish      | N.A              | N.A            | N.A               | Schönberger et al. [34] |
| Missense     | c.1834A>T         | Exon 19     | p.Lys612*         | Chinese      | 27 years         | Moderate        | Gently downsloping | Tan et al. [53] Vona et al. [54] |
| CNV          | Deletion 7689 bp (Ex7 to Ex11) | Exon 20 | p.Trp619Gly       | Chinese      | 25 years         | N.A            | N.A               | Xiao et al. [57] Shinagawa et al. [9] |
| CNV          | Deletion 9.5 Mb (Ex4 to Ex 20) | Exon 19 | p.Arg587*         | Belgian      | 6–40 years       | Mild to moderate | N.A              | Xin et al. [57] Shinagawa et al. [9] |
| CNV          | Deletion 2747 bp (Ex15 to Ex17) | Exon 19 | p.Glu406Arg       | Japanese     | 13 years         | Severe          | LF/HF             | Morin et al. [13] |
| CNV          | Deletion 9 Mb at 6q23.1–24.1 (Ex4–20) | Exon 19 | p.Glu406Arg       | Japanese     | 8 years          | Moderate        | Flat              | Morin et al. [13] |
| CNV          | Deletion 4846 pb incl. intron 9, exon 10 and partial intron 10 c.581_804del (In9, Ex10, part of In10) | N.A | N.A | N.A | N.A | N.A | Schönberger et al. [34] |
that resulted in loss of the EYA domain (eyaHR) and part of the variable region (eyaVR) was detected [32]. A heterozygous deletion of 2747 bp represented a copy variant loss encompassing exon 15 to exon 17 [13]. Additionally, in Japan, a novel hemizygous indel in the EYA4 gene was predicted to be p. (Val124_Pro323del) [14]. We now add a genomic rearrangement consisting of a deletion and a 10-bp insertion to this list (Fig. 3, Table 3).

The EYA4 gene is widely distributed in the inner ear, which includes otic vesicles, the Reissner membrane and the sensory epithelia of the vestibular system and Corti [12, 33]. Although the pathogenic mechanism of ADNSHL associated with EYA4 variants remains to be further investigated, haploinsufficiency is generally thought of as the major mechanism. Many reports indicate that EYA4 participates in important pathways in cardiac tissue. The physiological level of Eya4 phosphatase activity is thought to participate in normal cardiac gene regulation. Instead of most DCM genes that encode structural proteins, EYA4 is a transcriptional coactivator. Large deletions comprising the variable domain are most likely to affect cardiac functions. A patient carrying a de novo 9 MB interstitial deletion that disrupts the gene EYA4 presented a patent ductus arteriosus and aortic insufficiency. A family with a 4846-bp deletion was associated with DCM as well as NSHL. In the other 2 unrelated families, polymorphic loci on chromosome 6q23 to 24 were associated with DCM and NSHL. [30, 34]. For that reason, we performed electrocardiograms for all family members. None of the members exhibited a cardiac phenotype, and electrocardiograms showed no abnormalities. Including our work, many studies indicated that the genotype–phenotype correlation of large deletions in EYA4 and dilated cardiomyopathy was not very obvious [14]. One reason may be that previously reported cardiopathy was caused by other variants in the large deleted regions. Another reason is that the defect in a contiguous gene could account for the cardiac defects [34]. More experiments, including detecting EYA4 levels in nuclear and cytoplasmic components, may provide evidence for this theory. Increasing the yield of genetic testing among patients with both NSHL and DCM may allow for better detection of the EYA4 gene and cardiac pathology [35].

**Table 3** (continued)

| Variant type | Nucleotide Change | Exon/Intron Amino Acid Change | Origin | Age at HL onset | HL degree | Audiogram profile | References |
|--------------|-------------------|--------------------------------|--------|----------------|-----------|--------------------|------------|
| CNV          | Deletion 10.4 Mb promoter and exon 1,2 (Ex1–2) | Japanese 20-month-old | Moderate to severe | MF/flat | Abe et al. [31] |
| CNV          | Deletion 3.7 MB in 6q23.1q23.2 (Ex1–20) | Italian 12 years | N.A | N.A | Gana et al. [58] |
| CNV          | Deletion 12,835 bp (Ex6–10) | Japanese 23 years | Mild to severe | LF/HF/Flat | Ishino et al. [14] |
| CNV          | Deletion 17.4 kb and 10 bp insertion (Ex8–11) | Chinese 26–42 years | Moderate–severe | Flat | This work |

![Fig. 3 Overview of the CNVs identified in this study and those previously identified in EYA4](image-url)
Conclusions
A novel CNV deletion at 6q23 in exons 8–11 of EYA4 in a Chinese ADNSHL family was identified by WGS and Sanger sequencing. The phenotype of the family differed from that of previously reported pedigrees with CNV deletion of EYA4 variants. The phenotypes also differed between individuals with the same variant in the same family. Our results highlight the complexity of the EYA4 genotype and phenotype.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12920-022-01269-x.

Acknowledgements
We thank the patients and their family members for their cooperation in this study.

Author contributions
WZ, JS: original draft preparation, conducting experiments. JY and YY reviewed the manuscript. LG, MM, BT: conceptualization, supervision, methodology, reviewing and editing manuscript. All authors read and approved the final manuscript.

Funding
This project has received funding from the Natural Science Foundation of Shanghai (No 19ZR1408700), the Shanghai 2020 Science and Technology Innovation Action Plan One Belt One Road International Cooperation Project (20410740600) and the Clinical Research Plan of SHDC (SHDC2020CR1049B).

Availability of data and materials
The authors are not able to share the clinical data due to full anonymisation of the data is very difficult. The CNV in our study was called based on the human assembly GRCh38 (https://hgdownload.soe.ucsc.edu/downloads.html#human). This CNV has been submitted to LOVD under accession ID 00361730. All sequencing data used to support the findings of this study are available from the corresponding author upon request.

Declarations
Ethics approval and consent to participate
The study was conducted in accordance with the principles of the Declaration of Helsinki. Approval for the study was obtained from the Ethics Committee of the Eye & ENT Hospital, Fudan University for Human Studies. Written informed consent was obtained from the participants or the parents of minors.

Consent for publication
Not applicable.

Competing interests
None declared.

Author details
1 Department of Otolaryngology and Skull Base Surgery, Eye Ear Nose and Throat Hospital, Fudan University, Shanghai 200031, China. 2 Shanghai Clinical Medical Center of Hearing Medicine, Shanghai 200031, China. 3 Key Laboratory of Hearing Medicine of National Health Commission of the People’s Republic of China, Shanghai 20031, China. 4 Research Institute of Otolaryngology, Fudan University, Shanghai 200031, China. 5 Lateral Skull Base Diagnosis and Treatment Center, Eye Ear Nose and Throat Hospital, Fudan University, Shanghai 200031, China. 6 Department of Otorhinolaryngology Head and Neck Surgery, First Affiliated Hospital of Anhui Medical University, Jixi Road 218, Hefei 230022, Anhui, China. 7 ENT Institute and Department of Otorhinolaryngology, Eye & ENT Hospital, Fudan University, 83 Fenyang Road, Xuhui District, Shanghai, China.

Received: 8 October 2021 Accepted: 10 May 2022
Published online: 16 May 2022

References
1. World Health Organization. Global costs of unaddressed hearing loss and cost-effectiveness of interventions. Geneva: World Health Organization (WHO), 2018.
2. WHO. Deafness and hearing loss https://www.who.int/health-topics/hearing-loss#tab=tab_2. 20 Sept 2021.
3. Vona B, Nanda I, Hofrichter MAH, Shehata-Dieler W, Haaf T. Non-syndromic hearing loss gene identification: a brief history and glimpse into the future. Mol Cell Probes. 2015;29(5):260–70.
4. Stelma F, Bhutta MF. Non-syndromic hereditary sensorineural hearing loss: review of the genes involved. J Laryngol Otol. 2014;128(1):13–21.
5. Van Camp G, Smith RIH. Hereditary Hearing Loss Homepage. https://hereditaryhearingloss.org. 30 Aug 2021.
6. O’Neill ME, Marietta J, Nishimura D, Wayne S, Van Camp G, Van Laer L, Negrini C, Wilcox ER, Chen A, Fukushima K, et al. A gene for autosomal late-onset progressive non-syndromic hearing loss, DFNA10, maps to chromosome 6. Hum Mol Genet. 1996;5(8):853–6.
7. Borsani G, DeGrandi A, Ballabio A, Bullfome A, Bernard L, Banfi S, Gattuso C, Mariani M, Dixon M, Donnai D, et al. EYA4, a novel vertebrate gene related to Drosophila eyes absent. Hum Mol Genet. 1999;8(1):11–23.
8. Kito H, Komada S, Tago K, Tominaga S, Ozaki H, Sato S, Kawakami K. Cooperation of six and eya in activation of their target genes through nuclear translocation of eya. Mol Cell Biol. 1999;19(10):6815–24.
9. Shinagawa J, Moteki H, Nishio S-Y, Ohyama K, Otsuki K, Iwasaki S, Masuda M, Oshikawa C, Ohta Y, Arai Y, et al. Prevalence and clinical features of hearing loss caused by EYA4 variants. Sci Rep. 2020;10(1):3662.
10. Kim Y-R, Kim M-A, Sagong B, Bae SH, Lee H-J, Kim H-J, Choi JY, Lee K-Y, Kim U-K. Evaluation of the contribution of the EYA4 and GRHL2 genes in Korean patients with autosomal dominant non-syndromic hearing loss. PLoS ONE. 2015;10(3):e0119493.
11. Sun Y, Zhang Z, Cheng J, Lu Y, Yang C-L, Luo YY, Yang G, Yang H, Zhu L, Zhou J, et al. A novel mutation of EYA4 in a large Chinese family with autosomal dominant middle-frequency sensorineural hearing loss by targeted exome sequencing. J Hum Genet. 2015;60(6):299–304.
12. Wayne S, Robertson NG, DeClau F, Chen N, Verhoeven K, Prasad S, Tranelbarg J, Morton CC, Ryan AF, Van Camp G, et al. Mutations in the transcriptional activator EYA4 cause late-onset deafness at the DFNA10 locus. Hum Mol Genet. 2001;10(3):195–200.
13. Morin M, Borreguero L, Booth KT, Lachgar M, Huygen P, Willamar M, Mayo F, Barrio LC, Santos Serrão de Castro L, Morales C, et al. Insights into the pathophysiology of DFNA10 hearing loss associated with novel EYA4 variants. Sci Rep. 2020;10(1):6213.
14. Ishino T, Ogawa Y, Sonoyama T, Taruya T, Kono H, Hamamoto T, Ueda M, Ohto H, Kamada S, et al. Prevalence and clinical features of hearing loss caused by EYA4 variants. Sci Rep. 2020;10(1):3662.
15. Dong X, Deng L, Wang X, Zhao L, Yang J, Zhang H, Shi Q, et al. Novel genetic variation of EYA4 causing autosomal dominant non-syndromic hearing loss. PLoS ONE. 2018;13(9):e020602.
16. Hu S, Sun F, Zhang J, Tang Y, Qiu J, Wang Z, Zhang L. Genetic etiology study of ten Chinese families with nonsyndromic hearing loss. Neural Plast. 2018;2018:4902980.
17. Sang S, Ling J, Liu X, Mei L, Cai X, Li T, Li W, Li M, Wen J, Liu X, et al. Proband whole-exome sequencing identified genes responsible for autosomal recessive nonsyndromic hearing loss in 33 Chinese nuclear families. Front Genet. 2019;10:639.
27. Miyagawa M, Nishio SY, Usami S-I. Prevalence and clinical features of hearing loss in Asian Indians. J Genet. 2021;100:1–4.

28. Sloan-Heggen CM, Bierer AO, Shearer AE, Kolbe DL, Nishimura CJ, Frees KL, Ephraim SS, Shibata SB, Booth KT, Campbell CA, et al. Comprehensive genetic testing in the clinical evaluation of 1119 patients with hearing loss. Hum Genet. 2016;135(4):441–50.

29. Neveling K, Feenstra J, Gilissen C, Hoefstoot LH, Kamsteeg EJ, Mensen-kamp AR, Rodenburg RJT, Nteta MG, Spruit J, Vermeer S, et al. A post-hoc comparison of the utility of sanger sequencing and exome sequencing for the diagnosis of heterogeneous diseases. Hum Mutat. 2013;34(12):1721–6.

30. van Beelen E, Oonk AMW, Leijendieckers JM, Hoefstoot EHH, Penning RJF, Feenstra J, Deiker H-J, Huygen PLM, Snak AFM, Kremer H, et al. Audiometric characteristics of a Dutch DFNA10 family with mid-frequency hearing impairment. Ear Hear. 2016;37(1):1103–11.

31. Frykholm C, Klar J, Arnesson H, Rehnman A-C, Lodahl M, Wedén U, Dahl N, Tranberg J, Rendtorff ND. Phenotypic variability in a seven-generation Swedish family segregating autosomal dominant hearing impairment due to a novel EYA4 frameshift mutation. Gene. 2015;563(1):10–6.

32. Zhang et al. BMC Medical Genomics. 2022;15:113.

33. Nishio S-Y, Hattori M, Moteki H, Tsukada K, Miyagawa M, Naito T, Abe S, Ozaki A, Moteoti R, et al. Mid-frequency hearing loss is characterized by clinical features of OTOA-associated hearing loss. Genes (Basel). 2019;10(9):715.

34. Kremer H. Hereditary hearing loss; about the known and the unknown. Zentbl Gehor. 2013;14(2):125–38.

35. Rev Genet. 2014;6(5):37.

36. Chen S, Dong C, Wang Q, Zhong Z, Qi Y, Ke X, Liu Y. Targeted next-generation sequencing successfully detects causative genes in Chinese patients with hereditary hearing loss. Genet Test Mol Biomark. 2016;20(11):606–5.

37. Panigrahi I, Kumari D, Anil Kumar BN. Single gene variants causing deafness in Asian Indians. J Genet. 2021;100:1–4.
56. Iwasa Y-I, Nishio S-Y, Usami S-I. Comprehensive genetic analysis of Japanese autosomal dominant sensorineural hearing loss patients. PLoS ONE. 2016;11(12): e0166781.
57. Xiao S-Y, Qu J, Zhang Q, Ao T, Zhang J, Zhang R-H. Identification of a novel missense eya4 mutation causing autosomal dominant non-syndromic hearing loss in a Chinese family. Cell Mol Biol (Noisy-le-grand). 2019;65(3):84–8.
58. Gana S, Valetto A, Toschi B, Sardelli I, Cappelli S, Peroni D, Bertini V. Familial interstitial 6q23.2 deletion including Eya4 associated with otofaciocervical syndrome. Front Genet. 2019;10:650.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.