

Identification of novel cadherin 23 variants in a Chinese family with hearing loss

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Abstract. The aim of the present study was to elucidate the role of the non-syndromic autosomal recessive deafness 12 allelic variant of cadherin 23 (*CDH23*) in Chinese patients with non-syndromic hearing loss. The present study focused on a Chinese family with hearing loss in which there were two siblings with autosomal, recessive deafness, ranging from severe to profound hearing loss over all frequencies. DNA sequencing was used to assess the genetic factors in the disease etiology. The data revealed a compound heterozygous mutation of *CDH23* in both patients. Genetic *CDH23* variants are known to be responsible for non-syndromic hearing loss, and *CDH23* variants frequently occur in various populations, including Japanese and Republic of Korean. Results from the present study, indicated a significant contribution of *CDH23* variants to the non-syndromic hearing loss in Chinese patients.

Introduction

Hearing loss or hearing impairment refers to a partial inability to hear sounds in one or both ears, while deafness is defined by full inability to hear (1). The Global Burden of Disease Study has demonstrated that hearing loss is the fourth leading cause of global disability (2). In the United States, the prevalence of hearing loss doubles every 10 years with an increasingly aging population (3). Risk factors for hearing loss include genetic factors, aging, a noisy environment, ear trauma or infection, birth complications, certain medications and toxins (1). Genetic factors may be responsible for ~40% of childhood hearing loss, especially in children born of consanguineous marriages (4-6). Hundreds of gene mutations have been identified that lead to hearing loss either as an exclusive clinical

feature or in combination with extra-auditory symptoms as part of a syndrome (7). Non-syndromic forms of deafness account for 70% of cases, of which ~85% are inherited in an autosomal recessive manner (8,9). Owing to recent improvements in research of genetic factors, such as the identification of gene mutations involved in congenital hearing loss, genetic counseling has emerged and increased in availability (1). High-throughput DNA sequencing technologies, known as next-generation DNA sequencing or massively parallel sequencing, are used to detect multiple gene mutations, which results in improved detection and early treatment of childhood hearing loss (10).

Recessive genetic mutations of cadherin 23 (*CDH23*) have been associated with the allelic variants Usher syndrome type 1D (*USH1D*) and non-syndromic autosomal recessive deafness 12 (*DFNB12*) (11,12). *CDH23* is on chromosome 10q21-q22 and contains 69 exons that encode the *CDH23* protein. *CDH23* comprises 3,354 amino acids with 27 cadherin extracellular (EC) repeats, a single transmembrane domain and a short unique cytoplasmic domain (12). Each EC domain of the *CDH23* protein contains several cadherin-specific amino acid motifs, such as LDRE, DXD and DXNDN, and is highly conserved in sequence and spacing, which is required for cadherin dimerization and calcium-binding (13). *CDH23* mutations induce disorganization of the stereocilia in the hair cells of the inner ear in the 'waltzer' mouse model of the *USH1D* (14). Thus, the extracellular domains of *CAD23* are crucial for the correct morphogenesis of hair bundles in the inner ear neurosensory cells, whereas the cytoplasmic domain of the *CDH23* interacts with other hair bundle proteins, including myosin VIIA and harmonin (15,16). However, an increasing number of genetic variants of different genes have been reported, such as *USH1D* and *DFNB12* (17-20). Certain gene variants occur with a distinct frequency in different populations. For example, two variants were screened in patients with a Swedish background (17), whereas others were present in Japanese or Korean subjects (18-20). The aim of the present study was to elucidate the role of the *DFNB12 CDH23* mutation in Chinese patients with non-syndromic hearing loss. The nature of diverse *CDH23* mutations and their resulting phenotypes in hearing loss were studied using a comprehensive strategy that included DNA sequencing and bioinformatics, which provided information regarding hearing loss in Asian populations.

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Materials and methods

Study subjects. The study was approved by the Ethics Committee of the First Hospital of Jilin University (Changchun, China) and all participants provided written informed consent prior to being enrolled in the study. The study enrolled two patients (II:1 and II:2) with severe sensorineural hearing loss and their parents (I:1 and I:2). Clinical information and peripheral blood samples were acquired for DNA extraction and DNA sequencing. The patients were diagnosed using audiologic tests according to the American College of Medical Genetics and Genomics guidelines (21). Linkage analysis was not carried out owing to the small number of participants.

DNA extraction and DNA sequencing. Genomic DNA was extracted from the blood samples using a TIANamp Blood DNA Kit (TianGen Biotech Co., Ltd.) and quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, the genomic DNA samples were subjected to DNA sequencing focusing on nine gene loci: GJB2:35delG, 176del16, 235delC, 299delAT, GJB3:538C>T, SLC26A4:2168A>G, IVS7-2A>G, mitochondrial 12SrRNA:1494C>T and 1555A>G, using the LuxScan 10K Microarray Scanner (CapitalBio Corporation). A customized exome enrichment kit (MyGenostics, Inc.) was designed to identify genes that induce hearing loss. The prepared samples were sequenced using Illumina NextSeq 500 (Illumina, Inc.).

DNA libraries were constructed using the genomic DNA samples with a Library Preparation kit (MyGenostics, Inc.), according to the Illumina platform requirements. The enzymatic fragmentation of genomic DNA samples was end-repaired, and adapters were added. The length of the prepared libraries was ~350-400 bp, and they were amplified by PCR and analyzed using an Agilent 2100 Bioanalyzer (Agilent technologies Inc.) by MyGenostics, Inc. The aligned short reads were compared with the human reference genome (hg19) (<http://hgdownload.cse.ucsc.edu>) using the Burrows-Wheeler Aligner (version 0.7.15; <http://bio-bwa.sourceforge.net/>). The quality control assessment and variant calling were processed using the Genome Analysis Toolkit (GATK; version 3.6; <https://www.broadinstitute.org/gatk/>) following GATK best practices (22) and annotated using ANNOVAR (version 2016-02-01), similarly to a previous study (23). Potential damaging gene variants were screened for quality against the 1000 Genomes public variant databases (<ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp>), the Single Nucleotide Polymorphism database (<http://www.ncbi.nlm.nih.gov/projects/SNP>), Sorting Intolerant from Tolerant (SIFT; <http://sift.bii.a-star.edu.sg>) and Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2>). Considering the deduction of an autosomal recessive pattern of inheritance, only the variants that were homozygous or compound heterozygous were selected as candidates.

The variants that met the search criteria were validated by PCR-based Sanger sequencing using the following primers: *CDH23* exon 9, forward 5'-TACAACGTGCCCCATTCTGC-3', reverse 5'-GTCTAGGTTTCAGCTATGCCGTT-3'; *CDH23* exon 43, forward 5'-TCTTCCGTGGTGGTCCATT-3', reverse 5'-AGATGCCTACTGGCTCTCCTT-3'. The TransStart Taq DNA Polymerase kit (Beijing TransGen Biotech Co., Ltd.) was used according to the manufacturers

protocol and the thermocycling conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec, 68°C for 2 min. The amplification product was between 200 and 400 bp. The data were analyzed using Lasergene-SeqMan software (Lasergene 9; DNASTar, Inc.) and the DNA sequences were compared with the sequence of *CDH23* (NM_022124.5) and corresponding *CDH23* protein sequences (NP_071407.4). In total, three sequences were compared to establish a consensus.

In silico analysis. Pathogenicity of the missense variants of *CDH23* was predicted by using SIFT and Polyphen-2. The pathogenicity was determined using the following criteria: i) The variants were considered as 'likely damaging or damaging' by either SIFT or Polyphen-2; ii) the variants occurred at the residues that were highly conserved among various species; iii) the variants occurred in affected subjects in homozygous or compound heterozygous phenotypes.

A 3D molecular structure model of the extracellular domain was generated using I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>) and compared with the confidence score and TM-score using the predicted models. The confidence score is used to estimate the quality of the predicted models using I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. TM-scores are a recently proposed scale for measuring the structural similarity between two structures (24). The DeepView/Swiss-PdbViewer (<http://spdbv.vital-it.ch>) was used for structural analysis and visualization.

Results

Patient clinicopathological features. The proband was a 24-year-old female (II:1) that had bilateral hearing loss, and the sibling of the proband was a 17-year-old male (II:2) with severe deafness. Pure tone audiometry revealed profound sensorineural hearing loss at all frequencies. The patients' parents (I:1 and I:2) had normal hearing, and all other family members exhibited no impairment in movement, vestibular or visual functions.

Genetic analysis. Nine gene loci (GJB2:35delG, 176del16, 235delC, 299delAT, GJB3:538C>T, SLC26A4:2168A>G, IVS7-2A>G, mitochondrial 12SrRNA:1494C>T and 1555A>G) were analyzed and none was detected in either of the siblings or in the mother. However, mitochondrial 12SrRNA 1555A>G was detected in the father. Following exclusion of variants of these four genes by Sanger sequencing, the target region capture sequence was used to resolve the genetic causes. Compound heterozygous variants in *CDH23* from the proband were identified following DNA sequencing as one of the two shifts caused by a transition *CDH23:c.791A>T* in exon 9 (Fig. 1). This transition resulted in the substitution of a valine with an aspartic acid at position 264 (*p.D264V*). The other variant identified was *CDH23:c.5853T>A* in exon 43. This resulted in the substitution of an aspartic acid for a glutamic acid residue at position 1951 (*p.D1951E*; Fig. 1).

The associated regions of *CDH23* were subsequently sequenced in other family members (Fig. 2) and it was revealed that *p.D264V* also occurred in the patients' mother. *p.D1951E* was considered to be a paternal gene, and multiple sequence alignment of the two affected EC domains revealed that the amino acids residues were well-conserved among various species, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Danio rerio* (Fig. 2C).

In silico analysis. Two novel mutations of *CDH23* (*c.791A>T* and *c.5853T>A*) were identified in the coding region, both of which were missense variants. The data were subsequently scored on the compatibility with the 1000 Genome sequence database, the Single Nucleotide Polymorphism database, SIFT and PolyPhen2 score (Table I) and according to the scores, the two gene variants were damaged, which was indicated by the comparison with SIFT and Polyphen-2.

3D models of these variants were created and revealed that affected EC domains (containing aspartic acid residues) contained the highly conserved calcium-binding motifs, EC3 and EC18, in the location of the linking cadherin repeats (Fig. 3). The substitution at residue 264 was the second aspartic acid residue of the DXD calcium-binding motif in EC3. The substitution at residue 1,951 was the first aspartic acid residue of DXNDN calcium-binding motif in EC18. Modeling analysis of the mutated *CDH23* used in the present study revealed that these *CDH23* variants were related to DXD and DXNDN motifs. Consistently, DNFB12 mutations were more likely to be involved in the highly conserved regions, such as LDRE, DXNDN and DXD, compared with *USH1D* (Fig. 4).

Discussion

Previous studies have indicated that *CDH23* expression is vital for the bundle of hair cells in the cochlea and serves an important role in the establishment and maintenance of the proper organization of the stereocilia (25,26). *CDH23* forms a functional network with *USH1C*, *USH1G*, *CDH23* and *myosin VIIA*, and participates in mechanotransduction in auditory processing; therefore, *CDH23* variants may contribute to hearing loss. The present study identified two novel missense mutations of *CDH23*, *c.791A>T* and *c.5853T>A*, in the coding region.

The gene mutation spectrum can be distinguished across different ethnic origins. For example, *GJB2:c.35delG* occurs more frequently in people of Caucasian origin, whereas *GJB2:c.167delT* is prevalent in Ashkenazi Jews; *GJB2:p.R143W* was found in an African farmer, and *GJB2:c.235delC* is commonly reported in East Asian countries (27,28). The frequency of the gene mutations involved in hearing loss is due to the founder effect (1). In the present study, nine gene loci (*GJB2:35delG*, *176del16*, *235delC*, *299delAT*, *GJB3:538C>T*, *SLC26A4:2168A>G*, *IVS7-2A>G*, *mitochondrial 12SrRNA:1494C>T* and *1555A>G*) were assessed, but none of these mutations was identified in either affected sibling. However, using the target region captured DNA sequencing technology, two novel pathological *CDH23* variants were identified, which may be possible pathological variants and missense *CDH23* mutations; this was in agreement with previous studies (11,17,18). A previous study demonstrated nonsense, frame shift or splice mutations of *CDH23* in patients

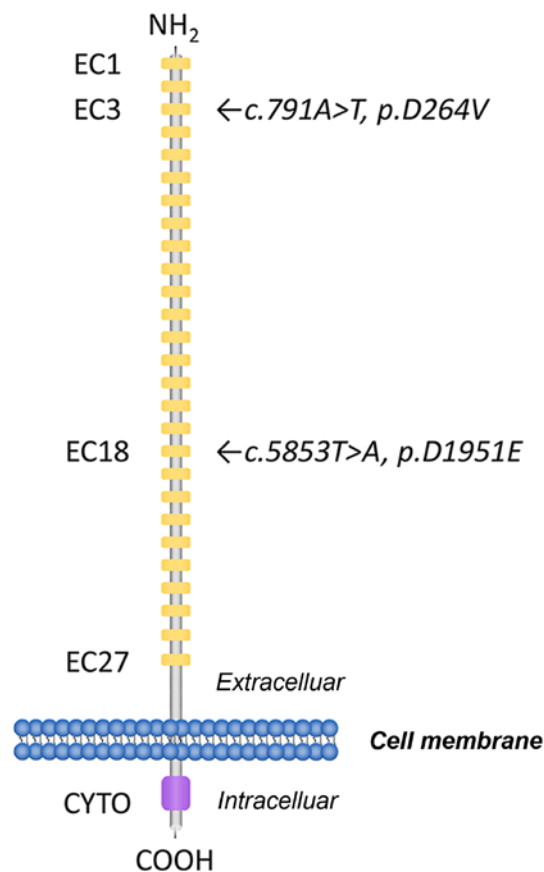


Figure 1. Molecular structure of the human cadherin 23 protein. Positions of the mutated nuclear acids are on the right and the corresponding extracellular repeats are on the left. CYTO, the cytoplasmic domain (purple); EC, extracellular repeat (yellow).

with *USH1D* (17). Aspartic acid is a medium-sized acidic residue, whereas, valine is hydrophobic. Substitution of the aspartic acid residues with valine strongly reduced the number of oxygen atoms binding to calcium, indicating that these variants may have an effect on *CDH23* structure. Thus, this valine substitution may be pathological in hearing loss. These motifs have been suggested to be essential for a calcium-binding ability, which may influence linearization, rigidification and dimerization of cadherin (29,30).

Calcium ions are usually enclosed by several oxygen atoms (31), and the absence of these atoms normally weakens calcium-binding capacity (32). Substitution of the aspartic acid residues with a valine impaired the interaction of the *CDH23* protein either with itself or with other protein, owing to the loss of oxygen atoms present in aspartic acid. The *c.791A>T* mutation involved an amino acid change from an aspartic acid to a hydrophobic valine (*p.D264V*), which altered the function of the *CDH23* protein. In addition, the results of Polyphen-2 and SIFT predicted the impact of these changes and indicated that they may pathogenically affect protein structure or function. Calcium ions serve a crucial role in the process of adaptation of mechanical transduction, frequency tuning, neurotransmitter release and efferent synaptic signaling in the auditory and vestibular systems (33). A previous study has also demonstrated that the *CDH23* protein associates with myosin-1c to form a protein complex that is

Table I. Pathogenicity prediction of cadherin 23 variants associated with hearing loss using *in silico* analysis.

Nucleotide change	Amino acid change	Exon	Domain	dbSNP	1000 Genomes	Polyphen-2 score ^a	SIFT score ^b
<i>c.791A>T</i>	<i>p.D264V</i>	9	3	-	N/A	0.998	0.00
<i>c.5853T>A</i>	<i>p.D1951E</i>	43	18	-	N/A	0.997	0.00

^aPrediction of a change being damaging (>0.85), possibly damaging (0.15-0.85) or benign (<0.15). ^bPrediction of a change being damaging (<0.05) or tolerated. RefSeq NM_022124.5, CDH23 transcript variant 1; NP_071407.4, CDH23 isoform 1 precursor. -, not found; N/A, not applicable; CDH23, cadherin 23.

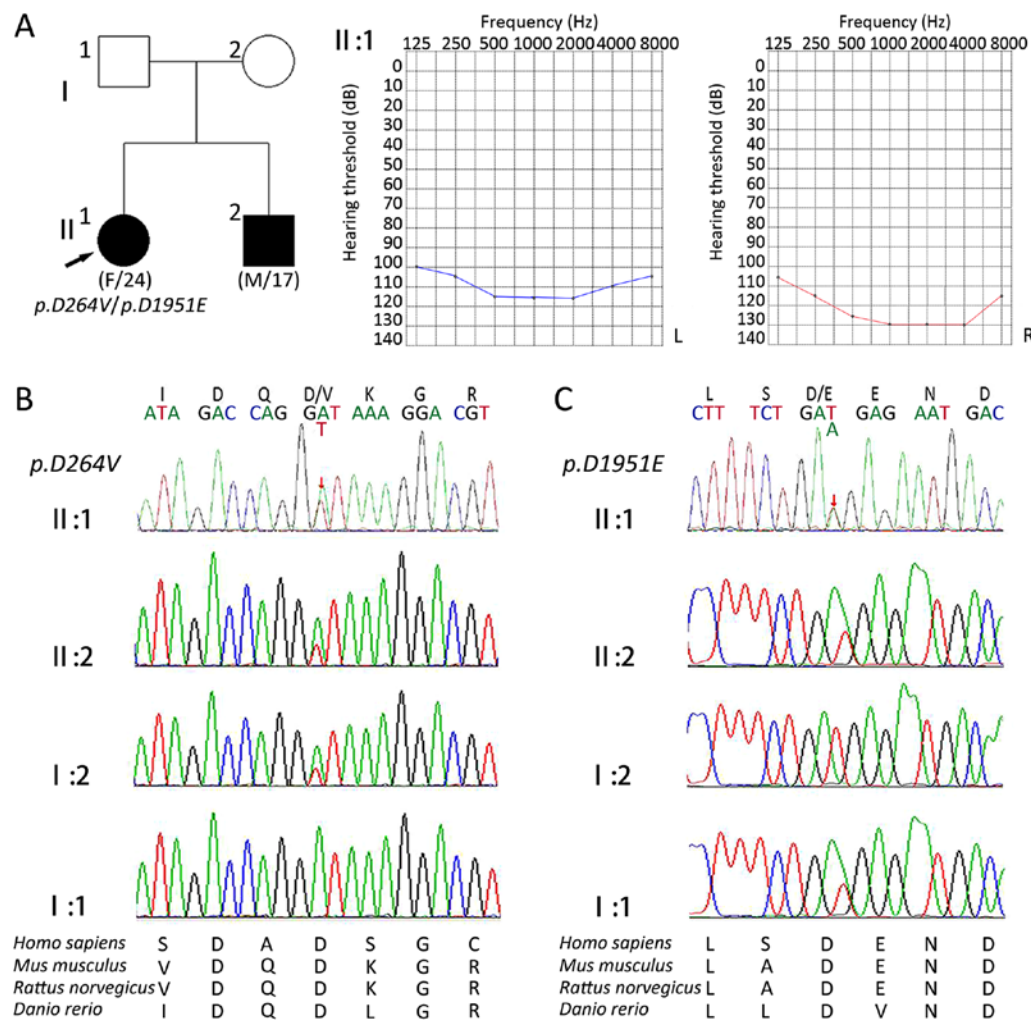


Figure 2. Identification of *CDH23* variants. (A) Patient genealogy. Filled symbols represent hearing-impaired individuals, clear symbols represent individuals with normal hearing ability. The proband, whose left and right ear audiograms are presented, is marked with an arrow. (B) DNA sequencing analysis of *CDH23* exon 9 with the heterozygous *c.791A>T* (*p.D264V*) variant and the alignment of the EC3 domain of the *CDH23* containing mutation. (C) DNA sequencing analysis of *CDH23* exon 43 with a heterozygous *c.5853T>A* (*p.D1951E*) variant and the alignment of the EC18 domain of the *CDH23* containing mutation. *CDH23*, cadherin 23; L, left; R, right.

a component of the mechanotransduction apparatus (34). This suggested that *CDH23* may participate in the activity of mechanically gated ion channels in hair cells. Missense variants of genes, including *CDH23*, may be a common cause of disease, depending on the mutation site within the protein. In the present study, two novel *CDH23* missense mutations were identified, and the results revealed that there may be an association between gene mutation and disease pathogenicity.

In addition, *in silico* analysis predicted pathogenic variants of a given gene (35), which was used to demonstrate that the two *CDH23* variants may contribute to the hearing impairment of the two patients. Previous studies have revealed that *CDH23* variants are associated with hearing loss in Chinese patients. For example, a homozygous *c.5985C>A* (*p.Y1995X*) variant has been linked to USH1D (36). Patients from the Chinese Jiangxi province were heterozygous with *p.E1006K*

Table II. Frequency of cadherin 23 variants in hearing loss.

Author, year	Country	Nucleotide change	Amino acid change	Number of patients			No. patients	(Refs.)
				-/-	Compound +/-	+/-		
Chai <i>et al.</i> , 2015	China	<i>c.5985C>A</i>	<i>p.Y1995X</i>	1	N/A	N/A	945	(36)
Lu <i>et al.</i> , 2014	China	<i>c.3016G>A</i> <i>c.4988A>T</i>	<i>p.E1006K</i> <i>p.D1663V</i>	N/A	2	N/A	9	(37)
Miyagawa <i>et al.</i> , 2012	Japan	<i>c.719C>T</i>	<i>p.P240L</i>	N/A	2	1	1,396	(19)
		<i>c.902G>A</i>	<i>p.R301Q</i>	N/A	3	N/A		
		<i>c.2866G>A</i>	<i>p.E956K</i>	N/A	1	2		
		<i>c.4103C>T</i>	<i>p.T1368M</i>	N/A	1	N/A		
		<i>c.4249C>T</i>	<i>p.R1417W</i>	1	N/A	2		
		<i>c.4877A>C</i>	<i>p.D1626A</i>	N/A	1	N/A		
		<i>c.5147A>C</i>	<i>p.Q1716P</i>	N/A	3	N/A		
		<i>c.6085C>T</i>	<i>p.R2029W</i>	2	2	6		
		<i>c.6861T>G</i>	<i>p.N2287K</i>	N/A	2	N/A		
		<i>c.7312G>A</i>	<i>p.E2438K</i>	N/A	1	N/A		
Woo <i>et al.</i> , 2014	Korea	<i>c.719C>T</i>	<i>p.P240L</i>	N/A	3	N/A	16	(20)
		<i>c.1025A>G</i>	<i>p.N342S</i>	N/A	2	N/A		
		<i>c.4783G>A</i>	<i>p.I595K</i>	N/A	1	N/A		
Present study	China	<i>c.791A>T</i>	<i>p.D264V</i>	N/A	2	N/A	2	
		<i>c.5853T>A</i>	<i>p.D1951E</i>	N/A	2	N/A		

N/A, not applicable.

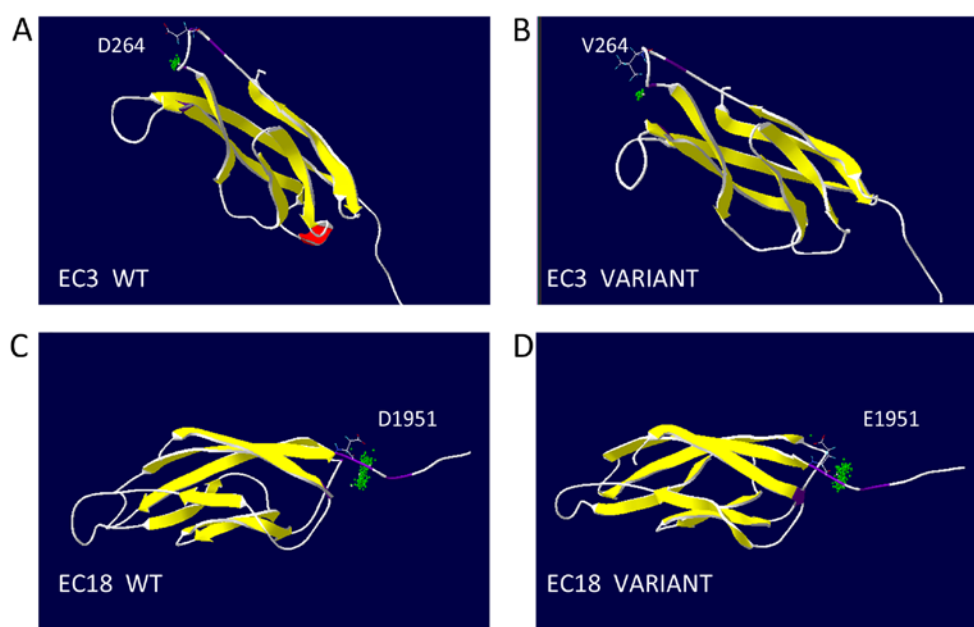


Figure 3. Molecular modeling of CDH23 extracellular domains 3 and 18. The CDH23 protein contains various conserved EC domains. The structure of the predicted 3D models reveals a typical folding pattern with several strands (yellow), coils (white) and helices (red). (A and B) Amino acids residues D264 and V264 are localized at EC3. (C and D) Amino acid residues D1951 and E1951 are localized at EC18. The structures of A and C are wild-type, whereas B and D are mutant. The two residues are associated with highly conserved EC calcium-binding sites (purple), whereas the green region represents the possible ligand calcium ion. EC, extracellular.

and *p.D1663V* in *CDH23* (37). Regarding the *CDH23* spectrum in the Japanese, the frequency was 3.7% (including the heterozygous phenotype) in the hearing loss population and

5.7% (including the heterozygous phenotype) among the recessive inherited cases (19). Two out of 13 (15%) Korean families with non-syndromic hearing loss were affected

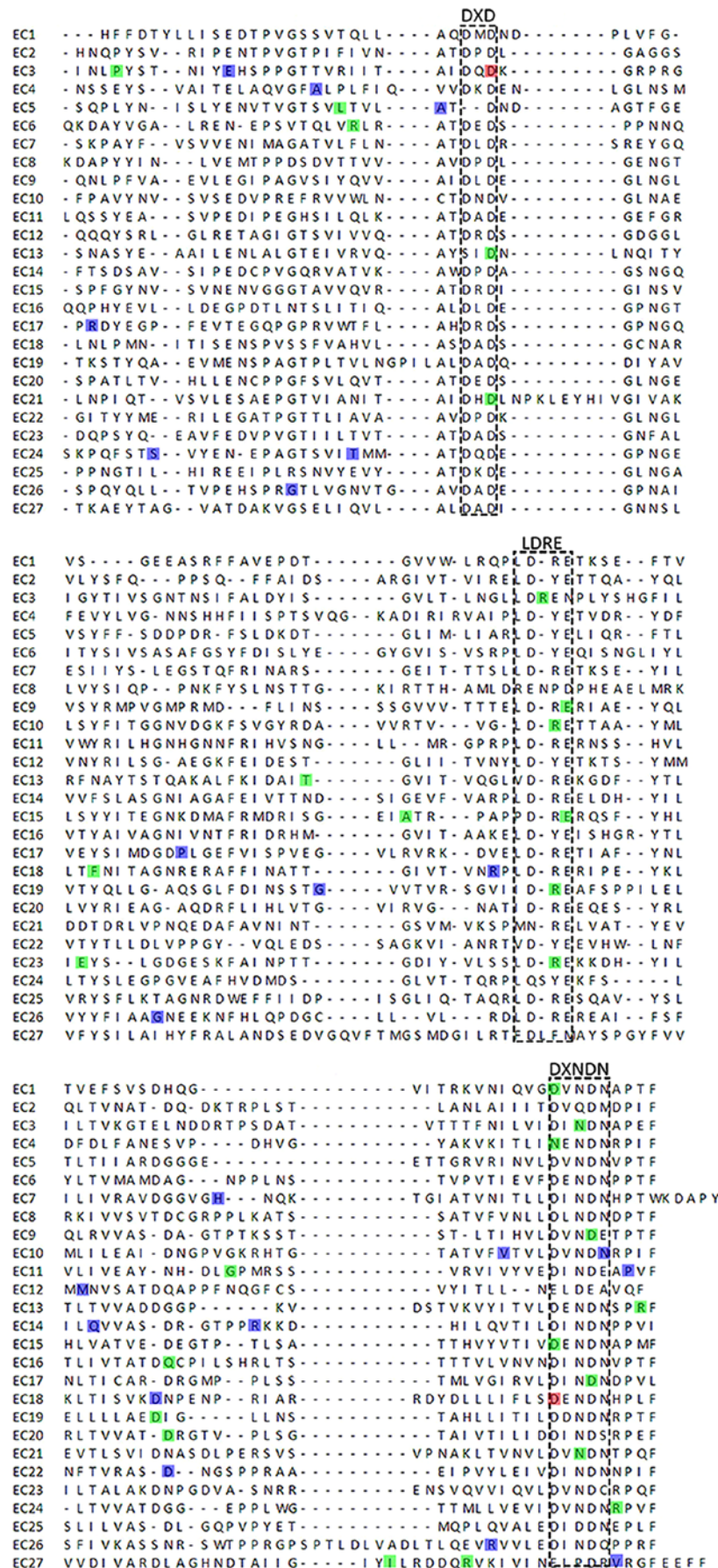


Figure 4. Alignment of CDH23 EC domains with mutations causing Usher syndrome type ID and recessive DBNF12. DNASTAR Lasergene analysis was used to align 27 EC domains. The corresponding EC domains are indicated on the left. The LDRE, DXNDN and DXD calcium-binding motifs are boxed. The sites of mutations identified in the present study are highlighted in red, whereas the sites of mutations identified by other studies are highlighted in green (DBNF12) and blue (Usher Syndrome type 1).

by *CDH23* mutations in an autosomal recessive pattern and three of 93 patients exhibited a heterozygous phenotype (20). Consequently, some *CDH23* variants frequently occurred in certain ethnic origins (19,20) and, based on these data, it was speculated that *CDH23* variants may be an important cause of hearing loss in Asian populations (Table II). Although the precise pathogenesis and molecular mechanism of hearing have not been defined, the data from the present study and the literature indicate that DFNB12 *CDH23* variants may impact the Chinese population with non-syndromic hearing loss.

In conclusion, the present study identified two novel *CDH23* mutations in one Chinese family with autosomal recessive non-syndromic hearing loss using the target region captured DNA sequencing. However, the exact frequency requires further investigation as only data from two generations of this family were obtained. Future studies with a larger cohort of patient samples are needed to confirm the present results, which may be used for early detection of hearing loss. In addition, target region captured DNA sequencing technology may be effective for detecting causative gene variants in patients with no mutations in *GJB2*, *GJB3*, *SLC26A4* and *mitochondrial 12SrRNA*.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

All authors made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. TX, HL and SY performed the experiments. TX drafted the article. PW and WZ critically revised the article. ZW reviewed the submitted version of manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Hospital of Jilin University (Changchun, China) and all participants provided written informed consent prior to being enrolled in the study.

Patient consent for publication

Written informed consent was obtained from the parent of the patient for the publication of this case report.

Competing interests

The authors declare that they have no competing interests.

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