


ORIGINAL ARTICLE

Identification of two novel mutations in POU4F3 gene associated with autosomal dominant hearing loss in Chinese families

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Abstract

Autosomal dominant non-syndromic hearing loss is genetically heterogeneous with 47 genes identified to date, including *POU4F3*. In this study, by using a next-generation sequencing panel targeting 127 deafness genes, we identified a pathogenic frameshift mutation c.704_705del and a missense mutation c.593G>A in two three-generation Chinese families with late-onset progressive ADNSHL, respectively. The novel mutations of *POU4F3* co-segregated with the deafness phenotype in these two families. c.704_705del caused a frameshift p.T235fs and c.593G>A caused an amino acid substitution of p.R198H. Both mutations led to an abnormal and incomplete protein structure. *POU4F3* with either of the two mutations was transiently transfected into HEI-OC1 and HEK 293 cell lines and immunofluorescence assay was performed to investigate the subcellular localization of mutated protein. The results indicated that both c.704_705del (p.T235fs) and c.593G>A (p.R198H) could impair the nuclear localization function of *POU4F3*. The p.R198H *POU4F3* protein was detected as a weak band of the correct molecular weight, indicating that the stability of p.R198H *POU4F3* differed from that of the wild-type protein. While, the p.T235fs *POU4F3* protein was expressed with a smaller molecular weight, implying this mutation result in a frameshift and premature termination of the *POU4F3* protein. In summary, we report two novel mutations of *POU4F3* associated with progressive ADNSHL and explored their effects on *POU4F3* nuclear localization. These findings expanded the mutation spectrum of *POU4F3* and provided new knowledge for the pathogenesis of *POU4F3* in hearing loss.

KEYWORDS

autosomal dominant, Chinese family, hearing loss, identification, novel mutation, *POU4F3*

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1 | INTRODUCTION

Hearing loss is the most frequent sensory impairment in humans, with a morbidity of 1/1000 in newborns.¹ Based on available evidence, approximately 60% of all cases are because of genetic factors. According to clinical characteristics, hearing loss can be divided into syndrome hearing loss and non-syndrome hearing loss (NSHL). Non-syndrome hearing loss accounts for about 70% of all hereditary cases.² The hereditary modes of NSHL include autosomal recessive, autosomal dominant and X chromosome-linked or mitochondrial inheritance, the incidences of which are, respectively, 80%, 20% and <1%.³ For autosomal dominant non-syndrome hearing loss (ADNSHL), only 72 loci and 47 corresponding genes have been identified as causative factors (<http://hereditaryhearingloss.org/>). The genetic aetiology of most patients with ADNSHL cannot be diagnosed. Furthermore, because of the high genetic heterogeneity of NSHL, using conventional Sanger sequencing to identify variants causing ARNSHL is extremely time-consuming and expensive. In contrast, next-generation sequencing (NGS), characterized by high throughput and low cost, has made it possible to identify nearly all mutations and genes involved in the onset and development of hearing loss.

POU4F3, characterized by two DNA-binding POU domains, was expressed exclusively in cochlear, vestibular hair cells and sensory.^{4,5} Function studies have demonstrated that *POU4F3* was required for the differentiation and maturation of hair cells and establishment of ear neural network, but not in fate determination of inner ear hair cells.⁶ For example, hair cells experienced a rapid, progressive apoptosis in *POU4F3* knockout mice during the late gestation and early postnatal period, which further reduced innervation and loss of sensory neurons and caused severe hearing loss and balance impairment.⁷ Other studies show that *POU4F3* is involved in the regulation of downstream targets, such as BDNF, NT-3, Lhx3, Gfi1 et al, which play important roles in inner ear development.⁸⁻¹¹ *POU4F3* might be a candidate for hereditary hearing impairment because of its important role in hair cells.

Clinicians have made great efforts for the identification of pathogenic mutations for hearing loss. *POU4F3* is the earliest discovered deafness-related gene to cause ADNSHL DFNA15. In 1998, Vahava et al first identified an 8-base pair deletion in *POU4F3* leading to progressive hearing loss.¹² Until now, more than 30 mutations of *POU4F3* were found to be associated with the pathogenesis of DNFA15, most of which occurred in DNA-binding domains. According to previous studies, *POU4F3*-related DFNA15 manifested as late-onset bilateral, progressive hearing loss with down-sloping audiometric configurations.¹³⁻¹⁵ For example, in a five-generation Dutch family linked with p.L289F on *POU4F3*, the progression rate for DNFA15 was 0.8-1.4 dB/year.¹⁶

Here, we performed mutation analysis on two Chinese families with ADNSHL by using NGS and Sanger sequencing. A missense mutation c.593G>A (p.R198H) and a frameshift mutation c.704_705del (p.T235fs) were identified as the causative factor for DNFA15 with a late-onset progressive symptom. Furthermore, we also investigated

the effects of these two mutations on the microscopic structure and subcellular location of *POU4F3* in cells.

2 | MATERIAL AND METHODS

2.1 | Clinical features

Two three-generation Chinese families, respectively, named as F052* and F493*, have been recruited through Shandong Provincial ENT Hospital Affiliated to Shandong University. The pedigrees of the families with autosomal dominant pattern of inheritance are shown in Figure 1A. Due to patients' privacy or other reasons, there were only six members (II-2, II-4, III-1, III-2 with hearing loss and II-3, II-5 with normal hearing) in F052* and 5 members (II-1, II-2, II-4, III-1 with hearing loss and III-2 with normal hearing) in F493* participating in our study (Figure 1).

The clinical examinations were performed in the Otologic Center, Shandong Provincial ENT Hospital Affiliated to Shandong University. After physical and otoscopic examinations, pure tone audiometry (PTA) was performed. Other diseases or ototoxic medication history that may cause hearing loss were ruled out. The study was approved by the ethics committee of the Shandong Provincial ENT Hospital and written informed consents were obtained.

2.2 | NGS of deafness gene

For mutation analysis, genomic DNA of the probands was extracted from peripheral blood using DNA extraction kit (Axygen) and subjected to a targeted NGS including 127 deafness-related genes (see Table S1). Data were analysed in accordance with NGS standard process. Sequence alignment was performed by using BWA 0.6.2-r126 software. UCSC hg19 Feb.2009 was used as reference genome. Mutation identification was performed by using GATK. dbSNP (snp137) as a reference. The pathogenicity of novel mutations was predicted by using 1000 genome database (phase I), HapMap database (combined data from phases II and III) and own databases as references. Guideline of American College of Medical Genetics and Genomics was used as the reference of data interpretation.¹⁷

2.3 | Mutation detection by Sanger sequencing on genomic DNA

Sanger sequencing of *POU4F3* was performed using those primers: forward 5'-CACCATCTGCAGGTTTCGAGT-3' and reverse 5'-CGAAATAG GCCTCGAGTGAAC-3' for c.704_705del mutation; forward 5'-CACAGATCCATCCACACCAC-3' and reverse 5'-CTT GCTGTTCTCTCTCGGTAG-3' for c.593G>A mutation. Lasergene-Seq Man software was used for data analysis. *POU4F3* protein sequence (NP_002691.1) and gene (NM_002700) were used as references for sequence alignment.

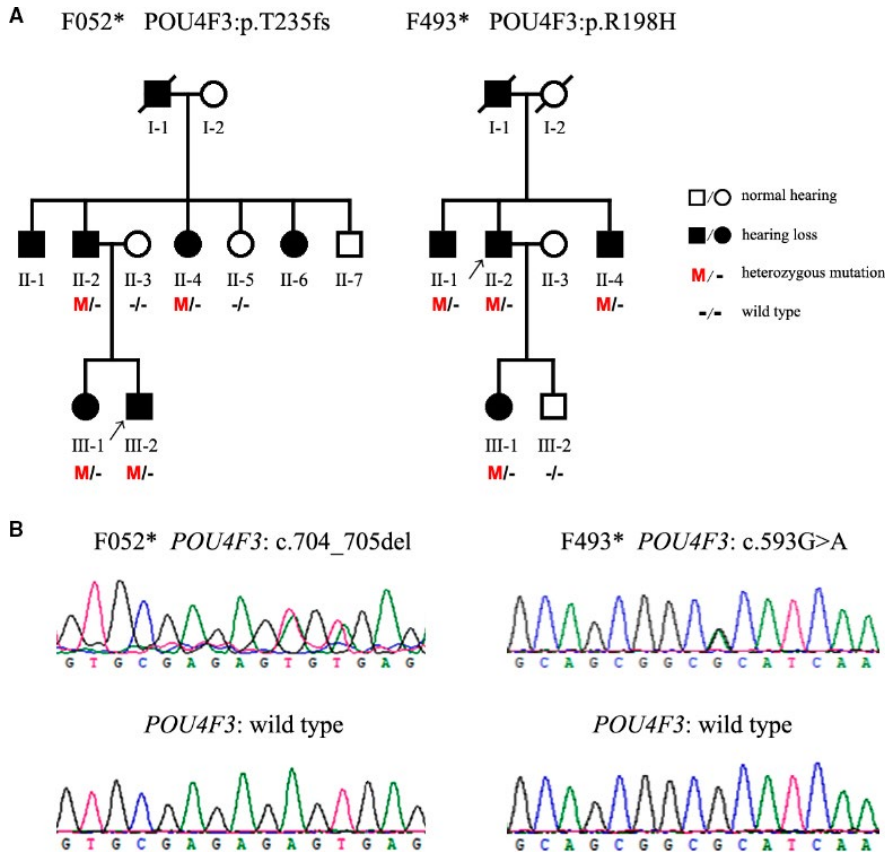


FIGURE 1 *POU4F3* mutations identified in the two Chinese families suffering from autosomal dominant hearing loss. A, Pedigrees of the Chinese families. Black squares and circles represent members with symptoms of DFNA15. The proband is indicated by an arrow. M and – indicate the mutant and wild-type alleles, respectively. Asterisks indicate the families with *POU4F3* mutations identified in the present study. B, Sanger sequencing showing the c.704_705del (p.T235fs) and the c.593G>A (p.R198H) mutations in *POU4F3*

2.4 | Bioinformatics analysis

The mutations detected by targeted NGS was predicted for potential pathogenicity on Mutation Taster (<http://www.mutationtaster.org/>).¹⁸ The molecular structure of wild-type (NP_002691.1, 338 amino acids) and mutant *POU4F3* was modelled using I-TASSER (<http://zhanglab.ccmb.med.umich.edu/>). Protein visualization were performed using Swiss-PdbViewer 4.1 software because of data from homology models.

2.5 | Cell culture

The culture condition was as follow: MEM medium (Gibco) containing 10% FBS (Gibco), 5% CO₂ at 37°C. Dr Federico Kalinec from University of California, Los Angeles friendly provided HEI OC1 auditory cells. The cells were maintained in DMEM medium with 10% FBS in 10% CO₂ at 33°C.¹⁹

2.6 | Plasmid construction

The plasmids loading *POU4F3* cDNA was obtained from Cusabio Biotech (Wuhan, China). The cDNA sequencing was confirmed by using Sanger sequencing (accessionnumber: BC112207). These *POU4F3* cDNA-loaded plasmids were then used to amplify wild-type *POU4F3* by PCR reaction. The PCR Primers for *POU4F3*

cDNA region were as follows: 5'-ATGCAGGATCCATGATGGCC ATGAACTCCAAGCAGCCTTTCG-3' and 5'-ACGCAGAATTCG TGGACAGCCGAATACTTCA-3'. The PCR products were digested by *Bam*H I and *Eco*R I and cloned into pCMV-Tag 2B plasmids (Agilent Technologies). To construct plasmids containing mutated *POU4F3*, the Quik Change site-directed mutagenesis kit (Stratagene) was used to introduce the mutations (c.704_705del or c.593G>A) into the wild-type vector.

2.7 | Transient transfection and immunofluorescence analysis

HEI OC1 and HEK 293 cells were plated into 24 well plates. When cells were cultured to 80% confluence, the wild-type or mutated *POU4F3* plasmids were transfected into both cell lines using Lipofectamine 3000 transfection reagent (Invitrogen). After transfection for 72 hours, cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilized in PBS containing 0.3% Triton X-100 for 10 minutes and then blocked in PBS containing 10% donkey serum for 1 hour at 37°C in a humid atmosphere.

The cells were then incubated with primary anti-FLAG antibody (Ca# F1804, Sigma, 1:800) for about 13 hours at 4°C. Sequentially, the secondary goat antimouse antibody (Sigma) and DAPI were incubated with cells with a dilution rate of 1:1000. Immunofluorescence imaging was performed by using a confocal microscope (TSC SPE, Leica).

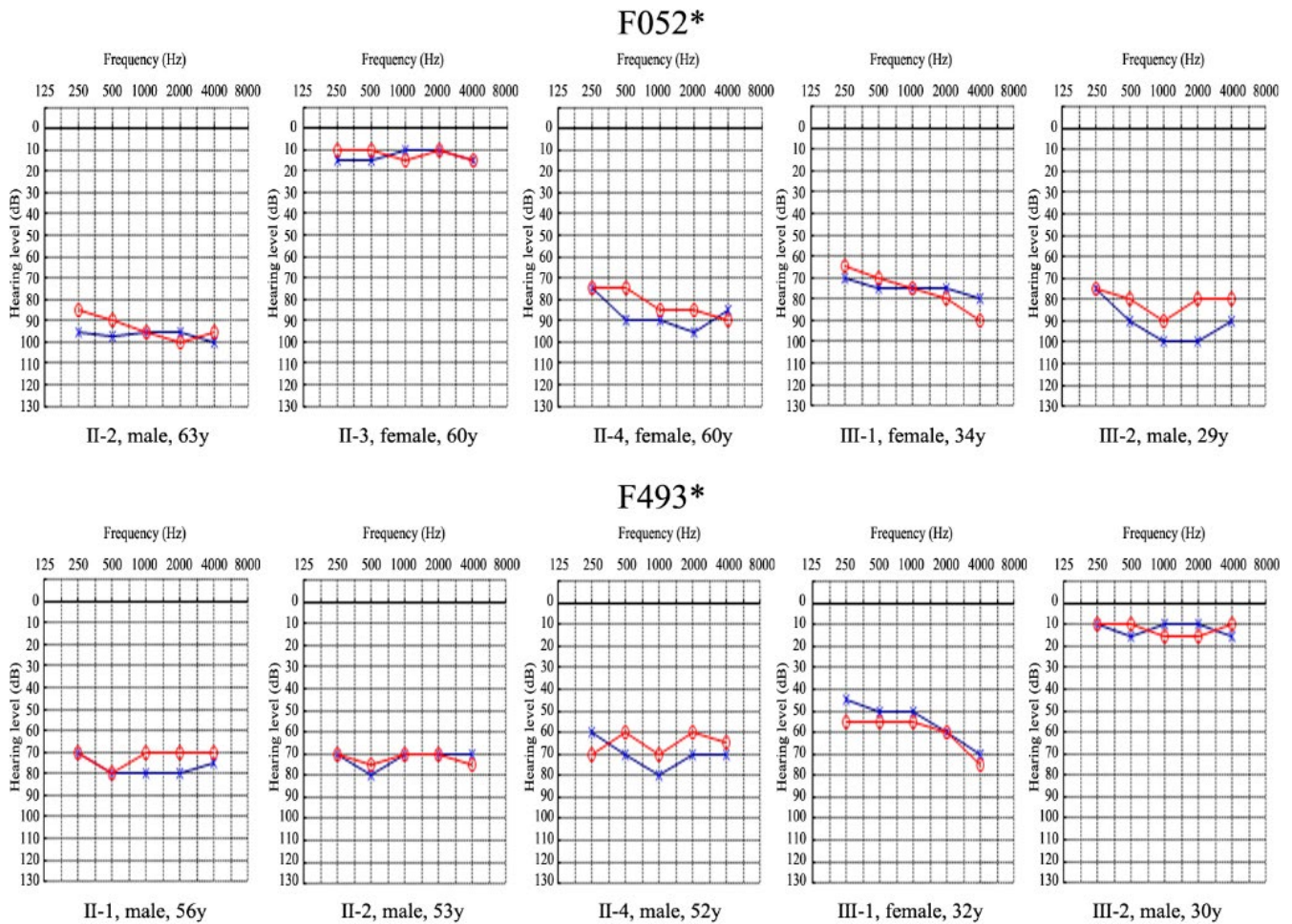


FIGURE 2 Audiograms of some members participating in this study in the two Chinese families. Blue crosses and red circles represent the air conduction hearing threshold levels of left and right ears, respectively. Asterisks indicate the families with *POU4F3* mutations identified in this study. Gender and age are shown below the audiogram of each individual

TABLE 1 Summary of clinical data for members in hearing loss families

| Patient | Gender | Age at test (y) | Age at onset | Use of aminoglycoside | PTA (dB) right ear | PTA (dB) left ear | Level of hearing impairment |
|-------------|--------|-----------------|--------------|-----------------------|--------------------|-------------------|-----------------------------|
| F052* II-2 | Male | 63 | 20 | No | 96.5 | 95 | Profound |
| F052* II-3 | Female | 60 | — | No | 12.5 | 12.5 | Normal |
| F052* II-4 | Female | 60 | 30 | No | 90 | 83.75 | Profound |
| F052* III-1 | Female | 34 | 15 | No | 78.75 | 76.25 | Severe |
| F052* III-2 | Male | 29 | 10 | No | 95 | 82.5 | Profound |
| F493* II-1 | Male | 56 | 17 | No | 78.75 | 72.5 | Severe |
| F493* II-2 | Male | 53 | 20 | No | 72.5 | 72.5 | Severe |
| F493* II-4 | Male | 52 | 12 | No | 72.5 | 63.75 | Severe |
| F493* III-1 | Female | 32 | 17 | No | 61.25 | 57.5 | Moderate |
| F493* III-2 | Male | 30 | — | No | 12.5 | 12.5 | Normal |

2.8 | Western blot

Total cell lysates extracted from the wild-type or mutated *POU4F3* plasmids transfected HEK 293 cells were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and examined by

Western blot with mouse monoclonal anti-Flag antibodies (ZSGB-Bio) and mouse monoclonal anti- β -actin antibodies (ZSGB-Bio), and then followed by antimouse IgG conjugated horseradish peroxidase (ZSGB-Bio). The signals were detected using the enhanced chemiluminescence system.

TABLE 2 Characteristics of POU4F3 variant, analysis of predicted protein structure and disease-causing effects

| Gene | Exon | Domain | Variation | | | Status | Polyphen-2 | Mutation Taster | SIFT | ExAC |
|--------|------|--------|-------------------------|-------------------------|------------|--------|------------|-----------------|------|-------|
| | | | Nucleotide ^a | Amino acid ^a | Type | | | | | |
| POU4F3 | 2 | POU | c.593G>A | p.R198H | Missense | Heter | 1 | 0.999848 | 0 | Novel |
| POU4F3 | 2 | POU | c.704_705del | p.235_235del | Frameshift | Heter | 0.842 | 0.999824 | 0 | Novel |

Abbreviations: c, variation at cDNA level; ExAC, Exome Aggregation Consortium; Heter, heterozygote; p, variation at protein level; POU4F3, POU class 4 homeobox 3 (NM_002700).

^aAll nucleotide and amino acid are abbreviated according to the International Union of Pure and Applied Chemistry (IUPAC).

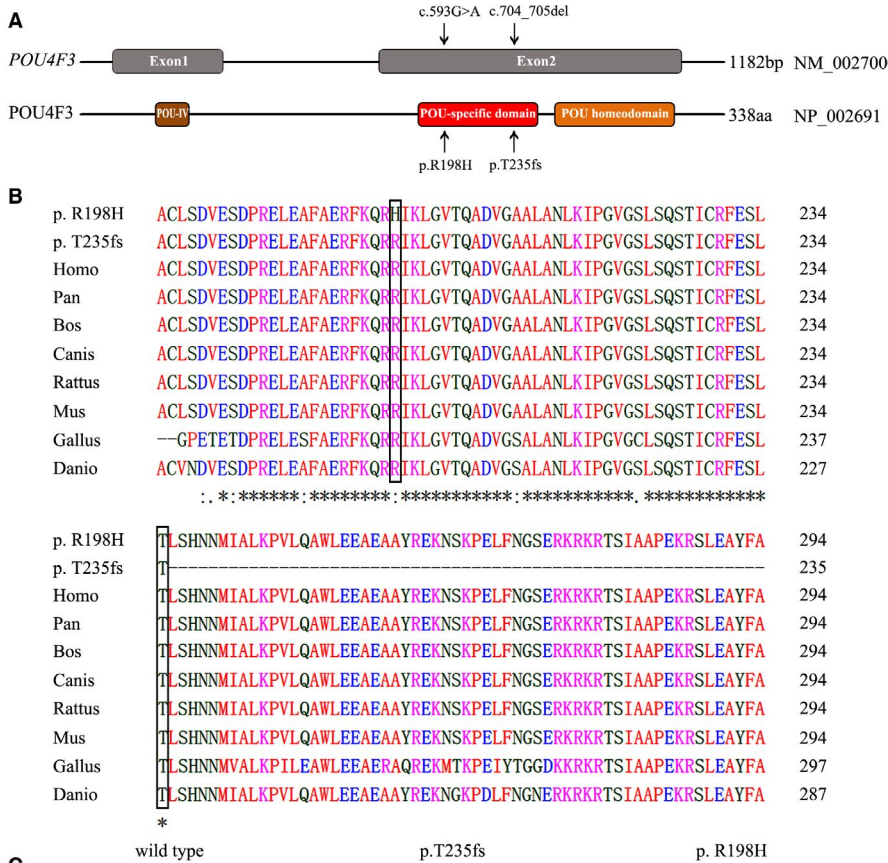
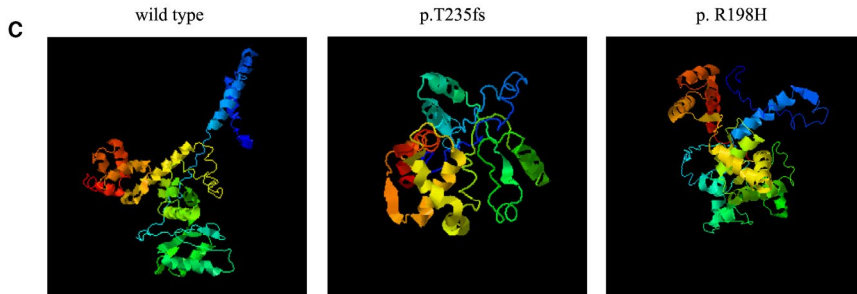


FIGURE 3 Genomic structure, conservation and 3D molecular model of POU4F3 mutations. A, Genomic structure of POU4F3 based on the open reading frame (NM_002700) containing two exons (grey rectangles). The positions of the c.704_705del (p.T235fs) and the c.593G>A (p.R198H) mutations are arrowed both at the gene (top) and the protein level (bottom). B, Protein alignment showing POU4F3 p.R198H occurred at evolutionarily conserved amino acids (in black box) across eight species. The mutation of c.704_705del (p.T235fs) caused a truncated protein. C, Three-dimensional molecular models revealed the abnormal incomplete structure of mutant-type protein



3 | RESULTS

3.1 | Clinical features

Two three-generation Chinese families with ADNSHL were enrolled in this study. The pedigree and disease statements of all participants were shown in Figure 1A. Totally, there were seven members in F052* family and five members in F493* family suffered from similar

hereditary progressive hearing loss, with 1 in F052* and 1 in F493* passed way at the time of investigation. As a result of patients' privacy and other special circumstances, we only got six members in F052* family and five members in F493* family to performed gene sequencing.

According to the audiograms in Figure 2, all the affected individuals in two families, ageing from 29 years to 63 years, had severe hearing loss with a relatively 'flat' audiometric profile affecting all frequencies.

Based on self-description of patients with c.704_705del in F052* and c.593G>A in F493*, the hearing loss associated with these two mutations was typically post-lingual, late onset and progressive (Table 1). For example, the proband III-2 in F052* recalled that the onset age of bilateral NSHL was 10 and symptoms became worse after then.

3.2 | Identification of pathogenic *POU4F3* mutations in two Chinese ADNSHL families by targeted NGS

Here, we aimed to screen possible pathogenic mutations in probands of another two Chinese ADNSHL families (F052*-III-2 and F493*-II-2, Figure 1A) by using targeted NGS of 127 deafness-related genes. As shown in Figure 1A, two novel mutations on *POU4F3*, c.704_705del (p.T235fs) and c.593G>A (p.R198H), were, respectively, identified as potential pathogenic variants in F052*-III-2 and F493*-II-2. These deleterious and pathogenic aspects of those two mutations are listed in Table 2.

Next, we used Sanger sequencing to confirm the two *POU4F3* variants in all the participating members. The results showed that c.704_705del and c.593G>A were the only *POU4F3* variants co-separating with hear loss symptoms in F052* and F493*, respectively (Figure 1A,B). By searching online, we confirmed that c.704_705del and c.593G>A on *POU4F3* were not reported so far. Furthermore, we also used Sanger sequencing investigate these two mutations in 200 control individuals and none of them had identical mutations.

We analysed the effects of screened mutations of *POU4F3* on its protein structure. As shown in Figure 3A, both c.704_705del and c.593G>A occurred on exon 2 of *POU4F3*. The resulted amino acids variations p.T235fs and p.R198H were located on the evolutionarily conserved POU-specific domain (Figure 3A).

The mutation of c.704_705del (p.T235fs) is a non-sense mutation, which leads to a truncated protein. While, the missense mutation c.593G>A was predicted to lead to arginine to histidine amino acid substitution at codon 198. The mutation of c.704_705del (p.T235fs) caused a truncated protein. The alignment of *POU4F3* from different species of the patients, *Homo*, *Pan*, *Bos*, *Canis*, *Rattus*, *Mus*, *Gallus* and *Danio* was shown in Figure 3B.

The three-dimensional structures of wild-type and mutant-type *POU4F3* were simulated according to the crystal structure. As shown in Figure 3C, compared with the wild-type structure, p.T235fs protein exhibited an abnormal truncated structure, resulted from the c.704_705del frameshift mutation. While, in p.R198H protein, the c.593G>A missense variant was predicted to perturb protein structure because of the substitution of arginine by histidine acid. In this respect, our prediction study revealed that these two novel mutations possibly lead to protein dysfunction.

3.3 | Variations of p.T235fs and p.R198H alter the subcellular localization of *POU4F3*

Accumulated evidence demonstrated that *POU4F3* functions as an important transcription factor for the functions of hair cells.^{13,20}

So the subcellular localization of *POU4F3* in nuclei is necessary for its function, because *POU4F3* needs to combine to target DNA sequence. To investigate whether the pathogenicity of *POU4F3* mutations was mediated by the alteration of protein subcellular localization, we overexpressed wild-type and mutant-type *POU4F3* by using pCMV-Tag2B plasmids in cell lines of HEK 293 and HEI OC1, an immortalized model of Corti-derived epithelial cell line.

As a FLAG-tag was fused to the N-terminal of *POU4F3* protein, the subcellular localization of *POU4F3* was detected by using anti-FLAG immunofluorescence analysis. As shown in Figure 4A, wild-type *POU4F3* protein was exclusively located in the cell nuclei in both cell lines, while mutant-type *POU4F3* exhibited a strong fluorescence signal in cytoplasm and a much weaker in nuclei. These results suggested that variations of p.R198H and p.T235fs caused a retreat of *POU4F3* from nuclei, which might explain the pathogenicity of screened mutations.

3.4 | Mutations of p.T235fs and p.R198H affect *POU4F3* protein expression

In order to detect the expression of mutant *POU4F3* protein, the wild-type *POU4F3* plasmid and two mutant plasmids were

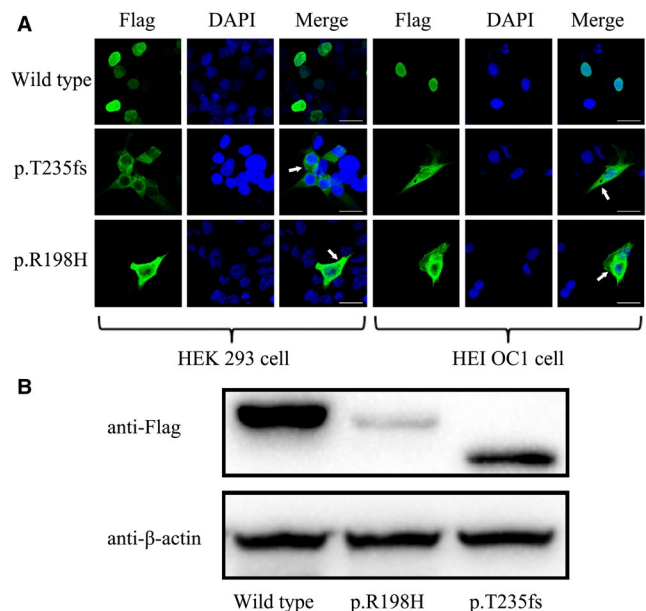


FIGURE 4 The *POU4F3* c.704_705del and c.593G>A mutations altered the subcellular localization and protein expression of transcription factor *POU4F3*. A, Immunofluorescence staining was performed after transient transfection in HEK 293 cells and HEI OC1 cells. Images display DAPI in blue, Flag-tagged protein in green, and merged pictures. Both in HEK 293 cells and HEI OC1 cells, mutant protein located mostly in cytoplasm while the wild-type protein was exclusively located in nuclei. The white arrows indicate *POU4F3* outside the nuclei. Scale bars = 30 μm. B, The expression of *POU4F3* protein and its mutants in transfected HEK 293 cells were detected by immunoblotting using anti-Flag and anti-β-actin (serving as an internal control) antibodies

TABLE 3 POU4F3 gene mutations found in patients with DFNB15

| Nucleotide change | Protein change | Exon | Domain | Ethnicity | Age of HL diagnosis | Severity of HL | Progression of HL | Audiometric configuration | Reference |
|--------------------------|----------------|------|--------------|-------------|---------------------|----------------------|-------------------|---------------------------|------------|
| Whole deletion of POU4F3 | | | | Brazil | 11-13 y | Moderate to profound | Yes | Flat and HF | 29 |
| c.74dupA | p.His25fs*18 | 1 | | Japan | 20's | Profound | Yes | HF | 21 |
| c.120+1G>T | | 1 | | China | 0-40 y | Moderate to profound | Yes | Flat | 31 |
| c.191A>T | p.Asp64Val | 2 | | Japan | 10-30 y | Moderate to profound | Yes | HF | 21 |
| c.337C>T | p.Gln113Ter | 2 | | China | 14-40 y | Moderate to severe | Yes | Flat and HF | 24 |
| c.367delA | p.Ile123fs*3 | 2 | | Japan | 40's | Moderate | Yes | MF | 21 |
| c.427C> | p.Gln143Ter | 2 | | Japan | 3 y | Moderate | NA | MF | 21 |
| c.491C>G | p.Pro164Arg | 2 | POU | China | NA | Mild to profound | NA | Flat and HF | 14 |
| c.574G>T | p.Glu192Ter | 2 | POU | Japan | 17-30's | Moderate | Yes | MF | 21 |
| c.581T>A | p.Phe194Tyr | 2 | POU | Japan | 10-20 y | Moderate | Yes | MF and HF | 21 |
| c.593G>A | p.Arg198His | 2 | POU | China | 28-56 y | Severe | Yes | Flat | This study |
| c.602T>C | p.Leu201Pro | 2 | POU | China | 10's | Mild to profound | Yes | MF | 25 |
| c.602delT | p.Leu201fs | 2 | POU | China | 16-30 y | Mild to profound | Yes | HF | 32 |
| c.603_604delGG | p.Val203fs_ | 2 | POU | China | NA | NA | NA | NA | 33 |
| c.662_675del14 | p.Gly221fs | 2 | POU | Korea | 20 y | Severe | NA | HF | 22 |
| c.665C>T | p.Ser222Leu | 2 | POU | Japan | 6 y | Moderate | Yes | HF | 21 |
| c.668T>C | p.Leu223Pro | 2 | POU | Netherlands | 4-44 y | Mild to severe | Yes | Flat, MF and HF | 13 |
| c.680delC | p.Thr227fs*13 | 2 | POU | Japan | Infant | Moderate to severe | Yes | MF and HF | 21 |
| c.694G>A | p.Glu232Lys | 2 | POU | Korea | 20's | Moderate to severe | NA | HF | 34 |
| c.704_705del | p.Thr235fs | 2 | POU | China | 27-63 y | Profound | Yes | Flat | This study |
| c.718A>T | p.Asn240Tyr | 2 | POU | Japan | 6 y | Moderate | Yes | MF | 21 |
| c.841A>G | p.Ile281Val | 2 | POU Homeobox | Japan | 50-54 y | Moderate | Yes | HF | 21 |
| c.865C>T | p.Leu289Phe | 2 | POU Homeobox | Netherlands | 13-20 y | Mild to profound | Yes | Flat, MF and HF | 13 |
| c.884_891del8 | p.Ile295fs | 2 | POU Homeobox | Israel | 18-30 y | Moderate to severe | Yes | HF | 12 |
| c.896C>T | p.Pro299Leu | 2 | POU Homeobox | Japan | 26-27 y | Moderate to profound | Yes | Flat, MF and HF | 21 |
| c.932T>C | p.Leu311Pro | 2 | POU Homeobox | China | 10-20 y | Moderate to profound | Yes | HF | 31 |
| c.976A>T | p.Arg326Ter | 2 | POU Homeobox | Japan | Childhood | Moderate | Yes | HF | 21 |
| c.977G>A | p.Arg326Lys | 2 | POU Homeobox | Korea | 10-50's | Mild to moderate | Yes | Flat and HF | 15 |
| c.982A>G | p.Lys328Glu | 2 | POU Homeobox | Taiwan | 10's | Profound | Yes | HF | 23 |
| c.1007delC | p.Ala336fs | 2 | POU Homeobox | Japan | 0 y | Moderate to severe | Yes | NA | 30 |

transfected into HEK 293 cells, respectively. Western blot analysis of lysates abstracted from transfected HEK 293 cells was performed by immunoblotting using anti-Flag and anti- β -actin antibodies. The result (Figure 4B) showed that the wild-type and the p.R198H POU4F3 proteins were expressed as a single protein of the correct molecular weight, meaning that the staining observed in the immunocytochemical analysis is specifically derived from the tagged POU4F3 proteins. While a weak band of p.R198H POU4F3 protein was detected, indicating that the stability of p.R198H POU4F3 differed from that of the wild-type protein. In addition, the p.T235fs POU4F3 protein was detected with a smaller molecular weight, implying this mutation result in a frameshift and premature termination of the POU4F3 protein.

4 | DISCUSSION

POU4 family of transcription factors, with three members of POU4F1 ~ 3, are well-known regulators in the development of sensory system. POU4F1 and POU4F2 are mainly expressed in retinal ganglion cells and involved in maintaining visual nervous system.¹² In contrast, POU4F3 is uniquely expressed in the hair cells.^{4,5} These three members all contain two common domains, POU homeodomain and POU-specific domain, which were responsible for their DNA binding activity. POU4F3 is involved in the proliferation and differentiation of hair cells by regulating the expression of neurotrophin, stress response proteins and so on.⁸⁻¹¹ In mice, targeted deletion of POU4F3 led to the elimination of hair cells and severe hearing loss.⁷ Correspondingly, mutations of POU4F3 also caused human late onset, progressive hearing loss (DFNA15) in multiple families.²¹⁻²⁴

Here, we identified c.704_705del and c.593G>A mutations of POU4F3 as pathogenic causes of ADNSHL in F052* and F493* families, respectively. Both families manifested later onset, progressive hearing loss with an autosomal dominant pattern. The mutation c.704_705del caused a frameshift mutation, which altered the amino acid sequence after T235 and c.593G>A was a missense mutation causing p.R198H. Both mutations were located on the POU-specific domain of POU4F3 and molecular model indicated that POU4F3 with either of them presented an abnormal structure. So mutations of c.704_705del and c.593G>A might disrupt the transcriptional activity of POT4F3 and further induced apoptosis of hair cells and hearing loss.

Since mutation of POU4F3 was first discovered as a pathogenic cause for hearing loss in 1998,¹² scientist and clinicians have identified 28 variants in POU4F3 (Table 3) and a complete loss of POU4F3 to be associated with ADNSHL. The affected individuals show a high degree of heterogeneity, including onset ages, disease levels and ethnicities. Epidemiological investigation have indicated that POU4F3 mutations were one of the most common cause of ADNSHL in Japanese (15/602) and Chinese (8/13) families.²⁵ In addition, as the severity of POU4F3 protein structural damage increases, patients' onset ages become earlier and disease progression slower.²¹

It is worth noting that most pathogenic mutations of POU4F3 are located on POU-domains, responsible for DNA-binding function of POU4F3.

Previous studies have demonstrated mutations that disrupted the nuclear localization sequence (NLS) resulted a defected trafficking of POU4F3 into cell nucleus, where it exerted transcriptional activity to regulate gene expression.^{13,24} POU4F3 contained two NLSs, including a monopartite (amino acids 274-278) and a bipartite (amino acids 314-331). Yin-Hung Lin et al reported that a missense mutation of p.Lys328Glu in bipartite NLS of POU4F3 could compromise transportation of POU4F3 from the cytoplasm to the nucleus.²³ Previously, we also found that c.337C>T (p. Gln113*) resulted into a truncated protein lacking the bipartite NLS and caused location of POU4F3 in cytoplasm.²⁴ In this study, we investigated the effects of diagnosed mutations of POU4F3 on its subcellular localization. In vitro experiments indicated that POU4F3 with either c.704_705del (p.T235fs) or c.593G>A (p.R198H) was mostly located in cytoplasm, while wild-type protein were immobilized in the nucleus. So we speculated that these two mutations (a frameshift and a missense mutation) disrupted the sequence or structure of POU4F3 NLS domain.

Although the mechanism of deafness caused by POU4F3 mutations is not well studied, the results of several studies point to haploinsufficiency. Our study strongly indicated that both missense mutations p.R198H as well as the frameshift mutation p.T235fs affect proper functioning of the POU4F3 protein. In terms of molecular biology, dominant inheritance involved three mechanisms: haploinsufficiency, function-gaining and dominant-negative effect.²⁶ As a dominant-negative effect have been ruled out, haploinsufficiency is the most likely mechanism so far.^{13,27} Although mice with Brn3c^{-/-} behaved healthily, several studies in humans supported the haploinsufficiency mode.²⁸ Freitas, E. L. et al reported that a complete deletion of POU4F3 caused ADNSHL.²⁹ Mutai, H. et al reported a frameshift variant of POU4F3 in a Japanese family, which would produce an extended mRNA because of the absence of in-frame stop codon.³⁰ The overlong mRNAs might be degraded in cells. In other words, these two mutations make POU4F3 a haplotype, which could only be explained by haploinsufficiency. Furthermore, the altered protein localization of POU4F3 in this study also supports haploinsufficiency mechanism.

In summary, by using a targeted NGS-base genetic test, we identified two novel variants of POU4F3, c.704_705del (p.T235fs) and c.593G>A (p.R198H), in two Chinese families with ADNSHL, respectively. Function analysis suggested that these two mutations caused an abnormal, incomplete protein structure and impaired nuclear localization of POU4F3. This study provided new knowledge of POU4F3 mutation spectrum associated with hearing loss and helps to identify the pathogenic mechanism of POU4F.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

XB and LX were in charge of the idea, project design and concept of the study; XB performed the in vivo experiments; FZ, YX and YJ performed DNA extraction, PCR amplification, sequencing and data analysis; HW and LX recruited the clinical patients and were in charge of the clinical assessments; XB wrote the manuscript; QZ and LX revised the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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