



## OPEN Oligogenic effect is associated with the clinical heterogeneity of autosomal dominant deafness-15

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Autosomal dominant deafness-15 which is caused by mutation in the *POU4F3* gene, has been reported with a wide degree of clinical heterogeneity, even between intrafamilial members. However, the reason is still elusive. In this study, A four-generation Chinese family with 11 patients manifesting late-onset progressive non-syndromic hearing loss was recruited. The phenotype of hearing loss in this family showed a large variability in terms of onset age and progression speed. A novel mutation (c.706 C>T, p.L236F) was identified by the whole exome sequencing, and its pathogenicity was confirmed by altering the subcellular localization of *POU4F3*. In addition, we found that two individuals with earlier age of onset and more rapid progression of hearing loss carry additional pathogenic variants in other deafness genes (III-7, *STRC*:c.4057 C>T; IV-1, *GJB2*:c.109G>A; *CDC14A*:c.935G>A). By using the real time quantitative PCR, western blot, luciferase assays and electrophoretic mobility-shift assay, *POU4F3* was proved to directly regulate the expression of *STRC*, *GJB2* and *CDC14A* respectively. ChIP-seq further revealed that *POU4F3* can also bind to a series of deafness genes. In summary we expanded the mutation spectrum of *POU4F3* by identifying a novel mutation and its pathogenicity. Meanwhile, three genes *STRC*, *GJB2* and *CDC14A* were validated as *POU4F3* new targets, implicating that the variants in the three genes may play a role of genetic modifier to generate a synergistic and enhancement effect on the progression of DFNA15.

**Keywords** Autosomal dominant deafness, *POU4F3*, Novel mutation, Oligogenic effect, Clinical heterogeneity

Autosomal dominant deafness-15 (DFNA15, OMIM #602459) is a form of progressive non-syndromic sensorineural hearing loss with postlingual onset between the second and sixth decades of life, which is caused by heterozygous mutation in the *POU4F3* gene<sup>1</sup>. It is one of the most common forms of autosomal dominant non-syndromic hearing loss (ADNSHL) in Chinese Hans population<sup>2,3</sup>. Lots of studies have revealed that audiometric profiles among the DFNA15 patients, even from one family with the same mutation show a large variability in terms of onset age, level of progression, and shape of the audiogram<sup>4,5</sup>, however, the mechanisms underlying this phenomenon remains unclear.

*POU4F3* has two exons and encodes a protein of 338 amino acids. It is a member of the POU family of transcription factors, which comprises two conserved DNA-binding domains (the POU-specific domain and the POU homo domain), and exclusively distributes in the nucleus. *POU4F3* mainly expresses in cochlear, playing an important role in the development of hair cells in the inner ear sensory epithelia<sup>6</sup>. Haploinsufficiency of *POU4F3* is the main underlying cause of DFNA15 deafness<sup>7</sup>.

More and more clinical and genetic evidence shows that even for classically monogenic disorders, the mutation of a single gene is not enough to explain all of the biological phenomena<sup>8</sup>. In recent years, with the progress of phenotype-genotype research, some hereditary disorders have been proved to be related to oligogenic or polygenic inheritance, such as inheritable cardiac disorders<sup>9</sup> and hypercholesterolemia<sup>10</sup>. In oligogenic disorders, a variant in one gene is sufficient to produce the phenotype, but an additional variant in a second gene like genetic modifiers (usually related to the same pathway/organ system) impacts the disease phenotype or

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alters the age of onset<sup>8,11</sup>. Although several syndromic deafness types are caused by the co-existence of variants in two different genes, which defined as a digenic inheritance, such as Usher syndrome<sup>12</sup>, the oligogenic or polygenic effect in deafness is still elusive.

In this study, we identified a novel mutation of *POU4F3* from a four-generation Chinese family with late-onset progressive non-syndromic hearing loss. Moreover, to explore the causes of clinical heterogeneity of deafness in this family, we proved that *STRC*, *GJB2* and *CDC14A* were the target genes of transcription factors *POU4F3*. Mutations of *STRC*, *GJB2* and *CDC14A* may impact the disease phenotype and alters the age of onset of DFNA15. This provides a new insight into the clinical heterogeneity of DFNA15 deafness.

## Materials and methods

### Subjects

A four-generation Chinese family named ADNSHL have been recruited through Hunan Jiahui Genetics Hospital. All the patients in this family represented bilateral late-onset progressive hearing loss. There were sixteen members (II-2, II-4, II-8, III-2, III-3, III-5, III-7, III-8, IV-1 with hearing loss and II-7, II-9, IV-2, IV-3, IV-4, IV-5, IV-6 with normal hearing) in this study. And 1 ml of peripheral blood was taken from all members for genetic examination. Only seven members (II-8, II-9, III-2, III-7, III-8, IV-1, IV-2) got detailed hearing information. Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of Center for Medical Genetics, Central South University, Hunan, China. We confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

### Whole exome sequencing and sanger sequencing

All the subjects' genomic DNA was extracted from peripheral blood samples using the Quickgene DNA Whole Blood Kit L (FUJIFILM, Tokyo, Japan) according to standard extraction methods. And 1 ml peripheral blood of each patient (III-2, III-7, IV-1) was performed the whole exome sequencing (WES, Berry Genomics Inc., Beijing, China). Sanger sequencing was performed in all the subjects in our study. The primers were designed using Primer Premier 5 and used to amplify the mutations filtrated from the WES data by polymerase chain reaction. Sequencing reactions were performed by Tsingke (Tsingke Biotechnology Co., Ltd). Data was analyzed using Lasergene-SeqMan software. GRCh38/hg38 assembly was used as the reference sequences. Guideline and standard of American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP)<sup>13</sup>, recommendations for ACMG/AMP mutation criterion<sup>14</sup> and the expert specification of the ACMG/AMP mutation interpretation guidelines for genetic hearing loss<sup>15</sup> were used as the reference of data interpretation.

### Transient transfection and immunofluorescence analysis

The wild type and mutant sequences of *POU4F3* cDNA and HA-tag were synthesized and cloned into the pcDNA3.1(+) vector respectively by Sangon (Sangon Biotech Co., Ltd. Shanghai, China). 293T cell lines obtained from the Center for Medical Genetics & Hunan Key Laboratory of Medical Genetics, School of Life Sciences, Central South University (Changsha, China). 293T cells were inoculated in 24-well plates with  $5 \times 10^4$  cells per well, when cells were cultured to 60% confluence, the wild type or mutated *POU4F3* vectors were transfected into 293T cell lines using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, Shanghai, China). After 48 h, cells were fixed in 4% paraformaldehyde for 30 min, permeabilized in PBS containing 0.5% Triton X-100 for 20 min and then blocked in 5%BSA for 30 min. The cells were then incubated with primary anti-HA antibody for 12 h at 4°C, secondary Dylight 488 Rabbit Anti-Goat antibody for 60 min and DAPI for 10 min. The immunofluorescence test was performed using TCS SP5 laser confocal microscopy (Leica).

### Real time fluorescence quantitative polymerase chain reaction (RT-qPCR)

Three small interfering RNAs (siRNA) were synthesized by GenePharma (Suzhou Genepharma Co.,Ltd, Suzhou, China). The siRNA, wild-type, mutated *POU4F3* vectors and empty vector were transfected into 293T cell. Total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA, USA) from 293T cells after 48 h transfection. RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, CA, USA). RT- qPCR was performed with an ASA-9600 Real-Time PCR System (Suzhou Baiyuan Gene Technology Co.,Ltd, Suzhou, China) using SYBR Premix Ex Taq (Thermo Fisher, CA, USA) with primers against *POU4F3*, *STRC*, *GJB2*, *CDC14A* and *GAPDH* (supplement Table 1). Three replicate wells were tested for each measurement experiment, and more than three replicate experiments were completed at different time periods.

### Western blot

293T cells were lysed in the mixture containing SDS buffer and protease inhibitor PMSF (Beyotime Biotechnology Co.,Ltd, Shanghai, China ) after 48 h transfection and the harvested protein was quantitated using the BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis with 8% acrylamide, then the transferred and blocked protein was incubated with rabbit polyclonal antibody to *STRC* (Biorbyt Ltd., Cambridge, UK), mouse monoclonal antibody to *GJB2* (Invitrogen, Carlsbad, CA, USA), rabbit polyclonal antibody to *CDC14A* (Invitrogen, Carlsbad, CA, USA) and rabbit polyclonal antibody to Vinculin(GeneTex, Irvine, CA, USA ) at 1:1000 dilution, followed by peroxidase-conjugated affinipure goat anti-mouse/rabbit IgG (Jackson ImmunoResearch, USA) at a 1:10000 dilution. Enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, USA) was used and then scanned to visualize the specific protein band. Three replicate wells were tested for each measurement experiment, and more than three replicate experiments were completed at different time periods.

### Dual luciferase reporter gene assay

The 2.8 kb upstream regulatory sequences of *STRC* (*STRC*-P2.8, chr15: 43618801–43621600), *GJB2* (*GJB2*-P2.8, chr13:20192939–20195738) and *CDC14A* (*CDC14A*-P2.8, chr1:100349706–100352505) were synthesized and cloned into the dual luciferase reporter gene vector pDuaLuc-Basic (GenScript (Nanjing) Co.Ltd., Nanjing, China) as promoter vectors. The promoter vectors *STRC*-P2.8, *GJB2*-P2.8 and *CDC14A*-P2.8 were co-transfected with wild type, mutated *POU4F3* vectors and empty vector into 293T cell, after 48 h, Cells were harvested and assayed using the Dual Luciferase Reporter Gene Assay Kit (Beyotime Biotechnology Co.,Ltd, Shanghai, China). Three replicate wells were tested for each measurement experiment, and more than three replicate experiments were completed at different time periods.

### Electrophoretic mobility shift assay (EMSA)

Six possible *POU4F3*-binding sites of the *STRC*, *GJB2* and *CDC14A* upstream regulatory sequence were predicted by using the JASPAR CORE (<http://jaspar.genereg.net/>). The sequences were extended by 10 bases at the upstream and downstream of the binding sites, and then labeled by biotin at the 5' end and synthesized by Sangon (Sangon Biotech Co., Ltd. Shanghai, China) as EMSA detection probes (supplement Table 1). The nuclear protein and cytoplasmic protein from the 293T cell which were transfected with wild type, mutated *POU4F3* vectors and empty vector were extracted by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology Co.,Ltd, Shanghai, China). The assay reactions of the detection probes and proteins were detected by Chemiluminescent EMSA Kit (Beyotime Biotechnology Co.,Ltd, Shanghai, China). Three replicate wells were tested for each measurement experiment, and more than three replicate experiments were completed at different time periods.

### Chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis

293T cells were lysed after 48 h transfected with wild type *POU4F3* vector. DNA were broken into 100–500 bp fragments by ultrasound. The DNA fragments that bind to the protein were pulled down by the HA antibody. After washing and purification, these fragments were sequenced by Novogene (Novogene Co., Ltd., Beijing, China). The interactions of *POU4F3* and genes identified from ChIP-seq data were analysed by the search tool for the retrieval of interacting genes/proteins (STRING, <http://string-db.org/>).

### Data statistics

The data differentiation analysis were conducted using GraphPad Prism software. significance was determined using Tukey's Multiple Comparison Test and Paired t test.

## Results

### Clinical features

The pedigree and disease statements of all individuals were shown in Fig. 1. There were thirty-one members in this family. Sixteen members (II-2, II-4, II-8, III-2, III-3, III-5, III-7, III-8, IV-1 with hearing loss and II-7, II-9, IV-2, IV-3, IV-4, IV-5, IV-6 with normal hearing) were recruited to perform WES or sanger sequencing. Based on the results of audiometric examination and description of proband (III-7), the onset age among the affected individuals in this family ranges from 8 to 31years. However, unlike other relative patients, III-7 and IV-1 manifested earlier age of onset and faster progression of hearing loss. The age of onset of proband (III-7) was 18 years old and developed moderately severe hearing loss at 28 years old. IV-1 had a much earlier age of onset (8 years old) and developed moderately severe hearing loss at the age of 12 (Table S2).

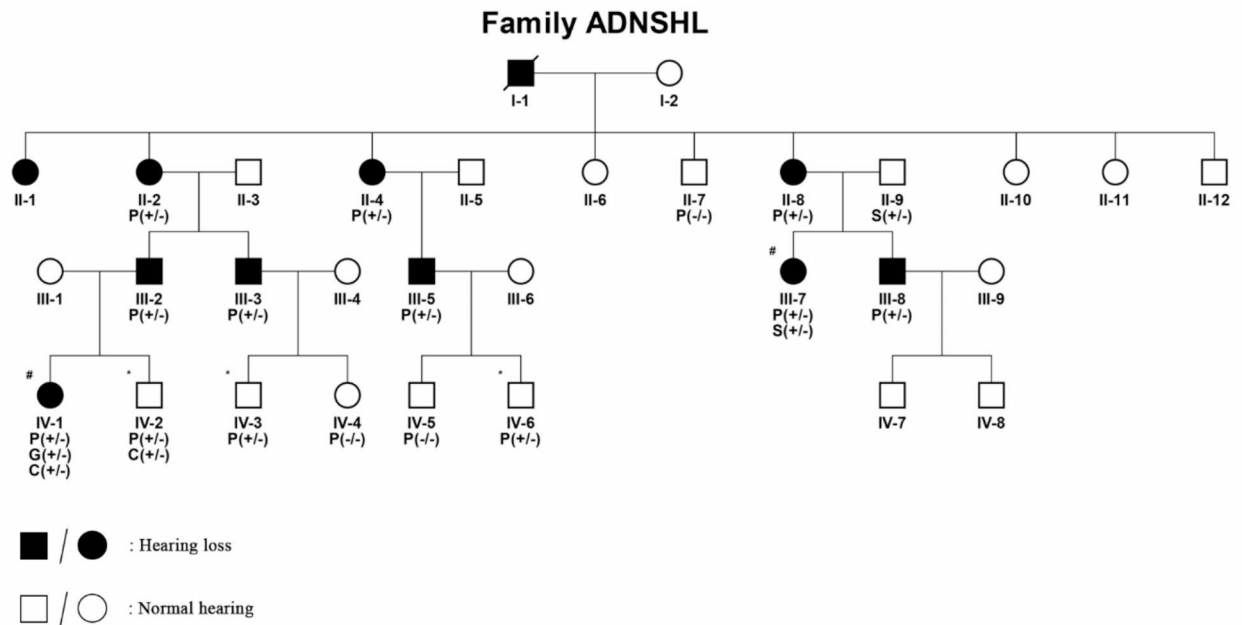
### A novel pathogenic variant in *POU4F3* accounts for the familial hearing loss

To uncover the genetic cause for the hearing loss in the family, WES was conducted based on the proband. After the bioinformatics analysis for the data, a novel heterozygous missense mutation in *POU4F3* gene (NM\_002700.3: c.706 C>T, p.L236F) was identified in the proband, which was inherited from her affected mother. The variant (c.706 C>T) was then tested in all members of this family by Sanger sequencing. The results showed that the variant was co-segregated among the patients (the affected individuals including II-2, II-4, II-8, III-2, III-3, III-5, III-7, III-8 and IV-1 all carried the mutation), while the unaffected members II-7, IV-4 and IV-5 were wild type (Fig. 1; Fig. S1). It should be noted that although the individuals IV-2, IV-3 and IV-6 also carried the variant, they did not present hearing loss, which may be because they were too young to reach the age of onset.

Given that *POU4F3* is a transcription factor and mainly locate in nucleus, we further performed functional tests for the subcellular localization of the mutant proteins to confirm the pathogenicity of the variant (c.706 C>T). Expression cassette encoding either the wild type or the mutant *POU4F3* protein was constructed to fuse with an HA-tag C-terminally. After transient transfection into the 293T cell lines, the subcellular localization of *POU4F3* was detected by using anti-HA immunofluorescence analysis. The results showed that almost all of the expression of wild-type *POU4F3* is located in the nucleus, while the mutants mostly distributed in the cytoplasm (Fig. 2). This indicates that the variant has altered the subcellular localization of the *POU4F3* protein, thus resulted in great loss of its transcriptional function. Together with the functional change, co-segregation results, the low frequency in population and potential damaging effect predicted by various softwares, the variant c.706 C>T in *POU4F3* can be classified as likely pathogenic, according to the standards and guidelines of ACMG/AMP (Table S3, Fig. S1), which would be the primary genetic cause for this familial hearing loss.

### Identification of additional pathogenic variants in *STRC*, *GJB2* and *CDC14A* in two patients

Considering a much faster progression of hearing loss and an earlier age of onset in III-7 and IV-1, we speculated that additional modifiers may contribute to such a high clinical variability other than the c.706 C>T in *POU4F3*. By analyzing the WES data of III-7 and IV-1, an extra novel heterozygous variant of *STRC* gene



**Fig. 1.** The pedigree and segregation pattern of the ADNSHL family. P(+/-): The heterozygous mutation of *POU4F3*. P(-/-): Wild type of *POU4F3*. S(+/-): The heterozygous mutation of *STRC*. G(+/-): The heterozygous mutation of *GJB2*. C(+/-): The heterozygous mutation of *CDC14A*. #Individuals (III-7, IV-1) with a faster progression of hearing loss and an earlier age of onset in this family. \*The individuals who carried the mutation of *POU4F3* but have not yet experienced hearing loss.

(NM\_153700.2: c.4057 C>T, p.Q1353\*) was identified in III-7, which is inherited from her unaffected father. In IV-1, two additional heterozygous variants (*GJB2*: NM\_004004.6: c.109G>A, p.V37I; *CDC14A*: NM\_003672.4: c.935G>A, p.R312Q) were detected, while her affected father (III-2) did not carry either of the variants. The variants *STRC*: c.4057 C>T and *CDC14A*: c.935G>A have not been reported in previous studies. According to the standards and guidelines of ACMG/AMP, the variants of *STRC* (c.4057 C>T), *GJB2* (c.109G>A) and *CDC14A* (c.935G>A) can be classified as pathogenic, pathogenic<sup>16</sup> and likely pathogenic respectively (Table S3, Fig. S1). The bi-allelic mutations in the *STRC*, *GJB2* and *CDC14A* can cause autosomal recessive deafness 16, 1 A and 15 respectively<sup>17,18</sup>.

### **POU4F3 regulates *STRC*, *GJB2* and *CDC14A* expression**

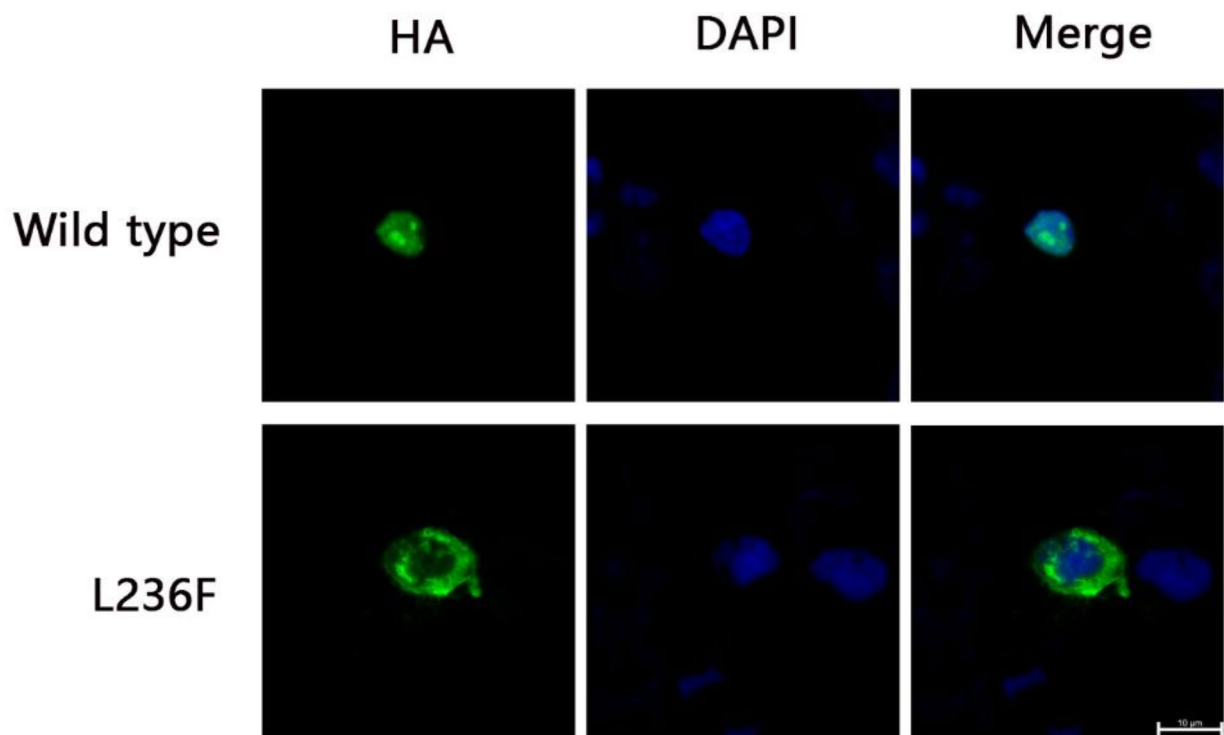
To investigate whether the *POU4F3* could regulate the *STRC*, *GJB2* and *CDC14A* expression, we firstly performed transient transfection assays to overexpress wild type and mutated *POU4F3* (L236F). By using the RT-qPCR, the results revealed that compared with the blank vector group, overexpression of wild type *POU4F3* in 293T cell significantly increased the mRNA transcription of endogenous *STRC* (1.83 fold,  $p < 0.001$ ), *GJB2* (1.41 fold,  $p < 0.001$ ) and *CDC14A* (1.31 fold,  $p < 0.05$ ), while overexpression of the mutant *POU4F3* did not change the mRNA levels of three genes (Fig. 3A).

In addition, the RNA interference was implemented to knock down the endogenous *POU4F3* and the sequence of siRNA2 demonstrated the highest interference efficiency (Fig.S2). After knock-down of the *POU4F3*, the mRNA transcription of *STRC*, *GJB2* and *CDC14A* was accordingly reduced to 63%, 79% and 80% of untreated 293T cells respectively (all  $p < 0.05$ ) (Fig. 3B). These results illustrated a positive correlation between the *POU4F3* amount and the transcription of *STRC*, *GJB2* and *CDC14A*.

Protein expression of endogenous *STRC*, *GJB2* and *CDC14A* were also tested by Western blot under the overexpression or knock down of *POU4F3*, but only the *CDC14A* could be detected and exhibited a similar tendency as the mRNA change (Fig. 3C and D). *STRC*, *GJB2* proteins failed to present may due to their low endogenous expression in 293T cells.

### **POU4F3 activated the promoters of *STRC*, *GJB2* and *CDC14A***

In order to verify whether the regulatory effect of *POU4f3* is related to the *STRC*, *GJB2* and *CDC14A* promoter, about 2.8 kb promoter regions flanked upstream of the coding sequence of each gene were cloned into dual luciferase plasmid. Each luciferase vector was co-transfected with wild-type, mutated *POU4F3* expression vectors and empty vector into 293T cell separately. The results suggested that wild type *POU4F3* significantly increased the promoter vectors' luciferase activity of *STRC* (2.45 fold,  $P < 0.01$ ), *GJB2* (2.40 fold,  $P < 0.001$ ) and *CDC14A* (2.18 fold,  $P < 0.001$ ). The mutant *POU4F3* did not change the luciferase activity under the promoter



**Fig. 2.** The variation L236F compromised transportation of POU4F3 from the cytoplasm to the nucleus. Immunofluorescence staining was performed after transient transfection in 293 T cells. Images display HA-tagged protein in green, DAPI in blue and merged pictures. Mutant protein located mostly in cytoplasm while the wild type protein was exclusively located in nucleus. Scale bars = 10  $\mu$ m. This experiment is repeated three times or more in biology.

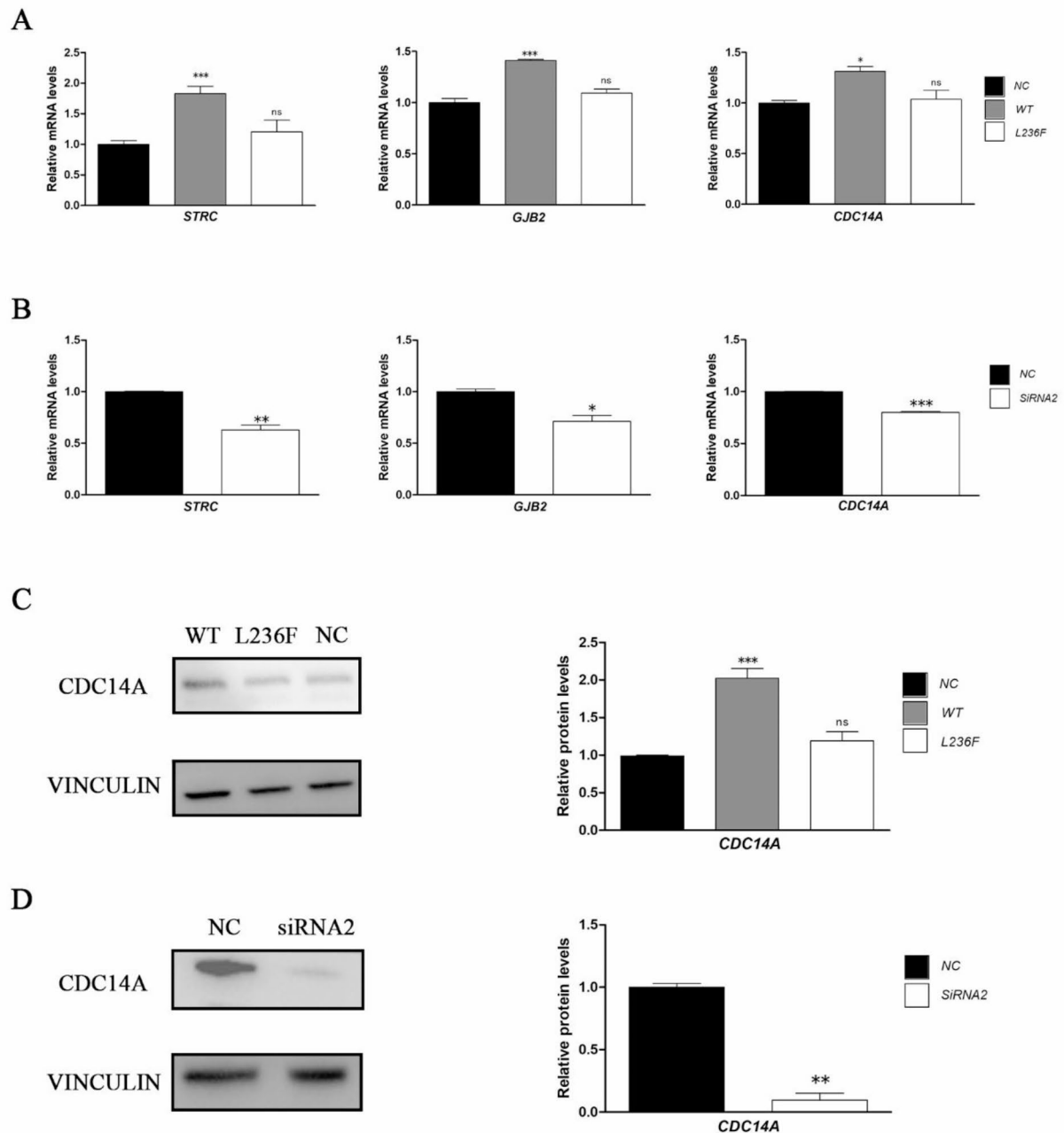
of *STRC* and *GJB2*, except an increase in the *CDC14A* group (Fig. 4). These data suggest that POU4F3 regulates the expression of *STRC*, *GJB2* and *CDC14A* by activating the promoter of them.

#### Six sites located on the promoters of *STRC*, *GJB2* and *CDC14A* were identified to bind to POU4F3

To further find the POU4F3 direct binding sites in *STRC*, *GJB2* and *CDC14A*, the putative binding sites were predicted by JASPAR CORE. Two of predicted sites in the promoter region of each gene with relative high score were selected and validated via EMSA. Compared with the only biotin-labeled probe group of each gene (*STRC*-P1, *STRC*-P2, *GJB2*-P1, *GJB2*-P2, *CDC14A*-P1, *CDC14A*-P2), a shifted band could be observed after co-incubation with nuclear proteins containing overexpression of WT POU4F3 (fused with HA), which would disappear when an overdosage of unlabeled probe competitively bound to POU4F3, while a super shifted band could also consequently display after adding HA antibody (Fig. 5A, C and E). These results demonstrated that POU4F3 indeed directly bind to *STRC*, *GJB2* and *CDC14A*, thus regulate the expression of three genes. Nevertheless, the mutant POU4F3 which located in the cytoplasm seemed to lose the DNA-binding ability (Fig. 5B, D and F).

#### POU4F3 may regulate more deafness genes

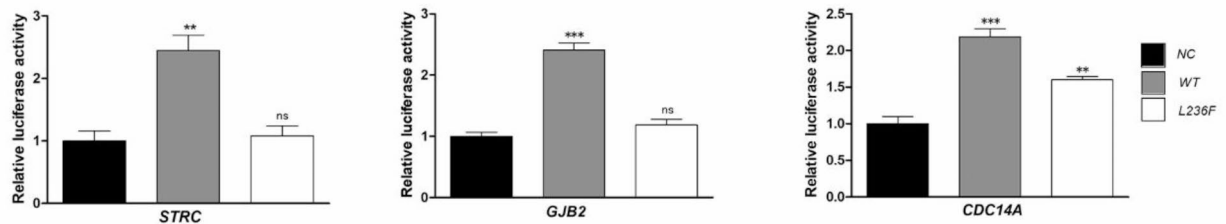
Since DFNA15 has a deep degree of clinical heterogeneity, except the *STRC*, *GJB2* and *CDC14A*, we inferred that POU4F3 as a transcription factor may regulate the expression of more extensive deafness genes. Hence, ChIP-seq was carried out to identify other POU4F3 binding genes. After transfection of the wild type POU4F3 vector into 293T cell, the fragmental DNA were pulled down by HA antibody and sequenced. According to the sequencing results, those genes which have been reported to be associated with deafness from the Hereditary Hearing Loss Homepage were analyzed. As expected, the results showed that POU4F3 could bind to the promoter regions of additional 17 deafness genes, including *MITF*, *USH2A*, *ADGRV1* and so on. The results of STRING analysis showed that 13 of them interacted with POU4F3 (Fig. 6). In addition, POU4F3 can also bind to the intron or exon regions of 33 deafness genes and 3' UTR regions of 4 deafness genes (supplement Table 4). These results suggest that these deafness genes may also be the modifiers for DFNA15.



## Discussion

Heterozygous mutation in the *POU4F3* can cause DFNA-15 and it is a form of progressive non-syndromic sensorineural hearing loss with postlingual onset between the second and sixth decades of life. In this study, we recruited a four-generation Chinese family with eleven individuals affected by hearing loss. By whole exome sequencing in the proband III-7 and sanger sequencing in her families, we found a novel mutation of *POU4F3* gene (c.706 C>T) co-segregating with hearing loss symptoms in this family. The resulted amino acids variation L236F was located on the evolutionarily conserved POU-specific domain. The POU-specific domain is required for high affinity sequence-specific DNA binding and confers DNA bending by interaction with the DNA<sup>19</sup>. Nuclear proteins usually have a common sequence NLS which is important for active protein transport into the nucleus. *POU4F3* contains two NSLs, the monopartite (amino acids 274 to 278 [RKRKR]) and the bipartite NLS (amino acids 314 to 331 [KKNVVRVWFCNLQRQKQKR])<sup>20</sup>. Previous studies have demonstrated that variation in bipartite NLS could compromise nuclear localization ability of *POU4F3*<sup>21</sup>. In this study, the results of immunofluorescence analysis showed that the mutant *POU4F3* proteins L236F were located in the cytoplasm. However, the variation L236F was not located on the monopartite or the bipartite NLS of *POU4F3*. Moreover, the results of the other reports showed that mutations which were not located on the monopartite or the bipartite

◀ **Fig. 3.** POU4F3 promoted the expression by activating the promoters of *STRC*, *GJB2* and *CDC14A*. (A) mRNA expression analyses of *STRC*, *GJB2* and *CDC14A* by RT-qPCR after POU4F3 overexpression. The results revealed that compared with the blank vector group, overexpression of wild type POU4F3 in 293T cell significantly increased the mRNA transcription of endogenous *STRC* (1.83 fold, P-values = 0.0007, F = 31.55, df = 8), *GJB2* (1.41 fold, P-values < 0.0001, F = 64.47, df = 8) and *CDC14A* (1.31 fold, P-values = 0.018, F = 8.44, df = 8), while overexpression of the mutant POU4F3 did not change the mRNA levels of three genes. (B) mRNA expression analyses of *STRC*, *GJB2* and *CDC14A* by RT-qPCR after POU4F3 knockdown by transfected with siRNA2. After knock-down of the POU4F3, the mRNA transcription of *STRC*, *GJB2* and *CDC14A* was accordingly reduced to 63% (P-values = 0.0048, t = 7.560, df = 3), 79% (P-values = 0.025, t = 4.178, df = 3) and 80% (P-values = 0.0004, t = 18.37, df = 3) of untreated 293T cells respectively. (C) Protein expression analyses of *CDC14A* by western blot after POU4F3 overexpression. The results revealed that compared with the blank vector group, overexpression of wild type POU4F3 in 293T cell significantly increased the protein expression of endogenous *CDC14A* (2.02 fold, P-values = 0.0009, F = 28.63, df = 8). D. Protein expression analyses of *CDC14A* by western blot after POU4F3 knockdown by transfected with siRNA2. After knock-down of the POU4F3, the protein expression of *CDC14A* was accordingly reduced to 90% (P-values = 0.0061, t = 12.79, df = 2). NC negative control, transfection of empty vector, WT transfection of wild type POU4F3 vector, siRNA2 transfection of wild type siRNA2, L236F transfection of mutant POU4F3 vector. Values represent mean  $\pm$  SD, significance was determined using 1way ANOVA Tukey's Multiple Comparison Test and Paired t test. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. This experiment is repeated three times or more in biology.

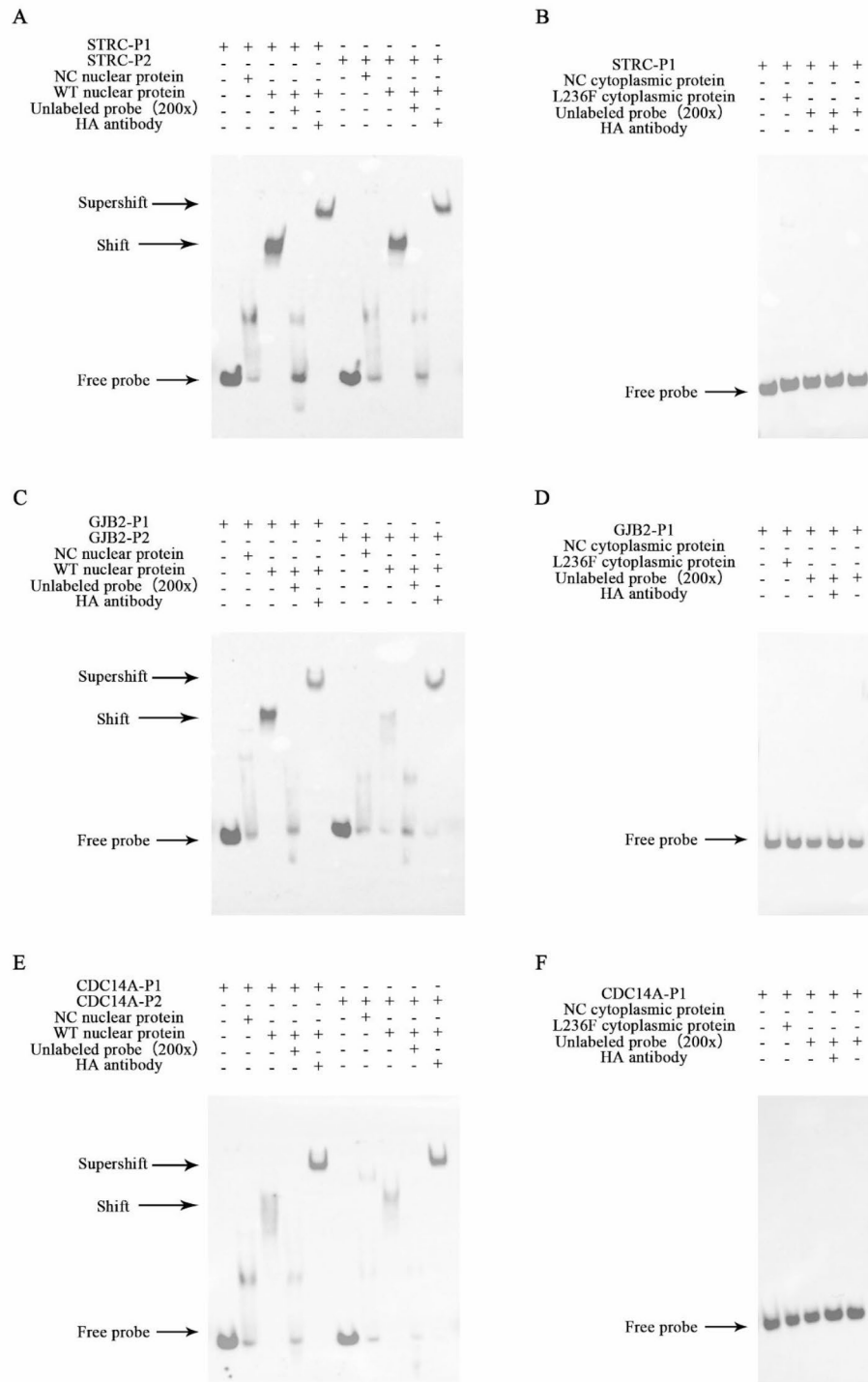


**Fig. 4.** Dual luciferase reporter gene assay of *STRC*, *GJB2* and *CDC14A* promoters. The results suggested that wild type POU4F3 significantly increased the promoter vectors' luciferase activity of *STRC* (2.45 fold, P-values = 0.0029, F = 18.03, df = 8), *GJB2* (2.40 fold, P-values < 0.0001, F = 66.55, df = 8) and *CDC14A* (P-values = 0.0002, F = 46.37, df = 8) NC: negative control, cotransfection of empty vector and the promoter vector; WT: transfection of wild type POU4F3 vector and the promoter vector; L236F: transfection of mutant POU4F3 vector and the promoter vector. This experiment is repeated three times or more in biology.

NLS of *POU4F3* could also impair the nuclear localization function<sup>5,22</sup>. The potential mechanism of this mutation compromised transportation of POU4F3 from the cytoplasm to the nucleus needs further exploration.

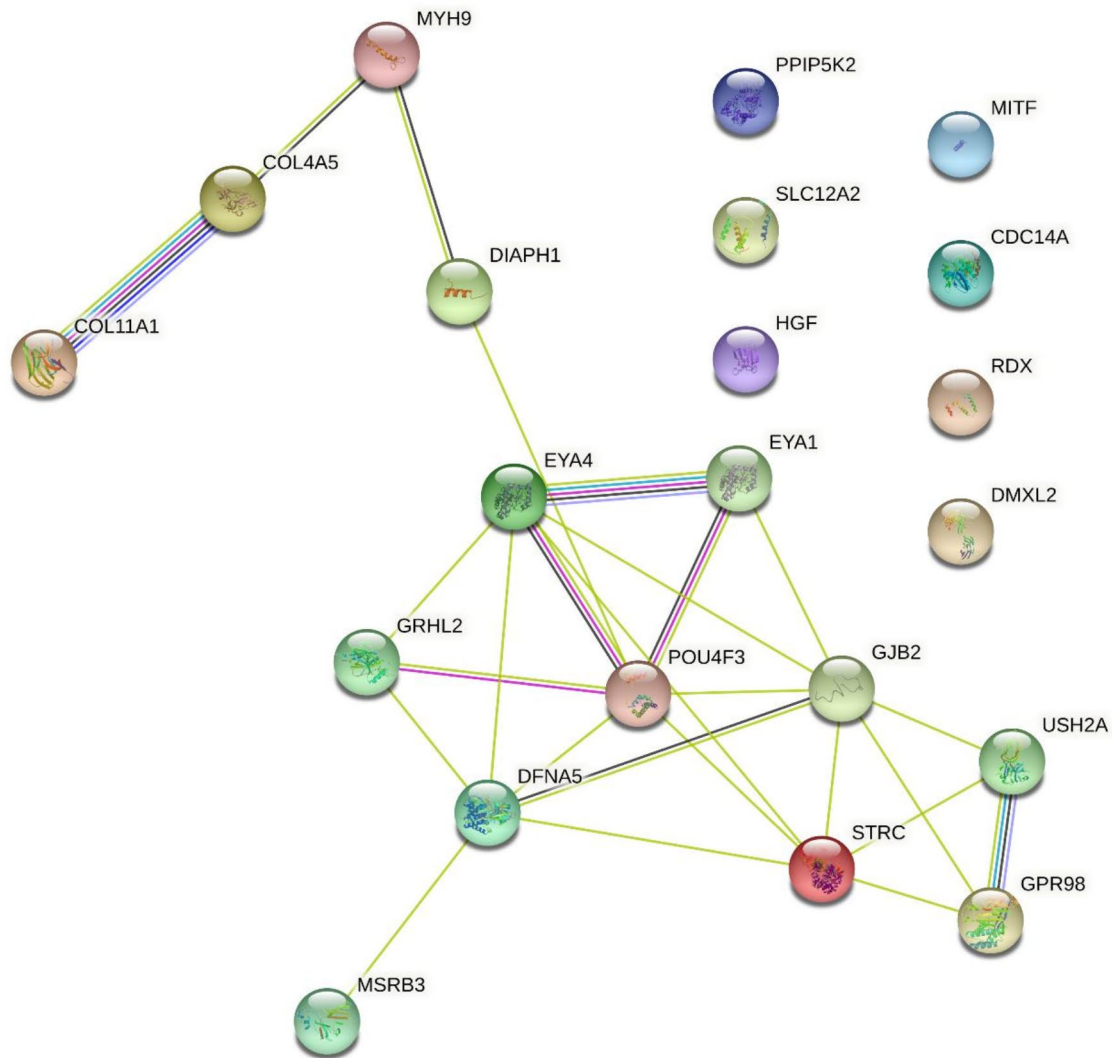
In deafness, digenic inheritance is a well know pattern, such as *GJB2*, *CDH23* and *SLC26A4* had been reported to cause hearing loss under digenic inheritance<sup>23–26</sup>. In these reports, variants at both genes are required for disease, but if only one of the two gene carrying the variants would not result in phenotype. However, the oligogenic inheritance in deafness is rarely reported, especially in those autosomal dominant non-syndromic hearing loss (ADNSHL) with the synergistic effect of other recessive pathogenic variants. In 2010, Yan X et al. once reported a Chinese family with autosomal dominant Waardenburg syndrome type 2 caused by a heterozygous mutation in *MITF*. The affected individuals in the family had moderate and mild sensorineural deafness, except for the proband, who presented with profound hearing loss as a newborn and had both *MITF* c.(742\_743delAAinsT;746\_747delCA) and *GJB2* mutations (c.109G > A and c.235delC)<sup>27</sup>. The author thought that digenic effect of *MITF* and *GJB2* genes may contribute to deafness of the proband, although they did not provide any further functional verification. To our knowledge, there is no report about digenic or oligogenic inheritance among *POU4F3* and other genes leading to more severe hearing loss. In our study, except for the mutation of *POU4F3*, the proband (III-7) and IV-1 carried extra mutations of *STRC*, *GJB2* and *CDC14A*. The results of RT-qPCR and western blot showed that POU4F3 promoted mRNA expression of *STRC*, *GJB2* and *CDC14A* and protein expression of *CDC14A*. POU4F3 bound to six sites which located on the promoter regions and activated the promoters of *STRC*, *GJB2* and *CDC14A*. All the above results suggested that *STRC*, *GJB2* and *CDC14A* were the target genes of POU4F3. However, the mutant POU4F3 significantly lose the regulatory effect. This is the first time that the target gene of POU4F3 has been identified in humans. In the inner ear of mature mammals, POU4F3 was expressed in hair cells<sup>28</sup> and *GJB2* was expressed in supporting cells<sup>29</sup>. However, during embryonic development, hair cells and supporting cells are generated from a common pool of postmitotic prosensory progenitors<sup>30</sup>. This suggests that the regulatory effects of POU4F3 and *GJB2* may occur in the early embryonic development. The oligogenic effect of *POU4F3* and *STRC/GJB2/CDC14A* may contribute to the onset age and progression level of hearing loss in III-7 and IV-1.

POU4F3 is the only POU4 transcription factor present in the sensory receptor hair cells of the cochlea. The regulation of POU4F3 on the expression of its target genes (*Caprin-1*, *Gfi-1*, *Lhx3* and *Nr2f2*) is essential for



**Fig. 5.** EMSA results of direct interaction between POU4F3 and the putative binding sites. STRC-P1, STRC-P2, GJB2-P1, GJB2-P2, CDC14A-P1 and CDC14A-P2: the detection probe which were labeled by biotin at the 5' end. NC nuclear/cytoplasmic protein: nuclear/cytoplasmic protein of empty vector transfected cell. WT nuclear/cytoplasmic protein: nuclear/cytoplasmic protein of wild type POU4F3 vector transfected cell. L236F nuclear/cytoplasmic protein: nuclear/cytoplasmic protein of mutant POU4F3 vector transfected cell. This experiment is repeated three times or more in biology.





**Fig. 6.** Interaction network of deafness genes that bind to POU4F3 in the promoter region from ChIP-seq data.

the development and survival of hair cells<sup>31–33</sup>. And most deafness genes specifically expressed in the hair cell, suggesting that POU4F3 may be the transcription factor of them. The results of ChIP-seq showed that POU4F3 could bind to the promoter regions of 17 deafness genes, intron or exon regions of 33 deafness genes and 3' UTR regions of 4 deafness genes.

More and more studies have shown that the clinical heterogeneity of genetic diseases is related to oligogenetic effects, and the genes that form oligogenetic effects often have interactive relationships or are located on the same signaling pathway. The results of this study indicate that POU4F3, as a transcription factor, regulates the expression of STRC, CDC14A, and GJB2 genes. When STRC, CDC14A, and GJB2 undergo heterozygous mutations, although they do not produce phenotypes alone, their functions may be more affected due to mutations in their transcription factor POU4F3, leading to premature or aggravated phenotypes. This study only validated the relevant functions in cell lines, lacking further confirmation from animal models, and the research results may have certain biases. Due to factors such as distance or inability of participants to cooperate, we did not obtain detailed hearing test results for IV-2, IV-3, and IV-6. Their hearing problems were reported orally by their parents or cousins, which is also a limitation of this study.

## Conclusions

In summary, in this study, we identified a novel mutation of *POU4F3* gene in Chinese patients with ADNSHL. Function analysis demonstrated that the mutation impaired nuclear localization of POU4F3 and compromised protein function. We proved that *STRC*, *GJB2* and *CDC14A* were the new target gene of POU4F3. This indicates

that oligogenic effect of *POU4F3* and *STRC/GJB2/CDC14A* may contribute to the clinical heterogeneity of DFNA15.

### Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2024), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA009740) that are publicly accessible at <https://ngdc.cnbc.ac.cn/gsa-human>.

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## Author contributions

JP, ZL, LW designed the study and collected the data. JP, FL, HT, SC, YT performed the experiments and analyzed the data. YL performed the clinical evaluation of the patients. JP drafted the manuscript. ZL, DL, and LW provided critical editing. All authors have read and agreed to the published version of the manuscript.

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## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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