



A novel missense mutation in *SMPX* causes a rare form of X-linked postlingual sensorineural hearing loss in a Chinese family

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Background: X-linked deafness-4 (DFNX4) caused by the functional loss of the *SMPX* gene is one form of nonsyndromic hearing loss with postlingual onset. This study aimed to investigate the cause of X-linked inherited sensorineural nonsyndromic hearing loss in a four-generation Chinese family and to explain the reason for extremely different hearing phenotypes between the proband and other family members.

Methods: Whole-exome sequencing (WES) and co-segregation analysis were used to identify the pathogenic variants. Furthermore, methylation differences among the androgen receptor genes were utilized to investigate whether the severe phenotype of the proband is related to X-chromosome inactivation (Xi).

Results: We described in detail the clinical characteristics of the family and identified a novel missense mutation (c.262C>G: p.Gln88Glu) in *SMPX* by WES. This variant was co-segregated with the postlingual hearing loss phenotype and was absent in 300 normal controls. Also, we found that the proband, a 4-year-old female, carries two new compound heterozygous mutations (c.9259G>A: p.Val3087Ile and c.8576G>A: p.Arg2859His) in the *USH2A* gene, but to date without any other symptoms except profound sensorineural hearing loss. Additionally, analysis of X-chromosome inactivation indicated moderate skewing in the proband, which is probably related to the heterogeneity of clinical characteristics.

Conclusions: This is the first study to report a missense mutation of *SMPX* in a Chinese family. Our findings have enriched the mutation and phenotypic spectrum of the *SMPX* gene.

Keywords: *SMPX*; X-linked hearing loss; novel variant; missense mutation

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Introduction

Hearing loss has always been a worldwide public health problem. It is estimated that 466 million people (6.1% of the world's population) suffer from hearing loss, and unaddressed hearing loss costs the global economy US\$750 billion annually (WHO data, www.who.int). Major causes of hearing loss include genetic etiological agents, chronic middle ear infections, noise-induced hearing loss, and ototoxic drugs.

Approximately 60% of hearing loss cases are hereditary hearing loss, which can be divided into syndromic hearing loss (SHL) and non-syndromic hearing loss (NSHL) (1). The X-linked forms are far rarer than autosomal or mitochondrial-inherited hearing loss, accounting for only 1–5% of all reported mutations associated with NSHL (2). Till now, in X-linked NSHL, only six loci (DFNX1–6) and five genes have been reported in the literature (3).

SMPX (DFNX4, OMIM 300066), mapping to Xp.22.1, is

composed of five exons and encodes a small muscle protein comprising 88 amino acids, which is originally cloned from skeletal muscle without known functional domains (4,5). Initial studies have shown that this gene is highly expressed in mammalian fetal and adult skeletal muscles and hearts, suggesting that it acts as a mechanical transducer during sensory changes in mechanical loads (6). However, neither *Smpx* gene knockout nor overexpression animal models have significant muscle dysfunction phenotypes (7). In the following study, it is confirmed that *SMPX* mutations are associated with X-linked hearing loss with obvious clinical and genetic heterogeneity (8-16). Moreover, *SMPX* has been identified in fetal and adult inner ears (9). Yoon *et al.* (17) reported the expression of mouse *Smpx* in the inner ears and vestibular hair cells. Ghilardi *et al.* (18) speculated that *smpx* localizes to the cuticular plate of the inner ear hair cells in zebrafish. On the other hand, *in vitro* studies suggested small muscle protein is mainly involved in regulation of cytoskeletal dynamics and promotion of myocyte fusion through the Rac1-p38 and IGF-1 pathways (6,7). Ferrán *et al.* (19) reported that the nuclear receptor NOR-1 positively regulates small muscle protein and myotube differentiation. Considering the expression pattern of *SMPX* and its response to mechanical force, it is believed that *SMPX* may play an important role in protecting the stereocilia of the sensory epithelium from mechanical stress during hearing (8).

To date, three nonsense mutations and seven frameshift mutations in *SMPX* have been reported to be associated with X-linked NSHL (Table 1). However, the missense mutation of *SMPX* has never been reported before. We investigated X-linked hearing loss in a four-generation Chinese family and identified a novel missense mutation (c.262C>G: p.Gln88Glu) in *SMPX* by whole-exome sequencing (WES). Also, we found the proband carries two new compound heterozygous mutations (c.9259G>A: p.Val3087Ile and c.8576G>A: p.Arg2859His) in the *USH2A* gene. Furthermore, skewed X-chromosome inactivation may be one of the reasons for phenotypic heterogeneity.

We present the following article in accordance with the MDAR checklist (available at <http://dx.doi.org/10.21037/tp-20-435>).

Methods

Subjects and clinical assessment

This study investigated X-linked hearing loss in a four-

generation Chinese family with a total of 10 people, including five patients. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This research was approved by the Institutional Ethics Committee of The Second Hospital of Jilin University. The study was conducted after receiving written informed consent from the patients. The proband (IV-2, Figure 1A), a 4-year-old female, was diagnosed with profound sensorineural hearing loss at the Department of Otolaryngology, the Second Hospital of Jilin University. The hearing loss began when she was approximately 3 years old, and it was more severe than that of other members of the family. A detailed clinical evaluation of all participating family members was obtained and collected by a questionnaire, which included age of onset, noise exposure, subjective degree of hearing loss, progression, treatment, and other relevant clinical manifestations. Physical and otoscopic examinations, acoustic immittance testing, pure tone audiometry (PTA), and vestibular testing were used to assess the function of the middle ear and vestibular system as well as hearing sensitivity. The proband underwent auditory brainstem response (ABR), multiple frequency auditory steady-state evoked responses (ASSR), distortion product otoacoustic emission (DPOAE), high-resolution computed tomography (HRCT), magnetic resonance imaging (MRI), ophthalmic examination, and routine blood examination. The degree of hearing loss (normal, mild, moderate, moderately severe, severe, and profound) was classified and defined according to the recommendations of the Global Burden of Disease Expert Group on Hearing Loss (20).

Deafness gene hotspot mutation screening

A mutation analysis of the proband was performed on 20 common variants of four genes related to hearing loss: *GJB2*, *GJB3*, *SLC26A4*, and mtDNA (*GJB2* c.35delG, c.167delT, c.176_191del16GCTGCAAGAACGTGTG, c.235delC, c.299_300delAT; *GJB3* c.538C>T, c.547G>A; *SLC26A4* c.281C>T, c.589G>A, C.919-2A>G, c.1174A>T, c.1226G>A, c.1229C>T, c.1707+5G>A, c.1975G>C, c.2027T>A, c.2162C>T, c.2168A>G, and *12S rRNA* A1555G, C1494T).

WES and bioinformatic analysis

Genomic DNA was extracted from the peripheral blood

Table 1 Overview of pathogenic variants identified in *SMPX* associated with nonsyndromic hearing loss.

Nucleotide	Amino Acid	Location	Type of variant	Country	Male			Female			Reference
					Onset	Severity	Progressive	Onset	Severity	Progressive	
c.109G>T	p.Glu37*	Exon3	Nonsense	Germany	3-7	Bilateral; moderate to profound	Yes	Second to third decades	Bilateral; moderate to severe	Yes	(8)
c.175G>T	p.Gly59*	Exon4	Nonsense	Spain	5-7	Bilateral; moderate to severe or profound	Yes	Fourth decades	Bilateral; moderate	Yes	(8)
c.130delG	p.Glu44Argfs*37	Exon3	Frameshift	Netherlands	4	Bilateral; severe	Yes	—	—	—	(9)
c.214G>T	p.Glu72*	Exon4	Nonsense	Netherlands	2-10	Bilateral; moderate to profound	Yes	3-48	Unilateral or bilateral; mild to profound	Yes	(9)
c.99delC	p.Arg34Glu fs*47	Exon3	Frameshift	Canada	First decade	Bilateral; severe	Yes	4-62	Unilateral or bilateral; variable severity	Yes	(11)
c.217dupA	p.Ile73Asnfs*5	Exon4	Frameshift	China	0-8	Bilateral; severe to profound	Yes	Third to fourth decades	Unilateral or bilateral; mild	—	(12)
c.87dupA	p.Gly30Argfs*12	Exon3	Frameshift	China	7	Bilateral; severe	Yes	>30	Unilateral or bilateral; severe	Yes	(13)
c.133-1G>A	p.Gly45Valfs*36	Intron3	Frameshift	United States	Childhood	Bilateral; profound	Yes	Childhood	Unilateral; severe	Yes	(15)
c.29insA	p.Asn10Lysfs*3	Exon2	Frameshift	China	5-10	Bilateral; profound	Yes	Second to third decades	Bilateral; moderate to profound; symmetric or asymmetric	Yes	(14)
c.132+1G>A	p.Met45Glyfs*16	Intron3	Frameshift	China	Newborn	Bilateral; severe to profound	Yes	>50	Bilateral; moderate to severe at high frequencies; mild at low frequencies	—	(16)
c.262C>G	p.Gln88Glu	Exon4	Missense	China	First or second decades	Bilateral; mild to moderately severe	—	3; third to fourth decades	Bilateral; mild to profound	Yes	This study

‘—’ means normal or unclear.

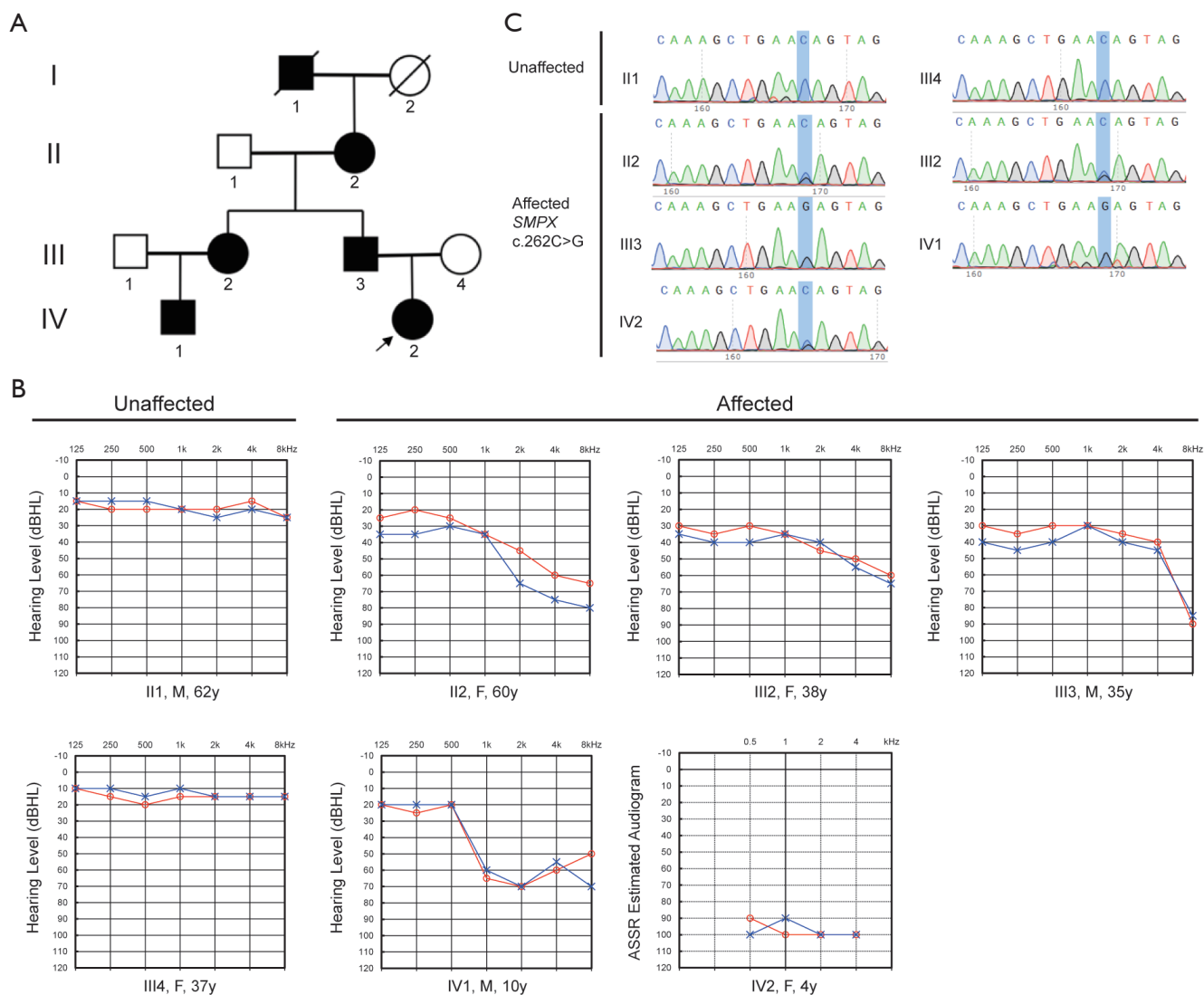


Figure 1 Pedigree, Audiograms and Sanger sequencing. (A) Pedigree of a Chinese family with X-linked post-lingual sensorineural hearing loss. Square: male; circle: female; open symbol: no hearing loss; solid symbol: hearing loss; slash: deceased individuals. (B) Audiograms of all participating family members. The air conduction results are represented by the blue “x” in the left ear and the red “o” in the right ear. (C) Sanger sequencing analysis of c.262C>G (p.Gln88Glu) mutation on *SMPX* in the family members.

sample of all participants through the standard phenol/chloroform method. WES was performed on two affected subjects (III-3 and IV-2) and one normal subject (III-4) by the Beijing Genomics Institution, China. The DNA sample was randomly broken into fragments with an average DNA size of 180–280 bp. Each targeted exon was sequenced using an Illumina HiSeq2000 platform after enrichment, hybridization, and capture. Mapping and analysis were based on the human reference genome (UCSC hg19).

The variants were filtered and identified by using public databases, including dbSNP, 1000 Genomes Project, ESP6500, ExAC, and the human gene mutation database (HGMD). Then, the synonymous variants and irrelevant gene variants that did not match clinical manifestations and inheritance patterns were further excluded to narrow down the number of the candidate variants. The functional prediction was carried out using PROVEAN, PolyPhen-2, Mutation Taster, and I-Mutant 3.0.

Table 2 Summary of the clinical data of all participants from the family

Subjects	Gender	Test (year)	Onset (year)	Noise exposure	Severity	Audiogram shape	Tinnitus	Vertigo
II-1	Male	62	—	No	Normal	—	No	No
II-2	Female	60	Fourth decade	No	Moderate (R), moderately severe (L)	Sloping	Yes	No
III-2	Female	38	Third decade	No	Moderate	Sloping	No	No
III-3	Male	35	Second decade	No	Mild (R), moderate (L)	Sloping	No	No
III-4	Female	37	—	No	Normal	—	No	No
IV-1	Male	10	6–7	No	Moderately severe	Sloping	No	No
IV-2	Female	4	3	No	Profound	Flat	No	No

Sanger sequencing

The *SMPX* (Gene ID: 23676) variant was amplified and sequenced to confirm if the gene co-separated with the hearing loss phenotype in the family (Forward primers: 5'-CAATGGATCTATGACTG-3'; Reverse primers: 5'-CCAAAGAGATCAAATGT-3'). Sanger sequencing was performed on the ABI 3730xl DNA Analyzer.

X-chromosome inactivation assay

X-chromosome inactivation analysis was performed on the proband (IV-2) and her parents (III-3 and III-4). An evaluation of X-chromosome inactivation based on HpaII methylation analysis, which is a polymorphic short tandem repeat (STR) in the X-linked *AR* gene of peripheral blood leukocytes (21) was performed. The peripheral blood DNA sample was digested with HpaII (R0171S, NEB) at 37 °C overnight, and then at 95 °C for 10 min for enzyme inactivation. For each sample, 200 ng of DNA was used for polymerase chain reaction (PCR) with specific primers of the methylation regions of the *AR* gene. The fluorescently labelled PCR products were analyzed by capillary gel electrophoresis to determine the Xi level of each allele by calculating the ratio between the heights of two peaks.

Results

Clinical characteristics

The proband (*Figure 1A*, IV-2) who passed the newborn hearing screening test (NHST) began to experience hearing loss in her 3rd year. A detailed audiological test and imaging examination were performed when she was 4 years old.

Neither ear passed DPOAE. An ABR test showed that the V wave response of both ears was normal, and the V wave response thresholds were 95 dB nHL (left) and 90 dB nHL (right). An ASSR test also showed that she exhibited a bilateral profound sensorineural hearing loss (*Figure 1B*), though HRCT as well as MRI showed no abnormalities. Ophthalmic examination and routine blood examination were performed at the same time, but no abnormal symptoms were found.

The audiological-related characteristics of all participants in the family, assessed by subjective and objective audiological examinations and summarized in *Table 2*, showed significant heterogeneity. While PTA data revealed that all affected family members exhibited binaural symmetrical sensorineural hearing impairment, and that high-frequency hearing loss occurred more severely than low-frequency hearing loss, the degree of hearing loss and the age of onset were quite different between male and female. Male patients suffered from hearing loss in the first to second decades, about 10 to 20 years earlier than female patients except the proband. The father (III-3) and cousin (IV-1) of the proband exhibited mild to moderately severe hearing loss, which was more severe than in female patients (II-2, III-2) of the same age. Subject II-2 had intermittent tinnitus, often accompanied by further hearing loss. None of the patients in the family had a history of vestibular dysfunction, noise exposure, or ototoxic medication use.

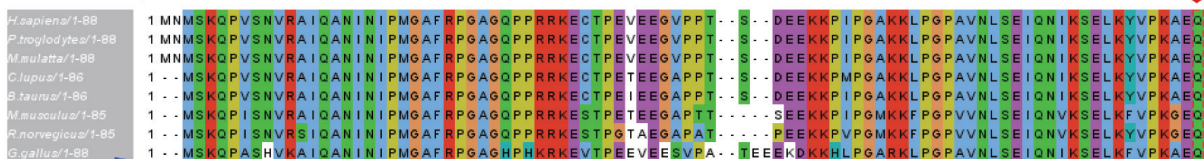
Mutation analysis

We obtained 5.08 Gb of raw data. The coverage of the targeted bases was 99.16%, and approximately 96.34% of the targeted region was covered at least 30x. After filtering

Table 3 In silico analysis of the specific mutations in *SMPX* and *USH2A*

Gene	Nucleotide	Amino Acid	Prediction information			
			PROVEAN	Polyphen2	Mutation taster	I-Mutant 3.0
<i>SMPX</i>	c.262C>G	p.Gln88Glu	Neutral	Benign	Disease causing	Neutral stability
<i>USH2A</i>	c.8576G>A	p.Arg2859His	Neutral	Possibly damaging	Disease causing	Large decrease of stability
<i>USH2A</i>	c.9259G>A	p.Val3087Ile	Neutral	Possibly damaging	Disease causing	Neutral stability

SMPX c.262C>G p.Gln88Glu



USH2A c.8576G>A p.Arg2859His



USH2A c.9259G>A p.Val3087Ile

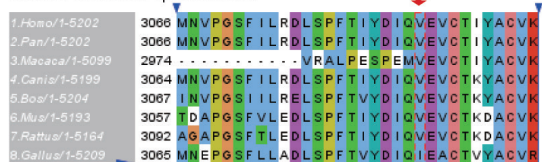


Figure 2 Amino acid conservation analysis. Conservation among multiple species at position 88 in *SMPX* and at position 2859 and 3087 in Usherin.

and analyzing the data according to the method described in the materials and methods, 32 variants remained, of which 16 variants followed autosomal dominant inheritance pattern and one followed X-linked dominant inheritance pattern. We then eliminated autosomal dominant genes including *DIAPH1*, *DIAPH3*, *DSPP*, *GRHL2* based on the family clinical phenotype. The variant in *SMPX* (c.262C>G: pGln88Glu) was finally identified as possibly related to the post-lingual hearing loss of the family. Furthermore, this missense mutation has not been reported before and was absent in 300 normal controls. Sanger sequencing validated that it was found in all of the affected members (II-2, III-2, III-3, IV-1 and IV-2), but absent in unaffected members (Figure 1C). In other words, c.262C>G located in exon 4 of *SMPX* was fully co-segregated with the hearing loss phenotype of this family. Also, we found that the proband carries two compound heterozygous mutations (c.9259G>A: p.Val3087Ile and c.8576G>A: p.Arg2859His) in the *USH2A* gene, but without any other symptoms except profound sensorineural hearing loss to date.

Table 3 shows the prediction results of the pathogenicity of these genetic alterations by online bioinformatics prediction tools (PolyPhen-2, Mutation Taster, and

I-Mutant 3.0). The 88 amino acids in human *SMPX* are conserved across several species (Figure 2).

Analysis of X-chromosome inactivation skewing

$X_i = a:b$, where *a* represented the ratio of the height of the parent peaks of HpaII-digested to HpaII-undigested, similarly, *b* denoted the ratio of the height of the two maternal peaks. The X-inactivation ratios of paternal (*a*) to maternal (*b*) allele of the proband was 29:71, which means that approximately 71% of the paternal X-chromosome including the defective *SMPX* gene was active in the proband (Figure 3). Briefly, it indicated moderate skewing in the proband, which may account for the phenotypic differences.

Discussion

X-linked non-syndromic hearing loss is highly heterogeneous in genetic and clinical phenotypes. DFNX4 (DFN6) was first reported in 1996 in a Spanish family with X-linked dominant progressive non-syndromic sensorineural deafness (22). High-frequency hearing loss

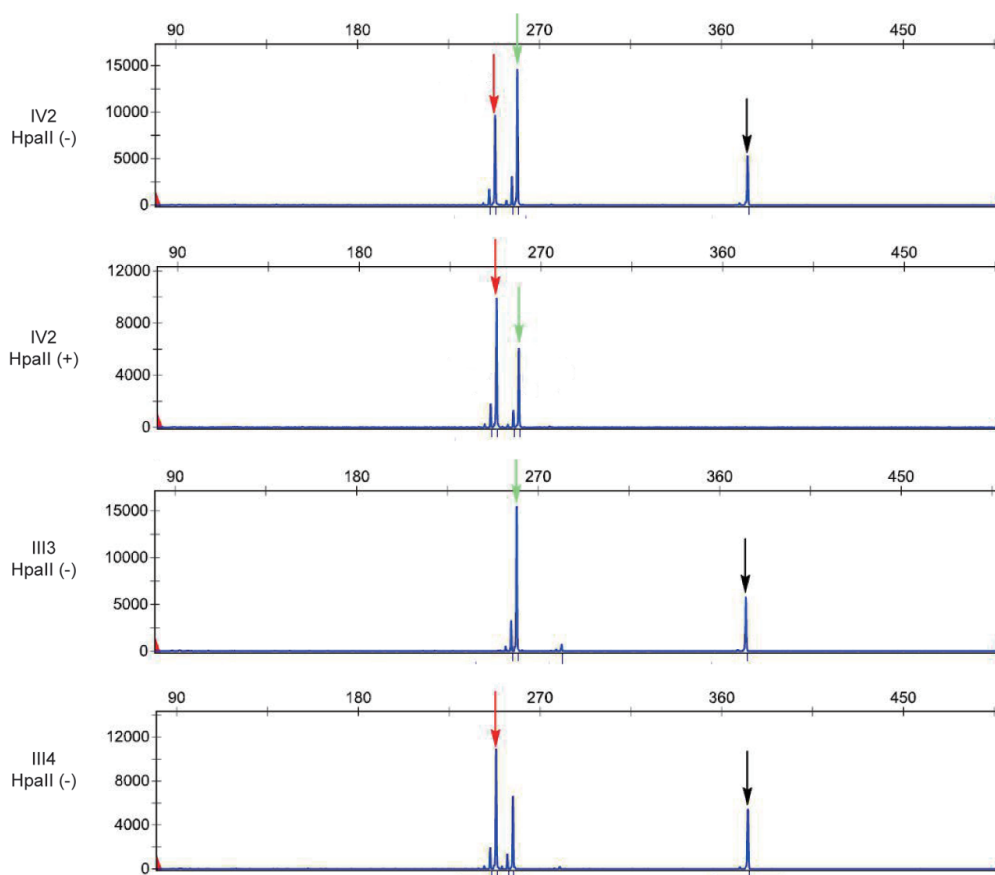


Figure 3 Analysis of X-chromosome inactivation skewing. Chromatograms of amplification products of AR STR are shown with and without HpaII digestion. The red arrow indicates the maternal peak. The green arrow indicates the paternal peak. The black arrow indicates the amplified product of the internal reference gene (MIC2).

occurred in the affected males at school age and gradually developed into full-frequency severe-to-profound deafness before adulthood, while the carrier females showed moderate high-frequency deafness in their fourth decade. In 2011, Huebner *et al.* (8) identified two nonsense mutations (*SMPX* c.109G>T: p.Glu37*; *SMPX* c.175G>T: p.Gly59*) in German and Spanish families, respectively. Males presented with progressive, moderate or above hearing loss and onset at the age of 3–7 years. The age of onset in female carriers ranged from the second to the third decade, and hearing loss progressed to severe within 10–15 years. In the same year, Schraders *et al.* (9) reported a nonsense mutation (*SMPX* c.214G>T: p.Glu72*) and a frameshift mutation (*SMPX* c.130delG: p.Glu44Argfs*37) in two Dutch families. The age of onset for male and female carriers was 2–10 years (average, 3.3 years) and 3–48 years (average, 28.2 years), respectively. The men showed a severe increase

in threshold values mainly during the first two decades, whereas women demonstrated significant inter-individual heterogeneity in age of onset, disease progression, degree of hearing loss, and inter-aural asymmetry in thresholds. In 2013, Abdelfatah *et al.* (11) confirmed a frameshift mutation (*SMPX* c.99delC: p.Arg34Glu fs*47) in two multiplex families from Newfoundland. Hearing loss in males appeared to be similar to previous studies in degree and age of onset, but congenital hearing loss was not definitely excluded due to a non-detectable mild hearing loss during newborn screening. Likewise, female carriers displayed highly variable phenotypes, including a widespread age of onset (4–62 years), variable severity, and unilateral or bilateral symmetric or asymmetric deafness. In 2017, Niu *et al.* (12) first reported a novel frameshift mutation (*SMPX* c.217dupA: p.Ile73Asn fs*5) in a Chinese population with late-onset progressive NSHL, as well as congenital

hearing impairment which had never been proven before. Subsequently, a North American family with a novel frameshift mutation creating a premature stop codon (c.133-1G>A: p.Gly45fs*36) of *SMPX* was described by Niu *et al.* (15). Meanwhile, Deng *et al.* (13), Gao *et al.* (14) and Lv *et al.* (16) successively identified three frameshift mutations of *SMPX* (c.87dupA: p.Gly30Argfs*12; c.29insA: p.Asn10Lysfs*3; c.132+1G>A: p.Met45Glyfs*16) from multiple Chinese lineages. To sum up, most pathogenic variants of *SMPX* caused post-lingual progressive NSHL, and a few led to congenital deafness. The affected males showed more severe hearing loss at a younger age, while female carriers manifested incomplete penetrance and variable expressivity probably due to skewed X-chromosome inactivation.

Generally, one of the two X chromosomes of female cells is randomly inactivated in mammals, resulting in most normal females being chimeras, that is, approximately half of the cells express the alleles on the paternal X chromosome, and the other half express the maternal. Non-random inactivation of the X chromosome, namely skewed X-chromosome inactivation, occurs when the ratio of paternal and maternal cell inactivation is highly deviated from 1:1, which might lead to the phenotypic heterogeneity of female carriers of X-linked inherited deafness. The human androgen receptor assay (HUMARA), based on HpaII methylation analysis of a polymorphic short tandem repeat (STR) in the X linked *AR* (androgen receptor) gene, is one of the most convenient, rapid and widely accepted methods for measuring X-chromosome inactivation (23).

In this study, we reported X-linked hearing loss in a four-generation Chinese family and identified a novel missense mutation (c.262C>G, p.Gln88Glu) in *SMPX* by WES. This variant was co-segregated with the post-lingual hearing loss phenotype and was absent in 300 normal controls. However, the female proband presented profound sensorineural deafness with early age of onset, which was significantly heterogeneous from other female carriers in the family. Analysis of the X-chromosome inactivation indicated moderate skewing in the proband, which was probably related to the heterogeneity of clinical characteristics.

In addition, we found the proband carries two new compound heterozygous mutations (c.9259G>A: p.Val3087Ile and c.8576G>A: p.Arg2859His) in the *USH2A* gene, but to date without any other symptoms except profound sensorineural hearing loss. *USH2A* (OMIM 608400), together with *ADGRV1* and *WHRN*, is known to be associated with Usher syndrome type II (USH2). *USH2A*,

located on chromosome 1q41, encodes the usherin which is primarily expressed on the basement membrane of the cochlea and retina and is involved in postnatal development of cochlear hair cells and retinal photoreceptors (24). *USH2A* variants are associated with autosomal recessive syndromic hearing loss with moderate to severe bilateral sensorineural hearing impairment and progressive visual loss that occurs during adolescence or beyond. So far, more than 600 mutations have been found in the *USH2A* gene, including nonsense mutations, frameshift mutations, splice-site mutations, missense mutations, and intron mutations (<http://www.lovd.nl/USH2A>). The mutation (*USH2A* c.9259G>A: p.Val3087Ile) has been reported in patients (25,26), while the variant (*USH2A* c.8576G>A: p.Arg2859His) has never been detected before. Whether the combination of *USH2A* and *SMPX* mutations causes profound hearing loss in the proband still needs long-term follow-up and further experimental research.

Conclusions

We reported X-linked hearing loss in a four-generation Chinese family and identified a novel missense mutation (c.262C>G, p.Gln88Glu) in *SMPX* by WES. This is the first study to report a missense mutation of *SMPX* in a Chinese family. Our findings have enriched the mutation and phenotypic spectrum of the *SMPX* gene. Furthermore, skewed X-chromosome inactivation may be one of the reasons for phenotypic heterogeneity.

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Footnote

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[org/10.21037/tp-20-435](https://doi.org/10.21037/tp-20-435)). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This research was approved by the Institutional Ethics Committee of The Second Hospital of Jilin University. The study was conducted after receiving written informed consent from the patients.

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